The function of DLL4, a ligand of Notch signaling, in regulating metabolic disease

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Directed by Professor Kyung-Hee Chun

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ABSTRACT

The function of DLL4, a ligand of Notch signaling, in regulating metabolic disease

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(Directed by Professor Kyung-Hee Chun)

Delta-like 4 (DLL4), one of ligands of Notch signaling, which is mostly known to regulate development, morphogenesis, cell differentiation or stem cell maintenance. There are several researches showing that Notch signaling has an important function in liver during obesity. When DLL4 was blocked by its antibody, glucose tolerance and insulin resistance got improved and weight loss in mice model. Also when the Notch inhibitor was injected to mice with NASH, liver fibrosis levels were improved. These previous studies indicate the relationship between Notch

signaling and metabolic diseases, but specific molecular mechanism study has not been done.

In this study, I've investigated whether DLL4 regulates metabolic diseases like Nonalcoholic Steatohepatitis (NASH) and Nonalcoholic Fatty Liver Disease (NAFLD). Liver tissues of high-fat diet (HFD) induced obese mice had several increased mRNA levels of Notch signal factors, but the expression level of DLL4 was highest. DLL4 TG x Albumin cre mice were fed HFD, and as a result, these mice had definitely higher body weight and liver sizes compared to the WT mice. AST and ALT, which are the indicators of liver toxicity, were higher in DLL4 TG x Alb cre mice and also the fibrosis and inflammation markers of their mRNA of liver. From the Sirius red staining, liver sections of DLL4 TG x Alb cre mice had massive distribution of collagen. Accordingly, these data show that NASH was induced in DLL4 TG mice only through the HFD. When knock down study of DLL4 was processed, lipid accumulation and lipid related gene expression levels were decreased. For the in vivo study, HFD fed obese mice were injected with DLL4 antibody, and these mice has shown improved metabolic rates. Taken together, DLL4 plays an important role in inducing inflammation and fibrosis on liver, and it is assumed that DLL4 could be a potent approach in treating patients with liver steatosis or NASH.

Key word: DLL4, Metabolic disease, Obesity, Liver Steatosis, NASH

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I. INTRODUTION

Obesity is a global health problem which leads to adverse effects in terms of both physical and mental health.[1] Obesity leads to the development of various kinds of metabolic diseases such as type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease.[2] NAFLD is closely linked with obesity, and according to the global reports, as the number of obese patients increases, NAFLD patients also increases.[3] NAFLD includes simple steatosis and nonalcoholic steatohepatitis (NASH), which might cause cirrhosis and even hepatocellular carcinoma.[4] NASH is an advance form of NAFLD and has typical characteristics of liver fibrosis and inflammation.[5] Free fatty acids, generated by excessive hepatic de novo lipogenesis, are critical in NASH pathogenesis.[6] Nevertheless, the patients with

NAFLD or NASH are increasing, there are no specific therapeutics for them and its medical needs are high.[7]

Delta-like 4 (DLL4), is one of the ligands of Notch signaling cascades, and is known to regulate development, morphogenesis, cell differentiation or stem cell maintenance.[8] This Notch signal has 4 kinds of receptor, Notch 1 through 4 and ligands like Jagged and DLL4 binds to it.[9] Along with the binding, a disintegrin and metalloprotease (ADAM) family and gamma-secretase gets cut, and Notch intracellular domains moves into the nucleus to bind with transcription factors.[10] This Notch signal is also known to have an important function in the liver during obesity.[11],[12] According to the previously published papers, Notch activation enhances pathologic glucose output and fatty liver in obese mice in conjugation with diminished Akt activation by insulin.[13] The Notch activation in obesity causes worsened diabetes and fatty liver, due to the increased lipogenesis and diminished insulin signaling.[14] Also, there was a previously reported studies about DLL4 regulating the overall metabolism of high-fat diet (HFD) induced obese mice.[15] When the DLL4 was blocked by its antibody, glucose tolerance and insulin resistance got improved and weight loss in mice model, and when DLL4 was activated, inflammation rates in adipose tissues and liver were increased.[15],[16],[17] These previous studies indicate the relationship between Notch signaling and metabolic diseases, but specific molecular mechanism study has not been done.

To investigate whether DLL4 regulates metabolic diseases like Nonalcoholic Steatohepatitis (NASH) and Nonalcoholic Fatty Liver Disease (NAFLD). Liver tissues

of high-fat diet (HFD) induced obese mice had several increased mRNA levels of Notch signal factors, but the expression level of DLL4 was highest. DLL4 TG x Albumin cre mice were fed HFD, and as a result, these mice had definitely higher body weight and liver sizes compared to the WT mice. AST and ALT, which are the indicators of liver toxicity, were higher in DLL4 TG x Alb cre mice and also the fibrosis and inflammation markers of their mRNA of liver. From the Sirius red staining, liver sections of DLL4 TG x Alb cre mice had massive distribution of collagen. Accordingly, these data show that NASH was induced in DLL4 TG mice only through the HFD. When knock down study of DLL4 was processed, lipid accumulation and lipid related gene expression levels were decreased. For the in vivo study, HFD fed obese mice were injected with DLL4 antibody, and these mice has shown improved metabolic rates. Taken together, DLL4 plays an important role in inducing inflammation and fibrosis on liver, and it is assumed that DLL4 could be a potent approach in treating patients with liver steatosis or NASH.

II. MATERIALS AND METHODS

1. Cell culture and transfection

AML12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 medium (1:1) with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, 10% fetal bovine serum (Corning Cellgro), and 1% antibiotics (Invitrogen) and maintained at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. 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 antibiotics. Confluent 3T3-L1 cells were incubated for 48 hours. Then, media was replaced DMEM supplement with 10% fetal bovine serum (FBS), dexamethasone (1 μM), insulin (1 μg/ml) and isobutylmethylxanthine (520 μM). After 48 hours, media was replaced DMEM supplemented with 10% FBS and insulin (1 μg/ml). After 48 hours, media was replaced DMEM supplemented with 10% FBS. AML12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 medium (1:1) with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, 10% fetal bovine serum (Corning Cellgro), and 1% antibiotics (Invitrogen) and maintained at 37℃ in a humidified incubator with a 5% CO_2 atmosphere.

Transfection with DLL4 overexpression vector and DLL4 siRNA were performed with Lipofectamine 2000 and Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad,

CA, USA) respectively, according to the manufacturer's instruction. DLL4 siRNA (5′-CCCAAGUUCUGCUGUUGGACU-3′) were purchased from COSMOGENETECH (GenePharma Co, Shanghai, China). Cells were harvested two days after the transfection for use in subsequent experiments.

2. Total RNA isolation, reverse transcription-polymerase chain reaction

RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a reverse transcription system (TOYOBO, Tokyo, Japan) and primers listed in Table 1. PCR was performed using instructions given in Ex-Taq (TaKaRa, Kyoto, Japan) manual. 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.

3. Western blotting

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 minutes on ice and centrifuged at 4℃ for 25 minutes at 13,200 rpm. 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 or Nitrocellulose membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk or BSA for 1 hour at room temperature. After blocking, membranes were incubated with primary antibodies. overnight at 4℃. The membranes were washed 3 times for 10 minutes with PBST and incubated with HRP-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA) for 1 hour at room temperature. The membranes were washed 3 times for 10 minutes with PBST. The FUSION SOLO S (Vilber, Eberhardzell, Germany) was used for image detection according to manufacturer's directions. All results were normalized by b-actin.

4. Immunohistochemistry

Tissue specimens were fixed in 4% paraformaldehyde (Biosesang, Seoul, Korea). The fixed tissues were embedded in paraffin blocks and were sliced into 0.4-μm-thick sections. α-SMA, DLL4, and F4/80 in liver slides were immunohistochemically detected using Vectastain ABC kit and DAB substrate kit (Vector Laboratories, Burlingame, Ca, USA) , according to the manufacturer's directions.

5. Oil red O staining

Cells were washed with DPBS and incubated in 10% formalin for 10 minutes. 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/100 ml) was diluted with

isopropanol to make 60% ORO working solution. The dried cells were stained with ORO working solution for 30 minutes and washed third time with distilled water.

6. Animal experiments

All animal experiments were approved by the Institutional Review Board of the Yonsei University College of Medicine and were performed in specific pathogen-free facilities according to the university's guidelines for the Care and Use of Laboratory Animals (2015-0376). 6 weeks old C57BL/6 mouse was purchased from Orientbio. After 1 week of stabilization of mice, fed with a high fat diet containing 60% fat for 10-12 weeks (12 hours light, 12 hours dark cycle).

7. Mouse Primary Hepatocytes

Hepatocytes were isolated from mice at 8–10 weeks of age. Under anesthesia, the abdominal cavity was opened, and the liver was perfused via the portal vein first with a Ca2+/Mg2+-free Hanks' buffered salt solution (HBSS) with EDTA, second with a Ca2+/Mg2+-free HBSS (without EGTA), and perfusion with Ca2+/Mg2+-free HBSS containing 0.195 mg/ml of type I collagenase (Sigma). The hepatocytes were then gently shaken in the collagenase solution for 10 minutes. The digested tissue was run through, sequentially, 100μm and 70μm nylon meshes. The suspension was centrifuge at 600 rpm (50 × g) for 5 minutes at 4℃ to wash and differentially sediment hepatocytes from other cell types. The resulting cell pellet was re-suspended, and an aliquot was taken to determine cell number and viability by the trypan blue exclusion test. The cells

were plated at a density of 2.0 × 105 cells/well onto collagen-coated 12-well plates (SPL Life Science, Pocheon-si, Korea) in Medium 199 (sigma) supplemented with 10% FBS and 1% penicillin/streptomycin and allowed to adhere for 4 hours. The media were then replaced with fresh ones and 1% antibiotics (Invitrogen) and maintained at 37℃ in a humidified incubator with a 5% CO_2 atmosphere.

8. Liver digestion

Livers were extracted and finely minced. Liver tissue was additionally blended with gentle MACS Dissociator (#130-093-235, miltenyi Biotec, Berghish Glatbach, Germany) and digested with MACS Miltenyi Liver Dissociation Kit for mouse (#130-105-807, miltenyi Biotec) according to manufacturer's instructions. Dissociated liver cells were washed with DMEM and passed through a 70M cell strainer (SPL Life Science) followed by lysis of red blood cells using RBC Lysis solution (Biosesang, Seongnam-si, Korea). And liver cells were then washed, and collected to analyze Flow cytometry.

9. Flow cytometry

Single-cell suspensions were made from the liver tissues. Livers were extracted and processed as described above before re-suspension in PBS buffer containing 2% FBS and 2mM EDTA for flow cytometric analysis. LIVE/DEAD™ fixable Near-IR Dead Cell Stain Kit (L-10119, Invitrogen) was applied to cells in combination with anti-mouse CD16/CD32 Fc blocker antibody (#14-0161-81, Invitrogen) for 15 minutes

on ice in the dark. Cells were washed and incubated with fluorochrome-conjugated antibody (anti-mouse Ly-6C APC, clone HK1.4, Biolegend cat. 128016; anti-mouse Ly-6G PE, clone 1A8, Biolegend cat. 1027607; anti-mouse F4/80 APC, clone BM8, Biolegend cat. 123115; anti-mouse I-A/I-E APC, clone M5/114.15.2, Biolegend cat. 107614; anti-mouse CD11b FITC, clone M1/70, Biolegend cat. 1001206) at manufacturer's recommended dilution for 30 minutes on ice in the dark. Cells were washed with PBS and resuspended in FACS buffer for flow cytometric analysis on BD LSRFortessa at the flow cytometry core of the Avision Biomedical Research Center in Yonsei College of Medicine. 10,000-1,000,000 cells were analyzed per sample using BD FACS Diva Software. The data was analyzed with Flow Jo software.

10. Sirius Red staining

To examine hepatic morphology and assess liver fibrosis, H&E staining and Sirius red staining were performed, respectively. Liver specimens were fixed in 10% neutral buffered formalin (Sigma), embedded in paraffin and cut into 4 μm sections. Next, the specimens were deparaffinized, hydrated and stained by standard methods.

11. Body fat composition analysis

Dual energy X-rat Absorptiometry (DEXA) measurements were used to compare body fat between HFD fed C57BL6J mice injected with vehicle or DLL4 antibody. DEXA measurements were performed after 12 weeks of administration using a total-body scanner. DXA measures one time with low energy and one time with high energy

to separate the images into bones and tissues in gram units by separating them into fat and lean before analysis. Radiography of body fat was displayed by three modes according to low density fat (blue color), medium density fat (yellow color) and high density fat (red color).

12. Statistical analysis

Statistical evaluations of the data were expressed as the mean ± SEM. The statistical significance of the differences be-tween the mean values for the treatment groups was analyzed by Student's t-tests and one-way analysis of variance (ANOVA) using the software GraphPad Prism software (version 6; GraphPad Software Inc., La Jolla, CA). Data were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Table 1. Primer lists and sequence for RT-PCR

primer	Sequence (5' to 3')
β-actin	Forward: CCAGTTGGTAACAATGCCATGT
	Reverse: GGCTGTATTCCCCTCCATCG
Notch1	Forward: TGAGAATGATGCCCGCACTT
	Reverse: CAGGTGCCCTGATTGTAGCA
Notch2	Forward: AGCAGGAGCAGGAGGTGATA
	Reverse: TGGGCGTTTCTTGGACTCTC
Notch3	Forward: CAGGCGAAAGCGAGAACAC
	Reverse: GGCCATGTTCTTCATTCCCA
Notch4	Forward: ATGACTCCTTGCCCTCTCTCT
	Reverse: CTCTCACCCTTTAGTCCCTCAGA
Jagged1	Forward: TGGACTGGCCCCACGTGTTC
	Reverse: GGGCGGGCACACACTTGAA
Vegf1	Forward: AAAGGCTTCAGTGTGGTCTGAGAG
	Reverse: GGTTGGAACCGGCATCTTTATC
Vegf2	Forward: TTAGAGCTCAACCCAGACACCTGTA
	Reverse: CCTGTGAAGCAGGGCCATAA
Hes1	Forward: ACACCGGACAAACCAAAGACA
	Reverse: AATGCCGGGAGCTATCTTTC
Hes3	Forward: TCAACGAGAGCCTAAGCCAGCT
	Reverse: CGCACAGTCATTTCCAGGATGTC
Hes5	Forward: CACCAGCCCAACTCCAAG
	Reverse: AGTAGCCCTCGCTGTAGTCC
Hey1	Forward: GCCGAAGTTGCCCGTTATCTG
	Reverse: TGTGTGGGTGATGTCCGAAGG
Hey2	Forward: TCCACCTCTCTTCTGTCCGAAGG
	Reverse: GACTGGAGGCTGCGGATACC
DLL1	Forward: CGGGCCAGGGGAGCTACACA
	Reverse: AGCTGTCCTCAAGGTCCGTGG

DLL3	Forward: TGCCCTTCCGCGATGCTTGG
	Reverse: CTCCCATGTGCCTGCGCT
DLL4	Forward: CAGCATCCCCTGGCAGTGTGC
	Reverse: GCTGGCACACTTGCTGAGTCCC
CD36	Forward: GATGACGTGGCAAAGAACAG
	Reverse: TCCTCGGGGTCCTGAGTTAT
FABP4	Forward: TGAAATCACCGCAGACGACAGG
	Reverse: GCTTGTCACCATCTCGTTTTCTC
CEBPb	Forward: AGAAGACCGTGGACAAGCACAG
	Reverse: CTCCAGGACCTTGTGCTGCGT
Pparg	Forward: GTACTGTCGGTTTCAGAAGTGCC
	Reverse: ATCTCCGCCAACAGCTTCTCCT
a-SMA	Forward: CTATGCCTCTGGACGCACAACT
	Reverse: CAGATCCAGACGCATGATGGCA
col1a1	Forward: CCTCAGGGTATTGCTGGACAAC
	Reverse: CAGAAGGACCTTGTTTGCCAGG
col1a2	Forward: ATCCAACTAAGTCTCCTCCCTTGG
	Reverse: CTCTGTGGAAGATAGTCAGATGG
col3a1	Forward: GGATCAGGCCAGTGGAAATGTAAAGA
	Reverse: CTTGCGTGTTCGATATTCAAAGACTGTT
col4a1	Forward: CTGGCACAAAAGGGACGAG
	Reverse: ACGTGGCCGAGAATTTCACC
IL-10	Forward: ATCGATTTCTCCCCTGTGAA
	Reverse: TTCCGGAGAGAGGTACAAACGA
INFg	Forward: GAGCCAGATTATCTCTTTCTACC
	Reverse: GTTGTTGACTCTCAAACTTGG
CCL2	Forward: TAAAAAACCTGGATCGGAACCAA
	Reverse: GCATTAGCTTCAGATTTACGGGT
TNFa	Forward: CGTCAGCCGATTTGCTATCT
	Reverse: CGGACTCCGCAAAGTCTAAG
TGFb	Forward: CGTCAGCCGATTTGCTATCT
	Reverse: CGGACTCCGCAAAGTCTAAG

III. RESULTS

Part 1.

1. In NASH patients and HFD induced obese DLL4 TG x Alb cre mice has elevated expression levels of DLL4

First, I've determined the expression levels of DLL4 in livers of control patients, healthy obese patients, steatosis patients, and NASH patients available in NCBI database (Fig. 1A). According to this data, all patient groups had higher DLL4 mRNA expression levels than normal healthy patients. Then, mRNA expression levels of Notch signal related genes were measured in the livers of control and HFD fed obese mice. Interestingly, the expression level of DLL4 was the highest among the Notch signal factors (Fig. 1B). Also, when DLL4 TG mice were fed high-fat diet for 20 weeks, male mice had extremely increased body weights compared to wild type mice group (Fig. 1C). Based on these results, I could assume that there is a relationship between DLL4 and liver metabolism.

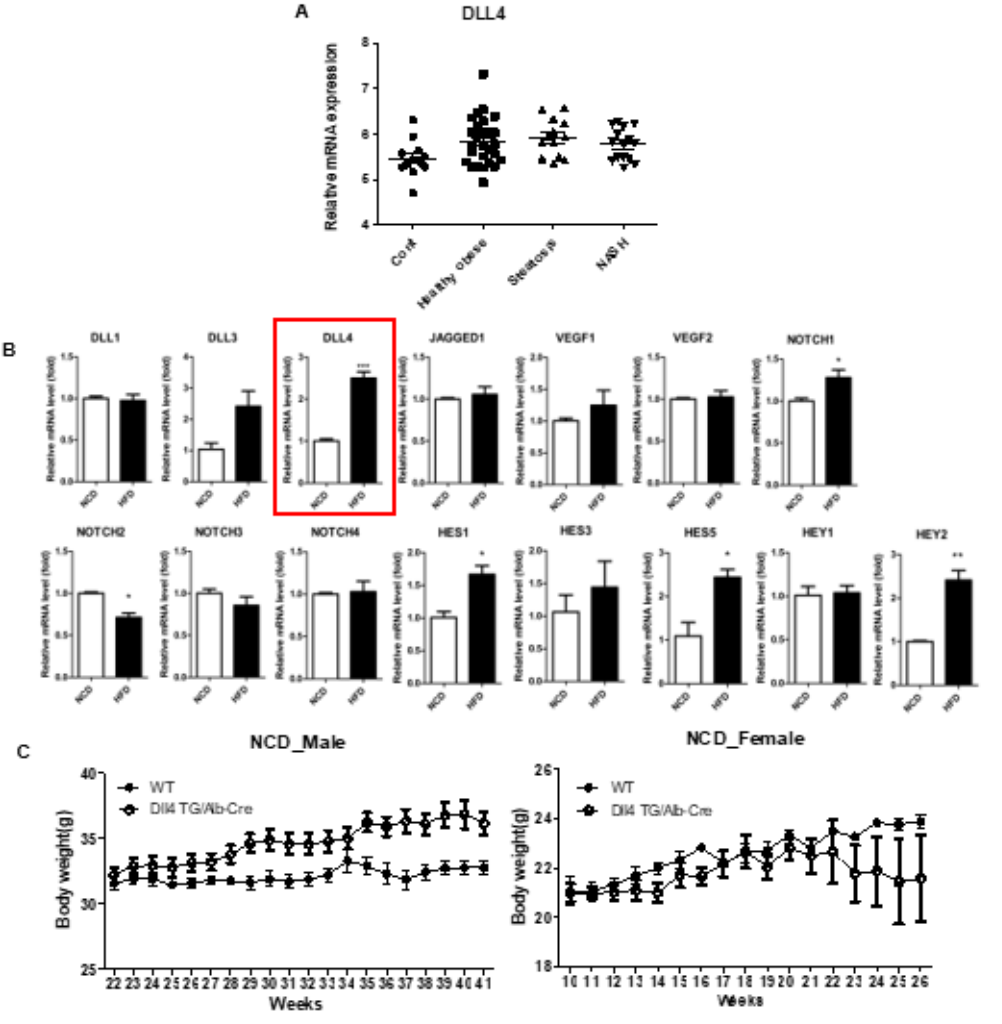


Figure 1. DLL4 is overexpressed in NASH patients and HFD DLL4 TG x Alb cre mice. (A) mRNA expression level of DLL4 of healthy control liver, healthy obese liver, steatosis liver, and NASH liver from patients are presented as diagrams. (B) mRNA expression levels were analyzed by qRT-PCR and b-actin was used as a normalization control. (C) Wild type and DLL4 TG male and female mice were fed high-fat diet for about 20 weeks, and their body weights were measured every week. Significant

differences are indicated by an asterisk (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), and p values were calculated using the Student's t test.

2. DLL4 overexpressed obese mice has higher body weight and aggravated metabolic phenotypes

To identify the role of DLL4 in mouse liver, DLL4 liver-specifically overexpressing mice (DLL4 TG x Alb cre) were generated, this model was fed with HFD to induce obesity and characterized the phenotypes (Fig. 2A). During the 22 weeks of HFD, male group of DLL4 TG x Alb cre mice had gained more weight gradually than WT mice group (Fig. 2B). After 22 weeks, DLL4 TG x Alb cre mice had significantly higher body weight than WT (Fig. 2C). Also, DLL4 TG x Alb cre mice liver had relatively more beige colored, larger and heavier weight (Fig. 2D – 2G). From the H&E staining of liver sections, DLL4 TG x Alb cre mice had more lipid droplets compared to the WT mice. Accordingly, their adipose tissues were also analyzed. Inguinal white adipose tissue (iWAT) of DLL4 TG x Alb cre mice were larger and heavier than those of WT mice (Fig. 2H, 2J and 2K). From the H&E staining of iWAT sections, DLL4 TG x Alb cre mice had relatively larger adipocyte size (Fig. 2I). gWAT had similar phenotypes as iWAT (Fig. 2L – 2O). Brown adipose tissue (BAT) of DLL4 TG x Alb cre mice were significantly larger and heavier than those of WT mice (Fig. 2F, 2R and 2S). From the H&E staining of BAT sections, DLL4 TG x Alb cre mice had larger and more lipid droplets (Fig. 2Q). These in vivo data suggests that liver-specifically DLL4 overexpressing obese mice is more vulnerable to lipid accumulation and weight gaining.

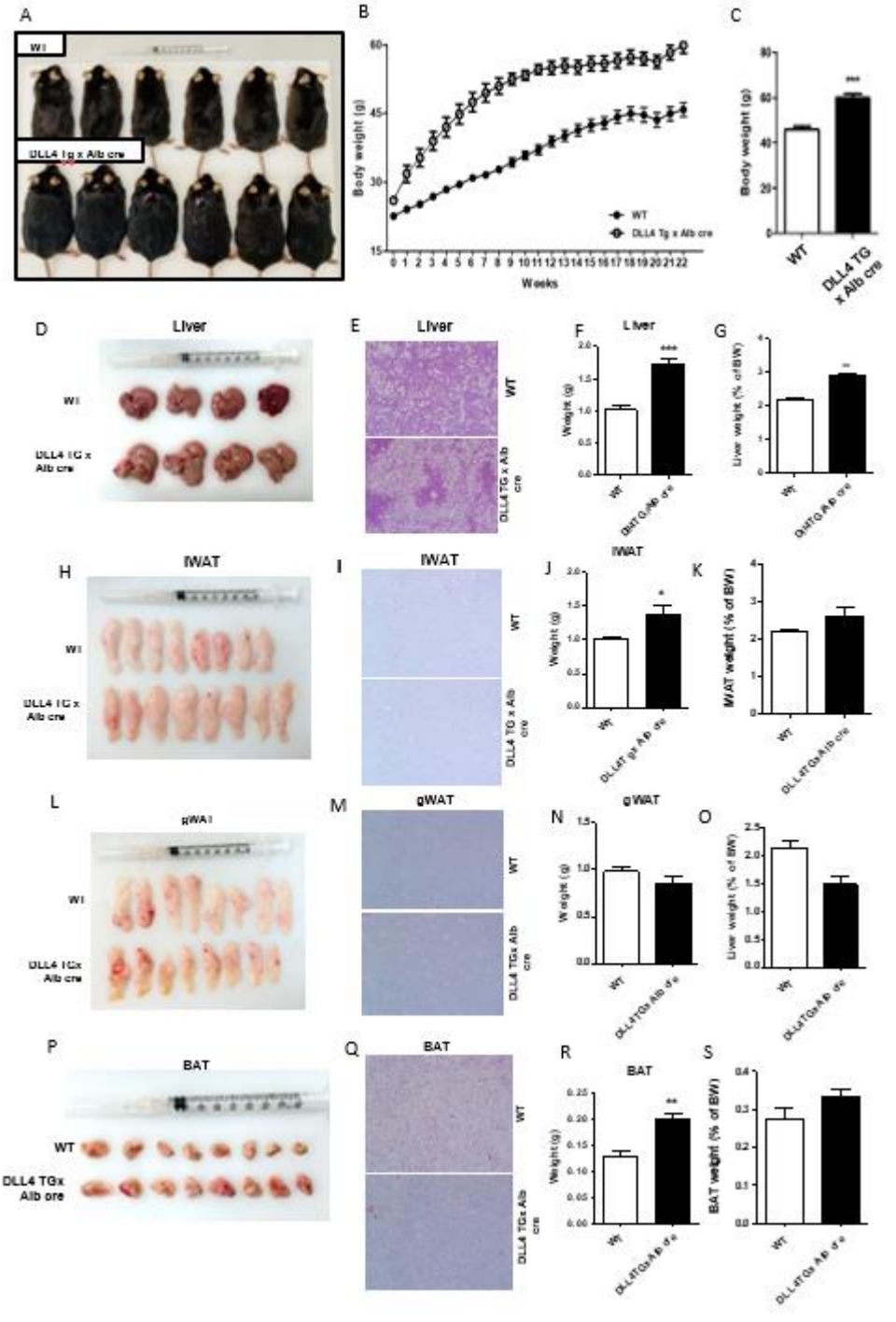


Figure 2. DLL4 overexpression aggravates phenotypes of HFD induced obese mice.

(A) Representative picture of mice fed HFD for 22 weeks. (B) Body weights of HFD fed WT and DLL4 TG x Alb cre mice for 22 weeks. (C) Final body weight after the 22 weeks of HFD. (D) Representative picture of liver. (E) H&E staining of liver sections. (F) Weight of liver. (G) Ratio of liver weight to body weight. (H) Representative picture of iWAT. (I) H&E staining of iWAT sections. (J) Weight of iWAT. (K) Ratio of iWAT weight to body weight. (L) Representative picture of gWAT. (M) H&E staining of gWAT sections. (N) Weight of gWAT. (O) Ratio of gWAT weight to body weight. (P) Representative picture of BAT. (Q) H&E staining of BAT sections. (R) Weight of BAT. (S) Ratio of BAT weight to body weight. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test.

3. DLL4 overexpressing mice develop NASH phenotypes after HFD feeding

To investigate the role of DLL4 in the liver of mouse model, blood serum of these mice were analyzed, and both ALT and AST were higher in DLL4 overexpressing mice, which indicates a liver injury (Fig. 3A). Moreover, total TG, glucose level, total cholesterol, and free fatty acids in DLL4 overexpressing mice had significantly higher levels than the WT mice Then, liver sections of 22 weeks HFD fed WT and DLL4 TG x Alb cre mice were used for the further analysis (Fig. 3B). According to the H&E and ORO staining, larger and more lipid droplets were observed. To analyze the fibrosis levels of liver, Sirius red staining was processed. According to the results, DLL4 TG x Alb cre mice had more distribution of collagen. Also IHC staining of α-SMA ensures that fibrosis have occurred and F4/80 staining indicates that more inflammation had occurred in DLL4 TG x Alb cre mice liver. Accordingly, mRNA expression levels of NASH markers of those livers were analyzed. Lipid accumulation, fibrosis and inflammation related gene expression levels were higher in DLL4 overexpressing mice (Fig. 3C – 3E).

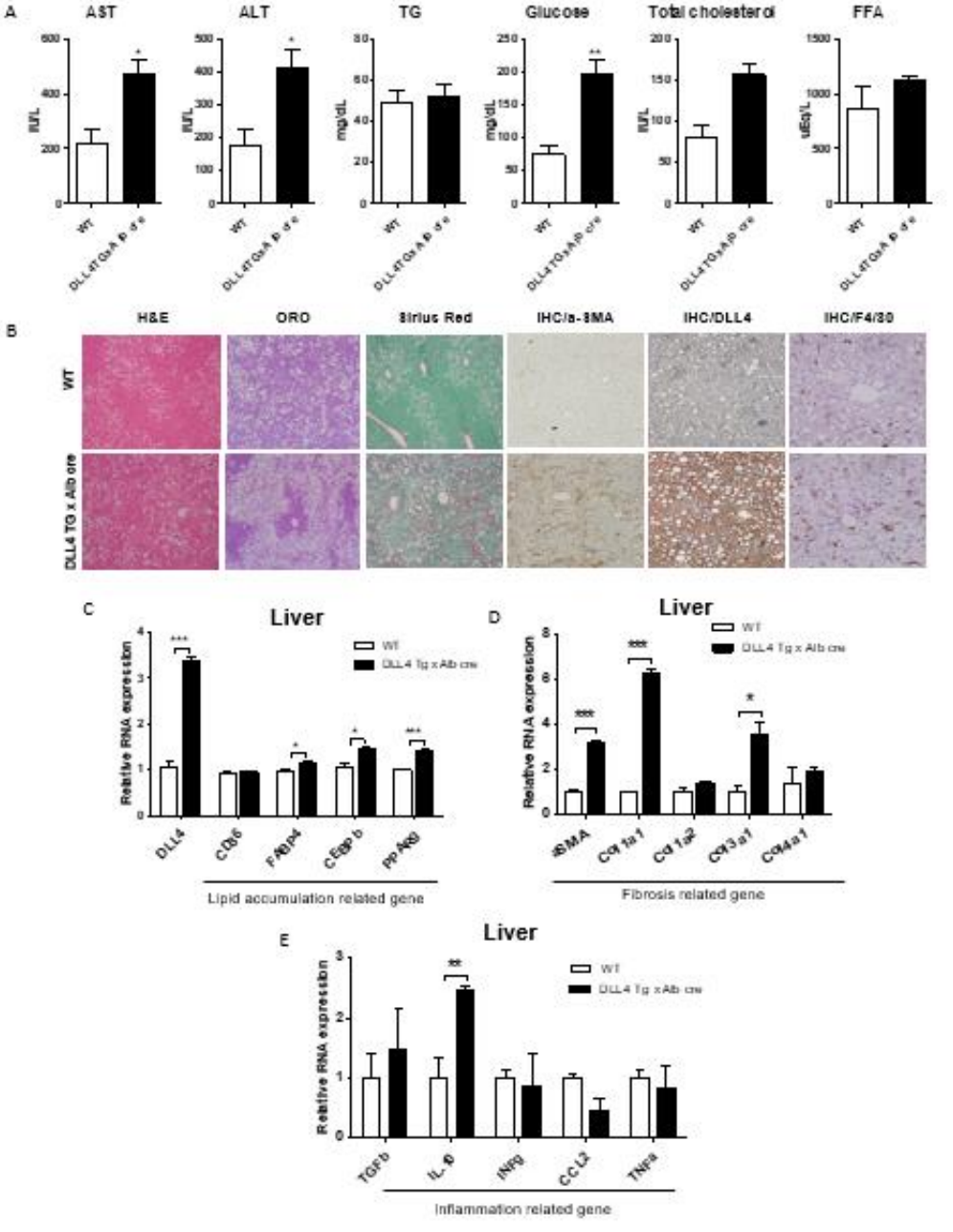


Figure 3. DLL4 overexpressed mice develop NASH. (A) Liver injury-related parameters of mice. ALT and AST activities were measured. Levels of total TG, glucose, cholesterol and FFA in mouse serum. (B) Liver sections of WT and DLL4 TG x Alb cre mice fed with HFD for 22 weeks were analyzed by H&E, ORO, Sirius red, and IHC with α-SMA, DLL4, F4/80 staining. (C - E) Real-time RT-PCR analysis of NASH marker genes in the liver. b-actin was used as a normalization control. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and *** $P < 0.001$), and p values were calculated using the Student's t test.

4. DLL4 overexpressed hepatocytes has elevated infiltrated immune cells and expression levels of NASH markers

Accordingly, HFD fed WT and DLL4 overexpressing mice liver cells were analyzed by FACS analysis to investigate their infiltrated inflammatory cells (Fig. 4A). Inflammatory factors like monocytes, neutrophils, macrophages, and dendritic cells were more existing in the adipose tissues of DLL4-liver specifically overexpressing (DLL4 TG x Alb cre) mice. Also mRNA expression levels fibrosis markers and inflammation markers, which are the characteristics of NASH, were also increased (Fig. 4B - 4D). These data suggest that HFD fed obese mice with overexpressed DLL4 in the liver has increased infiltrated inmmune cells and has developed NASH.

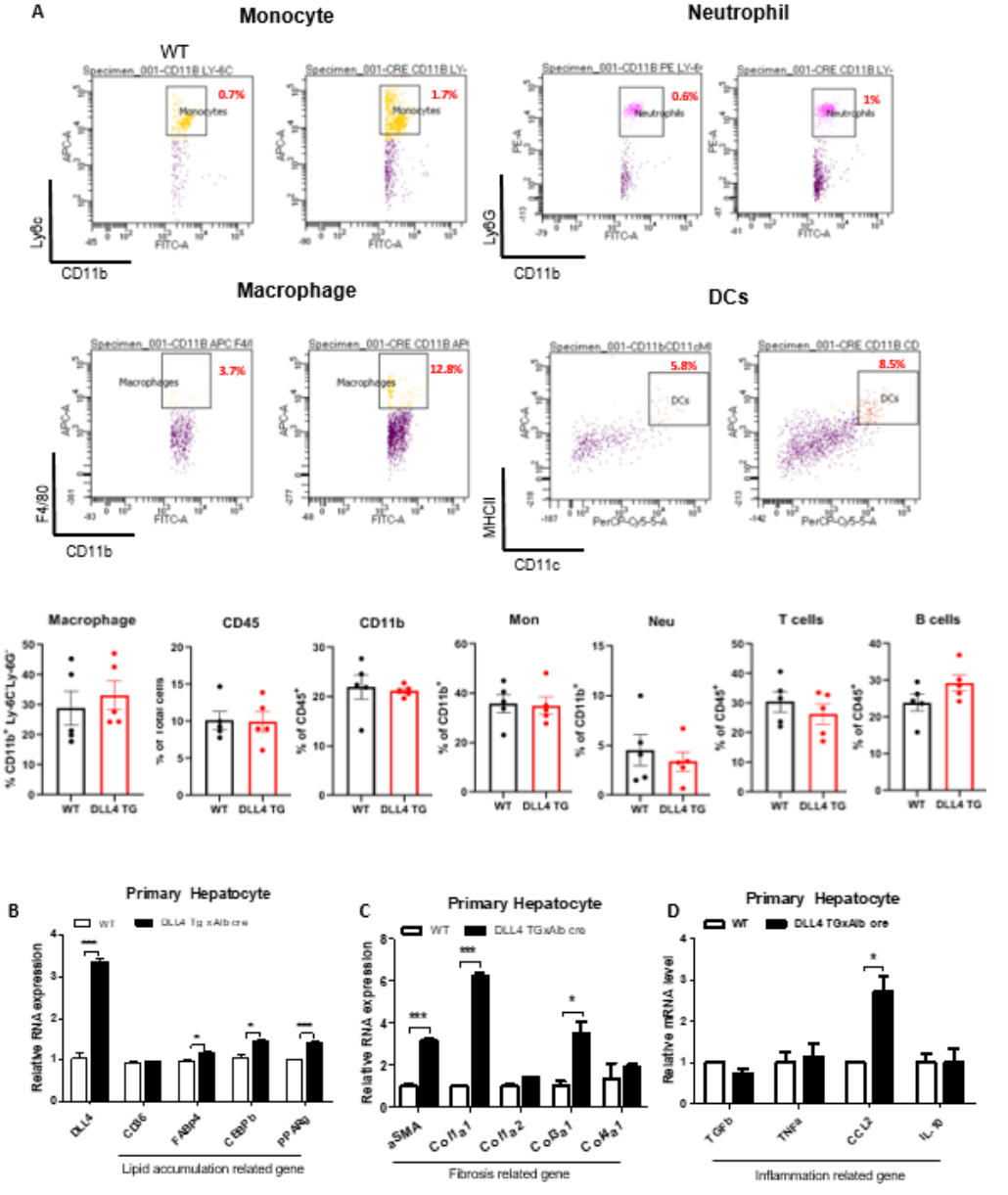


Figure 4. DLL4 overexpressed liver cells has more infiltrated immune cells and elevated mRNA NASH markers. (A) Monocytes, neutrophils, macrophages, and dendritic cells were isolated from WT and DLL4 TG x Alb cre mice adipose tissue and

examined by flow cytometry. FACS analysis gating strategies are CD11b+Ly6c+ for monocytes, CD11b+Ly6G+ for neutrophils, CD11b+F4/80+ for macrophages and CD11c+MHCII+ for DCs. (B –D) WT and DLL4 TG x Alb cre primary hepatocytes were isolated from mice fed with HFD for 12 weeks. Real-time RT-PCR analysis of genes in the primary hepatocytes. (C) mRNA expression levels of lipid accumulation genes were analyzed 48 hours later. (D) mRNA expression levels of fibrosis related genes. (E) mRNA expression levels of inflammation related genes. b-actin was used as a normalization control. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test.

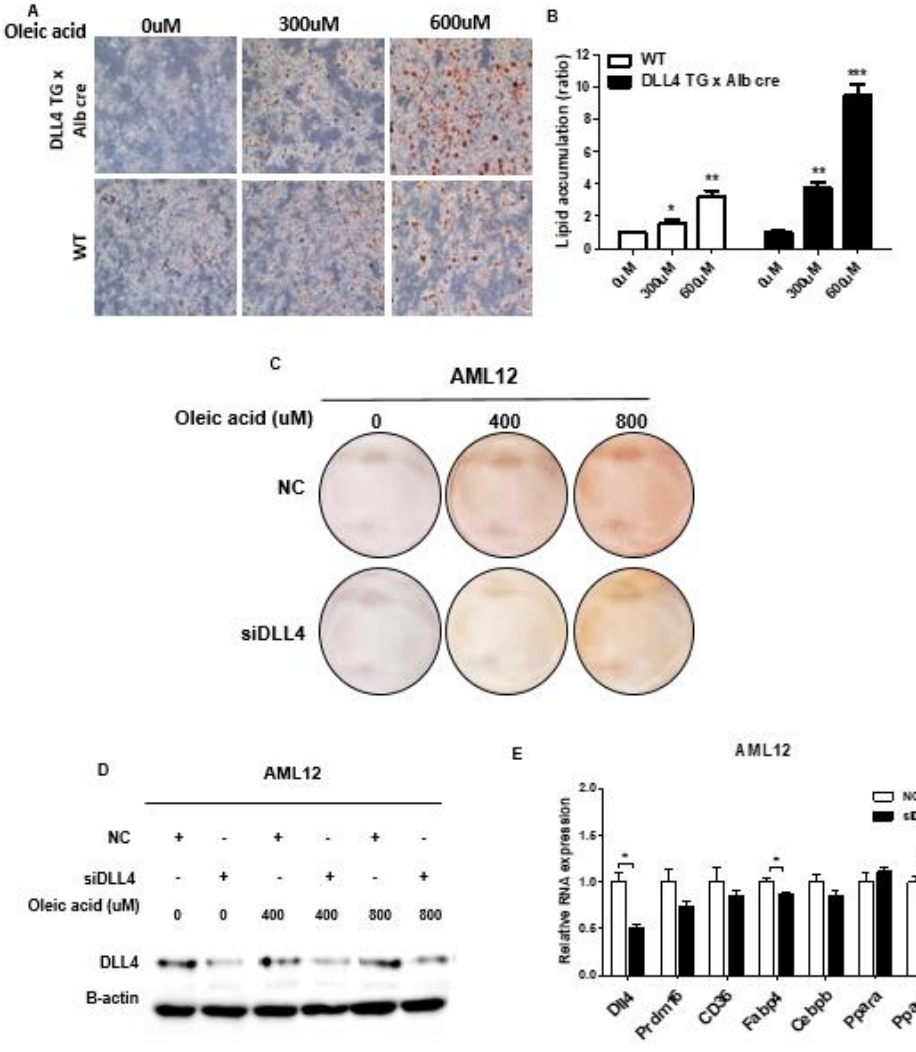
5. DLL4 knockdown reduces lipid accumulation and adipogenesis related marker expression levels

Next, I've isolated WT and DLL4 TG x Alb cre primary hepatocytes of 7-week old mice were isolated and gradual concentrations of oleic acid were treated. Interestingly, DLL4 overexpressing hepatocytes had more lipid accumulation than the control cells (Fig. 5A). The lipid accumulation rate of this data were analyzed (Fig. 5B). From these results, I could confirm that DLL4 overexpressing hepatocytes could accumulate more lipid than normal hepatocytes.

Next, to confirm the role of DLL4 in the lipid accumulation, DLL4 was knockdown by siRNA in AML12 cells and oleic acid was treated. Lipid accumulation was reduced when DLL4 was decreased (Fig. 5C). The decreased protein expression level of DLL4 was confirmed (Fig. 5D). mRNA expression of Pparɣ and FABP4 were also reduced when DLL4 was knockdown (Fig. 5E).

Subsequently, DLL4 overexpressing vector was tranfected to AML12 hepatocytes and its mRNA expression levels were analyzed. As a result, Pparɣ, FABP4 and CD36 expression levels were significantly increased (Fig. 5F).

Then DLL4 recombinant were treated to AML12 hepatocytes to induce the expression of DLL4. The lipid accumulation was increased and mRNA expression level of Pparɣ were increased by DLL4 induction (Fig. 5G and 5H). Therefore, DLL4 overexpression had induced lipid accumulation and lipogenic gene expression in hepatocytes, which could lead us to assume that DLL4 might be an important factor in lipid accumulation of liver.



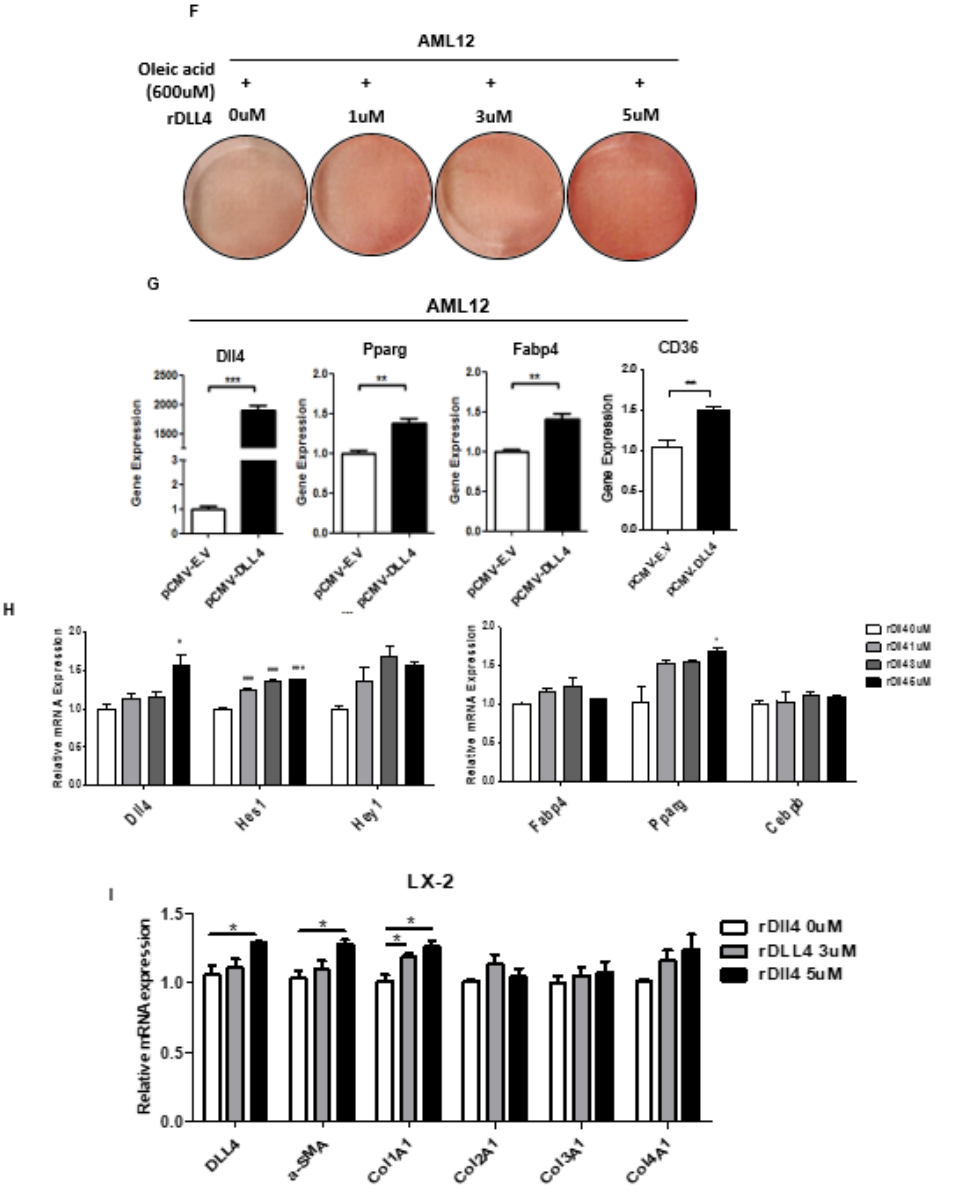


Figure 5. Lipid accumulation and adipogenic markers are decreased when DLL4 is reduced by siRNA. WT and DLL4 TG x Alb cre primary hepatocytes were isolated from mice fed with HFD for 12 weeks. (A) ORO stained and (B) Lipid accumulation.

AML12 cells were treated with 0, 400, and 800uM of oleic acids for 48 hours. (C) Oil Red O staining of AML12 cells. (D) Western blot analysis of AML12 cells after 48 hours treatment of oleic acid. (E) mRNA expression levels of indicated genes were detected by real-time RT-PCR. (F) qRT-PCR analysis of DLL4 overexpressed AML12 cells. DLL4 recombinant were treated in gradient concentration on AML12 cells and oleic acid were treated for the lipid accumulation. (G) Oil red o staining of AML12 cells. (H) mRNA expression levels were analyzed by qRT-PCR in AML12 cell. (I) mRNA expression levels were analyzed by qRT-PCR in LX-2 cell. b-actin was used as a normalization control. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test.

Part 2.

1. DLL4 inhibition by DLL4 antibody injection to obese mice improves its metabolic rates

To investigate whether DLL4 inhibition recover the negative effects of obesity, *in vivo* study was processed by injecting DLL4 antibody (2.5mg/kg) to HFD induced WT obese mice. These mice were fed with HFD and their body weight were monitored for 12 weeks and injection was started from the week 4 (Fig. 1A). After the 12 weeks, mice injected with DLL4 antibody had lean phenotypes and less body weight compared to the control group (Fig. 1B and 1C). To determine the effects of DLL4 antibody on HFD induced body composition, fat mass and their body weight were determined by DEXA. The DEXA scanning of the mice fed HFD with vehicle injection and DLL4 antibody injection are presented as radiography of body fat (Fig. 1D). The vehicle group mice presented more red color area than blue and yellow, which represents they had more high density fat than low and medium density fats. The DLL4 antibody injected group had significantly reduced fat deposition. Furthermore, their relative distribution of fat mass was notably decreased (Fig. 1E). Next, glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed on these mice groups. Blood glucose levels over the entire time course of GTT were lower in DLL4 antibody injected group than vehicle group. Both the rise and fall of blood glucose levels were faster (Fig. 1F). In response to insulin, vehicle group showed a deeper and longer lasting fall of blood glucose levels while DLL4 inhibited group displayed reduced blood glucose (Fig. 1G).

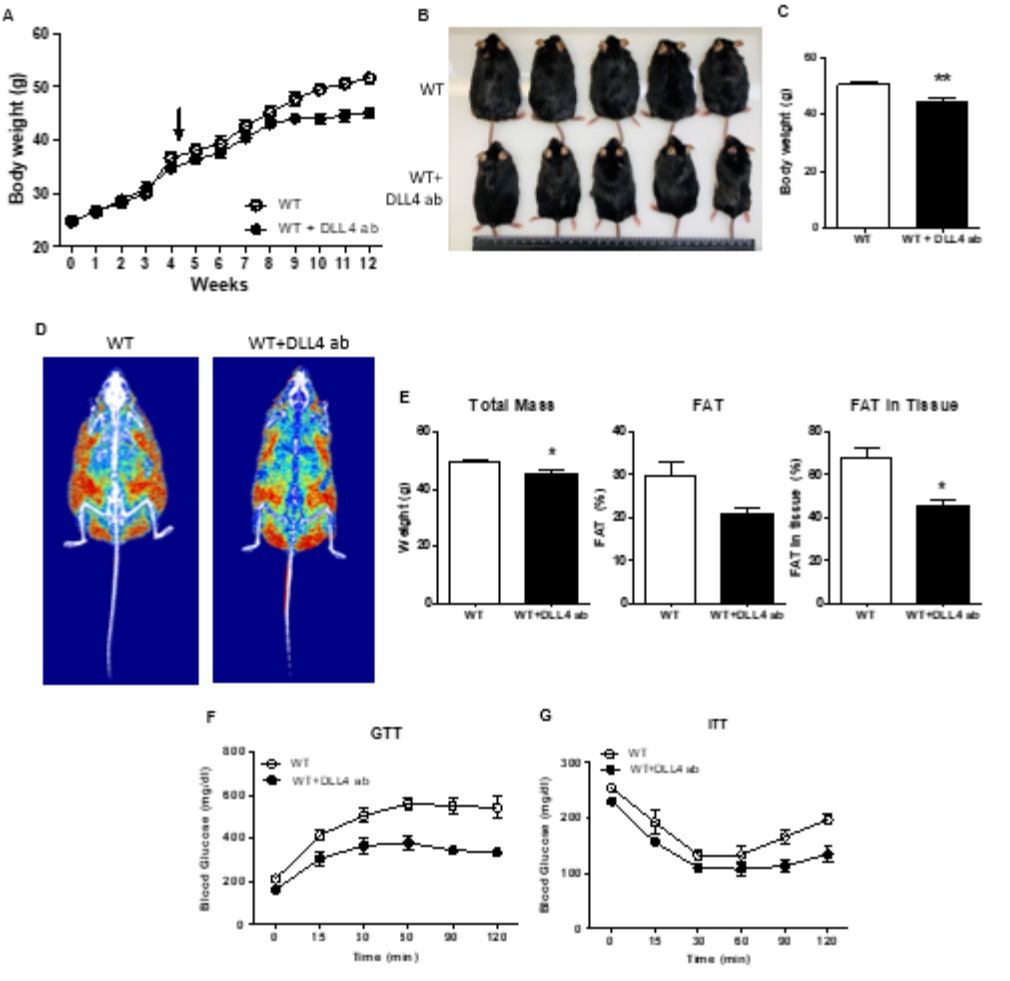


Figure 1. DLL4 inhibition in obese mice improves its metabolic rates. (A) Body weight (g) was determined in mice over 12 weeks of HFD in nonfasting condition. (B) Representative picture of mice fed with HFD for 12 weeks. (C) Final body weight after the 12 weeks HFD. (D) The radiography of body fat is displayed by three modes according to low density fat (blue), medium density fat (yellow), and high density fat (red). (E) The total mass, fat mass, and fat mass in tissue were measured by DEXA. (F)

Glucose tolerance test (GTT). Overnight fasted mice subject to an intraperitoneal injection of glucose (1g/kg). (G) Insulin tolerance test (ITT). ITT was performed by an intraperitoneal injection of insulin (1U/kg) to 6 hours fasted mice. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and *** $P < 0.001$), and p values were calculated using the Student's t test.

2. DLL4 inhibition by DLL3 antibody injection to obese mice improves its metabolism related tissue phenotypes

In addition, vehicle injected mice and DLL4 antibody injected mice fed with 12 weeks of HFD were sacrificed for the further investigation. In the vehicle treated mice liver, their livers were more beige colored which represents a lipid accumulation (Fig. 2A). Also liver weight of DLL4 inhibited mice were less than the control group (Fig. 2B). DLL4 inhibited mice exhibited smaller in the size of white adipose tissue (iWAT) and gonadal white adipose tissue (gWAT) (Fig. 2C and 2E). Accordingly, their tissue weights were lighter than the control group (Fig. 2D and 2F). Lastly, vehicle treated control group had significantly larger brown adipose tissue (BAT) in size than DLL4 inhibited group (Fig. 2G) and DLL4 inhibited group had lighter weight of BAT (Fig. 2H).

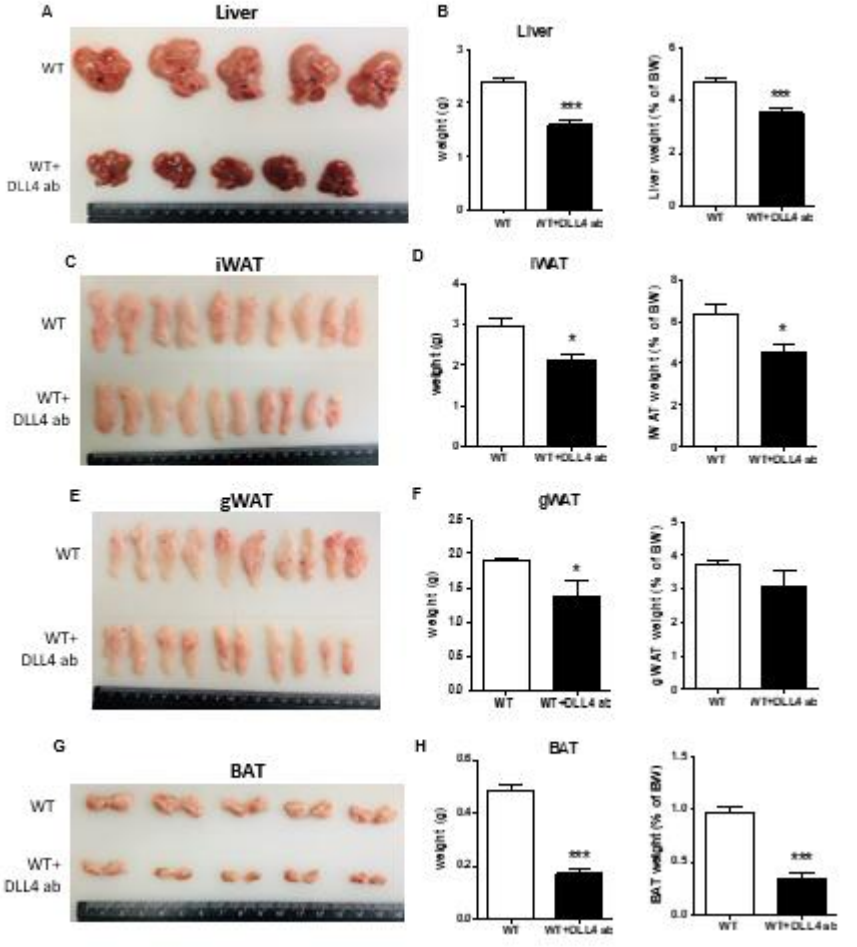


Figure 2. DLL4 inhibition in obese mice improves the phenotypes of metabolic tissues. Representative picture of mice Tissues and weight of each tissues and the ratio of tissue weight to body weight. (A) Liver. (B) Inguinal white adipose tissue (iWAT). (C) gonadal white adipose tissue (gWAT). (D) Brown adipose tissue (BAT). Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test.

3. Improved ALT, AST and metabolic tissue phenotypes in DLL4 antibody injected mice

Serum analysis of DLL4 antibody injected mice and wild type were processed. As a result, ALT and AST were significantly improved by DLL4 injection (Fig. 3A). But in case of serum glucose, TG, total cholesterol, and FFA levels were increased. Further studies regarding this phenomenon is needed. Additionally, gWAT, BAT and liver sections were analyzed. According to the H&E staining results, I could observe that the lipid droplets were significantly reduced in DLL4 injected mice. From the IHC result of DLL4, reduced expression levels of DLL4 were confirmed (Fig. 3B).

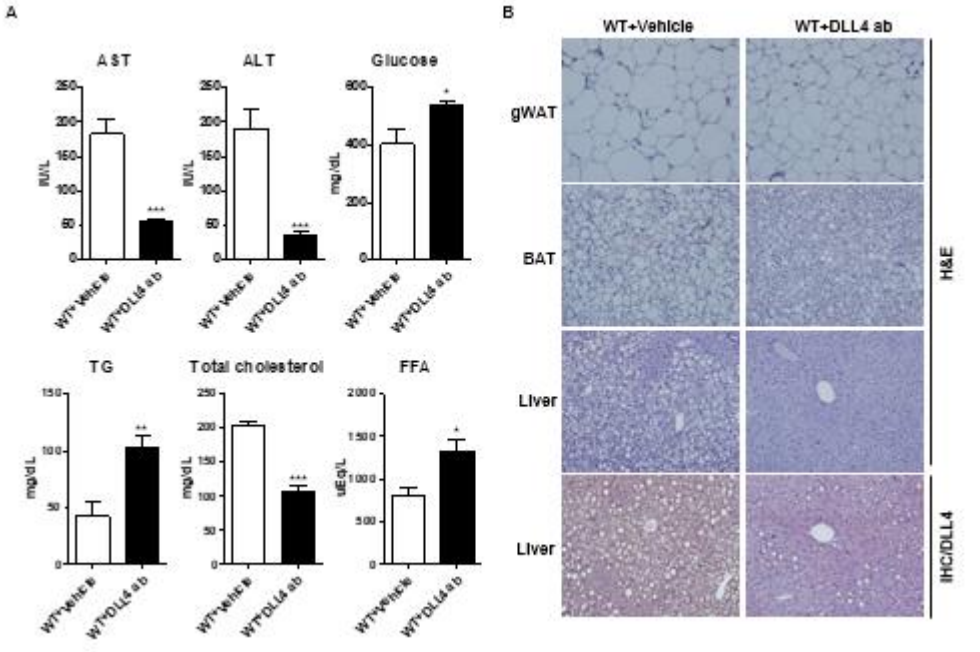


Figure 3. Increased DLL4 expression induces lipid accumulation and adipogeneiss related gene expression levels. (A) Serum analysis of WT and DLL4 overexpressing mice. (B) H&E staining and IHC staining of WT and DLL4 overexpressing mice liver sections. Significant differences are indicated by an asterisk (* p<0.05, ** p<0.01 and ***P < 0.001), and p values were calculated using the Student's t test.

4. Reduced adipogenesis markers in adipocytes by DLL4 antibody treatment

In addition, to confirm the anti-lipogenic effect of DLL4 inhibition, 3T3-L1 adipocytes were differentiated and treated with DLL antibody. As the cells become differentiated to mature adipocytes, DLL4 inhibition had decreased the mRNA expression levels of adipogenic markers and lipogenic markers such as Pparɣ, SREBP1, FABP4, FASN, and adiponectin (Fig. 4).

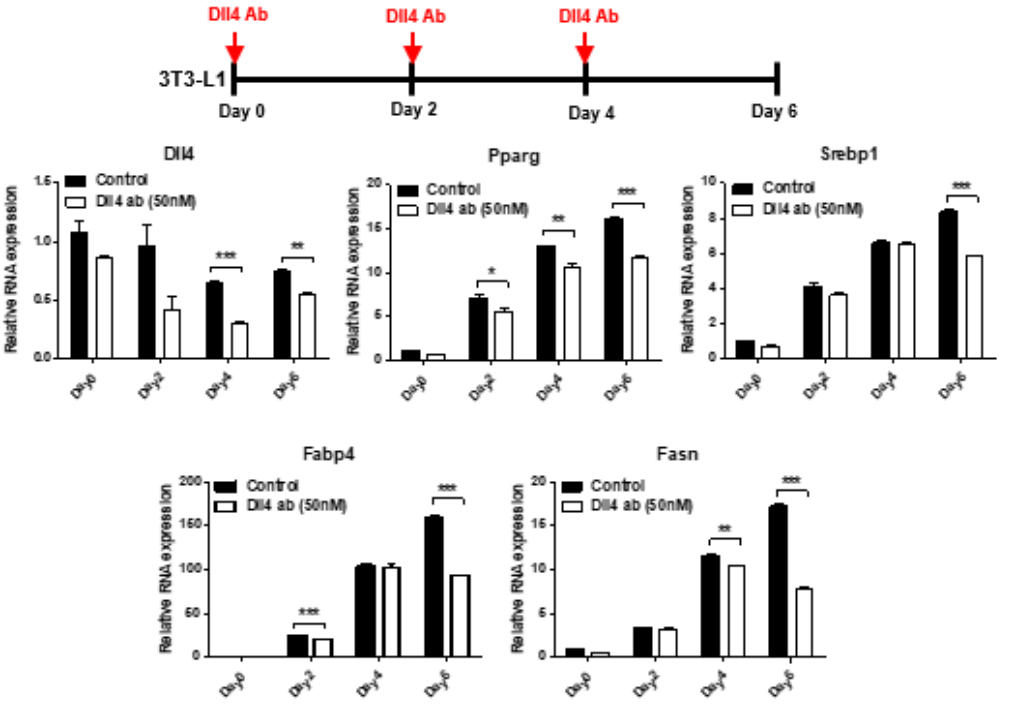


Figure 4. Expression levels of adipogenic markers are decreased in adipocytes by DLL4 inhibition. 3T3-L1 cells were differentiated and samples were harvested on day 0, 2, 4, and 6. DLL4 antibody was treated in every 48 hours. mRNA expression levels were analyzed by qRT-PCR. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test.

5. DLL4 inhibition by DLL4 antibody and GSI reduces lipid accumulation and lipid regulating gene expression levels

To further investigate the lipid accumulation regulating role of DLL4 in hepatocyte, I've decided to use DLL4 antibody (ABL001) which inhibits DLL4, and GSI which inhibits Notch signaling pathway. By treating DLL4 antibody and GSI to hepatocyte, DLL4 inhibition was confirmed in protein expression levels and mRNA expression levels (Fig. 5A and 5B). These results suggest that DLL4 inhibition degree was suitable for the further experiments.

When DLL4 was reduced by its antibody or GSI treatment on AML12 hepatocyte cell line, lipid accumulation was reduced, which the tendency is similar with the siRNA knockdown results (Fig. 5C). Accordingly, lipid synthesis related mRNA expression levels were significantly decreased as well (Fig. 5D). Then, DLL4 antibody were treated on AML12 in gradient concentrations and oleic acid were treated to induce lipid accumulation. When DLL4 were inhibited, lipid accumulation was also decreased (Fig. 5E). Also the decreased expression level of DLL4 was confirmed by western blot (Fig. 5F). RNA expression levels of Pparɣ and CD36 were also gradually decreased by gradual treatment of DLL4 antibody (Fig. 5G) and α-SMA was decreased in LX-2 cell (Fig. 5H)

In consequence, we could assume that DLL4 has lipid accumulation regulating role, which could cause the liver to build up fat and furthermore cause lipotoxicity

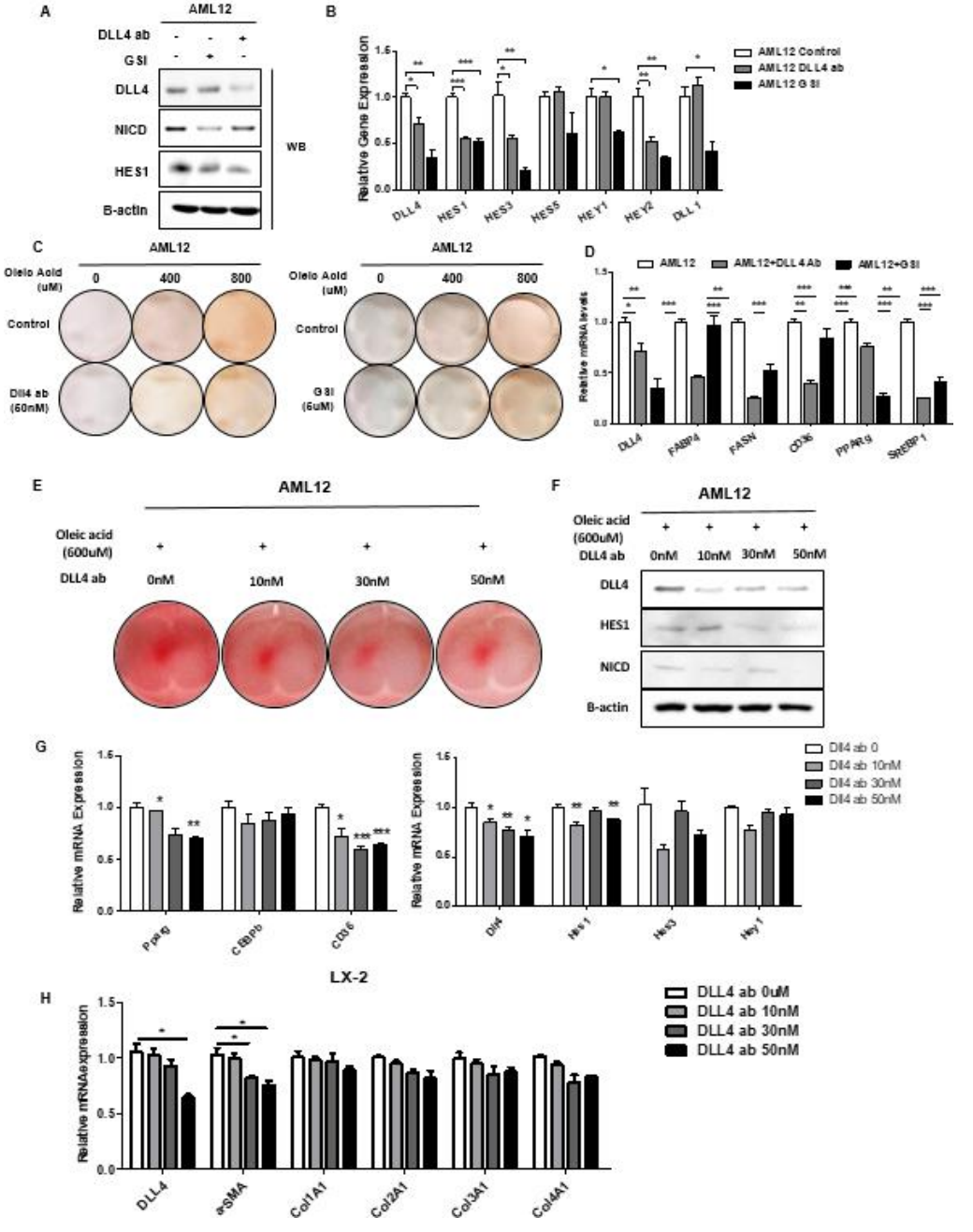
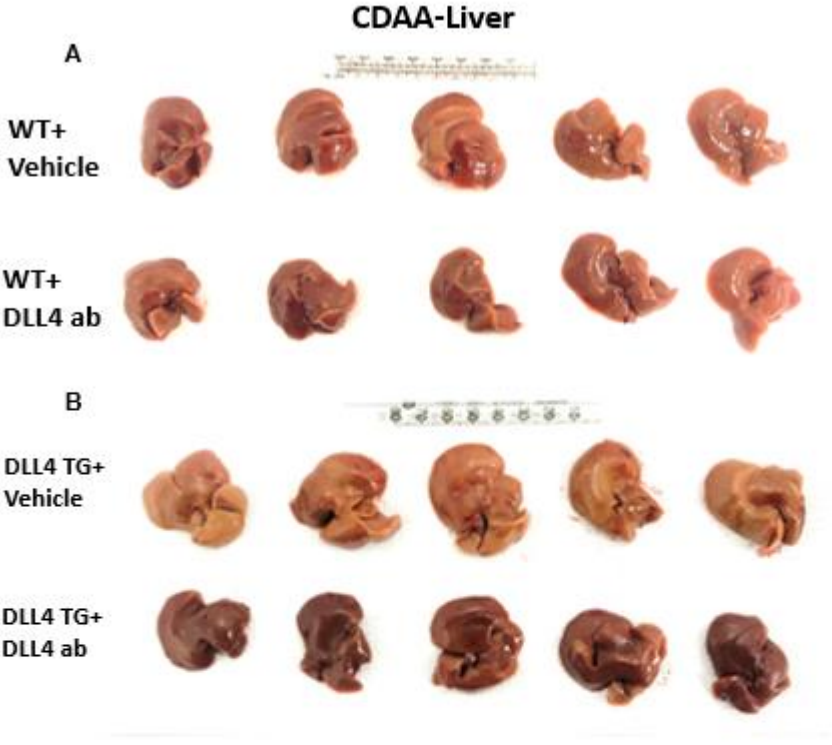


Fig 5. Lipid accumulation and expression levels of adipogenic markers are decreased by DLL4 inhibition. (A and B) DLL4 antibody (ABL001) and GSI was treated on AML12 cells. Protein expression level was detected by western blot analysis and mRNA expression level was detected by real-time RT-PCR. AML12 cells were treated with 0, 400, and 800uM of oleic acid for 48 hours. (C) Oil red o staining of AML12 cells after treating DLL4 antibody and GSI. (D) mRNA expression levels of lipogenic factors in AML12 cells were analyzed by qRT-PCR. DLL4 antibody were treated gradiently 0, 10, 30, and 50 uM and oleic acid were also treated. (E) Oil red o staining of AML12 cells after treating DLL4 antibody. (F) Western blot analysis. (G) mRNA expression levels were examined by qRT-PCR in AML12 cell. (H) mRNA expression levels were examined by qRT-PCR in LX-2 cell. b-actin was used as a normalization control. Significant differences are indicated by an asterisk (* $p<0.05$, ** $p<0.01$ and ***$P < 0.001$), and p values were calculated using the Student's t test**.**

6. CDAA diet fed DLL4 antibody treated mice has improved NASH phenotypes of liver

Finally, vehicle injected mice and DLL4 antibody injected WT mice and DLL4 TG mice fed with 17 weeks of CDAA diet were sacrificed for the further investigation. In the vehicle treated mice liver, their livers were more beige colored and severe NASH phenotypes than DLL4 antibody treated mice (Fig 6A and 6B). Also liver weight of DLL4 inhibited mice were less than the control group (Fig. 2B).



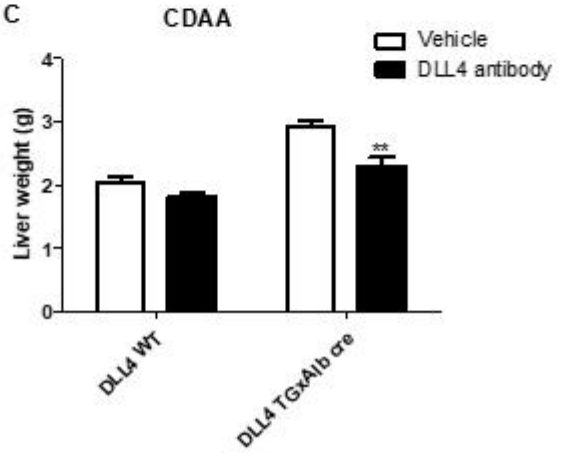


Figure 6. CDAA diet fed DLL4 antibody treated mice has improved NASH phenotypes of liver. WT and DLL4 TG x Alb cre mice were fed with CDAA diet for 17 weeks and DLL4 antibody were treated for 13 weeks. (A) Livers of WT control and DLL4 antibody treated mice. (B) Livers of DLL4 TG control and antibody treated mice. (C) Average liver weights of each groups. Significant differences are indicated by an asterisk (** $p<0.01$), and p values were calculated using the Student's t test.

IV. DISCUSSION

As the number of obese people increases, non-alcoholic fatty liver disease(NAFLD) is cited as the main cause of chronic liver disease.[19,20] The development of Non-alcoholic steatohepatitis(NASH) leads to fibrosis progression and hepatocellular damage.[21,22] Hepatic fibrosis resulting from accumulation of extracellular matrix proteins secreted by hepatic myofibroblasts plays an important role in disease progression.[23] Also Liver sinusoidal endothelial cells (LSECs) release inflammatory mediators and contribute to the recruitment of inflammatory cells, thus promoting liver injury and inflammation.[24] DLL4 is increased in LSECs isolated from NASH mouse liver.[25]

DLL4 has important role in vascular development, proliferation of cancer stem cells and immune system.[26,27,28] Studies have shown that the characteristics of DLL4 is important for regulating cancer stem cells,[17,29] and most of studies have been investigated on the role of DLL4 associated with cancer. Recently, studies have been conducted on the effect of the regulates of DLL4 on the metabolic disorder of obesity and liver diseases.[30,31] DLL4 improves atherosclerosis and metabolic disease indicating DLL4 signaling is involved in the crosstalk of inflammatory and metabolic pathways.[31,32] However, there are only few evidences of functional role of DLL4 in obesity and its associated liver diseases. So I have decided to investigate the role of DLL4 in adipogenesis and High fat induced obesity.

I ‘ve searched the expression level of DLL4 in healthy control liver, healthy obese liver, steatosis liver, and NASH liver from patients using public GSE data set. I

observed that DLL4 was upregulated in healthy obese liver, steatosis liver, and NASH liver. Then, I screened Notch signaling family's mRNA expression level in NFD and HFD mice liver and DLL4 has the most elevated level. Accordingly, liver specifically DLL4 overexpressing mice (DLL4 TG) were fed HFD for about 20 weeks and I could observe that DLL4 TG male mice had extremely elevated body weight than the control group. So I could assume that DLL4 expression level has an important role in regulating liver steatosis.

Next, 22 weeks of HFD fed WT and DLL4 TG mice were sacrificed and their liver, iWAT, gWAT, and BAT tissues were extracted. As a result, DLL4 TG mice had severely accumulated lipid droplets and increased liver weight. iWAT, gWAT and BAT tissues of DLL4 TG mice were similar with liver phenotype, which had larger lipid droplet sizes and heavier tissue weights. Their serum was analyzed and DLL4 TG mice had higher AST, ALT, Glucose, total cholesterol, and FFA, which refers that these mice had exacerbated liver homeostasis and glucose levels. From the liver sections, H&E, ORO, Sirius red, and IHC staining was processed. According to the H&E and ORO staining, larger and more lipid droplets were observed in DLL4 TG mice liver, and Sirius red staining result have shown the progressed fibrosis in the liver of DLL4 TG mice. Also from the IHC staining of α-SMA and F4/80, DLL4 TG mice had higher expression levels of them. Flowingly, these liver tissues were lysed and their RNA was extracted, and higher mRNA expression levels of lipid accumulation, fibrosis, and inflammation related genes were observed. According to these results, liver specifically

DLL4 overexpressing mice had severely altered glucose metabolism, fibrosis and more inflammation rates, which represents the typical characteristics of NASH.

Therefore, HFD fed mice livers were lysed and according to the FACS result, DLL4 TG mice had more infiltrated monocytes, neutrophils, macrophages, and DCs which shows an elevated levels of infiltrated immune cells. Then isolated primary hepatocytes were analyzed by qRT-PCR and this result have shown that DLL4 TG mice had increased lipid accumulation, fibrosis and inflammation related mRNA expression rates. Next, to investigate the specific role of DLL4 in lipid accumulation, oleic acid was treated to DLL4 TG mice primary hepatocyte. As a result, DLL4 TG mice liver had accumulated more lipid than WT. When DLL4 was down regulated by siRNA treatment, lipid accumulation was reduced and lipid regulating mRNA expression levels were also reduced. Then, DLL4 overexpressing vector was treated and accordingly, Pparɣ, Fabp4, and CD36 expression was down regulated. Also, when DLL4 recombinase was treated, more lipid accumulation and Pparɣ expression level was increased.

To investigate the effect of reduced DLL4 expression in vivo, DLL4 antibody was injected to HFD feeding mice. Surprisingly, DLL4 antibody injected mice had reduced body weight than the control group and less fat mass. Also, these mice had improved glucose tolerance and insulin tolerance rates. After the sacrifice of DLL4 antibody injected mice and control mice, I could observe improved phenotypes of liver, iWAT, gWAT, and BAT tissues compared to the control obese mice. According to the serum analysis results, DLL4 reduced mice also had notably improved AST and ALT

levels. Also from the H&E results of the liver and adipose tissue sections, DLL4 reduced mice had reduced lipid droplets in size and numbers.

Lastly, I've investigated the effect of reduced DLL4 expression in hepatocyte lipid accumulation. When DLL4 expression levels were reduced by DLL4 antibody or GSI treatment, AMl12 cells had decreased lipid accumulation and mRNA expression levels of lipid regulating genes were also down regulated. Then, I've treated DLL4 antibody in gradual concentration, and gradually decreased lipid accumulation was observed and also the mRNA expression levels of Pparγ, Cebpβ, and CD36 were decreased.

Taken together, this study demonstrates that DLL4 is a key regulator in regulating liver homeostasis and overall metabolism.

V. CONCLUSION

The level of DLL4 was elevated in obese, steatosis and NASH patients. Liver specifically DLL4 overexpressing mice had heavier body weights after the HFD diet and exacerbated metabolic tissues. These mice had more infiltrated inflammatory cells and progressed fibrosis. DLL4 overexpressing hepatocytes had higher capacity to accumulate more lipid and they had upregulated expression levels of lipid regulating, fibrosis, and inflammation related genes. Then, when DLL4 expression level was inhibited in mice, these mice had gained less body weights than the control group and improved insulin tolerance. DLL4 inhibited mice had smaller adipose tissues and comparatively normal liver phenotypes than the control obese mice. In addition, DLL4 inhibition by the treatment of antibody and GSI had resulted in less lipid accumulation in vitro. In conclusion, this study suggests that DLL4 might be the key regulator in treating metabolic diseases.

ABSTRACT (IN KOREAN)

Notch 신호의 ligand 인 DLL4 가 대사질환 조절에 미치는 기능에 대한 연구

< 지도교수 전 경 희 >

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DLL4 는 Notch 신호전달의 리간드 중 하나로 발생, 형태 형성, 세포분화 또는 줄기세포유지를 조절한다고 알려져 있다. Notch 신호전달과 관련하여 비만에 의해 유도되는 간질환 조절에 대한 연구가 최근에 활발히 진행되고 있다. DLL4 가 항체에 의해 차단되면 내당성 및 인슐린저항성이 향상되고 마우스 모델에서는 체중 감소가 확인되었다. 또 NASH 마우스에 Notch 저해제를 주사하면 간섬유화 정도가 향상됐다. 지금까지의 연구는 Notch 시그널전달과 대사성질환의 관계를 보여주고 있지만 특정 분자 기전 연구는 미비한 상황이다. 이러한 기존의 연구를 바탕으로 본 연구에서는

먼저 DLL4 가 비알코올성 지방간염(NASH)이나 비알코올성 지방간질환(NAFLD) 등의 대사성 질환을 조절하는지 여부를 조사하였다. 고지방식이(HFD) 유도 비만 마우스 간 조직에는 Notch 시그널과 관련된 유전자들의 mRNA 값이 여러 개 증가했는데, 그 중 DLL4 발현 수준이 가장 높았다. 고지방식이를 진행한 DLL4TG x Albumin cre 마우스는 WT 마우스보다 체중이나 간의 크기가 확실히 증가하였다. 간 독성의 지표인 AST 와 ALT 는 DLL4 TG×Alb cre 마우스에서 증가하였고 간의 mRNA 의 섬유화 지표나 염증과 관련된 지표에서도 증가하였다. Sirius red 염색을 통해 DLL4 TG×Alb cre 마우스의 간에서 다수의 증가한 콜라겐의 분포를 확인하였다. 이 연구결과를 통해 NASH 가 DLL4TG 마우스에 HFD 를 통해서만 유도된 것을 확인하였다. DLL4 의 knockdown 실험을 실시한 결과, 지질 축적과 지질 관련 유전자 발현 수준이 감소하였다. 생체 내 연구에서는, HFD 식이를 진행한 비만 마우스에 DLL4 항체를 주사한 결과, 마우스의 전반적인 대사율을 향상시켰다.

이러한 결과들은 DLL4 의 발현 증가가 비만을 유도하고 그에 따른 간의 염증이나 섬유증을 유발하는 데 중요한 역할을 하는 것을 의미한다. 따라서 간지방증이나 NASH 환자 치료에 있어서 DLL4 가 새로운 표적으로 제시 될 수 있다.

핵심되는 말: DLL4, 대사질환, 비만, 간지방증, 비알콜성 지방간염

VI. REFERENCES

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