# Regulation of adipogenesis by Galectins, as carbohydrate binding protein

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**Department of Medical Science**

**The Graduate School, Yonsei University**

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**Directed by Professor Kyung-Hee Chun**

The Doctoral Dissertation
submitted to the Department of Medical Science,
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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

**Jung-Hwan Baek**

**December 2017**

# This certifies that the Doctoral Dissertation of Jung-Hwan Baek is approved.

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**Regulation of adipogenesis by Galectins, as carbohydrate binding protein**

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(Directed by Professor **Kyung-Hee Chun**)

Galectin-1 and -3 are a member of the animal lectin family that contains a carbohydrate-recognition binding domain (CRD) that binds a *B*-galactoside. Many studies are reported about the role of galectin-1 and -3 in cancer and immune disorders. But, the role of galectin-1 and -3 in metabolic dysfunction is not fully understood. In this study, we investigated whether galectin-1 and -3 regulates adipocyte differentiation and high fat diet (HFD) induced obesity. The level of galectin-1 and -3 increased during adipocyte differentiation and was predominantly expressed in mouse fat tissues. Galectin-1 and -3 knockdown significantly attenuated adipocyte differentiation of 3T3-L1 cells and also decreased the expression of peroxisome proliferator-activated receptor (PPAR)-γ, ccaat enhancer binding protein (C/EBP) α, fatty acid binding protein (FABP) 4 and fatty acid synthase (FASN). Endogenous galectin-

3 interacted with PPARγ and galectin-3 ablation reduced nuclear accumulation of PPARγ and PPAR response element (PPRE) luciferase activity. Galectin-1 is ubiquitously localized in the nucleus, cytoplasm, and extracellular membrane. According to its cellular location, galectin-1 has intracellular and extracellular functions to regulate various biological processes. When lactose was treated to inhibit function of extracellular galectin-1, there was no effect on adipocyte differentiation. This result suggests that galectin-1 mediated regulation of adipocyte differentiation is not regulation through extracellular mechanism. After 10-12 week high-fat diet (60% fat), galectin-1 deficient ($lgals1^{-/-}$) mice and galectin-3 deficient ($lgals3^{-/-}$) mice had lower body weight and white adipose tissue (WAT) mass than wild type mice. Fasting glucose level was also lower in $lgals1^{-/-}$ mice. The expression levels of lipogenic genes were significantly down-regulated in liver and gonadal white adipose tissue (gWAT) of $lgals1^{-/-}$ and $lgals3^{-/-}$ mice. In addition, $lgals1^{-/-}$ mice had elevated expression of genes involved in thermogenesis in inguinal white adipose tissue (iWAT) and brown adipose tissue (BAT). We suggest that galectin-1 and -3 might be potential therapeutic target in obesity.

Key words : galectin-1, galectin-3, PPAR gamma, adipogenesis, obesity

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## I. Introduction

A major role of white adipose tissue (WAT) is the maintenance of energy homeostasis through energy storage [1]. When excessive food energy is taken in, the remaining energy is stored as triglycerides in WAT, increasing WAT mass. Too much WAT contributes to obesity, which is a metabolic disease that is associated with type 2 diabetes mellitus, hypertension, arteriosclerosis, and hyperlipidemia

Galectins are a family of proteins that recognize and bind to B-galactoside. All galectins have conserved carbohydrate-recognition binding domain (CRD), consisting of about 130 amino acids [2]. Galectins are ubiquitously localized in cellular compartments, and involved in cell adhesion, cell cycle, apoptosis, inflammation, and cell growth. Because major studies on galectins were focused on cancer and inflammatory disorders, the role of galectins in metabolic disease such as obesity and type 2 diabetes mellitus is still poorly understood. Among galectins, galectin-12 was known to regulate adipocyte differentiation for the first time in 2004 [3] and was most studied in adipocyte differentiation and obesity [3-5].

To investigate the relationship between obesity and other galectins other than galectin-12, we performed screening to find galectins that increase expression during adipocyte differentiation. We confirmed that the expression of galectin-1 and -3 elevates during adipocyte differentiation. Most studies on galectin-1 and -3 also focused on cancer and inflammation like other galectins [6,7].

Galectin-1 and -3 are overexpressed in a variety of cancer cells [8]. Overexpression of galectin-1 in tumor induces cell transformation by increasing the membrane anchorage and signal transduction of oncogenic H-RAS [8,9]. Galectin-3 binds to oncogenic K-RAS and activates PI3K signaling cascade [10]. Galectin-1 induces apoptosis of activated $CD4^+$ and $CD8^+$ T cells [11], and promotes angiogenesis through promoting vascular endothelial cell proliferation and migration [6,12]. There are few evidences of the relationship between obesity and galectin-1 and -3. In study for identification of proteins secreted during adipocyte differentiation, galectin-1 was newly identified as one of novel adipokine [13]. Galectin-1 was also known as a lipid droplet-associated protein in primary adipocyte of mouse [14]. Recent study has reported that TDG (thiodigalactoside), inhibitor of galectins treated 3T3-L1 cells exhibited retardation of adipocyte differentiation. In addition, TDG treated rats had reduced body weight and WAT mass compared with vehicle treated group, suggesting that TDG treatment increases resistance to high fat diet induced obesity [15]. Galectin-3 is up-regulated in growing adipose tissue and stimulates pre-adipocyte proliferation [16]. Serum galectin-3 is elevated in obesity and is negatively correlated with glycated hemoglobin in type 2 diabetes [17]. These findings suggested that galectin-1 and -3 might be positively associated with adipocyte differentiation and obesity. But mechanisms are not yet fully understood.

We confirmed that the expression level of galectin-1 and -3 was increased during adipocyte differentiation and highly expressed in adipose tissue of mice. Galectin-1 and -3 depleted 3T3-L1 cells exhibited retardation of adipocyte differentiation, and also decreased the expression of peroxisome proliferator-activated receptor (PPAR)-γ, ccaat enhancer binding protein (C/EBP) α fatty acid binding protein (FABP) 4 and fatty acid synthase (FASN). Lgals1$^{-/-}$ mice and lgals3$^{-/-}$ mice higher resistance to diet-induced obesity than wild type mice. The adipose tissues of lgals1$^{-/-}$ show lower expression of lipogenic genes and higher expression of thermogenic genes than those of lgals1$^{+/+}$. We suggest that galectin-1 and -3 might be potential therapeutic target in obesity.

## II. MATERIALS AND METHODS

### 1. Cell culture and adipocyte differentiation

3T3-L1 cells were kindly provided professor Jae-Woo Kim (Yonsei University). 3T3-L1 cells were maintained and differentiated as previously described [18]. 3T3-L1 cells were maintained in DMEM (Welgene) supplemented with 10% bovine serum (BS) and antibotics. Confluent 3T3-L1 cells were incubated for 48 hr. Then, media was replaced DMEM supplement with 10% fetal bovine serum (FBS), dexamethasone (1μM), insulin (1μg/ml) and isobutylmethylxanthine (520μM). After 48hr, media was replaced DMEM supplemented with 10% FBS and insulin (1μg/ml). After 48hr, media was replaced DMEM supplemented with 10% FBS. Mouse embryonic fibroblasts (MEFs) differentiation was induced with DMEM supplement with 10% FBS, dexamethasone (1μM), insulin (10μg/ml), isobutyl-methylxanthine (520 μM), and rosiglitazone (1 μM). MEF media was replaced every 2 days with high-glucose DMEM containing 10% FBS, insulin (10 μg/ml), and rosiglitazone (1 μM) until day 14.

### 2. Oil Red O staining

Differentiated 3T3-L1 cells were washed with DPBS and incubated in 10% formalin for 10min. The cells were washed with distilled water. Then, cells were washed with 60% isopropanol and completely dried. Oil Red O (ORO) stock solution (0.35g/100ml) was diluted with isopropanol to make 60% ORO working solution. The dried cells were stained with ORO working solution for 30 min and washed third time with distilled water.

### 3. Transfection of small interfering RNA

3T3-L1 cells were transfected with mouse galectin-1 siRNA (50nM) using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. After 24 h, media was replaced maintenance media supplemented with 10% bovine serum.

## 4. Western blot

Cell lysate extractions were prepared with RIPA buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0). Cell lysates were incubated for 20 min on ice and centrifuged at 4℃ for 25 min at 13,200rpm. The supernatant was transferred to a new microcentrifuge tube. The concentration of the supernatant was measured with protein assay reagent (Thermo Scientific, Waltham, MA, USA). Protein samples were loaded into wells of the SDS-PAGE gel and transferred to PVDF membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 1hr at room temperature. After blocking, membranes were incubated with primary antibodies (Galectin-1, Galectin-3, PPARγ, C/EBPα, FASN, FABP4, HA, B-actin from Santa Cruz Biotechnology, Dallas, TX, USA and Flag from Aldrich, St. Louis, Missouri, USA) overnight at 4℃. The membranes were washed 3 times for 10 min with PBST and incubated with HRP-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA) for 1hr at room temperature. The membranes were washed 3 times for 10 min with PBST. The FUSION SOLO S (Vilber, Eberhardzell, Germany) was used for image detection according to manufacturer's directions. B-actin was used loading control.

## 5. Immunoprecipitation

Cell lysate extraction were performed with immunoprecipitation buffer. After centrifugation (4°C for 25min at 13,200rpm), the supernatants were added to protein A/G agarose beads (Santa

Cruz Biotechnology, Dallas, TX, USA) and incubated at 4°C for 30 min in a rotor for preclearing. After centrifugation (4°C for 25min at 13,200rpm), anti-Flag beads (Sigma-Aldrich, St. Louis, Missouri, USA), anti-galectin-3, anti-PPARγ and normal IgG (negative control) were independently added to supernatants and were then incubated at 4°C for overnight in a rotor. Immunoprecipitates were washed twice in immunoprecipitation buffer, added to 2X sodium dodecyl sulfate sample buffer, and boiled at 95°C for 5 min. After centrifugation (4°C for 2smin at 13,200rpm), supernatants were analyzed by Western blot.

### 6. RNA isolation and real-time PCR

Total RNA was prepared with RNA-lysis reagent (Intron) following the manual. cDNA (1μg) was synthesized using quantitative RT-PCR master mix (TOYOBO, Osaka, Japan). The following primers were used.

Galectin-1, forward: 5'-CTCTCGGGTGGAGTCTTCTG-3' and
reverse: 5'-GCGAGGATTGAAGTGTAGGC-3'

Galectin-3, forward: 5'-CAGTGCTCCTGGAGGCTATC-3' and
reverse: 5'-ATTGAAGCGGGGGTTAAAGT-3'

PPARγ, forward: 5'-AGGGCGATCTTGACAGGAAA-3' and
reverse: 5'-CGAAACTGGCACCCTTGAAA-3'

C/EBPα, forward: 5'-GACATCAGCGCCTACATCGA-3' and
reverse: 5'-TCGGCTGTGCTGGAAGAG-3'

FASN, forward: 5'-TGGGTTCTAGCCAGCAGAGT-3' and
reverse: 5'-ACCACCAGAGACCGTTATGC-3'

FABP4, forward: 5'-CATCAGCGTAAATGGGGATt-3' and

reverse: 5’-TCGACTTTCCATCCCACTTC-3’

SREBP, forward: 5’-GATCAAAGAGGAGCCAGTGC-3’ and

reverse: 5’-TAGATGGTGGCTGCTGAGTG-3’

CD36, forward: 5’-TGATACTATGCCCGCCTCTCC-3’ and

reverse: 5’-TTTCCCACACTCCTTTCTCCTCTA-3’

ACC1, forward: 5’-ATGCGATCTATCCGTCGGTG-3’ and

reverse: 5’-TCCTCCAGGCACTGGAACAT-3’

ACLY, forward: 5’-GAAGCTGACCTTGCTGAACC-3’ and

reverse: 5’-CTGCCTCCAATGATGAGGAT-3’

SCD1, forward: 5’-GTACCGCTGGCACATCAACT-3’ and

reverse: 5’-AAGCCCAAAGCTCAGCTACTC-3’

UCP1, forward: 5’-GGGCCCTTGTAAACAACAAA-3’ and

reverse: 5’-GTCGGTCCTTCCTTGGTGTA-3’

PGC1α, forward: 5’-ATGTGTCGCCTTCTTGCTCT-3’ and

reverse: 5’-ATCTACTGCCTGGGGACCTT-3’

PRDM16, forward: 5’-CAGCACGGTGAAGCCATTC-3’ and

reverse: 5’-GCGTGCATCCGCTTGTG-3’

CIDEA, forward: 5’-CATACATCCAGCTCGCCCTT-3’ and

reverse: 5’-CGTAACCAGGCCAGTTGTGA-3’

Adiponectin, forward: 5’-TACTGCAACATTCCGGGACTC-3’ and

reverse: 5’-GAGGCCTGGTCCACATTCTT-3’

G6PC, forward: 5’-CCTGAGGTACCAAGGGAGGA-3’ and

reverse: 5’-GAAGGCGTTCCTCAGGTCAG-3’

PCK, forward: 5’-AGATCATCATGCACGACCCC-3’ and

reverse: 5’-TGTCCTTCCGGAACCAGTTG-3’

IL-10, forward: 5’-ATCGATTTCTCCCCTGTGAA-3’ and

reverse: 5’-TTCGGAGAGAGGTACAAACGA-3’

IFNγ, forward: 5’-GAGCCAGATTATCTCTTTCTACC-3’ and

reverse: 5’-GTTGTTGACCTCAAACTTGG-3’

TNFα, forward: 5’-CGTCAGCCGATTTGCTATCT-3’ and

reverse: 5’-CGGACTCCGCAAAGTCTAAG-3’

CCL2, forward: 5’-TAAAAAACCTGGATCGGAACCAA-3’ and

reverse: 5’-GCATTAGCTTCAGATTTACGGGT-3’

CCL3, forward: 5’-GTGACTCACCTTGTGGTCCT-3’ and

reverse: 5’-AGGGCAGATCCCAATTGTCAG-3’

F4/80, forward: 5’-CGTCAGCCGATTTGCTATCT-3’ and

reverse: 5’-CGGACTCCGCAAAGTCTAAG-3’

B-actin, forward: 5’-GGCTGTATTCCCCTCCATCG-3’ and

reverse: 5’-CCAGTTGGTAACAATGCCATGT-3’

Real-time PCR was performed using SYBR Premix Ex Taq (Clontech Laboratories, Mountain View, CA, USA) with ABI instruments (Applied Biosystems Inc, Foster City, CA, USA). All results were normalized by B-actin.

### 7. Luciferase reporter assay

PPRE-TK-Luc, shgalectin-3, and β-gal were co-transfected in HEK293 cells using

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hr, cells were harvested and luciferase activities were measured using the Luciferase Assay System (Promega, Madison, Wisconsin, USA) according to manufacturer's directions. Luciferase activities were normalized by a β-gal enzyme assay system (Promega, Madison, Wisconsin, USA).

### 8. Immunocytochemistry

Cells in chamber slides were fixed with 4% formaldehyde at 4°C for 30 min, washed with 1X PBS, and permeabilized in 0.5% Triton X-100 for 10 min. Cells were incubated with primary antibodies at 4°C and then were incubated with FITC anti-mouse and Cy5 anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA, USA) as well as DAPI staining solution (Vector Laboratories, Burlingame, CA, USA). Images were analyzed by confocal microscopy (LSM 700, Oberkochen, Germany).

### 9. Mouse studies

Galectin-3 deficient C57BL/6 mice were kindly provided from Dr. Fu Tong Liu (University of California, Davis). Galectin-1 deficient C57BL/6 mice were purchased from Knockout Mouse Project (KOMP, Oakland, CA, USA) Repository. Seven-week-old wild-type, galectin-1 deficient ($lgals1^{-/-}$) and galectin-3 deficient ($lgals3^{-/-}$) were fed a high fat diet containing 60% fat for 10-12 weeks (12h light, 12h dark cycle). Animal studies were approved by the Yonsei University Health System Institutional Animal Care and Use Committee.

### 10. Quantification of size of adipocytes in gonadal white adipose tissue

Mouse gWATs were fixed in 10% formalin and embedded in paraffin. gWAT paraffin sections were performed hematoxyline and eosin (H&E) staining. H&E stained sections were analyzed using ImageJ software (NIH, Bethesda, Maryland, USA).

### 11. Statistical analysis

Unpaired (two sample) t test was used to determine the p-values. P-values <0.05 were considered to be statistically significant. Statistical analyses were using Prism (GraphPad software, La Jolla, CA, USA).

## III. Results

### 1. Galectin-1 and -3 is elevated in the process of adipocyte differentiation and predominantly expressed in adipose tissue of mice

We measured the change of galectin family level during adipocyte differentiation. 3T3-L1 cells were induced with DMI and differentiated for 10 days. The expression of galectin-1 and -3 as well as PPARγ and FASN, markers of adipocyte differentiation increased during adipocyte differentiation (Figure 1A). To confirm the distribution of galectin-1 and -3 in mouse organs, we performed RT-PCR in various mouse organs. We determined that galectin-1 and -3 is highly expressed in adipose tissue, compared with other organs (Figure 1B). The mRNA level of galectin-1 and -3 in WAT was increased in mice fed high fat diet (Figure 1C). We hypothesized that galectin-1 and -3 might be a positive regulator of adipocyte differentiation and obesity.

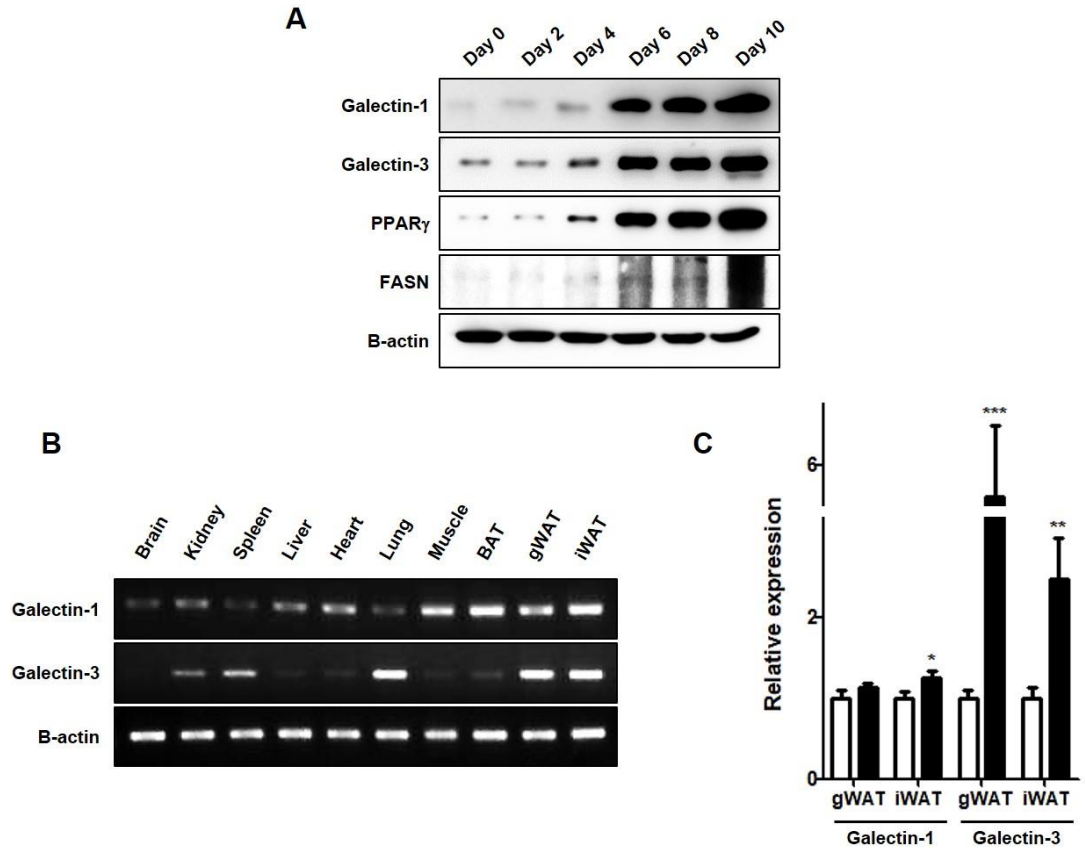


**Figure 1. Expression of galectin-1 and -3 in adipocyte differentiation and mouse adipose tissues.** (A) Level of galectin-1 and -3 protein during adipocyte differentiation. B-actin was used as the normalization control. (B) mRNA expression of galectin-1 and -3 in mouse tissues. Gaelctin-1 and -3 levels were analyzed by RT-PCR. B-actin was used as the normalization control. (C) Real-time RT-PCR analysis of galectin-1 and -3 expression in gonadal WAT and inguinal WAT of mice fed chow and high fat diet. Data are presented as means ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for NFD vs HFD.

**PART I**

**1. Seventeen-month-old lgals3$^{-/-}$ mice have reduced body weight and white adipose tissue**

Seventeen-month-old male lgals3$^{-/-}$ mice were significantly smaller than wild-type (lgals3$^{+/+}$) mice (Figure 1A). gWAT was also drastically lessened in lgals3$^{-/-}$ mice (Figure 1B). There was no significant difference in the size of liver and BAT between groups (Figure 1C and D). We measured the expression of genes related to adipogenesis or lipogenesis in the gWAT and in the liver. Interestingly, mRNA expression of PPARγ and FABP4 was reduced in gWAT in lgals3$^{-/-}$ mice (Figure 1E). Expression levels of IL-10, IFNγ, and TNFα were unchanged in gWAT of lgals3$^{-/-}$ mice, suggesting that the amplified inflammation did not occur in lgals3$^{-/-}$ mice (Figure 1F). Even though liver size was unchanged, mRNA expression of PPARγ and FASN was reduced in the liver of lgals3$^{-/-}$ mice (Figure 1F), indicating that galectin-3 deficiency down-regulates the expression of adipogenic and lipogenic genes in both gWAT and liver.

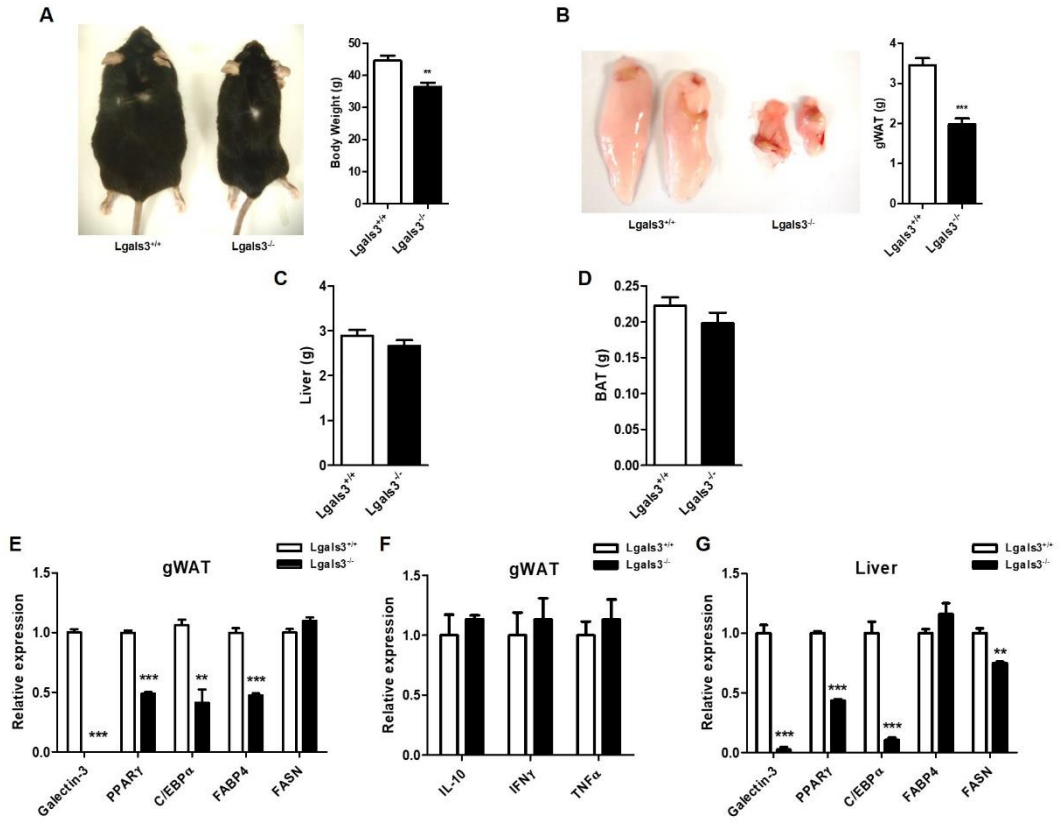


**Figure 1. Seventeen-month-old lgals3$^{-/-}$ mice have reduced body weight and white adipose tissue.** (A) Decreased body size and weight of lgals3$^{-/-}$ mice fed chow. Representative picture of lgals3$^{+/+}$ and lgals3$^{-/-}$ mice fed chow for 17 months. (B) Size and weight of gonadal WAT. (C) Weight of liver. (D) Weight of brown adipose tissue. (E, F) Real-time RT-PCR analysis of genes in gonadal WAT. (G) Real-time RT-PCR analysis of liver genes including galectin-3, peroxisome proliferator-activated receptor gamma (PPARγ), ccaat-enhancer-binding protein alpha (C/EBPα), fatty acid binding protein (FABP4), fatty acid synthase (FASN), interleukin 10 (IL-10), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) mRNA expression was normalized to β-actin. Data are presented as mean ± SEM

(n=5 for lgals3$^{+/+}$ and lgals3$^{-/-}$ mice fed chow) $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for lgals3$^{+/+}$ vs lgals3$^{-/-}$ mice.

**2. Lgals3$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit retardation of adipocyte differentiation**

As the expression of adipogenic and lipogenic genes was reduced in lgals3$^{-/-}$ mice, we hypothesized that galectin-3 might play an important role in adipogenesis. Lgals3$^{+/+}$ and lgals3$^{-/-}$ MEFs were treated with DMI and rosiglitazone, which induced adipocyte differentiation. The intensity of adipocyte differentiation was measured with oil red o (ORO) staining of lipid droplets. For up to 14 days, adipocyte differentiation in lgals3$^{-/-}$ MEFs was retarded compared to lgals3$^{+/+}$ MEFs (Figures 2A and B). Adipocyte-differentiated lgals3$^{-/-}$ MEFs had less total lipids than adipocyte-differentiated lgals3$^{+/+}$ MEFs (Figure 2C). The expression of PPARγ, C/EBPα, C/EBPβ and FABP4 was reduced in adipocyte-differentiated lgals3$^{-/-}$ MEFs (Figure 2D).

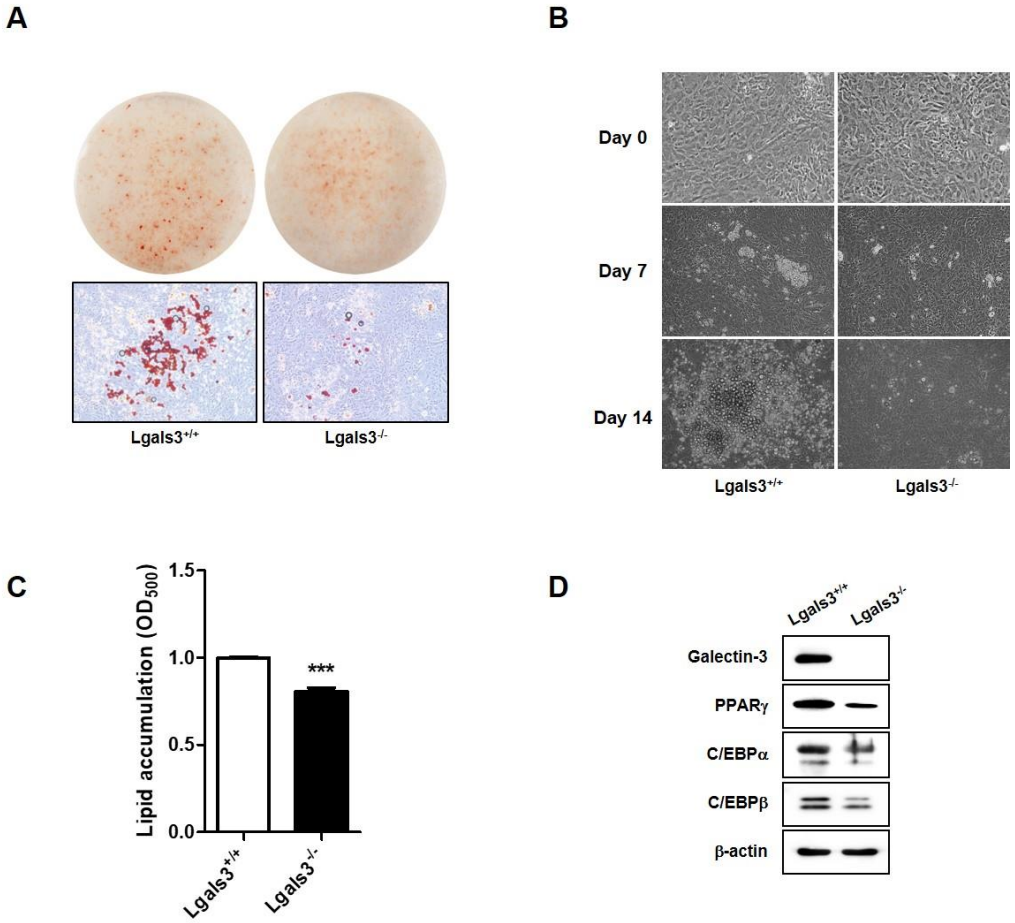


**Figure 2. Lgals3$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit retardation of adipocyte differentiation.** (A, B) Adipocyte differentiation of lgals3$^{+/+}$ and lgals3$^{-/-}$ MEFs. MEFs were differentiated with DMI and rosiglitazone for 14 days. We performed ORO staining on adipocytes. (C) Measurement of lipid accumulation. Stained ORO dye was eluted by 100% isopropanol and measured using the $OD_{500}$. (D) Western blot analysis of adipogenic factors in differentiated lgals3$^{+/+}$ and lgals3$^{-/-}$ MEFs. Protein expression of adipogenic factors was normalized to β-actin. Data are presented as mean ± SEM (n=3 for each lane) $^{***}P < 0.001$ for lgals3$^{+/+}$ vs lgals3$^{-/-}$ MEFs.

**3. Galectin-3 depleted 3T3-L1 cells exhibit retardation of adipocyte differentiation**

We determined the effect of galectin-3 on differentiation of pre-adipocyte 3T3-L1 cells. Levels of galectin-3 protein increased during adipocyte differentiation. To identify the role of galectin-3 in adipocyte differentiation, we stably silenced galectin-3 expression in 3T3-L1 cells. 3T3-L1 cells were infected with galectin-3 shRNA lentiviruses, and galectin-3 depletion was confirmed in shRNAs 2 and 5 (Figure 3A). 3T3-L1 cells were differentiated with DMI and differentiated for 8 days. Galectin-3 depleted 3T3-L1 cells showed significantly delayed adipocyte differentiation and lipid accumulation, compared to control 3T3-L1 cells (Figure 3B and C). Consequently, they had low expression of PPARγ, C/EBPα, C/EBPβ, and FABP4 (Figure 3D). These data suggest that galectin-3 might be a positive regulator of adipocyte differentiation.

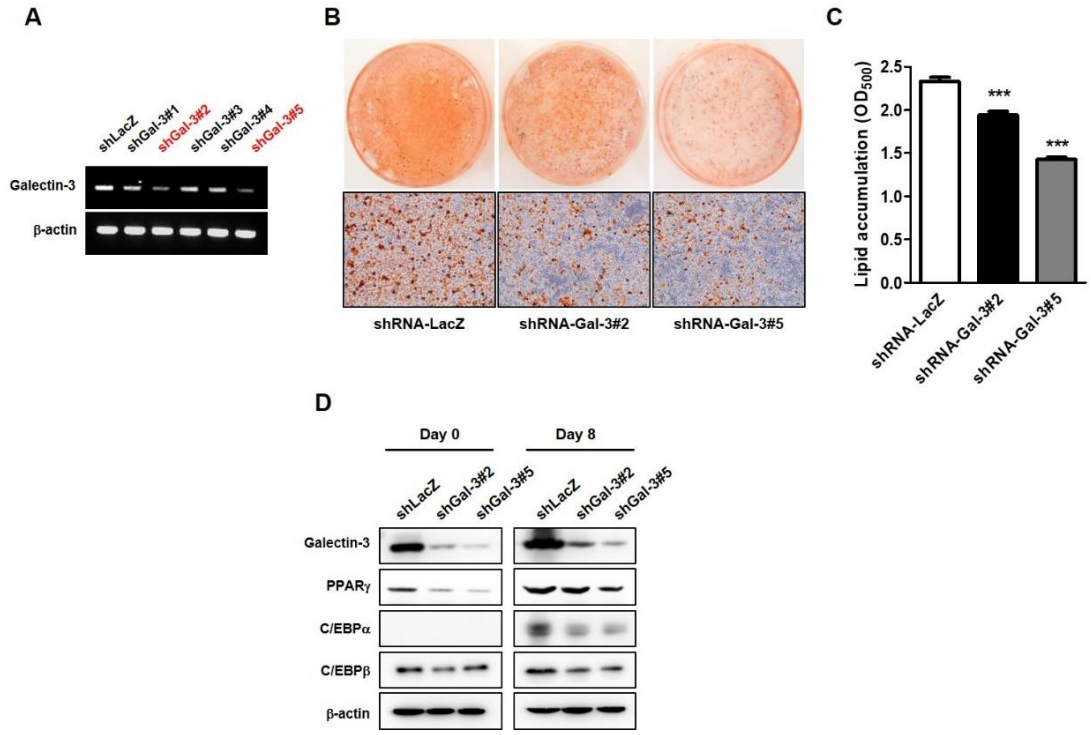


**Figure 3. Galectin-3-depleted 3T3-L1 cells exhibit retardation of adipocyte differentiation.** (A) Galectin-3 stably silenced 3T3-L1 cells. 3T3-L1 cells were infected with galectin-3 shRNA lentiviruses (pLKO.1-puro vector) and selected by puromycin. (B) Adipocyte differentiation of galectin-3 stably silenced 3T3-L1 cells. Cells were differentiated with DMI for 8 days, and we performed ORO staining on adipocytes. (C) Measurement of lipid accumulation. Stained ORO dye was eluted with 100% isopropanol and measured using the $OD_{500}$. (D) Western blot analysis of adipogenic factors in galectin-3 stably silenced 3T3-L1. Protein expression of adipogenic factors was normalized to β-actin. Data are presented as mean ± SEM (n=3 for each lane) $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for shLacZ vs. shgalectin-3 #2, #5.

**4. Galectin-3 interacts with PPARγ and increases its transcriptional activity**

We investigated the interaction between galectin-3 and PPARγ. HEK293 cells were co-transfected with FLAG-tagged galectin-3 and HA-tagged PPARγ. After 48 hr, cells were harvested and cell lysates were immunoprecipitated using anti-FLAG beads. We confirmed interactions between exogenously expressed Flag-galectin-3 and HA-PPARγ in HEK293 cells by western blot with anti-FLAG and anti-HA antibodies (Figure 4A). Furthermore, interaction between endogenous galectin-3 and PPARγ was confirmed in 3T3-L1 cells (Figure 4B). To determine the effect of galectin-3 on PPARγ transcriptional activity, galectin-3 was knocked down by shRNA in HEK293 cells with a transiently transfected PPRE reporter in the absence or presence of rosiglitazone, a PPARγ agonist (Figure 4C). Galectin-3 depletion significantly reduced PPARγ transcriptional activity with and without rosiglitazone compared to shLacZ. Using immunocytochemistry, we found that expression and nuclear localization of PPARγ were decreased by galectin-3 silencing in 3T3-L1 cells (Figure 4D). These data suggest that galectin-3 might positively regulate PPARγ expression and transcriptional activity by direct interaction.

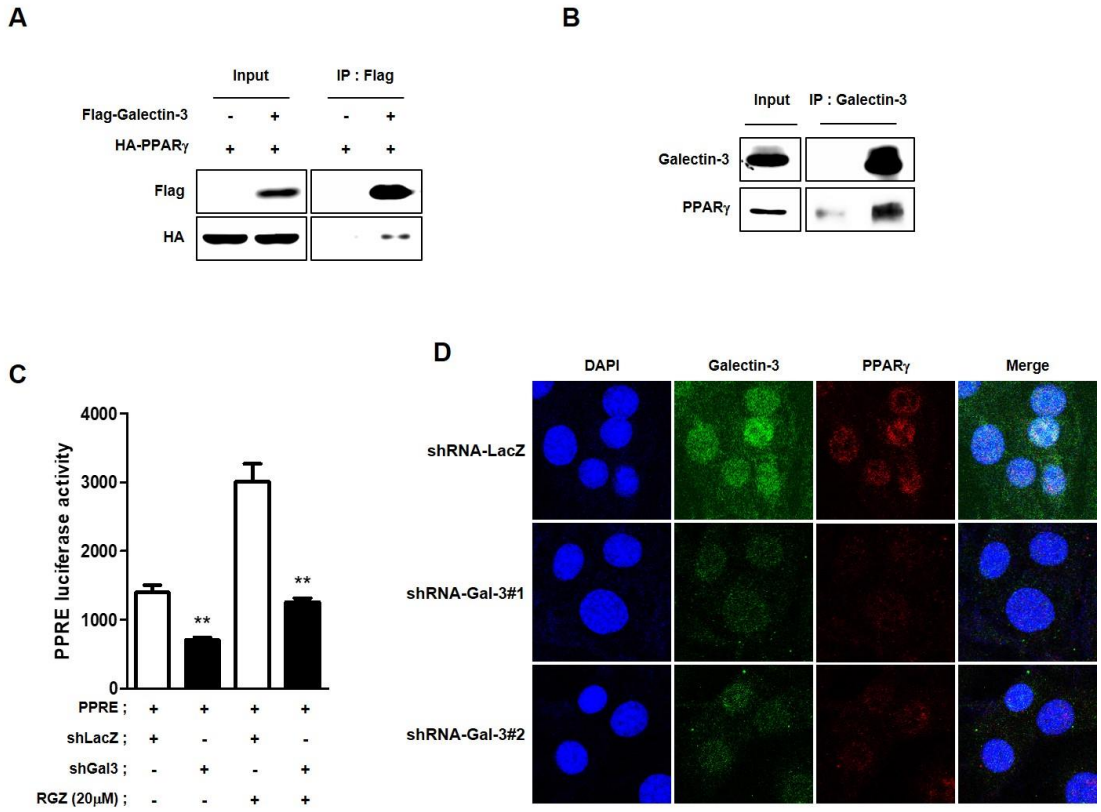


**Figure 4. Galectin-3 interacts with PPARγ and increases its transcriptional activity.** (A) Co-immunoprecipitation of exogenous Galectin-3 (Flag-tagged) and PPARγ (HA-tagged) in HEK293 cells. Cell lysates were immunoprecipitated using anti-FLAG beads. (B) Co-immunoprecipitation of endogenous Galectin-3 and PPARγ in 3T3-L1 cells. (C) Luciferase activities of PPRE in HEK293 cells. HEK293 cells were co-transfected with sh-galectin-3, PPRE and β-gal, and then treated with 20$\mu$M rosiglitazone. Luciferase activities were normalized to β-gal activity. (D) Immunocytochemistry of galectin-3 and PPARγ in galectin-3 stably silenced 3T3-L1 cells. Data are presented as mean ± SEM (n=3 for each lane) $^{*}P < 0.05$ and $^{**}P < 0.01$ for sh-LacZ vs sh-galectin-3.

**5. Lgals3$^{-/-}$ mice are resistant to high-fat diet-induced obesity**

We fed a high-fat diet containing 60% fat to male lgals3$^{+/+}$ and lgals3$^{-/-}$ mice (n=5) for 12 weeks, and characterized the phenotypes. Male lgals3$^{-/-}$ mice had a lower body weight than lgals3$^{+/+}$ mice (Figures 5A and B), even though food intake did not differ between these two groups (Figure 5C). Moreover, lgals3$^{-/-}$ mice exhibited less gWAT weight (Figure 5D). However, brown adipose tissues and liver weight were not statistically different between these two groups (Figures 5E and F). Many reports indicate that obesity is related to the risk of type 2 diabetes mellitus and hyperlipidemia [19,20]. We examined the regulation of blood glucose in lgals3$^{-/-}$ mice (Figures 5G). Despite increased body weight and adiposity in lgals3$^{+/+}$ mice, fasting blood glucose was not statistically different between lgals3$^{+/+}$ and lgals3$^{-/-}$ mice.

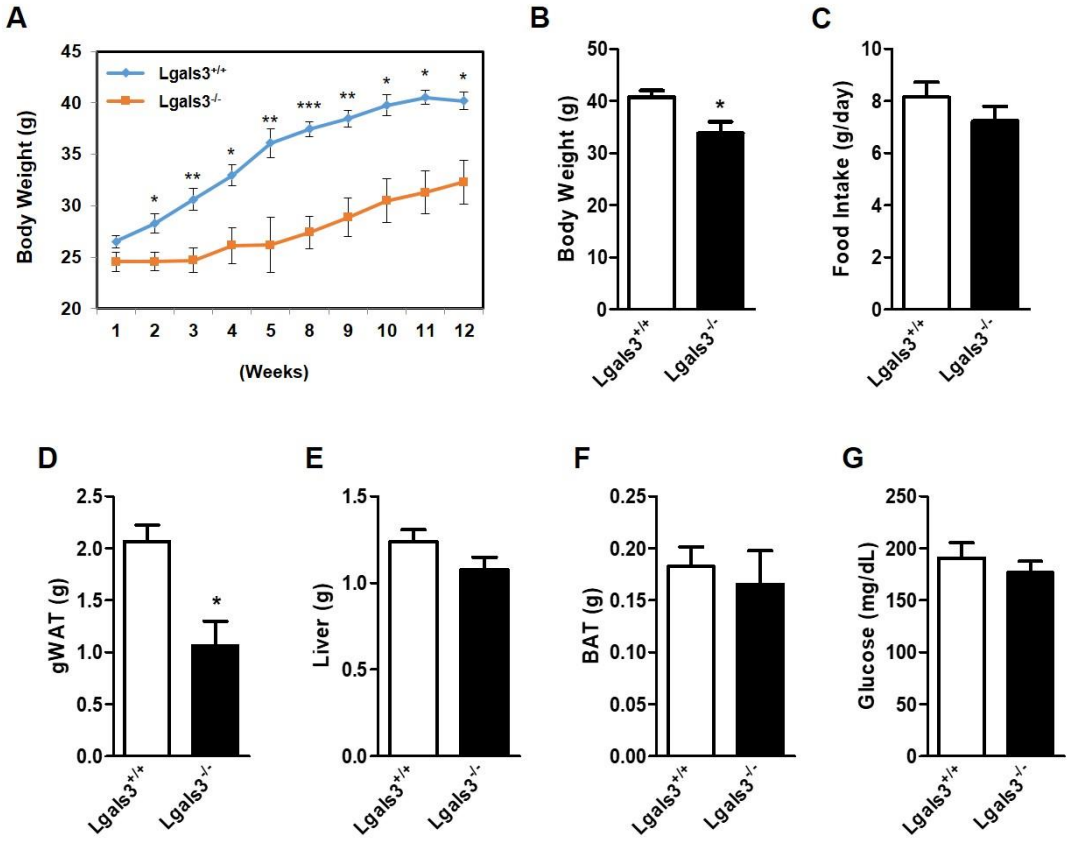


**Figure 5. Lgals3$^{-/-}$ mice are resistant to high-fat diet-induced obesity.** (A, B) Body weight of lgals3$^{+/+}$ and lgals3$^{-/-}$ mice fed a HFD (60% fat) for 12 weeks. (C) Grams of food pellets consumed per day. (D) Weight of gonadal WAT. (E) Weight of liver. (F) Weight of brown adipose tissue (G) Blood glucose levels, measured after 5 hr fasting. Data are presented as mean ± SEM (n=5 for lgals3$^{+/+}$ and lgals3$^{-/-}$ mice fed a HFD) $^{*}P < 0.05$ for lgals3$^{+/+}$ vs. lgals3$^{-/-}$ mice.

**6. Lgals3$^{-/-}$ mice exhibit decreased adiposity and have altered hepatic lipogenic gene expression**

We detected a reduced adipocyte size in gWAT of lgals3$^{-/-}$ mice fed a high-fat diet (Fig 5A), indicating that reduced adiposity in gWAT was not due to a decrease in adipocyte number. TG levels were also lower in the gWAT of lgals3$^{-/-}$ (Fig 5B).

Fatty liver diseases are often exhibited in obesity, but histological analysis of livers showed no difference between lgals3$^{+/+}$ and lgals3$^{-/-}$ mice in this study. Therefore, we measured expression of lipogenic genes in liver tissues by DNA microarray analysis. mRNA expression of PPARγ, C/EBPα, FABP4, FASN, Me1, Acaca, Acyl, and Slc25a1 was reduced in liver tissues of lgals3$^{-/-}$ mice (Fig 5C). We also used real-time RT-PCR analysis to confirm that mRNA expression of PPARγ, C/EBPα, FABP4, and FASN was relatively low in liver tissues of lgals3$^{-/-}$ mice (Figure 5D). This suggests that galectin-3 might influence lipogenic genes in the liver, stimulating systemic obesity.

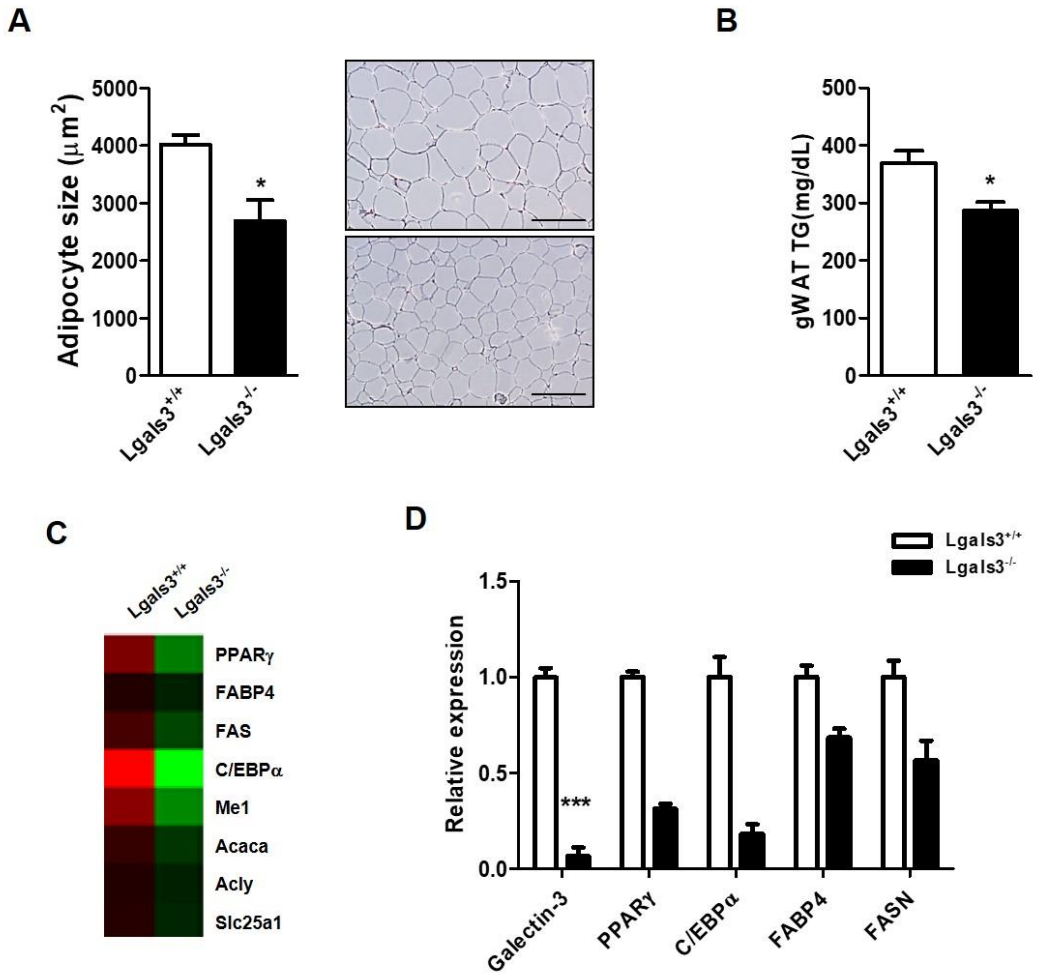


**Figure 6. Lgals3$^{-/-}$ mice exhibit decreased adiposity and have altered hepatic lipogenic gene expression.** (A) Adipocyte size of gWAT sections stained with H&E. Size measurement was performed using ImageJ software. (B) TG content in gWAT. TG accumulation was measured with a TG assay kit. (C) Microarray analysis of liver tissue showed decreased expression of PPARγ, C/EBPα, FABP4, FASN, Me1, Acaca, Acyl and Slc25a1. (D) Real-time RT-PCR analysis of genes in the liver, including galectin-3, peroxisome proliferator-activated receptor gamma (PPARγ), ccaat-enhancer-binding protein alpha (C/EBPα),

fatty acid binding protein (FABP4), and fatty acid synthase (FASN). mRNA expression of lipogenic factors was normalized to β-actin. Data are presented as mean ± SEM (n=5 for lgals3$^{+/+}$ and lgals3$^{-/-}$ mice fed a HFD) $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for lgals3$^{+/+}$ vs lgals3$^{-/-}$ mice.

**PART II**

**1. Galectin-1 depleted 3T3-L1 cells exhibits retardation adipocyte differentiation**

To identify the role of galectin-1 in adipocyte differentiation, we silenced galectin-1 expression in 3T3-L1 cells using galectin-1 siRNA (Figure 2A). The 3T3-L1 cells were differentiated for 6 days. Galectin-1 depleted 3T3-L1 cells show significantly delayed adipocyte differentiation and lipid accumulation, compared with control 3T3-L1 cells (Figure 2B and C). Lipid accumulation in 3T3-L1 cells was measured using Oil Red O staining on day 6. We test if galectin-1 knockdown affect expression of genes that regulates adipocyte differentiation and lipid metabolism. The expression of PPARγ, C/EBPα, FASN and FABP4 was attenuated by galectin-1 knockdown during adipocyte differentiation (Figure 2D and E). These suggest that galectin-1 is essential for adipocyte differentiation.

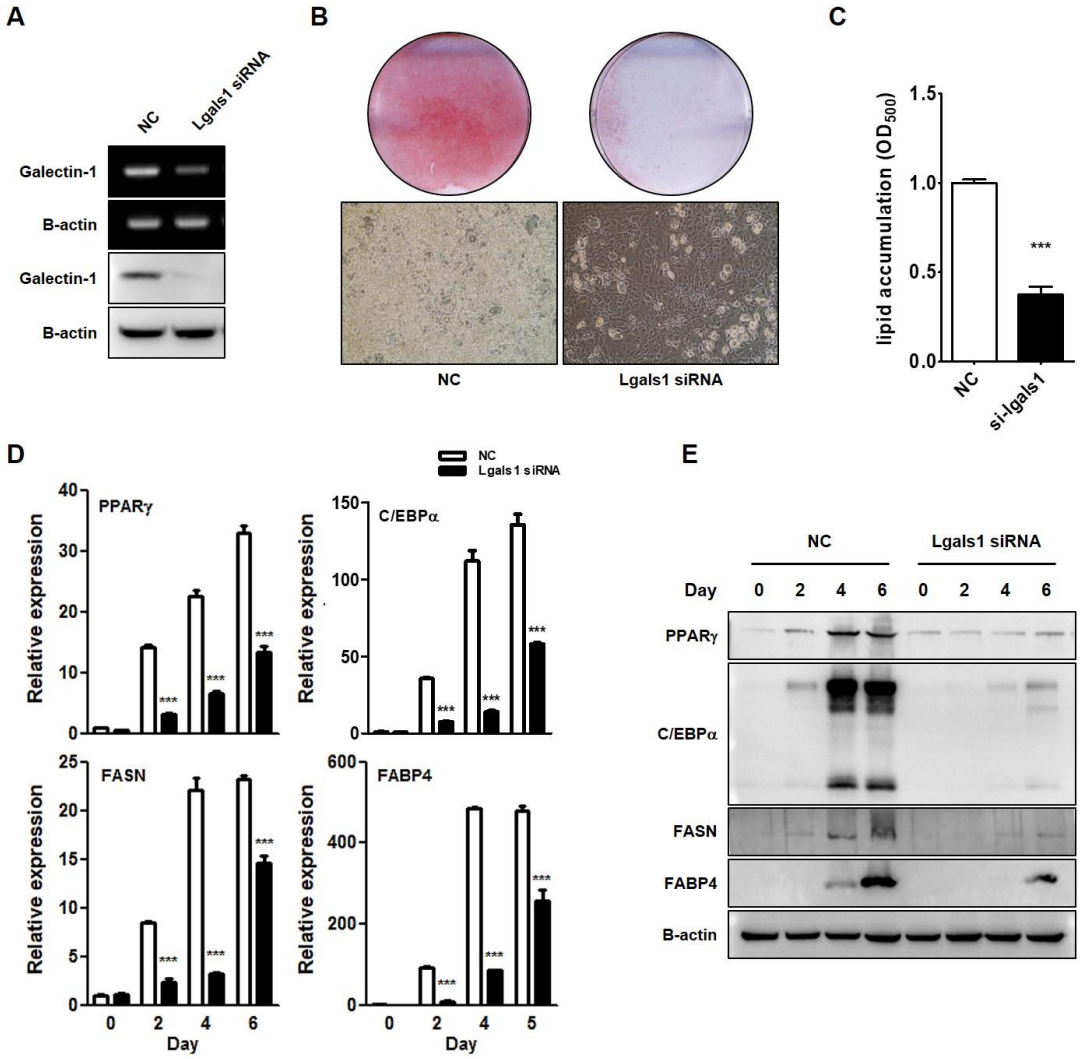


**Figure 1. Knockdown of galectin-1 inhibits adipocyte differentiation.** (A) Knockdown of galectin-1 using galectin-1 siRNA in 3T3-L1 cells. mRNA and protein were normalized by B-actin. (B) Adipocyte differentiation of galectin-1 knockdown 3T3-L1 cells. Cells were differentiated for 6 days, and performed ORO staining. (C) Measurement of lipid accumulation. Stained ORO dye was eluted with 100% isopropanol and measured using the $OD_{500}$. (D and E) Real-time RT-PCR and western blot analysis of adipogenic and lipogenic

genes during adipocyte differentiation. B-actin was used as the normalization control. Data are presented as means ± SEM (n=3 for each lane) *** $P < 0.001$ for NC vs Lgals1 siRNA.

**2. Inhibition of extracellular galectin-1 does not affect adipocyte differentiation**

Galectin-1 is ubiquitously localized in cellular compartment, and is particularly highly distributed in extracellular space. Therefore, many studies were focused on extracellular galectin-1, For example, extracellular galectin-1 was reported that stimulates angiogenesis in vitro and in vivo and this effect was inhibited by lactose treatment [12]. We examined whether extracellular galectin-1 affects adipocyte differentiation. When lactose was treated to inhibit function of extracellular galectin-1, there was no effect on adipocyte differentiation (Figure 3A and B). This result suggests that galectin-1 mediated regulation of adipocyte differentiation is not regulation through extracellular mechanism. Galectin-1 knockdown reduced cell proliferation, measured 48hr after adipogenic induction (Figure 3C).

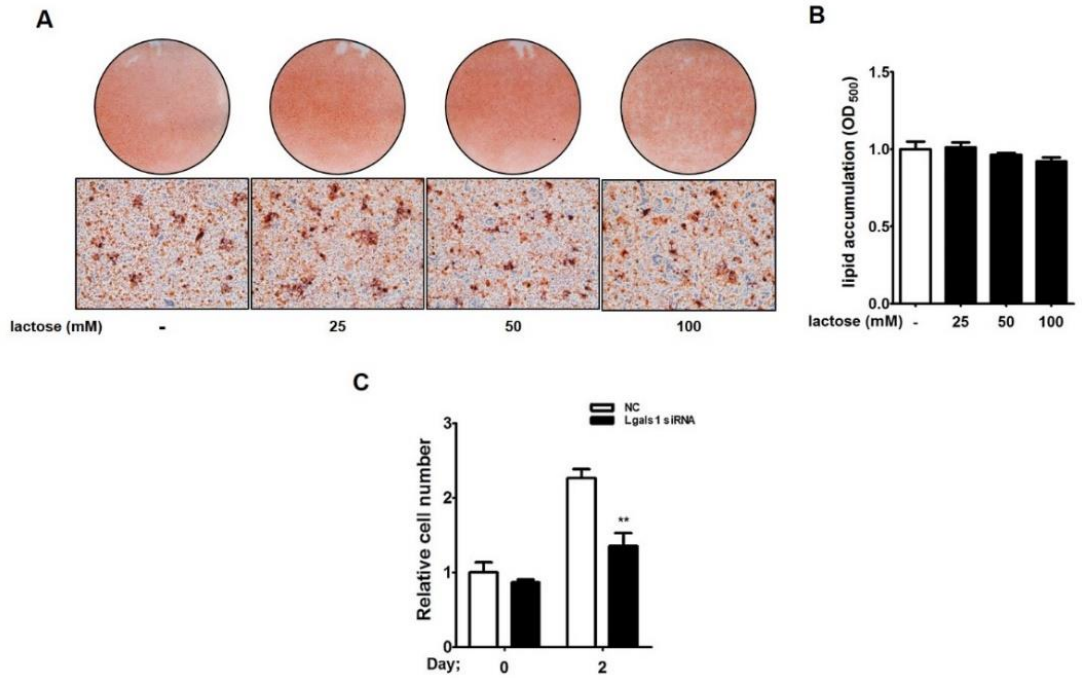


**Figure 2. Intracellular galectin-1 regulates adipocyte differentiation.** (A) Adipocyte differentiation in the absence or presence of lactose (25, 50 and 100mM). Lactose was treated for 6 days on adipocyte differentiation. (B) Measurement of lipid accumulation. Stained ORO dye was eluted with 100% isopropanol and measured using the $OD_{500}$. (C) Mitotic clonal expansion during adipocyte differentiation. Cells were counted 0 and 48h after induction of adipocyte differentiation. Data are presented as means ± SEM (n=3 for each lane) $^{**}P < 0.01$ for NC vs Lgals1 siRNA.

### 3. Lgals1$^{-/-}$ mice have resistance to high fat diet induced obesity

To confirm the role of galectin-1 in obese mouse model, we fed a high-fat diet containing 60% fat to male lgals1$^{+/+}$ and lgals1$^{-/-}$ mice (n=5) for 10 weeks. No significant difference in body weight was seen on normal chow. Lgals1$^{-/-}$ mice fed high fat diet had a lower body weight than lgals1$^{+/+}$ mice fed high fat diet (Figure 4A and B), even though food intake did not differ between lgals1$^{+/+}$ and lgals1$^{-/-}$ mice (Figure 4C). We measured the weight of mouse organs in involved in metabolism (Figure 4D). The visceral gonadal WAT and the subcutaneous inguinal WAT were smaller in lgals1$^{-/-}$ mice. But liver and BAT weight were not significantly different (Figure 4E). Because obesity is known the risk of type 2 diabetes mellitus (T2D) and hyperlipidemia, we detected levels of glucose, triglyceride and free fatty acids in serum. Fasting glucose was lower lgals1$^{-/-}$ mice in fed high fat diet (Figure 4F). Triglyceride and free fatty acids were not different between lgals1$^{+/+}$ and lgals1$^{-/-}$ mice. (Figure 4G and H).

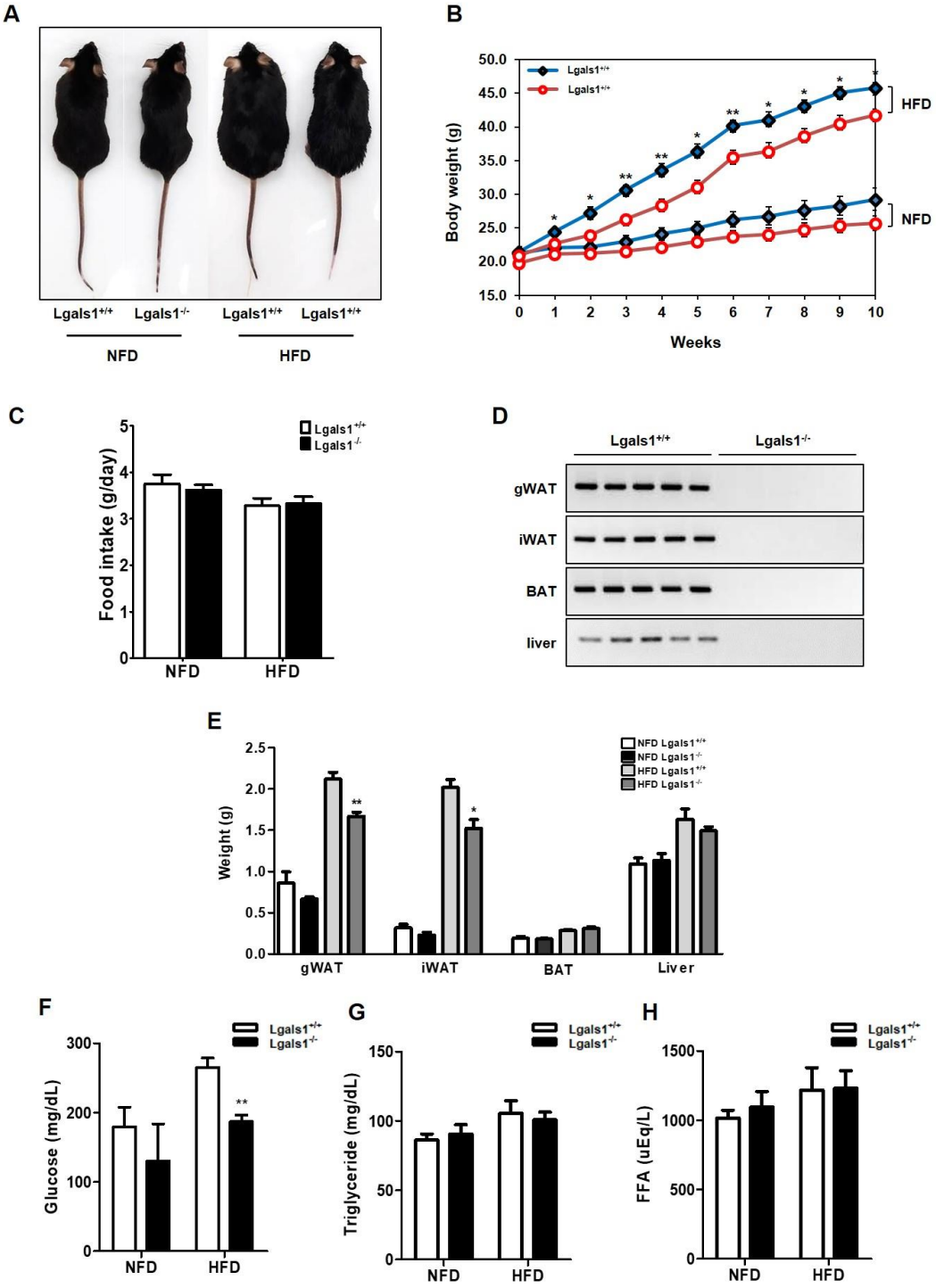

**Figure 3**. **Lgals1$^{-/-}$ mice are lean and have reduced WAT mass.** (A and B) Body size and weight of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice fed chow and high fat diet (60% fat) for 10 weeks. (C) Daily food intake of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice. (E) Expression of galectin-1 in gonadal WAT, inguinal WAT, BAT and liver of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice. Level of galectin-1 mRNA was analyzed by RT-PCR. (E) Weight of liver, gonadal WAT, inguinal WAT and BAT of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice fed chow and high fat diet for 10 weeks. (F, G, H) Levels of glucose, triglyceride and free fatty acid in serum. Data are presented as means ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ for lgals1$^{+/+}$ vs lgals1$^{-/-}$ mice.

**4. Lgals1$^{-/-}$ mice exhibit decreased adiposity and altered expression of genes involved in lipid metabolism and thermogenesis**

Gonadal WAT of lgals1$^{-/-}$ mice was smaller than those of lgals1$^{+/+}$ mice (Figure 5A). We detected a reduced adipocyte size in gonadal WAT of lgals1$^{-/-}$ mice fed a high-fat diet (Figure 5B and C). The expression of genes involved in lipid accumulation and synthesis significantly reduced in lgals1$^{-/-}$ mice (Figure 5D). Obesity causes infiltration of macrophage and elevation of pro-inflammatory cytokine in WAT. We measured the expression of macrophage markers and pro-inflammatory cytokine. But there was no significantly difference (Figure 5E). Thermogenesis as well as lipid accumulation and synthesis were reported to regulate the fat mass [21]. Therefore, we measured the expression of genes involved in thermogenesis in inguinal WAT and BAT. These genes were significantly elevated in both inguinal WAT and BAT (Figure 5F and G).

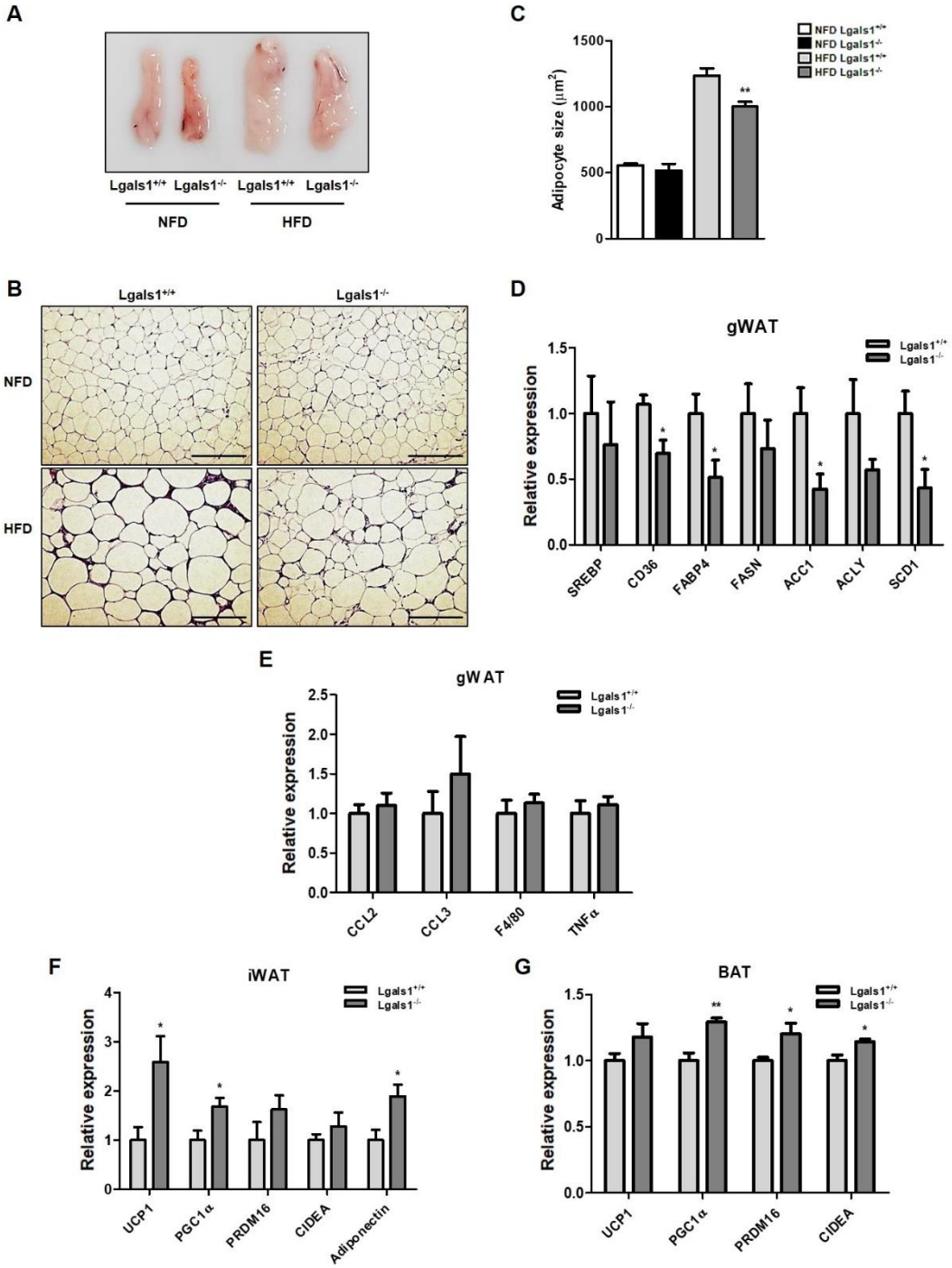

**Figure 4. Lgals1$^{-/-}$ mice have decreased expression of lipogenic genes and increased expression of thermogenic genes in adipose tissues**. (A) Gonadal WAT of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice. (B) Hematoxylin and eosin (H&E) section of gonadal WAT in chow and high fat diet fed mice. (C) Adipocyte size of gonadal WAT sections of chow and high fat diet fed mice. Size measurement was performed using imageJ software. (D) Real-time RT-PCR analysis of expression of genes involved in fatty acid metabolism in gonadal WAT of high fat diet fed mice. (E) Real-time RT-PCR analysis of pro-inflammatory cytokines and macrophage markers in gonadal WAT of high fat diet fed mice. (F and G) Real-time RT-PCR analysis of brown fat and thermogenic genes in inguinal WAT and BAT of high fat diet fed mice. Data are presented as means ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ for lgals1$^{+/+}$ vs lgals1$^{-/-}$ mice.

**5. Lgals1$^{-/-}$ mice have decreased expression of genes promoting hepatic gluconeogenesis and lipogenesis**

Because fatty liver diseases are often exhibited in obesity, we conducted histological analysis of liver. Although there was no difference between lgals1$^{+/+}$ and lgals1$^{-/-}$ mice fed chow, a phenotype of severe lesion was showed in the liver of lgals1$^{+/+}$ fed high fat diet (Figure 6A and B). We found that expression of genes in gluconeogenesis, such as glucose-6-phosphatase (G6PC) and Phosphoenolpyruvate carboxykinase (PCK), were significantly attenuated in lgals1$^{-/-}$ mice. Expression of FASN, ACC1, and SCD1 involved in lipogenesis was also down-regulated in lgals1$^{-/-}$ mice (Figure 6C).

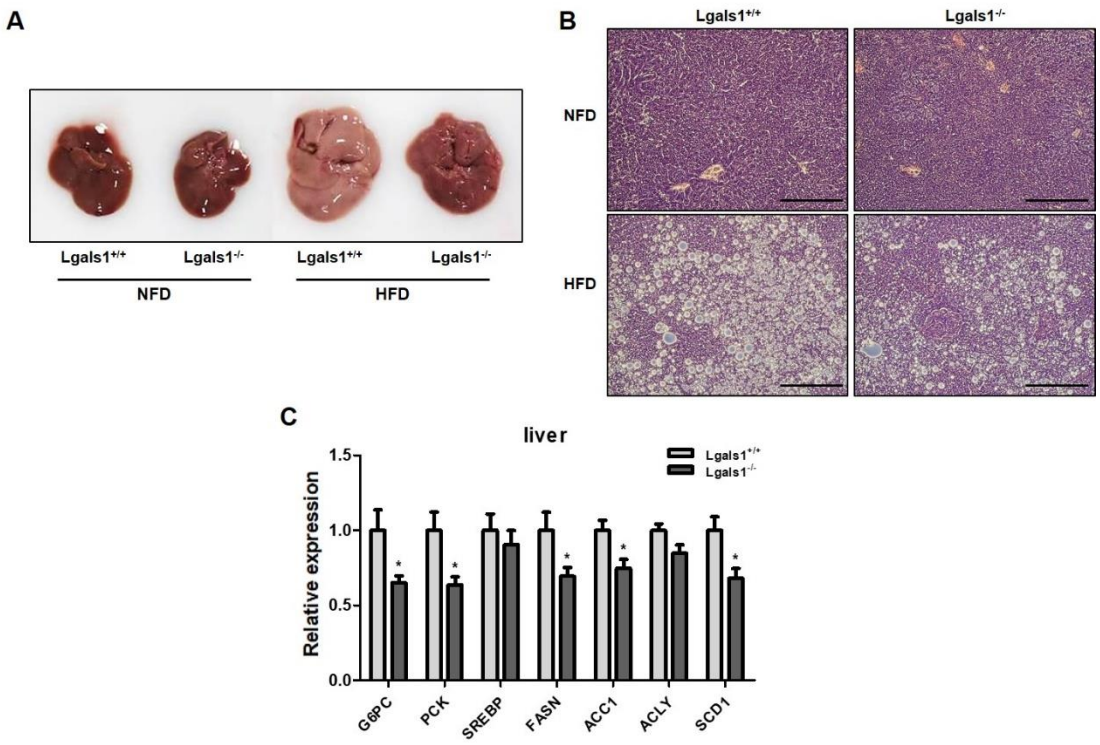


**Figure 5. Lgals1$^{-/-}$ mice improves fatty liver phenotype.** (A) Liver of lgals1$^{+/+}$ and lgals1$^{-/-}$ mice. (B) Hematoxylin and eosin section of liver in chow and high fat diet fed mice. (C) Real-time RT-PCR analysis of expression of genes involved in gluconeogenesis lipogenesis in liver of high fat diet fed mice. Data are presented as means ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ for lgals1$^{+/+}$ vs lgals1$^{-/-}$ mice.

## IV. DISCUSSION

Basic role of galectin family is to recognize glycosylation in glycoconjugates such as glycolipid and glycoprotein. Since glycoconjugates are mainly distributed on the cell surface [8], many studies have been investigated on the role of extracellular galectin family. Our group has focused on the role of intracellular galectin family in tumors [22-25]. Recently, the roles of galectin family in metabolic disorders have been investigated [4,18,26], but most of the studies on galectin family were focused on cancer and inflammatory diseases. Galectin-12 was reported to modulate adipocyte differentiation and HFD-induced obesity. However, the studies on other galectin family in obesity are still limited. There are only few evidences about function of galectin-1 and -3 in obesity [13,14]. We observed increase expression of galectin-1 and -3 in process of adipocyte differentiation, and investigated the role of galectin-1 and -3 in adipogenesis and HFD-induced obesity.

We confirmed that galectin-3-depleted pre-adipocyte 3T3-L1 cells exhibited delayed adipocyte differentiation. Lgals3$^{-/-}$ mice had reduced body weight and gWAT mass compared to wild-type (lgals3$^{+/+}$) mice, suggesting that galectin-3 deficiency increases resistance to high-fat diet-induced obesity. However, other groups reported that galectin-3 KO mice have increased adiposity. Young galectin-3 KO mice develop mild hyperglycemia followed by increased adiposity and systemic inflammation [27]. Galectin-3 deficiency induces obesity through systemic inflammation, increasing pro-inflammatory macrophages, type 1 T cells, and NKT cells while decreasing regulatory T cells and M2 macrophages [28]. Both studies observed amplified systemic inflammation in lgals3$^{-/-}$ mice. As galectin-3 deficiency may increase inflammation and control metabolism and obesity, it is possible that galectin-3

regulates the inflammatory response and consequently regulates obesity and glucose metabolism. However, these studies did not focus whether galectin-3 deficiency regulates the expression and activity of genes regulating adipocyte differentiation and fat accumulation. We examined the regulation of adipogenic factors by galectin-3. We did not observe symptoms of amplified inflammation in our lgals3$^{-/-}$ mice. Other group also reported that lgals3$^{-/-}$ mice were protected from inflammation [26].

DNA microarray analysis revealed that lipogenic genes were reduced in liver tissues of lgals3$^{-/-}$ mice. Up-regulation of lipogenic genes contributes to excessive TG accumulation in the liver, as well as fatty liver diseases. Previous reports demonstrated that galectin-3 deficiency inhibited hepatic fibrosis [29,30] and protected against nonalcoholic steatohepatitis (NASH) [31,32], suggesting that galectin-3 might be a therapeutic target in liver diseases, such as fatty liver, steatosis, and cirrhosis, through regulation of lipid accumulation and fibrosis. Although we could not detect any serious liver abnormalities in lgals3$^{+/+}$ mice, the change observed in lipogenic genes is sufficient evidence indicating that overexpression of galectin-3 in liver may induce liver disease.

We confirmed that adipocyte differentiation is reduced by galectin-1 knockdown. In addition, treatment with galectins inhibitor, TDG reduced adipocyte differentiation and improved obesity in HFD Rat [15]. TDG is a non-metabolized disaccharide known to bind to galectin-1, -3, -8 and -9, and inhibits function of intracellular and extracellular galectins [33,34]. Inhibition of extracellular galectins by lactose treatment did not affect adipocyte differentiation. This result suggests that the regulation of adipocyte differentiation by galectin-1 is due to the intracellular mechanism. The expression of nuclear galectin-1 in adipocyte differentiation also supports this finding [15].

Lgals1$^{-/-}$ mice showed a decrease in body weight and adipose tissue mass, and also had lower fasting glucose level, resulting improvement of diabetes mellitus. According to previous studies on galectin-1 and diabetes, treatment of soluble galectin-1 promotes apoptosis of pathological Th1 cells, causing pancreatic B-cell destruction in nonobese diabetic (NOD) mice model [35] Obesity cause infiltration of macrophage and elevation of pro-inflammatory cytokine in WAT [36]. The expression of macrophage markers and pro-inflammatory cytokine did not decrease in lgals1$^{-/-}$ mice, despite improved obesity. Galectin-1 inhibits the secretion of pro-inflammatory cytokine, such as TNFα and IFNγ [37]. TNFα is one of the typical cytokines that cause insulin resistance [38]. On the diabetic side, deficiency of galectin-1 has pro-diabetic effect that reduces pancreatic B-cell function and increase inflammation of adipose tissues. In our results, we focused on lipogenesis and lipid accumulation in adipose tissue and did not observe pancreatic B-cell function and insulin resistance. Further studies aimed at role of galectin-1 in glucose homeostasis are necessary to determine whether galectin-1 has pro- or anti-diabetic effects.

In addition, we confirmed increased expression of thermogenic genes in iWAT and BAT. Further studies should be conducted to confirm whether the increase of thermogenic genes by galectin-1 knockdown is due to the regulation of the B-adrenergic signal, which is a typical regulator of thermogenic gene expression.

We found that lgals1$^{-/-}$ liver has lower expression of genes involved in gluconeogenesis and lipogenesis. Accumulation of hepatic triglyceride was also reduced in lgals1$^{-/-}$ liver. It is unclear whether this result is a direct regulation of hepatic gene by galectin-1 or additional effect of obesity improvement. Recent study reported that the interaction of galectin-1 and neuropilin-1

promote liver fibrosis through activation of hepatic stellate cells [39]. These results suggest that galectin-1 can directly modulate not only obesity but also liver disease.

Taken together, this study demonstrates that galectin-1 and -3 are a positive regulator of adipocyte differentiation and development of obesity.

## V. CONCLUSION

The level of galectin-1 and -3 increased during adipocyte differentiation and was highly expressed in mouse WAT. Galectin-1 and -3 knockdown significantly reduced adipocyte differentiation in 3T3-L1 cells. Galectin-3 interacted with PPARγ and regulated transcriptional activity of PPARγ. Inhibition of extracellular galectin-1 by lactose did not affect adipocyte differentiation. After 10-12 week high-fat diet (60% fat), lgals1$^{-/-}$ and lgals3$^{-/-}$ mice had resistance to high fat diet induced obesity. The expression levels of genes in were significantly down-regulated in liver and gonadal WAT of lgals1$^{-/-}$ and lgals3$^{-/-}$ mice. In addition, galectin-1 deficient mice had elevated expression of genes involved in thermogenesis in inguinal WAT and BAT. We suggest that galectin-1 and -3 might be potential therapeutic target in obesity.

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## ABSTRACT (IN KOREAN)

# 당인식 단백질 갈렉틴에 의한 지방세포 분화 조절

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백정환

Galectin-1 과 -3 는 베타갈락토사이드에 결합하는 당 인식 결합 도메인을 가지고 있는 렉틴 계열의 단백질이다. 기존에 Galectin-1 과 -3 에 관련된 많은 연구는 암과 면역질환에서 진행되었다. 하지만 대사질환과 관련된 연구는 거의 진행되지 않았다. 본 논문에서는 Galectin-1 과 -3 가 지방세포 분화와 고지방식이로 유도되는 비만을 조절하는지에 대한 연구를 진행하였다. Galectin-1 과 -3 는 지방세포 분화과정에서 발현이 증가하였고, 마우스의 다른 조직에 비하여 지방 조직에서 상대적으로 높은 발현을 보였다. 또한 비만을 유도한 마우스의 지방 조직에서 galectin-1 과 -3 의 발현이 증가하는 것을 확인하였다. 3T3-L1 세포에서 galectin-1 과 -3 의 발현을 억제하면 지방세포의 분화가 감소하였고 지방세포 분화와 지방 축적에 관련된 유전자인 PPARγ, C/EBPα, FABP4 와 FASN 의 발현이 감소하였다. 세포 내부의 galectin-3 가 지방세포 분화 과정에서 중요한 인자인 PPARγ와 결합을 하고 galectin-3 의 발현을 억제하였을 경우에 PPARγ의 핵내 축적과 PPARγ의 전사조절 능력이

감소하는 것을 확인하였다. Galectin-1 은 세포의 핵, 세포질과 세포 외부 등에 고르게 분포되어 있고, 각각의 위치에서 다양한 역할을 수행한다. Galectin-1 에 결합할 수 있는 락토스를 처리하여 세포 외부에 있는 galectin-1 의 기능을 억제하였을 경우에 지방세포 분화는 감소하지 않았다. 이를 통하여 지방세포 분화를 조절하는 galectin-1 의 기능은 세포 외부가 아니라 세포 내부에서 이루어지는 것임을 알 수 있다. 마우스에 고지방식이를 하여 비만을 유도한 결과, Galectin-1 넉아웃 마우스와 galectin-3 넉아웃 마우스가 정상 쥐에 비하여 몸무게와 지방 조직의 양이 적게 증가하였다. 지방합성에 관여한 유전자의 발현이 Galectin-1 넉아웃 마우스와 galectin-3 넉아웃 마우스의 간과 지방 조직에서 발현이 감소하였다. 또한 열 발생에 관여하는 유전자의 발현이 Galectin-1 넉아웃 마우스의 갈색지방과 피하지방에서 증가하였다. 이러한 결과를 통하여 Galectin-1 와 -3 는 비만 치료를 위한 좋은 타겟이 될 가능성을 제시하였다.

핵심되는 말 : galectin-1, galectin-3, PPAR gamma, 지방세포 분화, 비만