博　士　論　文

Studies on biological functions of flavonoids that affect the transcription of genes related to lipid metabolism

（脂質代謝関連遺伝子の転写を調節するフラボノイド類の機能解析研究）

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Article Outline

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
ACATs	Acyl-CoA cholesterol acyltransferases
ACC	Acetyl-CoA carboxylase
ACOD	Acyl-CoA-Oxidase
ACS	Acyl-CoA-Synthetase
ALLN	N-acetyl-Leu-Leu-norleucinal
AOD	Ascorbate oxidase
ASCVD	Atherosclerotic cardiovascular disease
β-gal	β-galactosidase
BMI	Body mass index
BPB	Bromo phenol blue
bZIP	Basic leucine zipper
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
ChIP	Chromatin immunoprecipitation
CM	Chylomicrons
CoA	Coenzyme A
COUP-TF II	Chicken ovalbumin upstream promoter transcription factor II
CQ	Chloroquine
CT	Computed tomography
CYP7A1	Cholesterol 7alpha-hydroxylase
DAX-1	Dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome, gene 1

DGAT	Diacylglycerol acyltransferase
DR	Direct repeat
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
GOT	Glutamic-oxaloacetic transaminase
GPO	Glycerol-3-phosphate Oxidase
GPT	Glutamic-pyruvic transaminase
GR	Glucocorticoid receptor
GRIP1	Glutamate receptor-interacting protein 1
H-Chol	High-Cholesterol
HFD	High-fat diet
HMGCR	Hydroxy-3-methyl-glutaryl-CoA reductase
HMGCS	Hydroxy-3-methyl-glutaryl-CoA synthase
HNF1α	Hepatic nuclear factor 1α
HNF4α	Hepatic nuclear factor 4α
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRE	Insulin response element
LA	Linoleic acid
LAP	Liver-enriched transcriptional activator protein
LDL	Low density lipoprotein
LDLR	Low-density lipoprotein receptor
LIP	Liver-enriched transcriptional inhibitory protein
MODY1	Maturity on-set diabetes of the young type 1
MTP	Microsomal triglyceride transfer protein
NEFA	Non-esterified fatty acids
OAC	Oxaloacetate decarboxylase
OGTT	Oral glucose tolerance test

PDI	Protein disulfide isomerase
PGC-1α	Peroxisome proliferator-activated receptor γ co-activator 1-α
PI3K	Phosphatidylinositol 3-kinase
PIC	Protease inhibitor cocktail
PL	Phospholipid
PMSF	Phenylmethylsulphonyl fluoride
POD	Peroxidase
POP	Pyruvate oxidase
qRT-PCR	Quantitative real-time PCR
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SHP	Small heterodimer partner
SR-B1	Scavenger receptor class B member 1
SRC-1	Steroid receptor co-activator-1
SREBP	Sterol regulatory element-binding protein
T-BA	Total Bile Acod
T-Chol	Total-Cholesterol
TG	Transport of triglyceride
TOFA	Tetradecyloxy-2-furancarboxylic acid
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein

Chapter 1

Introduction

Obesity can be defined as an excess of body fat. A surrogate marker for body fat content is the body mass index (BMI), which is determined by weight (kilograms) divided by height squared (square meters). In clinical terms, a BMI of 25–29 kg/m^2 is called overweight; higher BMIs ($\geqslant$30 kg/m^2) are called obesity (1). The worldwide increase in obesity and related chronic diseases has largely been driven by global trade liberalization, economic growth and rapid urbanization (2). Globally, the prevalence of obesity increased from 4.8% in 1980 to 9.8% in 2008 for men, and from 7.9% to 13.8% in women, paralleled by an increase in mean BMI of 0.4 to 0.5 kg/m^2 per decade (equivalent to 1.2 to 1.4 kg per decade for a person 1.6 to 1.8 m tall). By 2008, mean BMI had reached 23.8 kg/m^2 for men and 24.1 kg/m^2 for women (3).

The foremost physical consequence of obesity is atherosclerotic cardiovascular disease (ASCVD) (4). A substantial portion of the ASCVD resulting from obesity is mediated by type 2 diabetes. But obesity is accompanied by several other risk factors for ASCVD. The sum of the risk factors that predisposes to ASCVD goes by the name of metabolic syndrome. In addition, obesity is accompanied by other medical complications other than ASCVD and diabetes; these include fatty liver, cholesterol gallstones, sleep apnea, osteoarthritis, and polycystic ovary disease. These disorders are commonly found in individuals who carry the metabolic syndrome (5). Besides, persons with BMI $\geq$27.0 kg/m^2 had a higher risk of ASCVD (6). High BMI was also associated with an increased incidence for ASCVD (7-10). Therefore it is crucial to find an approach to treat the diseases, which are caused by obesity.

1-1 Apolipoprotein B (Apo B)

1-1-1 Apo B and diseases caused by obesity

Apo B concentrations, which reflect the number of small dense LDL particles in plasma, are a significant predictor of cardiometabolic risk among adults with a high prevalence of metabolic syndrome (11). Plasma Apo B has recently been closely associated with metabolic syndrome (12) and elevated risk of developing coronary heart disease (CHD) (13). Apo B is increased in type 2 diabetes (14). Apo B-containing lipoprotein particles, which are secreted and cleared by the liver, are essential for the development of atherosclerosis (15,16).

1-1-2 Apo B secretion process

Apo B is synthesized in two isoforms. In humans, Apo B48 is synthesized in the small intestine and becomes a component of chylomicrons (CM). Apo B100 is synthesized in liver and becomes a component of both very low density lipoprotein (VLDL) and low density lipoprotein (LDL)(17). Apo B100 is an about 550 kD single chain polypeptide, and Apo B 48 represents an Apo B100 fragment encompassing the N-terminal 48% of the polypeptide that results from the selective editing of the Apo B encoding mRNA (18).

The assembly of lipoproteins containing Apo B is a complex process. The initial association of Apo B with lipid occurs during its translation by membrane bound ribosomes (19). The product of the co-translational phase of Apo B assembly is a small dense emulsion particle (20). The formation of large heterogeneous VLDL in liver or CM in intestine is thought to occur via a second, posttranslational step in which preformed, apparently protein-free TG droplets fuse with the nascent Apo B-containing particles formed during translation. The assembly of Apo B with lipid requires a dedicated endoplasmic reticulum (ER)-localized cofactor termed the microsomal triglyceride transfer protein (MTP) (21). Apo B 100 is regulated by intracellular degradation at both the co-translational and post-translational levels and that ubiquitin-proteasome-mediated and non-proteasome-mediated (such as autophagy) pathways are involved in this process (22).

1-1-3 Regulation of Apo B

The Apo B gene transcription is believed to be constitutive, and Apo B levels are thought to change primarily by co- and post-translational mechanisms. However, transcriptional mechanisms may also play a role in the control of Apo B secretion (23). In human Apo B gene promoter, there are the binding sites of C/EBP, HNF3, and HNF4 (24). Among the regulator factors during co- and post-translation, MTP is a significant one. It is now clear that MTP plays an obligatory role in the stage of Apo B assembly and secretion (25).

1-2 Microsomal triglyceride transfer protein (MTP)

1-2-1 Role of MTP on Apo B secretion

MTP is a heterodimer consisting of a 97-kDa unique subunit complexed with the ubiquitous ER-localized chaperone, protein disulfide isomerase (PDI), is able to accelerate the transport of triglyceride (TG), cholesteryl ester (CE), and phospholipid (PL) between membranes. MTP is required for the assembly and secretion of very low density lipoproteins by the liver and chylomicrons by the intestine (21). The role of MTP on Apo B secretion is as follows (Fig 1-1):

1. MTP transfers lipid from the ER membrane or some other donor site to Apo B during the co-translational phase of lipoprotein assembly (21).
2. In some studies, MTP has been shown to play a role in the posttranslational fusion of nascent Apo B-containing particles with TG droplets. MTP may be necessary for the trafficking of TG from the cytosol to the ER lumen, a process that occurs independently of Apo B (26).
3. MTP prevents co-translational degradation of Apo B100 by the proteasome (27).

1-2-2 Regulation of MTP

MTP gene mutation lead to a rare disease called abetalipoproteinemia, resulting in a complete lack of apo B–containing lipoproteins in the circulation. Because of its role in lipoprotein assembly, MTP inhibition is considered an important modality to treat

hyperlipidemia and reduce risk for atherosclerosis (28). In this regard, several MTP inhibitors have been identified that lower plasma lipids (29); however, they have not reached clinical use due to associated adverse events mainly related to accumulation of lipids in the liver.

MTP is regulated by macronutrients, hormones and other factors. High fat diet increases MTP mRNA in both of liver and intestine; high sucrose diet increases MTP mRNA in the liver but not in the intestine (30) whereas a fructose enriched diet increases MTP mRNA and activity in both the liver and intestine (31). Two different pathways regulating MTP expression by insulin have been proposed. In the MAPK signaling pathway, insulin increases phosphorylation of Raf-1, MEK1/2 and ERK1/2, which culminates in reduced expression of MTP in cells. This signaling arm is negatively regulated by MAPKp38 (32). In the PI3K pathway, insulin activates PI3-kinase and AKT/PKB. This leads to increased phosphorylation of fork head transcription factors, their export from the nucleus and reduced MTP gene transcription (33). MTP regulation is complex. Whereas the major mode of regulation occurs at the transcriptional level (34). Deletion analysis in hepatic cells revealed that the promoter sequence contains critical positive (hepatic nuclear factor [HNF] 1, HNF4, direct repeat [DR]1 and FOX) and negative regulatory sterol and insulin response element (SRE/IRE) (34). HNF4α transcription factor interacts with the HNF4 element (35). HNF4α knockout mice do not express MTP, indicating that it is absolutely required for expression (36).

1-3 Hepatic nuclear factor 4α (HNF4α)

1-3-1 Function of HNF4α

HNF4α (or NR2A1) is a highly conserved member of the nuclear receptor superfamily. It forms homodimers that bind to a direct repeat of the AGGTCA with one-base spacing (DR1) (37). HNF4α is expressed at the highest levels in the liver, kidney, intestine, and pancreas in mammals (36). HNF4α is the most abundant nuclear receptor expressed in the liver (38), with the function of regulating network required for maintenance of the

hepatocyte phenotype (39) as well as metabolic genes. HNF4α target genes are involved in lipid transport, fatty acid oxidation, bile acid synthesis and transport, lipoprotein metabolism, steroid metabolism, glucose metabolism, amino acid metabolism, blood coagulation and viral genome replication (40) (Fig 1-2 A, B).

Human HNF4α gene mutations cause maturity on-set diabetes of the young type 1 (MODY1), which has impaired glucose regulation of insulin secretion due to dysfunction of the pancreatic β-cells (41). Ablation of the Hnf4α gene is embryonic lethal (42). Liver-specific deletion of the hnf4α gene in adult mice reveals phenotypes of decreased serum cholesterol, triglycerides, increased bile acids, and accumulation of lipids in mouse livers, suggesting that HNF4α plays a critical role in maintaining lipid homeostasis (36). Pancreatic specific Hnf4α null mice have impaired insulin secretion and MODY-1 phenotypes (43). Intestine epithelial cell-specific Hnf4α null mice has the phenotypes of inflammatory bowel disease (IBD) (44).

1-3-2 Ligand of HNF4α

The existence of a ligand for HNF4α has not been clearly defined and is somewhat controversial. Initially, it has been reported that fatty acyl–CoA thioesters are endogenous ligands of HNF4α (45,46). The crystal structure of the ligand-binding domain of HNF4α reveal that the ligand-binding pocket contains a mixture of saturated and cis-monounsaturated C14 – 18 fatty acids (47,48). Recent study reveals that linoleic acid (LA) has been identified as the reversible endogenous ligand of native HNF4 expressed in mouse liver, however ligand occupancy does not appear to have a significant effect on HNF4α transcriptional activity (49).

1-3-3 Regulation of HNF4α

HNF4α expression is synergistically regulated by several liver-enriched transcription factors, including HNF4α, HNF1s, HNF3s and HNF6 (50,51). Besides, many NRs such as PXR, CAR, LXR, FXR, small heterodimer partner (SHP), Vitamin D receptor (VDR), chicken ovalbumin upstream promoter transcription factor (COUP-TF) and glucocorticoid

receptor (GR) interact with HNF4α to regulate the expression of the target genes in a complex fashion (Fig 1-2 C)(52).

The trans-activating activity of HNF4α is stimulated by co-activators and inhibited by ubiquitous nuclear receptor co-repressors, The HNF4α co-activators include peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α), Glutamate receptor-interacting protein 1 (GRIP1), p160 family co-activators, steroid receptor co-activator-1 (SRC-1), SRC-2 and SRC-3, and p300/CBP (cAMP response element binding protein) family co-activators (53-55). The co-repressors include Small heterodimer partner (SHP) (56), dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome, gene 1 (DAX-1) (57).

The trans-activating activity of HNF4α also can be regulated by post-translational modifications. There are 13 potential serine/threonine phosphorylation sites on HNF4α (40). Phosphorylation of HNF4α by protein kinase A, mitogen-activated protein kinases p38 or JNK/cJun reduces DNA binding activity (58). AMP kinase reduces HNF4α dimerization and protein stability (59). Phosphorylation of Serine78 by protein kinase C causes nuclear exclusion and degradation (60). CBP acetylates HNF4α at a lysine residue causing HNF4α nuclear retention and increasing DNA binding and trans-activating activity (61,62).

1-4 C/EBPβ

1-4-1 C/EBP family and C/EBPβ isoforms

The CCAAT-box/enhancer binding proteins (C/EBPs) belong to a larger family of basic leucine zipper (bZIP) transcription factors, which have a C-terminal leucine zipper domain for dimerization and a basic domain for binding to DNA. The C/EBP family has important roles in cellular proliferation and differentiation, survival and/or apoptosis, metabolism, inflammation and transformation, and oncogene-induced senescence and tumorigenesis (63). There are at least six members of this family, α, β, γ, δ, ε, and ζ, that can both homodimerize and heterodimerize with each other and bind to the same C/EBP regulatory

element in the promoters/enhancers of many genes (64). In addition, each family member can give rise to several isoforms by a process of selective use of translational start sites within each mRNA or by proteolysis of a larger precursor protein (65).

The C/EBPβ mRNA, for instance, gives rise to at least four isoforms corresponding to the following peptides, 38 kD, 34 kD, 30 kD and 20 kD. The 34 kD protein is often referred to as LAP (liver-enriched transcriptional activator protein) since it has been shown to be a potent transactivator of liver gene expression(66). The 20 kD polypeptide, however, can inhibit hepatic gene expression and is, therefore, referred to as LIP (liver-enriched transcriptional inhibitory protein)(66). This LIP isoform of C/EBPβ corresponds to the C terminal portion of the LAP protein that lacks the transactivation domain but contains the basic leucine zipper region. Consequently, LIP can act as a potent dominant negative repressor of C/EBPβ activity. The LIP/LAP ratio is a critical factor in C/EBPβ-mediated gene transcription (67).

1-4-2 Function of C/EBPβ

C/EBPβ is involved in the differentiation of a large variety of cell types, including keratinocytes, hepatocytes, mammary epithelial cells, ovarian luteal cells, adipocytes, B cells, and macrophages. Moreover, C/EBPβ regulates cell survival, apoptosis, metabolism, inflammation, and tumorigenic transformation (68).

1-4-3 Regulation of C/EBPβ

Post-translational modifications such as, phosphorylation, acetylation, methylation and sumoylation, play crucial roles in the regulation of C/EBPβ binding, transcriptional activity, protein–protein interactions and subcellular localization(63) . Phosphorylation of C/EBPβ is an essential mechanism in the regulation of C/EBPβ-dependent gene regulation (69). Phosphorylation within the inhibitory domains can abolish this repressive effect, and in many cases, leads to an increase in the transcriptional activity of C/EBPβ. C/EBPβ phosphorylation occurs on numerous residues and is regulated via numerous signaling pathways, which include: MAPK (70-72), growth factors and glycogen synthase kinase 3β

(GSK3β) (Refs (73-75), protein kinases A and C (76,77), and cAMP signaling(78).

1-5 Flavonoid

Flavonoids belong to a vast group of polyphenolic compounds that are widely distributed in all foods of plant origin. Flavonoids occur as aglycones, glycosides and methylated derivatives. The flavonoid aglycone consists of a benzene ring (A) condensed with a six membered ring (C), which in the 2-position carries a phenyl ring (B) as a substituent (Fig 1-3 A). On the basis of the molecular structures, flavonoids can be divided into six major subclasses, including the flavones (e.g., Apigenin, Luteolin), flavonols (e.g., Quercetin, Myricetin), flavanones (e.g., Naringenin, Hesperidin), catechins or flavanols (e.g., Epicatechin, Gallocatechin), anthocyanidins (e.g., Cyanidin, Pelargonidin), and isoflavones (e.g., Genistein, Daidzein)(79) (Fig 1-3 B, Tab 1-1).

Epidemiological evidence suggests that flavonoids may play an important role in the decreased risk of chronic diseases. Flavonoids are thought to have antiviral, anti-bacterial, anti-inflammatory, and anti-carcinogenic properties(80). Flavonoids exert some of their effect via interactions with nuclear receptors, making them a promising pharmaceutical and nutraceutical source of compounds for the treatment of metabolic disorders, but their precise mechanism of action is largely unknown (81).

1-5-1 Luteolin

Luteolin (Fig 1-3 C) is widely distributed in the plant kingdom. Dietary sources include celery, broccoli, green pepper, parsley, chamomile tea, carrots, olive oil, and rosemary(80). As other flavonoids, Luteolin is most often found in plant in the form of glycosides, which are eventually metabolized by intestinal bacteria, cleaved and glucuronated during uptake in the gut and metabolized in the organism(82). It is reported that after administration of Luteolin by gastric intubation in rat, free Luteolin, its conjugates and methylated conjugates are present in plasma. And the main conjugate is a monoglucuronide of the unchanged aglycone(83).

Luteolin has been reported to exert anti-inflammatory effects by inhibiting pro-inflammatory cytokines, chemokines and enzymes (e.g. TNF, IL-1, IL-6, IL8, COX-2, and iNOS) via decreasing NF-kappa B activity and AP-1 activation. Several studies suggest that Luteolin has cancer preventive potential by inhibiting angiogenesis, inducing apoptosis, and reducing tumor growth. Experimental data indicate that Luteolin, some of its glycosides or plants with these flavonoids may prevent cardiovascular disease by reducing blood pressure and cholesterol levels (84-86), and prevent diabetes by reducing glucose levels (87-89).

1-5-2 Quercetin

Quercetin-type flavonols (primarily as Quercetin glycosides) (Fig 1-3 D), the most abundant of the flavonoid molecules, are widely distributed in the plant. They are found in a variety of foods including apples, berries, Brassica vegetables, capers, grapes, onions, shallots, tea, and tomatoes, as well as many seeds, nuts, flowers, barks, and leaves (90,91).

Quercetin glycosides are found in far greater amounts in the diet than is Quercetin aglycone (92). Quercetin glucosides are efficiently hydrolyzed in the small intestine by beta-glucosidases to the aglycone form, much of which is then absorbed (93). In humans, Quercetin aglycone might be more bioavailable, or least more reliably bioavailable, than its glycosides (94).

Quercetin also has been reported to exhibit antioxidative (95,96), anticarcinogenic (97-99), anti-inflammatory effects (100) and protects against coronary heart disease (101,102)

1-6 Objective of this study

A worldwide increase in obesity is a big issue to be solved. Obesity is usually accompanied by cardiovascular diseases, metabolic syndrome, and diabetes. Therefore it is crucial to find an approach to treat the diseases caused by obesity.

Apo B concentrations are a significant predictor of cardiometabolic risk. Over-secretion

of Apo B is closely associated with metabolic syndrome, type 2 diabetes, and the development of atherosclerosis. Thus, inhibition of Apo B secretion has the potential as one of approaches to treat the diseases caused by obesity.

In addition, MTP plays an important role for the assembly and secretion of Apo B containing lipoproteins. The objective of this thesis was to study the biological function and molecular mechanisms of flavonoids that affect the transcription of MTP and Apo B.

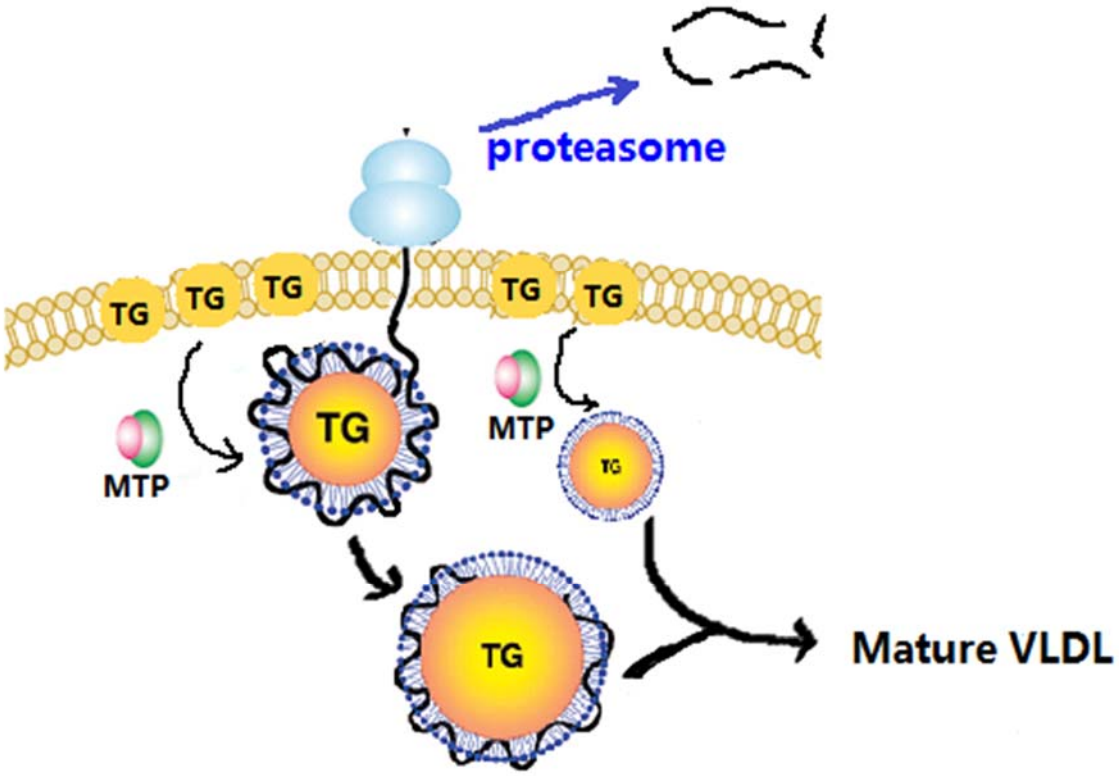


Figure 1-1 Role of MTP on Apo B secretion

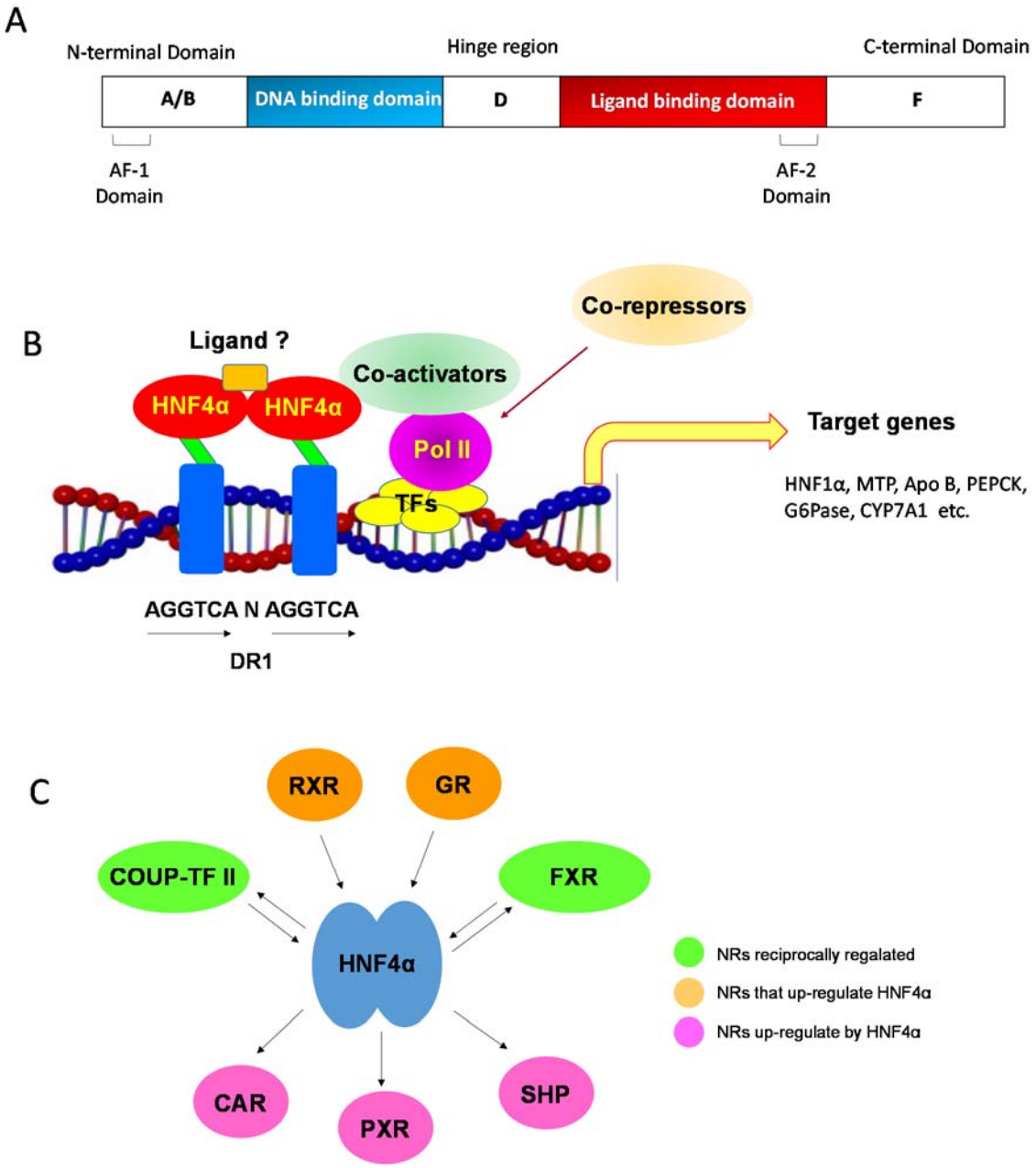


Figure 1-2 Structure and transcription mechanism of HNF4α

A, Structure of HNF4α. B, transcription mechanism of HNF4α. C, Interaction of HNF4α and other nuclear factors.

Table 1-1. Flavonoid Subclasses and Examples

Subclass	Selected Examples
Flavonols	Kaempferol, Myricetin, Quercetin
Flavones	Apigenin, Chrysin, Luteolin
Flavanones	Hesperidin, Naringenin
Flavanols (also called catechins)	Epicatechin, Gallocatechin
Isoavones	Genistein, Daidzein
Anthocyanidins	Cyanidin, Malvidin, Pelargonidin

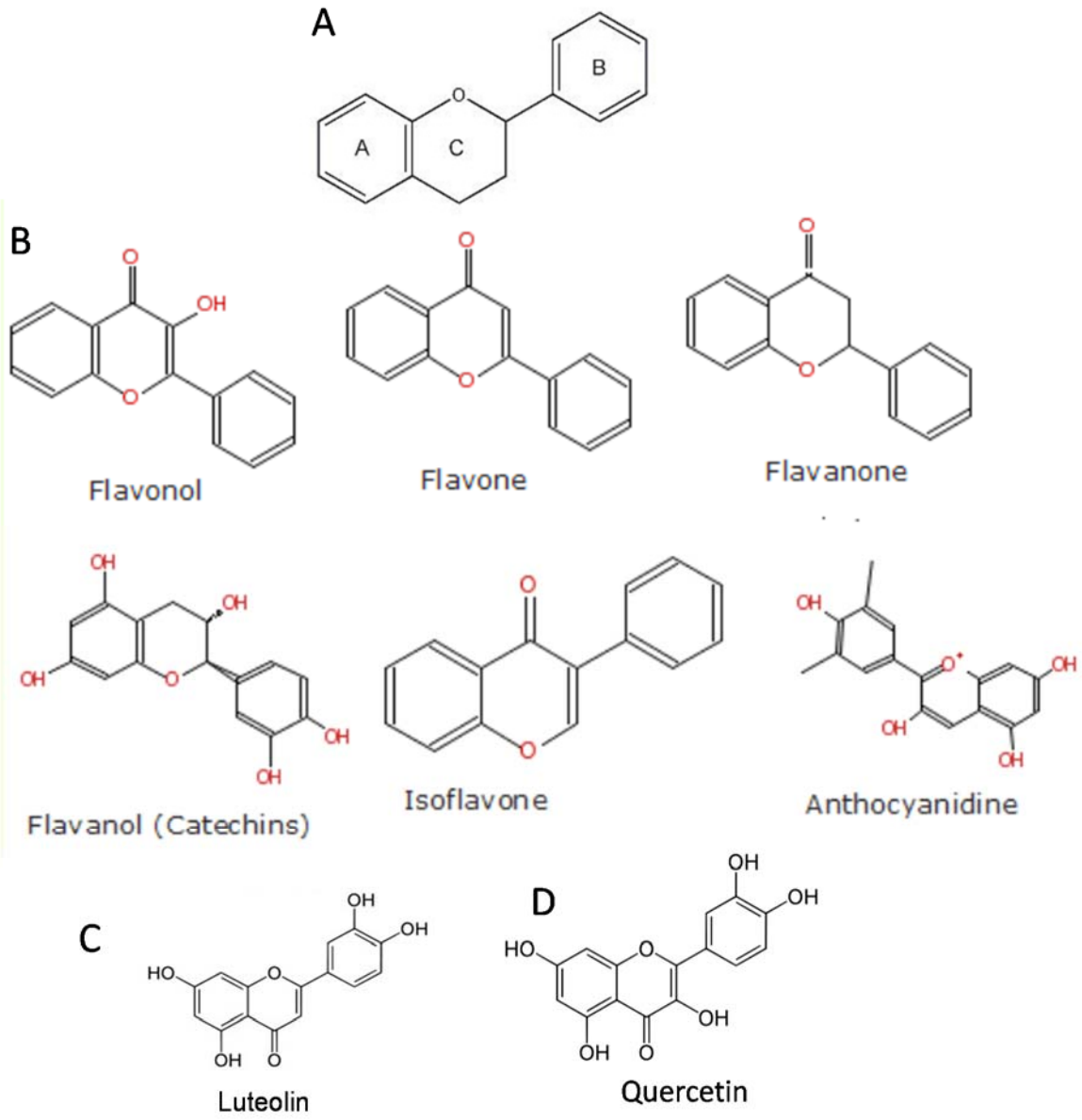


Figure 1-3 : Structures of flavonoids.

A, The basic structure of flavonoids. B, Structures of the major classes of flavonoids. C, Structure of Luteolin. D, Structure of Quercetin.

Chapter 2

Luteolin inhibited Apo B secretion through lowering MTP transcription, which is dependent on reduced HNF4α activity

2-1 Preface

Flavonoids are common dietary components of vegetables and fruits, with the function of anti-cancer, anti-inflammatory and antioxidant. Epidemiological studies show that the dietary intake of flavonoid is inversely associated with the mortality from coronary heart disease. In addition, food scientific researches indicate that consumption of flavonoids in foods and beverages may decrease the risk of atherosclerosis by improving the lipid metabolism. However, the precise mechanism is largely unknown.

Apo B concentrations, which reflect the number of small, dense LDL particles in plasma, are a significant predictor of cardiometabolic risk. Besides, MTP plays an important role for the assembly and secretion of Apo B containing lipoproteins. Thus, inhibition of MTP activity has the potential to treat the diseases caused by obesity.

In this chapter, an assay system for screening of food components and natural substances that can suppress the transcription of MTP was developed. Then the mechanism of declined MTP transcription was investigated.

2-2 Material and methods

Cell culture

HepG2 cells

HepG2 cells were maintained in DMEM (High Glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 5% CO2 atmosphere. Cells were passaged at a density of 2×10^6 cells/100 mm dish.

Caco-2 cells

Caco-2 cells were maintained in DMEM (Low Glucose) supplemented with 10% fetal bovine serum, 1%non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 5% CO2 atmosphere. Cells were passaged at a density of 2×10^6 cells/100mm dish.

Differentiated Caco-2 Cells

Caco-2 cells that had been cultured for 14 days after reaching confluence. The medium was changed every other day.

HEK293 cells

HEK293 cells were maintained in DMEM (High Glucose) supplemented with 10%fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 5% CO2 atmosphere. Cells were passaged at a density of 1×10^6 cells/100mm dish.

Cell Passage

Removed medium directly by aspiration, then wash cells with 5 mL 1xPBS, aspirate the PBS, and add 1 mL trypsin solution, incubate for about 5 minutes at 37 °C. Without removed trypsin add 5ml of fresh medium to the plate, detach cells from dishes by pipetting, and transfer to a 15 ml tube. Centrifuge at 1000rpm for 5 min, and aspirate the supernatant. Then Add appropriate volume of DMEM medium to suspend cell by pipetting, count the number of cells, seed cells at 1-2x10^6 cells (10 ml) per 100 mm culture dish, and incubate at 37 °C, 5% CO_2.

Plasmid

PGVB$_2$-hMTP (-204~+33)

PhMTP-AKO

PhMTP-BKO

PhMTP-C KO

pMTP-HNF1α KO

pMTP-B-HNF1α DKO

pCMV-3xFlag-hHNF4α (full length)

pCMV-β-gal

5xUAS

The plasmids were from Laboratory of Food Biochemistry, and purified using Plasmid Midi Kit (QIAGEN)

Plasmid purification

Transform

For transformation, 15 μl of TG1 competent cells were thawed on ice and mixed with 10 ng of the experimental plasmid DNA, incubated on ice for 20 min. After incubation, cells were heat-shocked in a 42°C water bath for 45 sec and then immediately transferred on ice for 2 min. Then, 200 μL of the LB medium was added to the cells and incubated at 37°C for 30min. Then, 100 μL of transformation mixtures were plated on LB agar plates containing 100 μg/mL ampicillin and incubated at 37°C overnight.

Qiagen Plasmid Midiprep

A single TG1 E. coli colony from a LB Ampicillin selective plate was picked to inoculate a starter culture of 2 mL LB medium (100 μg/mL ampicillin) to incubate for about 8 h at 37°C with vigorous shaking. The starter culture were used to inoculate 100 ml LB medium (100 μg/ml ampicillin) and incubated for 16 h at 37°C with vigorous shaking. The dense bacterial culture harvested by centrifugation for 10 minutes at 6000rpm at 4°C. The supernatant was removed and the pellet was resuspended in 4 ml of Buffer P1 (containing RNase A). After the pellet was completely resuspended, 4 ml of Buffer P2 (Lysis Buffer)

were added, mixed gently by swirling and incubated for 5min at RT. After the addition of 4ml of chilled Buffer P3 (Neutralization Buffer), the tube was inverted immediately 4-6 times, incubated in ice for 15min and then centrifuged for 30min at 11000rpm at 4°C. In the meantime a QIAGEN-tip 100 was equilibrated by applying 4ml Buffer QBT and allowing the column to empty by gravity flow. The cleared supernatant was transferred into equilibrated QIAGEN-tip by gravity flow. The QIAGEN-tip was washed twice with 10ml Buffer QC (wash Buffer). Afterwards the DNA was eluted with 5ml Buffer QF (Elution Buffer) into a new tube and precipitated by adding 3.5ml isopropanol. After mixing the precipitated DNA was centrifuged immediately at 11000rpm for 30min at 4°C. After the supernatant was carefully removed, 2ml of chilled 70% ethanol were added onto the pellet to remove precipitated salt. Then the tube was centrifuged at 11000rpm for 10 minutes at 4°C. The supernatant was removed completely and the pellet was dried and dissolved in TE Buffer stored at –20°C until use.

Luciferase Assay

Calcium phosphate transfection

Sub-confluent monolayers of HepG2 or Caco-2 cells in 12-well plates were transfected with 1μg plasmid DNA for one well. 1μg plasmid DNA was added to 50μL of a 0.25M $CaCl_2$ solution followed by addition of 50μL 2xHBSS solution, mixed by bubbling 20 times. The DNA-$CaCl_2$-2xHBSS mixture was incubated for 20 minutes at room temperature, then added drop wise to the well of cells which was gently swirled to favorite distribution of the transfecting solution. After the cells were incubated for 24h in an incubator with an atmosphere of 5% CO_2 at 37°C, fresh media was changed. Cells were then treated according to specific experimental protocol.

Plasmid DNA for MTP promoter activity (for 1 well)

PGVB$_2$-hMTP (-204~+33) or mutant	0.2μg
pCMV-β-gal	0.2μg

Plasmid DNA for HNF4α activity with HNF4α expression vector (for 1 well)

3xFlag/3xFlag- hHNF4α	0.2μg

PGVB$_2$-hMTP (-204~+33)	0.2μg
pCMV-β-gal	0.2μg

2xHBSS

280 mM	NaCl
10 mM	KCl
1.38 mM	$Na_2H_2PO_4$
11.2 mM	Glucose
42 mM	HEPES, pH 7.05

Preparation of Cell Lysate

After stimulation, cells were washed with 1xPBS followed by the addition of 100μL/well of 1x Luciferase Lysis Buffer. Plates were gently shaked for 20 min on ice followed by transferred to a fresh tube, and centrifuged at 15000rpm for 10 min at 4°C. Supernatants from lysates were assayed for luciferase activity and β-gal (β-galactosidase) activity.

1x Luciferase Lysis Buffer

25mM	Tris-phosphate (pH 7.8)
2mM	DTT
2mM	CDTA (trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid)
10%	glycerol
1%	Triton X-100

Luciferase Assay

This assay was performed with 10-20 μL (HEK293 cells 10μL, HepG2 or Caco-2 cells 20μL) of cell lysate, added to a 3.5mL tube (SARSTEDT Art. No.55.484) and 50 μl of Luciferase Substrate Reagent mixed in using the pipette. Readings were taken at room temperature with 2-10 s (HEK293 cells 2 s, HepG2 or Caco-2 cells 10 s) acquisition time using mini Lumat LB9560 (Berthold).

Luciferase Substrate Reagent

20 mM	Tricine

1.07 mM	$(MgCO_3)_4\ Mg(OH)_2 \cdot 5H_2O$
2.67 mM	$MgSO_4$
0.1 mM	EDTA
0.53 mM	ATP
33.3 mM	DTT
0.27mM	coenzyme A
0.47 mM	luciferine (Promega)

β-gal (β-galactosidase) Assay

20 μL of the lysates were transferred to an ice cold 96 well plate mixed with 90 μl of β-gal Substrate Reagent, incubated at 37°C until the solution turned yellow. The assay is determined with an absorbance reading taken at 415 nm (reference: 655nm).

Luciferase activity was normalized to β-gal activity and data was expressed as fold increase of stimulated cells over control.

β-gal Substrate Reagent

1.1mM	$MgCl_2$
1mg/ml	ONPG (o-nitrophenol-Beta-D-galactopyranoside)
82mM	Na_2HPO_4
18mM	NaH_2PO_4

mRNA quantitation

Cells RNA isolation:

Total RNA was isolated from cells using ISOGEN. Cells were cultured on 6-well plates and stimulated according to specific experimental protocol. After media were aspirated, cells were washed with 1mL 1xPBS, added with 0.5mL ISOGEN, and shacked at room temperature for 3 min. The ISOGEN cell mixture was transfer into a new tube added with 0.1mL chloroform, vortexed for 15s, then stored at room temperature for 3 min. After storage, the tubes were centrifuged at 12000 rpm for 15 min at 4°C. The uppermost aqueous layers containing the extracted RNA were transferred into new tubes, added with 0.25 ml of isopropanol, and stored at room temperature for 10 min to precipitate RNA. The

precipitated RNA was collected by centrifugation at 12000 rpm for 20 min at 4°C. All aqueous phase was discarded and the precipitated RNA was washed with 1 ml 70% ethanol. The tubes were again centrifuged at 12000 rpm for 10 min at 4°C. Alcohol was removed completely and the pellet was dried and eluted with 20μL DEPC (Diethyl pyrocarbonate) water stored at –80°C until use.

DNAse treatment

1μg RNA of each sample were mixed with 2μL1/30 DNase I (Roche) in a total reaction volume of 10μL and incubated for 30min at 37°C, 10min at 75°C in a thermo cycler. After DNase reaction, the solution was stored at –80°C or continued with reverse transcription.

Reverse transcription (RT)

Reverse transcription of total RNA was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 1μg RNA with DNAse treatment(10μL) of each sample were mixed with 10μL 2 x RT mater mix in a total reaction volume of 20μl and incubated for 10min at 25°C, 120min at 37°C and 5min at 85°C in a thermo cycler.

2 x RT mater mix

10 x RT buffer	2μL
25 x dNTP Mix	0.8μL
10 x Random Primer	2μL
RTase (200U/μL)	0.5μL
DEPC-ddH_2O	4.7μL

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time RT-PCR using SYBR Green PCR Master Mix (SYBR, Applied Biosystems) or Taqman Universal PCR Master Mix (Taqman, Applied Biosystems) was performed on ABI PRISM 7000 Sequence Detection Systems. Reaction master mix was made with SYBR/Taqman Master mix, primers/Gene Expression Assays and template. The total volume of reaction mixture for a single PCR reaction is 10 μL. PCR reaction was done with 96 well plates covered with optical adhesive covers. Reaction conditions were as

follows: one step of 50°C for 2 min, one 95°C for 10 min, and 40 cycles of 95°C for 10 s denaturation and 60°C for 1 min annealing and extension. The threshold cycle number for product detection (ΔCT value) was used to calculate the relative expression levels. Relative mRNA levels were determined by normalizing to the 36B4 transcript.

SYBR reaction mixture

DDW	2.6μL
SYBR Green PCR Master Mix	5μL
10μM Forward Primer	0.4μL
10μM Reverse Primer	0.4μL
Template cDNA	2μL

Taqman reaction mixture

DDW	2.5μL
Taqman Universal PCR Master Mix	5μL
Taqman Gene Expression Assays	0.5μL
Template cDNA	2μL

Taqman Gene Expression Assays

hMTP	Hs00165177_m1
hHNF4α	Hs00230853_m1
hFXR	Hs00231968_m1

Primer (5'----3')

h36B4	F TGCATCAGTACCCCATTCTATCA
	R AAGGTGTAATCCGTCTCCACAGA
hApoB	F GCCATTGCGACGAAGAAAATAh
	R TGACTGTGGTTGATTGCAGCTT
hHNF1α	F GAGGCAGAAGAACCCTAGCAA
	R CCCTCTCTGGATGCATTCC
hG6Pase	F CGACCTACAGATTTCGGTGCTTG

	R	AGATAAAATCCGATGGCGAAGC
hPEPCK	F	GTGCTGGAGTGGATGTTCAAC
	R	ACATCTGGCTTATTCTTTGCTTC

siRNA Transfection

siRNA Transfection (Lipofectamine RNAiMax)

6-well plates were used for siRNA transfection. HNF4α siRNA or Lamin A/C control siRNA (20μM stock 15μL, 150 pmol/well) were diluted in a volume of 500 μL/well of OptiMEM media. Then 6 μl/well of Lipofectamine RNAiMAX transfection reagent were added into OptiMEM media and mixed, incubated for 20 min at RT. During the incubation, prepared HepG2 cell as the protocol of cell passage, finally add appropriate volume of DMEM medium without P/St (Penicillin-Streptomycin) to suspend cell. After incubation, seed cells at $5x10^5$ cells/well into siRNA- RNAiMAX transfection reagent- OptiMEM media mixture, and incubate at 37 °C, 5% CO_2. After 24 h, media was aspirated and replaced with DMEM medium including P/St. Transfected cells were incubated for another 24 h to get desired siRNA effect, then cells were stimulated according to specific experimental protocol.

si Control (Lamin A/C):	5'- CUG GAC UUC CAG AAG AAC ATT-3'
	5'- UGU UCU UCU GGA AGU CCA GTT-3'
si HNF4α:	5'- GCA GGA AGU UAU CUA GCA AGC-3'
	5'-UUG CUA GAC AAC UUC CUG CUU-3'

Western blotting

Sample preparation

Whole cell lysate preparation

HepG2 or Caco-2 cell were cultured on 6-well plates and stimulated according to specific experimental protocol. Media were aspirated, cells were washed with 1mL 1xPBS, scraped off the plate, and subsequently lysed with 100μL/well of ice-cold RIPA buffer (1 mM PMSF (phenylmethylsulphonyl fluoride), 1/100 PIC (protease inhibitor cocktail), 50μM Calpain Inhibitor ALLN (N-acetyl-Leu-Leu-norleucinal), vortexed 3 x 5 seconds,

incubated for 30 min on ice, and centrifuged at 15000rpm for 10 min at 4°C. Supernatants were transferred to a fresh tube. Small aliquots were taken for protein quantification using Pierce BCA protein assay kit. Cell lysates were heat denatured with 6x Laemmli Sample buffer for 5 min at 95°C.

Secretory ApoB

HepG2 or Caco-2 cell were cultured on 6-well plates and stimulated according to specific experimental protocol. Media were harvested and heat denatured with 6x Laemmli Sample buffer for 5 min at 95°C.

SDS polyacrylamide gel electrophoresis and protein transfer

Cell lysates (10 μg)/media (15μL) were loaded onto a 5-15% SDS polyacrylamide gel and electrophoresed at 100 V in 1x Running buffer. A protein standard ladder was also added to confirm the sizes of proteins being investigated. Following electrophoretic separation, protein was transferred to PVDF (polyvinylidene fluoride) membranes (5.5cm x 9cm) using a wet transfer system (Bio craft) in 1x transfer buffer or CAPS buffer for 1 h at 120mA/membrane.

Immunoblotting

Following protein transfer, PVDF membranes were blocked with 5% (w/v) skim milk powder in PBS-T for 1 h with gentle shaking and then incubated with specific primary antibody in 5% skim milk/PBS-T solution or solution A for 1h at room temperature or overnight at 4°C. Membranes were washed 3 x 10 min in PBS-T with gentle shaking followed by incubation with secondary antibodies in 5% skim milk/PBS-T solution or solution B for 1 h at room temperature. Membranes were washed 3 x 10 min, placed in ECL substrate reagent for 15s and exposed to film (Fujifilm LAS-1000) for detection.

Antibodies used in SDS/PAGE immunoblotting.

Primary antibody	Maker	Dilution	Secondary antibody	Dilution
HNF4α	Santa cruz	1/1000, Solution A	Rabbit	1/3000, Solution B
ApoB	Abcam	1/1000, Solution A	Goat	1/5000, Solution B
Actin	Chemicon	1/1000, skim milk/PBST	Mouse	1/1000, skim milk/PBST

SDS-PAGE

Resolving Gel				Stacking Gel	
	5%	10%	15%		
1M Tris-HCl (pH8.8) (mL)	2.25	2.25	2.25	1M Tris-HCl (pH6.8) (mL)	0.35
30% Ac. Amide (mL)	1.00	2.00	3.00	30% Ac. Amide (mL)	0.3
dH_2O (mL)	2.69	1.69	0.69	dH_2O (mL)	1.65
10% APS (μL)	60			10% APS (μL)	40
TEMED (μL)	3			TEMED (μL)	2.5

Solutions:

1xPBS

1.47mM KH_2PO4

8.1mM $Na_2HPO4\text{-}12H_2O$

137mM NaCl

2.68mM KCl

PBS-T solution

1xPBS solution supplemented with 0.1% (v/v) Tween-20

RIPA Buffer

50 mM Tris−HCl (pH) 7.4

150 mM	NaCl
1nM	EDTA
1%	NP-40
0.25%	sodium deoxycholate

6xLaemmli Sample Buffer

2.8 mL	1M Tris-Cl pH 6.8
3mL	Glycerol
1g	SDS (sodium dodecyl sulfate / sodium lauryl sulfate)
A few	BPB (Bromo phenol blue)
DDW	up to 10mL

1x Running buffer

25 mM	Tris-base
0.1%	SDS (w/v)
192 mM	glycine

1x Transfer buffer

100 mM	Tris-base
192 mM	glycine
5%	methanol (v/v)

CAPS buffer

60mM	Tris
40mM	CAPS

2-3 Results

2-3-1 Luteolin is identified as a component that inhibits MTP activity

To screen the food components that inhibit MTP transcription, the luciferase reporter plasmid containing human MTP gene promoter (-204~+33) was used for analysis (Fig 2-1 A). In HepG2 cells, after reporter plasmid was transfected for 24h, 156 kinds of food components (100μM) were treated for another 24h. The results showed that flavones (Luteolin, Apigenin, Acacetin, Chrysin) and flavonols (Fisetin, Kaempferol) reduced MTP transcription, but not isoflavones (Genistein, Daidzein). Among all the components, Luteolin showed the strongest inhibitory activity (Fig 2-1 B).

Because high concentration of Luteolin (100μM) was used in screening, to investigate the effect of concentration on the activity, lower concentration was examined. Even 30μM of Luteolin had the inhibition effect; 50μM showed the strongest inhibition activity (Fig 2-2 A). According to the function of MTP in intestine for chylomicrons formation, the effect was then determined in Caco-2 cells. The same as HepG2, Luteolin also reduced MTP transcription in Caco-2 cells (Fig 2-2 B).

Next the effect of Luteolin on MTP expression was estimated by RT-PCR in HepG2 cells. With Luteolin treatment for 12h, even 10μM declined MTP mRNA level (Fig 2-3 A). 50 μM of Luteolin for 6h treatment also showed the inhibition activity (Fig 2-3 B). These results suggested that Luteolin inhibited MTP transcription and expression.

2-3-2 Luteolin decreases Apo B secretion in HepG2 and differentiated Caco-2 cells

To determine the effect of Luteolin on Apo B secretion, the media for incubating cell and cell lysates were analyzed by western blotting. The Apo B from cells and media was decreased by the treatment of Luteolin for 12h in both HepG2 and differentiated Caco-2 cells. Although MTP expression began to be inhibited by Luteolin from 6h treatment, surprisingly, secretory Apo B was inhibited in both of the cells with Luteolin even from 3h treatment. (Fig 2-4 A and B).

2-3-3 Luteolin decreases MTP promoter activity through HNF4α and HNF1α

Subsequently, the mechanism of MTP inhibition by Luteolin was studied. The MTP promoter (-204~+33) contains a pair of functional responsive elements for HNF4α and HNF1α. Mutant analysis (Fig 2-5 A) revealed that the MTP transcription activity of HNF1 KO, HNF4-B KO or HNF1-4B DKO was obviously decreased even in the absence of Luteolin treatment. Compared to WT promoter, Luteolin inhibitory effect lowered with the transfection of HNF1 KO, HNF4-B KO reporter gene, while Luteolin did not show any inhibition activity with HNF1-4B DKO (Fig 2-5 B). The result indicated that HNF1α and HNF4α sites were important for MTP transcription and Luteolin decreases MTP activity through HNF1α and HNF4α binding sites. Because HNF1α is a target gene of HNF4α, my study focused on the effect of Luteolin on HNF4α activity.

2-3-4 Luteolin declines HNF4α activity and its expression

To estimate the effect of Luteolin on HNF4α activity, the luciferase activity was analyzed in HEK293 cells, the reporter plasmid of hMTP promoter together with expression plasmid for human HNF4α were co-transfected (Fig 2-6 A). It was observed that the luciferase activity remarkably increased when co-transfected with HNF4α expression plasmid. However, the HNF4α activity was reduced by Luteolin treatment, while was not affected by Daidzein (Fig 2-6 B), which did not inhibit the MTP promoter activity during components screening (Fig 2-1).

HNF4α target genes were also determined to estimate the effect of Luteolin on HNF4α activity. Luteolin reduced the mRNA level of MTP in HepG2 cells (Fig 2-3 A, B). Besides of MTP and HNF1α, lipid related gene Apo B, gluconeogenesis related gene PEPCK and G6Pase are also the target genes of HNF4α. The mRNA levels of these target genes were measured in HepG2 cells with different concentration of Luteolin treatment for 12h. 10μM of Luteolin reduced the mRNA levels of Apo B, HNF1α, PEPCK and G6Pase. Higher concentration (30μM) showed stronger inhibition effect on HNF1α and G6Pase (Fig 2-7 A). Besides, Luteolin also decreased HNF4α mRNA level even at the concentration of 10μM,

and at 30μM the decrement was more observably (Fig 2-7 B). HNF4α can be regulated by its self-regulation factors, such as COUP-TFII and FXR, thus the expression of them was examined. The mRNA levels of COUP-TFII and FXR were reduced under the treatment of Luteolin, moreover the inhibition pattern of concentration was similar to HNF4α (Fig 2-7 C). Then the time-course effect of Luteolin inhibition activity on HNF4α and its expression was evaluated. For HNF4α target genes, Luteolin began to inhibit the mRNA levels of HNF1α and G6Pase from 2h, inhibited PEPCK from 3h, and inhibited Apo B only at 12h (Fig 2-8 A). For HNF4α itself, Luteolin inhibit it in a time-dependent manner from 2h (Fig 2-8 B). For HNF4α self-regulation factors, Luteolin inhibited the mRNA level of COUP-TFII from 2h and inhibited FXR from 6h (Fig 2-8 C).

In differentiated Caco-2 cells, Luteolin also inhibited HNF4α and its target genes. For HNF4α target genes, Luteolin began to inhibit the mRNA level of HNF1α from 3h, inhibited PEPCK at 6h, inhibited MTP at 12h, and Luteolin had an inhibitory trend for Apo B and G6Pase (Fig 2-9 A). For HNF4α itself, Luteolin inhibit it in a time-dependent manner from 6h (Fig 2-9 B). For HNF4α self-regulation factors, Luteolin inhibited the mRNA level of COUP-TFII at 3h and 6h, inhibited FXR at 6h (Fig 2-9 C). Although higher concentration (100μM) was used in differentiated Caco-2, the inhibition effect was weaker than that in HepG2 cells.

Subsequently, the effect of Luteolin on the protein level of HNF4α was studied. After HepG2 cells treated with 50 μM Luteolin for 12h, cellular HNF4α was measured by western blotting. The results showed that the protein level of HNF4α was reduced by Luteolin (Fig 2-10 A). The similar phenomenon was showed in Caco-2 cells (Fig 2-10 B).

2-3-5 There are other pathways for Luteolin inhibitory effect on Apo B secretion independent of HNF4α

Fig 2-4 A showed 3h Luteolin treatment inhibited Apo B secretion, but had no effect on MTP expression (Fig 2-3 B), hence, it is thought that there are other pathways except HNF4α. In order to confirm this hypothesis, HNF4α was knocked down by si-RNA. si-HNF4α treated HepG2 cells exhibited a significant decrease in cellular HNF4α, Apo B

and secretory Apo B in comparison to si-control transfected cells in the absence of Luteolin treatment (Fig 2-11 Lane1 and 2). In accordance with the above results, 9h Luteolin treatment reduced cellular HNF4α, Apo B and secretory Apo B in the cells transfected with si- control (Fig 2-11 Lane1 and 3). In the presence of Luteolin, si-HNF4α treated cells exhibited a further decrease in secretory Apo B comparison to si- control transfected cells (Fig 2-11 Lane3 and 4). These results confirmed that HNF4α was related to Apo B secretion. In si-HNF4α treated cells, there is a further decline of secretory Apo B with Luteolin treatment (Fig 2-11 Lane2 and 4). The result suggested there are other pathways for the inhibition of Luteolin on Apo B secretion independent of HNF4α.

2-3-6 Luteolin inhibits Apo B secretion not by proteasome pathway

Apo B can be regulated by degradation. It is reported that naringenin inhibits Apo B secretion in oleate-stimulated HepG2 cells and selectively increases intracellular degradation via a largely proteasomal, rapid kinetic pathway (103). Hence, it is presumed that Luteolin may inhibit Apo B secretion via proteasome pathway. To determine the hypothesis, proteasome inhibitor MG132 was used for studying in HepG2 cells. In accordance with the above results, 3h or 12h Luteolin treatment reduced secretory Apo B (Fig 2-12 Lane1, 2 and 3, 4). However the inhibited secretory Apo B by Luteolin was not recovered with the MG132 treatment (Fig 2-12 Lane3, 4 and 5, 6). Although only with MG132 treatment the secretory Apo B was a little increased compared to control (Fig 2-12 Lane1, 2 and 7, 8). The result indicated that Luteolin inhibits Apo B secretion not by proteasome pathway.

2-3-7 Luteolin's rapidly inhibitory effect on Apo B secretion is not lowered by autophagy inhibitor chloroquine

Apo B also can be degraded by autophagy. Moreover it is reported that Luteolin could cause autophagy. It is postulated that Luteolin may inhibit Apo B secretion via autophagy pathway. To determine this hypothesis, autophagy inhibitor chloroquine (CQ) was used for studying in HepG2 cells. Since p62 accumulates when autophagy is inhibited, and

decreased levels can be observed when autophagy is induced, p62 is used as a marker. (104). Firstly, to confirm the effect of Luteolin on autophagy, HepG2 cells were treated with Luteolin for 3h or 12h, Luteolin decreased p62 level for both 3h and 12h (Fig 2-13 A). It indicated that Luteolin caused autophagy.

Next the relation of autophagy pathway and Luteolin inhibitory effect on Apo B secretion was studied. Agree well with the above results, Luteolin decreased p62 and secretory Apo B level in HepG2 cells for 3h treatment (Fig 2-13 B Lane1, 2 and 3, 4). When the cells were co-treated with 30μM CQ, the decreased p62 was recovered, while there was no effect on secretory Apo B in the media (Fig 2-13 B Lane3, 4 and 5, 6). Only with the CQ treatment, there was little effect on p62 and secretory Apo B compared to control (Fig 2-13 B Lane1, 2 and 7, 8). The results indicated that Luteolin is rapidly inhibitory effect on Apo B secretion is not lowered by autophagy inhibitor CQ.

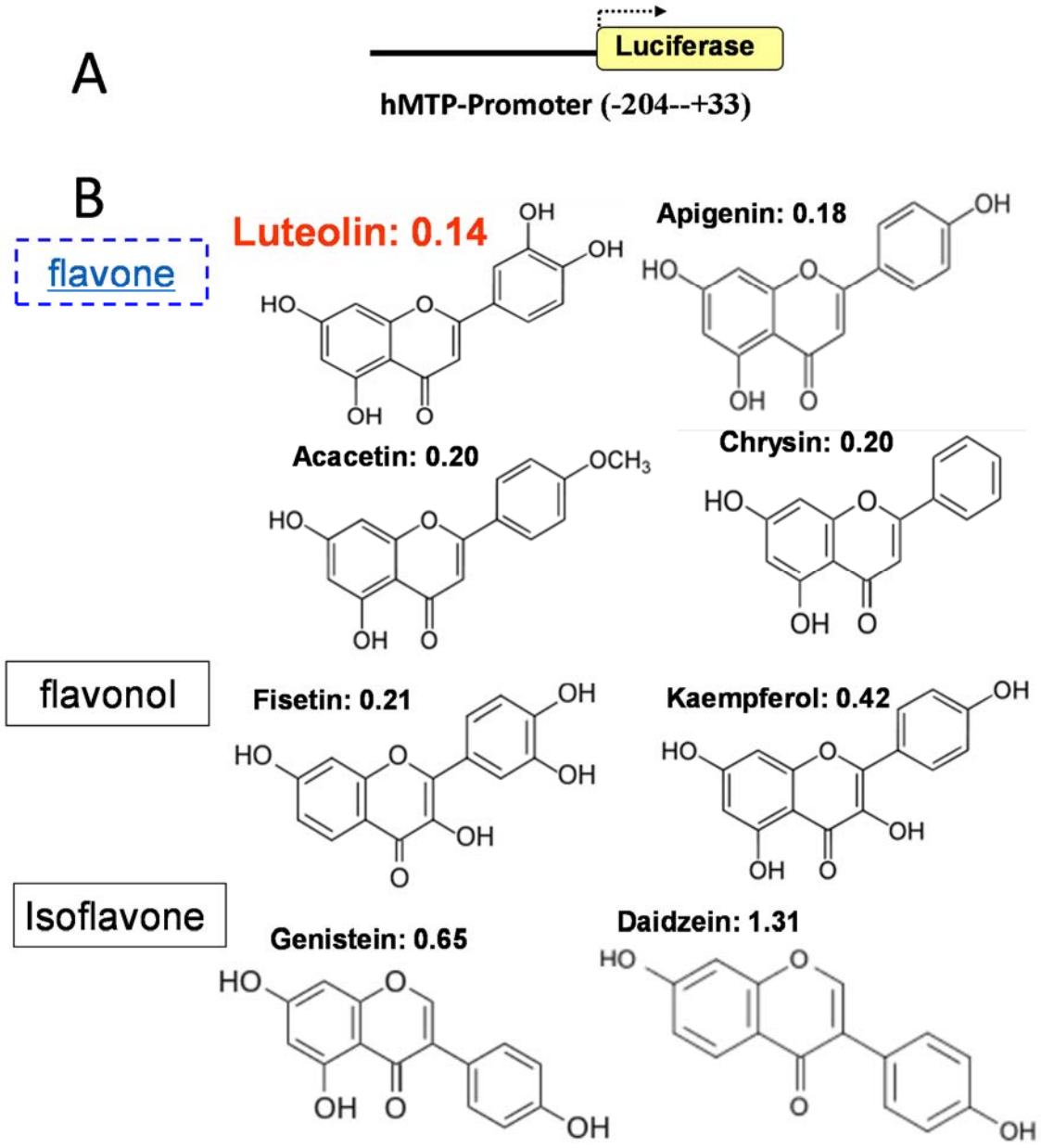


Figure 2-1 Screening results of food components on MTP transcription activity

A, reporter plasmids containing human MTP gene promoter (-204~+33) was used for analysis of MTP transcription activity. B, HepG2 cell were transfected with the report plasmid and an expression plasmid for β-galactosidase. After transfection, cells were treated with 100 μM 156 kinds of food components for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1.

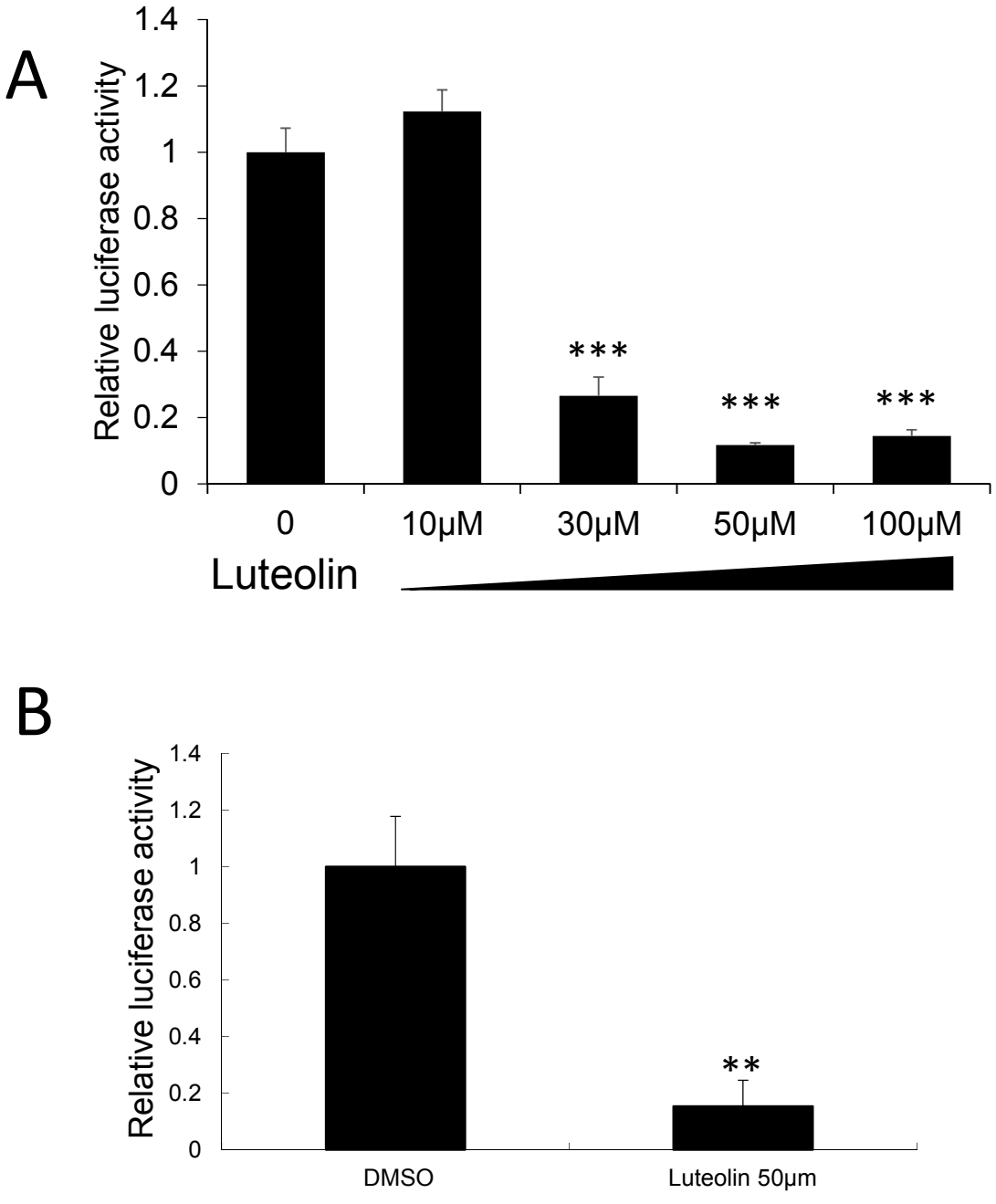


Figure 2-2 Luteolin inhibits MTP transcription activity in both HepG2 and Caco-2 cell

Cell were transfected with the report plasmid containing human MTP gene promoter (-204~+33) and an expression plasmid for β-galactosidase. After transfection, cells were treated with Luteolin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. A, in HepG2 cell. B, in Caco-2 cell. Results are means±S.D. (n =3). **P <0.01, ***P <0.001.

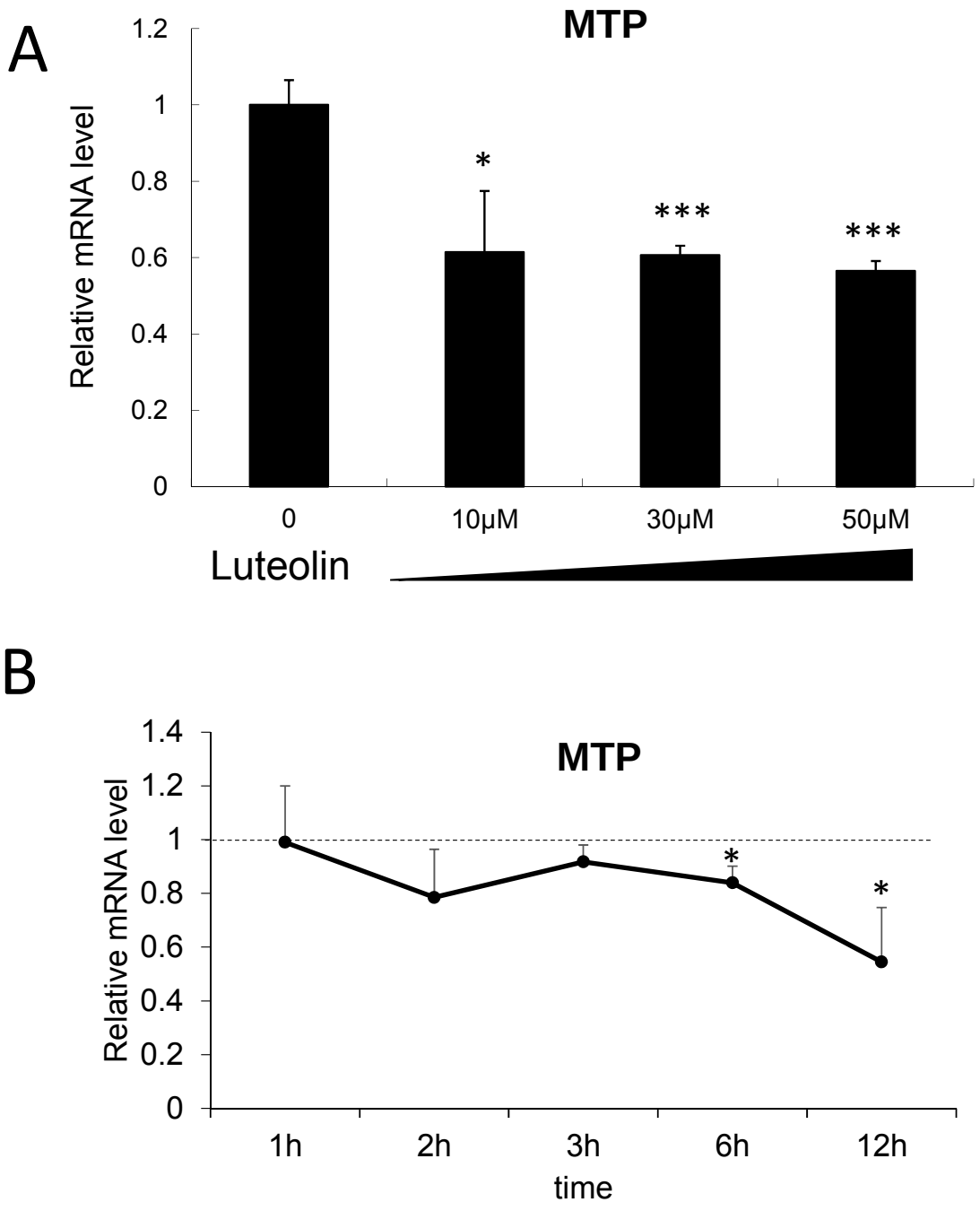


Figure 2-3 Luteolin inhibits MTP mRNA level in HepG2 cell

HepG2 cells were treated with Luteolin, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. A, different concentration of Luteolin treated for 12h. B, 50μM of Luteolin treated for different time. Results are means±S.D. (n =3). *P <0.05, ***P <0.001.

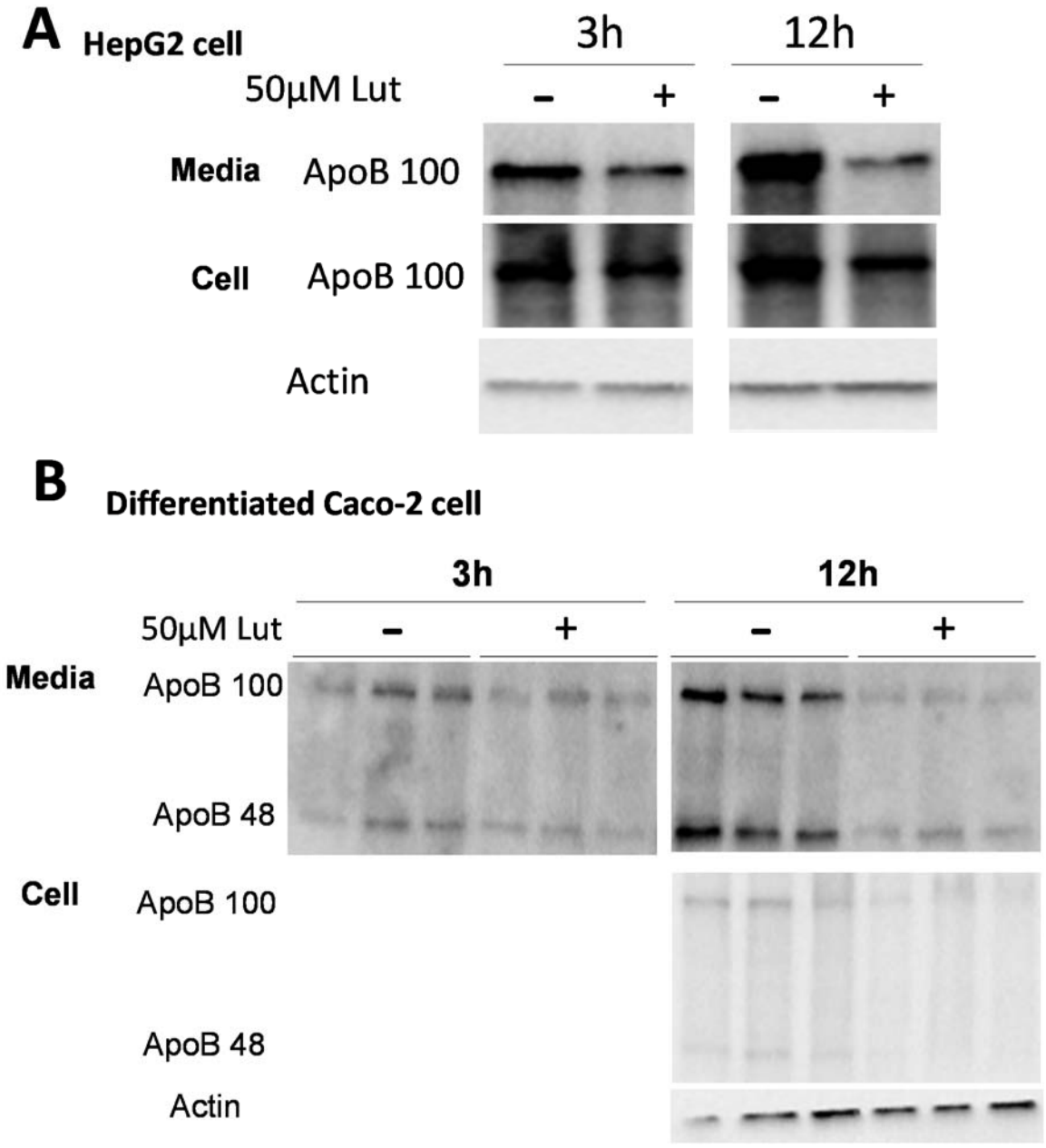


Figure 2-4 Luteolin decreases Apo B secretion in both HepG2 and differentiatedCaco-2 cell

Cells were treated with serum-free medium containing 50 μM Luteolin for 3 or 12h. Cellular protein (10 μg/lane) and the culture medium (15 μL/lane) were subjected to SDS-PAGE and Western blotting using antibodies against human Apo B or Actin. A, HepG2 cell (n=2, mixed). B, Differentiated Caco-2 cell (n=3). Caco-2 cells were differentiated by continuous culture for 14 days after they had reached confluence.

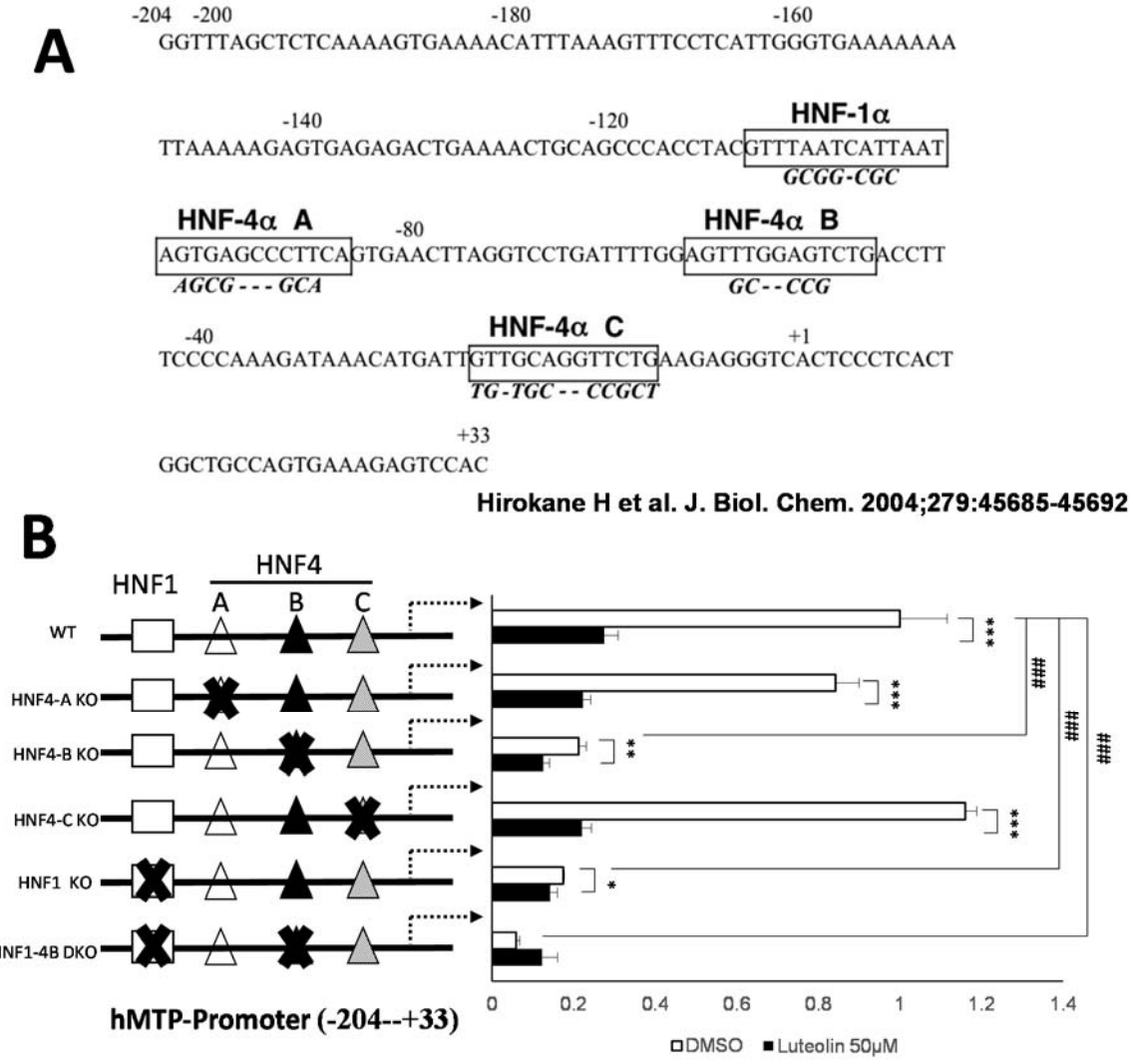
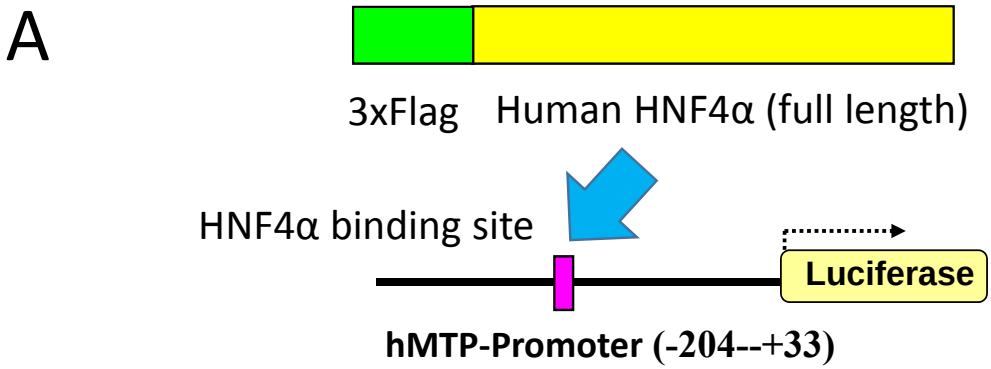


Figure 2-5 Luteolin declines MTP transcription through HNF4α and HNF1α

A, Human MTP promoter sequence. The transcription start site is position +1. The putative HNF-1 and HNF-4 recognition sites are boxed. The mutant sequences in their sites are shown under the individual sequences. B, HepG2 cell were transfected with the report plasmid containing mutant or wild-type hMTP gene promoter (-204~+33) and an expression plasmid for β-galactosidase. After transfection, cells were treated with 50μM Luteolin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities of wild-type reporter plasmid with DMSO treatment are considered as 1. Results are means±S.D. (n =3). *P <0.05, **P <0.01, ***P <0.001 vs. each of reporter plasmid. $^{###}$P <0.001 vs. wild-type reporter plasmid under DMSO treatment.



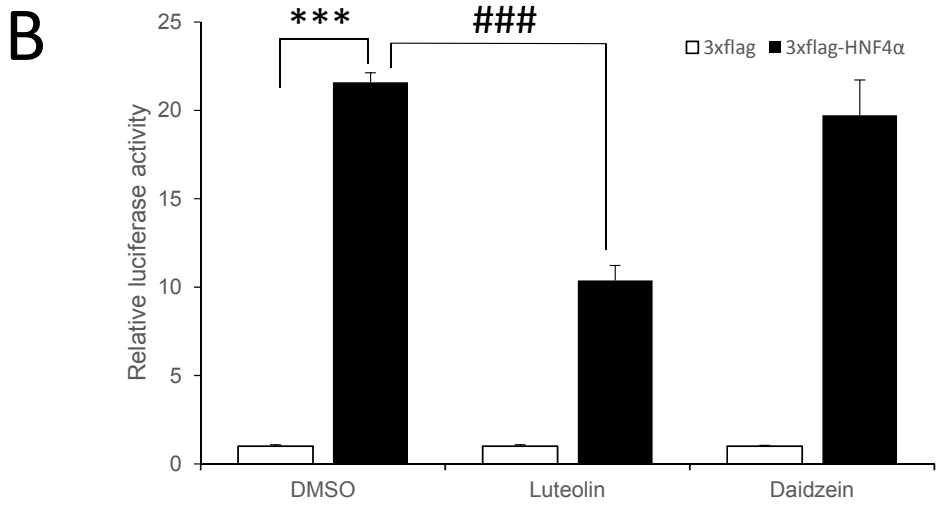


Figure 2-6 Luteolin decreases HNF4α activity

A, the structure of 3xFlag-hHNF4α expression plasmid. B, HEK293 cells were cotransfected the reporter plasmid of hMTP promoter (-204~+33) together with for β-galactosidase expression plasmid in the presence or absence of expression plasmid for human HNF4α. The cells were incubated with 30 μM Luteolin or Daidzein for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities of pMTP-204 in the absence of HNF4α under each component treatment are considered as 1. Results are means±S.D. (n =3). ***P <0.001 vs. in the absence of HNF4α under DMSO treatment. $^{###}$P <0.001 vs. in the presence of HNF4α under DMSO treatment .

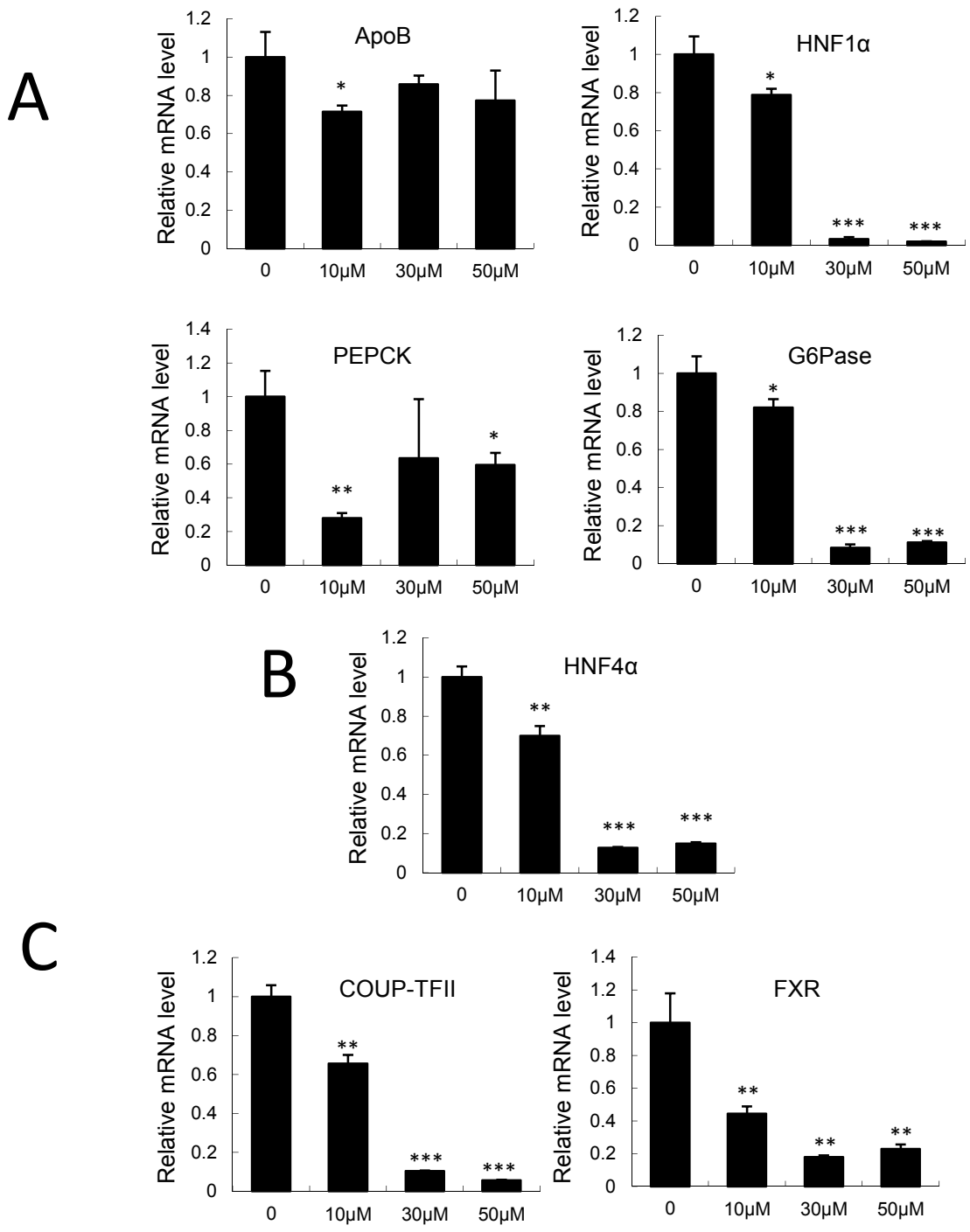


Figure 2-7 Luteolin reduces mRNA level of HNF4α target genes in HepG2 cell with different concentration

HepG2 cells were treated with different concentration of Luteolin for 12h in serum-free media, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. A, mRNA level of HNF4α target genes. B, mRNA level of HNF4α. C, mRNA level of HNF4α self-regulation factor. Results are means±S.D. (n =3). *P <0.05, **P <0.01, ***P <0.001.

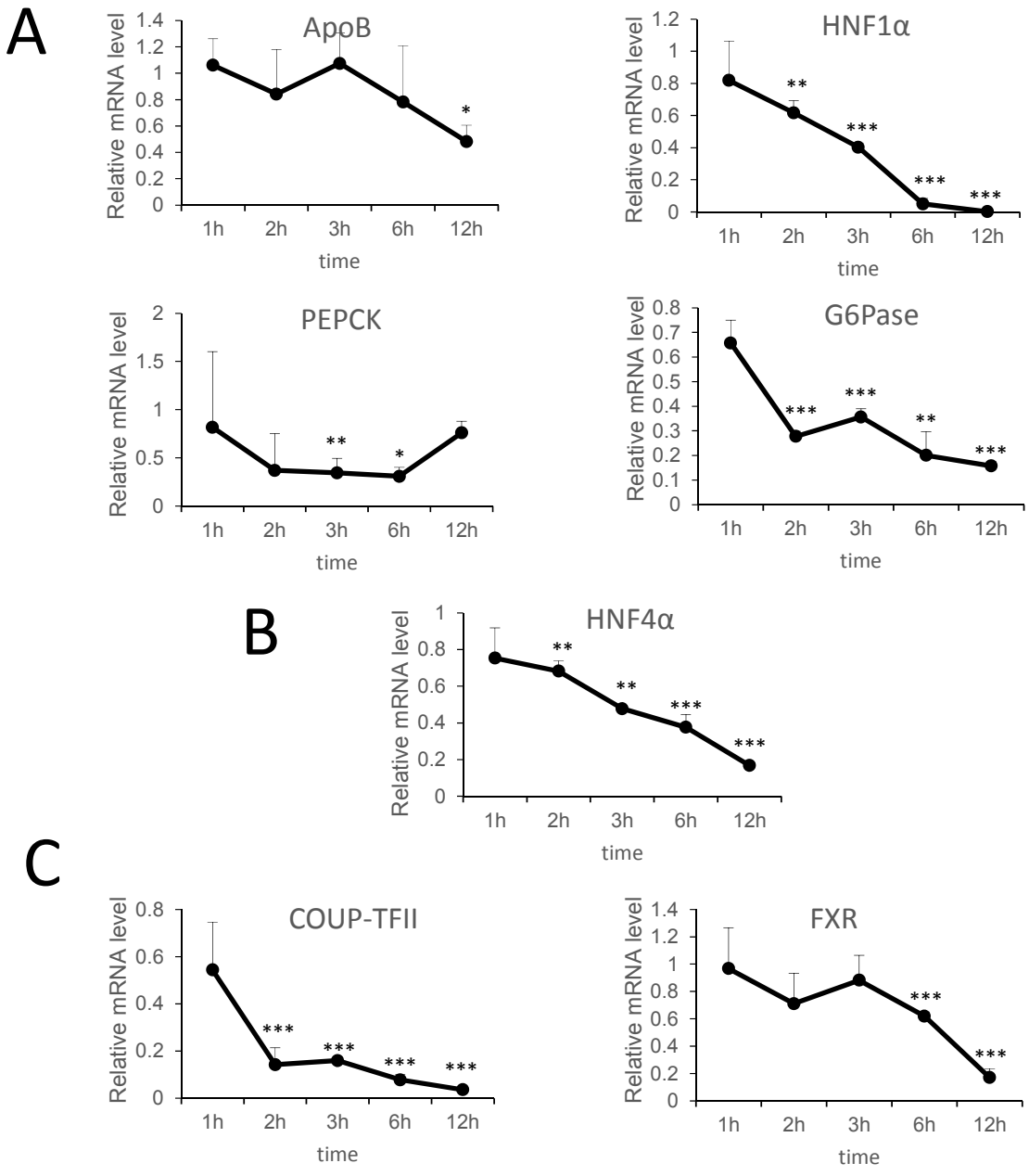


Figure 2-8 Luteolin reduces mRNA level of HNF4α target genes in HepG2 cell with different time

HepG2 cells were treated with 50μM of Luteolin for time-course studying in serum-free media, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells for each time were set to 1. A, mRNA level of HNF4α target genes. B, mRNA level of HNF4α. C, mRNA level of HNF4α self-regulation factor. Results are means±S.D. (n =3). *P <0.05, **P <0.01, ***P <0.001.

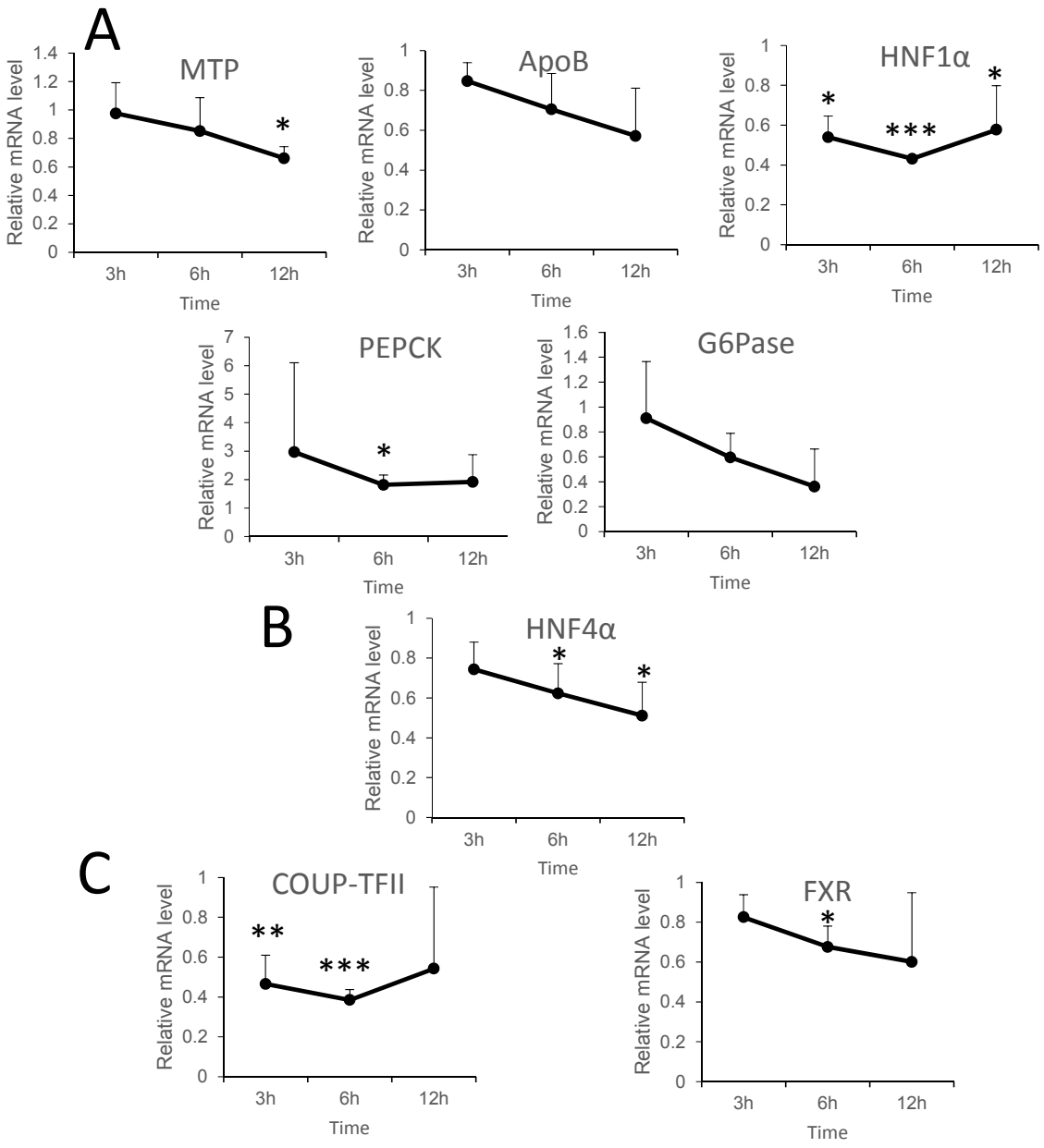


Figure 2-9 Luteolin reduces mRNA level of HNF4α target genes in differentiated Caco-2 cell with different time

Caco-2 cells were differentiated by continuous culture for 14 days after they had reached confluence. cells were treated with 100μM of Luteolin for time-course studying in serum-free media, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells for each time were set to 1. A, mRNA level of HNF4α target genes. B, mRNA level of HNF4α. C, mRNA level of HNF4α self-regulation factor. Results are means±S.D. (n =3). *P <0.05, **P <0.01, ***P <0.001.

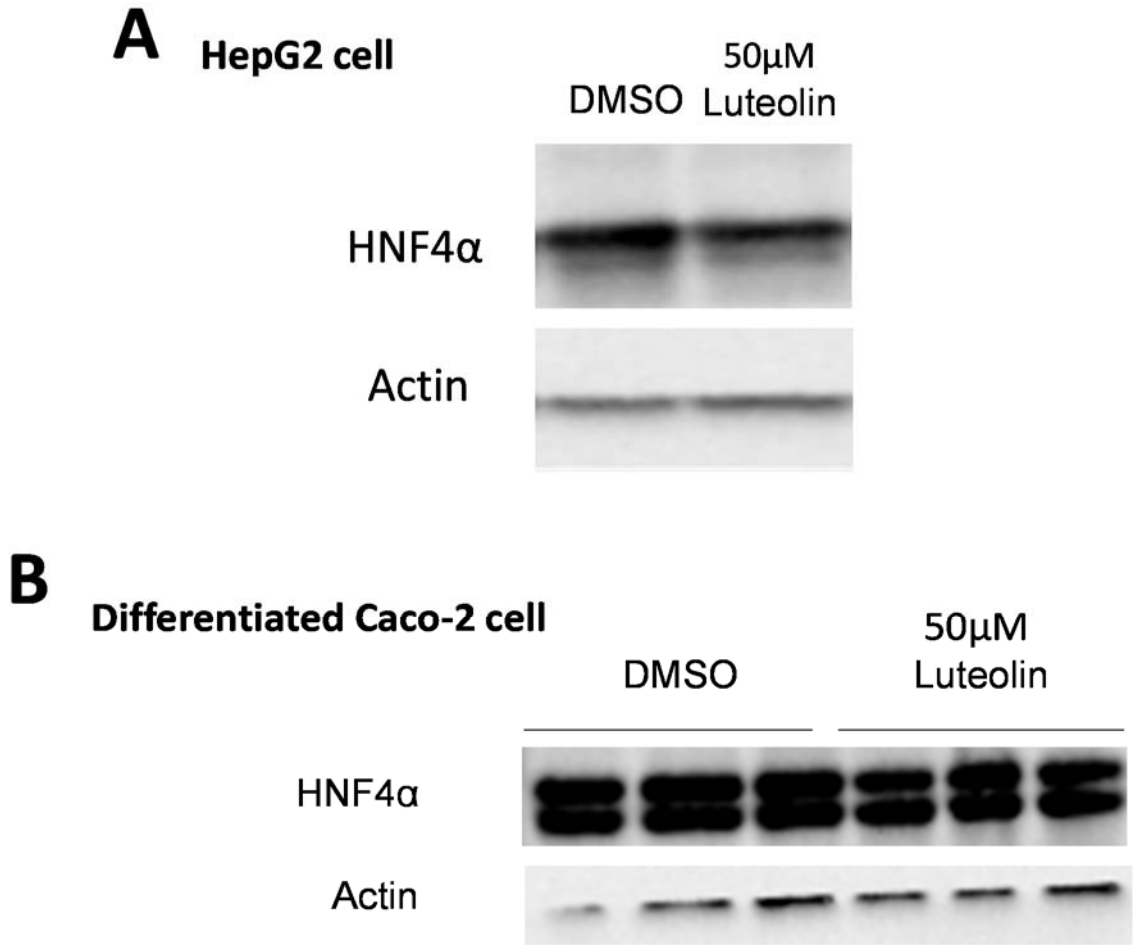


Figure 2-10 Luteolin declines HNF4α in both HepG2 and differentiatedCaco-2 cell

Cells were treated with serum-free medium containing 50 μM Luteolin for 12h. Cellular protein (10 μg/lane) was subjected to SDS-PAGE and Western blotting using antibodies against human HNF4α or Actin. A, HepG2 cell (n=2, mixed). B, Differentiated Caco-2 cell (n=3). Caco-2 cells were differentiated by continuous culture for 14 days after they had reached confluence.

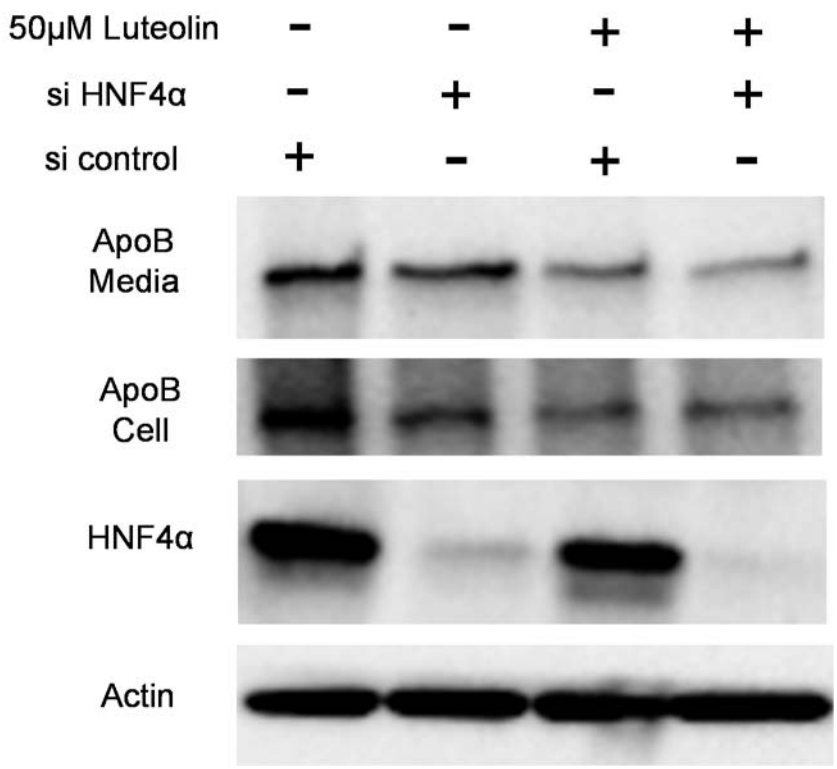


Figure 2-11 HNF4α is related to Apo B secretion and Luteolin inhibits Apo B secretion through HNF4α and other pathways

HepG2 cells transfected with HNF4α-siRNA or control-siRNA using Lipofectamine RNAimax and incubated for 24 h. After media changed, transfected cells were incubated for another 24 h followed by further incubation for 9 h in the presence or absence of 50 μM Luteolin in serum-free media. Cellular protein (10 μg/lane) the culture medium (15 μL/lane) were subjected to SDS-PAGE and Western blotting using antibodies against human HNF4α, Apo B or Actin

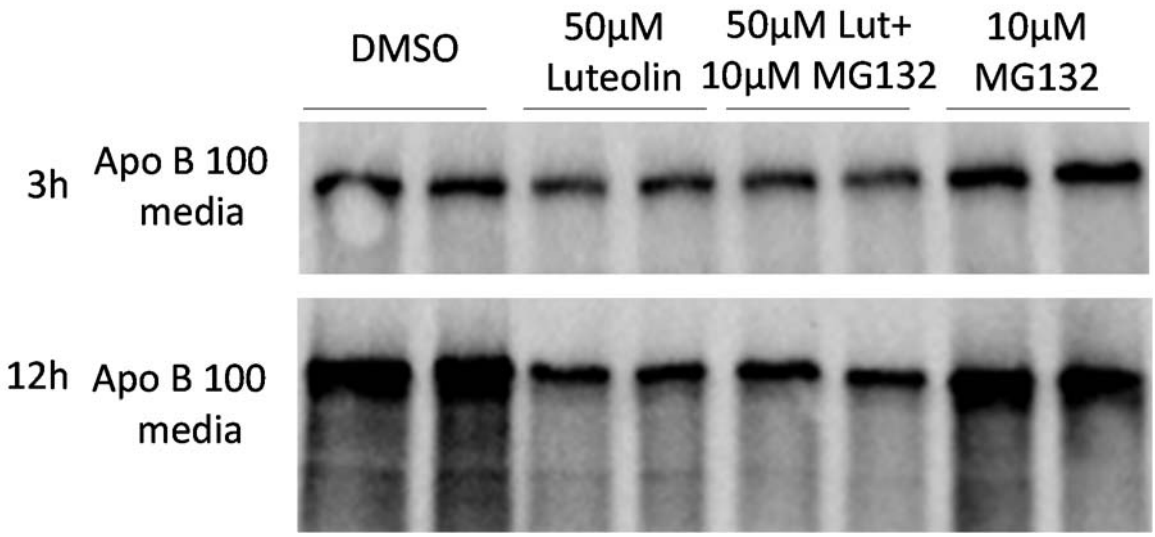


Figure 2-12 Luteolin inhibits Apo B secretion not by proteasome pathway

HepG2 Cells were treated with serum-free medium containing 50 μM Luteolin and 10 μM MG132 for 3 or 12h. Culture medium (15 μL/lane) were subjected to SDS-PAGE and Western blotting using antibodies against human Apo B.

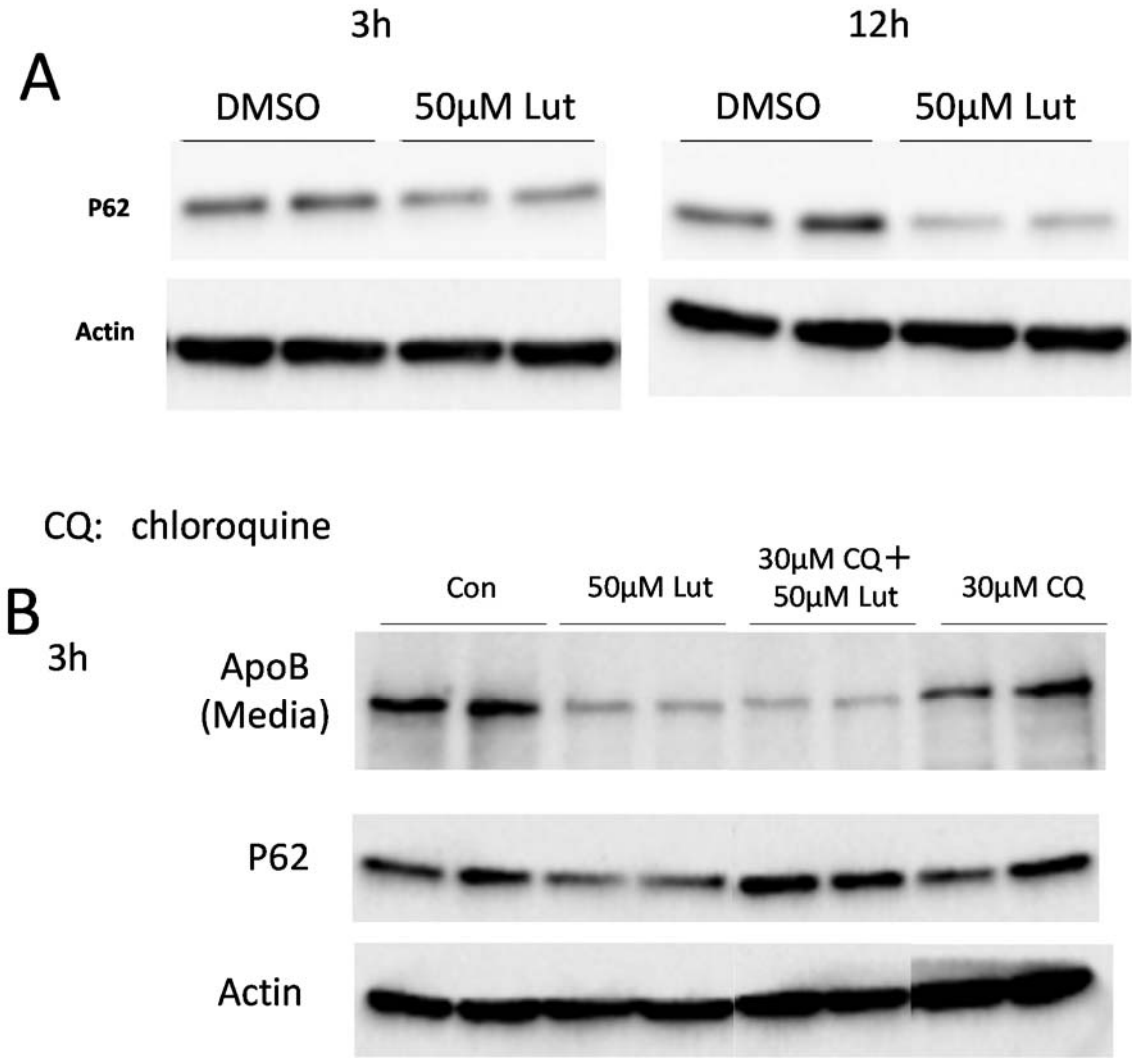


Figure 2-13 Luteolin rapidly inhibitory effect on Apo B secretion is not lowered by autophagy inhibitor chloroquine

HepG2 Cells were treated with serum-free medium containing Luteolin or MG132. Culture medium (15 μL/lane) were subjected to SDS-PAGE and Western blotting using antibodies against human Apo B. A, treated with 50 μM Luteolin for 3h or 12h. B, treated for 50 μM Luteolin and 10 μM MG-132 for 3h.

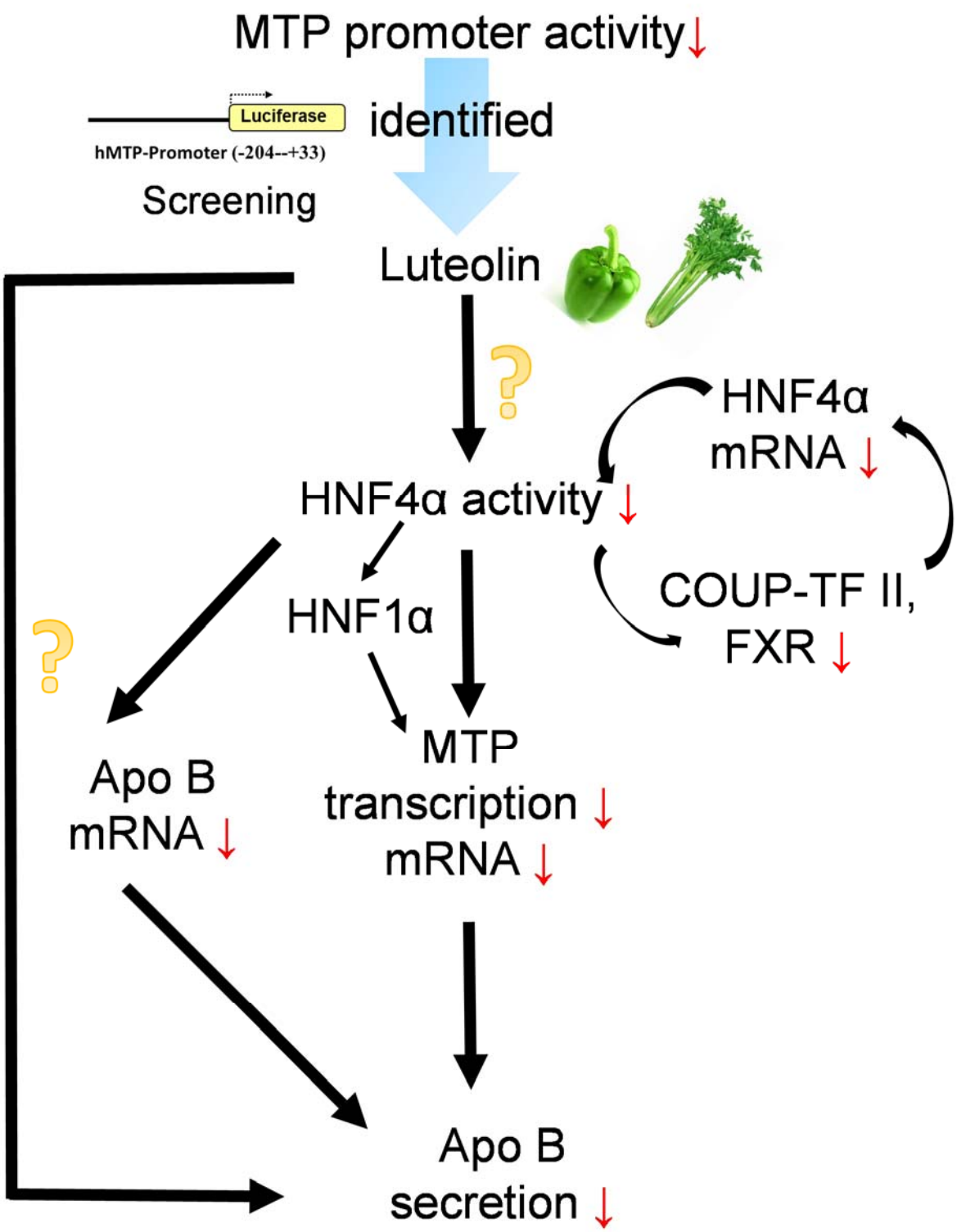


Figure 2-14 Summary of Chapter 2

2-4 Discussion

The results obtained in this chapter were summarized in Fig 2-14. It is clarified as follows.

[I]. Luteolin was identified as a functional food factor with the strongest inhibitory effect on MTP gene expression. The decreased MTP mRNA resulted in lowered Apo B secretion.

[II]. Luteolin decreased MTP promoter activity through reduced HNF4α and HNF1α transcriptional activities.

[III]. Luteolin lowered HNF4α activity and its expression.

[IV]. Luteolin hindered Apo B secretion independently of decreased HNF4α activity and expression. Intracellular degradation of Apo B was not caused by proteasome or autophagy.

I found that flavones (Luteolin, Apigenin, Acacetin, and Chrysin) and flavonols (Fisetin and Kaempferol) reduced MTP transcription, but not isoflavones (Genistein and Daidzein).

In the screening system, human MTP promoter (-204~+33) was used. This region contains most of critical elements required for MTP gene expression, a pair of response elements for HNF4 and HNF1, and an insulin response element, whereas a Foxo-binding element exists in the upstream region apart from this region. (For FoxO1 (33) and FoxA2 (105)). Therefore, this screening gave us the results that several types of flavonoids decreased the MTP promoter activity through its limited region (-204~+33).

Regarding aforementioned result [II], it has been reported that HNF1α is one of HNF4α target genes (106). Therefore, the finding that Luteolin suppressed HNF4α activity in this study strongly supports the idea that declined MTP promoter activity by Luteolin was partly caused by decreased HNF1α. In contrast, in HepG2 cells transfected with siHNF4α, HNF1α mRNA level was further reduced by Luteolin treatment (data not shown). Indeed, HNF1α gene expression was more rapidly and strongly inhibited by Luteolin than HNF4α (Fig 2-7, 2-8). These results indicate that Luteolin is capable of down-regulating HNF1α independently of declined HNF4α activity. Because HNF1α is one of critical transcription factors for human Apo B gene expression (107), it seems likely that Luteolin reduces Apo

B secretion by inhibiting both the MTP and Apo B transcription through reduced HNF1α activity. It was reported that Luteolin prevented production and release of high mobility group B-1 (HMGB1) (108), a co-activator of HNF1α (109). There is a possibility that Luteolin may suppress HNF1α activity by preventing the recruitment of HMGB1.

Regarding aforementioned result [IV], it appears that Luteolin directly hinders Apo B secretion without involving decreased HNF4α. Although the degradation of Apo B can be mediated by the ubiquitin-proteasome pathway (110), it was found that Luteolin decreased intracellular Apo B level not through the proteasome pathway.

Apo B can also be degraded by the autophagy pathway (18), which is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. To investigate the relation between Luteolin inhibitory effect on Apo B secretion and autophagy, HepG2 cells were cultured with chloroquine (an autophagy inhibitor) and/or Luteolin. Inhibition of lysosomal degradation did not alter the Apo B secretion, whereas the autophagy caused by Luteolin was abolished. Chloroquine inhibits autophagy as it raises the lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation (111). Autophagy is a complex process, it also can be regulated by Beclin1 pathway or mTOR pathway, which differ from the principle of chloroquine (112). Luteolin were recently shown to induce autophagy in both normal cells and several cancer cell types (113), however the mechanism is poorly understood.

Besides, Luteolin ameliorated lipid accumulation, and decreased FAS and SREBP-1c mRNA levels in HepG2 cells (114). In this study I obtained the same results (data not shown). A previous report demonstrated that 5-tetradecyloxy-2-furancarboxylic acid (TOFA), an inhibitor of acetyl-coA carboxylase, decreased Apo B secretion in hamsters (115). These findings imply a causal connection between decreased Apo B secretion and reduced fatty acid synthesis by Luteolin.

Diacylglycerol acyltransferase (DGAT) 1 and 2 are thought to be involved in Apo B-lipoprotein assembly and in the regulation of plasma TG concentrations. Acyl-CoA cholesterol acyltransferase (ACAT s) 1 and 2 catalyze the formation of CE from cholesterol

and long chain fatty acyl-coenzyme A (116). Although these enzymes are highly involved in Apo B-containing lipoprotein secretion, there have been no reports about the effect of Luteolin on them. Therefore further studies are needed.

Phosphatidylinositol 3-kinase (PI3K) activation by insulin increases sterol regulatory element-binding protein (SREBP)-1 and LDLR expression and inhibits Apo B secretion in hepatocytes (117). It appears that activation of PI3K/PKB/AKT may result in reduced Apo B secretion. Several studies have shown that Luteolin suppress phosphorylation of AKT (118, 119), consistent with results in HepG2 cells in this study (data not shown). However, it has been also reported that Luteolin enhanced AKT2 phosphorylation in an insulin-stimulated state in 3T3-L1 adipocytes (120). Hence, further investigation into a connection between the AKT pathway and Apo B secretion regulated by Luteolin is needed.

Chapter 3

Luteolin directly bound to HNF4α to decrease the activity through the effect on DNA binding activity of HNF4α and co-factor recruitment

3-1 Preface

HNF4α (or NR2A1) is a highly conserved member of the nuclear receptor superfamily and also functions as a transcription factor. It forms homodimers that bind to a direct repeat of the AGGTCA with one-base spacing (DR1) (37). HNF4α is the most abundant nuclear receptor expressed in the liver (38), with the function of regulating network required for maintenance of lipid and glucose metabolic genes. The trans-activating activity of HNF4α can be regulated by co-factors and post-translational modifications.

From Chapter 2 it is clear Luteolin inhibited Apo B secretion and MTP promoter activity by blocking HNF4α activity. In this chapter, the mechanism of inhibition of HNF4α activity by Luteolin was studied.

3-2 material and methods

Plasmid (protein purification)

Plasmid pET28a-hHNF4α (LBD) with a His tag at its N-terminal was constructed using the primer set of 5'- ATATGGATCCAGCCTGCCCTCCATCAATGCG -3' and 5'- TAAAGCGGCCGCTTAACCTCCCAGCAGCATCTCCTG -3'.

pGAL4-hHNF4α-LBD (116~455)

Luciferase Assay

Plasmid DNA for GAL4-HNF4α activity (for 1 well)

GAL4/GAL4-hHNF4α-LBD	0.2μg
5xUAS	0.2μg
pCMV-β-gal	0.2μg

As described in Chapter 2 were used in this experiment.

RT-PCR

As described in Chapter 2 were used in this experiment.

Western blotting

As described in Chapter 2 were used in this experiment.

Protein purification

Plasmid PET-28-hHNF4α (LBD) was transformed in E. coli strain BL21. Cells harboring the expression plasmid for PET-28-hHNF4α were grown in LB- kanamycin (50μg/ml) medium until OD_{600} reached 0.6-0.8 at 37℃. Then 0.25 mM β-isopropylthiogalactoside (IPTG) was added and the cells were grown overnight at 30℃. The cells were then collected and suspended in 50 mM Tris-HCl buffer, pH 8.0, disrupted by sonication (duty cycle 50%, output 5.0) and centrifuged at 11000rpm for 30 minutes at 4°C. After centrifugation, the supernatant was subjected to 3mL equilibrated HIS-Select Nickel Affinity Gel. The column was washed with 50 mM Tris-HCl buffer, pH 7.5, and then the proteins eluted with 50 mM Tris-HCl, 500 mM imidazole buffer, pH 7.5, were collected.

The eluted sample was checked by CBB staining.

Luteolin treatment

Protein solution with 1/100 volume of 100mM Luteolin was incubated overnight at 4°C, centrifuged at 11000rpm for 10 minutes at 4°C, the supernatant was washed twice by ultrafiltration.

Gel filtration

For absorption spectrum analysis and Trypsin analysis, gel filtration was performed, through a column (Hiload 16/60 Superdex 200 prep grade).

Gel filtration Buffer

20mM Tris-HCl (pH 7.5)

250mM NaCl

Absorption spectrum

Absorption spectra of HNF4α (0.8mg/mL) solution with Luteolin or no treatment were recorded with a JASCO V-560 spectrophotometer.

Trypsin treatment

8μg of HNF4α solution with Luteolin or no treatment was treated with 0.1% trypsin for 1, 10, and 30 min at 37 °C, subjected to SDS/PAGE, and visualized by Coomassie blue staining.

Surface plasmon resonance (By BIACORE)

Surface plasmon resonance experiments were performed with BIACORE (National Institute of Biomedical Innovation, Osaka). A dextran surface of sensor chips was activated with EDC/NHS Buffer, flowpath 2 was coated at 10 μL/min with 50μg/mL HNF4α protein solution in immobilization buffer (10 mM KAc, pH 5 with HAc), 70uL of ethanolamine was injected for blocking the activated NHS, 10 μL of 10 mM Gly-HCl (pH2.0) was injected to remove the un immobilized HNF4α. Measurements were done in running buffer (HBS-P, 1%DMSO, 50μM $ZnCl_2$) using a flowrate of 50 μL/min, 50μL of different concentration of Luteolin or Daidzein solution was injected.

Detection of HNF4α and Luteolin binding by Luteolin Beads

Luteolin immobilized on agarose beads through a photo-affinity linker to make the Luteolin beads (RIKEN). HepG2 cells were treated with Luteolin or DMSO (as control) for 3h, harvested and washed twice with PBS, then resuspended in binding buffer. After cell lysis by homogenization with a syringe, the insoluble material was removed by centrifugation, and the supernatant was collected as a cell lysate. After the cell lysate (1mg of protein) was precleared by incubation with control beads (10μL) for 1 h at 4°C, the cleared cell lysate was incubated with Luteolin beads (15 μL) for 12h at 4°C.The reacted beads were washed with binding buffer, and the co-precipitated proteins were subjected to SDS-PAGE and Western blotting using antibodies against human HNF4α.

Binding buffer

10 mM	Tris-HCl pH 7.6
50mM	KCl
5mM	$MgCl_2$
1mM	EDTA
1/100	PIC

Chromatin immunoprecipitation (ChIP) assay

HepG2 cells were set up on day 0 at a density of 3×10^6 cells per 100-mm dish in DMEM (High Glucose) medium supplemented with 10% fetal calf serum. On day 2, cells were refed serum-free medium without or with 50μM Luteolin for 3 h. Then cell were harvested and processed for ChIP assays using the ChIP Assay kit (Upstate) according to the manufacturer's instructions. Briefly, chromatin was crosslinked for 10 min at 37 ºC by adding 0.27mL formaldehyde to the culture medium. Cells were then harvested, washed with 2mL 1xPBS three times, centrifuged immediately at 8000rpm for 10min at 4°C. After the supernatant was carefully removed, 400 μL of SDS Lysis Buffer with protease inhibitors was added to the pellet, vortexed and incubated for 10 min on ice. Cell lysates were subjected to sonication on ice for 10 sec intervals seven times at 50% maximum power. After clearing insoluble debris by centrifugation, the lysates were diluted with ChIP

Dilution Buffer and incubated with 60μL salmon sperm DNA/protein An agarose slurry for 1h, centrifuged at 3000rpm for 10min at 4°C. Then 5 μg of the indicated antibody was added to the supernatant, incubated overnight at 4 °C. The antibodies used were: preimmune rabbit IgG as a negative control; anti-acetyl-histone H3 (Lys9) IgG as a possitive control; and polyclonal anti-HNF4α IgG. The following day, 30 μL of salmon sperm DNA/protein An agarose slurry was added, and the mixture was incubated for 1h at 4 °C. Then the beads were washed by Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and TE Buffer (twice). After washing the protein an agarose/antibody/chromatin complexes, complexes were eluted from the beads by incubating with Elution Buffer for 1h at RT. Then Protein/DNA crosslinks and diluted lysates were reversed by adding 10μL of 4M NaCl incubated at 65 °C overnight. The following day, the samples were treated 0.4μL of RNAase for 30min at 37 °C, then treated with 20 μg of proteinase K at 45 °C for 1 h, finally DNA was extracted using phenol–chloroform method. PCR analysis was performed using Real-Time PCR with purified DNA and the primers.

SDS Lysis Buffer

1%	SDS
10mM	EDTA
50mM	Tris-HCl pH 8.1

ChIP Dilution Buffer

0.01%	SDS
1.1%	Triton X- 100
1.2mM	EDTA
16.7mM	Tris-HCl pH 8.1
167mM	NaCl

Low Salt Immune Complex Wash Buffer

0.1%	SDS
1%	Triton X-100

2mM	EDTA
20mM	Tris-HCl pH 8.1
150mM	NaCl

High Salt Immune Complex Wash Buffer

0.1%	SDS
1%	Triton X-100
2mM	EDTA
20mM	Tris-HCl, pH 8.1
500mM	NaCl

LiCl Immune Complex Wash Buffer

0.25M	LiCl
1%	NP40
1%	deoxycholate
1mM	EDTA
10mM	Tris-HCl pH 8.1

TE Buffer

10mM	Tris-HCl pH 8.0
1mM	EDTA

Elution Buffer

1%	SDS
0.1 M	$NaHCO_3$
10mM	DTT

Primer (5'----3')

The following primers were used to amplify the HNF4α proximal promoter

hMTP	F GTGAGAGACTGAAAACTGCAGC
	R CATCCAGTGCCCAGCTAGGAG
hPEPCK	F CCCCAGTTAGGTTAGGCATT

	R GTTGGCAAAACACCACAGCT
hG6Pase	F AACCTACTGGTGATGCACCT
	R TGCTCTGCTATGAGTCTGTG
hHNF1α	F CATGATGCCCCTACAAGGTT
	R AGCTGGGGAAATTCTCCAAG
hApo B	F CTTCAAGGCTCAAAGAGAAGCC
	R AGGTCCCGGTGGGAATG

The following primers were used to amplify the HNF4α distal promoter

hMTP	F GGAGAACTGATACAGTGCCTTT
	R GAGAGGTCAAGAGATATAGAGGATAGA
hPEPCK	F CTTGTATGAGCAGTTCGAGG
	R TGCCACATAGACAGTTCCAG
hG6Pase	F TTAGAAGGAGATGGCGGGAA
	R TCTACATTGCCCAGACTGGT
hHNF1α	F CATATTGGTCAGGGTGGTCT
	R TGTGGCTACCAAGCACTTGA
hApo B	F GGGCACAGTTCCATCTACAA
	R CCTATCTCGTTTCTGCCTATGAC

3-3 Results

3-3-1 Luteolin functions within cells

It is postulated that Luteolin inhibited HNF4α activity from outside of cells through a specific receptor (indirect action) or through their direct interaction within cells (direct action) (Fig 3-1). In order to make clear how Luteolin inhibits the HNF4α transcription activity, Luteolin glucosides (Luteolin-7-glucoside and Isoorientin), which are thought to be poorly taken up by cells, were used (Fig 3-2). Different from Luteolin, neither of Luteolin glucosides suppressed the transcription of MTP using the human MTP gene promoter (-204~+33) in HepG2 cells (Fig 3-3 A). Furthermore, neither of them exhibited effect on Apo B secretion for 3h or 12h treatment (Fig 3-3 B). Afterwards the influence of Luteolin glucosides on HNF4α activity was examined by investigating the mRNA levels of HNF4α target genes in HepG2 cells. In contrast to Luteolin, neither of the two kinds of Luteolin glucosides reduced the target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase, COUP-TFII, and FXR) (Fig 3-4). These results indicate different from Luteolin, Luteolin glucoside did not inhibit the HNF4α activity. It is suggested Luteolin functioned within cells. Hence, it was thought Luteolin inhibited HNF4α activity through a direct interaction.

3-3-2 Luteolin is capable of being associated with HNF4α directly

The plasmid of PET-28-HNF4α (LBD) was transformed in *E.coli* BL21strain for expression. After *E.coli* lysate was purified by nickel affinity gel, preliminary purified HNF4α protein was obtained, followed by incubating with Luteolin or not (abbreviated as $HNF4\alpha_{+Lut}$, $HNF4\alpha_{-Lut}$ respectively). After HNF4α protein was subjected to gel filtration for removing the unconjugated Luteolin, fractionated $HNF4\alpha_{+Lut}$ turned yellow (Fig 3-5 A), as the same color of Luteolin, whereas fractionated $HNF4\alpha_{-Lut}$ remained colorless (Fig 3-5 B). It is suggested that HNF4α interact with Luteolin.

Purified HNF4α protein was also analyzed by the methods of absorption spectrum, trypsin cleavage and BIACORE to verify the interaction with Luteolin.

Absorption spectrum analysis showed that regardless of Luteolin incubation there is an absorption peak in about 280nm, which was the absorbance of protein. Additionally, it was observed that there was a little difference between HNF4α$_{+Lut}$ and HNF4α$_{-Lut}$ (Fig 3-6 B). The section enlarged showed an absorption peak in about 400nm of HNF4α$_{+Lut}$ (Fig 3-6 A). Because Luteolin had an absorption spectrum peak near 400nm. It is thought that Luteolin was bound to HNF4α.

Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine. Because there are many trypsin cleaves sites in protein, it is postulated that the hydrolysis by trypsin may be affected when something interacted with the protein (Fig3-7 A). Trypsin cleavage analysis showed that HNF4α protein was hydrolyzed into small fragments by trypsin with different speed in the absence or presence of Luteolin treatment. HNF4α$_{+Lut}$ was resistant to trypsin digestion (Fig3-7 B). These results suggest that there is an interaction between HNF4α and Luteolin.

BIACORE is a method for protein-ligand interaction. Purified HNF4α protein was immobilized on the sensor chip surface, then a series of concentrations of Luteolin and Daidzein solution flowed across the surface. When the binding interaction were detected, the response occurred (Fig 3-8 A). For Daidzein, even if 50μM concentration was used, there was little response. For Luteolin, with the increasing of concentration, the response became stronger. However, it did not converge upon a unique value (Fig 3-8 B).

Luteolin was immobilized on agarose beads through a photo-affinity linker (Fig. 3-9 A). HepG2 cells were treated with Luteolin or DMSO (as control) for 3h. One mg of HepG2 cells lysates were pre-incubated with control beads (con beads), and then the unbound fraction was further incubated with Luteolin-immobilized beads (Lut beads). The reacted beads were washed, and the co-precipitated proteins were subjected to SDS-PAGE and Western blotting using antibodies against human HNF4α. The results show that more HNF4α was recovered with Lut beads than with con beads from the lysates of cells that were cultured without Luteolin (Fig 3-9 C Lane3 and 5). When cells were incubated with Luteolin, the cell pellets turned yellow, probably due to the uptake of Luteolin. The amount

of HNF4α recovered with Lut beads decreased as compared with DMSO treated cell lysates (Fig 3-9 C Lane5 and 6). This decline seems to be caused by the competiton of free Luteolin and HNF4α to bind the Lut beads. All the results indicate Luteolin is capable of being associated with HNF4α directly.

3-3-3 Luteolin decreases DNA binding activity of HNF4α

Subsequently, the mechanism of inhibitory effect of Luteolin on HNF4α activity was investigated.

In order to verify the effect of Luteolin on DNA binding activity of HNF4α, ChIP assay was performed. Firstly, the time-course for Luteolin treatment was examined in HepG2 cells. It was observed that HNF4α protein level was not decreased by Luteolin until 3h (Fig 3-10 A).

Chromatin from the control and Luteolin treated cells were immuneprecipitated with anti-acetylated histone H3, anti-human HNF4α or rabbit Immunoglobulin G (IgG) antibodies. DNA precipitated by the antibodies was then subjected to real-time PCR with gene-specific primers or non-specific primers as negative control (Fig 3-10 B). ChIP assays revealed that HNF4α and acetyl-histon bound to the target gene promoters (Apo B, MTP, HNF1α, PEPCK and G6Pase), whereas Luteolin decreased the binding activity of HNF4α and acetyl-histon (Fig 3-11 A). ChIP assays using primer pairs covered the target gene distal region gave us negligible low PCR products, suggesting these regions did not interact with HNF4α and acetyl-histon. (Fig 3-11 B).

3-3-4 Luteolin has effect on the recruitment of co-factor(s)

In order to verify the effect of Luteolin on HNF4α co-factor(s) recruitment, GAL4 luciferase assay system was used (Fig 3-12 A). A GAL4-DBD-HNF4α-LBD fusion plasmid and the 5xUAS reporter gene were transfected in HepG2 cells. The luciferase activity was increased with GAL4-DBD-HNF4α-LBD transfection and reduced by Luteolin in a dose-dependent manner. This suggests that Luteolin may regulate the recruitment of co-factor(s) required for induction of HNF4α transcription activity.

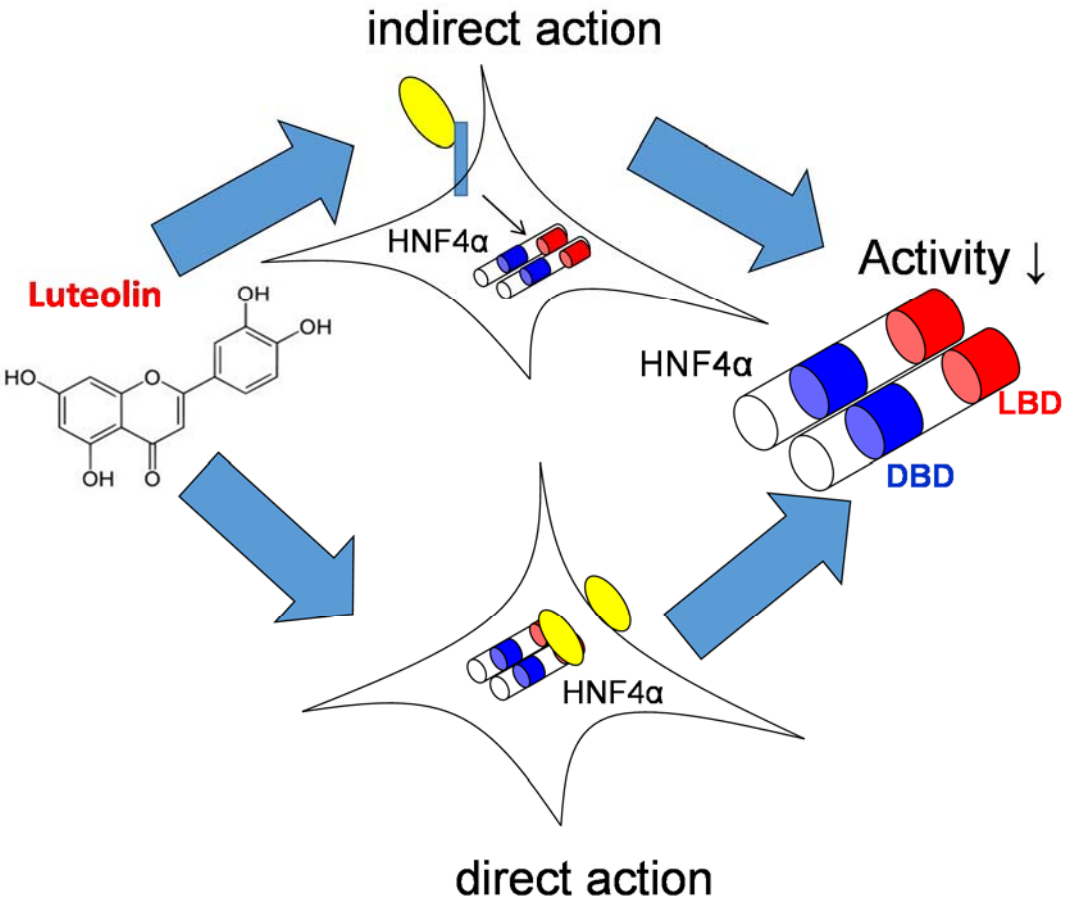


Figure 3-1 The postulated manners for Luteolin inhibiting HNF4α activity.

One is from outside of cells through a specific receptor (indirect action), the other one is direct interaction within cells (direct action).

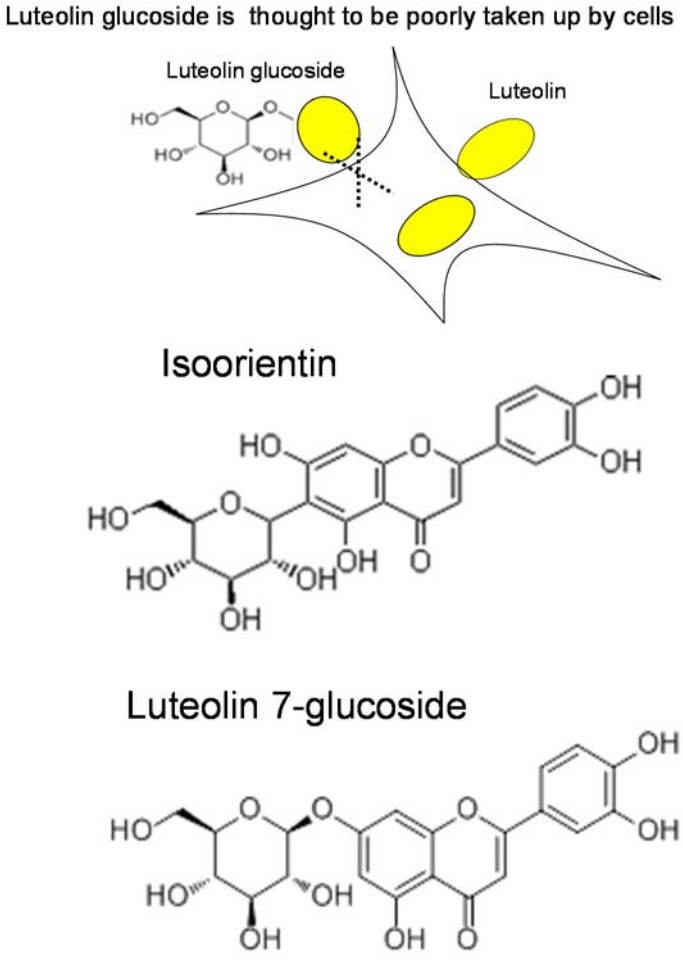


Figure 3-2 The structure of Luteolin glucoside

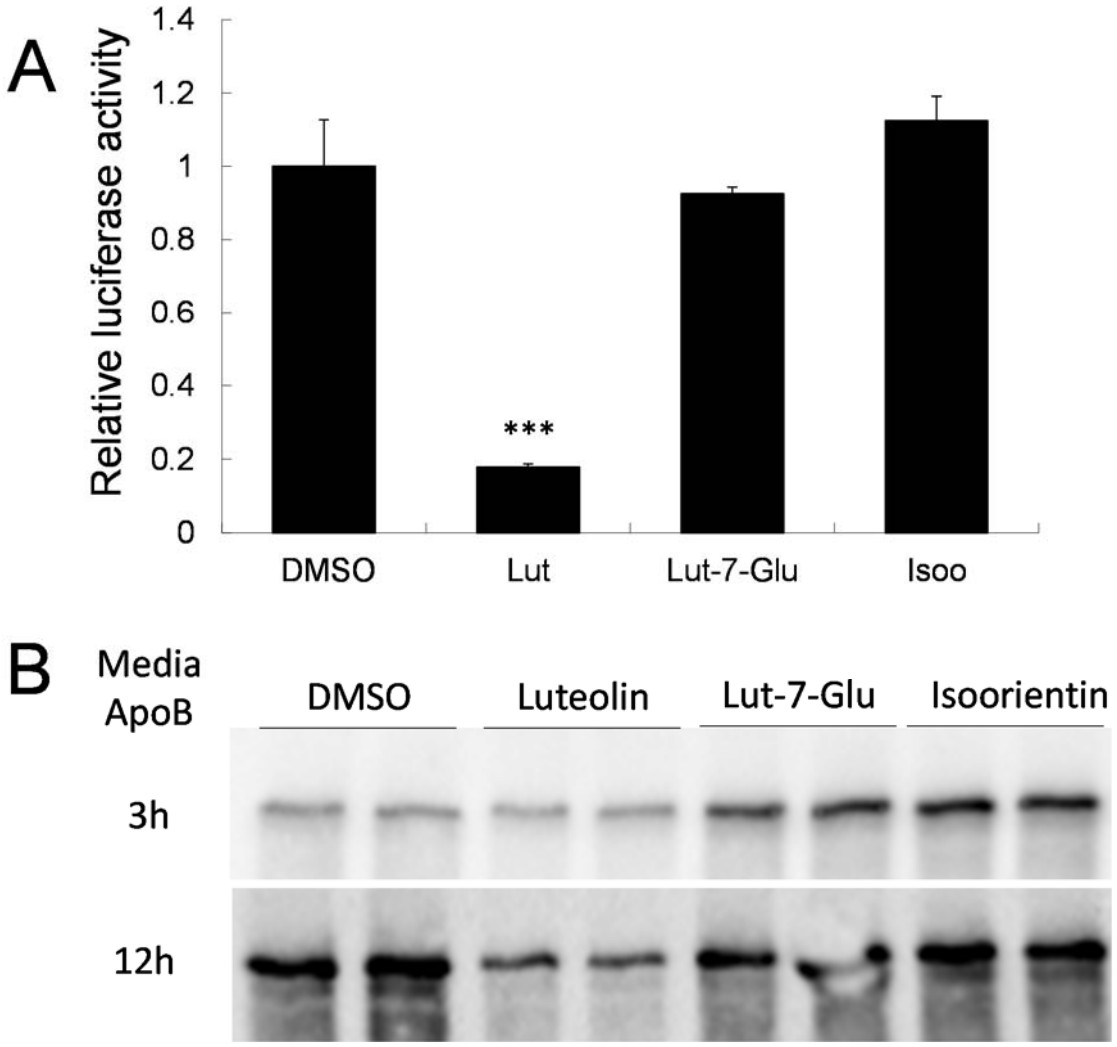


Figure 3-3 Luteolin glucosides have no effect on MTP transcription and Apo B secretion in HepG2 cell

A. HepG2 Cell were transfected with the report plasmid containing human MTP gene promoter (-204~+33) and an expression plasmid for β-galactosidase. After transfection, cells were treated with 50μM Luteolin, Luteolin-7-glucoside, and Isoorientin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. Results are means±S.D. (n =3). ***P <0.001. B. HepG2 cells were treated with serum-free medium containing 50μM Luteolin, Luteolin-7-glucoside, and Isoorientin for 3h or12h. The culture medium (15 μL/lane) was subjected to SDS-PAGE and Western blotting using antibodies against human Apo B.

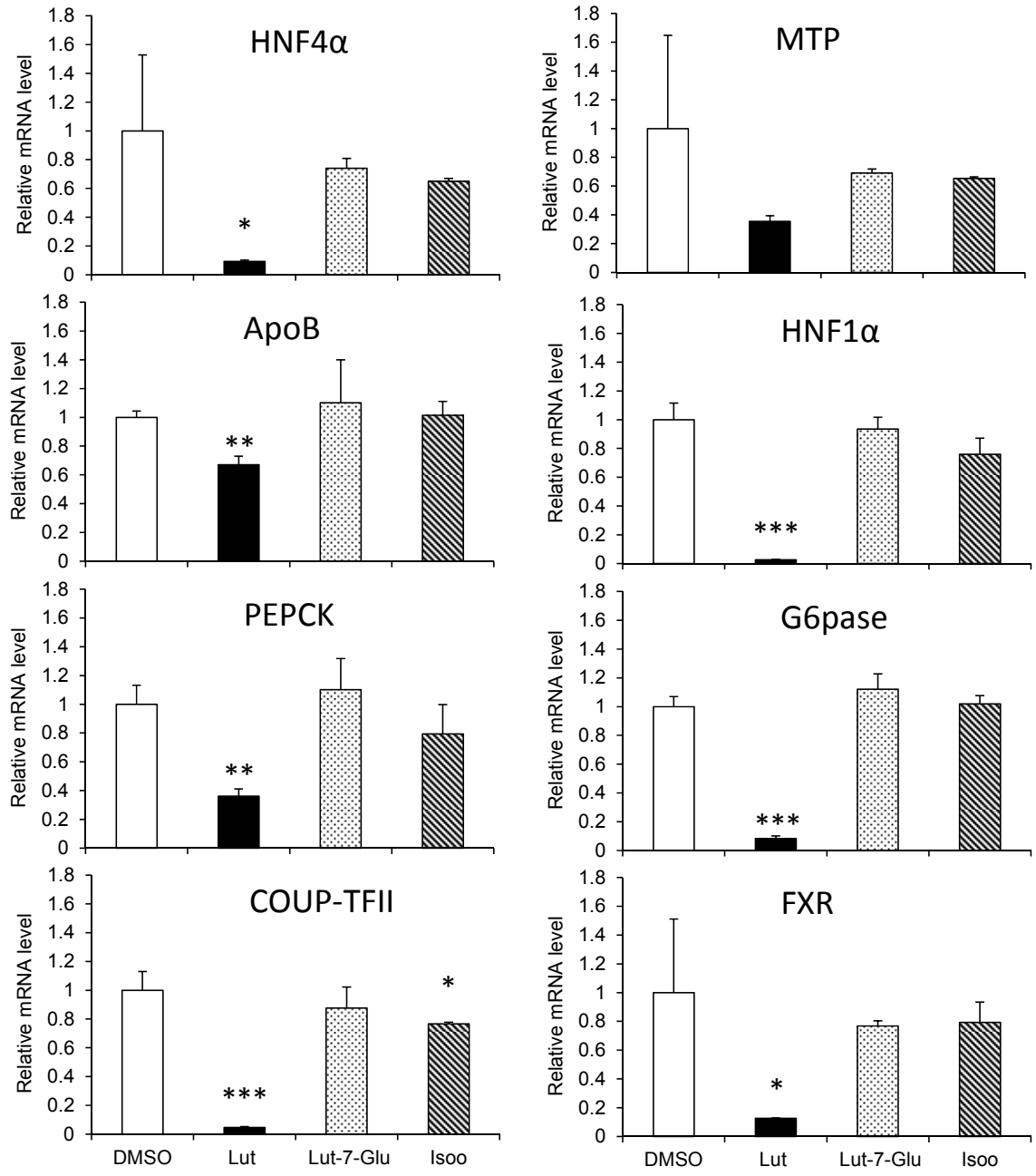


Figure 3-4 Luteolin glucosides have no effect on HNF4α related genes in HepG2 cell

HepG2 cells were treated with 50μM Luteolin, Luteolin-7-glucoside, and Isoorientin for 12h in serum-free media, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. Results are means±S.D. (n =3). *P <0.05, **P <0.01, ***P <0.001.

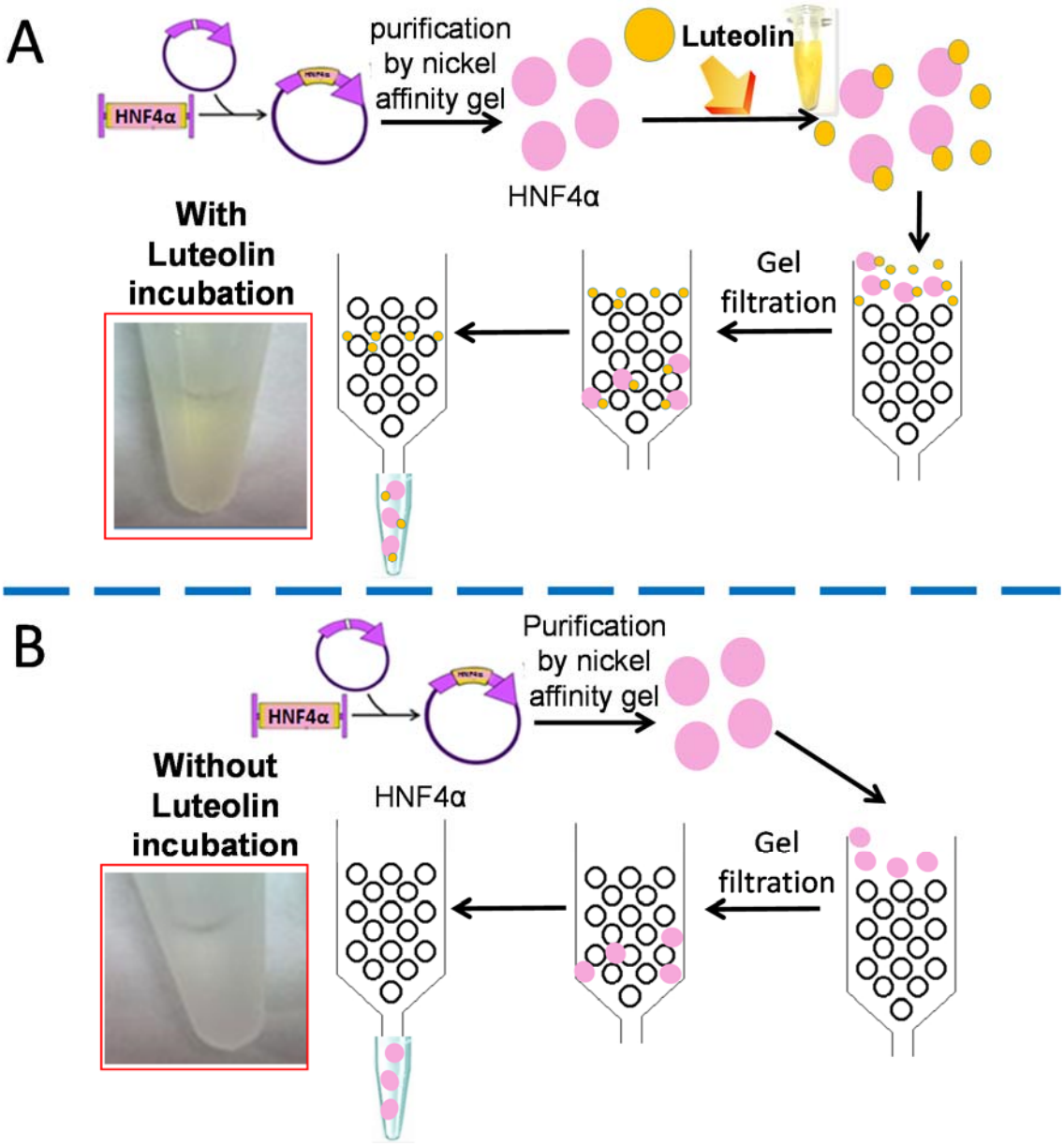


Figure 3-5 HNF4α purification and Luteolin treatment

Plasmid PET-28-hHNF4α (LBD) was transformed in E. coli strain BL21, then incubated in medium until OD_{600} reached 0.6-0.8 at 37℃. Then 0.25 mM IPTG was added and then incubated overnight at 30℃. The cells were then collected and suspended in 50 mM Tris-HCl buffer, pH 8.0, disrupted by sonication. After centrifugation, the supernatant was subjected to HIS-Select Nickel Affinity Gel, washed with 50 mM Tris-HCl buffer, pH 7.5, then eluted with 50 mM Tris-HCl, 500 mM imidazole buffer, pH 7.5. half of the protein solution with 1/100 volume of 100mM Luteolin was incubated overnight at 4 ℃. After centrifugation, the supernatant was washed twice by ultrafiltration and subjected to filtration.

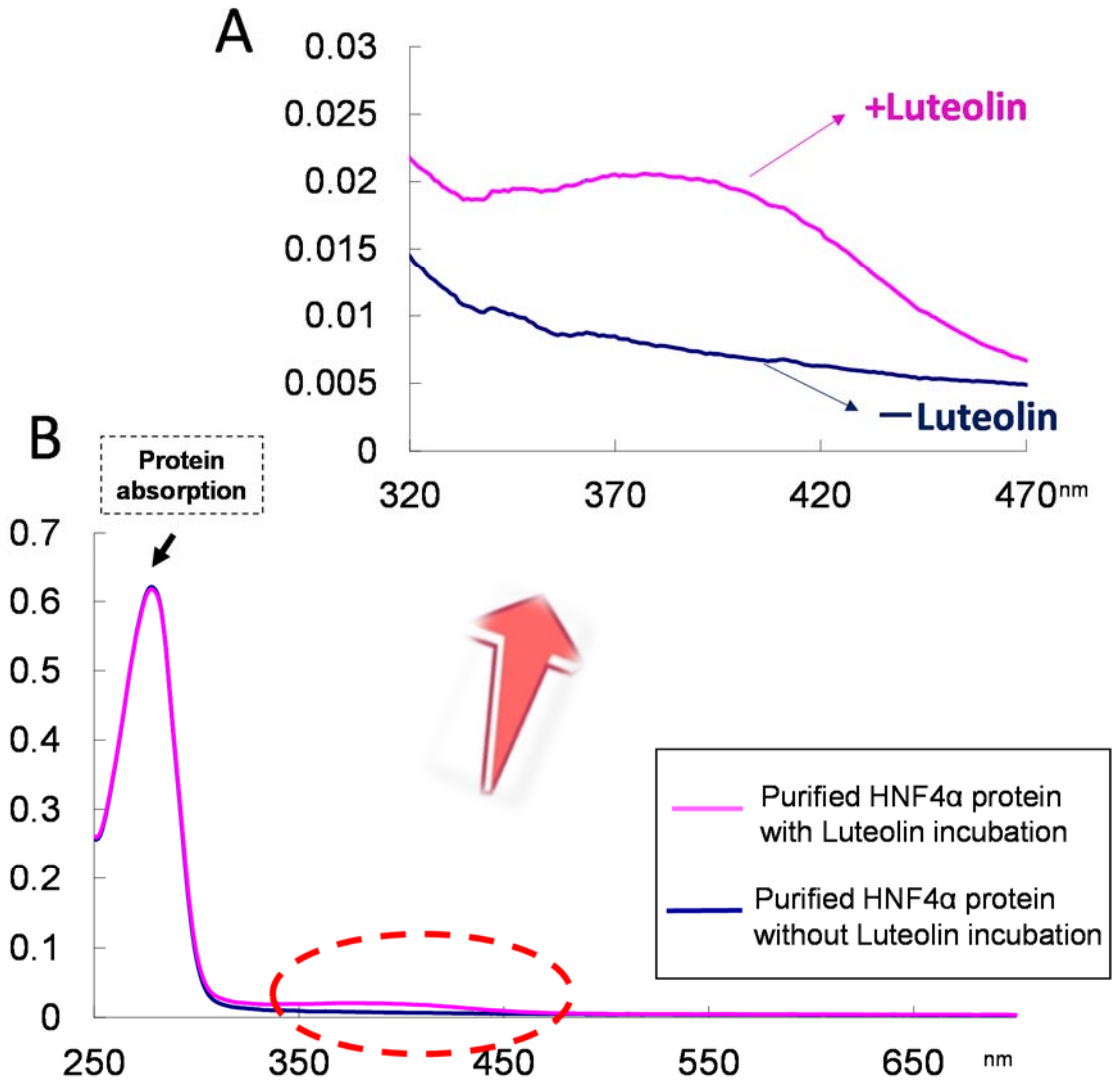


Figure 3-6 Absorption spectrum analysis of purified HNF4α protein

Purified HNF4α solution (0.8mg/mL) with Luteolin or no treatment were recorded with a JASCO V-560 spectrophotometer for Absorption spectrum analysis. A. Amplification of the spectrum between 350 and 450 nm. B. The absorption of HNF4α.

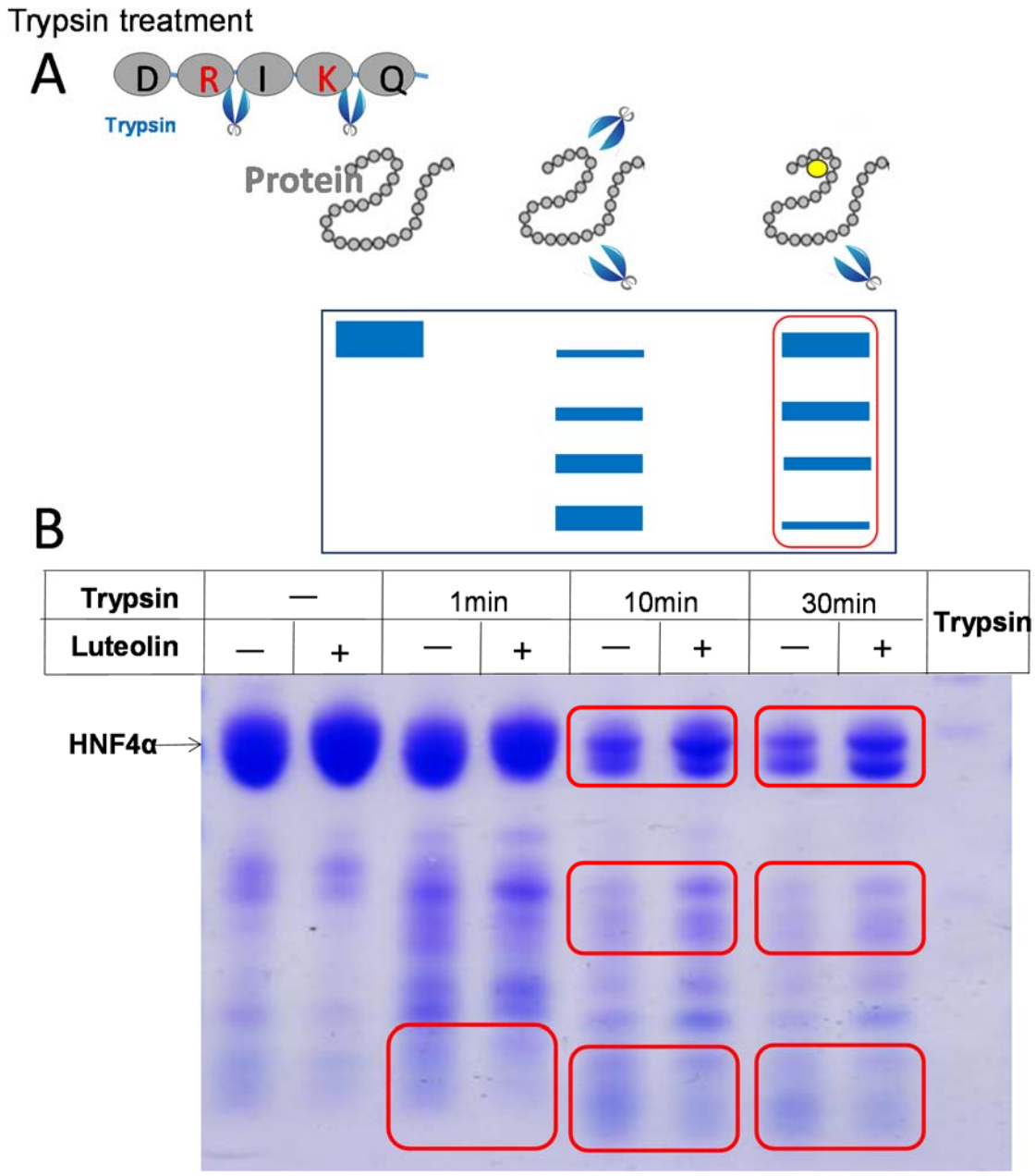


Figure 3-7 Trypsin cleavage analysis of purified HNF4α protein

A. Schematic diagramof trypsin hydrolysis. B. Purified HNF4α (after Gel filtration) (8μg) with Luteolin or no treatment was treated with 0.1% trypsin for 1, 10, and 30 min at 37℃, subjected to SDS/PAGE, and visualized by Coomassie blue staining.

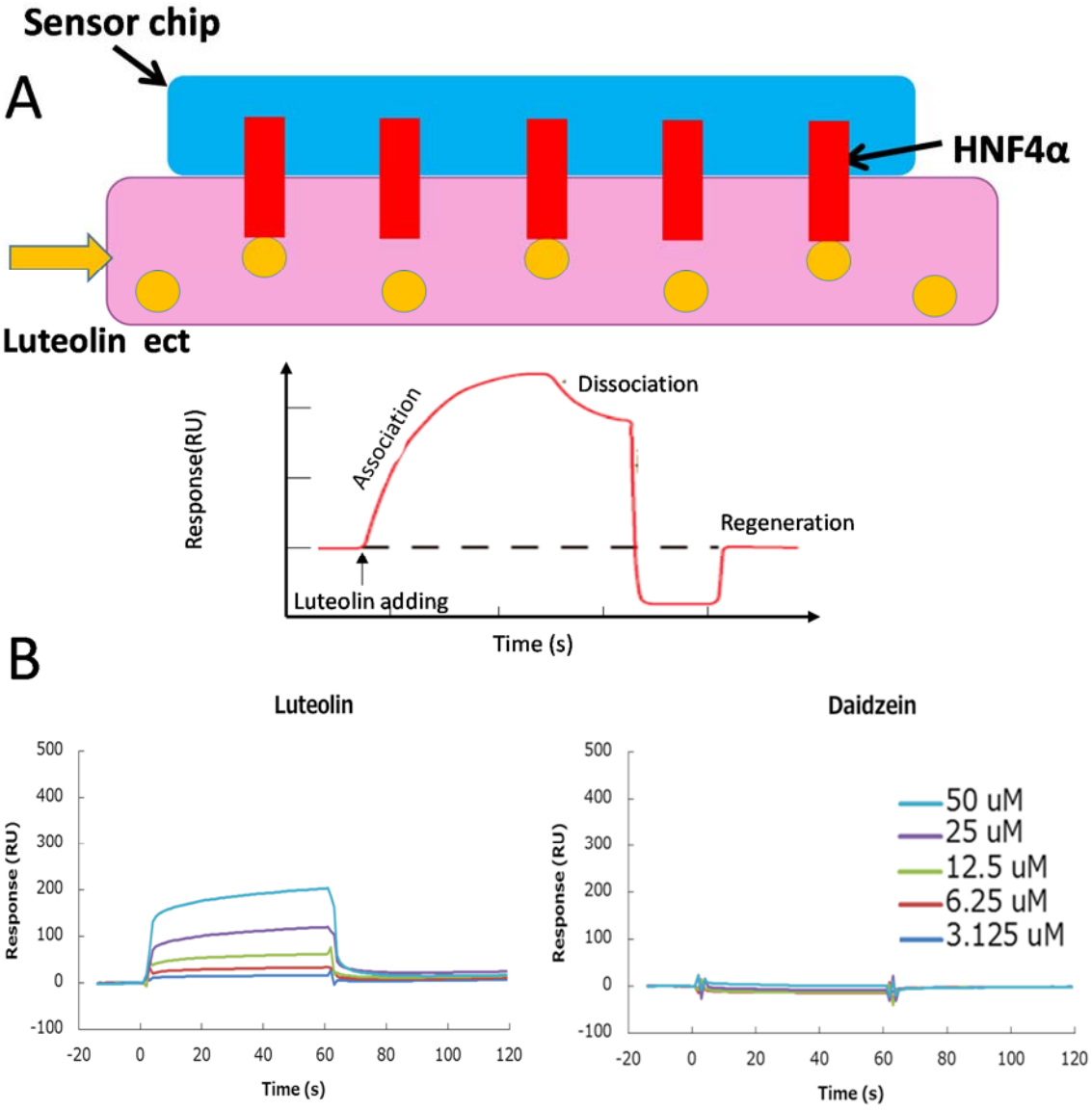


Figure 3-8 Surface plasmon resonance analysis of purified HNF4α protein by BIACORE

A. The schematic diagram of Surface plasmon resonance experiments. B. A dextran surface of sensor chips was activated with EDC/NHS Buffer, flow path 2 was coated at 10 μL/min with 50μg/mL HNF4α protein solution in immobilization buffer (10 mM KAc, pH 5 with HAc), 70uL of ethanolamine was injected for blocking the activated NHS, 10 μL of 10 mM Gly-HCl (pH2.0) was injected to remove the un immobilized HNF4α. Measurements were done in running buffer (HBS-P, 1%DMSO, 50μM $ZnCl_2$) using a flowrate of 50 μL/min, 50μL of different concentration of Luteolin or Daidzein solution was injected.

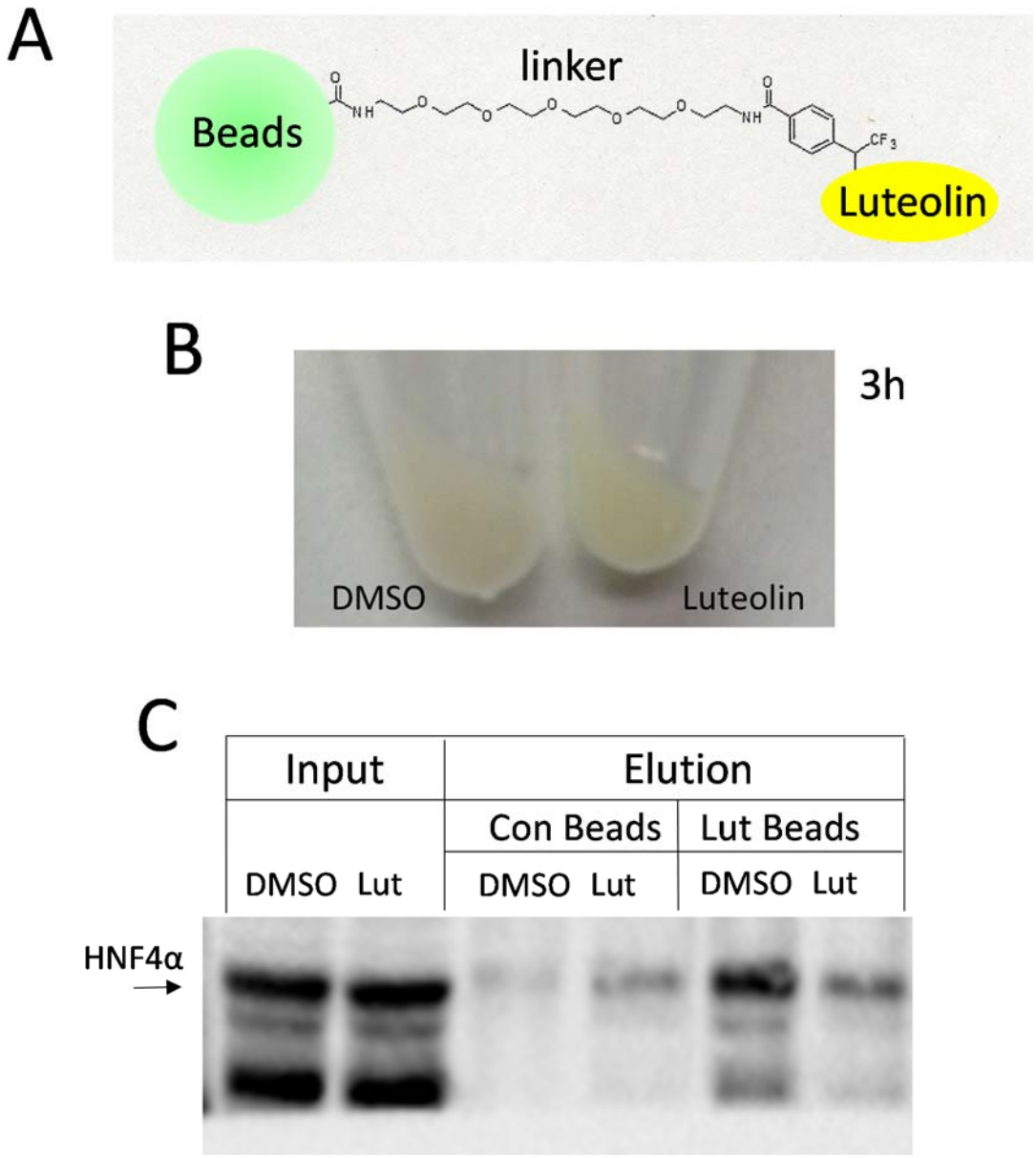


Figure 3-9 Analysis of the interaction of HNF4α and Luteolin by Luteolin beads

A. Model structure of Luteolin beads. Luteolin immobilized on agarose beads through a photo-affinity linker to make the Luteolin beads (RIKEN). B.HepG2 cells were treated with Luteolin or DMSO (as control) for 3h, harvested and washed twice with PBS. C. Harvested HepG2 cells were resuspended in binding buffer. After cell lysis by homogenization with a syringe, the insoluble material was removed by centrifugation, and the supernatant was collected as a cell lysate. After the cell lysate (1mg of protein) was precleared by incubation with control beads (10μL) for 1 h at 4 ℃, the cleared cell lysate was incubated with Luteolin beads (15 μL) for 12h at 4 ℃.The reacted beads were washed with binding buffer, and the co-precipitated proteins were subjected to SDS-PAGE and Western blotting using antibodies against human HNF4α.

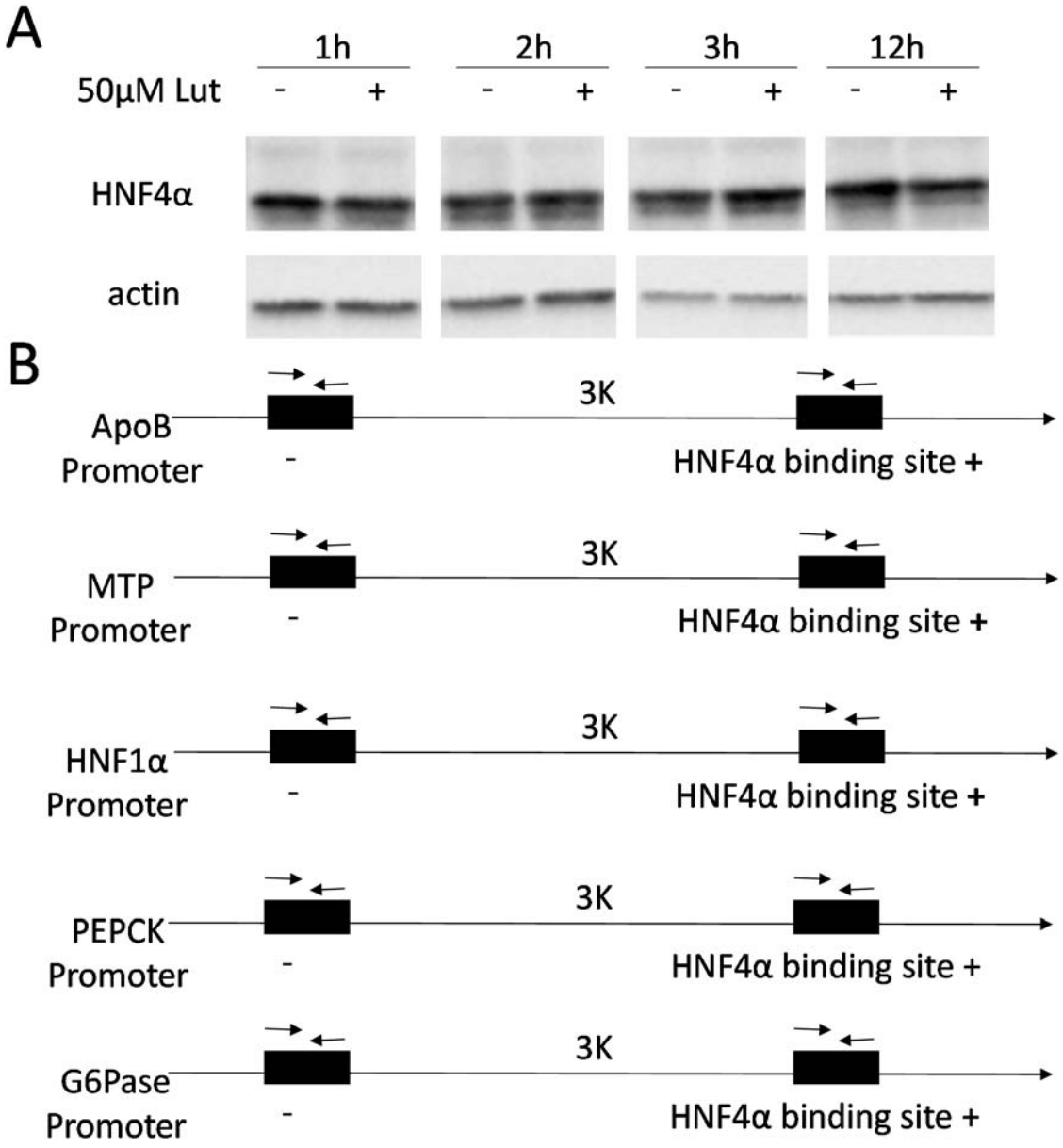


Figure 3-10 The primers designed for ChIP assay

A. The time-course study for Luteolin inhibitiory effect on HNF4α. B. Gene-specific or non-specific primer s for HNF4α binding sites.

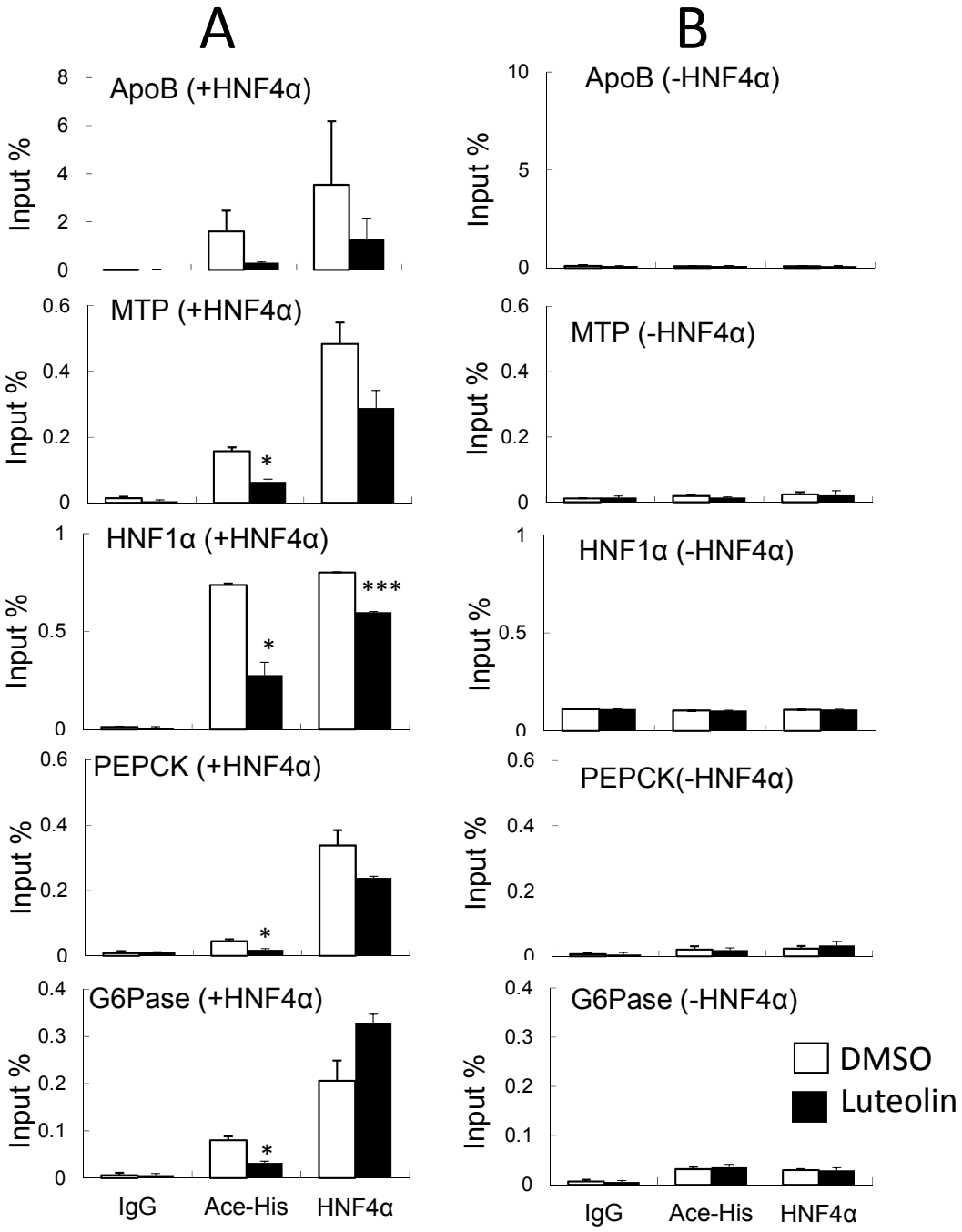


Figure 3-11 Luteolin decreases DNA binding activity of HNF4α and histon acetylation

HepG2 cells were refed serum-free medium without or with 50μM Luteolin for 3 h. Then cell were harvested and processed for ChIP assays using the ChIP Assay kit (Upstate) according to the manufacturer's instructions. The antibodies used were: preimmune rabbit IgG as a negative control; anti-acetyl-histone H3 (Lys9) IgG as a possitive control; and polyclonal anti-HNF4α IgG. PCR analysis was performed using Real-Time PCR with purified DNA and the primers. Results are means±S.D. (n =3). *P <0.05, ***P <0.001.

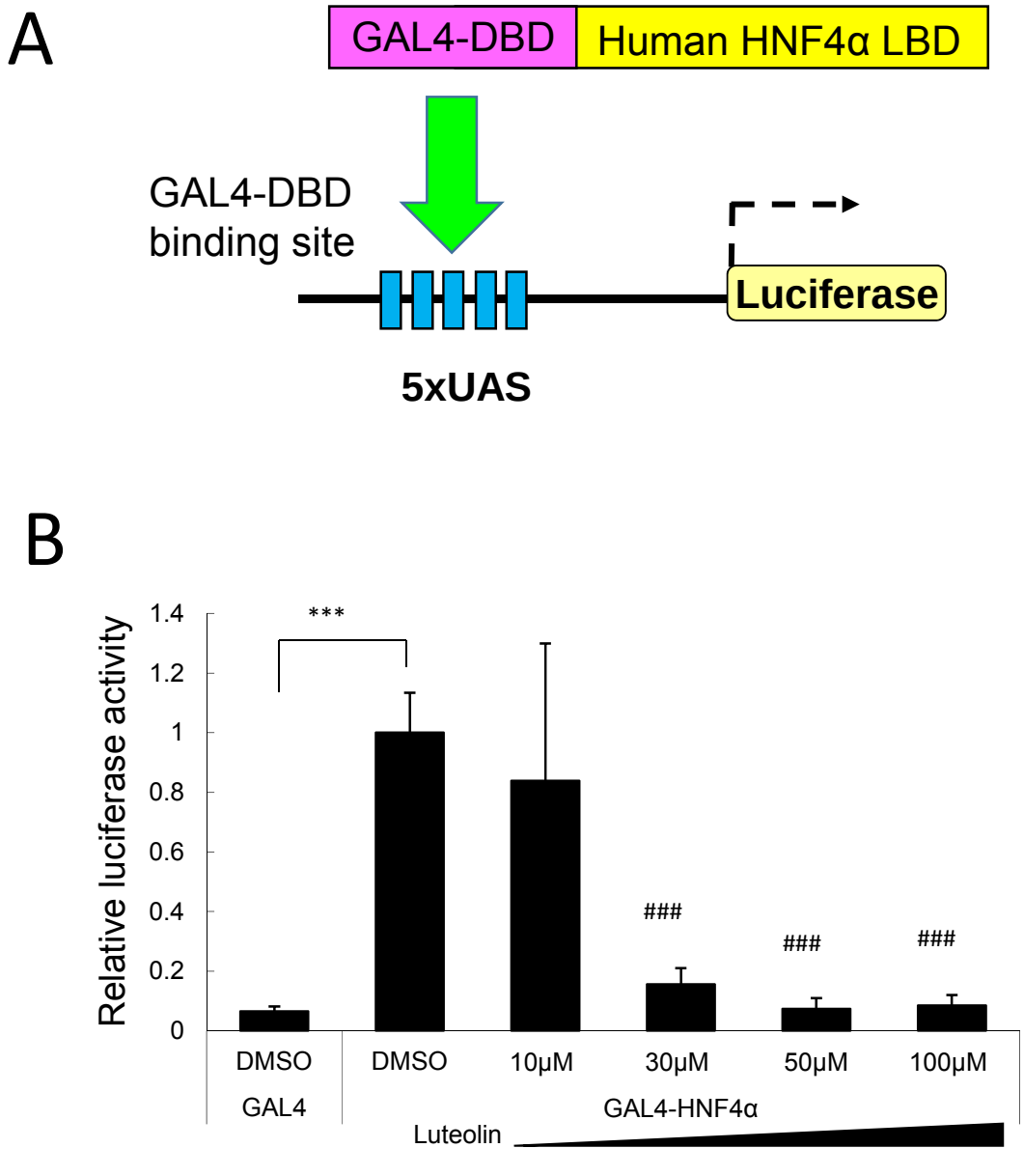


Figure 3-12 Luteolin has effect on the recruitment of co-factor(s)

A. The schematic diagram of GAL4-DBD-HNF4α-LBD system. B. HepG2 cells were transfected with the report plasmid 5xUAS, expression plasmids for β-galactosidase and GAL4/GAL4-DBD-HNF4α-LBD. After transfection, cells were treated with 30μM Luteolin or Daidzein for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities of GAL4-HNF4α with DMSO treatment are considered as 1. Results are means±S.D. (n =3). ***P <0.001 vs. in the absence of GAL4-HNF4α under DMSO treatment. $^{###}$P <0.001 vs. in the presence of GAL4-HNF4α under DMSO treatment.

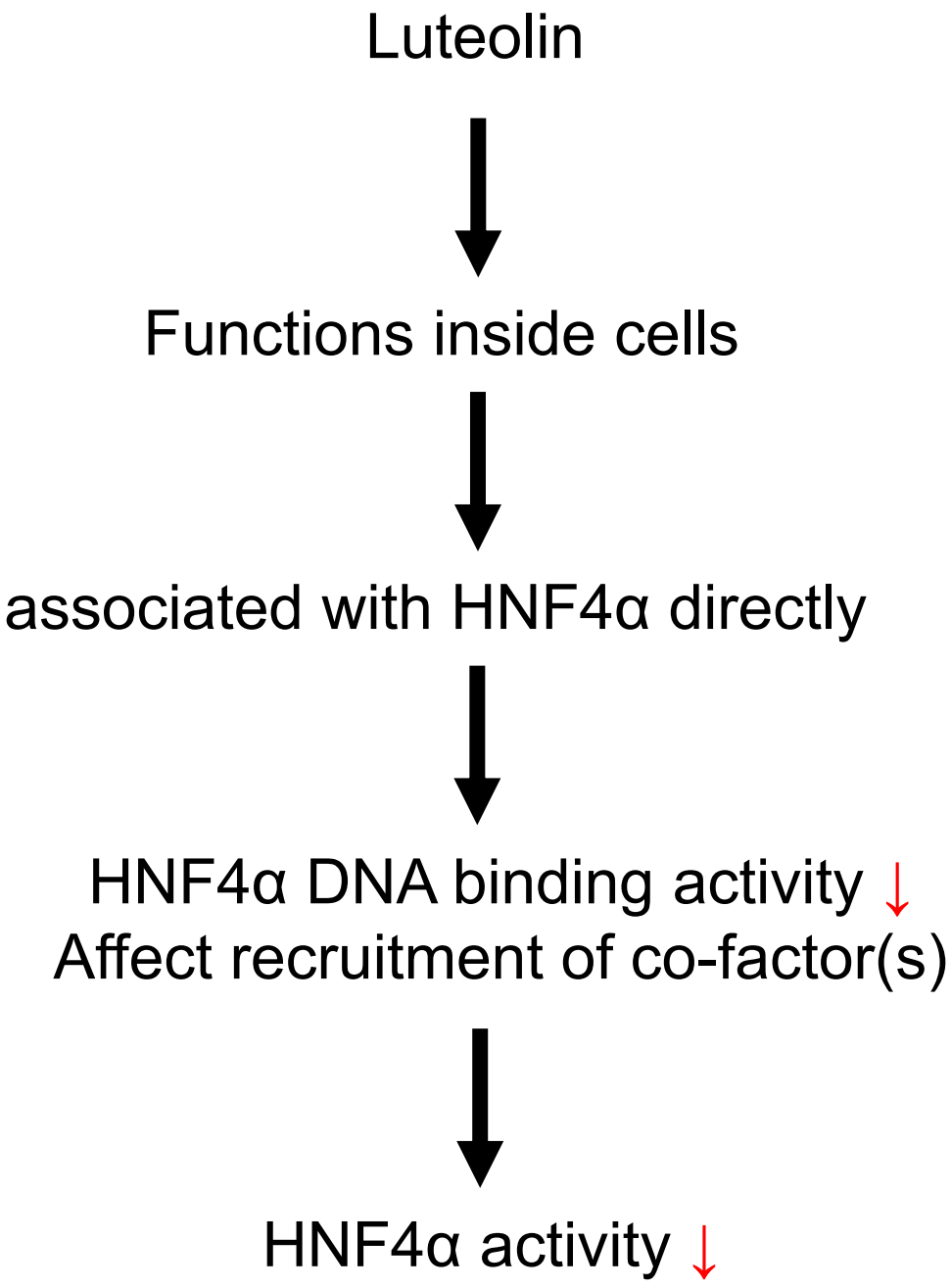


Figure 3-13 Summary of Chapter 3

3-4 Discussion

In this chapter, the mechanism of inhibition of HNF4α activity by Luteolin was studied. The results was summarized in Fig 3-13, and concluded as follows:

[I]. Luteolin functioned within cells and was capable of being associated with HNF4α directly

[II]. Luteolin decreased DNA binding activity of HNF4α and histon acetylation

[III]. Luteolin had effect on the recruitment of co-factor(s)

Regarding result [I], generally, flavonoid aglycones exhibit favorable membrane permeability, but the flavonoid glycosides was poor (121). Thus, Luteolin glucoside was used to investigate how Luteolin inhibited HNF4α activity. Luteolin glucoside showed no effect on MTP promoter activity, Apo B secretion, and HNF4α activity, these results suggested Luteolin may function within cells or through cell surface receptors, which can bind to Luteolin but not Luteolin glucoside.

Absorption spectrum analysis showed an absorption peak in about 400nm of HNF4α$_{+Lut}$, which is near to the absorption peak of Luteolin. In fact absorption peak of HNF4α$_{+Lut}$ was 402 nm, whereas Luteolin was 352nm (data not shown). In some researches, by adding progressively Luteolin to the human serum albumin solution, a significant broadening and strong bathochromic shift of Luteolin took place. It was caused by presence of a specific, non-covalent interactions between Luteolin and the amino acid residues of the binding site. Because of the absorption spectrum of Luteolin is strongly influenced by pH, resulting from the de-protonation of -OH groups inducing a strong bathochromic shift of the absorption band owing to extension of the conjugation (122). The same results was observed in this study.

Using BIACORE analysis of purified HNF4α, the interaction of HNF4α and Luteolin was detected, however, the response did not converge upon a unique value (Fig 3-8 B). It may result from the low solubility of Luteolin in flowing buffer. Although the interaction was confirmed, Luteolin binding site on HNF4α and binding specificity remain further investigation using other methods, such as crystallization analysis.

Many metabolic diseases have relation to the transcriptional level by a family of ligand-activated nuclear receptors. HNF4α target genes are involved in lipid transport, fatty acid oxidation, bile acid synthesis and transport, lipoprotein metabolism, steroid metabolism, and glucose metabolism (40). It is suggested through the regulation of HNF4α activity may affect glucose and lipid metabolism. However, the existence of a ligand for HNF4 has not been clearly defined and is somewhat controversial. Flavonoids are as regulators of many receptors, such as ERα/β (agonists: Daidzein, Genistein, Naringenin), FXR (agonists: EGCG), LXRα (agonists: Hesperetin), PPARα (agonists: Daidzein, Naringenin, Nobiletin, Quercetin, Tangeretin), PPARγ (agonists: Daidzein, Quercetin, Naringenin etc.), but there is not any research on flavonoid as ligands of HNF4α (81). Until recently, it is reported BI6015 (Millipore Bioscience), β, β-tetramethyl-hexadecanedioic acid (Medica 16) and β, β-tetramethyl-octadecanedioic acid act as an HNF4α antagonist (123). In this study, it is clarified Luteolin inhibited HNF4α activity and is capable of being associated with HNF4α directly. It is suggested Luteolin maybe a potential flavonoid as an antagonist of HNF4α.

Concerning result [II] and [III], GAL4 system and ChIP assay revealed Luteolin had an effect on HNF4α co-factor(s) recruitment and decreased DNA binding activity of HNF4α and histon acetylation. The trans-activating activity of HNF4α is stimulated by co-activators and inhibited by co-repressor. The HNF4α co-activators include PGC-1α, p160 family co-activators, SRC-1, SRC-2 and SRC-3, and p300/CBP (cAMP response element binding protein) family co-activators (53-55). Among the co-activators, CBP and SRC-1 possesses intrinsic histone acetyltransferase activity (54,124). Small heterodimer partner (SHP) interacts with HNF4α and inhibits HNF4α target gene transcription (56,125). SHP is known to inhibit HNF4α by three distinct mechanisms: interaction with HNF4α and to directly inhibit HNF4α activity, interaction with HNF4α to prevent its binding to DNA, and interaction with HNF4α to block its recruitment of PGC-1α (56). Therefore, decreased DNA binding activity of HNF4α may relate to hindering co-activator and inducing co-repressor. The effect of Luteolin on the interaction of HNF4α and its co-factor(s)

remains further studied.

Besides co-factor(s) regulation, DNA-binding activity is modulated posttranslationally by phosphorylation. Thirteen potential serine/threonine phosphorylation sites exist in HNF4α. HNF4α is phosphorylated by kinases including p38 kinase, ERK1/2, PKA, AKT, PKC and AMPK, and the phosphorylated forms have lower DNA binding, dimerization or transactivation activities (40). There is a possibility that Luteolin may suppress HNF4α activity by phosphorylation. Because Luteolin bound to HNF4α, it may alter the structure or charge density near the bind site, and result in directly affecting the phosphorylation of HNF4α. On the other hand, Luteolin may alter phosphorylation via kinase pathway.

Luteolin is reported to attenuate PKC in many studies. which indicated Luteolin inhibitory effect on HNF4α may be not through phosphorylated by PKC. Luteolin shows different effect on others kinase. Luteolin treatment increased AKT/PKB phosphorylation in diabetic rat kidneys compared with that in the untreated diabetic rat kidneys(126), but Luteolin reduced LPS-induced AKT phosphorylation in mice lung (127). Pretreatment of RAW 264.7 with Luteolin inhibited the LPS-induced ERK1/2 and p38 (128), whereas Luteolin activates ERK and p38 in human breast cancer cell lines (129). Hence, Luteolin inhibitory effect on HNF4α whether through AKT, p38 or ERK pathway needs further investigation.

However, Luteolin activates AMPK in HepG2 and 3T3-L1 cells (114); (130);(131), and activation of AMPK in hepatocytes greatly diminished HNF4α protein levels and the target gene expression, including Apo B and HNF1α(132). There is a possibility that Luteolin may inhibit HNF4α activity through activation of AMPK.

The post-translational modifications of HNF4α also includes methylation (133), ubiquitilation and acetylation (134). It is required further study about Luteolin effect on these post-translational modifications.

Chapter 4

Luteolin improved glucose and lipid metabolism in high fat diet induced obese mice

4-1 Preface

From Chapter 2 and 3, it is clear Luteolin inhibited Apo B secretion and MTP promoter activity resulting from reduced HNF4α activity, which is related to altering co-factor(s) recruitment and declining DNA binding activity of HNF4α.

Luteolin was identified as a component that inhibited MTP transcription by screening assay *in vitro*. In this chapter, I evaluate whether Luteolin have the same effect on MTP or HNF4α activity *in vivo*, meanwhile determine the influence on glucose, lipid metabolism.

4-2 material and methods

Animals and diets

Five-week-old male C57BL/6J mice were purchased from CLEA Japan. The mice were housed 3-4 per cage and fed ad libitum a high-fat diet (HFD) (60% kcal fat, D12492M). After HFD feeding for 11 weeks, the mice were divided to 3 groups by body weight and housed one cage for each. Mice were fed ad libitum for the next 8 weeks (n = 8/group) a HFD diet; a HFD diet+0.6% wt/wt Luteolin; or a HFD diet+1.5% wt/wt Luteolin. During the next 8 weeks, food intake and body weight were measured every other day. Mice were fasted for 4 h before sacrificed. Mice had free access to tap water during the experiments. Before the experiments all mice were kept on 1-week acclimatization period when mice were fed with HFD diet. All experiments were handled in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of Tokyo Animal Care Committee.

Blood glucose Assay

At 5 weeks of Luteolin diet, blood glucose levels were measured after a 4h period of fasting (10:00 a.m. to 13:00 p.m.). Blood glucose concentration was determined using 1 drop of blood from the tail using Ascensia BREEZE2 blood glucose meter (Bayer).

Abdominal fat composition and liver fat content analysis

At 6 weeks of Luteolin diet, abdominal fat and liver fat were analyzed by computed tomography (CT) scan. Mice were anesthetized by inhalation of Isoflurane and then scanned using a LaTheta (LCT-100) experimental animal CT system (Aloka, Tokyo, Japan). Abdominal scans refer to the area between the proximal end of the first vertebra and the distal end of the tibia. For abdominal fat composition analysis, CT scanning was performed at 2-mm intervals from the diaphragm to the bottom of the abdominal cavity. Visceral fat, subcutaneous fat, and muscle were distinguished and evaluated quantitatively using LaTheta software. Liver scan refer to the areas that include liver and spleen (between the cranial part of the diaphragm and the lumbar vertebra L3) and adipose tissue (narrow area in the region of the lumbosacral joint) were scanned. For liver fat content analysis,

contiguous 0.1-mm slice images between L3 and 12 were used for quantitative assessment using LaTheta software. Liver fat content was evaluated quantitatively.

Oral glucose tolerance test (OGTT)

At 7 weeks of Luteolin diet, mice were fasted for 16h and then given an oral gavage of D-(+)-glucose at 2 g per kg body weight (about 100μL glucose solution per mouse). Blood glucose was measured in tail vein blood using an Ascensia Breeze 2 meter just prior to gavage (time zero) and at 15, 30, 60, 90,120,150 minutes after glucose administration.

Serum and tissues Collection.

After a 4 hour fast, Mice were anesthetized by inhalation of ethyl ether. Blood was collected from inferior vena cava. Serum was obtained after allowing the collected blood to sit for 4h and centrifuged at 3000 x g for 15 minutes at 4°C, saved in tubes and immediately stored frozen at -80C until assay.

Livers were removed, flushed clean with 1 x PBS, weighted and snap-frozen in liquid nitrogen. The small intestine was divided into duodenum jejunum, ileum, flushed clean with 0.09% NaCl, and snap-frozen in liquid nitrogen. All tissues were stored at -80°C until time of processing.

Serum Insulin Assay

Serum Insulin was analyzed using insulin ELISA kit according to the manufacturer's recommended procedure. Antibody coated 96-well was washed with wash solution for four times. After the liquid remaining in the well was removed, 100μL of Pipette biotin-conjugated anti-insulin antibody was added, stirred well by micro-plate shaker, then 10μL of serum sample and standard were added, mixed, incubated for 2 hours at RT. After the reaction, the reaction solution was discarded and the plate was washed with wash solution for four times. Then 100μL of HRP-conjugated streptavidin was added, stirred, incubated for 30 min at RT. The plate was washed again, then 100μL of color reagent was added, mixed well. After incubation for another 30 min at RT, 100μL of reaction stop solution was added, measured with spectrophotometer at wavelength of 450 nm (reference: 655nm). Serum insulin content was calculated from the results of samples absorbance

according to the standard curve.

Serum ApoB Assay

Serum ApoB was analyzed by western blotting. Mix an equal volume of serum of mice, which were fed with the same diet. 10μL of serum mixture was dilute with 190μL of RIPA Buffer and heated denatured with 6x Laemmli Sample buffer for 5 min at 95°C.

Serum Triglyceride (TG) Assay

Serum TG levels were analyzed with a GPO・DAOS method according to the manufacturer's recommended procedure. 2 μL of serum sample, standard and blank were transferred to a 96 well plate mixed with 300 μL of Color Reagent, incubated at 37°C for 5min. Level of serum TG was calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 595nm.

Buffer	
Good's (PIPES) buffer, pH6.5	50mmol/L
Chromogen Substrate (when reconstituted)	
Lipoprotein lipase	99units/mL
Adenosine 5'-Triphosphate Disodium Salt Trihydrate (ATP)	1.7mmol/L
Glycerolkinase	30units/mL
Glycerol-3-phosphate Oxidase (GPO)	1.0unit/mL
Peroxidase (POD)	5.5units/mL
N-Ethyl-N (-2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS)	0.54mmol/L
4-Aminoantipyrine	0.11mmol/L
Ascorbate Oxidase	4.5units/mL
[before reconstitute] Contains Sodium Hydoroxide	8.01％.
Standard Solution	
Glycerol	31.2mg/dL
(Corresponding to 300mg/dL Triolein)	
Color Reagent	

Prepare Color Reagent by dissolving 52mg of Chromogen substrate to 15mL of Buffer. After reconstitution, the solution should be stored at 2-10°C.

Serum Total-Cholesterol (T-Chol) Assay

Serum T-Chol levels were analyzed with a Cholesterol Oxidase·DAOS method according to the manufacturer's recommended procedure. 2 μL of serum sample, standard and blank were transferred to a 96 well plate mixed with 300 μL of Chromogen Reagent, incubated at 37°C for 5min. Level of serum T-Chol was calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 595nm.

Buffer

MES Buffer	50mmol/L

Chromogen (After reconstitution)

Cholesterol esterase	1.6units/mL
Cholesterol oxidase	0.31units/mL
Peroxidase (HRP)	5.2units/mL
DAOS	0.95mmol/L
4-Aminoantipyrin	0.19mmol/L
Ascorbate oxidase	4.4units/mL

Standard solution

Cholesterol	200mg/dL

Chromogen Reagent

Chromogen reagent is prepared by dissolving 35mg of Chromogen with 30mL of Buffer.

Serum High-Cholesterol (H-Chol) Assay

Serum H-Chol levels were analyzed with a phosphotungstate-magnesium salt precipitation method according to the manufacturer's recommended procedure. 20 μL of serum sample was mixed with 20 μL of Precipitating Reagent, incubated at RT for 10min and centrifuged at 3000 rpm for 15 minutes at RT. 5 μL of supernant, standard and blank were transferred to a 96 well plate mixed with 300 μL of Colour reagent, incubated at 37°C for 5min. Level

of serum H-Chol was calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 595nm.

Precipitating Reagent

Phosphotungstic acid

Magnesium chloride

Color Reagent

Cholesterol esterase (from microbial)

Cholesterol oxidase (from microbial)

Peroxidase (from horseradish)

DAOS

4 - aminoantipyrine

Ascorbic acid oxidase (from pumpkin)

Buffer

Good's (MES) buffer pH6.1

Standard solution

Cholesterol 50.0mg/dL

(Corresponding to HDL-cholesterol 100mg/dL)

Color reagent solution

Dissolve 18mg of Color Reagent to 15mL of Buffer. After reconstitution, the solution should be stored at 2-10°C.

Measurement of lipid profiles.

Plasma lipoproteins were analyzed using a high-performance liquid chromatography system at Skylight Biotech (LipoSEARCH, Akita, Japan).

Serum non-esterified fatty acids (NEFA) Assay

Serum NEFA levels were analyzed with a ACS・ACOD method according to the manufacturer's recommended procedure. 15 μL of serum sample, standard and blank were added into 1.5ml eppendorf tubes mixed with 300 μL of Color reagent solution A,

incubated at 37°C for 10min. Then 600 μL of Color reagent solution B was added, mixed and incubated at 37°C for the next 10min. After incubation 300 μL of the mixture was transferred to a 96 well plate. Level of serum NEFA was calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 540nm.

Solvent A

Phosphate Buffer, pH 7.0	50 mmol/L

Color Reagent A

ACS (Acyl-CoA-Synthetase)	0.3 kU/L
AOD (Ascorbate oxidase)	3.0 kU/L
CoA (Coenzyme A)	0.6 g/L
ATP (Adenosine triphosphate)	5.0 mmol/L
4-Aminophenazone	1.5 mmol/L

Solvent B

MEHA (3-Methyl-N-Ethyl-N-(ß-hydroxyethyl) aniline)	1.2 mmol/L

Color reagent B

ACOD (Acyl-CoA-Oxidase)	6.6 kU/L
POD (Peroxidase)	7.5 kU/L

NEFA C Standard

Oleic acid	1mEq/L

Color reagent solution A

Dissolve contents of a bottle Color reagent A with 10 mL Solvent A and mix well. After reconstitution, the solution should be stored at 2-10°C.

Color reagent solution B

Dissolve contents of a bottle Color reagent B with 20 mL Solvent B and mix well. After reconstitution, the solution should be stored at 2-10°C.

Serum Total Bile Acod (T-BA) Assay

Serum T-BA levels were analyzed with an enzyme-colorimetric method according to the

manufacturer's recommended procedure. 20 μL of serum sample, standard and blank were transferred to a 96 well plate mixed with 50 μL of Enzyme solution or Blind enzyme solution, incubated at 37°C for 10min. Then 50 μL of Reaction stop solution was added to the 96 well plate, mixed and measured with spectrophotometer at wavelength of 570nm. Level of serum T-BA was calculated from the absorbance of $E-E_{BI}$ (E: absorbance of samples reacted with enzyme solution; E_{BI}: absorbance of samples reacted with blind enzyme solution).

Buffer

Phosphate Buffer, pH 7.3

Enzymes

3-α-hydroxysteroid dehydrogenase (3-α-HSD) (from microorganism)

β-nicotinamide adenine dinucleotide, oxidized form (β-NAD +) (from yeast)

Diaphorase (from microbial)

Nitro blue tetrazolium (NO_2-TB)

Blind enzyme

Diaphorase (from microbial)

β-nicotinamide adenine dinucleotide, oxidized form (β-NAD +) (from yeast)

Nitro blue tetrazolium (NO_2-TB)

Reaction stop solution

Hydrochloric acid

Standard solution

Glycocholate 50μmol / L

Enzyme solution

Dissolve contents of a bottle enzyme with 5 mL Buffer and mix well. After reconstitution, the solution should be stored at 2-10°C.

Blind enzyme solution

Dissolve contents of a bottle blind enzyme with 5 mL Buffer and mix well. After

reconstitution, the solution should be stored at 2-10°C.

Serum GOT GPT Assay

Serum GOT/GPT levels were analyzed with a POP·TOOS method according to the manufacturer's recommended procedure. 2 μL of serum sample, standard and blank were transferred to a 96 well plate mixed with 100 μL of GOT/GPT substrate chromogenic solution, which was already warmed at 37°C for 5min, incubated at 37°C for 20min. Then 200 μL of Reaction stop solution was added to the 96 well plate, mixed. Level of serum GOT/GPT levels were calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 555nm.

GOT enzyme agent

Pyruvate oxidase (POP) (from microorganism)

Oxaloacetate decarboxylase (OAC) (from microorganism)

4 - aminoantipyrine

Ascorbic acid oxidase (from pumpkin)

Catalase (from bovine liver)

GOT substrate buffer

Good's buffer pH7.0

L-aspartic acid

GPT enzyme agent

Pyruvate oxidase (POP) (from microorganism)

4 - aminoantipyrine

Ascorbic acid oxidase (from pumpkin)

Catalase (from bovine liver)

GPT substrate buffer

Good's buffer pH7.0

L-alanine

Color agent

Peroxidase (from horseradish)

Color agent dissolving solution

Good (HEPES) buffer pH7.0

α-ketoglutarate

N-Ethyl-N-(2 - hydroxy-3 - sulfopropyl) m-toluidine sodium (TOOS)

Reaction stop solution

Citric acid

Surfactant

GOT standard solution

Pyruvic acid containing potassium Corresponding to GOT100Karmen unit

GPT standard solution

Pyruvic acid containing potassium Corresponding to GPT 100Karmen unit

GOT substrate enzyme solution

Dissolve 60mg GOT enzyme agent to 10mL of GOT substrate buffer. After reconstitution, the solution should be stored at 2-10°C.

GPT substrate enzyme solution

Dissolve 60mg GPT enzyme agent to 10mL of GPT substrate buffer. After reconstitution, the solution should be stored at 2-10°C.

Chromogenic reagent

Dissolve 60mg Color agent to 10mL of Color agent dissolving solution. After reconstitution, the solution should be stored at 2-10°C.

GOT substrate chromogenic solution

Mix equal amounts of Chromogenic reagent and GOT substrate enzyme solution. After reconstitution, the solution should be stored at 2-10°C.

GPT substrate chromogenic solution

Mix equal volume of Chromogenic reagent and GPT substrate enzyme solution. After

reconstitution, the solution was stored at 2-10°C.

Hepatic lipid Assay

Hepatic lipid extraction

About 150mg piece of frozen liver tissue was immersed in 4mL chloroform:methanol (2:1, v/v) homogenized for 20s. Tissue homogenates were vortexed and stored at RT 30min. 1mL of 50mM NaCl was added to all samples. All samples were vortexed for 10s and centrifuged at 1500 g at 4°C for 30min. The upper aqueous phase was removed and discarded. The lower lipid-containing phase of each sample was mixed with 1mL of 0.36 mol/L CaCl2: methanol (1:1, v/v), vortexed and centrifuged at 1500 g at 4°C for 10min. The upper aqueous phase was removed. The lower lipid-containing phase was washed once more with 1mL of 0.36 mol/L CaCl2: methanol (1: 1), and lipid extracts were transferred to 5mL mess-flask and chloroform was added to the mark to prepare a sample.

Triglyceride and cholesterol assays

10μL of 50% Triton X-100 was added to 200μL of lipids extraction with 40μL of DDW, 40μL of standard in glass tube, vortexed, air dried in a fume hood overnight. 500 μL of Color Reagent for triglyceride /cholesterol was added, vorexed, incubated at 37°C for 5min, centrifuged at 13000rpm at RT for 3min. Supernatant was transfer to 96 well plate, the level of TG/Chol was calculated from the results of samples absorbance measured with spectrophotometer at wavelength of 595nm. Final the TG/Chol content was normalized to liver weight.

RT-PCR

Tissue RNA isolation

Total RNA was isolated from tissue using ISOGEN. A piece of frozen liver/ half of duodenum/ jejunum/ileum was immersed in 1mL (liver)/2mL (intestine) ISOGEN, homogenized for 20s and centrifuged at 15000 rpm for 5 min at 4°C. 1mL of supernatant ISOGEN solution was transfer into a new tube added with 0.2mL (liver)/0.3mL (intestine) chloroform, vortexed, stored at room temperature for 3 min. After storage, the tubes were

centrifuged at 12000 rpm for 15 min at 4°C. 0.3mL (liver)/0.4mL (intestine) of the uppermost aqueous layers containing the extracted RNA were transferred into new tubes, added with 0.24mL (liver)/0.32mL (intestine) of isopropanol, and stored at room temperature for 10 min to precipitate RNA. The precipitated RNA was collected by centrifugation at 12000 rpm for 20 min at 4°C. All aqueous phase was discarded and the precipitated RNA was washed with 1 ml 70% ethanol twice. The tubes were again centrifuged at 12000 rpm for 10 min at 4°C. Alcohol was removed completely and the pellet was dried and eluted with 250μL DEPC (Diethyl pyrocarbonate) water stored at –80°C until use.

DNAse treatment, Reverse transcription (RT) and Quantitative real-time PCR (qRT-PCR) described in Chapter 2 were used in this experiment.

Taqman Gene Expression Assays

mHNF4α	Mm00433964_m1
mMTP	Mm00435015_m1
mApo B	Mm01545150_m1
mPEPCK	Mm00440636_m1
mG6Pase	Mm00839363_m1
mCOUP-TFII	Mm00772789_m1
mACC	Mm01304280_m1
mFAS	Mm00662319_m1
mCPT1α	Mm00550438_m1
mHMGCS	Mm00524111_m1
mHMGCR	Mm01282509_g1
mLDLR	Mm00440169_m1
mSPEBP-2	Mm01306283_m1

Primer (5'----3')

m36B4	F CTGATCATCCAGCAGGTGTT
	R CCAGGAAGGCCTTGACCTTT

mHNF1α	F GACCTGACCGAGTTGCCTAATG
	R CCATCGTCATCCGTGTCATC
mFXR	F CCAACCTGGGTTTCTACCC
	R CACACAGCTCATCCCCTTT
mGLUT2	F GTCCAGAAAGCCCCAGATACC
	R GTGACATCCTCAGTTCCTCTTAG
mSGLT1	F TCTGTAGTGGCAAGGGGAAG
	R ACAGGGCTTCTGTGTCTTGG
mSREBP1c	F CACGGAGCCATGGATTG
	R CCGGGAAGTCACTGTCTTG
mPPARα	F CTCGCGTGTGATAAAGC
	R CGATGCTGTCCTCCTTG
mPGC1α	F TTCTGGGTGGATTGAAGTGGTG
	R TGTCAGTGCATCAAATGAGGGC
mSR-B1	F TCCCCATGAACTGTTCTGTGAA
	R TGCCCGATGCCCTTGA
mABCA1	F TCCTCATCCTCGTCATTCAAA
	R GGACTTGGTAGGACGGAACCT
mABCG5	F TGTCCTACAGCGTCAGCAACC
	R GGCCACTCTCGATGTACAAGG
mABCG8	F AGAGTTGCATCCCCCTAGCC
	R TCCTTGACACAGGCATGAAGC
mCYP7a1	F AGCAACTAAACAACCTGCCAGTACTA
	R GTCCGGATATTCAAGGATGCA
mNTCP	F CACCATGGAGTTCAGCAAGA
	R AGCACTGAGGGGCATGATAC
mOATP1	F GTCTTACGAGTGTGCTCCAGAT
	R GGAATACTGCCTCTGAAGTGGATT

mBSEP	F	GCTCGGGCCATTGTACGAGAT
	R	CACCACTCCTTGTGACATGACG

4-3 Results

4-3-1 Luteolin does not affect food intake but decreases body weight in high fat diet induced obese mice

The metabolic effects of Luteolin were initially evaluated in high fat diet induced obese mice. five-week-old C57BL/6 mice were fed with high fat diet (HFD) for 11 weeks to gain weight. Then the mice were separated into 3 groups (n=8) based on their body weights and blood glucose levels, and were fed with their respective diet: HFD diet, HFD+0.6% Luteolin diet, and HFD+1.5% Luteolin diet (Fig 4-1 A). Luteolin dose-dependently decreased body weight, 1.5% Luteolin diet markedly reduced body weight compared to HFD diet (Fig 4-1 B). The body weight decrease at 35, 42, and 49days was due to the performance of blood glucose, CT scan, oral glucose tolerance test (OGTT). Among groups, there were no significant differences in food intake (Table 4-1).

Fat accumulation were investigated by CT scan. 1.5% Luteolin diet significantly reduced abdominal fat, fat content, visceral fat, and subcutaneous fat accumulation induced by HFD feeding. 0.6% Luteolin only suppressed visceral fat accumulation, but did not affect others (Fig 4-2 B).

4-3-2 Luteolin decreases some of HNF4α target genes *in vivo*

In this research it has been proved that Luteolin decreases HNF4α and its activity *in vitro*. In this section, the effect of Luteolin on HNF4α activity *in vivo* was examined by investigating the target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase).

In liver, 1.5% Luteolin declined the mRNA levels of MTP ($P<0.05$), G6Pase ($P=0.056$), elevated PEPCK and had no change in other target genes (HNF1α, Apo B), HNF4α itself, and regulatory factors (COUP-TF II and FXR) (Fig 4-3).

In duodenum, 1.5% Luteolin decreased the mRNA levels of HNF1α and COUP-TFII, whereas had no effect on other genes related to HNF4α (Fig 4-4). In jejunum, Luteolin did not show any inhibitory activity, in contrast, increased the expression of MTP, HNF1α and

COUP-TFII (Fig 4-5). In ileum, 1.5% Luteolin decreased the mRNA levels of Apo B and G6Pase, whereas had no effect on other genes (Fig 4-6).

Luteolin, identified from the screening system, also suppress MTP expression *in vivo*.

4-3-3 Luteolin attenuates serum triglyceride, cholesterol, and Apo B

Luteolin at 1.5% decreased serum total-TG, LDL-TG, and HDL-TG by 67%, 48%, 45%, respectively, while CM-TG and VLDL-TG only showed decreasing trend. 1.5% Luteolin diet decreased serum total-Chol, VLDL-Chol, LDL-Chol, and HDL-Chol by 83%, 64%, 68%, and 90%, respectively compared with HFD group, while CM-Chol was almost unaffected. Serum Apo B 48 and Apo B 100 levels were lowered by 1.5%Luteolin administration (Fig 4-7 A). 0.6% Luteolin diet showed decreasing trend of triglyceride, while had no effect on cholesterol and Apo B (Table 4-1, Fig 4-7).

Serum levels of non-esterified fatty acid (NEFA) and total-bile acid (TBA) did not differ among the three groups. Compared with HFD group, serum levels of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) significantly decreased in 1.5% Luteolin diet while were not affected by 0.6% Luteolin (Table 4-1).

4-3-4 Luteolin prevents hepatic lipid accumulation and liver weight

Then liver lipids were examined. 1.5% Luteolin administration declined hepatic triglyceride, cholesterol, and lipid content by 45%, 84%, 76% respectively. Liver weight was also significantly reduced by 1.5% Luteolin. However, 0.6% Luteolin diet had no influence on hepatic lipid and weight (Table 4-1, Fig 4-2 A).

4-3-5 Luteolin decreases the mRNA levels of FAS and ACC does not affect the fatty acid oxidation factors

The obvious reduction in hepatic triglyceride and serum triglyceride (total and LDL) suggested that Luteolin may affect the fatty acid synthesis and oxidation. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are two sequential enzymes in the fatty acid biosynthetic pathway (135). And SREBP-1c activate the transcription of ACC and

FAS. In addition, PGC1α activation can initiate mitochondrial biogenesis. Enzymes involved in mitochondrial and peroxisomal fatty acid oxidation, such as CPT1α, can be upregulated by PPARα (136). It is shown 1.5% Luteolin significantly suppressed the mRNA levels of ACC and FAS in liver, whereas did not affect the oxidation related factors (PPARα, CPT1α, and PGC1α) (Fig 4-8). These results indicate the reduction of hepatic and serum triglyceride, may result from the declined ACC and FAS by Luteolin.

4-3-6 Luteolin suppresses the cholesterol in liver and serum

The obvious reduction in hepatic cholesterol and serum cholesterol (total, VLDL, and LDL) suggested that Luteolin may affect the cholesterol regulatory factors. SREBP-2 regulate the genes involved in cholesterol biosynthesis and metabolism, such as Hepatic 3-hydroxy-3-methyl-glutaryl-CoA synthase (HMGCS), Hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), low-density lipoprotein receptor (LDLR). HMGCS catalyzes the formation of the substrate HMG-CoA; HMGCR transforms HMG-CoA to mevalonic acid and is considered the rate-controlling enzyme in the pathway (137); LDLR mediates the clearance of cholesterol and cholesteryl ester containing LDL particles from the blood. The results indicated Luteolin had no effect on the mRNA levels of LDLR and SREBP2, whereas increased HMGCS and HMGCR in both 0.6% and 1.5% Luteolin diet (Fig 4-9). These results indicate Luteolin increase the factors related to cholesterol synthesis.

Subsequently, the factors related to cholesterol transport was studied, such as scavenger receptor class B member 1 (SR-B1), ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette sub-family G member 5 (ABCG5), and ATP-binding cassette sub-family G member 8 (ABCG8). ABCA1 and SR-B1 are critical mediators of reverse cholesterol transport; ABCG5/ABCG8, are necessary for the majority of sterols secreted into bile. The results indicated there was no change of the mRNA levels of ABCA1, SR-B1, ABCG5, and ABCG8 among the three groups (Fig 4-9). These results suggest Luteolin does not affect cholesterol transport factors.

Because Luteolin reduced the liver and serum cholesterol levels, but did not inhibit the

factors related to cholesterol synthesis and transport. Next, cholesterol 7alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in the bile acid biosynthetic pathway that converts cholesterol into bile acids in the liver, was investigated. The mRNA level of CYP7A1 was significantly elevated 3.6- fold by 1.5% Luteolin diet (Fig 4-9). These results suggest the reduction in hepatic and serum cholesterol by Luteolin may result from the increasing of CYP7A1 levels.

4-3-7 Luteolin improves glucose metabolism and insulin sensitivity

In this section, the effect of Luteolin on glucose metabolism was studied. Fasting serum glucose concentration was reduced by 1.5% Luteolin supplement (Table 4-1). For Oral Glucose Tolerance Test (OGTT), the blood samples were analyzed for glucose content at 0, 30, 60, 90,120 and 150 min, respectively after 16h fasting. The blood glucose levels of 1.5% Luteolin group at the time points of 30, 60, 90 min were significantly declined than those of the HFD group (Fig 4-10 A). In addition, a significant reduction of glucose AUC was also observed in 1.5% Luteolin diet (Fig 4-10 B). These results indicate Luteolin impair glucose tolerance and improve glucose metabolism. Moreover, serum insulin was elevated by the HFD and prevented by Luteolin in a dose-dependent manner. (Table 4-1). It is suggested Luteolin improve the insulin resistance induced by HFD.

It was noteworthy that 1.5% Luteolin suppressed the glucose level at 30min, the peak of glucose concentration in OGTT (Fig 4-10 A). It is suggest Luteolin may have an effect on glucose absorption. Hence, the energy-dependent sodium-hexose co-transporter sodium-glucose linked transporter 1 (SGLT1) and the facilitative hexose transporters Glucose transporter 2 (Glut2) was investigated in intestine (Fig 4-11 A). Luteolin did not affect the mRNA levels of SGLT1 and Glut2 in duodenum, jejunum, and ileum (Fig 4-11 B).

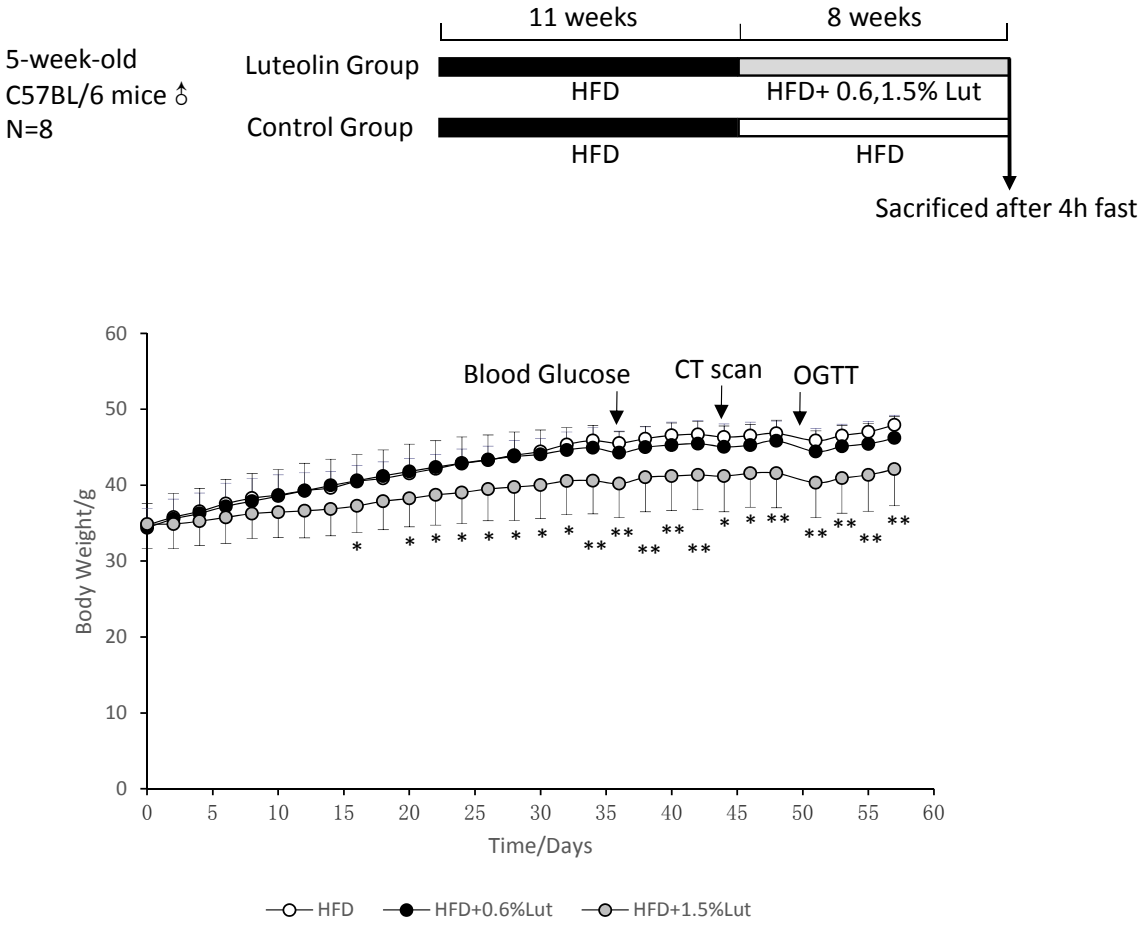


Figure 4-1 Luteolin decreases body weight in high fat diet induced obese mice

A. 5-week-old C57BL/6 mice were fed with high fat diet (HFD) for 11 weeks to gain weight. Then the mice were separated into 3 groups (n=8) based on their body weights and blood glucose levels, and were fed with their respective diet: HFD diet, HFD+0.6% Luteolin diet, and HFD+1.5% Luteolin diet. B. Body weight. Results are means±S.D. (n =8). *P <0.05, **P <0.01.

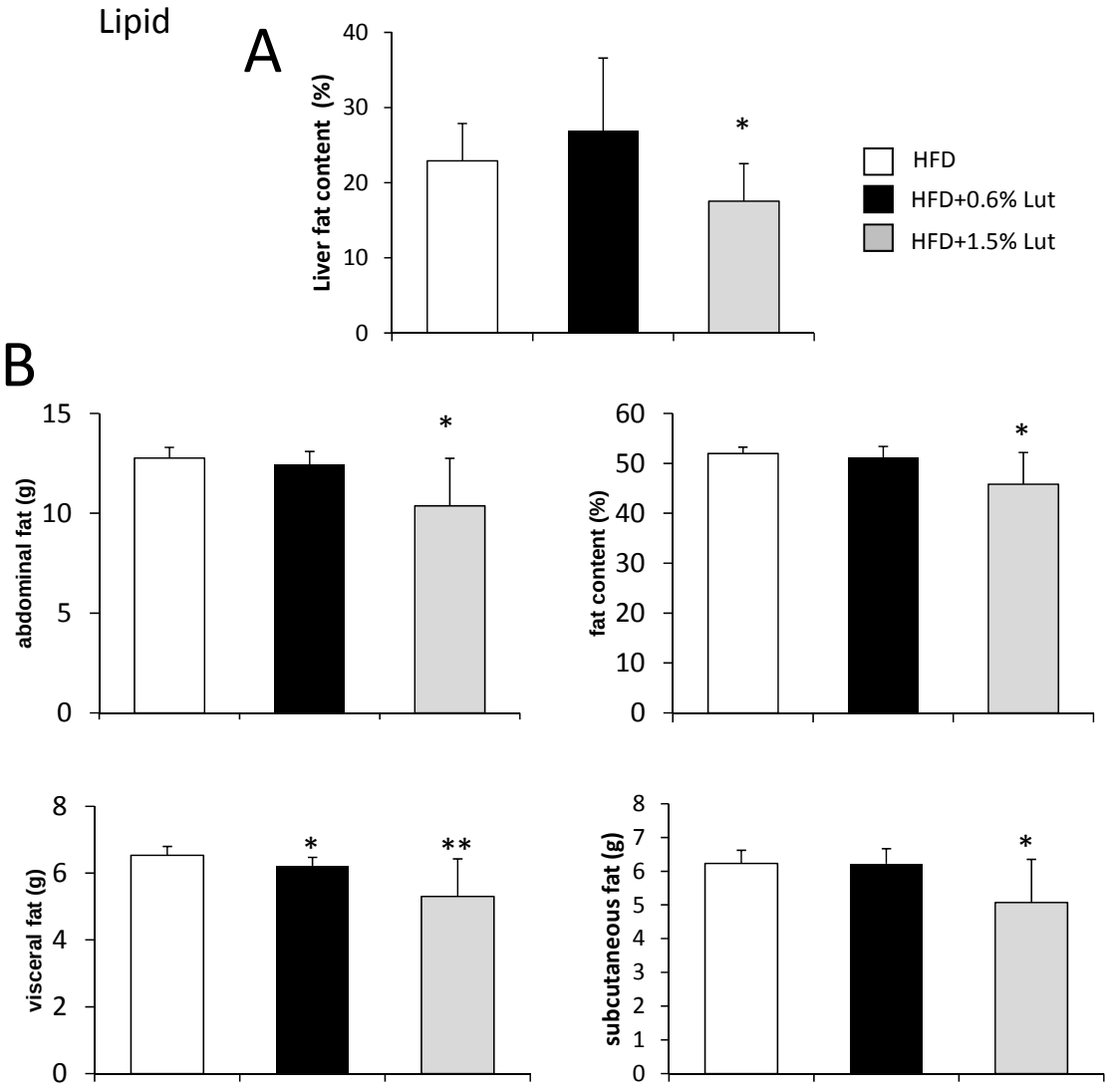


Figure 4-2 Luteolin decreases liver fat content and obesity induced by a high-fat diet

Abdominal, liver, visceral and subcutaneous fat were analyzed by computed tomography (CT) scan. Quantitative assessment using LaTheta software. A, liver fat percentage. B, Abdominal, visceral and subcutaneous fat. Results are means±S.D. (n =8). *P <0.05.

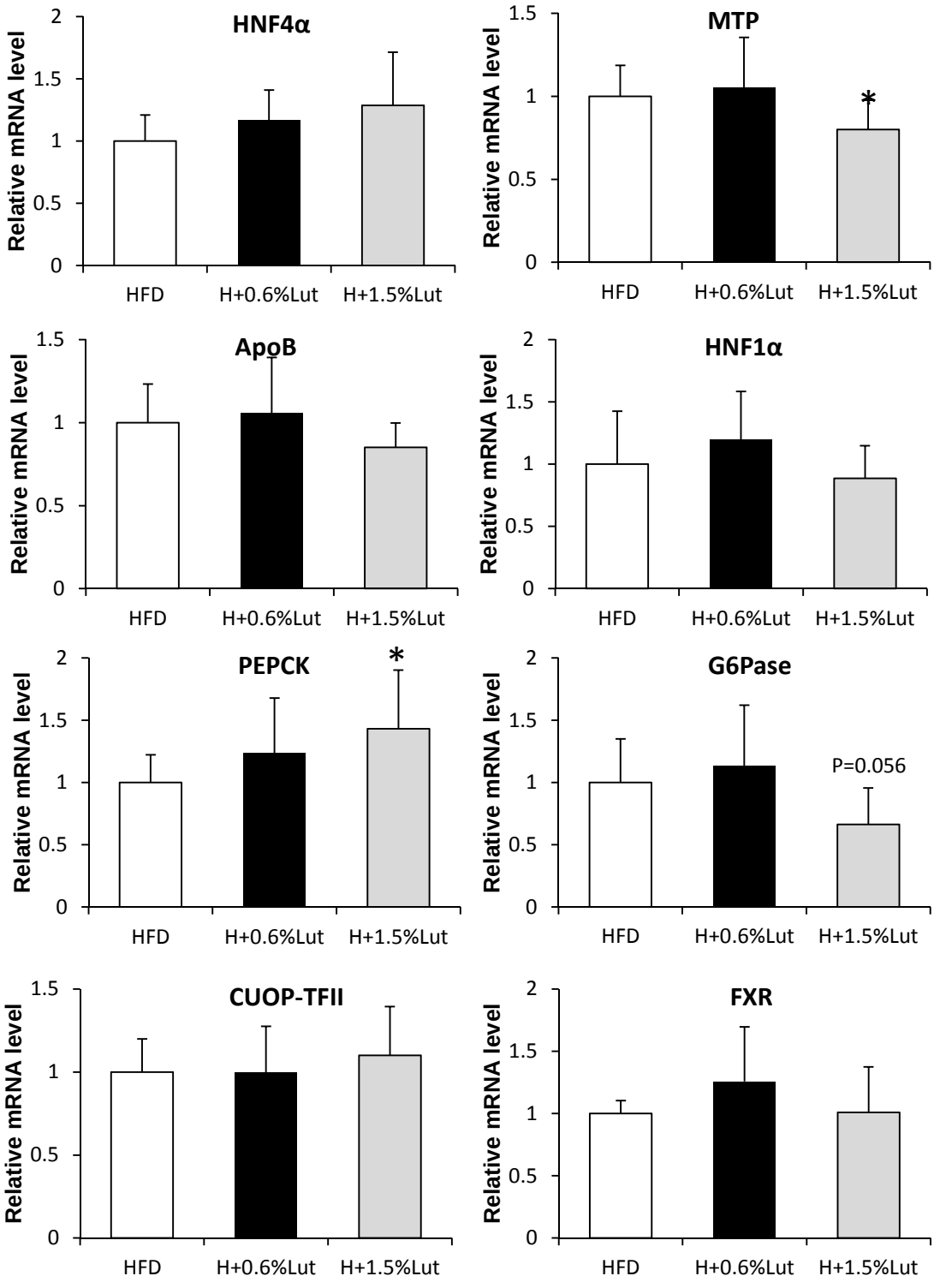


Figure 4-3 The effect of Luteolin diet on the HNF4α related factors in liver

Real-time PCR analysis of HNF4α target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase), HNF4α, and its self-regulation factors (COUP-TF II and FXR in liver. Results are means±S.D. (n =8). *P <0.05.

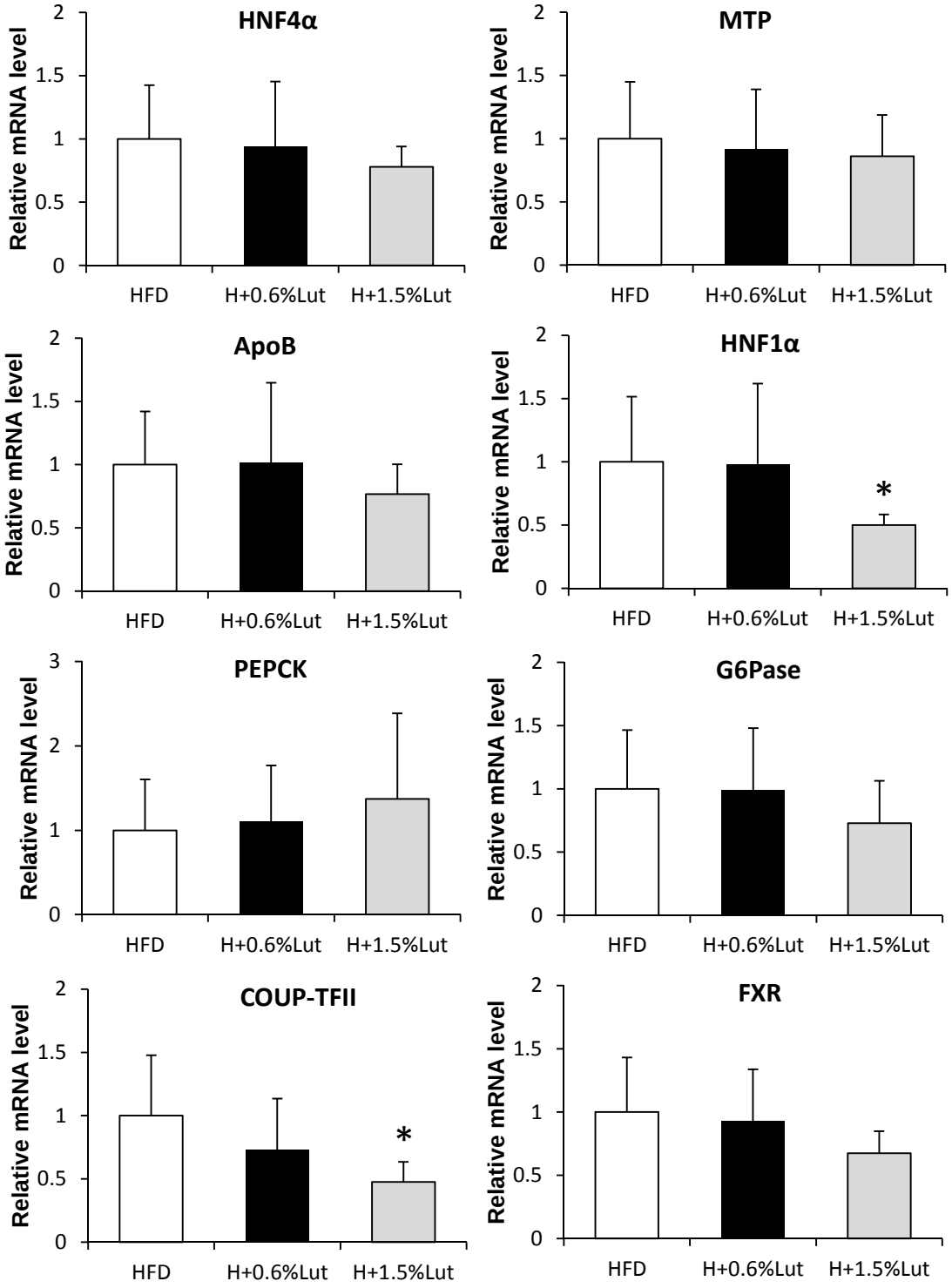


Figure 4-4 The effect of Luteolin diet on the HNF4α related factors in duodenum

Real-time PCR analysis of HNF4α target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase), HNF4α, and its self-regulation factors (COUP-TF II and FXR in duodenum. Results are means±S.D. (n =8). *P <0.05.

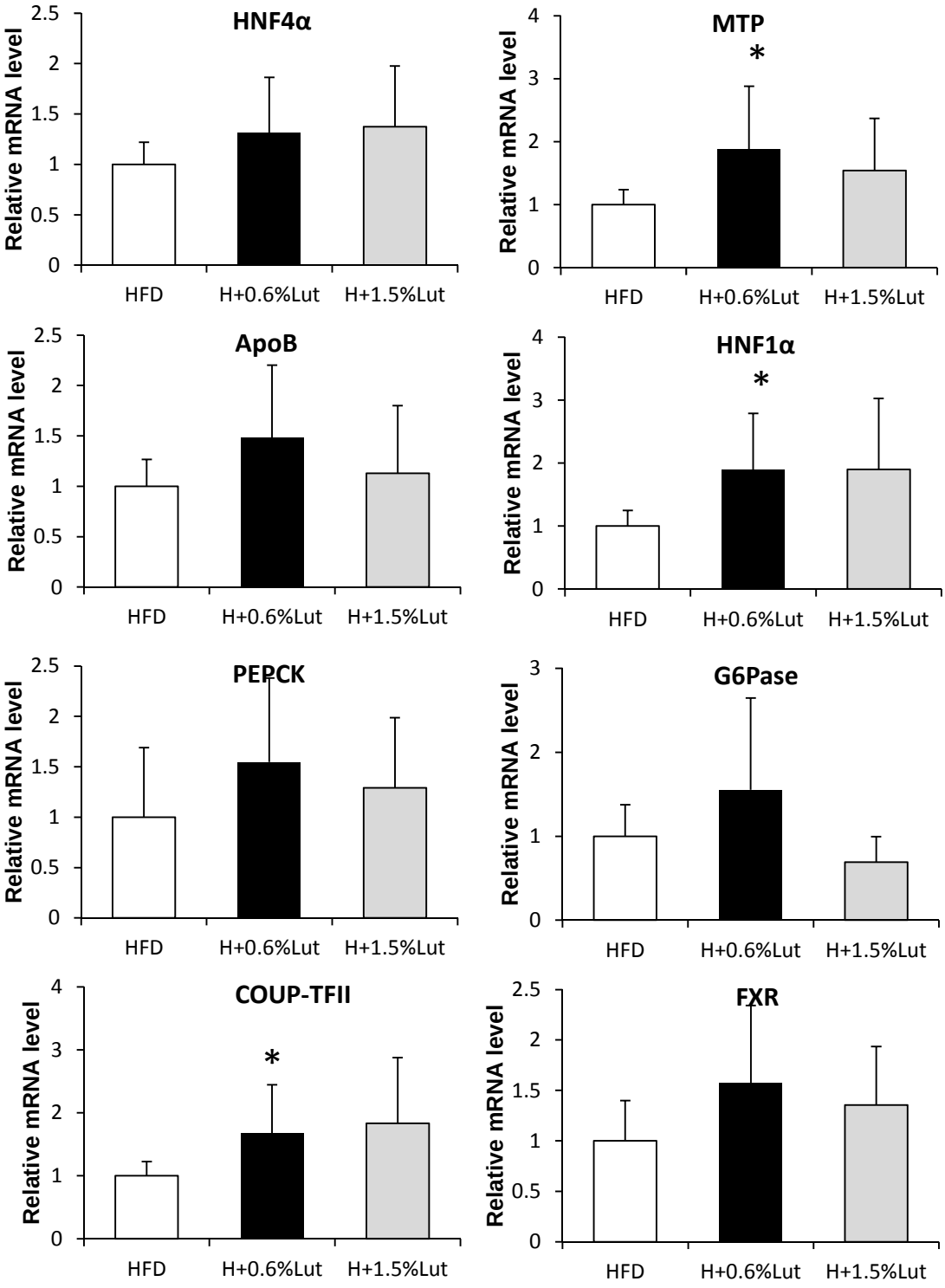


Figure 4-5 The effect of Luteolin diet on the HNF4α related factors in jejunum

Real-time PCR analysis of HNF4α target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase), HNF4α, and its self-regulation factors (COUP-TF II and FXR in jejunum. Results are means±S.D. (n =8). *P <0.05.

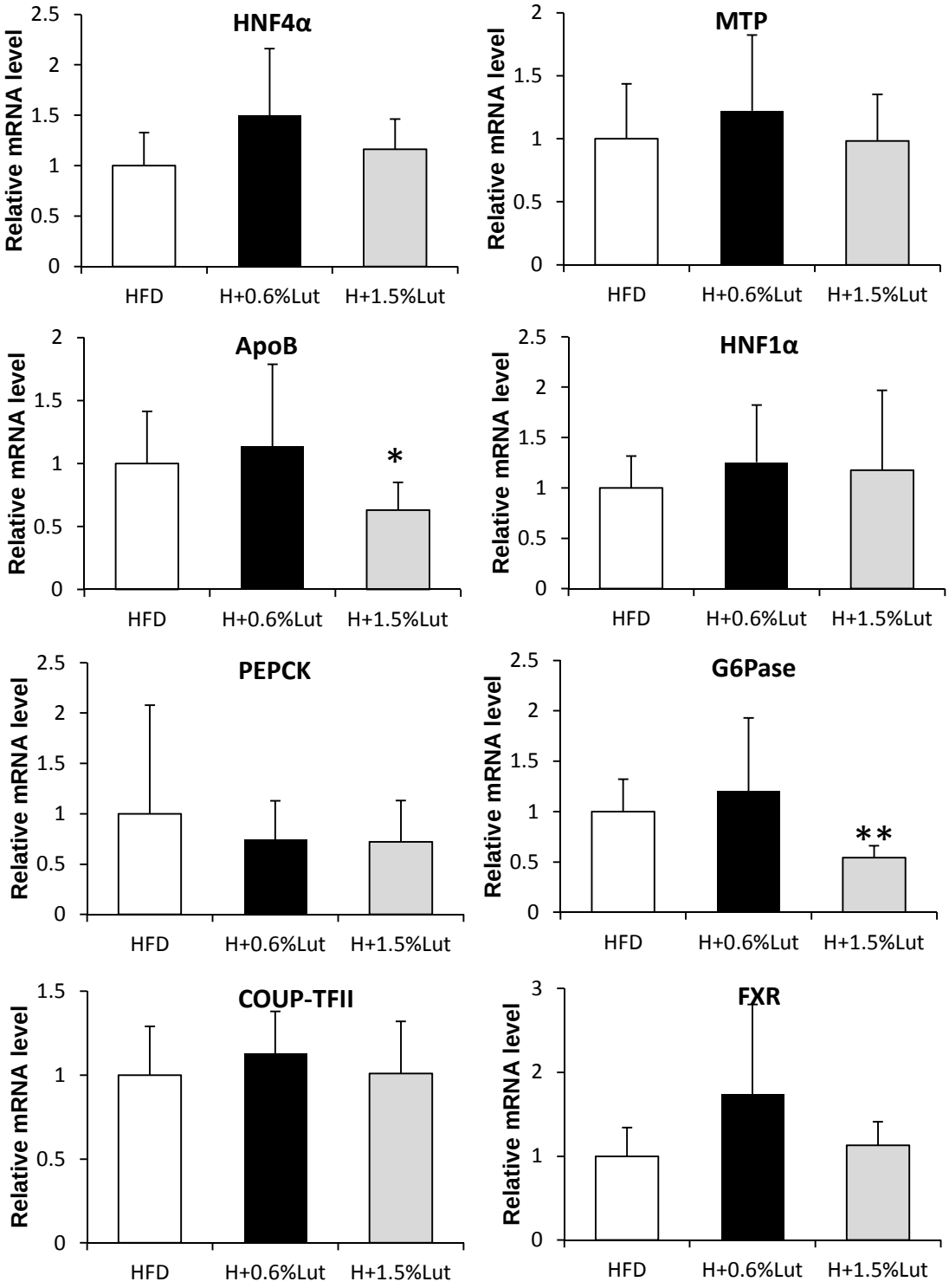


Figure 4-6 The effect of Luteolin diet on the HNF4α related factors in ileum

Real-time PCR analysis of HNF4α target genes (MTP, Apo B, HNF1α, PEPCK, and G6Pase), HNF4α, and its self-regulation factors (COUP-TF II and FXR in ileum. Results are means±S.D. (n =8). *P <0.05; * * P <0.01.

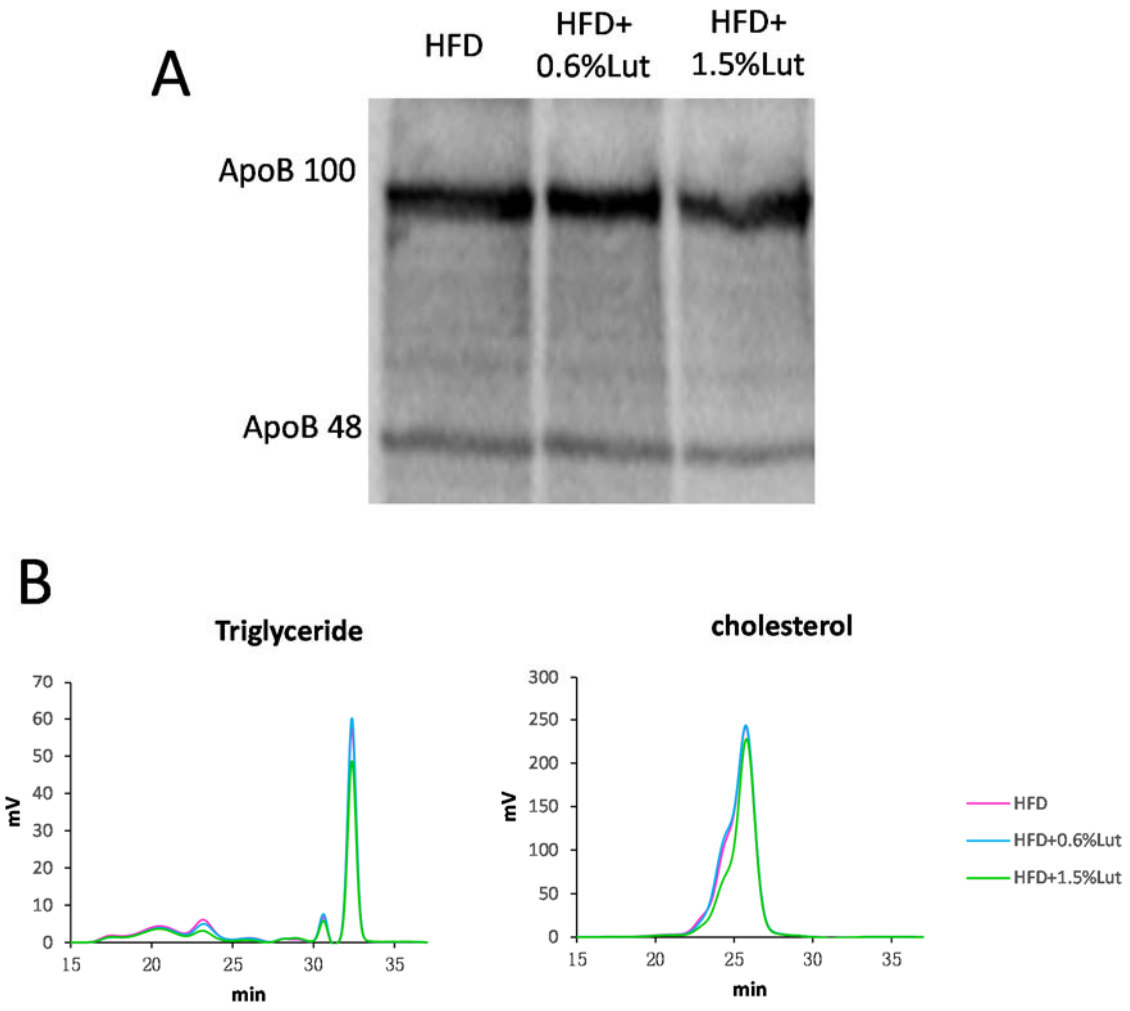


Figure 4-7 Luteolin prevents serum Apo B, triglyceride, and cholesterol

A, Serums for each group (n=8) were mixed together and diluted 20 fold by RIPA buffer. Serums (40 μL/lane) was subjected to SDS-PAGE and Western blotting using antibodies against mouse Apo B. B, Serums for each group (n=8) were mixed together, and serum lipoproteins were analyzed using a high-performance liquid chromatography system at Skylight Biotech (LipoSEARCH, Akita, Japan)

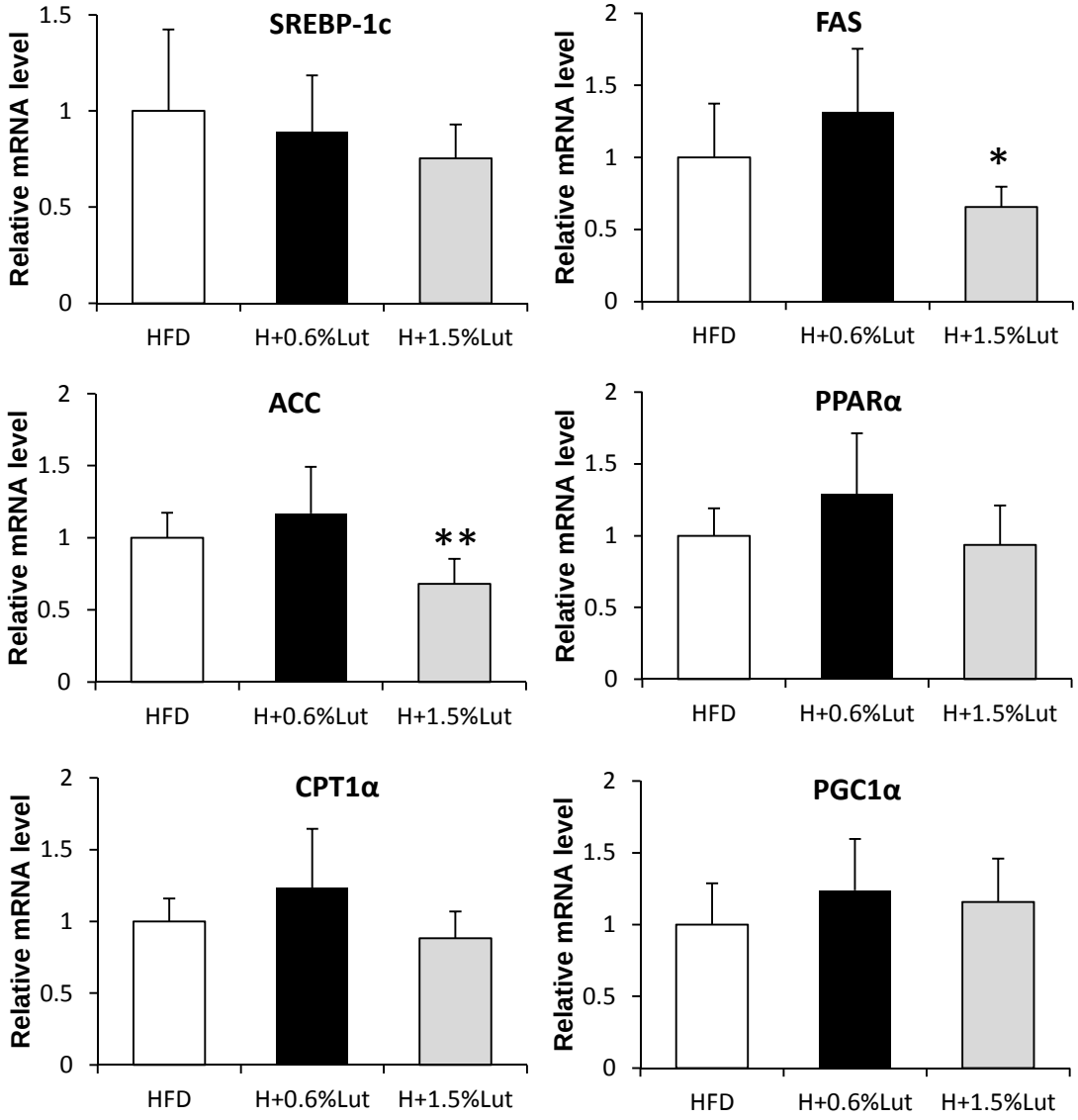


Figure 4-8 Luteolin decreases the mRNA levels of FAS and ACC does not affect the fatty acid oxidation factors in liver

Real-time PCR analysis of genes involved in fatty acid synthesis (SREBP-1c, ACC, and FAS.) and oxidation (PPARα, CPT1α, and PGC1α) in liver. Results are means±S.D. (n =8). *P <0.05; * * P <0.01.

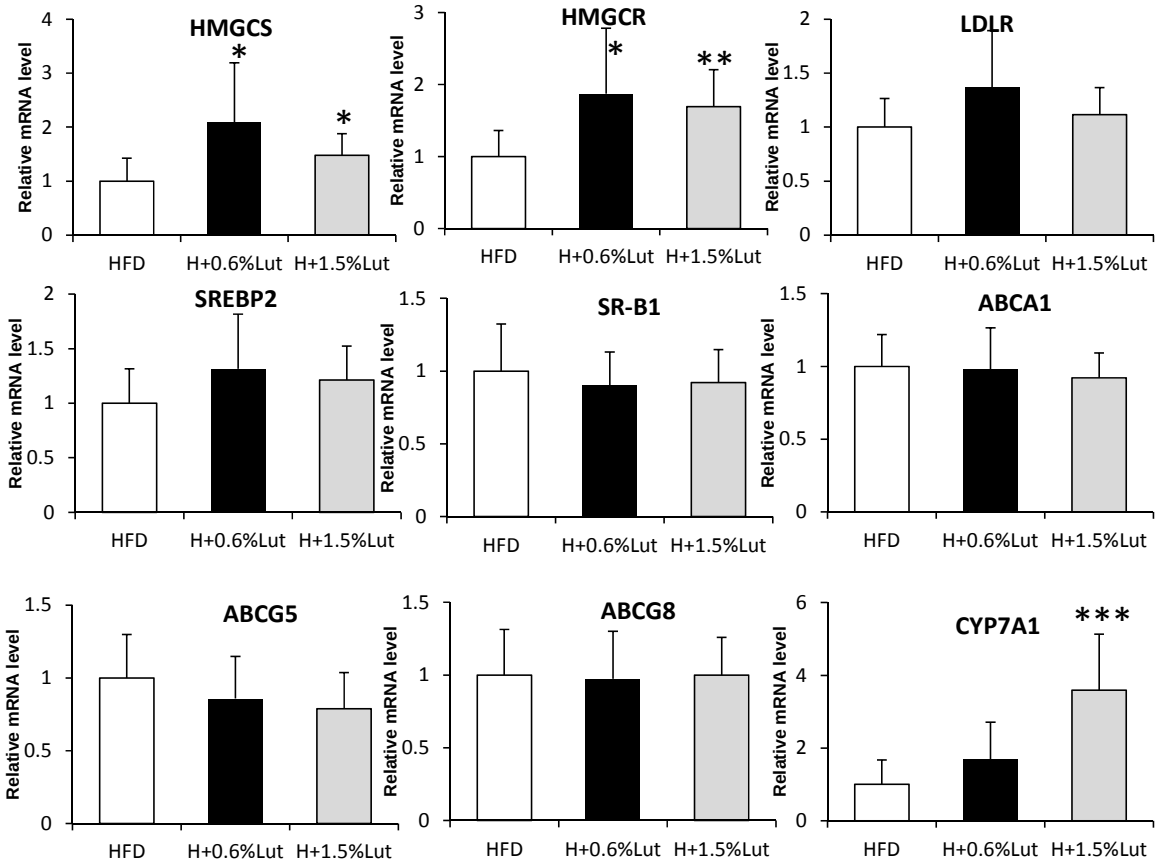


Figure 4-9 Luteolin elevates the mRNA levels of HMGCS, HMGCR, and CYP7A1 in liver

Real-time PCR analysis of genes involved in cholesterol synthesis (HMGCS, HMGCR, SQS, LDLR, and SREBP2), transport (SR-B1, ABCA1, ABCG5, and ABCG8) and consumption (CYP7A1) in liver. Results are means±S.D. (n =8). *P <0.05; ** P <0.01; ***P <0.001.

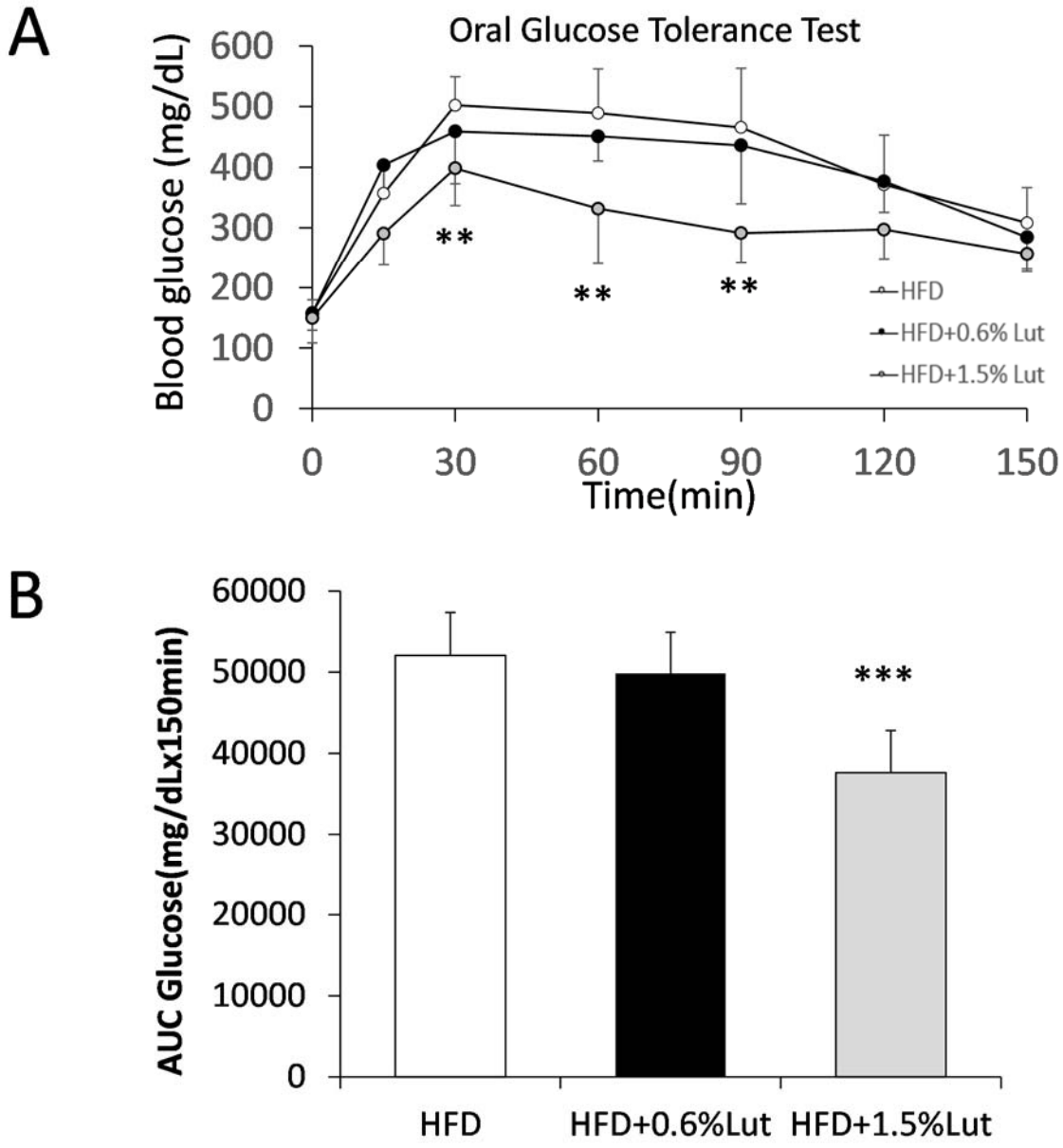


Figure 4-10 Luteolin impairs glucose tolerance and improve glucose metabolism

A, Plasma glucose concentrations during the oral glucose tolerance test (2 g/kg) following 16h. B, Area under the curve (AUC) for glucose. Results are means±S.D. (n =8). ** P <0.01; ***P <0.001.

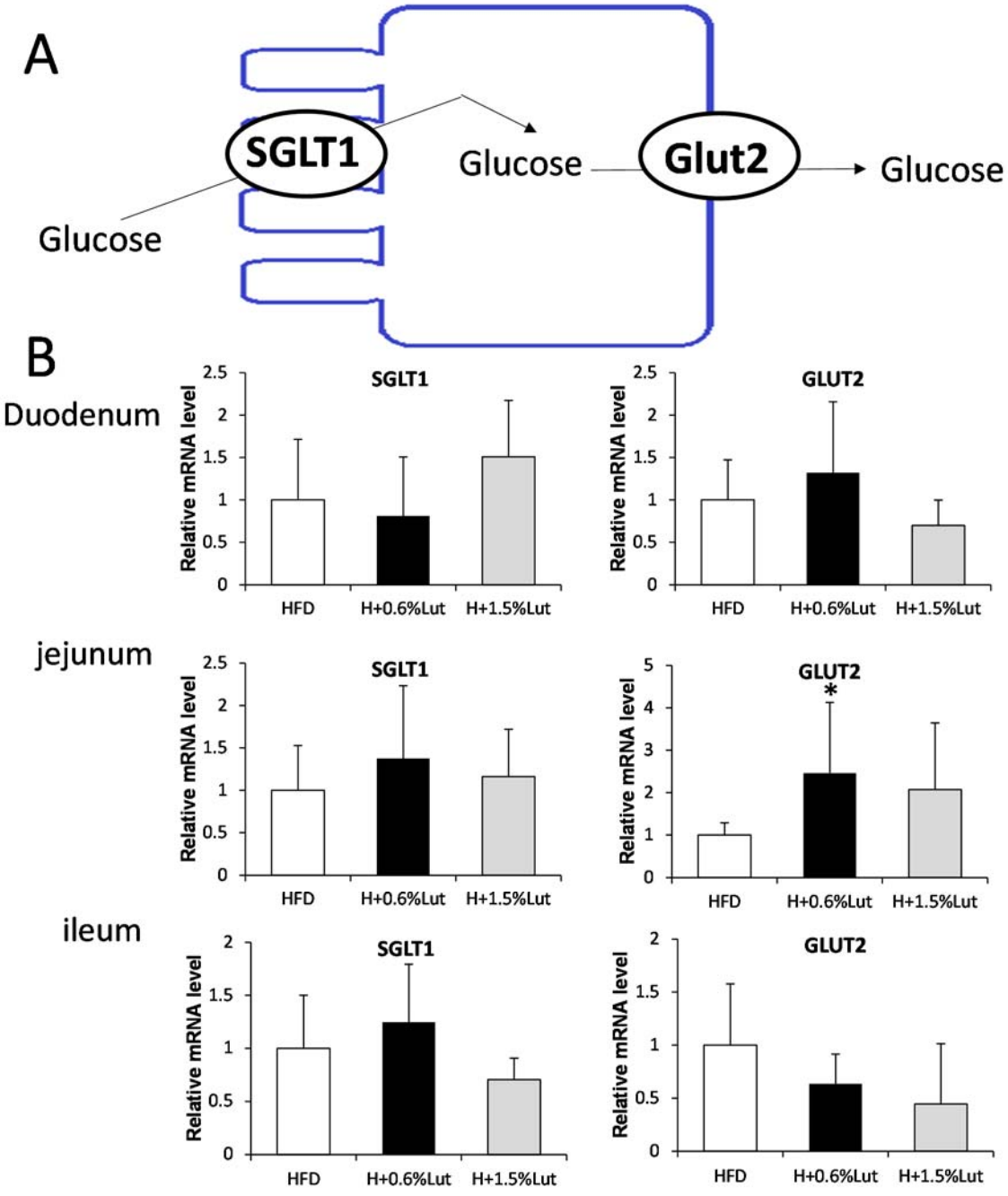


Figure 4-11 Luteolin does not inhibit the genes involved glucose absorption (SGLT1 and GLUT2) in intestine.

A, Glucose absorption is regulated by SGLT1 and GLUT2. B, Real-time PCR analysis of the mRNA levels of SGLT1 and GLUT2 in duodenum, jejunum, and ileum. Results are means±S.D. (n =8). *P <0.05.

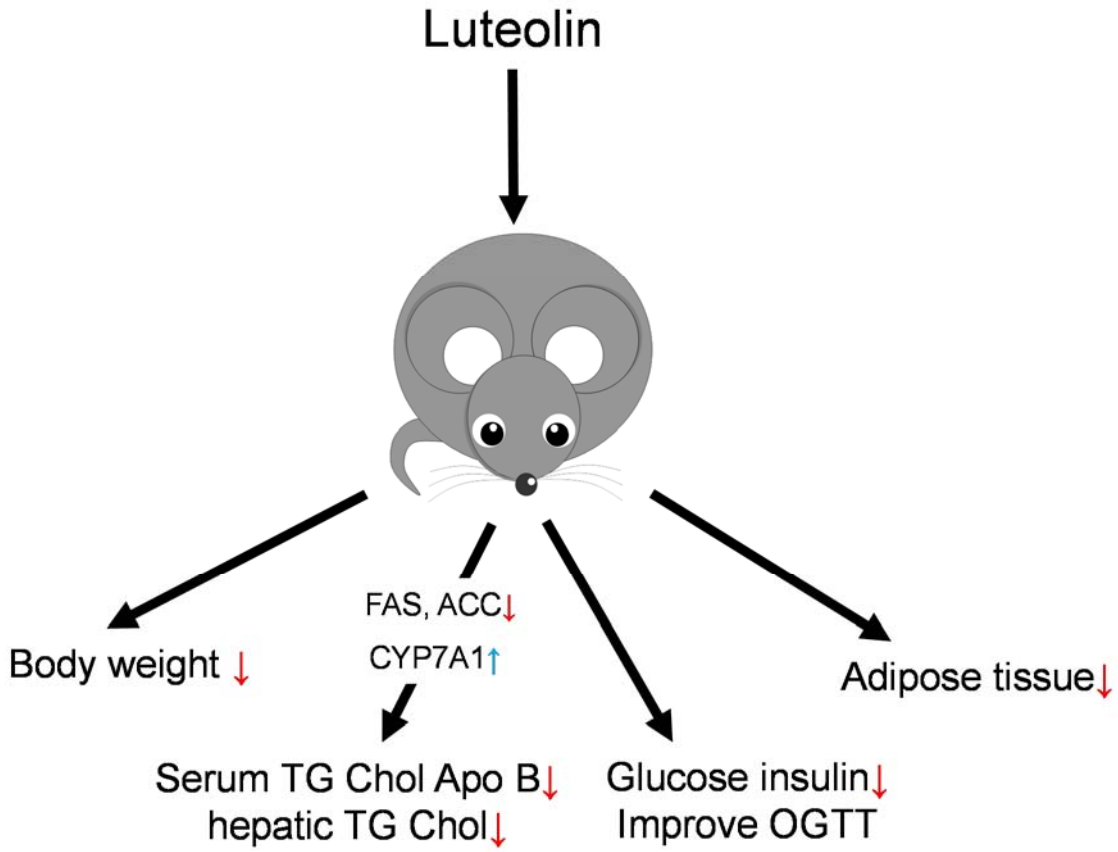


Figure 4-12 Summary of Chapter 4

	HFD	HFD+0.6% Lut	HFD+1.5% Lut (vs HFD)
Food Intake, g/day/mouse	2.85±0.03	2.79±0.07	2.84±0.11
Serum			
Glucose, mg/dL	281±11	246±13	214±14** (76%)
Insulin, ng/mL	9.47±3.88	4.90±1.91* (52%)	4.52±2.53** (48%)
Total-Triglyceride, mg/dL	24.7±4.4	20.3±2.5	16.5±4.6* (67%)
CM-Triglyceride, mg/dL	3.02±0.81	2.58±0.43	2.77±1.00
VLDL-Triglyceride, mg/dL	10.3±1.82	9.03±1.66	8.28±1.97
LDL-Triglyceride, mg/dL	9.32±1.83	7.09±1.21	4.48±1.33* (48%)
HDL-Triglyceride, mg/dL	2.13±0.58	1.57±0.52	0.95±0.47* (45%)
Total-Chol, mg/dL	205±8.8	201±4.5	171±18* (83%)
CM- Chol, mg/dL	0.42±0.13	0.36±0.04	0.34±0.12
VLDL-Chol, mg/dL	2.38±0.37	1.97±0.42	1.53±0.41* (64%)
LDL- Chol, mg/dL	56.0±1.8	54.4±5.3	37.8±10.4* (68%)
HDL- Chol, mg/dL	146±6.6	144.7±1.8	132±9.4* (90%)
NEFA, mEq/L	1.00±0.06	0.90±0.06	0.87±0.05
Total-Bile Acid, μmol/L	50.1±4.2	58.0±6.5	40.3±4.7
GOT, Karmen	103±25	104±34	71.6±25.9* (70%)
GPT, Karmen	63.2±16.0	66.2±28.1	39.5±22.9* (63%)
Liver			
Weight, g	2.18±0.10	2.20±0.19	1.59±0.12** (73%)
Triglyceride, mg/g	916±119	910±70	409±109** (45%)
Cholesterol, mg/g	7.38±0.24	6.43±0.45	6.20±0.36* (84%)

Results are means±SEM (n =8)
*P<0.05; **P<0.01.

Table 4-1. Food intake, serum parameters, hepatic weight and lipid of mice.

4-4 Discussion

In this chapter, the effect of Luteolin on HNF4α activity and glucose, lipid metabolism *in vivo* was studied. The results was summarized in Fig 4-12, and concluded as follows:

[I]. Luteolin decreased body weight without influence on food intake in high fat diet induced obese mice.

[II]. Luteolin attenuated serum and hepatic triglyceride, cholesterol, decreased serum Apo B 100 level.

[III]. Luteolin improved glucose metabolism and insulin sensitivity.

In this study, I provided evidence that the anti-obesity effects of Luteolin. These results suggest that Luteolin had preventive effects against the development of metabolic syndrome, including obesity, fatty liver, dyslipidemia, hyperglycemia, and insulin resistance in mice.

As other flavonoids, Luteolin is most often found in plant materials in the form of glycosides, which are eventually metabolized by intestinal bacteria, cleaved and glucuronated during uptake in the gut and metabolized in the organism (82). Although the absorption of Luteolin was not measured in this research, it is reported that after administration of Luteolin by gastric intubation in rat, free Luteolin, its conjugates and methylated conjugates are present in plasma. And the main conjugate is a monoglucuronide of the unchanged aglycone (83).

In order to analyse the effect of Luteolin on HNF4α activity, HNF4α target genes mRNA levels were investigated. In liver, 1.5% Luteolin administration declined the mRNA levels of MTP and G6Pase, had no effect on HNF1α and Apo B, but significantly elevated PEPCK and CYP7A1 mRNA levels (CYP7A1 is also a target gene of HNF4α). Other than the target genes, ACC, FAS mRNA levels lowered, HMGCR, HMGCS mRNA levels elevated, and had no effect on expression of cholesterol transport factors (ABCA1, SR-B1, ABCG5, ABCG8), oxidation related factors (PPARα, CPT1α, and PGC1α), LDLR, SREBP-1c, SREBP-2.

In hepatic HNF4α-deficient mice, mRNA levels of genes involved in VLDL secretion

(MTP, Apo B), de novo cholesterol biosynthesis (HMGCR, HMGCS, SREBP-2), cholesterol catabolism (CYP7A1, CYP8B1), cholesterol esterification (ACAT2, LCAT), and cholesterol uptake (LDLR, SR-BI) were all significantly reduced (138).

In hepatic HNF4α-deficient mice, plasma bile acid levels were increased, plasma levels of triglycerides and cholesterol were reduced, food intake, body weight, glucose tolerance, or insulin sensitivity were not affected, but of fatty liver was developed (138). In contrast, 1.5% Luteolin diet markedly reduced total body weights, serum levels of cholesterol, triglyceride, Apo B, glucose, and insulin, also significantly lowered hepatic cholesterol and triglyceride levels.

In intestine, 1.5% Luteolin diet depressed HNF1α expression in duodenum (Fig 4-3), the mRNA levels of Apo B and G6Pase in ileum (Fig 4-5), whereas, increased the expression of MTP, HNF1α in jejunum (Fig 4-4). HNF4α expression was not affected by Luteolin in both of liver and intestine.

Comparing the phenotype of Luteolin diet and hepatic Hnf4α deficiency mice, from this research it is difficult to determine whether Luteolin suppress HNF4α activity *in vivo*. In order to make clear, further research is required by short-time Luteolin administration or using hepatic HNF4α-deficient mice.

1.5% Luteolin supplement decreased hepatic triglyceride and cholesterol. Triglyceride content is related to fatty acid synthesis and oxidation. 1.5% Luteolin diet decreased the mRNA levels of ACC and FAS, which are two sequential enzymes in the fatty acid biosynthetic pathway, but did not alter SREBP-1c expression, which activate the transcription of ACC and FAS. Luteolin had no effect on the oxidation related factors (PPARα, CPT1α, and PGC1α) (Fig 4-7). Hence, it seems likely that the reduction of hepatic triglyceride by Luteolin administration may result from the declined fatty acid synthesis, not oxidation.

Hepatic cholesterol content is related to cholesterol biosynthesis, catabolism, and transport. HMGCS catalyzes the formation of the substrate HMG-CoA; HMGCR catalyzes the rate-controlling step in cholesterol production. LDLR mediates the clearance of cholesterol and cholesteryl ester containing LDL particles. SREBP-2 regulates the

transcription of HMGCS, HMGCR, and LDLR. Surprisingly, Luteolin elevated the expression of HMGCS and HMGCR, had no influence on LDLR and SREBP-2 and cholesterol transport factors (ABCA1, SR-B1, ABCG5, and ABCG8). For cholesterol catabolism, CYP7A1 is the rate-limiting enzyme in the bile acid biosynthetic pathway that converts cholesterol into bile acids in the liver. The mRNA level of CYP7A1 was significantly elevated by 1.5% Luteolin diet. These results suggest the reduction in hepatic cholesterol by Luteolin may result from the increased CYP7A1 expression.
The regulation mechanism of Luteolin for ACC, FAS, HMGCS, HMGCR, and CYP7A1 is required further study.

Declined hepatic lipid levels, MTP expression caused by Luteolin may contribute to the decreased serum triglyceride, cholesterol and Apo B. Serum CM-TG and Chol was not altered with Luteolin supplement, it may be due to the unaffected MTP expression in intestine. HDL-TG and Chol also lowered by Luteolin, but liver ABCA1 was not changed. However, mouse HDL content is higher than human due to lack of cholesteryl ester transfer protein (CETP), thus Luteolin may have different effect on human HDL TG and Chol. In this research, the inhibitory mechanism of Luteolin for serum HDL level is required further investigation.

Serum glucose level was lowered by 1.5% Luteolin. It may have relation to the G6Pase mRNA level in liver and ileum. It is well known that a high-fat diet leads not only to increased body fat accumulation, but also to insulin resistance. From the results of serum insulin concentration and OGTT, Luteolin improved insulin resistance and glucose metabolism. In addition, from the result of OGTT, 1.5% Luteolin suppressed the glucose level peak at 30min, it is thought Luteolin may have an effect on glucose absorption. However, Luteolin did not decrease the mRNA levels of SGLT1 and Glut2 in intestine.

Luteolin has no influence on the food intake and not elevated serum GOT, GTP level (markers of liver damage). The reduced body weight in 1.5%Luteolin group may due to the declined weight of adipose tissue. It is suggested that Luteolin may block the fat accumulation in adipose tissue, but the mechanism remains unknown.

Chapter 5

Quercetin decreased Apo B secretion via C/EBPβ

5-1 Preface

Quercetin, a flavonol categorized as flavonoid, is widely distributed in fruits, vegetables, tea and grains. Quercetin has been reported to have physiological functions as an antioxidant, or by lowing blood pressure and improving metabolic syndrome. Besides, Quercetin intake is inversely correlated with plasma total and LDL-cholesterol, and Quercetin inhibits hepatic and intestinal Apo B secretion. However, few studies have been done on the mechanism. In this chapter, the inhibitory mechanism of Quercetin for Apo B secretion was investigated.

5-2 material and methods

Luciferase Assay

Plasmid DNA for Apo B promoter activity (for 1 well)

$PGVB_2$-hApoB-Luc	0.5μg
pCMV-β-gal	0.5μg

Plasmid DNA for C/EBPβ activity with C/EBPβ expression vector (for 1 well)

pcDNA/pcDNA-C/EBPβ	0.5μg
$PGVB_2$-hApoB-Luc/ $p(C/EBP)_4$-Luc	0.5μg
pCMV-β-gal	0.5μg

Plasmid DNA for GAL4-C/EBPβ activity (for 1 well)

GAL4/GAL4-hC/EBPβ	0.5μg
5xUAS	0.5μg
pCMV-β-gal	0.5μg

As described in Chapter 2 were used in this experiment.

RT-PCR

As described in Chapter 2 were used in this experiment.

ChIP Assay

As described in Chapter 3 were used in this experiment.

Primer (5'----3')

The following primers were used to amplify the hC/EBPβ proximal promoter

hApo B	F CTTCAAGGCTCAAAGAGAAGCC
	R AGGTCCCGGTGGGAATG

The following primers were used to amplify the hC/EBPβ distal promoter

hApo B	F GGGCACAGTTCCATCTACAA
	R CCTATCTCGTTTCTGCCTATGAC

Detection of C/EBPβ and Quercetin binding by Quercetin Beads

As described in Chapter 3 (Detection of HNF4α and Luteolin binding by Luteolin Beads) were used in this experiment.

5-3 Results

5-3-1 Quercetin decreases MTP and Apo B mRNA levels without influence on their mRNA stability

It is postulated Quercetin may affect gene expression of MTP and Apo B, which are tightly related to secretion of Apo B-containing lipoproteins. Firstly, the effect of Quercetin on the Apo B and MTP mRNA levels in differentiated Caco-2 cells was examined. Quercetin suppressed Apo B gene expression in a dose-dependent manner, and decreased MTP mRNA level at 100 μM (Fig 5-1 A). Next, the effect of Quercetin on mRNA stability was examined in the presence of a transcription inhibitor actinomycin D. It is showed that Quercetin had no effect on Apo B and MTP mRNA levels. Hence, it seems likely that Quercetin decreases transcription of MTP and Apo B genes (Fig 5-1 B).

5-3-2 Quercetin suppresses Apo B transcription through declined C/EBP activity

Subsequently, to investigate the influence of Quercetin on transcription, reporter gene assays were carried out by using the luciferase reporter plasmids containing the human MTP gene promoter (-204 ~ +33 nt) or Apo B gene promoter (-1800 ~ +42 nt). In Caco-2 cells Quercetin significantly lowered the Apo B promoter activity, whereas no change in the MTP promoter activity was observed (Fig 5-2). No response to Quercetin is assumed to be due to the short promoter sequence (-204 ~ +33 nt) in the MTP reporter used in this experiment. In the following experiments I focused on the regulation of Apo B transcription by Quercetin.

To identify a specific sequence responsible for the Quercetin-mediated suppression of Apo B gene expression, luciferase assays were performed in Caco-2 cells using different 5' -serially deleted human Apo B promoter-reporter constructs. Figure 5-3 A shows that Quercetin suppressed the luciferase activity of -1298/+42-Luc, -574/+42-Luc, -140/+42-Luc, -73/+42-Luc, but had no effect on -29/+42-Luc. This suggests that the region of human Apo B promoter between -73 and -29 b was essential for

Quercetin-mediated suppression of Apo B gene expression. This region contains a C/EBP-binding motif that is conserved among human, mouse, and rat genes (Fig 5-3 B).

An expression plasmid for C/EBPβ was used for estimating the effect of Quercetin on the C/EBPβ transcriptional activity in Caco-2 cell (Fig 5-4). Overexpression of C/EBPβ increased the luciferase activity of -1298/+42-Luc, -574/+42-Luc, -140/+42-Luc, and -73/+42-Luc, which contains the C/EBP-binding motif, but did not increase that of -29/+42-Luc (Fig 5-4 A). When luciferase assays were performed using the p$(C/EBP)_4$-Luc reporter plasmid containing 4 repeats of the C/EBP-binding motif, Quercetin was found to decrease the luciferase activities in a dose-dependent manner (Fig 5-4 B).

It has been reported that both C/EBPα and C/EBPβ function as crucial factors for Apo B transcription in liver and intestine, respectively. Overexpression of C/EBPα or C/EBPβ significantly increased the luciferase activity of Apo B reporter gene and Quercetin suppressed their activities (Fig 5-5). These results suggest that Quercetin inhibited both C/EBPα and C/EBPβ transcriptional activities.

5-3-3 Quercetin functions within cells and binds to C/EBPβ directly

It is postulated that Quercetin inhibited C/EBPβ activity from outside of cells through a specific receptor (indirect action) or through their direct interaction within cells (direct action) (Fig 5-6). In order to make clear how Quercetin hinders the C/EBP transcriptional activity, Quercetin-3-glucuronide, which is thought to be poorly taken up by cells, was compared with Quercetin (Fig 5-7 A). When HepG2 or Caco-2 cells were cultured with Quercetin, Apo B mRNA level was reduced, but with Quercetin-3-glucuronide, no decline was observed (Fig 5-7 B). This result implies that Quercetin, which is efficiently taken up by cells, affects C/EBPβ activities within cells.

Quercetin was immobilized on agarose beads through a photo-affinity linker (Fig. 5-8 A). HEK293 cells were transfected with exogenous expression plasmid of pcDNA-C/EBPβ, and treated with 50 μM Quercetin or DMSO (control) for 12 h. One mg of Caco-2 cell lysates were pre-incubated with control beads (con beads), and then the unbound fraction was further incubated with Quercetin-immobilized beads (Que beads). The reacted beads

were washed, and the co-precipitated proteins were subjected to SDS-PAGE and Western blotting using antibodies against human C/EBPβ. The results show more C/EBPβ was recovered with Que beads than with con beads from the lyasates of cells that were cultured without Quercetin (Fig 5-8 B lanes 3 and 5). When cells were incubated with Quercetin, the cell pellets turned yellow (Fig 5-8 B), probably due to the uptake of Quercetin. The amount of C/EBPβ recovered with Que beads decreased as compared with DMSO-treated cell lysates (Fig 5-8 B lanes 5 and 6). This decline seems to be caused by the competiton of free Quercetin and C/EBPβ to bind to Que beads. When C/EBPβ was exogenously expressed in HEK293, the same results were observed using the Que beads. (Fig 5-8 C). These results suggest that C/EBPβ binds to Quercetin directly.

5-3-4 Quercetin has no effect on the DNA binding activity of C/EBPβ but regulates the co-factor recruitment required for C/EBPβ activation

Next the mechanism of the inhibitory effect of Quercetin on C/EBPβ activity was investigated.

In order to verify the effect of Quercetin on DNA binding activity of C/EBPβ, ChIP assay was performed. Firstly, time-course experiments to examine whether Quercetin affects the C/EBPβ mRNA levels in Caco-2 cells were carried out. Quercetin didn't change the C/EBPβ mRNA levels for 10 h, but decreased the Apo B mRNA levels within 9 h (Fig 5-9).

Chromatin from the control and Quercetin-treated cells were immuneprecipitated with anti-acetylated histone H3, anti-human C/EBPβ or rabbit Immunoglobulin G (IgG) antibodies. DNA precipitated by the antibodies was then subjected to real-time PCR with gene-specific primers or non-specific primers as negative control (Fig 5-10 A). ChIP assays revealed that C/EBPβ bound to the Apo B proximal promoter, but Quercetin didn't affect the activity. ChIP assays using primer pairs covered the Apo B distal region gave us negligible low PCR products, suggesting that this region didn't interact with C/EBPβ (Fig 5-10 B).

In order to verify the effect of Quercetin on C/EBPβ co-factor(s) recruitment, GAL4

luciferase assay system was used (Fig 5-11 A). A GAL4-C/EBPβ fusion plasmid and the 5xUAS reporter gene were transfected in HEK293 cells. The activity was increased with GAL4-C/EBPβ transfection and decreased by Quercetin in a dose-dependent manner. This suggests that Quercetin may regulate the recruitment of co-factor(s) required for induction of C/EBPβ transcription activity.

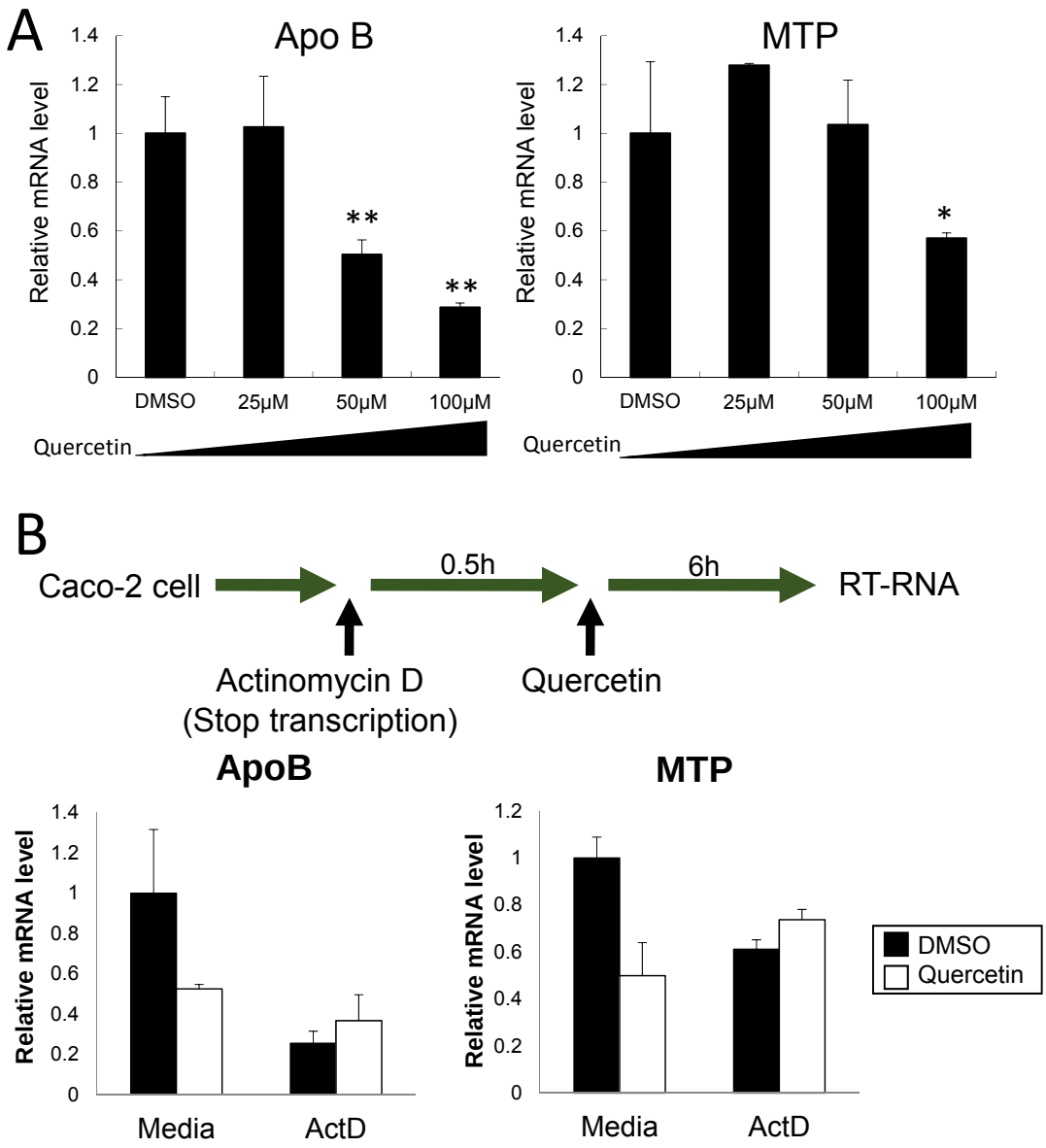


Figure 5-1 Quercetin inhibits Apo B and MTP mRNA level without effect on mRNA stability in Caco-2 cells

Caco-2 cells were treated with Quercetin, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. A, different concentration of Quercetin treated for 12h. B, followed by pre-treatment of Actinomycin D, 100μM of Quercetin treated for another 6h. Results are means±S.D. (n =3). *P <0.05, **P <0.01.

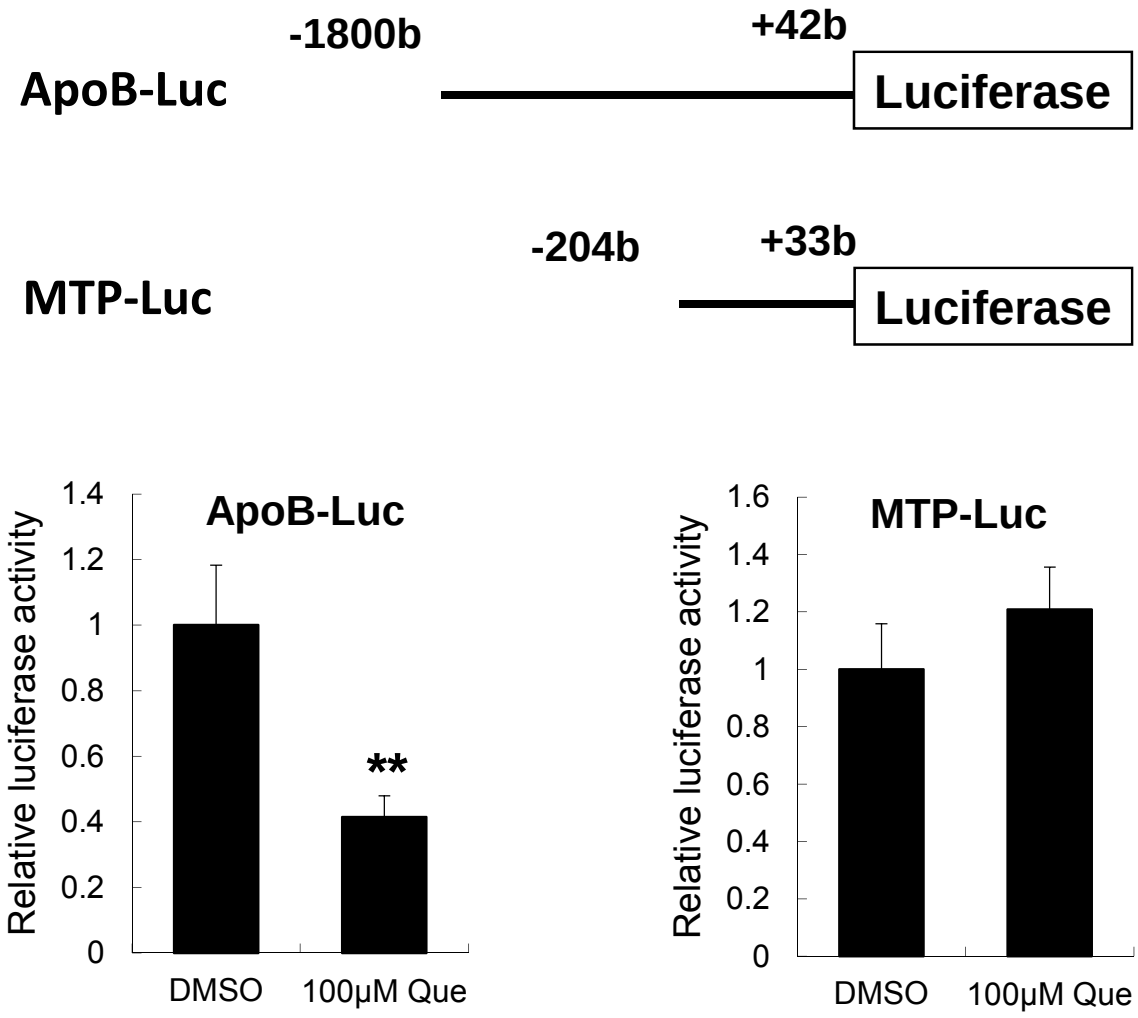


Figure 5-2 Quercetin inhibits Apo B promoter activity in Caco-2 cells

Caco-2 cells were transfected with the report plasmid containing human Apo B gene promoter (-1800~+42) or human MTP gene promoter (-204~+33) and an expression plasmid for β-galactosidase. After transfection, cells were treated with 100μM of Quercetin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. Results are means±S.D. (n =3). **P <0.01.

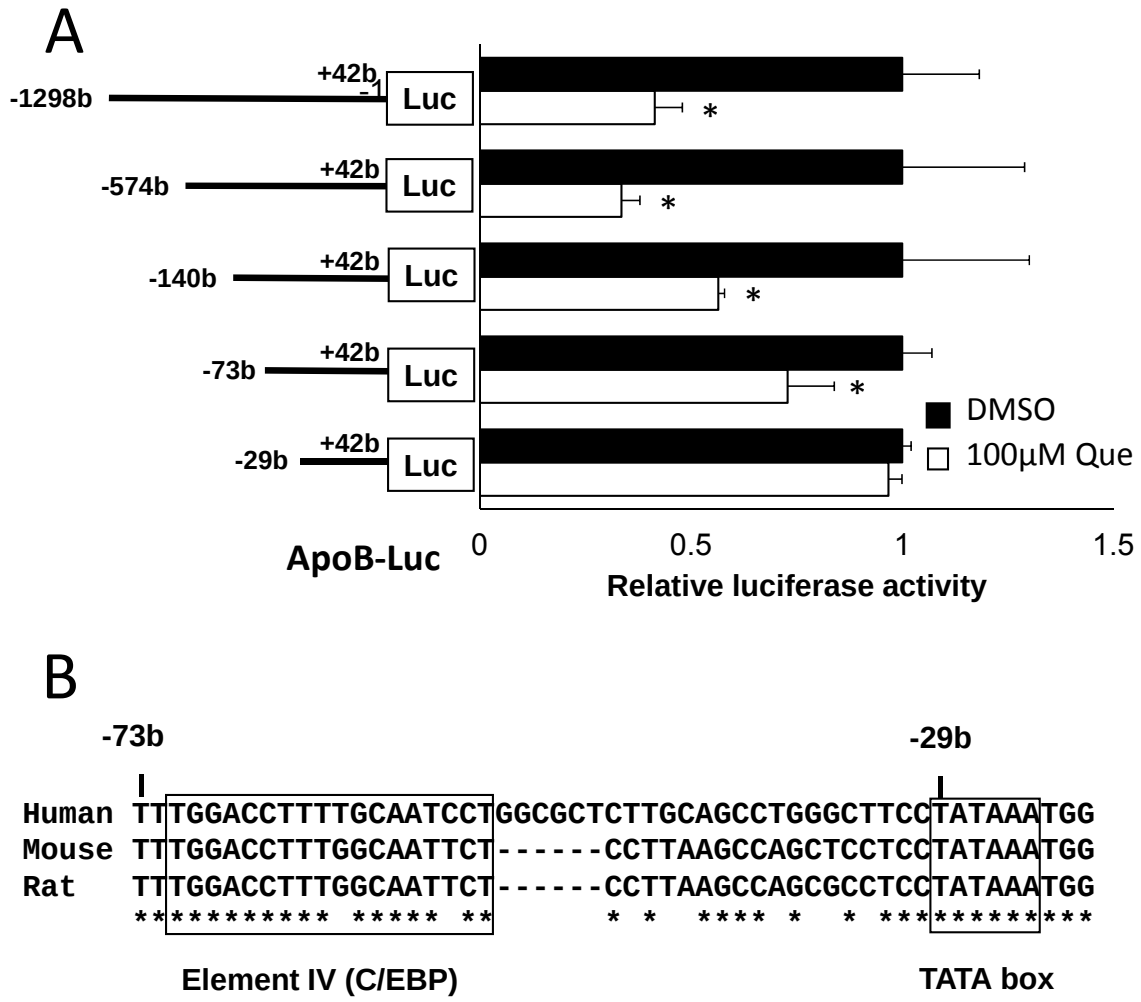


Figure 5-3 C/EBP-binding motif is required for Quercetin-mediated suppression of Apo B transcription

A, Caco-2 cells were transfected with the indicated reporter genes and an expression plasmid for β-galactosidase. After transfection, cells were treated with 100μM of Quercetin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. Results are means±S.D. (n =3). *P <0.05. B, DNA sequences of the human, mouse, rat Apo B promoters around the C/EBP was shown. Conserved DNA sequences among species are marked by asterisks.

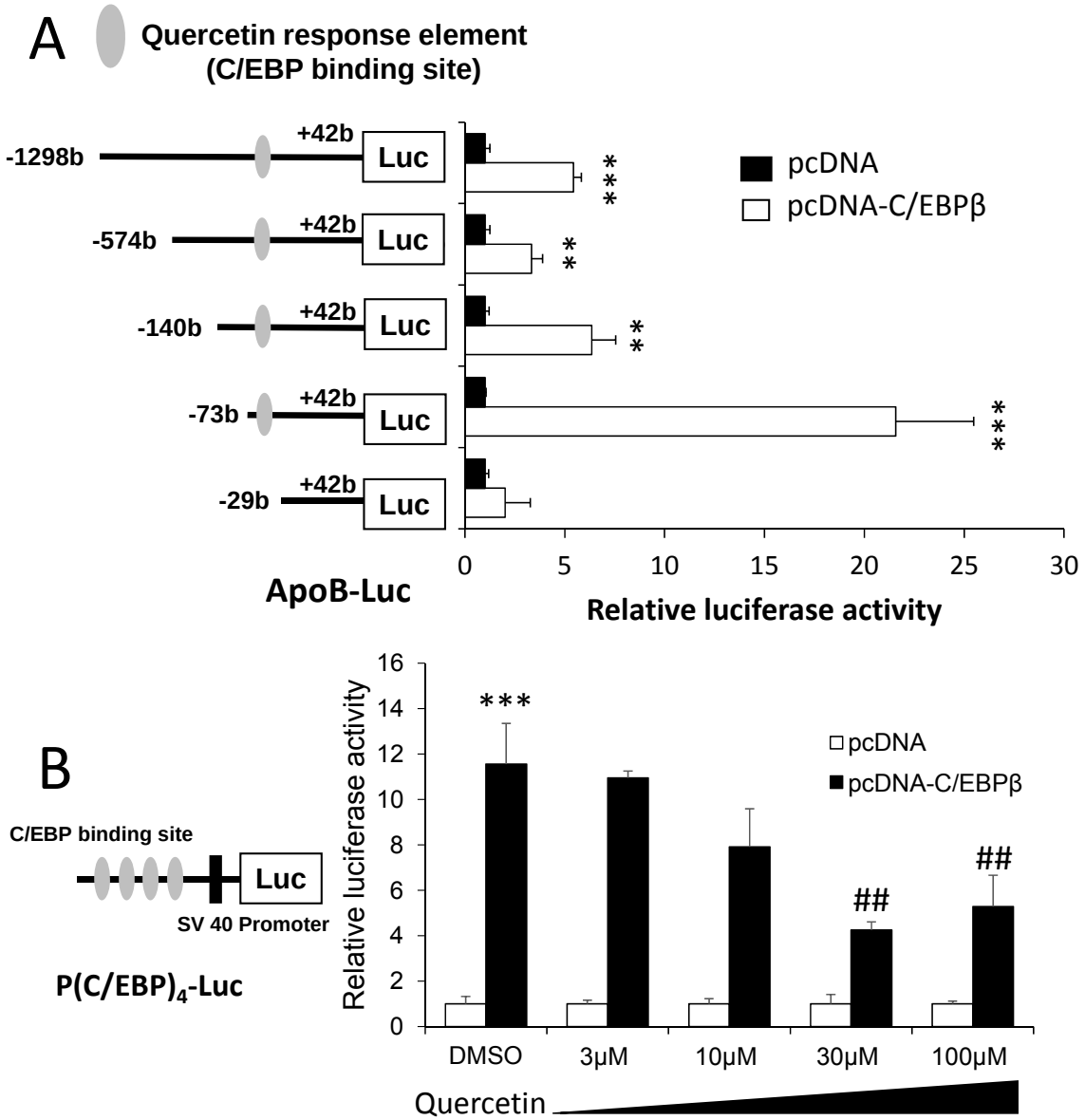


Figure 5-4 Quercetin inhibits C/EBPβ activity.

A, Caco-2 cells were transfected with the indicated reporter genes and an expression plasmid for β-galactosidase and pcDNA-C/EBPβ. B, Caco-2 cells were transfected with the reporter genes of $P(C/EBP)_4$-Luc and an expression plasmid for β-galactosidase and pcDNA-C/EBPβ. After transfection, cells were treated with different concentration of Quercetin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. Results are means±S.D. (n =3). **P <0.01, ***P <0.001, vs. in the absence of C/EBPβ under DMSO treatment . $^{\#\#}$P <0.01 vs . in the presence of C/EBPβ under DMSO treatment .

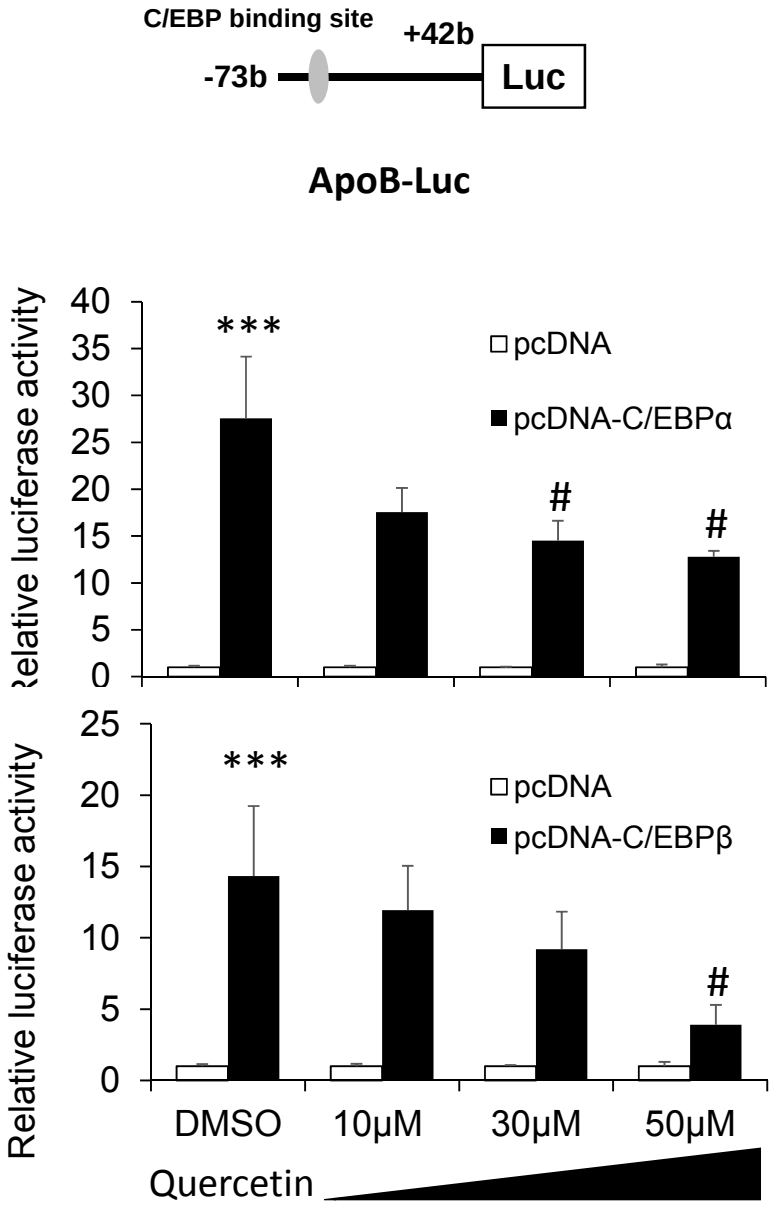


Figure 5-5 Quercetin inhibits C/EBPα and C/EBPβ activity.

Caco-2 cells were transfected with the reporter genes of Apo B-Luc (-73~+42) and an expression plasmid for β-galactosidase and pcDNA-C/EBPα/pcDNA-C/EBPβ. After transfection, cells were treated with different concentration of Quercetin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities with DMSO treatment are considered as 1. Results are means±S.D. (n =3). **P <0.01, ***P <0.001, vs. in the absence of C/EBPα/β under DMSO treatment. $^{\#\#}$P <0.01 vs. in the presence of C/EBPα/β under DMSO treatment.

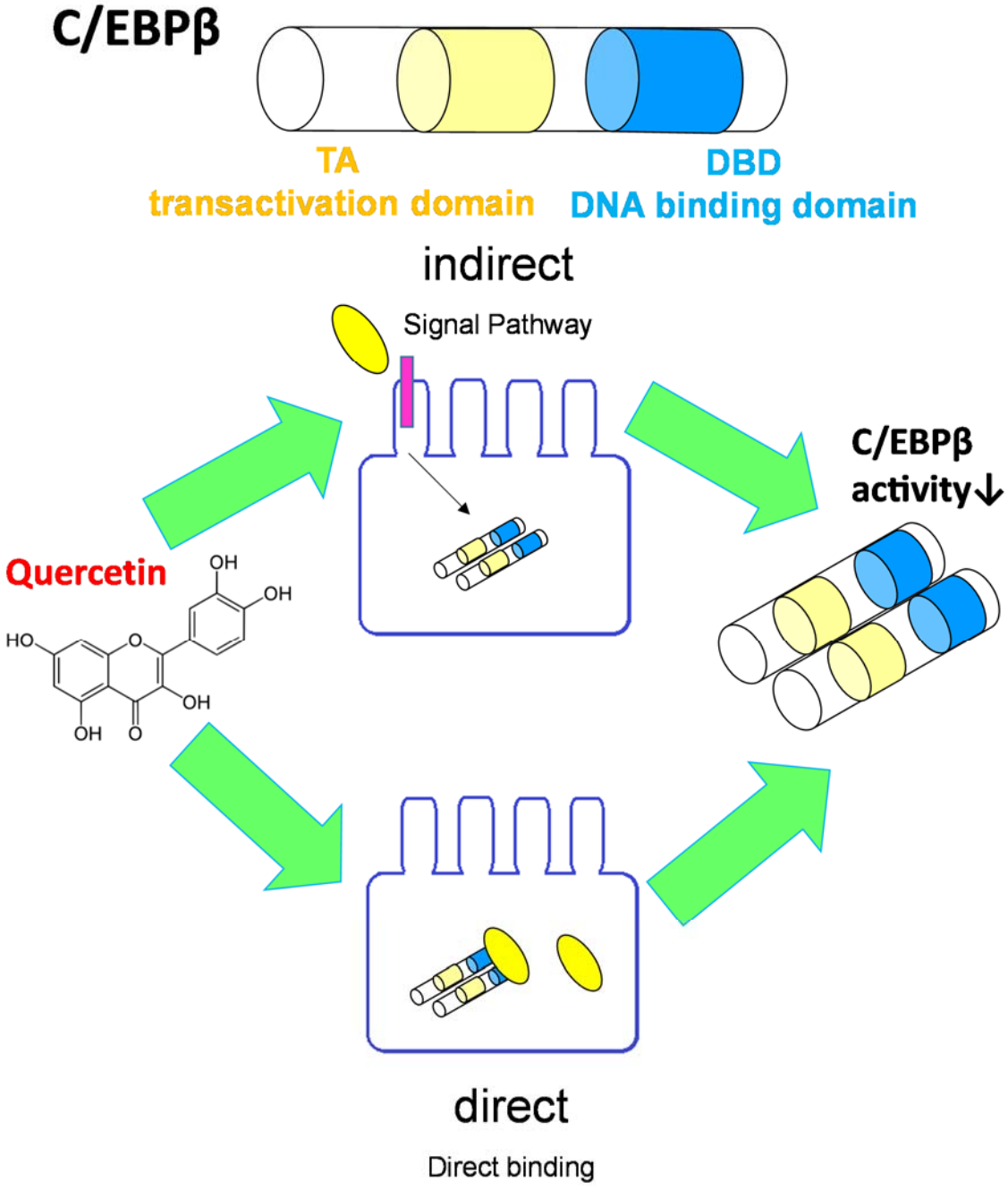


Figure 5-6 The postulated manners for Quercetin inhibiting C/EBPβ activity.

One is from outside of cells through a specific receptor (indirect action), the other one is direct interaction within cells (direct action).

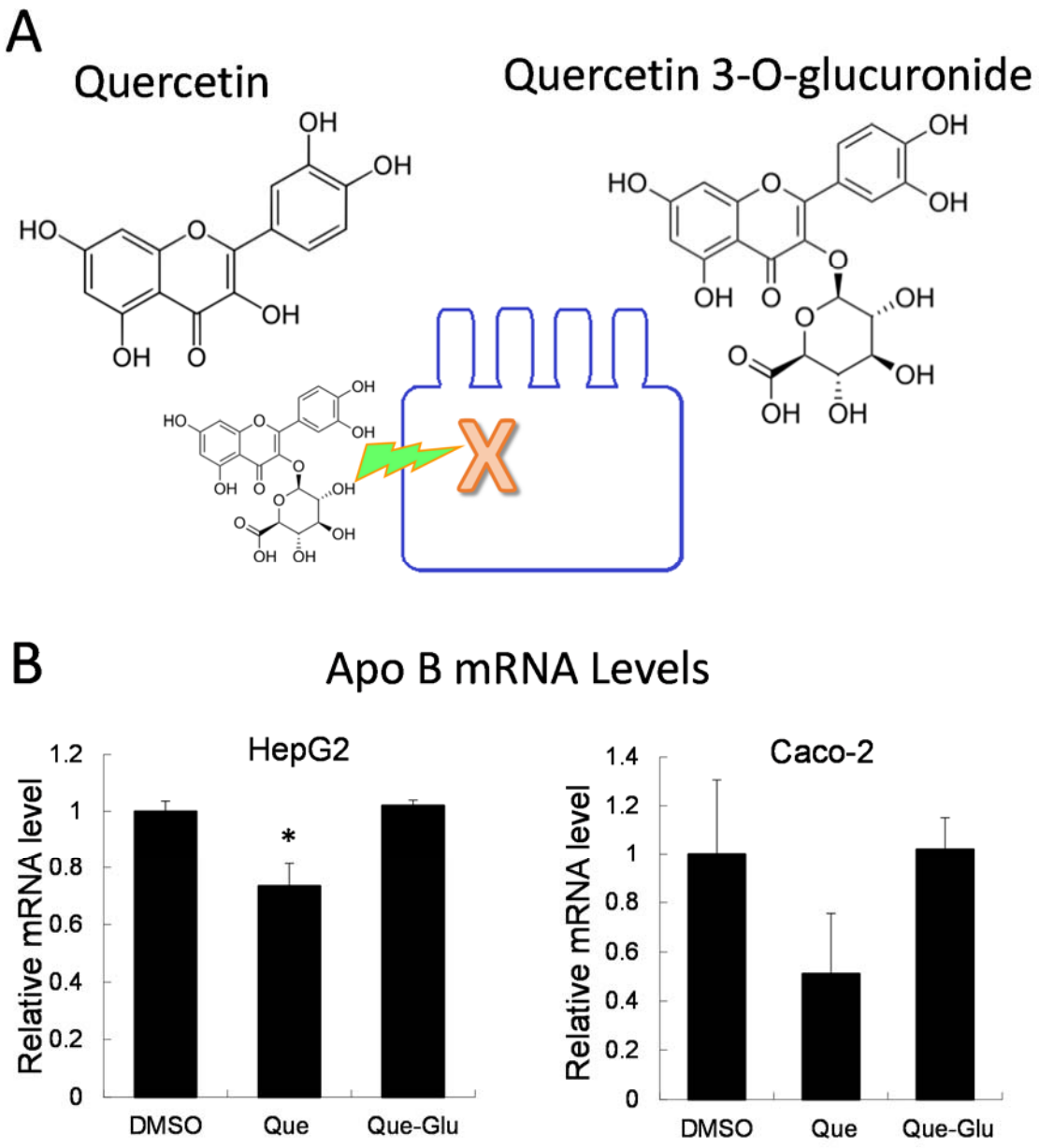


Figure 5-7 Quercetin 3-O-glucuronide have no effect on Apo B mRNA level in HepG2 and Caco-2 cells

A, The structure of Quercetin and Quercetin 3-O-glucuronide. B, HepG2 and Caco-2 cells were treated with 50μM Quercetin and Quercetin 3-O-glucuronide for 12h, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. Results are means±S.D. (n =3). *P <0.05.

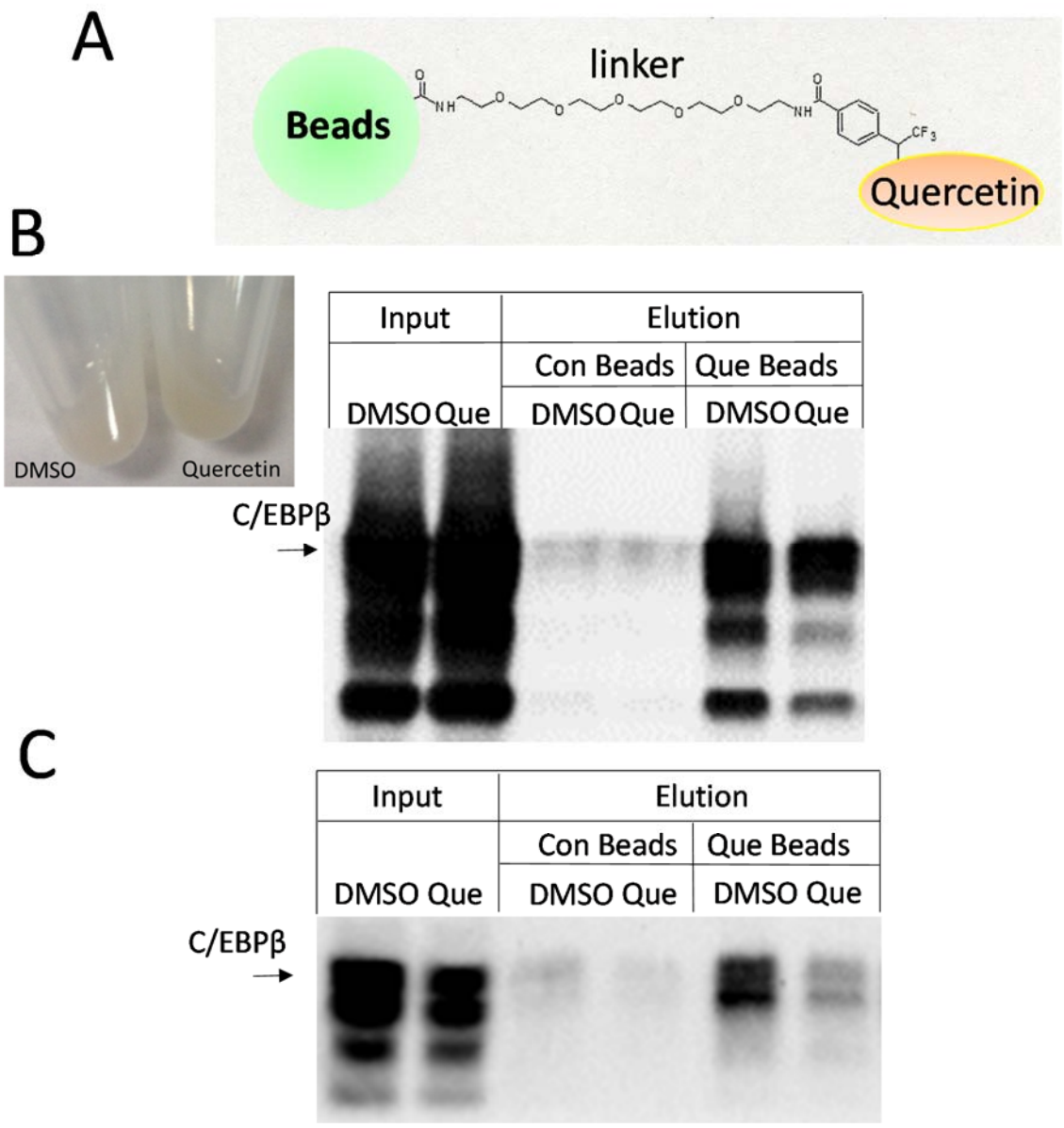


Figure 5-8 C/EBPβ binds to Quercetin directly

A. Model structure of Quercetin beads. Quercetin immobilized on agarose beads through a photo-affinity linker to make the Quercetin beads (RIKEN). B, HEK291 cells were transfected with the expression plasmid for pcDNA-C/EBPβ. After transfection, cells were treated with of 50 μM Quercetin for 12 h. C, Caco-2 cells treated with 100 μM Quercetin or DMSO (as control) for 12h. Cells harvested and washed twice with PBS. C, Caco-2 cells were resuspended in binding buffer. After cell lysis by homogenization with a syringe, the insoluble material was removed by centrifugation, and the supernatant was collected as a cell lysate. After the cell lysate (1mg of protein) was precleared by incubation with control beads (10μL) for 1 h at 4 ℃, the cleared cell lysate was incubated with Que beads (15 μL) for 12h at 4 ℃.The reacted beads were washed with binding buffer, and the co-precipitated proteins were subjected to SDS-PAGE and Western blotting using antibodies against human C/EBPβ.

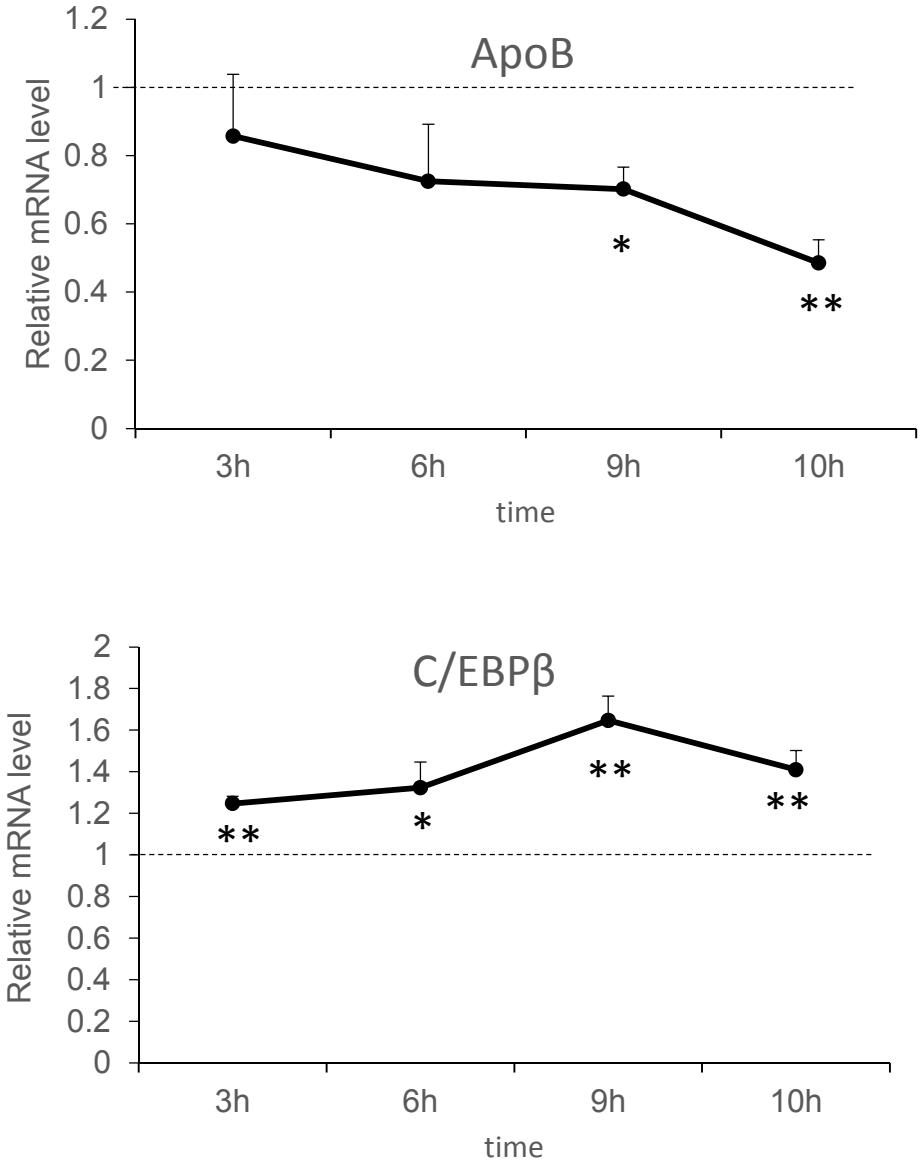


Figure 5-9 The time-course study for Quercetin effect on C/EBPβ.

Caco-2 cells were treated with 100μM Quercetin for 3, 6, 9, 10h, after which total RNA was isolated. mRNA levels were determined by real-time PCR and given as relative expression using36B4 mRNA for normalization. The relative mRNA levels in DMSO treated cells were set to 1. Results are means±S.D. (n =3). *P <0.05, **P <0.01.

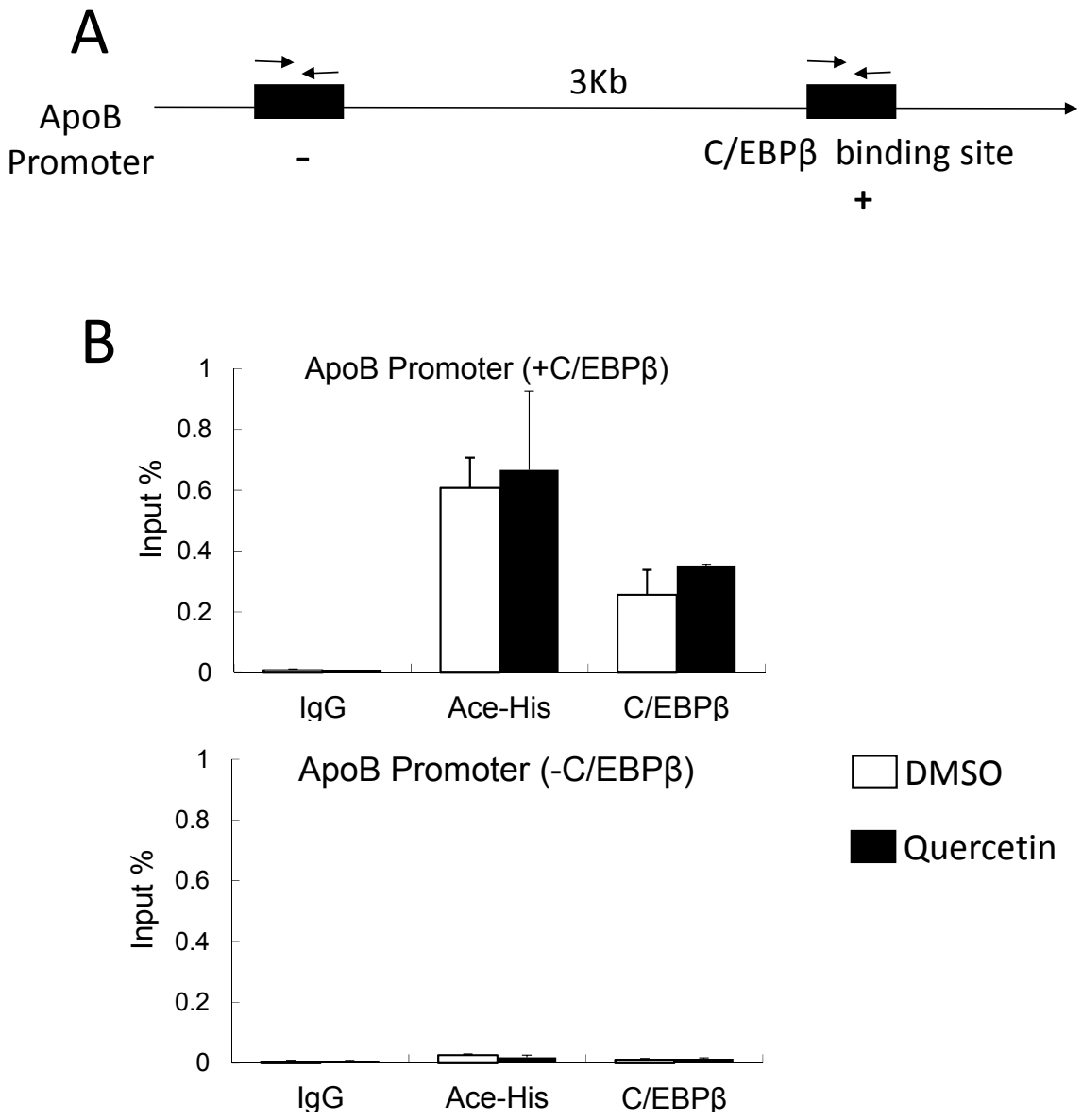


Figure 5-10 Quercetin has no effect on DNA binding activity of C/EBPβ

A, Gene-specific or non-specific primer s for C/EBPβ binding sites. B, Caco-2 cells were treated without or with 100μM Quercetin for 10 h. Then cell were harvested and processed for ChIP assays using the ChIP Assay kit (Upstate) according to the manufacturer's instructions. The antibodies used were: preimmune rabbit IgG as a negative control; anti-acetyl-histone H3 (Lys9) IgG as a possitive control; and polyclonal anti-C/EBPβ IgG. PCR analysis was performed using Real-Time PCR with purified DNA and the primers. Results are means±S.D. (n =3).

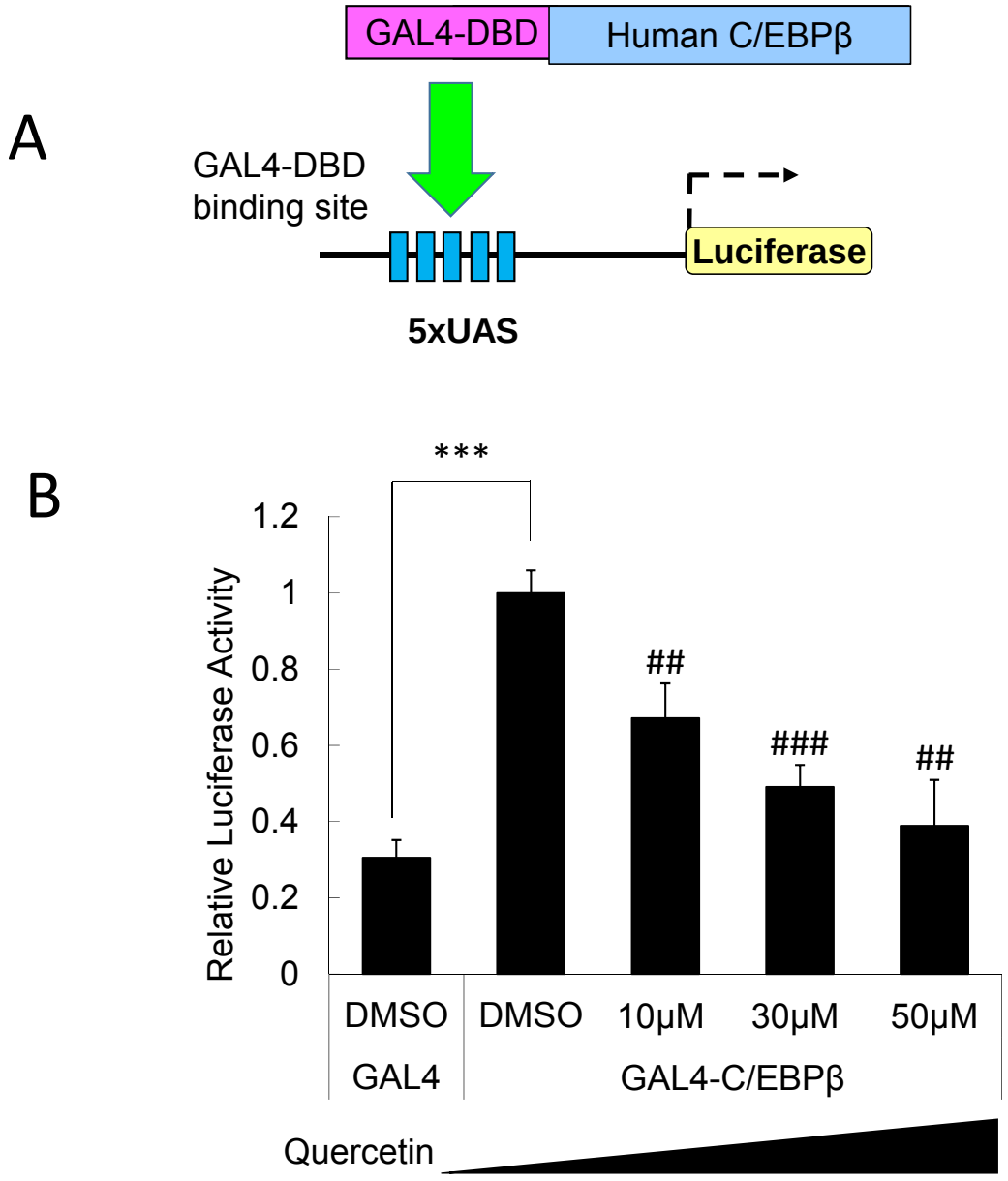


Figure 5-11 Quercetin has effect on the recruitment of co-factor(s)

A. The schematic diagram of GAL4-DBD-C/EBPβ system. B. HEK293 cells were transfected with the report plasmid 5xUAS, expression plasmids for β-galactosidase and GAL4/ GAL4-DBD-C/EBPβ. After transfection, cells were treated with different concentration of Quercetin for 24 h. Luciferase activities were normalized to β-galactosidase activities. The luciferase activities of GAL4-C/EBPβ with DMSO treatment are considered as 1. Results are means±S.D. (n =3). ***P <0.001 vs. in the absence of GAL4-C/EBPβ under DMSO treatment. $^{\#\#}$P <0.01, $^{\#\#\#}$P <0.001 vs. in the presence of GAL4-C/EBPβ under DMSO treatment.

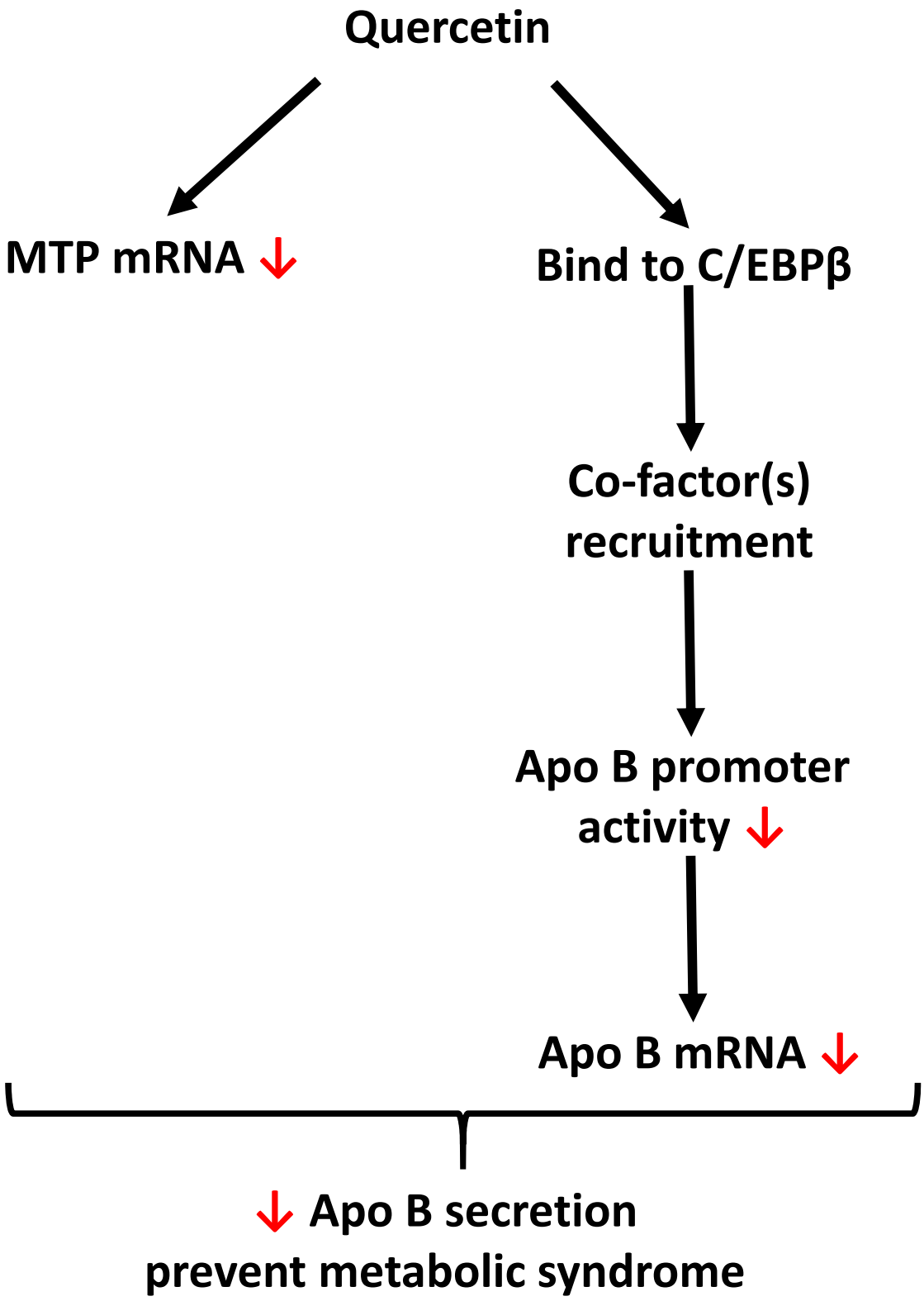


Figure 5-12 Summary of Chapter 5

5-4 Discussion

In this chapter, the inhibitory mechanism of Quercetin for Apo B secretion was investigated. The results were summarized in Fig 5-12. It is clarified as follows.

[I]. Quercetin decreased MTP and Apo B mRNA levels without influence on their mRNA stability.

[II]. Quercetin suppressed Apo B transcription through declined C/EBP activity.

[III]. Quercetin functioned within cells and binds to C/EBPβ directly.

[IV]. Quercetin had no effect on the DNA binding activity of C/EBPβ but reduced the co-factor recruitment required for C/EBPβ activation.

Concerning result [1], Quercetin declined MTP mRNA level in differentiated Caco-2 cells, experiments on mRNA stability using actinomycin D revealed no effect of Quercetin, it is suggested that Quercetin may decline MTP gene transcription. However, the luciferase activity of human MTP gene promoter (-204~+33) was not affected by Quercetin. The region of human MTP promoter between -204 and +33 b contained the responsive elements for HNF4, HNF1, and SRE/IRE (139), whereas located in -499~-473 nt region, there is a responsive site for FoxO1. It seems likely that Quercetin may regulate MTP transcription via FoxO1. MTP expression is induced by FoxO1, and FoxO1 transcriptional activity is dependent on its phosphorylation state. Un-phosphorylated FOXO1 is localized in nucleus, where it binds to the promoter and increases its rate of transcription. However, when FOXO1 is phosphorylated by AKT, it is excluded from the nucleus and degraded. This suggests phosphorylation of FOXO1 by AKT may decrease the transcription of MTP. However, Quercetin induces FOXO1 expression in HSC-3 cells (140). In contrast, it is reported AKT phosphorylation increased after Quercetin postconditioning in myocardial ischemia/reperfusion (I/R) injury rat (141). Hence, it needs further investigation for the relation of Quercetin and FOXO1.

Regarding results [II],[III], and [IV] the trans-activating activity of C/EPβ is stimulated by co-activators and inhibited by co-repressor. The C/EPβ co-activators include transcriptional intermediary factor 1 beta (TIF1β), CBP, and p300. TIF1β functions as a

co-activator for C/EBPβ and is required for induced differentiation in the myelomonocytic cell line U937 (142). CBP and p300 are reported as the co-activator in many researches (143,144). SMRT is as a co-repressor of C/EBPβ (145). The effect of Quercetin on the interaction of C/EBP-β and its co-factor(s) remains further studied.

Additionally, it has been demonstrated that post-translational site-specific phosphorylation of C/EBP-β is an essential mechanism in the regulation of C/EBP-β-dependent gene regulation (69). Phosphorylation of C/EBP-β at Ser299 and Ser277 by protein kinase C or by M-kinase resulted in an inhibition of binding to CCAAT oligodeoxynucleotide *in vitro* (146). Phosphorylation of C/EBP-β by cAMP-dependent protein kinase A or by protein kinase C at Ser240 within the DNA-binding domain resulted in an attenuation of DNA binding (147), whereas phosphorylation at Ser105 by protein kinase C within the activation domain of C/EBP-β enhances its transcriptional efficacy (76). Hence, whether Quercetin has an effect on the phosphorylation of C/EBP-β requires further research.

Chapter 6

Comprehensive discussion

Metabolic diseases such as diabetes, obesity, and dyslipidemia are a rising cause of mortality worldwide. Apo B concentrations, which reflect the number of small, dense LDL particles in plasma, are a significant predictor of cardiometabolic risk. Thus, inhibition of Apo B secretion has the potential to treat the diseases caused by obesity. Flavonoids are thought to have anti-atherosclerotic, anti-inflammatory, and anti-carcinogenic properties but their precise mechanism of action is largely unknown. In this research, I focused on the inhibitory mechanisms of Luteolin and Quercetin on Apo B secretion, the results was summarized as follows:

Luteolin was identified as a component with the inhibitory activity on MTP transcription by screening system, often found in celery, green peppers, and olive oil. Luteolin functioned within cells, and directly bound to HNF4α to decrease the transcription activity through the effect on DNA binding activity and co-factor recruitment, resulting in blocking the transcription and expression of MTP, and decreasing Apo B expression and secretion. *In vivo*, Luteolin had preventive effects against the development of metabolic syndrome, including obesity, fatty liver, dyslipidemia, hyperglycemia, and insulin resistance in mice.

Quercetin functioned within cells, and directly bound to C/EBPβ to decline the transcription activity via regulating co-factor recruitment, resulting in decreasing the transcription and expression Apo B.

6-1 The inhibitory mechanisms and activity of Apo B vary with the kind of flavonoids

Apo B secretion can be regulated by the transcription or translation of MTP and Apo B. In this research, Luteolin was identified from the screening systems of inhibiting MTP promoter activity. But some studies report the different mechanisms of Flavonoids for inhibiting Apo B secretion.

Luteolin significantly decreased MTP promoter activity, whereas Quercetin exhibited no effect. In contrast, in Caco-2 cells, Quercetin lowered C/EBPβ activity, but Luteolin had no effect (data not shown). Daidzein blocked Apo B secretion by decreasing the activity and mRNA level of MTP in HepG2 cells (148), but in my research Daidzein showed no effect on MTP promoter activity. In HepG2 cells, Naringenin and Hesperetin reduced activities of ACAT1 and ACAT2, and a decrease in ACAT2 expression (149). Genistein and daidzein also inhibited the activities of ACAT1 and ACAT2, but not affect the mRNA levels of ACATs in HepG2 cells (148). Taxifolin reduced Apo B secretion by limiting TG availability via DGAT and MTP activity (150). *In vivo*, 3% Naringenin administration increases hepatic fatty acid oxidation related factors (CPT1α, and PGC1α) in $LDLR^{-/-}$ mice fed with a western diet (136), but in this research Luteolin showed no effect. These findings indicate the inhibitory mechanisms of Apo B secretion vary with the kind of flavonoids.

Lin *et al*. investigated the mechanisms of action for blood lipid lowering effects of citrus flavonoids (Hesperidin, Naringin, Tangeretin, and Nobiletin) and their methoxylated analogues in HepG2 cells. Results show that two polymethoxylated citrus flavonoids (PMFs), Tangeretin and Nobiletin, potently inhibited Apo B secretion and modestly inhibited Chol and TG synthesis, without affecting LDL-receptor activity. Other PMFs (e.g., Sinensetin) and non-PMFs (e.g., Hesperetin and Naringenin) had only weak effects on Chol and TG syntheses and Apo B secretion. The structure activity analysis indicated that a fully methoxylated A-ring of the flavonoid structure was associated with a potent inhibitory activity on hepatic Apo B secretion. These findings suggest the inhibitory activity of Apo B vary with the structure of flavonoids (151).

6-2 Flavonoids has the potential as an antagonist of HNF4α

Until now the existence of a ligand for HNF4 is somewhat controversial. HNF4 was originally classified as an orphan receptor that exhibits constitutive transactivation activity apparently by being continuously bound to a variety of fatty acids. It has been reported that fatty acyl-CoA thioesters, a mixture of saturated and cis-monounsaturated C14~18 fatty acids and linoleic acid are endogenous ligands of HNF4α.

It is reported Luteolin exhibited weak partial agonist/antagonist activity. Luteolin inhibited PPARγ several target genes in 3T3-L1 cells and PPARγ-dependent adipogenesis but activated another target gene GLUT4. Interestingly, IL-8 production was increased by the PPARγ antagonist GW9662 and preincubation with Luteolin reversed this effect. The individual effects of Luteolin and GW9662 on IL-8 release are attributable to their respective partial activation and suppression of PPARγ activity. The crystal structure of the PPARγ LBD revealed that Luteolin occupies buried ligand binding pocket (LBP) but binds an inactive PPARγ LBD conformer and occupied a space far from activation helix (H12) consistent with partial agonist/antagonist actions (152).

Many researches reveal that flavonoids are important for regulators of nuclear receptor activity, such as ERα/β (agonists: Daidzein, Genistein, Naringenin), FXR (agonists: EGCG), LXRα (agonists: Hesperetin), PPARα (agonists: Daidzein, Naringenin, Nobiletin, Quercetin, Tangeretin), PPARγ (agonists: Daidzein, Quercetin, Naringenin etc.), but there is not any research on flavonoid as ligands of HNF4α (81).

In this research, it is cleared Luteolin interacted with HNF4α directly, and reduced HNF4α activity. But it is required further research to determine whether Luteolin binds to HNF4α specifically, where the binding site is, and whether other flavones (Apigenin, Acacetin, Chrysin) bind to HNF4α.

6-3 Flavonoids may be an approach for preventing life style-related diseases

With plentiful supplies of inexpensive foods and sedentary jobs, obesity is becoming alarmingly common worldwide. But several of the current drug-based treatments on weight

management are either lack efficacy or with side-effects. Therefore, an opportunity to develop functional foods which are both nutritionally beneficial and also aid in health maintenance. Recently, much attention has been focused on flavonoids that might be beneficial in reducing the risk of obesity and obesity-associated metabolic disorders. This research would provide some evidences for researchers in further investigations, for industries in developing practical health agents, and open an avenue for potential application of flavonoids to health maintenance.

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論文題目

Studies on biological functions of flavonoids that affect the transcription of genes related to lipid metabolism

（脂質代謝関連遺伝子の転写を調節するフラボノイド類の機能解析研究）

Abstract

Chapter 1 Introduction

A worldwide increase in obesity and related chronic diseases is a big issue to be solved. Globally, the prevalence of obesity increased from 4.8% in 1980 to 9.8% in 2008 for men, and from 7.9% to 13.8% in women. Obesity is usually accompanied by cardiovascular diseases, metabolic syndrome, and diabetes. Therefore it is crucial to find an approach to treat the diseases caused by obesity.

Apolipoprotein B (Apo B) concentrations, which reflect the number of small, dense LDL particles in plasma, are a significant predictor of cardiometabolic risk. Over-secretion of Apo B is closely associated with metabolic syndrome, type 2 diabetes, elevated risk of coronary heart disease, and the development of atherosclerosis. Thus, inhibition of Apo B secretion has the potential as one of approaches to treat the diseases caused by obesity.

Flavonoids are common dietary components of vegetables, fruits, wine, and tea, with the function of anti-cancer, anti-allergic, anti-inflammatory, antioxidant, and anti-microbial. Epidemiological studies show that the dietary intake of flavonoid was inversely associated with the mortality from coronary heart disease. In addition, food scientific researches indicate that consumption of flavonoids in foods and beverages may decrease the risk of atherosclerosis by improving the lipid metabolism. However, the precise mechanism is largely unknown.

In this study, I have developed *in vitro* assay systems for screening food components and natural substances that suppress the transcription of microsomal triglyceride transfer protein (MTP), which plays an important role for the assembly and secretion of Apo B containing lipoproteins. Then I focused on the function of Luteolin, which was identified as the strongest flavonoid that suppressed MTP

transcription. On the other hand, it was reported that Quercetin intake was inversely correlated with plasma total and LDL-cholesterol. I analyzed Quercetin functions and found its inhibitory effect on Apo B synthesis.

Chapter 2 Luteolin inhibited Apo B secretion through lowering MTP transcription, which is dependent on reduced HNF4α activity

MTP plays an obligatory role in the stage of Apo B assembly and secretion. Firstly, we screened 156 kinds of food components that suppress the transcription of MTP using the human MTP gene promoter (-204~+33) in HepG2 cells. The results showed that flavones (Luteolin, Apigenin, Acacetin, Chrysin) and flavonols (Fisetin, Kaempferol) reduced MTP transcription activity, but not isoflavones (Genistein, Daidzein). Among all the components, Luteolin showed the strongest inhibition activity. In addition, Luteolin also decreased the MTP mRNA level and Apo B secretion in HepG2 and Caco-2 cells. These results suggested that Luteolin inhibited Apo B secretion by lowering MTP transcription. Next the mechanism of Luteolin was investigated.

The MTP promoter used in the screening system contained a pair of functional responsive elements for HNF4 and HNF1. Mutant analyses revealed that suppression of MTP transcription by Luteolin was observably declined when the MTP promoter had a mutation at the HNF4α-B or HNF1α site. Because HNF1α is a target gene of HNF4α, it is postulated that Luteolin inhibited MTP transcription via decreased HNF4α activity.

To verify the hypothesis, the effect of Luteolin on HNF4α activity was studied. It was observed the luciferase activity increment by overexpression of HNF4α was decreased with the treatment of Luteolin. Besides Luteolin depressed the mRNA level of HNF4α target genes (Apo B, PEPCK, G6Pase, and HNF1α). Surprisingly, Luteolin also lowered HNF4α mRNA and protein levels, resulting from the inhibition of HNF4α self-regulation factors (COUP-TFII and FXR). In order to determine the relationship between HNF4α activity and Apo B secretion, siHNF4α was used in HepG2 cells. The results showed that Apo B secretion reduced by HNF4α knockdown was further decreased with Luteolin treatment. These results suggest that Luteolin decreased MTP transcription by inhibiting HNF4α activity. In contrast, it is different to neglect distinct pathways other than an HNF4α pathway for the inhibitory effect of Luteolin on Apo B secretion.

Chapter 3 Luteolin directly bound to HNF4α to decrease the activity through the effect on DNA binding activity of HNF4α and co-factor recruitment

In this chapter, the mechanism of inhibition of HNF4α activity by Luteolin was studied. It is postulated that Luteolin inhibited HNF4α activity from outside of cells through a specific receptor

(indirect action) or through their direct interaction within cells (direct action). When HepG2 cells were cultured with one of Luteolin glucosides (Luteolin-7-glucoside and Isoorientin), which are thought to be poorly taken up by cells, both of them did not decrease MTP transcription, HNF4α activity, and Apo B secretion. These results suggest Luteolin functioned within cells. Hence, it was thought Luteolin inhibited HNF4α activity through a direct interaction.

HNF4α protein purified from E.coli BL21strain was incubated with Luteolin and subjected to gel filtration. Fractionated HNF4α protein turned yellow, due to the interaction with yellow colored Luteolin, whereas fractionated HNF4α without Luteolin remained colorless. Purified HNF4α protein was also analyzed by the methods of absorption spectrum, trypsin cleavage and BIACORE. Absorption spectrum analysis showed that there was an absorption peak in about 400nm (near the absorption spectrum peak of free Luteolin) only when HNF4α was incubated with Luteolin. Trypsin cleavage analysis showed that Luteolin treated HNF4α protein was resistant to trypsin hydrolysis. BIACORE analysis revealed HNF4α protein interacted with Luteolin. Using the Luteolin conjugated beads, it was observed that HNF4α from HepG2 bound to Luteolin. All the results indicate Luteolin is capable of being associated with HNF4α directly.

Subsequently, ChIP assay showed the DNA binding activity of HNF4α to the target gene promoter region was reduced by Luteolin. GAL4 luciferase assays revealed that Luteolin decreased the HNF4α transcription activity via its ligand-binding domain. This suggests that Luteolin may hinder the recruitment of co-factor(s) required for induction of HNF4α transcription activity.

Chapter 4 Luteolin improved glucose and lipid metabolism in high fat diet induced obese mice

In this chapter the effect of Luteolin *in vivo* on lipid metabolism was studied. 5-week-old C57BL/6 mice were fed with high fat diet (HFD) for 11 weeks to gain weight. Then the mice were separated into 3 groups (n=8) based on their body weights and blood glucose levels, and were fed with their respective diet: HFD diet, HFD+0.6% Luteolin diet, and HFD+1.5% Luteolin diet. 1.5% Luteolin diet markedly reduced total body, liver, abdominal fat, visceral fat, and subcutaneous fat weights. Serum levels of cholesterol (total, VLDL, LDL, and HDL), triglyceride (total, LDL, and HDL), and Apo B (Apo B 100, Apo B 48) were also significantly decreased by 1.5% Luteolin diet. Additionally, 1.5% Luteolin significantly lowered hepatic cholesterol and triglyceride levels. The rise in serum glucose and insulin caused by HFD was significantly suppressed by 1.5% Luteolin while improving glucose tolerance. These results indicate Luteolin improved glucose and lipid metabolism in high fat diet induced obese mice.

Chapter 5 Quercetin decreased Apo B secretion via C/EBPβ

Based on the finding that Quercetin intake was inversely correlated with plasma total and LDL-cholesterol, it was postulated Quercetin may affect gene expression of MTP and Apo B, which are tightly related to plasma cholesterol levels. Firstly, Quercetin declined MTP and Apo B mRNA levels in differentiated Caco-2 and HepG2 cells. Experiments on mRNA stability using actinomycin D (a transcription inhibitor) revealed no effect of Quercetin. Hence, it seems likely that Quercetin may decrease MTP and Apo B transcription. The luciferase assay using the human MTP gene promoter (-204~+33) or the human Apo B gene promoter (-1800~+42) were performed. Quercetin significantly lowered Apo B transcription.

Using several versions of deleted reporter genes, it was found that the region of human Apo B promoter between -73 and -29b was required for Quercetin-mediated suppression of Apo B gene expression. This region contained a C/EBP-binding motif that was conserved among human, mouse, and rat. It has been reported that both C/EBPα and C/EBPβ are crucial factors for Apo B transcription in liver and intestine, respectively. Overexpression of C/EBPα or C/EBPβ significantly increased the luciferase activity of Apo B reporter gene and Quercetin suppressed it. These results suggest that Quercetin inhibit C/EBPα or C/EBPβ activity.

When HepG2 or Caco-2 cells were cultured with Quercetin-3-glucuronide, which is thought to be poorly taken up by cells, no decline in Apo B mRNA level was observed. This result implies that Quercetin, which is efficiently taken up by cells, affects C/EBPβ activities within cells. Using Quercetin-conjugated beads it was observed C/EBPβ from Caco-2 cells bound to Quercetin directly.

Next the mechanism of the inhibitory effect of Quercetin on C/EBPβ activity was investigated by ChIP assay and GAL4 luciferase system. ChIP assay using differentiated Caco-2 cells showed the C/EBPβ association with its binding motif in the Apo B promoter was not affected by Quercetin treatment. In contrast, GAL4 luciferase assay revealed that Quercetin reduced C/EBPβ transcriptional activity in a dose-dependent manner. These results indicate that Quercetin hinders the recruitment of co-factor(s) required for induction of C/EBP transcriptional activity.

Chapter 6 Conclusion

In this research, biological functions of flavonoids that affect the transcription of genes related to lipid metabolism were studied. It is concluded that Luteolin inhibited Apo B secretion through an HNF4α pathway and improved glucose and lipid metabolism *in vivo*. In contrast, the mechanism by which Luteolin directly inhibited Apo B secretion remains to be further investigated. Besides, Quercetin inhibited Apo B gene expression through a C/EBP pathway *in vitro*. These results would provide us a new approach for preventing life style-related diseases and open an avenue for potential application of flavonoids to health maintenance.

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