EFFECT OF ADIPOSE DIFFERENTIATION-RELATED PROTEIN ON CELL PROLIFERATION AND SURVIVAL VIA INSULIN SIGNALING PATHWAY IN HUH7 CELLS

By

Siripun Tipluechar

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree

MASTER OF PHARMACY

Program of Biopharmaceutical Sciences

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ผลของ Adipose Differentiation - Related Protein ต่อการเพิ่มจำนวนและรอดชีวิตของเซลล์ผ่านวิถีการนำส่งกระแสสัญญาณอินซูลินในเซลล์เพาะเลี้ยง Huh7

โดย
นางสาวศิริปุณณ์ ทิพลือชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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Adipose differentiation-related protein (ADRP) regulates triglyceride turnover in hepatocytes and its expression level increases in hepatic steatosis. It has been demonstrated that triglyceride (TG) accumulation in hepatocytes is a causative factor on the development of hepatic insulin resistance. The objectives of this study are to investigate the effect of ADRP on hepatic insulin responsiveness in cells proliferation, survival, extracellular signal-regulated kinases (ERK), and product of the akt protooncogene (AKT) phosphorylation in human hepatoma Huh7 cell line. ADRP expression in Huh7 cells were stably downregulated with short hairpin siRNA. The ADRP protein expression of stable knockdown cells was reduced by approximately 40% compared to control cells. Cell proliferation and survival of these cells were three fold better than Control cells to in medium supplemented with 1% charcoal-stripped fetal bovine serum (CHX-FBS) and two fold in serum-free medium (SFM). Moreover, insulin increases cell viability in ADRP downregulated cells. In addition, insulin-induced AKT phosphorylation of these cells was increased by approximately 2-fold compared to control cells, but no difference of insulin-induced ERK phosphorylation between the two cells. These results suggested that ADRP downregulation increased cell proliferation and survival via phosphatidylinositide-3 kinase (PI3K) cascade, in our model. These findings imply that regulation of ADRP could be a therapeutic target on insulin resistance, metabolic syndromes and cardiovascular diseases.

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Student's signature .....
Thesis advisors' signature ……..…….....

50355202 : สาขาวิชาเภสัชชีวภาพ
คำสำคัญ : ADIPOSE DIFFERENTIATION-RELATED PROTEIN / ภาวะต้านต่ออินซูลิน / ความอยู่รอด / PHOPHATIDYLINOSITOL-3 KINASE / PROTEIN KINASE B

ศิริปุณณ์ ทิพลือชา : ผลของ adipose differentiation - related protein ต่อการเพิ่มจำนวนและรอดชีวิตของเซลล์ผ่านวิถีการนำส่งกระแสสัญญาณอินซูลินในเซลล์เพาะเลี้ยง Huh7. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภก. ผศ. ดร. วิสิฐ ตั้งเคียงศิริสิน. 60 หน้า.

Adipose differentiation-related protein (ADRP) เป็นโปรตีนที่ควบคุมปริมาณไตรกลีเซอไรด์ในเซลล์ตับและพบว่าโปรตีนดังกล่าวมีปริมาณเพิ่มมากขึ้นในโรคไขมันคั่งในตับ ผลการวิจัยพบว่าในเซลล์ตับที่มีการสะสมไตรกลีเซอไรด์จะทำให้เกิดภาวะต้านอินซูลินได้ งานวิจัยฉบับนี้มีวัตถุประสงค์เพื่อศึกษาผลของ adipose differentiation-related protein (ADRP) ต่อการเพิ่มจำนวนเซลล์และการรอดชีวิตของเซลล์ รวมทั้งการทำงานของ extracellular signal-regulated kinases (ERK) และ product of the *akt* protooncogene (AKT) ในเซลล์ตับเพาะเลี้ยง Huh7 โดยการนำเซลล์ Huh7 มาทำให้โปรตีน ADRP ลดลงด้วย short hairpin siRNA พบว่าเซลล์ดังกล่าวมีการสร้างโปรตีน ADRP ลดลงประมาณ 40% เมื่อเปรียบเทียบกับเซลล์ปกติ และเมื่อเลี้ยงเซลล์ที่มีปริมาณ ADRP ลดลงในอาหารทั้ง 2 แบบคือ 1% charcoal-stripped fetal bovine serum (CHX-FBS) และ serum-free medium (SFM) พบว่ามีการเพิ่มจำนวนและรอดชีวิตของเซลล์ดีกว่าเซลล์ปกติเป็นจำนวนสามเท่าและสองเท่าตามลำดับ นอกจากนี้อิซูลินสามารถเพิ่มจำนวนเซลล์ในเซลล์ที่มีปริมาณ ADRP ลดลง นอกจากนี้การศึกษาในระดับโมเลกุลพบว่าเซลล์ Huh7 ที่มีการสร้าง ADRP ต่ำมีการกระตุ้น AKT ด้วยอินซูลินมากกว่าเซลล์ปกติ 2 เท่า แต่ไม่พบความแตกต่างในการกระตุ้น ERK ด้วยอินซูลินระหว่างเซลล์ทั้งสองชนิด ดังนั้นการลดลงของ ADRP จึงทำให้เซลล์มีการเพิ่มจำนวนและการรอดชีวิตผ่าน phosphatidylinositide-3 kinase (PI3K) ในการศึกษานี้ จากผลการทดลองนี้สามารถกล่าวได้ว่าการควบคุมปริมาณ ADRP สามารถเป็นเป้าหมายในการรักษาภาวะต้านอินซูลิน กลุ่มอาการเมตาบอลิกและโรคหลอดเลือดหัวใจได้

สาขาวิชาเภสัชศาสตร์ชีวภาพ บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2554
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รายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ .....

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CHAPTER 1

INTRODUCTION

1. Statements and significance of the problem

Hepatic steatosis or non-alcoholic fatty liver disease (NAFLD), associated with metabolic syndrome, is characterized by the excess intracellular accumulation of lipid droplets in hepatic parenchyma. Hepatic steatosis is the most common liver disease in industrialized countries and becomes continuously increasing in nutrient availability and more sedentary lifestyles, including in Thailand [1-3]. In the United States of America, the prevalence of hepatic steatosis is found in 50-70% of type 2 diabetes patients and 90% of obese people. In addition, it is also seen in children, approximately 10% at age 10-14 years and 18% at age 15-19 years. Sixty percent of them were obese and twenty percent were overweight [1, 4]. The prevalence of hepatic steatosis is continuously increasing, not only in developed countries, but also in developing nations. It was seem to be a major public health concern in Asia-Pacific region, estimated prevalence of hepatic steatosis in this region range from 5 to 30%,[2] and 12 to 24% [3]. Thus, physicians and other healthcare professionals worldwide are well aware of the growing burden of hepatic steatosis. Recently, attention has been focused on the excessive triglyceride accumulation in the liver as a part of the metabolic syndrome. It remains unclear what extent NAFLD is a cause rather than a consequence of the metabolic syndrome. Several evidences indicate that hepatic triglyceride accumulation is a causative factor that involve in hepatic insulin resistance [5]. This fact shows the necessity to improve the knowledge on NAFLD in molecular mechanisms to approach towards prevention and therapeutic possibilities.

Hepatocytes are responsible for maintenance of glucose and lipid homeostasis via insulin regulation. Therefore, hepatic insulin resistance is sufficient to induce several component of metabolic syndromes including hyperglycemia and hyperlipidaemia, and also promote progression to cardiovascular diseases [6]. The precise mechanism of hepatic insulin resistance development is unclear. Strong evidence demonstrates

triglyceride accumulation in hepatocytes, also called hepatic steatosis, initially found in early sign of metabolic syndromes, is a causative factor for development of hepatic insulin resistance [5, 7-10].

Adipose differentiation-related protein (ADRP, ADFP or adipophilin) is discovered by Ginette Serrero and colleagues in 1992 [11]. ADRP is a lipid droplets-association protein, and is ubiquitously expressed in several types and regulates triglyceride turn over not only in hepatocytes but also in adipocytes, monocytes, skeleton muscles, trophoblasts and in several cell types [12-17].

ADRP level is positively correlated with the amount of LDs in the cells [18]. ADFP knockout mice ($ADFP^{-/-}$) does not have any dramatic phenotype. The major effect was seen in the liver, with reduced liver triglyceride content. Furthermore, $ADFP^{-/-}$ mice were found to be resistant to diet-induced hepatic steatosis. There were no effects on adipocyte differentiation or lipolysis. Taken together, ADRP plays a critical role in LDs regulation in hepatocytes

2. Goals and objectives

1) To study the downregulation effect of ADRP on cell proliferation in Huh7 cells.

2) To study the downregulation effect of ADRP on cell survival in Huh7 cells.

3) To study the downregulation effect of ADRP on ERK and AKT phosphorylation responded to insulin activation in Huh7 cells.

3. The research hypotheses

1) Downregulation of ADRP is related to cell proliferation and survival in Huh7 cells.

2) Downregulation of ADRP has effect on the cell proliferation and survival via insulin signaling pathway.

CHAPTER 2

LITERATURE REVIEWS

1 Pathophysiology of hepatic steatosis

Liver is a vital organ in vertebrates that plays a central role in the regulation of whole body energy homeostasis. Usually, liver stores excess energy by converting free fatty acid (FFA) into triglyceride (TG) and stores lipid droplets (LDs) within the cell for energy preservation in absorptive state. Liver hydrolyzes triglyceride into free fatty acid (FFA) which can be secreted into the blood in the form of very-low-density lipoprotein (VLDL) TG for whole body energy preservation.

The pathological condition of abdominal obesity shows visceral adipose tissue (VAT) releases a large number of FFAs and adipokines into the portal vain for direct transport to the liver then increases TG content in hepatocytes. The excessive TG accumulation in liver called hepatic steatosis. The positive correlation between central abdominal obesity and hepatic steatosis has been confirmed, as well as hepatic steatosis, is related to increasing delivery of FFAs [7, 19-21]. The causative factor for hepatic steatosis development is not only the increasing of liver FFA uptake, but also triglyceride content. [22]. Their study shows that triglyceride content accounted for in the liver of NAFLD patients, 60% arise from non-esterified fatty acid (NEFA), a little over 10% from the diet, and close to 30% came from *de novo* lipogenesis, underling the important of fat synthesis in the pathology of NAFLD. In addition, It was shown that hepatic steatosis can be developed by increasing of *de novo* lipogenesis in high-sucrose diet rat [23, 24]; inhibition of fatty acid oxidation with etomoxir, a carnitine O-palmitoyl transferase-1 (CPT-1) inhibitor, in the liver [25]; and increasing of hepatic VLDL production in obese *db* and *ob* mice and microsomal TG transfer protein inhibitor treated mice [26, 27]. Furthermore, liver is an insulin sensitive organ that plays a key role in regulating the whole body carbohydrate and lipid metabolism under tightly metabolic control by circulating insulin.

Hepatocytes carried out gluconeogenesis, glycogenolysis and lipogenesis, which

were activated by insulin. Significant evidences show the positive correlation between hepatic steatosis and hepatic insulin resistance development. It was shown that high-fat diet rat occurred hepatic fat accumulation associated with decreases insulin activation of glycogen synthase and increases gluconeogenesis [8], as well as a hormone-sensitive lipase (HSL) knockout mice reduces TG stores, resulting an increase in suppressive effect of insulin on hepatic glucose production [20] a liver-lipoprotein lipase overexpression increases liver TG content and impairs ability of insulin to suppress endogenous glucose production in mice model [28]. Patients with high-level of intrahepatic triglyceride decreased hepatic insulin sensitivity index [29, 30], and increased endogenous glucose production [7, 19, 31]. In addition, hepatic steatosis inversely correlated with systemic insulin resistance that reported by whole body glucose disposal (Rd) during euglycemic-hyperinsulinemic clamp studies [9], a homeostasis model of insulin resistance (HOMA-IR) and oral glucose tolerance test [32]. Furthermore, the disruption of insulin signaling in hepatocytes induced severe insulin resistance. The liver-specific insulin receptor knockout (LIRKO) mice showed dramatic insulin resistance, severe glucose intolerance, and a failure of insulin to suppress hepatic glucose production, paralleled by hyperinsulinemia form a combination of increased insulin secretion and decreased insulin clearance [33]. In human, it was shown that the increasing of steatosis in hepatocytes decreases metabolic insulin clearance rate [34, 35]. Interestingly, hepatic steatosis does not progress only to hepatic and systemic insulin resistance, but also develop into cardiovascular diseases [36-40].

In summary, hepatic steatosis that initially found in early sign of metabolic syndromes, is a critical role for hepatic insulin resistance development that is progressing to serious complication, such as type 2 diabetes, cardiovascular diseases and others. Thus, the mechanism of hepatic insulin resistance development needs to be further elucidated.

2. Mechanisms of insulin signaling pathway and metabolic disorders

Insulin is an anabolic hormone which produced and secreted from pancreatic β-cells in the responses to the elevated blood glucose in absorptive state. This hormone acts as a plasma glucose regulator by increasing glucose uptake in skeletal muscles

and adipocytes, and inhibiting hepatic glucose production. Insulin also regulates lipid/protein metabolism and mitogenic response and promotes cell proliferation, differentiation and survival. These functions of insulin appear via its receptor activation and subsequently trigger a second messenger through the insulin signaling pathway, as shown in Figure 1 [41].

The receptor of insulin is called insulin receptor. It is a tyrosine kinase receptor. It consists of a heterotetrameric complex protein; two α- and two β-subunits. The α-subunit includes an insulin binding site, whereas β-subunit includes tyrosine kinase domains. In resting state, the α-subunits of the receptor act as a tyrosine kinase inhibitor of β-subunits. Conversely, in the active state, insulin binds the α-subunits, leads to intermolecular autophosphorylation of the two β-subunit drives its structural-conformation change, strongly kinase activity at specific tyrosine residues on the β subunits. These residues act as docking sites for proteins containing phosphotyrosine binding (PTB) domains, including the IR substrates, IRS1 and IRS2 [42].

A number of important proximal substrates include the number of insulin receptor substrate family (IRS 1/2/3/4), the Shc adapter protein isoforms, and Grb-2 associated binding protein 1 (Gab-1). IRS family knockout animal model showed that IRS-1 and IRS-2 play a critical role of physiologic functions. The homozygous IRS-1-knockout mice developed mild state of peripheral insulin resistance and impaired glucose tolerance, as well as exhibition of growth retardation, but it does not led to the development of type 2 diabetes [43, 44]. On the other hand, the homozygous IRS-2-knockout in both mice and mouse

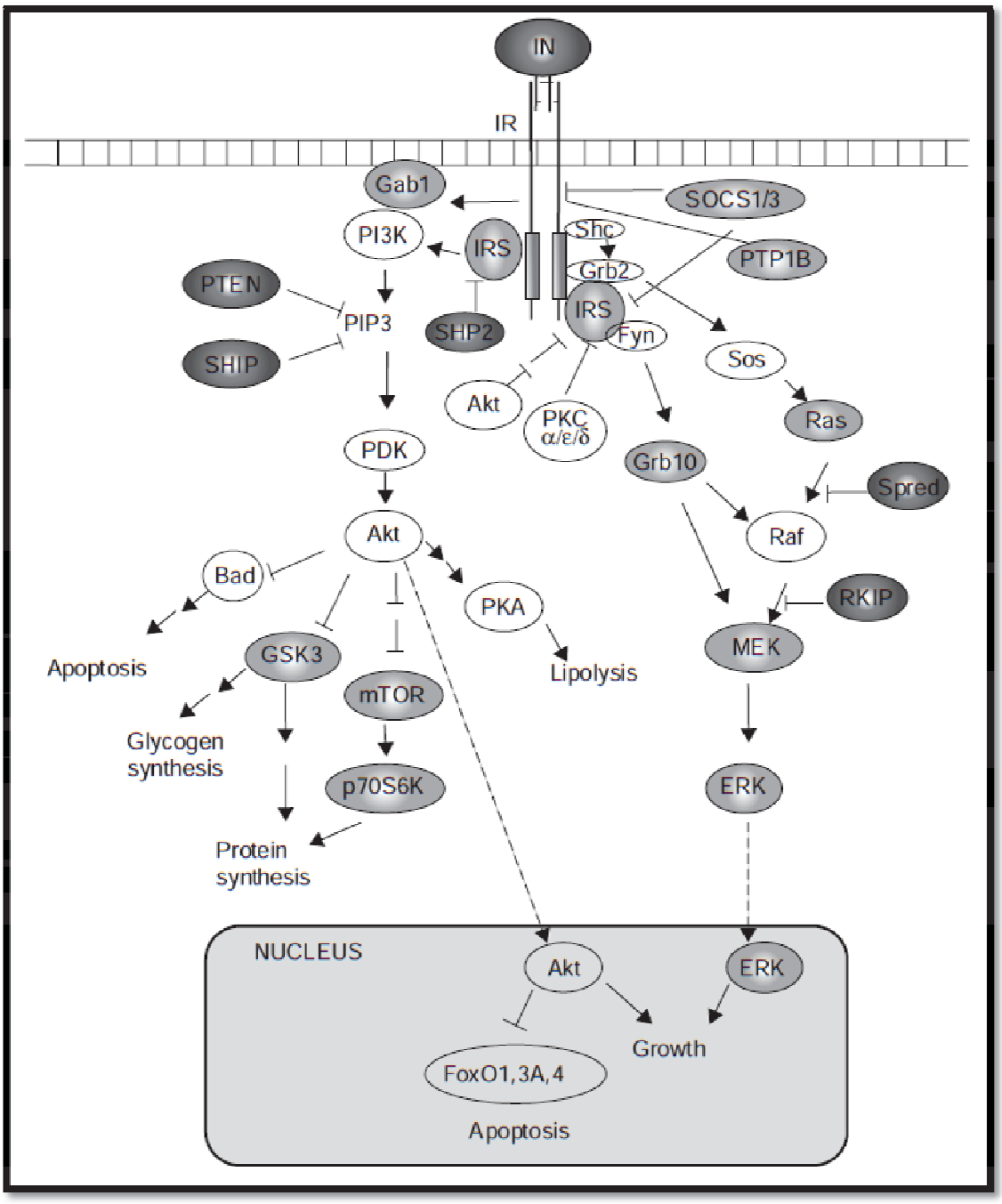

Figure 1 Diagram of insulin signaling pathway.

The major signaling pathway of insulin activation includes PI3K and MAPK cascades [41].

also occurred peripheral and liver insulin resistance associated with decreases β-cells mass and led to development of type 2 diabetes, whereas in mitogenic response of IRS-2$^{-/-}$showed defects growth in only some tissue, including brain, islets and retina [45, 46]. Conversely, IRS-3- and IRS-4- knockout mice do not statistically significant difference in both metabolic and mitogenic responses [47]. Furthermore, tyrosine phosphorylated IRS-1 and IRS-2 interact with Src-homology-2 (SH-2) domain of downstream signaling molecules that are divided into two cascades, including phosphatidylinositol-3-kinase (PI3K) cascade, metabolic regulation, mitogenic responses, and mitogen-activated protein kinase (MAPK) cascade which regulates only mitogenic response. In addition, MAPK cascade is activated not only by IRS-1 and IRS-2, but also by Gab-1 and Shc-Grb-2-SOS complex. Taken together, the major signaling pathway of insulin activation includes PI3K and MAPK cascades.

3. Phosphatidylinositide-3 kinase (PI3K) cascade

PI3K has a pivotal role in the metabolic and mitogenic actions of insulin. In preadipocytes model, the effect of insulin on PI3K cascade promotes glucose transportation, glycogen and lipid synthesis [48]. PI3K is a dimer protein complex that consists of a p110 catalytic subunit and a p85 regulatory subunit. The p85 subunit interacts with tyrosine-phosphorylated motif in IRS proteins. Then, IRS catalyzes tyrosine phosphorylation in p110 subunit [49]. However, PI3K cascade consists of several downstream signaling molecules, including the AGC family of serine/threonine protein kinases [50], guanine nucleotide-exchange proteins of the Rho family of GTPases [51], and the TEC family of tyrosine kinases [52]. Extensive evidence indicated that PKB/Akt, activated by phosphoinositide-dependent kinase 1 (PDK1) which is one of the major targets of PI3K cascade. In addition, PKB/Akt plays a critical role in cell growth, proliferation, survival, glucose and lipid metabolism [53-56]. Mammalian genome encodes three isoforms of PKB/Akt: PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3, which are tissue specific expression. The PKBα/Akt1 mRNA present at low level in pancreas and skeletal muscles [57]. PKBβ/Akt2 mRNA is highly abundant in insulin-responsive tissues such as skeletal muscles, adipocytes, and hepatocytes [58]. PKBγ/Akt3 mRNA levels are high in brain, testis, lung, mammary gland, and adipose tissue. PKB/Akt family is fully activated and exerted its biological function by

phosphorylation at two sites. The first site is the threonine at 308 (Thr308) in PKBα/Akt1 located within the activation loop of the kinase domain. The second site is the serine at 473 (Ser473) located within regulatory phosphorylation hydrophobic motif. The mice model shows that PKBβ/Akt2 deletion produce insulin resistance in insulin-sensitive tissues; skeletal muscles, adipocytes, and hepatocytes [59-61]. The downstream signaling molecules of PKB/Akt are a large number of metabolic and mitogenic regulators. Furthermore, insulin promotes mitogenic response not only via PI3K cascade, but also via MAPK cascade.

4. Mitogen-activated protein kinase (MAPK) cascade

MAPK plays a role in growth factor-induced cell growth, proliferation, and differentiation. The upstream signaling molecule of MAPK cascade is Grb2 [62]. The Grb2 is an adapter protein which contains a SH2 domain. The domain binds to tyrosine phosphorylated motif in the IRS proteins, and two SH3 domains that constutively associated with mSOS, a guanine nucleotide exchange protein that stimulates GDP/GTP exchange on ras proto-oncogene (Ras, $p21^{ras}$) [62-65]. During insulin stimulation, the tyrosine phosphorylation of IRS proteins and/or SH2 domain-containing proteins (Shc) engages Grb2/mSOS. This complex is recruited to the plasma membrane containing Ras for MAPK cascade activation [65]. In MAPK pathway, Ras stimulates a serine kinase cascade though the stepwise activation of Raf, MEK, and ERK. The phosphorylated ERK is subsequently translocated into the nucleus for catalysis the transcription factor which leads a cellular proliferation and differentiation [66]. Mitogen-activated protein kinase kinase (MEK) inhibition by dominant negative mutants or pharmacological inhibitors prevents the stimulation of cell growth by insulin, but has no effect on the metabolic actions of insulin [67].

Insulin signaling pathway does not constitutively activate in both PI3K and MAPK cascades in activated state. Strong evidences suggest that SHP2 mediates opposing signal essential for insulin action. SHP2 is a phosphotyrosine phosphatase with two SH2 domains that is expressed in most mammalian cells [68]. During insulin stimulation, SHP2 binds two tyrosine residues of IRS-1 [69, 70], IRS-2 [71] and also phosphorylate tyrosine of signaling molecules in district pathway such as the insulin

receptor [72]. The result from mice model showed that downregulation of SHP2 increases IRS-1 tyrosine phosphorylation and its associated PI3K activity responses to insulin activation [73]. Conversely, the activation of Ras also requires stimulations of the SHP2. The dominant negative mutant of SHP2 inhibits insulin-stimulated MAPK cascade in intact cells [74-76].

In summary, insulin signaling pathway plays crucial roles in regulating metabolism and mitogenic responses. Thus, this pathway is important in regulating glucose and lipid turnover in insulin-sensitive cells, including adipocytes, skeletal muscles and hepatocytes. also whole body level, as well as tightly associated with cell growth, proliferation, differentiation, and survival. Taken together, the insulin signaling pathway becomes a core of pathophysiology in enormous diseases, such as metabolic syndromes, insulin resistance, type 2 diabetes and cancer.

5. Adipose differentiation-related protein (ADRP)

Adipose differentiation-related protein (ADRP, ADFP, adipophilin or perilipin 2) is a lipid droplet-associated protein. It was isolated and characterized in 1992 by Ginette Serrero and colleagues. The full-length cDNA for ADRP is 1.7 kb and contains an open reading frame of 1375 bp that encodes a 425-amino acid and has a molecular mass of 50 kDa. ADRP mRNA and its protein expression are positively correlated with ongoing adipocytes differentiation, which is localized in microsomal fraction as a membrane-associated protein [11]. ADRP expresses not only in adipocytes, but also in lung, liver, testes, spleen, brain, heart, skeletal muscle and kidney. In addition, immunofluorescence analysis revealed that ADRP is a lipid droplet-associated protein in culture murine 3T3-L1 adipocytes, Chinese hamster ovary (CHO) fibroblast, human HepG2 hepatoma cells and murine MA-10 Leydig cells [12]. During adipocytes differentiation, ADRP mRNA and protein expression increased at the transcriptional level in time- and dose- dependent of long-chain fatty acid activation on preadipocytes [14].

Lipid droplet-associated proteins play a crucial roles in emulsifies intracellular triglyceride and other neutral lipid, which form into lipid droplets (LDs) in aqueous cytosolic. LDs are essential organelles in mammalian cells because of its energy

preservations for cell survival mechanism. The major of lipid droplet-associated proteins called PAT protein family. The PAT family comprises of five members, perilipin (perilipin 1), ADRP (perilipin 2), the tail-interacting protein of 47 kDa (TIP47 or perilipin 3), S3-12 (perilipin 4) and perilipin 5, including myocardial lipid droplet protein (MLDP), lipid storage droplet protein 5 (LSDP5) and OXPAT. They all share a sequence similarity and lipid droplet binding characteristics. However, they show the different tissue distribution, subcellular localization, and lipid binding properties. This protein family is categorized according to protein stability in cytosol. First, constitutively TAG-associated PAT proteins (CPATS), including perilipin and ADRP, are targeted to proteasome for degradation in free form. In general, CAPTS bound to LDs [77, 78]. Second category is stable cytosolic PAT proteins, including TIP47, S3-12, MLDP, LSDP5 and OXPAT. These proteins response to rapid TG synthesis and emergence of new cytosolic droplets [79-81]. Perilipin plays a major role in adipocytes and steroidogenic cells, but ADRP is a ubiquitous CPATS expression in most other tissue [12]. Furthermore, this study shows ADRP expression found on small LDs in preadipocytes and early differentiated adipocytes, but not maturing adipocytes. In contrast, perilipin is absent from early during differentiation, but it is found on small and large lipid droplet at later stages. The function of perilipin in adipocytes has been described as a hydrolysis protecting protein [82], whereas ADRP acts as perilipin analogous in non-adipocytes [83].

In hepatocytes, ADRP and TIP47 proteins usually expressed and localized around LDs in the cells, but theirs expression are difference in ratios. The unique characteristic of LDs localization and the different ratios of these two proteins were due to difference essential molecular segment. However, ADRP and TIP47 were shown the highest similarity with regard to their N-terminal PAT-1 domain, as shown in Figure 2 [81]. The LDs localization of ADRP controlled by the PAT-1 domain, whereas TIP47 were controlled by PAT-2 domain [84]. A molecular segment of protein study shows that the N-terminal 28 amino acids of human ADRP, which contained within PAT-1 domain, are necessary for LDs localization. In addition, the C-terminal region, from amino acid 139 to 437, located in PAT-2 domain, is important in efficient targeting, but PAT-2 domain alone is not capable of directing the ADRP to LDs [85]. In Huh7 cells, it was shown that only 10-20% of the cells appeared TIP47-positive LDs, whereas all cells

present of ADRP-positive LDs. In addition, ADRP knockdown cells shows residual TIP47 presents in the ADRP-negative LDs, whereas ADRP-positive LDs in same cell are devoid of TIP47. Conversely, TIP47 knockdown did not affect ADRP labeling [84].

ADRP level is positively correlated with the amount of LDs in the cells [18]. ADFP knockout mice (ADFP $^{-/-}$) does not have any dramatic phenotype. The major effect was seen in the liver, with reduced liver triglyceride content. Furthermore, ADFP$^{-/-}$ mice were found to be resistant to diet-induced hepatic steatosis. There were no effects on adipocyte differentiation or lipolysis. Taken together, ADRP plays a critical role in LDs regulation in hepatocytes

6. ADRP and hepatic steatosis

In normal human liver, LDs in hepatocytes were positively ADRP and TIP47 stained around the few small LDs. However, hepatocellular steatosis was found not only ADRP and TIP47 but also perilipin. The staining intensity of ADRP and perilipin positively correlated with the LDs amount of the steatotic state. In addition, TIP47 is associated with smaller LDs, whereas ADRP and perilipin are present in larger LDs. Besides, hepatocellular carcinoma cell lines, such as Huh7 and HepG2 shown ADRP and perilipin on LDs, but could not detect TIP47 [86]. It was also shown the difference pattern of ADRP and perilipin expression in hepatic steatosis, perilipin expression was unexpected pattern in late stage of steatosis, whereas ADRP expression is increased gradually along with steatosis progression. The relationship of ADRP localization to progressive nonalcoholic steatohepatitis (NASH), but not perilipin, was found in small lipid droplets, especially in ballooned hepatocytes in NASH, and the frequency of oxidized phosphotidlycholine-positive ballooned cells [87]. Furthermore, ADRP mRNA and its protein is upregulated in hepatic steatosis mice, which is induced by high-fat diet and in *ob/ob* mice, as well as in fatty liver patients [11, 88]. Taken together, ADRP is a crucial role of hepatic steatosis development and progression.

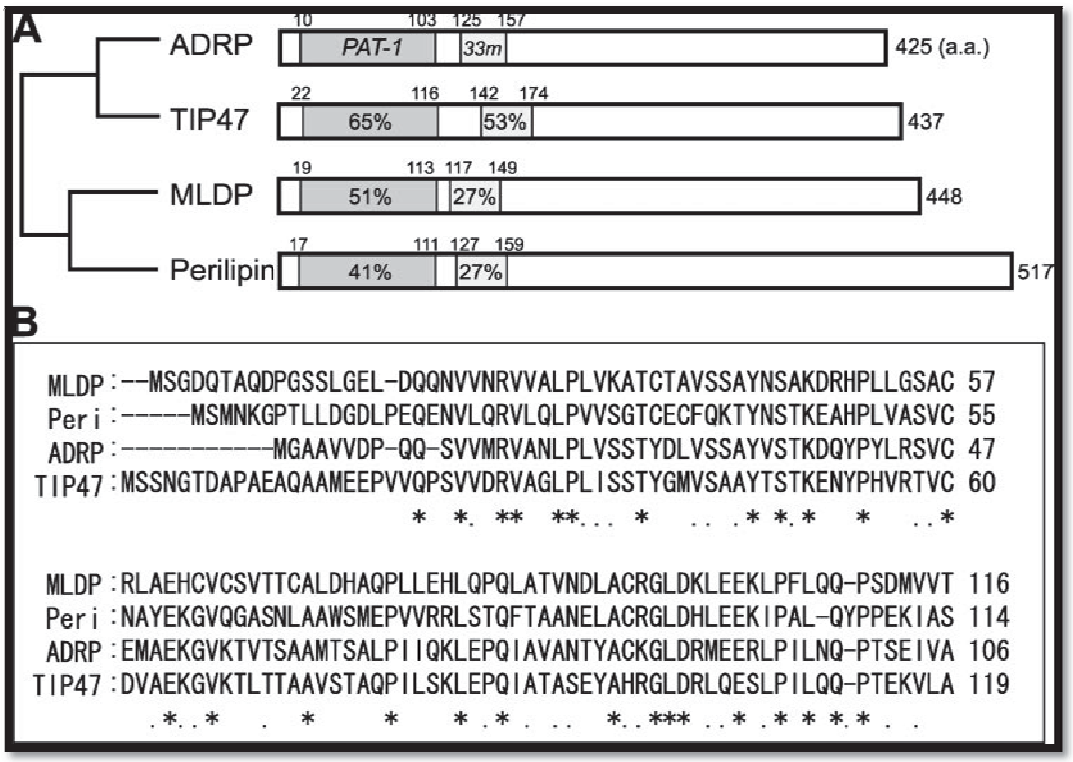

Figure 2 PAT protein family amino acid sequences.

A) A highly conserved region at PAT1 domain of PAT protein family, especially between ADRP and TIP47. B) Amino acid sequence alignment of PAT1 region of MLDP, perilipin, ADRP, and TIP47 [81].

7. Hepatic steatosis and insulin resistance

Insulin resistance, a pathologic metabolic state, is due to impaired action of insulin on whole body glucose homeostasis regulation. High level blood glucose is due to the unresponsive to insulin in multiple organs. The organs include adipose tissues, skeletal muscles, and liver. In physiologic state, glucose is absorbed from intestine into blood and distributed to multiple organs. The β-pancreatic cells uptake glucose and secrete insulin into bloodstream. Insulin increases glucose uptake in adipocytes, skeletal muscles, and liver via glucose transporters (GLUTs), that insulin induces translocate GLUTs to the plasma membrane. Skeletal muscle oxidizes glucose for energy. Liver not only oxidizes glucose but also esterifies FFA to triglyceride for energy storage. The excessive free-fatty acids are uptake into adipocytes. In addition, insulin also inhibits endogenous glucose production of liver and lipolysis of adipocytes, causing the decrease of blood glucose level. Thus, insulin resistance state shows many metabolic disorders in the body. In this state, adipocytes do not response to lipolysis suppressed by insulin causes releasing of FFAs into bloodstream. FFAs are uptaken and esterified to triglyceride by hepatocytes. Strong evidence suggest that triglyceride accumulation in hepatocytes, also called hepatic steatosis, is a causative factor for development of insulin resistance [5, 7-10]

8. ADRP and insulin resistance

ADRP upregulation correlated with steatogenic cells, such as hepatic steatosis [86, 88]; NASH [87]; atherosclerotic plaques [13, 89, 90]; milk-secreting mammary epithelial cells [91]; fatty acids loading trophoblasts [17]; fatty acid loading pancreatic islets [92]; type Ⅰ skeletal muscle fibers, higher capacity to store intramyocellular lipid (IMCL) [93]; pulmonary lipofibroblasts [94]; and malignant tumors [95]. Thus, ADRP plays a role in LDs accumulation in several cell types. However, the mechanism of ADRP on this process is unclear. For instance, recent studies suggest that ADRP-induced LDs accumulation correlated with cellular dysfunction, especially insulin sensitivity disorder [16, 87, 88, 96-100]. Systemic insulin resistance characteristics are cellular function defects in insulin-secreting cells (β-cells) and insulin-responsive cells (myocytes, cardiomyocytes, and hepatocytes) [101-104]. Mice fed with high-fat diet

increases ADRP expression and downregulation of ADRP in this model by antisense oligonucleotides (ASO) suppresses the accumulation of triglycerides in pancreatic islets [92]. In skeletal muscle, ADRP protein content negatively correlated with insulin-stimulated glucose uptake and rosiglitazone-induced downregulation of ADRP is beneficial for insulin sensitivity [16]. Conversely, mice fed with olive or safflower oil showed a higher ADRP expression and a lower insulin resistance index than palm oil [98]. In agreement with this study, obese non-diabetic subjects undergoing weight loss increased ADRP content in skeletal muscle associated with decreased triglycerides and improved insulin action. In addition, ADRP expression in obese treated with troglitazone or metformin was upregulated [99]. However, the positive correlation between accumulation of IMTG and insulin resistance is not absolute. IMTG elevation relationship with improves aerobic capacity and insulin sensitivity in endurance-trained athletes, highly insulin-sensitive subjects [105]. In human diabetic, it was shown that upregulation of ADRP associated with LDs accumulation in hepatocytes [86]. Downregulation of ADRP improved hepatic insulin sensitivity in high-fat diet and leptin-deficient mice [96, 100]. Overall, ADRP may affects the insulin resistance development of LDs accumulation in insulin-responsive cells, such as hepatic steatosis.

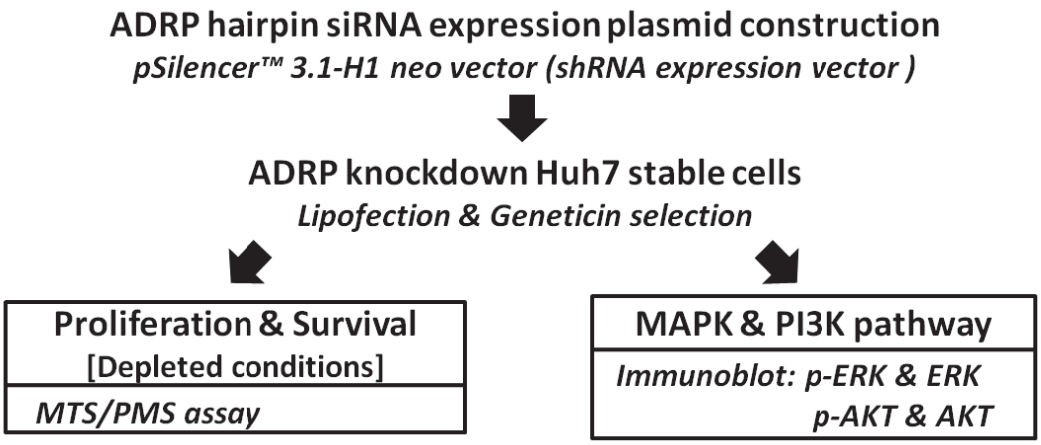

Figure 3 Conceptual framework of this study.

CHAPTER 3

MATERIALS AND METHODS

1. Tissue culture cultivation

The Huh7 cells (the human hepatocellular carcinoma cell line) were maintained in minimum-essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% non-essential amino acid. Cells were incubated at 37°C in a 5% CO_2 incubator. Passage number of cell that was used in the experiments was less than 20, due to phenotypic changes concerning. The 10-cm stock plates were washed once with 10 ml phosphate buffered solution (PBS) and then 0.5 mL of 1X trypsin-EDTA solution was added in the plate for the brief coverage on this surface. The solution was removed and the plate was incubated at 37°C for 15 minutes. Three milliliters of completed medium were added to stop the trypsin-EDTA reaction. The stock plates were cultured in 10-cm plates at a density between 2 x 10^6 to 5 x 10^6 cells per plate. The medium was replaced every 3-4 days. Typically, cells were sub-cultured when it reached to 70-80% confluence of plate surface.

2. Preparation of ADRP-shRNA and Control-shRNA plasmid DNA

ADRP- and Control- shRNA plasmids were prepared according to the protocol from manufacturer's recommendation (Ambion, Austin, TX). First, a shRNA targeted to ADRP mRNA was designed within the coding sequence of ADRP mRNA using siRNA finder program that was available on Ambion webpage. The reference sequence was retrieved from NCBI database. It is named Perilipin 2 (NCBI ID: 001122). A BLAST search with these potential target sequences is performed to ensure that only the ADRP gene was targeted. After shRNA target was selected, the complementary of shRNA template sequences was designed by adding *Bam*HI restricted sequence at 5' end (GAT CC), sense strand (GCT AGA GCC GCA AAT TGC A), loop (TTC AAG AGA), antisense strand (TGC AAT TTG CGG CTC TAG C), RNA polymerase III terminator

(TTT TTT), and *Hin*dIII restricted sequences at 3' terminal (GGA AA), respectively. The sense and antisense shRNA template oligonucleotides were synthesized (Ward Medic, Malaysia). The synthesized oligonucleotides were reannealed in the same microcentrifuge tube. Briefly, the reaction was carried out by adding the following components;

Sense shRNA template oligonucleotides	1	μL
Antisense shRNA template oligonucleotides	1	μL
1X DNA annealing solution	23	μL

The mixture was incubated at 90°C in a water bath for 3 minutes and slowly cooled down in the water bath after turned off about 1-2 hours to room temperature.

The annealed shRNA template insert and Negative-Control insert (non-targeting shRNA, Ambion) were ligated into pSilencer 3.1™ H1 *neo* vector. For ligation, the reactions were carried out by adding the following components;

shRNA or Negative-control inserts	1	μL
Nuclease-free water	6	μL
10X T4 DNA ligase buffer	1	μL
pSilencer vector	1	μL
T4 DNA ligase (5 U/μL)	1	μL

The mixture was incubated at 16°C overnight. The ADRP shRNA- and Negative Control shRNA- pSilencer™ 3.1 H1 *neo* plasmids were named ADRP-shRNA plasmid and Negative control shRNA plasmid, respectively.

Both ligated reactions were transformed into *Escherichia coli* (*E.coli*) strain DH5α by electroporation (Eppendorf) using 1500 voltage. Then, the transformed clones were selected by plating on Luria Bertani (LB) agar containing 100 μg/ml ampicillin and further incubated at 37°C overnight. Three selected colonies were propagated and lyzed to collect the plasmid DNA for identification. The preliminary process was performed by restriction endonuclease digestion with *Bam*HI enzyme and analyzed size of product by electrophoresis in 1% agarose in Tris-acetate EDTA buffer and compared with Negative-Control shRNA plasmid product. After that the correct sized of linearized).

ADRP-shRNA plasmid were verified its nucleotides sequence by DNA sequencing (Ward Medic, Malaysia

3. Stable transfection

The cells were seeded at a density 1 x 10^6 cells/well (6-well plate) in MEM supplemented with 5% FBS and incubated for 16 hours. The cells were washed twice with serum-free medium (SFM). The transfection solution was prepared as followed:

Solution A: Two μg of DNA in 100 μL of SFM

Solution B: Six μL of Lipofectamine2000™ solution in 100 μL of SFM

Solution A and B were gently mixed together and incubated at room temperature for 20 minutes. 800 μL of MEM was added into the complexes. One milliliter of the transfection solution was gently overlaid into each well. The cells were incubated with the complexes for 5 hours at 37°C. One milliliter of 10% FBS supplemented MEM was added to the cells to bring the final concentration of FBS at 5%. The cells were passaged onto 10-cm plate in 5% FBS medium and cultured for 24 hours. The stable transfected cells were selected with 800 μg/ml geneticin (G418) for 3 weeks. After colony selection, the cells were maintained in 200 μg/ml G418 for further experiments.

4. Cell lysate

The cells were washed twice with cold PBS and lyzed in lysis buffer (30 mM Tris-HCl pH 6.8, 10 mg/ml SDS, 1 μg/μL aprotinin, 1 μg/μL pepstatin, 1 μg/μL leupeptin, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1mM benzamidine) on ice. Cell lysates were prepared by sonication five times at 130 watts, 40% output of five seconds each and wait five seconds each, the insoluble material was removed by centrifugation at 12,000 x g, 4°C for ten minutes. The cell lysates were determined protein concentration by Micro BCA™ Protein Assay Kit and determined ADRP expression by immunoblotting technique.

5. Protein quantification by MicroBCA

Protein concentration of cell lysate was determined by Micro BCA™ Protein Assay Kit (Pierce Biotechnology, Bockford, IL). A standard curve was constructed by the following protocol. Bovine serum albumin (BSA) standard solution (2 mg/mL) was diluted with water to 100, 200, 400, 600, 800 and 1000 μg/mL. Ten microliters of each dilution was pipetted into a flat-bottom well microtitre plate containing 90 μL of dH_2O in duplicate. For sample determination, ten μL of protein sample was added in duplicate to the plate with 90 μL of dH_2O. The samples were diluted with dH_2O in the ratio 1:2 before protein quantification. The working reagent was prepared by mixing 50 parts of Reagent A, 48 parts of Reagent B and two parts of Reagent C. 100 μL of the working solution was added to each well, mixed plate thoroughly and the plate was sealed by Saran Wrap and incubated at 50°C for 30 minutes in the hybridization oven. Plate was cooled down at room temperature for ten minutes and the absorbance at 562 nm was measured by microplate reader (Biohit, Finland).

6. SDS-PAGE and immunoblotting

The cell lysates, equivalent to 50 μg total protein, were separated onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) base on method of Laemmil [106] and transferred to polyvinylidene fluoride (PVDF) membrane with transfer buffer (10 mM cyclohexylaminopropanesulfonic acid (CAPS), 10% methanol, pH 11) for two hours. Then, the membranes were blocked with 5% bovine serum albumin (BSA) in PBST buffer (10 mM sodium phosphate pH 7.2; 0.9% NaCl; 0.05% Tween®-20) at room temperature for two hours. The membranes were incubated with primary antibody (rabbit anti-ADRP, mouse anti-GAPDH, rabbit anti-p-ERK (Santa Cruz Biotech (Santa Cruz, CA) or rabbit anti-p-AKT (Cell Signaling Technology, Danver, CA)) in 1% BSA, PBST at 4°C overnight. After the excess-antibodies were washed with PBST five times at room temperature for ten minutes each, the membranes were incubated with secondary antibody (anti-mouse Cy3 or anti-rabbit Cy5 (GE Healthcare, Buckinghamshire, UK)) in PBST at room temperature for an hour. The membranes were washed five times with PBST at room temperature for ten minutes. Then, the membranes were air-dried, followed by detection of fluorescence bands of proteins by

Ettan DIEG Imager (GE Healthcare, Buckinghamshire, UK). In insulin-induced ERK and AKT phosphorylation, the membranes were reprobed with stripping buffer (7 μL/mL β-mercaptoethanol, 2% SDS in PBST) at 50°C for 30 minutes. The membranes were washed three times in PBST for five minutes each. Then, the membranes were incubated with primary antibody (anti-rabbit ERK or anti-rabbit AKT) and followed by the appropriate secondary antibody.

7. Determination of ADRP protein expression

The expression of ADRP protein of ADRP-shRNA and Control- shRNA cells were determined by immunoblotting. The cells were plated at 1×10^6 cells in MEM supplemented with 5% FBS and incubated overnight. Then, the cells were cultivated in SFM for 48 hours. Relative ADRP expression was calculated by using the following equation.

$$\text{Relative ADRP expression (fold)} = \frac{\left(\frac{\text{ADRP intensity of ADRP-shRNA}}{\text{GAPDH intensity of ADRP-shRNA}}\right) \times 1}{\frac{\text{(ADRP intesity of Control-shRNA)}}{\text{(GAPDH intensity of Control-shRNA)}}}$$

8. Preparation of charcoal-stripped FBS (CHX-FBS)

The effect of ADRP on cell proliferation and survival was determined in growth factor depleted medium. This medium was prepared from MEM supplemented FBS that stripped out growth factors by activated charcoal particle. Briefly, the charcoal, placed in a Pyrex glass tube, was activated by heating on Bunsen burner until the particles tune red. Then one gram of activated charcoal was added in each 100-mL FBS. The mixture was rolled at 55°C for 30 minutes in a hybridization oven. The activated charcoal particle was removed by centrifugation at 2000 g for 30 minutes at 4°C and filtered with a 0.2 μm pore size cellulose nitrate filter membrane. The CHX-FBS was aliquoted and stored at -20°C until use. Working serum was kept at 4°C.

9. Measurement of cell viability

The effect of ADRP on cell proliferation and survival in deprived medium was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/Phenazine methosulfate (PMS) colometric assay (Promega, Madison, WI). The concept of method was to detect the absorbance of MTS-formazan, which produced from bioreduction of MTS within viability cells, in cultivated medium. Briefly, the assay consists of two components, MTS and PMS solutions freshly mixed. PMS solution was prepared to a final concentration to 0.92 mg/mL and stored in −20°C, and protect from light. MTS was freshly prepared in PBS solution to final concentration two mg/mL and protected from light. At time of the experiment, the cells were preformed in 100 μL cultured medium by using 96-well plate. Twenty μL of MTS/PMS combined solution, including 20 μL MTS and 1 μL PMS solutions, was adding in each well and mixed thoroughly with the medium. The 96-well plate was incubated at 37°C for 90 minutes. Finally, the MTS-formazan products were detected by a spectrophotometer at wavelength 490 nm in a microplate reader (Biohit, Finland).

Bioreduction

PMS (electron coupling agent)

MTS-tetrazolium ----------> MTS-Formazan

(slightly yellow) (dark red, soluble)

10. Cell optimization study

The cell numbers were used to determine the cell proliferation and survival in the experiment. Cell type and cultivated condition are the major factors that affect MTS reduction to be MTS formazan product. Therefore, standard curve that represented relation between absorbance and viable cells was developed for further study. The cells were seeded onto 96-well plate in MEM supplemented with 1% CHX-FBS at 0, 3125, 6250, 12500, 25000, 50000, and 100000 cells for overnight. Then the absorbance was measured by MTS/PMS assay. Regression equation of the relationship between cell number and absorbance was constructed.

11. Determination of cell proliferation and survival

To determine the effect of ADRP on insulin induced proliferation, cells were seeded at 1 × 10^4 cells onto 96-well plates in MEM supplemented with 1% CHX-FBS medium for 24 hours. The cells were treated with 0.1, 1, 10 μg/ml insulin or without insulin for 3 days in 1% CHX-FBS or serum-free medium (SFM). The cell viable was determined by MTS/PMS assay. Each time points were performed in triplicate. Relative ADRP expression was calculated by using the following equation.

$$\text{Relative cell number (fold)} = \frac{\text{(cell number at each point)} \times 1}{\text{cell number of Control-shRNA at 1\% CHX-FBS or SFM}}$$

12. Determination of long term proliferation and survival assay

The further investigation the effect of ADRP on long term viability was determined in 5% CHX-FBS medium up to 8 days. The cells were seeded at 1 × 10^4 cells onto 96-well plates in MEM supplemented with 5% CHX-FBS medium for 24 hours. Then, the medium were replaced with 5% CHX-FBS medium supplemented with 10 μg/ml insulin or without insulin for 2, 4, 6 and 8 days. The medium were refreshed at day 4^{th}. The cell numbers were determined by MTS/PMS assay. Each time point was performed in triplicate.

13. Determination of ERK and AKT phosphorylation

In order to determine the effect of ADRP on insulin induced ERK and AKT phosphorylation, cells were plated onto 6-well plates at a density of 1 × 10^6 cells/well in MEM supplemented with 1% CHX-FBS, and 2 mM L-glutamine. After 24 hours of incubation, the cells were washed with PBS for three times. The insulin signaling pathway was performed after serum-starvation in SFM for 48 hours. The cells were activated with 1 μg/ml insulin for 0, 5, 15, and 30 minutes. Insulin-activated reaction was stopped in ice-cooled PBS, followed by cell lysate preparation, as described in materials and methods; cell lysate. Relative ADRP expression was calculated by using the following equation.

$$\text{Relative p-ERK expression (\%)} = \frac{\left(\frac{\text{p-ERK intensity at each point}}{\text{ERK intensity at each point}}\right) \times 100}{\frac{\text{(p-ERK intesity of Control-shRNA at 0 minute)}}{\text{(ERK intensity of Control-shRNA at 0 minute)}}}$$

$$\text{Relative p-AKT expression (\%)} = \frac{\left(\frac{\text{p-AKTtensity at each point}}{\text{AKT intensity at each point}}\right) \times 100}{\frac{\text{(p-AKT intesity of Control-shRNA at 0 minute)}}{\text{(AKT intensity of Control-shRNA at 0 minute)}}}$$

14. Statistical analysis and data presentation

Experiments were performed in at least triplicate manner and repeated at least twice. The results were showed as mean ± SEM as indicated. Student's two-tail *t test* was used to determine different between two groups. Significance was accepted at *p*-value < 0.05.

CHAPTER 4

RESULTS

1. Hepatic insulin sensitivity and ADRP knockdown

To investigate the role of ADRP in hepatic insulin sensitivity, the Huh7 cell, an human hepatocellular carcinoma cell line was used. The Huh7 cells were derived from human hepatoma and have a large number of lipid droplets (LDs) under standard culture conditions. These LDs were positive for ADRP in all cells [107]. In addition, ADRP expression in Huh7 cells is enough to be a model of human liver tissue with severe steatosis [86]. The hepatocytes developed in this study expressed ADRP below the baseline level. These characteristics are advantage in development of a constitutively harbor of low level ADRP in hepatoma. In summary, the model systems in this study contain low and high (assumed from normal cells) baseline level ADRP expression in Huh7 cells. To investigate the effect of ADRP on hepatic insulin resistance, these cells were constructed by stable transfection of shRNA-ADRP. The technical concept was performed by transfecting recombinant plasmid DNA into cells, which constitutively produces shRNA to destruct the target mRNA, resulting in lower amount of the target protein.

First, a 21-oligonucleotides strand was selected within the coding sequences of ADRP mRNA strand. As shown in Figure 4, ADRP siRNA in this study was flanked between nucleotide position 211 and 231.

In Figure 5, siRNA strand was used for shRNA design. The sense and antisense siRNA template complementary oligonucleotide strands were synthesized. The hairpin siRNA template insert was constructed by reannealing both synthesized DNA strands in the same microcentrifuge tube and ligating with linear-pSilencer™ 3.1 H1 *neo* vector. Negative Control shRNA plasmid, provided in pSilencer™ 3.1 H1 *neo* kit, was used as a control. These constructions were transformed into in *E.coli* strain DH5α by electroporation. The transformed cells were selected and propagated in ampicillin-containing medium. Constructed plamids were examined in preliminary correction by

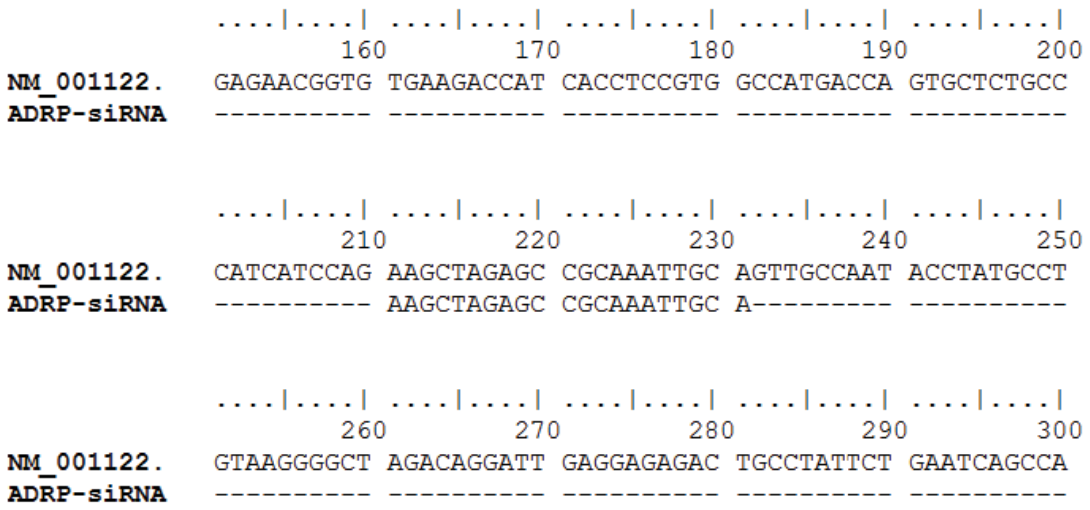

Figure 4. Selection of siRNA target site.

ADRP siRNA target site locates on coding sequence of ADRP mRNA.

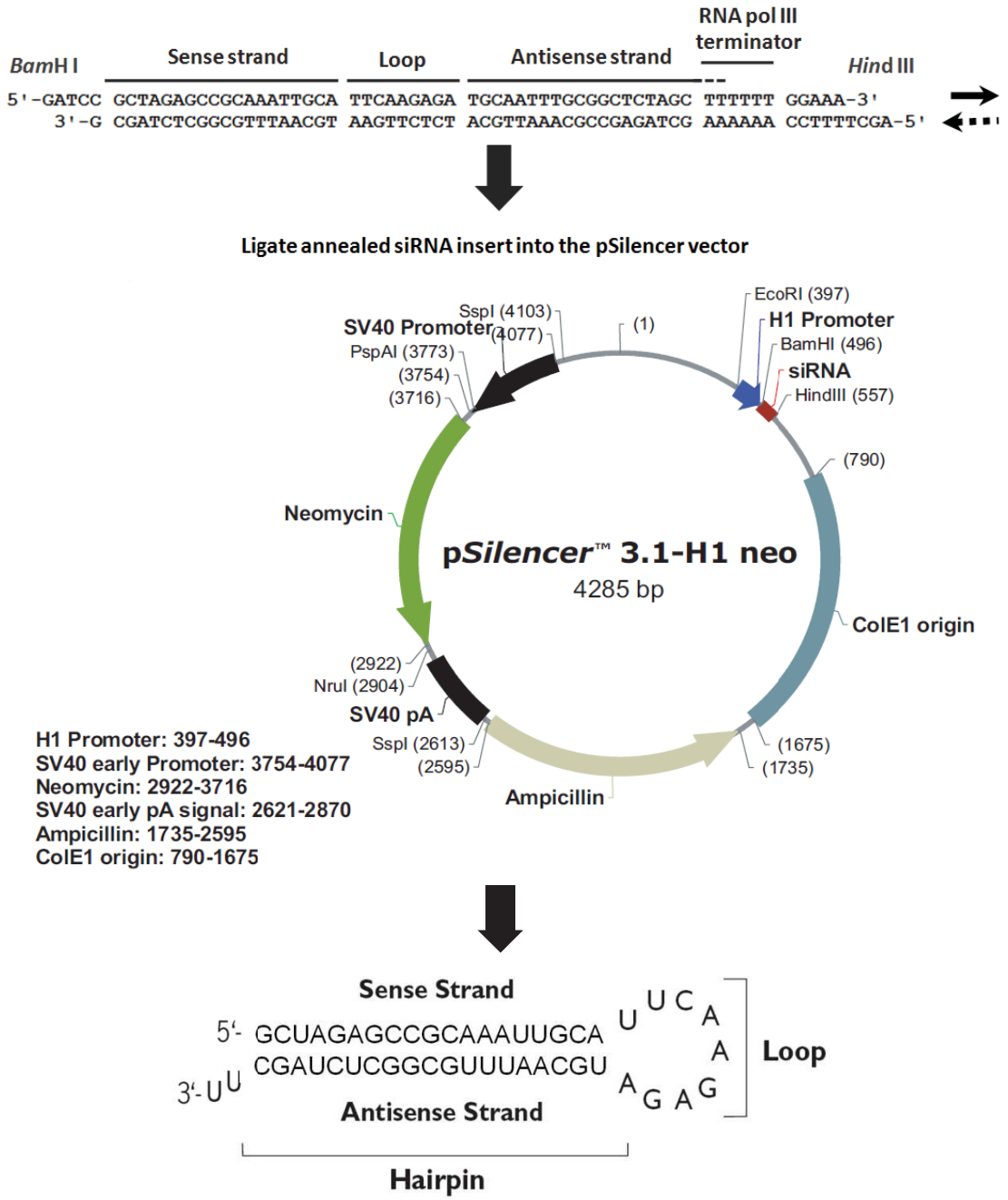

Figure 5. Short hairpin siRNA template design.

Design and cloning of short hairpin siRNA (shRNA) insert into pSilencer vector.

restriction digestion. As shown figurein Figure 6, the constructed plasmids: ADRP-shRNA plasmid clone 1 (C1), ADRP-shRNA plasmid clone 2 (C2), ADRP-shRNA plasmid clone 3 (C3), and Negative Control plasmid (NC) were single site cut with *Hin*dIII restriction enzyme. The corrected size was 4.3 kb. Then, the sequences of hairpin siRNA template insert within the plasmid were confirmed by oligonucleotides sequencing technique, as shown in Figure 7. The purified ADRP shRNA plasmid were transfected into Huh7 cells. Finally, the colonies of stable-transfected cells have been selected with 800 μg/ml G418 for 3 weeks. The stable knockdowned ADRP Huh7 cells (ADRP-shRNA) were larger than the stable Negative Control Huh7 cells (Control-shRNA), as shown in Figure 8.

2. Expression of ADRP in ADRP-shRNA and Control-shRNA cells

After cell selection, ADRP protein expression level was compared in ADRP-shRNA and Control-shRNA cells by immunoblotting technique. As shown in Figure 9, ADRP expression in ADRP-shRNA cells were knockdowned approximately 40%, compared to Control-shRNA cells. In other study, ADRP expression has been knockdowned approximately 45-50% by shRNA [107].

3. Viable cells determination by MTS/PMS assay

Cell number was determined by MTS/PMS assay. The viable cells converted MTS/PMS into MTS/formazan product, which was detected by spectrophotometer at 490 nm. The standard curve between absorbance and cell number was optimized for cell number reporting. As shown in Figure 10, the result showed a linear-response relationship between cell number and absorbance at 490 nm. The correlation coefficient of the line was 0.998.

4. Cell proliferation and survival assay in ADRP-shRNA and Control-shRNA cells

ADRP-shRNA and Control-shRNA cells were determined mitogenic response to insulin. The proliferation assay was performed in insulin-depleted medium, using CHX-FBS instead of FBS. CHX-FBS is adapted-serum to reduce large non-polar molecules, such

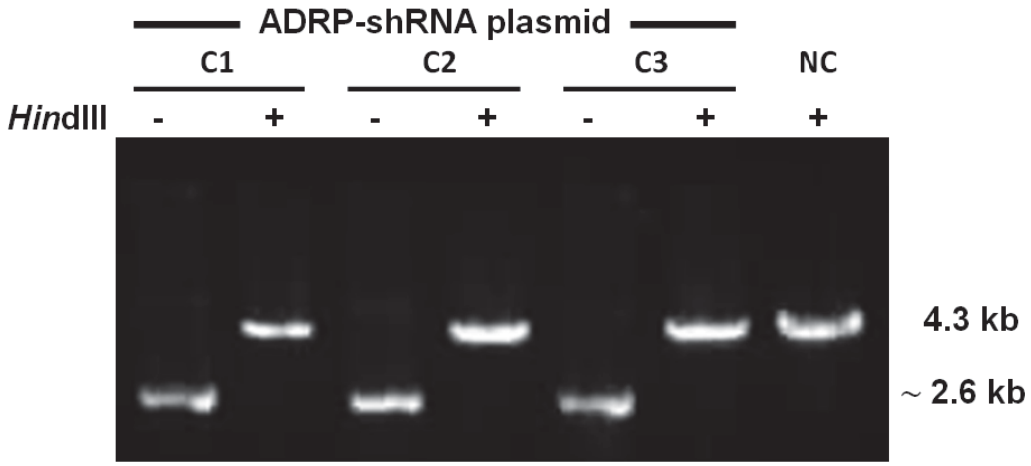

Figure 6. Determination of DNA size.

Restriction digestion analysis of ADRP-shRNA plasmid; clone 1 (C1), clone 2 (C2), clone 3 (C3) and Negative Control-shRNA plasmid (NC), the plasmids were digested with *Hin*dIII. Digested-DNA strands were determined molecular size by 1% agarose gel electrophoresis.

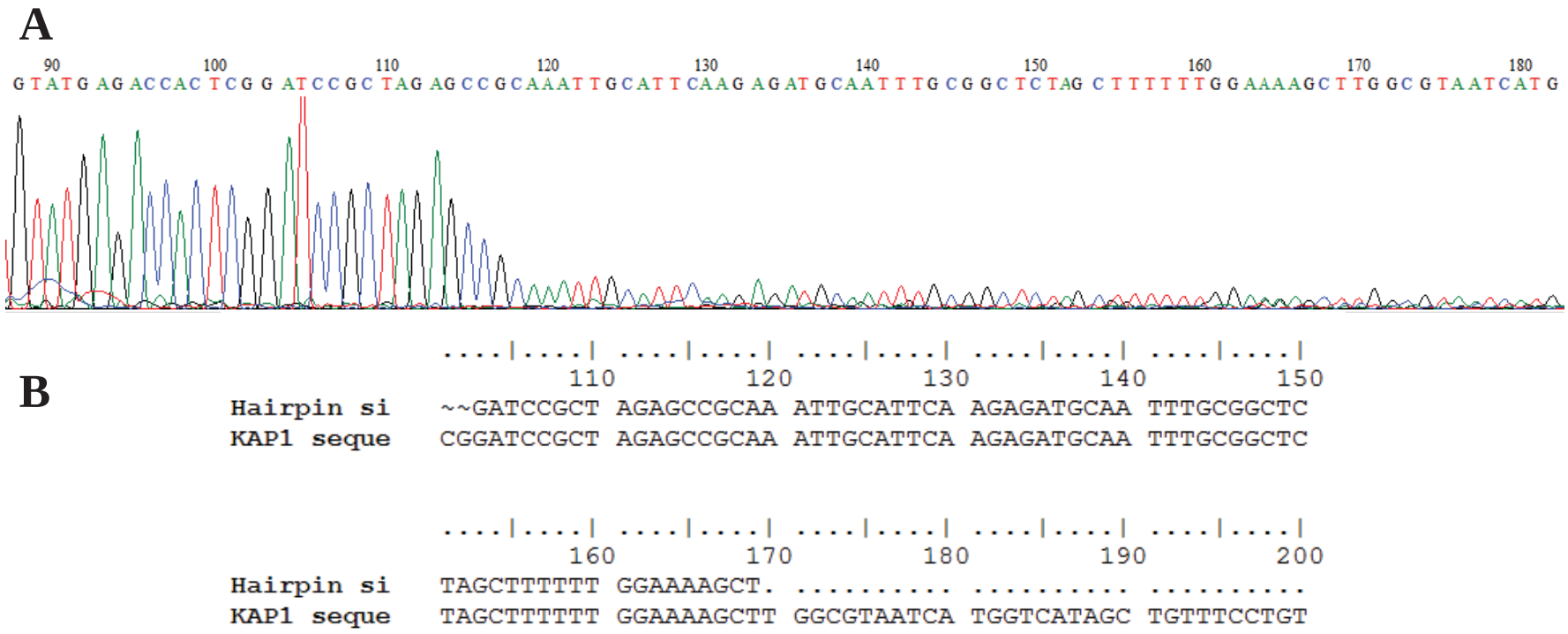

Figure 7. Oligonucleotides sequences of the ADRP-shRNA insert in pSilencer™ plasmid.

ADRP-shRNA plasmid was confirmed the corrected sequences by DNA sequencing. A) The sequencing signal of ADRP-shRNA plasmid clone 1 (KAP1) with M13F (-29) primer. B) The ADRP hairpin siRNA sequences were aligned with KAP1.

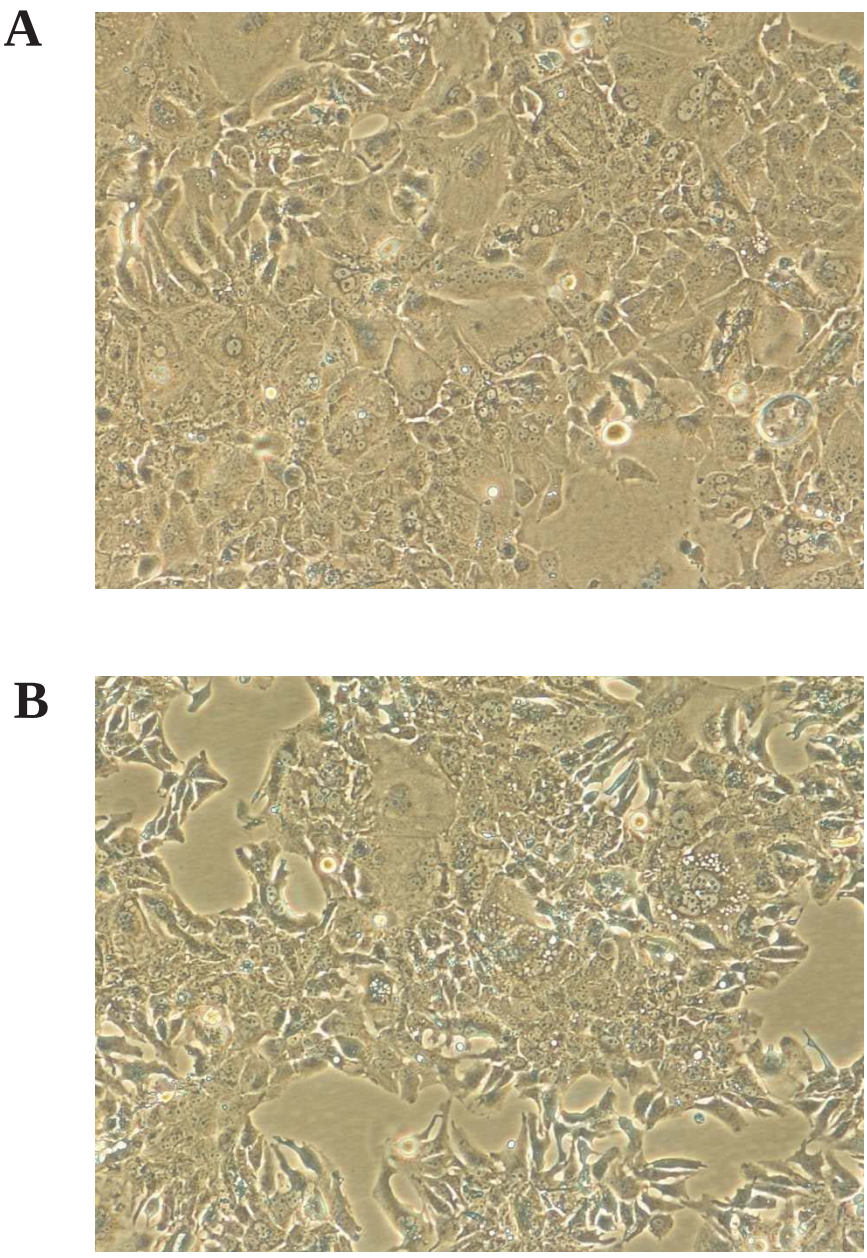

Figure 8. Cell morphology of ADRP-shRNA and Control-shRNA cells.

Cells were cultivated in MEM supplemented with 5% FBS for 48 hours. Cells were visualized under a phase contrast microscope, magnification was 400X. A) ADRP-shRNA cells, B) Control-shRNA cells.

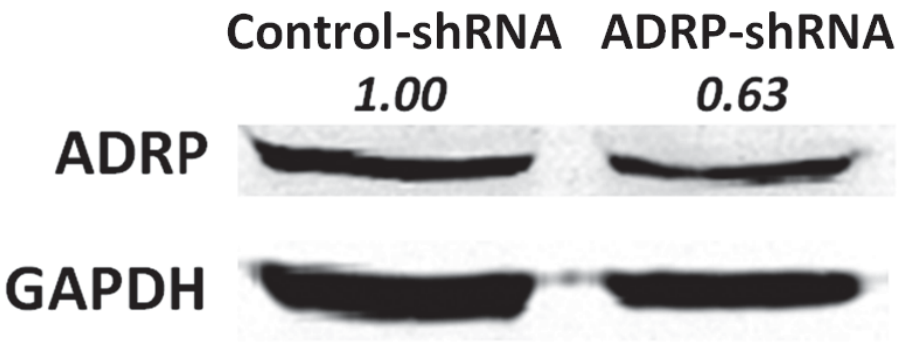

Figure 9. Immunoblotting of ADRP protein expression in ADRP-shRNA and Control-shRNA cells

Cells were cultivated in SFM for 48 hours. Total protein of cell lysate was prepared and quantified by MicroBCA method. Then, ADRP expression was determined in the 50 μg total proteins by immunoblotting technique and GAPDH was an internal control. Western blot image was quantified by ImageQuant TL software and expression of ADRP was normalized with GAPDH expression. ADRP expression is represented as relative ratio (fold), compared to Control-shRNA cells.

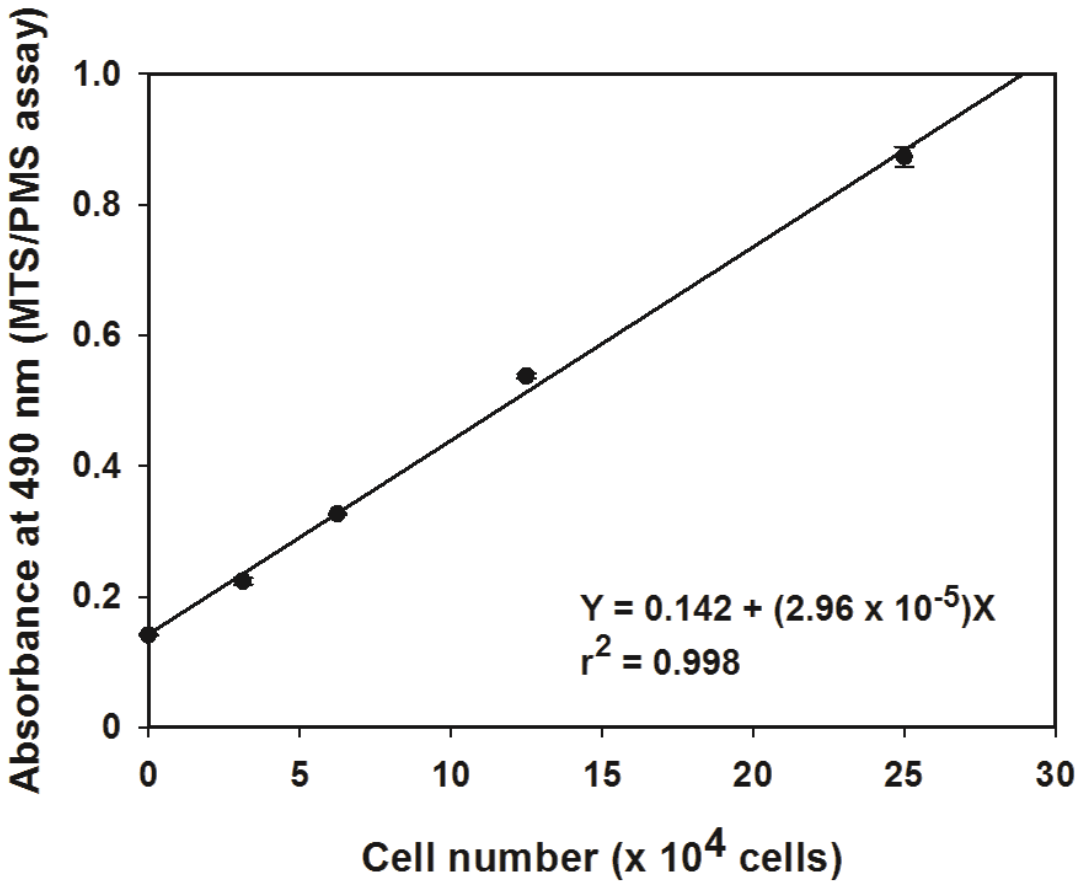

Figure 10. Cell number optimization.

Various cell numbers of ADRP-shRNA and Control-shRNA cells were plated in 96-well plate and were cultivated with MEM supplemented with 1% CHX-FBS for 20 hours. MTS/PMS assay was performed by reading the absorbance at 490 nm. The correlation coefficient between cell number and absorbance at 490 nm was 0.998. The equation, which presented on graph, was using to calculate the relative cell number in this study.

as hormones, cytokines, and growth factors, including insulin in FBS by activated charcoal. The advantage of CHX-FBS in this study was to minimize the amount of insulin and other growth factors [108]. This study was performed in 1% CHX-FBS in supplemented medium with or without insulin for 3 days. As shown in Figure 11A), the cell number of Control-shRNA cells was increased in response to insulin activation. Conversely, the cell numbers of ADRP-shRNA cells in the presence or absence of insulin were similar. Interestingly, the cell number of ADRP-shRNA cells was approximately 3-fold more than Control-shRNA cells in 1% CHX-FBS condition. However, both of them were no difference in 5% FBS condition.

Furthermore, ADRP-shRNA and Control-shRNA cells were preformed in SFM for 3 days. As shown in Figure 11B), the cell number of Control-shRNA cells in SFM condtion was approximately 2-fold lower than ADRP-shRNA cells. These results indicated that the proliferation and survival of ADRP-shRNA cells were better than Control-shRNA cells in depleted medium.

5. Long term proliferation assay in ADRP-shRNA and Control-shRNA cells

In previous experiment, cells with low baseline ADRP level had the ability to survive in nutrient-deprivation, but there was no difference between presence and absence of insulin supplemented medium, whereas a high-level baseline ADRP expression cells were difference response in insulin additive. It was possible that nutrient-deprivation medium was enough for short term survival of ADRP-shRNA cells. Thus, the next experiment was going to determine cell proliferation response to insulin activation in long term duration up to 8 days. In this experiment, the cultivation medium was changed from 5% FBS to 5% CHX-FBS to maintain the cells for long term study. In this medium, long term proliferation assay up to 8 days was investigated. As shown in Figure 12, the cell number of Control-shRNA and ADRP-shRNA cells was increased continuously until day 6 then the cell number of them was decreased. The cell number of ADRP-shRNA cells was more than that of the Control-shRNA cells in all time points. Interestingly, ADRP-shRNA cells were proliferated continuously up to 8 days in insulin adding condition, whereas in absence insulin medium, the proliferation was decreased. This study indicated that ADRP-shRNA cells were more insulin responsiveness in proliferation than in the control cells.

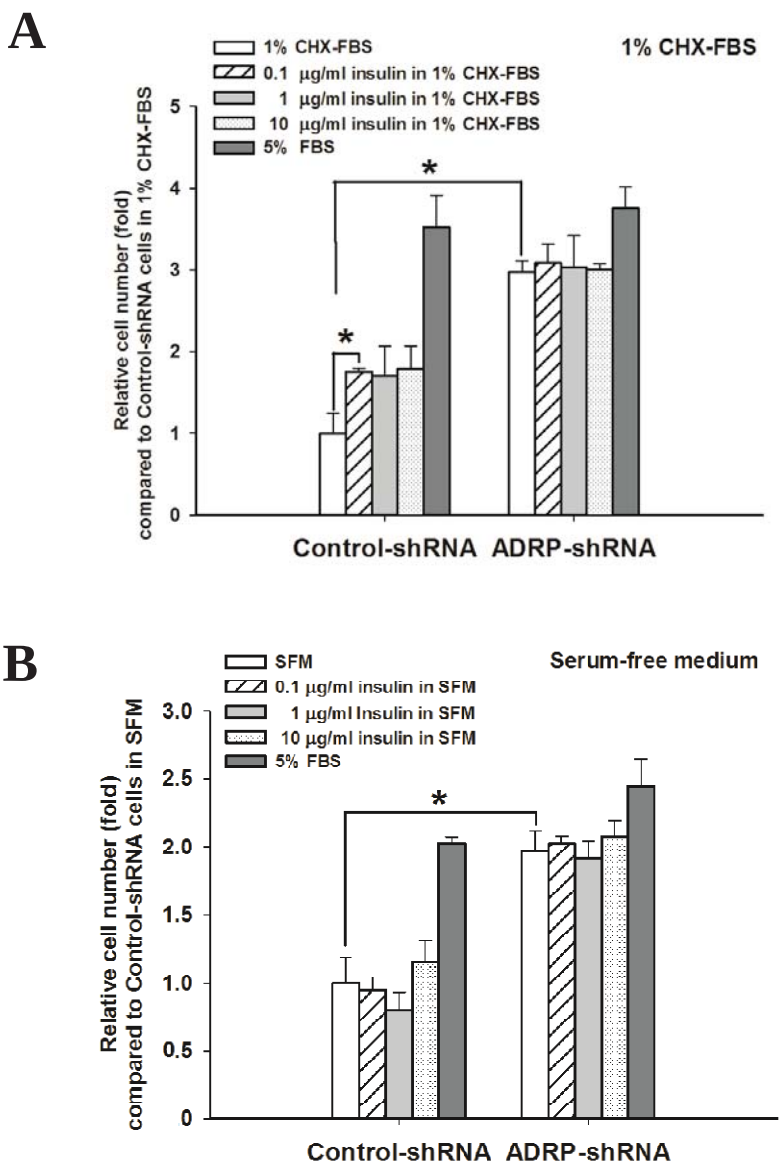

Figure 11. Cell number of cell proliferation and survival assay in ADRP-shRNA and Control-shRNA cells.

ADRP-shRNA and Control-shRNA cells were seeded onto 96-well plate in MEM supplemented with 1% CHX-FBS for 24 hours. The cells were treated with or without various concentrations insulin in refresh medium, A) 1% CHX-FBS, B) serum-free medium as negative control condition. The cells were cultivated for 3 days before determined relative cell number by MTS/PMS assay. Experiments were preformed in triplicates, ∗; $p<0.05$. Raw data is shown in Appendix.

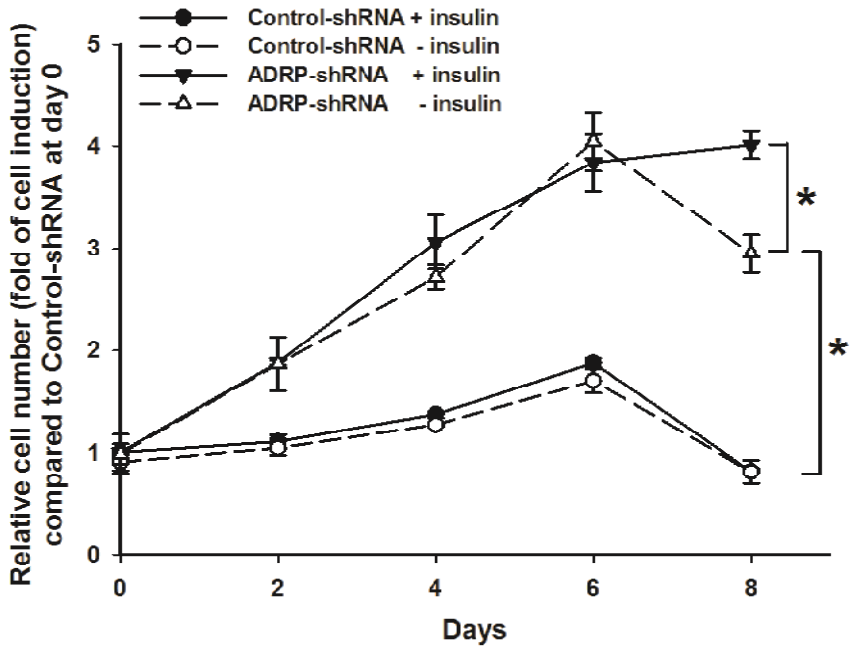

Figure 12. Cell number of long term proliferation study in ADRP-shRNA and Control-shRNA cells.

ADRP-shRNA and Control-shRNA cells were seeded onto 96-well plate in MEM supplemented with 5% CHX-FBS for 24 hours. The cells were treated presence or absence of 10 μg/ml insulin in refresh medium. The relative cell number was determined by MTS/PMS assay at 2, 4, 6 and 8 days. The medium were refreshed in the 4^{th} day. Experiments were preformed in triplicates, *; $p<0.05$.

9. ERK and AKT phosphorylation assay

Since ADRP-shRNA cells better survives in depleted medium, further experiment is to investigate the effect of ADRP depletion on insulin activation via both MAPK and PI3K pathways. ERK and AKT are second messenger proteins which are activated by MEK and PI3K protein on MAPK and PI3K cascade, respectively. Phosphorylation of these proteins has been widely used for investigation of insulin sensitivity. The concept of this study is phosphorylation activation is rapidly (within minutes) and time-dependent manner. Thus these cells cultivate into serum-free medium for 48 hours. These cells were not entered into S phase, but starved at G_0/G_1 phase in this condition. After insulin activation, the ERK and AKT phosphorylation signal via growth factor stimulation were activated at the same time, which is advantage in protein phosphorylation study. As shown in Figure 13, insulin can activate ERK phosphorylation in both cells at 5 min, while phosphorylated ERK (p-ERK) was not different between Control-shRNA cells and ADRP-shRNA cells at all time points. On the other hand, insulin-induced AKT phosphorylation of ADRP-shRNA cells was significantly higher than Control-shRNA cells at 5 and 15 min. These results indicated that the reduced ADRP expression promotes cell viability in depleted medium involves AKT phosphorylation.

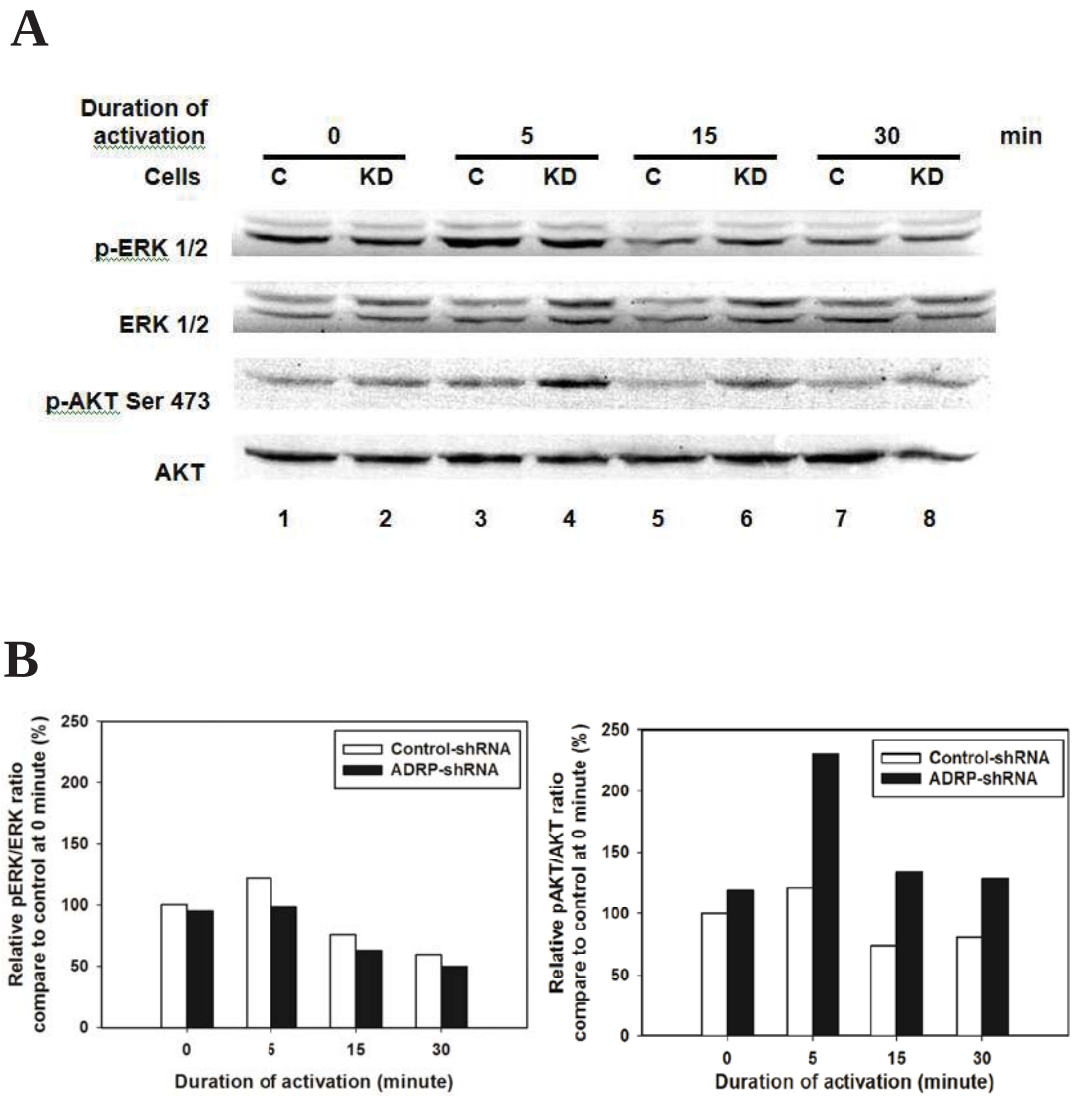

Figure 13. ERK and AKT phosphorylation assay.

A) ADRP-shRNA cells (KD) and Control-shRNA cells (C) were seeded onto 6-well plate in MEM supplemented with 5% CHX-FBS for 24 hours. The medium was replaced with SFM for 48 hours. The cells were activated with 1 µg/mL insulin for 0, 5, 15 and 30 minutes. Total protein was isolated and quantified by MicroBCA. The p-ERK, p-AKT, ERK, and AKT were determined by Western blot analysis. B) Relative insulin-induced ERK and AKT phosphorylation obtained by ImageQuant TL software. Western blot images were quantified by ImageQuant TL software and expression of p-ERK and p-AKT was normalized to ERK and AKT, respectively.

CHAPTER 5

DISCUSSIONS AND CONCLUSIONS

1. Address to the hypotheses

Adipose differentiation-related protein (ADRP) was discovered by Ginette Serrero in 1992 [11]. After that, this group found that ADRP played a role in management of intracellular lipid storage in abundant cell types [12]. Recently, ADRP is constitutively associated with lipid droplets and play roles in sustained fat storage and regulation of lipolysis [109]. ADRP is up-regulated in hepatic steatosis and hepatitis. In addition its expression level is positive regulation with lipid droplets amounts [86]. In skeletal muscle, ADRP content negatively correlates with insulin-induced glucose uptake [16]. Overall, ADRP may affects the insulin resistance development of lipid droplets (LDs) accumulation in insulin-responsive cells, such as hepatic steatosis.

The objectives of this study are to investigate the effect of ADRP on hepatic insulin responsiveness in cells proliferation, survival, extracellular signal-regulated kinases (ERK), and product of the akt protooncogene (AKT) phosphorylation in human hepatoma Huh7 cell line.

This study shows that ADRP knockdowned by shRNA in hepatocellular carcinoma cell lines, Huh7 cells, is not only survives in nutrient-deprivation medium, but also proliferates continuously insulin activation. In molecular mechanism, a downregulated of ADRP increases insulin-induced AKT phosphorylation, but not insulin-induced ERK1/2 phosphorylation.

2. ADRP knockdown promotes cell survival via AKT phosphorylation

We found that ADRP downregulation promotes cell survival in hepatocytes. Experiments in this study revealed that in up to 6 days downregulation of ADRP in hepatocytes better proliferated and survived in depleted medium. In addition, the cell

proliferation and survival are no difference between presence and absent insulin in short term. In contrast, the cell survival responses to insulin activation in the 8^{th} day. We propose that the downregulation of ADRP increases cell proliferation and survival via both insulin-dependent and insulin-independent pathway. In this study show that ADRP downregulation in Huh7 cells increases the responsiveness insulin-induced AKT phosphorylation, but not ERK phosphorylation. AKT is one of the major targets of PI3K-generated signal. AKT regulates cell growth, proliferation, anti-apoptosis, glucose metabolism, angiogenesis, and migration [53-56]. The phosphorylated AKT induces the number and size of thymocytes, cardiomyocytes[110], pancreatic β-cells[111], prostate epithelial cells[112], and hepatocytes[113]. In addition, phosphorylated AKT acts as anti-apoptosis signal [114, 115]. A mechanism connecting AKT with anti-apoptosis has recently been described. The target of AKT in anti-apoptosis is BAD, which is a negative regulator of prosurvival Bcl-2 family protein. Phosphorylated AKT induces BAD phosphorylation. Phosphorylated BAD forms complex with a 14-3-3 protein. This complex appears to prevent BAD association with Bcl-X_L or Bcl-2 and prevent the release of cytochrome c and activation of the caspase cascade [114]. These observations suggest that downregulation of ADRP promote the AKT phosphorylation, which would promote BAD phosphorylation mediating cell survival.

In addition, we propose that ADRP downregulated cells are more sensitive to starvation-induced autophagy. In starvation, cells induce autophagy to preserve cellular energy homeostasis [116, 117]. In the liver, autophagy is a prominent survival mechanism under nutrient deprivation [118]. The function of autophagy within hepatocytes is the degradation of lipid droplets. During nutrient deprivation, inhibition of autophagy, that was constructed in hepatocytes of tissue culture and mouse model, increased triglyceride storage in LDs associated with increased ADRP expression [119]. Furthermore, *trans*-10,*cis*-12 conjugated linoleic acid-induced ADRP protein levels are blocked by rapamycin, mammalian target of rapamycin (mTOR) inhibitor. Increasing of ADRP protein expression is mediated by mTOR pathway [120]. Thus, downregulation of ADRP induce transient cell proliferation and survival, because ADRP may be a major factor of lipid droplet degradation response to nutrient deprivation-induced autophagy. These findings suggest that ADRP would be a key protein in regulating lipid

accumulation, autophagy and survival. However, the molecular mechanism needs to be further elucidated.

3. ADRP knockdown improves hepatic insulin resistance

ADRP is upregulated during triglyceride accumulation, which induces insulin resistance in hepatocytes [10]. Conversely, ADRP downregulation in Huh7 cells increased insulin-induced AKT phosphorylation, in this study. Thus, the downregulation of ADRP improved insulin sensitivity. This is in the agreement with the previous reports in mice model and primary rat hepatocytes culture [15, 100]. They found the inverse relationship between ADRP level and hepatic insulin sensitivity. In addition, ADRP- and leptin deficient mice improved hepatic and muscle insulin resistance, which associated with increased VLDL secretion rate [96]. Furthermore, in a muscle cell line, Zucker diabetic fatty (ZDF) rat and T2DM patient studies has been shown ADRP protein expression correlated with increasing of intramyocellular lipid (IMCL) content and skeletal muscle insulin resistance [16, 98, 121]. In addition, weight loss and troglitazone therapy increased ADRP expression associated with improvement of insulin sensitivity [99]. In this study, our results support that downregulation of ADRP expression improves hepatic insulin sensitivity.

Hepatic insulin resistance is pathophysiological condition that is less sensitive to the effect of insulin on the production of hepatic glucose, very low-density lipoprotein (VLDL) and triglyceride (TG). In primary rat hepatocytes, it is shown that an increased ADRP reduces secretion of VLDL and TG by diverting fatty acid from the VLDL assembly pathway into cytosolic TGs, whereas a decreased ADRP increases the sorting of fatty acid to β-oxidation and promotes the secretion of apoB-48 VLDL1 [15]. ADRP overexpression suppresses the formation of Apo-B crescents, apolipoprotein B-100 deposited in a region around lipid droplets (LDs), whereas knockdowned ADRP by RNAi increases the number of Apo-B crescents in Huh7 cells [122]. Taken together, ADRP may play a critical role in LDs lipolysis, subsequently fatty acid form into VLDL, which exports fatty acid into bloodstream. In this condition, hepatocytes do not show dyslipidaemia, as hepatic steatosis, is associated with the improvement of hepatic insulin sensitivity, which is the advantage in metabolic syndrome.

4. Conclusion

To investigate the role of ADRP on cell proliferation, survival, ERK and AKT phosphorylation, ADRP-shRNA and Control-shRNA cells were used. The results showed that downregulation of ADRP better proliferated, survived in depleted medium and increased insulin-induced AKT phosphorylation, but not insulin-induced ERK1/2 phosphorylation. As mentioned above, this study provides evidence about the significant role of ADRP on insulin resistance in hepatocytes. These results are implied that ADRP inhibits hepatic insulin sensitivity, promotes cell death and mitigates the AKT phosphorylation in PI3K signaling pathway. Our results support the hypothesis that downregulation of ADRP related to cell proliferation and survival via insulin signaling pathway in Huh7 cells. Thus, ADRP would be a potential target for prevention of systemic insulin resistance and its associated consequences.

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APPENDIX

LIST OF ABBREVIATIONS

ADRP	Adipose differentiation-related protein
Amp	Ampicillin
Av	Average
bp	Base pair
BSA	Bovine serum albumin
CHX-FBS	Charcoal-stripped fetal bovine serum
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FFA	Free fatty acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
hr	Hour
IRS	Insulin receptor substrate
k	kilo
L	Liter
LB	Luria Bertani
LDs	Lipid droplets
M	Molarity

MAPK	Mitogen-activated protein kinase
MEM	Minimal essential medium
mg	milli gram
min	Minute
mL	milli liter
mM	milli molar
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAFLD	Nonalcoholic fatty liver disease
NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween®20
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolution per minute
S.D	Standard deviation
S.E	Standard error
SDS	Sodium dodecyl sulphate
sec	Second

SH-2	Src-homology-2
siRNA	Small interfering ribonucleic acid
TG	Triglyceride
Tris	Tris (hydroxymethyl) aminomethane
VLDL	Very-low-density lipoprotein
µg	micro gram
µL	micro liter

Table 1. Data record in experiment of cell proliferation and survival assay in 1% charcoal-stripped fetal bovine serum (CHX-FBS).

Absorbance at 490 nm										
Cells	Control-shRNA					ADRP-shRNA				
Conditions	1% CHX-FBS	0.1 μg/ml Ins	1 μg/ml Ins	10 μg/ml Ins	5% FBS	1% CHX-FBS	0.1 μg/ml Ins	1 μg/ml Ins	10 μg/ml Ins	5% FBS
Sample 1	0.269	0.372	0.372	0.404	0.768	0.660	0.712	0.702	0.678	0.780
Sample 2	0.345	0.448	0.474	0.472	0.696	0.649	0.641	0.601	0.661	0.781
Sample 3	0.343	0.457	0.487	0.500	0.833	0.696	0.711	0.732	0.683	0.859
Cell number (cells)										
Sample 1	4290.54	7770.27	7770.72	8851.35	21148.65	17500.00	19256.76	18918.92	18108.11	21554.05
Sample 2	6858.11	10337.84	11216.22	11148.65	18761.22	17128.38	16858.11	15506.76	17533.78	17128.38
Sample 3	6790.54	10641.89	11655.41	12094.59	23344.59	18716.22	19222.97	19932.43	18277.03	18716.22
Relative cell number (fold)										
Mean ± SEM	1.00 ± 0.24	1.75 ± 0.04	1.71 ± 0.36	1.79 ± 0.28	3.52 ± 0.39	2.97 ± 0.14	3.08 ± 0.23	3.03 ± 0.39	3.01 ± 0.07	3.76 ± 0.26

Table 2. Data record in experiment of cell proliferation and survival assay in serum-free medium (SFM).

Absorbance at 490 nm										
Cells	Control-shRNA					ADRP-shRNA				
Conditions	SFM	0.1 μg/ml Ins	1 μg/ml Ins	10 μg/ml Ins	5% FBS	SFM	0.1 μg/ml Ins	1 μg/ml Ins	10 μg/ml Ins	5% FBS
Sample 1	0.446	0.426	0.405	0.485	0.694	0.696	0.704	0.680	0.747	0.859
Sample 2	0.442	0.414	0.350	0.487	0.719	0.642	0.718	0.704	0.723	0.833
Sample 3	0.369	0.375	0.337	0.414	0.700	0.724	0.687	0.631	0.684	0.781
Cell number (cells)										
Sample 1	10270.27	9594.59	8885.14	11587.84	18648.65	18716.22	18986.49	18175.68	20439.19	24222.97
Sample 2	10135.14	9189.19	7027.03	11655.41	19493.24	16891.89	19459.46	18986.49	19628.38	23344.59
Sample 3	7668.92	7871.62	6587.84	9189.19	18851.35	19662.16	18412.16	16520.27	18310.81	21587.84
Relative cell number (fold)										
Mean ± SEM	1.00 ± 0.16	0.95 ± 0.10	0.80 ± 0.13	1.16 ± 0.15	2.03 ± 0.05	1.97 ± 0.15	2.03 ± 0.06	1.91 ± 0.13	2.08 ± 0.11	2.46 ± 0.14

Table 3. Data record in experiment of long term cell proliferation assay.

Absorbance at 490 nm																				
Cells	Control-shRNA										ADRP-shRNA									
Condition	5% CHX-FBS					5% CHX-FBS + insulin					5% CHX-FBS					5% CHX-FBS + insulin				
Day	0	2	4	6	8	0	2	4	6	8	0	2	4	6	8	0	2	4	6	8
Sample1	0.510	0.539	0.613	0.737	0.396	0.517	0.545	0.658	0.865	0.450	0.351	0.522	0.690	0.864	0.744	0.296	0.502	0.790	0.937	0.896
Sample2	0.502	0.556	0.624	0.780	0.470	0.528	0.539	0.668	0.845	0.458	0.330	0.539	0.646	0.925	0.713	0.357	0.502	0.695	0.830	0.904
Sample3	0.436	0.505	0.623	0.826	0.472	0.508	0.586	0.644	0.830	0.435	0.314	0.444	0.661	0.975	0.673	0.351	0.510	0.710	0.878	0.945
Cell number (x 10^4 cells)																				
Sample1	1.243	1.341	1.591	2.010	0.858	1.267	1.361	1.743	2.443	1.041	0.706	1.283	1.851	2.439	2.034	0.520	1.216	2.189	2.686	2.547
Sample2	1.216	1.399	1.628	2.155	1.108	1.304	1.341	1.777	2.375	1.068	0.635	1.341	1.703	2.645	1.929	0.726	1.216	1.868	2.324	2.574
Sample3	0.993	1.226	1.625	2.311	1.115	1.236	1.500	1.696	2.324	0.990	0.581	1.020	1.753	2.814	1.794	0.706	1.243	1.919	2.486	2.713
Relative cell number (fold)																				
Mean ± SEM	0.9 ± 0.11	1.0 ± 0.07	1.3 ± 0.02	1.7 ± 0.12	0.8 ± 0.12	1.0 ± 0.03	1.1 ± 0.07	1.4 ± 0.03	1.9 ± 0.05	0.8 ± 0.03	1.0 ± 0.10	1.9 ± 0.26	2.7 ± 0.12	4.0 ± 0.29	3.0 ± 0.18	1.0 ± 0.17	1.9 ± 0.02	3.1 ± 0.27	3.8 ± 0.28	4.0 ± 0.14

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