USP15 regulates lipid accumulation in hepatocyte through FABP4 and PPARγ dependent mechanism

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USP15 regulates lipid accumulation in hepatocyte through FABP4 and PPARγ dependent mechanism

Directed by Professor Kyung-Hee Chun

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Nam-Jun Kim

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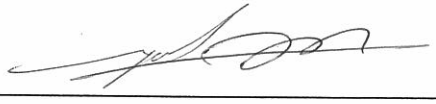
This certifies that the Master's Thesis of Nam-Jun Kim is approved.

Thesis Supervisor: Kyung-Hee Chun



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ABSTRACT

USP15 regulates lipid accumulation in hepatocyte through FABP4 and PPARγ dependent manner

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The Ubiquitin Specific Peptidase 15 (USP15) is an enzyme that is encoded by the *USP15* gene. USP15 is a hydrolase that removes conjugated ubiquitin from target proteins. It regulates various pathways such as TGF-β receptor signaling and NF-κB pathways.

Especially, USP15 acts as a key regulator of TGF-beta receptor signaling pathway and promotes deubiquitination of R-SMADs. Therefore, it alleviates inhibition of R-SMADs and promotes activation of TGF-β target genes. There have been a number of USP15 studies as to cancer and cancer immunology. However, correlation between USP15 and metabolic diseases is poorly understood. In this study, we demonstrate that considerably high expression level of USP15 in liver of healthy obese and steatosis patients. Also, we confirmed expression level of USP15 is up-regulated liver tissue of high fat diet fed C57BL/6 wild type mice and palmitic acid treated mouse hepatocyte cell line. Knock down of USP15 expression retarded lipid accumulation in mouse hepatocyte and expression of non alcoholic fatty liver diseases (NAFLD) related factors such as FABP4, perilipin and PPARγ. In contrast, overexpression of mUSP15 increased lipid accumulation in mouse hepatocyte and expression of NAFLD related factors. We also confirmed that liver tissue specific USP15 KO mice have less hepatic lipid accumulation than that of USP15 WT although PPARγ is overexpressed

in liver.Moreover, we determined that USP15 interacted with FABP4 and PPARγ and then up-regulated their protein expressions. Additionally, we identified that up-regulated protein stabilty of FABP4 and PPARγ was due to retardation of ubiquitination of FABP4 and PPARγ by USP15. Taken together, USP15 induces deubiquitination of NAFLD associated factors and protein stability of them is upregulated. We proposed that USP15 has positive role of lipid accumlation in hepatocyte and ablation of USP15 expression prohibits NAFLD.

Keyword: NAFLD, USP15, FABP4, PPARγ, deubiquitination, hepatocyte

Ⅰ. INTRODUCTION

As an important metabolic organ in the human, the live plays a major role in the regulation of lipid metabolism and glucose metabolism such as gluconeogenesis.[1,2] Hepatic steatosis is an early pathological step of the liver diseases and can cause steatohepatitis, cirrhosis, hepatocellular carcinoma, and serious cardiovascular diseases.[3-5] Some evidence indicates that hepatic steatosis occurs in individuals with obesity and insulin resistance.[6] In the liver, overloaded free fatty acid into hepatocyte, which involve in the fatty acid binding protein4 (FABP4), peroxisome proliferator-activated receptor gamma (PPARγ), and perilipin, results in lipid accumulation in hepatocyte.[7-9] In short, excessive uptake of free fatty acid into hepatocyte, insulin resistance, and systemic pro-inflammatory response[10] are interconnected pathological events that are usually found in NAFLD patients. Recently, TRAF3, which has E3 ligase function[11] and is involved in ubiquitination,[12] induces hepaticsteatosis and insulin resistance in hepatocyte by interacitng with TAK1.[13] Although extensive research about NAFLD has been conducted, the specific and complex mechansim of causing and progressing NAFLD are not fully understood.

The ubiquitin specific peptidases (USPs) consists of 54 members (USP1-USP54) that can fuction as deubiquitination[14] of each target proteins and promotes their stabilities.[15] Among USP members, USP15 plays role as a major component of the transforming growth factor-β (TGF-β) signaling pathway. It binds to R-SMADs and promotes TGF-beta cell signal. *USP15* gene is mostly found in glioblastoma, breast cancer and ovarian cancer.[16,17] Furthermore, It is known that USP15 stabilizes MDM2, E3 ubiquitin ligase, in cancer cells and prohibits anti tumor T cell responses.[18] Most of the USP15 studies are involved in pathological function in cancer and cancer immunology. In the recent years, USP10 interacts with AMPK, which is associated with insulin resistance,[19] and regulates glucose metabolism.[20] Addtionally, USP7 interacts with histone acetyltransferase TIP60 and regulates early adipogenesis.[21] However, correlation between USP15 and metabolic disease is poorly understood.

In this study, we confirmed that more than three PPARγ binding sites were located in human and mouse USP15 promoter region based on genomatix online data (Data not shown) and also identified that USP15 interacts with FABP4 according to GeneMania diagram (Graph not shown). Therefore, we aimed to investigate the correlation between USP15 and NAFLD associated factors such as USP15 and PPARγ in terms of hepatic lipid metabolism *in vitro* and *in vivo*. We demonstrated that USP15 interacts FABP4 and PPARγ then increased protein stability of them by deubiquitination. Therefore, we proposed that ablation of USP15 expression can ameliorate lipid accumulation in liver.

Ⅱ. MATERIAL AND METHODS

1. Chemicals

Palmitic acid, N-ethylmaleimide and ORO powder were purchased from Sigma Chemicals (St. Louis, MO, USA).

2. Cell culture

The AML12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 medium (1:1) with 0.005mg/ml insulin, 0.005mg/ml transferring, 5ng/ml selenium, 40ng/ml dexamethasone, 10% fetal bovine serum (FBS), and 1% antibiotics and maintained at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. Palmitic acid was dissolved in NaCl and treated in AML12 with 0, 400, 600μM dose in F12 medium with 0.5 % bovine serum albumin (BSA). The HEK293 were cultured in DMEM with 10% FBS and 1% antibiotics.

2. siRNA and vector transfection

Mouse USP15 siRNA transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen) following manufacturer's instructions[22]. Overexpression of USP15, FABP4, PPARɣ, and ubiquitin plasmid DNAs was performed with Viafect Transfection Reagent (Promega, Madison, WV, USA). The mouse USP15 siRNA sequences were as follows and purchased from Shanghai GenePharma (Shanghai, China). USP15 siRNA#2: cccgucauauacugcuuauaacuc (sense), gaguuauaagcaguauaugacggg (anti-sense) USP15 siRNA#3: ugagaggugaaauagcuauuucaccucuca (sense), gagauuuagcuauuucaccucuca (anti-sense).

3. RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For the RT-PCR experiments, total mRNA was isolated from Liver, gWAT, iWAT, BAT tissues using an RNA lysis reagent (easy-BLUE™, iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. cDNA (1μg) was synthesized from RNA by qPCR RT master mix (Takara Bio, Otsu, Japan). RT-PCR was performed using 2X TOP simple DyeMIX-nTaq (Enzynomics, Daejeon, Korea) with a thermal cycler purchased from Applied Biosystems (Foster City, CA, USA). The primer sequences were as follows. USP15: F_5'-cccaggtgcatccaattt-3', R_5'-ttgctcaaacactgaatggct-3', PPARγ: F_5'-agggcgatcttgacaggaaa-3', R_5'-cgaaactggcacccttgaaa-3', FABP4: F_5'-catcagccttaaatggggatt-3',

R_5'-tcgactttccatcccacttc-3', perilipin: F_5'-ggcatcggatagggacatgg-3' R_5'-tgcagaacactctcchhaac-3', β-actin: F_5'-ggctgtattcccctccatcg-3', R_5'-ccagttggtaacaatgccatgt-3'.

4. MTT assay

AML12 were plated in 12-well plates and incubated until 60-70% of confluence. Then, AML12 cells were treated with oleic acid for the indicated time. Cell viability was measured using EZ-Cytox (Daeil Lab Services, Wonju, Korea) according to the manufacturer's protocol.

5. Western blot assay

Cell lysate extractions and tissues were prepared with radio immunoprecipitation assay buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150mM NaCl; 50mM Tris-HCl, pH 7.5; and 2mM EDTA, pH 8.0) as described previously[23]. Antibodies against USP15, PPARγ, A-FABP were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against perilipin was obtained from Cell Signaling Technology (Danvers, MA, USA). The normalization control was anti-β-actin. (Santa Cruz Biotechnology, Dallas TX, USA).

6. ORO staining

AML12 cells were incubated with 10% formalin for 10 min and washed with distilled water. Then, cells were stained with ORO in 60% isopropanol. Finally, the ORO stain that bound with the cells was eluted with 100% isopropanol and measured using the OD_{500}.

7. Immunoprecipitation assays

HEK293 cell lysates containing about 700μg of protein was precleared by incubation with 35μl of protein-A/G linked agarose beads (Santa Cruz Biotechnology, Dallas TX, USA) 1 hour at 4℃. After the beads were spun down, the supernatant was incubated with 1μg of a specific antibody (anti-FLAG and HA) overnight at 4℃, followed by incubation with 35μl of protein-A/G linked agarose beads. MouseIgG (SantaCruz) was used as the negative control After incubation, beads were washed 3 times in diluted RIPA buffer before being dissoloved in SDS-PAGE loading buffer. Westernblot analysis was performed as mentioned above.

8. Ubiquitination assay

Ubiquitination assay was investigated under denaturing condition. Cells were lysed in PBS containing 5 mM N-ethylmaleimide, (Sigma-Aldrich, St Louis, MO, USA) to hinder deubiquitination. We diluted the suspension with 0.9ml of non denaturing lysis buffer. Then, fragment the viscous chromation complexes by passing the lysed suspension 3-5 times through a needle attached to 1ml syringe followed by incubation on ice for 5 min. Later, we spun down 13,000 rpm for 10 min in 4℃ followed by proceeding with the immunoprecipitation as above mentioned.

9. Animal experiments

Liver tissue specific *USP15* KO mice were generated by breeding animals harboring a Floxed *USP15* allele with transgenic mice expressing Albumin-Cre recombinase. Mice were maintained on standard chow diet. For hepatic lipid accumulation studies, Adenoviruses encoding PPARγ were infected in *USP15* WT and liver tissue specific *USP15* KO mice by tail-vein injection. All mouse tissues were frozen in liquid nitrogen and stored at −80 °C before performing experiments.

10. Morphological analysis of tissues

Liver tissues were fixed in 4% paraformaldehyde for 24–48 hr at 4 ℃ and processed for paraffin embedding and were stained with hematoxylin and eosin.

11. Statistical analysis

We employed unpaired *t*-tests to analyze comparisons between two or three groups. Statistical analysis was performed using Prism 5 (GraphPad, La Jolla, CA). *P* values < 0.05 were considered to be significant.

Ⅲ. RESULTS

1. Analysis of USP15 expression in liver samples of steatosis patients

We determined the expression level of USP15 in liver samples from healthy obese and steatosis patients that is opened in GEO data base (Figure 1) using the online resourse http://www.ncbi.nlm.nih.gov/geo/. We collected three datasets of patients and compared the expression level of USP15 in control and NASH and steatosis livers.

There were statistical differences between control and steatosis but not NASH. Based on the data, the expression level of USP15 was up-regulated in liver of steatosis patients.

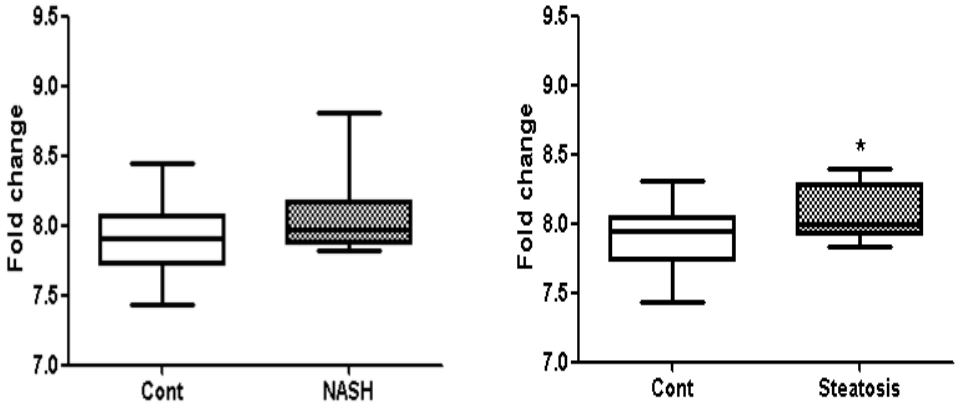

Figure 1. Correlation between NASH, steatosis and USP15 expression in patients. mRNA expression level of USP15 from liver tissues of NASH and steatosis patients are presented as a diagram (GSM1178970-1178974, GSM1178976-1178979, GSM1178981-1178986, GSM1178988-1178994, GSM1178997-1179000, GSM1179005, GSM1179007, GSM1179009-1179014, GSM1179016, GSM1179018-1179025, GSM1179027-1179032, GSM1179034, GSM1179037-1179038, GSM1179040-1179042). 55 samples of human liver grouped into C (control=14), N (NASH=18), S (steatosis=14).

2. The expression level of USP15 appears to wild type mice liver tissue

At first, we identified the expression level of USP15 in ten tissue samples of mice (Figure 2). Among of them, liver, heart, muscle, BAT, gWAT were expressed more than fifty percent of USP15 mRNA. Therefore, we decided to study USP15 in liver based on Figure 1 and Figure 2A.

Furthermore, we confirmed the USP15 level in normal chow diet and high fat diet (60% kcal as fat) fed mice. Especially, protein level of USP15 was most high among other USPs family such as USP1, 10, 11 in liver of HFD-fed mice (Figure 3A). The factors related to NAFLD such as FABP4, perilipin were increased as expression of USP15 was up-regulated (Figure 3B). Taken together, we suggested that expression level of USP15 was augmented in non alcoholic fatty liver.

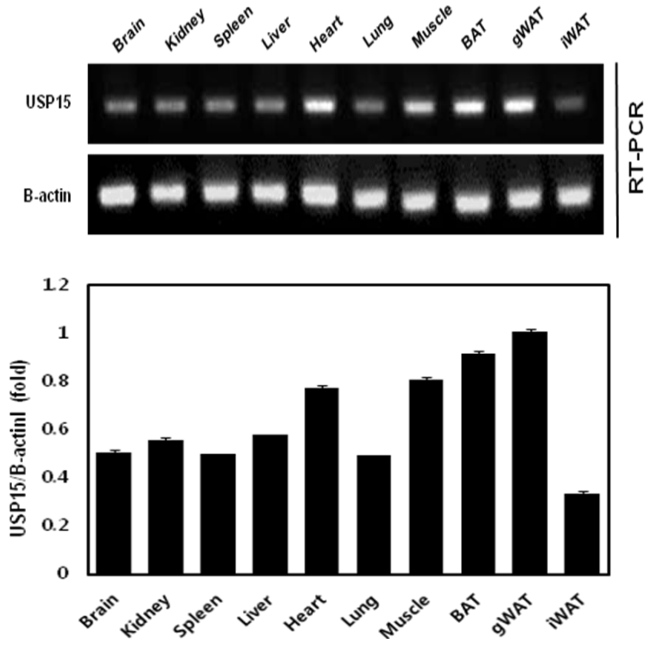

Figure 2. The up-regulated expression of USP15 in mouse metabolic organ. mRNA expression levels of USP15 in ten mice tissues detected by RT-PCR.β-actin was used as a normalization control.

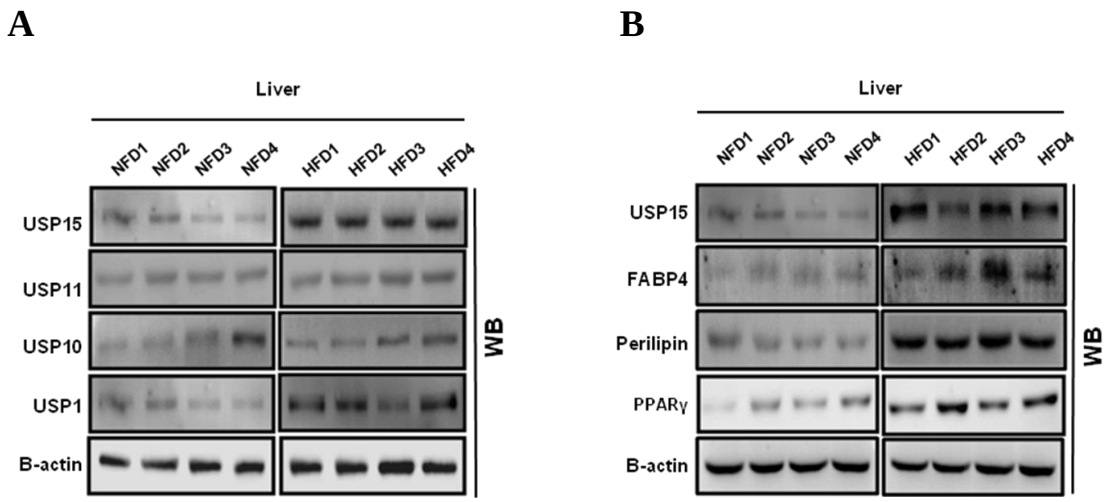

Figure 3. The up-regulated expression of USP15 and NAFLD related factors in HFD-fed mice liver. (A), (B) Protein expression of USP1, 10, 11, 15, FABP4 and perilipin in liver tissues was detected by western blotting. β-actin was used as a loading control.

3. Expression of USP15 is correlated with lipid accumulation in hepatocyte

To induce lipid accumulation in AML12 cells, we treat 0, 100, 200, 400, 600, 800μM of palmitic acid by dose dependent manner. At first, we performed cell viability assay to draw region of adequate concentration of oleic acid. Due to 800μM of palmitic acid was toxic to AML12 cell, we decided to treat 0, 100, 200, 400, 600μM of palmitic acid to induce lipid accumulation in AML12 cells (Figure 3A). We performed ORO staining at the AML12 cells treated with palmitic acid, and the results showed that lipid accumulation in AML12 cells were increased (Figure 3B). Protein level of USP15 was increased in AML12 cells by dose dependent manner but not USP1, 10, 11 (Figure 3C). Palmitic acid induces protein expression of USP15 and NAFLD related factors such as FABP4, perilipin, and PPARγ. (Figure 3D). Taken together, USP15 was correlated with lipid accumulation in hepatocyte.

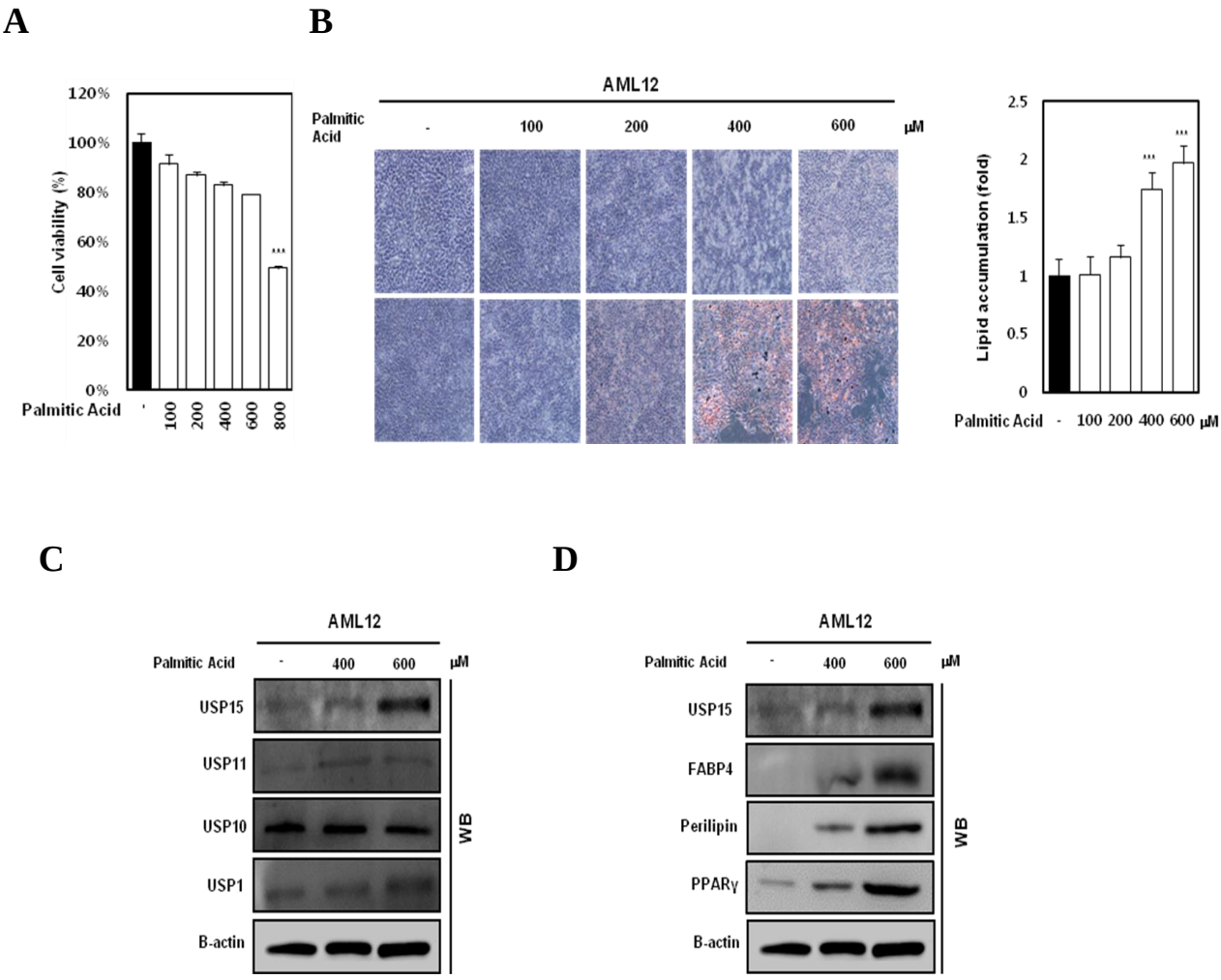

Figure 4. Lipid accumulation and USP15 expression is augmented in AML12 cells by dose dependent manner. (A) Cytotoxicity in AML12 treated with palmitic acid at doses up to 800μM by MTT assay. (B) AML12 was treated with palmitic at 0, 100, 200, 400, 600μM and Oil Red O staining was performed as described in the "Materials and Methods". Lipid accumulation in AML12 was measured using spectrophotometry as described in the "Materials and Methods". (C) Protein expression of USP1, 10, 11, 15 in AML12 cell lysates was detected by western blotting. β-actin was used as a loading control. (D) Protein expressions of USP15, FABP4, perilipin, PPARγ were detected by westernblotting. β-actin was used as a loading control. Data are presented as mean ± SD; ***P < 0.001 for untreated control *vs* 400, 600 μM of Palmitic acid.

4. Ablation of USP15 reduces lipid accumulation in hepatocyte

After knock-down of USP15 by 10μM of USP15 specific siRNA, we treat 0, 400, 600 μM of Palmitic acid in AML12 for 24 hours. We performed ORO staining at the AML12 cells treated with or without USP15 specific siRNA, and the results showed that lipid accumulation in USP15 specific siRNA-treated AML12 cells were decreased (Figure 5A). However, we wondered that reduced lipid accumulation in USP15 specific siRNA-treated cells was due to death of AML12. To investigate the possibility of cell death by USP15 specific siRNA, we performed cell viability assay (Figure 5B). The results showed that there were no differences in cell viability between negative control group and siUSP15 treated group. Furthermore, we identified the protein expression in AML12 with or without USP15 specific siRNA and MG132, a proteasome inhibitor (Figure 5C). The expression of USP15, FABP4, perilipin, and PPARγ was down-regulated if siUSP15 were treated in AML12. However, the expression of USP15, FABP4, perilipin, and PPARγ were restored when treating MG132. To sum up, ablation of USP15 was associated with reduced lipid accumulation in hepatocyte by proteasome dependent mechanism.

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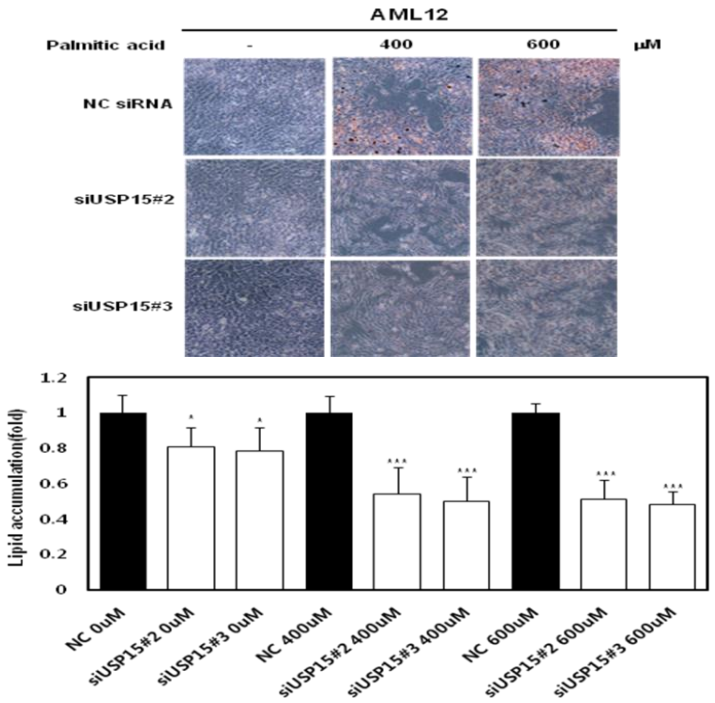

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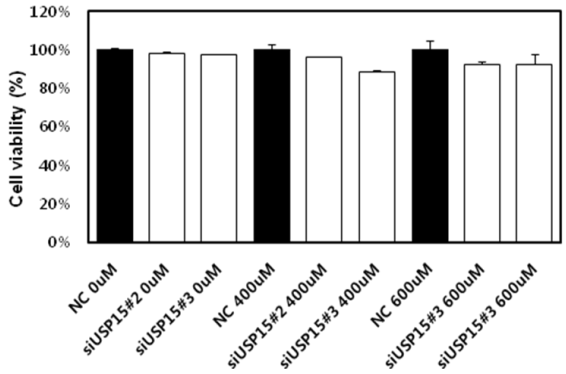

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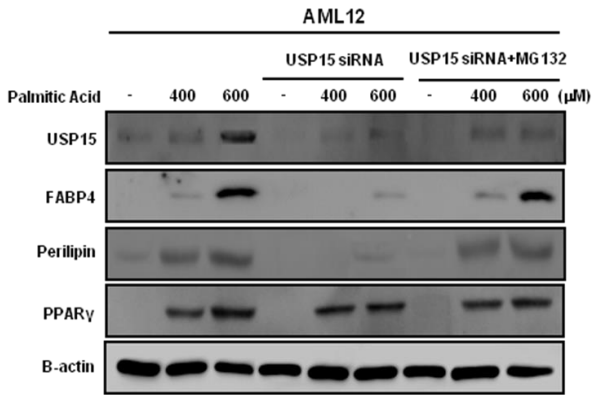

Figure 5. Loss of function of USP15 reduces lipid accumulation in AML12. (A) AML12 was treated with or without siUSP15 and palmitic acid then Oil Red O staining was performed as described in the "Materials and Methods". Lipid accumulation in AML12 was measured using spectrophotometry as described in the "Materials and Methods". (B) Cytotoxicity in AML12 treated with or without USP15 specific siRNA by MTT assay. (C) Protein expression of USP15, FABP4, perilipin, and PPARγ in AML12 cell lysates with or without USP15 siRNA and MG132 was detected by western blotting. β-actin was used as a loading control. Data are presented as mean ± SD; **P* < 0.05, and ****P* < 0.001 for negative control *vs.* siUSP15#2, #3.

5. Overexpression of mUSP15 induces lipid accumulation in hepatocyte

To determine whether mUSP15 overexpression induces lipid accumulation, we prepared the pcDNA3.0_mUSP15 (mUSP15 overexpression vector) and transfected them into AML12 cells. The lipid accumulation was analyzed by performing ORO staining (Figure 6A and B). Over–expression of mUSP15 induced lipid accumulation but over-expression of mutant vector could not induce lipid accumulation as mUSP15 vector did.

The expression level of protein of NAFLD molecules went up as mUSP15 over-expression vector was transfected (Figure 6C). Based on these data, we suggest that expression level of mUSP15 was related with induction of lipid accumulation in hepatocyte.

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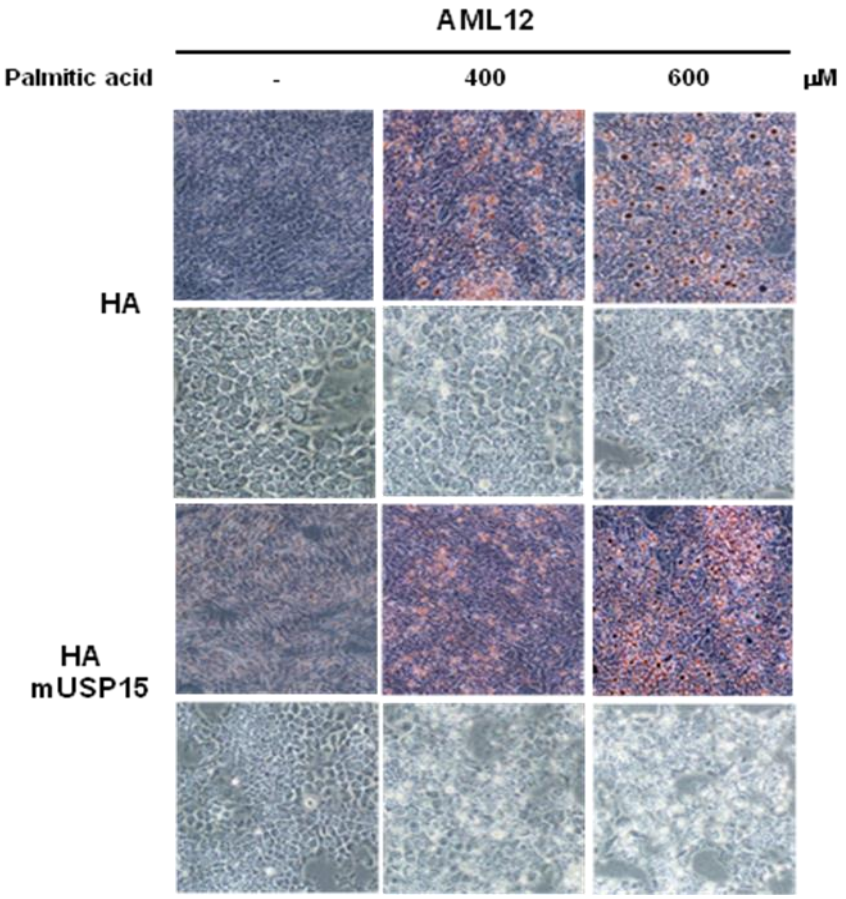

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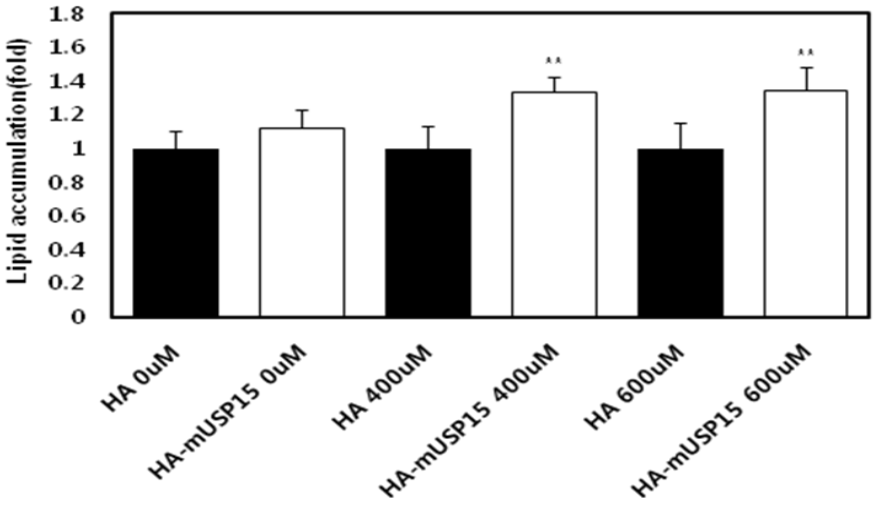

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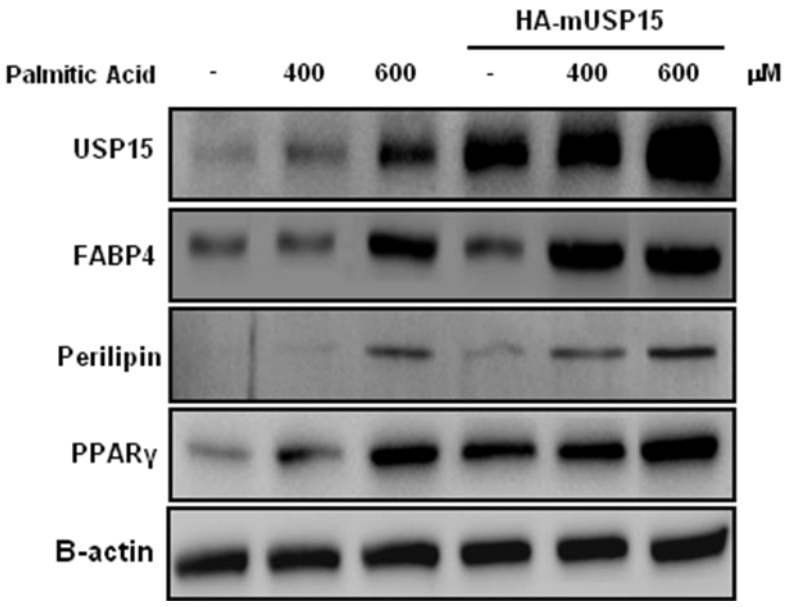

Figure 6. Gain of function of USP15 reduces lipid accumulation in AML12. (A) AML12 was treated with mUSP15 and palmitic acid then Oil Red O staining was performed as described in the "Materials and Methods". (B) Lipid accumulation in AML12 was measured using spectrophotometry as described in the "Materials and Methods". (C) Protein expression of USP15, FABP4, perilipin, and PPARγ in AML12 cell lysates with mUSP15 was detected by western blotting. β-actin was used as a loading control. Data are presented as mean ± SD; $^{**}P < 0.01$ for negative control *vs.* pcDNA3.0_mUSP15.

6. Liver tissue specific USP15 KO mice have less lipid accumulation than USP15 WT although PPARγ overexpression in liver

To determine whether liver tissue specific USP15 KO mice have less lipid accumulation in liver, we crossed USP15 conditional floxed/floxed KO mice with albumin-cre mice in order that USP15 was liver tissue specific knocked out. First of all, we confirmed USP15 was knocked out in liver but not gWAT, iWAT, and BAT by RT-PCR analysis (Figure 7A). To induce lipid accumulation in liver, we infected adenovirus-PPARγ into USP15 WT and KO mice by tail vein injection. To check PPARγ was overexpressed in liver tissue, we performed RT-PCR in liver, gWAT, iWAT, and BAT. RNA level of PPARγ was augumented in liver of adenovirus-PPARγ infected mice (Figure 7B). After one week later from adenovirus infection, we checked body weight of AdGFP USP15 WT, AdGFP USP15 KO, AdPPARγ USP15 WT, and AdPPARγ USP15 KO mice followed by sacrifice. There were no differences of body weight between two groups (Figure 7C). However, liver weight of AdGFP USP15 WT and AdPPARγ USP15 WT was heavier than that of USP15 KO (Figure 7D).

To perform histological analysis, we implemented H&E staining of sectioned liver tissues. As a result, the lipid ratio was reduced in liver sections of AdPPARγ USP15 KO mice (Figure 7E). Furthermore, we confirmed protein expression of USP15, FABP4, and PPARγ in liver of AdGFP USP15 WT, AdGFP USP15 KO, AdPPARγ USP15 WT, and AdPPARγ USP15 KO by westernblot assay. Protein level of USP15, FABP4, and PPARγ was increased in AdPPARγ USP15 WT compared to AdPPARγ USP15 KO (Figure 7F). We identified that liver tissue specific USP15 KO mice have less lipid accumulation than that of USP15 WT although PPARγ overexpression in liver. Taken together, USP15 is positive regulator of hepatic lipid accumulation *in vivo*.

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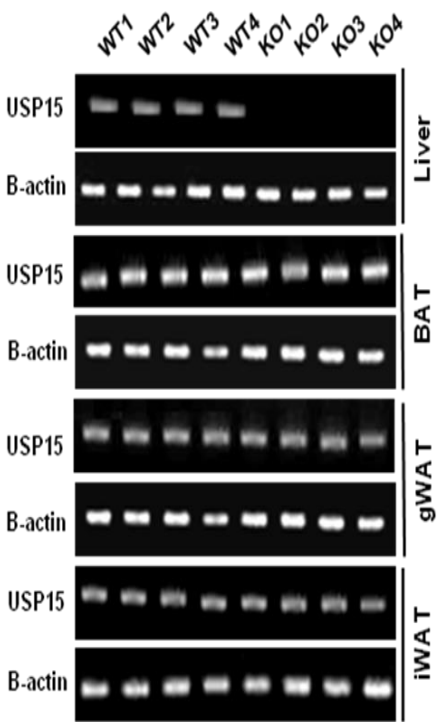

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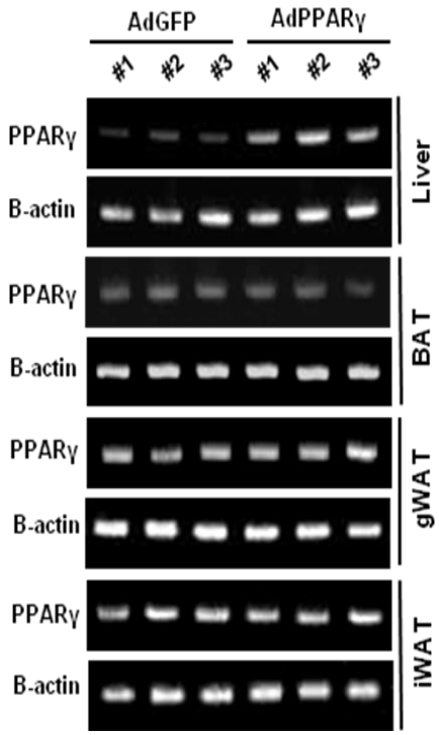

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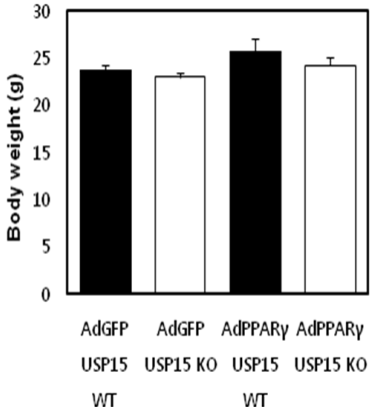

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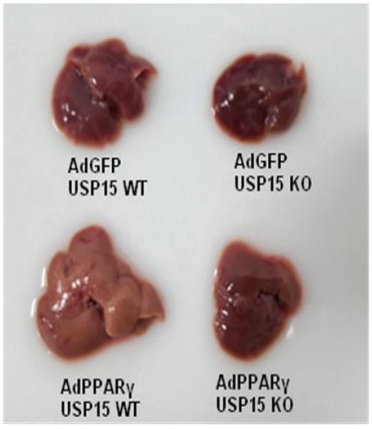

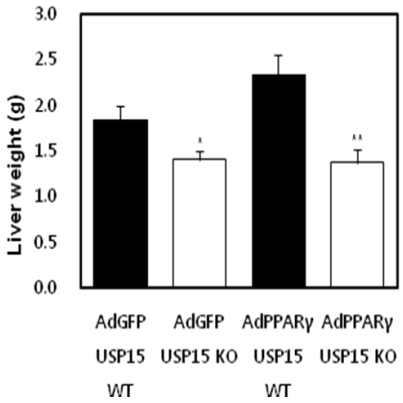

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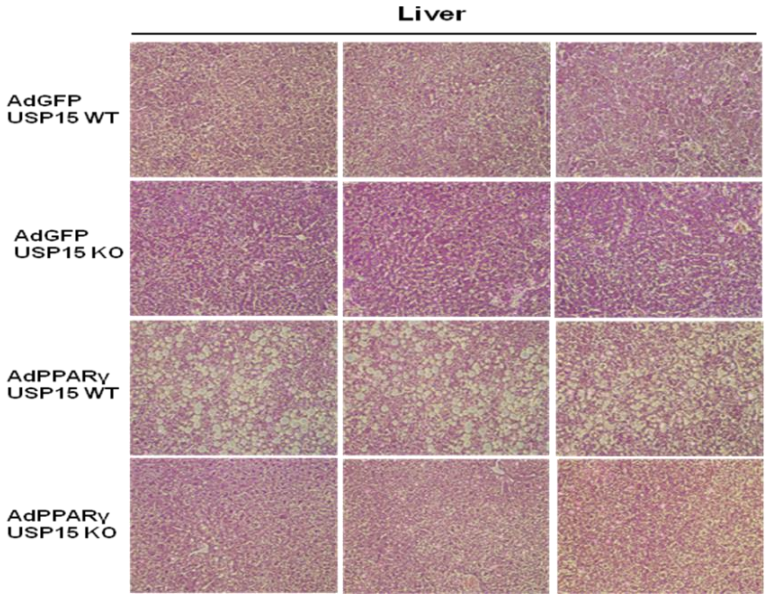

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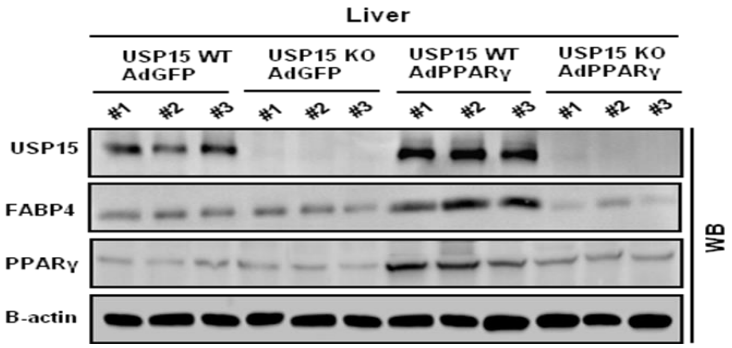

Figure 7. Hepatic lipid accumulation in AdPPARγ USP15 WT is more than that of USP15 KO. (A) RNA level of USP15 in liver, BAT, gWAT and iWAT was detected by RT-PCR analysis. (B) RNA level of PPARγ in liver, BAT, gWAT and iWAT was detected by RT-PCR anaylsis (C) Body weight of AdGFP USP15 WT, AdGFP USP15 KO, AdPPARγ USP15, AdPPARγ USP15 KO. (D) Liver images and Liver weight of AdGFP USP15 WT, AdGFP USP15 KO, AdPPARγ USP15 WT, and AdPPARγ USP15 KO. (E) H&E staining of liver sections was performed in each indicated group as described in the "Materials and Methods". (F) Protein expression of USP15, FABP4 and PPARγ in liver tissure was detected by western blotting. β-actin was used as a loading control. Data are presented as mean ± s.e.m.; $*P < 0.05$, and $**P < 0.01$ for AdGFP USP15 WT *vs.* AdGFP USP15 KO and AdPPARγ USP15 WT *vs.*AdPPARγ USP15 KO

7. USP15 interacts with FABP4 and PPARγ and regulates their protein expression

To determine how USP15 regulates lipid accumulation in cells, we prepared the pcDNA3.0_hUSP15 (hUSP15 overexpression vector), pcDNA3.0_hFABP4 (hFABP4 overexpression vector) and pcDNA3.0_hPPARγ (hPPARγ overexpression vector). At first, we co-transfected pcDNA3.0_hFABP4, pcDNA3.0_hPPARγ (constant level), and pcDNA3.0_hUSP15 (0, 0.5, 1, 2μg each) into HEK293 cells (Figure 8A and B). We confirmed that protein level of FABP4 and PPARγ were up-regulated as USP15 over-expression level. Plus, we performed IP analysis and detected interaction between USP15 and FABP4, PPARγ in HEK293 (Figure 8C and D). We also wondered their interaction between USP15 was detected in mouse hepatocyte. Therefore, we performed IP analysis endogenously and confirmed their interaction between USP15 in AML12.

Taken together, we suggested that USP15 interacts with NAFLD related molecules such as FABP4 and PPARγ and then it regulates their protein expression.

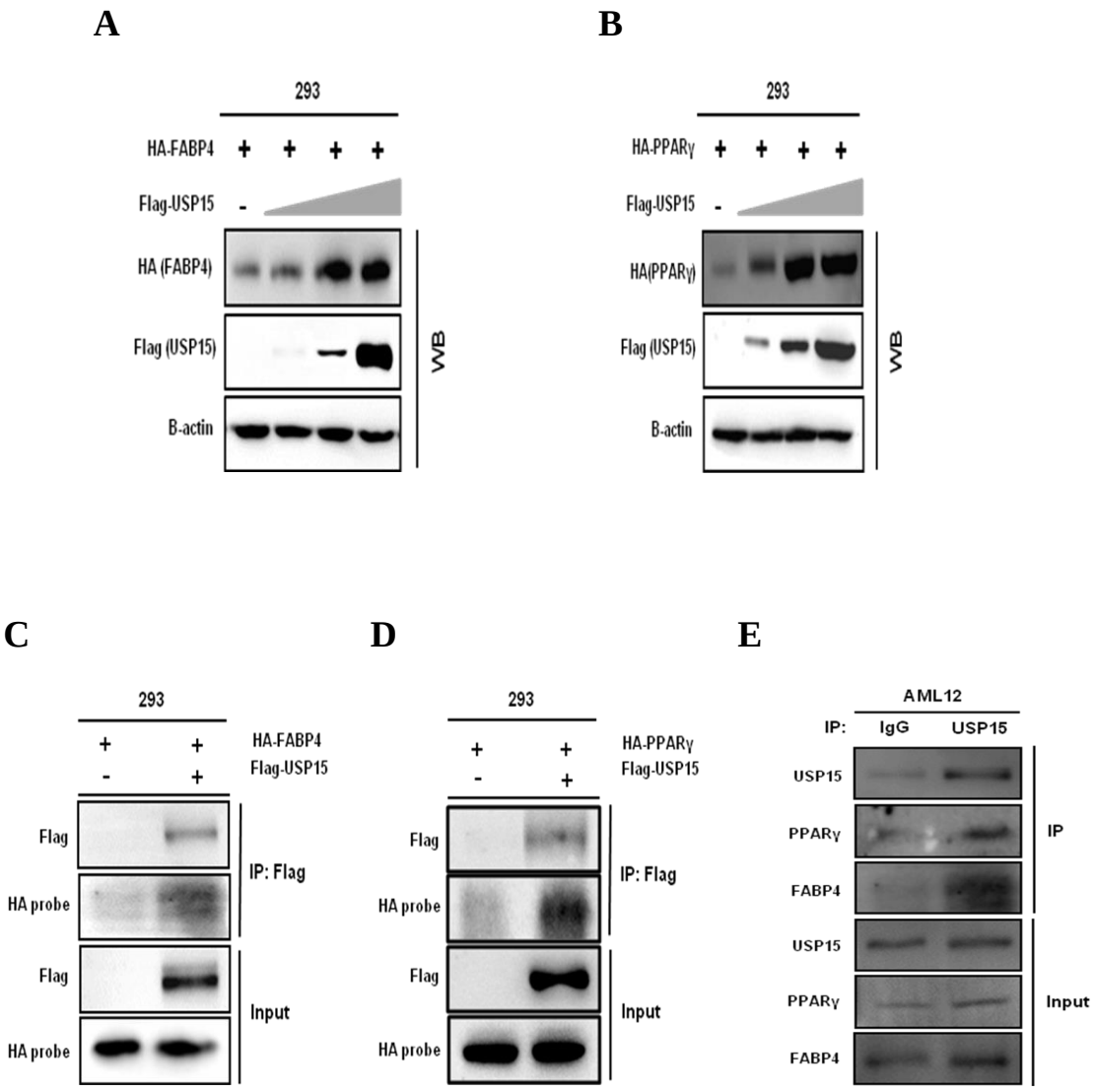

Figure 8. USP15 interacts with FABP4 and PPARγ as well as regulates their protein expressions. (A), (B) pcDNA3.0_hUSP15, pcDNA3.0_hFABP4 and pcDNA3.0_hPPARγ were co-transfected in HEK293 cells and protein level was detected by western blotting. β-actin was used as a loading control. (C), (D) pcDNA3.0_hUSP15, pcDNA3.0_hFABP4 and pcDNA3.0_hPPARγ were co-transfected in HEK293 cells and their interaction with USP15 was detected by immunoprecipitation assay. (E) PPARγ and FABP4 interaction with USP15 in AML12 was detected by immunoprecipitation ssay. IgG was used as negative control.

8. USP15 upregulates protein stability of FABP4 and PPARγ by deubiquitination

To assess whether USP15 upregulates protein stability of FAB4 and PPARγ by deubiquitination, we prepared the pcDNA3.0_hUSP15 (hUSP15 overexpression vector), pcDNA3.0_hFABP4 (hFABP4 overexpression vector), pcDNA3.0_mUSP15 (mUSP15 overexpression vector), and pcDNA3.0_mPPARγ (hPPARγ overexpression vector) for ubiquitination assay. At first, we co-transfected pcDNA3.0_hFABP4, pcDNA3.0_mPPARγ with pcDNA3.0_hUSP15, pcDNA3.0_ mUSP15, respectively (Figure 9A and B). We confirmed that FABP4 and PPARγ were deubiquitinated by USP15 when MG132 was treated. Based on these data, we suggested that USP15 upregulates protein stability of NAFLD related molecules such as FABP4 and PPARγ by deubiquitinaton.

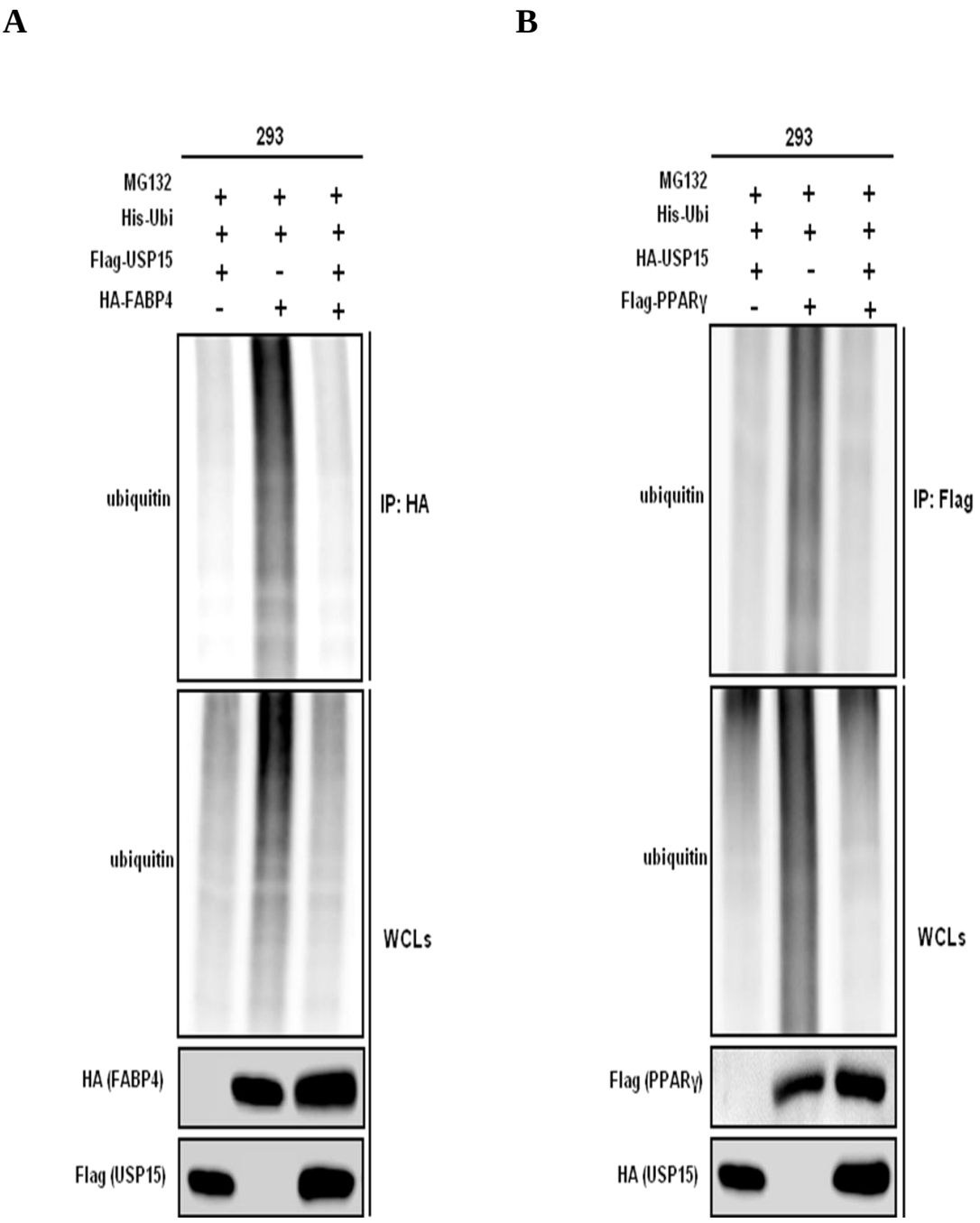

Figure 9. USP15 deubiquitinates FABP4 and PPARγ. (A), (B) pcDNA3.0_hUSP15, pcDNA3.0_ hFABP4, pcDNA3.0_ mUSP15, pcDNA3.0_mPPARγ and pcDNA3.0_His-ubi were co-transfected in HEK 293 cells and MG132 was treated 10μM for 8 hours. ubiquitination assay was performed as described in the "Materials and Methods".

Ⅳ. DISCUSSION

The obesity has led to the occurence of fatty liver and steatosis. Simple steatosis is a benign condition, but almost 30% of patients will be suffered from fibrosis, hepatocyte death, hepatocellular carcinoma, and cardiovascular diseases.[3-5,24,25] Despite the clinical importance of hepatosteatosis, many gaps remain in our understanding of fatty liver. In this context, the finding that deubiquitination of FABP4 and PPARγ, NAFLD associated factors,[9,26] regulated by USP15 provides new insight into NAFLD and may suggest another reason of fatty liver.

The deubiquitinase activity of USP15 has been related with transcription factor NF-κB, parkin-mediated mitochondrial ubiquitination and mitophagy[27], the Nrf2 pathway in anti-oxidant response.[28] Furthermore, USP15 has been known to regulate TGF-β signaling and been correlated with the signal transducer SMAD3, the E3 ligase SMURF2, and type 1 TGF-β receptor by deubiquitination.[16,17,29,30] Furthermore, USP15 has been associated with regulation of the immune system *in vitro* and *in vivo*. USP15 also regulates T_H1 responses in CD4+ T cells through E3 ubiquitin ligase MDM2[18] and contributes to the regulation of type 1 interferon response.[31] In recent studies, it was identified that USP10 regulates AMPK signaling involved in glucose metabolism in liver[20]. Additionally USP7 regulates early adipogenesis by deubiqutination of TIP60[21] as well as regulates insulin signaling by modulating the insulin receptor substrate 1 (IRS1).[32] However lipid and hepatic metabolism studies about USP15 is poorly understood. In this study, we confirmed more than three PPARγ binding sites in human and mouse USP15 promoter region (data analyzed from genomatix online). Secondly, we also identified mRNA expression level of USP15 in both healthy obese and steatosis liver tissues from patients (Data analyzed from the GEO dataset in NCBI). There were differences in mRNA expression levels of hUSP15 between normal and healthy obese and steatosis. In short, mRNA level of USP15 is augumented in healthy obese and steatosis patients.

First, we confirmed expression of USP15, FABP4, and PPARγ in mouse liver was increased in HFD-fed group. Plus, we treated oleic acid, one of the fatty acids, into AML12 mouse hepatocyte and

confirmed expression level of USP15, FABP4, perilipin, and PPARγ. As a result, thier protein levels were agumented in mouse hepatocyte. Secondly, we investigated the effect of USP15 by loss-of function and gain of function analysis on palmitic acid treated AML12. Knock down of USP15 significantly reduced the lipid accumulation and protein expression of NAFLD associated factors; conversely, overexpression of USP15 increased them. We also indentified that AdPPARγ USP15 WT mice have more liver weight and hepatic lipid accumulation than thoes of AdPPARγ USP15 KO mice. To sum up, USP15 expression in mouse hepatocyte and liver tissue were associated with expression patterns of NAFLD realted factors. Moreover, we determined how USP15 regulates lipid accumulation in cells. We identified that protein level of FABP4 and PPARγ were increased as USP15 over-expression level. We also performed IP analysis and confirmed their interaction between USP15 and FABP4, PPARγ. Our results suggested that USP15 interacts with FABP4 and PPARγ to regulate protein stability of them. We wondered whether protein stabilty of FABP4 and PPARγ was due to deubiquitination mechanism by USP15. Finally, we identified that protein stability of FABP4 and PPARγ was gained by deubiquitination mechanism of USP15. Taken together, we considered that high expression of USP15 leads to hepatosteatosis in liver of patients. Ablation of USP15 significantly reduced lipid accumulation in liver and inhibited protein exprssion of one of NAFLD associated factors. Futhermore, the expression and protein stability of FABP4 and PPARγ was regulated by USP15. Therefore, this study suggests that USP15 could be potent molecules in terms of NAFLD and liver metabolism. Further studies are needed to investigate their clinical implications.

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ABSTRACT (IN KOREA)

USP15 는 FABP4 와 PPARγ 의존적인 기작을 통해 간세포에서의 지방축적을 조절한다.

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유비퀴틴 단백질 분해효소 15 (USP15) 는 *USP15* 유전자로부터 암호화 되어서 생성되는 효소이다. USP15 는 표적 단백질에 접합해 있는 유비퀴틴을 가수분해 시키는 기능을 한다. USP15 는 TGF-베타 수용체 신호와 NF-kappa-B 신호전달체제를 다양하게 조절한다. 특히, USP15 는 TGF-베타 수용체 신호전달경로의 주요 조절인자이며 R-SMAD 들의 탈유비퀴틴화를 촉진한다. 그러므로 USP15 는 R-SMAD 들의 저해를 경감시키고 TGF-베타의 표적 유전자의 활성을 촉진한다. 지금까지 USP15 와 관련된 연구는 대다수가 암 그리고 암 면역분야에 관한 것이 대다수이다. 하지만 USP15 와 대사질환과의 관계에 대한 연구는 미흡한 실정이다. 본 연구에서 우리는 건강한 비만환자 그리고 지방간 환자의 간에서 USP15 의 발현 정도가 높다는 것을 확인하였다. 또한, 우리는 고지방식이를 시킨 C57BL/6 쥐의 간조직과 팔미틱산을 처리한 쥐의 간세포에서 USP15 의 발현이 증가한다는 것을 확인하였다. USP15 의 발현저하는 쥐의 간세포에서 지방축적과 FABP4, perilipin, PPARγ 와 같은 비알콜성 지방간에 관여하는 인자들의 발현을 모두 저해하였다. 반면에 USP15 의 과발현은 쥐의 간세포에서 지방축적과 비알콜성 지방간에 관여하는 인자들의 발현을 촉진시켰다. 우리는 또한 간 조직 특이적으로 USP15 가 KO 된 쥐에서 간 내 PPARγ 가 과발현 되었음에도 불구하고 간의 지방 축적이 USP15 WT 쥐에 비해 감소하다는 것을 알게 되었다.

게다가, 우리는 USP15 가 FABP4 와 PPARγ 와 단백질 간 상호작용을 하고 두 단백질의 발현을 증가시킨다는 것을 밝혔다. 추가적으로, 우리는 또한 FABP4 와 PPARγ 단백질의 발현증가는 USP15 에 의한 두 단백질의 탈유비퀴틴화로 인한 것임을 밝혔다. 종합적으로, USP15 는 비알콜성 지방관과 관련된 단백질 인자들의 탈유비퀴틴화를 유도함으로써 관련 단백질 인자들의 안정성을 촉진한다. 우리는 본 연구를 통해서 USP15 가 간세포내의 지방축적에 관여하는 역할을 하고 USP15 의 억제는 비알콜성 지방간 발병을 완화시키는 기능을 한다고 제안하는 바이다.

핵심되는 말: 비알콜성 지방간(NAFLD), USP15, FABP4, PPARγ, 탈유비퀴틴화, 간세포