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ORIGINAL ARTICLE

Embelin attenuates adipogenesis and lipogenesis through activating canonical Wnt signaling and inhibits high-fat diet-induced obesity

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BACKGROUND: Recent studies suggest that Embelin, a natural plant extract might have the potential to prevent body weight gain in rats. However, the mechanisms involved remain to be elucidated.

METHODS: Effects of Embelin on adipocyte differentiation and lipogenesis were studied in murine ST2 stromal cells and C3H10T1/2 mesenchymal cells. The mechanisms through which Embelin regulates adipogenic differentiation and lipogenesis were explored. The *in vivo* anti-obesity effects of Embelin in high-fat diet (HFD)-induced obesity mice and possible transcriptional impact were investigated.

RESULTS: Embelin treatment suppressed ST2 and C3H10T1/2 cells to proliferate, and differentiate into mature adipocytes, along with the inhibition of adipogenic factors peroxisome proliferator-activated receptor γ, CCAAT/enhancer binding protein-α, adipocyte protein 2 and adipsin. Embelin treatment also decreased the expression levels of lipogenic factors sterol regulatory element-binding protein 1, fatty acid synthase, acetyl-CoA carboxylase 1 and stearoyl-Coenzyme A desaturase 1. Embelin promoted the translocation of β-catenin from the cytoplasm into the nucleus in C3H10T1/2. The nuclear protein levels of β-catenin and TCF-4 were increased following Embelin treatment. Furthermore, Dickkopf-1 (Dkk1) expression was downregulated by Embelin, and overexpression of Dkk1 in C3H10T1/2 reversed the inhibition of adipogenesis and lipogenesis by Embelin. *In vivo* studies showed that Embelin treatment reduced the gain of body weight and fat, decreased the serum level of triglycerides, free fatty acid and total cholesterol, and improved glucose tolerance and insulin resistance in HFD-fed mice. Moreover, Embelin blocked induction of adipogenic and lipogenic factors and Dkk1 in adipose tissue in HFD-fed mice.

CONCLUSIONS: The present work provides evidences that Embelin is effective in inhibiting adipogenesis and lipogenesis *in vitro* and the mechanisms may involve canonical Wnt signaling. Embelin has the potential to prevent body weight gain and fat accumulation, and to improve obesity-related glucose tolerance impairment and insulin resistance in the HFD-fed mice.

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INTRODUCTION

Obesity is a major risk factor for cardiometabolic diseases, including diabetes, hypertension, dyslipidemia and coronary heart disease. It is characterized by an increase of adipocyte cell size and cell number, leading to an abnormal increase of fat mass and too much fat accumulation in mesentery, epididymis and other organs. In mammals, the adipocyte number of an adult individual remains relatively constant, but adipocyte size increases under nutritional oversupply. During lifetime, adipocyte number extension happens mainly during two periods: embryogenesis and infancy, and early adolescence. Owing to the rapid increase in the prevalence of overweight and obesity worldwide and the lack of efficacy of current medical therapies, efforts to develop novel pharmacological therapies for obesity have intensified.

Adipocytes are derived from mesenchymal lineage. Differentiation of adipocytes from mesenchymal stem cells is governed by the activity of several key transcriptional regulators, including peroxisome proliferator-activated receptor γ (PPARγ) and members of the CCAAT/enhancer-binding protein (C/EBP) family.[1–4] PPARγ is essential both for adipocyte differentiation and for maintenance of mature adipocytes, and PPARγ-deficient embryonic stem cells lost the capability to differentiate into adipocytes.[5,6] Three members of C/EBP family, that is, C/EBPα, C/EBPβ and C/EBPγ, are also critical for differentiation and metabolism of adipose tissue.[7–11] In addition, sterol regulatory element-binding protein 1 (SREBP1) also has a role in adipogenesis and lipogenesis by regulating the expression of lipogenic proteins including acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1).[12] Besides the canonical factors and signaling pathways, emerging studies of miRNAs in adipocyte commitment provide new insights into understanding the molecular basis of adipogenesis. We recently reported the contributions of miR-223, miR-140 and miR-20a to adipogenesis from progenitor cells.[13–15]

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Embelin is a naturally occurring alkyl substituted hydroxy benzoquinone and a major constituent from all the parts of Embelia ribes Burm plant (Myrsinaceae), which is a medicinal plant used traditionally as anti-inflammatory agent to treat rheumatism and fever.[16] Embelin was discovered as an X-linked inhibitor of apoptosis (XIAP) inhibitor[17] and reported to possess anti-inflammatory, analgesic,[18,19] antioxidant,[20] hepatoprotective,[21] wound healing,[22] antibacterial[23] and antidiabetic[24–26] properties. Besides, Embelin also inhibits cell migration, and invasion and induces apoptosis in pancreatic, breast, prostate and lung cancer cells, and acute leukemia and multiple myeloma cells.[27–31] It can also negatively modulate cell survival pathways in cancer cells.[32] When used *in vivo*, Embelin is reported to have a LD50 as high as 2000 mg kg^{-1} body weight in rats and mice, conferring a wide margin of safety for it.[33]

Recently Chaudhari *et al.*[33] studied the preventive effect of embelin against hyperlipidemia and oxidative stress in high-fat diet (HFD)-induced obesity in rats. Twenty-one days of Embelin (50 mg kg^{-1}) treatment reduced body weight gain, blood pressure, visceral fat pad weight, serum lipid levels, as well as coronary artery risk and atherogenic indices in HFD-fed rats. Embelin treatment also improved free radical scavenging activity in hepatic tissue in the obese rats. In spite of these findings, the cellular and molecular mechanisms by which Embelin inhibit obesity remain to be explored.

The present work shows that Embelin blocks adipocyte differentiation and lipogenesis *in vitro* and the underlying mechanisms may involve canonical Wnt signaling. *In vivo* evidences show that Embelin inhibits weight gain and obesity in HFD-fed mice.

MATERIALS AND METHODS

Cells

C3H10T1/2 was obtained from ATCC (Manassas, VA, USA) and ST2 from Riken Cell Bank (Tsukuba, Japan). Mycoplasma contamination tests revealed no contamination. Cells were maintained in DMEM containing 10% FBS. Confluent cells were pretreated for 4 h with various concentrations of Embelin (1, 5, 10 μmol l^{-1}) or Vehicle (DMSO), then cultured in α-MEM containing 10% FBS or adipogenic medium (α-MEM containing 10% FBS, 0.5 μM dexamethasone, 0.25 mM methylisobutylxanthine, 5 μg ml^{-1} insulin, and 50 μM indomethacin) for 3 days. Then the cells were cultured in medium with or without 5 μg ml^{-1} insulin for 2 more days. Embelin or DMSO was supplemented when the medium was replaced.

Cell growth analysis

Cells were plated in 96-well plates at 10^4 per well and grown to 70% confluence. Then the cells were treated with Embelin for 24 h. The effect of Embelin on cell growth and viability was determined by using a CCK-8 assay kit (Dojindo Molecular Technology, Kumamoto, Japan).

Quantitative RT-PCR

The PCR primers used are listed in Supplementary Table 1. Briefly, RNA was extracted using a total RNA isolation kit (Omega Bio-Tek, Norcross, GA, USA). After reverse transcription, the cDNAs were PCR-amplified on a real-time PCR system using a SYBR green real-time PCR kit (Thermo Scientific, Rockford, IL, USA). The qRT-PCR consisted of 40 cycles (95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s) after an initial denaturation step (95 °C for 2 min). The expression levels of the target genes were normalized to that of β-actin, and measured by the comparative Ct (ΔΔCt) method.[34]

Oil-red O staining

Fully differentiated adipocytes were gently washed twice with phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde for 10 min. The samples were then washed twice with deionized water, and 60% saturated oil-red O staining was carried out for 5 min. For oil-red O quantification, 4% IGEPAL CA 630 in isopropanol was added to each well. Light absorbance was measured at 520 nm.[35]

Western blot analysis

Total proteins were extracted with RIPA lysis buffer and nuclear proteins were extracted with a nuclear protein extraction kit (Sangon Biotech, Shanghai, China). Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL, USA). Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated overnight with primary antibodies that include rabbit monoclonal antibodies by Abcam (Cambridge, MA, USA, anti-C/EBPα (ab40764), anti-β-catenin (ab32572), and anti-TCF-4 (ab76151); rabbit monoclonal antibodies by Cell Signaling Technology (Danvers, MA, USA): anti-perilipin (#9349), and anti-PPARγ (#2443); mouse monoclonal antibody by Abgent (San Diego, CA, USA): anti-PPARα (AM8425b); rabbit polyclonal antibodies by Proteintech (Wuhan, China): anti-aP2 (12802-1-AP), anti-SREBP1 (14088-1-AP), anti-FASN (10624-2-AP), anti-ACC1 (21923-1-AP), anti-Lamin B1 (12987-1-AP) and anti-β-actin (66009-1-Ig). This was then followed by incubation with the corresponding horseradish peroxide-labeled IgG (1:5000) for 1 h. Finally, chemiluminescence reagent (Advansta, Menlo Park, CA, USA) was used to visualize the results.

Immunofluorescence staining

ST2 stromal cells were seeded in a 24-well plate at a density of 15 000 cells per well. After 24 h, Embelin or DMSO was added for an additional 24 h and the cells were subsequently washed, and fixed with 4% PFA for 10 min. Cells were permeabilized with 0.2% Triton X-100 for 15 min and incubated for 30 min with 5% BSA blocking solution. The cells were incubated with β-catenin antibody (ab32572, Abcam, Cambridge, MA, USA) overnight at 4 °C and then washed with PBS three times. Cells were then incubated with Alexa Fluor 488-conjugated IgG secondary antibody (Proteintech) for 1 h, followed by incubation with 49, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St Louis, MO, USA) for 5 min. After mounting, the fluorescence signal was captured under fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

High-fat diet-induced obesity

Mice were purchased from Hua Fu Kang Biological Technology (Beijing, China) Four-week-old male healthy C57bl6/J mice with similar body weights (14–15 g) were maintained with controlled room temperature (22 ± 2 °C) and humidity (55 ± 5%) with 12:12-h light: dark cycle. Five mice were housed per cage and all the mice had free access to water. Equal quantity of food was given to each group per day. Our study was approved by the Animal Ethics Committee of Tianjin Medical University Metabolic Diseases Hospital, Tianjin, China. Sample size was estimated according to recently published literatures.[36–40] After 1-week acclimation, the mice were numbered based on body weights and then randomly assigned to one of the four groups ($n = 10$ per group): the normal-fat diet (NFD), HFD, HFD supplemented with 5 mg kg^{-1} Embelin (HFD +5 mg kg^{-1}), and HFD supplemented with 10 mg kg^{-1} Embelin (HFD +10 mg kg^{-1}). The body weights among the four groups did not show significant difference. HFD food (45% calories from fat) was purchased from Beijing Hua Fu Kang Biological Technology. To make Embelin solution, Embelin was firstly dissolved in DMSO, then diluted with corn oil. A total of 5 mg kg^{-1} or 10 mg kg^{-1} Embelin was subcutaneously injected to the treated groups every other day. Equal amount of DMSO mixed in corn oil was injected to NFD and HFD groups. The body weight was measured weekly. After 12 weeks, mice were fasted for 12 h. Blood was collected from the retro-orbital sinus. Adipose tissues (epididymal and Inguinal) were collected. No blinding was done for the animal study.

Fat composition

Fat composition was determined by using dual-energy X-ray absorptiometry (DEXA) with software Encore 2011 (Lunar Prodigy bone densitometer, General Electric Medical Systems, Milwaukee, WI, USA) as described by the manufacturer. The value is expressed as percentage of fat mass to body weight (%).

Serum biochemical assays

Serum triglyceride, glucose, free fatty acids (FFA) and total cholesterol were quantified with the enzymatic colorimetric assay kits (Jiancheng Biotechnologies, Nanjing, China) on a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA). The level of insulin was measured with an ELISA kit (RayBiotech, Norcross, GA, USA). Homeostasis model-assessment of insulin resistance (HOMA-IR) was calculated as fasting glucose level (mmol l^{-1}) × fasting insulin level (μU ml^{-1})/22.4.

Glucose tolerance test

Mice were fasted for 12 h with free access to water. For the glucose tolerance test (GTT), glucose (2 g kg^{-1}) was administered by gavage, and blood glucose was measured with the Accu-Check active (Roche Applied Science, Mannheim, Germany) at 0, 15, 30, 60 and 120 min.

Histology and hematoxylin and eosin staining

The epididymal fat pads were excised and fixed in 10% PBS-buffered formalin for 24 h. Following paraffin embedding and sectioning (5 μm), the tissues were stained with hematoxylin and eosin (H&E). To determine adipocyte size, pictures of the H&E staining were obtained using the digital microscope camera (Olympus Optical, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± s.d. Statistical analysis was performed with SPSS. Tests of homogeneity of variances were done before the one-way ANOVA. If the one-way ANOVA was significant, a *post-hoc* comparison was performed using the Student–Newman–Keuls test. All the tests are two-sided. The experiments were repeated three times. Differences are regarded as significant if the value of $P < 0.05$.

RESULTS

Embelin inhibited cell proliferation, blocked adipocyte differentiation and lipogenesis in mesenchymal C3H10T1/2 cells

As shown in Figure 1a, Embelin dose dependently inhibited cell proliferation in C3H10T1/2, with maximal inhibition of 20% at 10 μM (Figure 1a). Embelin blocked adipocyte differentiation from C3H10T1/2 in a dose-dependent manner, with maximal inhibitory effect of 85% at 10 μM in oil-red O staining compared to adipogenic treatment (Figures 1b and c). Accordingly, the mRNA levels of adipogenic transcription factors and marker genes, including PPARγ, C/EBPα, aP2 and adipsin, were decreased by Embelin in a dose-dependent manner 48 h after adipogenic treatment (Figures 1d–g). Consistent with this, the protein levels of PPARγ, C/EBPα and aP2 were significantly reduced in Embelin-treated C3H10T1/2 cells 72 h after adipogenic treatment as compared with the cells treated with adipogenic agent alone (Figure 1h).

SREBP1 is regulated in early stage of adipocyte differentiation, which can activate the critical lipogenic enzymes FASN, ACC1 and SCD1. To be specific, FASN facilitates the synthesis and cytoplasmic storage of massive amounts of triglyceride,[41] ACC1 controls the synthesis of malonyl-CoA from acetyl-CoA[42] and SCD1 regulates the biosynthesis of unsaturated fatty acids.[43]

We examined the effects of Embelin on expression levels of lipogenic and lipolytic genes in C3H10T1/2. As expected, the mRNA levels of lipogenic genes Srebp1, Fasn, Acc1 and Scd1, and lipid droplet-containing protein Perilipin were decreased by Embelin in a dose-dependent manner 48 h after adipogenic treatment (Figure 1i). As a secondary effect, the lipolytic genes examined, including peroxisome proliferator-activated receptor α (PPARα), lipoprotein lipase (LPL) and acyl-Coenzyme A oxidase 1 (Acox1), were also decreased after Embelin treatment (Figure 1j). Consistently, the protein levels of SREBP1, FASN, ACC1 and Perilipin were reduced in Embelin-treated cells 72 h after adipogenic treatment (Figure 1k). These suggest that Embelin decreases lipid accumulation mainly through inhibiting lipogenesis.

Embelin inhibited cell proliferation, blocked adipocyte differentiation and lipogenesis in stromal ST2 cells

As shown in Figure 2a, Embelin dose dependently inhibited cell proliferation in stromal ST2 cells, with maximal inhibition of 28% at 10 μM. We further demonstrated that Embelin also dramatically inhibited adipogenic differentiation from ST2 (Figures 2b and c). Consistently, the mRNA levels of PPARγ, C/EBPα, aP2 and adipsin were decreased, respectively, 48 h after adipogenic treatment (Figures 2d–g). Moreover, Embelin also substantially decreased the protein levels of PPARγ, C/EBPα and aP2 72 h after adipogenic treatment as compared with the adipogenic treatment alone (Figure 2h).

Embelin also affected expression levels of lipogenic and lipolytic factors in ST2. As expected, Embelin dose dependently decreased the mRNA levels of all the lipogenic and lipolytic genes examined 48 h after adipogenic treatment (Figures 2i and j). Consistently, Embelin decreased the protein levels of SREBP1, FASN, ACC1 and Perilipin at 72 h versus adipogenic treatment alone (Figure 2k).

Embelin acted as an activator of canonical Wnt signaling

Immunofluorescence staining studies showed that Embelin promoted the translocation of β-catenin from the cytoplasm into the nucleus in C3H10T1/2 cells (Figure 3a), suggesting that Embelin may activate the canonical Wnt signaling. Consistent to this, western blotting showed that the nuclear protein levels of β-catenin and TCF-4 were increased after Embelin treatment versus adipogenic treatment alone (Figure 3b). To further identify the upstream molecules through which Embelin affects β-catenin and TCF-4 activity, we performed qRT-PCR to detect the expression levels of several factors. Secreted frizzled-related protein 1 (Sfrp1) and dickkopf-1 (Dkk1), two soluble inhibitors of Wnt signaling, were increased, whereas low-density lipoprotein receptor-related protein 5 (Lrp5) and Wnt10b were decreased after adipogenic treatment versus vehicle treatment. However, Dkk1 was the only one downregulated by Embelin as compared with the adipogenic treatment alone. By contrast, Embelin does not affect the expression of Sfrp1, Lrp5 and Wnt10b (Figure 3c). This suggests that Embelin activates Wnt pathway by repressing Dkk1 expression.

We then carried out Dkk1 gain-of-function studies and investigated if Dkk1 overexpression altered regulation of adipogenesis and lipogenesis by Embelin. Transfection of the Dkk1 expression plasmid in C3H10T1/2 substantially increased Dkk1 mRNA, suggesting that the construct works well (Figure 3d). Upon adipogenic treatment, Dkk1 overexpression promoted adipocyte differentiation and lipogenesis and increased mRNA expression levels of adipogenic factors and lipogenic factors (Figures 3e–h). Transfection of the Dkk1 construct along with Embelin treatment attenuated Embelin inhibition of adipogenesis and lipogenesis program, and reversed inhibition of adipogenic and lipogenic factors by Embelin (Figures 3e–h).

Embelin treatment prevented body weight gain and obesity in mice

As mentioned above, Embelin blocked adipocyte differentiation *in vitro* and the mechanisms might involve canonical Wnt signaling. We then explored if Embelin affects adipose tissue accrual *in vivo*. A significant increase in body weight gain was observed in the HFD group as compared with the NFD group at all the indicated time points (4 weeks to 12 weeks, Figure 4a). By 12 weeks, the body weight of HFD group was 1.46-fold of NFD-fed mice (Figure 4b). However, treatment with either 5 mg kg^{-1} or 10 mg kg^{-1} of Embelin markedly attenuated the gain of body weight at all the indicated time points (Figure 4a). By 12 weeks, the body weight in Embelin-treated groups was significantly reduced as compared to HFD group, but unchanged versus NFD group (Figure 4b).

We further measured body fat composition in the mice using bone densitometer. Consistent to the change in body weight, by 12 weeks, the fat composition of HFD group increased dramatically, being 78% greater than NFD group. After treatment with Embelin, the fat composition in Embelin-treated groups showed substantial decrease as compared to HFD group, whereas did not show significant change versus NFD group (Figure 4c). Consistently, the Inguinal fat weight in the Embelin-treated groups was

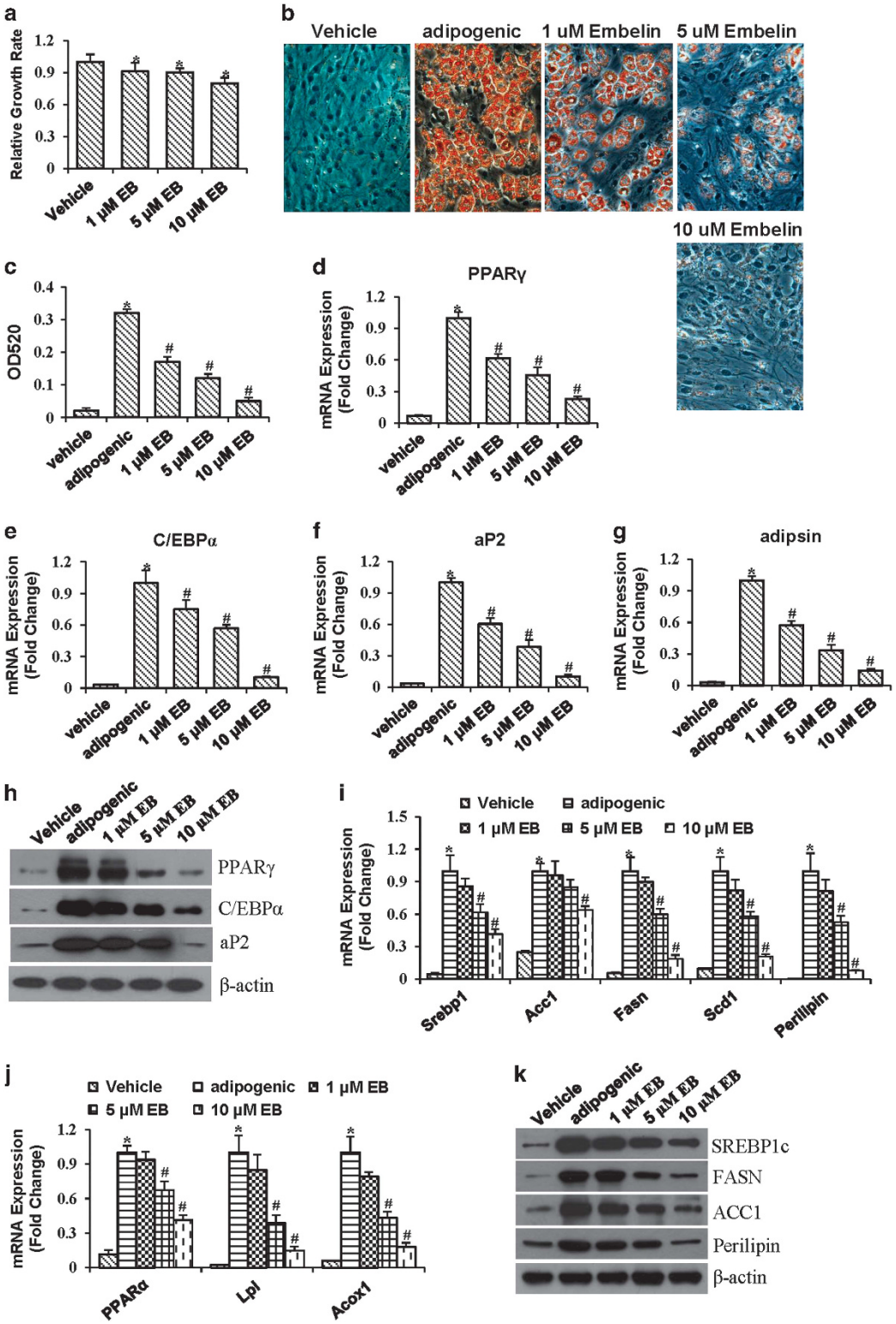

Figure 1. Embelin inhibited cell proliferation, adipogenic differentiation and lipogenesis in C3H10T1/2. Embelin inhibited proliferation of C3H10T1/2 (**a**), $n=8$. Embelin dose dependently inhibited adipocyte differentiation (**b**, **c**). Embelin inhibited the mRNA levels of PPARγ, C/EBPα, aP2 and adipsin, $n=3$ (**d**–**g**). Embelin inhibited the protein levels of PPARγ, C/EBPα and aP2 (**h**). Embelin inhibited the mRNA levels of lipogenic genes Srebp1, Fasn, Acc1 and Scd1, lipid droplet-containing protein perilipin (**i**), and lipolytic genes PPARα, LPL and Acox1 (**j**). Embelin inhibited the protein levels of SREBP1, FASN, ACC1 and Perilipin (**k**). Image magnification in **b**: ×200. Values are mean ± s.d. *Significant versus vehicle, $P<0.05$, #Significant versus adipogenic treatment, $P<0.05$. The values of adipogenic treatment are set as 1 (**c**–**g**, **i**, **j**).

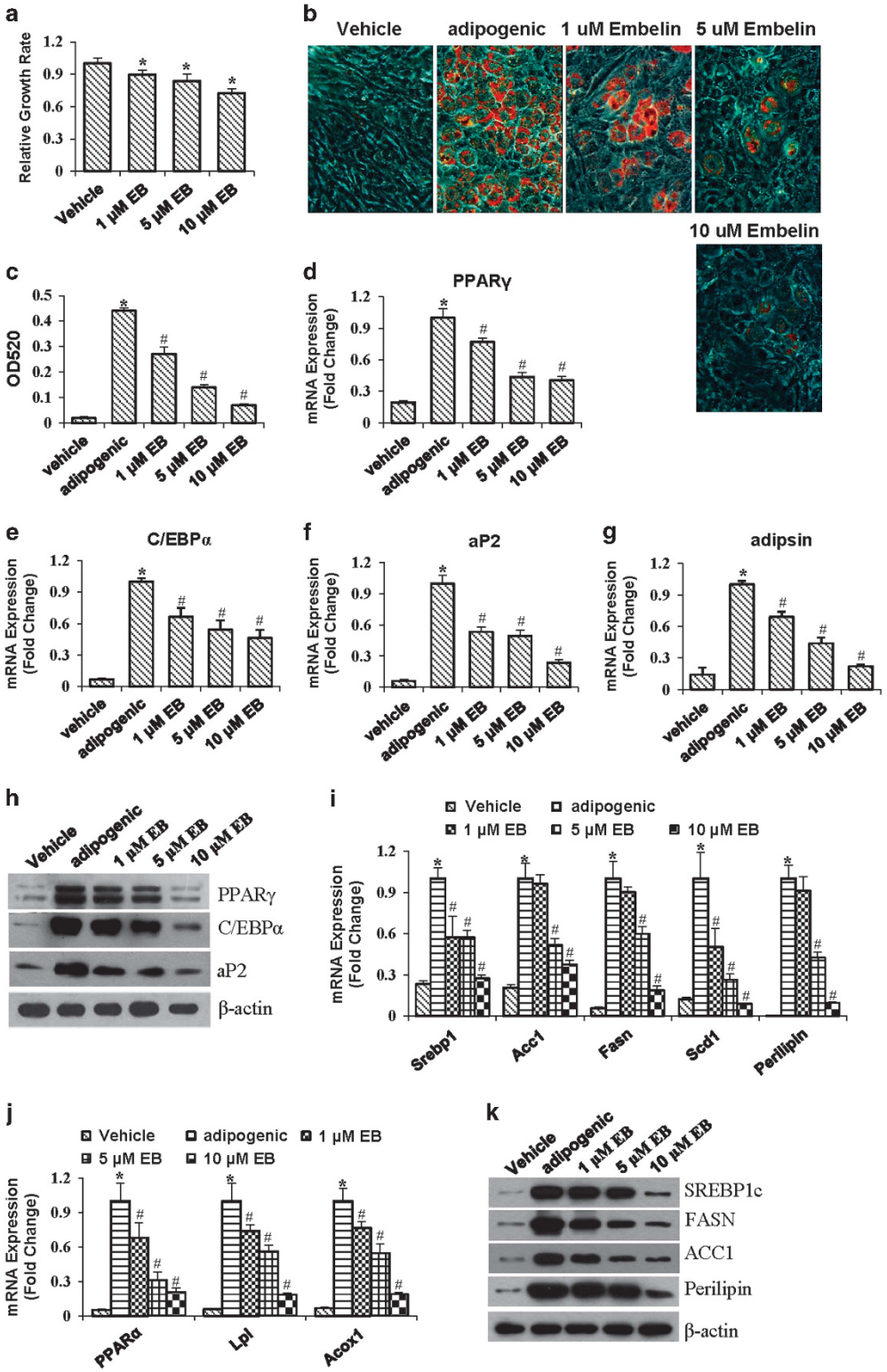

Figure 2. Embelin inhibited cell proliferation, adipogenic differentiation and lipogenesis in ST2. Embelin inhibited proliferation of ST2 (**a**), $n=8$. Embelin dose dependently inhibited adipocyte differentiation (**b**, **c**). Embelin inhibited the mRNA levels of PPARγ, C/EBPα, aP2 and adipsin, $n=3$ (**d**–**g**). Embelin inhibited the protein levels of PPARγ, C/EBPα and aP2 (**h**). Embelin inhibited the mRNA levels of lipogenic genes Srebp1, Fasn, Acc1 and Scd1, lipid droplet-containing protein perilipin (**i**), and lipolytic genes PPARα, LPL and Acox1 (**j**). Embelin inhibited the protein levels of SREBP1, FASN, ACC1 and Perilipin (**k**). Image magnification in **b**: ×200. Values are mean ± s.d. *Significant versus vehicle, $P<0.05$, #Significant versus adipogenic treatment, $P<0.05$. The values of adipogenic treatment are set as 1 (**c**–**g**, **i**, **j**).

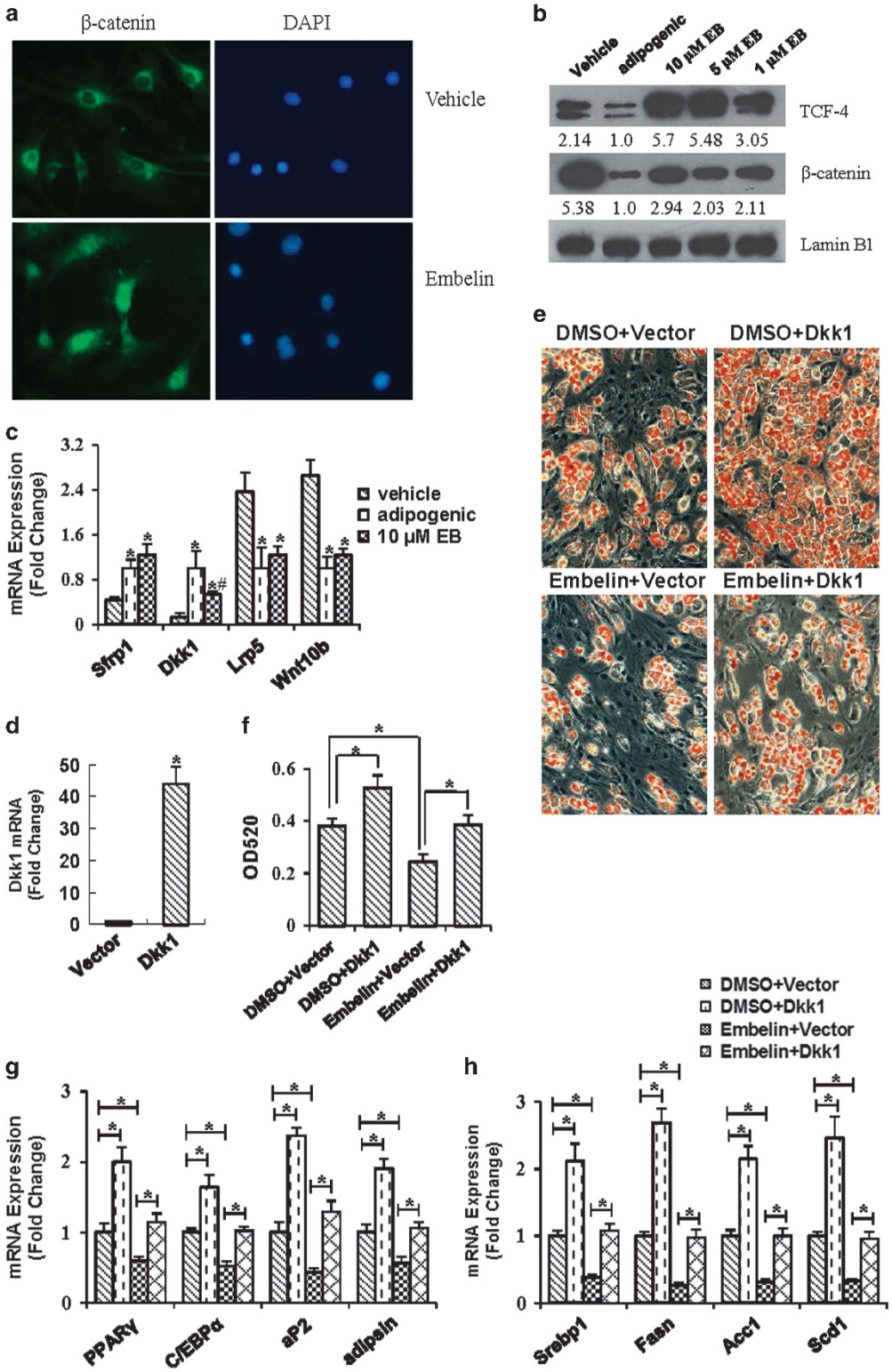

Figure 3. Embelin activated canonical Wnt signaling. Immunofluorescence staining revealed increased nuclear translocation of β-catenin by Embelin in C3H10T1/2 (**a**). Western blotting revealed increase of nuclear protein levels of β-catenin and TCF-4 by Embelin (**b**). Expression levels of several components of canonical Wnt signaling after Embelin treatment were examined by using qRT-PCR, $n = 3$ (**c**). Transfection of the Dkk1 expression plasmid substantially increased Dkk1 mRNA (**d**). Overexpression of Dkk1 along with Embelin treatment attenuated Embelin inhibition of adipocyte formation (**e**, **f**) and related genes expression, $n = 3$ (**g**, **h**). Values are mean ± s.d. *Significant versus vehicle or vector, $P < 0.05$, #Significant versus adipogenic treatment, $P < 0.05$.

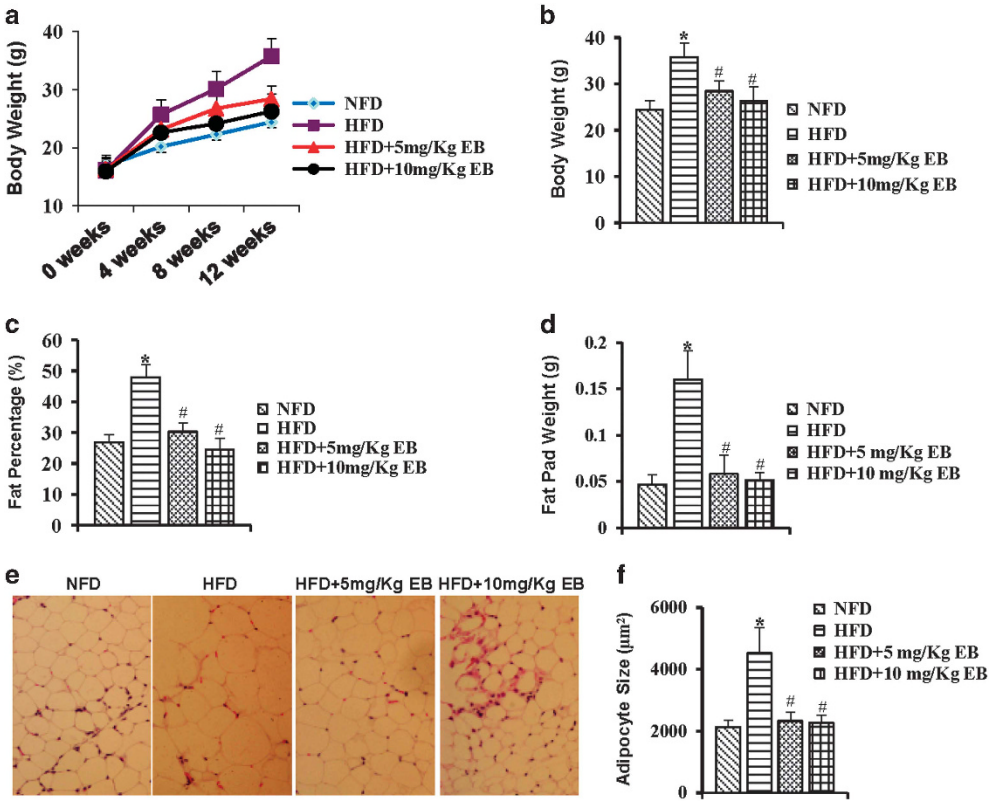

Figure 4. Embelin treatment prevented obesity in mice. HFD increased body weight, and treatment with Embelin attenuated body weight gain at all the indicated time points (**a**, **b**). Embelin treatment reduced fat composition in HFD-fed mice revealed by using DEXA ($n = 8$) (**c**). Embelin treatment decreased inguinal fat weight in HFD-fed mice ($n = 8$) (**d**). HE staining revealed enlarged size of adipocytes in HFD group versus NFD group, whereas Embelin attenuated the enlargement of adipocyte size (**e**, **f**). *Significant versus NFD, $P < 0.05$, #Significant versus HFD, $P < 0.05$.

substantially decreased as compared to HFD group (9 weeks after treatment; Figure 4d).

Consistently, HE staining of the Paraffin sections showed that the size of adipocytes in HFD group was enlarged versus that in NFD-fed mice. After 12 weeks of treatment with either 5 mg kg^{-1} or 10 mg kg^{-1} of Embelin, the enlarged adipocyte cell size was greatly reduced as compared to HFD group (Figures 4e and f). These suggest that the lipid accumulation was largely inhibited in the Embelin-treated mice.

Disturbed serum lipid and glucose were improved in HFD-fed mice after embelin treatment

As shown in Figure 5, the levels of triglyceride, glucose, total cholesterol and FFA in the HFD group mice were significantly higher than in the NFD group (Figures 5a–d). After 12 weeks of treatment with either 5 mg kg^{-1} or 10 mg kg^{-1} of Embelin, the increased levels of triglyceride, glucose, total cholesterol and FFA were brought down significantly. Treatment with 10 mg kg^{-1} Embelin reduced serum triglyceride by 55%, glucose by 47%, total cholesterol by 50% and FFA by 39% versus HFD group mice.

It was reported that Embelin might improve glucose tolerance in HFD-fed rats and in diabetic rats.[26,33] In our study, serum insulin level was increased in HFD group mice, while it was significantly reduced in the mice treated for 9 weeks with Embelin (Figure 5e). The impaired glucose tolerance in HFD-fed mice was also substantially improved after treatment with either 5 mg kg^{-1} or 10 mg kg^{-1} of Embelin (Figure 5f). The higher level of HOMA-IR in the HFD-fed mice was also substantially decreased in the Embelin-treated mice (Figure 5g). These suggest that Embelin could improve insulin sensitivity and glucose homeostasis in HFD-fed mice.

Embelin treatment inhibited adipogenic and lipogenic factors in mice

As shown in Figure 6, the mRNA level of Dkk1 was increased in epididymal adipose tissue in the HFD group as compared to NFD-fed mice, whereas Embelin treatment largely attenuated the induction of Dkk1 (Figure 6a). Moreover, the mRNA levels of PPARγ, C/EBPα and aP2 were increased in adipose tissue in HFD group versus NFD group. After treatment with either 5 mg kg^{-1} or 10 mg kg^{-1} of Embelin, the mRNA levels of PPARγ, C/EBPα and aP2 were substantially decreased (Figures 6b–d).

We also detected the expression levels of the lipogenic factors in epididymal adipose tissue. As expected, the mRNA levels of Srebp1, Fasn and Scd1 were increased in HFD group mice versus NFD group, whereas they were significantly reduced after Embelin treatment (Figures 6e–g).

DISCUSSION

Embelin is a potent, nonpeptidic, and cell-permeable small molecular XIAP inhibitor that targets the third baculoviral IAP repeat domain (BIR3) of XIAP.[17] XIAP is the most potent member of the inhibitors of apoptosis proteins (IAP) gene family. XIAP binds to and inhibits caspase 3, 7 and 9 and therefore suppresses various agent-induced cancer cell apoptosis.[44] As an inhibitor of XIAP activity, Embelin induces cell growth inhibition and apoptosis in different human cancers,[27–31] showing the potential as an antitumor agent.

In the current study, we investigated whether Embelin has any effect on obesity. We first performed *in vitro* study and tested the effects of Embelin on cell proliferation of adipogenic progenitor cells, and on adipogenic differentiation and lipogenesis. Our data

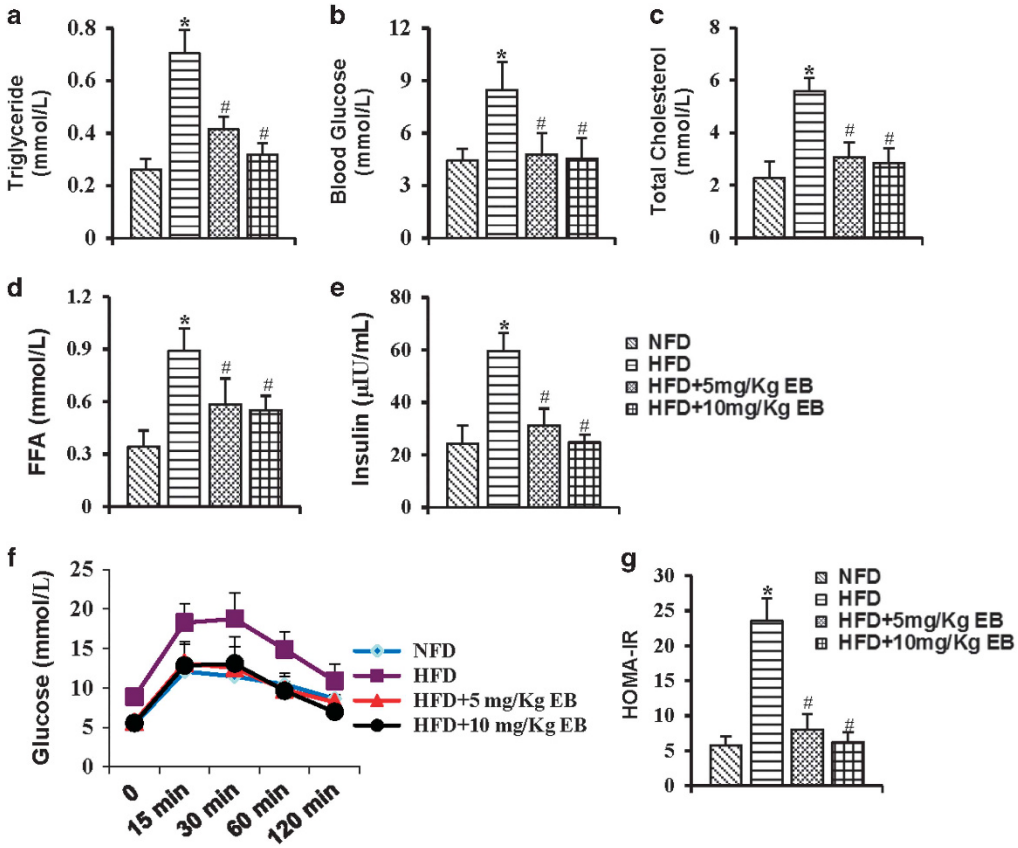

Figure 5. Embelin treatment improved glucose and lipid in HFD-fed mice. Embelin treatment attenuated the increase of triglyceride (**a**), glucose (**b**), total cholesterol (**c**) and FFA (**d**) in HFD-fed mice, $n = 10$ (**a**–**d**). Embelin treatment reversed the increase of fasting insulin, impairment of glucose tolerance and increase of HOMA-IR, $n = 7$ (**e**–**g**). *Significant versus NFD, $P < 0.05$, #Significant versus HFD, $P < 0.05$.

showed that treatment of undifferentiated adipogenic progenitors ST2 and C3H10T1/2 with Embelin attenuated cell proliferation, blocked the formation of adipocytes in a dose-dependent manner. Embelin substantially inhibited the expression levels of adipogenic and lipogenic factors.

Embelin can inhibit the activation of canonical Wnt signaling in tumor cell, which may at least partially mediate its inhibitory effect on tumor cell growth. In prostate cancer cells, Embelin activated glycogen synthase kinase (GSK)-3β by preventing phosphorylation and suppressed β-catenin expression, which subsequently attenuated TCF transcriptional activity and gene transcription of downstream target genes such as cyclin D1, c-myc and matrix metalloproteinase (MMP)-7.[28] However, in non-tumor cells, Embelin is able to activate canonical Wnt signaling. Xue *et al.*[45] reported that Embelin suppressed dendritic cell functions and limited autoimmune encephalomyelitis partially through activating the β-catenin signaling pathway. On the other hand, canonical Wnt signaling was long believed to be a major inhibitor of adipogenesis, we therefore reasoned that Embelin might activate canonical Wnt signaling in the adipogenic progenitors. Therefore, in the current study, we investigated if the anti-adipogenic activity of Embelin in adipogenic progenitors involves canonical Wnt signaling. Immunofluorescence staining studies showed that Embelin treatment promoted the nuclear translocation of β-catenin from the cytoplasm. Consistently, western blotting showed that the nuclear protein levels of β-catenin and TCF-4 were upregulated after Embelin treatment. To further identify the upstream molecules through which Embelin affects β-catenin and TCF-4 activity, we detected the expression levels of several factors. Embelin significantly suppressed Dkk1 expression, whereas it had no effect on the expression of Sfrp1, Lrp5 and Wnt10b. This suggests that Embelin may activate the Wnt pathway by repressing the expression of Dkk1.

Adipocyte hyperplasia is grossly divided into two stages of mesenchymal stem cells: commitment to preadipocytes and preadipocytes differentiation to adipocytes. Both canonical and noncanonical Wnt inhibit adipogenesis in both stages through deacetylation of PPARγ and C/EBPα promoter and blocking of their expressions.[46,47] As a Wnt antagonist, Dkk1 has the capability to regulate adipogenesis.[48,49] To clarify if Dkk1 is involved in the regulation of adipogenesis and lipogenesis by Embelin, we carried out Dkk1 gain-of-function studies along with Embelin treatment. Dkk1 overexpression reversed the inhibition of adipocyte formation and expression of adipogenic and lipogenic factors by Embelin treatment. These data suggest that activated canonical Wnt signaling may be involved in the inhibition of adipogenesis and lipogenesis by Embelin, although our study so far cannot rule out the possibility that other signaling pathways or mechanisms may contribute to the effect of Embelin as well.

We then explored if Embelin has any effect on obesity *in vivo*. Mice fed a high-fat diet gained much more weight and fat than those fed a normal diet. However, Embelin treatment substantially reduced the gain of body weight and fat along with decreased adipocyte size in HFD-fed mice, suggesting that Embelin is effective in preventing obesity. In addition, triglyceride and total cholesterol levels were all decreased, suggesting Embelin to be a potential natural compound for the treatment of obesity and disturbed lipid profiles.

Obesity is associated with an impaired ability of tissue to respond to insulin and effectively store and utilize glucose. This

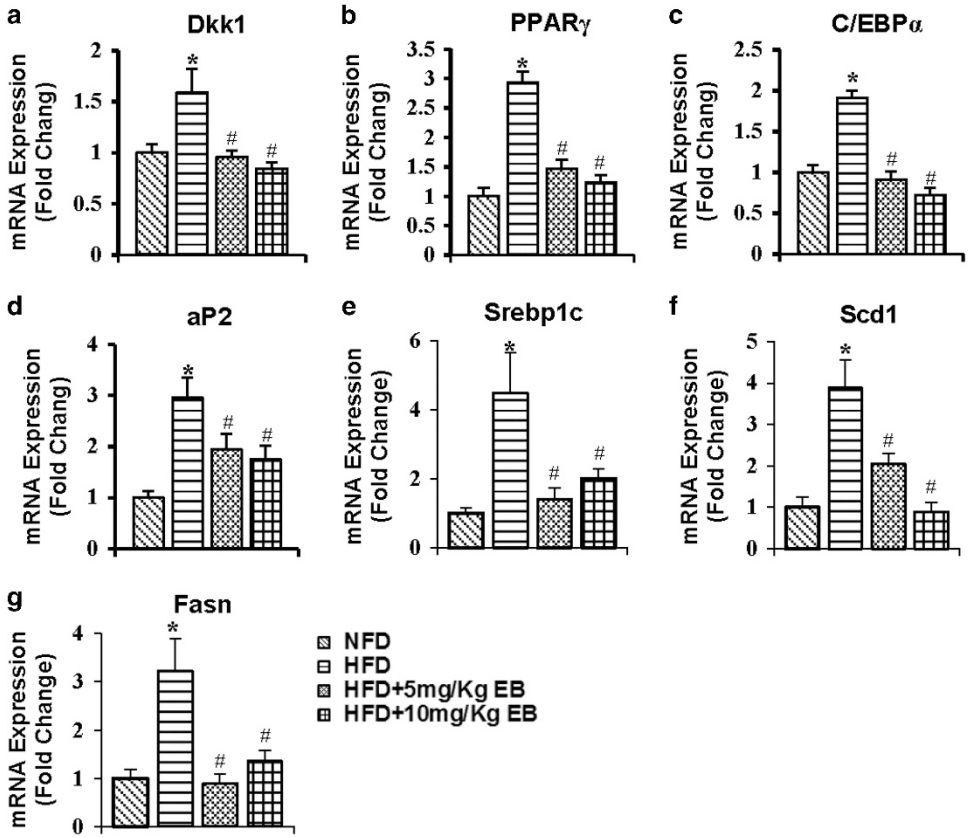

Figure 6. Embelin treatment inhibited adipogenic and lipogenic factors in mice. Embelin treatment attenuated the induction of Dkk1 mRNA level in epididymal adipose tissue of HFD-fed mice, $n = 8$ (**a**). Embelin treatment reversed the increase in the mRNA levels of adipogenic factors in epididymal adipose tissue of HFD-fed mice, $n = 8$ (**b**–**d**). Embelin treatment also attenuated the increase in the levels of lipogenic factors, $n = 3$ (**e**–**g**). *Significant versus NFD, $P < 0.05$, #Significant versus HFD, $P < 0.05$. The values of NFD are set as 1.

leads to the development of insulin resistance, hyperinsulinemia, hyperglycemia, and ultimately type 2 diabetes. It was reported that Embelin might improve glucose tolerance in HFD-fed rats and in diabetic rats.[26,33] Consistently, in our study, a significant decrease in the serum glucose and insulin levels and the calculated HOMA-IR values were observed after treatment with Embelin in HFD-fed mice. Moreover, glucose tolerance was improved. Although it is not clear if the amelioration of serum lipids and glucose levels were due to Embelin treatment per se or secondary to the adiposity of the mice, the results indicate that Embelin can improve obesity-related metabolic dysfunctions and insulin sensitivity in obese mice.

We further explored the molecular basis underlying the anti-obesity effect of Embelin. The expression levels of both adipogenic and lipogenic factors were increased in the adipose tissue of the HFD group mice, but were substantially reduced after treatment with Embelin. Furthermore, Embelin treatment attenuated the induction of Dkk1 in adipose tissue in HFD-fed mice, which was consistent to our *in vitro* findings. Combined with the observations that Embelin treatment prevented the faster gain of body weight and fat mass, and ameliorated dyslipidemia and impaired glucose tolerance and insulin sensitivity, the data demonstrated that Embelin effectively prevents diet-related lipid accumulation. The data also suggests that downregulation of Dkk1 and subsequent activation of canonical Wnt signaling might be involved in the anti-obesity effect of Embelin.

In summary, the present work provides evidences that Embelin is effective in inhibiting adipogenesis and lipogenesis *in vitro* and the effect is possibly based on the activation of canonical Wnt signaling. Embelin is also effective in preventing body weight gain and fat accumulation, improving dyslipidemia and obesity-related impairment in glucose tolerance and insulin sensitivity in the HFD-fed mice. The data suggest that Embelin may have excellent pharmacological potential to prevent obesity and disturbed lipid profiles.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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