

# Gremlin 2 inhibits adipocyte differentiation through activation of Wnt/ $\beta$ -catenin signaling

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**Abstract.** The primary function of white adipose tissues is to store excess energy. The current study aimed to investigate the roles of Gremlin 2 (Grem2), a glycoprotein in adipogenesis. Using polymerase chain reaction-based microarrays, it was determined that Grem2 was markedly downregulated in adipose tissues from obese animals and humans. In addition, 3T3-L1 cells were used to investigate the details of the mechanisms underlying the anti-adipogenic effects of Grem2. Grem2 expression was markedly decreased upon the induction of adipocyte differentiation, as demonstrated by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Notably, Grem2 overexpression inhibited adipogenesis, while knockdown of Grem2 led to an increase in adipogenesis. At the molecular level, Grem2 promotes nuclear translocation of  $\beta$ -catenin, an integral Wnt signaling component. Consistently, inhibition of Wnt/ $\beta$ -catenin signaling using a retrovirus targeting the  $\beta$ -catenin coding region attenuated the anti-adipogenic effects of Grem2. Therefore, to the best of our knowledge, the current study shows for the first time that Grem2 may be an important regulator of adipocyte differentiation.

## Introduction

When dietary nutrient intake chronically exceeds energy expenditure, obesity occurs due to an increase in the size and/or number of white adipocytes (1). Obesity is closely associated with type 2 diabetes, fatty liver, hyperlipidemia, cardiovascular diseases and tumorigenesis (2,3). Thus, investigating the molecular and cellular mechanisms regulating adipocyte

differentiation is crucial for designing potential therapies to prevent obesity and the associated diseases.

Decades of studies have identified a number of transcription factors involved in adipocyte differentiation, including peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and members of the C/EBP family (4,5). Additionally, a number of other factors or signaling molecules are involved in the control of adipocyte differentiation, such as Wnt/ $\beta$ -catenin, cAMP/CREB, and the family of kruppel-like factor (KLF) proteins (6-8). Furthermore, recent studies have indicated that modification of these proteins has an important role in adipogenesis. It has been shown that phosphorylation, acetylation and small ubiquitin-like modifier (SUMO)ylation of PPAR $\gamma$  affects adipocyte differentiation, though regulation of its transcriptional activity (9-11). In this regard, the process of adipogenesis is rather complex, suggesting that identification of the additional factors may provide novel insights into the mechanisms regulating the initiation of fat cell differentiation and obesity.

In the present study, polymerase chain reaction (PCR)-based microarrays were performed in obese and lean mice to screen potential genes involved in adipogenesis.

## Materials and methods

**Mice.** Male C57BL/6, *ob/ob* and *db/db* mice, aged 10-12 weeks, were purchased from the Shanghai Laboratory Animal Company (Shanghai, China). All mice were housed and bred according to international standard conditions, with a 12-h dark/light cycle. For induction of obesity, mice aged 10 weeks were placed on a high-fat-diet (50 kcal% fat, 25 kcal% protein and 25 kcal% carbohydrate) for 12 weeks. The normal diet consisted of 10 kcal% fat, 15 kcal% protein and 75 kcal% carbohydrate. The animal protocol was reviewed and approved by the Animal Care Committee of Chongqing Ninth People's Hospital (Chongqing, China).

**Human tissue samples.** Human subcutaneous fat tissues from obese (BMI>25; n=25) and lean (BMI<23; n=20) adults were collected at the Department of Endocrinology at Chongqing Ninth People's Hospital. All subjects had a stable weight, defined as the absence of fluctuations of >1% of body weight for at least 2 months prior to surgery. Adipose tissue was

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immediately frozen in liquid nitrogen following extraction. All samples were obtained with informed consent and the study was approved by the Chongqing Ninth People's Hospital Institutional Review Board.

**Cell culture.** 3T3-L1 fibroblasts were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco-BRL). Adipocyte differentiation was induced by treating the cells at 80-90% confluency with standard differentiation inducers [100  $\mu$ M IBMX (Beyotime Institute of Biotechnology, Nantong, China), 100 nM dexamethasone (Beyotime Institute of Biotechnology) and 10  $\mu$ g/ml insulin (Eli Lilly & Co., Indianapolis, IN, USA)] for 48 h (from day 0 to day 2). Cells were treated with DMEM supplemented with 10  $\mu$ g/ml insulin and 10% FBS for another 48 h (from day 2 to day 4). Subsequently, the medium was replaced by DMEM and 10% FBS, which was changed daily, and the cells were cultured for a further 4-6 days.

**RNA extraction, cDNA microarrays, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from tissues or cells using the TRIzol reagent (Invitrogen Life Technologies, Shanghai, China), and reverse transcription was performed using the RT kit from Promega Corporation (Madison, WI, USA), according to the manufacturer's instructions. Affymetrix arrays were performed using Mouse Genome 2.0 chips from Gene Tech Company Limited (Shanghai, China). In order to quantify the transcripts of the interest genes, qPCR was performed using a SYBR Green Premix Ex Taq kit (Takara Bio Inc., Dalian, China) on a Light Cycler 480 (Roche, Basel, Switzerland) with 5 mg RNA. The following primer sequences from Bioyare Biotechnology Company (Shanghai, China) were used: Mouse Grem2, forward 5'-TGTGCTGGTAAAGGTAGCTGA-3' and reverse 5'-CCACCTCTCTGAGTTGTTGCT-3'; human Grem2, forward 5'-ATCCCCTCGCCTTACAAGGA-3' and reverse 5'-TCTTGCACCACTCTTGA-3'; C/EBP $\alpha$ , forward 5'-CAAGAACAGCAACGAGTACCG-3' and reverse 5'-GTCACCTGGTCAACTCAGCAC-3'; PPAR $\gamma$ , forward 5'-CTCCAAGAATACCAAGTGCGA-3' and reverse 5'-GCCTGATGCTTTATCCCCACA-3'; LPL, forward 5'-ATGGATGGACGGTAACGGGAA-3' and reverse 5'-CCCGATACAACCACTCTACTACA-3'; PEPCK, forward 5'-ATGAGCCGCAAACTGGGTCTC-3' and reverse 5'-AGAGCCGAAGTCCACAATCTC-3'; CD36, forward 5'-AGCATTCAACGCCAGGTTC-3' and reverse 5'-CGAGTCTGTGCTCAGTTCAATACCAA-3'; aP2, forward 5'-AAGGTGAAGAGCATCATAACCCT-3' and reverse 5'-TCACGCCTTTTCATAACACATTCC-3'; mouse HPRT1, forward 5'-TCAGTCAACGGGGGACATAAA-3' and reverse 5'-GGGGCTGTACTGCTTAACCAG-3'; human HPRT1, forward 5'-CCTGGCGTCGTGATTAGTGAT-3' and reverse 5'-AGACGTTCACTGCTGTCCATAA-3'. The PCR cycling conditions were as follows: Initial period at 94°C for 3 min, followed by a two-step PCR program consisting of 95°C for 5 sec and 60°C for 30 sec for 55 cycles. Gene expression

was calculated relative to the mRNA expression of HPRT1, and relative quantitation analysis of gene expression data was performed using the  $2^{-\Delta\Delta C_t}$  method.

**Retrovirus, small interfering (si)RNA and transfection.** Retroviruses containing empty vector or Grem2 cDNA were constructed by Invitrogen Life Technologies. siRNA oligos targeting Grem2 or  $\beta$ -catenin were purchased from Gene Pharma (Shanghai, China) and transfected into 3T3-L1 cells using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. Histological analysis was conducted using Oil Red O staining. Cells were fixed with 4% paraformaldehyde for 30 min, followed by incubation with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) for 45 min.

**Western blot analysis.** Cells or tissues were harvested and lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl and 1% NP-40; pH 7.5). Proteins were quantified and separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Bioscience, Buckinghamshire, UK). After blocking with 10% non-fat milk in phosphate-buffered saline, membranes were immunoblotted with the following rabbit antibodies from Abcam (Cambridge, MA, USA): Polyclonal anti-Grem2 (ab102563; 1:1,000), monoclonal C/EBP $\alpha$  (ab40764; 1:1,000), polyclonal PPAR $\gamma$  (ab19481; 1:1,000), monoclonal P-P38 (ab178867; 1:2,000), polyclonal P-AKT (ab66138; 1:1,000), monoclonal P-CREB (ab32096; 1:2,000), monoclonal CREB (ab32515; 1:2,000), polyclonal  $\beta$ -catenin (ab6302; 1:1,000), polyclonal lamin B (ab16048; 1:2,000) and monoclonal GAPDH (ab181602; 1:1,000). The blots were then incubated with horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Inc. Danvers, MA, USA). The signals were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

**Statistical analysis.** The results are expressed as the mean  $\pm$  standard error of the mean and analyzed using Student's t-test or one way analysis of variance with GraphPad Prism software, version 5.0.1 (GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Downregulation of Grem2 in obese mice and humans.** To identify potential genes involved in adipogenesis and obesity, male C57BL/6 mice aged 10 weeks were placed on high-fat or normal diets for 12 weeks. The mice were sacrificed by cervical dislocation and gene expression arrays were performed using epididymal adipose tissues. The clustering analysis of the Affymetrix arrays revealed that 1,174 genes were upregulated and 1,572 genes were downregulated in the adipose tissues of mice fed a high-fat-diet compared with those fed a normal diet ( $P < 0.05$ ; data not shown). Among these genes, it was determined that Grem2 was significantly downregulated ( $P < 0.001$ ). Its downregulation was further confirmed by RT-qPCR and western blot analysis (Fig. 1A and B).

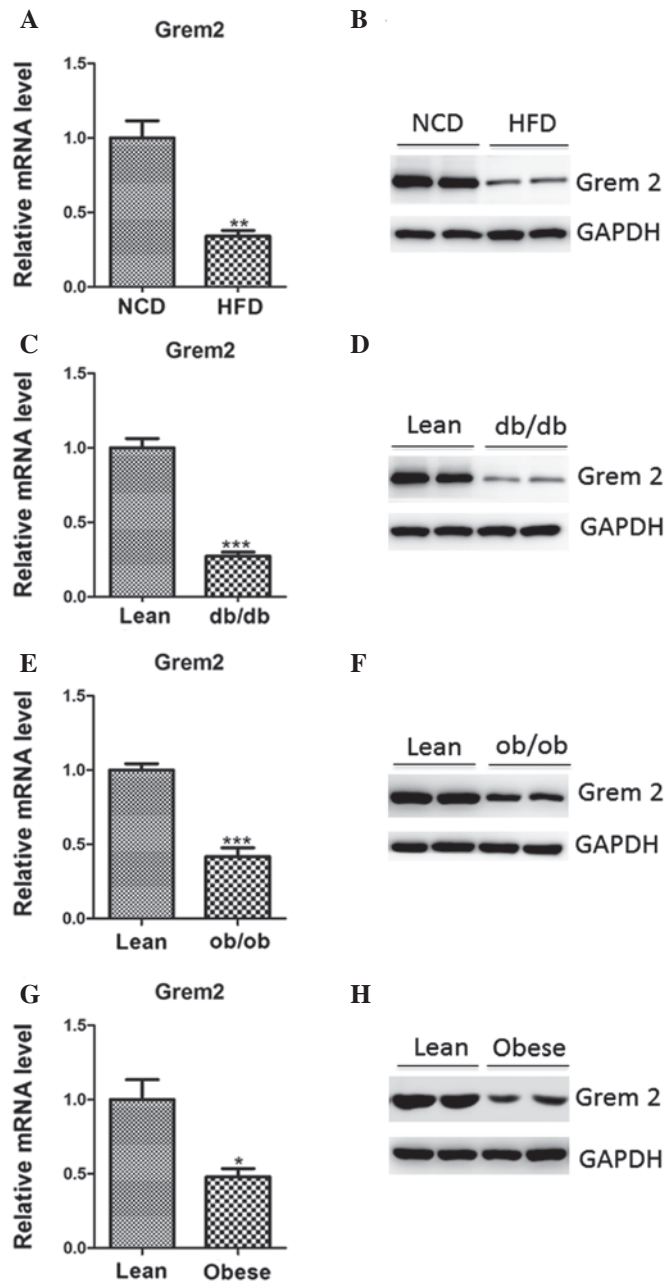


Figure 1. Downregulation of Gremlin 2 (Grem2) in adipose tissues from obese mice and humans. (A-F) mRNA and protein levels of Grem2 were determined in epididymal adipose tissues from obese mice. C57BL/6 mice were fed a high-fat-diet (HFD) or normal chow diet (NCD) for 12 weeks. After fasting for 8 h, mice were sacrificed and tissues were collected. n=5-8. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. (G and H) mRNA and protein levels of Grem2 were determined in subcutaneous adipose tissues from obese and lean human subjects.

To determine whether the inhibition of Grem2 represents a common feature of obesity, *db/db* and *ob/ob* mice were employed as models for monogenic-induced obesity. Indeed, Grem2 mRNA and protein levels were found to be reduced in the epididymal adipose tissues of these mice, as compared with lean controls (Fig. 1C-F). Notably, Grem2 mRNA and protein levels were significantly reduced in subcutaneous fat from obese humans (Fig. 1G and H), further suggesting that the downregulation of Grem2 expression may be a conserved feature in obese mice and humans.

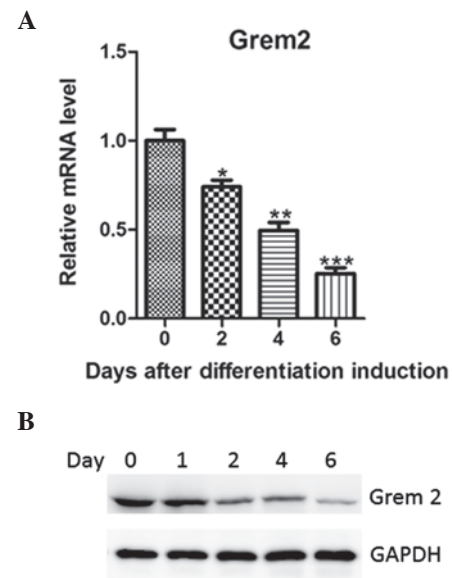


Figure 2. Gremlin 2 (Grem2) expression during 3T3-L1 adipocyte differentiation. Expression of Grem2 mRNA and protein during differentiation of 3T3-L1 cells was analyzed using (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis at the indicated times \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared with day 0.

*Grem2 suppresses the differentiation of 3T3-L1 preadipocytes into adipocytes.* To explore the functions of Grem2 in adipogenesis, its gene expression was investigated at numerous time points during 3T3-L1 differentiation. The RT-qPCR results revealed that Grem2 mRNA gradually reduced from day 0 post-induction (Fig. 2A). The protein levels for Grem2 were consistent with its mRNA levels (Fig. 2B).

The role of Grem2 in adipogenesis was tested using retroviruses carrying Grem2 cDNA or empty vector, which resulted in forced overexpression of Grem2 in 3T3-L1 preadipocytes (Fig. 3A). These cells were cultured to confluence and differentiated using the standard induction cocktail for 8 days. Cell morphology indicated that cells overexpressing Grem2 accumulated significantly less lipids than the control cells (Fig. 3B). In addition, the expression levels of adipogenic regulators such as C/EBP $\alpha$  and PPAR $\gamma$  were reduced (Fig. 3C and D), which was consistent with the Oil Red O staining. Furthermore, markers of mature adipocytes LPL, PEPCK, CD36 and aP2, which are downstream targets of C/EBP $\alpha$  and PPAR $\gamma$ , were repressed by Grem2 overexpression (Fig. 3E).

*Grem2 knockdown promotes adipocyte differentiation.* To further examine the roles of Grem2, its specific siRNA was transfected into 3T3-L1 cells (Fig. 4A). As a result, knockdown of Grem2 levels showed a clear increase in the adipogenic potential of the 3T3-L1 cells compared with that of the cells treated with the negative control siRNA, as illustrated by cell morphology (Fig. 4B). Furthermore, knockdown of Grem2 increased expression levels of the adipocyte markers including C/EBP $\alpha$ , PPAR $\gamma$ , LPL, PEPCK, CD36 and aP2 (Fig. 4C and D). Together, these results indicate that Grem2 is a critical negative regulator in the adipogenesis.

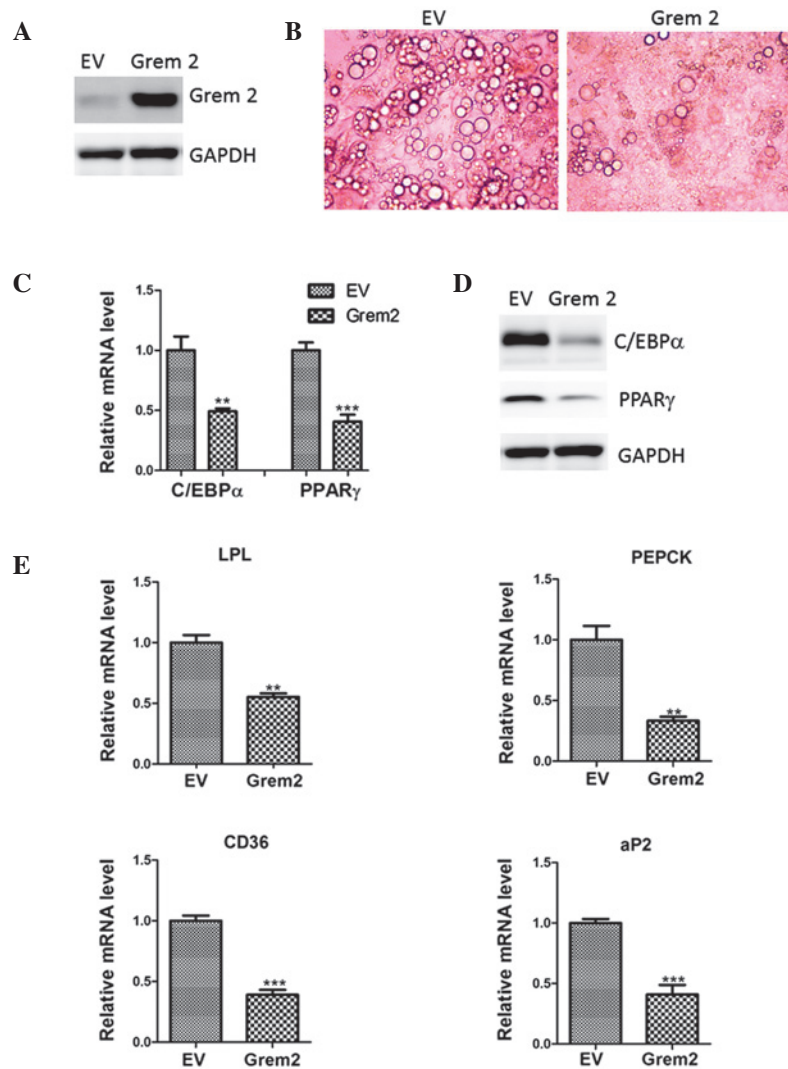


Figure 3. Gremlin 2 (Grem2) overexpression inhibits differentiation of 3T3-L1 preadipocytes into adipocytes. (A) Grem2 protein levels in 3T3-L1 cells after transfection with retrovirus containing empty vector (EV) or Grem2 cDNA. (B) 3T3-L1 cells were infected with retrovirus containing empty vector (EV) or Grem2 cDNA. On day 8 after induction of differentiation, cell morphology was determined with Oil Red O staining and a microscope at x200. (C and D) mRNA and protein levels of C/EBPα and PPARγ in 3T3-L1 cells on day 8 after induction of differentiation. (E) mRNA levels of LPL, PEPCK, CD36 and aP2 in 3T3-L1 cells \*\*P<0.01, \*\*\*P<0.001, compared with EV.

*Grem2 activates Wnt/β-catenin signaling in adipocytes.* Finally, the molecular basis for the observed phenotypic changes in 3T3-L1 cells with Grem2 overexpression was investigated. As shown in Fig. 5A, Wnt signaling was highly activated by Grem2 overexpression, as evidenced by nuclear accumulation of β-catenin, while other signaling molecules, including p38, AKT and cAMP/CREB, remained unaffected (Fig. 5B). Subsequently, it was investigated whether ablation of β-catenin reversed the roles of Grem2. A retrovirus targeting a β-catenin coding region (sh-catenin) was generated using pAD\_BLOCK\_IT\_DEST vectors (Invitrogen Life Technologies, Grand Island, NY, USA) to knockdown endogenous β-catenin expression in 3T3-L1 cells (Fig. 5C). As a result, sh-catenin largely attenuated the inhibitory roles of Grem2 on the adipogenesis, as evidenced by gene expression of adipogenic markers (Fig. 5D), suggesting that the role of Grem2 in the adipogenesis, at least in part, relies on Wnt/β-catenin activation.

## Discussion

In the present study, it was determined that the Grem2 gene was significantly downregulated in white adipose tissue from obese mice and humans. Using 3T3-L1 cells cultured *in vitro*, it was demonstrated that Grem2 inhibited adipocyte differentiation. Thus, for the first time to the best of our knowledge, these results identify Grem2 as a key regulator in adipose formation and obesity.

Members of the Gremlin family, including Grem2, have been shown to be bone morphogenetic protein (BMP) antagonists (12). Indeed, Grem2 regulates BMP signaling in cardiomyocyte and osteoblast differentiation (13,14). Furthermore, a recent human study revealed that genetic variants in Grem2 are associated with bone mineral density (15). Since certain members of the family of BMPs support white and brown adipocyte differentiation (16,17), whether Grem2 regulates BMP-induced adipogenesis requires further investigation.



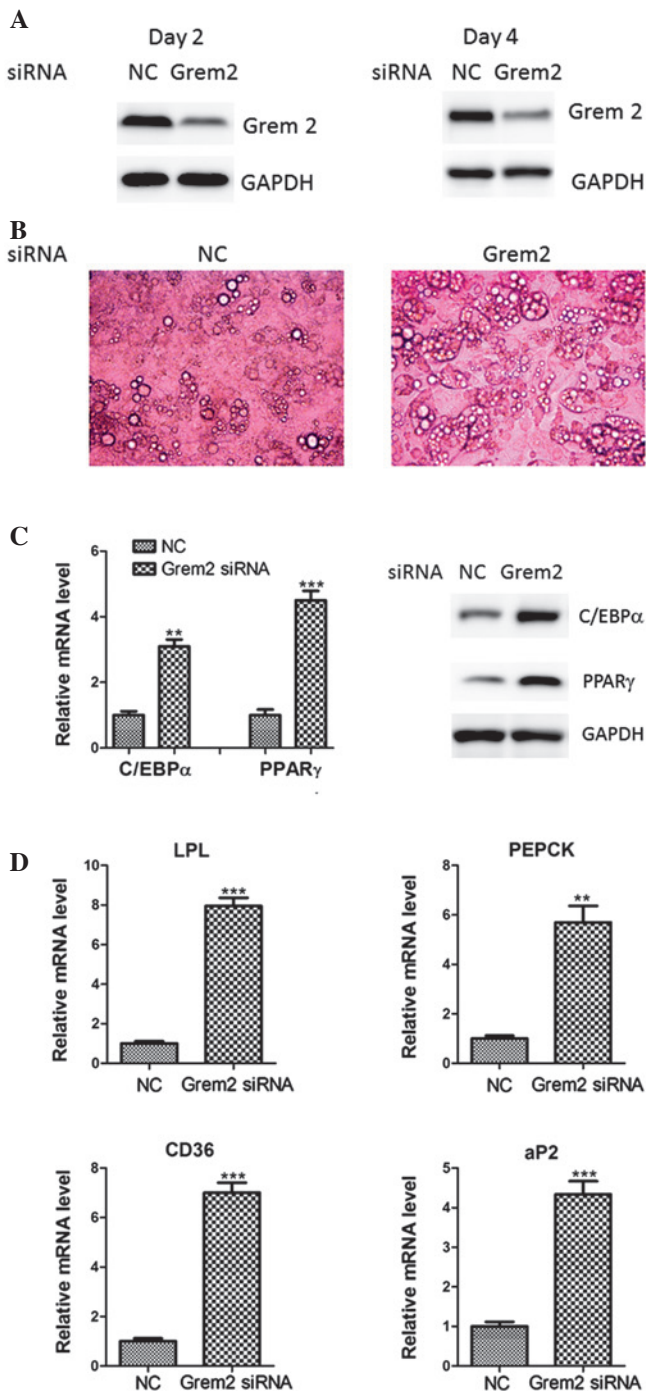


Figure 4. Gremlin 2 (Grem2) knockdown promotes 3T3-L1 cell differentiation. (A) Grem2 protein levels in 3T3-L1 cells after transfection with small interfering (si)RNA oligos targeting Grem2 or negative controls. Cells were harvested at day 2 and day 4 after differentiation induction for western blot analysis. (B) 3T3-L1 cells were infected with siRNA oligos targeting Grem2 or negative controls. On day 8 after induction of differentiation, cell morphology was determined with Oil Red O staining and a microscope at x200. (C and D) Expression levels of C/EBPα, PPARγ, LPL, PEPCK, CD36 and aP2 in 3T3-L1 cells. \*\*P<0.01, \*\*\*P<0.001, compared with NC.

At the molecular level, it was revealed that Grem2 activates Wnt/β-catenin signaling, as evidenced by its nuclear accumulation. Initial studies have demonstrated that ectopic expression of Wnt1, an activator of Wnt/β-catenin signaling, may inhibit adipogenesis (6,18,19). Concurrently, pharmacological compounds that activate Wnt/β-catenin signaling or

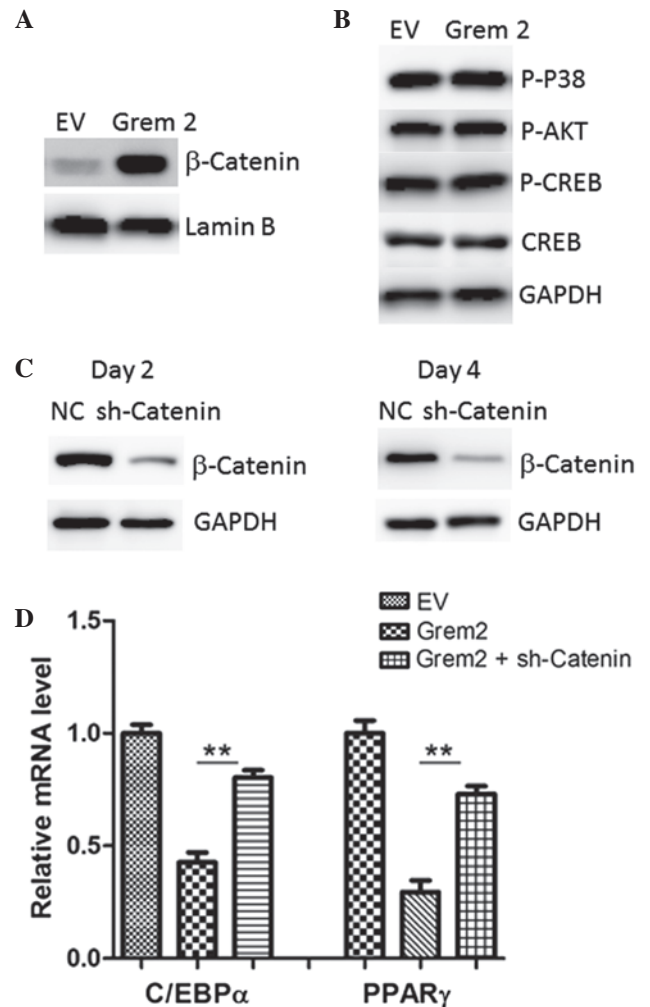


Figure 5. Gremlin 2 (Grem2) activates Wnt/β-catenin signaling to inhibit adipogenesis. (A) Protein levels of nuclear β-catenin was determined by western blot analysis in 3T3-L1 cells after transfection of Grem2 or empty vector (EV). (B) Protein levels of phosphorylated p38, AKT and CREB were examined by western blot analysis in 3T3-L1 cells after transfection of Grem2 or EV. (C) Grem2 protein levels in 3T3-L1 cells after transfection with siRNA oligos targeting β-catenin or negative controls. Cells were harvested at days 2 and 4 after differentiation induction for western blot analysis. (D) Expression levels of C/EBPα and PPARγ in 3T3-L1 cells. \*\*P<0.01.

transfection of stable β-catenin block preadipocyte differentiation (18). However, inhibition of Wnt signaling promotes cell differentiation (18,20), indicating that Wnt signaling has a negative role in the adipocyte differentiation. In accordance with this conclusion *in vitro*, transgenic mice overexpressing *Wnt10b*, an endogenous factor for Wnt signaling, have a 50% reduction in adiposity under a normal chow diet (21). These mice resist expansion of adipose tissue under conditions of high-fat-diet-induced and genetic-induced obesity (21). Furthermore, recent genetic research indicated the importance of Wnt/β-catenin signaling in human obesity. Polymorphisms of the *Wnt10b* gene may be associated with obesity in human subjects of European origin (22). Together, these data suggest that modulators of Wnt/β-catenin pathway may represent a useful therapeutic target for obesity (23).

In conclusion, the results of the current study highlight a pivotal role for Grem2 in white fat-cell differentiation by controlling Wnt/β-catenin signaling. Further studies are

required to investigate the functions and mechanisms of Grem2 *in vivo*, using knockout or transgenic animals.

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