

T.R.
GEBZE INSTITUTE of TECHNOLOGY
GRADUATE SCHOOL of ENGINEERING and SCIENCES

THE EFFECTS of VEGF-C and SIK2 on POLARIZATION
and RECRUITMENT of MACROPHAGES

HAMZA FURKAN ALKAN
A THESIS SUBMITTED for THE DEGREE of
MASTER of SCIENCE
DEPARTMENT of MOLECULAR BIOLOGY and GENETICS

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ASSOC. PROF. DR. FERRUH ÖZCAN

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**GEBZE YÜKSEK
TEKNOLOJİ ENSTİTÜSÜ**

YÜKSEK LİSANS JÜRİ ONAY FORMU

GYTE Mühendislik ve Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 20.01.2014 tarih ve 2014/04 sayılı kararıyla oluşturulan jüri tarafından 18/02/2014 tarihinde tez savunma sınavı yapılan Hamza Furkan Alkan'ın tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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SUMMARY

Obesity, of which prevalence has reached 10% in the entire globe, consists of great risks for human health via contributing to the development of other diseases such as type 2 diabetes, metabolic syndrome and cardiovascular diseases. High levels of Endoplasmic Reticulum (ER) stress and inflammatory macrophage accumulation in adipose tissue is commonly associated with insulin resistance in obesity. Increased level and activity of SIK2 in adipose tissues of obese subjects along with the involvement of SIK2 in both endoplasmic reticulum stress response and polarization of macrophages suggests a potential critical regulatory role for this kinase in development of chronic low-grade inflammatory state associated with obesity.

In our study, using Simpson-Golabi-Behmel Syndrome (SGBS) human adipocyte cell line and THP-1 human monocytes/macrophages, we investigated the effects of SIK2 and Endoplasmic Reticulum stress to the inflammatory profile of human adipocytes at transcriptional level and the effects of conditioned medium collected from stressed and SIK2 inhibited adipocytes on macrophage polarization. Furthermore, in VEGF-C overexpressing FVB background mice on HFD, we also try to characterize whether SIK2 is taking place in downstream of VEGF-C/VEGFR3 pathway which is shown to recruit inflammatory macrophages into the adipose tissue resulting increased levels of inflammation.

In conclusion, our results may point out a critical role of SIK2 for the development of chronic inflammation in obesity via supporting the pro-inflammatory (IL-6) and suppressing the anti-inflammatory (IL-10 and IL-5) cytokine production in human adipocytes. We hope that our study bears a potential extent to SIK2 to be targeted in various inflammatory diseases including obesity, immune diseases, arthritis, dementia etc.,

Key Words: Obesity, Metabolic Inflammation, Endoplasmic Reticulum (ER) Stress, SIK2, IL-10, IL-5, IL-6, VEGF-C, Macrophage Polarization.

ÖZET

Dünya üzerindeki rastlanma oranı artık %10a erişmiş obezite, Tip-2 Diyabet, Metabolik Sendrom ve Kardiyovasküler hastalıklar gibi diğer birçok hastalığın da oluşmasına ve gelişmesine katkıda bulunmasıyla birlikte, insan hayatını tehlikeye sokan en önemli sağlık problemlerinden biri olarak değerlendirilebilir. Yüksek düzeyde Endoplazmik Retikulum stresi ve yağ dokudaki artan enflammatuvar makrofaj birikmesi genel olarak obezite durumunda ortaya çıkan insülin direnciyle ilişkilendirilir. Obez bireylerin yağ dokularında SIK2'nin miktar ve aktivite olarak artışı ve SIK2'nin hem Endoplazmik Retikulum stres cevabında hem de makrofaj polarizasyonunda rol alıyor olması, bu kinazın obeziteye bağlantılı olarak ortaya çıkan kronik enflammasyonun gelişiminde potansiyel kritik bir rolü olabileceği fikrini bizlere vermiştir.

Bu çalışmamızda, Simpson-Golabi-Behmel Sendromu (SGBS) insan adiposit hücre hattını ve THP-1 insan monosit/makrofajlarını kullanarak; SIK2'nin ve Endoplazmik Retikulum stresinin transkripsiyonel düzeyde insan adipositlerinin enflammatuvar pfofillerine etkisini ve strese ve SIK2 inhibisyonuna maruz kalmış hücrelerden alınan besiyerinin makrofaj polarizasyonu üzerine etkisini inceledik. Buna ek olarak, VEGF-C'yi overeksprese eden FVB fareleri yüksek yağlı diyetle tabi tutarak, SIK2'nin yağ dokuya enflammatuvar makrofaj çağrılmasında görevli olduğu da bilinen VEGF-C/VEGFR3 yolağında bulunup bulunmadığına baktık.

Sonuçlarımız, SIK2'nin, insan yağ hücrelerinden pro-enflammatuvar (IL-6) sitokin üretimini desteklerken anti-enflammatuvar (IL-5 ve IL-10) sitokinlerin üretimini baskılayarak obezite durumundaki kronik enflammasyon halinin gelişmesinde önemli bir düzenleyici rolü olabileceğini göstermektedir. Umuyoruz ki çalışmalarımız SIK2'nin potansiyel kapsamı hakkında bize yeni ufuklar açacak ve obezite, immün hastalıklar, eklem yangısı gibi çeşitli enflammatuvar hastalıklar için hedef alınarak bu hastalıkların tedavisine olanak sağlayacak.

Anahtar Kelimeler: Obezite, Metabolik Enflammasyon, Endoplazmik Retikulum (ER) Stres, SIK2, IL-10, IL-5, IL-6, VEGF-C, Makrofaj Polarizasyonu.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Assoc. Prof. Ferruh Özcan for trusting me enough to let me work on one of his valuable projects and supporting me during whole my study. His directly goal-oriented, calm and supportive personality made my master work more efficient and less painful. Due to the way of his critical thinking and technical knowledge, it was also a very didactic experience to work with him.

I would also like to thank my committee members Assoc. Prof Ayhan Çelik and Assoc. Prof Uygar Halis Tazebay for their important criticism and ideas. I am thankful to Prof Martin Wabitsch for SGBS cells and Assoc. Prof. Nesrin Özören for THP-1 cells. In addition, I am also thankful to Prof Michael Detmar and Dr. Sinem Karaman for my works in ETH Zürich.

Of course, I would like to appreciate the helps of and the friendly environment provided by all the current, previous and temporal members of OBEDIA LAB including Tuğçe Keskiner, Fehime Şeyma Özen, Onur Çalışkaner, Meryem Topçu, Betsi Köse, Nazlı Erciyas, İlkay Göksu Polat, Fatih Altıntop, Mustafa Çoban, Özge Arslan. It would be very hard to maintain and to finish my work without them. I am also grateful to other members of our institute whose helps I cannot ignore: Zeynep Özcan, Hüseyin Gül, Fatma Sağır, Emrah Özçelik, Nurettin Ayvalı, Veysel Süzerer, Mehmet Sarıhan, Gülşife Candemir, Ebru Turhanlar, Ekin Kestevur, Zeynep Yılmaz, Ayça Arslanhan, Nesil Han Altaca, Zuhale Bayram, Abdallah Waheed, Metin Çetin, Türkan Çakar.

I would like to express my special thanks to the inventor of coffee and Red-bull, both helped me to go through the sleepless nights in the lab so many times.

Last but not least, my most sincere gratitude would go to my mother and father who believed in me more than I believed in myself.

This thesis was supported by Turkish Scientific and Research Council Fund, 112T274.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
μL	: microliter
μM	: micromolar
AMPK	: AMP-activated Kinas
APS	: Amonium Persulfat
ATP	: Adenosine Triphosphate
BAT	: Brown Adipose Tissue
BCA	: Bicinchoninic Acid
BiP	: Binding Immunoglobulin Protein
BMI	: Body Mass Index
bp	: basepair
BSA	: Bovine Serum Albumin
CaCl ₂	: Calcium Chloride
CC	: Compound C
CD11b	: Cluster of Differentiation Molecule 11b
CD163	: Cluster of Differentiation Molecule 163
CD206	: Cluster of Differentiation Molecule 206
cDNA	: complementary DNA
CEBPβ	: CCAAT-enhancer-binding proteins
CRTC	: cAMP response element binding protein
Dex	: Dexamethasone
DMEM/F12	: Dulbecco's Modified Eagle Medium/ Ham's F12 Medium
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
ER	: Endoplasmic Reticulum
FBS	: Fetal Bovine Serum
FFA	: Free Fatty Acid
HFD	: High-Fat Diet
HOMA-IR	: Homeostatic Model Assessment of Insulin Resistance
g	: gram

IBMX	: 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione
IFN γ	: Interferron gamma
IL-10	: Interluekin 10
IL-18	: Interluekin 18
IL-1 β	: Interluekin 1beta
IL-5	: Interluekin 5
IL-6	: Interluekin 6
IR	: Insulin Resistance
JNK	: c-Jun N-terminal kinase
kDa	: kiloDalton
kg	: kilogram
LKB1	: Liver Kinase B1
MACS	: Magnetic Assissted Cell Sorting
MCP-1	: Monocyte Chemoattractant Protein 1
mg	: miligram
MgCl ₂	: Magnesium Chloride
mL	: mililiter
mM	: milimolar
mRNA	: messenger RNA
Na ₃ VO ₄	: Sodium Orthovanadate
NaCl	: Sodium Chloride
NAFLD	: Non-Alcholic Fatty Liver Disease
NF- κ B	: Nuclear Factor kappa B
nm	: nanometer
nM	: nanomolar
PCR	: Polymerase Chain Reaction
Pen/Strep.	: Penicilin/Streptomycin
PMA	: phorbol 12-myristate 13-acetate
PMSF	: Phenylmethanesulfonylfluoride
PPAR γ	: Peroxisome Proliferator-Activated Receptors
Pref1	: Preadipocyte factor 1
PVDF	: Polyvinylidene Fluoride
qPCR	: quantitative PCR
RNA	: Ribonucleic Acid

rpm	: Rotations Per Minute
RPMI	: Roswell Park Memorial Institute medium
SC	: Subcutaneous
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: SDS- Polyacrylamide Gel Electrophoresis
Ser	: Serine
SGBS	: Simpson-Golabi-Behmel Syndrome
shRNA	: small hairpin RNA
SIK	: Salt Inducible Kinase
SIK2	: Salt Inducible Kinase 2
sXBP-1	: spliced X-Box Binding Protein 1
T2DM	: Type-2 Diabetes Mellitus
T3	: triiodothyronine
TBS	: Tris Buffered Saline
TBST	: Tris Buffered Saline Tween
TEMED	: Tetramethylethylenediamine
TG	: Transgenic
THA	: Thapsigargin
Thr	: Threonine
TLR-4	: Toll-like receptor 4
T _m	: Melting Temperature
TNF α	: Tumor Necrosis Factor alpha
UBA-	: Ubiquitin Associated -
UPR	: Unfolded Protein Response
UV	: Ultraviolet
VEGF-C	: Vascular Endothelial Growth Factor C
VEGFR3	: Vascular Endothelial Growth Factor Receptor 3
VS	: Visceral
WAT	: White Adipose Tissue
WT	: Wild-Type

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1. INTRODUCTION

1.1. Aim of the Study

As its prevalence has reached 10% globally and being the major risk factor for diseases such as diabetes, coronary heart disease, dyslipidemia, hypertension and stroke; obesity has become a crucial topic of investigation in the recent years [3], [5]. Obesity constitutes the sixth greatest health risk underlying numerous metabolic diseases and reducing the life expectancy [2].

Although some of the obese individuals have been shown to be metabolically healthy, insulin resistance –defined as a metabolic deficiency that causes type 2 diabetes mellitus (T2DM) and metabolic syndrome– is the most common and inevitable risk of obesity [11], [14]. By altering the cytokine production profile of adipocytes, obesity-induced insulin resistance leads to metabolic inflammation in the adipose tissue that later worsens the insulin-resistant state of adipocytes. Moreover, obese subjects have increased number and percentage of macrophages in adipose tissue [7], which are known to play a major role in the development of inflammation and insulin resistance.

Endoplasmic Reticulum stress is one invariably associated with metabolically active tissues of obese subjects and animals. Increased level and activity of SIK2 in adipose tissues of obese subjects along with the involvement of SIK2 in both endoplasmic reticulum stress response [79] and polarization of macrophages suggests a potential critical regulatory role for this kinase in development of chronic low-grade inflammatory state associated with obesity.

In this study, using Simpson-Golabi-Behmel Syndrome (SGBS) human adipocyte cell line [86], [87] and THP-1 human monocytes/macrophages [88], we investigate the effects of SIK2 and Endoplasmic Reticulum stress to the inflammatory profile of human adipocytes at transcriptional level and the effects of conditioned medium collected from stressed and SIK2 inhibited adipocytes on macrophage polarization. Furthermore, in VEGF-C overexpressing FVB background mice on HFD, we also try to characterize whether SIK2 is taking place in downstream of VEGF-C/VEGFR3 (Vascular Endothelial Growth Factor C and its receptor) pathway which is shown to recruit inflammatory macrophages into the

adipose tissue resulting increased levels of inflammation. We hope that our study bears a potential extent to SIK2 to be targeted in various inflammatory diseases including obesity, immune diseases, arthritis, dementia etc.,

2. BACKGROUND

2.1. Obesity and its Outcomes

“Obesity is not only a disease itself, but also the harbinger of others”
Hippocrates (BC 460 – BC 375).

2.1.1. Obesity

Obesity is simply defined as a body-mass index (BMI: weight (kg)/height-squared (m^2)) greater than or equal to 30 kg/m^2 whereas BMI greater than or equal to 25 kg/m^2 is considered as overweight. Excessive caloric intake and sedentary lifestyle along with susceptible genetic backgrounds are considered to play significant role in the development of obesity (reviewed in [1]). Higher BMI has shown to be the sixth most crucial risk factor in the entire globe for causing various metabolic diseases [2] including diabetes, hypertension, dyslipidemia and even several related cancers [3]. In addition, excess body weight has also been indicated to shorten the life expectancy by approximately 7 years [4]. According to the latest statistics of World Health Organization (WHO), the prevalence of obesity has dramatically increased over the last three decades and reached over 10% of the global population [5].

Obesity occurs when the long-term caloric intake is higher than the energy expenditure (reviewed in [6]). The energy imbalance affects adipose tissue –as well as liver and skeletal muscle– and triggers an “insulin-resistant” state, which leads to metabolic disorders such as type 2 diabetes mellitus (Figure 2.1) [7]. It has been reported that obese subjects have larger and increased number of adipocytes in the adipose tissue [8]. Furthermore, anatomical location of fat has a critical role in metabolic diseases as well. For instance, expanded visceral fat induces crucial risks for metabolic diseases; however, expanded subcutaneous fat in the thighs and hips does not increase the risk to develop metabolic disorders [9].

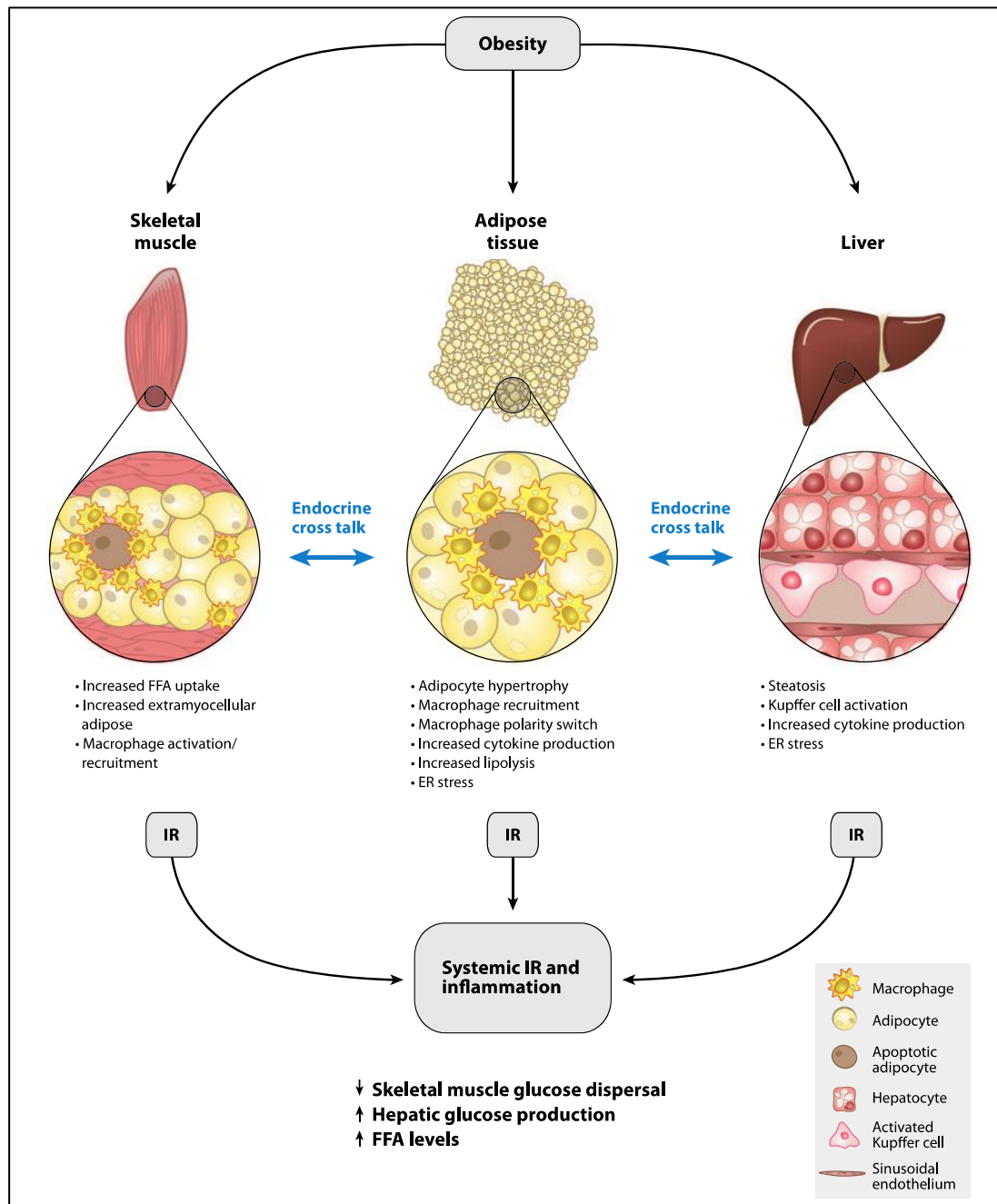


Figure 2.1: Summary of the major effects of obesity on liver, skeletal muscle and adipose tissue.

2.1.2. Metabolic Syndrome, Type 2 Diabetes and Insulin Resistance

Metabolic syndrome, which is defined by the International Diabetes Federation as having at least two of the following four features: abnormal concentration of triglycerides, HDL cholesterol and fasting glucose and hypertension [10], is one of the most common outcomes diagnosed in obese individuals [11].

Likewise, another metabolic disorder called type 2 diabetes mellitus (T2DM) that involves higher levels of circulating glucose due to the lack of insulin production is highly associated with obesity [3]. Both T2DM and metabolic syndrome are results of obesity-induced insulin resistance [3], [10], [11].

Insulin resistance develops following the excessive fat deposition and secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin 6 (IL-6) by the enlarged adipocytes in obesity (reviewed in [3]). Increase in these factors results in decreased production of adiponectin, which is known to increase insulin sensitivity [12], [13]. The spillover of excess lipids into the pancreatic islet cells diminishes their ability to produce adequate insulin in long-lasting insulin resistance, which causes glucose intolerance and later premature type 2 diabetes (reviewed in [3]). On the other hand, the fact that obese people without insulin resistance happen to have smaller adipocytes compared to metabolically unhealthy obese people suggests a positive correlation between adipocyte size and insulin resistance [14].

In a healthy individual, insulin has two main roles in energy metabolism: (1) promoting the glucose uptake and lipogenesis and (2) inhibiting the gluconeogenesis and inducing the break-down of complex biomolecules such as lipids, proteins and glycogen [15]. Insulin regulates the cellular glucose uptake by increasing the concentration of glucose transporters (Glut 4 and Glut 1; in adipocytes and skeletal muscle cells) on the cell membrane (Figure 1.2). Glut 4 molecules normally reside in the vesicular compartments in the cytoplasm in the absence of insulin. When insulin stimulates the insulin receptors, Glut 4 vesicles fuse within the cell membrane as a result of insulin signaling pathways and glucose can be taken through those Glut 4 transporters that are translocated to the cell membrane (reviewed in [16]). In insulin-resistant states or in complete lack of insulin, as in type 2 diabetes, glucose cannot be taken into the cells leading to long-term increase in blood glucose level (hyperglycemia) [15]. Furthermore, impaired insulin action results in ectopic fat accumulation in skeletal muscle and liver due to the spillover of free fatty-acids that can no longer be stored in the adipocytes [17], [18]. In order to quantify the level of insulin resistance, Matthews et al. suggested a method described as Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) based on fasting glucose and insulin levels, in 1985 [19]. Intriguingly, insulin resistance does not affect its downstream pathway equivocally, selective insulin resistance in mice's livers in

which insulin fails to suppress gluconeogenesis but continues to activate lipogenesis, leading to the fatal combination of hyperglycemia and hypertriglyceridemia [20].

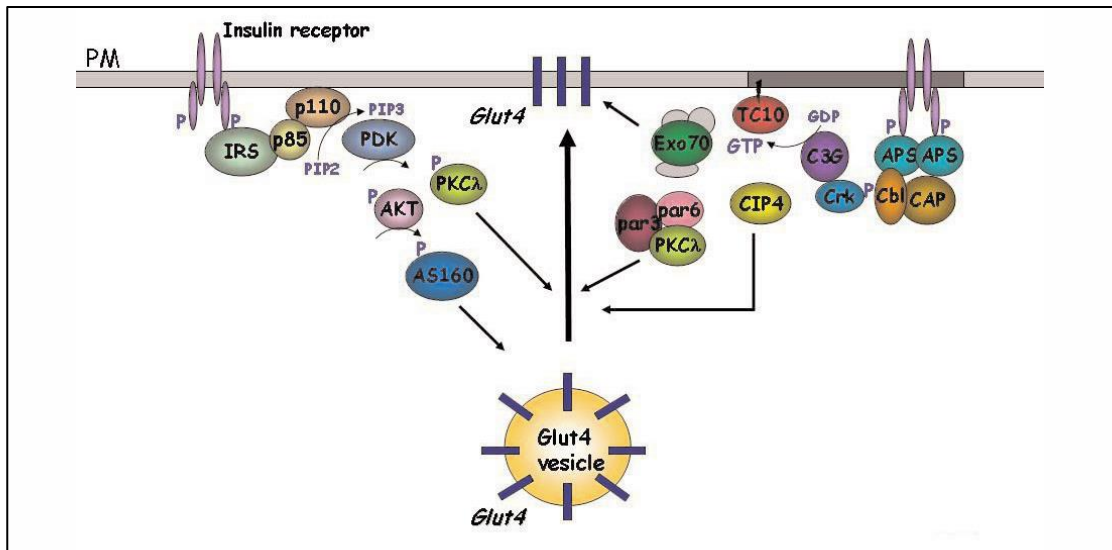


Figure 2.2: Fusion of glucose transporter Glut 4 vesicles with the cell membrane through insulin signaling pathways.

2.1.3. Metabolic Inflammation: Metaflammation

Inflammation is also a part of obesity-related insulin resistance. Adipose tissue of obese individuals has been shown to contain higher percentage of macrophages compared to lean subjects ([21] and reviewed in [7]). In adipose tissue, the production of pro-inflammatory cytokines such as TNF α and IL-6 also increases in diabetic and insulin-resistant subjects whereas suppression of TNF α has been revealed to decrease insulin resistance in obese rodents [22], [23].

As it will be explained more detailed (in section 5.2), adipose tissue is not only an energy storage unit but also an endocrine organ that secretes free fatty-acids, hormones, cytokines and chemokines [23]. Overnutrition alters the adipocyte metabolism and gene expression, causing increased production of pro-inflammatory free fatty-acids and chemokines that either recruit or activate pro-inflammatory, so called “classically activated” (M1) macrophages into adipose tissue whereas in lean adipose tissue anti-inflammatory alternatively activated (M2) macrophages are more abundant. M1 macrophages secrete substantial amounts of pro-inflammatory cytokines that reduce the insulin sensitivity of adipocytes in the tissue [7]. This starts

a positive feedback that increases inflammation and insulin-resistance even further (reviewed in [7]).

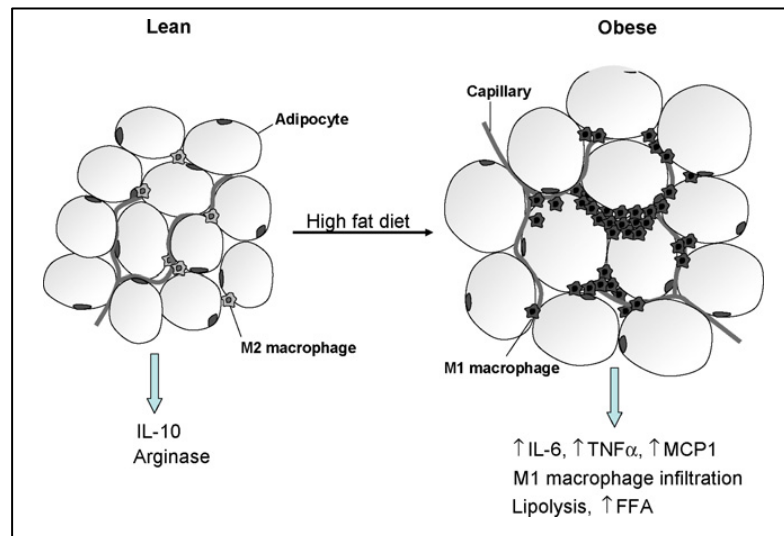


Figure 2.3: The cytokine profiles of lean and obese adipose tissue.

2.2. Adipose Tissue in Obesity

Adipose tissue is a loose composition of pre-adipocytes, fibroblasts, endothelial cells, immune cells such as macrophages and lymphocytes (together called Stromal Vascular Fraction) embedded in between adipocytes [24]. Adipose tissue can be found in two main subcutaneous (anterior and posterior) and numerous visceral adipose tissue depots in mammals [25]. In addition to its regulatory role in fat and nutrient balance and being the largest energy storage, adipose tissue is now considered as a highly active endocrine organ that also controls homeostasis, blood pressure, lipid and glucose metabolism, inflammation and atherosclerosis by secreting large amounts of free fatty-acids and precursor molecules called adipokines from adipocytes [23]. Therefore, adipose tissue and its functions are needed to be well-understood in order to investigate obesity, insulin resistance, type 2 diabetes mellitus and metabolic inflammation.

2.2.1. Types of Adipose Tissues

Adipose tissue is generally classified into two major types: white adipose tissue (WAT) and brown adipose tissue (BAT). All adipose tissue depots consist of brown and white adipocytes and the adipose tissue is considered as brown or white depending the abundance of those adipocytes in the tissue. Although they are both called “adipocytes” and store various amounts of lipids, brown and white adipocytes have several morphological differences. For instance, white adipocytes are generally larger (30 - 70 μm in adult rats) and have single lipid droplet (“uni-locular”, Figure 2.4); while, brown adipocytes appear to be much smaller (20 - 30 μm in adult rats) and contain several lipid droplets (“multi-locular”, Figure 2.4). In addition to morphological differences, BAT and WAT also have quite different functions: WAT is mainly responsible for energy storage and regulation of metabolism by secreting factors such as adipokines; whereas, BAT is responsible for thermogenesis with its ability to remove free fatty-acids from multi droplets in high speed [24]. Recent studies has shown that white and brown adipocytes can convert into each other when needed. This physiological transdifferentiation is important because the brown phenotype of the organ associates with resistance to obesity and related disorders [26].

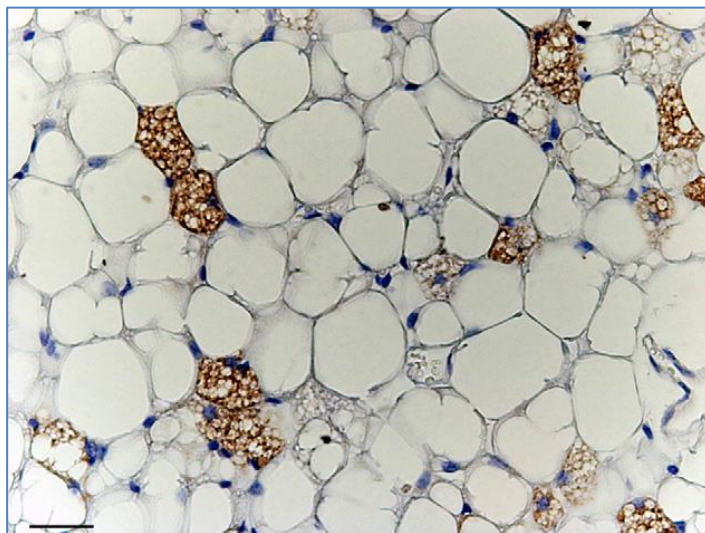


Figure 2.4: Brown and white adipocytes in subcutaneous adipose tissue.

Since they show contrary expression patterns for many genes, “subcutaneous” and “visceral” compartments can also be classified as two different types of adipose tissue [27]. Furthermore, subcutaneous adipose tissue can expand limitlessly; whereas, visceral adipose tissue has a limited growing capacity and de novo adipogenesis [28]. Besides, it has also been demonstrated that enlargement of the visceral adipose tissue carries higher risks for metabolic deficiencies than subcutaneous fat, which has led to substantial amount of to discriminate and characterize metabolic parameters in between subcutaneous and visceral fat depots [9].

2.2.2. Endocrine Function of Adipose Tissue

Extended amount of free fatty acids in enlarged adipose tissue and association with development of insulin resistance in peripheral tissues such as liver and skeletal muscle are the most common features of obesity [29]. The role of adipose tissue as the main source of energy established about 50 years ago [30]. However, after the initial studies with genetically obese ob/ob mice (leptin-deficient) [31] in the following decades, it is now quite clear that adipose tissue also acts like an endocrine organ by secreting factors called adipokines to regulate systemic metabolism, homeostasis and inflammation (Figure 2.5) (reviewed in [23]-[27]).

Leptin is one of the first discovered secreted factors from adipocytes (reviewed in [27]). It is a 16 kDa protein and was shown to be secreted also from the brain [32], [33]. Mice that genetically lack leptin (ob/ob mice) have shown to be obese regardless of the diet [27]. The role of leptin is basically to suppress the appetite by stimulating leptin receptors in the brain and prevent weight gain (reviewed in [23], [30]). On the contrary to the findings in ob/ob mice, plasma leptin levels have been shown to be increased in obese individuals [34]. Since the size of adipocytes are expanded up to 2 - 4 times, secreted leptin amount per cell also increases in obese subjects compared to lean subjects [35]. In fact, Lonnqvist et al has declared a positive linear correlation ($r = 0.8$) between total body fat mass and serum leptin levels [36]. The contradiction between leptin deficient obese mice and high plasma leptin levels in diet-induced obese subjects is explained with the leptin-resistant state in obesity (reviewed in [27]). Other known roles of leptin are to control

hepatic gluconeogenesis and promote the fatty acid oxidation in liver and skeletal muscle [23].

Adiponectin is another important protein that is highly produced by adipocytes. Although adiponectin monomer is 30 kDa, it circulates in the blood as low molecular weight trimers to high molecular weight dodecamers [27]. The secretion of adiponectin begins at an intermediate stage of adipogenesis and it is the most abundant adipokine released by adipose tissue. Levels of adiponectin in plasma are negatively correlated with circulating leptin concentrations [24]. In line with this, plasma adiponectin levels were shown to be decreased in obese and insulin-resistant subjects [24]. In further investigations, it has also been shown that rodents overexpressing adiponectin have enhanced glucose uptake and fatty acid oxidation in muscle, reduced gluconeogenesis in liver and increased whole-body insulin sensitivity. Adiponectin is also known to suppress the expression of inflammatory cytokines such as TNF α and IL-6 [23].

Resistin is a peptide hormone expressed by white adipocytes in rodents and adipose tissue macrophages in humans ([37], reviewed in [27]). Although its function is not completely understood, resistin is considered to be an inducer of hepatic and skeletal muscle insulin resistance in obesity by stimulating the expression of inflammatory cytokines TNF α and IL-6. However, there are still contradictory findings about the connection of resistin with the glucose metabolism [23].

TNF α and IL-6 are two pro-inflammatory cytokines expressed by adipose tissue and both involved in development of insulin resistance. Deletion of TNF α and its receptor diminished the insulin resistance in both diet-induced and leptin-deficient mice [24]. Moreover, expression of TNF α in adipose tissue shows a correlation with BMI and whole-body fat ratio. Likewise, fasting plasma TNF α levels are also shown to be increased in insulin-resistant individuals. On the other hand, the effect of IL-6 on insulin resistance is still in debate. However, its serum levels are increased in obesity and insulin-resistant states [23].

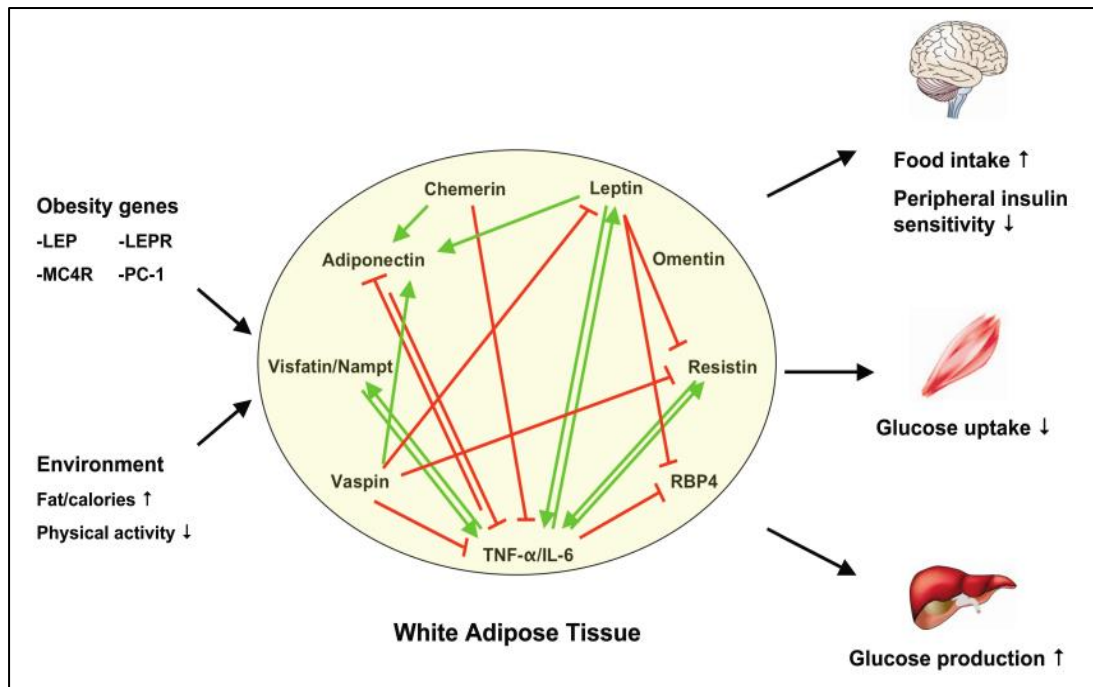


Figure 2.5: Interactions of adipokines in obesity. Green arrows represent stimulation, red lines stand for suppression.

Insulin resistance should be investigated not only through the effects of adipokines individually, but also based on the interactions between nutrition, glucose, insulin and adipokine levels. For instance, adiponectin and TNF α suppress each other's expression; whereas, overnutrition activates inflammatory response resulting in diminished adiponectin expression and insulin sensitivity. On the other hand, TNF α and IL-6 induce the expression of leptin and resistin leading to a leptin and insulin resistant state; in the same way leptin and resistin stimulate TNF α and IL-6 expression and trigger an inflammatory response [23] (Figure 2.5).

2.2.3. Adipose Tissue Macrophages

In a healthy individual, about 10% of total cell number in adipose tissue consists of macrophages; whereas, this number reaches up to almost 40% in obesity [20]. Macrophages have quite significant roles in the development of insulin resistance and chronic low-grade inflammation state in obesity through infiltration into the tissue and production of cytokines [38]. Macrophages, in general, can be classified into two general phenotypes: (1) classically activated macrophages (M1) that secrete high amounts of pro-inflammatory cytokines such as iNOS, TNF α , IL-1

and IL-6 and (2) alternatively activated macrophages (M2) that express the mannose receptor CD206, Arginase and secrete the anti-inflammatory cytokine “IL-10” [39]. M1 macrophages are more cytotoxic and basically responsible for innate immune responses; while, M2 macrophages are mainly associated with tissue repair and angiogenesis [29], [40]. These two phenotypes both have the ability to polarize (type-switching) into the other type by exposure to various chemokines and cytokines (for instance interferon-gamma (IFN γ) for M1 polarization or IL-4 and IL-13 for M2 polarization) [37]. In addition to the increased ratio of macrophages in obesity [21], Lumeng et al. showed that adipose tissue macrophages in obese individuals express increased levels of pro-inflammatory cytokines “TNF α ” and “iNOS” –associated with M1 macrophages– whereas they act more M2-like in lean individuals and generally express IL-10 that is known to suppress inflammation [41]. Although the mechanisms behind it are not completely clear yet, it has been proposed that expanded adipocytes secrete chemotactic agents that recruit macrophages –such as Macrophage Chemoattractant Protein 1 (MCP-1)– into the tissue or polarize them into pro-inflammatory M1-type macrophages [36], [37]. For instance, excessive amount of free fatty acids secreted by adipocytes in obesity might be recognized by Toll like receptor 4 (TLR-4) and stimulate the activation of inflammatory macrophages [35]. On the other hand, increased macrophage migration into the adipose tissue might also be the result of risen number of death adipocytes triggered by obesity [36].

As in obesity, M1 / M2 ratio of macrophages in adipose tissue is also positively correlated with both tissue-specific and whole-body insulin resistance (reviewed in [43]). In lean subjects, M2 macrophages might improve insulin sensitivity of adipocytes indirectly by suppressing the accumulation and polarization of M1-like macrophages in the tissue through the production of anti-inflammatory cytokines [44]. It has also been suggested that inducing a Th2 type response results in decreased inflammation and insulin resistance in adipose tissue [45]. M1 macrophages, on the other hand, directly induce insulin resistance by secreting high levels of pro-inflammatory cytokines such as TNF α and IL-6 (see section 5.3.2) [40]. Moreover, the suppressing effect of inflammatory cytokines on adipocyte differentiation causes adipocyte enlargement and decreases the insulin sensitivity indirectly [46]. In the same direction, deficiency of CD11c⁺ cells, which are

considered as M1 macrophages, has shown to reverse insulin resistance in obesity [47].

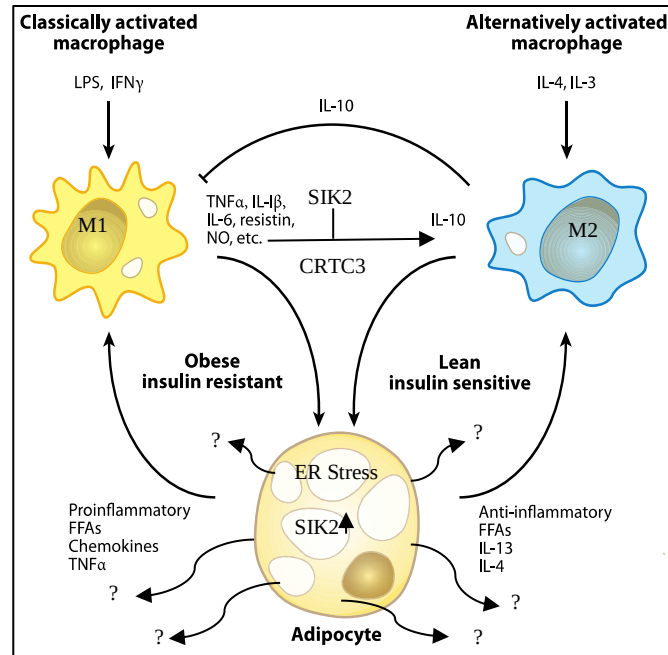


Figure 2.6: The connection between adipose tissue macrophages and adipocytes in both obese (insulin resistant) and lean (insulin sensitive) states.

2.2.4. Endoplasmic Reticulum Stress in Obesity

The endoplasmic reticulum is the organelle which is responsible for much of a cell's protein synthesis and folding by chaperones inside [48]. When the amount of the proteins synthesized in ER at a unit of time is beyond the capacity of ER the unfolded proteins accumulate as aggregates in lumen due to their hydrophobic residues. Deposition of those unfolded proteins in ER is toxic and incline to ER stress [49]. In reply to ER stress, eukaryotic cells activate a mechanism called unfolded protein response (UPR) to maintain the ER homeostasis under stress conditions [49], [50].

In obesity, high metabolic processes depending on the high nutritional intake cause an ER and mitochondrial stress especially in adipocytes, liver and skeletal muscle cells [51]. Recent findings propose that unfolded protein response following the ER stress can ignite an inflammatory response which later result to a chronic inflammatory state [48]. In fact, inflammatory cytokines TNF α and IL-6 are up-

regulated in response to free-fatty acid (FFA)-mediated reactive oxygen species (ROS) induced ER stress in 3T3-L1 adipocytes [52].

2.3. Effect of Obesity on Peripheral Tissues

Although, in obese subjects and animal models insulin resistance and inflammation initially develop in adipose tissue (or skeletal muscle in some cases) and later spread into peripheral tissues and internal organs. Inflamed and insulin-resistant adipose tissue secretes increased amounts of free fatty-acids and inflammatory cytokines and produce reduced amount of insulin sensitizer adipokines such as “adiponectin” resulting in alteration of the whole-body glucose metabolism [53]-[55]. Since they take an important part in glucose metabolism, liver and skeletal muscle are directly affected by the insulin resistance [56]. Lack of insulin signaling as a result of obesity-induced insulin resistance causes lipid accumulation in liver and muscle through the increased lipogenesis and inhibition of the oxidative consumption of glucose respectively [57]. It has been shown that hepatic lipid content and fat accumulation in skeletal muscle, as well as BMI, are negatively correlated with insulin sensitivity in type 2 diabetes patients [17], [18]. In fact, Non Alcoholic Fatty Liver Disease (NAFLD) is closely associated with obesity and insulin resistance [58].

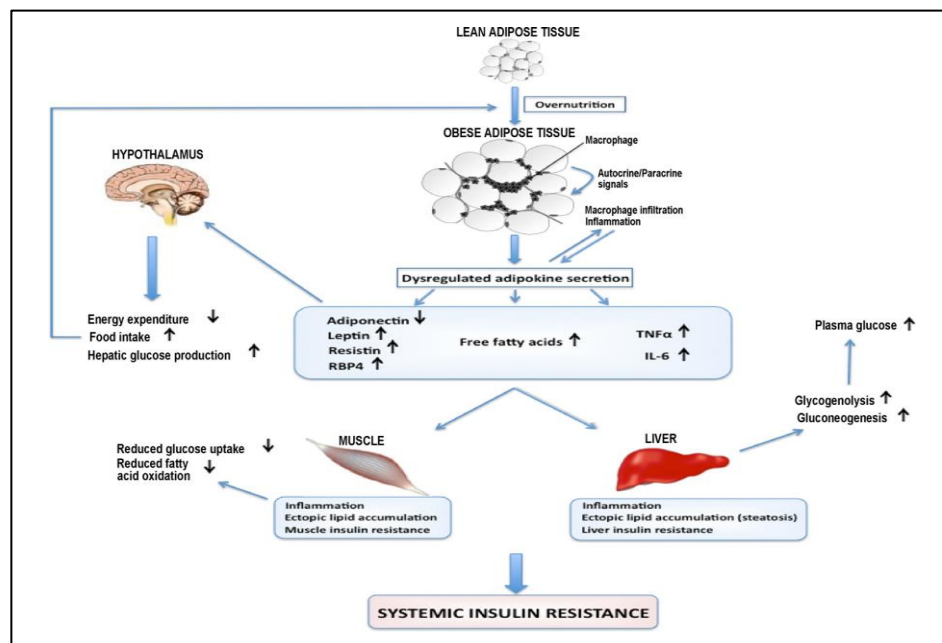


Figure 2.7: Systemic effects of obesity.

2.4. Vascular Endothelial Growth Factors

“Vascular Endothelial Growth Factors (VEGFs) are secreted regulatory glycoproteins that play vital roles in the modulation of vasculogenesis in embryonic stage and angiogenesis in adults. In mammals, there are five members of the VEGF family –VEGF A, B, C, D and placenta growth factor (PLGF), which have numerous splice variants [59]. VEGFs circulate as dimers and bind to specific VEGF Receptors (VEGFRs): VEGF-A, VEGF-B and PLGF to VEGFR-1; VEGF-A and VEGF-E to VEGFR-2; VEGF-C and VEGF-D to VEGFR-3. However, there are some processed forms of human VEGF-C and VEGF-D that can bind to VEGFR-2 in low affinity [59].

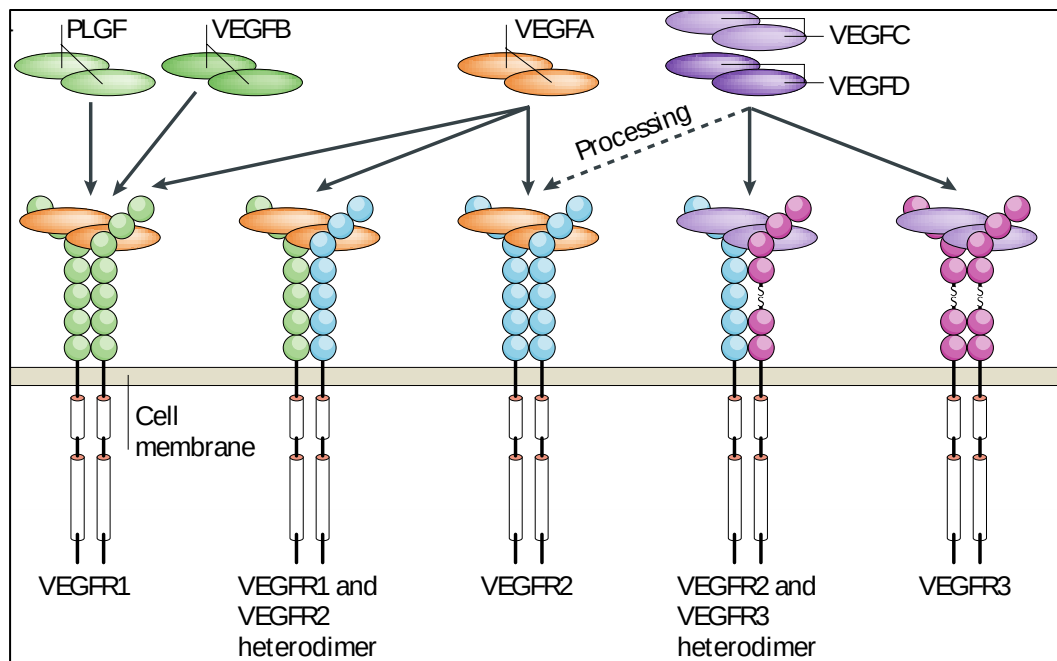


Figure 2.8: Vascular Endothelial Growth Factors and their receptors.

VEGF-C is essential for lymphatic vessel formation in embryonic state through regulating proliferation, migration and survival of lymphatic endothelial cells [60]. It is also shown that overexpression of VEGF-C resulted in increased lymphatic endothelial cell proliferation and lymphatic vessel enlargement [61]. In addition to its importance for vascular development, Skobe et al. revealed the chemotactic effect of VEGF-C on macrophages both in vivo and in vitro, suggesting that VEGF-C could also be considered as an immunomodulator [62]. It was recently

shown that overexpression of VEGF-C under Keratin 14 promoter increased the migration of inflammatory macrophages into the inflamed skin [63]. VEGF-C has also been suggested to be associated with obesity. Silha and colleagues showed that serum VEGF-C levels were increased in obese and overweight individuals [64]. Furthermore, it has also been proposed that increased circulating VEGF-C levels positively correlate with insulin resistance in obesity [65]. During my training in Dr. Detmars' Lab (ETH Zürich, Switzerland) we have also observed that blocking the VEGF-C and VEGF-D ligands using soluble VEGFR-3 improved metabolic parameters in mice under high-fat diet [66].

2.5. Salt-Inducible Kinase Family

Salt-inducible kinase (SIK) subfamily consists of three isoforms of a Serine/Threonine kinase that was first identified in adrenocortical tissue of high-salt treated rats [67]. SIKs are considered as members of AMP-dependent protein kinases (AMPK)-related family, although they lack the AMP and glycogen sensitivity comparing to the other AMPK members [68], [69].

SIKs contain very well conserved homologous N-terminal kinase subdomain and a ubiquitin-associated (UBA) subdomain right adjacent to it. It has been reported that the activation of SIKs increases upon the phosphorylation of their kinase domain by Liver Kinase B 1 (LKB1). In addition, next to the conserved Arginine/Lysine rich domain before the C-terminal, all three isoforms of SIK have a Protein Kinase A (PKA) phosphorylation site of which phosphorylation leads to translocation of SIK from cytoplasm into the nucleus [70]. Furthermore, Protein Kinase B (PKB, also known as AKT) also phosphorylates SIK2 on a S358 residue near the UBA domain to activate SIK for insulin signaling pathways [71].

In mammals SIK1 is up-regulated in brain, testes, adrenal glands and skeletal muscles and down-regulated in liver, heart and adipose tissue [72]. On the other hand, SIK2 is shown to be abundantly expressed in adipose tissue, liver and brain where SIK3 is normally expressed in all tissues [73].

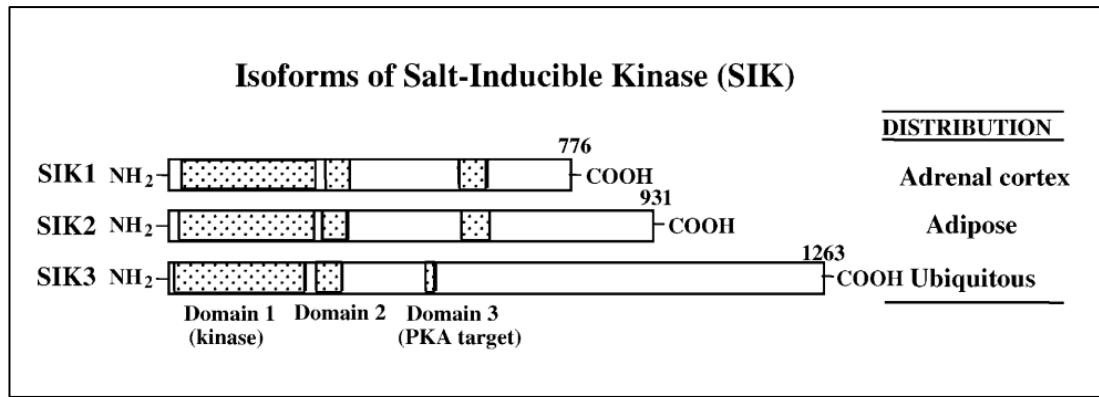


Figure 2.9: Salt-inducible kinase isoforms.

2.5.1. SIK2 (Salt-Inducible Kinase-2)

Salt-inducible kinase subfamily (SIK2) is a 926 amino acids long protein (in humans) which consists of a kinase domain, (20-271), and an UBA domain (295-553) (UNIPROT, Q9H0K1). It shares high sequence similarity with the two other isoforms [70]. SIK2 is abundant in both brown and white adipocytes on mRNA level and also upregulated in pre-adipocytes during the adipogenesis [72], [74].

Since SIK2 is mainly expressed in liver and adipose tissues, it is not a surprise that it is involved in several metabolic pathways such as insulin signaling, lipogenesis, gluconeogenesis etc. In fact, Insulin Receptor Substrate 1 (IRS1) in adipocytes is the first identified SIK2 substrate of which phosphorylation at Ser⁷⁹⁴ (in humans) diminishes the effectiveness of the downstream of insulin signaling pathway [70], [72], [75]. Expectedly, SIK2 levels and activity has been found to be increased in white adipose tissue of diabetic mice, which may suggest a critical function of SIK2 in regulation of insulin signaling pathway and development of type-2 diabetes [72]. Moreover, it is known that SIK2 regulates gluconeogenesis and lipogenesis by phosphorylating CRTC2 (in liver) and CRTC3 (adipose tissue and other tissues) respectively by sequestering these CREB-regulated Transcription Co-activators in the cytoplasm via 14-3-3 proteins [76], [77].

Since its discovery, SIK2 has been the subject of considerable attention for translational medicine by harboring great potential for therapeutic modalities in various metabolic disorders including obesity, T2D, cancer, and neurological disorders [78]-[83]. Last but not least, most recently, a novel role for SIK2 in macrophage polarization has been discovered. It has been established by Clark &

MacKenzie that active SIK2 suppresses IL-10 production and support inflammatory phenotype by making CRTC3 being held in the cytoplasm by 14-3-3 proteins where inhibition SIK2 results in a polarization into regulatory/ anti-inflammatory phenotype [84]-[85].

3. MATERIALS

3.1. Cell Lines

In this study, we used PLAT-A, SGBS (from University of Ulm) and THP-1 (from Bosphorus University) cell lines.

3.2. Kits

RNA isolations, cDNA synthesis and quantitative Real-Time PCR experiments were performed using the following kits. For RNA from adipocytes (primary cells and SGBS) RNeasy Lipid Tissue Kit were used whereas, RNA isolations from THP-1 cells were performed according to NucleoSpin RNA Mini Kit.

Table 3.1: Kits used.

RNeasy Lipid Tissue Kit	Qiagen
Dynamo cDNA Reverse Synthesis Kit	Thermo Scientific
Maxima SYBR Green qPCR Master Mix (2X)	Thermo Scientific
NucleoSpin RNA Mini Kit	(MACHEREY-NAGEL)

3.3. Equipments

Table 3.2: Equipments used.

Fluorescent Microscope	Nikon, Eclipse Ti-S)
PVDF Western Blotting Membrane	Amersham Biosciences, Hybond-P
Microcentrifuge	Beckman Coulter 22R
Microcentrifuge	Eppendorf Mini Spin
Centrifuge	Beckman Coulter Allegra X-22,
Shaker	BioSan, ES-20
Thermal Cycler	BioRad
Real Time Thermal Cycler	Roche, LC480II
0.2 ml, 15 ml and 50 ml centrifuge tubes	Isolab
1.5 ml centrifuge tubes	Axygen
0.2 µm filter tips	TPP
0.2 µm vacuum filter	TPP
-80°C Freezer	Symphany
Cell culture flasks and cryotubes, TPP	
Glass Materials	Isolab
pH Meter	Mettler Toledo
Gel Imaging System	BioRad, ChemiDoc XRS+
Vortex	Yellowline TTS2
Liquid Nitrogen Tank	Arpege 140
Pipettor	Gilson
Micropipettor	Gilson, Thermo
Filtered Micropipette Tips	Thermo
Serologic Pipette	Isolab, Precicolor, Lamtek
Pasteur Pipette	Isolab
Orbital Shaker	IKA, Yellow line
Heat blocks	VWR
Magnetic Stirrer	Heidolph

4. METHODS

4.1. Experimental Setup

In the present study, we used SGBS and THP-1 cell lines for mimicking the inflamed adipose tissue. We differentiated SGBS pre-adipocytes into mature adipocytes (protocol will be discussed in Chapter 4.2.1) and induced ER stress and inhibited SIK2 during the last day of differentiation. In order to induce the ER Stress, we used Thapsigargin (Santa Cruz), which inhibits Ca^{+2} -ATPase pumps in ER lumen [100]. As SIK2 inhibitor, we used Compound C (Sigma, 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine). Then we checked both protein and mRNA levels of some ER stress or inflammation markers as well as adipokines in each situation. In addition, we used the conditioned media from SGBS cells treated with Compound C in the presence and absence of Thapsigargin on THP-1 macrophages during their differentiation period and measured the mRNA levels of macrophage markers and cytokines. In the meantime, we established shRNA mediated SIK2 stable SGBS cell lines since Compound C's effects also on AMPKs (Figure 4.1).

Furthermore, in Detmar's lab (Pharmaceutical Sciences, ETH Zürich, Switzerland) we also fed 4-week-old FVB Background K14-VEGF-C mice (which overexpress Vascular Endothelial Growth Factor C in the skin) and their wild-type littermates either with control diet (chow; 11% kcal from fat, Provimi-Kliba, Kaiseraugst, Switzerland) or with high-fat diet (HFD, 60% kcal from fat, Research diets, Inc., NJ, USA) for 16 weeks and checked their inflammatory profile as well as the possible effects on SIK2.

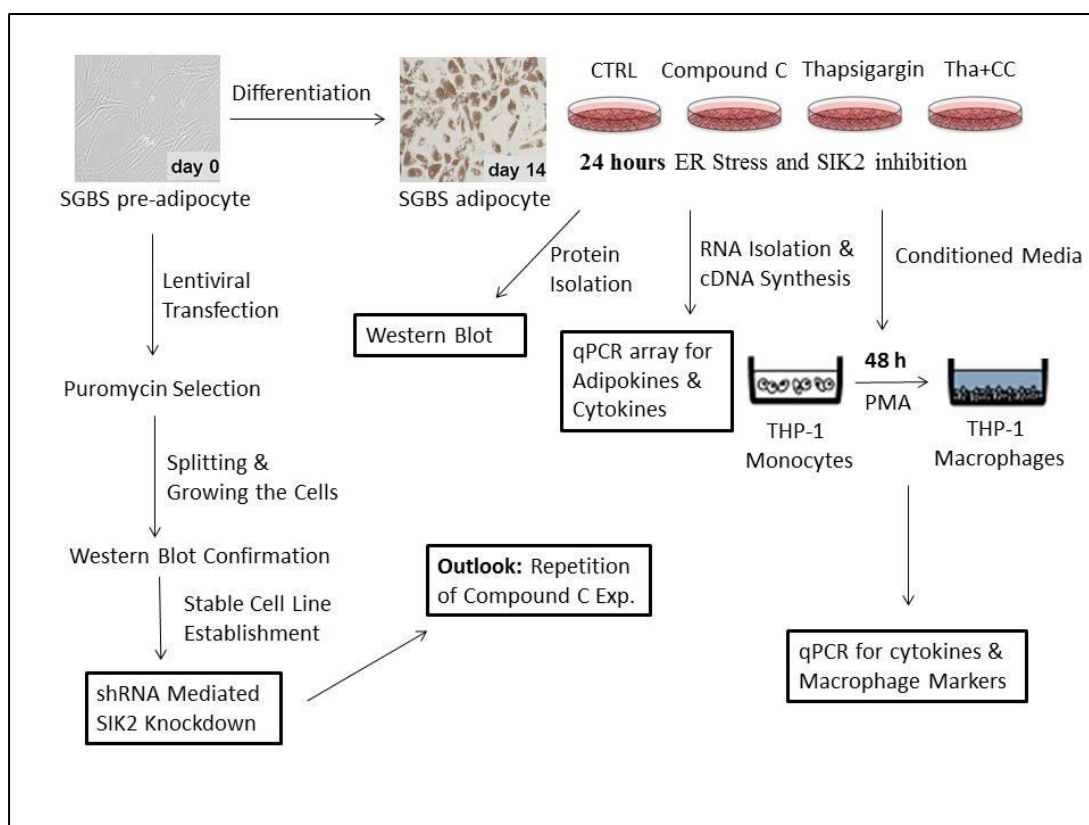


Figure 4.1: Experimental setup of our study.

4.2. Cell Culture

4.2.1. SGBS Maintenance and Differentiation

SGBS pre-adipocyte cell line is derived from subcutaneous white adipose tissue of a male infant with Simpson-Golabi-Behmel Syndrome. SGBS cells have been characterized with a high capacity of differentiation in presence of some hormones and PPAR γ agonist [64].

SGBS pre-adipocytes were kindly obtained from Prof Martin Wabitsch of University of Ulm (Department of Pediatrics). SGBS cell were maintained in DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F12) mixture medium (Lonza) supplemented with 10% Fetal Bovine Serum (BioWest) and 1% Penicilin/Streptomycin (Lonza), 3.3mM Biotin and 1.7mM Panthotenat. The medium is refreshed every 3-4 days, depending on the intensity. The cells were trypsinized (10X Trypsin, diluted in PBS, Lonza) before they reach 60% confluency. After trypsinization, cells were centrifuged at 200xg for 10 minutes. For later use, cells

were frozen in 10% glycerol (AppliChem) in regular growth medium and stored either in -80°C or vapor phase liquid nitrogen. The differentiation protocol was initiated once the cells reach 80-90% of confluency.

In order to induce the differentiation, the cells first washed with PBS three times and differentiation medium containing 0,01mg/mL Transferrin, 20nM human insulin, 100nM cortisol and 0,2nM T3 (Triiodothyronine) prepared in serum free growth medium. For the first 4 days of differentiation, 25µM Dexamethasone, 250mM IBMX and 10mM Rosiglitazone added in differentiation medium. Then, differentiation medium was refreshed two times a week until the day 14. In our experimental setup, during the 14th day of the differentiation, we induced ER stress using 1µM Thapsigargin and inhibited SIK2 using 15µM Compound C. After 24 hours of incubation, we collected the media and continued with either RNA or protein isolation.

4.2.2. THP-1 Maintenance and Differentiation

THP-1 cells are derived from human monocytes and can be differentiated into activated macrophages by phorbol 12-myristate 13-acetate (PMA). The cells grow in suspension until they are induced and they become adherent when they turn into macrophages. THP-1 cells were gifted by Prof. Nesrin Özören (Molecular Biology and Genetics Department, Bosphorus University, Istanbul) and maintained in 10% serum containing RPMI 1640 medium. They grow between the density of 1×10^5 cells/ml to 1×10^6 cells/ml and split into 5-10 vials before reaching 2×10^6 cells/ml. The medium was refreshed every 2-3 days and each time cells were collected into a falcon tube, centrifuged at 1000rpm for 5 minutes. The cells were frozen in 10% DMSO containing growth medium and stored in liquid nitrogen vapor phase for later use.

For differentiation, around 2×10^5 cells/ml were exposed to 100ng/mL PMA (Sigma) dissolved in DMSO. In our experimental setup, we added 1:1 SGBS conditioned media onto PMA containing THP-1 medium. As the control, we induced THP-1 cells in 1:1 DMEM/F12 : RPMI 1640 mixture. Then we performed RNA isolation to check the altered cytokine production and polarization of macrophages.

4.2.3. shRNA Mediated SIK2 Knockdown in SGBS

Cells were seeded around 50% confluency in complete medium, considered as Day 1. In the second day, medium was replaced with 5 µg/mL Polybrene containing growth medium and lentiviral virus particles were added. After 24 hours, the medium was again switched back to regular growth medium and cells were incubated one more day. The next day, cells were split 1:4 and let grow 2 more days in their regular growth medium. Then, in order to select the stable cell lines, cells were treated with 1 µg/mL puromycin (the puromycin concentration had been already determined by making a killing curve for SGBS and the concentration that killed all the cells in around 5-7 days was used in the experiment) and incubated for one week with wild-type SGBS cells for control. After all the cells were died in the control group, the puromycin-resistant cells were collected and seeded onto new plates where they were maintained with 0,1-0,5 µg/mL puromycin (shRNA Lentiviral Particles Transduction Protocol, Santa Cruz). One of the plates from that passage were spared for protein isolation to confirm the SIK2 knockdown with Western Blot. The other cells were either maintained or frozen for later use.

4.3. Mouse Protocol

4.3.1. Tissue Collection

FVB background, 4-week-old male K14-VEGF-C mice (which overexpress Vascular Endothelial Growth Factor C in the skin) and their wild type littermates were fed ad libitum either with control diet (chow; 11% kcal from fat, Provimi-Kliba, Kaiseraugst, Switzerland) or with high-fat-diet (HFD, 60% kcal from fat, Research diets, Inc., NJ, USA) for 16 weeks under conventional specific pathogen free (SPF) conditions. Mice were weighed on alternative weeks and on 16th week, they were sacrificed for tissue collection. Subcutaneous (SQ) and Visceral (VS) fat tissues, liver and tail samples were collected from each mice for further analysis. All animal experiments were approved by Kantonales Veterinäramt Zürich.

4.3.2. Adipocyte Isolation

Following the dissection, both left and right SQ and VS adipose tissues were dissected and weighed. The left (of the mouse) ones initially collected in ice cold PBS for adipocyte isolation; whereas, the right ones were snap frozen in liquid nitrogen and kept for RNA or protein isolation in -80°C . After fixation, fat tissues were chopped with fine scissors and suspended in 1mL collagenase (Sigma, 2mg/mL; in PBS). Thereafter, they were incubated at 37°C for one hour, transferred into falcon tubes with Teflon tips and neutralized in 2 mL RPMI (Gibco) medium. The solutions were then centrifuged at 4°C and 1200 rpm for 4 minutes. Finally, the adipocytes were collected from the top of the supernatant into a new eppendorf tube and after snap freezing, they were kept in -80°C for RNA isolation. In addition, two (one from right and one from left) biopsy samples (with Biopsy needles, Acuderm) were collected from both SQ and VS adipose tissue for histology studies with paraffin embedding and whole-mount staining and fixed in 4 % PFA and kept in 4°C .

4.3.3. Isolation of Macrophages with M.A.C.S. Sorting

14-week-old female K14-VEGF-C transgenic mice and their wildtype littermates on chow diet (n=4 per group) were sacrificed and subcutaneous fat pads from both sides were collected and kept in PBS on ice until the macrophage isolation process started. First, the fat pads were minced in 1 mL of Collagenase II (2mg/mL in PBS, Sigma) and incubated at 37°C for one hour and agitated every 10 minutes by pipetting. Then, they were suspended with 2 mL of RPMI (with 1% FBS) in fresh falcon tubes. After 5 minutes of centrifugation at 1000 rpm at 4°C , the floating adipocytes together with the supernatant were removed and cells at the bottom (the stromal-vascular fraction) were resuspended with freshly prepared erythrocyte lysis buffer and incubated on ice for 10 minutes. After another centrifugation, the supernatants were removed and the samples were resuspended in 100 μL of MACS Buffer per sample. Thereafter, the cells were incubated with 20 μL of CD11b (general macrophage marker) MACS beads (Miltenyi Biotech, CD11b Microbeads, human & mouse) for 15 minutes on ice. After incubation, the cells were washed with

5 mL MACS buffer and after the samples were centrifuged, the supernatants were discarded and 500 μ L MACS Buffer was pipetted into each tube. After this, the MACS columns (Miltenyi Biotech GmbH) and filters were set on the MACS device (Multi Stand) and they were equilibrated by washing with 3 mL MACS buffer. Then, all 500 μ L of cell suspension was pipetted onto the columns and after all the liquid have passed through, the columns were washed with 3 mL of MACS buffer three more times. After the last wash, the columns were taken out from MACS device and fit onto fresh falcon tubes. 2 mL MACS buffer was added onto each column and all the liquid was pushed down into falcon tubes with the plunger of the column. Finally, the cells were counted (with Countess, Invitrogen) from 20 μ L of representative samples.

The CD11b+ cells were centrifuged once again and resuspended with RPMI. Later, 50,000 cells from each mice were seeded in 6-well-plate with serum-free RPMI (2 mL final volume per well), and after 24 hour conditioned media were collected, aliquoted and kept at -20°C for in vitro adipocyte differentiation experiments and cytokine analyses (Cytolab). Additionally, 10^4 macrophages were seeded per well of 8-well-chambered glasses (company) with 500 μ L of RPMI (supplemented with 1% FBS) per well for ovalbumin processing experiments. Remaining cells were lysed with 350 μ L RLT buffer separately and kept -80°C until RNA isolation.

4.4. Quantitative Real-Time PCR

4.4.1. RNA Isolation

RNA isolation from pure adipocytes (both subcutaneous and visceral) and SGBS cells was performed with RNeasy Lipid Tissue kit (Qiagen). For isolation, approximately 1 mL of pure adipocytes was collected into fresh eppendorf tubes by filtering to remove undigested tissue. First, the adipocytes were mixed with 1 mL QIAzol Lysis reagent from the same kit and were homogenized with stainless steel beads using Tissue Lyzer (Qiagen) two times for 2 minutes at 30 frequency (Instead of homogenization step, cells were lysed in the plates with cell scraper). After incubating the tubes at room temperature for 5 minutes, 200 μ L chloroform was

added into each tube and the tubes were shaken vigorously for 15 seconds. Following another 3 minutes of incubation, the lysates were centrifuged at 12000 g for 15 minutes at 4°C. The supernatants were then transferred into fresh tubes and 1 volume of 70% Ethanol (approximately 550 µL for each sample) was added to the supernatants. Afterwards, the solutions were transferred into RNeasy spin columns placed in 2 mL collection tubes and centrifuged at 8000 g for 15 seconds. The flow-through was discarded and the same process was performed for the remaining samples. Later, samples were washed with 350 µL Buffer RW1 and centrifuged for 15 seconds. Before the second wash, columns were incubated with 80 µL DNaseI solution (RNase free DNase kit, Qiagen: 10 µL DNaseI with 70 µL RDD buffer) for 15 minutes on the bench. Thereafter, the samples were washed with 500 µL buffer RPE twice and the flow-throughs were discarded after 2 minutes of centrifugation. Finally, RNeasy spin columns were placed onto fresh 1.5 mL eppendorf tubes and the RNA was eluted by addition of 40 µL RNase-free water and centrifugation at 8000 g for 1 minute. The RNAs were then kept at –80°C until cDNA synthesis.

RNA Isolation from livers, macrophages and THP-1 cells, were performed according to a similar protocol in NucleoSpin RNA Mini Kit (MACHEREY-NAGEL).

4.4.2. cDNA Synthesis

The isolated RNAs were then used for cDNA synthesis. The cDNA synthesis was done using approximately 1 µg RNA according to the Table 4.1 with Dynamo cDNA Reverse Synthesis Kit (Thermo Scientific) and the PCR program in Table 4.2, cDNAs were diluted with 30 µL RNase-free water and kept in –20°C until quantitative real-time PCR (qPCR) analysis.

Table 4.1: Components for cDNA Synthesis from isolated RNA.

Name of Component	Amount (20 µL total)
10X RT Buffer	10 µL
Random Hexamer Primers	1 µL
ddH ₂ O	0-2 µL
H ⁺ reverse transcriptase	2 µL
1 µg RNA in 10 µL water (min 200 ng)	6-8 µL

Table 4.2: PCR Program for cDNA Synthesis.

Temperature	Duration
25°C	10 minutes
37°C	60 minutes
85°C	5 minutes
4°C	Hold

4.4.3. qPCR

After cDNA synthesis, the qPCR analysis was performed for SQ, VS adipocytes and liver cells from WT Chow, WT HFD and TG Chow mice as well as SGBS and THP-1 cells using Thermo Scientific's Maxima SYBR Green qPCR Master Mix (2X). Approximately 1 μ L of diluted cDNA and 9 μ L of Master Mix (Primer mixes + Maxima SYBR Green qPCR Master Mix) were put into each well. Ct values of different genes (see Appendix A for primer sequences) were measured for each mouse with three replicates. RPLP0 was used as the housekeeping gene and Δ Ct values of all the other genes were calculated by subtracting RPLP0's Ct value from the Ct value of the gene of interest. The $\Delta\Delta$ Ct values were calculated using the average Δ Ct value of untreated (control) cells or WT Chow group for each gene. Finally, the foldchanges were calculated using the $2^{-\Delta\Delta C_t}$ method [89]. Gene expression foldchanges were always normalized to WT Chow mice or untreated group in SGBS and THP-1 cells; therefore setting their gene expression foldchange to 1. P values were calculated according to student t-test if there were two groups or One-Way ANOVA if there were more. Mice experiments consisted of 8 independent experiments. SGBS experiments were repeated 2 times for two independent experiments. THP-1 experiment was only performed once.

4.5. Western Blot

4.5.1. Bicinchoninic acid (BCA) Assay

For determination of the concentration of proteins, BCA Assay kit was used. Unknowns and bovine serum albumin (BSA) dilutions ranging from 0.025 to 2

mg/ml were mixed with 200 µl of 50:1 diluted BCA Working Solution. After 30 minutes incubation at 37°C and 5 minutes incubation at room temperature (18-25°C), absorbance measured at 592 nm. Unknown sample concentrations were extrapolated from the standard curve.

4.5.2. SDS-PAGE and Western Blotting

Proteins ruptured on 10% polyacrylamide gels (10% acrylamide:bisacrylamide (29:1), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine) with 8% resolving gel (6% acrylamide:bisacrylamide (29:1), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% N,N,N',N'-tetramethylethylenediamine). Samples were boiled in blue 4X loading dye (240 mM Tris-Cl, pH 6.8, 5 % beta-mercaptoethanol, 8% SDS, 0.04% bromophenol blue, 40% glycerol) at 95°C for 5 minutes to be prepared. The gels were used in 25 mM Tris-HCl, 250 mM glycine and 0.2% SDS buffer at 100-150 V.

For Western blotting, the samples fragmentized in polyacrylamide gels were electro-blotted into PVDF membranes (GE, USA) in transfer buffer (200 mM glycine, 25 mM Tris.HCl, 15% methanol) at 110 V for 1-1.5 hours at 4°C. To deactivate the membrane, it was washed in tris buffered saline-tween (TBS-T) solution (150 mM NaCl, 20 mM Tris.HCl, pH 8.0, 0.1 % Tween 20) three times for total of 30 minutes.

The membrane was incubated in blocking solution (1% BSA or 1%-5% skimmed milk powder in TBS-T) for 1 hour at room temperature (or longer at 4°C) with gentle shaking. The membrane was left overnight in the blocking solution containing appropriate antibodies at 4°C, where the antibodies were diluted as given in Appendix B.

Following, the membrane was washed with TBS-T three times for 5 minutes each to remove unbound antibody and incubated in secondary antibodies matching to the primer antibody. Finally, after another washing step, the membrane was incubated in ECL Solution (Cell signalling, USA) for 1 minute and images were acquired using ChemiDoc visualizing system with 5 minutes of exposure time.

5. RESULTS

5.1.ER Stress and SIK2 inhibition in mature SGBS adipocytes

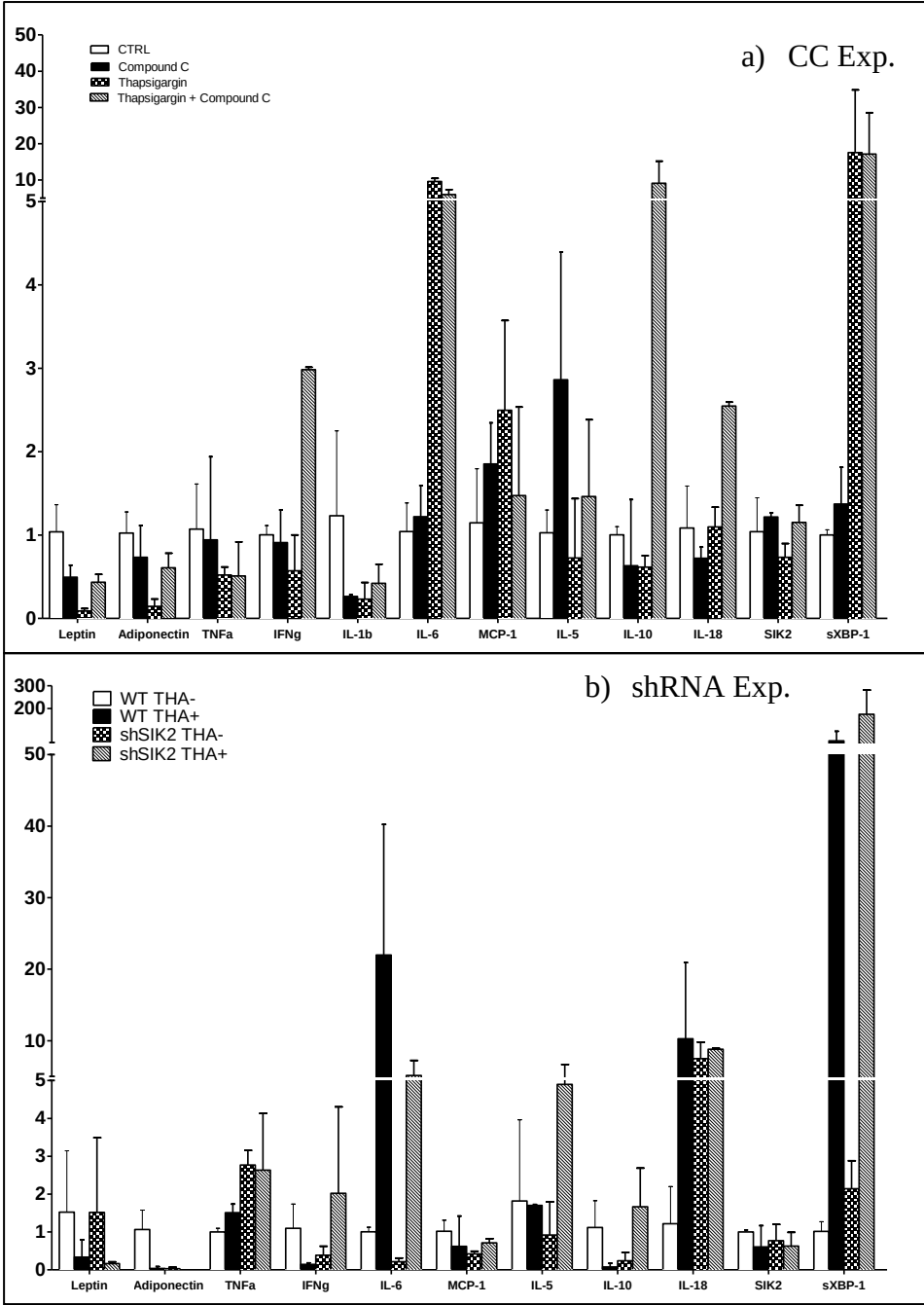


Figure 5.1: Gene expression changes of SGBS cells upon ER stress and SIK2 inhibition.

Upon ER Stress induced by Thapsigargin; Leptin, Adiponectin, TNF α , IFN γ , IL-1 β , IL-5, IL-10 genes were down-regulated where IL-6, MCP-1 and spliced form of XBP-1 was up-regulated. However, in the presence of Compound C, Leptin, Adiponectin, MCP-1 expressions were a little normalized. In addition, when SIK2 inhibited during the ER stress, IL-5, IL-10, IFN γ and IL-18 expressions increased even compared to untreated controls (Figure 5.1, a). IL-10 showed very similar profile in the shRNA mediated SIK2 knockdown experiments. IL-6 gene was again up-regulated in THA treatments but, in contrast with CC experiment, SIK2 inhibition also diminished its expression regardless of ER stress. In addition, similar to IL-10, SIK2 inhibition increased IL-5 expression when it is combined with ER stress (Figure 451, b).

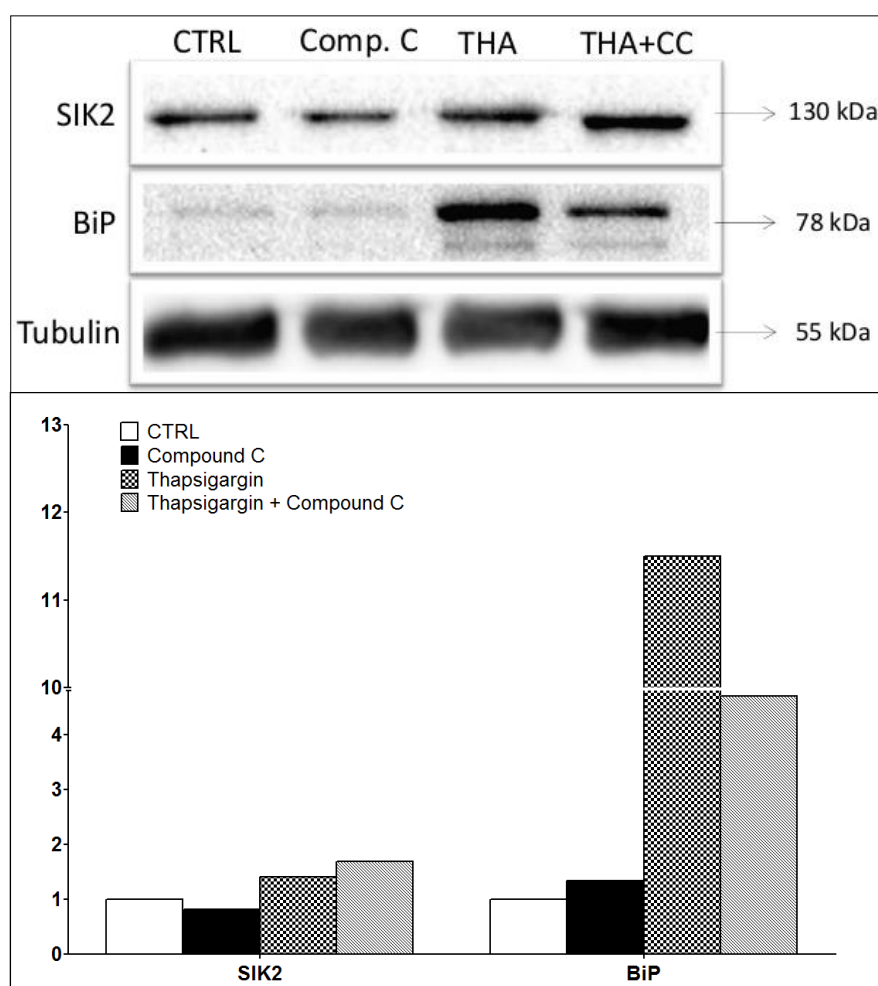


Figure 5.2: Changes in protein levels of BiP and SIK2 upon ER stress.

In our experiment, Bip protein and sXBP-1 mRNA were used as an ER stress marker [67]. As expected, both Bip and sXBP-1 expressions were increased upon ER

stress. On the other hand, its expression seemed to be diminished a little in the presence of Compound C during the ER stress. Compound C alone did not affect the levels of both SIK2 and Bip. However, SIK2 band appeared to be unexpectedly got stronger when Compound C combined with Thapsigargin effect.

5.2. THP-1 cells differentiation

During the induction of THP-1 cells to PMA in order to differentiate from monocytes to macrophages they were treated with SGBS conditioned media (C.M.) from the previous experimental setup (see Figure 4.1). mRNAs were isolated and used for quantitative Real-Time experiments. M2 related genes including CD206, IL-5 and IL-10 were more than 10 times up-regulated in the cells that were incubated with the conditioned media from Thapsigargin and Compound C treated SGBS cells. On the other hand, the expressions of M1 associated genes such as, TNF γ , MCP-1, IL-1 β and IL-6 were relatively lower than M2 related genes. For the control, THP-1 cells incubated with untreated SGBS conditioned media during two days of PMA induction were used.

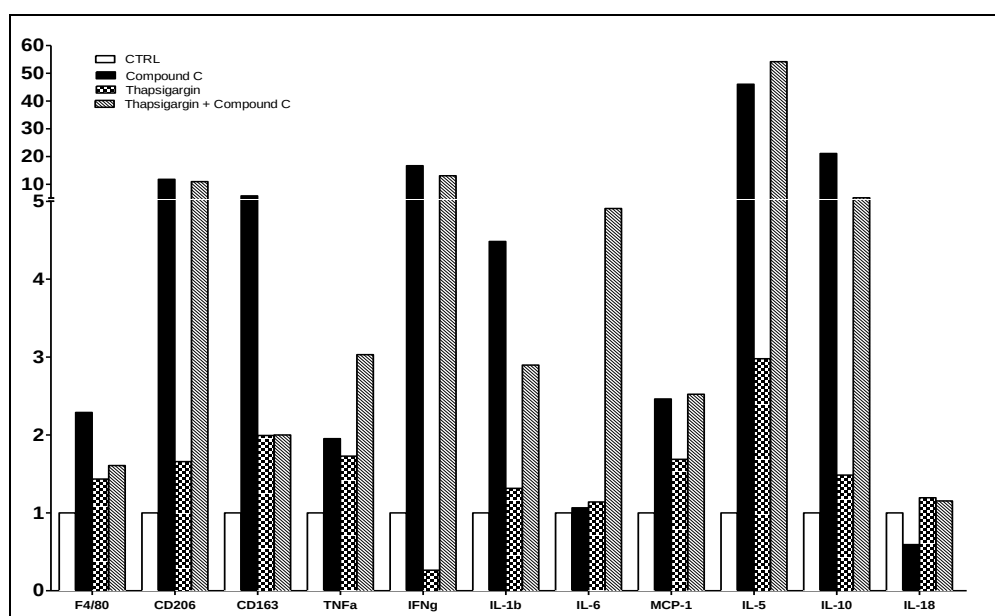


Figure 5.3: Gene expression changes of THP-1 cells upon SGBS C.M. treatment.

5.3. shRNA-mediated SIK2 knockdown

After the second passage, puromycin-resistant SGBS cells were lysed and SIK2 protein levels of transfected cells were compared to wild-type SGBS' with Western Blot.

Although SIK2 levels were slightly decreased in random shRNA transfected cells (shCTRL), the SIK2 band was almost disappeared in SIK2 shRNA transfected ones (shSIK2).

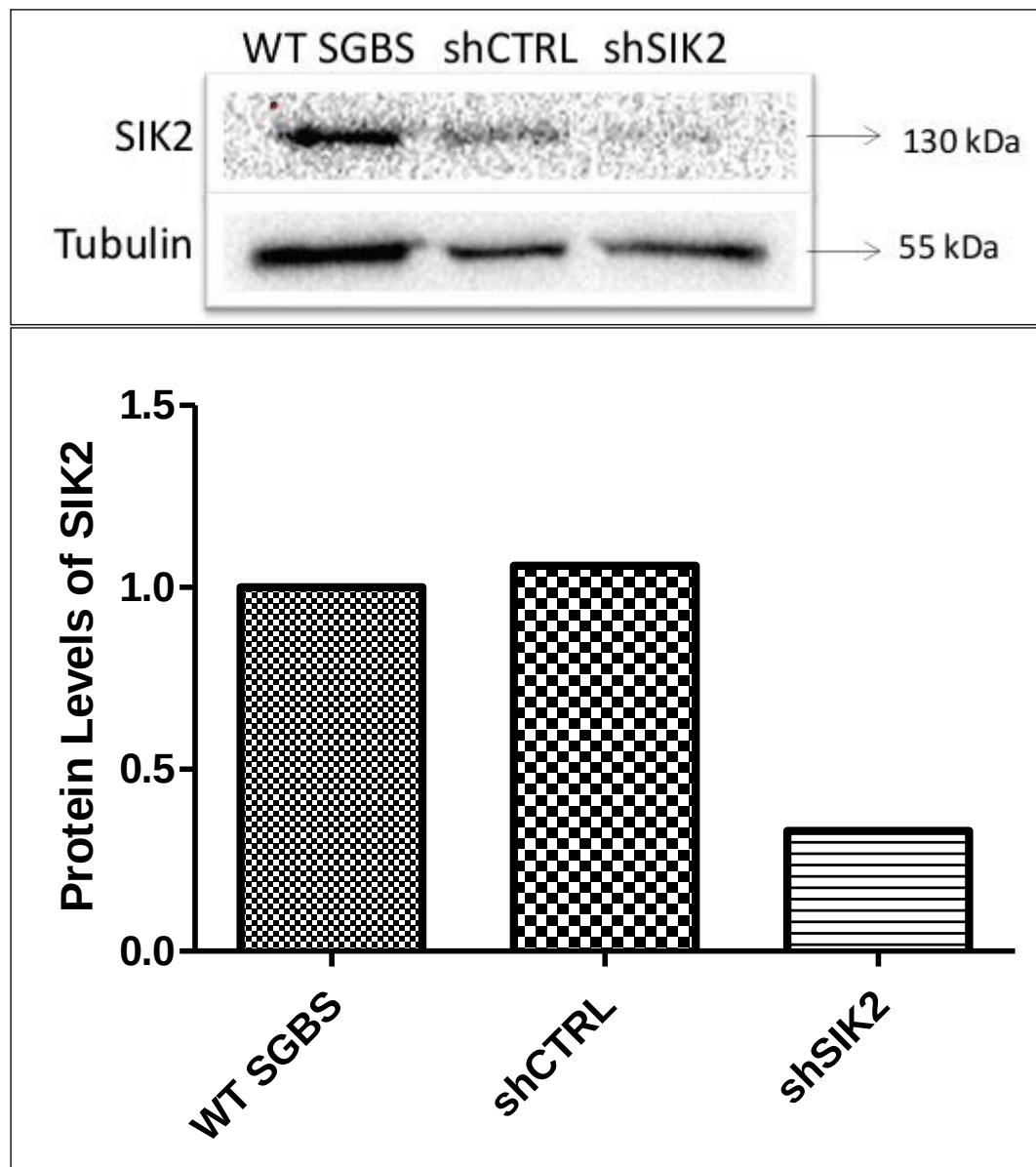


Figure 5.4: Confirmation of SIK2 knockdown with Western Blot.

5.4. Inflammatory phenotype of Adipose Tissue Macrophages of K14-VEGF-C transgenic mice

In order to confirm the inflammatory phenotype of VEGF-C overexpressing mice, gene expression levels of particular cytokines or cell surface markers –which are frequently used as M1 or M2 markers– of isolated macrophages from the same mice were measured with quantitative real-time PCR analysis. As expected, M1 markers such as CD11c, IL-6 and TNF α were up-regulated in K14-VEGF-C macrophages whereas the gene expression levels of M2 markers (such as CD206, CD163 and F4/80 -although it is a common macrophage marker, it is expressed higher in M2 macrophages [91] were down-regulated (Figure 5.5). Expectedly, the VEGFR-3 mRNA levels were also up-regulated in K14-VEGF-C macrophages, which might be involved in their recruitment into the tissue.

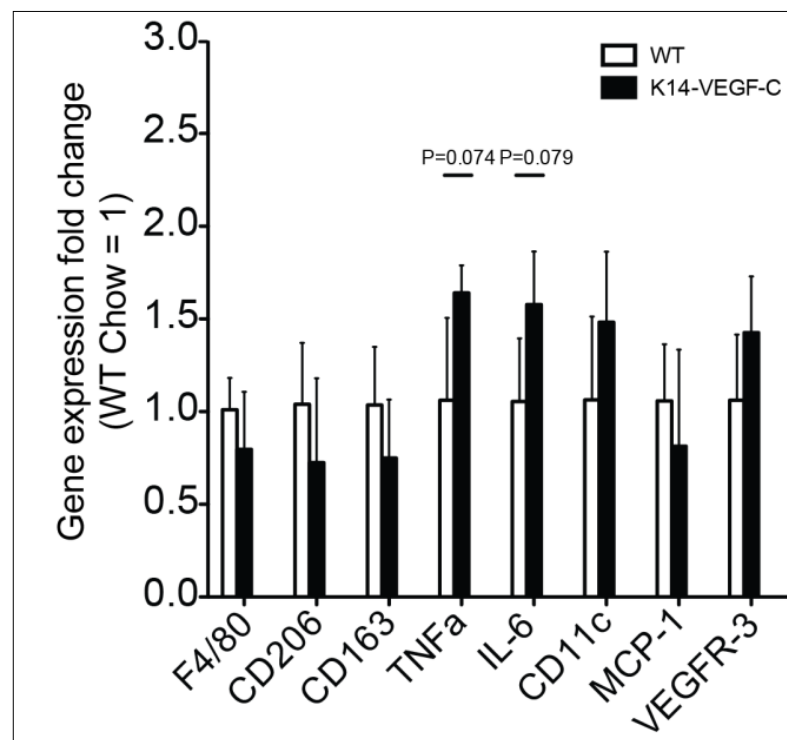


Figure 5.5: qPCR analysis of particular markers (up-regulation in M1 genes TNF α , IL-6 and CD11c and down-regulation in M2 genes CD206 and CD163) supported the tendency towards M1 phenotype in TG macrophages.

5.5. Real-time PCR of subcutaneous isolated adipocytes

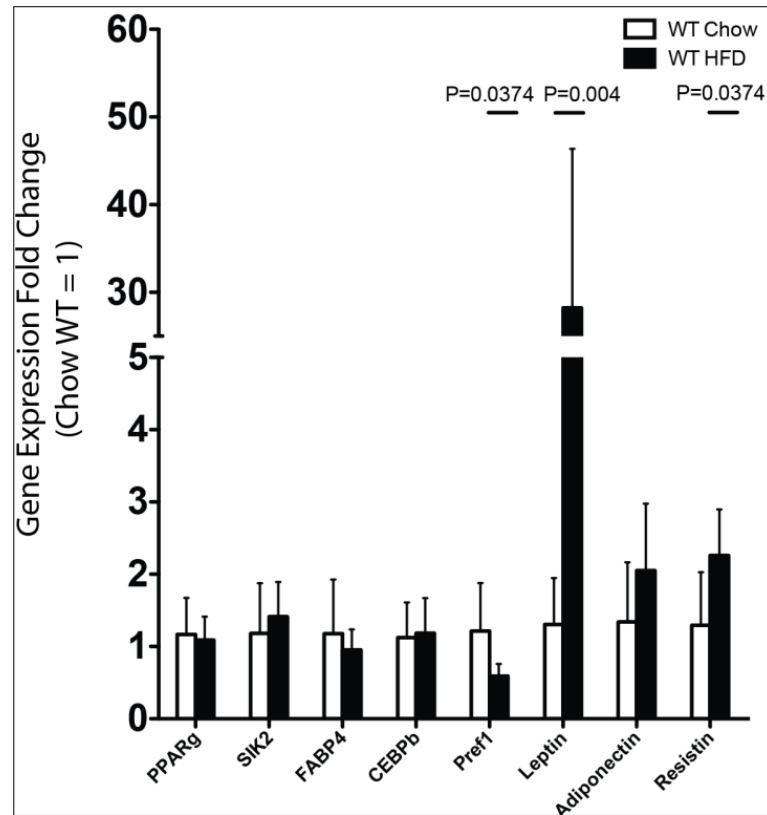


Figure 5.6: qPCR analysis of adipogenic genes in SC white adipocytes of wild-type mice on high-fat diet.

Upon HFD, expression levels of certain adipokines and adipocyte differentiation genes were altered. In line with the literature, Leptin, Adiponectin and Resistin levels were at least two-fold increased. In addition, pre-adipocyte marker Pref1's expression was significantly down-regulated which may point to an increased number of adipocytes. SIK2 mRNA levels were also increased in subcutaneous adipocytes of mice on high-fed diet.

5.5.1 In response to VEGF-C overexpression

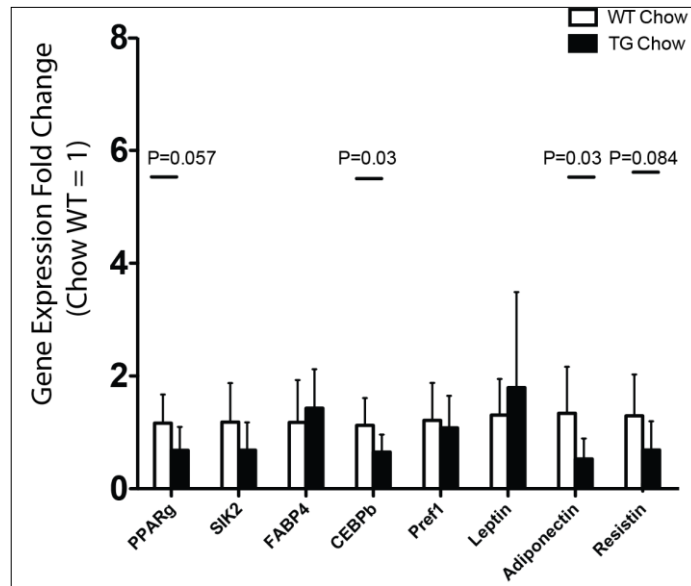


Figure 5.7: qPCR analysis of adipogenic genes in SC white adipocytes of K14-VEGF-C mice on chow diet.

In SC adipocytes, some adipocyte differentiation genes were down-regulated in K14-VEGF-C transgenic mice such as PPAR γ and CEBP β . Likewise, Adiponectin and Resistin levels were also decreased in transgenic mice which may be associated with insulin resistance. On the other hand, leptin mRNA levels were increased upon VEGF-C overexpression.

6. DISCUSSION and CONCLUSIONS

Obesity has become a serious threat for human health via contributing to the development of other diseases such as type 2 diabetes, metabolic syndrome and cardiovascular diseases. The prevalence of obesity has been sharply increasing over the last few decades; therefore, understanding the mechanisms underlying obesity and related metabolic diseases are at great importance for human health in the coming century. In efforts to unravel the mechanism of obesity, Endoplasmic Reticulum stress and chronic inflammation has been center of immense investigations.

Although there are signs of an association between development of endoplasmic reticulum stress and low-grade chronic inflammation in obese animals and human subjects, the mechanisms behind it are not yet well-described. Under nutritional stress, unfolded protein response can activate JNK and/or NF- κ B signaling, as well as other potential pathways, to induce inflammatory mediators [92]. Recent studies indicated SIK2 (Salt-inducible kinase) in both adipocytic ER stress and inflammatory cytokine production in macrophages. This information led us to think that there might be a regulatory role of SIK2 in the link between ER stress and secretom profiles of adipocytes that may in turn affect the phenotype of activated macrophages in inflamed adipose tissue.

In a recent study, SIK2 has been shown to down-regulate anti-inflammatory gene expressions via CREB-CRTC3 proteins in macrophages; however we do not know if this interconversion is mediated by external cues in the obese animals. Here, we generated an in vitro model to test if this conversion is mediated by secreted molecules from adipocytes under stress conditions.

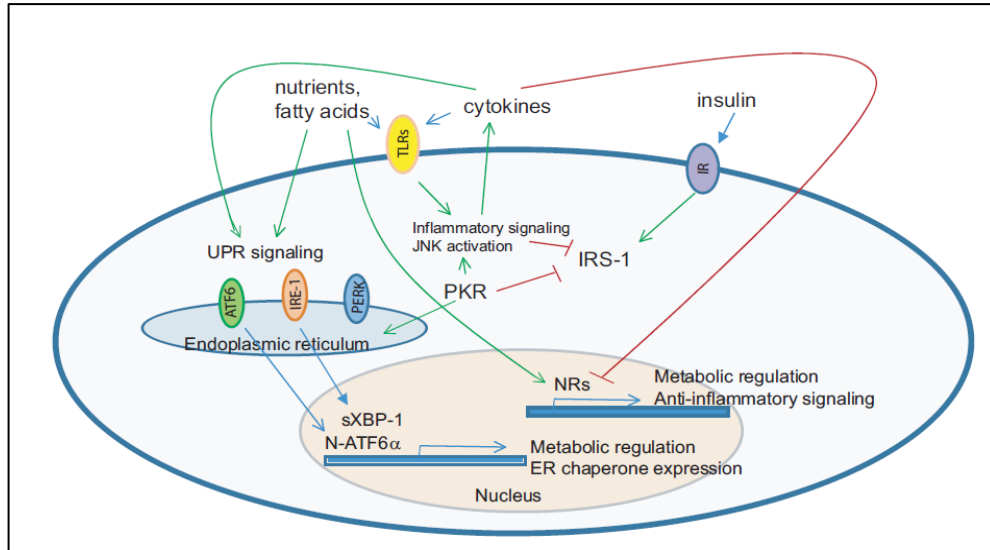


Figure 6.1: Association of ER stress, inflammation and metabolic pathways.

When we treat SGBS mature adipocytes with Thapsigargin, the increase in both mRNA levels of the spliced X-box binding protein and protein levels of BiP confirmed that the ER stress were initiated successfully [90]-[93]. Upon ER stress, adiponectin and leptin were around five-fold down-regulated, but SIK2 inhibition recovered this down-regulation a bit. ER stress is known to decrease adiponectin expression [94] and cause leptin resistance [95] which is correlated with our findings and the compensatory effect of Compound C during ER stress on production of both adipokines might point out a regulatory role of SIK2 in these pathways. Although the slight decrease in mRNA levels of some inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL1-}\beta$ was contradictory to findings of other *in vivo* studies, up-regulation of IL-6 and MCP-1 was supported the inflammatory profile which is in line with the literature. Except $\text{IFN}\gamma$, SIK2 inhibition during Thapsigargin treatment did not have major effect on gene expressions alterations of inflammatory cytokines, comparing to only Thapsigargin treatment. On the other hand, decreased anti-inflammatory cytokines upon ER stress (such as IL-5 and IL-10) were sharply up-regulated when ER stress was combined with SIK2 inhibition. Inhibition of SIK2 is shown to increase IL-10 production and induce anti-inflammatory (M1 to M2) type switching in macrophages [84], thus our findings can propose a similar role of SIK2 in SGBS mature adipocytes. In both qPCR and Western Blot results, we observed that SIK2 levels were slightly increased which seemed unexpected at first. However, once activity of SIK2 was diminished, cells might try to compensate and produce more

SIK2 than usual. In order to be sure that SIK2 activity is still lower than control groups, a kinase activity assay for SIK2 should be performed in the future.

Stable cell line establishment of shRNA-mediated SIK2 knockdown of SGBS pre-adipocytes seemed promising according to Western Blot results and can be tested in a similar experimental setup after testing the differentiation efficiency. Thus, due to the ambiguity of results obtained from chemical inhibition of SIK2 in fully differentiated human adipocytes, we repeated the same experiment with stable SGBS cell lines expressing shSIK2. Compound C is a selective inhibitor for AMPK and of probably few other kinases such as mTOR and BMP. Therefore the discrepancy between Compound C treated and shSIK2 mediated expression profiles of some adipocytes secreted genes including Adiponectin, Leptin, TNF α and MCP-1 are expected. Among those, Leptin's expression did not change at all in SIK2 knockdown cells in both stressed and unstressed conditions which may be concluded that it is not regulated by SIK2. In addition, IL-5, IL-18, IL-6 genes in shSIK2 cells showed a slight alteration comparing to the Compound C experiment which may point out an AMPK effect, as well. For instance, SIK2 specific knockdown definitely had a positive effect on the expression of IL-5 but only during the ER stress, however, Compound C had increased its expression only without the stress. On the other hand, IL-18 was up-regulated in shSIK2 cells in both conditions while its expressions were increased in stress condition via Compound C. Expectedly, Thapsigargin treatment up-regulated IL-6 expressions however, upon the Compound C treatment, IL-6 expressions did not change both during ER stress or normal conditions. In contrast, SIK2 inhibition via shRNA down-regulated IL-6 regardless of the ER stress which suggests a role of on positive regulation of inflammatory cytokine IL-6. Only IL-10 showed very similar expression profile in all conditions of both Compound C and shRNA experiments. As it was explained above, this data confirms the fact that SIK2 inhibition favors the M2 phenotype via supporting IL-10 production in macrophages also exists in mature human adipocytes.

If one only focuses on the results from shSIK2 mediated inhibition experiment, one can suggest the following conclusions about SIK2. First of all, SIK2 is required for Adiponectin expression in unstressed adipocytes and does not correlate with exogenous ER stress while decreases Adiponectin level. Second, SIK2 down-regulates TNF α level significantly; upon SIK2 knockdown its level increase at least two times. SIK2 also seems to act on INF γ by up-regulating its level at least 2-

times in unstressed cells. Expectedly, SIK2 up-regulate IL-6 level by more than two fold in stressed cells. However, when combined with ER stress which by itself rev up IL-6 level 20-40 fold down-regulates by four fold. Moreover, SIK2 is also required for the maintenance of IL-5 in stressed adipocytes such that upon SIK2 knockdown IL-5 levels increased about 3-times under stress conditions. Finally, SIK2 is also required for IL-10 expression which is down-regulated in the presence of ER stress. There must be a cross-talk between ER stress and SIK2 level (or activity) in order to explain increased IL-10 expression in SIK2 knock-down cells under exogenous stress condition.

These results all together point out a critical role of SIK2 for the regulation of both pro-inflammatory (IL-6) and anti-inflammatory (IL-10 and IL-5) cytokines in human adipocytes. Inhibition of SIK2 up-regulated IL-10 expression during the ER stress and inhibited IL-6 regardless of the stress. In conclusion, SIK2 should now be considered and investigated as a major regulator for obesity-induced inflammation and controlling its activity during ER stress may diminish the contributions of ER stress into chronic inflammation and insulin resistance.

In theory, ER stress initiates couple of unknown pathways leading to an inflammatory response by inhibiting anti-inflammatory cytokine production via SIK2 at least in macrophages and triggering up-regulation of inflammatory ones. These two pathways might be independent from each other and according to our findings; SIK2 is both involved in suppressing anti-inflammatory cytokine (IL-5 and IL-10) production only during the ER stress and supporting pro-inflammatory one (IL-6) regardless of the stress. When SIK2 was inhibited during the ER stress, mRNA levels of inflammatory cytokine IL-6 were also diminished comparing the SIK2 wild-type cells in stress conditions, however the cells were able to produce anti-inflammatory IL-10 and IL-5 cytokines more than even usual to compensate the inflammatory response. In addition, relatively decreased Bip and sXBP-1 expressions in both Thapsigargin & Compound C and Thapsigargin & shSIK2 treated groups might suggest that SIK2 inhibition may also cure the ER stress as well as recovering the inflammatory response in mature adipocytes.

After obtaining conditioned media (C.M) from SGBS cells (CTRL, CC, THA, THA+CC), we treated THP-1 cells with those C.M. during their differentiation via PMA. After the RNA isolation and q-RT PCR experiments from those THP-1 macrophages, we have observed that M2 related genes such as CD206, CD163, IL-

10 and IL-5 were more than 10-times up-regulated in the groups that were treated with Compound C treated SGBS medium. However, the difference of ER stress during the SIK2 inhibition was only observed in CD163 expression. Interestingly, IFN γ was also around 20-times up-regulated even though it is a M1 associated gene, and even down-regulated in THA group. On the other hand, the gene expressions of all other cytokines associated with M1 phenotype were also increased, however they were slight increases comparing to M2 genes. Especially we would expect that IL-6 to be down-regulated in THA+CC group comparing to THA treatment. Nevertheless, we can still mention an anti-inflammatory profile due to the fact that M2-related genes were more up-regulated than the M1-related ones.

Since there are more M2 macrophages in a regular adipose tissue and we are using untreated SGBS conditioned media as the reference group, we would expect that M2 genes to change their expressions less than M1s. Furthermore, it would be more logical to see increased levels of M1 associated genes and decreased M2s in Thapsigargin according to our previous finding on SGBS cells. However, the major increase in M2 gene expressions may point out that SIK2 inhibition altered the secretion profile from SGBS which later changed the macrophage profile regardless of the ER stress. In any case, this experimental setup shall be repeated to get more trustworthy results.

In my previous study in Detmar's lab (ETHZ), we had shown that VEGF-C recruits inflammatory macrophages into the adipose tissue. Real-time PCR analysis of macrophages isolated from subcutaneous adipose tissue macrophages showed more inflammatory-like (M1-like) profile and expressed increased levels of VEGFR-3 which confirms the idea that those macrophages responded VEGF-C. We checked if SIK2 and other adipogenic genes are either involved or were affected in downstream of VEGF-C signaling response in adipocytes by performing real-time PCR from subcutaneous (SC) adipocytes. We observed an up-regulation in Leptin and Resistin expression upon HFD, which is in line with the literature [96]. In addition, Pref1 was down-regulated in HFD, which suggests an increased adipocyte differentiation upon HFD. However, contradictory with this finding, the adipogenic differentiation genes were also downregulated in the K14-VEGF-C transgenic mice compared to the wildtype mice, which also correlates with our previous findings about the adipocyte sizes (data not shown in this thesis). Finally, Adiponectin expression in the SC adipocytes of K14-VEGF-C transgenic mice was also

significantly decreased, which might explain the mechanism behind the diminished insulin sensitivity (data not shown). SIK2 expression did not dramatically change upon HFD but seemed a little decreased in K14-VEGF-C transgenic mice.

We also had observed lipid accumulation in livers of both VEGF-C transgenic and especially HFD-fed mice (data not shown). SIK2 is known to be involved in the regulation of lipid synthesis and gluconeogenesis in liver, so we decided to check SIK2 mRNA levels as well as other metabolic genes that can potentially be responsible with the altered lipid accumulation. We performed qPCR experiments with the liver samples. Interestingly, overexpression of VEGF-C in the skin, increased the expression of some of the lipid synthesis genes (FAS, PPAR γ 2 and Acc1) in liver under chow diet. The significant up-regulation of SIK2 in the same condition might be the cause of this result. On the other hand, although it was not up-regulated under chow diet, the expression of SREBP-1c, another lipid synthesis gene, was increased in K14-VEGF-C mice under HFD. These findings may suggest that the mechanism behind lipid accumulation based on HFD and VEGF-C overexpression might be different. It has also been found that gluconeogenic genes were not altered upon VEGF-C overexpression, which means that the transgene does not affect gluconeogenesis. Since it has been shown that higher levels of leptin inhibits the lipid synthesis genes of liver in order to compensate the increased lipid accumulation and worsened metabolism in obesity [97], we expected to see decreased expression levels of these genes upon HFD. Not surprisingly, most of the lipid synthesis genes were down-regulated in both genotypes upon HFD. However, we did not see a similar profile in gluconeogenic genes, which is conflicting with the literature [98], [99]. Likewise, upon HFD, we did not observe any major alterations in SIK2 expression levels.

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BIOGRAPHY

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APPENDICES

Appendix A: Primer List

Table A1.1: Primers used for quantitative Real-Time PCR Experiments.

Gene Name	Forward Primer (5' to 3') / Tm	Reverse Primer (5' to 3') / Tm
Leptin	TGCCTTCCAGAAACGTGATCC /62.4	CTCTGTGGAGTAGCCTGAAGC /61.9
Adiponectin	AACATGCCCATTCGCTTTACC /61.3	TAGGCAAAGTAGTACAGCCCA /60.2
RPLP0	AGCCCAGAACACTGGTCTC /60.9	ACTCAGGATTCAATGGTGCC /60.3
TNFα	CCTCTCTCTAATCAGCCCTCTG /60.8	GAGGACCTGGGAGTAGATGAG /60.2
IFNγ	TCGGTAACTGACTTGAATGTCCA /61.2	TCGCTTCCCTGTTTTAGCTGC /62.9
IL-1β	ATGATGGCTTATTACAGTGCAA /60.0	GTCGGAGATTCGTAGCTGGA /60.8
IL-6	ACTCACCTCTTCAGAACGAATTG /60.2	CCATCTTTGGAAGGTTCAAGTTG /61.3
MCP-1	CAGCCAGATGCAATCAATGCC /62.3	TGGAATCCTGAACCCACTTC /60.4
F4/80	CCAGTGTTAATGCCGAAGTCT /60.0	GTGAACAGGTAAGCCATGACA /60.0
CD206	GGGTTGCTATCACTCTCTATGC /60.0	TTTCTTGCTGTGCGGTAGTT /60.1
CD163	TTTGCAACTTGAGTCCCTTCAC /60.6	TCCCGCTACACTTGTTTTAC /60.5
IL-5	TCTACTCATCGAACTCTGCTGA /60.0	CCCTTGACAGTTTGAAGTCTC /60.8
IL-10	GACTTTAAGGGTTACCTGGGTTG /60.5	TCACATGCGCCTTGATGTCTG /63.0
IL-18	TCTTCATTGACCAAGGAAATCGG /60.5	TCCGGGGTGCATTATCTCTAC /60.8
sXBP-1	GGTCTGCTGAGTCCGCAGCAGG	GGGCTTGGTATATATGTGG
SIK2	CTGGACATCTGGAGTATGGG	GAATCTTCCTTCCAGAACCCT

Appendix B: Antibodies

Table B1.1: Antibodies dilutions for Western Blot experiments.

Antibody Name	Dilution	Origin
SIK2 (1°)	1:1000 (in 5% BSA)	Rabbit
BiP (1°)	1:1000 (in 5% BSA)	Rabbit
Tubulin (1°)	1:1500 (in 5% dry, not-fat milk)	Mouse
Anti-rabbit (2°)	1:5000 (in 5% dry, not-fat milk)	Goat
Anti-mouse (2°)	1:5000 (in 5% dry, not-fat milk)	Goat