Targeting cellular dysfunction with green tea polyphenols to treat the underlying basis of the metabolic complications of obesity

A thesis submitted for the Degree of Doctor of Philosophy

by

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Certificate of Original Authorship

I, Wei Guo Lao declare that this thesis, is submitted in fulfilment of the requirements for the award of Degree of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution. This research is supported by UTS Research Excellence Scholarship (RES) commencing Autumn 2013. This scholarship comprises of an Australian Postgraduate Award (APA) Scholarship funded by the Australian Government.

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Abstract

Obesity is a metabolic disease with high risk of severe complications, including type 2 diabetes, cardiovascular disease, and cancer conditions. To develop natural approaches to obesity-associated metabolic complications a series of *in vitro* studies were conducted to evaluate effects of green tea polyphenols (GTP) on cellular dysfunction related to the development of obesity and its complications.

Increased differentiation of adipocytes from preadipocytes contributes to fat mass expansion and leads to obesity and type 2 diabetes. The effects of GTP on fat cell differentiation and lipids accumulation were investigated in Chapter 3 using 3T3-L1 preadipocytes. The results demonstrated that GTP suppressed the differentiation of 3T3-L1 preadipocytes into mature adipocytes through down-regulating adipogenic regulators of PPARγ, C/EBPα, and SREBP-1c at gene expression and post-transcriptional level. The findings from this study suggest that GTP can combat unnecessary adipogenesis at the cellular level to prevent obesity and subsequently reduce the risk of type 2 diabetes and cardiovascular disease.

Accumulating evidence demonstrated an increase of adipogenesis accompanied by osteoporosis in obese populations. Adipocytes and osteoblasts originate from a common ancestor, pluripotent mesenchymal stem cells. Based on the anti-adipogenic effect of GTP, the study in Chapter 4 investigated whether GTP possesses the ability to limit human adipose tissue-derived stem cells (hADSCs) differentiation to adipogenic lineage and concomitantly enhance osteogenesis. The study utilising hADSCs and PPARγ agonist identified PPARγ and Runx2 as essential regulators involved in adipogenesis and osteogenesis, respectively. GTP treatment inhibited ADSCs differentiation to mature adipocytes and promoted osteogenesis through suppressing the PPARγ-induced adipogenesis and upregulating Runx2-Bmp2

mediated osteogenic pathway. Therefore, the enhancement of osteogenesis with preventing adipogenesis with GTP may provide a rational approach towards developing GTP as multifaceted therapeutic goods for the intervention of obesity-associated osteoporosis.

Polycystic Ovarian Syndrome (PCOS) is a complex of the metabolic and reproductive disorder. Obesity associated with insulin resistance plays an important role in excessive androgenesis by ovarian theca cells. Based on our observations that GTP is capable of enhancing insulin-mediated glucose and lipid metabolism with anti-obese effect study in Chapter 5 investigated whether GTP would be able to alternate ovarian cell dysfunction led abnormal steroidogenesis using dexamethasone-induced hyperandrogenism in primary thecal cells from female mice. GTP treatment inhibited over-secretion of testosterone in theca cells through downregulation of the expression of the cytochrome P450 17a-hydroxylase (CYP17A1) additionally reduced CYP11A1 expression at the high concentration (25 µg/mL). These findings provide scientific evidence to support the use of GTP for ovarian dysfunction leading to hyperandrogenism in PCOS.

DNA methylation is essential for healthy development and is also involved in aging and carcinogenesis. The recent study using genome-wide DNA methylation analysis has identified hypermethylation as a potential epigenetic change of obesity-related cancer. Based on the antiadipogenic effect of GTP during ADSCs differentiation, the study in Chapter 6 evaluated the effects of GTP on DNA methylation in deferent passages of ADSCs. This study discovered GTP enhanced DNA methyltransferase 1 (biomarker of DNA methylation) expression without changing MYC expression at early stage of ADSCs passages and downregulated DNMT1 expression at passage 12 of ADSCs culture. These findings suggest GTP possesses dual regulatory effects on DNA methylation during ADSCs expansion with genomic stability, which highlights the potential use of this naturally occurring compound in

ADCSs expansion for regenerative medicine without carcinogenic risk.

In summary, *in vitro* studies conducted in this thesis have demonstrated beneficial effects of and molecular mechanisms of GTP on obesity and obesity-associated osteoporosis and PCOS. To gain deeper insight into therapeutic application of GTP for obesity and its metabolic complications, the animal studies and clinical trials are warranted in the future study.

Publications and communications

Refereed publications arising from this thesis

Lao WG., Jin X., Tan Y., Xiao L., Padula M. P., Bishop D.P., Reedy B., Ong M., Kamal M.A. and Qu X., Characterisation of Bone Beneficial Components from Australian Wallaby Bone, Medicines 2016, 3(3), 23; doi:10.3390/medicines3030023

Lao WG., Tan Y., Jin X., Xiao L., Kim JK. and Qu X., Comparison of Cytotoxicity and the Anti-Adipogenic Effect of Green Tea Polyphenols with Epigallocatechin-3-Gallate in 3T3-L1 Preadipocytes, Am. J. Chin. Med. 43, 1177 (2015). https://doi.org/10.1142/S0192415X15500676

Tan Y., Jin X., Lao WG., Kim JK., Xiao L. and Qu X., Anti-Resistin RNA Oligonucleotide Ameliorates Diet-Induced Non-Alcoholic Fatty Liver Disease in Mice through Attenuating Proinflammatory Cytokines, BioMed Research International, Volume 2015 (2015), Article ID 414860, 13 pages

Tan Y., Lao WG., Xiao L., Wang ZZ., Xiao W., Kamal M.A., Seale J.P. and Qu X., Managing the Combination of Nonalcoholic Fatty Liver Disease and Metabolic Syndrome with Chinese Herbal Extracts in High-Fat-Diet Fed Rats, Evidence-Based Complementary and Alternative Medicine, Volume 2013 (2013), Article ID 306738, 10 pages, http://dx.doi.org/10.1155/2013/306738

Xiao L., Lao WG., Tan Y. and Qu X., *In vitro* Investigation of Anti-diabetic Effect of Taxus cuspidate Extracts by Ultrasound Assisted Method. Am.J Chin Med. 2012, 40:1-11.

Papers presented at scientific conferences

Lao WG., Tan Y., Jin X., Xiao L., Kim JK. and Qu X., Green tea polyphenols inhibit adipogenesis in 3T3-L1 preadipocytes through regulating expressions of PPARγ, C/EBPα and SREBP-1c, New Horizons 2014 Conference, The Kolling Building, RNSH, Sydney, Australia, 17th - 19th November 2014, NH2014-8745

Lao WG., Ong M., Jin X. and Qu X., The effects of Green Tea Polyphenols on ovarian steroidogenesis in ovarian cells, The New Horizon 2016 Conference,33rd Combined Health Science Conference, City - Haymarket, CB08 Dr Chau Chak Wing Building, Building 8, UTS, Sydney, Australia, 21-22 November, 2016, NH2016-105317

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List of Abbreviations

5mC   5-methylcytosine

AMM   adipocyte maintenance medium

ABS   Australian Bureau of Statistics

ADA   American Diabetes Association

ADSCs   Adipose-derived stem cells

ALP   Alkaline phosphatase

AS   Atherosclerosis

BFR   The Body Fat Ratio

BGP   Osteocalcin

BMD   Bone mineral density

BMI   Body Mass Index

Bmp2   Bone morphogenetic protein 2

NBS   Bovine Calf Serum

CVC   Cardiovascular complications

CVD   Cardiovascular Disease

C/EBP   CCAAT/enhancer-binding protein-a

CHD   Coronary heart disease

CYP11A   Cytochrome P450 family 11 subfamily A

CYP17A   Cytochrome P450 family 17 subfamily A

DEX   Dexamethasone

DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyl-transferase
EGCG	(−)-epigallocatechin-3-gallate
EM	Expansion Medium
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFAs	Free fatty acids
GLUT-4	Glucose transporter 4
GT	Green tea
GTE	Green tea extract
GTP	Green tea polyphenol
HDACs	Histone deacetylase
IBMX	Isobutyl-1-methyl-xanthine
IDF	International Diabetes Federation
IOF	International Osteoporosis Foundation
IR	Insulin resistance
IRS-1	Insulin receptor 1
IL-6	Interleukin- 6
LC-MS	liquid chromatography-mass spectrometry
MHO	Metabolically healthy obesity
MS	Metabolic Syndrome
NAFLD	Non-alcoholic fatty liver disease
NIDDM	Noninsulin-dependent diabetes mellitus

NWO	Normal weight obesity
OECD	Organization for Economic Co-operation and Development
PAI-1	Plasminogen activator inhibitor 1
PCOS	Polycystic ovary syndrome
PPARγ	Peroxisome proliferator-activated receptor γ
PPRE	PPARγ response element
PPC	Polyphenols and L-carnitine
qRT-PCR	Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction
RunX2	Runt-related transcription factor 2
RXR	Retinoid X receptors
SAS	Sleep apnoea syndrome
SAM	S-adenosylmethionine
SREBP-1c	Sterol regulatory element-binding protein 1c
TGF-β1	Transforming growth factor beta 1
TNFα	Tumor necrosis factor
TZD	Thiazolidinedione compounds
T2D	Type 2 diabetes
WHF	World Heart Federation
WHR	Waist-to-hip ratio
WHO	World Health Organization
TCM	Traditional Chinese medicine

Chapter One

General Introduction

1.1 Obesity and health burden

1.1.1 The epidemiological status of Obesity

Obesity is the accumulation of excess body fat to the extent that it affects the general health of the individual (Keenan *et al.,* 2013). The global proportion of overweight and obese adults rose by 27.5% between 1980 and 2013, while that of overweight and obese children increased by 47.1%. During this period, the international population of overweight and obesity increased from 921 million in 1980 to 2.1 billion in 2013 (Lancet, 2014). Men and women living in developing or developed countries have different age patterns for obesity. In developed countries, the proportion of overweight and obesity in adult men is generally higher than that of women (Lau *et al*., 2007). The peak age of obesity is 55 years old, the proportion of overweight is over 33%, and the proportion of obesity is around 25%. Contrarily, in developing countries, the proportion of overweight and obese adult women is higher than men. And peak age of onset is 60 years old, by over 32.2% overweight and 31.3% obese (OECD 2017).

The World Health Organization (WHO) has reported a more severe global obesity condition. At least one-third of adults in the world are overweight and almost one-tenth of them are physically obese. In addition, 40 million children under five years of age who are overweight or physically obese suffer serious health consequences (WHO 2007). The U.S. and Australian governments also report similar trends.

A survey study from the Australian Government and the Organisation for Economic Co-operation and Development (OECD) reported that the number of obese children in Australia is close to 25% and that of adults is up to 63% (OECD, 2014). This means that the obesity level in Australia is comparable to that of in the United States, and only

slightly lower than that in New Zealand. The overweight rate in Australia is 65%, and the obesity rate in Australia is 30% (Australian Bureau of Statistics 2008). In 2000, the latter proportion was only 19.8%. Among them, one-quarter of Australians over 15 are overweight or obese. In the 2017 Health at a Glance pointed out that the issue of obesity is urgent and requires immediate attention (OECD, 2014).

An additional OECD analysis（OECD, 2017）estimated the direct and indirect medical and nonmedical cost of obesity patients in Australia is around $4708 per person in 2016, accumulatively exceeding 9.6% of Australian GDP.

In addition to the financial burden obesity places on society, it also has an alarming prevalence. Another OECD health report (OECD, 2017) found that obesity is the main cause of the onset of diabetes and cardiovascular diseases and that the government needs to adopt stronger policies to reverse this trend. This does not only apply to Australia, but also for many other countries around the world, both developed and developing.

In fact, it is expected that this number of people suffering from obesity to increase in both economically developed and developing countries, which creates a major health challenge in Australia and even the world. It is also a major risk factor for the spread of chronic diseases.

1.1.2 Health burden of Obesity

A global open report titled " Overcoming obesity: an initial economic analysis " presented by the McKinsey Global Institute in 2014 reported 14 major economic, political, social, and military issues caused by human development, then analyzed and evaluated the impact of these issues on human social development in the future.

Remarkably, obesity considered to be amongst the top three social burdens in the world, second only to smoking, armed violence, war, and terrorism (McKinsey Global Institute 2014).

In fact, obesity is currently considered to be a chronic metabolic disease affecting adults and children around the world and has become one of the leading causes of death (Barness *et al.,* 2007, The GBD 2013 Mortality and Causes of Death 2014).

Two studies related to weight and mortality are worth nothing for this allegation. The first is a prospective study from the Global BMI Mortality Collaboration in 2016, with 10.6 million participants in 32 countries over a time period of 13.7 years (Angelantonio *et al.,* 2016). The results suggested that there was a direct relationship between the mortality rate and the increase in the degree of overweight or obesity. The second one is a retrospective study that analyzed the results of 228 studies involving multiple diseases, which found that patients who were not overweight or obese had longer follow-up times. In other words, people who were not overweight or obese lived longer than those who were, regardless of the disease they suffered from.

In 2015, high body mass index contributed to 4.0 million deaths globally which represented 7.1% of deaths from any cause, since obesity is known to be the main risk factor for a number of non-communicable diseases, including *MS*, non-alcoholic fatty liver, PCOS, type 2 diabetes, ischemic cardiovascular disease, osteoporosis, cancer and certain types of cancer (Sharma *et al.,* 2015).

Although it is not yet clear that overweight and obesity must cause a health problem, it has been ascertained that the increase of an individual's weight accompanies that of the possibility of health problems, as well as the risk of developing obesity-related diseases in the overweight and obese. This can result in long-term pain, and even death, for

individuals and families (OECD 2013).

Therefore, body weight should be actively controlled and since the increase in the incidence of morbidity and mortality due to obesity and obesity-related diseases, the American Medical Association and several international medical organizations, including the World Obesity Federation, have made the decision to formally recognize obesity as a disease.

1.2 Definition and diagnostic criteria of Obesity

1.2.1 Definition of Obesity

Obesity refers to the physical state that causes excessive accumulation of body fat and has a negative impact on health. It may lead to reduced life expectancy and various health problems (Haslam *et al.*, 2005, WHO, 2000, OECD 2017). Fat was considered to be the tissue for storing energy in the past, but recent studies have found that fat cells also secrete leptin, adiponectin, resistin, inflammation-associated interleukins and many other substances that regulate physiological functions. When the ratio is out of balance, the body's metabolic functions becomes irregular and can be considered to be a systemic disease (Heilbronn 2008, Kadowaki *et al*., 2005).

1.2.2 Diagnostic criteria of Obesity

a. The Body Mass Index (BMI)

The current standard of obesity is often measured using the Body Mass Index (BMI). A

BMI of 18.5 to 24.9 is normal, 25 to 29.9 is overweight, 30 to 35 is a first-class obesity, 35 to 40 is second-class obesity, and 40 is extremely obese (The Global BMI Mortality Collaboration 2016).

b. The Waist-Hip Ratio (WHR)

Waist-to-hip ratio (WHR) is also a standard used to determine obesity. It refers to the ratio of waist circumference to hip circumference. According to the definition of WHO, men who are more than 0.9m or women who are more than 0.85m are obese (WHO 2000). Some studies have pointed out that WHR can predict cardiovascular disease risk more accurately than BMI (Mørkedal *et al.*, 2011). However, if WHR was used to define obesity instead of BMI, the global population of those classified as having a high risk of heart disease would be increased by threefold (Yufuf *et al.*, 2005).

c. The Body Fat Ratio (BFR)

Body fat percentage, also called body fat ratio (BFR) is the percentage of fat content in total body weight. Male body fat greater than 25% or female body fat greater than 33% is considered obese (Gallagher *et al.*, 2000). Since the direct measurement of BFR has a certain degree of difficulty, there are too many artificial interference factors, and the accuracy is difficult to be unified, it is not recommended by some organizations as a viable replacement for BMI.

In addition, healthy weight in children has different criteria based on gender and age. This standard needs to be determined by comparison with other children of the same race, age, and gender. In general, obesity in children and adolescents is defined as 95% greater than the same class of samples with BMI (Center for disease control and prevention 2009).

1.3. The metabolic complications of obesity

Obesity is a breeding ground for many diseases. Diseases caused by obesity can be described throughout the body in various systems. These include Metabolic Syndrome (MS), Non-Alcoholic Fatty Liver Disease (NAFLD), Polycystic Ovarian Syndrome (PCOS), Type 2 Diabetes (T2D), Cardiovascular Disease (CVD), osteoporosis, and cancer, especially include metabolic diseases such as diabetes, osteoporosis, PCOS and others closely related to obesity (Haslam *et al.*, 2005), which will be further studied in this research project.

The development of these complications and obesity appear to be interactive. If the patient's weight is reduced by 5-10%, the symptoms of their complications can be better controlled, which indicates that the improvement of complications is proportional to weight loss (American Diabetes Association (ADA) 2007).

1.3.1 Obesity and Metabolic Syndrome (MS)

a. The definition and diagnostic criteria of MS

Metabolic syndrome (MS) is not only a group of clinical syndromes with obesity, hyperglycemia (diabetes or impaired sugar canker), dyslipidemia (high TG hyperlipidemia and/or low HDL-C hyperlipidemia), and high blood pressure, which affect the body's health, but also a combination of metabolically related risk factors that directly contribute to the increase in atherosclerotic cardiovascular disease, type 2 diabetes (T2D), and all-cause mortality. Its components also include chronic inflammatory reactions, hyperuricemia, and hypercoagulability.

It is currently believed that obesity, especially abdominal obesity (central obesity) and insulin resistance, are central parts of the MS and are closely related to its occurrence.

From 2006, the International Diabetes Federation (IDF) made relevant adjustments to the diagnostic criteria for MS according to the opinions of WHO and several international health organizations. Abdominal obesity was listed as the focus of diagnosis in the diagnostic criteria of IDF (IDF 2012). The MS is central obesity in addition to any two of the following:

*Raised triglycerides: > 150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality; *Reduced HDL cholesterol: < 40 mg/dL (1.03 mmol/L) in males, < 50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality; *Raised blood pressure (BP): systolic BP > 130 or diastolic BP >85 mm Hg, or treatment of previously diagnosed hypertension; *Raised fasting plasma glucose (FPG): >100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes.

b. The mechanism of MS

There are many pathophysiologic causes of MS. Amongst these, it is currently believed that obesity, especially central obesity, induces insulin resistance and hyperinsulinemia, and is the etiology and pathogenesis of MS (Alan *et al*., 2017).

Abdominal obesity

Abdominal obesity, also known as central obesity, occurs when excessive abdominal fat around the stomach and abdomen has built up to the extent that it can have a negative impact on health. This is measured by the waist circumference.

Hypertrophy and hyperplasia of adipocytes result in obesity. The progressive increase in obesity and adipocytes results in ischemic and hypoxic adipose tissue, further leading to the necrosis of adipocytes, macrophage infiltration, and adipocytokines - including free fatty acids (FFAs), glycerol, inflammatory mediators (TNFα, leukocytosis), and a large number of plasminogen activator inhibitor 1 and C-reactive protein release - mediate a variety of reaction processes involving insulin sensitivity, oxidative stress, energy metabolism, coagulation function, and promote atherosclerosis and thrombosis form.

Assessment of obesity using BMI has many limitations because it does not reflect fat distribution and metabolic characteristics. Waist circumference can be a good indicator of local fat accumulation and has a strong ability to predict diabetes and cardiovascular disease. Studies have found that when waist circumference increased by 1cm and WHR increased by 0.01, the risk of cardiovascular disease increased by 2% and 5%, respectively (Rutherford *et al*., 2010, Luksiene *et al*., 2015).

In a strict sense, obesity is not the same as metabolic abnormalities. There are subpopulations of metabolically healthy obesity (MHO) in obese populations, and subpopulations of abnormal metabolic components are also present in normal weight obesity (NWO) populations. 20% to 30% of obese people belong to the MHO population, and the definition of MHO is BMI $\geq$ 30 kg/m (Van Vliet-Ostaptchouk *et al*., 2014). NWO is defined as the population with normal body mass and BMI (BMI < 25 kg/m2), but with an increased body fat percentage, the NWO population is a high-risk group of MS, with a prevalence rate four times higher than that of the population with normal BMI and body fat ratio. It is also a high-risk group for cardiovascular disease and type 2 diabetes.

MHO was considered to be one of the main reasons for the maintenance of normal metabolic capacity because of the low accumulation of peripheral fat in the liver when

compared with obese patients with metabolic abnormalities. However, a recent meta-analysis (Van Vliet-Ostaptchouk *et al*., 2014) showed that although there was no significant increase in the risk of cardiovascular events and related mortality in the MHO population, there was still an increasing trend suggesting that even the MHO population had an increased subclinical cardiovascular burden.

Insulin resistance (IR)

Insulin resistance (IR) is a pathological condition in which cells fail to respond normally to the hormone insulin, and the physiological effects of uptake and utilization of glucose by peripheral target tissues (fat, muscle, liver) are significantly attenuated (De Souza *et al*., 2001, Boden 2011, Wensveen *et al.,* 2015).

It is believed that Free fatty acids (FFAs) secreted by adipocytes, tumor necrosis factor (TNFα) secreted by inflammation, leptin, resistin, and adiponectin are closely related to IR mechanisms. Among them, FFAs and TNFα are two of the important mechanisms of obesity-induced insulin resistance (IR) (Chiu *et al*., 2007).

FFAs: Due to the body's self-stabilizing effect, the increased adipose tissue tends to be more metabolically degraded after the increase of adipose tissue, resulting in further increase of FFAs. High FFAs hyperlipidemia is an important pathogenic factor for obesity-induced IR. Under the stimulation of obesity, excess free fatty acids released by lipolytic activity increase the expression of SREBP-1c and PPARγ, thereby also increasing the accumulation of lipids. Meanwhile, elevated plasma FFAs level increases hepatic gluconeogenesis, promotes basal insulin secretion, and reduces hepatic clearance ability for insulin, resulting in hyperinsulinemia and decreasing adipose tissue glycogen synthase activity (Kadowaki *et al.,* 2007, Guilherme *et al*., 2008, Boden 2011).

The expression of insulin receptor 1 (IRS-1) and glucose transporter 4 (GLUT-4) are then downregulated, resulting in mobilization of FFAs into the circulation and uptake into skeletal muscle. This not only leads to excess body fat but also decreases GLUT4 transference from the cytoplasm to the cell membrane, which in turn reduces the body's sensitivity to insulin (Yan *et al*., 2016) and triggering IR.

At the same time, the phosphorylation of insulin receptor β subunit and IRS-1 lowers under a high FFAs environment. This suggests that FFAs inhibits the activity of insulin receptor tyrosine kinase and IRS-1 while also promoting IR (Guilherme *et al*., 2008, Cohenet *et al*., 2011).

Inflammation activity: The progressive increase of obesity and adipocytes can induce inflammation and oxidative stress, increase adipocyte production, up-regulate mRNA expression of PPARγ, C/EBPα, and transcription factors closely related to lipid synthesis (SREBP-1c), which causes hypertrophy and hyperplasia of adipocytes. This results in ischemic and hypoxic adipose tissue, further leading to necrosis of adipocytes, macrophage infiltration. Furthermore, adipocytokines including FFAs, glycerol, and inflammatory mediators (TNFα, IL-6), a large number of plasminogen activator inhibitor 1 (PAI-1) and the release of C-reactive protein (CRP) mediate a variety of reaction processes involving insulin sensitivity, oxidative stress, energy metabolism, coagulation function, and promote atherosclerosis and thrombosis form (Lee *et al*., 2014, Abd El-Haleim *et al*., 2016).

TNFα plays a key role among those regular factors. The effect of TNFα on insulin signaling pathways is such that it mediates serine phosphorylation of the insulin receptor substrate and makes it an insulin receptor tyrosine kinase, thus inhibiting the tyrosine phosphorylation of IRS-1, leading to IR (Alan *et al*., 2017).

At the same time, high concentrations of TNFα inhibit insulin-stimulated glucose transport by inhibiting the mRNA expression of GLUT4, and high concentrations of TNFα can, in turn, promote the breakdown of adipocytes, release large amounts of FFAs, and aggravate the occurrence of IR (Wensveen *et al*., 2015, Esser *et al*., 2014).

1.3.2 Obesity and Non-Alcoholic Fatty Liver Disease (NAFLD)

a. The definition and diagnostic criteria of NAFLD

Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic steatohepatitis, non-alcoholic steatohepatitis. Cirrhosis, a group of chronic non-alcoholic steatohepatitis, and any possible liver damage could be the first cause of NAFLD (Rinella 2015). The incidence of MS and obesity, especially the latter, is positively correlated with the prevalence of worldwide NAFLD (Esser *et al*., 2014).

NAFLD is associated with metabolic diseases such as obesity, diabetes, and hyperlipidemia. The diagnostic criteria of American College of Gastroenterology and American Gastroenterological Association therefore listed obesity as part of the criteria for NAFLD, in addition to the following (Chalasani *et al*., 2012): Evidence of hepatic steatosis in imagery or histology, and no other factors contributing to the accumulation of hepatic fat, including excess alcohol, drugs, and genetic diseases.

NAFLD mainly involves the pathogenesis aspects in the following three theories: lack of HDL function, dysregulation of the intestinal bacterial population, and insulin resistance and "second strike" doctrine. After the in-depth exploration of the occurrence and pathogenesis between NAFLD and obesity, it is speculated that there may be a common pathogenesis in both (Rinella 2015).

b. The mechanism of Non-alcoholic fatty liver disease (NAFLD)

Insufficient function of High-Density Lipoprotein (HDL)

High-Density Lipoprotein (HDL) is a macromolecular complex with a density of 1.063~1.210 g/mL. The composition includes 45% to 55% of apolipoproteins (mainly apoA-I apoA-II), 26% to 32% of phospholipids, 15% to 20% Esterified cholesterol, 3% to 5% free cholesterol and less than 5% triacylglycerol (CDC 2017).

HDL synthesis mainly occurs in the liver, and the macromolecules involved in the physiological processes of HDL mainly include ATP binding transporter A1 (ABCA1) which is also known as cholesterol efflux regulator protein since helps with cholesterol outflow from extrahepatic tissue; lecithin-cholesterol acyltransferase (LCAT); cholesteryl ester transfer protein (CETP); phospholipid transporter; apoA-I and scavenger receptor SR-BI (Zvintzou *et al.*, 2014, CDC 2017).

Based on the role of HDL in lipid metabolism, it has been inferred that there are common points in the pathogenesis of obesity and NAFLD. The HDL analysis of patients after weight-loss surgery found that not only did the body mass rapidly decline, but the patient's predominant component was also changed from ApoE and ApoC-HI to apoA-I in HDL apolipoprotein. Plasma LCAT and CETP activity were also observed to increase, suggesting that obesity may be associated with insufficient HDL function (Zvintzou *et al.*, 2014).

Intestinal bacterial population disorders

There are more than 2 000 kinds of bacteria living in the intestine of normal adults, and most of them colonize in the large intestine. The total mass amounts to over 1 kg. Genome analysis showed that there are several dominant bacteria in the endophyte:

Streptomyces, Bacteroides, Actinomyces, and metamorphosis. Studies in the investigation of NAFLD pathogenesis have found that intestinal dysbacteriosis was associated with the occurrence of various diseases such as obesity, autoimmune diseases, and NAFLD (Clemente *et al*., 2012). Animal experiments also found that both the intestinal flora and the number of Bacteroides were reduced in obese rats when compared to lean rats. Therefore, some scholars have referred to these bacteria that increase body mass as "obesity bacteria" (Turnbaugh *et al*., 2009).

It is generally believed that overgrowth of small bacterial colonies results in the destruction of the tight junctions between the intestinal endothelium cells, as well as increased intestinal endothelium permeability, intestinal enterotoxins, and cell breakdown products entering the blood circulation to activate the body's inflammatory response, possibly related to liver fat deposition (Turnbaugh *et al*., 2009, Ratziu *et al.*, 2010).

Insulin Resistance and "Second Strike" Doctrine

Adipose tissue is one of the insulin effector organs. Insulin stimulates the deposition of glycerol in adipocytes through stimulating the differentiation of preadipocytes into adipocytes, increasing the uptake of sugars and fatty acids in the blood circulation by up-regulating the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) and angiopoietin-like protein 2 (ANGPTL2), and inhibiting fat degradation. Furthermore, it increases the quality of adipose tissue and promotes further development of obesity by increasing the expression of sterol regulatory element-binding protein 1 (SREBP-1c) and PPARγ (Wu *et al*., 1999, Hsuy *et al*., 2006, Keuper *et al*., 2013).

Much evidence has showed that adipose tissue is also involved in insulin resistance and plays a regulatory role by secreting various adipocytokines and cytokines, such as

adiponectin, interleukin 18, IL-18, angiopoietin-like protein 2 (ANGPTL2), etc. (Gastaldelli *et al*., 2017, Stafeev *et al*., 2017), and up-regulating the expression of PPAR-γ and PPAR-α. The role of insulin resistance in relieving insulin level and regulating IL-18 is still unclear (Tan *et al*., 2010), though that of ANGPTL2 and PPAR-γ in promoting inflammation and insulin resistance is clear (Tabata *et al*., 2009).

Fatty decomposed products of fatty acids and their metabolites can interfere with the insulin signaling pathways mediated by MAPK, PKC NF-KB, etc., thereby affecting the normal physiological effects of insulin and leading to insulin resistance. Saturated fatty acids induce insulin resistance by increasing ceramide synthesis. In obese patients, increased free fatty acids in the blood circulation can enter the liver through the portal vein, thus increasing hepatic triglyceride synthesis and mediating insulin resistance (Lee *et al*., 2007, Anisha *et al*., 2013, Ratziu *et al*., 2010). Based on the conclusions of those researches, lipid deposition and insulin resistance are the first attack in the "second strike" pathogenesis theory in the NAFLD. Such as inflammatory factors, adipokines, and mitochondrial dysfunction could induce oxidative stress, which is "the second attack".

The pathogenic factors of NAFLD has become more complicated; the "two strikes" theory has gradually shifted to the "multiple strikes" doctrine, but a lot of scientists still advocate that insulin resistance is the first attack on the pathogenic mechanism of NAFLD (Polyzos *et al*., 2012), indicating that insulin resistance is the most important part of NAFLD in pathogenesis.

1.3.3. Obesity and Polycystic Ovarian Syndrome (PCOS)

a. The definition and diagnostic criteria of PCOS

Polycystic ovary syndrome (PCOS) is a common metabolic and reproductive disorder, characterized by obesity, insulin resistance and androgen (Sam 2007, Faghfoori *et al.*, 2017). Obesity is not only considered as a characteristic of PCOS but may also be one of the major causes of PCOS, as it has been showed that 30% to 75% of PCOS patients have excess body weight, even though the exact cause of PCOS remains unclear (Ehrmann 2005).

"Rotterdam 2003" is the current recommended international PCOS diagnostic criteria, which indicates the presence of PCOS if any two out of the provided three criteria are met in the absence of other entities that might affect the findings: oligo-ovulation and/or anovulation, excess androgen activity, polycystic ovaries diagnosed by gynecologic ultrasound (Chalasani *et al.*, 2017).

Obesity, especially abdominal type obesity, is a major cause of menstrual disorders in women. It is also a major factor in insulin resistance, affecting up to 70% of the PCOS population (Marshall *et al.*, 2012, Lim *et al.*, 2012). Hyperinsulinemia is caused by insulin resistance and leads to excess androgen, which in turn causes menstrual disorders in women. Since 1952, several studies have reported around 27% to 43% of PCOS patients as overweight or obese (Rogers *et al.*, 1952, Teede *et al.*, 2010, Faghfoori *et al.*, 2017), and a significant decline of PCOS symptoms after weight loss (Reinehr *et al.*, 2017).

Thus, overweight or obese women have a higher risk of developing PCOS compared to normal-weight individuals. Therefore, obesity may be part of the pathophysiological

mechanism in PCOS formation.

b. The mechanism of PCOS

Insulin resistance and compensatory hyperinsulinemia are now recognized as playing a large part in the pathogenesis of PCOS and stimulating ovarian androgen production (Zhang *et al*., 2000, Cadagan *et al*., 2016), and obesity is thought to be the trigger for that process (Legro *et al*., 2012). Insulin resistance in PCOS patients is mainly caused by the reduction of adiponectin resulting from obesity (Sam 2007, Faghfoori *et al*., 2017), which is an insulin sensitizer secreted by fat self-organization and directly related to androgen levels (Escobar-Morreale *et al*., 2006).

Obesity stimulates an increase in excess free fatty acids released by lipolytic activity and the expression of SREBP-1c and PPARγ, thereby increasing the accumulation of lipids and triggering insulin resistance (IR), which ultimately reduces the body's sensitivity to insulin (Yan *et al*., 2016). The expression of insulin receptor 1 (IRS-1) and glucose transporter 4 (GLUT-4) are then downregulated, resulting in an increased androgen secretion and disrupted insulin signaling (Qu *et al*., 2009). In order to maintain normal blood glucose levels, the body compensates for excess insulin in order to ensure the body's blood glucose intake and utilization, which results in hyperinsulinemia. The ensuing high insulin concentrations also have a strong promoting effect on androstenedione and testosterone. Insulin is generally believed to bind to IGF-1 receptors and act on theca cells. Therefore, it increases the availability of testosterone and insulin-like growth factors in target tissues and upregulates CYP11A and CPY7A expression and stimulates the secretion of androgen in the ovaries and adrenal glands (Guilherme *et al*.,2008), which facilitates the synthesis of androstenedione and testosterone

(Dadachanji *et al*., 2018).

Briefly, the starting point of PCOS mechanism is insulin resistance. Since hyperinsulinemia caused by insulin resistance leads to excess androgen, which conduces menstrual disorders in women and eventual PCOS, obesity is thought to trigger the entire process.

1.3.4. Obesity and Type 2 Diabetes

a. The definition and diagnostic criteria of Type 2 Diabetes

Diabetes, also called diabetes mellitus (DMs), is a metabolic disease characterized by long-term hyperglycemia and blood glucose levels above the standard value (WHO 2007). The symptoms of hyperglycemia include polyphagia, polydipsia, polyuria, and lower body weight.

Thus, the WHO definition for both type 1 and type 2 diabetes is a single raised glucose reading with symptoms or raised values on two occasions (WHO 2007): 1. Fasting plasma glucose $\geq$ 7.0 mmol/l (126 mg/dl); 2. Or with a glucose tolerance test, two hours after the oral dose a plasma glucose $\geq$ 11.1 mmol/l (200 mg/dl).

The theme of "World Diabetes Day 2004" is diabetes and obesity, both of which have been shown to be closely related. However, obesity is usually considered to be more closely related to type 2 diabetes, since type 2 diabetes is caused by lifestyle, and type 1 diabetes is often caused by autoimmune diseases or viral infections and is not related to lifestyle/obesity.

A higher incidence of obesity indicates a higher incidence of diabetes. IDF has reported

that Nauru had experienced a dramatic increase in living standards and cause up to 70%

obese population after the Second World War, and nearly half of the entire population

suffer from diabetes (IDF 2014).

Another study has also shown that the prevalence of diabetes in obese people is 4 times

higher than those with normal weight, and as much as 30 times higher in people with

severe obesity, which shows that obesity has a significant relationship with the incidence

of diabetes, particularly type 2 diabetes (The GBD 2013 Obesity Collaboration, 2014).

Among type 2 diabetes patients over 40 years old, about two-thirds were at least 10%

over the standard weight, which also indicates that obesity is closely related to diabetes.

Most scholars believe that obesity is one of the important causes of the type 2 diabetes,

and type 1 diabetes has nothing to do with obesity (Wu *et al*., 2014).

b. The mechanism of Type 2 Diabetes

Thus, type 2 diabetes is considered to be the most common **related** metabolic disease of

obesity, and impaired glucose tolerance is very common in obese people because of the

presence of insulin resistance. The exact pathogenesis of type 2 diabetes remains unclear.

Despite this, it is generally accepted that two characteristics are particularly important

for diabetes. The first is insulin resistance, where skeletal muscles lose their

responsiveness to insulin. The second is the failure of pancreatic β-cells to secrete the

required levels of insulin. Insulin resistance, therefore, seems to be the major cause of

diabetes, particularly type 2 diabetes（Mohammed *et al.* 2007）.

Insulin resistance means that cells cannot respond properly to normal concentrations of

insulin. As the previous discussion (Section 1.3.1), excess free fatty acids were released

by lipolytic activity under the stimulation of obesity, thereby increasing the expression of SREBP-1c, PPARγ, and adipose tissue accumulation. This can lead to a decrease in FFAs storage capacity of muscle cells and the uptake of glucose by adipocytes, enhancing the activity of TNFα, and then the muscles, liver, and adipose tissues ultimately cannot respond properly to normal concentrations of insulin (Wu *et al*., 1999, Hsuy *et al*., 2006, Keuper *et al*., 2013).

Another feature is that isolated beta cells cannot secrete the required level of insulin. It is generally thought to be due to leptin resistance and decreased adiponectin levels caused by obesity, increased triglyceride content in skeletal muscle cells by up-regulation of SREBP-1c and PPARγ expression, and then the excessive accumulation of cholesterol in β cells causes its function decline, which also leads to a decrease of insulin levels (Ewelina *et al.,* 2015, Abd El-Haleim *et al*., 2016).

c. Complication of Diabetes

Clinical trials have shown that diabetes is the most destructive possibility in several diseases associated with obesity (Helle *et al*. 2010). Obesity, especially abdominal obesity, is by far the most important factor in the development of diabetes, in addition to genetic background (Ginter *et al*. 2013).

As the previous discussion, at least two-thirds of diabetes patients were overweight or obese. Diabetes with obesity can increase the complications of diabetes including macroangiopathy (stroke, coronary artery disease), microangiopathy (retinopathy, nephropathy) and diabetic neuropathy (Frank *et al*. 2011). Among those, cardiovascular complications (CVC) are the most common causes of death in patients with diabetes

(Avramoglu *et al*. 2013).

Diabetes and obesity seem to be interactive, and clinicians advise that the symptoms of diabetes could be better controlled if obese diabetic patients lost 5-10% body weight, suggesting that improvement of diabetes is proportional to that of weight loss (ADA 2007). Initial treatment of obese diabetic patients begins with physical activity and nutritional intervention, with psychological support if necessary (Rorive *et al*. 2005).

1.3.5 Obesity and Cardiovascular Disease (CVD)

a. The definition of CVD

According to the definition from World Heart Federation (WHF), CVD refers to diseases related to the heart or blood vessels, also known as circulatory system diseases （WHF 2017）. Common cardiovascular diseases include coronary syndrome, stroke, hypertensive heart disease, rheumatic heart disease, aneurysm, myocardial disease, atrial fibrillation, congenital heart disease, endocarditis, peripheral arterial obstructive disease, etc.

Hypertension, Atherosclerosis (AS) and abnormal glucose metabolism (impaired glucose regulation or diabetes) are recognized risk factors and mechanisms for CVD. Studies found that insulin resistance caused by central obesity plays a central role in the occurrence of CVD （Gastaldelli *et al*., 2017）.

b. The mechanism of CVD

Obesity and hypertension of CVD

Most hypertensive patients are overweight or obese, and the prevalence of hypertension in the obese population is 6 times more than that of the normal population (Colosia *et al.*, 2013). Weight gain during teenage years often predicts the risk of developing high blood pressure in the future.

Each 10 kg increase in body weight can increase systolic blood pressure by 3 mmHg and diastolic blood pressure by 2.3 mmHg. This increase in blood pressure can, in turn, increase the risk of CVD by 12%, and the risk of stroke by 24 %(Xie *et al.*, 2012).

The results of the meta-analysis showed that the prevalence of hypertensive disease increased with BMI value in both males and females (Sharma *et al.*, 2015). The main mechanisms of obesity-induced hypertension include: (1) The direct influence of obesity on hemodynamic; (2) More insulin resistance, hyperinsulinemia, inflammation and oxidative stress make vascular endothelial cell dysfunction, sympathetic increased nerve tension, retention of sodium and water, and eventually leading to vasoconstriction, increased peripheral vascular resistance and blood pressure.

Obesity and Atherosclerosis (AS) of CVD

AS is the main pathological change of CVD. The formation of AS is the result of various metabolic risk factors such as abnormal glucose metabolism, hypertension, and dyslipidemia.

Obesity and its resulting insulin resistance are still considered to be the starting point of

atherosclerosis (Lovren *et al.*, 2015): impaired endothelial cell function, hypercoagulable state of blood, formation of terminal glycosylation products resulting from disorders of glucose metabolism, which cause macrophage phagocytosis, and then lead to LDL-C Oxidation Modification and deposition of vascular sub-endothelial cells. The eventual product of this process is lipid streaks and atheromatous plaques. Excess insulin as a specific smooth muscle cell growth factor can accelerate the occurrence of AS.

As the previous discussion (Section 1.3.1), obesity leads to insulin resistance, glucose and lipid metabolism disorders, which then causes hyperglycemia, hyperlipidemia and high levels of tumor necrosis factor alpha (TNFα). Hyperlipemia, leptin resistance, and inactive PPARγ system are also closely related to the formation of AS (Monnier *et al.*, 2013).

Obesity and abnormal glucose metabolism of CVD

Cardiovascular disease caused by abnormal glucose metabolism (impaired glucose regulation or diabetes), commonly known as diabetes cardiovascular disease, results in excessive blood sugar levels that will continue to affect the patient's cardiovascular health. A number of studies have found that people with diabetes have a two to four times higher risk of cardiovascular disease than the average person and that around a quarter of all diabetics eventually die of cardiovascular disease. Therefore, the medical community has advocated the control of sugar in recent years, and had to concern the patient's cardiovascular health at the same time.

The direct effect of obesity on the CVD

A clinical study found that the incidence of left ventricular hypertrophy in obese people is significantly higher than that of non-obese people. It is reported that about 50% of severe obese people have left ventricular hypertrophy, suggesting that obesity itself can cause changes in heart structure and function, which was a statistically significant correlation with body weight. This kind of effect is not only direct but can also be fatal （Movahed *et al.*, 2008）.

Obesity is positively correlated with the incidence of cardiovascular disease. The Framingham heart study showed that risk of heart failure in men increases by 5% for every increase in BMI, whilst that of women increases by 7%, and the more severe the degree of obesity, the higher the risk of heart failure. The study also showed that the yearly incidence of spontaneous sudden death in obese people is 40 times higher than that of those with average weight (Mahmood *et al.*, 2014).

A large number of studies (Gower *et al.*, 2002, Schenk *et al.*, 2009, Clamp *et al.*, 2017) have also confirmed that weight loss can increase the body's sensitivity to insulin, reduce insulin resistance, and improve cardiovascular risk factors such as blood pressure, blood glucose and blood lipids. In the British Diabetes Prospective Study, the diabetes-related mortality, all-cause mortality, and cardiovascular complication rate in the obese subgroup were significantly lower in the metformin-intensive treatment group than in the conventional treatment group.

1.3.6 Obesity associated with Osteoporosis

a. The definition and diagnosis of Osteoporosis

Osteoporosis, the most common reason for a broken bone, is a medical condition in which the bones become brittle and fragile with increased risk of bone fracture (WHO Scientific Group 2003). Typically, more than half of the osteoporotic population are directly or indirectly linked to the obesity epidemic (Zhao *et al*. 2007), while hormonal changes and deficiency in calcium or vitamin D form the secondary cause of osteoporosis (Christodoulou *et al*. 2003). These increase the likelihood of complications such as fractures, which most commonly occur in the spine, wrist, and hips, but can also affect the arm or pelvis.

Osteoporosis can be diagnosed using methods such as conventional radiography and by measuring the bone mineral density (BMD). The most popular method of measuring BMD is dual-energy X-ray absorptiometry, which is also considered the gold standard for osteoporotic diagnosis. Osteoporosis is diagnosed when bone mineral density is less than or equal to 2.5 standard deviations below a 30 to 40 years old healthy female reference population (Guglielmi *et al*., 2010). This reference standard value is described as T-score.

According to the International Osteoporosis Foundation (IOF), it is estimated that there are approximately 200 million osteoporotic patients worldwide, with a particularly high prevalence in females and the elderly (Cole *et al*. 2008). Australia not only has an aging population but also relatively high rates of vitamin D deficiency. Moreover, osteoporosis heavily contributes to hospitalization and related expenses, costing approximately $1.9 billion per annum (Cole *et al*. 2008). Osteoporosis and related fractures are therefore detrimental to physical and mental health and a financial burden on society.

b. The mechanism of Osteoporosis

Mechanical force and obesity

Mechanical force is one of the popular mechanisms used to explain the complex relationship between fat and bone mass. The greater the amount of fat, the greater the mechanical force on the bone, and an increase in fat also leads to an increase in body weight. In order to withstand these larger loads, the bone mass also increases in a vicious cycle (Baker *et al.* 2013). However, the amount of fat accounts for only a small part of the body mass, so this mechanism is not sufficient to explain the complex effects of fat mass on bone mass.

Fatty peptides

Adipose tissue is not only an inert organ for energy storage but also can express and secrete a variety of biologically active molecules, such as sex hormones, resistin, leptin, adiponectin, interleukin- 6 (IL-6) etc. These molecules affect the body's energy balance, and also participate in bone metabolism and help explain the complex relationship between fat mass and bone mass. The most important of these bioactive molecules are sex hormones (Kershaw *et al.*, 2004, Heilbronn *et al.*, 2008, Gastaldelli *et al.*, 2017).

Aromatase is usually present in gonadal tissues and adipocytes. Aromatase is an enzyme that converts androgens (such as testosterone) into estrogens. It is also used to synthesize estrogens. Higher amounts of adipose tissue in obese patients can increase aromatase activity, which in turn results in higher circulating estrogen levels. Extragonadal estrogen synthesis in adipose tissue is the major source of estrogen, especially for middle-aged and elderly patients. Therefore, as a producer of estrogen, adipocytes become an

important influencing factor on bone metabolism in the elderly (Reid 2002) and may be the cause of PCOS (Escobar-Morreale *et al.,* 2006). Estrogen can inhibit bone turnover by reducing bone resorption and stimulating osteoblast-mediated bone formation. Increased concentrations of estrogen in bone marrow mesenchymal stem cells can directly stimulate bone formation and inhibit adipocyte differentiation (Salamanna *et al*. 2015). Estrogen replacement therapy can also prevent fat accumulation and reduce the incidence of osteoporosis (Jia *et al*. 2015), and drugs that can block aromatase activity can also be used as an estrogen replacement therapy.

Differentiation of Bone Marrow Stem Cells

Adipocytes and osteoblasts originate from a common ancestor - pluripotent mesenchymal stem cells (Horowitz *et al.* 2004). Their tendency is the same in the process of differentiation of stem cells into adipocytes or osteoblasts, except that the balance of their differentiation is regulated by several interacting pathways.

It is currently believed that the differentiation of bone and adipose tissue in the bone marrow supports a negative correlation between fat mass and bone mass. Such as PPARγ is conducive to the activation of bone marrow mesenchymal cells into adipocytes instead of osteoblasts (Hasegawa *et al.* 2008). However, the activity of Transforming growth factor beta 1 (TGF-β1), Runt-related transcription factor 2 (RunX2) and Bone morphogenetic protein 2 (Bmp2) which regulate the osteogenesis process is also affected by PPARɤ, especially as RunX2 is directly inhibited by PPARɤ (Liu *et al*. 2010).

In contrast, Wnt signaling inhibits fat formation and promotes osteogenesis (Kobayashi *et al.* 2015). Based on these findings, it has been proposed that the inverse of the "seesaw

paradigm" describes the relationship between fat and bone mass in the bone marrow (Gimble 2004). However, it should be noted that the above relationship is limited to the bone marrow microenvironment.

Insulin resistance affects the synthesis of collagen

Insulin receptors exist on the surface of osteoblasts. Obesity-induced insulin deficiency can lead to decreased maturation and turnover of the bone matrix, decomposition of bone matrix, loss of calcium salts, and osteoporosis (Einhorn *et al.* 1988).

Collagen synthesis by osteoblasts is affected by insulin deficiency, which accelerates collagen metabolism and increases bone resorption. A clinical study showed that bone mineral density (BMD) declined in the lumbar vertebrae in 4 years after diagnosed with type 1 diabetes (Williams *et al*., 2015). Another clinical study also confirmed that a decrease in serum procollagen I carboxy-terminal propeptide (PICP) levels and in lumbar vertebrae BMD in 6 months after diagnosed as type 1 diabetes (Cunezler *et al*. 2001).

Insulin deficiency inhibits the synthesis of osteocalcin (BGP) by osteoblasts, which results in greater bone resorption than bone formation and a reduced bone turnover rate. BGP is a non-collagen protein synthesized and secreted by non-proliferative osteoblasts. It is mainly deposited in the bone matrix and consists of 49 to 50 amino acids. Its main physiological role is to regulate the bone turnover process and maintain the bone mineralization rate. Insulin can promote the synthesis of 1.25 dihydroxy vitamin D [1-25 (OH)D], while 1.25 (OH) D3 can promote osteoblast synthesis and secretion of BGP, diabetic insulin deficiency, so 1-25 (OH) D reduction could lead to BGP decline (Verhaeghe *et al*. 1990).

The Negative balance of calcium, phosphorus, and magnesium metabolism

The Negative balance of calcium, phosphorus, and magnesium metabolism will appear in obesity, particularly obesity with hypomagnesemia, which present calcium, phosphorus, and magnesium reabsorption in renal tubular, leading to the loosing of bone substance. In 2000, 104 cases of type 2 diabetes with osteoporosis study found that a negative balance of calcium in a diabetic state leads to a decrease in BMD (Suzuki *et al*. 2000). It has been confirmed that magnesium metabolism disorders play an important role in the process of bone loss.

Parathyroid hormone increased PTH in diabetes may be associated with increased urinary calcium excretion caused by secondary hyperparathyroidism, especially diabetes. Kidney disease, renal dysfunction, VitD insufficient, resulting in reduced intestinal calcium absorption and decreasing of serum calcium, which stimulates PTH secretion, and increases bone resorption (Nikodimopoulou M and Liakos S 2011).

1.3.7. Obesity and risk for cancer condition

a. The definition and Epidemiology of Osteoporosis

Cancer, also known as the malignant tumor, refers to an abnormal proliferation of cells, which may invade other parts of the body (Zaveri 2006; Yu *et al*., 2017). Therefore, cancer is considered to be a special disease caused by a malfunction in the control of cell division and proliferation.

Obesity has become the second most important carcinogenic factor since smoking. In the past few decades, there have been many pieces of epidemiological evidence indicating

that obesity can increase the incidence and mortality of cancer. It is an important risk factor for many kinds of cancer, particularly liver cancer, pancreatic cancer, and colorectal cancer (Pix 2017).

The occurrence of cancer is related to many factors, such as genetics, environment, clinical diagnosis, lifestyle and psychological factors. Smoking can significantly increase the risk of lung cancer, which is both a consensus and a clearer cause of cancer. However, obesity can also cause cancer, and its occurrence and development are secretive and underlying, and the resulting risk of carcinogenesis is very serious and widespread (Pix 2017). At present, research on obesity-causing cancer is still under development, and many unclear mechanisms need to be further explored.

A retrospective study in 2008 evaluated the relationship between obesity and cancer (Renehan *et al*., 2008). The results showed that for every $5kg/m^2$ increase in BMI, the risk of esophageal adenocarcinoma increased by 52%, the risk of thyroid cancer increased by 33%, the risk of colon cancer increased by 24%, and the risk of kidney cancer increased by 24%.

At the same time, obesity can increase the risk of cancer-related death. The overall risk of cancer death in male and female patients increased by 1.5 and 1.6 times respectively when BMI exceeded 40 kg/m^2, (Renehan *et al*., 2008). A subsequent study in the United States also found that cancer mortality increased by 10% with a BMI increase of 5 kg/m^2 (Basen-Engquist and Chang 2011). In addition, follow-up studies of patients undergoing bariatric surgery for bariatric surgery have shown that weight loss can reduce the incidence of cancer and related mortality, which is particularly evident in women (Basen-Engquist and Chang 2011). The findings of these three surveys are not consistent, but they all indicate that obesity increases cancer mortality.

The latest report on the relationship between cancer and obesity also highlights cancers associated with overweight and obesity, including thyroid, liver, kidney and ovarian cancer, and accounts for 40% of cancers diagnosed in the United States. The study also reviewed 2005-2014 US cancer statistics and found that 55% of all cancers diagnosed by women were associated with overweight and obesity, compared with 24% for men (Pix 2017).

b. The mechanism of cancer occurrence

Obesity can trigger an inflammatory reaction. In normal-weight human fat cells, macrophages tend to anti-inflammatory phenotypes. However, with obesity, macrophages up-regulate mRNA expression of PPARγ, C/EBPα, transcription associated with lipid synthesis (SREBP-1c) and increase adipocyte production due to oxidative stress induced by obesity. mRNA expression of these factors makes macrophages prone to inflammatory phenotypes. Currently, abundant macrophages can produce pro-oncogenes, including excess tumor necrosis factor (TNFα) and IL-6 (Qin *et al.*, 2016). The expression of these regulatory genes can promote the proliferation, survival, and angiogenesis of cells, and simultaneously enhance the invasiveness, metastasis and pluripotency of cells, thereby promoting the occurrence and development of cancer.

Obesity may be the cause of insulin resistance (IR). As previously mentioned, fat cells can produce excess free fatty acids by lipolytic activity, increase the expression of SREBP-1c and PPARγ, thereby increasing the accumulation of lipids and triggering IR. Ultimately, the body's sensitivity to insulin is reduced (Yan *et al.*, 2016). In order to maintain normal blood glucose levels, the body compensates excess insulin to ensure its

blood glucose intake and utilization, resulting in hyperinsulinemia. In addition, hyperglycemia activates hypoxia-inducible factor-1 (HIF-1), which increases cancer cell survival under hypoxic conditions and enhances glycolytic enzyme expression, providing sufficient energy for cancer cell growth (Hu *et al*., 2003).

Other factors include the loss of immune surveillance ability and the mutation of genetic loci. Cytotoxic T cell (CTC) and natural killer cells (NK) are decreased, and macrophages, mononuclear leukocytes, PPARγ and C/EBPα expression are increased, which then upgrades the production of fat cells, and in turn causes a decreased immune response ability and immunity damage (Wensveen *et al*., 2015, Chang *et al*., 2017). As a result, the immune surveillance ability that inhibits the growth of early tumor cells is lost (Andersen *et al*., 2016). In addition, when some genetic loci related to obesity, diabetes, insulin resistance was mutated, which could cause corresponding symptoms and promote cancer (Su *et al*., 2017).

Thus, although there is no evidence that obesity is the direct cause of cancer, it is clear that obesity and cancer have many of the same regulatory factors and regulatory pathways.

1.4. Cellular dysfunction of obesity

Peter Arner's study published in Nature magazine found that the cause of obesity is the impaired metabolism of fat cells. The study found that the fats stored in fat cells are completely updated every six years in a healthy population. In obesity patients, such as those suffering from diabetes, fat metabolism is impaired and stored fats are difficult to consume (Arner *et al*. 2011), meaning that the therapeutic direction of obesity is to treat

the cellular dysfunction.

1.4.1 Adipocytes and insulin resistance

Adipose tissue was treated as hypertrophy of fat mass. According to recent studies, it is no longer purely considered as an energy storage organ, but also an extremely important part of the endocrine system (Adamczak *et al.* 2013). It plays an important role in insulin regulation and whole-body metabolism control by sequestering fat (Konige *et al.* 2013).

Clinical and animal studies have proved that there is a high correlation between obesity and T2D. There is also a causal relationship between obesity and insulin resistance, as weight loss and gain correlate with increasing and decreasing insulin sensitivity respectively (Yaturu 2011). However, it remains unclear what factor, or factors, are crucial in mediating insulin resistance. At least one important mediator, FFAs, has been identified (Yaturu 2011, Oakes *et al.* 2013).

Human studies have demonstrated that elevated levels of circulating FFAs could cause peripheral insulin resistance (Boden 2011, Mashili *et al.* 2013). Corresponding to these studies, another human study showed that using polyphenols and L-carnitine (PPC) to lower FFAs could enhance glucose uptake, as well as reduce triglyceride and insulin levels in the periphery (Radler *et al.* 2011). These studies support the hypothesis that high circulating FFAs mediate insulin resistance (Savage 2007, Guilherme *et al.* 2008). Under the stimulation of obesity, excess FFAs released by lipolytic activity by increasing of SREBP-1c and PPARγ expressions, especially the expression of SREBP-1c, and lead to the accumulation of lipids and trigger IR, which ultimately reduces the body's sensitivity to insulin (Yan *et al.*, 2016). The expressions of insulin receptor 1 (IRS-1) and

glucose transporter 4 (GLUT-4) are then downregulated in this stage, resulting in mobilization of FFAs into the circulation and uptake into skeletal muscle, which not only leading to excess body fat but also promoting IR (Guilherme *et al*. 2008, Cohen *et al*. 2011). Meanwhile, animal studies have also shown that a lack of adipose tissue leads to elevated circulating concentrations of triglycerides and fatty acids, and thereby insulin resistance (Laustsen *et al*. 2002).

Adipose tissue seems to have two key roles: secreting optimal levels of adipokines to influence whole-body metabolism and neuroendocrine function; and sequestering lipids as adipose triglyceride stores, which then attenuates the deleterious effects of both circulating FFAs and ectopic triglyceride stores (Guilherme *et al*. 2008). Thus, functional adipose tissue in proper proportion to individual body size could help to maintain normal insulin sensitivity and glucose homeostasis.

1.4.2. The role of adipocyte dysfunction in obesity

Adipose tissue is the master regulatory tissue in controlling whole-body lipid flux and modulating glucose homeostasis. Adipocytes are the base unit for processing these functions, not only synthesizing and storing triglycerides in feeding states but also hydrolyzing and releasing triglycerides as FFAs and glycerol in fasting states (Rosen *et al*. 2006).
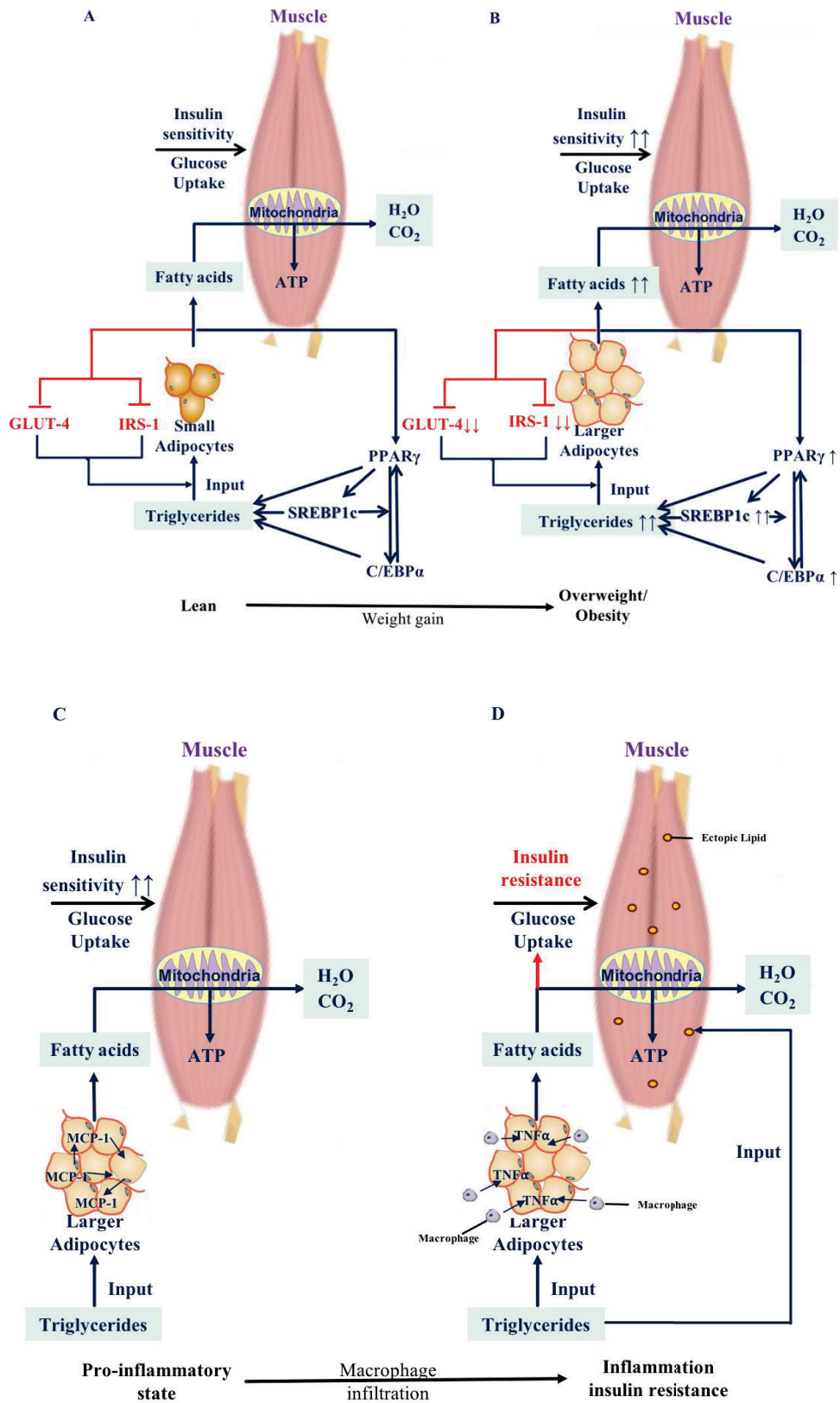
Figure 1.1 Adipocyte dysfunction triggers insulin resistance in skeletal muscle

There is a dynamic equilibrium between the release of fatty acids into the circulation and

their uptake and oxidation by peripheral tissues, especially the skeletal muscle (Guilherme *et al*. 2008). In this state, skeletal muscle insulin sensitivity and glucose uptake are normal. Figure 1A is a lean state model; fatty acyl-CoA levels within muscle cells are low due to their rapid oxidation in mitochondria (Guilherme *et al*. 2008).

Because of the body's self-stabilizing effect, the increased adipose tissue tends to be more metabolically degraded after the increase of adipose tissue, resulting in further increase of FFAs. High FFAs hyperlipidemia is an important pathogenic factor for obesity-induced insulin resistance (lR). Under the stimulation of obesity, excess free fatty acids released by lipolytic activity increase SREBP-1c and PPARγ expression, especially that of SREBP-1c, and thereby increasing lipid accumulation. Meanwhile, elevated plasma FFAs levels increase hepatic gluconeogenesis, promote basal insulin secretion and reduce hepatic clearance ability for insulin, resulting in hyperinsulinemia and decreasing adipose tissue glycogen synthase activity (Kim *et al*., 1998b, Boden 2011, Oakes *et al*., 2013).

Adipocytes enlarge when caloric intake increases, because of the increased triglyceride deposition (Qatanani *et al*.2007). Figure1.1B showed in the early stages, the triglyceride and lipolytic rates are close to the normal ratio due to the large capacity of adipocytes and skeletal muscle maintaining a high insulin sensitivity (Robert *et al*, 2003, Guilherme *et al*. 2008).

The expression of insulin receptor 1 (IRS-1) and glucose transporter 4 (GLUT-4) are then downregulated, resulting in mobilization of FFAs into the circulation and uptake into skeletal muscle. This not only leads to excess body fat but also decreases GLUT4 transference from the cytoplasm to the cell membrane, which in turn ultimately reduces the body's sensitivity to insulin (Yan *et al*., 2016) and triggers insulin resistance (IR). At

the same time, the phosphorylation of insulin receptor β subunit and IRS-1 under high FFAs environment lowers, suggesting that FFAs promotes IR whilst inhibiting the activity of insulin receptor tyrosine kinase and the expression and activity of IRS-1 (Guilherme *et al.*, 2008, Cohenet *et al.*, 2011).

The increase in adiposity affects the ability of adipocytes to function as endocrine cells and secrete multiple biologically active proteins. Hypertrophied adipocytes have been found to secrete large amounts of monocyte chemo attractant protein‑1 (Curat *et al.* 2004), which is a pro-inflammatory state (Figure1.1C) (Guilherme *et al.* 2008).

Obesity itself can trigger an inflammatory response (Iantorno *et al.*, 2014). A large number of studies suggest that macrophages are the most important inflammatory cells in adipose tissue and play a key role in insulin resistance caused by obesity.

Hotamisligil found that the secretion of tumor necrosis factor-α (TNF-α) in adipose tissue of obese animal models increased, and for the first time, obesity was associated with inflammation in 1993 (Hotamisligil *et al.*, 1993). Xu's study discovered that there was a large amount of macrophage infiltration in the adipose tissue of the obese animal model, which opened the survey on the relationship between adipose tissue macrophages (ATM) and obesity in 2003 (Xu *et al.*, 2003). The subsequent experimental studies also proved this point (Lee *et al.*, 2016; Amano *et al.*, 2014).

Macrophages in normal mouse and human adipose tissue are called tissue macrophage, which are small round cells dispersed in adipose tissue and is involved in maintaining the homeostasis of adipose tissue. When obesity occurs, the number of ATMs increases dramatically (Murray *et al.*, 2011; Weisberg *et al.*, 2003).

Macrophages exhibit greater variability in different microenvironments, so there are

views on dividing macrophages into classically activated (also known as M1) and alternatively activated (also known as M2). This name is derived from Th1 and Th2 type immune responses. Th1 type immune responses such as interferon gamma (IFN-γ) cytokines can stimulate macrophage activation to M1 type, while Th2 type immune response IL-4 And IL-13 activate signal transducers and activators of transcription 6, STAT6 to induce M2-type activation (Martinez *et al*., 2014).

M1 macrophages are thought to mediate insulin resistance, and their mechanisms involved in insulin resistance are associated with elevated free fatty acids (FFAs), which may be the predisposing factor for ATM-enhancing insulin resistance. Nuclear factor-κB inhibits protein kinase β/NF-κB (IKKβ/NF-κB) and c-Jun N-terminal kinase/activator protein 1 (JNK/AP-1) Phosphorylation of the inflammatory signaling pathway leads to increased expression of inflammatory genes in cells (Maruo *et al*., 2018; Ying *et al*., 2017; Xu *et al*., 2015).

Under physiological conditions, insulin-mediated tyrosine phosphorylation of the insulin receptor substrate (IRS) through insulin receptors, thereby activating downstream phosphatidylinositol 3-kinase/serine/threonine protein kinases (The phosphoinositide 3-kinase/AKT serine/threonine kinase (PI3K/Akt) signaling pathway promotes glucose uptake by cells and exerts hypoglycemic effects of insulin. However, activated IKKβ, and JNK can phosphorylate the serine of IRS, block the tyrosine phosphorylation of IRS and the downstream PI3K/Akt pathway, leading to insulin resistance (Solinas *et al*., 2010).

M2 macrophages are thought to improve insulin sensitivity. Contrary to M1, adipose tissue M2 macrophages secrete the anti-inflammatory factor IL-10 to inhibit inflammation and improve insulin resistance (Riki *et al*., 2016). Krüppel-like factor 4

(KLF4) is a cytokine closely related to the M2 macrophage phenotype, which is a subtype of the zinc finger structure of the transcription factor. Experiments have shown that knocking out KLF4 from obese mouse macrophages will lead to a decrease in the proportion of M2 macrophages in adipose tissue of obese mice and a worsening of insulin resistance and hyperglycemia (Saha *et al*., 2015; Date *et al*., 2014).

At present, most of the literature still uses the M1 and M2 type two classification methods to summarize the ATM characteristics. But in fact, the ATM in the body presents a complex and unique continuous phenotype spectrum. A study found that ATM in obese patients does not express markers of classically activated M1 macrophages in human chronic infectious inflammation. The high-sugar, high-fat, high-insulin environment induces activation of macrophages, and pathogen-stimulated activated macrophages have different gene expression (Kratz *et al*., 2014).

Therefore, the macrophage phenotypic changes and corresponding functions in the development of obesity remain to be studied. Currently, there is no uniform marker for human macrophage spectrum, and the cell signals involved in the expression of macrophage markers are not yet clear. Pathway, which limits the development of research.

In general, macrophages tend to possess anti-inflammatory phenotypes in normal-weight human adipocytes, and pro-inflammatory phenotypes in obese human adipocytes according to current studies. Abundant macrophages can produce pro-cancer factors, including excess TNFα and IL-6. These related regulatory genes not only promote SREBP-1c and PPARγ expression as the previous discussion (Section 1.3.1) but also decrease the expression of insulin receptor 1 (IRS-1) and glucose transporter 4 (GLUT-4). The entire process increases lipid accumulation, which causes fat cell dysfunction

and decreases the body's sensitivity to insulin, and triggers IR (Yan *et al*., 2016).

The developing inflammatory state in adipose tissue is associated with insulin resistance in skeletal muscle (Figure1.1D). Because the inflammatory response can, in turn, lead to adipocyte dysfunction and affect adipocyte metabolism by cytokines such as TNFα and IL-6, which play a key role among those regular factors, especially TNFα. The effect of TNFα on insulin signaling pathways is mediating serine phosphorylation of the insulin receptor substrate and making it into an insulin receptor tyrosine kinase, thus inhibiting tyrosine phosphorylation of IRS-1, leading to IR (Guilherme *et al*.2008, Alan *et al*., 2017).

This process may lead to increasing of fatty acid synthesis, and very-low-density lipoprotein synthesis in the liver and skeletal muscle (Simopoulos 2013).

In addition to the inflammatory response effects, high concentrations of TNFα inhibit insulin-stimulated glucose transport by preventing mRNA expression of GLUT4. High concentrations of TNFα can then in turn promote adipocyte breakdown, release large amounts of FFAs, and aggravate the occurrence of IR as well as the inflammatory effect itself (Wensveen *et al*., 2015, Esser *et al*., 2014).

The studies have reported that extensive macrophage infiltration had been observed in human omentum adipose tissue of obese patients, which support the hypothesis that the inflammatory response contributes to metabolic dysfunction in obesity in type 2 diabetes. Inflammation could also cause IR by a direct action of TNFα on muscle insulin signaling (Cancello *et al*. 2005).

Thus, FFAs and TNFα could be two major insulin signalling in skeletal muscle caused by adipocyte dysfunction (Guilherme *et al*.2008, Bao *et al*. 2014).

1.4.3. Adipocyte dysfunction and adipogenesis/lipogenesis

Adipocytes control whole-body lipid flux and modulate glucose level, as discussed above. Their dysfunction may lead to high levels of circulating fatty acids being released into peripheral tissues such as the skeletal muscle, and cause/enhance macrophage infiltration into adipose tissues, increasing insulin resistance and thereby resulting in adipogenesis.

The differentiation from preadipocyte to mature adipocyte is called adipogenesis. This process consists of three stages: the mitotic clonal expansion phase, the terminally differentiated phase, and the mature adipocyte phase.

Adipogenesis is controlled by a tightly regulated transcriptional cascade wherein transcription factors activate or repress each other's expressions in a sequential manner. In this process, the up-regulation in PPARγ, C/EBPα, and SREBP-1c expression play a decisive role in the differentiation from preadipocytes to mature adipocytes (Siersbæk *et al.* 2012).

Mitotic clonal expansion phase: In this stage, the fibroblast-like preadipocytes start to integrate and undergo growth arrest. The cell morphology changes from fibrous to round, Pref-1 (preadipocytes marker gene) expression is decreased, and the β and δ isoforms of CCAAT/enhancer binding protein (C/EBP β and C/EBP δ) begin to express and initiate cellular differentiation (Gregoire *et al.* 1998).

Terminally differentiated phase: Cell cycle arrests and exit from mitotic clonal expansion. The proliferation of vast amounts transcription factors, such as PPAR-γ and C/EBP α, express then activate glucose metabolism-related gene expression. Lipid droplets gradually begin to form and accumulate, while immature adipocytes transform

into mature adipocytes. Adipocytes become further rounded and start to hydrolyze triglycerides with the stimulation of catecholamine's substances (Feve 2005).

Mature adipocyte phase: Immature adipocytes differentiate into normal mature functional adipocytes after the last stage (Rosen 2006).

So, errors or dysfunctions in any of these three phases can result in the release of high levels of circulating fatty acids into peripheral tissues and cause or enhance macrophage infiltration into adipose tissue, increasing insulin resistance, leading to fat formation. Based on this point of view, the adipocyte dysfunction is the major problem in obesity and metabolic syndrome and treat this issue is the critical therapeutic direction of obesity.

1.5. Current therapy for obesity

Mainstream treatments for obesity include behavioral therapy, drug therapy, and surgery. Behavioral therapy is the most important form of treatment, and all successful cases are based on or combined with behavioral therapy. But no matter what kind of treatment plan, the stage goals and plans must be set up based on individual conditions (Bloom 1959, Colosia *et al*., 2013, Yanovski *et al*., 2014).

1.5.1. Behavioural therapy

Behavioural therapy is the most important treatment method, achieving weight loss by changing lifestyle, diet structure, and persistent exercise (Lau *et al.,* 2007, Naude *et al.,* 2014). After early excesses and dietary weight loss had been proven to affect intelligence and lead to endocrine disorders in 1959 (Bloom 1959), modern behavioral therapy

believes that reasonable dietary control can significantly reduce body weight in the short term (Strychar 2006). This can be beneficial for risk of heart disease and diabetes complications, especially in conjunction with 30 to 60 minutes of moderate-intensity physical exercise daily (Shick *et al.,* 1998). However, it is often difficult to maintain the results of weight loss. Generally, only less than 20% of people can maintain their weight without rebound for a long time (Guo *et al.,* 2010).

1.5.2 Pharmaceutical therapy

As the previous discussion, obesity has become a severe global medical and social problem. For the treatment of obesity, it is to reduce the body's excess fat and body weight by using drugs with weight loss effects. Clinical trial results show that the effects of anti-obesity medication and bariatric surgery on permanent weight loss are better than the impact of lifestyle changes alone. Accordingly, the US official guidelines recommend that patients with BMI values greater than 30 kg/m2 or greater than 27 kg/m2 and have weight-related complications be considered for drug treatment.

Although such products have significant effects, according to the clinical trials and reports, there are still some side effects. So, to be safety and health, the process of administration should be under the supervision of the medical officer. Drugs couldn't change the behavioral characteristics of obesity such as eating habits, exercise, work habits, and other environmental factors, so we should weigh the pros and cons when choosing medication.

At present, commonly used drugs can be divided into the following categories according to their mechanism of action (Adan 2013): Suppressing appetite; increasing water

discharge; increasing gastrointestinal motility and accelerate excretion; increasing calorie expenditure.

Suppressing appetite drug: When the appetite is relatively strong, and the diet is not tolerated, use some anti-appetite drugs. The principle is mainly to stimulate the hypothalamic sensory center, control the appetite center, and then suppress the appetite through the action of nerves so that obese people can readily accept the control of diet.

Increasing water discharge drug: These drugs use diuretic and defecation to reduce weight and suppress thirst, thereby achieving weight loss.

Increasing gastrointestinal motility drug: Such drugs are used to increase the gastrointestinal motility, accelerate the excretion of the diet, reduce the residence time of the food in the stomach, so that the food has been excreted before being absorbed into fat, and achieve the purpose of losing weight.

Increasing calorie expenditure drug: Such drugs can dissipate heat in the human body, promote catabolism in the body, and inhibit anabolism, thereby reducing the body weight of obese people, to achieve the purpose of weight loss. You can also enjoy a cup of tea after dinner every day. It can help detoxify and lose weight.

Currently commonly used drugs on the market include Sibutramine, Orlistat, Lorcaserin, Phentermine/topiramate, Naltrexone/bupropion.

Among these drugs, Sibutramine is a serotonin and norepinephrine reuptake inhibitor, and it can reduce appetite and produce a feeling of fullness in a deprived state, thus discouraging eating. And Sibutramine was also the first weight-loss drug approved for long-term treatment of obesity, but in 2010 due to cardiovascular risks was withdrawn. (Yanovski et al., 2014).

Orlistat is the only weight-loss-pills on the market that works by reducing fat absorption in the gut. Orlistat was developed by Roche. It was first approved by the European EMA in 1998, and then approved by the US FDA in 1999. It is marketed by Roche in Europe and the US. Xenical®. Orlistat is a gastrointestinal lipase inhibitor that reduces body fat by blocking the body's absorption of fat in food to achieve weight loss. This medicine is combined with a mild low-calorie diet for weight control in obese patients with a body mass index (BMI) greater than or equal to 30 kg/m2 or overweight patients with a body mass index >28 kg/m2 with associated risk factors(Al-Tahami et al., 2017).

Lorcaserin was developed by Arena and was approved by the US FDA on June 27, 2012. It is marketed in the US by Eisai under the trade name Belviq®. Chlorcarberin hydrochloride is a 5-HT2C receptor agonist that reduces food intake and enhances satiety. The drug is used as an adjunct to low-calorie diets and exercise for long-term weight control in adults. Moreover, in clinical studies, lorcaserin was found to promote sustained weight loss, and there was no higher major cardiovascular event than placebo (Bohula et al., 2018).

Phentermine/topiramate: The US FDA approved the compound diet drug Qsymia (a sustained release agent containing phentermine and topiramate) (formerly known as Qnexa) on 2012. The drug is approved for obesity with an adult body mass index (BMI) $\geqslant$ 30 or overweight with a BMI $\geqslant$ 27, and these patients have at least one weight-related disease, such as hypertension, type 2 diabetes, or hyperlipidemia. In a clinical trial, Phentermine significantly reduces patient weight, and patients are generally well tolerated during drug use (Hollander et al., 2017). So far, the most popular weight loss drug prescription in the US is still phentermine according to the annual marking report.

Naltrexone/bupropion: The US FDA approved Contrave (a sustained release tablet of

naltrexone hydrochloride and bupropion hydrochloride) as adjunctive therapy combined with a low-calorie diet and physical exercise for chronic weight management since 2014. This drug is approved for use in adult patients with a body mass index (BMI) of 30 or greater (obesity), or a BMI of 27 or greater (overweight), and at least one weight-related disorder such as hypertension, type 2 diabetes or adult patients with high cholesterol (dyslipidemia), which is a new option for chronic weight management(Sherman et al., 2016).

Additionally, due to the side effects of western medicine in weight loss treatment, there are some who prefer natural therapies with fewer side effects, most prominently traditional Chinese medicine.

Modern clinical research (Li et al., 2004) found that ephedra, hawthorn, rhubarb, tea, orange peel, mountain wax, and other traditional Chinese medicines have a slimming and rouge effect. Clinical and experimental studies have found more, and the weight loss effect is outstanding. In addition to Chinese medicine treatment by itself, there also exist TCM syndrome differentiation treatment and Chinese medicine compound treatment.

1.5.3. Surgical therapy

The bowel resection was common surgical therapy in the early stage. Afterward, it was found that side effects and mortality rates were high after operation, and this has been modified into gastrectomy.

Surgical therapy is the last and most effective method, usually considered by patients with severe obesity who have not improved after receiving the above treatment. Patients undergo long-term weight loss after surgery, improving obesity-related problems (Chang

et al., 2014) and reducing mortality (Sjöström *et al.,* 2007). There are also risks in the surgery itself, but the latest research shows that the mortality rate of morbidly obese patients was 0.68% and that of un-operated patients was 6.17% after 5 years of follow-up (Ma *et al.,* 2011). Therefore, the risk of untreated morbid obesity is much higher than that of surgery.

1.6. Green tea polyphenols (GTPs)

Traditional Chinese medicine (TCM) has a history of more than three thousand years; boasting obvious advantages such as less toxicity, better effect and cheaper cost, which make it one of the most important therapies in the world (Shi *et al.* 2012). Tea is part of TCM, and, as the previous researches, is beneficial to the treatment of obesity (Hsu *et al.*, 2008, Kim *et al.*, 2013, Hayakawa *et al.*, 2016, Pan *et al.*, 2017).

Tea is from the plant *Camellia sinensis*. According to the different production processes and methods, teas from all around the world can be divided into black, green, and Oolong tea (McKay *et al.* 2002). Among those, Green tea has the most significant effects on human health. Thousands of years ago, green tea was recorded to be a treatment for easing the effects of alcohol in China, in addition to being a stimulant, promoting digestion, and improving urinary and brain function (Cabrera *et al.* 2006).

The role of tea is far more than just part of Traditional Chinese Medicine, according to modern scientific and clinical researches the efficacy and effects of green tea mainly include anti-sunburn, anti-radiation, lipid-lowering, anti-bad breath, anti-aging, antibacterial, anti-gastric, anti-cancer, and promote digestion, as follows:

Anti-sunburn: Recently, a global study pointed out that catechins in green tea have a

potent antioxidant function. Applying skin care products containing green tea ingredients on the skin can prevent sunburn and sag, even being exposed to the sun (Hajra and Yang 2015).

Anti-Radiation: The tea is rich in provitamin A, which can be quickly converted into vitamin A after being absorbed by the body. Vitamin A can synthesize rhodopsin, making it easier for the eyes to see things in the dark. Therefore, green tea not only eliminates computer radiation but also protects and enhances vision (Zhou *et al.,* 2015; Liang *et al.,* 2017).

Lipid-lowering: Drinking tea can lower blood lipids and cholesterol and make your body lighter. This is because the comprehensive coordination of phenolic derivatives, aromatic substances, amino acids and vitamins in tea, especially the combination of tea polyphenols and tea and vitamin C, can promote fat oxidation, help digestion, reduce fat and lose weight. These are considered to be the main effects of green tea and will be discussed further in later sections (Pan *et al.,* 2017; Sharifzadeh *et al.,* 2017; Onakpoya *et al.,* 2014).

Prevent halitosis: Green tea contains fluorine, of which catechin can inhibit the action of sputum bacteria and reduce the occurrence of plaque and periodontitis. The tannic acid contained in the tea has a bactericidal effect and can prevent food slag from propagating bacteria, so it can effectively prevent halitosis (Gaur and Agnihotri 2014; Morin *et al.,* 2015).

Anti-Aging: The antioxidants contained in green tea help to fight to age. Because the body's metabolism process, if peroxidized, will produce a lot of free radicals, natural to age, and also cause cell damage. SOD (superoxide disproportionation) is a free radical scavenger that effectively removes excess free radicals and prevents free radical damage

to the human body. The catechins in green tea can significantly increase the activity of SOD and scavenge free radicals (Afzal *et al.,* 2015; Fei *et al.,* 2017).

Antibacterial: Studies have shown that catechins in green tea have an inhibitory effect on some bacteria that cause human disease, and at the same time do not harm the reproduction of beneficial bacteria in the intestine (Sun *et al.,* 2014; Shahid-Ul-Islam *et al.,* 2018; Chen *et al.,* 2018).

Anti-gastric disease: A study shows that drinking green tea can prevent chronic gastritis and stomach cancer. The study found that people who drink green tea for a longer period have a lower rate of chronic gastritis and stomach cancer, and those who drink green tea have a 48% lower rate of stomach cancer than those who do not like tea (Sato *et al.,* 2002).

Anti-cancer: Green tea has an inhibitory effect on certain cancers, but its principles are limited to the inference stage. For the prevention of cancer, drinking more tea must have its positive encouragement (Hayakawa *et al.,* 2016; Yu *et al.,* 2017; Shirakami *et al.,* 2018).

Promote digestion: Recent studies have shown that green tea can help improve indigestion, such as acute diarrhea caused by bacteria, and drink a little green tea to alleviate the condition (Fallon *et al.,* 2008).

1.6.1 The Major Polyphenols in green tea

The chemical composition of green tea is complex, consisting of proteins (15-20% dry weight), amino acids (1-4% dry weight), carbohydrates (5-7% dry weight), minerals and

trace elements (5% dry weight) (Flier *et al.* 2004).
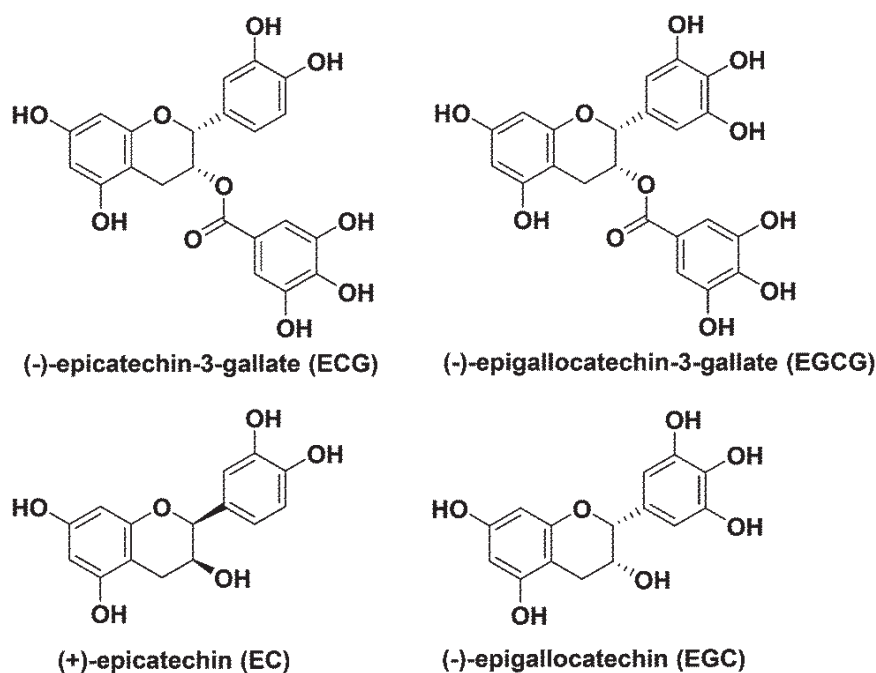
Figure 1.2: The Major Polyphenols in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and (−)-epigallocatechin-3-gallate (EGCG)

The pharmacological efficacies of green tea extract are polyphenols and caffeine. The proportion of polyphenols (45-90%) and caffeine content (0.4-10%) in green tea varies, depending on whether the extracts are in liquid or powder. The polyphenols include flavanols, flavandiols, flavonoids, and phenolic acids. Most the green tea polyphenols (GTPs) are flavonols, more commonly known as catechins (Vinson *et al.* 2000). There are four kinds of catechins mainly found in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and (−)-epigallocatechin-3-gallate (EGCG), which is considered as the most effective catechin for anti-obesity action (Sano *et al.* 2001, Bigelow *et al.,* 2006). EGCG was considered to be the main component and its purify in GTP is about 68% (Kim *et al.* 2013).

Caffeine (1,3,7-trimethylxanthine) is another main component of green tea, a naturally

occurring alkaloid that is found in varying quantities in the beans, leaves, and fruits of more than 60 plants (Weinberg *et al.* 2001). However, as there is no report about the effects of synthetic caffeine on glucose and lipid metabolism, it will not be used for further study.

1.6.2 Health benefits of GTP

Recently studies show that the health benefits of green tea polyphenols (GTP) include antiproliferative activity (Vanessa *et al.* 2004), prevention of many types of cancer (Min *et al.* 2005, Zaveri 2006), and antifungal activity (Hirasawa *et al.* 2004). GTP also has antioxidant, antimutagenic, and anticarcinogenic effects (Mukhtar *et al.* 1992, Sano *et al.* 1995, Sharifzadeh *et al.,* 2017). But the most common and popular research focuses on the effects of GTP on diabetes and obesity (Zheng *et al*. 2004, Ku *et al.* 2009, Zhang *et al.* 2011, Shen *et al.* 2012, Pan *et al.,* 2017).

These GTP mechanisms may be considered to certain pathways, including modulations of energy balance, endocrine systems, food intake, lipid and carbohydrate metabolism, and oxidation-reduction status (Yang *et al.* 2001). However, the exact details of the mechanism remain unclear.

An animal study was utilized to investigate whether green tea (GT) inhibits the expression of genes regulating hepatic lipogenesis and intestinal lipid transport in fructose-fedovariectomized(OX) rats. After 6 weeks treatment, the green tea groups showed markedly lowered plasma and liver TG, meanwhile, the expressions of SREBP-1c, fatty acid synthase, and stearoyl-CoA desaturase 1 mRNA in the liver were significantly decreased by GT treatment groups. The inhibition of hepatic lipogenesis by

GT may involve SREBP-1c and its responsive genes without affecting lipoprotein assembly (Shrestha *et al.* 2009).

Generally, excess hepatic lipid accumulation and oxidative stress may cause non-alcoholic fatty liver disease (NAFLD). A green tea extract (GTE) study was investigated with hepatic lipids and serum alanine aminotransferase (ALT) in obese mice and lean control models. Data indicated that steatosis livers of obese mice had the extensive accumulation of tumor necrosis factor-α (TNFα), whereas GTE at 1% decreased hepatic TNFα protein and inhibited adipose TNFα mRNA expression. GTE could increase hepatic catalase and glutathione peroxidase activities, and these activities were inversely correlated with ALT and liver lipids. In this way, GTE mitigated NAFLD and hepatic injury in ob/ob mice by decreasing the release of fatty acids from adipose and inhibiting hepatic lipid peroxidation as well as restoring antioxidant defenses and decreasing inflammatory responses. The results suggest that GTE could be used to mitigate obesity-triggered NAFLD (Park *et al.* 2011).

Another animal study investigates the long-term effects of GTPs on body composition and bone properties. Obese rats were divided into 2 groups: with or without GTP (0.5%) in drinking water. After 8 months, the GTP treatment group showed an increased percentage of fat-free mass, bone mineral density and strength, and GPX protein expression, and a decreased percentage of fat mass, serum insulin-like growth factor I, leptin, adiponectin, and proinflammatory cytokines. The results demonstrate that GTP mechanism possibly acts by enhancing antioxidant capacity and suppressing inflammation (Shen *et al.* 2012).

EGCG is the main component of GTPs, which is considered to be the modulator of insulin-stimulated mitogenesis, 3T3 preadipocyte was utilized to investigate the

pathways involved. The results demonstrated that EGCG not only inhibited insulin stimulation of preadipocyte proliferation in a dose- and time-dependent manner, but also tended to increase insulin-stimulated associations between the 67LR and IR, IRS1, IRS2, and IRS4 proteins. These data suggest that EGCG mediates anti-insulin signaling in preadipocyte mitogenesis via the 67LR pathway (Ku *et al.* 2009).

A meta-analysis of 14 randomized controlled clinical trials was performed at human adults with green tea intake. Weighted mean differences were calculated for net changes in lipid concentrations by using fixed-effects or random-effects models. Jadad score and a meta-analysis were utilized to assess the quality of research. Total 1136 subjects had enrolled in this meta-analysis. The meta-analysis of this study demonstrated that the green tea extracts could significantly reduce the blood TC and LDL-cholesterol concentrations. However, there was no effect on HDL cholesterol had been observed in those trials (Zhang *et al.* 2011). Recently, green tea and green tea extracts have demonstrated to modify glucose metabolism beneficially in experimental models of type 2 diabetes mellitus (Wu *et al.* 2004, Roomi *et al.* 2007)

In summary, the above studies have proven that GTP has significant value in assisting the management of obesity.

1.7. Hypothesis and aim of the study

The aim of this study was to test how green tea polyphenols (GTP) possesses anti-obesity properties. Previous studies have greatly improved our understanding of the diversity of roles and functions associated with GTP in natural remedies. However, there are still questions about the specific types of GTP and their potential functions that have not yet

been answered. The overall goal of the project is to further evaluate the effects of GTP on different obesity and related diseases using methods such as biochemical detection, immunofluorescence staining, genomics, and meta-proteomics.

We hypothesized that GTP can benefit cellular dysfunction and inhibit the production of adipocytes, promote bone cell production by deacetylating Runx2 and suppressing PPARγ. Meanwhile, GTP treatment can inhibit over-secretion of testosterone in theca cells through downregulation of CYP11A1 and CYP17A1 protein expression.

The comprehensive approach will help to further explore the causes and mechanisms of the effects of GTP on obesity, as well as the metabolic complications of osteoporosis and PCOS, and even use the latest DNA methylation to solve and discover the potential role of GTP. The results of this study may lead to a natural therapy for obesity in Australia.

1.8. Chapter Synopsis

Chapter 3: The aim of this study was to test whether GTP possesses anti-adipogenic properties. This was performed by investigating the effects of various concentrations of GTP and EGCG on 3T3-L1 preadipocytes. The effects tested included cell growth, the adipogenic process, storage of intracellular lipids in mature adipocytes and the effects on signal pathways. To elucidate the mechanisms of GTP on adipogenesis, the regulatory factors of adipogenesis, including C/EBPα, PPARγ, and SREBP-1c, were determined with immunoblotting and a semi-quantitation of mRNA expression. The results of this study are used to establish the GTP research model for obesity in further studies of natural therapy.

Chapter 4: GTP could be used for the treatment of inhibition in 3T3-L1 adipogenesis

differentiation, which proved that GTP could inhibit the differentiation from preadipocytes to adipocytes (adipogenesis). Has this inhibition appeared at another cell line, particularly the stem cell, like ADSC? The results of the study showed that not only could GTP be used for 3T3-Li cell line, but that it could also benefit ADSCs or any other cell line. Since the first chapter of this study, GTP has been verified to be able to inhibit the differentiation of adipocytes from adipocytes, and this result was only obtained in the cell line of an obese GTP cell model. It is not yet clear whether this result can be applied to other cell lines, especially stem cells which can be pluripotent and multi-differentiated. Therefore, the ADSC cellular model was established and validated in the second part of the research project (Chapter 4), then used to observe the effect of GTP on obesity.

Osteoporosis is one of the major metabolic complications of obesity. More than half of osteoporotic cases are directly or indirectly caused by obesity. GTP has been proved to be useful in the treatment of obesity. The previous chapter's study of regulatory pathways showed that GTP inhibits fat formation in adipose tissue stem cells (ADSCs) by modulating PPARγ expression. At the same time, literature studies showed that PPARγ can inhibit osteoblasts. In order to understand the mechanisms involved in the relation between adipocyte and osteoblast differentiation and to attempt to develop natural therapeutic approaches to osteometabolic diseases, the second half of this research project (Chapter 4) set out to investigate the underlying mechanisms of PPARγ and biomarkers in adipogenesis and osteogenesis pathways during the differentiation of human adipose tissue-derived stem cells (hADSCs) into mature adipocytes and osteoblasts with or without GTP treatment.

Chapter 5: Polycystic Ovarian Syndrome (PCOS) is a complex and heterogenous disorder, present in up to 15% of women of reproductive age, hyperandrogenism is a key

clinical feature of the disorder. The disease mechanism of PCOS still reminds unclear but the reports in the literature have suggested a stronger correlation between PCOS and metabolic syndrome, including obesity, hyperinsulinemia, dyslipidemia, insulin resistance and impaired glucose tolerance. GTP was proved to be beneficial to obesity and insulin resistance as the previous experiments (Chapter 3 and Chapter 4). Thus, it was hypothesized that GTP may increase progesterone and decrease testosterone levels and may be beneficial for the obesity-related disease PCOS. Therefore, in the third research project (Chapter 5), a PCOS cell model was established with theca cell treated by dexamethasone (DEX) and verified the effect of GTP on PCOS cell models at different concentrations. To define the molecular mechanism of GTP on ovarian hormone production, expressions of steroidogenic enzymes (CYP17A1 and CYP11A1) were detected in the cell in the presence and absence of GTP treatment.

Chapter 6: Just like other clinical and basic research studies, the three previous research projects in this study have only studied the different beneficial effects that the consumption of green tea has on obesity and obesity-related metabolic diseases. Despite this, they did not explore mechanisms on a deeper level, for example, e.g. whether there was a change in epigenetics of ADSCs throughout the entire process was not mentioned. Therefore, the 4th research project (Chapter 6) was established to indicate the mechanism of GTP regulation on DNA methylation in ADSC. There is a lack of reagents to control genomic stability during ADSCs differentiation. Previous studies of this thesis have shown that treatment of ADSCs with GTP inhibited lipid accumulation by arresting adipocyte differentiation into fat cells through downregulating the adipogenic transcriptional factor PPARγ, which indicated that GTP would be capable of regulating the differentiation ability and genomic stability of ADSCs. The experiment not only observed the effect of different concentrations of GTP on DNA methylation, but also

observed the methylation status of cells in different batches and age groups by comparing differential methylation biomarkers, including DNMT-1, DNMT-3a, and DNMT-3b. The results can be separately and comprehensively evaluated on the effects of age and GTP on cell methylation.

Finally, the overall conclusions of the project were discussed and possible future work was proposed to further understand natural treatments for GTP-related metabolic diseases.

Chapter Two

General Materials and Methodology

2.1. Experimental materials

2.1.1. Green Tea Polyphenols (GTP)

The tea polyphenols (98% in purity) were extracted from green tea leaves (Camellia sinensis) grown in Guizhou province, south-western China, by Zuyilushen Kangyuan Co (Guizhou, Meitan, China). Green tea contains a family of catechins (Figure. 1.2), including (−)-epigallocatechin-3-gallate (EGCG), which accounts for about 68–69% of polyphenols, followed by (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), and (-)-epicatechin (EC, *circa*) (Chen *et al.,* 2001), identified using liquid chromatography-mass spectrometry (LC-MS).

With the increased popularity of using dietary polyphenols/ EGCG for obesity and obesity-related chronic diseases, it is important to determine the optimal formulation, e.g., a highly purified EGCG or a polyphenol mixture for the development of anti-obesity products, which is the research target for first project performed in chapter 3.

2.1.2. Analysis and Purity of Cell Treatments

a. Green Tea Polyphenols (GTP)

The purity of Green Tea Polyphenols (GTP) were determined by Liquid chromatography–mass spectrometry (LC-MS) (Del Rio *et al*., 2004, Bravo *et al*., 2007, Kim *et al*., 2013). Initial analysis of GTP samples supplied by Zuyilushen Kangyuan Company (Guizhou, Meitan, China) highlighted the need to use LC-MS methods to initially separate the catechin-derived compounds.

The polyphenols extracted from green tea were analysed using an Agilent 1200 series

liquid chromatograph/mass selective detector equipped with QTOF 6510 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The liquid chromatographic system consisted of a quaternary pump 1290, on-line vacuum degasser, autosampler 1200 and thermostatic column compartment, connected in line to a DAD 1290 before the mass spectrometer. Data acquisition and analysis was carried out in an Agilent MassHunter. Sample (2µl) was injected into the HPLC system, and separation was per formed on an Agilent Eclipse XDB-C18 reversed-phase column (110mmx4.6mm, 2µm). The HPLC was performed under binary gradient elution, and mobile phases A (0.5% acetic acid in H_2O) and B (0.5% acetic acid in ACN) were used. The flow rate was 0.4ml/min, injection volume 2µl. The samples were filted with 0.2µm Nylaflo membrane filter. The LC time program (linear gradient) was 5-12% B (0-5min); 12-40% B (5-30min); 40-70% B (30-35min); 70% B (35-36min); 70-5% B (36-38min); 5% B (38-42min). The wavelength of DAD detector was set at 280nm. The electrospray ionization (ESI) source operated in negative ion mode. The electrospray capillary voltage was set to 3500V, with a nebulising gas flow rate of 5L/min and drying gas temperature of 350°C. Mass spectrometry data were acquired in the Scan mode (mass range m/z 100-1700). Quantification was done by comparison with GTP and EGCG standard. The concentration of other polyphenols was calculated using the calibration curves of the GTP and EGCG standard.

Table 2.1 LC-MS characteristics of phenolic compounds in green tea product

Compound	RT(min)	Polyphenolic content %	[M-H]$^-$	Fragment ions	Proposed structure
1	15.845	67.83	457	457,493,915	EGCG
2	20.142	0.95	441	441,477,217	ECG
3	11.644	7.39	305	305,341,387	EGC
4	15.384	18.34	289	289,325,579	EC
5	16.603	3.08	457	457,493,255	EGCG (Isomer)
6	9.395	1.66	305	305,341,387	EGC (Isomer)
Total		99%			

The results showed that the polyphenols contents including (-)-epigallocatechin (EGC, *circa* 7%), (-)-epicatechingallate (ECG, *circa* 1%), and (-)-epicatechin (EC, *circa* 18%), (−)-epigallocatechin-3-gallate (EGCG, *circa* 68%) and total Green Tea Polyphenols (GTP, 99%), were all quantified by reference to standard calibration curves obtained with diode array detection at λ_{max} values were shown in Table 2.1. The results from the LC-MS analysis confirm that GTP for using in this thesis is standardized to 99% purity.

b. Purity of other treatments

Other treatments used in this thesis were obtained directly from the supplier at very high purity suitable for the project and did not need further purification analysis.

(−)-epigallocatechin-3-gallate (EGCG)(Purity≥95%，Cat #E4143), Pioglitazone (Pio) (Purity≥98%，Cat # E6910) and Dexamethasone (Dex) (Purity≥97%，Cat # E4902) was provided by Sigma Aldrich (Missouri, USA).

2.2. Cell Lines

2.2.1. 3T3-L1

3T3-L1 is a cell line derived from (mouse) 3T3 cells and is generally used for the biological study of adipose tissue (Cristancho *et al.* 2011). 3T3-L1 cells themselves have a fibroblast-like morphology, but under appropriate conditions, cells can differentiate into adipocyte-like phenotypes. The 3T3-L1 cells with adipocyte morphology increase the synthesis and accumulation of triglycerides, form intracellular fat droplets and obtain the signet appearance of adipocytes (Zebisch *et al.* 2012). These cells are also sensitive lipolytic hormones and drugs, including epinephrine, isoproterenol and insulin, and can therefore be used to observe and evaluate the effects of drug intervention (Loo *et al.* 2009). This is why we chose 3T3-L1 cells as a model for the first phase of the study.

3T3-L1 mouse embryonic fibroblasts obtained from the American Type Culture Collection (Manassas, VA) were grown in a cell culture flask with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum, 1% streptomycin and penicillin at $37^{o}C$, 5% CO_2 until 80% confluence. 3T3-L1 pre-adipocytes were seeded at 2×10^4 cells per well in a 24-well plate until confluence was reached. For adipocyte differentiation induction, the cell culture was incubated with a differentiation medium (DMEM containing 1.0µM dexamethasone, 0.5mM methylisobutylxanthine, and 1µM insulin) supplemented with 10% fetal bovine serum (FBS) for 48 h, then replaced with adipocyte maintenance medium (DMEM with 10% FBS and 1 µM insulin).

2.2.2. Adipose-derived stem cells (ADSCs)

Adipose-derived stem cells (ADSCs) are stem cells isolated from adipose tissue that have multi-directional differentiation potential. They have the ability to differentiate into and repair various organs and tissues. They are a subset species of mesenchymal stem cells. The study found that ADSCs cells can stably proliferate *in vitro* and have a low mortality rate (Woo *et al.* 2016). At the same time, ADSCs cells can be obtained easily, and a small amount of tissue can be used to obtain a large number of stem cells. With a wide range of sources and a large amount of internal reserves, the ADSCs cells also have the advantages of suitable large-scale culture and minimal damage to the donor (Zuk *et al.* 2001 Beane *et al.* 2014). Whilst they are suitable for the research. The use of stem cells and drugs and can also be used for autologous transplantation.

The cells used here were donated from an existing research project approval under Macquarie University human research ethics committee (Ref #: 5201100385). ADSC isolation and culture was performed using previously published methods as previously described (Bunnell *et al.* 2008). Adult ADSCs were derived from abdominal lipo-aspirates and subsequent steps were conducted under sterile conditions in a class II laminar flow hood (Clyde-Apac BH2000 series). Lipo-aspirates were rinsed twice in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco), connective tissue digested with collagenase type 1 (Gibco) for 45 minutes at 37ºC before centrifugation at 1600×g for 10 minutes at 4ºC to separate adipocytes from the stromal vascular fraction (SVF). The pellet was resuspended in 3 ml of D-MEM and layered on top of 3 ml of Ficoll Paque PLUS (Sigma-Aldrich) to remove red blood cells from the SVF. The resulting purified stromal vascular fraction (SVF) was aliquoted into a T25 culture flask (Nunc) in Delbucco's modified eagle medium (D-MEM) Glutmax/F12 (Gibco) with 10% Fetal

bovine serum (FBS) (Gibco, Auckland, NZ) and 1% antibiotics/antimycotics (ABAM) (Invitrogen, California, USA) and incubated at 37ºC at 5% CO2 for 48 hours until ADSCs adhered to the culture flask. Non-adherent cells were eliminated by replacing the media. All isolations were confirmed CD45 negative and CD90 positive (data not shown). ADSCs were passaged 3~5 times by detaching cells with TrypLE Express (Gibco) and before being utilized in differentiation experiments.

2.2.3. Theca cells

Rat ovaries were collected at a local procedure room at UTS Ernst facility, placed into cold PBS (4°C) supplemented with penicillin (500IU/ml), streptomycin (500g/ml) and amphotericin B (3.75g/ml), maintained in a freezer bag and transported to the laboratory within 1h. After two washes with PBS and ethanol (70%), granulosa cells were aseptically harvested by aspiration from follicles with a 26-gauge needle and released in medium contain heparin (50IU/ml), centrifuged for pelleting and then treated with 0.9% pre-warmed ammonium chloride at 37°C for 1min to remove red blood cells. Cell number and viability were estimated using a hemocytometer under a phase contrast microscope after vital staining with trypan blue (0.4%). Cells were seeded at different plating densities (see below) in culture medium composed of McCoy's 5A medium (Sigma) supplemented with supplemented with 10% FBS and 0.1% Penicillin-Streptomycin.

After passage 3, cells were seeded in 24-well plates at 2×10^4 cells/well in 0.5mL of McCoy's 5A medium (Sigma), supplemented with 10% FBS and 0.1% Pen-Strep. After 48 hours incubation, cells medium was then collected and replaced with either McCoy's 5A medium with 1% BSA or medium supplemented with 10µmol/L dexamethasone.

GTP was then added to the medium at various concentrations (1.0μg/mL, 5.0μg/mL and 10μg/mL). Cells were left to incubate at 37ºC for 24 hours in 5% humidity. At this time, medium was collected and replaced with the same treatment medium. Medium was further collected and replaced at 48 and 72 h.

The samples of media were then assayed for levels of estrogen, progesterone and testosterone hormones.

2.3. Ethics approval

This study does not involve any animal research, so we were not required to obtain ethics approval from the University of Technology, Sydney (UTS) Animal Care & Ethics Committee (ACEC).

2.4. Histology and Immunology stains

2.4.1. Oil red O stain

Cells stained with Oil Red O (Sigma-Aldrich, St Louis, MO, USA) were used to detect lipid droplets in adipocytes. 3 parts of Oil Red O stock solution mixed with 2 parts *Deionized water* (DI water) and incubate 10 minutes at room temperature. A piece of Whatman filter paper (Sigma-Aldrich, MO, USA) was applied in a funnel above a vessel, then get the fresh Oil Red O working solution through the filter funnel.

Cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formalin at room temperature for 1 hour. After fixation, the formalin was removed from

each well and cells were washed once with PBS. One millitre of 60% isopropanol was added to each well and removed after 5 minutes. Cells were allowed to dry completely at room temperature.

They were then stained with filtered Oil Red O solution (60% isopropanol, 40% water) for 30 min. After staining the lipid droplets, the Oil Red O staining solution was removed, and the plates were rinsed with water to remove unbound dye. One millilitre of Mayer's haematoxylin stain was added to each well and incubated for 10 minutes at room temperature. The haematoxylin stain solution was removed and rinsed with *Deionized water* (DI water) until the rinse became clear and the slides were then dried at room temperature.

The stained lipid droplets were viewed with an Olympus microscope (Tokyo, Japan) and images were captured with digital camera (DP70, Tokyo, Japan), then quantitated using Image J software (Media Cybernetics, Inc. MD, USA) from 4 individual experiments.

2.4.2. Immunofluorescence stain

In a 24 well plate, cells were grown on coverslips until 80% confluence, and then underwent differentiation. Following this, wells were washed with cold DPBS and then fixed with 2% PFA. Cells were then washed with PBST solution (PBS, 0.06% Tween 20, 0.04% Triton 100) and then blocked with 5% BSA. After blocking, cells were incubated with 1:100 primary antibodies in 2% BSA in PBS overnight at 4°C. Cells were washed with fresh PBS and then 1:200 anti-mouse FITC IgG (secondary Antibody) was added to the cells and mixed gently on a shaker. Cells were then mounted with 0.1ug/mL PI. The stained cells were viewed with an Olympus microscope (Tokyo, Japan) and

images were captured with digital camera (DP70, Tokyo, Japan). The staining channels of Cells were quantitated using FIJI (Image J) software (Schindelin *et al.,* 2012 from 4 individual experiments.

2.4.3. SYBR-DNA labeling assays.

To determinate cell cycle arrest or cell proliferation, DNA labelling with SYBRH Green I (SYBR) was used. DNA content can be accurately measured by fluorescence spectrometry, in a SYBR-DNA labelling assay (or SYBR green fluorescent assay).

Cell proliferation was determined in two steps according to previous studies (McGowan *et al.* 2011). Cells were cultured in a T75 flask, and then seeded at 5 concentrations (0 cells [unseeded], 300 cells, 600 cells, 900 cells) per well in triplicate. The unseeded wells without were used for background readings that were subtracted from the standard curve for statistical accuracy. The well containing 600 cells is the optimal density and was used in further experiment.

Cell culture as above was repeated and at 600 cells/well in 96 well plate. The cells were treated with a series of concentrations of GTP (0 µg/ml, 0.1µg/ml, 1 µg/ml, 10µg/ml and 100µg/ml) 4 x 96 well plate. The 4 plates were subjected to treatment for 24 hours, 48 hours, 72 hours and 96 hours. Medium was removed from plates after the specified time and the plate was stored for analysis at -80C. The cold plates were probed with SYBR I Green DNA stain diluted 1:8000 in 200µl hypotonic lysis buffer (10mM Tris pH 8.0,2.5mm EDTA, 0.1% Triton X-100) and incubated at 4˚C for 72 hours in the dark. Absorbance were read on a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) by using Tecan plate reader at 485nm excitation and 530nm

emission filter. The signal is proportional to number of nuclei and indicative of cells number (McGowan *et al.* 2011, Dilda *et al.* 2005). And data is analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA). To present the data the program PRISM (GraphPad Software Inc., La Jolla, CA, USA) can be used.

2.5. Western blot analysis

Protein concentration were measured for each treatment using the Quick Start Bradford Dye Reagent (Bio-Rad, Hercules, CA). Concentration total protein (20 µg) extracted from cell lysates was calculated and were then loaded onto a 12% SDS-PAGE for protein separation and electro blotted onto 0.45 µM polyvinyldenedifluoride (PVDF) membrane. Blotted membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20. Primary antibodies from Santa Cruz Biotechnology (Dallas, TX, USA) were diluted at 1:1000and incubated overnight at 4°C. After three washes in PBS-Tween, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1: 10,000) for 2 h at room temperature. Protein expressions were visualized by the ECL system (Pierce, Rockford, IL) and analysed using Quantity One 4.6.1 software of the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA).

2.6 Quantitative Real-time Reverse Transcription Polymerase Chain reaction (qRT-PCR)

Cells ready to be harvested were treated in cold PBS. Residual culture medium was removed by three repeats of washing and centrifugation at 500 × g for 3 minutes. Cells

(1×10^5) were subjected to RNA extraction using an RNeasy Mini kit (Qiagen, MD, USA) according to manufacturer's instructions. The concentration of RNA was measured with a Nano Drop, record the A260/A280 and A260/A230 ratios as well as the amount of RNA recovered (in ng/µL), which used for calculation in the elution step of RNA purification. The RNA was purified with RQ1 RNase-Free DNase Kit (Promega, NSW, AU). Reverse transcription was performed in a total of 20 µL volume containing 4 mM MgCl$_2$, PCR buffer, 0.5 mM dNTPs, 2.5 µM random decamers, RNA and nucleotide-free water. Negative controls were included for all reactions (ie without reverse transcriptase or without the RNA) were used to test for any RNA or DNA contamination. An internal positive control was used to test for expressions of housekeeper gene *Actb*. Amplification of cDNA with the NCBI designed target primers (as per instructions from web site http://www.ncbi.nlm.nih.gov/). cDNA was amplified on a Stratagene MXPro-Mx3000P (Agilent, NSW, Australia) with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The PCR cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s, and then 10 min at 72°C. The Delta Ct (= Ct[Target Primers] - Ct[*Actb*]) was a measure of relative changes of Target mRNA content of the cells from 3 individual experiments. Data was normalized to the control treatment mRNA levels. A plot of $2^{-(\text{normalized DeltaCt})}$ was shown.

2.7. Biochemical assays

2.7.1. Measurement of cellular triglyceride content

Cellular triglyceride contents were measured on day 10 after differentiation induction

and treatment. Cells were grown in 24 well plate (the cell seeding number depending on the cell line) and then medium was removed and washed with PBS. Differentiated cells were harvested with 200 µl PBS from each well, then centrifuged at $1000 \times g$ for 10 min. The pellet was dissolved in 1 ml lipid extracting solution (chloroform: methanol = 2:1). After drying under N_2 gas, the triglyceride extract was reconstituted in a known volume of absolute ethanol and a sample (5µl) of triglyceride contents were quantified using the commercial triglyceride assay kit (Wako Pure Chemical Industries, Osaka, Japan), the results spectrophotometrically by measuring the absorbance of the sample at 490 nm on a microplate reader (Model 3550-UV. Bio-Rad Laboratories, Hercules, CA, USA) and compared it to that of known amounts in a standard curve. The final concentration was calculated using cells number counted after harvest. Data was analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA) and graphs were drawn up using PRISM 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

2.7.2. Alkaline phosphatase (ALP) activity Quantitative assay

Cells in culture within 24 well plate had medium removed and washed three times with DPBS. Cells from each well were harvested by scraping and were suspended in 1 ml saline solution. The cells were then completely lysed by sonicator for 30 seconds with 50% power (Sonics & Materials $^{@}$ Vibra Cell, USA). The sonicates were centrifuged for 10 minutes at $10000 \times g$, and the supernatants were subjected for the ALP activity assay using automatic biochemistry instrument (ARCHITECT, Japan). The data was analyzed and drawn up in PRISM 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

2.7.3. Calcium（Ca）Quantitative assay

Cells in culture had medium removed and were washed twice with DPBS. After addition of 200µl 0.5M HCl in plastic tubes, the tubes were sealed and rotated overnight at room temperature. All the mixtures were sonicated for 30 seconds with 50% power (Sonics & Materials $^{@}$ Vibra Cell, USA), then centrifuged at 5000× g for 10 minutes (Ungrin *et al.* 1999, Davies *et al.* 1954). The supernatant was collected for the calcium assay using automatic biochemistry instrument ARCHITECT c System (Abbott ARCHITECT, Japan) by commercially available Arsenazo Dye methodology (Abbott Calcium 7D61).

2.7.4. Hormone levels tests Laboratory

Theca cells isolated from chow-fed rats were used from passage 3. Cells were seeded in 24-well plates at $2x10^4$ cells/well in 0.5mL of McCoy's 5A medium (Sigma), supplemented with 10% FBS and 0.1% Pen-Strep. After 48 hours incubation, the medium was collected and replaced with either McCoy's 5A medium with 1% BSA or medium supplemented with 1.5µmol/L dexamethasone that contained three concentrations of Green tea polyphenols (GTP) (0.1µg/ml, 1µg/ml and 10µg/ml). Cells were left to incubate at 37ºC for 24 hours in 5% humidity. Culture media of treated cells was collected and replaced at 0 hour and 24 hours, the collected media was assayed for hormone levels of estrogen, progesterone and testosterone. In brief, the hormone assays were carried out using the automatic biochemistry instrument (ARCHITECT, Abbott Park, Illinois, U.S.A.) according to manufacturer's instruction. The reading of results was blanked with the mean of control groups.

2.8. Statistical analysis

All values are expressed as the means ± SE. Comparisons across the three groups were performed using one-way ANOVA followed by Tukey's test to determine significant differences between the two treatments using PRISM version 6 (GraphPad Software Inc, CA, USA). P-value < 0.05 was considered statistically significant.

Chapter Three

Green tea polyphenols attenuate adipogenesis in 3T3-L1 preadipocytes through down-regulating expressions of C/EBPα and SREBP-1c

3.1 Introduction

Obesity markedly increases the risk of developing type 2 diabetes, cardiovascular diseases, cancer, and many other illnesses. Therefore, the current obesity pandemic is placing a huge burden on public health worldwide. Obesity is defined by increased adiposity, which, on a cellular level, is driven by both hypertrophy (adipocyte size increase) and hyperplasia (adipocyte number increase) (Drolet *et al.* 2008; Drolet R *et al.* 2008) Hyperplasia results from the differentiation of new adipocytes from preadipocytes. This process, called adipogenesis, is important for adipose tissue turnover and expansion in normal and obese individuals (Spalding *et al.* 2008). Adipose tissue is traditionally viewed as a passive reservoir for excess energy intake. Recently, extensive experimental studies identified it as a dominant regulator of whole-body lipid and glucose homeostasis (Kershaw *et al.* 2004). Indeed, hyperplasia and hypertrophy of adipocytes not only increases fat storage in adipose tissue but also accompanies adipose dysfunction, including the secretion of abnormal levels of cytokines linked to low-grade chronic inflammation, impairment of triglyceride storage and an increase in lipolysis (Guilherme *et al.* 2008; Savage *et al.* 2007; Flier 2004). These abnormalities can contribute to increased fatty acids in the circulation and lead to an overload of fatty acids in the skeletal muscle (glucose disposal), liver (glucose disposal, gluconeogenesis and lipoprotein production) and pancreas (insulin secretion), which, in turn, exacerbates insulin resistance and promotes the development of type 2 diabetes. Therefore, one of the key targets to prevent or treat insulin resistance and type 2 diabetes in obesity is to reduce preadipocyte differentiation and control adipose dysfunction.

Currently, there is a lack of pharmaceutical drugs for adipose dysfunction. Antihyperglycemic agents, such as thiazolidinediones (TZDs), improve peripheral

insulin resistance; however, their impact on adipocytes leads to a marked increase in weight gain in diabetic patients and animals (Smith *et al.* 2006). To discover and develop new agents to control obesity and insulin resistance syndrome, naturally occurring compounds have received great attention in recent years as they portray minimal side effects and are cost efficient and readily available in our society. Green tea polyphenols (GTP) have been found to have health-promoting metabolic effects to ameliorate insulin resistance, type 2 diabetes and obesity (Kao *et al.* 2006). Recent studies have shown that GTP and its major active compound epigallocatechin gallate (EGCG) have beneficial effects on both hyperlipidemia and hyperglycemia (Kao YH *et al.* 2000). Experimental and clinical studies showed that GTP effectively controls weight gain associated with decreasing body fat mass (Kao *et al*. 2006;Liao *et al.* 2001; Lin *et al.* 2006). These studies suggest that possible mechanisms of GTP and EGCG in targeting obesity include stimulating energy expenditure and fat oxidation as well as increasing faecal lipid excretion (Dulloo, Duret, Rohrer, Cirardier*, et al.* 1999). Despite this, however, whether GTP is capable of regulating adipogenesis at a molecular level or not has not been studied.

Adipogenesis is controlled by a tightly regulated transcriptional cascade in which a variety of proteins, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein-α (C/EBPα), sterol regulatory element-binding protein-1c (SREBP-1c) and other factors, play a decisive role in the differentiation from preadipocytes to mature adipocytes (Tang *et al.* 2003; Darlington *et al.* 1998; Eberle *et al.* 2004; Farmer 2006). In spite of our and other previous studies having shown the beneficial effects of GTP on body weight and lipid metabolism (Lin and Lin-Shiau 2006; Dulloo, Duret, Rohrer, Cirardier, *et al*. 1999; Kim *et al.* 2013), the molecular mechanisms by which GTP controls adipogenesis through regulating adipogenic regulators remains to be elucidated. The hypothesis was that GTP is capable

of controlling adipogenesis and reducing *de novo* synthesis of triglycerides in adipocytes, thus diminishing adipose hyperplasia and hypertrophy to prevent fat mass formation. This was examined by observing 3T3-L1 preadipocytes differentiation with a variety of dosages of GTP treatment. To elucidate the mechanisms of GTP on adipogenesis, the regulatory factors of adipogenesis, including C/EBPα, PPARγ and SREBP-1c, were determined with immunoblotting and a semi-quantitation of mRNA expression.

3.2. Research Plan and Methods

3.2.1. Green Tea Polyphenols, (-)-epigallocatechin-3-gallate and pioglitazone

The tea polyphenols (99% purity) were extracted from green tea leaves (*Camellia sinensis*) grown in Guizhou province, south-western China, by Zuyi Lushen Kangyuan Co (Guizhou, Meitan, China). The polyphenolic compounds identified using liquid chromatography-mass spectrometry (LC-MS) were composed of 68% EGCG, 7% epigallocatechin, 1% epicatechingallate and 18% epicatechin in GTP (w/w) as previously described [(Kim JJ *et al.* 2013). In order to determine the effect of GTP as only attributed to EGCG or total polyphenols, pure EGCG (Sigma-Aldrich, MO, USA) was used as a control and pioglitazone (Sigma-Aldrich, MO, USA) was used as a positive drug control.

3.2.2. Cell culture, differentiation and GTP Treatment

3T3-L1 preadipocytes obtained from ATCC (VA, USA) were grown in Dulbecco's

Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum, 1% streptomycin and penicillin at 37°C, 5% CO_2. For differentiation, 3T3-L1 preadipocytes were cultured in a 24-well plate to reach confluence and induced with DMEM supplemented with 10% foetal bovine serum (FBS), 1.0 µM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM methylisobutylxanthine (IBMX, Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/ml insulin. After 2 days of induction, cells were maintained in DMEM containing 10% FBS and insulin with or without treatment. Treatment agents were first dissolved in DMEM and then added into the corresponding wells, with GTP at concentrations of 0.1, 1 and 10 µg/ml and pioglitazone at 100 µM. The medium was changed every 2 days. After 9 days of differentiation induction and treatment, the 3T3-L1 adipocytes were collected in order to count the fat cell number, measure lipid accumulation and cellular triglyceride contents, and extract total protein for Western blotting assay and RNA for gene expression assay.

3.2.3. Cell Viability Assay

To determine the cytotoxicity of GTP and EGCG after induction and treatment, cell cultures were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5mg/ml) for 4h at 37 C. Subsequently, the MTT medium was replaced with DMSO. The optical density of the resulting supernatant was measured photometrically at 570 nm using a micro-plate reader (Bio-Tek Instruments Inc., Winooski, VT) (Stoddart 2011). Cell viability was determined to the control cells.

3.2.4. Oil Red O staining

After the differentiation induction and treatment described in 2.2, cells were stained with Oil Red O (Sigma-Aldrich, St Louis, MO, USA) to detect lipid droplets in adipocytes. Cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formalin at room temperature for 1 h. After fixation, cells were washed once with PBS and stained with filtered Oil Red O solution (60% isopropanol, 40% water) for 30 min. After staining the lipid droplets, the Oil Red O staining solution was removed, and the plates were rinsed with water to remove unbound dye, dried and photographed. The stained lipid droplets were viewed and quantitated with an Olympus microscope (Tokyo, Japan) and images were captured with digital camera (DP70, Tokyo, Japan) using Image-Pro6.2 software (Media Cybernetics, Inc. MD, USA).

3.2.5. Measurement of Cellular Triglyceride Content

Quantitation of cellular triglyceride contents were performed on day 9 after differentiation induction and treatment. After washing with PBS buffer, differentiated cells were harvested with 200 µl PBS buffer then centrifuged at $1000 \times g$ for 10 min. The pellet was dissolved in 1 ml lipid extracting solution (chloroform: methanol = 2:1). After drying under N_2, lipids were lysed in 100 µl ethanol. The triglyceride content in the cell lysates were quantified using the commercial triglyceride assay kit (Wako Pure Chemical Industries, Osaka, Japan). The concentration was collaborated using the cells number counted after harvest.

3.2.6. Immunoblotting analysis

To detect the molecular mechanism of GTP on preadipocyte differentiation and adipogenesis, cells were lysed on day 9 after differentiation induction with a variety of treatments. Protein concentration was measured for each treatment using the Quick Start Bradford Dye Reagent (Bio-Rad). Protein expressions of PPARγ, C/EBPα and SREBP-1c were measured with immunoblotting. In brief, the total protein (20 μg) extracted from cell lysates was separated by 12% SDS-PAGE and electroblotted onto a 0.45 μM polyvinyldene difluoride (PVDF) membrane. Blotted membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20. The primary antibodies from Santa Cruz Biotechnology (CA, USA) for anti-PPARγ (SC-7273), anti-C/EBPα (SC-166258), anti-SREBP-1c (SC-367) and anti-ß-actin (SC-47778) were diluted at 1:1000 and incubated overnight at 4°C. After three washes in PBS-Tween, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1: 10,000) for 2 hours. Protein expressions were visualized with the ECL system (Pierce, Rockford, IL) and analyzed using Quantity One 4.6.1 software of the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.7. Quantitative Real-time Reverse Transcription Polymerase Chain reaction (qRT-PCR)

To further clarify whether GTP treatment affected expressions of adipogenic genes, transcription of C/EBPα, PPARγ and SREBP-1c genes were determined by qRT-PCR in preadipocytes treated with varying concentrations of GTP after day 9 of the differentiation induction. 3T3-L1 cells were treated in cold PBS to discard the culture medium by three repeats of washing and centrifugation at $500 \times g$ for 3 minutes. 1×10^6

cells were subject to RNA extraction by manufactory instruction of RNeasy Mini kit (Qiagen, Cat. No. 74104). The RNA was purified with RQ1 RNase-Free DNase Kit (Promega, Alexandria NSW, Australia). Reverse transcription was performed in a total of 20 μL volume containing 4 mM MgCl2, PCR buffer, 0.5 mM dNTPs, 2.5 μM random decamers (Applied Biosystems/Ambion), RNA and nucleotide-free water. Negative controls for all reactions were without reverse transcriptase or without the RNA sample (to test for any RNA or DNA contamination, respectively). An internal positive control was to test for the expression of housekeeper gene *Actb*. Amplification of cDNA with primers (Thermo Fisher Scientific, Massachusetts, USA) showing in the Table 3.1. cDNA was carried out using Stratagene MXPro-Mx3000P (Agilent Technologies, Waldbronn, Germany) with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The PCR cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s, and then 10 min at 72°C. The Delta *Ct* (= *Ct*[*Cebpa, Pparg* or *Srebp-1c*] - *Ct*[*Actb*]) was a measure of the relative changes in *Cebpa*, *Pparg* and *Srebp-1c* mRNA content of the cells. Data were normalized to the control treatment mRNA levels. A plot of $2^{-(\text{normalized Delta}Ct)}$ was shown.

Table 3.1. Primer sequences used in real-time PCR

Symbol*	Forward primer	Reverse primer
Cebpa	AGCAACGAGTACCGGGTA	TGTTTGGCTTTATCTCGG
Pparg	CAAGAATACCAAAGTGCGATCAA	GAGCTGGGTCTTTTCAGAATAATAAG
Srebp-1c	GATCAAAGAGGAGCCAGTGC	TAGATGGTGGCTGCTGAGTG
Actb	TCACCCACACTGTGCCCATCTA	TTGCTGATCCACATCTGCTGG

Real-time PCR primer set for adipose (mouse) were obtained from Thermo Fisher Scientific Inc.

3.2.8. Statistical Analysis

All values are expressed as the means ± SE. Comparisons across the three groups were performed using a one-way ANOVA followed by Tukey's test to determine significant differences between the two treatments using Prism version 6 (GraphPad Software Inc, CA, USA). P-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1. Effect of GTP on Cell Viability

GTP treatments (0.1, 1 and 10 µg/ml) and EGCG (0.68 µg/ml) did not affect cell viability, with the cell number being similar to control group. In contrast, pioglitazone had a pronounced anti-proliferative effect, with the cell number falling to 88.6 ± 1.3% ($p <$ 0.05) of control values (Figure 3.1).
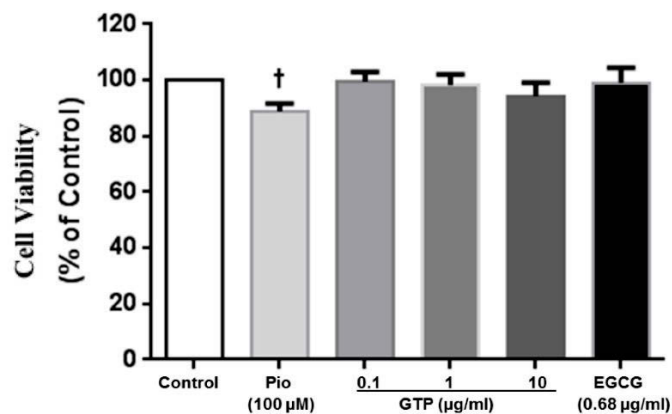
Figure 3.1 Effect of GTP and EGCG on cell viability during differentiation induction.
3T3-L1 preadipocytes were treated with cell culture medium (Control), pioglitazone (Pio, 100 µM), GTP (0.1, 1, 10 µg/ml) or EGCG (0.68 µg/ml) during differentiation induction, and cell viability was measured by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Values are expressed as mean ± SE (n = 5). †$p <$ 0:05 vs. control.

3.3.2. Effects of GTP on lipids accumulation during 3T3-L1 pre-adipocytes differentiation

To examine effects of GTP on adipocyte differentiation and lipids accumulation, 3T3-L1 pre-adipocytes were treated with 0.1, 1 and 10 µg/ml GTP in presence of DM. Cells accumulated intracellular lipids gradually from day 0 to day 9 after the addition of induction reagents. 3T3-L1 cells were differentiated into mature adipocytes, which were accumulated with a quantity of oil droplets, as detected by Oil-red O staining on day 9 (Figure 3.2A). The quantity of extracted intracellular Oil Red O was shown in Figure 3.2B. 100 µM of pioglitazone significantly increased lipids by $39.6 \pm 3.6\%$ ($p < 0.001$) compared to the control. GTP concentrations at 0.1, 1 and 10 µg/ml reduced lipid content in 3T3-L1 cells by $13.5 \pm 2.5\%$ ($p < 0.05$), $24.9 \pm 0.9\%$ and $36.0 \pm 1.5\%$ (both $p < 0.01$) respectively compared to the control. Overall, Oil Red O staining revealed a significant dose–dependent decrease in lipid droplet formation in GTP-treated 3T3-L1 cells.
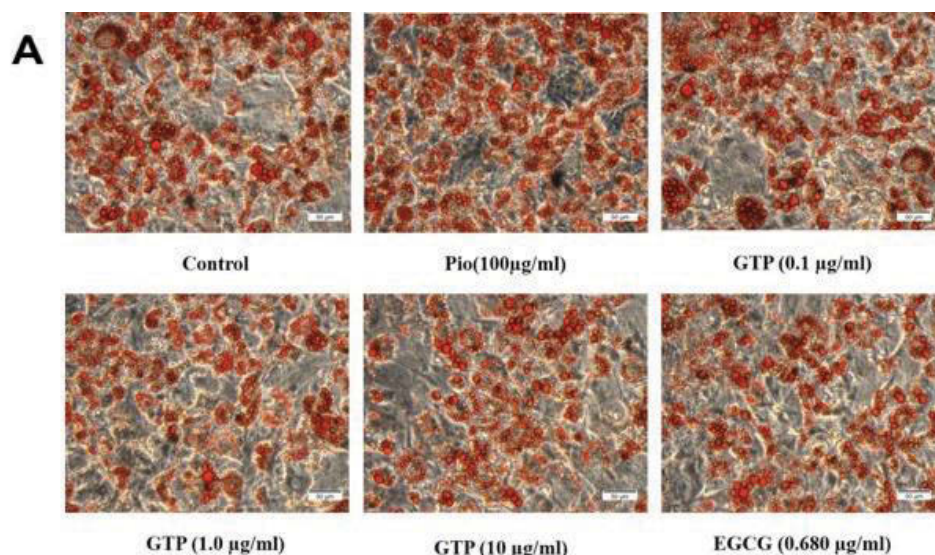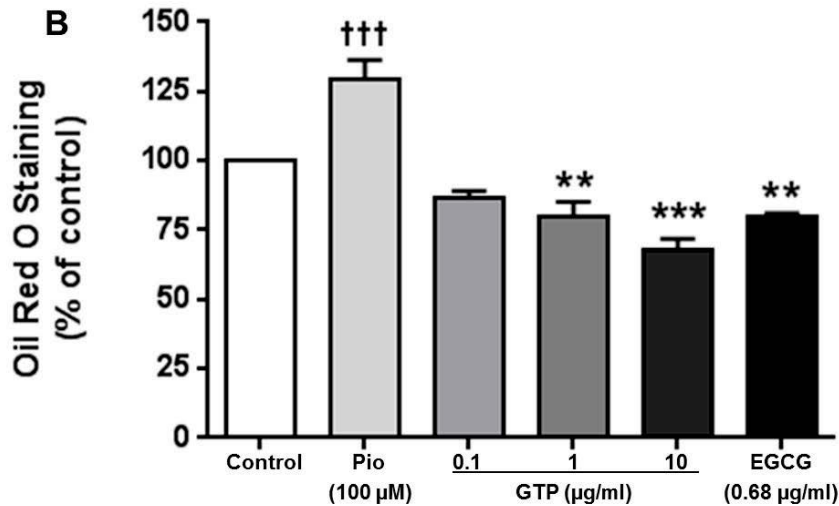
Figure 3.2 Effects of GTP on lipids accumulation during 3T3-L1 pre-adipocytes differentiation. The lipid droplets were stained with Oil Red O (A). Analysed results of lipid areas (B) are shown as means ± SE of four independent experiments. *$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$ GTP and EGCG treatments versus the control. $^{†††}p < 0.001$ pioglitazone treatment versus the control.

Concordant with the reduced lipid staining, intracellular triglyceride contents were reduced by 13.6 ± 1.9% ($p < 0.05$), 27.8 ± 3.7% and 38.6 ± 2.2% ($p < 0.01$) respectively in GTP treated cells, and EGCG also significantly reduced triglycerides by 21.7 ± 2.0% ($p < 0.01$) compared to the control cells. In contrast to the GTP treatment, 100 µM pioglitazone increased triglyceride contents by 27.1 ± 2.6% ($p < 0.01$) when compared with the control cells (Figure 3.2).
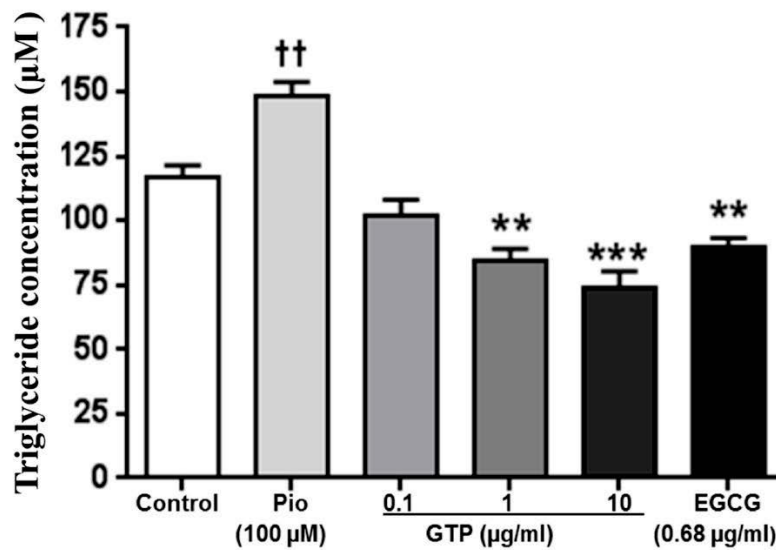
Figure 3.3 Effect of GTP, EGCG and pioglitazone treatments on cellular triglyceride content. On day 9 of 3T3-L1 preadipocytes differentiation. Results are means ± SE of four independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ GTP and EGCG treatments versus the control. ††$p < 0.01$ pioglitazone treatment versus the control.

3.3.3. Effects of GTP on C/EBPα, PPARγ and SREBP-1c expression during 3T3-L1 pre-adipocytes differentiation

Differentiation of 3T3-L1 pre-adipocytes to adipocytes are regulated by key transcription factors, including C/EBPα, PPARγ and SREBP-1c. To elucidate the mechanism of action for decreasing pre-adipocyte differentiation with GTP, the protein expression of C/EBPα, PPARγ and SREBP-1c were measured with Western blotting on day 9 after differentiation induction and treatments. Figure 4 showed that 100 μM pioglitazone significantly increased expressions of C/EBPα, PPARγ and SREBP-1c by 41.1 ± 5.5%, 36.0 ± 4.8% and 92.6 ± 4.5 % (all $p < 0.001$) respectively, compared to the control. GTP treatment at doses 0.1 μg/ and 1 μg/ ml (containing 0.68 μg/ ml of EGCG) reduced

expression of C/EBPα, PPARγ and SREBP-1c but all of the results did not achieve statistical significance when compared to the control. EGCG treatment (0.68 µg/ ml) did not significantly alter the expression of C/EBPα, PPARγ and SREBP-1c (Figure 3.4). The cells treated with 10 µg/ml GTP significantly decreased the expressions of C/EBPα, PPARγ and SREBP-1c by $23.8 \pm 6.8\%$, $(p < 0.01)$ $21.7 \pm 6.1\%$ $(p < 0.05)$ and $31.1 \pm 8.0\%$ $(p < 0.05)$ respectively, compared to the control cells (Figure 3.4), suggesting that the effect of GTP on the expressions of adipogenic mediators is dose-dependent.
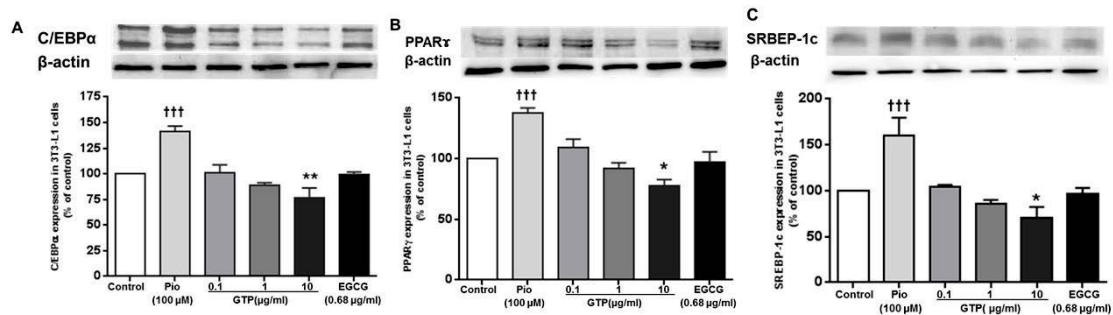
Figure 3.4 Effects of GTP, EGCG and piolitazone on protein expression of C/EBPα (A), PPARγ (B) and SREBP-1c (C) in 3T3-L1 adipocytes using immunoblots. Results of quantitative analysis are shown as means ± SE of four independent experiments. *$p < 0.05$ and **$p < 0.01$ GTP treatments versus the control. †††$p < 0.001$ pioglitazone treatment versus the control.

3.3.4. Effects of GTP on *Cebpa, Pparg* and *Srebp-1c*mRNA expression

Adipocyte transcription factors such as C/EBPα, PPARγ and SREBP-1c play a crucial role in adipocyte differentiation, adipogenesis, and the accumulation of cellular lipid droplets. The fact that GTP reduced adipocyte differentiation prompted us to investigate the effects of GTP on the transcription of *Cebpa, Pparg* and *Srebp-1c*. Treatment with pioglitazone caused significantly increased expression of *Cebpa, Pparg* and *Srebp-1c* (all $p < 0.001$). Preadipocytes treated with 0.1, 1 or 10 µg/ml GTP during differentiation

demonstrated a significant reduction in *Cebpa* transcript compared to control adipocytes (Figure 3.5A). Similarly, a significant ($p < 0.01$) reduction in *Srebp-1c* mRNA was observed in GTP (1 or 10 µg/ml) and EGCG treated cells (Figure 3.5C). The low dose of GTP (0.1 µg/ml) reduced amount of *Pparg* transcript by $68.7 \pm 10.2\%$ when compared to control adipocytes ($p < 0.05$) (Figure 3.5B). Despite this, however, the transcription of *Pparg* gene in preadipocytes treated with 1.0 - 10 µg/ml GTP and EGCG during differentiation was not significantly different compared to control cells (Figure 3.5B).
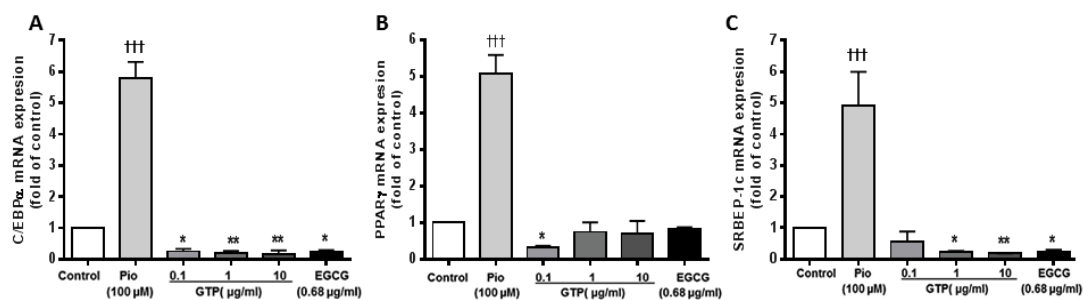
Figure 3.5 Effects of GTP, EGCG and piolitazone on mRNA expression of Cebpa (A), Pparg (B) and Srebp-1c (C) gene expressions using real-time quantitative RT-PCR. Mean ± SE of four independent replicates of relevative mRNA expression to the control. Each reaction contained 2.5 ng RNA to be tested. *$p < 0.05$ and **$p < 0.01$ GTP treatments versus the control. $^{†††}p < 0.001$ pioglitazone treatment vs. the control.

3.4. Discussion

Adipogenesis is the process by which undifferentiated precursor cells (pre-adipocytes) differentiate into fat cells (mature adipocytes). Increased adipogenesis contributes to adipose tissue expansion and the development of obesity. Moreover, impaired adipogenesis in obesity is implicated in the development of metabolic complications, such as type 2 diabetes and cardiovascular diseases. Hence, early intervention into impaired adipogenesis is important to normalizing adipose tissue biology and preventing

obesity and associated metabolic diseases. Previous studies have shown that green tea-derived polyphenols have beneficial effects on obesity and type 2 diabetes by blocking lipid absorption in the gastrointestinal tract, thus stimulating thermogenesis and fat oxidation (Hsu *et al.* 2008; Fukino *et al.* 2008; Rains *et al.* 2011; Chen *et al.* 1997; Dulloo, Duret, Rohrer, Girardier*, et al.* 1999). In this study, we demonstrated that GTP processes a direct anti-adipogenic effect, as evidenced by the significantly reduced number and size of oil droplets in GTP treated 3T3-L1 cells at day 9 of the differentiation induction. The inhibitory effects of GTP on adipogenesis were in a dose-dependent manner, showing the lowest lipid accumulation and triglyceride contents in 10 µg/ml of GTP treated cells (P<0.001). These results suggest that GTP can prevent adipogenesis and the accumulation of cytoplasmic lipid droplets during the differentiation stage of 3T3-L1 preadipocytes. Adipogenesis was slightly lower in 1 µg/ml of GTP (containing 0.68 µg/ml EGCG and 0.32 µg/ml other polyphonic compounds) treated cells when compared with EGCG control (0.68 µg/ml), suggesting that EGCG is the principal active compound for inhibiting adipogenesis within the four polyphonic compounds. Primary obesity is an enlargement of adipose tissue mass not only through an increase in fat cell volume (hypertrophy) by lipid accumulation, but also by the proliferation of preadipocytes with subsequent differentiation into mature adipocytes (hyperplasia). Our results suggest that GTP can directly regulate the adipogenic process, thereby reducing body fat mass through the dedifferentiation of preadipocytes.

To understand the mechanism by which GTP attenuated adipogenesis, we detected the expressions of C/EBPα and PPARγ, which are critical transcription factors involved in adipocyte differentiation and lipid accumulation (Rosen *et al.* 2002). Our data indicates that GTP possibly promotes the dedifferentiation of maturing preadipocytes via downregulating C/EBPα and PPARγ expression. To our knowledge, this is the first study

to elucidate the effects of GTP on adipocyte differentiation together with analyzing the adipogenic transcription factors at protein and gene levels. The CCAAT/enhancer binding proteins include C/EBPα, C/EBPβ, and C/EBPδ. These subtypes play different roles in adipocyte differentiation (Chen L *et al.* 2013). C/EBPβ and C/EBPδ induce low levels of PPARγ and C/EBPα, which are then able to induce each other's expression in a positive feedback loop that promotes and maintains the differentiated state (Rosen *et al.* 2002). PPARγ can promote adipogenesis in C/EBPα-deficient cells, but C/EBPα has no ability to promote adipogenesis in the absence of PPARγ, indicating that C/EBPα and PPARγ participate in a single pathway of fat cell development with PPARγ being the proximal effector of adipogenesis (Rosen *et al.* 2002). GTP significantly reduced both C/EBPα and PPARγ in a high dosage (10 µg/ml) and this data showed a correlative relationship between lipid droplets accumulation and the expressions of C/EBPα and PPARγ during 3T3-L1 preadipocyte differentiation. The lesser the expressions of C/EBPα and PPARγ, the greater adipogenesis is reduced. Furthermore, the level of mRNA encoding the adipocyte differentiation marker C/EBPα was markedly decreased in the treated cells inside all tested doses of GTP. In contrast, inhibitory transcription of the PPARγ gene was only observed in a low dose of GTP (0.1 µg/ml) and the treatments with higher doses of GTP and EGCG did not affect the transcription. This different inhibitory ability of GTP in the regulation of the transcription and translation of PPARγ gene may suggest a negative feedback requirement for transcription regulation while the protein synthesis is markedly blocked.

An additional mechanism for reduced adipogenesis by GTP may be through regulating SREBPs expression. SREBPs have three subtypes, SREBP-1a, SREBP-1c and SREBP-2. Among them, SREBP-1c is most highly expressed in brown fat, followed by the liver, white fat, and the kidney. SREBP-1c can promote hepatic lipogenesis as well as the

differentiation of 3T3-L1 preadipocytes (Hsuy *et al.* 2006). SREBP-1c and PPARγ cooperate with each other, and a synergistic effect has been observed in lipid homeostasis *in vivo* (Rosen *et al.* 2000). In this study, the protein expressions of SREBP-1c protein and PPARγ were significantly reduced in GTP-treated 3T3-L1 adipocytes, indicating that the suppressed adipocyte differentiation and TG accumulation by GTP is through the inhibition of the SREBP-1c-PPARγ pathway.

Interestingly, in this study, pioglitazone promoted adipogenesis associated with significantly enhanced C/EBPα, SREBP-1c and PPARγ at the protein level with increased transcription of C/EBPα, SREBP-1c and PPARγ by 12-fold, 4-fold, and 3-fold respectively. Pioglitazone is a PPARγ agonist and can improve insulin sensitivity by increasing adipogenesis and thereby attenuating lipotoxicity, though leading to secondary weight gain [30]. Recent studies indicate that partial PPARγ antagonism by various plant extracts may be beneficial in improving insulin sensitivity and may also inhibit adipocyte differentiation and lipid accumulation (Huang *et al.* 2006; Christensen *et al.* 2009). In the current study, GTP significantly inhibited adipogenesis in differentiating preadipocytes with a concomitant marked reduction in C/EBPα and SREBP-1c at both the translation and transcription levels, and also reduced PPAR protein expression. These results provide important insights into normalizing adipose tissue biology via a combination of GTP with a low dose of pioglitazone. A further *in vivo* study is needed to confirm that the combined therapy has a synergetic effect with less adverse reactions for controlling obesity and type 2 diabetes.

In summary, our results indicate that GTP suppressed the differentiation of 3T3-L1 preadipocytes through down-regulating C/EBPα and SREBP-1c at the gene expression and post-transcriptional level, as well as a reduction of PPARγ protein expression. This

in vitro study also demonstrated the inhibitory effects of GTP on adipogenesis in a dose-dependent manner. A relevant high dose (10 µg/ml *in vitro*) is essential for a therapeutic effect with alteration of adipogenic regulators. Increased differentiation of adipocytes from preadipocytes contributes to fat mass expansion and leads to obesity and type 2 diabetes. Combating unnecessary adipogenesis at the cellular level will prevent the development of obesity and subsequently reduce the risk for type 2 diabetes and cardiovascular disease. It is common sense that green tea beverage is a healthier drink than sweet soft drinks. Despite this, however, tea beverage ingested daily does not provide sufficient amount of GTP for the reduction of body weight and fat mass in humans. With the dramatically increasing prevalence of obesity population, the traslational studies from experimental obeservations *in vitro* and in animal to human clinical trials with appropriate dosages of GTP are needed to develop health products for controlling obesity and associated metabolic diseases at the early stage.

Chapter Four

Potential therapeutic effects of green tea polyphenols on obesity associated osteoporosis

4.1. Introduction

Osteoarthritis (OA) and osteoporosis (OP) are highly prevalent health problems, associated with considerable mortality worldwide (AE 2018). The increase of subchondral bone loss is a characteristic feature of both OP and the early stages of OA, the latter of which is the leading cause of physical disability and reduced quality of life for the aged population. A recent epidemiological study in the Unite state reports that the prevalence of knee OA has doubled since the mid-20th century, affecting at least 19% of American adults aged 45 years and older alongside epidemic proportions of obesity (Wallace IJ *et al.* 2017). Substantial evidence has indicated that a high body mass index (BMI) is a well-known risk factor for knee OA, likely due to the combined effects of joint overloading and adiposity-induced inflammation (Sridhar MS *et al.* 2012; Sartori-Cintra AR *et al.* 2014) . Conversely, weight loss may reduce the risk of OA in overweight subjects, as suggested by the Framingham study in which women who lost an average of 11 pounds decreased their risk of knee OA by 50% (Felson DT *et al.* 1992). The deeper underlying causes of OA's high prevalence in obesity remain unclear, however, hindering prevention and treatment efforts.

Experimental and clinical studies have revealed that fat hypertrophy in obesity increases the expression and release of adipokines (Gierman LM *et al.* 2012; Wu J *et al.* 2018; Toussirot E5 *et al.* 2017) such as interleukin (IL) 1β, IL-6, resistin, leptin and tumor necrosis factor alpha (TNFα), that promote adipocyte differentiation and impact on haematopoiesis, such as interleukin (IL) 1β, IL-6, resistin, leptin and TNFα (Gierman LM *et al.* 2012; Wu J *et al.* 2018; Toussirot E5 *et al.* 2017). Bone and fat cells share a common mesenchymal stem cell (MSC) within the bone marrow, and bone marrow adipose tissue (MAT) accounts for approximately 10% of the total fat mass in healthy

adult humans (Fazeli PK *et al.* 2013). Increases in bone marrow MAT with obesity and diabetes suggest that some adpokines and transcription factors influence MSC differentiation into osteoblasts or adipocytes.

Adipocytes and osteoblasts originate from a common ancestor – pluripotent mesenchymal stem cells (Horowitz *et al.* 2004). Their tendency is the same in the process of differentiation of mesenchymal stem cells (MSCs) into adipocytes or osteoblasts, except for several interacting pathways that regulate the balance of their differentiation. Bone and fat cells share a common mesenchymal stem cell (MSC) within the bone marrow, and bone marrow adipose tissue (MAT) accounts for approximately 10% of the total fat mass in healthy adult humans (Fazeli PK *et al*. 2013). Increases in bone marrow MAT with obesity and diabetes suggest that some adpokines and transcription factors influence MSC differentiation into osteoblasts or adipocytes. Peroxisome proliferator-activated receptor-γ (PPARγ), a ligand activated transcription factor, has acquired a great deal of attention as it is involved in cell differentiation. How it influences the differentiation of MSCs into adipocytes or osteoblasts, however, remains to be defined.

The previous study in chapter three has demonstrated that green tea polyphenols (GTP) inhibit adipogenesis in 3T3-L1 adipocytes. Chani *et al*. demonstrated that epigallocatechin gallate (EGCG), a major component of GTP, can prevent the differentiation of mouse MSCs into their adipogenic lineage (Chani B *et al.* 2016). In order to understand the mechanisms involved in the relation between adipocyte and osteoblast differentiation and to attempt to develop natural therapeutic approaches to osteometabolic diseases, the present study investigates the underlying mechanisms of PPARγ and biomarkers in adipogenesic and osteogenesic pathways during the differentiation of human adipose tissue-derived stem cells (hADSCs) into mature

adipocytes and osteoblasts with or without GTP treatment.

4.2. Research Plan and Methods

4.2.1. Green Tea Polyphenols, (–)-Epigallocatechin-3-Gallate and Pioglitazone.

Green Tea Polyphenols (GTP, 99% in purity) were extracted from green tea leaves (*Camellia sinensis*) grown in Guizhou, Southwestern China, by Zuyilushen Kangyuan Co. (Guizhou, Meitan, China). The polyphenolic compounds were identified using Liquid Chromatography-Mass Spectrometry (LC-MS) and were found to be 68% EGCG, 7% EGC, 1% ECG and 18% EC in GTP (w/w). Pioglitazone(Pio) was purchased from Sigma-Aldrich (St. Louis, MO) and were used as a positive drug control.

4.2.2. Human adipose tissue-derived stem cells isolation and culture

Human adipose tissue-derived stem cells (ADSCs) were kindly provided by Dr Jerran Santos from an existing research project approval under the Macquarie University human research ethics committee (Ref #: 5201100385). ADSCs were isolated with the method previously described (Bunnell *et al.,* 2008). Lipoaspirates were briefly washed three times with phosphate buffered saline (PBS) and digested with same volume of PBS-supplemented 0.2% collagenase type Ⅰ (Gibco, Auckland, NZ) for 45 minutes at 37ºC before centrifugation at $1600 \times g$ for 10 minutes at 4ºC to separate adipocytes from the stromal vascular fraction (SVF). The SVF pellet was resuspended in 3 ml of DMEM and layered on top of 3 ml of Ficoll Paque PLUS (Sigma-Aldrich, St Louis, MO, USA) to remove red blood cells from the SVF. The purified SVF was cultured in growth media

containing DMEM Glutmax/F12 with 10% Fetal bovine serum (FBS), 1% [v/v] 100 units/mL of penicillin, and 100 ng/ mL streptomycin [Gibco BRL, Auckland, NZ]) for 48 hours until cells adhered to the culture flask. Non-adherent cells were eliminated by replacing the media. Human ADSCs were confirmed by CD45 negative and CD90 positive with fluorescein isothiocyanate kits (BD Biosciences, San Jose, CA, USA) using a FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). ADSCs were sub-cultured to passages 5 to 6 for the following *in vitro* studies.

4.2.3. ADSCs differentiation and treatment

To observe the differentiation of ADSCs into adipogenic cells and osteogenic cells, 5 x 103 ADSCs were seeded into 24 well plates to expand to 70% confluence before adding reagents for either adipogenic or osteogenic induction. A DEME medium containing Glutmax/F12 (Gibco®, Auckland, NZ) with 0.5 mM isobutyl-methylxanthine (IBMX), 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin and 10% FBS was used for the adipogenic induction, and a DEME medium with 0.1μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate and 10% FBS was used for the osteogenic induction (Bunnell *et al.*, 2008). To evaluate the effect of GTP on adipogenesis and osteogenesis, GTP (1 and 10 μg/ml) was added into the induction medium in the presence or albescence of 100 μM of pioglitazone (de Souza *et al.*, 2014). The fresh media with treatment reagents were changed every two days.

To determine the optimal time from the differentiation of primary ADSCs into adipogenic cells, the cultures were observed on days 1, 7, 14 and 21 via visual inspection of the lipid droplet formation under light microscopy (Olympus, BX51 microscope, Tokyo, Japan) followed by Oil Red O staining (Sigma-Aldrich, St Louis, MO, USA).

Osteogenic differentiation was analyzed by a measurement of alkaline phosphatase (ALP)

activity and calcium content at each experimental point.

4.2.4. Determination of Lipid Accumulation by Oil Red O Staining

ADSCs culture was harvested at each experimental point for measurement of

intracellular lipid accumulation. Cells were washed three times with PBS and fixed with

10% formalin at room temperature for 1 hour at room temperature. Fixed cells were

washed once with PBS and stained with filtered Oil Red O solution (60% isopropanol,

40% water) for 30 minutes. After staining the lipid droplets, the Oil Red O staining

solution was removed and the plates were rinsed with distilled water to remove unbound

dye, then dried and photographed. The stained lipid droplets were viewed with an

Olympus microscope (Tokyo, Japan) and images were captured with digital camera

(DP70, Tokyo, Japan), then quantitated using Image J software (Media Cybernetics, Inc.

MD, USA) from 4 individual experiments.

4.2.5. Measurement of Cellular Triglyceride Content

Cellular triglyceride contents were measured in the cells collected on day 21 of the

adipogenic differentiation study. Harvested cells were suspended in 200 µl PBS from

each well, then centrifuged at $1000 \times g$ for 10 minutes. The pellet was dissolved in a 1

ml lipid extracting solution (chloroform: methanol = 2:1, v/v). After drying under N2,

lipids were lysed in 100 µl ethanol. The triglyceride content in the cell lysates was

quantified using the commercial triglyceride assay kit (Wako Pure Chemical Industries,

Osaka, Japan) by measuring the optical absorbance at 540 nm with a Microplate reader

(Bio-Rad Laboratories, Hercules, CA, USA). The concentrations were calibrated with the cell numbers counted in each well.

4.2.6. Alkaline phosphatase activity assay

The alkaline phosphatase enzyme (ALP) is expressed in the early stages of differentiation and is a marker of osteoblastic phenotype. ADSCs were cultured in osteogenic differentiation media with variety of treatments for 1, 7, 14 and 21 days at 37oC under 5% CO2 95% air. At each experimental point, cells were harvested from each well with 0.05% trypsin-0.53 mM EDTA for 10 min at 37oC and washed with PBS, lysed in 1 ml of 0.2% Triton X-100 aqueous solution then lysed using a Vibra-Cell sonicator (VXC 500 series, Sonic and Materials Inc., CT, USA) for 30 seconds. The sonicates were centrifuged for 10 minutes at 10000 $\times$g and the supernatants were collected for ALP activity assay using automatic biochemistry instrument (ARCHITECT, Japan). ALP activity was calibrated with the total protein content, determined using the Bradford method. Statistical data analysis was achieved by calculating mean and standard errors for each experimental point performed in triplicate.

4.2.7. Quantitative estimation of intracellular calcium

To detect intracellular calcium content, the cells harvested at each experiment point were dissolved in 200 µl of 0.5M HCl then vigorously shaken for 16 hours at room temperature, all the mixtures were sonicated for 30 seconds then centrifuged at 5000 $\times$g for 10 minutes. The supernatant was collected for calcium assay using automatic biochemistry instrument (ARCHITECT, Japan). The calcium contents were calibrated with cells

number counted in the each well.

4.2.8. Immunofluorescence staining

Immunofluorescence staining was used to determine specific protein expression involved in adipogenesis and osterogenesis. Cells were grown on sterile coverslips in a 24 well plate then underwent 14 days of osterogenic induction and 21 days of adipogenic induction. Before applying immunofluorescence staining, cells were washed twice with PBS and fixed with 4% paraformaldehyde. Following this, cells were permeabilised in PBS with Tween-20 and Triton X-100 (0.06% Tween-20 and 0.04% Triton X-100). Cells were washed twice again with PBS and blocked with a 5% BSA solution containing 10% goat serum.

Cells were then stained with primary antibodies (1:100, PPARγ or RUNX2, Santa Cruz Biotechnology, Texas, USA) in 2% BSA-PBS and incubated overnight at 4°C. BSA-PBS solution was used for negative control. After incubation with the primary antibody, cells were washed with fresh PBS followed by staining with secondary antibodies (1: 400 goat anti-rabbit Alexa Fluor 488 and 1:400 goat anti-mouse IgG Alexa Fluor 568). Cells were washed twice with PBS before counterstaining the cell nuclei with Hoescht®33342 (10 μg/ml), followed by a final wash with PBS. Coverslips were mounted on slides with mounting medium and visualized under an epifluorescent microscope (Olympus BX51, Tokyo, Japan). Images were captured with a digital camera (Olympus DP72, Tokyo, Japan) using a 40×Plan Fluor objective (NA 0.75). Fluorescence intensity of the antibody labelled regions within cells were quantitated using Image J software (Media Cybernetics, Inc. MD, USA).

4.2.9. Quantitative Real-time Polymerase Chain reaction (qRT-PCR)

To further clarify the effects of GTP on ADSCs differentiation, the gene expression of *Pparg*, *Cebpa* and *Creb* which regulate adipogenesis, as well as *Runx2 and Bmp2* genes which regulate osteogenesis, were determined by qRT-PCR.

Total RNA of cell culture at the end of experiment was extracted using a RNeasy Mini kit (Qiagen, MD, USA) and RNA concentration was measured with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity of RNA (A260/A280) was >1.90. To obtain cDNA, reverse transcription was performed in a 20 µl volume containing 4 mM MgCl2, PCR buffer, 0.5 mM dNTPs, 2.5 µM random decamers, 4 µl of total RNA and nucleotide-free water. Amplification of cDNA with primers (Thermo Fisher Scientific, Massachusetts, USA) showing in the Table 4.1. cDNA was carried out using Stratagene MXPro-Mx3000P (Agilent Technologies, Waldbronn, Germany) with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The PCR cycles were carried out at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, one cycle at 60°C for 30 seconds, 72°C for 45 seconds then the last cycle at 72ºC for 10 minutes. The Delta Ct value (Ct [targeted gene] - Ct[Actb]) was a measure of relative changes mRNA of *Cebpa, Pparg, Creb, Runx2* and *Bmp2* in GTP treated cells and compared with mRNA levels of positive and negative controls.

Table 4.1. Primer sequences used in real-time PCR

Symbol*	Forward primer	Reverse primer
Cebpa	TATAGGCTGGGCTTCCCCTT	AGCTTTCTGGTGTGACTCGG
Pparγ	CCGTGGCCGCAGATTTGA	AGATCCACGGAGCTGATCCC
Creb	TTCAAGCCCAGCCACAGATT	AGTTGAAATCTGAACTGTTTGGAC
Runx2	CACCGAGACCAACAGAGTCA	TGGTGTCACTGTGCTGAAGA
Bmp2	TTTCAATGGACGTGTCCCCG	AGCAGCAACGCTAGAAGACA
Actb	CTCACCATGGATGATGATATCGC	AGGAATCCTTCTGACCCATGC

Real-time PCR primer set for ADSCs (Human) were obtained from Thermo Fisher Scientific Inc.

4.2.10. Statistical Analysis

All values are expressed as the mean ± SE. Comparisons across the three groups were performed using one-way ANOVA followed by Tukey's test to determine significant differences between the two treatments using Prism version 6 (GraphPad Software Inc, CA, USA). P-value < 0.05 was considered as statistically significant.

4.3. Results

4.3.1. Time Course Experiments

The mature adipocyte is characterized by increased intracellular triglyceride content, and intracellular calcium content is a biomarker of osteocyte. To determine the optimal time of differentiation of ADSCs towards the osteogenic and adipogenic lineages, cells were collected at the time points of 1, 7, 21 and 28 days of differentiation induction for analysis of triglyceride and calcium contents. Figure 4.1 showed that triglyceride and calcium

contents gradually increased. Calcium level reached almost 10-fold higher than the baseline level in cells on day 14 after osteogenic induction (Figure 4.1B) and triglyceride contents was increased more than 10 times on day 21 of adipogenic induction (Figure 4.1A). Based on these results, the optimal differentiation time of ADSCs into adipocytes was 21 days, and a 14-days induction was used for the osteogenic experiment.
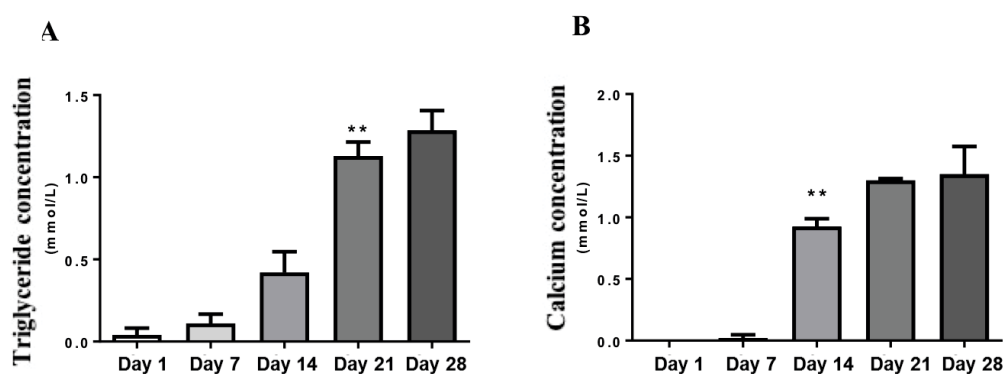
Figure 4.1 Time course study of ADSCs differentiation.   Triglyceride and calcium measurements were performed at each experiment time point. Data are expressed mean ± SME (n=3). **$p<0.01$ v.s day 14 results for triglyceride and day 7 result for calcium, respectively.

4.3.2. The effects of GTP on ADSCs adipogenic differentiation

a. GTP decreased lipid accumulation during ADSCs adipogenic differentiation

To examine the effects of GTP on adipocyte differentiation, lipid accumulation was determined on day 21 of differentiation cells with 1 and 10 µg/ml of GTP alone or a combination with 100 µM of pioglitazone, which is an insulin sensitizer and used as a drug control. Figure 4.2 showed that intracellular lipids were stained and quantified by the Oil Red O. Pioglitazone significantly increased the total amount of lipids and GTP prevented lipid accumulation. Interestingly, cells treated with the combination of GTP

and pioglitazone presented significantly lower lipid contents compared with pioglitazone treatment alone, indicating that GTP attenuated pioglitazone-induced adipogenesis during ADSCs differentiation.
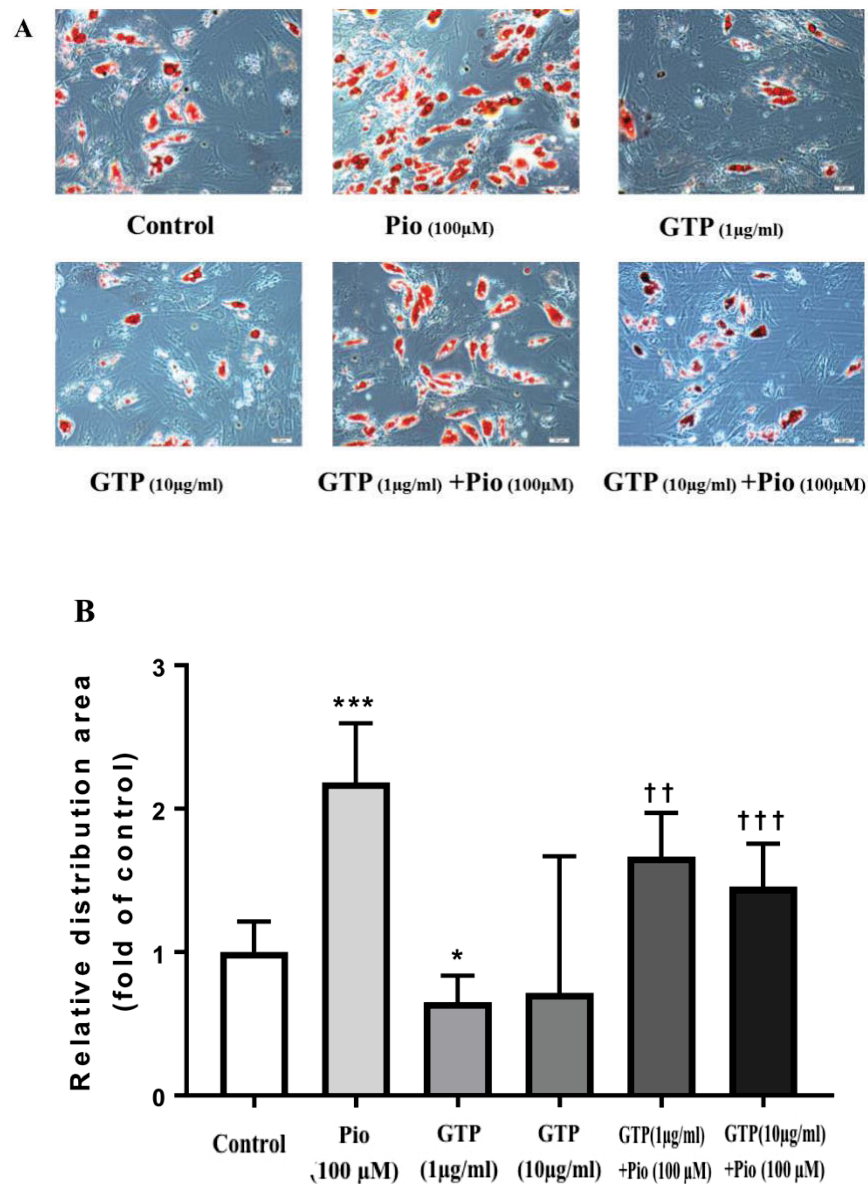
Figure 4.2 Effect of GTP on lipids accumulation during ADSCs Adipogenic differentiation. The lipid droplets were stained with Oil Red O (A) after 21 days of adipogenic induction and (B) quantitative results of lipid droplets analyzed using image J software with one-way ANOVA analysis. Data are presented as mean ± SEM (n=5). *$p < 0.05$ and ***$p < 0.001$ GTP and pioglitazone vs. The control. †† $p < 0.01$ and †††$p < 0.001$ GTP plus pioglitazone versus pioglitazone treatment alone.

b. GTP reduced the cellular triglyceride content on ADSCs adipogenic differentiation

In concordance with reduction of total lipid contents, intracellular triglyceride levels were reduced by $10.5 \pm 5.9\%$ and $18.3 \pm 3.3\%$ in GTP treated cells compared with the control cells. Pioglitazone promoted triglyceride production but this was markedly attenuated by the addition of GTP (Figure 4. 3).
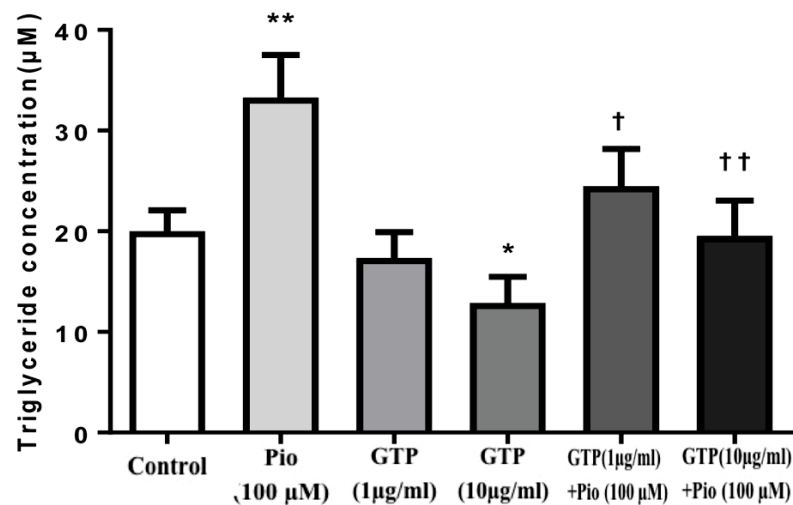
Figure 4.3 GTP decreased the cellular triglyceride content during ADSCs Adipogenic differentiation. The quantitative results of triglyceride were statistically analyzed with one-way ANOVA. Data are presented as mean $\pm$ SEM (n=5). *$p < 0.05$ and **$p < 0.01$ GTP and pioglitazone vs. the control. †† $p < 0.05$ and $^{\dagger\dagger}p < 0.001$ GTP plus pioglitazone versus pioglitazone treatment alone.

c. The inhibitory effects of GTP on PPARɣ protein expression during ADSCs adipogenic differentiation

PPARγ is a key transcription factor that is upregulating genes expression involved in adipogenesis. To elucidate the mechanism by which GTP inhibits adipogenesis during ADSCs differentiation, PPARγ expression was detected with immunofluorescence staining and the density of PPARγ images was quantitatively analysed using Image J

software. PPARγ agonist (pioglitazone) was used as a reference drug.

Figure 4.4 shows that100 μM pioglitazone increased expression of PPARγ by 11.7 ± 10.5% compared to the control. GTP treatment at 1 and 10 μg/ml downregulated PPARγ expression by 17.3 ± 7.1% (p< 0.01) and 27.7 ± 8.0% (p < 0.001) respectively, when compared with the control. When GTPs were added on pioglitazone, the increased PPARγ expression was diminished and it achieved by 23.8 ± 7.8% with 10 μg/ml of GTP, suggesting that GTP prevented pioglitazone-induced PPARγ overexpression.
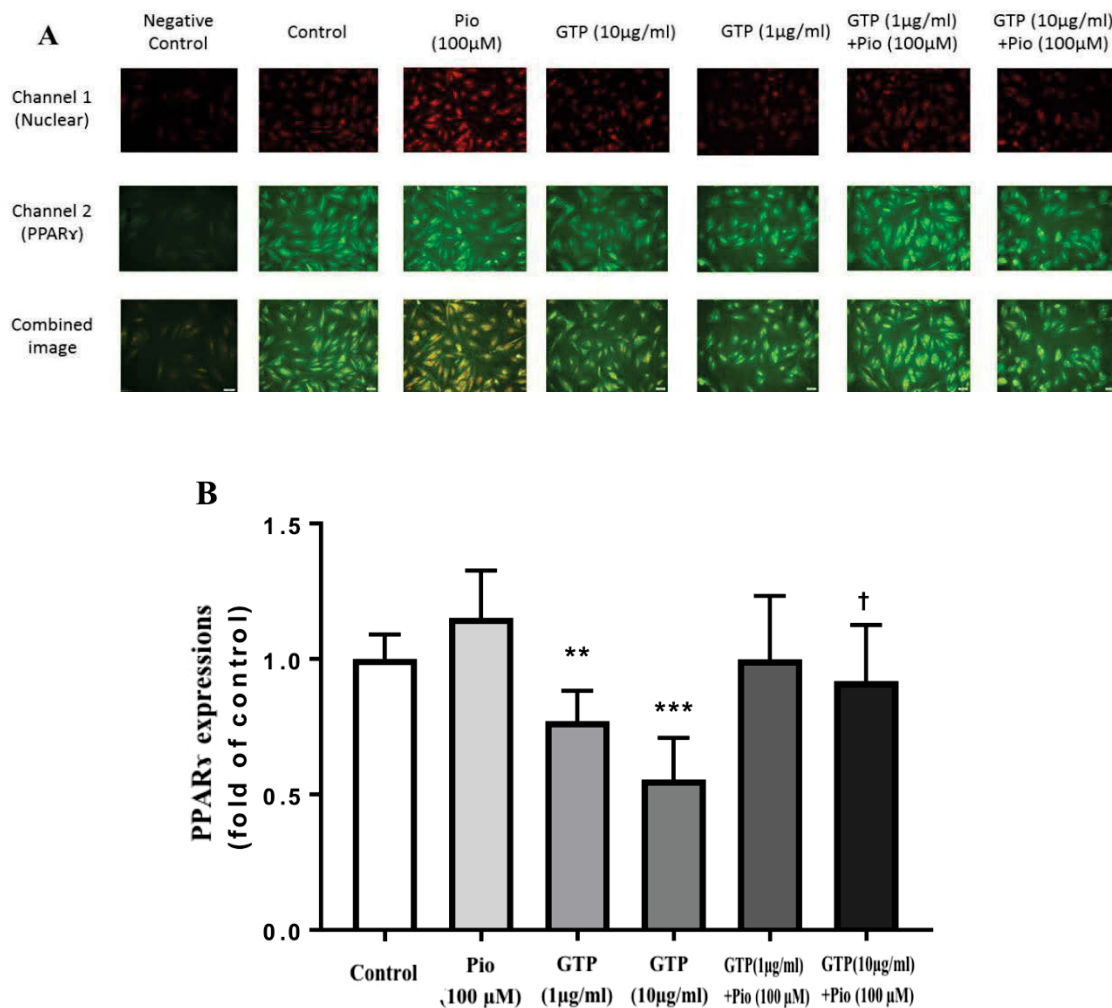
Figure 4.4   The inhibitory effect of GTP on PPARɤ expression during ADSCs adipogenic differentiation.   Images of immunofluorescence staining (A) showed increased PPARɤ protein contents stained as green colour in pioglitazone treated cells (Pio, 100 μM) and decreased PPARɤ protein expression with GTP treatment.

Quantitative data (B) are expressed as mean ± SEM. **$p< 0.01$ and ***$p < 0.001$ GTP and pioglitazone treatments vs. the control. $^{†}p< 0.05$ GTP plus pioglitazone vs. pioglitazone alone.

d. GTP down-regulated mRNA expression of Pparg, Cebpa and Creb

PPARγ, C/EBPα and CREB play a crucial role in adipogenic pathways. To define the molecular mechanism by which GTPs negatively regulate ADSCs towards adipocyte differentiation, the transcription factors of Pparg, Cebpa and Creb, which play a crucial role in adipogenic pathway, were analyed with qRT-PCR.

Pioglitazone significantly increased the expression of Pparg ($p< 0.01$) and Cebpa ($p<0.05$) but did not significantly affect the mRNA of Creb. GTP at low dose (1 µg/ml) did not affect the expression of these transcription factors but 10 µg/ml of GTP only downregulated Pparg ($p<0.05$) when compared to the control (Figure 4.5 A). The experiment also revealed that GTP can be utilised against pioglitazone-induced overexpression of Cebpa mRNA (Figure 4.5 B).
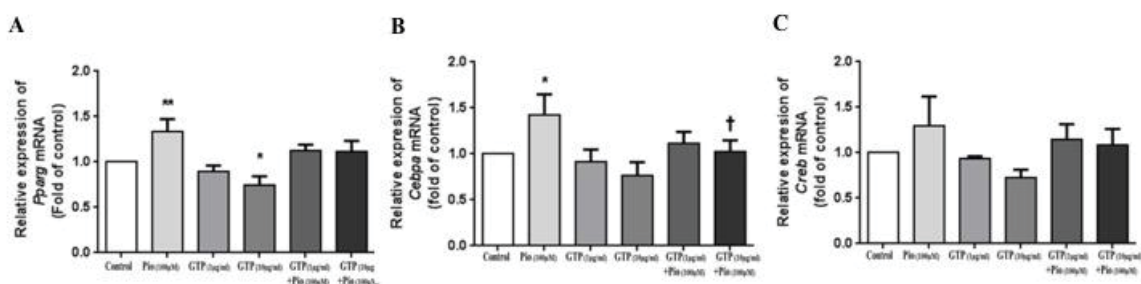
Figure 4. 5 Effect of GTP on mRNA expression of *Pparg Cebpa and Creb.* Gene expression of *Pparg* (A)*, Cebpa* (B) *and Creb* (C) were analysed by qRT-PCR in the cell underwent 21 days of adipogenic differentiation. Data from three separate experiments are expressed as mean ± SEM. **$p< 0.01$ and *** $p < 0.001$ GTP and pioglitazone treatments vs. the control. $^{†}p< 0.05$ GTP plus pioglitazone vs. pioglitazone alone.

4.4.3. The effects of GTP on ADSCs towards Osteogenic differentiation

a. GTP increased calcium contents and ALP activity during ADSCs osteogenic differentiation

Calcium contents and ALP activity were used to characterize ADSCs towards osteogenic differentiation. Figure 4.6 showed that the insulin sensitizer pioglitazone treatment led to a significant reduction of calcium contents collected in the cells at 14 days after osteogenic induction, and this was reversed by 10µg/ml of GTP treatment. GTP at 1µg/ml did not change intracellular calcium levels but 10µg/ml of GTP treatment significantly increased calcium contents ($p < 0.05$ vs. control cells) in mature osteogenic cells.
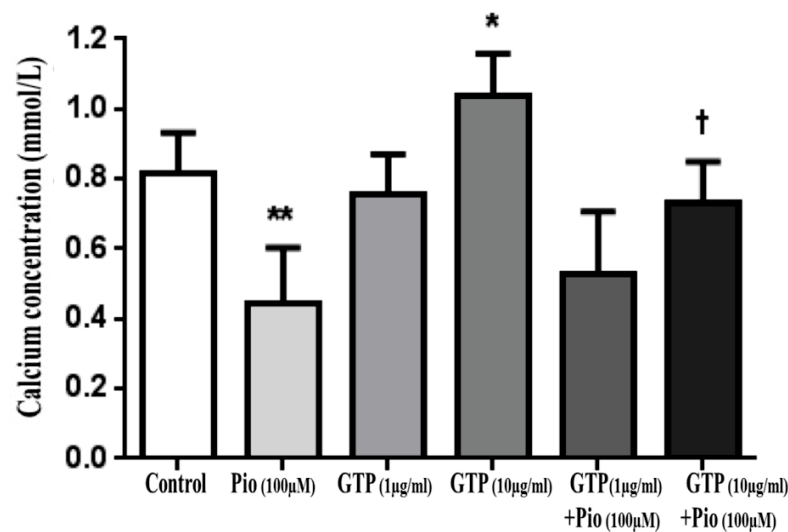
Figure 4.6 Effect of GTP calcium contents in mature osteocytes derived from ADSCs. The quantitative assay of calcium level was performed by an automatic biochemistry instrument (ARCHITECT, Japan) and data from three separate experiments were analyzed with one-way ANOVA and are presented as mean ± SEM. *$p < 0.05$ and **P < 0.01 pioglitazone and GTP vs. the control. † P < 0.05 GTP plus pioglitazone vs. pioglitazone treatment alone.

The results of ALP activity were shown in Figure 4.7. 100 µM of pioglitazone

significantly decreased ALP activity by $37.3 \pm 11.2\%$ when compared to the control ($p<0.01$) and this reduction was reversed and normalized by adding on 1 and 10 µg/ml of GTP. Furthermore, GTP treatment alone increased APL activity but did not achieve significant difference when compared to the control.
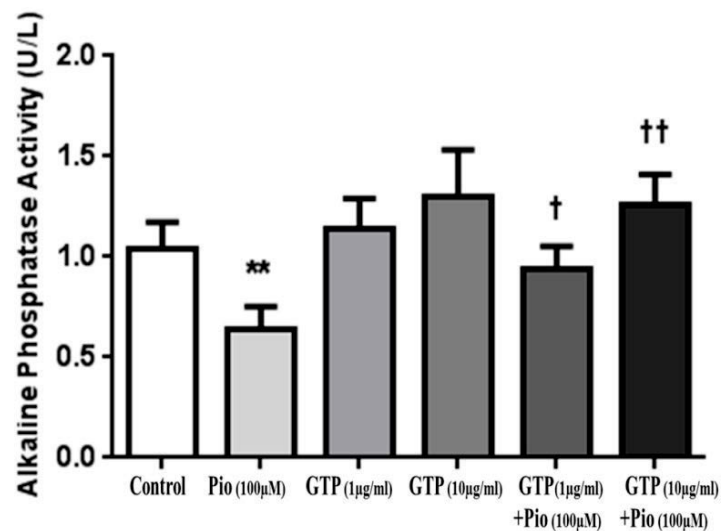
Figure 4.7 Effects of GTP on ALP activity in mature osteocytes derived from ADSCs. The quantitative assay of ALP activity was performed by an automatic biochemistry instrument (ARCHITECT, Japan) and data from three separate experiments were analyzed with one-way ANOVA and are presented as mean ± SEM. **$p< 0.01$ pioglitazone vs. the control. † $p < 0.05$ †† $p < 0.01$ GTP plus pioglitazone vs. pioglitazone treatment alone.

b. GTP up-regulated RunX2 expression during ADSCs towards osteogenic differentiation

The osteogenic differentiation process is upregulated by key transcription factors, including RunX2 and Bmp2. To elucidate the mechanism by which GTP enhances osteogenic differentiation, as evidenced by increased calcium contents and APL activity, protein expression of Runx2 were measured with immunofluorescence staining on day 14 after differentiation induction and treatments.

Figure 4.8 shows that pioglitazone slightly decreased Runx2 protein expression, and GTP treatments (1 and 10 µg/ml) significantly enhanced Runx2 expression by $19.1 \pm 7.3\%$ ($p < 0.01$) and $32.2 \pm 5.7\%$ ($p < 0.001$) compared to the control. Adding GTP to pioglitazone, however, did not affect RunX2 expression.
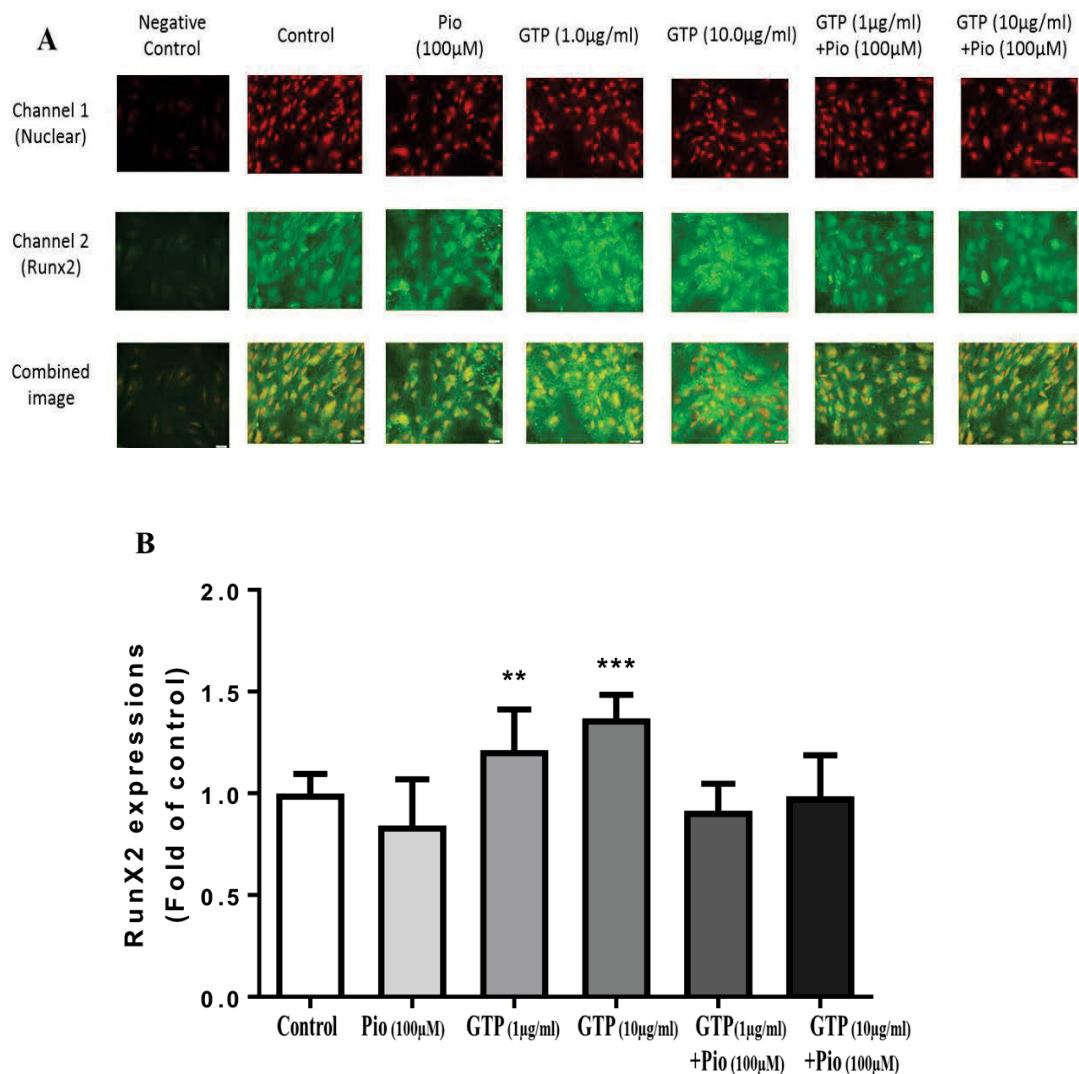
Figure 4.8 GTP up-regulated Runx2 expression during ADSCs towards oestrogenic differentiation. Images of immunofluorescence staining (A) showed runx2 protein contents stained as green colour in cells treatment with pioglitazone (Pio, 100 µM), 1 and 10 µg/ml GTP alone or the combination of GTP and pioglitazone. Quantitative data (B) from three separate experiments are expressed as mean ± SEM. $**p < 0.01$ and $***p < 0.001$ GTP treatments vs. the control.

c. Up-regulation effects of GTP on mRNA transcription of Bmp2 and Runx2

To understand the molecular mechanism by which pioglitazone and GTP influence ADSCs differentiation into osteocytes, osteogenic transcription factors Bmp2 and Runx2 were analysed with qRT-PCR.

Treatment with pioglitazone significantly decreased the expression of Runx2 ($p < 0.05$) but did not affect Bmp2 expression (Figure 4.9). Contrastingly, ADSCs treated with 10 µg/ml GTP during differentiation significantly increased Bmp2 ($p<0.05$) and Runx2 ($p<0.01$) compared to the control (Figure. 4.9). GTP (10 µg/ml) reversed pioglitazone-reduced Runx2 mRNA.
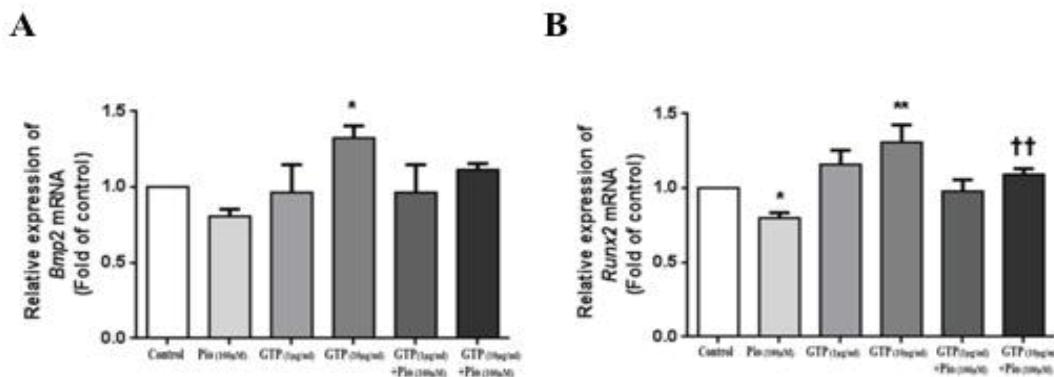
Figure 4.9 Effects of GTP on mRNA expression of *Bmp2* and *Runx2* in mature osteocytes. Gene expression of *Bmp2* (A) *and Runx2* (B) analysed with qRT-PCR in the cell underwent 14 days of oestrogenic induction. Data from three separate experiments are expressed as mean ± SEM. *$p< 0.05$ and ** $p < 0.01$ pioglitazone and GTP treatments vs. the control. [††]$p< 0.01$ GTP plus pioglitazone vs. pioglitazone alone.

4.4. Discussion

Accumulating evidence supports a complex relationship between adiposity and osteoporosis in overweight/obese individuals, with an increase of adipogenesis

accompanied by decreasing bone formation in obese populations. Numerous studies have demonstrated that there is a reciprocal relationship between the adipogenesis and osteogenesis of mesenchymal stem cells (MSCs). Based on the previous results of this thesis that GTP can inhibit adipogenesis during 3T3-L1 preadipocytes proliferation (Lao *et al.*, 2015), present study evaluates whether GTP was capable of alternating the differentiation of adipose-derived stem cells (ADSCs) shifting towards osteogenic lineage and preventing adipogenesis.

The present study demonstrated that GTP prevents lipid accumulation and reduces triglyceride content in ADSCs destined to differentiate in adipocytic cells following exposure to an adipogenic differentiation medium. This finding is consistent with previous reports that EGCG, a major component of GTP, has the ability to suppress adipogenesis through downregulating adipsin gene expression in mice MSCs (Chani B *et al.* 2016). Human ADSCs used in this study possess a higher stem cell population compared to other MSC sources (Zuk PA *et al.* 2002) and its pluripotency behaviour and pathophysiology is more correlative to human obesity than MSCs from mice. The inhibitory effect of GTP on hADSCs adipocyte-differentiation may highlight the potential for translational study. Furthermore, PPARγ agonist pioglitazone was used as a control drug in this study. We found that pioglitazone enhances adipogenesis, and this was attenuated by adding 10 μg/ml of GTP to pioglitazone. It is well known that pioglitazone enhances insulin sensitivity for type 2 diabetes but adversely affects body composition and bone metabolism (Derosa G *et al.* 2012). Thus, the combination of GTP with PPARγ agonist may provide a novel therapy for obesity associated with insulin resistance and hyperglycaemia (Figure 4.10).
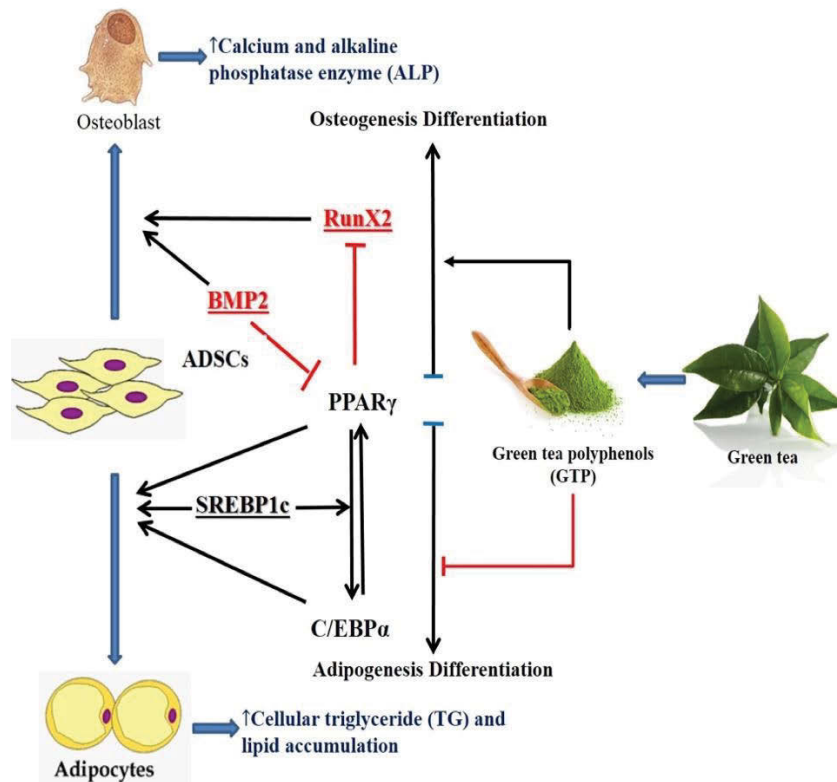
Figure 4.10 The Pathway of Adipogenic and Osteogenic differentiation.

Besides its anti-adipogenic effect, treatment with 1 and 10 µg/ml of GTP facilitated ADSCs to differentiate into osteoblasts, as evidenced by two measurements. One is a cellular calcium determination and the other is an ALP activity assay in ADSCs after 14 days of exposure to osteogenic differentiation medium. ALP is produced by the liver and mature osteoblasts. It has been solidified as a specific osteogenic marker to be detected in the early stages of osteogenic differentiation from MSCs and is a specific marker for mineralization. This study showed that GTP increased not only ALP activity but also enhanced cellular contents of calcium which are an essential substance for mineralization and bone strength. Interestingly, PPARγ agonist pioglitazone negatively regulated the differentiation of ADSCs into osteocytes, and GTP also normalized reduced calcium levels, as well as ALP activity via pioglitazone. Shen *et al*. have reported that green tea supplementation prevented body weight gain and improved bone

microstructure and strength in obese female rats fed with a high-fat diet (Shen CL *et al.* 2011; Shen CL *et al.* 2015). The results of the present study together with findings of *in vivo* studies indicates that the consumption of green tea may simultaneously prevent fat formation and enhance bone formation. Thus, GPT may result in an optimal therapy for obese patients with osteoporosis (Figure 4.10).

It is reported that PPARγ, C/EBPα and CREB are master regulators involved in transcriptional control of the early stages of adipocyte-differentiation (Wu Z *et al.* 1999; Zhang JW *et al.* 2004). To understand the underlying mechanisms by which GTP attenuated adipogenesis, expression of PPARγ, C/EBPα and CREB was detected in mature adipocytes in both the presence and absence of GTP in the induction medium. The result showed that pioglitazone stimulates the expression of PPARγ and C/*ebp*α. GTP treatment at 10 µg/ml significantly reduced the expression of PPARγ mRNA and proteins, indicating an inhibition of *Pparg* gene and/or a reduction of the transcription from mRNA to PPARγ protein with GTP.   GTP did not affect the expression of *Cebpa* and *Creb*, however, and reversed diminished-*Cebpa* by pioglitazone. These results critically revealed a mechanism of GTP inhibiting the differentiation of ADSCs into adipocytes by suppressing the master regulator of adipocyte-differentiation PPAR*γ*.

Recent work with gene silencing and overexpression has revealed that PPARγ is not only a crucial factor to controlling the transcriptional pathways of adipocyte differentiation, but also has a role in controlling osteogenic differentiation with Runx2 (Zuk PA *et al.* 2002).   In present studies, PPARγ promoted adipogenesis and inhibited osteogenesis with decreased expression of RUNX2 when activated by pioglitazone. GTP treatment enhanced not only the gene expression of master regulators *Runx2* but also *Bmp2* normalized reduced-*Runx2* by pioglitazone. In addition, it also increased *Bmp2*

expression. *Runx2* and *Bmp2* are the critical molecular switches involved in the osteogenic differentiation of mesenchymal stem cells (Zuk PA *et al*. 2002). These results indicate that GTP is capable of promoting osteogenic differentiation via up-regulating the gene expression of *Runx2* and *Bmp2*, and inhibiting adipogenic differentiation of ADSCs by down-regulating the expression of *Pparg*.

In summary, through utilizing hADSCs and PPARγ agonist, this study identified PPARγ and RUNX2 as important regulators in adipogenesis and osteogenesis. The findings of present studies suggest that GTP possesses the ability to limit ADSCs differentiation into adipogenic lineage and simultaneously enhance osteogenesis through suppressing PPARγ-induced adipogenesis and upregulating the RUNX2-BMP2 mediated osteogenic pathway (Figure 4.10). Therefore, the enhancement of osteogenesis alongside the prevention of adipogenesis with GTP may provide a rational approach towards developing GTP into multifaceted therapeutic goods for the intervention of obesity associated osteoporosis.

Chapter Five

Can Green Tea Polyphenols Alternate Ovarian Cell Dysfunction Leading to Polycystic Ovary Syndrome?

5.1. Introduction

Polycystic Ovarian Syndrome (PCOS) is a complex and heterogenous disorder, affecting up to 15% of women at their reproductive age (Papalou and Diamanti-Kandarakis, 2017). Biochemical and/or clinical hyperandrogenism is a key clinical feature of this disorder. PCOS is characterized by ovulatory dysfunction and polycystic ovarian morphology (Legro *et al.,* 2013). The aetiology of PCOS still reminds unclear but the reports in the literature suggested a stronger correlation between PCOS and metabolic syndrome, including obesity, hyperinsulinemia, dyslipidaemia, insulin resistance and impaired glucose tolerance (Caserta *et al.,* 2014). The relationship between the two syndromes is mutual: PCOS women have a higher prevalence of metabolic syndrome and women with metabolic syndrome commonly present the reproductive/endocrine trait of PCOS. Clinical observation showed that insulin resistance in women with PCOS is positively correlated with high levels of androgen (Luotola *et al*., 2018). Although the causative relationship still needs to be clarified, but it is generally believed that obesity is associated with insulin resistance and triggers excessive steroidogenesis in ovarian theca cells. It has been reported that under hyperinsulinemic status, insulin binds to insulin-like growth factor-1 (IGF-1) on theca cells*,* thereby upregulating the expression of key steroidogenic enzymes, cholesterol side-chain cleavage (CYP11A1) and 17α-hydroxylase (CYP17A1), to facilitate the synthesis of androstenedione and testosterone in obese people that has hypercholesterolemia (Guilherme *et al.,* 2008, Dadachanji *et al.,* 2018).

Our previous studies demonstrated that green tea polyphenols (GTP) is capable of enhancing insulin-mediated glucose and lipid metabolism and processes anti-obesity effect and prevent fatty liver disease (Kim *et al*, 2014, Lao *et al*., 2015, Tan *et al*., 2017).

Based on these observations, it was speculated that GTP would be able to alternate ovarian cell dysfunction and result in abnormal steroidogenesis. To verify this hypothesis, effect of GTP on ovarian hormone production in a PCOS cellular model-induced by dexamethasone was evaluated. To evaluate the molecular mechanism of GTP on ovarian hormone production, the expression of steroidogenic enzymes (CYP17A1 and CYP11A1) were measured in the cell in the presence and absence of GTP treatment.

5.2. Research Plan and Methods

5.2.1. Green Tea Polyphenols (GTP)

The tea polyphenols (99% in purity) were extracted from green tea leaves (Camellia sinensis) grown in Guizhou province, south-western China, by Zuyilushen Kangyuan Co (Guizhou, Meitan, China). The polyphenolic compounds identified using liquid chromatography-mass spectrometry (LC-MS) were 68% EGCG, 7% EGC, 1% ECG and 18% EC in GTP (w/w).

5.2.2. Cell culture with dexamethasone and GTPtreatment

Primary theca cells isolated from 4-week-old, non-pregnant female mice were kindly provided by Reproductive Medicine Team at *Kolling Institute of Medical* Research (University of Sydney, NSW Australia) and maintained in complete McCoy's 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin (pH 7.2). Cells were seeded at a density of 1×10^6 in T25 flasks and maintained in an incubator at $37^\circ C$, 5% CO_2 until passage 3. Following this, cells were seeded into 24-well plates at a density

of 1×10^5 cells for 48 h to allow for anchorage, at which stage, media was replaced with either fresh media (control), media with dexamethasone (10 µM) or dexamethasone (10 µM) with various concentrations of GTP (1, 10 and 100 µg/mL) for a further 24 h. Cell media was then collected and stored at -20°C for subsequent hormone analysis.

5.2.3. Cell Proliferation Assay

2.5×10^4 cells isolated above were seeded in 96-well plates for 48 h. Media was removed and replaced with complete medium and GTP (0, 0.1, 1.0, 10, 25 and 100 µg/ml). After 24 h of treatment media was 'flicked-off' and frozen at -80°C as previously described (McGowan, Eileen M. *et al*., 2011) . This was also carried out at 48 h. Frozen plates were then thawed and SYBR Green I (1:8000) (cat # S-7567, Thermo Fisher Scientific，Massachusetts, USA) in a lysis buffer (10 mM Tris, 2.5 mm EDTA, 0.1% Triton X-100) was added to each well. Plates were then left for a further 72 h in the dark at 4°C. Fluorescence intensity was measured at 485 nm excitation and 530 nm emission filters using a microplate reader and Magellan Software (Tecan, Zürich, Switzerland).

5.2.4. Testosterone and Progesterone Measurement

The sexual hormones were released during ovary culture. Therefore, measurement of the hormone concentration in the culture medium can directly reflect secretion of the ovarian cells. After 24 h treatment with GTP at 1, 5, and 10 µg/ml, media was collected and analysed using the Cobas 8000 modular analyzer (Hoffman-La Roche Ltd, Basel, Switzerland) for testosterone (T) and the ARCHITECT i2000SR immunoassay analyzer (Abbott, Illinois, USA) for progesterone (P) measurement. For P measurement, cell

culture media was diluted 1:5 times with sterile Milli-Q® water before measurement. Culture media was used as a background and results were expressed as a percentage of control.

5.2.5. Immunofluorescence staining

To determine the cell type and observe the morphology of theca cells, immunofluorescence staining was used. Cells were grown on sterile coverslips after treatment, washed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. Following this, cells were permeabilised in PBS with Tween-20 and Triton X-100 (0.06% Tween-20 and 0.04% Triton X-100). Cells were washed twice again with PBS and blocked with 5% BSA solution with 10% goat serum.

Cells were then stained with primary antibodies (1: 500 anti-mouse CYP11A1 and 1: 500 anti-rabbit CYP17A1) (Abcam, Cambridge, UK) or PBS only (for negative control), followed by staining with secondary antibodies (1: 400 goat anti- rabbit Alexa Fluor 488 and 1:400 goat anti-mouse IgG Alexa Fluor 568). Cells were then washed twice with PBS before counterstaining the cell nuclei with Hoescht® (1: 1000). This was followed by a final wash with PBS. Coverslips were mounted on slides with mounting medium and visualized under an inflorescent microscope (Olympus BX51, Tokyo, Japan). Images were captured with a digital camera (DP70, Tokyo, Japan), using a 40× objective (NA 0.75). FIJI (Image J) was used to threshold, segment and determine fluorescence intensity of the antibody labelled regions within cells (Schindelin *et al.,* 2012).

5.2.6. Western Blotting

Cells were treated as previously described. After 24 h, cells were harvested, washed and centrifuged at 10,000 ×g. RIPA buffer was added to the pellets and frozen in liquid nitrogen followed by quick thawing in water, vortexing and centrifuging. This process was repeated another two times to facilitate cell lysis as previously described (Jin and O'Neill, 2011).

Following protein determination by Bradford assay, total protein (8 µg) was separated on 10% SDS-page gels by electrophoresis for 90 min at 20 V. Gels were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked in 5% skim milk in PBS with Tween-20. Membranes were then probed with primary antibodies (CYP11A1, CYP17A1 and β-actin) overnight before washing and incubating with secondary antibodies (1: 10,000). Protein bands were detected using Pierce Chemiluminescent HRP Substrate and the Amersham Imager 600 (Illinois, USA). Protein bands were subsequently analyzed using Image J software. The data were normalized to housekeeping protein, β-actin and expressed as a relative density to the control group's band density.

5.2.7. Quantitative Real-time Reverse Transcription Polymerase Chain reaction

To further clarify molecular mechanism of GTP on hormone production, *Cyp11a1* and *Cyp17a1* gene were detected by qRT-PCR. Thecal cells were treated in cold PBS to discard the culture medium by three repeats of washing and centrifugated at 500 ×g for 3 mins. 1×10^5 cells were subject to RNA extraction by manufactory instruction of RNeasy Mini kit (Qiagen, MD, USA). The RNA was purified with RQ1 RNase-Free DNase Kit (Promega, NSW, AU). Reverse transcription was performed in a total of 20

μL volume containing 4 mM MgCl2, PCR buffer, 0.5 mM dNTPs, 2.5 μM random decamers, RNA and nucleotide-free water. Negative controls for all reactions were without reverse transcriptase or without the RNA sample (to test for any RNA or DNA contamination, respectively). An internal positive control was to test for expression of housekeeper gene *Actb*. Amplification of cDNA with the primers (Sigma-Aldrich, Missouri, USA) as shown in Table 5.1. cDNA was amplified on Stratagene MXPro-Mx3000P (Agilent Technologies, Waldbronn, Germany) with FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The PCR cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s, and then 10 min at 72ºC. The Delta *Ct* (= *Ct*[*Cyp11a1, Cyp17a1*] - *Ct*[*Actb*]) was a measure of relative changes *Cyp11a1* and *Cyp17a1* mRNA content of the cells from 3 individual experiments. Data were normalized to the control treatment mRNA levels. A plot of $2^{-(normalized\ Delta Ct)}$ was shown.

Table 5.1. Primer sequences used in real-time PCR

Symbol*	Forward primer	Reverse primer
Cyp17a1	CACATGTGTGTCCTTCGGGA	CACATGTGTGTCCTTCGGGA
Cyp11a1	AAAGGGAGCTGGTACCTCTACT	AAACTGACTCCAAAGTGCCCA
Actb	CTTTGCAGCTCCTTCGTTGC	CCATCACACCCTGGTGCCTA

Real-time PCR primer set for Theca cell (mouse) were obtained from Sigma-Aldrich, Inc.

5.2.8. Statistical Analysis

All statistical data are expressed as the mean ± SEM and analysed using one-way ANOVA followed by post-hoc analysis with Dunnett's multiple comparison test to either the control or dexamethasone group with GraphPad Prism software version 6 (GraphPad

Software, Inc., La Jolla, CA, USA). Data from hormone assays are represented as a percentage (%) of control group. Statistical significance was set at $p<0.05$.

5.3. Results

5.3.1. GTP extract does not affect theca cell proliferation at low dosage.

Cell proliferation was evaluated by SYBR Green assay to determine the safety of Green tea polyphenols (GTP) on theca cells. Cells were treated with 0, 0.1, 1.0, 10, 25 and 100 µg/mL of GTP. Results showed that the GTP extractions below 25 µg/mL did not exhibit any significant changes to fluorescence intensity at 24h and 48h compared to the control group (no treatment) as shown in Figure 5.1, while 100µg/mL GTP treatment group showed significant changes in 24 hours and 48 hours ($p < 0.05$ and $p < 0.01$ respectively).
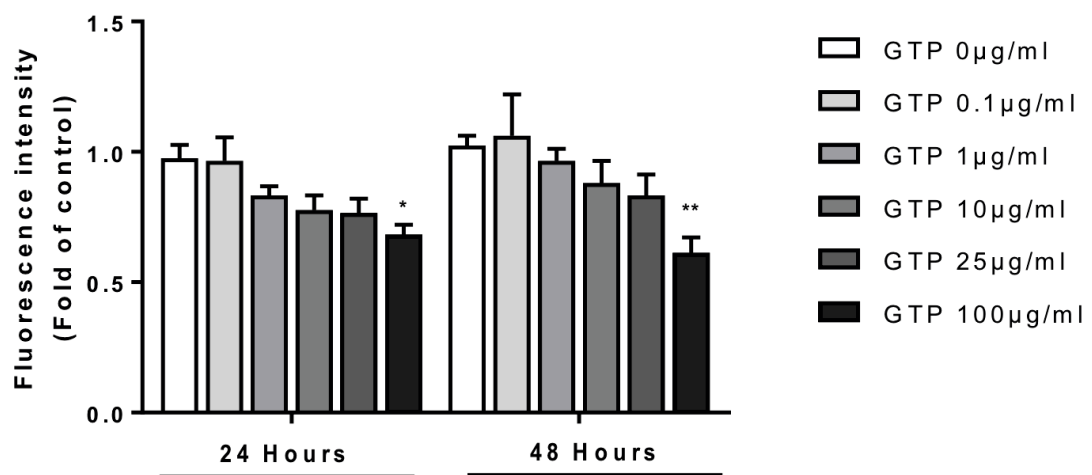

Figure 5.1. SYBR green proliferation assay of Green tea polyphenols (GTP) treated theca cells. Following treatment at time points, SYBR solution were added into cell cultures and incubated for 72 h. Fluorescence intensity was measured at 485 nm to determine effect of GTP on cell proliferation at time points. Data are presented as Mean

± SEM from 3 separate experiments (n=3). *$p < 0.05$ and **$p < 0.01$ GTP versus the negative control.

5.3.2. GTP ameliorated dexamethasone-induced hormonal changes in theca cells

To determine the effects of dexamethasone on ovarian hormone secretion from murine theca cells, testosterone and progesterone levels in cell media were analysed. The experimental data was normalized to cell number each cultured well. Cells treated with dexamethasone alone exhibited significant increases in testosterone level compared to the normal control ($p<0.05$) in Figure 5.2A. Furthermore, progesterone levels were suppressed by dexamethasone as shown in Figure 5.2B ($p<0.05$).

Hyperandrogenism is a characteristic of PCOS. This result confirmed that dexamethasone treated murine theca cells emulated PCOS *in vitro*, so this cellular model was used in further experiments. Green tea polyphenols (GTP) significantly lowered the testosterone level of Dexamethasone (DEX)-treated cells at 10 μg/mL ($p<0.05$) and 25 μg/mL ($p<0.01$). Additionally, the highest dose of Green tea polyphenols (GTP) (25 μg/mL) significantly increased progesterone levels ($p<0.05$) back to levels similar to that of control in dexamethasone (DEX)-treated cells (Figure 5.2).
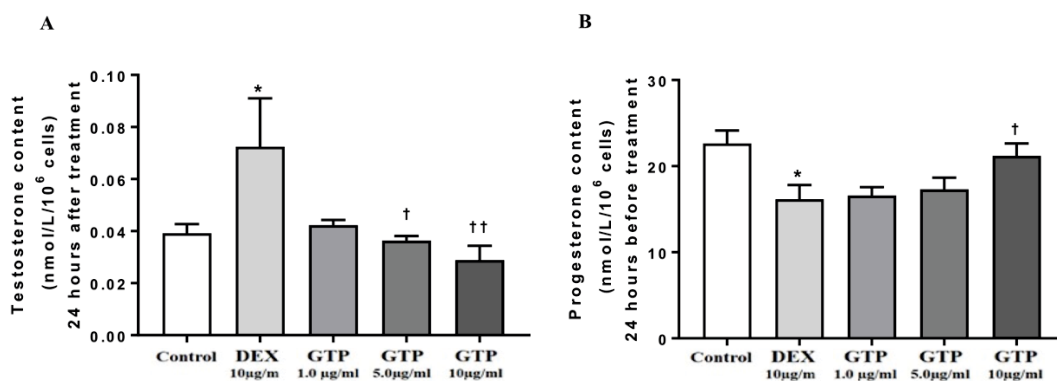
Figure 5.2 Testosterone and progesterone analysis of green tea polyphenols (GTP) treated theca cells. Cells were treated with complete media as the normal

control, treated with dexamethasone (DEX, 10 µM) and DEX in the presence of GTP

(1, 5 and 10 µg/mL). Media was collected 24 h after treatment for assay of

testosterone (A) and progesterone (B). Results were represented as the mean ± SEM

from 3 separate experiments (n=3). *$p< 0.05$ DEX versus the normal control. [†]$p <$

0.05 and [††]$p< 0.01$ GTP treatment versus DEX treatment.

5.3.3. Theca cell morphology and confirmation of CYP17A1 and CYP11A1 expression

The immunofluorescence images allowed for a visual confirmation of the cell type as

ovarian cells. CYP11A1 is a specific murine theca cell gene (Tian *et al.*, 2015). Cells

stained with both primary antibody and secondary antibody showed high fluorescence

compared to cells stained with secondary antibody alone,images of thecal cell culture

was showed in Figure 5.3. DEX significant enhanced CYP11A1 expression and high-

dose GTP treatment (10 µg/mL) appeared to decrease protein expression of CYP11A1,

indicated by the lower fluorescent intensity. Results were further quantified by western

blotting.
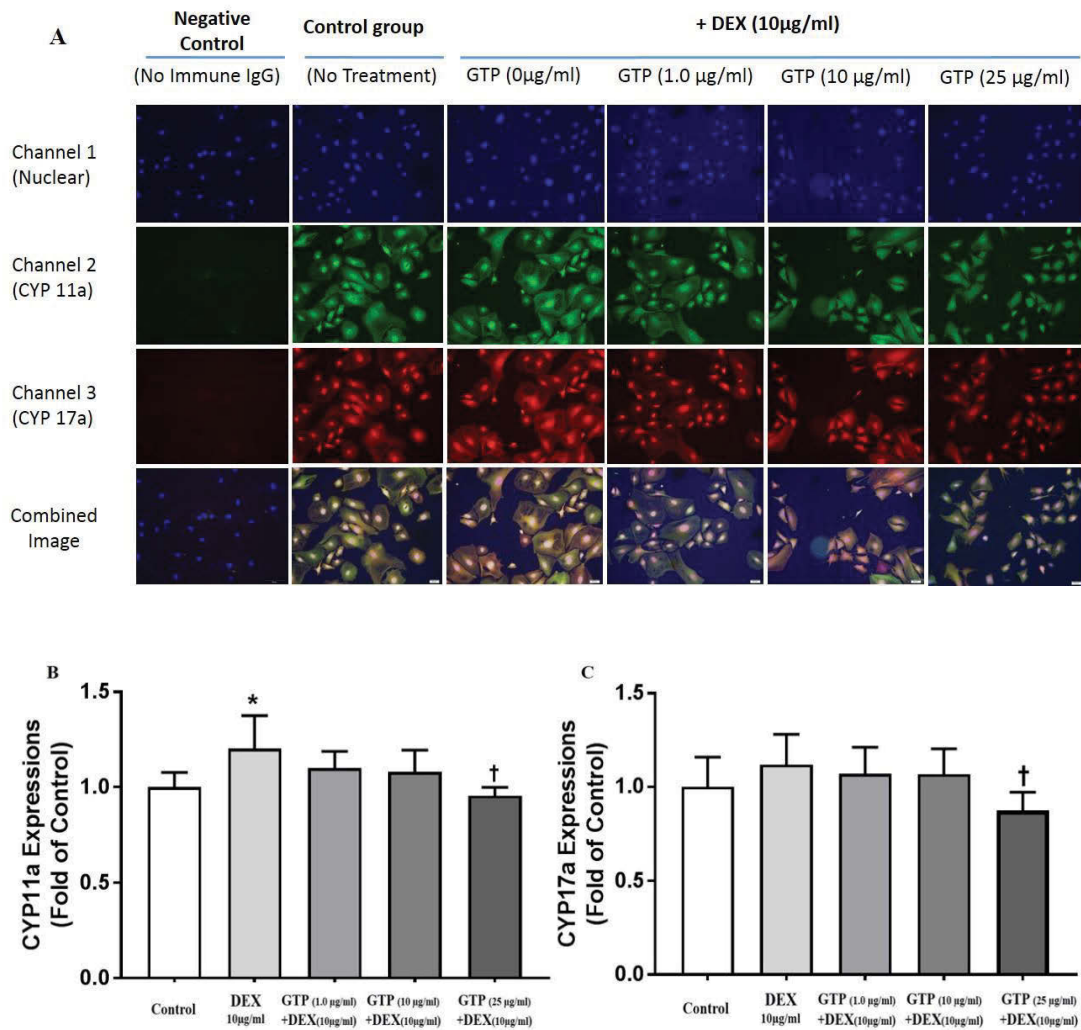
Figure 5.3 Immunofluorescence staining of theca cells. Cells were treated with control media (control), dexamethasone (DEX) media (10 μM) or DEX with GTP (1, 10 and 25 μg/mL), and then stained with CYP11 (green), CYP17 (red) antibodies or no antibodies for negative control (A). Cells were visualised under an Olympus BX51 fluorescent microscope (Tokyo, Japan) and captured with DP70 digital camera (Tokyo, Japan), using a 40×objective. FIJI (Image J analysis) was used to analyse the CPY11A1. The quantitative analysis of fluorescence intensity of CPY11A1 (B) and CPY17A1 (C) was conducted using FIJI Image J (Schindelin *et al.,* 2012) Data are represented as the mean ± SEM from three replicates (n=3). *$p<0.05$ DEX versus the control. †$p < 0.05$ GTP versus DEX treatment.

5.3.4. GTP inhibited CYP17A1 and CYP11A1 expression in theca cells

Western blot analysis revealed that DEX significantly increased the protein expression of CYP11A1 and CYP17A1 compared to the control group ($p<0.05$). GTP treatments (1, 10 and 25 µg/mL) significantly decreased protein expression of CYP11A1 ($p<0.05$, $p<0.01$ and $p<0.001$), respectively, compared to DEX (Figure. 5.4A). GTP treatment decreased protein expression of CYP17A1 when compared to DEX treatment alone (Figure. 5.4 B).
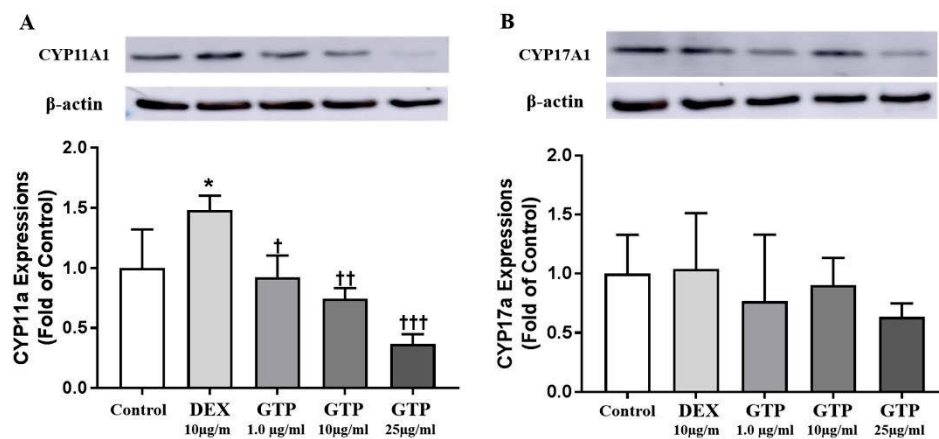
Figure 5.4: **Effect of GTP on CYP11A1 and CYP17A1 expression in theca cells.** Cells were treated with control media (control group), dexamethasone (DEX) media (10 µM) or DEX with GTP (1, 10 and 25 µg/mL). After treatment, protein expression of CYP11A (A) and CYP17 (B) were detected by western blotting and quantitated using Image J software. Data are expressed as relative density of the control group after normalising to housekeeping protein βactin (n=4). *$p< 0.05$ DEX treatment versus the control; †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.01$ GTP treatments versus the DEX treatment.

5.3.5. GTP treatments down-regulated mRNA expression of *Cyp11a1* and *Cyp17a1* in theca cells

CYP11A1 and CYP17A1 are cytochrome P450 enzymes which are widely present in

ovarian cells, adrenal glands, and they play a key role in the androgen biosynthetic pathway (Figure 5.6). qRT-PCR was used to verify the effect of GTP on mRNA expression of *Cyp11a1* and *Cyp17a1* in theca cells with variety of treatments. DEX significantly increased expression of *Cyp11a1* compared to the control group ($p < 0.05$ Figure 5.5A). GTP treatment significantly reduced the expression of mRNA *Cyp11a1* compared with DEX treatment ($p < 0.05$, Figure 5.5A). The expression of *Cyp17a1* was increased by *DEX and* GTP treatments prevented the overexpression of *Cyp17a1* in DEX induced cells (Figure 5. 5B).
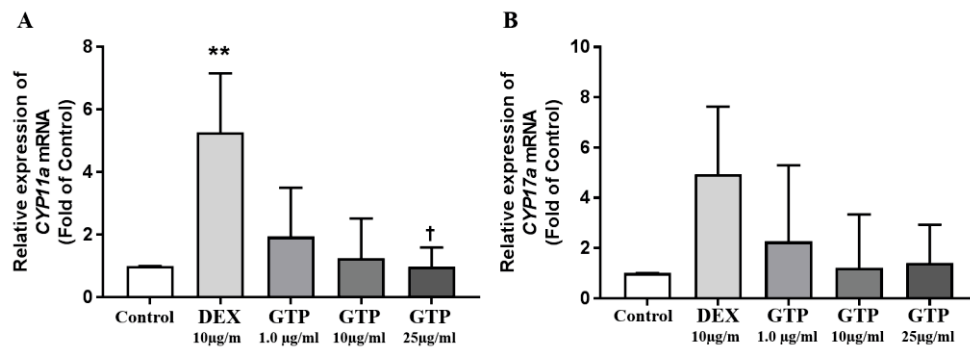
Figure 5.5: **Effect of GTP on the expression of *Cyp11a1* and *Cyp17a1* in theca cells.** Cells treated with media only (control group), dexamethasone (10 µM, DEX) group and GTP (1, 10 and 25 µg/mL) in the presence of DEX (10 µM). Expression of *Cyp11a1* (A) and *Cyp17a1* (B) were determined by qRT-PCR. Data are represented as the mean ±SEM from three replicates (n=3). *$p<0.05$ and **$p < 0.01$ DEX) treatments versus the control. †$p < 0.05$ GTP treatment versus DEX treatment group.

5.4. Discussion

Polycystic ovarian syndrome is a heterogeneous disorder with multiple phenotypes meaning there is often complexity when it comes to treatment. To discover natural approaches to hyperandrogenism in PCOS, this study was evaluated whether GTP can

reduce testosterone secretion in a cellular model of hyperandrogen-induced by dexamethasone and further clarified the molecular mechanisms by which GTP alternates steroidogenic synthesis in primary murine theca cells.

Firstly, the optimal dosages of GTP were determined by SYBR green assay which uses fluorescent DNA-labelling to closely represent cell proliferation and cell cycle arrest (McGowan *et al.,* 2011). GTP treatments did not significantly affect cell proliferation when compared to control and did not exhibit apoptotic effects, except for high concentration of 100 μg/mL. Therefore, three dosages of GTP (1.0, 10 and 25 μg/mL) were chosen to study the effects and mechanism of GTP on dexamethasone-induced testosterone over-secretion in theca cells. Secondly, the present study found that DEX significantly increased testosterone secretion in murine derived ovarian theca cells. This result was similar to a previous study, where the results showed that 1 μM dexamethasone treated porcine theca cells increased androgen secretion and disrupted insulin signalling by downregulation of insulin-receptor 1 (IRS-1) and glucose-transporter 4 (GLUT-4) expression (Qu *et al.,* 2009). Insulin resistance and hyperinsulinemia are now recognised to play an important role in the pathogenesis of PCOS and can stimulate ovarian androgen production (Zhang *et al.,* 2000, Cadagan *et al.,* 2016).
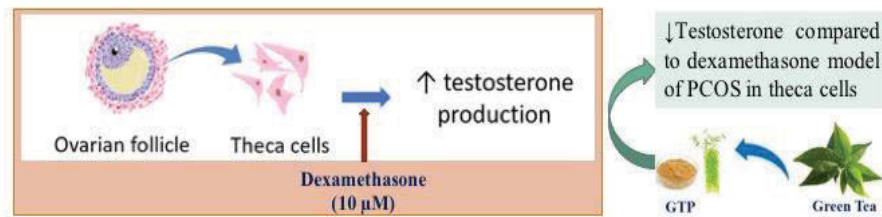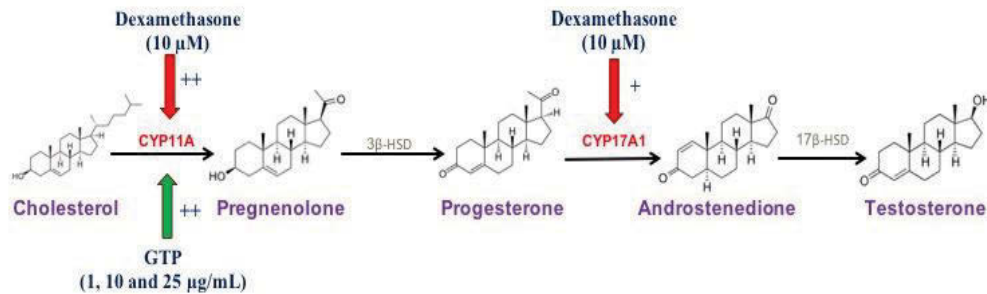
Figure 5.6 GTP reverses dysfunction of ovarian theca cell induced by dexamethasone

Furthermore, in this study, dexamethasone significantly increased protein expression of CYP11A1 in theca cells (Figure 5.6). CYP11A1 is the steroidogenic enzyme responsible for the synthesis of Pregnenolone from Cholesterol and shown to be increased in theca cells from women with PCOS (Nelson *et al*., 2001; Wickenheisser *et al*., 2005). Dexamethasone also modestly increased CYP17A1 protein expression in this study; however, this was not statistically significant and therefore dexamethasone may increase testosterone secretion in murine theca cells mainly through upregulation of CYP11A1 protein expression.

Importantly, this study demonstrated that GTP reduced over-secretion of testosterone-induced by DEX in murine theca. This result was consistent with previous study that green tea suppressed testosterone: androstenedione ratio in rat ovaries (Veri *et al.,* 2015, Sadat *et al.,* 2015). This study also revealed that effects of GTP on testosterone production was in a dose-dependent manner. GTP at all concentrations (1, 10 and 25 µg/mL) was shown to be protective for dexamethasone-induced elevation of testosterone through the downregulation of CYP11A1 protein expression. In addition, the highest

concentration of GTP (25 µg/mL) also showed decrease protein expression of CYP17A1, the gene that is responsible for the regulation of ovarian steroidogenesis. Therefore, GTP at 25 µg/mL downregulated both CYP11A1 and CYP17A1 at protein and mRNA expression (Figure 5.6). This dual mechanism may explain the dose-dependent manner in which GTP significantly reduced testosterone expression in DEX-treated theca cells. To my best knowledge, this is the first time GTP has been described to have an effect on CYP11A1 and CYP17A1 protein expression.

Another finding of this study was that dexamethasone (DEX) had an inhibitory effect on progesterone secretion. A previous study has also demonstrated that dexamethasone (DEX) in pre-ovulatory rat follicles, inhibited progesterone secretion by modulating cholesterol transport mediated by steroidogenic acute regulatory protein (StAR) (Huang and Shirley Li, 2001). GTP protected against the effect of suppression of progesterone expression induced by dexamethasone. The reverse on the inhibitory effect of dexamethasone on progesterone may, correspond to the suppression of CYP11A1 expression induced by GTP, however exact mechanisms are warranted to be investigated.

In summary, dexamethasone treatment altered thecal cell hormone profile to resemble characteristics of hyperandrogenism in PCOS and therefore may be considered as a useful *in vitro* model in murine ovarian cells. These effects were, at least in part, due to increased protein expression of CYP11A1. GTP treatment inhibited over-secretion of testosterone in theca cells through the downregulation of CYP11A1 expression and additionally reduced CYP17A1 expression at the higher concentration of GTP (25 µg/mL). These findings provided scientific evidence to support the use of the GTP for ovarian dysfunction leading to hyperandrogenism in PCOS.

Chapter Six

Effects of Green Tea Polyphenols on DNA Methylation in Deferent Passages of Human Adipose Derived Stem Cells

6.1. Introduction

Human adipose-derived stem cells (hADSCs) refer to the plastic-adherent and multipotent cell population isolated from collagenase digests of adult adipose tissue. hADSCs can be induced to differentiate along several mesenchymal tissue lineages, including adipocytes, osteoblasts, myocytes and chondrocytes. The differentiation ability of hADSCs has attracted research interest because hADSCs are easier to obtain, have relatively lower donor site morbidity, a higher yield and rapid ex-vivo expansion. When cultured under defined *in vitro* condition differentiation of hADSCs may have potential use in regenerative medicine. The differentiation stability of hADSCs is a crucial factor for clinical therapy because long-term culture may cause the rick of the malignant transformation.
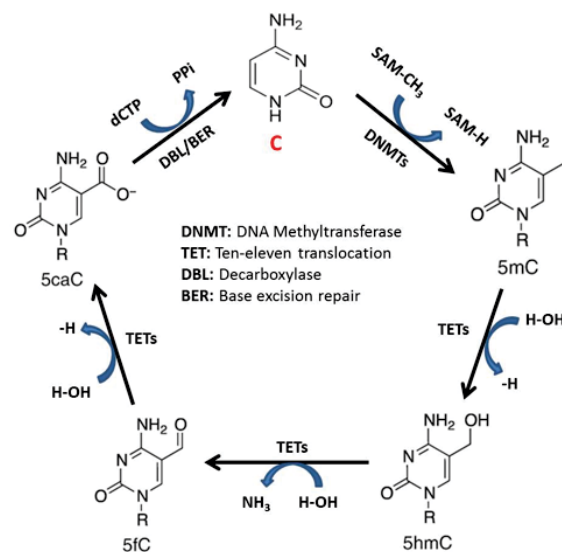
Figure 6.1 The pathway of DNA methylation. DNMT-mediated methylation of cytosine, stepwise TET-mediated 5mC oxidation to 5hmC/5fC/5caC, and the DNMTs mainly regulate the synthesis of 5MC.

Gene expression potential in ADSCs differentiation is regulated by epigenetic mechanisms that alter the transcriptional permissiveness of chromatin, of which DNA

methylation (DNAm) is the best characterized component (Reik 2007). DNA methylation is a process by which methyl groups are added to the cytosine base of DNA molecule. Methylation can change the activity of a DNA segment without changing the sequence. DNA methylation is a common epigenetic modification catalysed by DNA methyltransferase (DNMT). Therefore, expression of DNMTs, including DNMT-1, 3a, 3b, can be used as biomarkers of DNA methylation in ADSCs differentiation (Bentivegna *et al.,* 2013). 5Mc is a methylated form of the DNA base cytosine that may be involved in the gene transcriptional function of DNMTs, so it is believed that the up-regulated the DNMTs may result in the increasing of 5Mc (Figure 6.1) (Kim *et al.,* 2016).

DNA methylation is essential for normal development and is also involved in aging and carcinogenesis. Consistent with these important roles, a growing number of human diseases was found to be associated with aberrant DNA methylation. Recent study using genome-wide DNA methylation analysis has identified hypermethylation as a potential epigenome mark of obesity-related human colorectal cancer (Crujeiras *et al.,* 2018). Thus, agents for DNA methylation maintenance could be useful not only for safety of hADSCs expand for therapeutic applications but also for cancer prevention.

Currently, there is the lack of reagents to control genomic stability during hADSCs differentiation. Previous study of this thesis has shown that treatment of hADSCs with green tea polyphenols (GTP) inhibited lipid accumulation by arresting adipocyte differentiation into fat cells through downregulating adipogenic transcriptional factor PPARγ. It has been well documented that GTP processes anti-cancer effects though anti-oxidant species and induction of apoptosis (Yu *et al.,* 2017, D'Angelo *et al.,* 2017, Hayakawa *et al.,* 2016). Based on these observations, it was speculated that GTP would be capable of regulating differentiation ability and genomic stability of hADSCs,

therefore, to reduce the risk of oncogenic gene expression potential during ADSCs expansion. To verify this hypothesis, this study observed effects of GTP on DNA methylation by comparing differential methylation biomarkers of DNA methylation, including DNMT-1, 3a, 3b, between early and late passages of ADSCs.

6.2. Research Plan and Methods

6.2.1. Green Tea Polyphenols

The tea polyphenols (99% in purity) were extracted from green tea leaves (Camellia sinensis) grown in Guizhou province, south-western China, by Zuyilushen Kangyuan Co (Guizhou, Meitan, China). The polyphenolic compounds identified using liquid chromatography-mass spectrometry (LC-MS) were 68% EGCG, 7% EGC, 1% ECG and 18% EC in GTP (w/w).

6.2.2. Adipose tissue-derived stem cells (ADSCs) culture and collection

ADSCs isolation was described previously (Section 4.2.2). ADSCs at passage 6 were sub-cultured for DNA methylation study. To observe effect of GTP on stability during ADSCs expansion, primary ADSCs at passage 6 was defined as starting point (passage 0) then continuously cultured for next 6 passages.   The cells at passage 0, 2, 4 and 6 were collected for following protein and mRNA analysis.

6.2.3. ADSCs Proliferation Assay

SYBR® Green I-based fluorescence assay for ADSCs proliferation was used to determine appropriate seeding density of ADSCs in 96 well plat followed by screening test with a series of GTP concentrations. Firstly, the cells were seeded at various concentrations (0, 300, 600, 900 cells/well) in triplicate. Cells were incubated in 200 μL completed medium/well for 24, 48, 72 and 96 h. At each experimental time point, plates were taken out from the incubator, and media was removed out and frozen at -80°C. Frozen plates were then thawed and 100 μL of SYBR Green I (1:8000) in a lysis buffer (10 mM Tris, 2.5 mm EDTA, 0.1% Triton X-100) was added to each well. Plates were then left for a further 72 h at 4°C in the dark. Fluorescence intensity was then measured at 485 nm excitation and 530 nm emission filters using a microplate reader and Magellan Software (Tecan, Zürich, Switzerland). The result of fluorescence intensity in each well was calculated and presented as percentage of the control.

To determine effect of GTP on ADSCs viability, 600 cells/well was seeded in 96 well plate in the presence of vehicle (medium) or GTP (0.1, 1, 10 and 100μg/ml) and cultured as above. ADSCs proliferation with variety of GTP treatments was determined by measuring fluorescence intensity and presented as percentage of the control (vehicle treatment).

6.2.4. GTP treatment and Immunofluorescence stain

5×10^4 ADSCs defined as passage 0, 2, 4 and 6 were seeded on coverslips in a 24 well plate. After reaching 80% confluence, cells were treated with GTP (0.1, 1, 10 μg/ml) and medium treatment as the control. After 24 h of treatment, plate was washed twice with

PBS and fixed with 4% paraformaldehyde. Following this, cells were permeabilised in PBS with Tween-20 and Triton X-100 (0.06% Tween-20 and 0.04% Triton X-100). Cells were washed twice again with PBS and blocked with 5% BSA solution with 10% goat serum. To determine effect of GTP on expression DNA methylation markers immunofluorescence stain with primary antibodies (1:200 dilution, 150 µl of rabbit polyclone antibodies against DNMT1, DNMT3a and DNMT3b, Sigma-Aldrich, MO, USA) and mouse-monoclonal antibody against 5Mc, Sigma-Aldrich, MO, USA) was applied followed by washing twice with PBS before counterstaining the cell nuclei with Hoescht® (1: 1000). Images of immunofluorescence staining and quantitative data were achieved by methods described previously (Section 5.2.5).

6.2.5. Immunoblotting analysis

Protein expression of Dnmt1, Dnmt3a, Dnmt3b and MYC were measured by immunoblotting. MYC is a family of regulator genes and proto-oncogenes that code for transcription factors. In the human genome, MYC is located on chromosome 8, which is believed to regulate expression of up to 15% of all genes by binding on enhancer box sequences (Gearhart *et al*., 2007). The most importance finding about MYC was activated upon multi-mitogenic signals such as WNT, SHH and EGF by the MAPK/ERK pathway (Boucherat *et al.*, 2014). So, the function of MYC could be used to detect the cell pluripotency in DNA methylation experiment.

The ADSCs culture and GTP treatment were same as immunofluorescence staining assay. Cells were harvested for protein extraction to determine protein expression of Dnmt1, Dnmt3a, Dnmt3b and MYC by immunoblotting described previously (Section 5.2.6). In brief, total protein (20 µg) extracted from cell lysates was separated by 12% SDS-PAGE

and electro blotted onto 0.45 µM polyvinyldenedifluoride (PVDF) membrane. Blotted

membranes were blocked with 5% skim milk in PBS with 0.05% Tween 20. Primary

antibodies from Santa Cruz Biotechnology (Dallas, TX, USA) for anti- Dnmt1 (H-12),

anti-Dnmt3a (H-295), anti-Dnmt3b (H-230) and anti-Myc (C-33) were diluted at 1:500

and incubated overnight at 4°C. After three washes in PBS-Tween, the blots were

incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1:

10,000) for 2 h at room temperature. Protein expression were visualized by the ECL

system (Pierce, Rockford, IL) and analysed using Quantity One 4.6.1 software of the

ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA).

6.2.6. Quantitative Real-time Reverse Transcription Polymerase Chain reaction

To further clarify the effects of GTP treatment on DNA methylation, mRNA expression

of *Dnmt1*, *Dnmt3a* and *Dnmt3b* was determined by qRT-PCR described previously

(Section 5.2.7). Amplification of cDNA with the following primers (Life technology, Au)

in Table 6. Quantitative data of mRNA expression were normalized to mRNA levels of

the control treatment and presented as $2^{-(normalized\ Delta Ct)}$.

Table 6.1. Primer sequences used in real-time PCR

Symbo*	Forward primer	Reverse primer
Dnmt1	ACCAAGCAGGCATCTCTGAC	GCAGGATGTTGCAGTCCTCT
Dnmt3a	AAGCAGCGCCAGGTGGA	TATCGTGGTCTTTGGAGGCG
Dnmt3b	GGAGATTCGCGAGCCCAG	CTCCCTTCATGCTTTCCTGC
Actb	CTCACCATGGATGATGATATCGC	AGGAATCCTTCTGACCCATGC

Real-time PCR primer set for Theca cell (mouse) were obtained from Sigma-Aldrich, Inc.

6.2.7 Statistical Analysis

All values are expressed as the mean ± SE. Comparisons across the three groups were performed using one-way ANOVA followed by Tukey's test to determine significant differences between the two treatments using Prism version 6 (GraphPad Software Inc, CA, USA). P-value < 0.05 was considered statistically significant.

6.3. Results

6.3.1. Effect of GTP treatments on ADSCs proliferation

ADSCs were cultured in 96 well-plates and cproliferation at 24, 48, 72 and 96 h were assayed with the fluorescence dye (SYBR Green I). Fluorescence density provides relative quantification of cultured cells. Figure 6.2 showed that well-seedings using 600 cells, comparing to 900 cells, gave similar cellular growth after 96 days of incubation, suggesting that 600 cells seeding perwell is sufficient for the purpose of the assay.

Initially seeded 600 cells was similar to fluorescence density in the well seeded 900 cells initially, indicating 600 cells as initial seeding density was sufficient, which was used in the cell culture with GTP treatments.
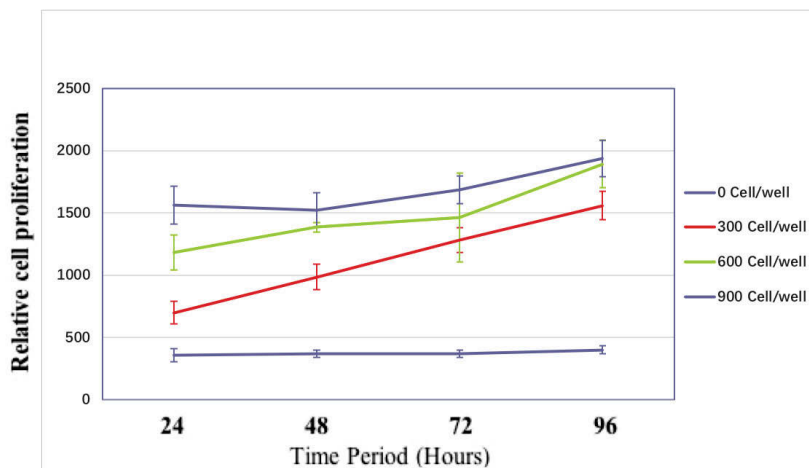
Figure 6.2 The ADSCs proliferation on various seeding density by SYBR green assay.
The proliferation of ADSCs on 0 cells, 300 cells, 600 cells, 900 cells per well in triplicate stained by SYBR green I after 24, 48, 72, and 96 hours of culture.

Results in Figure 6.3 indicated that GTP at 0.1, 1.0, 10 µg/ml did not affect ADSCs proliferation compared with the vehicle treatment. 100 µg/ml of GTP significantly inhibited ADSCs proliferation at all experiment time points (Figure 6.3), indicating cytotoxicity occurred at this dosage.
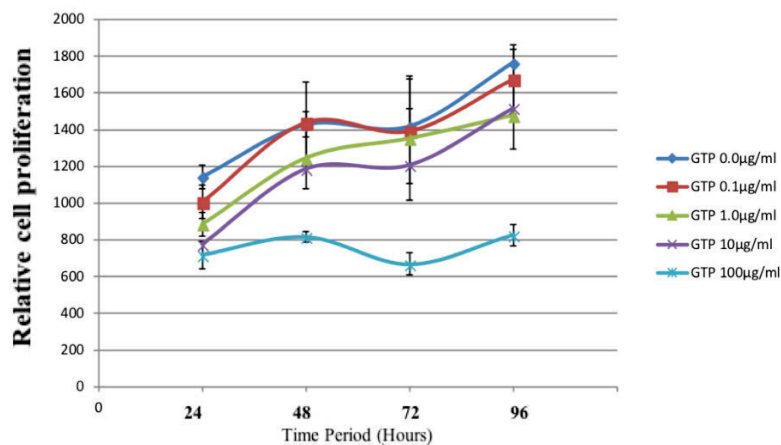
Figure 6.3 The ADSCs proliferation with different concentrations of GTP (0.1, 1, 10 µg/ml) and medium only treatment. ADSCs were initially seeded in 96 well plate, 600 cells per well, and evaluated the effect of GTP on ADSCs proliferation at 24, 48, 72 and 96 h. Cell number was quantified with the fluorescence dye (SYBR Green I). Data are presented as Mean ± SEM from 3 separate experiments (n=3). *$p < 0.05$ GTP versus the control.

6.3.2. The inhibitory effects of GTP on Dnmt1 and 5Mc expression during ADSCs culture in Immunofluorescence stain

The DNA methylation process is upregulated by key transcription factors, such as 5Mc and Dnmts including Dnmt1, Dnmt3a and Dnmt3b (Figure 6.1). To elucidate whether GTP affect DNA methylation during ADSCs passage, protein expression of Dnmt1, Dnmt3a and Dnmt3b and 5Mc were measured with immunofluorescence staining at passage 6 of ADSCs.   Dnmt1 and 5Mc were detected but there was no detection of Dnmt3a and Dnmt3b, suggesting Dnmt1 and 5Mc are major biological markers for DNA methylation during ADSCs passage.   GTP treatment at 10 µg/ml significantly decreased Dnmt1 and 5Mc expression.
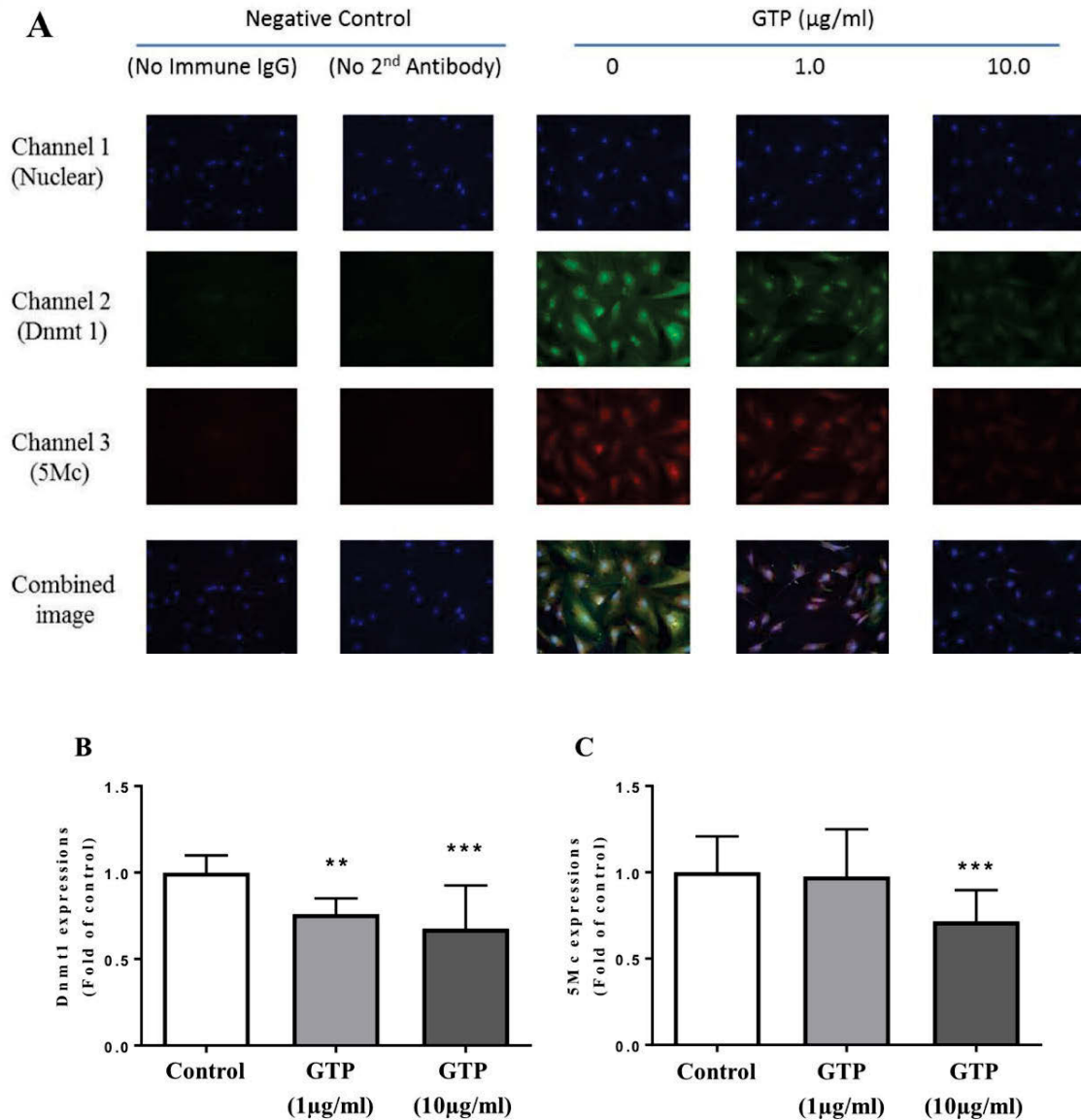
Figure 6.4 Dnmt1 and 5Mc expression in passage 6 of ADSCs with GTP treatments **(**1μg/ml and 10 μg/ml**)** and medium treatment as the control (A) Images of immunofluorescence staining with primary antibodies against Dnmt1 and 5Mc in ADSCs culture with or without GTP treatments, medium only samples were used as control. Quantitative fluorescence intensity of Dnmt1 (B) and 5Mc (C). Data are presented as Mean ± SEM from 3 separate experiments (n=3) $**p<0.01$, $***$ $p<0.001$ v.s the control.

6.3.3. GTP inhibited DNMTs expression in human ADSC detected by immunoblotting

Immunofluorescence study showed that the ADSC cells had DNMTI expression. Therefore, protein expression of DNMT1, DNMT3A AND DNMT3B were verified in passage 0, 2 4 and 6 of ADSCs cultures by western blotting. Consistent with immunofluorescence staining, Dnmt1 was detected only at passage 6 of ADSCs but also in samples collected from other passages. Interestingly, two bands at 250 and 183 kDa were revealed in all samples, and both of bands were increased with GTP treatment in a dose-dependent manner at first 4 passages but DNMT1 expression decreased at passage 6 of ADSCs treated with GTP treatment, especially with high dosage of GTP (Figure 6.5A). DNMT3A and DNMT3B were not detected by Western blotting. There was the error on experimental data of Passage 0 (High concentration of GTP), which is statistically meaningless. This set of data is retained as part of the experimental results, but not used for the experimental results and discussion.

MYC is a family of regulator genes and proto-oncogenes that code for transcription factors. In the human genome, MYC is located on chromosome 8, which is believed to regulate expression of up to 15% of all genes by binding on enhancer box sequences (Gearhart *et al.,* 2007). The most importance finding about MYC was activated upon multi-mitogenic signals such as WNT, SHH and EGF by the MAPK/ERK pathway (Boucherat *et al.,* 2014). In this experiment, MYC was used to detect the cell pluripotency, and the result showed that MYC expression was not affected by GTP, indicating ADSCs culture from passage 0 to passage 6 reminded as similar stem cell pluripotency (Figure 6.5A).

A   **GTP effects on DNMTs in human ADSCs**
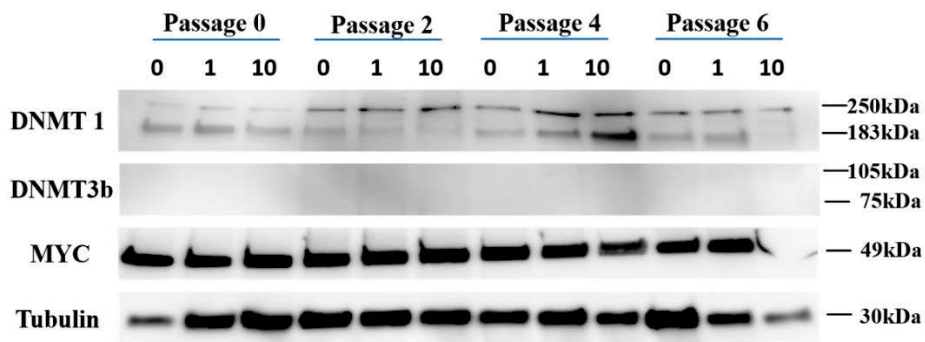
B
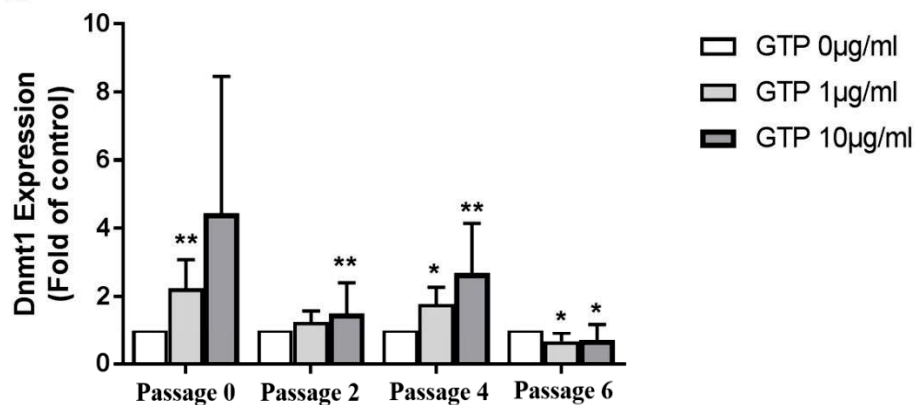
Figure 6.5 Dnmts and MYC expression during ADSCS culture with GTP or medium treatment. Protein expression of Dnmt1 and MYC was detected showing as immunoblotting band (A) and quantitative data (B). All values are presented as mean ± SE (n = 6). *$p < 0.05$ and **$p < 0.01$, GTP treatment versus the control.

6.3.4. GTP treatments down-regulated mRNA of *Dnmt1* in human ADSC

qRT-PCR analysis was used to verify gene expression of Dnmts. Consistent with protein detection, mRNA of Dnmt3A and Dnmt3B was not revealed. Similar to the result of immunoblotting, Dnmt1gene expression increased in the first four passages of ADSCs culture with GTP treatment in a dose-dependent manner. However, mRNA expression of Dnmt1 in passage 6 of ADSCs culture was downregulated by GTP treatment at 10 µg/ml, suggesting that DNA methylation was upregulated at the early passage of ADSCs and downregulated at the late passages of the culture.
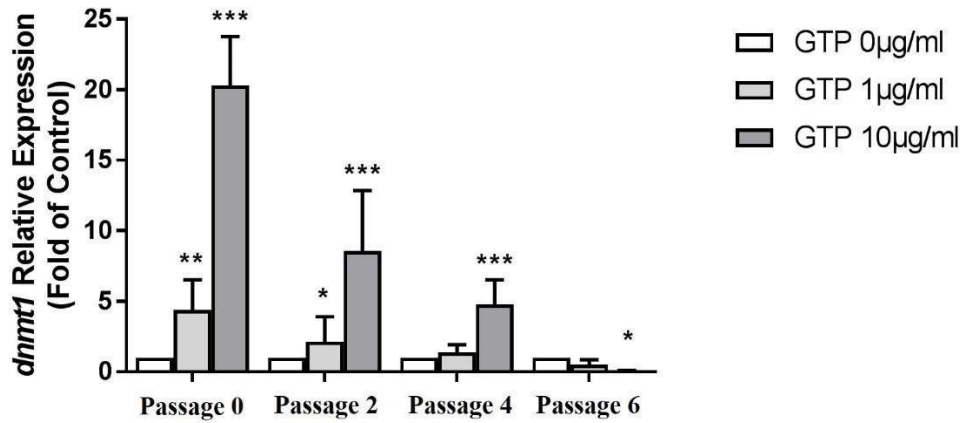
Figure 6.6   mRNA expression of *Dnmt1* in variety of ADSCS passages with GTP or medium (the control) treatment. All values are presented as mean ± SME (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.001, GTP treatment versus the control.

6.4. Discussion

The present study demonstrated that GTP processes potential to regulate DNA methylation during ADSCs expansion by alternating DNMT1 expression. Primary ADSCs used in the study was from passage 6 (termed P0 in this study). During the cell expansion from passage 6 to passage 12 (termed P6 in this study), GTP treatment enhanced expression of DNMT1 at protein and mRNA levels at the early passages of ADSCs (P2 and P4) but downregulated DNMT1 expression at the late passage of ADSCs culture (P12).

DNA methylation is a common epigenetic modification, which plays a pivotal role in the new development as well as an important aetiology of cancer (Fakhr *et al.,* 2013, Koch *et al.,* 2018). The DNA methylation occuredby covalently adding a methyl group to the 5′ carbon of the cytosine ring, resulting in 5-methylcytosine (Breiling and Lyko 2015). The addition of a methyl group to the cytosine residence is catalysed by DNA methyltransferases (DNMTs). DNMT family includes four active enzymes: DNMT1,

DNMT3A, DNMT3B, and DNMT3L (DNMT2 which potentially methylate RNA instead of DNA). DNMT1 is the proposed maintenance methyltransferase responsible for copying DNA methylation patterns to newly biosynthesized DNA during replication. In this study, enhancement of DNMT1 without changing MYC expression in GTP treated ADSCs suggests that GTP may promote biosynthesized DNA during early passages of ADSCs, meanwhile, it can maintain certain the cell pluripotency. The result of this study suggests that prior to 4th generation of ADSCs culture, GTP enhanced DNMT1, which in turn, promotes young cells from copying DNA methylation patterns to newly biosynthesized DNA during replication. This funding suggests that GTP may be beneficial to ADSCs induction for regenerative medicine.

Interestingly, this study found that GTP downregulated DNMT1 expression at passage 12 of ADSCs culture, indicating an inhibitory effect on DNA methylation at late stage of ADSCs expansion.   GTP is able to stabilize DNA methylation when ADSCs become ageing (passage 12). By this way, GTP maintains the pluripotency and self-renewal of stem cells and reduces the risk of malignant mutations due to DNA hypermethylation. Aberrant DNA methylation-mediated epigenetic gene silencing has constantly engaged in the development of anti-cancer therapies that work by inhibiting DNA methylation and restore normal epigenetic landscape by reprogramming of genes involved in disease mechanisms (Agrawal *et al.*, 2018).

In summary, the current study discovered GTP enhanced DNMT1 expression without changing MYC expression at early stage of ADSCs passage and downregulated DNMT1 expression at late passage 12 of ADSCs culture. The dual regulatory effects of GTP on DNA methylation at different stages of ADSCs expansion highlight the potential this naturally occurring compound in ADCSs expansion for regenerative medicine.

Chapter Seven

Conclusions and Future Direction

7.1 Summary and conclusions

The prevalence of obesity has become a health burden in economically developed countries and in countries that continue to face economic challenges. Obesity is defined as an excess of body adiposity and now regarded as a chronic metabolic disease affecting adults and children worldwide. The current obesity pandemic is placing a huge burden on public health globally and become one of the leading causes of death. In 2015, high body mass index contributed to 4.0 million deaths globally, which represented 7.1% of the deaths from any cause (Kushner and Kahan 2018) as obesity is known to be the primary risk factor for a number of non-communicable diseases, including insulin resistance syndrome, type 2 diabetes, ischemic cardiovascular disease and certain type of cancers.

Currently, there is the lack of pharmaceutical drugs for satisfactorily controlling obesity and prevention its metabolic complications. To discover and develop new agents to manage obesity and insulin resistance syndrome, naturally occurring compounds have received great attention in recent years as they portray minimal side effects, are cost-efficient and readily available in our society. Green tea polyphenols (GTP) have been found to have health-promoting metabolic impacts to ameliorate insulin resistance, type 2 diabetes and obesity (Cao *et al.*, 2000; Kao *et al.,* 2006). Recent studies have shown that GTP and its primary active compound epigallocatechin gallate (EGCG) have beneficial effects on both hyperlipidemia and hyperglycemia (Rains *et al.*, 2011).

Our laboratory has obtained 99% purity of GTP that was extracted from green tea leaves (*Camellia sinensis*) grown in Guizhou province, south-western China by Zuyi Lushen Kangyuan Co (Guizhou, Meitan, China). The polyphenolic compounds identified using

liquid chromatography-mass spectrometry (LC-MS) were 68% EGCG, 7% epigallocatechin, 1% epicatechin gallate and 19% epicatechin in GTP (w/w). Our previous studies have demonstrated that GTP possesses effects on the high fat diet-induced metabolic syndrome and attenuates fat accumulation in the liver and enhance insulin sensitivity (Kim *et al*., 2013; Tan *et al.* 2017). Studies in this thesis aimed to evaluate effects of GTP on cellular dysfunction related to the development of obesity and its complications.

Because increasing differentiation of adipocytes from preadipocytes contributes to fat mass expansion and leads to obesity, the first study of this thesis evaluated effects and molecular mechanism of GTP on the dysfunction of adipocytes. The results demonstrated that GTP suppressed the differentiation of 3T3-L1 preadipocytes into mature adipocytes through down-regulating adipogenic regulators of PPARγ, C/EBPα, and SREBP-1c at gene expression and post-transcriptional level. The findings from this study suggest that GTP can combat unnecessary adipogenesis at the cellular level to prevent obesity and subsequently may reduce the risk of type 2 diabetes and cardiovascular disease.

Study in Chapter 4 of the thesis investigated whether GTP possesses the ability to limit human adipose tissue-derived stem cells (hADSCs) differentiation to adipogenic lineage and concomitantly enhance osteogenesis. The study was utilizing pioglitazone (PPARγ agonist) as a controled drug identified that stimulating PPARγ activity promoted adipogenesis and diminished osteogenesis. GTP treatment alone inhibited ADSCs differentiation to mature adipocytes and promoted osteogenesis. Besides, GTP also ameliorated pioglitazone-enhanced adipogenesis, through suppressing the PPARγ expression and upregulating Runx2-Bmp2 mediated osteogenic pathway.

Obesity associated with insulin resistance plays an essential role in the development of PCOS, which is a metabolic and reproductive disorder affecting more than 15% of women at childbirth age. The study in Chapter 5 utilizing primary murine theca cells has demonstrated that GTP can normalize ovarian cell dysfunction-induced by dexamethasone attenuates over-secretion of testosterone through downregulation of the expression of CYP17A1 and reduced CYP11A1 expression. These findings provide scientific evidence to support the use of GTP for ovarian dysfunction leading to hyperandrogenism in PCOS.

The recent study using genome-wide DNA methylation analysis has identified hypermethylation as a potential epigenetic change of obesity-related cancer. Based on the antiadipogenic effect of GTP during ADSCs differentiation, the study in Chapter 6 evaluated the effects of GTP on DNA methylation in deferent passages of ADSCs. This study discovered GTP enhanced DNA methyltransferase 1 expression without changing MYC expression at early stage of ADSCs passages and downregulated DNMT1 expression at passage 12 of ADSCs culture. This primary study suggests that GTP may have dual regulatory effects on DNA methylation during ADSCs expansion, which may be able to maintain epigenetic stability ADSCs culture for regenerative medicine without carcinogenic risk.

In conclusion, the *in vitro* studies conducted in this thesis have reveled GTP is capable of correcting cellular dysfunction against adipogenesis, enhancement of osteogenesis and ameliorate excessive androgenesis by ovarian theca cells. The cellular studies have illustrated that the beneficial effects of GTP achieved through regulating gene and protein expression involved in adipogenesis, osteogenesis, androgenesis and DNA methylation.

7.2 Future Directions

The findings of the thesis highlighted a thoughtful insight towards developing GTP as multifaceted therapeutic goods for the intervention of obesity-associated osteoporosis, POCS and utilizing GTP as a natural agent to maintain epigenetic stability ADSCs culture for regenerative medicine without carcinogenic risk.

There are two main directions for future research. Firstly, to gain depth understanding of molecular mechanisms by which GTP inhibit the development of obesity and prevent obesity associated with metabolic complications proteomic profiling may be applied to reveal more and precise proteins and enzymes involved in GTP-mediated metabolic pathways. Secondly, therapeutic doses of GTP from the cellular study translating to clinical use should be optimized either in health volunteers (Phase I clinical trial) or subjects with obesity (Phase II clinical trial).

Furthermore, study of this thesis demonstrated GTP possesses ability against pioglitazone-induced adipogenesis, thus adding GTP on this insulin sensitizer may provide the synergetic effect on obesity associated with insulin resistance without body weight again. Further study to investigate the interaction between GTP and TZD medication may be an interesting research area.

Overall, to gain deeper insight into the therapeutic application of GTP for obesity and its metabolic complications, the animal studies and clinical trials are warranted in the future study.

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