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## The Effects of Retinoic Acid on the Expression of Glucose Transporter 4 in Differentiating L6 Myocytes and 3T3-L1 CAR $\Delta$ 1 Adipose Cells

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To the Graduate Council:

I am submitting herewith a thesis written by Yan Zhang entitled "The Effects of Retinoic Acid on the Expression of Glucose Transporter 4 in Differentiating L6 Myocytes and 3T3-L1 CAR $\Delta$ 1 Adipose Cells." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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We have read this thesis and recommend its acceptance:

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The Effects of Retinoic Acid on the Expression of  
Glucose Transporter 4 in Differentiating L6 Myocytes  
and 3T3-L1 CAR $\Delta$ 1 Adipose Cells

A Thesis Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville

Yan Zhang  
December 2017

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## ABSTRACT

The epidemicity of metabolic diseases such as obesity and diabetes globally has become a public health concern. Therefore, understanding interactions of macronutrients such as glucose and micronutrients such as vitamin A (VA) has become urgent. Both skeletal muscle and adipose tissues are critical for the regulation of glucose homeostasis. Retinoid acid (RA) is an active metabolite of retinol which mediates the major functions of VA. RA and insulin signals have been shown to regulate glucose metabolism. However, their roles in glucose metabolism in skeletal muscle and adipocytes remain to be revealed. We postulate that insulin and RA signals may work together to regulate glucose metabolism in skeletal muscle cells and adipocytes. In the first study, the glucose transporter 4 (GLUT4) expression were measured in L6 myocytes treated with increasing doses of RA in the absence or presence of insulin for 6 days. RA and insulin were found to inhibit the expression levels of GLUT4 in L6 cells after 4 and 6 days of treatment. The synergy inhibition effects of RA and insulin on GLUT4 can be observed at Day 4 with RA at 1  $\mu$ M RA. To study the RA effects on adipocytes, recombinant adenoviruses were used to overexpress retinoid X receptor  $\alpha$  (RXR $\alpha$ ) and chicken ovalbumin upstream transcription factor II (COUP-TFII), two transcription factors with the potential to mediate RA signaling. 3T3-L1 CAR $\Delta$ 1 cells were transfected with ad- $\beta$ -gal, ad-RXR $\alpha$  or ad-COUP-TFII and treated with increasing RA doses during differentiation. Lipid accumulation and GLUT4 protein level were examined. Interestingly, overexpression of RXR $\alpha$  enhanced the inhibitory effects of RA on lipid accumulation. Moreover, the inhibitory effect of RA on GLUT4 expression can be attenuated in the presence of RXR $\alpha$  overexpression. The overexpression of COUP-TFII may enhance the inhibitory effects of RA on the expression of GLUT4. The fact that RA and insulin synergized to inhibit the GLUT4 expression in L6 cells seems to indicate that cautions should be taken when VA supplement is recommended for patients with diabetes or obesity.

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## LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Name</u>
GLUTs	Glucose Transporters
FAs	Fatty Acids
BMI	Body Mass Index
T1DM	Insulin-dependent Type 1 Diabetes
T2DM	Insulin-independent Type 2 Diabetes
VA	Vitamin A
CNS	Central Nervous System
RA	Retinoic Acid
RARs	Retinoic Acid Receptors
RXRs	Retinoid X Receptors
COUPTFs	Chicken Ovalbumin Upstream Promoter-Transcription Factors
RE	Retinyl Esters
RAE	Retinol Activity Equivalents
RARE	Retinoic Acid Responsive Element
mcg	microgram
IOM	Institute of Medicine
RBP	Retinol Binding Protein
CRBP-I	Cellular Retinol-binding Protein Type I
CRBP-II	Cellular Retinol-binding Protein Type II
LRAT	Retinol Acyl Transferase
RDAs	Recommended Dietary Allowances
ADH	Cytosolic Medium-Chain Alcohol Dehydrogenases
SDR	Membrane-Bound Short-Chain Dehydrogenase/ Reductase
RALDH	Retinal Dehydrogenase
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
RDR	Relative Dose Response
DBD	DNA Binding Domain
LBD	Ligand Binding Domain
PPAR	Peroxisome Proliferator-Activated Receptor
Cpt1	Carnitine Palmitoyl Transferase I
ER	Endoplasmic Reticulum
RER	Rough Endoplasmic Reticulum
Vmax	Maximal Velocity
Km	Michaelis Constant
PI3K	Phosphatidylinositol 3'-kinase
PI3K/Akt	3'-Kinase-Akt signaling pathway
IRS	Insulin Receptor Substrate
PI(4,5)P2	Phosphatidylinositol 4,5-Bisphosphate

PDK1	Phosphatidylinositol-Dependent Protein Kinase 1
GPCR	G Protein-Coupled Receptors
PH	Pleckstrin Homology
MAPK	Ras-Mitogen Activated Protein Kinase
DHA	Dehydroascorbic Acid
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
HS	Horse Serum
P/S	Penicillin/Streptomycin
BSA	Bovine Serum Albumin
dd H <sub>2</sub> O	Distilled Deionized Water
DMEM	Dulbecco's Modified Eagle Medium
HS	Horse Serum
P/S	Penicillin/streptomycin
BSA	Bovine Serum Albumin
μL	Microliter
M	Mole
μM	Micromole
nM	Nanomole
ml	Milliliter
MRFs	Myogenic Regulatory Factors
MCK	Myosin light Chain Kinase
ES cells	Pluripotent Embryonic Stem Cells
MHC	Myosin Heavy Chain
Embryoid Bodies	Embryo-like Aggregates
GTP	Guanosine Triphosphate
GDP	Guanosine Diphosphate
Grb2	Growth Factor Receptor-Bound Protein 2
SOS	Son of Sevenless
IRS	Insulin Receptor Substrate
Shc	SHC-Transforming Protein
Gab1	GRB1-Associated-Binding Protein
BCA	Bicinchoninic Acid

## CHAPTER ONE

### INTRODUCTION

Glucose metabolism is of great importance to be completely understood. Glucose is a common fuel for a variety of cells. For example, red blood cells in the circulation are responsible for oxygen transport in the body. They can only use glucose as an energy source.<sup>1</sup> Neurons also prefer glucose as an energy source, while other types of cells may obtain energy through protein and fat metabolism.<sup>2</sup> In addition, the abnormal glucose metabolism in the brain may lead to the development of brain diseases like Alzheimer's disease.<sup>3</sup>

Another important function of cellular glucose metabolism is to regulate the immune function of lymphocytes.<sup>4</sup> The inefficient uptake of glucose in lymphocytes can result in cell death, while excessive uptake of glucose will lead to over activation of immune responses which may cause the development of immune pathology.<sup>4</sup> If we can understand the regulation of glucose metabolism, it may be helpful for us to develop intervention methods for the prevention and treatment of diseases related to immunology such as cancer<sup>5</sup> or autoimmunity.<sup>6</sup>

What's more, glucose metabolism is an essential part of the whole-body glucose homeostasis. The pancreatic hormones, insulin, glucagon, and somatostatin work together to regulate glucose homeostasis.<sup>7</sup> Alterations of glucose metabolism are associated with endocrine or nutritional chronic diseases such as diabetes<sup>8</sup> and obesity<sup>9</sup>. Changes in glucose metabolism such as hyperglycemia in fasting and postprandial states are important biomarkers for the early detection of myocardial infarction.<sup>10</sup> In summary, the regulation of glucose metabolism has constantly been studied to help us understand how to prevent and treat chronic or acute diseases such as myocardial infarction, diabetes, and obesity.

Muscle cells are critical players in the whole-body glucose metabolism. This is because muscle makes up to one third of human body mass.<sup>11</sup> Skeletal

muscle is one important type of muscle, which contributes significantly to glucose homeostasis. Skeletal muscle can either store glucose or use it directly to generate energy under different metabolic conditions after the uptake of glucose.<sup>12</sup> Skeletal muscle is the major place for glucose utilization.<sup>12</sup> It uses a significant amount of glucose for energy production in physical activities. In addition, it also converts excessive glucose into glycogen for the use as a quick source of energy intracellularly.<sup>12</sup> Therefore, understanding the muscle glucose metabolism is relevant to the intervention of the development of metabolic diseases.

In healthy subjects, the blood glucose level is controlled in a narrow range from 4.0 to 5.9 mmol/L.<sup>13</sup> This is achieved through the balance of glucose uptake and usage. As an aldose, glucose could not enter cells freely without the help of carrier proteins. One type of these proteins is known as glucose transporters (GLUTs).<sup>14</sup> Glucose uptake is mediated by GLUTs in cells including skeletal muscle cells. GLUT1, GLUT4, and GLUT5 are 3 isoforms of GLUTs that mediate the uptake of glucose in skeletal muscle.<sup>14</sup> Insulin can stimulate the uptake of glucose in skeletal muscle in the postprandial state.<sup>12</sup>

Insulin is important for the transport and disposal of glucose especially in peripheral tissue such as muscle and adipose tissues.<sup>15</sup> The sensitivity of muscle in response to insulin is important in maintaining normal glucose level. Insulin was first found to be able to stimulate the movement of a GLUT from intracellular pool to the plasma membrane in 1980.<sup>16</sup> It has been thought that the main GLUT mediating insulin-stimulated glucose uptake is GLUT4.<sup>17</sup> GLUT4 locates specifically in cells of the striated muscle and adipose tissues.<sup>15</sup> GLUT4 moves rapidly from the intracellular storage locations to the plasma membrane when cells are stimulated by insulin.<sup>17</sup> Once present on the cell membrane, GLUT4 begins to mediate the uptake of glucose into the skeletal muscle cells. Insulin-mediated translocation of GLUT4 has been considered as a critical step for the glucose uptake in skeletal muscle.<sup>17</sup>

The insulin-induced GLUT4 translocation also exists in adipocytes.<sup>18</sup> Adipose tissues are not only a storage place for fat but also an endocrine organ for the regulation of lipid and glucose homeostasis.<sup>18</sup> They play critical roles in the maintenance of body health. The major storage form of fat in adipocytes is triglyceride, which is a glycerol backbone esterified to three fatty acids (FAs).<sup>18</sup> FAs can be derived from dietary fat or synthesized de novo from proteins or carbohydrates in the body.<sup>18</sup> Adipocytes include white adipocytes<sup>19</sup> and brown adipocytes.<sup>20</sup> White adipocytes are responsible for the storage of fat<sup>19</sup>, whereas brown adipocytes are responsible for the generation of heat to maintain the body temperature.<sup>20</sup> Enzymes in adipocytes catalyze the hydrolysis of triglycerides for the generation of free FA and glycerol in the body. Adipocytes are not a simple site for fat storage; they secrete important endocrine hormones such as leptin and adiponectin to regulate metabolism in the whole body.<sup>19</sup> Both leptin and adiponectin regulate metabolism to control the body mass in healthy subjects.<sup>19</sup> However, the regulatory functions of leptin and adiponectin towards body mass begin to have problems when the body mass of human subjects increases.<sup>20</sup>

Due to the economic development and improvement of education, people begin to pay more attention to the prevalence of overweight or obesity in America and other parts of the world.<sup>21</sup> Increased food consumption and decreased exercise are the common causes of overweight or obesity.<sup>21</sup> Individuals in a population categorized as underweight, normal weight, overweight or obese group based on body mass index (BMI).<sup>22</sup> BMI is determined as the body mass (kg) divided by the square of the body height (meters). kg/m<sup>2</sup> is the generally unit of BMI. BMI categories<sup>22</sup> are (1) underweight: <18.5, (2) normal weight: 18.5-25, (3) overweight: 25-30, (4) obese: >30. Obesity is a chronic metabolic disease which is related to cardiovascular disease, diabetes or cancers. As a result, people generally hold negative attitudes when they consider the role of adipocytes in obesity.

Diabetes mellitus is another one common metabolic disease, which is defined by the elevation of blood glucose level due to alteration of metabolism.<sup>23</sup>

Insulin plays key roles in regulating glucose homeostasis. Insufficient insulin secretion or low sensitivity of insulin stimulation of the body can lead to diabetes.<sup>23</sup> Patients with diabetes can be divided into two types: insulin-dependent type 1 diabetes (T1DM) and insulin independent type 2 diabetes (T2DM). Diabetes has become prevalent in both developed and developing countries. Take America as an example of the developed countries, the total number of people diagnosed with diabetes has increased dramatically for the past 40 years. The number of patients with diabetes is expected to increase 50% by 2030.<sup>23</sup> Take China as an example of the developing countries, based on the current trend, the number of patients with diabetes is expected to nearly double by 2030.<sup>23</sup> The rates of blindness, renal disease or cardiovascular disease increase in those people who are diagnosed with diabetes. Glycemic control is a term used to describe the ability of maintaining blood glucose levels under normal ranges. The impaired glycemic control of patients with diabetes further harms the body health of patients.<sup>23</sup> Although more and more people begin to focus on how to control the prevalence of diabetes, the roles of micronutrients in diabetes have not been well studied.

Nutrients are necessary for the growth and development of organisms. Macronutrients, such as carbohydrate, lipids, and proteins, provide the basic energy need for maintaining the normal metabolic state in the body. Micronutrients act as cofactors in metabolic pathways in the body. Vitamin A (VA) is a family of compounds which have the biological activity of retinol. VA plays a key role in the development of animals.<sup>24</sup> Retinoic acid (RA) is a metabolite of retinol. The major functions of VA are mediated by RA. First, VA is necessary for the development of nervous system.<sup>24</sup> Second, VA can also influence the differentiation and proliferation of cells. For example, the differentiation of epithelial cells can be influenced by VA status.<sup>24</sup> Third, the visual cycle needs retinal, an oxidized production of retinol.<sup>24</sup>

One key role of RA is to regulate gene expression. RA can bind to nuclear receptors in the cell to regulate gene expression. Both retinoic acid receptors



(RARs) and retinoid X receptors (RXRs) can be activated by RA. RAR/RXR or RXR/RXR heterodimers can bind to RA response element (RARE) located in the promoters of target genes. Then, the gene expression can be regulated following ligand binding. Recently, it has been shown that chicken ovalbumin upstream promoter-transcription factor II (COUP-TF II) and hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) can also influence the expression of RA-regulated gene recently.<sup>25</sup>

In summary, glucose metabolism are important in the regulation of metabolic homeostasis. Muscle and adipose tissues are critical to the regulation of glucose metabolism. The roles of VA and its signaling pathways in the regulation of muscle and adipocyte glucose uptake have not been revealed completely. It is important to understand RA function because RA signals have effects on both energy metabolism and glucose metabolism. The aim of this thesis is to understand how RA can influence the glucose metabolism in both skeletal muscle cells and adipocytes. The data shown in this thesis may help to learn how to control the prevalence of diabetes and obesity worldwide.

## CHAPTER TWO

# REVIEW OF THE LITERATURE ON VITAMIN A, INSULIN SIGNALING, MUSCLE METABOLISM AND 3T3-L1 METABOLISM

## 2.1 Vitamin A

### 2.1.1 Discovery of Vitamin A

Nutrients are needed for the growth and development of humans and animals. For our energy need, macronutrients, carbohydrate, protein, and lipids, are metabolized to generate ATP. Micronutrients are vitamins and minerals that are used as cofactors to regulate biomedical pathways and building blocks to sustain the body health, respectively. The human body only needs small or trace amount of micronutrients compared with a large amount of macronutrients. VA is known as a micronutrient.

In 1881, G. Lunin showed that mice could not live when fed pure casein, fat, sucrose, minerals and water. Interestingly, he found when fed whole dried milk, mice grew well.<sup>26</sup>In 1906, F. C. Hopkins hypothesized that “minimal qualitative factors” in the diet were essential for growth and survival.<sup>27</sup>McCollum showed that the existence of an important fat-soluble factor in milk was necessary for the growth and development of rats. He successfully proved that an ether extract of alfalfa leaves or liver could improve growth of rats.<sup>28</sup>This unknown fat-soluble nutrient was termed as “fat-soluble factor A”. Osborne and Mendel further isolated and identified the fat-soluble factor. They successfully subtracted the yellow oil from butter fat.<sup>29</sup>However, they failed to subtract active substance from lard or olive oil.<sup>30</sup> P. W. Boutwell hypothesized that yellow pigment ( $\beta$ -carotene) of fat-soluble factor A can be converted to the active form (VA or retinol).<sup>31</sup> This hypothesis was proved by T. Moore in 1930.<sup>32</sup> In brief, VA is one important micronutrient which deserve to be understood.

### 2.1.2 Structure and Physical Characteristics of VA

Retinol is the major form of VA which possesses a hydroxyl group at C-15.<sup>24</sup> Retinoids are a big family of compounds that have a 20-carbon structure with a  $\beta$ -ionone ring and an isoprenoid side chain containing different chemical groups at the acyclic terminus.<sup>24</sup> Retinol can be oxidized to retinal which possesses an aldehyde group (-CHO) at the acyclic terminus. Retinol and retinal can be interconverted. RA can be generated in the body by two oxidation steps in a sequence which convert retinol to retinal, and then, to RA. Once RA is produced, it cannot be reduced back to retinal. RA possess a carboxylic acid group (-COOH).<sup>24</sup>VA possess double bonds which are not stable. VA is soluble in ethanol but almost insoluble in water. It can be freely solubilized in organic solvents such as fats and oils. The side chain bonds of retinoids and carotenoids can go through slow conversion between *cis-form* and *trans-form*. Light, heat, and iodine can stimulate this conversion. Although retinol has a variety of isomers, carotenoids present almost exclusive in the all-*trans* form in both plants and animals. Most forms of VA are crystallizable but with low melting points. For example, the melting point of retinol is between 62-64°C. VA and provitamin A carotenoids are very sensitive to oxygen, light, and heat. It is important to exclude air and add antioxidant when isolating VA and provitamin A carotenoids.<sup>24</sup>

### 2.1.3 VA Sources

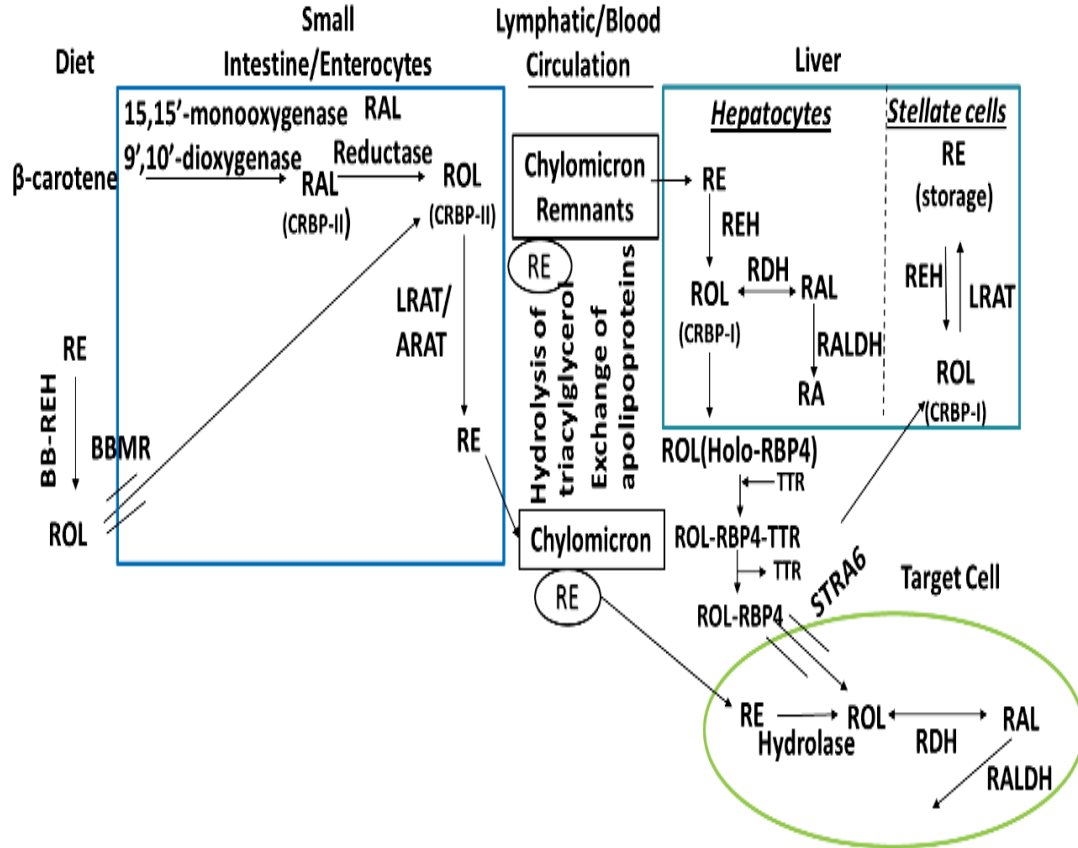
VA is an essential fat-soluble micronutrient because animals cannot synthesize it. For humans and animals, it should be taken from foods. Dietary VA exists in two forms: provitamin A carotenoids and preformed VA.<sup>24</sup> Provitamin A carotenoids are acquired from plant sources, while preformed VA retinyl esters (REs) and retinol are derived from animal products.<sup>24</sup> The main sources of provitamin A carotenoids are fruit and vegetables. Lots of orange, dark green colored fruits and vegetables are rich in carotenoids. Representative vegetables and fruits of provitamin A include carrots, pumpkin, winter squash, and apricots, etc. Carotene has 3 isomers:  $\alpha$ -carotene,  $\beta$ -carotene, and  $\gamma$ -carotene.<sup>24</sup>  $\beta$ -

carotene is one type of carotene which can be converted to functional VA in the body. Cryptoxanthins is also one type of carotene which only possess half of the biological activity of  $\beta$ -carotene.<sup>24</sup> Preformed VA is commonly provided by foods of animal products such as liver, dairy products, and fish.<sup>24</sup> The best way to get enough VA is to ingest a broad type of food.

It is important for us to ingest suitable amount of VA for our body health. The international unit (IU) was used to measure VA activities in the past. VA has different forms which make it complex to be measured in diet. People try to use retinol as a common standard form of VA when measuring total amount of VA content in diets. The amount of every types of VA needs to be transferred to the amount of retinol. In brief, retinol activity equivalent (RAE) was set up to measure amounts of VA sources. One RAE is equal to 1 microgram (mcg) of retinol. Take carotene as an example, 2 mcg of  $\beta$ -carotene in diet or supplement is equal to 1 RAE. The Recommended Dietary Allowances (RDAs) for VA is 900 mcg RAE for male and 700 mcg RAE for female between age 19 to 50 according to the Institute of Medicine (IOM) in America.<sup>33</sup>

#### **2.1.4 VA Uptake and Secretion**

Different forms of VA in diets are absorbed. In the small intestine, enterocytes can uptake carotenoids via passive diffusion. The provitamin A carotenoids taken into cells are cleaved into retinal via two mechanisms: central cleavage and eccentric cleavage. Both mechanisms work together to convert carotene into retinal, which is reduced into retinol. In Figure 1, in the central cleavage mechanism, 15,15' carbon double bond can be cleaved by  $\beta$ ,  $\beta$ -carotene-15, 15'-monooxygenase to generate two retinals.<sup>34</sup> In the eccentric cleavage mechanism,  $\beta$ ,  $\beta$ -carotene-9', 10'-dioxygenase catalyze carotenoids to generate two molecules of  $\beta$ -apocarotenals with different molecular weights.<sup>34</sup> Intestinal retinal reductase works later to reduce retinal to retinol.<sup>34</sup> Retinol and RE are absorbed differently. RE can be hydrolyzed to retinol and FAs by enzyme in intestinal lumen, which are then absorbed by the enterocytes. Two enzymes have been identified in the hydrolysis process: intestinal brush border



**Figure 1 VA Absorption, secretion and portal circulation in body.** The animal source of retinoids is RE. The plant sources of retinoids are carotenoids. Both RE and carotenoids can be converted to retinol in intestine. Retinol is esterified to RE by enzymes in enterocytes of small intestine. RE can be packed in chylomicrons which can be later secreted to the lymph circulation to enter the general circulation. Chylomicrons can be converted to chylomicrons remnants after several modifications of lipid components and lipoproteins. Liver parenchymal cells play a key role in VA storage. Chylomicrons remnants are taken by liver parenchymal cells. RE can be hydrolyzed to generate retinol which can be transported to endoplasmic reticulum to bind with retinol-binding protein (RBP) in parenchymal cells. ROL- RBP later can be secreted to the blood vessel or taken by the perisinusoidal stellate cells. In stellate cells, it can be stored in the form of RE and release in the form of ROL- RBP complex to help maintain the homeostasis of plasma ROL level between 1-2uM.

enzyme phospholipase B and pancreatic triglyceride lipase. Then retinol was taken up by the enterocytes.<sup>34</sup>

In the small intestine, after retinol is taken by the enterocytes, it can bind to retinol-binding protein type II (CRBP-II). This protein helps to solubilize retinol and to prevent its degradation. Long chain FAs and most retinol re-esterified later to generate RE. This process is catalyzed by retinol acyl transferase (LRAT) in enterocytes. Newly made RE can be incorporated in chylomicrons which carry different VA molecules such as carotenoids, RE, retinol, etc. They are packed together with triacylglycerol and phospholipids. Those chylomicrons are secreted in the lymph duct to enter circulation process eventually.<sup>34</sup>(Figure 1)

### **2.1.5 VA transport and storage**

After chylomicrons are secreted in the lymph system, they eventually enter the general circulation system in the body. Hydrolysis of triacylglycerol and exchange of apolipoproteins help to generate chylomicron remnants. Chylomicron remnants with RE arrive at the liver via blood capillary. Although tissues can extract most lipids and some carotenoids from circulating chylomicrons, most RE are derived from the chylomicron remnants. Liver parenchymal cells can basically uptake chylomicron remnants. Another type of liver cell which is important for VA metabolism and storage is perisinusoidal stellate cell. Parenchymal cells are responsible for the clearance of chylomicron remnants with RE. RE is hydrolyzed in parenchymal cells again to generate free retinol and FA. If retinol is not needed immediately in the cells, it can be re-esterified in the perisinusoidal stellate cells and retained in it. Most retinol in perisinusoidal stellate cells present in the form of RE packaged together with lipid droplets. The storage mechanism of VA in the liver helps to maintain normal plasma retinol level which is at 1-2  $\mu\text{M}$ . Stored RE can be hydrolyzed again by hepatocytes. The released retinol binds with retinol binding protein 4 (RBP4) to be released. RBP4 is mainly located in the ER of hepatocytes. RBP4 possesses the ability to bind with free retinol to form a stable RBP-retinol complex. RBP-

retinol complex can translocate to Golgi apparatus before the complex is secreted in plasma.<sup>35</sup>

After the RBP-retinol complex is secreted into the plasma, it can further bind with transthyretin. Transthyretin is a larger protein synthesized and secreted by hepatocytes. It interacts with thyroid hormone T4 and mediates T4 transports in the blood. The larger transthyretin-T4-RBP-retinol complex can be recycled by the kidney, a process that prevents the loss of retinol in the urine. This complex circulates in the blood to deliver the lipophilic retinol to extrahepatic cells. The complex can release transthyretin-T4 to form RBP-retinol complex again in plasma. In the extrahepatic cells, RBP4 receptors on cell membrane can bind with RBP-retinol complex. As a result, retinol can be taken by the target cells.<sup>35</sup>

### **2.1.6 Generation of Retinal and Retinoid Acid in Nonvisual Cells**

Retinol can be taken directly from plasma and converted to the active metabolites to exhibit multiple physiology functions inside target cells. In addition to the retinol from the bloods, cells can also generate active retinoid metabolites from those VA sources stored directly inside them or in the neighboring cells.

All-*trans* RA is the main active retinoid metabolite which is a ligand for the activation of a transcriptional factor. RA can be generated from retinol in two oxidation steps. *all-trans* retinol is converted to *all-trans* retinal first. This step is a rate limit step.<sup>35</sup> In the second step, *all-trans* retinal can be further oxidized to *all-trans* RA. Cytosolic medium-chain alcohol dehydrogenases (ADH) such as ADH1, ADH3 and ADH4 play a key role in the first oxidation step. ADH4 is the most efficient one. However, it seems that ADHs cannot catalyze the first oxidation step if retinol is bound to CRBP-I.<sup>24</sup> Membrane-bound short-chain dehydrogenase/ reductase (SDR) is also important for the first oxidation step. SDR family of microsomal enzymes such as RDH1, RDH5 and RDH11 oxidize *all-trans* retinol to *all-trans* retinal.<sup>36</sup> SDR can use *all-trans* retinol even it is bound to CRBP-I.<sup>29</sup> Retinal dehydrogenases (RALDHs) are responsible for the second oxidation step. For example, RALDH2 oxidizes *all-trans* retinal to *all-trans* RA in several cell types during embryonic development.<sup>24</sup>

### **2.1.7 VA Assessment, Deficiency and Toxicity**

The standard of VA level in the human body is not easy to be established. There are lots of potential influencing factors such as culture, geology, and individuals.<sup>37</sup> To assess different VA status in the body, plasma retinol and blood RBP4 levels are widely used as indicators of VA status. Currently, VA sufficiency is defined as a plasma retinol level  $> 1.05$   $\mu\text{mol/L}$ . VA deficiency is defined at retinol level  $< 0.7$   $\mu\text{mol/L}$ . Mass spectrometry (MS) is used for testing plasma retinol levels in the research and clinical labs nowadays. The relative dose response (RDR) test is another method to determine the VA status in the human body. In the first step, intravenous serum retinol concentration is measured. Next, 1000 mcg of retinyl palmitate is injected intravenously. Five hours later, intravenous blood retinol level is measured again. When liver VA content is less than 20 mcg/g liver, RDR values found are greater than 20%. When liver vitamin A content is greater than 20 mcg/g liver, RDR values found are less than 10%.<sup>37</sup>

Different VA statuses have considerable influence on the body health of human being. Both VA deficiency and VA toxicity (known as hypervitaminosis A) have negative effects on lots of physiology processes in the body. VA deficiency is not common in developed countries because people always ingest enough amount of meat products. However, VA deficiency is prevalence in developing countries. Take the data of children as an example, over one million children are affected by VA deficiency worldwide.<sup>38</sup> Low frequency and an insufficient amount of dairy and meat products consumption in meals can be used to explain why VA deficiency is prevalence in developing countries.<sup>38</sup> VA toxicity is commonly found in people with liver dysfunction typically. Other probable reasons leading to VA toxicity include drug effect, and malnutrition.<sup>39</sup> Absorption of excessive amount of preformed VA can lead to VA toxicity, which is not the case of over consumption of provitamin A carotenoids.<sup>39</sup>



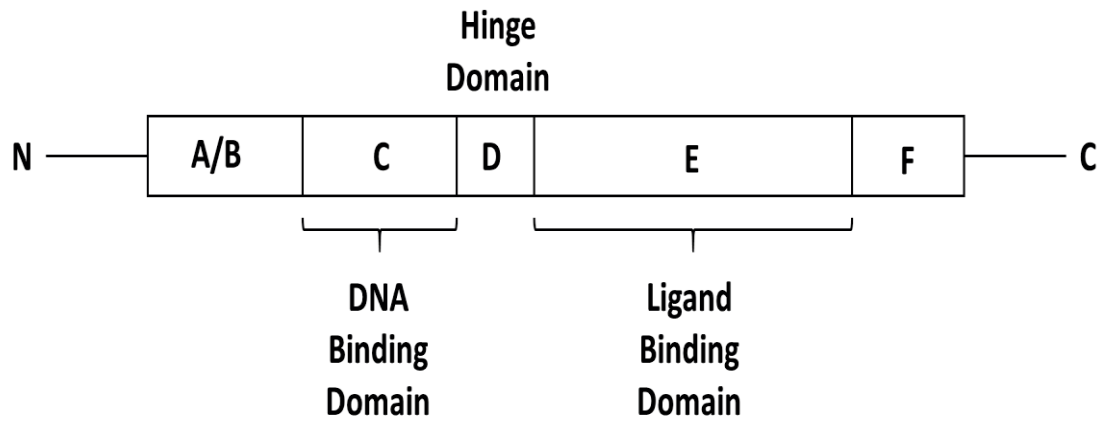
## **2.2 Retinoic Acid Signaling in the Regulation of Metabolism**

### **2.2.1 Overview**

Obesity and T2DM are two prevalent chronic metabolic diseases worldwide. These diseases are associated with abnormal glucose and lipid metabolism. This is in part attributed to the alteration of the expression levels of genes in metabolic active tissues. VA metabolism is possible to influence the macronutrient metabolism.<sup>24</sup>RA can regulate glucose and lipid metabolism.<sup>25</sup>This is through the control of gene expressions, which includes those genes for the glucose and lipid metabolism. The classic RA signaling system is mediated through the binding of RA to transcription factors such as RARs and RXRs in the nucleus, two family members of nuclear receptors.<sup>41</sup>Additional nuclear receptors have been shown to mediate RA signaling.<sup>41</sup>HNF4 $\alpha$  and COUP-TFII have been thought to act as a nuclear receptor that can mediate RA signals to regulate gene expression. RXRs, COUPTFII and others may play roles in mediating RA signals in metabolic active cells. We believe that RA signaling and these transcription factors may influence cellular metabolism which is associated with obesity and T2DM.<sup>41</sup>

### **2.2.2 Nuclear Receptor**

Lipophilic molecules like RA regulate cell differentiation, gene expression and general physiology processes through the modulation of the activities of nuclear receptors. Nuclear receptors interact with DNA sequences in the regulatory regions of their target genes. The expression levels of target gene can be regulated by nuclear receptors. Ligands bind to the nuclear receptors and cause the conformation changes. The allosteric change results in recruitments of other transcription factors for suppression or expression of the target genes. A typical nuclear receptor protein is shown in Figure 2. The DBD allows the receptors to bind to response elements on DNA. The conformation of transcriptional activation domain can be changed after binding with ligands. The LBD can bind to different ligands such as FAs, RA and steroids. Additionally, another term called nuclear orphan receptor is used specifically to describe those



**Figure 2 Common Structure of Nuclear Receptor.** A typical nuclear receptor molecule commonly contains five domains: N-terminal domain, DBD domain, hinge region, LBD domain and C-terminal domain.

nuclear receptors whose physiological ligands have not been identified. Although the ligands of nuclear orphan receptors are not identified, they still interact with specific elements on DNA. As a result, nuclear orphan receptors are still able to up- or down-regulate target gene expression.<sup>42</sup>

RA can bind to RARs or/and RXRs nuclear receptors to form a complex with changes of conformations. This RA and nuclear receptor complex are associated with the specific DNA sequence called RARE. This binding complex also interacts with other proteins in the genes' promoters to regulate gene expression. The cloning and identification nuclear receptors mediating RA signaling systems have helped to understand RA signaling pathways.<sup>42</sup>

### **2.2.3 Retinoid X Receptors**

Retinoids are important for cell growth, cell differentiation and nervous system development, etc. Complex signal pathways help to explain the diverse regulatory functions of retinoids. The first identified class of nuclear hormone receptors is RARs. RAR has three isoforms, RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ . RARs can bind with *all-trans* RA with high affinity. They share high level of structure conservations.<sup>43</sup>

RXRs possess different basic structure and specific ligands compared with RARs. RXRs cannot bind with *all-trans* RA. *9-cis* RA is identified to be the first ligand of RXR.<sup>44</sup> The identification of RXR and its ligand (*9-cis*-RA) facilitate the development of nuclear receptor area. First, it encourages researchers to identify the ligands of orphan nuclear receptor. Second, it encourages people to identify the nuclear receptors which can form heterodimers with RXRs.<sup>45</sup> For example, Peroxisome proliferator-activated receptor (PPAR) was proved to be the first class of orphan nuclear which can heterodimerize with RXR. RXRs can not only bind with RARs to form RAR/RXR heterodimers, but also bind with RXRs or other nuclear receptors to form varieties of homodimers or heterodimers. They can regulate transcription of target genes after binding to RARE which located in the promoters of target genes.<sup>45</sup>

RXRs can regulate the expression of genes involved in VA metabolism because RXRs are expressed in a variety of metabolically active organs such as small intestine, liver, and kidney.<sup>46</sup> For example, CRBP II gene is a potential target gene of RXRs. RXRs are also important for energy metabolism in mice. For example, the expression of carnitine palmitoyl transferase I gene increased in diabetic rat treated with LG100268 which is an RXR-specific agonist.<sup>47</sup> Moreover, RXR-selective agonists can induce adipose differentiation in preadipocytes. Mice with specific deletion of RXR $\alpha$  in hepatocytes show a lower amount of food intake and higher body weight with increased glucose tolerance than their controls.<sup>42</sup> RXR $\alpha$  is also proved to be related with lipogenesis. For example, in adipocytes, deletion of RXR $\alpha$  results in the resistant to obesity of mice.<sup>42</sup>

#### **2.2.4 The Chicken Ovalbumin Upstream Promoter-Transcription Factors**

COUPTF II is one transcriptional factor that can influence the expression of RA-regulated gene.<sup>25</sup> DNA sequences, regulatory factors and RNA polymerase can interact with each other to modulate gene expression at transcription level. Both recombinant DNA and gene transfer techniques help to characterize the promoters for RNA polymerase II-transcribed gene. Both *cis*-acting elements in DNA and the regions located further upstream in DNA are necessary to initiate DNA transcription. Experiments were conducted to test initiation efficiency of DNA transcription. Take the ovalbumin gene as an example, the TATA box is one of the *cis*-acting DNA elements which is located between -24 to -32 base pairs from the initiation site of the transcription. The efficiency and accuracy of DNA transcription initiation is supported by the TATA box. CAAT box which is a specific upstream sequence from TATA box is also necessary for initiating transcription of genes with high efficiency. CAAT box contains direct repeat of GTCAA sequence which is located around 80 base pairs away from the cap site. The cap site located on a DNA template where transcription begins. The result of 5' deletion mapping method first proved the existence of a distal DNA element in the promoter. Transcription competition assays were performed to test

the existence of a potential transcription factor that can interact with the promoter element. As a result, it is proved that CAAT box was an important DNA sequence which can bind with a transcription factor. To further identify a specific protein which can bind to CAAT box in ovalbumin gene, exonuclease footprinting experiments were conducted. In conclusion, a specific type of transcription factors called COUP-TFs were identified. COUP-TFs can bind with the direct repeat of GTCAAA sequence in the upstream promoter region of the ovalbumin gene.<sup>48</sup>

COUP-TFs include COUP-TFI, COUP-TFII and COUP-TFIII.<sup>25</sup> COUP-TFs are still orphan nuclear receptors, which have been studied extensively. All of them belong to the NR2F subfamily of nuclear receptors.<sup>25</sup> COUP-TFII plays a key role in embryonic development and can influence the reproductive function of mice. During embryonic development, COUP-TFII gene (Nr2f2) is widely expressed in tissues and organs of mice. COUP-TFII homozygous knockout (Nr2f2<sup>-/-</sup>) mice had defects in heart development.<sup>49</sup> COUP-TFII can also regulate insulin synthesis and secretion in pancreatic  $\beta$ -cells. Higher amount of insulin is required to keep the stable of glucose level for those people with low insulin sensitivity. In general, low insulin sensitivity indicates the abnormal metabolism of glucose in body. Impaired insulin sensitivity, abnormal insulin secretion and insulin resistance were found in mice specifically deleted of Nr2f2 in pancreatic  $\beta$ -cells.<sup>50</sup>

## **2.3 Insulin Signaling and Glucose Transporters**

### **2.3.1 Discover of Insulin**

The discovery of insulin led to its use for the treatment of diabetes mellitus. In 1893, the link between pancreas (an organ) and diabetes (a metabolic disease) was established through the research work by Oskar Minkowski and Joseph von Mering.<sup>51</sup> In the beginning of the first two decades of the twentieth century, people failed to extract functional substance from pancreas for the treatment of diabetes. This special substance was believed to be able to regulate blood glucose level in the body. Frederick G. Banting initiated attempts

to extract components from dog pancreas to test its ability to control the blood glucose level in diabetic dogs.<sup>52</sup> Banting and his team members successfully extracted and purified insulin for the treatment of patients with diabetes and were awarded the Nobel Prize.<sup>53</sup> The studies of insulin and its action have led many great discoveries and breakthrough in biomedical sciences. For example, the determination of insulin amino acid sequence led to the development of method for protein sequencing by Frederick Sanger.<sup>53</sup>

### **2.3.2 Insulin Synthesis**

In pancreatic  $\beta$ -cells, insulin is synthesized as preproinsulin which contains 110 amino acids. Preproinsulin is processed and secreted as mature insulin with the C-peptide into the blood circulation. The secreted insulin exists as a monomer to regulate metabolism through the binding to its receptor. It is stored in the form of hexamers in the secretory granules of pancreatic  $\beta$ -cells. Monomer form of insulin contains one A and one B chain. They are linked together by three disulfide bonds. Two disulfide bonds link A chain with B chain, the other one is located within A chain. For the secondary structure, A chain is made up of two antiparallel  $\alpha$ -helices while B chain is made up of both  $\alpha$ -helices and  $\beta$ -sheets.<sup>54</sup>

In pancreatic  $\beta$ -cells, preproinsulin is first translated from mRNA transcribed from insulin gene. Preproinsulin contains a specific N-terminal signal peptide. Preproinsulin can later translocate from the rough endoplasmic reticulum (RER) to the lumen. N-terminal signal peptide is cleaved to produce proinsulin in the lumen of RER. Cleavage leads to the formation of disulfide bonds in proinsulin and its folding. In trans-Golgi apparatus, proinsulin is further cleaved to produce the active mature insulin and the C-peptide. This process is catalyzed by cellular endopeptidases prohormone convertases and the exoprotease carboxypeptidase E. As a result, mature insulin only contains A chain and B chain. The mature insulin and C-peptide are packaged inside secretory granules.<sup>54</sup>

### 2.3.3 Insulin Secretion

In response to the ingestion of nutrients, insulin is released to control the metabolic homeostasis. Glucose is the major stimulus of insulin secretion. Digestion of starch, the main dietary component, results in absorption of glucose and leads to the rise of blood glucose level. Maximal Velocity ( $V_{max}$ ) and Michaelis constant ( $K_m$ ) are the two factors which help to explain the function of GLUTs.<sup>55</sup>  $V_{max}$  is termed to describe the rate of catalyze reaction when substrates fill up all the activity sites of GLUTs.  $K_m$  is the concentration of glucose at half of the  $V_{max}$ . Glucose metabolism is needed for glucose-stimulated insulin secretion. Glucokinase, also known as hexokinase D, is also a high  $K_m$  enzyme and is a rate-limiting step for glucose-stimulated insulin secretion.<sup>55</sup> Comparing with other type of hexokinase, hexokinase possesses lower affinity for glucose. The  $K_m$  of hexokinase is 6 mmol/L. The normal blood glucose range is between 4 mmol/L to 10 mmol/L. Moreover, the function of glucokinase cannot be suppressed by-products of this reaction, glucose 6-phosphate. As a result, glucokinase only works at high glucose level to start glycolysis.<sup>55</sup> The high  $K_m$  values of GLUTs and hexokinase D in pancreatic  $\beta$ -cells ensure that insulin secretion only occurs when glucose concentration is high. GLUT2 is a high  $K_m$  glucose transporter expressed in pancreatic  $\beta$ -cells. This allows that glucose only enters pancreatic  $\beta$ -cells when blood glucose level is high.

The end product of glycolysis is pyruvate, which is further converted into acetyl CoA in mitochondria. In the tricarboxylic acid cycle, acetyl CoA is oxidized to generate ATP in the mitochondria of pancreatic  $\beta$ -cells. This pathway can couple with the ATP-sensitive potassium channel-dependent pathway to stimulate insulin secretion. The oxidation of pyruvate can lead to the increase of ATP/ADP ratio. The ATP-sensitive potassium channel will be closed because of the increase of ATP/ADP ratio. Then potassium ions ( $K^+$ ) is prevented from leaving the cell. The accumulation of  $K^+$  can lead to the depolarization of the cell membrane. Then voltage-sensitive calcium ( $Ca^{2+}$ ) ions channels open because

of the accumulation of  $K^+$ . When the concentration of  $Ca^{2+}$  accumulates to a high level in pancreatic  $\beta$ -cells, insulin can be released.<sup>56</sup>

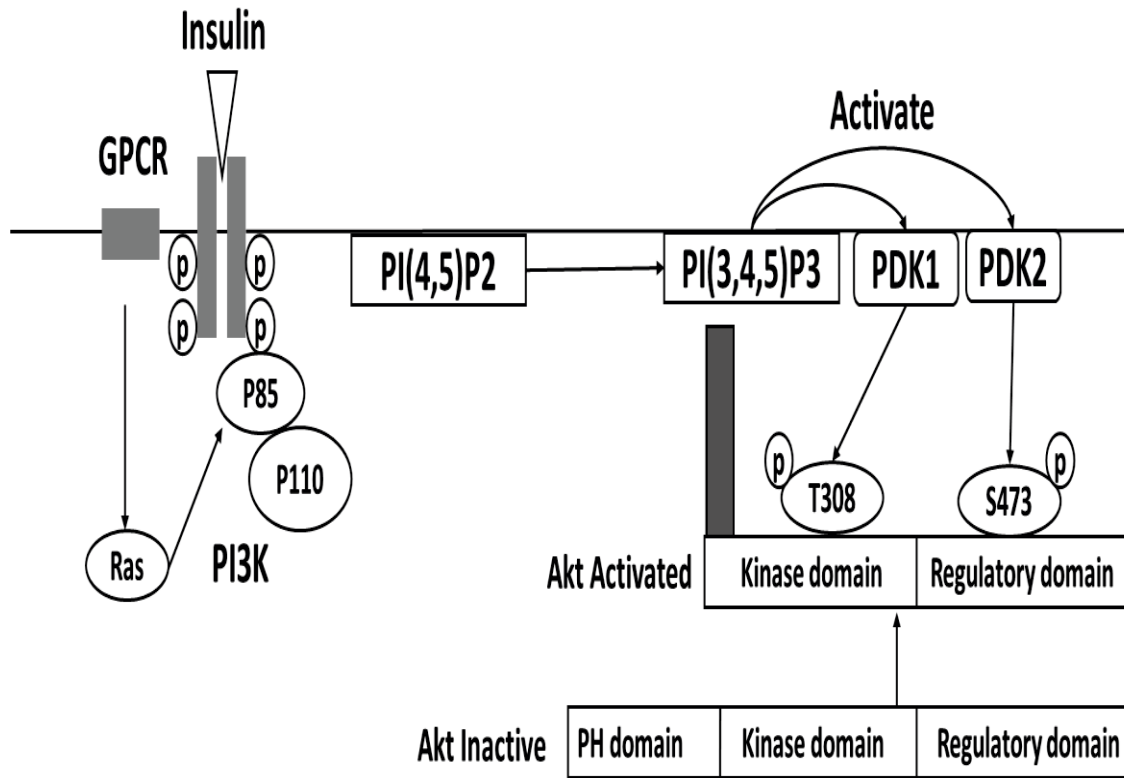
### **2.3.4 Insulin Signal Transduction**

Insulin initiates its physiological actions through the binding to insulin receptor on cell surface. Insulin receptor contains  $\alpha$ -subunits and  $\beta$ -subunits, which are linked by disulfide bonds. When insulin binds with the  $\alpha$ -subunits, two  $\beta$ -subunits come close to each other. The  $\beta$ -subunit of insulin receptor is a tyrosine kinase, which can phosphorylate each other in close proximity. This phenomenon is termed as autophosphorylation.<sup>56</sup> The phosphorylated  $\beta$ -subunits recruit insulin receptor substrates (IRS) and phosphorylate them. Phosphorylated IRSs can recruit additional signal transduction components and activate a signal cascade which mediates insulin signal transduction.<sup>56</sup>

The phosphatidylinositol 3'-kinase (PI3K)-Akt signaling pathway (PI3K/Akt) are important in mediating insulin signal. (Figure 3) Serine/threonine kinase Akt/PKB is one important enzyme in this pathway. Phosphatidylinositol 3'-kinase (PI3K) can convert phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3). This is important for insulin action. PI3K is made of two subunits: P110 and P85. P110 is a catalytic subunit while P85 is the regulatory subunit. PI3K can be activated by both receptors with protein tyrosine kinase activity and G protein-coupled receptors (GPCR). Pleckstrin homology (PH) domain can mediate the effect of PIP3. Akt is a serine/threonine kinase. Production of PI(3,4,5)P3 leads to the activation of Akt. Akt includes PH domain, a kinase domain, and regulatory domain. PH domain of Akt can interact with PIP3. As a result, the conformation of Akt changes. Thr308 and Ser473 of Akt are phosphorylated. Phosphatidylinositol-dependent protein kinase 1 (PDK1) are important for the activation of Akt. It can phosphorylate Thr308 in the kinase domain of Akt which helps to stabilize the conformation of Akt. Phosphorylation of residues in C-terminal region is also necessary to fully activate Akt.<sup>57</sup> (Figure 3)

Insulin can also activate the Ras-Mitogen activated protein kinase (MAPK)





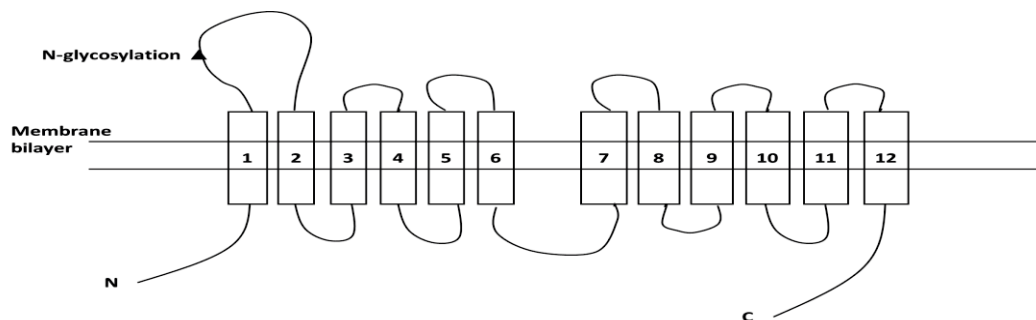
**Figure 3 Phosphatidylinositol 3'-kinase(PI3K)-Akt signaling pathway (PI3K/Akt).**

Phosphoinositol 3'-kinase (PI3K) converts PI(4,5)P2 to PI(3,4,5)P3. PI3K is made up of two subunits: P110 and P85. P110 is the catalytic subunit. P85 is the regulatory subunit. PI3K can be activated by either receptors with protein tyrosine kinase activity or G protein-coupled receptors (GPCR). Pleckstrin homology(PH) domain mediate the effect of PIP3. PI(3,4,5)P3 by PI3K leads to the activation of Akt. PH domain of Akt can interact with PIP3 which leads to the conformation of Akt changed. Thr308 and Ser473 are phosphorylated.

pathways as well. Ras belongs to the family of GTPases. Ras can bind to guanosine triphosphate (GTP) as well as hydrolyze GTP. As a result, GTP/ guanosine diphosphate (GDP) exchange rate can be regulated. The Ras–MAPK pathway is activated by insulin first. Then growth factor receptor-bound protein 2 (Grb2) and the guanyl nucleotide-exchange factor Son of Sevenless (SOS) come to cognate with IRS proteins, SHC-transforming protein (Shc) and GRB1-associated-binding protein (Gab1).<sup>58</sup> Raf is activated to trigger a pathway in which the dual-specificity kinases MEK1 and MEK2 are activated. Dual-specificity kinase is a term used to describe a kinase which possesses characteristics of both tyrosine kinase and serine/threonine kinase. Dual-specificity kinase MEK1 activates downstream MAPK such as ERK.<sup>58</sup>

### 2.3.5 Glucose Transporters (GLUTs)

The concept of glucose transporters appeared in 1948 when it was found that glucose could not pass through the cell membrane freely.<sup>59</sup> GLUTs are membrane proteins. They can mobilize glucose through the cell membrane. Based on the molecular cloning results, a total of 14 GLUTs have been identified in the human genome. All of them are encoded by the members of soluble carrier family 2 (SLC2) gene family. All GLUTs have near 500 amino acid residues arranged in a 12 membrane-spanning helical structure. As described in Figure 4, there is an N-terminal glycosylation between transmembrane helices 1 and transmembrane helices 2.<sup>59</sup>



**Figure 4 Structure of glucose transporters.** Glucose transporters contain 12 transmembrane helices with N-terminal and C-terminal. There is an N-terminal glycosylation between transmembrane helices 1 and transmembrane helices 2.

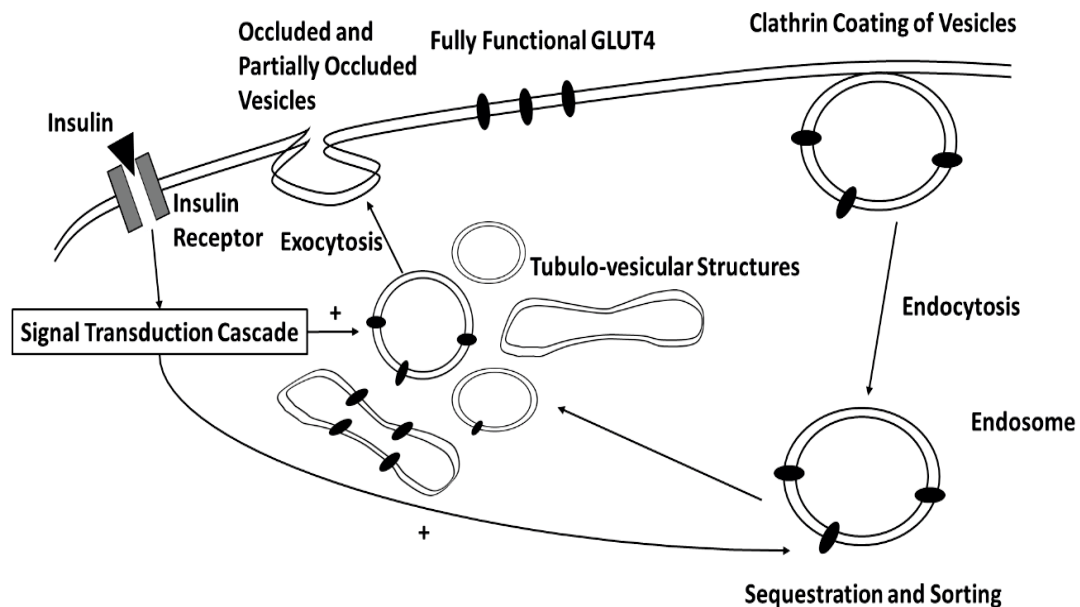
GLUT4 is an insulin-stimulated glucose transporter which is encoded by SLC2A4 gene in humans.<sup>17</sup> Insulin can regulate both the expression of GLUT4 and the translocation of GLUT4. GLUT4 is one of the most well studied types of GLUT. GLUT4 can transport glucose, glucosamine and dehydroascorbic acid (DHA) to cross the membrane. GLUT4 is important in glucose metabolism and glucose homeostasis. The impairment of GLUT4 regulation is associated with chronic diseases, such as obesity and T2DM. In the skeletal muscle, exercise helps to improve insulin sensitivity as well as stimulates the express of SLC2A4 gene at transcription level.<sup>60</sup>

In general, the substrate binding site of GLUTs can be identified in amino acid residues located on both outside and inside of the cell membrane. The transport of glucose by GLUTs is bi-directional. The binding of glucose to GLUTs will lead to the conformation change of GLUTs. Glucose can be released on the other side of the membrane as a result. In insulin-responsive tissues, insulin can stimulate the translocation of GLUT4. As described in Figure 5, GLUT4 is distributed major in tubulo-vesicular structures, an intracellular structure. GLUT 4 can be sequestered to the plasma membrane via exocytosis and recycle back to the tubulo-vesicular structures via endocytosis. GLUT4 vesicles arrive on the plasma membrane first and then fuse with the membrane. Insulin stimulates the exocytosis rate of GLUT4. Exocytosis rate of GLUT4 can be increased by 2.8 folds when stimulated by insulin. The endocytosis will continue with or without insulin. GLUT4 is recycled by clathrin-coated vesicles, which prepares to be sorted and sequestered again via exocytosis.<sup>60</sup>

## **2.4 Skeletal Muscle Cells**

### ***2.4.1 Physiology Role of Skeletal Muscle***

In adults, the muscle makes up to nearly the half of the human body mass. There are mainly three types of muscle: skeletal muscle, cardiac muscle, and smooth muscle. Cardiac muscle is a type of involuntary striated muscle found in the heart. Cardiac muscle plays a key role in maintaining the rhythmic contractions of the heart.<sup>61</sup> Contractions pump the blood from the heart to the



**Figure 5 Translocation of glucose via GLUT4.** GLUT 4 is present in intracellular tubulo-vesicular structures with no existence of insulin. Exocytosis rate is increased after insulin stimulation. GLUT 4 can be sequestered to the plasma membrane via exocytosis and recycle back to the tubulo-vesicular structures via endocytosis. GLUT 4 vesicles arrive in the plasma membrane first and then fuse with the membrane. Insulin has considerable influence on the exocytosis rate of GLUT 4. Exocytosis rate of GLUT 4 can increase to 2.8 folds when stimulated by insulin. The endocytosis will continue with or without insulin. GLUT 4 is recycled by clathrin-coated vesicles and sorted and sequestered again via exocytosis.

rest part of the body. Smooth muscle is an involuntary non-striated muscle which is different from skeletal muscle and cardiac muscle. It is found in the walls of an internal organ or in the tracts of digestive/reproductive system. Smooth muscle contracts automatically with a lower speed compared with striated muscle.

Skeletal muscle is a voluntary striated muscle which is connected by connective tissue. It is the most common type of muscle distributed in the human body.<sup>61</sup>

Sarcolemma is a term used to describe the cell membrane of a skeletal muscle cell.<sup>61</sup> Muscle fibers include myofibrils that are made up of sarcomeres. In the cytoplasm, fibers are assembled as a striated pattern contain thick and thin filaments that form myofibrils. The thick filaments are mainly composed of the protein myosin. The thin filaments are mainly composed of actin, troponin and tropomyosin. These proteins and other necessary factors like calcium work together to activate cross-bridges, which leads to the contraction of muscle. Skeletal muscle plays important roles in lots of physiology functions in the human body. The essentiality of skeletal muscle is associated with body movement and body gesture maintenance. Additionally, skeletal muscle is important for supporting the function of digestive and urinary systems. For example, sphincters are made up of skeletal muscle. Sphincters can support swallowing and urination. Moreover, skeletal muscle helps to regulate the body temperature. Contraction of skeletal muscle can lead to the generation of heat which helps body to maintain a constant body temperature.<sup>61</sup>

#### **2.4.2 L6 Rat Skeletal Muscle Cells(L6 cells)**

L6 rat skeletal muscle cells (L6 cells) were originally derived from thigh tissue of baby rat. It was first isolated and established by David Yaffe in 1965.<sup>62</sup> Primary skeletal muscle cultures were used to establish L6 rat cell line. The recipe of culture medium was medium 199(22.5%) and Dulbecco's modified Eagle medium (67.5%) containing horse serum (10%) and chick embryo extract (1-3%). Cells were kept in humid incubator at 37°C and 10% CO<sub>2</sub>. Next, several passages of myoblasts were conducted to select those cells which can attach to the plate. Clonal cells were isolated. As a result, L6 cell line was successfully

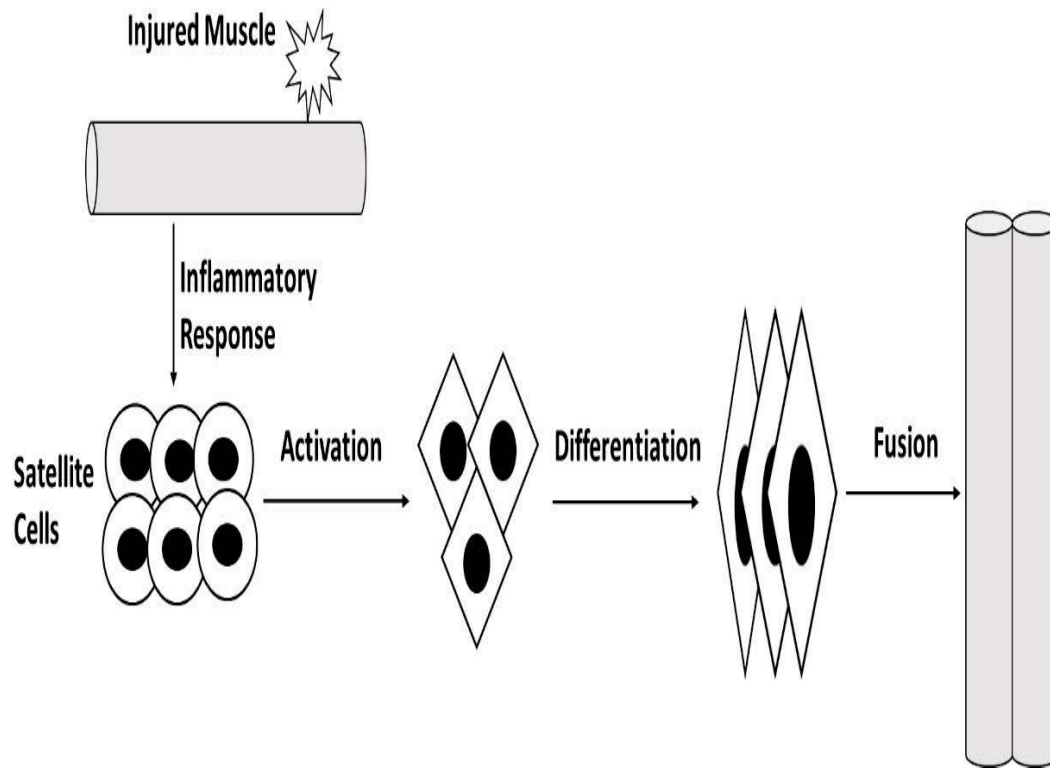
established.<sup>63</sup> In 1970, Yaffe proved that L6 cells can still maintain their myoblastic shape and can be differentiated to fuse with multinucleated fibers under standard culture conditions.<sup>63</sup>

The differentiation of skeletal muscle from myoblasts into myotubes completes by birth. Then only the size of muscle fiber can change in adults. It was proved that L6 cells present high differentiation rate when the basal medium containing high serum level is changed to a medium containing low level of horse serum (2%).<sup>64</sup> Under some specific conditions when muscle is damaged, muscle satellite cells begin to proliferate as myoblasts. Newly formed myoblasts can further differentiate and fuse into myotubes. This process is termed as muscle regeneration. Muscle regeneration is activated by the inflammatory response which is caused by the muscle damage.<sup>65</sup> Take the proinflammatory monocytes as an example, they can differentiate into proinflammatory macrophages in the damaged site. Then proinflammatory macrophages become M1 macrophages when muscle is damaged. M1 macrophages can secrete cytokines which can activate satellite cells to facilitate the differentiation and fuse of myoblasts into myotubes(Figure 6).<sup>55</sup>

Several important studies have been done using L6 cells as a research model. L6 cells have been used for studying the proliferation and differentiation of myogenic cells. In 1989, it was proven that growth factors and hormones could influence the proliferation and differentiation of L6 skeletal muscle.<sup>66</sup> L6 cells are used to study the influence of insulin on glucose utilization. It has been shown that insulin treatment for 24 h can lead to up to 10-fold activation of glucose transport activity after glucose deprivation.<sup>67</sup> In summary, L6 cells are a good model for studying the cell proliferation, cell differentiation, and glucose metabolism.

#### **2.4.3 The Role of Skeletal Muscle in Glucose/Glycogen Metabolism.**

Skeletal muscle plays a key role in glucose metabolism. Glucose and fat are the basic energy sources of skeletal muscle. During exercise and physical activities, glucose uptake is necessary to provide energy for skeletal muscle.



**Figure 6 Skeletal muscle differentiation and regeneration.** When muscle is damaged, muscle satellite cells begin to proliferate as myoblasts. Newly made myoblasts can further differentiate and fuse into myotubes. This process is termed as muscle regeneration. Muscle regeneration is activated by the inflammatory response which is caused by the muscle damage. Take the proinflammatory monocytes as an example, they can differentiate into proinflammatory macrophages in the damaged site. Then proinflammatory macrophages convert into M1 macrophages when muscle is damaged. Cytokines can be secreted by M1 macrophages to facilitate the differentiation and fuse of myoblasts into myotubes.

Glucose uptake is also important for the storage of glycogen in muscle after exercise. Skeletal muscle, liver, and pancreatic  $\beta$  cells cooperate with each other to control the blood glucose level in a narrow range from 4.0 to 5.9 mmol/L in healthy subjects.<sup>13</sup> Any defect in skeletal muscle, liver and pancreatic  $\beta$ -cells will lead to some problems in glucose homeostasis in the body. For example, the decrease in muscle cell number and impaired function of muscle cells have negatively influence on whole body glucose homeostasis in mice.<sup>17</sup>

Insulin regulates glucose and glycogen metabolism in the skeletal muscle. The sensitivity of muscle in response to insulin is important in maintaining normal glucose level. Glucose transport is a rate-limiting step in glucose metabolism.<sup>17</sup> Insulin can stimulate the movement of a GLUT4 from intracellular pool to the plasma membrane. GLUT4 locates specifically in cells of the striated muscle and adipose tissue. They can move rapidly from the intracellular storage locations to the plasma membrane when cells are stimulated by insulin. Once present on the cell membrane, GLUT4 begins to mediate the uptake of glucose into the skeletal muscle cells. The decreased responsiveness of tissues including skeletal muscle to the circulating insulin is termed as insulin resistance. Skeletal muscle insulin resistance is prevalent in patients with diabetes. Skeletal muscle insulin resistance generally begins in a small part of muscle mass. When skeletal muscle resistance develops to a significant percentage of muscle mass, disturbance of glucose homeostasis shows up. In T2DM patients, skeletal muscle resistance is the key reason to cause glucose homeostasis disturbance. Insulin resistance is specifically harmful to the early stage diabetes individuals. At the late stage, T2DM diabetic patients typically suffer from decreased insulin secretion and insulin resistance.<sup>13</sup>

## **2.5. Adipocytes**

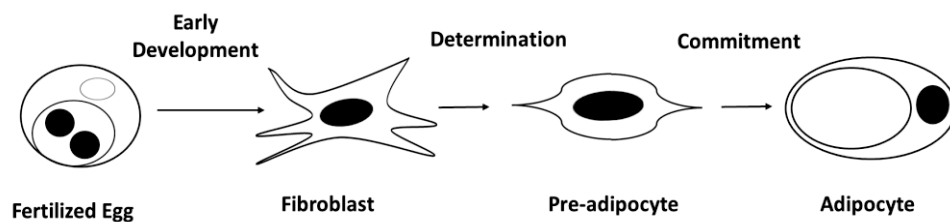
### **2.5.1 Adipocytes and Adipogenesis**

Adipose tissues are mainly consisted of adipocytes and found under the skin, between muscles and surrounding organs. There are two types of adipocyte tissues in mammals: white adipose tissue (WAT)<sup>19</sup> and brown adipose tissue



(BAT).<sup>20</sup> WAT is the most common type of adipose tissue. WAT can protect organs as well as provide energy under conditions like starvation. Lipolysis is a term used to define the triacylglycerol hydrolysis mediated by lipases, a process that generates FAs and glycerol. FAs can be oxidized in mitochondria for energy production in other tissues. The hydrolysis of triacylglycerol can be activated by hormones such as epinephrine, glucagon, etc. WAT is not a simple site for energy storage; it exhibits endocrine functions through secreting cytokines such as adiponectin, leptin, and resistin.<sup>19</sup> These cytokines also called adipokines, which play key roles in energy metabolism.<sup>19</sup> BAT is another type of adipose tissue responsible for heat production due to the presence of abundant mitochondria in brown adipocytes. BAT is generally found in new born animals.<sup>20</sup> The portion of BAT decreases with the increase of age in human.<sup>20</sup>

Adipogenesis, the growth and differentiation of adipocytes, has become a hot topic in recent years. The major health problem related to adipogenesis is obesity. There are several conversion stages from fertilized egg to mature adipocyte. As shown in Figure 7, fertilized eggs possess the ability to differentiate into different cell types. The stem cells which possess mesodermal origins can differentiate to preadipocytes.<sup>68</sup> In the late stage of embryonic development in human, preadipocytes begin to differentiate into adipocytes.<sup>69</sup> On the other hand, rat preadipocytes cannot begin to differentiate into adipose tissue until after birth.<sup>70</sup>



**Figure 7 Differentiation process from a fertilized egg to mature adipocyte.** There are several conversion stages from fertilized egg to mature adipocyte. Fertilized eggs can differentiate into different cell types. The stem cells which possess mesodermal origins can differentiate to preadipocytes. Preadipocytes can further differentiate into adipose tissue.

Adipose tissues possess key roles in controlling whole-body metabolism. The lack of adipose tissue mass leads to the increased circulating concentrations of triacylglycerol and FAs in both mice and humans. The presence of adipose tissue is necessary for normal secretion of adipokines such as leptin and adiponectin.<sup>19</sup> Both leptin and adiponectin can enhance insulin sensitivity.<sup>19</sup> The secretion of appropriate levels of adipokines and storage of lipids contribute to the function of adipose tissues in regulating whole body metabolism. Adipocytes can synthesize and store triacylglycerol during feeding, and release FAs and glycerol from hydrolysis of triacylglycerol during fasting.<sup>71</sup>

### **2.5.2 3T3-L1 *CARD1* Adipocytes**

3T3-L1 cells have been used as a cell model to study the differentiation and physiology of adipocytes. It is a good model to study the transformation of preadipocytes into adipocytes. 3T3-L1 cells were isolated from mice embryonic cells. They have been used in biological research on adipocyte differentiation. 3T3 cells are cells that need to be subcultured every 3 days to inoculum  $3 \times 10^5$  cells per well.<sup>72</sup> Before differentiation, 3T3-L1 cells grow like a fibroblast. These cells can be differentiated into mature adipocytes under specific conditions. 3T3-L1 preadipocytes can be induced to differentiation by a mixture of reagents promoting abiogenesis under confluency state. The mixture of insulin, glucocorticoid, and fetal bovine serum leads to the high differentiation rate of 3T3-L1 preadipocytes to adipocytes.<sup>73</sup> MDI is an abbreviation to describe the mixture of insulin, dexamethasone and methylisobutylxanthine (MIX). Dexamethasone (DEX) is a glucocorticoid agonist which can activate the pathways for glucocorticoid receptor. Mix is a cAMP-phosphodiesterase inhibitor which is can activate the cAMP-dependent protein kinase pathway.<sup>73</sup>

Preadipocytes begin to differentiate after the treatment with MDI. Preadipocytes under differentiating status go through a postconfluent mitosis stage after the induction with MDI for 24 hours.<sup>73</sup> Postconfluent mitosis plays a key role in unwinding DNA. Transcription factors can bind with the regulatory response elements in genes which modulate the phenotypes of mature

adipocytes as a result.<sup>73</sup> Then, cells complete the postconfluent mitosis status to enter a stage called growth arrest by day 2. Growth arrest stage is necessary for continuing the differentiation process. Lipogenic enzyme, lipolytic enzymes and important proteins begin to be expressed in differentiating cells. Those expressed proteins are important for modulating the phenotypes of mature adipocytes. By day 5 to day 7, the shape of cells becomes round and lipids begin to accumulate in cells.<sup>74</sup>

3T3-L1 cells are also used to study growth control and action of oncogenic viruses.<sup>75</sup> It is important to understand the mechanisms related to adipocyte conversion and lipid homeostasis. Analysis of the expression profiles of exogenous proteins in 3T3-L1 cells helps to study the proteins functions in adipose tissue. Coxsackievirus and adenoviruses are human pathogens. Coxsackie B viruses are members of the picornavirus family which are non-enveloped RNA viruses.<sup>75</sup> Adenoviruses can cause infections in respiratory and gastrointestinal systems. Adenoviruses are non-enveloped DNA viruses.<sup>67</sup> Recombinant adenovirus has been considered as a useful tool for introduction of exogenous genes into cells. However, 3T3-L1 cells cannot be transfected effectively by recombinant adenovirus.<sup>76</sup> There are generally two methods to increase the infection efficiency: modifying the viral particles of adenovirus or modifying gene expressions of 3T3-L1 cells. It is relatively convenient to modify gene expressions in 3T3-L1 cells. Coxsackie and adenovirus receptor (CAR) can bind with a protein of adenovirus to initiate the viral infection process. The efficiency of infection or transduction can increase significantly if CAR is expressed exogenously in Chinese hamster ovary cells.<sup>77</sup> The cytoplasmic tail of CAR is not necessary for the transfection of adenovirus. CAR was modified by deletion of the cytoplasmic tail, CAR  $\Delta$ 1 construct. CAR  $\Delta$ 1 expression construct was transfected into 3T3-L1 cell. 3T3-L1 cells with the expression of CAR  $\Delta$ 1 is termed as 3T3-L1 CAR  $\Delta$ 1 cells. The efficiency of transfection of recombinant adenovirus in 3T3-L1 CAR  $\Delta$ 1 cells increased significantly comparing with that in

parental 3T3-L1 cells while the accumulation of lipids did not change significantly.<sup>75</sup>

## 2.6 Conclusion

Skeletal muscle and adipocytes play key roles in glucose metabolism and energy homeostasis. VA and insulin may work together to regulate macronutrient metabolism in cells. RA exhibits the major physiological functions of VA. We aimed to find the influence of RA and insulin on the GLUT4 expression levels in L6 muscle cells and 3T3-L1 CAR $\Delta$ 1 adipocyte. Understanding the mechanism of how RA influences macronutrient metabolism will help us to better control diabetes and obese.

## CHAPTER THREE

### RA AND INSULIN INHIBIT THE EXPRESSION OF GLUT4 IN DIFFERENTIATED L6 MUSCLE CELLS

#### 3.1 Introduction

VA is a group of compounds which exhibit the biological activity of retinol. VA is one fat-soluble micronutrient which plays key role in the growth and development of animals.<sup>24</sup> The major functions of VA are mediated by RA. RA regulates many aspects of cell differentiation and proliferation. This is done via regulation of the gene expression levels associated with these processes. RA signaling pathway is mainly mediated by transcription factors in nuclear receptor super family.<sup>42</sup>

Skeletal muscle is one important type of muscle, which contributes significantly to glucose homeostasis. Depending on the metabolic states, skeletal muscle can utilize either glucose or FA as an energy source. It can also store glucose as glycogen or oxidizes it directly to generate energy under different metabolic conditions after the uptake of glucose.<sup>24</sup> Skeletal muscle uses a significant amount of glucose for energy production in physical activities. Additionally, it also converts excessive glucose into glycogen for the use as a quick source of glucose intracellularly.<sup>24</sup> Therefore, understanding the muscle glucose metabolism is relevant to the intervention of the development of metabolic diseases, such as obesity or diabetes.

L6 muscle cells were established to study the muscle differentiation.<sup>62</sup> As a muscle cell line, L6 muscle cells have been used as a tool to study cell proliferation, cell differentiation, and glucose metabolism. Several important studies have been done using L6 cells as a research model. L6 cells have been used for studying the proliferation and differentiation of myogenic cells. In 1989, it was proven that growth factors and hormones could influence the proliferation and differentiation of L6 skeletal muscle.<sup>66</sup> L6 cells is a good model to study

glucose utilization. It has been shown that a 24-hour insulin treatment can lead to up to 10-fold activation of glucose transport activity in differentiated L6 cells after glucose deprivation.<sup>67</sup>

There are only limited studies focusing on the effect of RA on glucose or energy metabolism in L6 cells. RA possesses a key role in skeletal muscle differentiation. Based on the studies before, pluripotent embryonic stem cells (ES cells) can be cultivated under different conditions which lead to cardiogenesis or myogenesis.<sup>78</sup> In 1994, Victor Maltsev conducted an experiment to test the effects of RA doses on ES cells.<sup>78</sup> ES cells were incubated with  $10^{-8}$  and  $10^{-7}$  mole(M) RA. He found that cardiogenesis was inhibited while myogenesis was induced between Day 2 and Day 5. He further observed that transcription of skeletal muscle-specific myogenin was induced while transcription of cardiac-specific  $\alpha$ - and  $\beta$ -cardiac myosin heavy chain (MHC) genes were inhibited.<sup>78</sup> This result proved that RA is important in the differentiation process from ES cells to skeletal muscle cells.

Glucose uptake is mediated by GLUTs in muscle cells. The impairment of GLUT4 regulation is associated with chronic disease such as obesity and T2DM. Insulin is the major stimulus for the increased glucose uptake and usage in skeletal muscle after a meal. This process has been thought to be mediated by the insulin-induced GLUT4 translocation.<sup>17</sup> On the other hand; physical activities also promote the translocation of GLUT4. Exercise increased the GLUT4 in plasma membrane.<sup>79</sup> In the skeletal muscle; exercise helps to improve insulin sensitivity as well as stimulates the expression of SLC2A4 gene at the transcription level.<sup>60</sup> Physiological factor like fiber type of muscle can also influence the GLUT4 protein level. GLUT4 protein level is higher in red muscle fibers than in white muscle fibers.<sup>80</sup> Those results proved to us that GLUT4 protein level is a crucial factor in skeletal muscle glucose metabolism.

It is important to understand RA function because RA signals affect both energy metabolism and glucose metabolism. The hypothesis of this thesis is that RA and insulin have a synergistic influence on GLUT4 expression in L6 cells.

Here, the GLUT4 expression in L6 cells treated with increasing doses of RA or 10 nM insulin for 6 days were tested. The effects of RA alone and in combination with insulin on GLUT4 expression in L6 rat muscle cells were also tested in Day 4 and Day 6. Finally, the phosphorylation status of Akt and the expression levels of GLUT4 in L6 cells following one-hour treatment with increasing concentrations of insulin were checked.

## 3.2 Materials and Methods

### 3.2.1 Reagents

For L6 rat skeletal muscle cell culture and differentiation, Dulbecco's Modified Eagle Medium (DMEM) was purchased from Mediatech (Manassas, VA, U.S.A.). Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY, U.S.A.). Horse serum (HS) was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). Penicillin/Streptomycin(P/S) was purchased from Mediatech (Manassas, VA, U.S.A.). To treat cells with insulin and RA, insulin and all-trans RA and were purchased from Sigma Aldrich Corporation(St. Louis, MO, U.S.A.). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology, Inc (Rockford, IL, U.S.A.) to test the protein concentration. Immobilon-PSQ PVDF membrane was purchased from EMD Millipore Corp (Billerica, MA, U.S.A.). Non-fat dry milk of Food Club was purchased from a grocery store and was used to block membrane and to dissolve the first antibodies (GLUT4,  $\beta$ -Actin, Akt, phospho-Akt, total Akt). Bovine serum albumin (BSA) protease-free powder was purchased from Fisher BioReagents™ to make the second antibody. For immunoblotting, GLUT4 antibody (C-terminus) was purchased from EMD Millipore Corp (Billerica, MA, U.S.A.).  $\beta$ -Actin (#4970), total Akt (#9272), phospho-Akt (Ser473) antibodies and horseradish-conjugated goat anti-rabbit IgG (#7074) secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). ECL Western Blotting Substrate was purchased from Pierce Pierce Biotechnology, Inc (Rockford, IL, U.S.A.), which was used to visualize the recognized proteins.

### **3.2.2 L6 Rat Skeletal Muscle Cell Culture and Differentiation**

L6 cells were maintained in 60 mm dishes with DMEM with 4.5 g/L glucose supplemented with 10% FBS + 1% P/S in the incubator at 37°C, 5% CO<sub>2</sub> and 95% air. The medium was changed to DMEM containing 4.5 g/L glucose with 2% HS + 1% P/S to induce differentiation when cells reached near 100% confluence.

For the insulin dosage experiment (one-hour treatment), when cells reached near 100% confluence, culture medium was changed to differentiation medium (DMEM+ 2% HS + 1% P/S) for 6 days. Differentiation medium was changed every 2 days. On Day 6, the cells were divided into five groups to be treated with the differentiating media containing increasing concentrations of insulin for one hour. The insulin concentrations were: (1) 0 nM, (2) 0.1 nM, (3) 1 nM, (4) 10 nM and (5) 100 nM insulin. Then cells were lysed to obtain total protein. After indicated treatments, L6 cells in 60 mm dishes were washed once with 3 ml PBS and scrapped from the dish into 400 µl of whole-cell lysis buffer. The lysates were placed on ice for at least 20 min before they were subjected to centrifugation at 12000 x g for 20 min.

For the RA dosage experiment (4 or 6 days), when cells reached near 100% confluence, medium was changed to differentiation medium (DMEM+ 2% HS + 1% P/S) containing (1) 0 µM RA, (2) 0.3 µM RA, (3) 1 µM RA, (4) 3 µM RA, (5) 10 µM RA, (6) 0 µM RA+10nM Insulin, (7) 0.3µM RA+10 nM Insulin, (8) 1 µM RA+ 10 nM Insulin, (9) 3 µM RA+ 10 nM Insulin, (10) 10 µM RA+10 nM Insulin. Cells were treated for 4 days or 6 days. RA was dissolved in 200 proof ethanol to prepare RA stock. The media were changed to fresh media every 2 days. At the end of the 4-day or 6-day treatments, cells were lysed and subjected for further analysis by immunoblotting.

For the long-term RA and insulin experiment (4 or 6 days), when cells reached near 100% confluence, medium was changed to differentiation medium (DMEM+ 2% HS + 1% P/S) containing (1) 0 nM Insulin/0 µM RA (control group), (2) 1µM RA, (3) 10nM Insulin or (4) 1µM RA/10nM Insulin. Cells in each group



were incubated in the indicated media for 4 days or 6 days. At the end of the 4-day or 6-day treatments, cells were lysed and subjected for further analysis by immunoblotting.

### **3.2.3 Protein Isolation and Immunoblotting**

Cell dishes were kept on the ice and then culture medium was pumped out through aspiration. Cells were washed once with 3 ml PBS. Then cells were lysed in 400 microliters ( $\mu\text{L}$ ) lysis buffer per dish. Whole cell lysates were scraped into different centrifugation tubes (1.5 ml). Centrifugation tubes with whole cell lysates were put on ice for more than 20 minutes. Then whole cell lysates were centrifuged at  $12,000 \times g$  and  $4^\circ\text{C}$  for 15 minutes.

The protein concentration in the cell lysate supernatant was determined with PIERCE BCA protein assay kit (Rockford, IL). This was achieved by first mixing reagent A with reagent B (50:1) to make working reagent. Five  $\mu\text{L}$  of each unknown sample was then pipetted into 495  $\mu\text{L}$  dd H<sub>2</sub>O in separate labeled tubes. The standard protein stock (2 mg/mL) was diluted to 80, 40, 20, 10, 5 and 0  $\mu\text{g}/\mu\text{L}$  for making standard curve according to the manufacture's protocol. In 1 ml reaction, 500  $\mu\text{L}$  of the working reagent was then added to each sample and standard protein sample.

OD reading at 560 nm was measured using a 96 plate reader (560 nm). A standard curve was prepared by plotting the average Blank-corrected 560 nm OD readings vs. the concentrations in  $\mu\text{g}/\text{mL}$ . A formula derived from regression was obtained and used to determine the total protein concentration of each sample.

To make the resolving gel, 5.9 mL ddH<sub>2</sub>O, 5 mL 30:0.8% w/v acrylamide:bisacrylamide, 3.8 mL 1.5M Tris-Cl pH 8.8, 150  $\mu\text{L}$  10% SDS were mixed together and 150  $\mu\text{L}$  ammonium persulfate and 6  $\mu\text{L}$  of TEMED were added. To make the stacking gel, 4.1 mL ddH<sub>2</sub>O, 1 mL 30:0.8% w/v acrylamide:bisacrylamide, 750  $\mu\text{L}$  1.0M Tris-Cl pH 6.8, 60  $\mu\text{L}$  10% SDS were mixed together and 60  $\mu\text{L}$  ammonium persulfate and 6  $\mu\text{L}$  of TEMED were added finally.

After quantification, 40 µg total cell proteins were separated on 8% SDS-PAGE gel. Then separated proteins were transferred from gel to Immobilon®-PSQ PVDF membrane, which was blocked with 5% milk diluted in 1x TBST at room temperature for one hour. The primary antibodies were diluted in TBST containing 5% BSA or dry milk according to the manufactures' instruction. The membrane was incubated in the first antibody at 4 °C for overnight. The membranes were washed three times with 1x TBST (5 minutes each time) later after overnight incubation. Goat anti-rabbit IgG conjugated to horseradish peroxidase (second antibody) was diluted in 5% milk or 5% BSA in 1x TBST (1:1000). The membrane was incubated in the second antibody for one hour. The membrane was washed for another three times with 1x TBST (5 minutes each time). Next, ECL Western Blotting Substrate was used to cover the membrane for visualization of the protein bands. Konica SRX 101A Film Processor was used to develop the film. Quantification analysis was performed on Image J software (NIH, Bethesda, Maryland, USA). The density of a protein band was calculated by subtracting the background in an area of the same size near it. Then, the ratio of the densities of the indicated protein to that of the control protein (protein of interest/ $\beta$ -actin) was calculated for quantification.

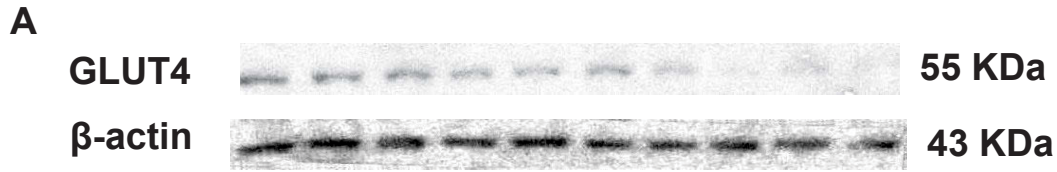
#### **3.2.4 Statistical Analysis**

All experiments were repeated for three times. One-way ANOVA with LSD post-hoc statistical analysis was conducted using SPSS version 22 statistical software (IBM, Armonk, NY, U.S.A.). Data were presented as means  $\pm$  S.E.M. The difference was considered significant when the p-value was less than 0.05.

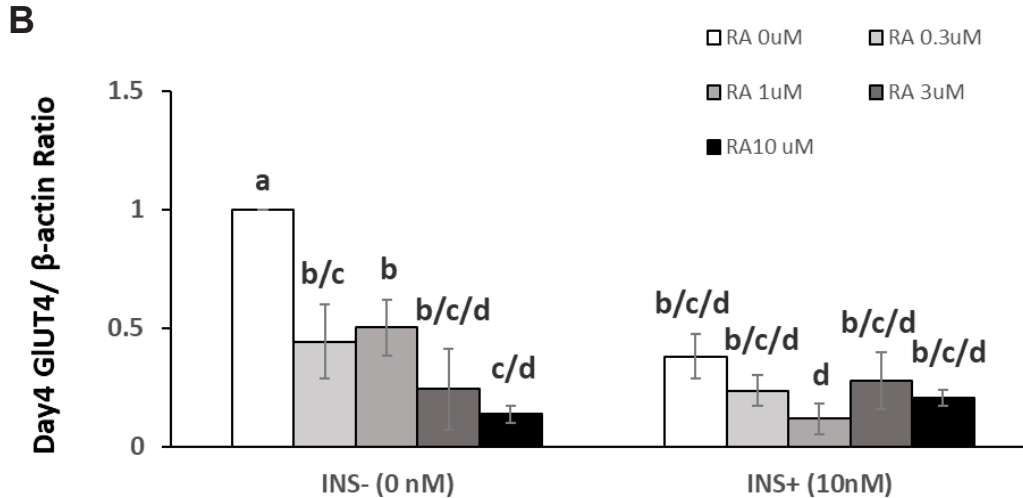
### **3.3 Results**

#### **3.3.1 RA treatment Inhibits the GLUT4 expression in L6 Cells on Day 4**

L6 cells were used here to research the effects of RA doses and insulin on the protein expression of GLUT4 in differentiating L6 cells. Figure 8 A shows a representative blot of GLUT4 in L6 cells treated with RA doses or 10 nM insulin for 4 days. Figure 8 B shows the summarized data of three independent studies. As shown in Figure 8 B, GLUT4 expression level on Day 4 in L6



RA(uM)	0	0.3	1	3	10	0	0.3	1	3	10
Insulin(nM)	-	-	-	-	-	10	10	10	10	10



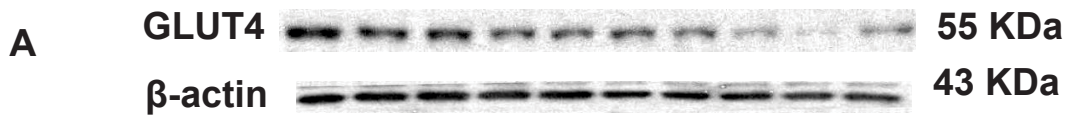
**Figure 8 Changes in GLUT4 expression in the medium of L6 cells treated with increasing doses of RA and 10 nM insulin on Day 4.** A: A representative blot of protein levels of GLUT4, and β-actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 uM), and RA + insulin for 4 days and 6 days. B: Quantification analysis of GLUT4. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (0.3-10 uM), or 2% HS + insulin(10nM) + RA (0.3-10 uM). The medium in each plate was replaced every 2 days with its respective treatment condition. On day 4 of treatment, whole cell lysate was collected as described in Materials & Methods. The ratio of the densities of GLUT4 to β-Actin was used to analyse data with control group set as 1. All p-values  $\leq 0.05$ ,  $a>b>c>d$ , One-way ANOVA is used to analyse data.(means  $\pm$  S.E.M (n=3))

control cells (RA 0  $\mu$ M, insulin 0 nM) is significantly higher than that in other groups treated with RA at 0.3, 1, 3, or 10  $\mu$ M RA or 10 nM insulin. This result proved that RA treatment can inhibit the expression of GLUT4. GLUT4 expression of Day 4 in L6 cells of the control group is also significantly higher than that in the insulin only treatment group (RA 0  $\mu$ M, insulin 10 nM). This result proved to us that insulin can inhibit the expression of GLUT4. GLUT4 expression of L6 cells treated with 0.3  $\mu$ M RA was significantly lower than that of L6 cells treated with 0.3  $\mu$ M RA and 10 nM insulin. This result indicates that insulin and RA may have synergistic effect on the expression of GLUT4 when the dose of RA was 0.3  $\mu$ M. Its expression in L6 cells treated with 0.3  $\mu$ M RA was significantly different from the insulin only group (RA 0  $\mu$ M, insulin 10 nM). As a result, 10 nM insulin has significant influence on the expression of GLUT4 on Day 4. GLUT4 expression of L6 cells on Day 4 treated with 0.3  $\mu$ M RA was significantly higher than those treated with 1  $\mu$ M RA and 10 nM insulin. GLUT4 expression of L6 cells treated with 1  $\mu$ M RA was significantly different from L6 cells treated with 10  $\mu$ M RA which also proves to us that different doses of RA (1  $\mu$ M and 10  $\mu$ M) have different effects on GLUT4 expression of Day 4. On day 4, the GLUT4 expression of L6 cells treated with 1  $\mu$ M RA were significantly higher than those treated with 10  $\mu$ M RA and 10 nM insulin. This result proved to us that insulin (10 nM) and RA (1  $\mu$ M) synergized to inhibit the expression of GLUT4 in L6 cells on Day 4. GLUT4 expression of L6 cells treated with 3  $\mu$ M RA was significantly different from L6 cells treated without RA and insulin (RA 0  $\mu$ M, insulin 10 nM).

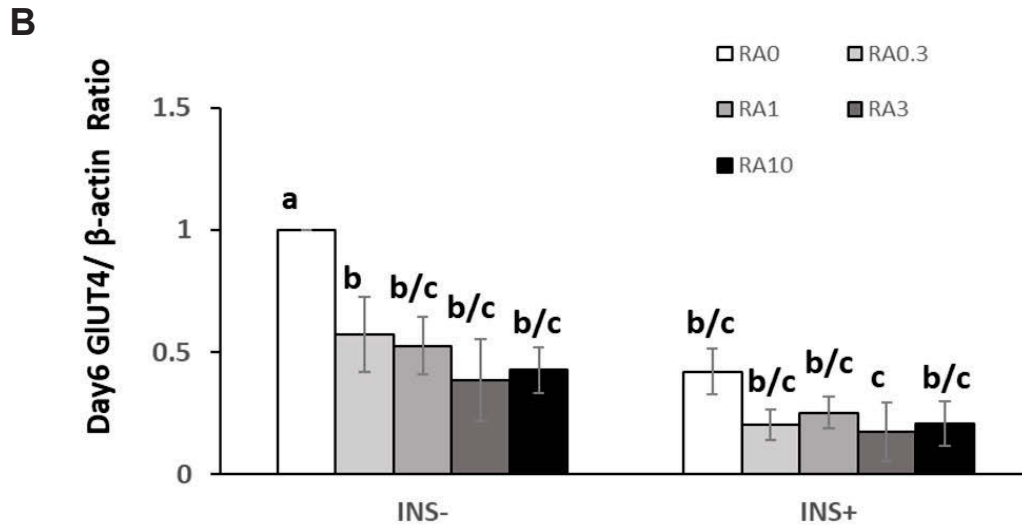
In summary, the results shown in 8 B indicate that RA can dose-dependently inhibit the expression of GLUT4 in the differentiating L6 cells after the treatment for 4 days. Insulin can also inhibit the expression of GLUT4. Insulin (10 nM) and RA (1  $\mu$ M) have synergized to inhibit the expression of GLUT4.

### **3.3.2 RA treatment Inhibits the Expression of GLUT4 in L6 Cells on Day 6**

Figure 9 A shows a representative blot of GLUT4 in L6 cells treated with increasing concentrations of RA in the absence or presence of 10 nM insulin for



RA(μM)	0	0.3	1	3	10	0	0.3	1	3	10
Insulin(nM)	-	-	-	-	-	10	10	10	10	10



**Figure 9 Changes in GLUT4 expression in the medium of L6 cells treated with increasing doses of RA and 10 nM insulin on Day 6.** A: A representative blot of protein levels of GLUT4, and β-actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μM), and RA + insulin for 6 days. B: Quantification analysis of GLUT4. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (0.3-10 μM), or 2% HS + insulin(10nM) + RA (0.3-10 μM). The medium in each plate was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. The ratio of the densities of GLUT4 to β-Actin was calculated to do data analysis with control group set as 1. All p-values ≤ 0.05, a>b>c>d, One-way ANOVA was used to analyse data( means ± S.E.M (n=3)).

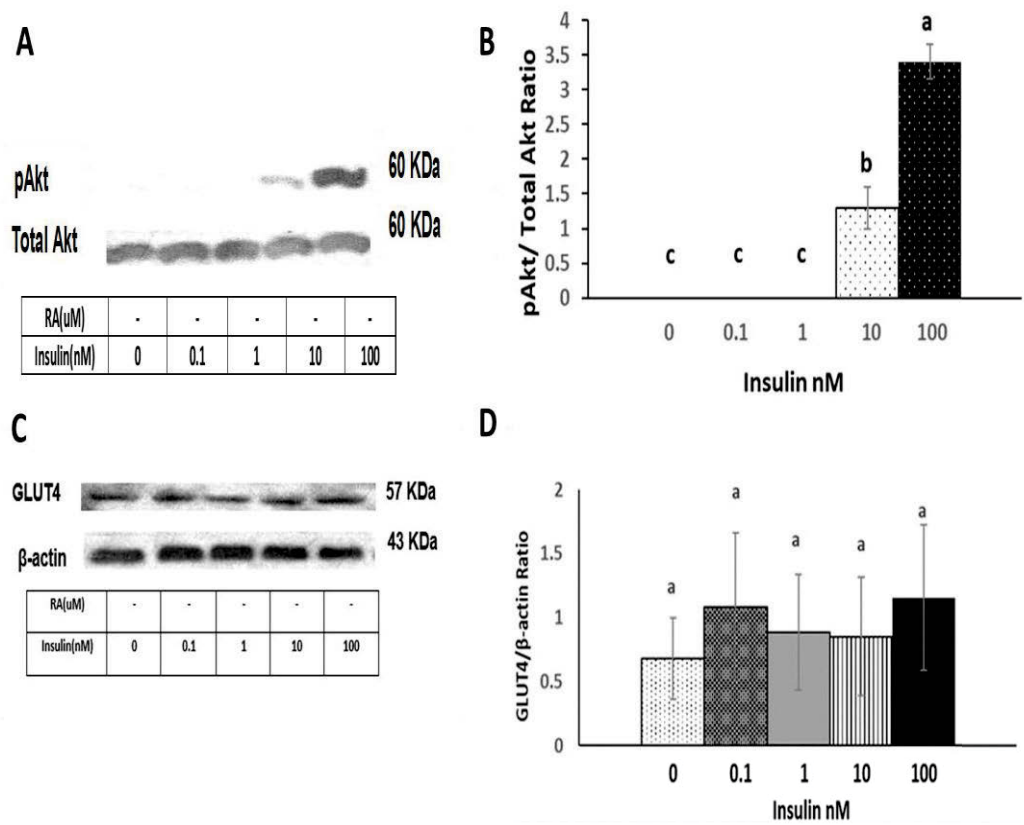
6 days. Figure 9 B shows the data from three independent studies. As shown in Figure 9 A, GLUT4 expression level on Day 6 in L6 cells of the control group was significantly higher than the other groups treated with RA (0.3  $\mu$ M, 1  $\mu$ M, 3  $\mu$ M, 10  $\mu$ M) and/or insulin (10 nM). This result proved that RA can inhibit the expression level of GLUT4. GLUT4 expression level in L6 cells of the control group on Day 6 is also significantly higher than the insulin treatment group (RA 0  $\mu$ M, insulin 10 nM). This result proved to us that insulin can inhibit the expression of GLUT4.

In summary, based on the data shown in Figures 8 A&B and 9 A&B, both RA and insulin inhibit the expression levels of GLUT4 in L6 cells after 4 and 6 days of treatment. The synergy between RA and insulin can be seen at Day 4 with 1  $\mu$ M RA.

### **3.3.3 Insulin influence the Phosphorylation of Akt in L6 Cells**

To determine whether the insulin treatment leads to changes of insulin signal transduction in L6 cells, we measured the phosphorylated Akt (pAkt) at serine 473. As shown in Figure 10 A&B, protein levels of pAkt in L6 cells following one-hour treatment of 100 nM insulin were significantly higher than pAkt expression in L6 cells following one-hour treatment of 10 nM insulin. Protein levels of pAkt in L6 cells following one-hour treatment of 10 nM insulin doses were significantly higher than pAkt expression in control group (insulin 0 nM). This result indicates that insulin treatment for one hour caused phosphorylation of Akt in L6 cells. Cells treated with 100 nM insulin had higher pAkt than 10 nM insulin. At one hour after the treatment, pAkt protein levels in L6 cells of 0 nM, 0.1 nM and 1 nM insulin groups were not different. As described in Figure 10 C&D, protein levels of GLUT4 in L6 cells after one-hour treatment of insulin doses were not significantly different from each other.

In summary, both 10 nM and 100 nM insulin exhibit its function in L6 cells following one-hour treatment. However, GLUT4 expression in 10nM and 100 nM insulin treatment groups were almost the same with the control group, indicating that one-hour insulin treatment will not affect GLUT4 expression in L6 cells on



**Figure 10 pAkt and GLUT4 protein levels in L6 cells following one-hour treatment of insulin doses.** Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS for 6 days. Following differentiation, L6 cells were treated with 2% HS (control), insulin (0 nM, 0.1 nM, 1nM, 10 nM, 100 nM) for 1 hour. Following treatment, whole cell lysate was collected as described in Materials & Methods. The ratios of the densities of pAkt to total Akt and GLUT4 to β-actin were calculated to do data analysis with control group set as 1. A&C: A representative blot of protein levels of pAkt, GLUT4 and β-actin. B&D: Quantification analysis of pAkt and GLUT4. All p-values ≤ 0.05; a>b>c. One-way ANOVA was used to analyse data(means ± S.E.M (n=3)).

Day 6 in the current experimental setting. Insulin does not have a significant influence on the GLUT4 protein level.

### **3.3.4 Insulin and RA Inhibit the Expression of GLUT4 Protein Level**

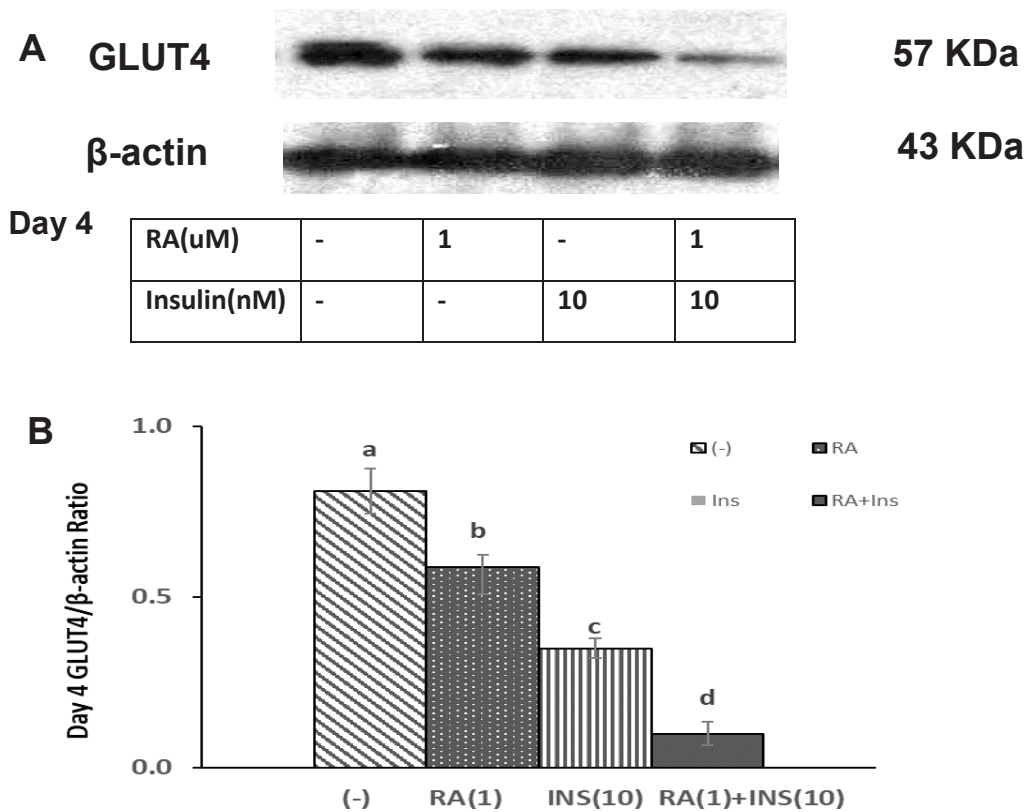
L6 cells were treated with 1  $\mu$ RA, 10 nM insulin and their combination for 6 days again to test the GLUT4 expression. As shown in Figure 11 A&B, GLUT4 expression on Day 4 in L6 control cells was significantly higher than that in cells treated with 1  $\mu$ M RA or 10 nM insulin. This result proved that RA or insulin alone can significantly inhibit the expression of GLUT4 protein level. GLUT4 expression on Day 4 in L6 cells treated with 1  $\mu$ M RA and 10 nM insulin was significantly lower than the RA or insulin alone group, demonstrating a synergy between them. In summary, according to the results shown in Figure 11 A&B, in the 4-days, in the 4-day RA and insulin experiment, we found 1  $\mu$ M RA and 10 nM insulin can inhibit the expression of GLUT4. RA and insulin have a synergistic effect to inhibit the expression of GLUT4 at the protein level in L6 cells treated with RA and insulin for 4 days.

As shown in Figure 12 A&B, the GLUT4 protein level in L6 control cells after differentiation for 6 days were significantly higher than L6 cells treated with 1  $\mu$ M RA, 10 nM insulin, and RA + insulin. This result proved that RA and insulin significantly alone inhibit the expression of GLUT4 protein level.

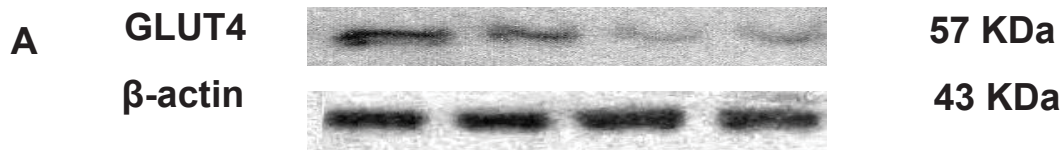
As described in Figure 12 A&B, the GLUT4 protein level in L6 cells treated with 1  $\mu$ M RA for 6 days was significantly higher than cells treated with 1  $\mu$ M RA and 10 nM insulin. GLUT4 protein level in L6 cells treated with 10 nM insulin for 6 days were not significantly different from L6 cells treated with 1  $\mu$ M RA and 10 nM insulin.

In summary, according to the results shown in Figure 11 A&B, we found 1  $\mu$ M RA and 10 nM insulin can inhibit the expression level of GLUT4 on Day 4. RA and insulin do not have a synergy to inhibit the expression of GLUT4 protein level on Day 6.



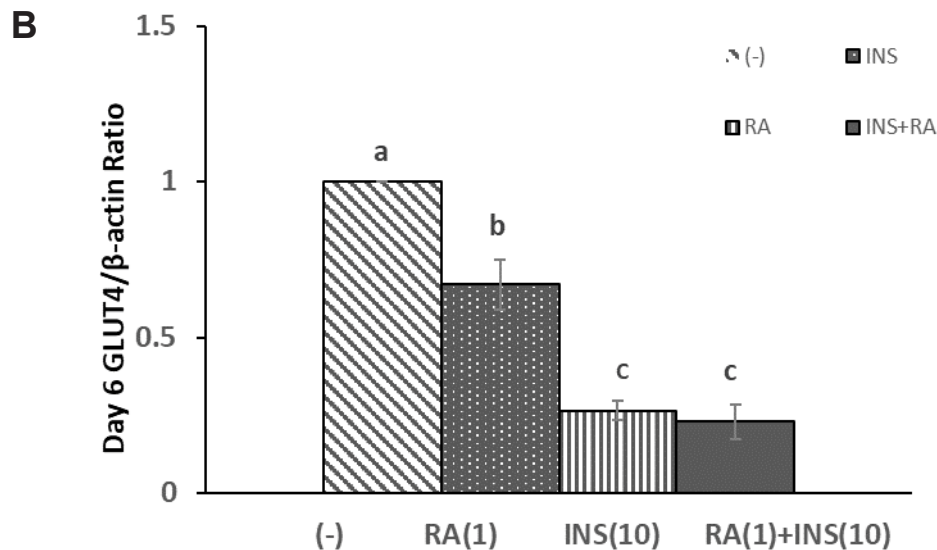


**Figure 11 Decrease of GLUT4 protein levels in L6 cells treated with insulin and RA for 4 days.** A representative blot of protein levels of GLUT4 and β-actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 uM), and RA + insulin for 4 days. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 uM), and RA + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 4 of treatment, whole cell lysate was collected as described in Materials & Methods. B: Quantification analysis of GLUT4 and β-actin in L6 cells on Day 4. The ratio of the densities of specified proteins to β-actin was calculated to do data analysis with control group set as 1. All p-values ≤ 0.05; One-way ANOVA was used to analyse data.



**Day 6**

RA(μM)	-	1	-	1
Insulin(nM)	-	-	10	10



**Figure 12 Decreases of GLUT4 protein levels in L6 cells treated with insulin and RA for 6 days.** A representative blot of protein levels of GLUT4 and β-actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μM), and RA + insulin for 6 days. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μM), and RA + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. B: Quantification analysis of GLUT4 and β-actin in L6 cells on Day 6. The ratio of the densities of specified proteins to β-actin was calculated to do data analysis with control group set as 1. All p-values ≤ 0.05; One-way ANOVA statistical analysis was performed using SPSS software.

### 3.4 Discussion

The aim of this part of the thesis was to understand how RA can influence the glucose metabolism in skeletal muscle cells. In this study, GLUT4 expression in L6 cells were tested. Insulin is an important hormone that regulates skeletal glucose metabolism. Both the expression of GLUT4 and the translocation of GLUT4 can be regulated by insulin. Insulin can stimulate the mobilization of the GLUT4. It has been proved that acute insulin treatment via hind limb perfusion or in viva injection can increase GLUT4 in plasma membrane fraction but decrease GLUT4 in the intracellular membrane fraction.<sup>79</sup> Those results suggest that GLUT4 protein expression is a crucial factor in skeletal glucose metabolism.

In this study, insulin can inhibit the expression of the GLUT4 protein in L6 skeletal muscle cells on Day 4 and 6 as shown in Figure 8 and 9. These results indicate that insulin treatment actually can decrease the GLUT4 protein expression levels in the differentiated L6 skeletal muscle cell. The results shown here suggest that insulin treatment for more than 4 days probably triggered a feedback mechanism by which GLUT4 protein level begins to be reduced. This finding may have physiological meanings as hyperinsulinemia is observed in patients with insulin resistance. Whether the high plasma insulin level in those patents initiates the drop of GLUT4 expression remains to be determined.

RA also dose-dependently inhibits the GLUT4 expression in L6 cells on Day 4 and 6. This finding is important because there were only limited studies about the effect of RA on differentiated skeletal muscle cells. The inhibitory effects of RA on GLUT4 expression in differentiated L6 cells also indicate that caution needs to be taken when obese or diabetes patients are given dietary advice regarding VA supplement. Based on our study, supplying the system with more RA seems not to help the control of glucose if the expression of the GLUT4 protein is inhibited. Excessive intake of VA may lead to more RA production which may cause the decrease of the expression level of GLUT4 protein in obese or diabetic patients. This may have negative effects on the regulation of skeletal glucose homeostasis in those patients.

The effects of RA and insulin combination on GLUT4 protein level in L6 cells was also conducted in this study. GLUT4 protein level of L6 cells treated with 10 nM insulin for 4 days were significantly higher than L6 cells treated with 1  $\mu$ M RA and 10 nM insulin. This result indicates that 1  $\mu$ M RA can further inhibit the expression of GLUT4 in the present of 10 nM insulin. The synergistic effect of RA and insulin further proved to us the negative influence of VA supplement if it was recommended to patients with obese or diabetes. According to the results shown in Figure 11, RA and insulin have a synergistic effect to inhibit the expression of GLUT4 at protein level. Matt Goff who worked in the same lab as me had found that the glucose usage of differentiated skeletal muscle cells treated with RA and insulin for 4 days increase significantly.<sup>81</sup> The decrease protein level of GLUT4 cannot explain the increase of glucose usage of L6 cells. However, GLUT4 is not the only type of glucose transporter found in skeletal muscle, GLUT1 and GLUT3 were also expressed in skeletal muscle.<sup>73</sup> Matt has observed the reduction of GLUT1 in L6 cells treated the same way. On the other hand, he has observed the induction of GLUT3 and GLUT6 expression levels after RA or insulin treatment.<sup>81</sup> The expression levels of GLUT1, GLUT3 and GLUT6 were not tested in this study. Since the L6 cells treated with RA and insulin have the increase of glucose usage, glucose probably gets into the cells through other GLUTs. Whether the changes of other GLUTs in L6 cells contribute to the elevated glucose usage remains to be investigated.

### **3.5. Conclusion**

In this study, RA inhibited GLUT4 expression in L6 cells treated with 1  $\mu$ M RA and/or 10 nM insulin. Moreover, our data also show the synergism of RA and insulin on GLUT4 protein expression. These results suggest that obese or diabetes patient should be cautious when they are given dietary recommendations such as VA supplements. This is because the GLUT4 expression is important for the uptake of glucose in skeletal muscle.

## CHAPTER FOUR

### EFFECTS OF RA ON LIPID ACCUMULATION AND GLUT4 EXPRESSION IN 3T3-L1 CARΔ1 ADIPOCYTES

#### 4.1 Introduction

VA is one important type of fat-soluble micronutrient which is important for the growth and development of animals.<sup>24</sup>RA is an active metabolite of retinol which mediates the major functions of VA. One key role of RA is to regulate gene expression. RA can bind to nuclear receptors in the cell to regulate gene expression. RA can activate both RARs and RXRs in the nuclear receptor super family. Additionally, COUPTF II and HNF4 $\alpha$  are transcriptional factors that can influence the expression of RA-regulated gene recently.<sup>25</sup>

Excess lipid accumulation can lead to obesity. Exploring of the regulation of adipogenesis can help us overcome obesity and associated pathologies. The main function of adipocytes is to balance energy in body.<sup>19</sup> Moreover, adipocytes can secrete important endocrine hormones such as leptin and adiponectin to regulate metabolism in the whole body. Both leptin and adiponectin regulate metabolism to control the body mass in healthy subjects.<sup>19</sup>

Studies on the mechanisms associated with adipogenesis have been done in cultured cell models, such as 3T3-L1 fibroblast cells. Insulin, glucocorticoid receptor agonist, and phosphodiesterase inhibitor can initiate the differentiation of 3T3-L1 fibroblast cells. Phosphodiesterase inhibitor can elevate adenosine 3',5'-cyclic monophosphate (cAMP) levels. Differentiation of 3T3-L1 cells can be triggered because of the treatment with this mixture.<sup>73</sup> In contrast, RA can negatively regulate adipocyte differentiation.<sup>82</sup> It has been shown that RA inhibits adipocyte differentiation of 3T3-L1 preadipocytes.<sup>82</sup> GLUT4 is an insulin-stimulated glucose transporter. Insulin stimulation leads to the movement of GLUT4 from an intracellular location to the cell membrane, a process that leads to elevation of glucose entry into adipocytes. On the other hand, GLUT4

protein expression increases with the differentiation process.<sup>83</sup> The mechanism of this induction of GLUT4 expression in association with differentiation is still unclear.

The aim of this part of the thesis is to understand how RA can influence the lipid and glucose homeostasis in adipocytes. We took advantage of 3T3-L1 CAR $\Delta$ 1 cells, a cell line allowing for transfection of recombinant adenoviruses.<sup>75</sup> The levels of RXR $\alpha$  and COUP-TFII were overexpressed in 3T3-L1 CAR $\Delta$ 1 cells via transfection of ad-RXR $\alpha$  and ad-COUP-TFII. We found that RA treatment and overexpression of RXR $\alpha$  in differentiated 3T3-L1 CAR $\Delta$ 1 cells resulted in the reduction of lipid accumulation. We also observed that RA treatment reduced the GLUT4 expression in differentiating 3T3-L1 CAR $\Delta$ 1 cells, a phenomenon that can be attenuated in the presence of RXR $\alpha$  overexpression.

## 4.2 Materials and Methods

### 4.2.1 Reagents

3T3-L1 CAR $\Delta$ 1 cells were provided by Dr. D. Orlicky (University of Colorado, Denver, CO).<sup>75</sup> For 3T3-L1 CAR $\Delta$ 1 adipocytes culture and differentiation, 3T3-L1 differentiation medium (DMEM+10%FBS+1% 3-isobutyl-1-methylxanthine +0.1% Insulin+0.1% dexamethasone+ P/S) and 3T3-L1 maintenance medium (DMEM + 10%CS + P/S) were purchased from Zenbio Company (Research Triangle Park, NC, U.S.A.).

Insulin and *all-trans* RA and were purchased from Sigma Aldrich, Corp (St. Louis, MO, U.S.A.). BCA protein assay kit was purchased from Pierce Biotechnology, Inc (Rockford, IL, U.S.A.) to test the protein concentration. Immobilon®-PSQ PVDF membrane was purchased from EMD Millipore Corp (Billerica, MA, U.S.A.). Food Club Non-fat dry milk were purchased from a grocery store (St. Abingdon, VA, U.S.A) to block the membrane and to make the first antibodies (GLUT4,  $\beta$ -Actin, RXR $\alpha$ , COUP-TFII). Protease-free bovine serum albumin (BSA) was purchased from Fisher BioReagents™ to prepare primary antibodies from Cell Signaling Technology. For immunoblotting, GLUT4 antibody (C-terminus) was purchased from EMD Millipore Corp (Billerica, MA, U.S.A.).  $\beta$ -

Actin (#4970), total Akt (#9272), phospho-Akt (Ser473) antibody, RXR $\alpha$ , COUPTFII, and horseradish peroxidase conjugated-goat anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). ECL Western Blotting Substrate were purchased from Pierce Biotechnology, Inc (Rockford, IL, U.S.A.).

#### **4.2.2 Preparation of Recombinant Adenoviruses**

Recombinant adenoviruses, Ad- $\beta$ -gal, Ad-RXR $\alpha$ , and Ad-COUPTFII have been used and published previously.<sup>84</sup> The methods of recombinant adenovirus generation have been demonstrated before.<sup>84</sup> When HEK 293 cells grown to 80% confluence in 150 mm tissue culture plates, confirmed original crude lysate was then used to infect HEK 293 cells. The ratio of the medium to crude lysate is 10 to 1 (v/v). After around 48-hour post infection, culture media and cells were collected, which was designated as the crude lysate. New crude lysates of ad-RXR $\alpha$  and ad-COUPTFII were stored at -80°C until being used.

#### **4.2.3 3T3-L1 CAR $\Delta$ 1 Adipocyte Cell Culture and Differentiation**

3T3-L1 cells with the expression of CAR  $\Delta$ 1 is termed as 3T3-L1 CAR  $\Delta$ 1 cells. The efficiency of transduction of recombinant adenovirus in 3T3-L1 CAR  $\Delta$ 1 cells increased approximately 100-fold comparing with than in parental 3T3-L1 cells. 3T3-L1 CAR $\Delta$ 1 adipocytes were maintained in 60 mm dishes in the incubator (37°C, 5% CO<sub>2</sub>, and 95% air) with 3T3-L1 maintenance medium (DMEM+10%CS+P/S). 3T3-L1 CAR $\Delta$ 1 adipocytes were seeded in 12-well plates with inoculum 3 $\times$ 10<sup>5</sup> cells per well. When cells reached near 100% confluence in the 12-well plates (Day 0), the medium was changed to 3T3-L1 differentiation medium supplemented with RA and crude lysate of adenovirus (ad- $\beta$ -gal, ad-RXR $\alpha$  or ad-COUPTFII). Cells were separated into different groups as follow.

On Day 3, the media of cells with different treatments were changed to fresh differentiation media once. On Day 5, differentiation medium was changed to maintenance medium with the same doses of RA. On Day 7, cells in the 12-well plates were fixed to for Oil Red O Staining. Then cells were observed under

**Table 1 Different treatment groups of 3T3-L1 CARΔ1 adipocytes tranfected with or without adenovirus.**

A. Non viral Control	Non-DM+0 μM RA	DM+0 μM RA	DM+0.3 μM RA	DM+1 μM RA	DM+3 μM RA	DM+3 μM RA
B. Ad-β-gal	Non-DM+0 μM RA+ Ad- β-gal	DM+0 μM RA+ Ad-β- gal	DM+0.3 μM RA+ Ad-β-gal	DM+1 μM RA+ Ad-β- gal	DM+3 μM RA+ Ad-β- gal	DM+10 μM RA+ Ad-β- gal
C. Ad-RXRα	Non-DM+0 μM RA+ Ad- RXRα	DM+0 μM RA+ Ad- RXRα	DM+0.3 μM RA+ Ad-RXRα	DM+1 μM RA+ Ad- RXRα	DM+3 μM RA+ Ad- RXRα	DM+10 μM RA+ Ad- RXRα
D. Ad- COUPTFII	Non-DM+0 μM RA+ Ad- COUPTFII	DM+0 μM RA+ Ad- COUPTFII	DM+0.3 μM RA+ Ad- COUPTFII	DM+1 μM RA+ Ad- COUPTFII	DM+3 μM RA+ Ad- COUPTFII	DM+10 μM RA+ Ad- COUPTFII

Note: cells in groups A to D in non-differentiated (1) or differentiated (2 to 5) conditions were treated without (1 and 2) or increasing concentrations (3 to 6) of RA for 7 days.



the optical microscope (Phase Contrast Objectives: LWD PH, 10 × 0.25). Then pictures were taken under a microscope (LWD PH, 20 × 0.4) via Micro Software (Pittsburgh, Pennsylvania USA). Oil red O retained in the cells was dissolved in isopropanol. Optical Density at 500 nm of these lysates was read for quantification.

For the long-term RA dosage curve experiment, when cells reached near 100% confluence (Day 0) in the 60 mm dishes, 3T3-L1 maintenance medium was changed to 3T3-L1 differentiation medium with increasing concentrations of RA in the presence of Ad- $\beta$ -gal, Ad-RXR $\alpha$  or Ad-COUPTFII. Cells were separated into different groups as described in Table 4.1. On Day 3, the media of cells with different treatments were changed to differentiation media containing the indicated reagents once. On Day 5, differentiation medium was changed to maintenance medium with the same doses of RA. On Day 7, cells were collected for analysis of protein levels using immunoblotting.

#### **4.2.4 Oil Red O Staining**

3T3-L1 CAR $\Delta$ 1 adipocyte were seeded in 12-well plates. After the cell differentiation process, old medium was removed, and 1 ml 10% formalin was added to fix cells in each well. Cells were incubated in 10% formalin for overnight. On the next day, 10% formalin in each well was removed, and 1 ml distilled deionized water (dd H<sub>2</sub>O) was added to wash the cells for 3 times. 0.35 g Oil-Red-O was dissolved in 100 ml 100% isopropanol to mix well for at least 20 minutes to make Oil-Red-O stock solution. Then Oil-Red-O stock was filtered with 0.2  $\mu$ m syringe filter. Three parts of the Oil-Red-O stock solution to two parts of dd H<sub>2</sub>O were mixed well together to make Oil-Red-O working solution. Then Oil-Red-O working solution was filtered with 0.2  $\mu$ m syringe filter. dd H<sub>2</sub>O in the 12-well plates was removed, and Oil-Red-O working solution was added to each well of the plate. The fixed cells were incubated in Oil-Red-O (1 ml per well) working solution for 20 minutes. Cells were washed 3 times again with dd H<sub>2</sub>O and then, maintained in dd H<sub>2</sub>O to cover cells. Then, cells were observed under

the microscope immediately after doing Oil-Red-O Staining and pictures of cells were taken.

Water in the 12-well plate with differentiated cells was removed, and cells were let dry. Then, 400  $\mu$ L 100% isopropanol was added to each plate and incubated for 20 minutes to elute Oil-Red-O at room temperature. To make sure that all Oil-Red-O in the solution was eluted, the isopropanol solution was pipetted several times to mix. The solubilized Oil-Red-O solution was transferred to 96-well plate to read OD 500 nm using a Glomax® Multi+Detection System.

#### **4.2.5 Protein Isolation and Immunoblotting**

Cell dishes were kept on ice and then culture medium pumped out through aspiration. Cells were washed once with 3ml ice cold PBS. Then cells are lysed in 400  $\mu$ l lysis buffer per dish. Whole cell lysates were scraped into different centrifugation tubes (1.5 ml). Centrifugation tubes with whole cell lysates were put on ice for more than 20 minutes. Then whole cell lysates were centrifuged at 12,000 x g and 4°C for 15 minutes. Supernatants were collected into new 1.5 ml centrifugation tubes and stored in the freezer (-80°C).

The protein concentration in the cell lysate supernatant was determined with PIERCE BCA protein assay kit (Rockford, IL). This was achieved by first mixing reagent A with reagent B (50:1) to make working reagent. Five  $\mu$ L of each unknown sample was then pipetted into 495  $\mu$ L dd H<sub>2</sub>O in separate labeled tubes. The standard protein stock (2 mg/mL) was diluted to 80, 40, 20, 10, 5 and 0  $\mu$ g/ $\mu$ L for making standard curve according to the manufacture's protocol. In 1 ml reaction, 500  $\mu$ L of the working reagent was then added to each sample and standard protein sample.

OD reading at 560 nm was measured using a 96 plate reader (560 nm). A standard curve was prepared by plotting the average Blank-corrected 560 nm OD readings vs. the concentrations in  $\mu$ g/mL. A formula derived from regression was obtained and used to determine the total protein concentration of each sample.

To make the resolving gel, 5.9 mL ddH<sub>2</sub>O, 5 mL 30:0.8% w/v acrylamide:bisacrylamide, 3.8 mL 1.5M Tris-Cl pH 8.8, 150 µL 10% SDS were mixed together and 150 µL ammonium persulfate and 6 µL of TEMED were added. To make the stacking gel, 4.1 mL ddH<sub>2</sub>O, 1 mL 30:0.8% w/v acrylamide:bisacrylamide, 750 µL 1.0M Tris-Cl pH 6.8, 60 µL 10% SDS were mixed together and 60 µL ammonium persulfate and 6 µL of TEMED were added finally.

After quantification, 40 µg total cell proteins were separated on 8% SDS-PAGE gel. Then, separated proteins were transferred from gel to Immobilon®-PSQ PVDF membrane, which was blocked by 5% milk diluted in 1x TBST at room temperature for one hour. The primary antibodies were diluted in 1x TBST containing 5% BSA. Then, the membrane was incubated in the first antibody at 4 °C for overnight. Then, the membrane was washed three times with 1x TBST (5 minutes each time). Horseradish peroxidase-conjugated goat anti-rabbit IgG (second antibody) was diluted in 1x TBST containing 5% milk (1:1000). Then, the membrane was incubated with the second antibody for one hour. The membranes were washed for another three times with 1x TBST (5 minutes each time). Next, ECL Western Blotting Substrate was used to cover the membrane, which was exposed to X-ray film. Konica SRX 101A Film Processor was used to develop the film. Quantification analysis was performed on Image J software (NIH, Bethesda, Maryland, USA). The density of a protein band was calculated by subtracting the background in an area of the same size near protein band. Then, the ratio of the density of the indicated protein to that of the control protein (protein of interest/ $\beta$ -actin) was calculated for quantification.

#### **4.2.6 Statistical Analysis**

All experiments were repeated three times. One-way ANOVA with LSD post-hoc statistical analysis was conducted using SPSS version 22 statistical software (IBM, Armonk, NY, U.S.A.). Data were presented as means  $\pm$  S.E.M. p-value less than 0.05 was considered statistically significant.

## 4.3 Results

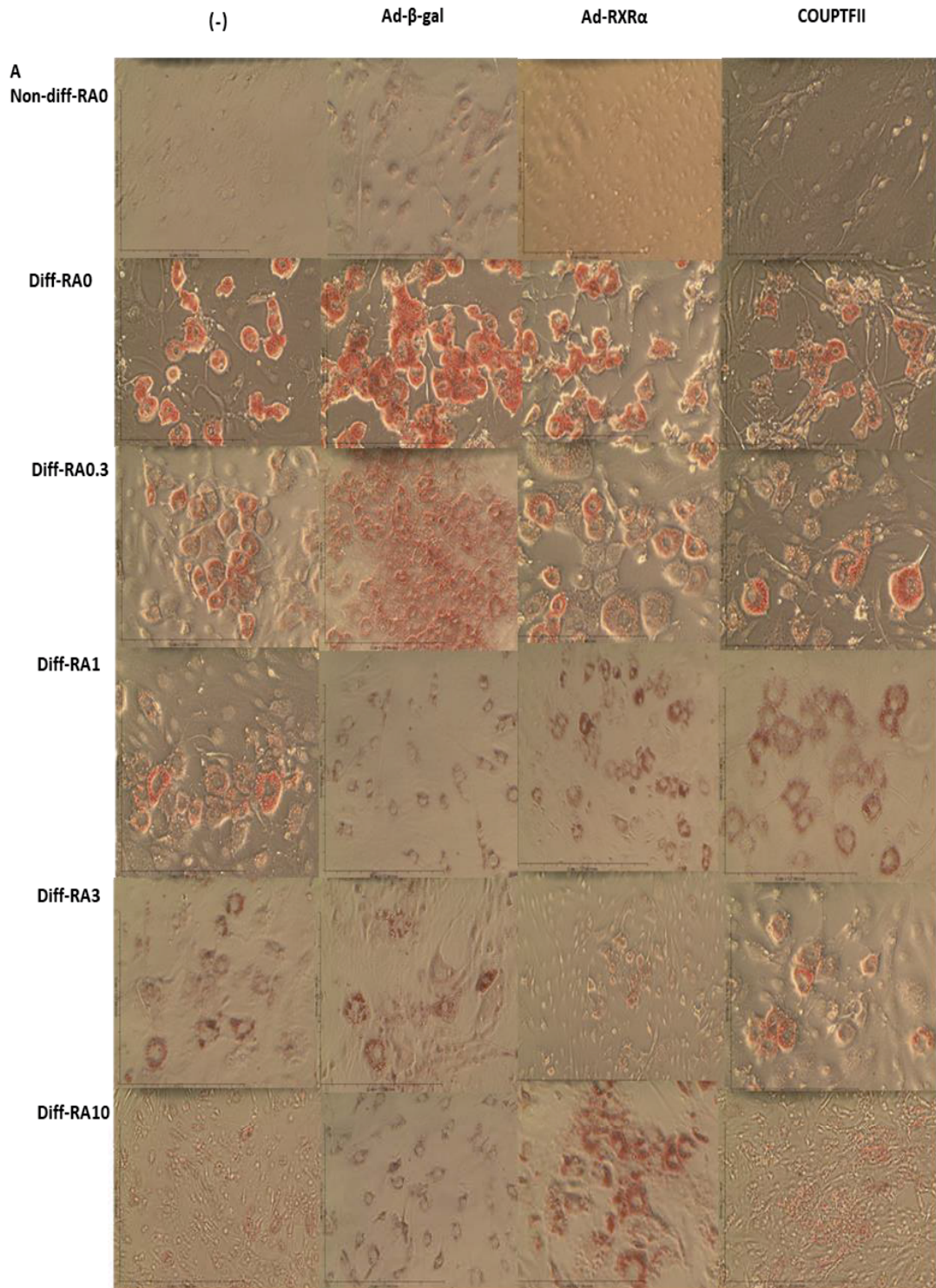
### 4.3.1 RA Inhibits Lipid Accumulation in 3T3-L1 CAR $\Delta$ 1 Cells

To study the RA effects on lipid accumulation, 3T3-L1 CAR $\Delta$ 1 cells were differentiated in RA doses without or with Ad- $\beta$ -gal transfection. In Figure 13 A, lipid accumulation decreased with the increase of RA concentration. Figures 13 B, C&D show the quantification of the lipid accumulation data.

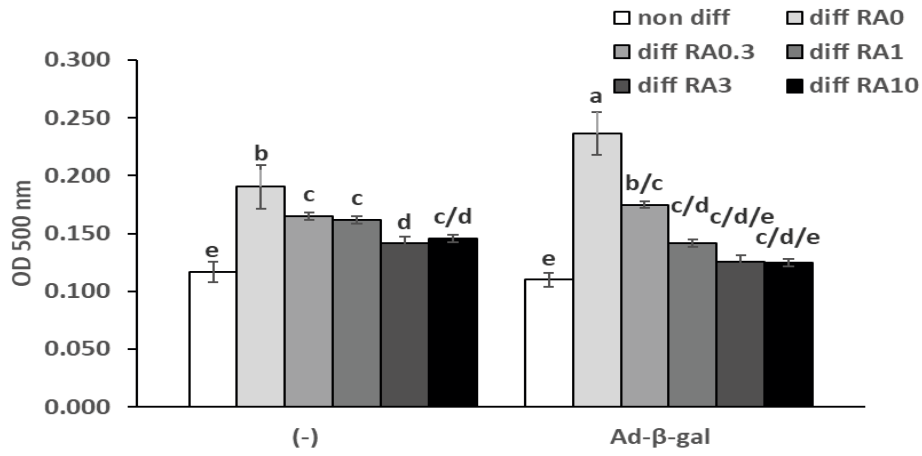
As shown in Figure 13 B, in the RA doses experiment, the control 3T3-L1 CAR $\Delta$ 1 adipocytes treated with differentiated medium had significantly higher lipid accumulation than the 3T3-L1 CAR $\Delta$ 1 cells treated with maintenance medium. This result indicated that 3T3-L1 CAR $\Delta$ 1 adipocytes were well differentiated in differentiation medium. The amounts of lipid accumulation in those differentiating 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3, 1, 3, and 10  $\mu$ M were significantly lower than that in differentiating 3T3-L1 CAR $\Delta$ 1 cells treated without RA. This result proved to us that RA can significantly inhibit lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells during the differentiation process. There were no significant differences of lipid accumulation between 3T3-L1 cells treated with RA at 0.3  $\mu$ M and 1  $\mu$ M. The lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3  $\mu$ M or 1  $\mu$ M was significantly higher than that in those treated with RA at 3  $\mu$ M. This result indicates that RA at 3  $\mu$ M can further inhibit the lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells compared with those treated with RA at 0.3  $\mu$ M or 1  $\mu$ M. As a result, RA at 3  $\mu$ M reached the highest inhibitory effect on lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells. The lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3, 1, 3 or 10  $\mu$ M RA was significantly higher than in those cells cultured in non-differentiation medium. Although RA can inhibit the lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells, certain amount of lipid can still accumulate in 3T3-L1 cells at a significant level.

As described in Figure 13 B, lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with differentiated medium was significantly higher than those treated with maintenance medium. This result indicates that the 3T3-L1 CAR $\Delta$ 1 cells transfected with ad- $\beta$ -gal differentiated well. The amounts of lipid accumulation in

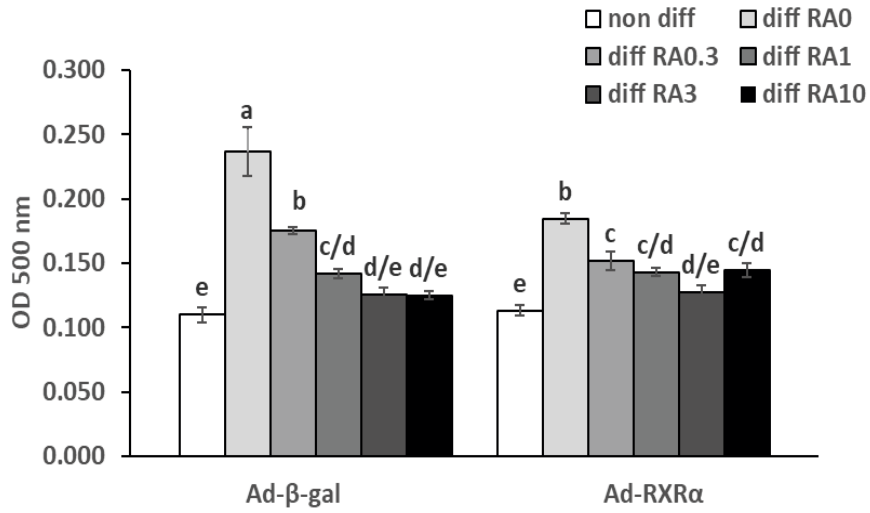
**Figure 13: RA Inhibits the Lipid Accumulation in 3T3-L1 CAR $\Delta$ 1 adipocyte cell treated with or without ad- $\beta$ -gal.** A: lipid accumulation in differentiating 3T3-L1 cells treated with RA doses with or without ad- $\beta$ -gal. B, C&D: Quantification of lipid accumulation in 3T3-L1 CAR $\Delta$ 1 adipocyte cell. The cells were cultured in triplicates for 120 h (5 days) in 3T3-L1 differentiation medium containing different doses of RA. Ad- $\beta$ -gal was added on Day 0 of differentiation. 3T3-L1 differentiation medium was changed to 3T3-L1 maintenance medium containing different concentration of RA for 48 hours (2 days).



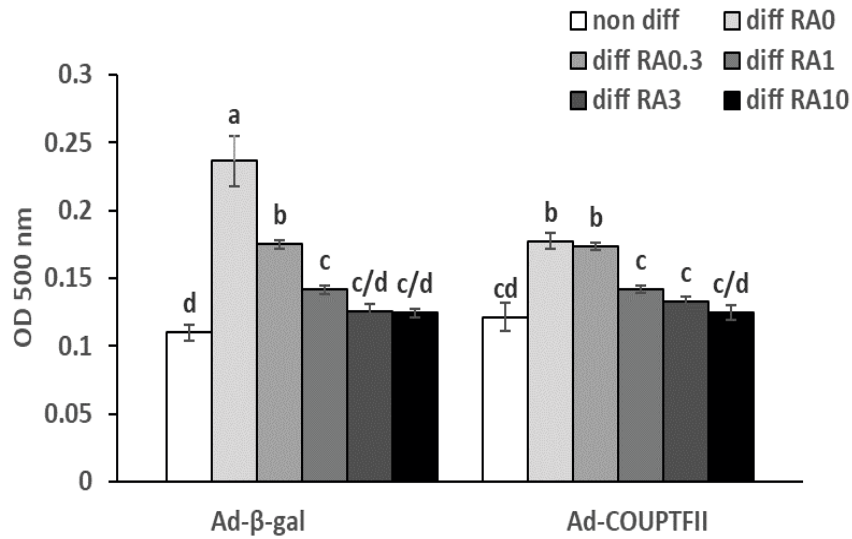
**B**



**C**



**D**



3T3-L1 cells treated with RA at 0.3, 1, 3 and 10  $\mu\text{M}$  with differentiation medium were significantly lower than that in 3T3-L1 CAR $\Delta$ 1 cells treated without RA in differentiation medium. This result proved to us that RA can significantly inhibit lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells infected with ad- $\beta$ -gal during differentiation process. There was no significant difference among differentiating 3T3-L1 cells treated with different doses of RA. The lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 3  $\mu\text{M}$  or 10  $\mu\text{M}$  was not significantly different from that treated with the non-differentiation medium. There was almost no lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 3  $\mu\text{M}$  or 10  $\mu\text{M}$ , which indicates that RA at 3  $\mu\text{M}$  or 10  $\mu\text{M}$  can effectively inhibit the lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells transfected with Ad- $\beta$ -gal. As a result, the inhibition of RA on lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells is more obvious in cells transfected with ad- $\beta$ -gal. The transfection of ad- $\beta$ -gal does not affect the inhibitory effect of RA on the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells.

When comparing the differentiating 3T3-L1 CAR $\Delta$ 1 cells treated with RA doses in the absence of recombinant adenovirus transfection with that transfected with ad- $\beta$ -gal and treated with RA, there was no significant difference of lipid accumulation in cells treated with RA at 0.3, 1, 3 or 10  $\mu\text{M}$ . The lipid accumulation in differentiating 3T3-L1 cells transfected with ad- $\beta$ -gal was significantly higher than that without viral transfection in RA 0  $\mu\text{M}$  treatment group. It suggests that transfection of recombinant adenovirus or overexpression of  $\beta$ -gal may cause more lipid accumulation in the differentiation process. This result demonstrated the importance to use ad- $\beta$ -gal as a control group to study the overexpression of other proteins.

In summary, RA can inhibit the lipid accumulation in differentiating 3T3-L1 cells in the absence or presence of ad- $\beta$ -gal infection. In the groups of differentiating 3T3-L1 cells treated without ad- $\beta$ -gal transfection, RA at 3  $\mu\text{M}$  presented the highest inhibitory effect. In the groups of differentiating 3T3-L1 CAR $\Delta$ 1 cells transfected with ad- $\beta$ -gal, RA at 0.3, 1, 3 and 10  $\mu\text{M}$  presented similar inhibitory effects on lipid accumulation. Either recombinant adenovirus or



overexpress of  $\beta$ -gal may influence the function of RA to inhibit lipid accumulation in differentiating 3T3-L1 cells. Therefore, 3T3-L1 cells infected with ad- $\beta$ -gal can serve as the controls for 3T3-L1 infected with ad-RXR $\alpha$  or COUPTFII.

#### **4.3.2 RA Inhibits Lipid Accumulation in 3T3-L1 CAR $\Delta$ 1 Cells Transfected with Ad-RXR $\alpha$**

The influence of RXR $\alpha$  overexpression on the lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells treated with increasing concentrations of RA were assessed by Oil-Red-O staining as shown in Fig. 13 A. Quantification of lipid accumulation in cells transfected with ad- $\beta$ -gal or ad-RXR $\alpha$  and treated with RA after differentiation was done by the measurement of OD 500 nm. (Fig. 13 C).

Here, we conducted RA dosage experiment in 3T3-L1 CAR $\Delta$ 1 adipocytes transfected with ad- $\beta$ -gal or ad-RXR $\alpha$ . Figure 13 A also presents microscopic pictures of the lipid accumulation in 3T3 cells treated with ad- $\beta$ -gal or ad-RXR $\alpha$ . As shown in Figure 13 C, lipid accumulation in ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 adipocytes treated with differentiated medium without RA was significantly higher than those with only maintenance medium. This result indicated that the 3T3-L1 CAR $\Delta$ 1 cells were well differentiated when transfected with ad-RXR $\alpha$ . The lipid accumulation in those differentiating 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3,1,3, or 10  $\mu$ M was significantly lower than differentiating 3T3-L1 CAR $\Delta$ 1 cells treated without RA. This result proved to us that RA can significantly inhibit lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells transfected with ad-RXR $\alpha$  during the differentiation process.

The lipid accumulation of the ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells treated without RA was significantly lower than those ad- $\beta$ -gal-transfected cells treated without RA. This result indicates that the overexpression of RXR $\alpha$  may inhibit the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells with no RA. The lipid accumulation in ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3  $\mu$ M had significantly lower lipid accumulation than the ad- $\beta$ -gal-transfected cells treated with 0.3  $\mu$ M RA. The overexpression of RXR $\alpha$  may help

to mediate the inhibitory influence of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells.

In summary, RA can significantly inhibit lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells transfected with ad-RXR $\alpha$  during the differentiation process. Overexpression of RXR $\alpha$  may inhibit the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells in the absence or presence of RA.

#### ***4.3.3 RA Inhibits Lipid Accumulation in 3T3-L1 CAR $\Delta$ 1 Cells Transfected with Ad-COUPFII***

The changes of lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells transfected with ad- $\beta$ -gal or ad-COUPFII and treated with increasing concentrations of RA were assessed by Oil-red-O staining as shown in Fig. 13 A. Quantification of lipid accumulation changes in ad- $\beta$ -gal-transfected or ad-COUPFII-transfected cells were detected by measurement of OD 500 nm, as shown in Fig. 13 D.

Figure 13 A presents microscopic pictures of the lipid accumulation in cells transfected with ad- $\beta$ -gal or ad-COUPFII. As described in Figure 13 D, the ad-COUPFII-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with differentiation medium without RA had significantly higher lipid accumulation than those treated with only maintenance medium in the absence of RA. This result indicates that the 3T3-L1 CAR $\Delta$ 1 cells were well differentiated with ad-COUPFII. There was no significant difference between 3T3-L1 cells treated with RA at 0  $\mu$ M and 0.3  $\mu$ M in the ad-COUPFII-transfected 3T3-L1 CAR $\Delta$ 1 cells with differentiation medium. There was no significant difference in 3T3-L1 cells treated with RA at 1  $\mu$ M, 3  $\mu$ M or 10  $\mu$ M. The lipid accumulation levels of ad-COUPFII-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0  $\mu$ M and 0.3  $\mu$ M were significantly higher than those treated with RA at 1  $\mu$ M, 3  $\mu$ M or 10  $\mu$ M. As a result, RA can still inhibit the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells with the overexpression of COUPFII.

The lipid accumulation of ad-COUP-TFII-transfected cells was significantly lower than ad- $\beta$ -gal-transfected cells treated with differentiation medium in the absence of RA. This result indicates that the overexpression of COUPFII may

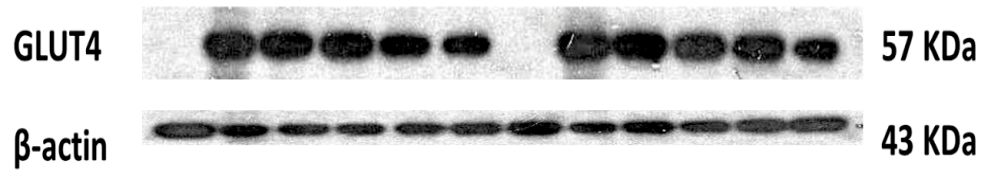
inhibit the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells. The lipid accumulation levels of ad- $\beta$ -gal-transfected and ad-COUP-TFII-transfected cells treated with increasing concentrations of RA were not significantly different from each other.

#### **4.3.4 Differentiation process Increases GLUT4 Protein Level in 3T3-L1 CAR $\Delta$ 1 Adipocytes**

To test the influence of RA treatment on GLUT4 expression and the potentials of using recombinant adenovirus to alter the expression levels of transcription factors, 3T3-L1 CAR $\Delta$ 1 adipocytes in differentiation process were treated with RA doses and ad- $\beta$ -gal. Fig 14 shows the expression levels of GLUT4 and  $\beta$ -actin protein levels. The cells were cultured for 120 h (5 days) in 3T3-L1 differentiation medium containing 0, 0.3, 1, 3, or 10  $\mu$ M RA. Ad- $\beta$ -gal was added at Day 0 of differentiation. 3T3-L1 differentiation medium was changed to 3T3-L1 maintenance medium containing 0, 0.3, 1, 3, or 10  $\mu$ M RA after 48 hours (2 days). All cultured cells on Day 7 were lysed for western blot analysis of protein expression.

As shown in Figure 14 on the left panel, in the non-differentiating group without ad- $\beta$ -gal transfection, there was almost no GLUT4 protein detected. GLUT4 protein levels in differentiating 3T3-L1 CAR $\Delta$ 1 cells were obviously higher than that in non-differentiating 3T3-L1 CAR $\Delta$ 1 cells in the absence of ad- $\beta$ -gal transfection. This result proved that the differentiation process increases GLUT4 protein level in 3T3-L1 CAR $\Delta$ 1 cells. We can observe a slightly decrease trend of GLUT4 expression in the differentiating 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0, 0.3, 1, 3 or 10  $\mu$ M.

As shown in Fig 14 on the right panel, the Ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells had almost no expression of GLUT4, which is similar to the cells without any recombinant adenovirus transfection. The GLUT4 expression level in ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells was also slightly reduced with the increase of RA concentration. We conclude that RA can inhibit the GLUT4 protein expression in differentiating 3T3-L1 CAR $\Delta$ 1 cells. Adenovirus-mediated



(-)	-	-	-	-	-	-	-	-	-	-	-	-
$\beta$ -gal	-	-	-	-	-	-	+	+	+	+	+	+
RA	0	0	0.3	1	3	10	0	0	0.3	1	3	10
Insulin	-	+	+	+	+	+	-	+	+	+	+	+

**Figure 14 Changes in GLUT4 expression in 3T3-L1 CAR $\Delta$ 1 Adipocyte Cell transfected with or without Ad- $\beta$ -gal.** The cells were cultured in triplicates for 120 hours (5 days) in 3T3-L1 differentiation medium containing different doses of RA. Ad- $\beta$ -gal was added on Day 0 of differentiation. 3T3-L1 differentiation medium was changed to 3T3-L1 maintenance medium containing different doses of RA for 48 hours (2 days). Whole cell lysate was collected on Day 7 as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate. The ratio of the densities of GLUT4 to  $\beta$ -Actin was calculated to do data analysis with control group set as 1.

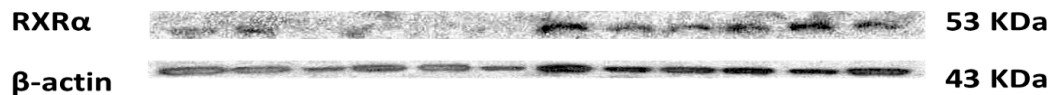
transfection has no significant influence on the GLUT4 protein levels in 3T3-L1 CAR $\Delta$ 1 cells.

#### **4.3.5 Overexpression of RXR $\alpha$ was achieved by ad-RXR $\alpha$ in Differentiating 3T3-L1 CAR $\Delta$ 1 Adipocytes**

To explore the transcription factors that may mediate the RA-suppressed GLUT4 protein expression in differentiating 3T3-L1 CAR $\Delta$ 1 cells, we compared the RXR $\alpha$  and  $\beta$ -actin expression levels in 3T3-L1 cells transfected with ad- $\beta$ -gal or ad-RXR $\alpha$  as shown in Fig.4.3. The cells were cultured in triplicates for 120 hours (5 days) in 3T3-L1 differentiation medium containing 0, 0.3, 1, 3, or 10  $\mu$ M RA. Ad- $\beta$ -gal and ad-RXR $\alpha$  were added at Day 0 of differentiation. 3T3-L1 differentiation medium was changed to 3T3-L1 maintenance medium containing 0, 0.3, 1, 3, or 10  $\mu$ M RA after 48 hours (2 days). All cultured cells on Day 7 were lysed to do immunoblotting.

In Fig 4.3, RXR $\alpha$  protein level in ad- $\beta$ -gal transfected 3T3-L1 CAR $\Delta$ 1 cells treated with differentiation medium without RA was slightly higher than non-differentiating 3T3-L1 cells in the absence of RA. There was almost no RXR $\alpha$  detected in the Ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0.3, 1, 3 or 10  $\mu$ M in differentiation medium. As shown in Fig 4.3, RXR $\alpha$  protein was detected in all groups of 3T3-L1 CAR $\Delta$ 1 cells in the presence of ad-RXR $\alpha$  transfection. RA-suppressed RXR $\alpha$  expression is absent when RXR $\alpha$  is overexpressed by ad-RXR $\alpha$ .

In the RA at 0.3, 1, 3 and 10  $\mu$ M groups, RXR $\alpha$  protein level in ad-RXR $\alpha$ -transfected 3T3-L1 cells with differentiation medium were obviously higher than ad- $\beta$ -gal transfected 3T3-L1 cells in respective treatment groups. In the RA at 0  $\mu$ M differentiation groups, RXR $\alpha$  protein level in ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells with differentiation medium was higher than ad- $\beta$ -gal transfected 3T3-L1 cells with differentiation medium. In the RA at 0  $\mu$ M non-differentiation groups, RXR $\alpha$  protein level in ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells with differentiation medium was obviously higher than ad- $\beta$ -gal transfected 3T3-L1 cells with differentiation medium.



<b>β-gal</b>	+	+	+	+	+	+	-	-	-	-	-	-
<b>RXRα</b>	-	-	-	-	-	-	+	+	+	+	+	+
<b>RA</b>	<b>0</b>	<b>0</b>	<b>0.3</b>	<b>1</b>	<b>3</b>	<b>10</b>	<b>0</b>	<b>0</b>	<b>0.3</b>	<b>1</b>	<b>3</b>	<b>10</b>
<b>Insulin</b>	-	+	+	+	+	+	-	+	+	+	+	+

**Figure 15 RXRα protein levels in 3T3-L1 CARΔ1 adipocytes.** The cells were cultured in triplicates for 5 days in 3T3-L1 differentiation medium containing 0, 0.3, 1, 3, or 10 μM RA. Ad-β-gal or ad-RXRα was added at Day 0 of differentiation. 3T3-L1 differentiation medium was changed to 3T3-L1 maintenance medium containing RA after treatment for 2 days. Whole cell lysate was collected on Day7 as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate. The ratio of the density of RXRα to that of β-Actin was calculated to do analyze data with control group set as 1.

Those results proved to us that recombinant adenovirus mediated the overexpression of targeted proteins 3T3-L1 CAR $\Delta$ 1 cells in both non-differentiated and differentiated conditions. This established the foundation for us to use 3T3-L1 CAR $\Delta$ 1 for studies of protein functions through recombinant adenovirus-mediated overexpression.

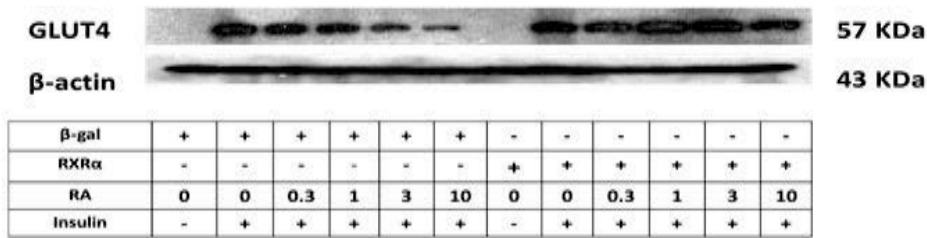
#### **4.3.6 RA-mediated Reduction of GLUT4 Protein Level was Attenuated by Recombinant Adenovirus-mediated RXR $\alpha$ Overexpression in Differentiating 3T3-L1 CAR $\Delta$ 1 Adipocytes**

Fig 16 shows that GLUT4 protein levels in ad- $\beta$ -gal-transfected or ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells treated with non-differentiation medium or differentiation medium containing increasing concentrations of RA. The cells and treatment were the same as Fig 15.

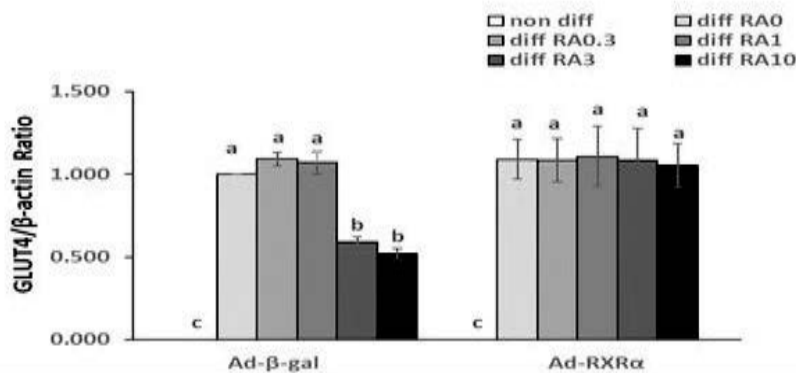
The GLUT4 protein levels in ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with differentiation medium containing RA at 0, 0.3, 1, 3 or 10  $\mu$ M were significantly higher than that treated with non-differentiation medium. There were almost no GLUT4 detected in the non-differentiating cells treated without RA. This result further proved that the differentiation process led to the induction of GLUT4 protein expression in 3T3-L1 CAR $\Delta$ 1 cells. GLUT4 protein level in differentiating cells treated with RA at 0, 0.3 or 1  $\mu$ M were almost same with each other. GLUT4 protein level in differentiating cells treated with RA at 3 or 10  $\mu$ M were significantly lower than differentiating cells treated without or with 0.3  $\mu$ M RA. We conclude that RA inhibits the GLUT4 protein level in ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells during the process of differentiation.

Similar to the results in the groups of 3T3-L1 cells with  $\beta$ -gal transfection, GLUT4 protein levels in the non-differentiating cells without RA treatment was significantly lower than the differentiating 3T3-L1 cells with RXR $\alpha$  overexpression. GLUT4 protein levels in differentiating cells treated with RA at 0, 0.3, 1, 3 and 10  $\mu$ M were almost same with each other in the 3T3-L1 cells with RXR $\alpha$  overexpression. Moreover, when compared GLUT4 levels in differentiating 3T3-L1 CAR $\Delta$ 1 cells with  $\beta$ -gal transfection with that with RXR $\alpha$  transfection in

A



B



**Figure 16 GLUT4 protein levels in 3T3-L1 CARΔ1 adipocytes transfected with Ad-β-gal or Ad-RXRα.** The cells were cultured in triplicates for five days in 3T3-L1 differentiation medium containing 0, 0.3, 1, 3, or 10 μM RA. Ad-β-gal or ad-RXRα was added at Day 0 of differentiation. Differentiation medium was changed to maintenance medium after two days. Whole cell lysate was collected on Day7 as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate. The ratio of the density of GLUT4 to that of β-Actin was calculated to do data analyze with control group set as 1. A: A representative blot of protein levels of RXRα and β-actin in 3T3-L1 CARΔ1 adipocytes. B: Quantification analysis of RXRα. All p-values ≤ 0.05, a>b>c>d>e, One-way ANOVA was used to analyze data ( means ± S.E.M (n=3))

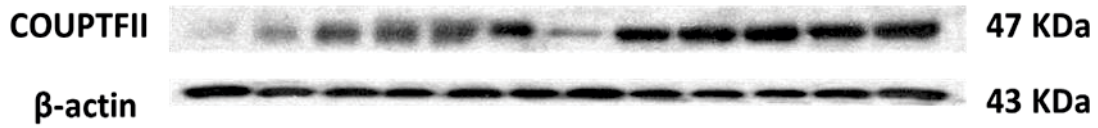


in the RA at 3  $\mu\text{M}$  or 10  $\mu\text{M}$  treatment group, we found that the protein level of GLUT4 in ad-RXR $\alpha$  transfected 3T3-L1 cells were significantly higher than that in ad- $\beta$ -gal-transfected 3T3-L1 cells treated with RA at the respective dosages. We already proved that RXR $\alpha$  was overexpressed successfully by ad-RXR $\alpha$  in both non-differentiated and differentiated 3T3-L1 cells as shown in Figure 15. We conclude that RA inhibits the expression of GLUT 4 in differentiated 3T3-L1 CAR $\Delta$ 1 cells with  $\beta$ -gal transfection. Interestingly, when RXR $\alpha$  was overexpressed in 3T3-L1 CAR $\Delta$ 1 cells, it attenuated the inhibitory effects of RA on GLUT4 expression. GLUT4 protein level was increased as a result. This finding is novel and very important to understand the glucose metabolism in 3T3-L1 CAR $\Delta$ 1 cells.

#### ***4.3.7 COUPTFII was Overexpressed Successfully by recombinant adenovirus in 3T3-L1 CAR $\Delta$ 1 Adipocytes***

We have shown that COUP-TFII also affected the RA signaling.<sup>84</sup> Therefore, we also overexpressed COUP-TFII to study its effect on GLUT4 expression in 3T3-L1 CAR $\Delta$ 1 cells. Fig 17 shows that GLUT4 protein levels in ad- $\beta$ -gal-transfected or ad-RXR $\alpha$ -transfected 3T3-L1 CAR $\Delta$ 1 cells treated with non-differentiation medium or differentiation medium containing increasing concentrations of RA. Cells were treated the same as that in Fig. 16. COUPTFII protein levels in ad- $\beta$ -gal-transfected 3T3-L1 cells treated with differentiation medium presented a trend of elevation with the increasing of RA doses. RA may help to increase the induction of COUPTFII in differentiating 3T3-L1 CAR $\Delta$ 1 cells. COUPTFII protein levels in differentiating ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with RA at 0, 0.3, 1, 3, and 10  $\mu\text{M}$  were all higher than the non-differentiating ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells without RA. This result proved to us that differentiation process may increase the protein level of COUPTFII.

The COUPTFII protein levels in ad-COUP-TFII-transfected 3T3-L1 CAR $\Delta$ 1 cells treated with differentiation medium were obviously higher than that treated with non-differentiation medium as shown Fig. 17 right panel. The cells treated



$\beta$ -gal	+	+	+	+	+	+	-	-	-	-	-	-
COUPTFII	-	-	-	-	-	-	+	+	+	+	+	+
RA	0	0	0.3	1	3	10	0	0	0.3	1	3	10
Insulin	-	+	+	+	+	+	-	+	+	+	+	+

**Figure 17 COUPTFII protein levels in 3T3-L1 CAR $\Delta$ 1 adipocytes.** The cells were cultured in triplicates for five days in 3T3-L1 differentiation medium(DM) containing 0, 0.3,1,3, or 10  $\mu$ M RA. Ad- $\beta$ -gal or ad- COUPTFII was added at Day 0 of differentiation. DM was changed to maintenance medium for two days. Whole cell lysate was collected on Day7 as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate. The ratio of the density of COUPTFII to that of  $\beta$ -Actin was calculated to do data analysis with control group set as 1.

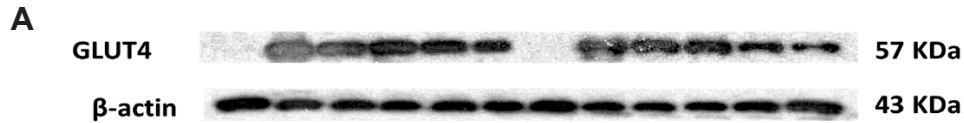
with RA at 0, 0.3, 1, 3, and 10  $\mu\text{M}$  had similar amount of COUP-TFII expression in differentiating ad-COUP-TFII-transfected 3T3-L1 cells. This result further proved to us that differentiation medium which contains insulin may induce the expression of COUPTFII.

When compared the expression levels of COUP-TFII in ad- $\beta$ -gal - transfected 3T3-L1 CAR $\Delta$ 1 cells with that in the ad-COUP-TFII-transfected one, we found that the protein level of COUPTFII in non-differentiated 3T3-L1 cells with COUPTFII overexpression was a little lower than that in differentiating ad-COUPTFII 3T3-L1 CAR $\Delta$ 1 cells. In the RA at 0, 0.3, 1, 3, and 10  $\mu\text{M}$  treatment groups, protein levels of COUPTFII in differentiating ad- $\beta$ -gal-transfected 3T3-L1 cells was obviously higher than that in the non-differentiating ad- $\beta$ -gal-transfected 3T3-L1 CAR $\Delta$ 1 cells respectively. This result proved that COUPTFII can also be induced during the process of differentiation. This result is very important because it proved to us that ad-COUPTFII was successfully overexpressed by ad-COUP-TFII in differentiated 3T3-L1 CAR $\Delta$ 1 cells which established the based for our next research.

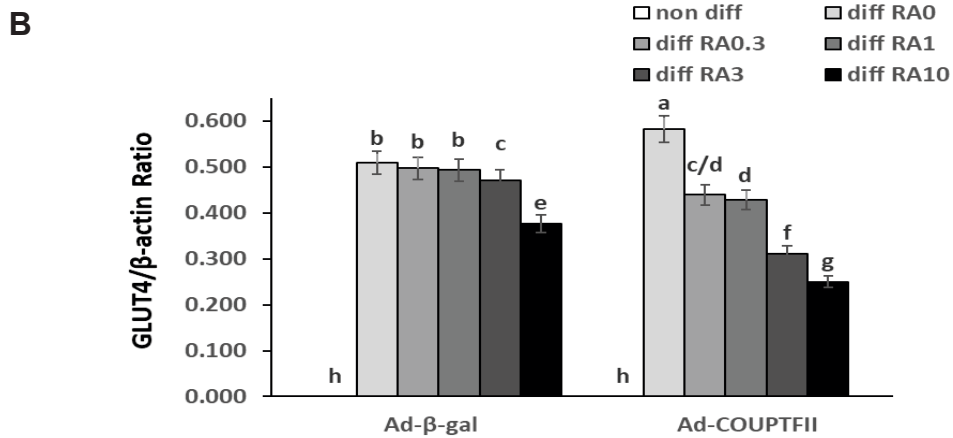
#### ***4.3.8 Differentiating ad-COUPTFII-transfected 3T3-L1 CAR $\Delta$ 1 Adipocytes Treated with RA Further Decreases GLUT4 Protein Level***

Fig 18 A shows the GLUT4 protein levels in ad- $\beta$ -gal-transfected or ad-COUPTFII -transfected 3T3-L1 CAR $\Delta$ 1 cells treated with non-differentiation medium or differentiation medium containing increasing concentrations of RA. The cells and treatment were the same as Fig 17. Fig 18 B described the quantification of GLUT4 protein in those cells.

The results of GLUT4 protein levels in  $\beta$ -gal-transfected 3T3-L1 cells were the same as described in Fig. 16 A. Similar to the results in the groups of 3T3-L1 CAR $\Delta$ 1 cells with  $\beta$ -gal overexpression, GLUT4 protein levels in the non-differentiating cells without RA was significantly lower than that in the differentiating cells with COUPTFII overexpression. In the Ad-COUP-TFII-transfected 3T3-L1 CAR $\Delta$ 1 cells, the GLUT4 protein level in differentiating cells treated with RA at 0, 0.3, 1, 3 or 10  $\mu\text{M}$  were significantly lower than non-



$\beta$ -gal	+	+	+	+	+	+	-	-	-	-	-	-
COUPTFII	-	-	-	-	-	-	+	+	+	+	+	+
RA	0	0	0.3	1	3	10	0	0	0.3	1	3	10
Insulin	-	+	+	+	+	+	-	+	+	+	+	+



**Figure 18 GLUT4 protein levels in 3T3-L1 CAR $\Delta$ 1 adipocytes transfected with Ad- $\beta$ -gal or ad- COUPTFII.** The cells were cultured in triplicates for five days in 3T3-L1 differentiation medium (DM) containing 0, 0.3, 1, 3, or 10  $\mu$ M RA. Ad- $\beta$ -gal or ad- COUPTFII was added at Day 0 of differentiation. DM was changed to maintenance medium after two days. Whole cell lysate was collected on Day 7 as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate. The ratio of the density of GLUT4 to that of  $\beta$ -Actin was calculated to do data analysis with control group set as 1. A: A representative blot of protein levels of GLUT4 and  $\beta$ -actin in 3T3-L1 CAR $\Delta$ 1 adipocyte cell. B: Quantification analysis of GLUT4. All p-values  $\leq$  0.05, a>b>c>d>e>f>g>h, One-way ANOVA were used to analyse data (means  $\pm$  S.E.M (n=3))

differentiating cells treated without RA. GLUT4 protein level in differentiating cells treated with RA at 0.3 or 1  $\mu\text{M}$  were significantly higher than that in differentiating cells treated with RA at 3 or 10  $\mu\text{M}$ . GLUT4 protein level in differentiating cells treated with 3  $\mu\text{M}$  RA was significantly higher than differentiating cells treated with RA at 10  $\mu\text{M}$ . We conclude that RA may inhibit the GLUT4 protein level in a dose dependent manner in Ad-COUP-TFII-transfected 3T3-L1 CAR $\Delta$ 1 cells.

Interestingly, we found protein levels of GLUT4 in 3T3-L1 CAR $\Delta$ 1 cells with COUPTFII overexpression were significantly lower than the 3T3-L1 cells with  $\beta$ -gal transfection in RA at 3 or 10  $\mu\text{M}$ , respectively. We demonstrated that COUPTFII was overexpressed successfully in differentiated 3T3-L1 CAR $\Delta$ 1 cells. We conclude that the COUPTFII overexpression may facilitate the inhibitory effects of RA on the expression of GLUT4.

## 4.4 Discussion

### ***4.4.1 The inhibitory effect of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells may be mediated by RXR $\alpha$***

The effects of RA depend on its concentration and duration of action. It has been shown that RA may block adipogenesis when introduced at early stages of differentiation (24h).<sup>82</sup> Moreover, it was shown that the inhibition of RA on adipocyte differentiation via RA is mediated by RARs.<sup>82</sup> In the study, RA was found to inhibit adipogenesis which was presented in Figure 13. Interestingly, we found the adipogenesis was further inhibited in ad-RXR $\alpha$ -infected 3T3-L1 cells. Overexpression of RXR $\alpha$  may help to mediate the inhibitory effect of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells. This finding was different from the results of Mitchell Lazar's study that the inhibition influence of RA on adipocyte differentiation were likely to be mediated by RARs rather than RXRs.<sup>82</sup> We hypothesize that the expression of RXR $\alpha$  in 3T3-L1 CAR $\Delta$ 1 cell may be too low to present significant inhibit effects on adipocyte differentiation or RXR $\alpha$  expression may be suppressed by the differentiation process.<sup>85</sup>

Here, we showed that overexpression of RXR $\alpha$  was achieved by ad-RXR $\alpha$  in differentiating 3T3-L1 CAR $\Delta$ 1 adipocytes as described in Figure 15. 3T3-L1

CAR  $\Delta$ 1 cells can be transduced effectively by recombinant adenovirus than general 3T3-L1 cells. The overexpression of RXR $\alpha$  supports the finding that RXR $\alpha$  may help to mediate the inhibitory effect of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells.

Another difference between my study and Mitchell Lazar's research is the difference of RA concentrations used. We found that the overexpression of RXR $\alpha$  may help to mediate the inhibitory effect of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells when the concentration of RA was at 0.3  $\mu$ M. Whereas, Mitchell Lazar only examined the influence of 10  $\mu$ M RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells.<sup>85</sup>

#### ***4.4.2 Overexpression of COUPTFII enhances the inhibitory effect of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells***

COUP-TFII expression is important in adipocyte differentiation. It has been proved by Evan Rosen that overexpression of COUP-TFII in 3T3-L1 preadipocytes inhibits adipogenesis.<sup>86</sup> As described in Figure 17 in my study, COUPTFII was overexpressed successfully by recombinant adenovirus in 3T3-L1 CAR $\Delta$ 1 adipocytes. We further proved that overexpression of COUPTFII may inhibit the lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells. Previous results of our lab have shown that overexpression of COUPTFII inhibited the RA-induced glucokinase gene expression in primary hepatocytes.<sup>81</sup> However, in my study, overexpression of COUPTFII did not attenuate the inhibition influence of RA on lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells as described in Figure 13 D.

#### ***4.4.3 RA-suppressed expression of GLUT4 is mediated by the expression of RXR $\alpha$ proteins in 3T3-L1 cells***

To understand the molecular pathogenesis of obesity and specifically the role of RXR $\alpha$  in differentiation process of 3T3-L1 CAR $\Delta$ 1 cells, ad-RXR $\alpha$  was used to transfect 3T3-L1 CAR $\Delta$ 1 cells and analyzed the expression levels of GLUT4 protein. When RXR $\alpha$  was overexpressed in 3T3-L1 CAR $\Delta$ 1 cells, it attenuated the inhibitory effects of RA on GLUT4 expression. GLUT4 protein

level remains constant in the presence of RA. This result is totally new. It shows that the RA-suppressed expression of GLUT4 probably is mediated by the expression of RXR $\alpha$  proteins in 3T3-L1 cells. Although it is acknowledged that total GLUT4 protein expression does not reflect the member-bound GLUT 4 which is considered the functional form to transport glucose into adipocytes, the underlying mechanism about the change of total GLUT4 protein expression still deserves to be further investigated.

#### ***4.4.4 RA-suppressed expression of GLUT4 is enhanced by the expression of COUPTFII proteins in 3T3-L1 cells***

To understand the role of COUPTFII in differentiation process of 3T3-L1 CAR $\Delta$ 1 cells, ad-COUPTFII was used to overexpression it in 3T3-L1 CAR $\Delta$ 1 cells. Our results showed a reduction of GLUT4 expression, which is similar to what has been observed by Evan Rosen. Their group also reported that the GLUT4 protein level in differentiating adipocyte significantly decreased when COUPTFII was overexpressed.<sup>86</sup> Interestingly, different from RXR $\alpha$ , overexpression of COUPTFII seems to facilitate the inhibitory effects of RA on the expression of GLUT4. The inhibitory effects of RA on the expression of GLUT4 was enhanced as a result. These results seem to indicate that the relative amounts of RXR $\alpha$  and COUP-TFII in 3T3-L1 CAR $\Delta$ 1 cells probably play an important role in mediating the RA effects on GLUT4 expression. Whether this is the case remains to be investigated.

### **4.5 Conclusion:**

Here, we found that the overexpression of RXR $\alpha$  or COUP-TFII inhibited lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells, RA-mediated inhibition of lipid accumulation in differentiating 3T3-L1 CAR $\Delta$ 1 cells may be facilitated by overexpression of RXR $\alpha$ , but not COUP-TFII. Interestingly, the RA-inhibited GLUT4 expression is attenuated by overexpression of RXR $\alpha$  and potentiated by overexpression of COUP-TFII. The findings shown here will help us to understand the roles of nutrients and hormones in the control of functions of adipocytes, and in turn, the pathogenesis of obesity.

## CHAPTER FIVE

### CONCLUSIONS AND FUTURE DIRECTIONS

#### 5.1 Conclusions

Skeletal muscle and adipose tissues play key roles in lipid and glucose metabolism. These are achieved through their responses to nutritional and hormonal stimulation. We demonstrated in this thesis that VA and insulin may work together to regulate macronutrient metabolism in cells. As a metabolite of VA and the main mediator of its actions, RA regulates transcription of genes. We aimed to find the effects of RA on the GLUT4 expression levels in L6 muscle cells and 3T3-L1 CAR $\Delta$ 1 adipocytes with or without insulin. Understanding the mechanism by which how RA influences macronutrient metabolism helps us to better control obesity and type 2 diabetes. This thesis contains two parts. Studies in the first part were conducted in L6 myocytes, which is a good model to study glucose metabolism and differentiation process in skeletal muscle. Studies in the second part were conducted in 3T3-L1 CAR $\Delta$ 1 cells, which is a good model to study adipocyte metabolism and preadipocyte differentiation. Compared with the parental 3T3-L1 cells, 3T3-L1 CAR  $\Delta$ 1 cells allow the transduction of genetic materials through the use of recombinant adenoviruses, which is an important feature for us to study the functions of transcription factors affecting RA signaling.

We showed here that both RA and insulin inhibit the expression levels of GLUT4 in L6 cells after 4 and 6 days of treatment. Interestingly, the synergy effects between RA and insulin can be seen at Day 4 with RA at 1  $\mu$ M. In the differentiating 3T3-L1 CAR $\Delta$ 1 cells, RA treatment leads to the decrease of lipid accumulation in 3T3-L1 CAR $\Delta$ 1 cells during the cell differentiation in this study. Additionally, overexpression of RXR $\alpha$  enhanced the inhibitory effects of RA on lipid accumulation in RA 0.3  $\mu$ M group. RA treatment reduced the expression levels of GLUT4 and RXR $\alpha$  in differentiating 3T3-L1 CAR $\Delta$ 1 cells.

Overexpression of RXR $\alpha$  attenuated the RA-inhibited GLUT4 expression. Other



the other hand, the inhibitory effects of RA on GLUT4 expression can be enhanced with COUPTFII overexpression.

We conclude that GLUT4 expression in both L6 and 3T3-L1 CAR $\Delta$ 1 cells can be reduced in the presence of RA. In addition, RA synergizes with insulin to reduce GLUT4 expression in L6 cells. RA-inhibition of GLUT4 expression in 3T3-L1 CAR $\Delta$ 1 cells may be mediated by the reduction of RXR $\alpha$  expression by RA.

## 5.2 Future Directions

We hope that our finding about the synergistic effects of RA and insulin on GLUT4 expression in L6 cells will bring attention of clinicians regarding the potential effects of VA supplement in patients with obesity and diabetes. Insulin resistance is commonly found in patients with obesity and diabetes . There is a possibility that the insulin in plasma level is significantly high. If the patients with diabetes or obesity intake VA supplements, their insulin in plasma and VA may have a synergistic effect to inhibit the GLUT4 protein level in skeletal muscle which is not good for the entry of glucose into the tissue.

Previous results from the lab showed that glucose usage of differentiated skeletal muscle cells treated with RA and insulin for 4 days increase significantly.<sup>81</sup> The decrease protein level of GLUT4 cannot explain the increase of glucose usage of L6 cells. The reason may be that GLUT1 and GLUT3 were also expressed in skeletal muscle. It was found GLUT1 decreased in L6 cells treated the same way. Whereas, he has observed the induction of GLUT3 and GLUT6 expression levels after RA and insulin treatments. I have not measured the expression levels of these GLUTs. We hypothesize glucose probably gets into the cells through other GLUTs since the L6 cells treated with RA and insulin have the increase of glucose usage. In the future, we plan to study the GLUT1, GLUT3 and GLUT6 expression in L6 cells. However, GLUT4 is not the only type of GLUTs expressed in skeletal muscle.

The GLUT4 expression is inhibited by RA in 3T3-L1 CAR $\Delta$ 1 cells. We have not examine whether there is a synergy of RA and insulin in the regulation

of GLUT4 expression in adipocytes. Future experiment to determine what the role of insulin is in the RA-inhabited GLUT4 expression are needed.

In addition, we also demonstrated that 3T3-L1 CAR $\Delta$ 1 cells is an effective cell line to test the roles of RXR $\alpha$  and COUP-TFII in GLUT4 expression. These cells will also be used to investigate the functions of other transcription factors to mediate insulin and RA responses in adipocytes.

### **5.3 Summary**

The data presented here in my study help us to understand the influence of RA in expression levels of proteins for glucose metabolism in both skeletal muscle and adipocytes. The synergistic effect of RA and insulin on GLUT4 expression suggests that the use of VA supplements in people with diabetes or obesity should be cautious. The results of GLUT4 expression and RXR $\alpha$  overexpression in 3T3-L1 CAR $\Delta$ 1 cells demonstrate the potential to use this cell line for the study of regulation of gene expression in adipocytes. In summary, the work presented here lays the foundation for understanding the glucose metabolism and pathology of chronic disease like diabetes or obesity in the body.

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