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不同能量狀態下肝臟 SIRT1 對動物能量代謝之調控

The role of hepatic SIRT1 on metabolic regulation under
different energy status

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中文摘要

　　Sirtuin 1，又稱之為SIRT1，是屬於Sirtuin哺乳動物蛋白質家族成員之一。SIRT1與其非脊椎動物同源蛋白Sir2 (silent information regulator 2 gene) 皆為菸鹼醯胺腺嘌呤二核苷酸依賴型去乙醯酵素，以菸鹼醯胺腺嘌呤二核苷酸 (NAD+) 與蛋白質上乙醯態之離胺酸為受質，進行去乙醯化作用。先前研究發現，此二種酵素都可因低能量情況或能量限制時而增加其表現量。後續其他研究發現，哺乳動物之SIRT1可以藉由調控其目標蛋白而改變身體代謝、神經及免疫功能，進而產生抗老化與延長壽命之效果。本研究目的在於了解不同代謝能濃度對於豬隻組織中SIRT1及其相關基因表現之影響，並探討高脂飼糧下對於豬隻與小鼠體內SIRT1與能量代謝之影響。

　　在試驗第一部分，使用十週齡蘭嶼豬進行十週的營養試驗，於期間分別餵飼每公斤2500 (L) 、2700 (M) 及2900 (H) 大卡之飼糧。結果顯示，三組間體重、飼料轉換率及平均日增重皆無差異。而L組別血液中具有較高之三酸甘油酯、高密度脂蛋白及血糖，並且在肝臟中發現其具有較高之SIRT1與脂質合成基因表現。L組別肝臟中合成較多三酸甘油酯，並在血液中觀察到具有較高的血脂現象，於此推斷其具有較高的能量利用效率。另一部分，使用五月齡蘭嶼豬進行六個月的營養試驗，於期間分別餵飼每公斤2700 (CON) 與3700 (HFD) 大卡之飼糧。結果顯示，HFD組具有較高的體重與背脂厚度，但試驗後兩組血脂分析則無出現差異。同時在肝臟中發現HFD組別具有較高的SIRT1與脂質分解基因表現，其餘基因表現皆無差異。據此推斷SIRT1在HFD餵飼下具有避免肥胖造成傷害之功能。

　　為了更加確定SIRT1是否可以保護身體在肥胖初期避免肥胖飼糧下造成之代謝傷害。此部分利用七週齡小鼠飼食一般飼糧 (CON) 與肥胖飼糧 (HFD) 探討各時期的結果。結果顯示，在試驗初期 (兩週) 肝臟中SIRT1於HFD組有增加的現象，但在長期間餵飼之下則沒有顯著差異，然而在年紀較大之動物上發現SIRT1有增加的現象。體重與血脂分析部分，在第二週時便可發現體重、三酸甘油酯、總膽固醇與血糖皆有顯著提升的效果。於此試驗推論，SIRT1可能在肥胖初期扮演保護的角色，隨著年紀增加其作用活性降低或無法抵禦老化造成之影響，因此仍然出現老化與肥胖現象。

綜觀上述，本研究指出豬隻肝臟中SIRT1表現在能量限制的情況下增加，此點與其他物種具有相同的結果。高能量飼糧餵飼兩週，小鼠肝臟中SIRT1也有提升的現象，在此推斷，SIRT1在肥胖初期具有保護的效果。然而，延長餵飼時間SIRT1的功效則失去保護，其相關機制有待進一步釐清。

關鍵字：SIRT1、能量限制、高脂飼糧、能量代謝、蘭嶼豬、小鼠

Abstract

Sirtuin 1, also called SIRT1, is one member of sirtuin family proteins in mammals. SIRT1 and its invertebrate homologue, silencing information regulator 2 (Sir2), both are nicotinamide adenine dinucleotide (NAD+) -dependent enzymes that exert deacetylating action on acetyl-Lysine of proteins. Previous studies showed that SIRT1 and Sir2 were up-regulated under low energy conditions or calorie restriction (CR). Furthermore, increasing SIRT1 expression regulated its target proteins to modulate metabolic, neuronal and immunological functions, and therefore lead to anti-aging and longevity. This study was focused on the link between metabolic energy (ME) level and related gene expressions SIRT1 in various tissues of swine, and in mouse models in response to high fat diet (HFD).

In the first part of this study, 10-week-old Lanyu miniature pigs were fed with diet with various ME levels (2500 (L), 2700 (M) or 2900 (H) kcal/kg) for 10 weeks. There was no difference on body weight, feed conversion ratio and average daily gain among three groups. L group had a higher plasma level of triglyceride (TG), high density high density lipoprotein (HDL) and glucose than H group. Increased mRNA expression of SIRT1 and lipogenic genes were found in liver of L group. And hepatic triglyceride content was no different among three groups. These results suggested that low energy diet induced high efficiency by SIRT1, and fluxed to peripheral tissues for further use. In the second part of this study, 5-month-old Lanyu miniature pigs were fed with control (CON) or HFD, as 2700 or 3700 (HFD) kcal ME/kg, for 6-month period. HFD pigs had a higher body weight and backfat thickness, but no difference in blood parameters, including TG, total cholesterol, HDL and LDL level between two groups was observed. After 6 month experiment period, Lanyu pig was not induced obesity. HFD pigs had an increase in hepatic transcript and protein levels of SIRT1 and lipolytic

genes, suggesting a protective role of SIRT1 in HFD-induced metabolic damage of porcine model.

In order to elucidate whether SIRT1 protects mice from HFD-induced metabolic damages in early stage of obesity, the third experiment was designed using 7-week-old mice as an animal model in response to high fat diet (HFD) for various duration. Results showed that SIRT1 mRNA expression increased with advancing age. Compared with CON mice, HFD mice had higher hepatic SIRT1 mRNA expression at 2-week period, while there was no difference after 25-week feeding. In addition, body weight, plasma TG, total cholesterol and glucose level were elevated since 2 week by HFD. These results suggested that SIRT1 might play a protective role in early obesity stage, and its activity and protection decline with advancing age.

Taken together, this study indicated that porcine hepatic SIRT1 expression was induced in CR as well as the rodent model. While in the high energy status induced by HFD-feeding 2 weeks, the hepatic SIRT1 expression of mice was elevated as well. These results suggest a protective role of SIRT1 in early obesity stage. However, the related mechanism needs further elucidation.

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LIST OF ABBREVIATIONS

ACO	acyl-Co A oxidase
BMI	body mass index
CON	pigs with 2700 kcal/kg diet or mice with chow diet
CPT1	carnitine palmitoyl transferase 1
CR	calorie restriction
CVD	cardiovascular diseases
DGAT	diacylgycerol acyltransferase
FAS	fatty acid synthase
FFA	free fatty acid
G6Pase	glucose 6-phosphatase
GK	glucokinase
H	2900 kcal/kg
HDL	high density lipoprotein
HFD	pigs with 3700 kcal/kg diet or mice with high fat diet
HMGR	3-hydroxy-3-methyl-glutaryl-Co A reductase
L	2500 kcal/kg
LDL	low density lipoprotein
LPK	pyruvate kinase in liver
M	2700 kcal/kg
ME	metabolic energy
PEPCK	phosphoenolpyruvate carboxykinase
PG	pituitary gland
SAT	subcutaneous adipose tissue
TBP	TATA-box binding protein
VAT	visceral adipose tissue

The list only shows the important abbreviation.

Chapter 1: Introduction

Obesity is a global public health concern. Body mass index (BMI) is a clinical measurement to evaluate human body condition, and the formula is weight (kg) / hight square (m^2). According to World Health Organization (WHO) definition, BMI $\geqq$ 25 kg/m^2 is considered as overweight, and BMI $\geqq$ 30 kg/m^2 is considered as obesity. The BMI value for obesity is changed with species. For example, overweight is BMI $\geqq$ 24 kg/m^2, and obesity is BMI $\geqq$ 27 kg/m^2 in Taiwan. In addition, the waist circumference, which is associated with abdominal obesity, is another major clinical concern. The goals of waist circumference are less than 40 inch for women and 35 inch for men.

Reports of WHO showed that over 200 million men and nearly 300 million women were obese in 2008. Bureau of Health Promotion, Department of Health, R.O.C. (Taiwan) revealed that 50% of Taiwan adult men were obese in 2005-2008. Therefore, it is worth paying attention on obesity issue.

I. *Energy Intake*

Animals cannot survive without food. In other words, food is necessary for life. The major function of food is to provide energy for metabolism. Carbohydrate and fat are the major energy sources, and they are further converted into simple molecules, such as mono-carbohydrate and fatty acid. Acetyl-Co A is synthesized during catabolism and oxidation, and it can enter the citric acid cycle (TCA cycle) to produce nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$). Then, NADH and FADH$_2$ attend electron transport chain (ETC) involve in adenosine triphosphate (ATP) production, which is an energy currency in cell. On the other hand, acetyl-Co A synthesizes fatty acid and triglyceride by fatty acid synthase and acryltransferase to

store energy as fat. There was a systemic oxidative metabolism overview about glucose, fatty acids and amino acids (Fig 1).
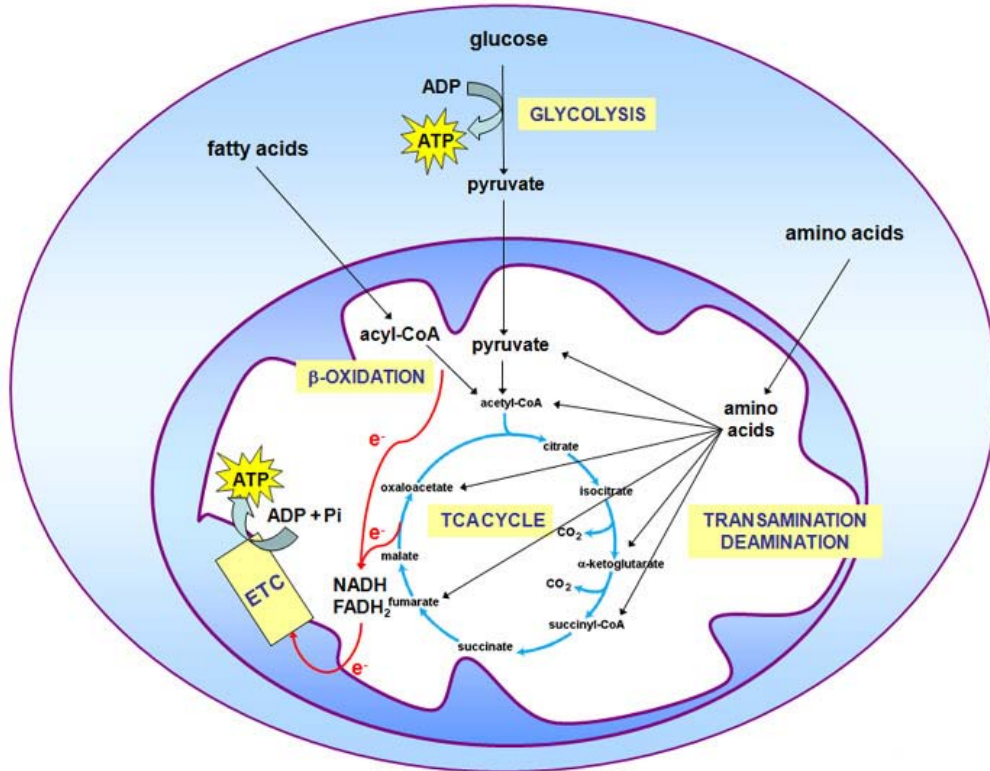
Fig 1 A schematic representation of oxidative metabolism and substrate level phosphorylation from glucose, fatty acids and amino acids.

(Da Poian et al. 2010)

A. *Calorie restriction*

Calorie restriction (CR) is a reduction of 10-50 % energy intake without causing malnutrition (Houtkooper et al. 2012). First CR-related experiment shower that CR retarded rat growth, depressed body size and extended life span (McCay et al. 1935). From this discovery, researchers have focused on the effect of CR. There were many studies showed that CR altered metabolism and caused longevity, in *Saccharomyces cerevisiae* (Lin et al. 2004), *Drosophila* (Partridge et al. 2005) and mice (Harper et al. 2006), In human, long term of CR caused a decrease in body weight, blood glucose, insulin and lipid profiles and showed anti-aging physiological characteristic (Walford et

al. 1992; Walford et al. 2002). Furthmore, the rhesus monkey treated with 20-year CR revealed had longer life span and caused fewer diseases, such as cardiovascular disease and diabetes (Colman et al. 2009).

As we all know, mitochondrion is an organelle for ETC and ATP production. Energy expenditure causes increasing NADH and $FADH_2$ production. NADH and $FADH_2$ are electron donors in ETC, electrons are channelled into the ETC and protons (H+) are exported from the mitochondrial matrix to the cytoplasm by complex protein. Thereby, the proton gradient generates between proton gradient of matrix and cytoplasm. Sometimes, electrons leak out of the ETC and generate reactive oxygen species (ROS), and the occurring ratio is 0.2-2% electrons upon ETC (Cadenas 1989; Halliwell and Gutteridge 2007). Superoxide anion ($O_2\cdot-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\cdot$) are main ROS in cells. Natural antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, can clear most ROS forming H_2O. There was a schematic model of ROS generation and scavenging in Fig 2. Some studies indicated that the reason of longevity by CR was reducing oxidative stress. Taking less calories might prolong life by slowing metabolism and reducing oxygen radicals and oxidative damage in organism (Harman 1983; Sohal and Weindruch 1996).

Excessive ROS can impair cell components, including lipids, proteins, and DNA, and even cause cell death (Apel and Hirt 2004). Oxidative stress is an imbalance between ROS production and ability of antioxidant. Excessive food intake increases NADH and $FADH_2$ production abundantly, therefore oxidative stress is elevated. Oxidative stress plays a core role in the pathogenesis of various diseases (Brownlee 2001). CR reduced H_2O_2 generation and lowered respiration rate (Bevilacqua et al. 2004; Bevilacqua et al. 2005) and increased uncoupling proteins (UCP) in mitochondria. The

responses above contribute to the benefit of CR on health.
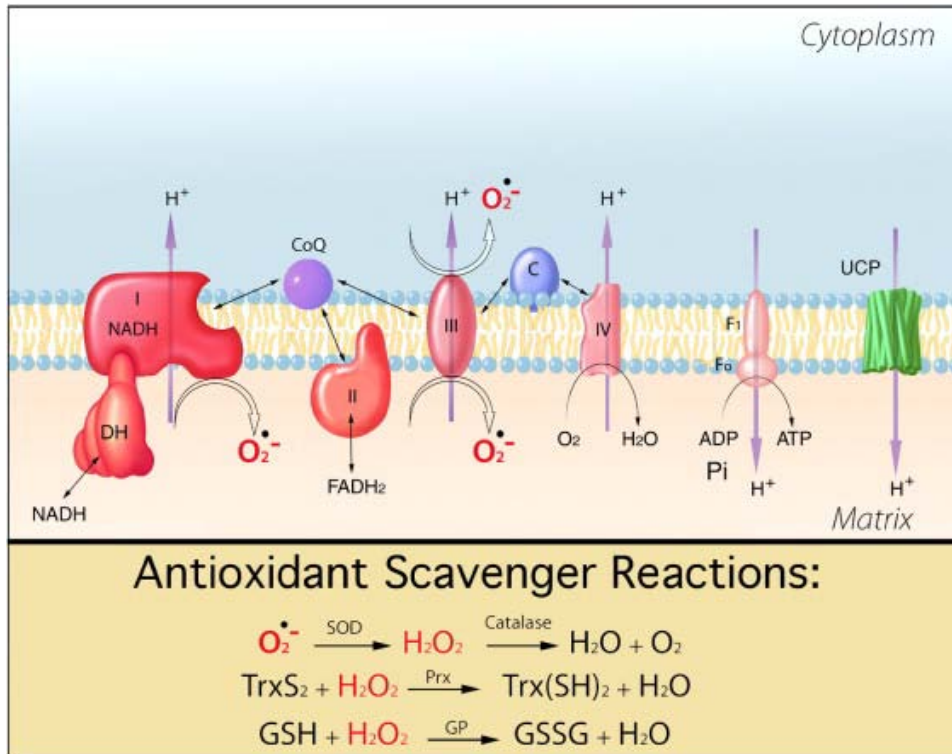
Fig 2 A schematic model of ROS generation and scavenging in the mitochondria. DH, dehydrogenases; C, cytochrome c; SOD, superoxide; TrxS$_2$, thioredoxin; Prx, peroxiredoxin; GSH, glutathione; GP, glutathione peroxidase.

(Balaban et al. 2005)

B. Excessive energy supply

Nutrients intake and absorption excessively raise acetyl-Co A generation, and fatty acid and triglyceride are synthesized to store energy as fat. Obesity is excessive fat accumulation in body, and overweight is the early stage of obesity. The phenotype of obesity is the hypertropy of lipid droplet, which stores excessive energy in tissue. Lipid usually accumulates in adipose tissues; however, fat accumulation in other tissue, as ectopic fat, occurs in obese condition. Briefly, obesity is an imbalance between intake and energy expenditure.

There was association with obesity, metabolic syndrome and oxidative stress. A study pointed out that increasing oxidative stress from adipose tissue led to early metabolic syndrome (Furukawa et al. 2004). Therefore, obesity not only increases fat

accumulation but also increases health problems. About obesity-related diseases will be discussed later.

II.   *Obesity and Metabolic Syndrome*

In 1920s, Kilyn physician found some relations between hypertension and high circulating glucose (Kilyn 1923). It was a beginning of gather syndromes on disease. Metabolic syndrome is defined as metabolic abnormalities, including glucose intolerance, insulin resistance, obesity, dyslipidaemia and hypertension in recent studies. When risks occurring together, it might develop cardiovascular diseases (CVD) (Isomaa et al. 2001; Lakka et al. 2002). CVD is a class of diseases, which involve the heart or blood vessels, with high death rate. Therefore, metabolic syndrome and CVD have been one of the health concerns recent years. There are various definitions of metabolic syndrome in many organizations, the most mentioned definition was WHO (1999). The criteria were as below:

Obesity
   BMI $\geqq$ 30 and/or waist:hip ratio > 0.9 (male) or > 0.85 (female)
Insulin resistance (identified by 1 of the following)
   Type 2 diabete
   Impaired fasting glucose
   Impaired glucose tolerance
Dyslipidaemia
   Plasma triglycerides $\geq$ 150mg/dL
   HDL cholesterol $\leq$ 35 mg/dL (male) or $\leq$ 39 mg/dL (female)
Hypertension
   Blood pressure > 140/90 mm-Hg
Microabluminuria
   Urinary albumin excretion > 20 μg/min
The characteristic will be introduced as following.

A. Obesity

The common obesity factor is due to diet with high energy content of poor nutrition, which causes fat accumulation. Although the objective definition of obesity by WHO is BMI $\geqq$ 30 kg/m^2. Body fat distribution is more important than BMI. Fat below skin, as subcutaneous adipose tissue (SAT), is different characteristic from visceral adipose tissue (VAT) surrounding abdominal viscera in mesentery and omentum (Marieb and Hoehn 2008; Ibrahim 2010). One of physiological functions of SAT is as a storehouse for energy deposition. SAT has high avidity for excess fatty acids and glycerol to synthesis triglycerides (Freedland 2004). While, the normal physiological function of VAT is around internal organs to prevent impact. Energy deposit in SAT firstly, when the storage capacity of SAT overloads or new adipocyte generates under stress or damage, excess fat was beginning to deposit in areas outside the subcutaneous tissue, such as VAT (Ibrahim 2010).

Abdominal obesity is emphasized in metabolic syndrome. Abdominal fat consists with SAT and VAT. It was suggesting that SAT was one part of abdominal fat, and VAT has a strong association with insulin resistance (Abate et al. 1995; Goodpaster et al. 1997). Moreover, studies found that there was high association between VAT and metabolic abnormalities, such as hyperglycemia, hypertension, insulin resistance and atherogenesis (Mårin et al. 1992; Lebovitz and Banerji 2005). Also, there was negative relation between glucose deposal rate and SAT, indicating SAT playing an important regulated role in obesity-related insulin resistance (Abate et al. 1995). Therefore, VAT is an important consist of abdominal obesity, as center obesity, which has been a popular issue with metabolic syndrome (Fig 3).

Adipose tissue can store energy in oil droplet, and adipocyte can produce adipokines, which are hormones secreted by adipose tissue, and act not specifically on adipose tissue. Adipokine includes plasminogen activator inhibitor-1 (PAI-1)

(Shimomura et al. 1996), tumor necrosis factor-alpha (TNF-a) (Mohamed-Ali et al. 1997; Uysal et al. 1997), resistin (Steppan et al. 2001; Banerjee et al. 2004), leptin (Friedman and Halaas 1998; Unger 2003), adiponectin (Arita et al. 1999; Matsuzawa et al. 2004) and interleukin-6 (IL-6) (Mohamed-Ali et al. 1997). IL-6, TNF-a and resistin from VAT were proved to induce insulin resistance and diabetes (Xu et al. 2003; Lafontan 2005). Adipokines might be the major factor causing metabolic syndrome and ectopic fat accumulation in liver, pancreas, muscle, heart and kidneys (Table 1). In other hand, adiponectin, also secreted by adipose tissue, improves glucose sensitivity and anti-diabetes, however, the secretion decreased in obesity (Park et al. 2004; Kobayashi and Inoguchi 2005; Lau et al. 2005; Trujillo and Scherer 2005). Although obesity is one risk of CVD, not every obese subject patient has insulin resistance or high risk of diabete and CVD (Abbasi et al. 2002; Després and Lemieux 2006)

Table 1 Overview of key adipokines on metabolic syndrome

Adipokine	Key properties	Secretion in abdominal obesity
Adiponectin	Anti-atherogenic, reduces risk of developing diabetes ↓ Differentiation of macrophages into foam cells ↓ Atherogenic vascular remodelling ↓ Hepatic glucose output ↑ Insulin sensitivity	↓
IL-6	Promotes inflammation, pro-atherogenic, promotes diabetes ↑ Vascular inflammation ↑ Hepatic C-reactive protein production ↓ Insulin signalling	↑
TNFα	Pro-atherogenic/pro-diabetic Paracrine role in the adipocyte ↓ Insulin signalling ↑ Secretion of other pro-inflammatory mediators	↑
C-reactive protein	Promotes inflammation, pro-atherogenic Marker of chronic low-grade inflammation Predicts adverse cardiovascular outcomes	↑
PAI-1	Pro-atherogenic, pro-coagulant ↑ Atherothrombotic risk	↑
Resistin	Exacerbates insulin resistance ↓ Insulin signalling ↓ Endothelial function ↑ Vascular smooth muscle proliferation	↑

(Després 2006)
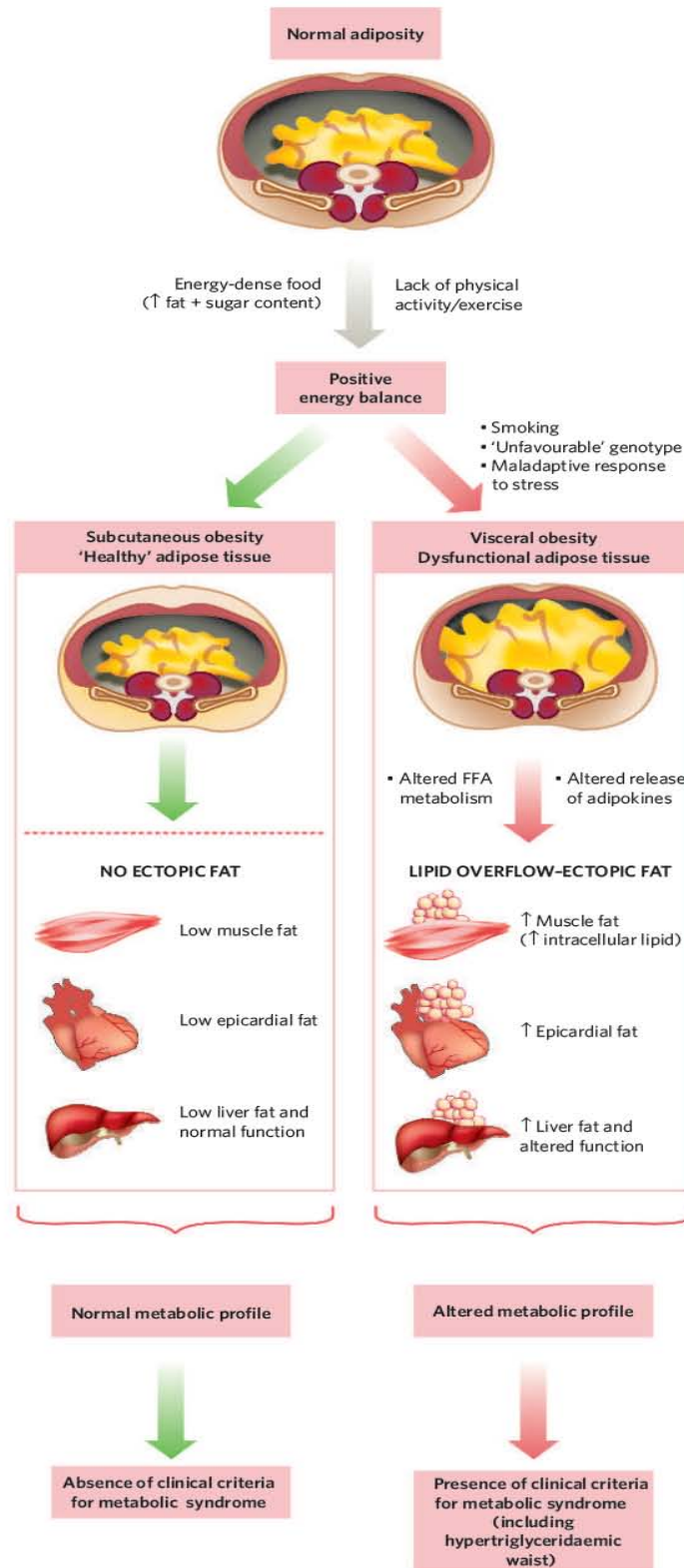
Fig 3 The lipid overview of abdominal fat
Compare with subcutaneous and visceral fat on metabolic syndrome.

(Després and Lemieux 2006)

B. Insulin resistance

Insulin is an important hormone, which is produced by beta cells and secreted into circulatory system regulate energy utilization, such as carbohydrate and lipid. It can stimulate glucose uptake and inhibit lipolysis. Insulin resistance is a condition, which the cells become resistant to insulin, stimulation and therefore can't use carbohydrate normally. As a result, there are high levels of insulin and glucose in blood. Because glucose deposal is broken down, cells use lipid for energy source instead, and cause hyperlipidemia.

Metabolic syndrome is also named as insulin resistance syndrome, it refers that metabolic syndrome is related to insulin resistance. Bergman et al. indicated that VAT was the major contributor of insulin resistance, and free fatty acids (FFA), the lipolytic products of VAT, and the anatomical position of VAT played an important role (Bergman et al. 2006). Insulin resistance was caused by large amounts of circulating FFA released from VAT, which flow into the portal vein and transported to the liver (Despres et al. 1990). Increasing circulating FFA inhibited insulin functions such as depressing hepatic glucose production and stimulating peripheral glucose uptake, which further stimulated insulin secretion and led to insulin insensitivity (Boden 1997; Boden and Shulman 2002). Overall, it is a vicious circle for insulin resistance.

C. Dyslipidaemia

There is high risk of dyslipidaemia in obese subject. Obese adults usually had increasing triglycerides and low density lipoprotein (LDL) and decreasing high density lipoprotein (HDL) (Evans et al. 1984). Acute hyperinsulinemia decreased triglyceride, FFA, very low density lipoprotein (VLDL) triglyceride and VLDL apo B production in health human (Lewis et al. 1993; Lewis et al. 1995). While chronic hyperinsulinemia increased triglyceride-rich VLDL and apo B production in obesity subject (Vega 2001;

Carr and Brunzell 2004). Triglycerides of VLDL was hydrolysis by lipoprotein lipase and VLDL transformed into LDL (Havel 1984). Triglycerides of VLDL and LDL exchanged for esterified cholesterol with HDL, as reverse cholesterol transport, therefore low HDL sometimes occurred in obese subjects (Evans et al. 1984; Fielding and Fielding 1995).

In addition, FFA stimulated mitochondrial ROS production (Inoguchi et al. 2000; Subauste and Burant 2007), which could oxidize LDL ((Steinberg 1997). The oxidize LDL has been known as a pathogenic factor in atherosclerosis. Therefore, dyslipidaemia plays an important role for the occurrence of CVD.

III.   *Sirtuin 1 (SIRT1)*

First function of Sir2 (silence information regulator 2) was that it could extend life span in *Saccharomyces cerevisiae* (Kaeberlein et al. 1999). Further studies revealed that yeast needed Sir2 to prolong longevity within CR (Lin et al. 2000). In invertebrate and vertebrate,   Sir2 played an important role in cell survival and metabolism (Kaeberlein et al. 1999; Lin et al. 2000; Tissenbaum and Guarente 2001; Rogina and Helfand 2004; Wood et al. 2004; Guarente 2005; Wang and Tissenbaum 2006). In mammals, there was a Sir2 homologue, SIRT1, which was also belonged to sirtuin family. The mammal sirtuin family has seven sirtuins proteins (SIRT1-7) that regulate different metabolic and stress response pathways as Table 2.

Table 2 Sirtuin localization and function

Sirtuin	Class	Localization	Activity	Targets
SIRT1	I	Nucleus	Deacetylation	PGC-1a, FOXO1, FOXO3, p53, Notch, NF-kB, HIF1a, HIF2a, LBK1, LXR, FXR, SREBP1c, eNOS and more
SIRT2	I	Cytosol	Deacetylation	Tubulin, PEPCK, FOXO1, FOXO3, PAR3
SIRT3	I	Mitochondria	Deacetylation	LCAD, HMGCS2, GDH, OXPHOS complex, SOD2, IDH2, NDUFA9, ATP5A, GDH, OTC, CypD (PPIF). ALDH2 and more
SIRT4	II	Mitochondria	ADP-ribosylation	GDH
SIRT5	III	Mitochondria	Deacetylation, demalonylation, desuccinylation	CPS1
SIRT6	IV	Nucleus	Deacetylation, ADP-ribosylation	H3K9, H3K56, HIF1a
SIRT7	IV	Nucleus	Unknown	p53

(Webster et al. 2011; Houtkooper et al. 2012)

A. Characteristics of SIRT1

Both SIRT1 and Sir2 are class III histone deacetylases and NAD-dependent deacetylases. The reaction requirs NAD+ and acetyl-lysine of protein as substrates, and the products are 2'-*O*-acetyl-adenosine diphosphate ribose, nicotinamide and lysine of protein (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000) (Fig 4). The activator and inhibitor for SIRT1 are resverstrol (Howitz et al. 2003) and nicotinamide (Bitterman et al. 2002), respectively.
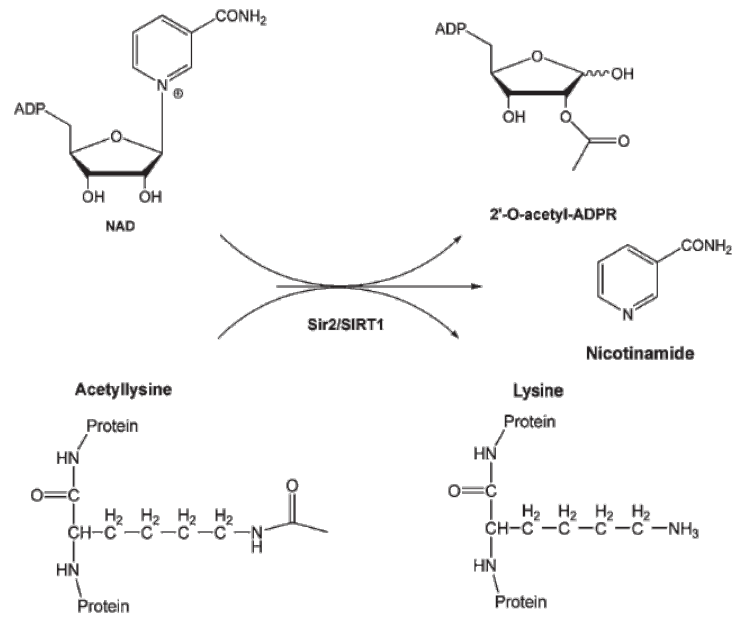
Fig 4 A stoichiometry of the Sir2/SIRT1 reaction

<div align="right">(Yang and Sauve 2006)</div>

B. Regulation of SIRT1 activity

SIRT1 activitys are modulated according to the energy status of animal. SIRT1 was increased in low energy status, such as fasted (Nemoto et al. 2004) and CR (Cohen et al. 2004), and decreased in high energy status (Coste et al. 2008).

There are several transcription factor binding sites in SIRT1 promoter, including forkhead box protein O1 (FOXO1) (Nemoto et al. 2004), cAMP response element-binding (CREB), carbohydrate response element-binding protein (CHREBP) (Noriega et al. 2011) and peroxisome proliferator activated receptors (PPARs) (Han et al. 2010; Hayashida et al. 2010), which regulate SIRT1 expression to respond to the stimulation. FOXO1, PPARalpha, PPARdelta and CREB promote SIRT1 expression (Nemoto et al. 2004; Hayashida et al. 2010; Noriega et al. 2011); in contrast, PPARgamma and CHREBP depresses SIRT1 expression (Han et al. 2010; Noriega et al. 2011).

SIRT1 activity was also regulated by forming complexes with other proteins. The active regulator of SIRT1 (AROS) bound with SIRT1, and positive regulated SIRT1 function and suppressed SIRT1 target p53 (Kim et al. 2007). There were several negative regulators, such as nuclear receptor co-repressor 1 (NCoR1) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). NCoR1, SMRT and SIRT1 generated a complex protein, then bound with PPARgamma to inhibit adipogenesis and promote fat mobilization (Picard et al. 2004). Lys-specific demethylase 1 (LSD1) and SIRT1 coregulated and depressed Notch target genes (Mulligan et al. 2011).

NAD+ is the substrate and cofactor of SIRT1. NAD+ levels were raised in muscle, liver and adipose tissue upon low energy condition and exercise (Cantó et al. 2009; Cantó et al. 2010). SIRT1 activity was affected by NAD+ content. The physical sources of NAD+ were nicotinic acid and nicotiamide though *De no* pathway and salvage. The NAD+ synthesis and supplement influenced SIRT1 activity (Imai 2009).

C. SIRT1 in metabolism

In the liver, SIRT1 increased upon fasting, and it deacetylated and activated PPARgamma coactivator 1 alpha (PGC-1a) and FoxO1 to increase gluconeogenesis and inhibit glycolysis (Frescas et al. 2005; Rodgers et al. 2005; Banks et al. 2008). Hypoxia-inducible factor 1 (HIF1a) suppressed by SIRT1, led to decrease glycolysis and increase oxidative metabolism (Kim et al. 2006). Liver X receptor (LXR) was a factor related to hepatic cholesterol and triglyceride synthesis. SIRT1 deacetylated LXR to increase transcriptional activity and further activated SREBP1c to increase fatty acid synthesis (Li et al. 2007). However, SIRT1 also deacetylated LXR and SREBP1c, and reduced lipogenesis (Ponugoti et al. 2010; Walker et al. 2010). SIRT1 also activate

PPARalpha and its cofactor, PGC-1a, to increase gene expression involved fatty acid uptake and/or beta-oxidation (Purushotham et al. 2009).

In liver SIRT1 increased gluconeogenesis and inhibit glycolysis by deacetylated and activated PGC-1a and FoxO1, and there were similar mechanism in muscle (Frescas et al. 2005; Rodgers et al. 2005; Banks et al. 2008). And further, SIRT1 repressed the UCP2 to reduce glucose-stimulated insulin secretion and body glucose tolerance in beta cells (Bordone et al. 2005; Moynihan et al. 2005). SIRT1 reduced lipolysis in liver, and it also found in other tissue. SIRT1 regulated PGC-1a to induce fatty acid oxidation in muscle (Gerhart-Hines et al. 2007), and SIRT1 inhibited PPAR-gamma to increase fat mobilization and reduce lipogenesis and adipogenesis in adipose tissue (Picard et al. 2004) (Fig 5).

In addition, SIRT1 not only affected metabolism but also affected feeding behavior. In the brain, SIRT1 increased in the hypothalamus upon fasting and decreased FoxO1 acetylation to modulate the central melanocortin system and decrease satiation (Giannakou and Partridge 2004; Ramadori et al. 2008).

Overall, activating SIRT1 can alters appetite and metabolism to increase energy expenditure and decrease gluconeogenesis, lipogenesis and adipogenesis.
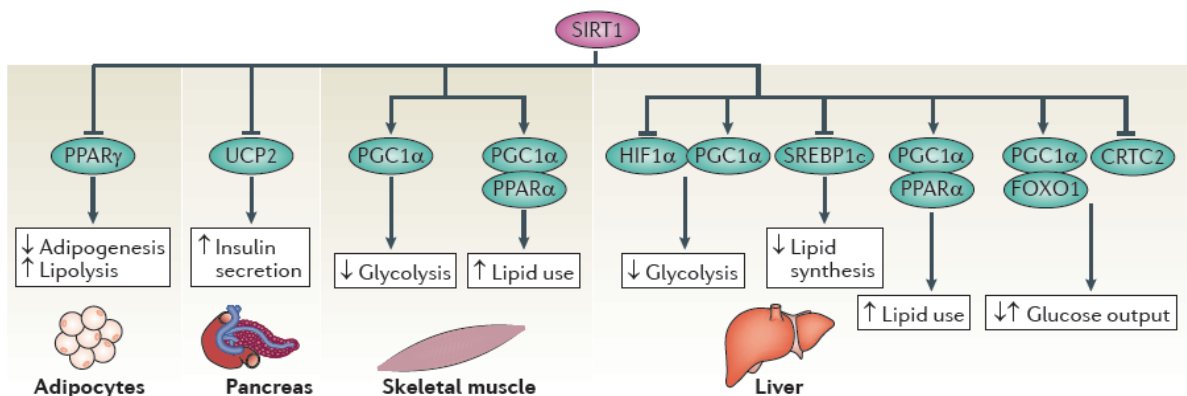
Fig 5 SIRT1 mediate metabolic response in several tissues
(Houtkooper et al. 2012)

14

Chapter 2: Materials and Methods

I.   *Experimental design and purpose*

A. *Experiment 1*

The purpose of this experiment was focused to the link between metabolic energy (ME) level and SIRT1-regulated gene expressions of various tissues in swine model.

B. *Experiment 2*

The purpose of this experiment was to examine the effect of high fat diet (HFD) -feeding on SIRT1 gene expression in swine and mouse model.

II.   *Animals and diets*

Lanyu miniature pig, which is distinctive animal of Taiwan, is an indigenous pig breed from Lanyu (Orchid) Island off the south-east coast of Taiwan. The characteristics of Lanyu pigs are a narrow head, long straight snout, small erect ears and coarse dark hairs. The adult body weight is about 70 kg and is categorized as a miniature pig (Jiang et al. 2008).

Experiment 1

Eight-week-old Lanyu miniature pigs were raised in Taitung Animal Propagation Station (TAPS), Livestock Research Institute of Council of Agriculture. The average initial weights were 7.12 ± 0.18 kg. Pigs were randomly divided into three groups (4 gilts and 4 barrows each group) and fed with different diet with three ME levels (2500 (L), 2700 (M) or 2900 (H) kcal/kg), respectively. Feed compositions were provided in Table 3.

In the 10-week experimental period, daily feeding rate was 5 % of body weight, and the weight and body length (crown–rump length) were recorded weekly. Blood sample was collected in the beginning, week 5 and the end of experiment.

A. *Experiment 2*

Porcine model

Five-month-old Lanyu miniature pigs were raised in TAPS. Initial weight of pigs was 12.61 ± 0.79 kg and the pigs were randomly divided into two groups (4 barrows each group) and were fed with diet with two ME levels (2700 (CON) or 3700 (HFD) kcal/kg), respectively. Feed composition was provided in Table 4.

In 6-month experimental period, pigs were fed *ad libitum*, and the weight and body length were recorded and blood sample was collected monthly. The backfat thickness was surveyed in the end of experiment (Ultrasound for Ultra Profits, RENCO, Minneapolis, MN, USA).

Mouse model

Five-week-old C57B/6J male mice were purchased from Laboratory Animal Center, Nation Taiwan University, and mice were kept under 12-hour day and 12-hour night conditions. Initial weight of mice was 21.60 ± 0.24 g. Mice were fed with standard chow diet (LabDiet 5001, St. Louis, MO, USA) for 2 weeks for adaptation. Then mice were divided into two groups; control (chow diet) or HFD (TestDiet), respectively for 28-week. Feed and water were free accessed. Feed composition was provided in Table 5.

In the 28-week experimental period, body weight was recorded weekly, blood and tissue samples were collected in the beginning.

III.   *Blood parameter analysis*

Prior to blood sample collection in pigs and mice went fasted for 16 hours and 12 hours, respectively. Plasma and serum were stored at -80 ℃ for further analysis.

Blood parameters were measured by using commercial kits: plasma glucose (GL2603, Rando, Antrim, UK), triglycerides (BXC0272C, Fortress, Antrim, UK), total

cholesterol (BXC0261B, Fortress, Antrim, UK), high density lipoproteins (HDL) (BXC0442A, Fortress, Antrim, UK) and non-esterified fatty acids (FFA) (K612, Bio-vision, Milpitas, CA, USA). Values of low density lipoproteins (LDL) were calculated by total cholesterol minus HDL. The porcine circulating hormones were measured by using commercial kits: leptin (E90084Po, Uscn, Wuhan, China) and insulin (AKRIN-013T, Shibayagi, Gunma, Japan).

All the procedures were followed according to the manufacturer's instructions. The absorbance was measured by the colorimetric assay (Spectra max190, Molecular Devices, CA, USA). The R^2 of standard curve $\geq$ 0.99 was accepted in each time. All samples were measured in triplicate.

IV.   Sample and collection

A. Porcine models

In the end of experiment, pigs were slaughtered by electrical stunning and bleeding. Tissues, including liver, muscle, back fat, visceral fat and pituitary gland, were collected, washed by saline, and snap-frozen into liquid nitrogen and stored at -80 ℃ for further analyses.

B. Mouse models

In the end of experiment, mice were sacrificed with CO2. Tissues, including brain, liver, and adipose tissue, were collected, washed by phosphate buffered saline (PBS; 135mM NaCl, 4.7mM KCl, 10mM Na2HPO4 and 2mM NaH2PO4, pH7.4) , and snap-frozen into liquid nitrogen and stored at -80 ℃ for further analysis.

V.   Protein concentration

After appropriate dilution of samples, the protein concentrations were measured by

Braford reagent (B6916, Sigma, St. Louis, MO, USA). The R2 of standard curve≥ 0.99 was accepted in every time. All samples were measured in triplicate and calculated protein concentrations according to average absorbance.

VI.   *Triglyceride content in tissue*

Tissue (0.1 g) was weighted and homogenized with 1 mL 5% NP-40. Sample was heated up at 85 ℃ in 3 minutes slowly and then was cooled down to room temperature. The heating procedure was repeated triplicate until the sample became cloudy. Sample was centrifuged (14000 × g for 2 minutes). The supernatant was removed to a new tube for use and diluted 10-fold with water for use.

The tissue triglyceride concentration was measured by commercial kits (BXC0272C, Fortress, Antrim, UK), and the procedure was followed blood sample described above. The formula as below:

$$\text{Triglyceeide content}\left(\frac{mg}{mg\ protein}\right)$$
$$= \text{triglyceride concentration}\left(\frac{mg}{dL}\right) \div \text{protein content}\left(\frac{mg}{dL}\right)$$

VII.   *Oxygen radical absorbance capacity (ORAC)in tissue*

Tissue (10 mg) was weighted and homogenized with 1 mL phosphate buffer (PB; 67.5 mM NaHPO4 and 7.5 mM KH2PO4, pH7.4), and the sample was centrifuged (5000 × g at 4 ℃ for 10 minutes). The supernatant was removed to a new tube and diluted to appropriate fold with PB before use.

Firstly, 100 μL of β-PE solution (βPE; 3.78 mg β-phycoerythrin (P1286, Sigma, St. Louis, MO, USA) in 1 litter water) were loaded in black plate. Secondly, 30 μL of PB, 20 μM Trolox (93510, Fluka, St. Louis, MO, USA) or diluted sample were loaded as

blank, control and sample, respectively. Finally, 30 μL of 80 mM 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH; 440914, Sigma, St. Louis, MO, USA) were added in each well. The fluorescent microplate reader (BioTek, Synergy H1, Ashton Vale, BRS, USA) was set up 420 nm as excitation and 576 nm as emission. The fluorescence was recorded immediately (0 minute) and every 5 minutes till the value was stable.

The area under curve (AUC) was calculated from initial to final minutes in each line. The relative ORAC value calculation as below:

$$\frac{ORAC_{sample}}{ORAC_{control}} = \frac{Net\ AUC_{sample}}{Net\ AUC_{control}} = \frac{AUC_{sample}\ - AUC_{blank}}{AUC_{control}\ - AUC_{blank}}$$

The ORAC formula as below:

$$ORAC_{sample}\left(\mu mole/g\ protein\right)$$

$$= \frac{Net\ AUC_{sample}}{Net\ AUC_{control}} \times ORAC_{control}\ (\mu M) \div \text{protein content}\ (mg/mL)$$

VIII. *Real-time quantitative polymerase chain reaction (PCR) analysis*

A. *Total RNA extraction and cDNA synthesis*

Tissue (0.1-0.2 g) was weighted and homogenized with 1 mL TRIzol (15596-018, Invitrogen, Grand Island, NY, USA) to extracted total RNA according the manufacturer's instructions. Sample was on ice for 15 minutes and then mixed with 100 μL of 1-bromo-3-chloropropane (BCP; BP151, Molecular research center, California, OH, USA). Next, sample was on ice for 15 minutes and then centrifuged (12000 × g at 4 ℃ for 15 minutes). Following centrifugation, the aqueous phase was removed to a new tube, and gently mixed with 500 μL of isopropanol. The samples were incubated at

-20 ℃ overnight. RNA pellets were precipitated by centrifugation (12000 × g at 4 ℃ for 10 minutes) and washed by 75% ethanol with 0.1% diethyl pyrocarbonate (DEPC; 1609-47-9, Bio Basic, Canada) in deionised water. RNA pellets were air-dried for 10 minutes and dissolved in 0.1% DEPC.

RNA samples contained some DNA, which were digested by DNase-free kit (AM1907, Ambion, Austin, TX, USA). After DNA digestion, RNA samples were reverse-transcribed to complementary DNA (cDNA) with High Capacity cDNA Reverse Transcription (RT) kit (4368813, Applied Biosystems, Foster city, CA, USA). All the procedures were followed according to the manufacturer's instructions.

B. Real-time RT- PCR

Real-time quantitative PCR reactions were performed on real-time PCR machine (CFX96, Bio-Rad, Hercules, CA, USA) using DyNAmo Flash SYBR Green qPCR Kit (F415L, Finnzymes, Espoo, Finland). The PCR program was as below:

Step 1   95.0℃ for 7 min

Step 2   95.0℃ for 10 sec

Step 3   Annealing temperature for 30 sec, go to Step 2 and repeat 40 times

Step 4   60.0℃ for 10 sec

Step 5   Melting curve, 65 ℃ to 95 ℃ by increasing 0.5℃ per 5 sec.

Primers and related informations were provided in Table 6 and Table 7.

C. Quantitative data analysis of real-time PCR

Value of threshold cycle (Ct) was detected after Real-time PCR. The TATA-box binding protein (TBP) and the beta-actin were used as the reference gene in pig and mouse model, respectively. Relative RNA gene expression was calculated based on the formula: $2^{-(\Delta\Delta Ct)}$.

The calculation as below:

$$\Delta\Delta Ct = \Delta Ct_{treat} - \Delta Ct_{control}$$

$$= \left[Ct_{target} - Ct_{reference} \right](treat) - \left[Ct_{target} - Ct_{reference} \right](control)$$

IX. *Protein expression and quantitation*

A. *Total protein extraction*

Tissue (0.1-0.2 g) was weighted, homogenized by liquid nitrogen, and mixed with 1 mL of extraction buffer to extract total protein according the manufacturer's instructions. Sample was on ice for 15 minutes and centrifuged (14000 × g at 4 ℃ for 30 minutes). The supernatant was collected to a new tube for use. The extraction buffer prescriptions were provided in Table 8.

B. *Polyacryamide gel electrophoresis*

After protein concentration determination, samples were diluted to appropriate concentration by PBS and denatured at 95 ℃ for 5 minutes with sample buffer. Samples were loaded in 10% SDS-polyacryamide gel, and the electrophoresis was run in running buffer at 150 V for 90 minutes. The prescriptions of sample buffer, running buffer and 10% SDS-polyacryamide gel were provided in Table 9 - 11.

C. *Western blot and immunostaining*

Protein was transferred from gel to PVDF membrane in blotting buffer at 400 mA for 1.5 hour. The 5% bovine serum albumin (BSA; A2153, Sigma, St. Louis, MO, USA) in PBST (PBS containing 0.1% Tween-20) was used as a blocking buffer. After blocking for 1 hour, the membrane was incubated with the primary (1st) antibody solution at 4 ℃ for overnight. Then, the membrane was washed with PBST triplicate, 10 minutes each, and then incubated with the 2nd antibody solution at room temperature

for 1 hour. Finally, after washing with PBST triplicate, the membranes were incubated in the chemiluminescence reagent (#WBKLS0500, Millipore, Billerica, MA, USA) with slow shaking, and exposed to the Imaging Systems (BioSpectrum Imaging Systems, UVP, Upland, CA, USA). The prescription of blotting buffer and antibody were provided in Table 12 - 13.

D. Quantitative data analysis of protein expression

Target bands were quantified using the BioSpectrum Imaging Systems (UVP, Upland, CA, USA) and corrected for the intensity of beta-actin in the same sample.

X. *Statistical analysis*

Data were expressed as mean ± SEM. Results were analyzed by SAS 9.2. Statistical significance among different experimental groups was determined by Duncan's multiple range tests. P-value ≤ 0.1 was considered pattern, and the P-value ≤ 0.05 was considered statistically significant.

Table 3 Diet composition for porcine in experimental 1

	L	M	H
Ingredient (kg)/1000 kg			
Yellow corn	370	495	601.2
Soybean meal	140	153	166
Fish meal	29	26	0
Alfalfa meal	50	105	50
Molasses	50	50	50
Wheat Bran	100	68	17
Soybean shell	250	90	70
Composition (calculated)			
ME (kcal/kg)	2510	2721	2892
CP (%)	19.0	19.0	18.5

ME, metabolic energy; CP, crude protein.

Table 4 Diet composition for porcine in experimental 2

	CON	HFD
Ingredient (kg) /1000kg		
Yellow corn	510	410
Soybean meal	124	245
Alfalfa meal	117.5	0
Molasses	30	30
Wheat bran	97	0
Soybean hull	95	0
Palm oil	0	138
Sucrose	0	130
Composition (calculated)		
ME (kcal/kg)	2713	3714
CP (%)	14.44	14.25

ME, metabolic energy; CP, crude protein.

Table 5 Diet composition for mouse in experimental 2

	Control*	HFD**
Energy from		
Protein (%)	28.5	18.3
Fat (%)	13.5	45.7
Carbohydrate (%)	58.0	35.3
Composition		
ME (kcal/gm)	3.02	4.65
Protein (%)	23.9	21.3

ME, metabolic energy.
*, LabDiet 5001, St. Louis, MO, USA
**, TestDiet 58V8 DIO Rodent Purified Diet, Richmond, IN, USA.

Table 6 Porcine primer sequences of selected genes for real-time RT- PCR analysis

Gene	Reference sequence	Forward primer (5'→3')	Reverse primer (5'→3')	Length (bp)	Annealing Temperature (℃)
TBP	DQ178129	GATGGACGTTCGGTTTAGG	AGCAGCACAGTACGAGCAA	123	60
SIRT1	NM_001145750.1	ACCAGCTTCTTCAGTGACTCTCCC	ACTGCAGGCAACATTCTTCAAACCT	184	60
GK	XM_003484067.1	TTGAAGAAGGTGATGAGGCGGATG	CCAGGTCCAGGGAGAGGAAGTC	151	60
LPK	XM_003481437.1	GTGCTGCTGCCATCATTG	CTGGAGGTTCACGGTAGAG	165	60
G6Pase	NM_001113445.1	CGGCTTTCGGTGCTTGAATGTC	AGGCTGGCGTTGTAGATACTCTG	180	60
PEPCK	NM_001161753.1	GCAGCCGAACACAAAGGGAAG	GCGACGGAACCAGTTGGCATG	159	60
ACO	NM_001101028.1	TGAGTGACCTAGACCTGCAGCA	AGGCCCAGGATGTCCTCGCT	176	64
CPT1	NM_001129805.1	GTGTCAGCGTAGCAAGTGGA	TCAGCGAGTGTGCCAGATAC	224	60
HMGR	NM_001122988.1	GCACCATGTCAGGGGTGCGT	GGACGACCCTCGCGGCTTTC	148	64
FAS	NM_001099930.1	AGGCCAGGCAGGTCCGAGAG	GTTCGCGGTGCCGCTGTACT	230	64
DGAT	NM_214051.1	ACCGGGACTGGTGGAACTCGG	GCGGAACATGCGCAGAGGGA	200	60

TBP, TATA-binding protein; SIRT1, sirtuin 1;GK, glucokinase; LPK, pyruvate kinase of liver; G6Pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; ACO, acyl-Co A oxidase; CPT1, carnitine palmitoyl transferase 1; HMGR, 3-hydroxy-3-methyl-glutaryl-Co A reductase; FAS, fatty acid synthase; DGAT, diacylgycerol acyltransferase.
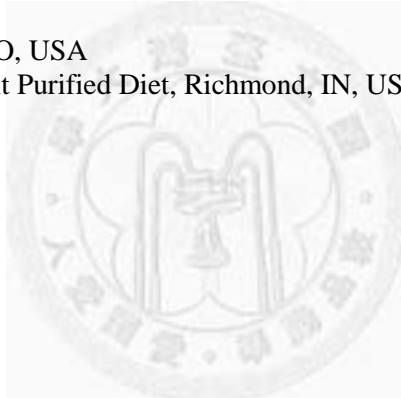
Table 7 Mouse primer sequences of selected genes for real-time RT- PCR analysis

Gene	Reference sequence	Forward primer (5'→3')	Reverse primer (5'→3')	Length (bp)	Annealed Temperature (℃)
beta-actin	NM_007393.3	CATGTACGTAGCCATCCAGG	CTCTCAGCTGTGGTGGTGAA	225	60
SIRT1	NM_019812.2	TTGGCACCGATCCTCGAAC	CCCAGCTCCAGTCAGAACTAT	209	60
CPT1	NM_013495.2	GGTCTCAAGTAATGGGTGC	GAATACCAAACGGAGTTGC	102	62
G6Pase		GCCTTCTATGTCCTCTTTCCC	AACAGAATCCACTTGAAGACAGA		62
PEPCK	NM_028994.2	TTGGAGAGAATGCTCGTGTG	TGGAGAACAGCTGACTGGTG	150	62

SIRT1, sirtuin 1; CPT1, carnitine palmitoyl transferase 1, G6Pase, glucose 6-phosphatase;PEPCK, phosphoenolpyruvate carboxykinase.

Table 8 Composition of extraction buffer

	1X extraction buffer
RIPA buffer (#9806, Cell signaling)	8 mL
Proteinase inhibitor (04693116001, Roche, Basel, Switzerland)	1 mL
Phosphatase inhibitor (04906845001, Roche, Basel, Switzerland)	1 mL
Total volume	10 mL

Proteinase inhibitor / phosphatase inhibitor, 1 tablet dissolved in 10 mL of each ddH2O

and diluted 10-fold with RIPA buffer for use.

Table 9 Prescription of sample buffer

4X sample buffer	
1 M Tris-HCl	20.0 mL
2- Mercaptoethanol	10.0 mL
100% Glycerol	20 mL
SDS (17-1313-01, GE, Piscataway , NJ, USA)	4.0 gm
Bromophenol blue	20.0 mg
ddH2O	20.0 mL
Total volume	50 mL

4X sample buffer were adjusting the pH value to 6.8.
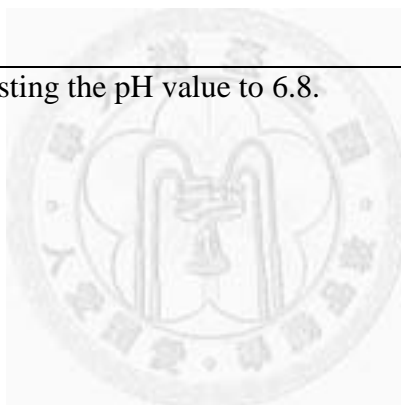
Table 10 Prescription of 10% SDS-polyacryamide gel

	Separation gel	Stacking gel
percentage	10%	4%
30% Acrylamide/Bis Solution (#161-0154, Bio-Rad, Hercules, CA, USA)	6.7 mL	0.66 mL
1.5 M Tris, pH8.8	5.0 mL	-
1.5 M Tris, pH6.8	-	1.24 mL
10% SDS (17-1313-01, GE, Piscataway , NJ, USA)	0.2 mL	0.05 mL
5% APS (A3678, Sigma, St. Louis, MO, USA)	0.1 mL	0.1 mL
TEMED (T9281, Sigma, St. Louis, MO, USA)	10 μL	50 μL
ddH2O	7.99 mL	2.9 mL
Total volume	20 mL	5 mL

Table 11 Prescription of running buffer

	5X running buffer
Tris (17-1321-01, GE, Piscataway , NJ, USA)	30.3 mL
Glycine (17-1323-01, GE, Piscataway , NJ, USA)	142.6 gm
Total volume	1000 mL

5X running buffer were adjusting the pH value to 8.3.

1X running buffer: 5 mL 10% SDS, 100 mL 5X running buffer and 395 mL ddH2O

Table 12 Prescription of blotting buffer

	10X blotting buffer
Tris (17-1321-01, GE, Piscataway , NJ, USA)	60.6 gm
Glycine (17-1323-01, GE, Piscataway , NJ, USA)	288 gm
total volume	2000 mL

10X blotting buffer were adjusting the pH value to 8.3.

1X blotting buffer: 100 mL 10X blotting, 200 mL methanol, 700 mL ddH2O

Table 13 The list of immunostaining antibody

	Name	Molecular weight (kDa)	Host source
1st	SIRT1 (#8469, Cell signaling)	120	Mouse
1st	beta-actin (Santa cruze)	~43	Mouse
2nd	Goat anti-mouse IgG (Abcam, Cambridge, MA, USA)	-	

Diluted factors were according the manufacturer's instructions.

Chapter 3: Results

I.   ME vs. SIRT1

A. The effect of different ME levels on growth performance and blood parameters

In experiment 1, we compared the effect of three ME levels (2500 (L), 2700 (M) or 2900 (H) kcal/kg) on growth performance and energy metabolism during 10-week experimental period. Daily feeding rate was 5 % of body weight, so the energy intake amounts were increased with higher energy group. Weight curve was similar among three groups, and there was no difference among three groups at the end of experiment (Fig 6A). In addition, there was no difference in feed conversion ratio, average daily gain and BMI among three groups (Fig 6B - 6D). Organ weight was recorded at the end of experiment. Liver weight was not different among three treatments. In addition, a similar result of heart, and spleen weight were observed among treatments (Table 14).

Blood parameters, including triglyceride, total cholesterol, HDL and LDL, were analyzed in the initial and at the end of experiment. Lipid profile was similar in the initial of experiment, but L group tended to have a higher level of triglyceride and HDL ($P < 0.1$) than H group after 10-week experimental period (Fig 7). In addition, there was a higher insulin concentration in L pigs compared with H pigs ($P < 0.05$), while leptin was not different among three groups (Table 15).

B. The effect of different ME levels on SIRT1 gene expression in tissues

SIRT1 gene expression in liver, subcutaneous adipose tissue, visceral adipose tissue, muscle and pituitary gland, were analyzed. L pigs had higher SIRT1 expression in the liver than H pigs ($P < 0.1$), whereas no differences were observed in other tissues (Fig 8A).

C. The effect of different ME levels on hepatic energy metabolism

L pigs also had higher diacylgycerol acyltransferase (DGAT) expression than other groups (P < 0.1). Transcript abundances of acyl-Co A oxidase (ACO), carnitine palmitoyl transferase 1 (CPT1), fatty acid synthase (FAS) and 3-hydroxy-3-methyl-glutaryl-Co A reductase (HMGR) were not different among three treatments (Fig 8B).There was no difference in hepatic triglyceride content among three groups. (Table 16)

L group had higher pyruvate kinase (LPK) expression in the liver than H group (P<0.1), and L pigs trended to have higher phosphoenolpyruvate carboxykinase (PEPCK) gene expression (P=0.1861). Glucokinase (GK) and glucose 6-phosphatase (G6Pase) expression were not different among three groups (Fig 8C).
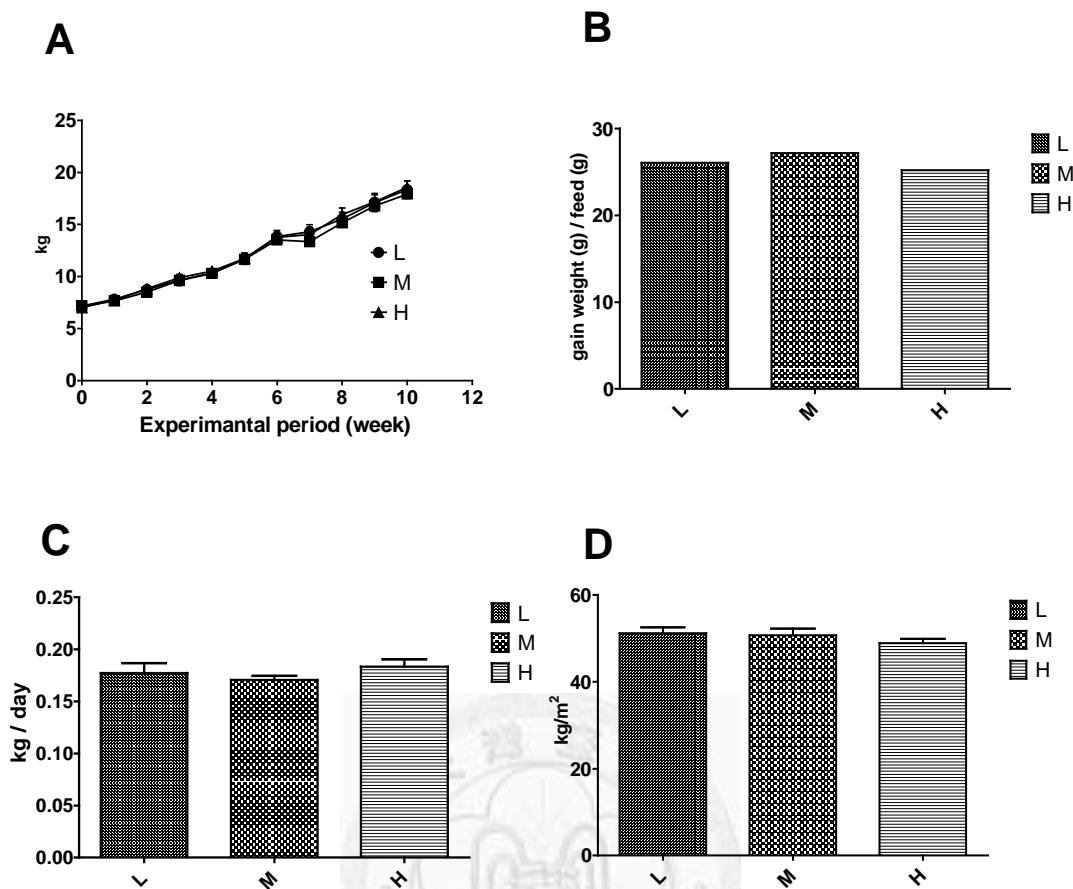
Fig 6 Growth performance of pigs in experiment 1

A, body weight. B, feed conversion ratio (weight gain / total feed intake). C, average

daily gain (body weight gain / average daily feed intake). D, BMI (body weight / body

length (crown–rump length) square). All results are expressed as mean ± SEM. N=8 per

group. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M

treatment, and 2900 Kcal/kg for H treatment.
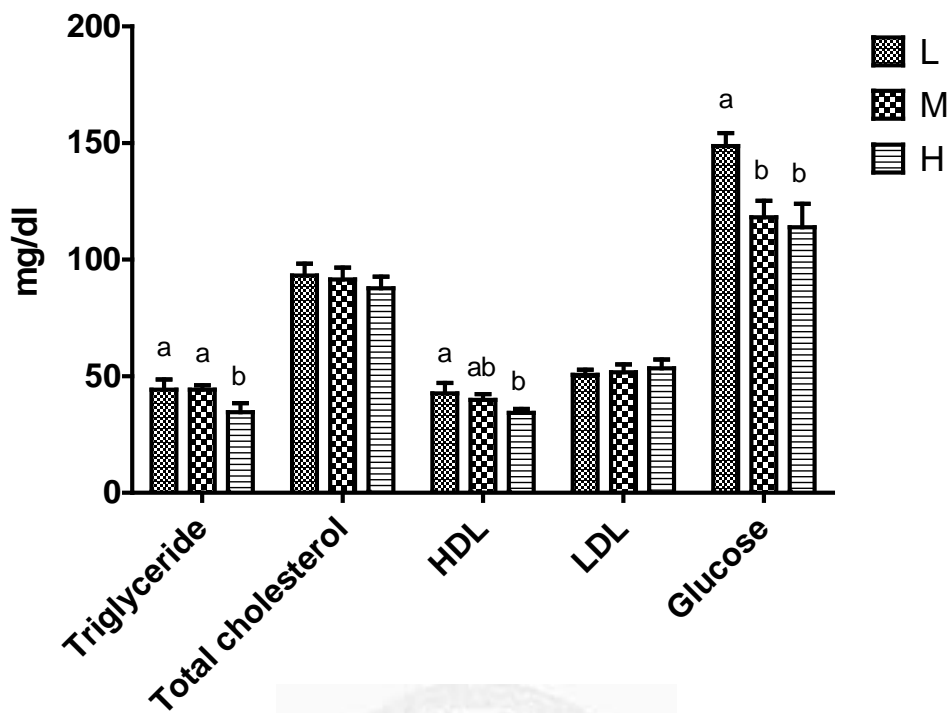
Fig 7 Blood parameters of pigs in experiment 1

Plasma triglycerides, total cholesterol, HDL, LDL and glucose were measured at the end of experiment. All results are expressed as mean ± SEM. Different lowercase letters indicate a significant difference at $P < 0.1$ and N=8 per group. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M treatment, and 2900 Kcal/kg for H treatment.

Table 14 Organ weight of pigs in experiment 1

	L	M	H
Heart (g)	73.5 ± 5.6	68.8 ± 2.9	79.8 ± 3.6
Liver (g)	391.0 ± 20.7	359.5 ± 7.1	401.3 ± 17.7
Spleen (g)	34.0 ± 2.7	32.3 ± 1.1	38.3 ± 2.8
Kidneys (g)	81.0 ± 2.8[a]	71.5 ± 3.4[b]	74.3 ± 3.6[ab]

All results are expressed as mean ± SEM. Different lowercase letters indicate a significant difference at P < 0.1 and N=4 per group. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M treatment, and 2900 Kcal/kg for H treatment.

Table 15 Circulating hormone of pigs in experiment 1

	L	M	H
Insulin (ng/mL)	3.6 ± 0.9^a	1.9 ± 0.3^b	1.5 ± 0.3^b
Leptin (ng/mL)	1.7 ± 0.3	2.3 ± 0.4	1.4 ± 0.3

All results are expressed as mean ± SEM. Different lowercase letters indicate a significant difference at $P < 0.05$. N=5 per group in insulin assay, N=8 per group in leptin assay. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M treatment, and 2900 Kcal/kg for H treatment.

Table 16 Hepatic triglyceride content of pigs in experiment 1

	L	M	H
Triglyceride (mg / mg protein)	0.6 ± 0.1	0.8 ± 0.0	0.8 ± 0.2

All results are expressed as mean ± SEM. N=4 per group. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M treatment, and 2900 Kcal/kg for H treatment.

Fig 8 SIRT1, glucose- and lipid- related mRNA expression of pigs in experiment 1

A, SIRT1 mRNA expression in liver, SAT, VAT, muscle and PG. B, hepatic lipid metabolic mRNA expression C, hepatic glucose metabolic mRNA expression. Gene expression was normalized to reference gene (TBP) and expressed relative to H group. All results are expressed as mean ± SEM. Different lowercase letters indicate a significant difference at P < 0.1. N=4 per group. Metabolic energy of diet is 2500 Kcal/kg for L treatment, 2700 Kcal/kg for M treatment, and 2900 Kcal/kg for H treatment.
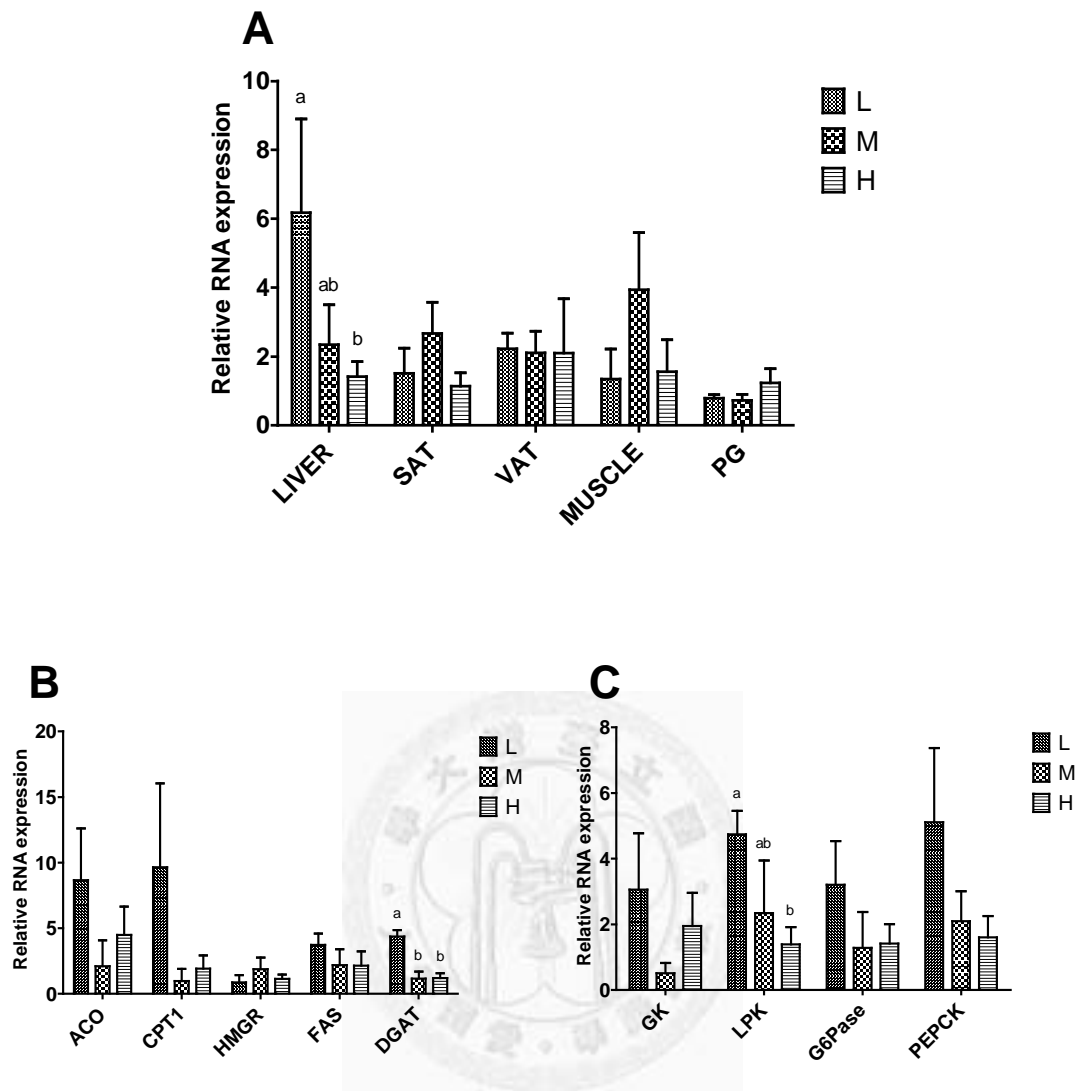
II.   HFD in porcine model vs. SIRT1

A.   The effect of HFD on growth performance and blood parameters

In experiment 2, we had fed HFD (3700 kcal/kg) or CON (2700 kcal/kg) with pigs for 6 months. In the beginning of experiment, body weight was similar between treatments. Body weight of HFD group was higher than CON pigs after 3-month experiment ($P < 0.1$) (Fig 9A), and average daily gain was higher in HFD pigs than CON pigs did ($P < 0.1$) (Fig 9B) HFD pigs also had a thicker back fat than CON pigs did ($P < 0.05$) (Fig 9C). In addition, HFD pigs had a larger spleen weight than CON pigs did (Table 17). Whereas, heart, lung and kidneys, were not different between two treatments.

In the beginning of experiment, plasma lipid profiles were similar between CON and HFD pigs. Unexpectedly, HFD did not affect the blood lipid profile after 6-month experiment (Fig 9D).

B.   The effect of HFD on SIRT1 gene expression in tissues

To confirm whether SIRT1 expression was regulated by HFD, SIRT1 gene expression was detected in energy metabolic related-tissues, including liver, muscle and pituitary gland. HFD pigs had an increase in hepatic mRNA expression of SIRT1 as compared with CON pigs ($P < 0.05$) (Fig 10), and hepatic SIRT1 protein level of HFD group was higher than CON group ($P < 0.05$) (Fig 11A-B).

C.   The effect of HFD on hepatic carbohydrate and lipid metabolism

HFD treatment increased CPT1 and ACO gene expression in the liver as compared with CON ($P < 0.1$). HMGR was slightly elevated in HFD group ($P=0.1863$). While, there were no difference on gene expression of FAS and DGAT between treatments (Fig 10C). Hepatic triglyceride content revealed that hepatic fat accumulation was not

changed by HFD treatment (Table 18). GK, LPK, G6Pase and PEPCK, were not different between two groups (Fig 10D).

   D. *The effect of HFD on hepatic antioxidative capacity*

The results revealed that the hepatic antioxidative capacity was not different between two treatments (Table 18).
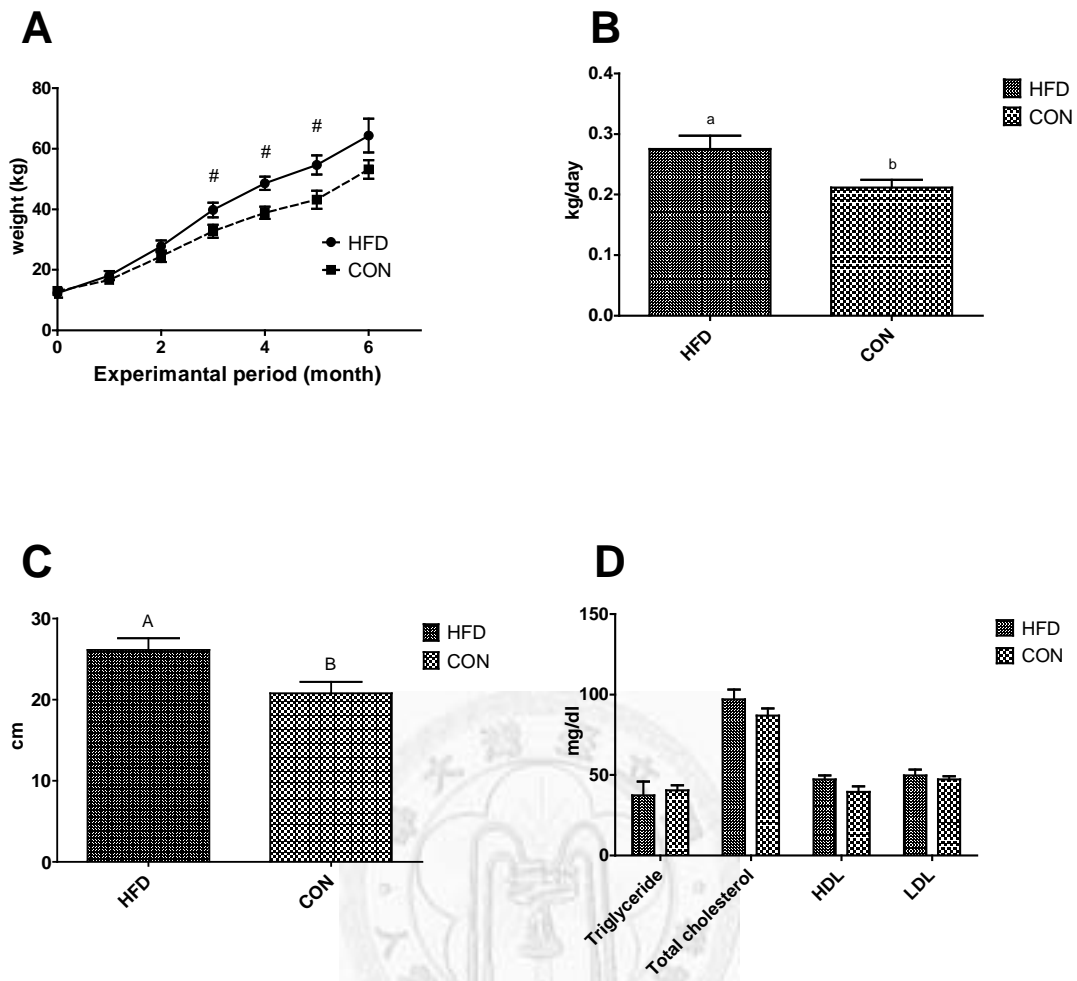
Fig 9 Growth performances and blood parameters of pigs in experiment 2

A, body weight. B, average daily gain. C, backfat thickness. D, plasma blood lipid profile in the end of experiment. All results are expressed as mean ± SEM. # indicate a significant difference between two groups and different lowercase letters indicate a significant difference at P < 0.1. Different lowercase or uppercase letters indicate a significant difference at P < 0.1 or P < 0.05, respectively. N=4 per group. Metabolic energy of diet is 3700 Kcal/kg for HFD treatment and 2700 Kcal/kg for CON treatment.

Table 17 Organ weight of pigs in experiment 2

(g)	HFD	CON
Heart	210.0 ± 19.1	192.3 ± 13.7
Liver	695.3 ± 76.6	647.0 ± 25.2
Spleen	115.5 ± 11.3[A]	80.0 ± 6.2[B]
Kidneys	193.8 ± 17.9	158.3 ± 7.8

All results are expressed as mean ± SEM. Different uppercase letters indicate a significant difference at P < 0.05. N=4 per group. Metabolic energy of diet is 3700 Kcal/kg for HFD treatment and 2700 Kcal/kg for CON treatment.
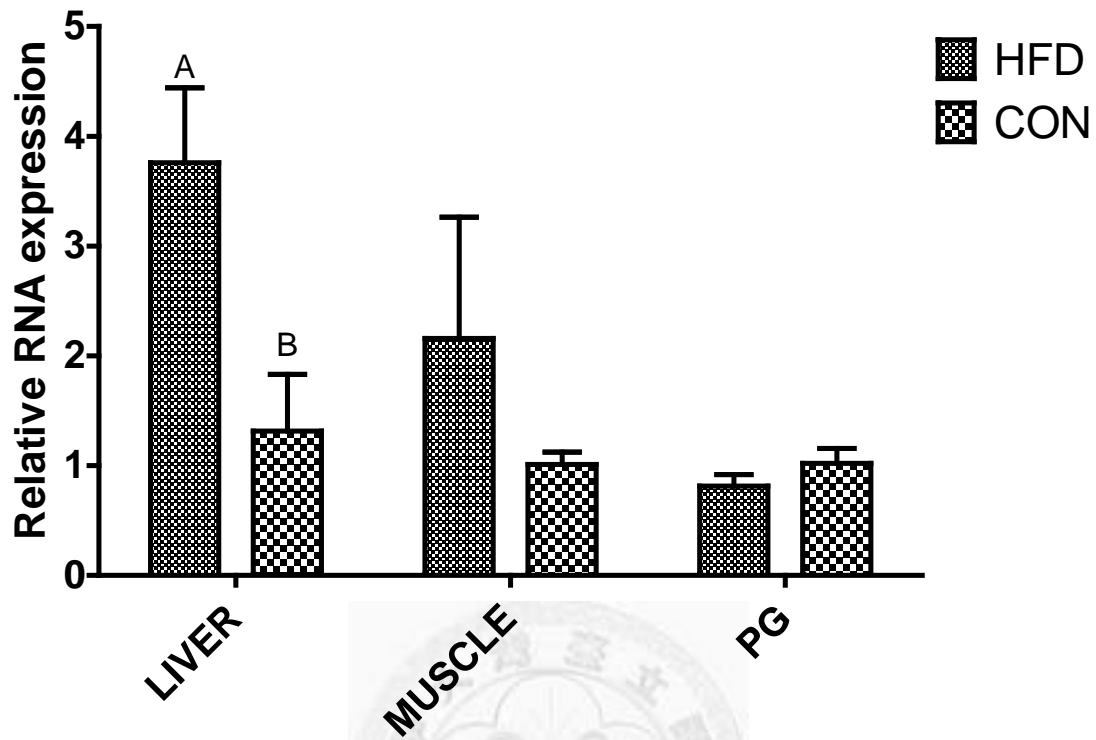
Fig 10 SIRT1 mRNA expression in tissues of pigs in experiment 2

SIRT1 mRNA expression in liver, muscle and PG. Gene expression was normalized to reference gene (TBP) and expressed relative to CON group. All results are expressed as mean ± SEM. Different uppercase letters indicate a significant difference at $P < 0.05$. N=4 per group. Metabolic energy of diet is 3700 Kcal/kg for HFD treatment and 2700 Kcal/kg for CON.
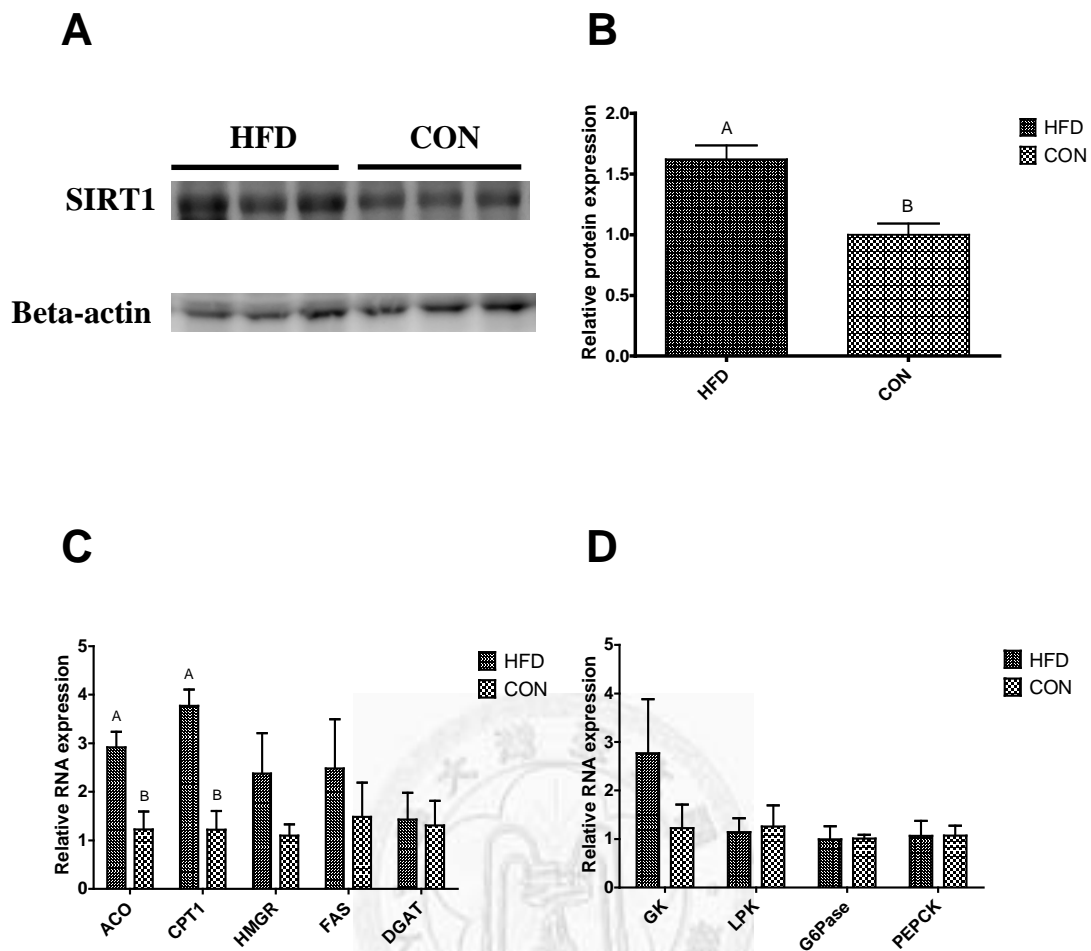
Fig 11 Hepatic SIRT1 protein level, lipid- and carbohydrate-metabolic related mRNA expression of pigs in experiment 2

A, western blot analysis of hepatic SIRT1 expression. B, quantitation of protein expression level. C, hepatic lipid metabolic mRNA expression. D, hepatic carbohydrate metabolic mRNA expression. Beta-actin was used as a loading control for normalization and expressed relative to CON group in western blot analysis. Gene expression was normalized to reference gene (TBP) and expressed relative to CON group. All results are expressed as mean ± SEM. Different uppercase letters indicate a significant difference at $P < 0.05$. N=4 per group. Metabolic energy of diet is 3700 Kcal/kg for HFD treatment and 2700 Kcal/kg for CON.

Table 18 Hepatic triglyceride content and antioxidative activity of pigs in experiment 2

	HFD	CON
Triglyceride (mg / mg protein)	0.9 ± 0.3	0.8 ± 0.2
Trolox (μmole / g protein)	45.1 ± 3.6	53.1 ± 4.9

All results are expressed as mean ± SEM. N=4 per group. Metabolic energy of diet is

3700 Kcal/kg for HFD treatment and 2700 Kcal/kg for CON treatment.

III.   HFD-feeding duration in mouse model vs. SIRT1

A. The effect of HFD-feeding duration on growth performance and blood parameters

In the experiment 2, we found that pigs fed HFD for 6 months didn't exhibit metabolic syndrome, and the HFD pigs had a higher hepatic SIRT1 expression. The results suggested that hepatic SIRT1 and its related regulation may protect pig from metabolic syndrome in response to HFD. Pig model has a longer life cycle, while mouse model has a shorter life and is easier to handle. Therefore, this study was designed to investigate the effect of HFD duration on SIRT1-regulated metabolism. Body weight curve showed that HFD mice had significantly increasing body weight since week 2 of experiment compared with CON mice ($P < 0.05$) (Fig 12).

At week 2 of experiment, there were no difference on tissue weights, including liver and heart between two groups. While HFD mice had significantly greater liver and heart weight than CON mice after 25 weeks of feeding ($P < 0.05$) (Table 19).

HFD mice had a higher fasting glucose than CON mice after week 2 of experiment ($P < 0.05$) (Fig 13A). Plasma total cholesterol and HDL levels were elevated by HFD feeding with advancing age ($P < 0.05$) (Fig 13B - 13C). HFD mice had a higher plasma triglyceride level than CON mice at week 2 of experiment ($P < 0.05$), while plasma triglyceride was decreased after week 8 of experiment and no differences were observed between treatments after week 20 (Fig 13D).

B. The effect of HFD-feeding duration on SIRT1 gene expression in liver

Hepatic SIRT1 expression was upregulated at week 2 in HFD mice ($P < 0.05$) (Fig 14A). However, with the prolonged HFD feeding, SIRT1 expression was similar between 2 groups at 25-week. Hepatic SIRT1 mRNA expression was lower at week 2 than that at week 25 ($P < 0.05$) (Fig 14B).

C. The effect of HFD-feeding duration on hepatic metabolism

There was no difference on hepatic CPT1, G6Pase and PEPCK expression between two treatments at week 2 of experiment. HFD mice had lower CPT1 ($P = 0.1256$) and G6Pase ($P < 0.05$) mRNA expression, and a higher PEPCK expression ($P < 0.05$) than CON group at week 25 of experiment (Fig 15A - 15C).
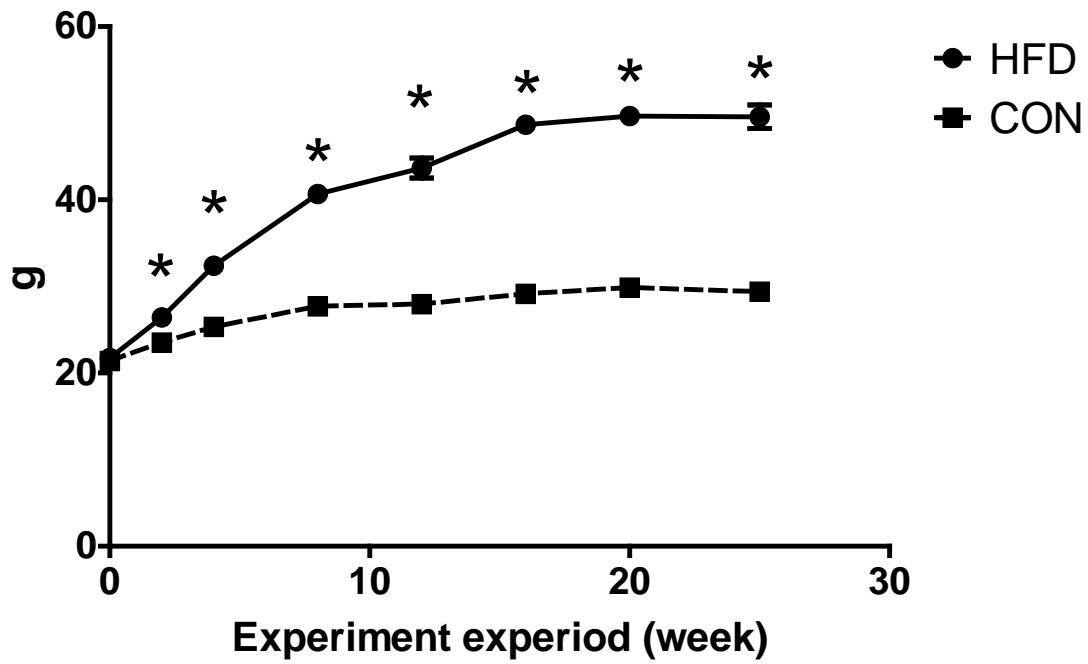
Fig 12 Body weight change of mice in experiment 2

Body weight was recorded on 0-, 2-, and every 4 weeks throughout the experiment. All results are expressed as mean ± SEM. * indicate a significant difference between two groups at *P* < 0.05. N=5 per group. HFD: mice with high fat diet; CON: mice with chow diet.
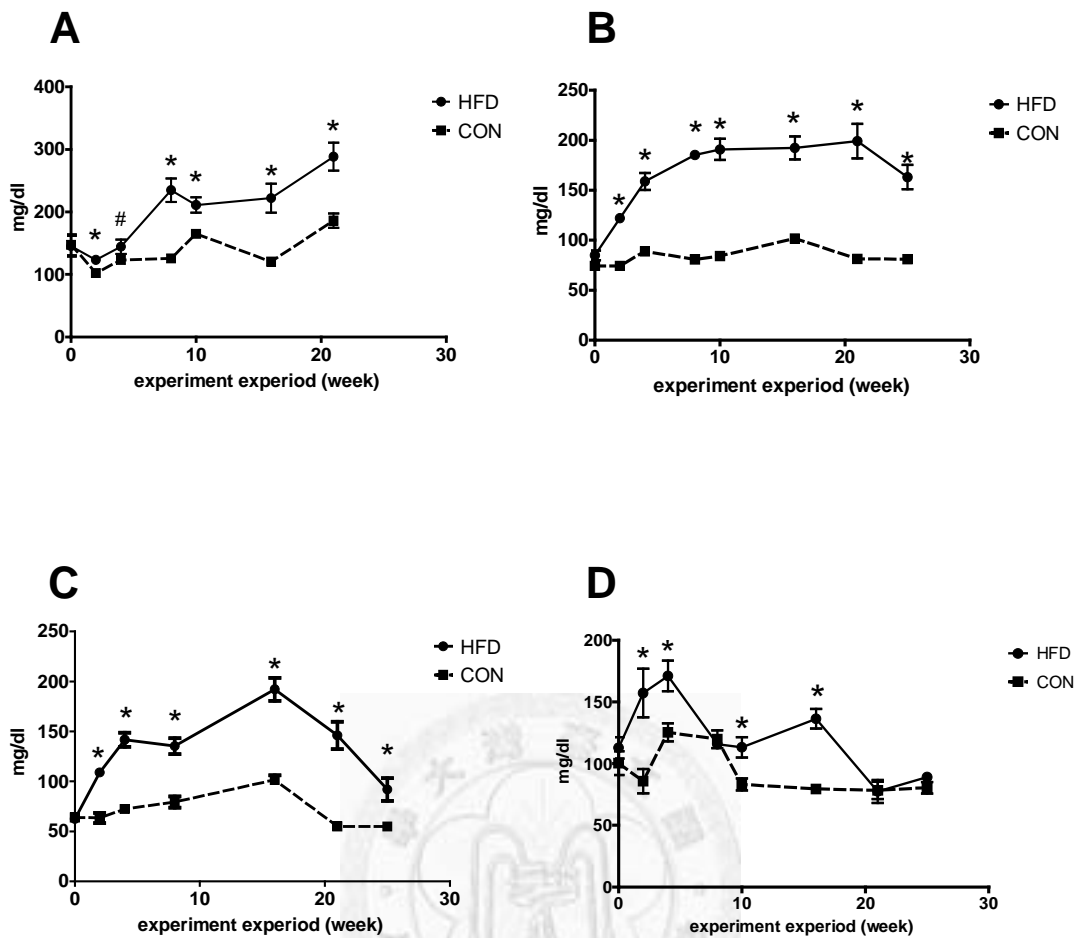
Fig 13 Blood parameters of mice in experiment 2

Plasma Glucose (A), total cholesterol (B), HDL (C) and triglyceride (D) were measured on 0-, 2-, 4- 8, 10- 20- and 25-week of experiment. All results are expressed as mean ± SEM. # indicate a significant difference between two groups at $P < 0.1$; * indicate a significant difference between two groups at $P < 0.05$. N=5 per group. HFD: mice with high fat diet; CON: mice with chow diet.

Table 19 Organ weight of mice in experiment 2

Experimental period	(g)	HFD	CON
2wk	Heart	0.14 ± 0.00	0.15 ± 0.01
	Liver	0.97 ± 0.05	0.97 ± 0.06
25wk	Heart	0.18 ± 0.01^A	0.14 ± 0.01^B
	Liver	1.75 ± 0.06^A	1.21 ± 0.05^B

All results are expressed as mean ± SEM. Different uppercase letters indicate a significant difference at $P < 0.05$. N=5 per group. HFD: mice with high fat diet; CON: mice with chow diet.
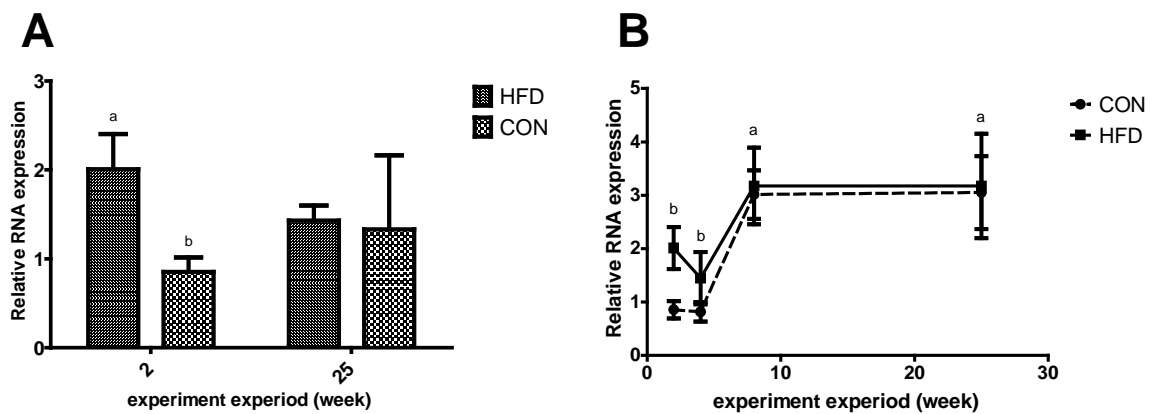
Fig 14 Hepatic SIRT1 mRNA expression of mice in experiment 2

A, Hepatic SIRT1 mRNA expression at week 2 and week 25 of experiment. B, SIRT1 mRNA expression change in liver. Gene expression was normalized to reference gene (beta-actin) and expressed relative to SIRT1 expression in CON group (A), or expressed relative to SIRT1 expression in CON group of week 2 (B). All results are expressed as mean ± SEM. Different lowercase letters indicate a significant difference at $P < 0.05$. N=5 per group. HFD: mice with high fat diet; CON: mice with chow diet.

Fig 15 The metabolic mRNA expression of mice in experiment 2

CPT1 (A), G6Pase (B) and PEPCK (C) mRNA expression in liver on week 2 and week

25 of experiment. Gene expression was normalized to reference gene (beta-actin) and

expressed relative to CON group. All results are expressed as mean ± SEM. Different

uppercase letters indicate a significant difference at P < 0.05. N=5 per group. HFD:
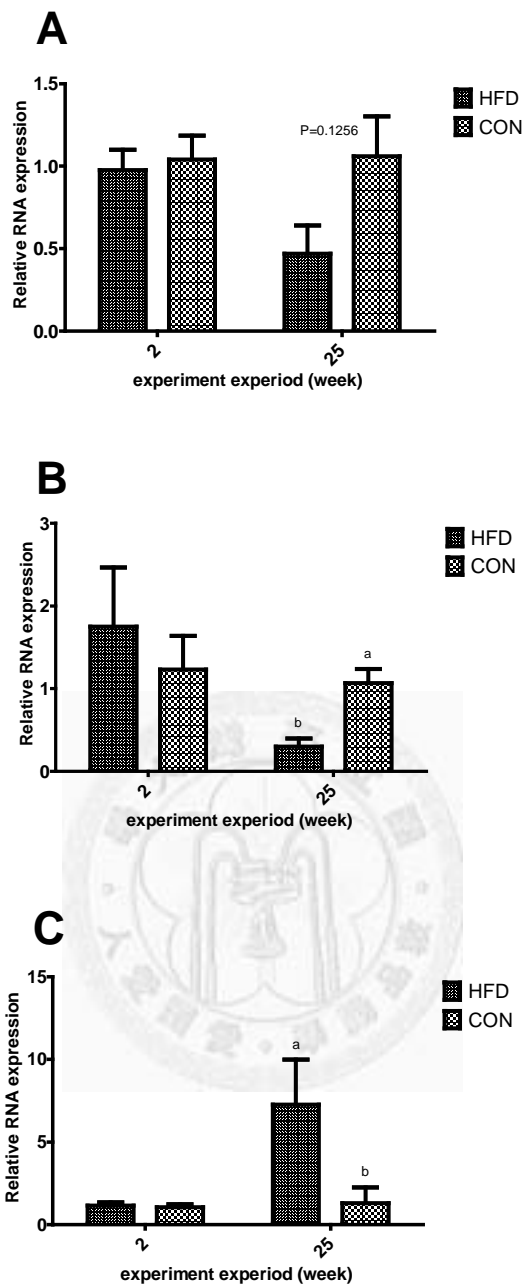
mice with high fat diet; CON: mice with chow diet.

Chapter 4: Discussion

I.   *Low ME vs. SIRT1*

Previous study revealed that porcine average daily gain and feed conversion ratio (weight gain / feed) was reducing with decreased dietary energy level (Campbell and Dunkin 1983). In our study, L diet did not cause growth retardation in pigs. Instead, L pigs had similar growth performance as H pigs did. In addition, L diet caused high SIRT1 expression and triglyceride synthesis in porcine liver. These results suggested that there were some relations between SIRT1 and a higher efficiency of energy utilization induced by L diet.

SIRT1 expression was modulated by energy status. SIRT1 expression were upregulated in mice and mammal cells by starvation (Nemoto et al. 2004). SIRT1 mRNA and protein levels were induced with CR in various tissues, including brain, adipose tissue, kidney and liver, and therefore improved mitochondrial oxidative stress and stress-induced apoptosis (Cohen et al. 2004; Nisoli et al. 2005; Civitarese et al. 2007). Studies showed that SIRT1 expression was increased in 40% CR, whereas decreased in 60% CR (Cohen et al. 2004; Chen et al. 2008), suggesting a limiting range of CR to promote SIRT1 activity in mammals. In this study, we supposed that 2900 kcal/kg was appropriate for growing Lanyu pig, and 2500 kcal/kg was 14% CR roughly. Hepatic SIRT1 increased with 14% CR in the porcine model, while SIRT1 expression in adipose tissue and muscle were similar among 3 groups. These results showed that, liver is a sensitive organ for energy restriction, and increased SIRT1 expression might modulate the cellular functions against oxidative stress and apoptosis.

Some studies proved that CR-induced SIRT1 expression elevated lipid usage in liver and muscle (Frescas et al. 2005; Rodgers et al. 2005; Gerhart-Hines et al. 2007; Banks et al. 2008). In our study, L pigs had high triglyceride synthesis in the liver and

elevated plasma triglyceride and glucose levels, suggesting that L diet increased triglyceride synthesis and nutrient flux to peripheral tissue for further use. In addition, low energy diet caused more HDL to carry cholesterol back to liver. Furthermore, SIRT1 expression was the highest in L pigs. Taken together, low energy diet (14% CR) increased hepatic SIRT1 expression, which might partially promote the efficiency of energy utilization in porcine.

II.   HFD vs. SIRT1

Miniature pig had been widely used in study of metabolic syndrome and CVD for several years. Gottingen miniature pigs fed with HFD for 6 weeks, exhibited hyperlipidemia (Johansen et al. 2001). Ossabaw pigs occurred metabolic syndrome and coronary artery disease after 9-week treatment by atherogenic diet (Dyson et al. 2006; Neeb et al. 2010). In our study, plasma lipid profiles were not different between two groups after 6 month-period , and HFD pigs did not cause metabolic syndromes and any clinical signs. These results demonstrated that Lanyu pigs might not be a suitable model for metabolic syndrome because of its metabolic characteristics.

Some studies proved that elevated SIRT1 activity by activator or overexpression protected animals from metabolic damages, including diet-induced obesity, diabetes, and insulin resistance (Baur et al. 2006; Banks et al. 2008; Pfluger et al. 2008). We found that HFD pigs displayed higher transcript and protein abundance than CON pigs. It suggested that promoted hepatic SIRT1 expression and its regulating pathway in response to HFD may protect pig against obesity or metabolic syndrome. Moreover, Lanyu pigs might be a potential therapeutic target in anti-metabolic syndrome animal model.

In the murine model, HFD mice had higher hepatic SIRT1 mRNA expression than CON mice on week 2 of experiment, whereas no difference in hepatic SIRT1 expression at week 25 was observed. Besides, HFD mice had low lipolysis and high glucose output in liver on week 2 of experiment. Mice exhibited similar hepatic SIRT1 protein levels with HFD feeding bewteen 4 and 19 weeks (Pfluger et al. 2008; Escande et al. 2010). However, prolonged HFD-feeding for 4 months, SIRT1 in muscle was decreased (Coste et al. 2008). In addition, SIRT1 protein levels in aorta were decreased with HFD at 6 months (Zhang et al. 2008). These results implied that SIRT1 gene expression in tissue might be decreased with prolonged HFD feeding. In our study, SIRT1 expression elevated on short-term (2 weeks) HFD feeding, suggesting the role of SIRT1 in response to HFD-induced dysregultaion. However, SIRT1 expression decreased with prolong HFD feeding, which may reduce its protective ability and increase the risk of metabolic syndrome.

III.   *Age vs. SIRT1*

We found that hepatic SIRT1 was increased with age in both HFD and CON mice. Aging causes metabolic malfunction. Old mice had lower insulin sensitivity (Blüher et al. 2002) and more body fat accumulation, such as inguinal white adipose tissue (Gyeong-Min Do 2008; Seo et al. 2009). Older mice fed with HFD displayed a higher risk of steatohepatitis and liver fibrosis (Kamari et al. 2011). Ferrara et al. (2008) found that SIRT1 activity decreased in the heart of old rats. Whereas, adequate activating SIRT1 prevented age-related disorders, such as oxidative stress (Alcendor et al. 2007), inflammation(Pearson et al. 2008), diabetes (Baur et al. 2006; Milne et al. 2007) and cardiovascular diseasees (Gyeong-Min Do 2008). We found that SIRT1 gene expression was higher in older mice than young mice. According to the studies mentioned above,

older mice had a higher risk of metabolic damages. The possible mechanism was that aging-induced damage may exceed the protection by SIRT1, or insufficient SIRT1 activity was generated in aging animal.

Taken together, this study indicated that porcine hepatic SIRT1 expression was induced in CR as the rodent model. While in the high energy status of mice induced by HFD-feeding 2 weeks, the hepatic SIRT1 expression was elevated as well. These results suggest a protective role of SIRT1 in early obesity stage. However, the related mechanism needs further elucidation.

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