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The Bile Acid Induced Hepatokine Orosomucoid Suppresses Adipocyte Differentiation

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Abstract

Bile acids have recently emerged as key metabolic hormones with beneficial impacts in multiple metabolic diseases. We previously discovered that hepatic bile acid overload distally modulates glucose and fatty acid metabolism in adipose tissues to exert anti-obesity effects. However, the detailed mechanisms that explain the salutary effects of serum bile acid elevation remain unclear. Here, proteomic profiling identified a new hepatokine, Orosomucoid (ORM) that governs liver-adipose tissue crosstalk. Hepatic ORMs were highly induced by both genetic and dietary bile acid overload. To address the direct metabolic effects of ORM, purified ORM proteins were administered during adipogenic differentiation of 3T3-L1 cells and mouse stromal vascular fibroblasts. ORM suppressed adipocyte differentiation and strongly inhibited gene expression of adipogenic transcription factors such as C/EBPβ, KLF5, C/EBPα, and PPARγ. Taken together, our data clearly suggest that bile acid-induced ORM secretion from the liver blocks adipocyte differentiation, potentially linked to anti-obesity effect of bile acids.

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Keywords

bile acid; hepatokine; orosomucoid; adipogenesis; lipogenesis; obesity

INTRODUCTION

The increasing incidence of obesity accelerates the prevalence of type 2 diabetes mellitus, fatty liver disease, and cardiovascular disease [1,2]. The liver is one of the largest internal organs in the human body and plays a central role in energy metabolism. Recent studies revealed that liver-derived bile acids are not only natural surfactants emulsifying dietary lipids, but also important metabolic regulators of glucose and lipid homeostasis [3]. Pharmacological modulation of bile acid signaling pathways generally exhibits metabolic benefits in obesity and diabetes [4,5]. Dietary supplementation with bile acids slows body weight gain in mice and improves metabolic parameters [6]. In addition, we showed that hepatic bile acid accumulation in liver-specific farnesoid X receptor (FXR) and small heterodimer partner (SHP) double knockout (L-DKO) mice is coupled with systemic metabolic improvements in aging-induced obesity. These observations suggest bile acid-induced inter-organ crosstalk prevent obesity and insulin resistance [7].

The liver is an important secretory organ that synthesizes and secretes the majority of the serum proteome including carrier proteins, apolipoproteins, coagulation factors, as well as hormones [8]. To this end, many liver-secreted hormones (*i.e.*, hepatokines) such as fetuin-A/B, retinol-binding protein 4, and selenoprotein P, fibroblast growth factor 21 (FGF21), Tsukushi, and apolipoprotein J link hepatic signaling to systemic metabolic regulation [9,10,11,12]. Among them, FGF21 is known to be associated with hepatic bile acid signaling, which improves lipid and carbohydrate metabolism. FGF21 is induced mainly by peroxisome proliferator-activated receptor alpha (PPARα) [13] and by other nuclear receptors like FXR [14], that respond to hepatic bile acids. However, since hepatic *Fgf21* expression in aged L-DKO mice is not altered [7], it cannot explain the metabolic benefit of L-DKO mice and likely requires additional factors that mediates bile acid effects.

In this study, we profiled the serum proteome in global FXR/SHP double knockout (DKO) and L-DKO mice to identify novel mediators of hepatic bile acid signaling. We discovered Orosomucoid (ORM) was abundantly expressed and highly induced by hepatic bile acids in the liver. We find that ORM inhibits adipocyte differentiation through suppression of critical adipogenic transcription factors. Taken together, our studies reveal bile acid regulation of the hepatokine ORM exerts anti-adipogenic activity during adipocyte differentiation. These findings are an important advance in the understanding of how bile acid acids can signal via liver-secreted hormones to coordinate adipocyte function.

MATERIALS AND METHODS

Animal Study

Global FXR/SHP double knockout (DKO) and liver-specific FXR/SHP double knockout (L-DKO) mice were previously described [7]. C57BL/6J WT (BCM Center for Comparative

Medicine) and littermate control mice having no *Alb-cre* transgene were used as controls for DKO and L-DKO, respectively. Mice were maintained on a normal chow (NC) diet (TD.00217, ENVIGO). Dietary bile acid overload mice were generated by feeding 0.5% cholic acid (CA) diet for 1 day (TD.110811, ENVIGO). All mice were humanely euthanized by isoflurane inhalation followed by cervical dislocation. All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine.

Cell Culture and Adipocyte Differentiation

Orosomucoid protein isolated from bovine plasma (G3643, Sigma Aldrich) was used for *in vitro* experiments. 3T3-L1 mouse preadipocytes (SP-L1-F, Zen-Bio) were maintained in DMEM (10–013-CV, Corning) supplemented with 10% bovine calf serum (BCS;16777–206, HyClone) and penicillin-streptomycin (30–002-CI, Corning). Post-confluent 3T3-L1 cells were induced by adding differentiation medium (MDI) containing DMEM/F12 (10–090-CV, Corning), 10% fetal bovine serum (FBS; 35011-CV, Corning), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; I5879, Sigma-Aldrich), 1 μM dexamethasone (D1756, Sigma-Aldrich), 5 μg/mL insulin (I9278, Sigma-Aldrich) and 0.5 μM rosiglitazone (71740, Cayman Chemical). Three days post-differentiation, medium was replaced with DMEM/F12, 10% FBS, and 5 μg/mL insulin for 2 days and maintained in DMEM/F12, 10% FBS thereafter.

SVF preadipocyte differentiation

The stromal vascular fraction (SVF) was isolated from inguinal white adipose tissue of 8-week-old male mice. Fat pads were digested with Collagenase Type I (17100–017, Gibco) in a 37°C water bath for 1h. After digestion, cells were centrifuged at 700g for 5 min and supernatant was discarded. The cell pellets were resuspended with DMEM/F12 containing 10% FBS and penicillin/streptomycin and passed through 100 μm cell strainer to remove debris. Centrifugation was repeated to wash the pellet and then cells were plated in 24-well plates. Once 80% confluence was reached, the cells were induced to differentiate by adding MDI and maintained as described for 3T3-L1 cells.

Oil Red O staining

Cells were washed with 1X Phosphate-Buffered Saline (PBS; 21040-CV, Corning) once and then fixed with 10% neutral buffered formalin (89370–094, VWR) for 20 min at room temperature. After washing with 1X PBS, fixed cells were pre-incubated with 100% propylene glycol (P4347, Sigma-Aldrich) for 5 min and then stained with Oil Red O solution (I1516, Sigma-Aldrich) for 15 min. Then, cells were washed with 85% propylene glycol once and followed with 1X PBS twice. Cell images were taken using an inverted microscope. To quantify the amount of accumulated lipids, Oil Red O was extracted from the stained cells using 250 μl of 100% isopropanol and two aliquots of 100 μl were transferred to a 96-well assay plate (9017, Corning). The OD values were measured at 520 nm using a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific).

Western Blotting

Whole-cell lysates were prepared using RIPA buffer (20mM Tris-HCL pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS) supplemented with Xpert Protease Inhibitor Cocktail (P3100, GenDEPOT) and Phosphatase Inhibitor Cocktail (P3200, GenDEPOT) for 30 min at 4°C followed by centrifugation at 14,000 rpm for 10 min. Supernatants were denatured in 2x Laemmli Sample buffer (1610737, Bio-Rad Laboratories) at 95°C for 5 min and loaded in 12% Mini-PROTEAN TGX Precast Protein Gels (4561046, Bio-Rad Laboratories). Proteins were transferred to PVDF membrane (162–0177, Bio-Rad Laboratories) by electrophoresis for 30 min at 100V. Primary antibody was incubated overnight at 4°C and followed by secondary anti-rabbit IgG HRP-conjugated antibody (7074, Cell Signaling) incubation for 1h. Immunoreactive bands were visualized by SuperSignal West Pico or Femto (34087 or 34095, Thermo Scientific) reagents using AZURE c600 IMAGING SYSTEM (Azure Biosystems). Detailed information regarding antibodies can be found in Supplementary Table 2.

Real-time Quantitative PCR

Total RNA was isolated by PureXtract RNAsol reagent (R6101, GenDEPOT) and converted into complementary DNA by the qScript Reverse Transcriptase Kit (95047, Quanta Bio). Relative mRNA expression was measured by quantitative PCR using the Light Cycler 480 Real-Time PCR System (Roche) with KAPA SYBR FAST qPCR Master Mix (2X) (KK4611, KAPA Biosystems) and quantified by the comparative cycle threshold method ($\Delta\Delta$Ct method) with normalization to β-actin. Primer information is listed in Supplementary Table 1.

Serum Secretome Analysis

Mouse serum was pre-cleared by Proteome Purify 2 Mouse Serum Immunodepletion resin (MIDR002, RnD Systems) to remove albumin and immunoglobulins. Depleted serum was subjected to Mass Spectrometry-based whole proteome analysis [15,16]. Briefly, trypsinized serum peptides were extracted by 50% acetonitrile/0.1% formic acid and 80% acetonitrile/ 0.1% formic acid solution. The proteome was profiled by nano-liquid chromatography-tandem mass spectrometry analysis with a nano-LC1000 coupled to a Thermo Q-Exactive (Thermo Fisher Scientific). The relative amount was normalized as the intensity-based absolute quantification algorithm and normalized to the intensity-based fraction of the total (iFOT).

Statistical Analysis

All data are presented as mean ± SEM. Independent *t*-test was used for comparisons between two groups, otherwise a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used. Statistical analysis was performed using Prism 8 software (GraphPad Software). A probability value of $p < 0.05$ was considered statistically significant, unless otherwise indicated.

RESULTS

Bile Acid Overload induces Hepatokine ORM Expression.

Liver-specific farnesoid X receptor (FXR) and small heterodimer partner (SHP) double knockout (L-DKO) in mice results in hepatic bile acid accumulation that is associated with systemic metabolic improvements in aging-induced obesity [7]. To screen for novel hepatokines that mediate the systemic metabolic effects during bile acid accumulation, we performed serum proteomics in DKO and L-DKO mice. We identified 84 and 25 serum proteins that were increased in DKO and L-DKO, respectively (Fig. 1A). Overlap of the elevated proteins revealed 15 proteins in common between DKO and L-DKO (Fig. 1B), consisting of many known liver-secreted proteins (Fig. 1C). Among them, ORM1 and ORM2 were the most abundant in both datasets (Fig. 1D). ORM, also known as alpha1-acid glycoprotein, is an acute phase protein found in blood [17]. While serum ORM is exclusively synthesized by the liver, other tissues such as heart, stomach, lung, and adipose tissue secrete ORM locally [17,18]. ORM was abundant in serum from DKO mice compared to control (Supplementary Fig. S1), consistent with the liver proteomics data.

To confirm the origin of serum ORM during bile acid accumulation, we quantified mRNA expression in the liver in genetic and dietary bile acid overload. In DKO, hepatic expression of all *Orm* isoforms were induced (Fig. 2A), whereas L-DKO markedly increased the *Orm2* isoform. (Fig. 2B). Notably, these changes were also consistent in the dietary bile acid overload induced by 0.5% cholic acid diet feeding (Fig. 2C). Next, we analyzed *Orm* expression in adipose tissues, which express low levels of *Orm1* [18]. In control mice, *Orm1* and *Orm2* were highly enriched in the liver compared with white and brown adipose tissues (Fig. 2D). Importantly, bile acid accumulation in L-DKO mice induced *Orm2* expression in the liver but had no effect in adipose tissues. Of note, although *Orm3* was increased in the liver and adipose tissues, the absolute level of *Orm3* was extremely low compared to *Orm1* and *Orm2* [19] and was undetectable in our serum proteome data. Therefore, these results suggest hepatic bile acid accumulation induces liver-specific expression of ORMs, which might contribute to metabolic functions in other tissues.

Hepatokine ORM Directly Inhibits Adipogenesis.

Bile acids exert strong anti-obesity effects [6,20]. To investigate whether ORM mediates these effects through modification of adipocyte formation, we exposed preadipocytes to ORM. We utilized 3T3-L1 preadipocytes, the best characterized *in vitro* model of adipocyte differentiation [21], and treated them with differentiating medium (MDI) in the absence or in the presence of purified ORMs at various concentrations. Oil Red O staining demonstrated decreased lipid accumulation with ORM treatment in a dose-dependent manner, indicating reduced adipogenesis (Fig. 3A). Quantification of intracellular lipid droplets confirmed that ORM treatment dramatically reduced the adipogenic potential of 3T3-L1 cells (Fig. 3B). Furthermore, the anti-adipogenic activity of ORM protein was validated in mouse SVF preadipocytes from inguinal white adipose tissue (Figs. 3C and 3D). Taken together, these results demonstrate that ORM treatment suppresses adipocyte differentiation.

Adipogenesis consists of 2 phases, an initial commitment step of preadipocytes (day 0 to day 2) and the subsequent differentiation (day 2 to day 7), which are controlled by temporal expression of multiple transcription factors [22,23]. To test which phase is impacted by ORM, we added purified ORM protein to the differentiation media at various time points as indicated in Supplementary Fig. S2A. Interestingly, ORM suppressed lipid accumulation when administered early in adipogenesis (Supplementary Fig. S2B and C). However, ORM treatment after Day 3 did not inhibit adipogenesis or lipid accumulation. These results suggest that ORM primarily impairs the early commitment steps of adipocyte differentiation.

ORM Coordinately Regulates Adipogenic Transcription Factors.

Adipocyte differentiation is regulated by timely activation and inhibition of multiple transcription factors, including activation of CCAAT/enhancer binding protein beta and delta (C/EBPβ and C/EBPδ) (3) and Kruppel-like factor 4 and 5 (KLF4 and KLF5) in the early phase (day 1 and 2) [24,25], and two master adipocyte transcription factors, C/EBPα and PPARγ to accelerate adipocyte differentiation in the late stage (day 2 to 7) [26]. Conversely, other transcription factors including KLF2, Pref-1, and GATA2/3 inhibit adipogenesis [27,28,29]. To test the impact of ORM on expression of early adipogenic transcription factors, we treated 3T3-L1 preadipocytes with purified ORM during MDI differentiation medium for 2 days. Intriguingly, ORM specifically inhibited the positive regulators *Cebpb* and *Klf5* by day 2 of differentiation (Fig. 4A), although it did not affect anti-adipogenic transcription factors at this time (Fig. 4B). We further examined ORM effects on the two master regulators of adipogenesis, C/EBPα and PPARγ. In accord with the strong effects on differentiation, mRNA (Fig. 4C) and protein levels (Fig. 4D) of C/EBPα and PPARγ were dramatically inhibited by ORM treatment on day 2, with persistent effects until terminal differentiation. Interestingly, ORM treatment to mature adipocytes also significantly suppressed both adipogenic and lipogenic genes (Supplementary Fig. S3). These data indicate that ORM potently suppresses early adipogenic transcription factors C/EBPβ and KLF5, resulting in loss C/EBPα and PPARγ activation, and ultimately inhibiting adipocyte differentiation.

DISCUSSION

Bile acids are important signaling molecules that impact glucose and fatty acid metabolism. We previously demonstrated that hepatic bile acid overload in DKO and L-DKO mice has beneficial metabolic effects [7]. Intriguingly, L-DKO fully phenocopied the DKO mice showing anti-obesity and insulin sensitizing effects, uncovering a specific role of hepatic bile acid signaling in the regulation of systemic energy homeostasis, particularly in adipose tissues. These results raise the possibility of undiscovered liver-adipose tissue crosstalk that is mediated by hepatic bile acids.

To identify liver-secreted hormones, we analyzed the serum proteome in DKO and L-DKO and nominated ORM as a novel bile acid-regulated hepatokine. ORM is an abundant plasma protein, accounting for approximately 1% of total plasma protein [30]. In our model, ORM is primarily induced by bile acid overload in the liver, suggesting the liver is the major organ that produces circulating ORM. Although the biological function of ORM has not been

clearly defined, it has been suggested that ORM binds to and carries lipophilic drugs, which might affect drug delivery [31]. A limited number of studies suggest a metabolic function of ORM. In muscle, ORM expression is increased by fatigue to enhance exercise endurance through regulation of glycogen metabolism [32,33]. Recently, ORM1 was identified as an adipokine that acts centrally and locally to decrease food intake and adipose tissue inflammation, respectively [18,34]. However, ORM signaling, particularly ORM2, as a mediator of bile acid function has not been studied.

Our results define metabolic functions of ORM as a novel bile acid-regulated hepatokine, acting on adipocyte. During adipocyte differentiation, ORM antagonizes adipogenic signaling by inhibition of adipogenic transcription factors at early stages of adipogenesis. We next asked whether ORM directly affects mitotic clonal expansion, which is one of the earliest events and an essential step for preadipocyte commitment [30]. However, ORM treatment did not affect cell proliferation of 3T3-L1 cells during early adipogenesis (Supplementary Fig. S4), suggesting that ORM may affect another pathway to suppress adipogenic transcription factors. Detailed mechanisms of ORM function during adipogenesis remain unclear.

At the molecular level, ORM activates AMP-activated kinase (AMPK) to increase muscle glycogen accumulation [32]. AMPK is a crucial cellular energy sensor that is activated at low cellular energy level (increasing AMP:ATP and ADP:ATP ratios) or by certain hormones (*e.g.*, adiponectin) [35]. In adipose tissues, AMPK activation promotes energy-producing processes such as fatty acid oxidation and inhibits energy-storing processes like lipid synthesis [36]. Direct AMPK stimulation suppresses 3T3-L1 adipocyte differentiation and reduces adipose tissue mass [37], which is consistent with ORM effects. Therefore, it is likely that ORM effects are partly mediated by AMPK activation. Further studies are needed to determine how ORM acts on AMPK signaling in adipocytes.

In conclusion, we discovered a novel bile acid-regulated hepatokine ORM and characterized its metabolic function in adipocytes. The primary impact of ORM is to decrease C/EBPβ and KLF5 expression at an early stage of adipogenesis and subsequently abolish the expression of key adipogenic transcription factors C/EBPα and PPARγ. Taken together, our data suggest that hepatic bile acid overload induces secretion of the hepatokine ORM to modulate adipocyte differentiation and adipose tissue function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

Abbreviations

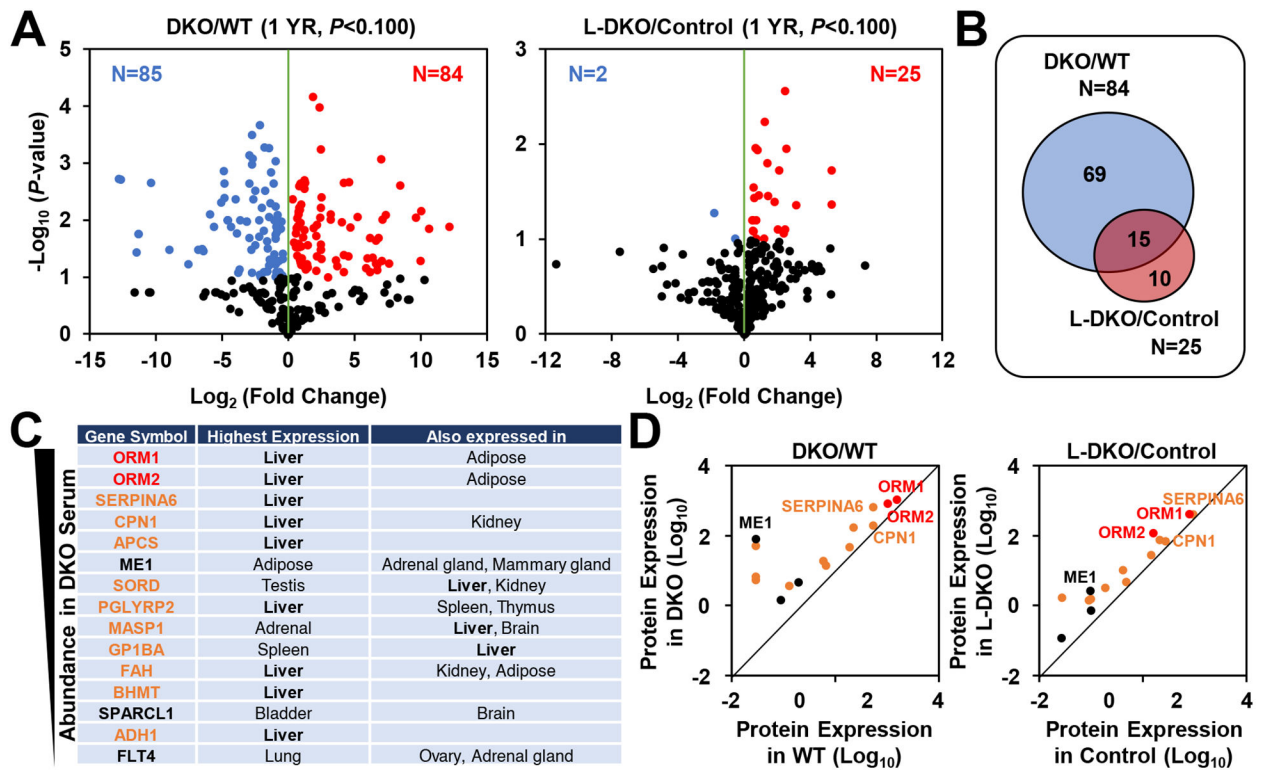|	
C/EBP	CCAAT-enhancer-binding proteins
DKO	FXR/SHP double knockout
FGF21	fibroblast growth factor 21
FXR	farnesoid X receptor
KLF	kruppel-like factor
L-DKO	liver-specific FXR/SHP double knockout
ORM	orosomucoid
PPAR	peroxisome proliferator-activated receptor
SHP	small heterodimer partner
WAT	white adipose tissue

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HIGHLIGHTS

- Hepatic bile acid overload stimulates hepatokine orosomucoid secretion.

- Hepatokine orosomucoid distally acts on adipocytes to suppress adipogenesis and lipogenesis, potentially exerting anti-obesity effect.

- Orosomucoid directly inhibits multiple early-adipogenic transcription factors, interfering C/EBPα and PPARγ activation.

A DKO/WT (1 YR, *P*<0.100) L-DKO/Control (1 YR, *P*<0.100)

B DKO/WT N=84

C

| Gene Symbol	Highest Expression	Also expressed in
ORM1	Liver	Adipose
ORM2	Liver	Adipose
SERPINA6	Liver	
CPN1	Liver	Kidney
APCS	Liver	
ME1	Adipose	Adrenal gland, Mammary gland
SORD	Testis	**Liver**, Kidney
PGLYRP2	Liver	Spleen, Thymus
MASP1	Adrenal	**Liver**, Brain
GP1BA	Spleen	**Liver**
FAH	Liver	Kidney, Adipose
BHMT	Liver	
SPARCL1	Bladder	Brain
ADH1	Liver	
FLT4	Lung	Ovary, Adrenal gland

D DKO/WT L-DKO/Control

Figure 1. Differentially Expressed Circulating Proteins in DKO and L-DKO serum
(A) Quantitative proteomic analysis. Volcano plot display of the results from statistical analysis of the serum proteome in 1 year-old DKO and L-DKO serum. (B) Venn diagram showing the distribution of upregulated proteins. (C) List of commonly-upregulated proteins in DKO and L-DKO serum. Among them, ORMs were highly-induced and mainly secreted from the liver. (D) Relative protein expression in DKO and L-DKO. ORMs are the most abundant. Relative protein expression was expressed as iFOT (intensity fraction of total).
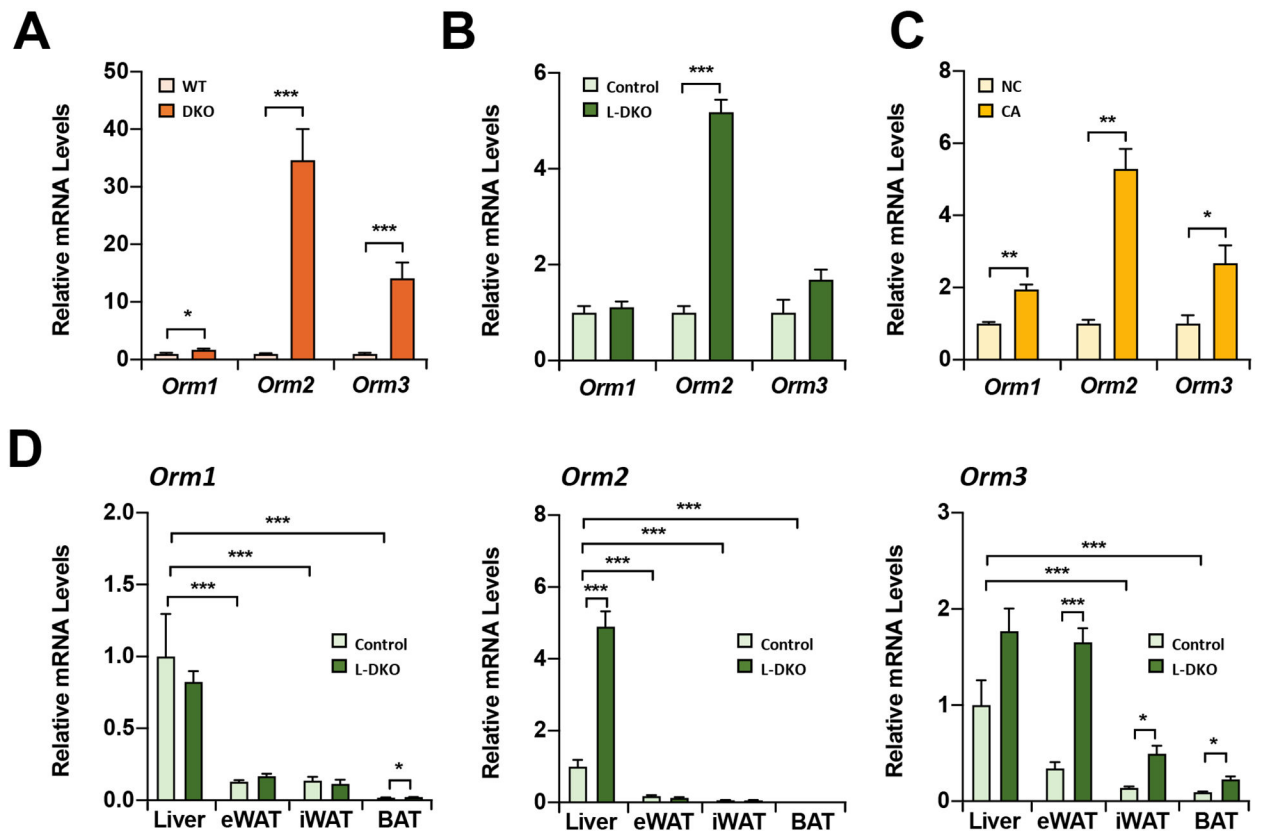
**Figure 2. Hepatic Orm Induction by Hepatic Bile Acid Overload**
Orm expression in the liver of (A) 1 year-old DKO and (B) 1 year-old L-DKO mice. (C) Hepatic *Orm* expressions after cholic acid diet (0.5%) feeding for one day (CA). NC, normal chow. (D) The comparison of *Orm* expressions in the liver and adipose tissues of 1 year-old L-DKO mice. eWAT, epididymal white adipose tissue. iWAT, inguinal WAT. BAT, brown adipose tissue. *$p<0.05$, **$p<0.01$, and ***$p<0.001$
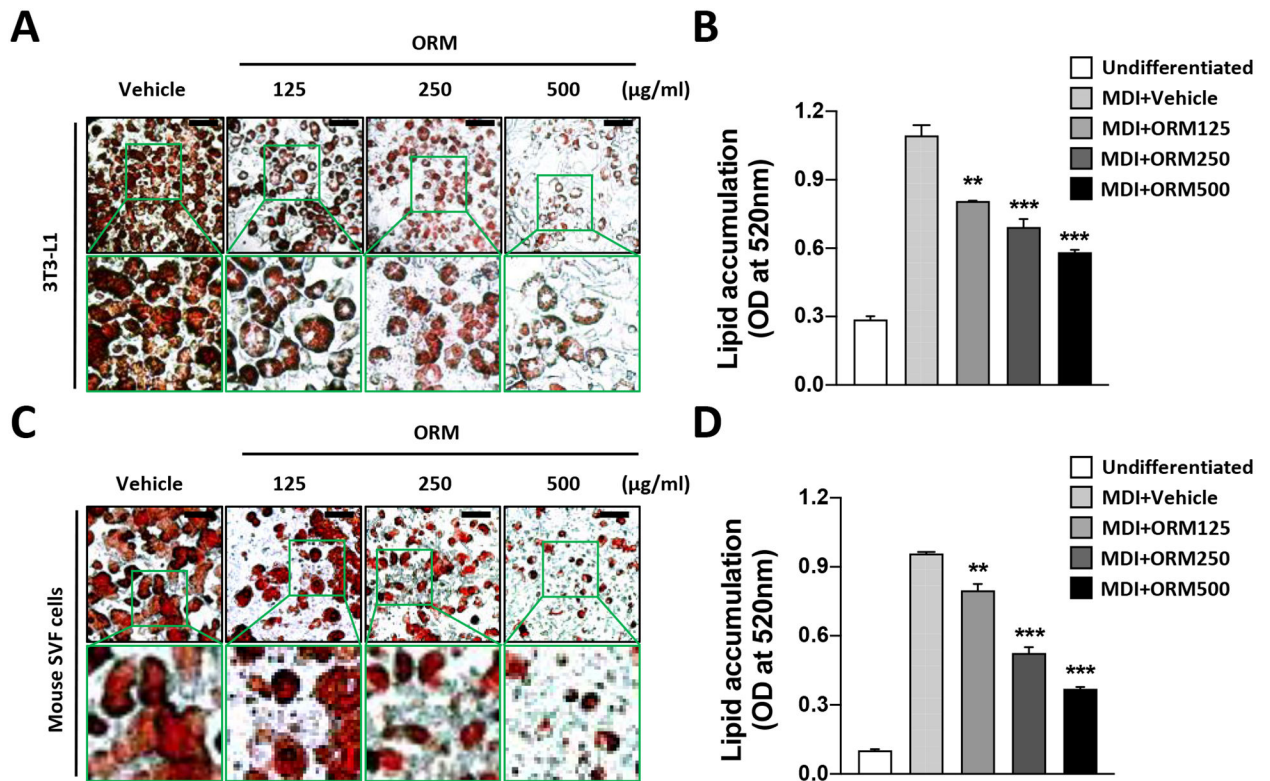
Figure 3. Suppression of Adipogenesis by ORM

(A) and (B) Differentiation of 3T3-L1 preadipocytes were induced by differentiation medium (MDI) with various concentrations of ORM protein (0, 125, 250, and 500 μg/ml). After 7 days, (A) Oil Red O staining of differentiated adipocytes. (B) Quantification of accumulated lipids by lysis of Oil Red O. (C) and (D) SVF preadipocytes from iWAT were differentiated (MDI) with various concentrations of ORM (0, 125, 250, and 500 μg/ml) for 7 days. (C) Visualization and (D) quantification after Oil Red O staining. Scale bar= 100 μm. *p<0.05, **p<0.01, and ***p<0.001 as compared to vehicle-treated differentiated cells.
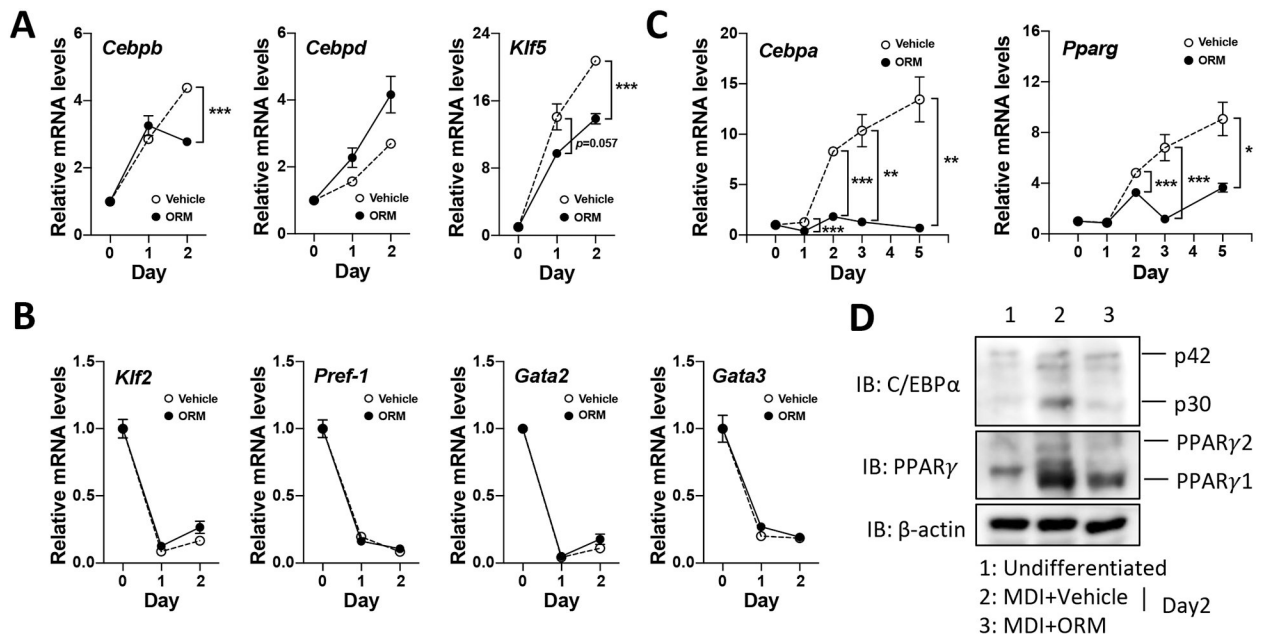
Figure 4. Decreased Expressions of Adipogenic Transcription Factors by ORM
(A) Changes of early adipogenic transcription factor expression by ORM treatment (500 μg/ml) at day 1 and 2. (B) Expression of anti-adipogenic regulators. qPCR was performed measure gene expression. (C) The mRNA expression of master adipogenic genes (*Cebpa* and *Pparg*) were analyzed by qPCR (up to day 5). *$p<0.05$, **$p<0.01$, and ***$p<0.001$ as compared to vehicle-treated differentiated cells. (D) The protein level of C/EBPα and PPARγ was examined at day 2 by Western blotting. β-actin was used as a loading control.