MECHANISM OF ACTIONS OF BILIMBI FRACTIONS AND ITS NATURAL COMPOUNDS IN STIMULATING BROWN ADIPOCYTE DIFFERENTIATION

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by

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LIST OF SYMBOLS

α	alpha
β	beta
δ	delta
γ	gamma
-	negative
+	positive
$\bar{x}$	average
%	percentage
˚C	degrees Celsius
°C/min	degrees Celsius per minute
Δ	change of difference
<	less than
≤	less than or equal to
>	greater than
:	ratio
/	per
™	trademark
µg	microgram
µL	microlitre
µL/well	microlitre/well
µm	micrometre
µM	micro-molar
µg/L	microgram per litre
µg/µL	microgram per microlitre
µg/mL	microgram per millilitre

bp	basepair
cells/mL	cells number per millilitre
cm	centimetre
cm^2	square centimetre
Da	dalton
eV	electron volt
g	gravity force
g	gram
Gae/g	gallic acid equivalents per gram
h	hour
kb	kilobase
kDa	kilo dalton
kg/m^2	kilograms per square metre
L	litre
M	molar
m	metre
m/z	mass to charge ratio
Mbar	millibar
mg	milligram
mg/kg	milligram per kilogram
mg/L	milligram per litre
mg/mL	milligram per millilitre
min	minute
mL	millilitre
mL/min	millilitre per minute
mm	millimetre
mM	millimolar

Mm/Rn	*Mus musculus/ Rattus norvegicus*
MPa	megapascal
nm	nanometre
nM	nanomolar
R^2	coefficient of determination
rpm	rotation per minute
v/v	volume per volume
w/v	weight per volume
X	concentration factor

LIST OF ABBREVIATIONS

A.bilimbi	*Averrhoa bilimbi*
Ab	Antibody
ABTS	2,2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid
ADC 2	Automatic Development Chamber 2
amu	Atomic mass unit
ANOVA	Analysis of variance
APS	Ammonium persulfate
ASC-1	Asc-type amino acid transporter-1
A-T	Adenine-Thymine
ATCC	American Type Culture Collection
ATPs	Adenosine Triphosphates
ATS4	Automatic TLC Sampler 4
BAT	Brown Adipose Tissue
BMI	Body mass index
BMP-7	Bone morphogenetic protein-7
BSA	Bovine Serum Albumin
BSC II	Biosafety cabinet class II
cAMP	Cyclic adenosine monophosphate
CIDEA	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
CO_2	Carbon dioxide
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9
C_t	Threshold cycle
Cyto C	Cytochrome C
DAPI	4′,6-diamidino-2-phenylindole

DEX	Dexamethasone
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et.al	et alia / and others
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF21	Fibroblast growth factor 21
FITC	Fluorescein isothiocyanate
FRAP	Ferric Reducing Antioxidant Power
G-C	Guanine-cytosine
GC-MS	Gas chromatography-mass spectrometry
HDL	High-density lipoprotein
HP-5 ms	5% phenyl methyl silicone
IBMX	Isobutyl methylxanthine
IC_{50}	Half maximal Inhibitory concentration
IgG	Immunoglobulin G
IgH	Immunoglobulin H

L	Large (size)
LC_{50}	Lethal Concentration 50
LC_{90}	Lethal Concentratio 90
mAb	Monoclonal antibody
MAPK	Mitogen-activated Protein kinase
$MgCl_2$	Magnesium chloride
M-MLV Rnase [H-]	Moloney Murine Leukaemia Virus Reverse Transcriptase, RNase H Minus
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
Myf5	Myogenic factor 5
Myf5-	Myogenic factor 5 negative
Myf5+	Myogenic factor 5 positive
NCBI	National Centre for Biotechnology Information
NTC	No template control
OD	Optical density
oWAT	Omental white adipose tissue
P13K-PKB/Akt	Phosphoinositide-3-kinase-protein kinase B/Akt
P2X5	Purinergic receptor
PAT2	Proton-coupled Amino acid transporter-2
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline with 0.1% Tween-20
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
Pen-strep	Penicillin-streptomycin

PFA	Paraformaldehyde
PGC1-α	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha.
pgWAT	Perigonadal white adipose tissue
PHY	Phytol
PPAR-γ	peroxisome proliferator-activated receptor γ
PRDM16	PR Domain containing 16
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rcf	relative centrifugal force/(g-force)
RFU	Relative Fluorescence Unit
RNase	Ribonuclease
ROSI	Rosiglitazone
rRNA	Ribosomal ribonucleic acid
RT	Retention Time
SD	Standard deviation
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid.
SIRT-1	Sirtuin-1
SQ	Squalene
SREBF-1	sterol regulatory element-binding transcription factor-1
STZ	Streptozotocin
sWAT	Subcutaneous white adipose tissue
T_a	Annealing temperature
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
TE	Trolox Equivalent
TEMED	N, N,N', N'- tetramethylethylenediamine
TLC	Thin layer chromatography

T_m	Melting temperature
UCP-1	Uncoupling protein 1
USA	United States of America
UV	ultraviolet
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WHO	World Health Organization
XXT	(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

MEKANISME TINDAKAN PECAHAN BILIMBI DAN SEBATIAN SEMULAJADINYA DALAM MERANGSANG PEMBEZAAN ADIPOSIT COKLAT

ABSTRAK

Averrhoa bilimbi, atau bilimbi, adalah salah satu tumbuhan perubatan dalam sistem perubatan Ayurveda dengan pelbagai aktiviti farmakologi. Daun bilimbi kebanyakannya dilaporkan digunakan secara tradisional dalam merawat hiperglikemia, diabetes, dan penyakit yang seumpamanya. Dalam kajian terdahulu, pecahan heksana bilimbi telah menunjukkan sifat anti-obesiti yang memberangsangkan, dengan merangsang pembezaan adiposity perang. Seterusnya, sebatian semulajadi utama dalam pecahan bilimbi, termasuk fitol dan skualen, juga dilaporkan mempunyai aktiviti pemerangan. Walau bagaimanapun, pemahaman kita tentang mekanisme tindakan ini masih terhad hingga kini. Oleh itu, kajian ini memberi tumpuan kepada satu siri kajian transkrip dan translasi untuk menjelaskan mekanisme tindakan pecahan bilimbi dan sebatian semulajadinya dalam merangsang pembezaan adiposit perang. Dalam kajian ini, pecahan heksana bilimbi (F7, F8, dan F9) dan sebatian semulajadinya (fitol dan skualen) telah merangsang pembezaan adiposit perang dengan ketara pada 50 µg/ml dan 100 µM, masing-masing, dalam ujian berasaskan sel. Berdasarkan analisis qRT-PCR, rawatan dengan pecahan bilimbi, fitol dan skualen menginduksi gen penanda adiposit perang UCP-1, PRDM16, dan PGC1-α secara signifikan. Sehubungan itu, ekspresi protein yang lebih tinggi bagi CIDEA, penanda khas protein adiposit perang, dipamerkan pada peringkat translasi melalui ujian Immunofluoresensi. Rawatan ini mengakibatkan penghasilan bersama miotub dan adiposit perang melalui pengurangan gen PRDM16. Menariknya, PRDM16 adalah

faktor penting dalam program pembezaan adiposit perang, namun didapati boleh diabaikan dengan penginduksian UCP-1 dan PGC1-α. Data yang dikumpulkan di sini mencadangkan aplikasi berpotensi tumbuhan ini sebagai agen anti-obesiti berasaskan semulajadi untuk mengawal obesiti dan komplikasinya.

MECHANISM OF ACTIONS OF BILIMBI FRACTIONS AND ITS NATURAL COMPOUNDS IN STIMULATING BROWN ADIPOCYTE DIFFERENTIATION

ABSTRACT

Averrhoa bilimbi, or bilimbi, is one of the medicinal plants in the Ayurvedic medicine system with various pharmacological activities. The bilimbi leaves were mainly reported for their traditional uses in treating hyperglycemia, diabetes, and related disorders. In previous studies, hexane bilimbi fractions have demonstrated promising anti-obesity properties by stimulating brown adipocyte differentiation. Similarly, major natural compounds of the bilimbi fractions, including phytol and squalene, have also been reported to possess browning activity. However, our understanding of this mechanism of action is still limited to date. To that end, this study focused on a series of transcriptional and translational studies to elucidate the mechanism of action of bilimbi fractions and their corresponding natural compounds in stimulating brown adipocyte differentiation. In the present study, hexane bilimbi fractions (F7, F8, and F9) and their natural compounds (phytol and squalene) substantially stimulated brown adipocyte differentiation at 50 μg/ml and 100 μM, respectively, on the cell-based assay. Based on qRT-PCR analysis, treatment with bilimbi fractions, phytol, and squalene significantly induced the brown-adipocyte marker genes of UCP-1, PRDM16, and PGC1-α. Correspondingly, a higher protein expression of CIDEA, the brown-adipocyte-specific protein marker, was exhibited on a translational level via Immunofluorescence assay. The treatment led to the co-development of myotubes and brown adipocytes upon gene knockdown of PRDM16. Interestingly, PRDM16 is a vital factor for the brown adipocyte differentiation

program, yet it was found to be dispensable by the induction of UCP-1 and PGC1-α. The data amassed herein suggests the potential application of this plant as a natural-based anti-obesity agent for the restriction of obesity and its complications.

Chapter 1

INTRODUCTION

1.1 Background of Research

Obesity is a common, concerning, and major public health issue that negatively impacts the quality of human life. People with obesity, compared to those with a healthy weight, have a greater risk of having several debilitating and life-threatening disorders, including diabetes, cancer, and cardiovascular diseases (Al-Goblan et al., 2014). Obesity is a multifactorial disease that fundamentally occurs due to energy intake exceeding energy expenditure. Other factors, including dietary habits, sedentary lifestyles, genetics, medical reasons, and environmental and physiological conditions, may all contribute to body fat deposition and obesity (Fonseca et al., 2018). There are several suggested treatments for obesity. Lifestyle modification, including a healthy diet and regular physical activity, is considered the first-line treatment for weight loss. Unfortunately, the long-term success rate is low and maintaining a healthy lifestyle can be challenging, often leading to weight regain. Some obese individuals may have contraindications to physical activities due to comorbidities such as heart diseases and orthopaedic disorders. Consequently, medications such as phentermine and orlistat appear to be the second weight-loss intervention. These medications work by suppressing appetite, inducing a feeling of fullness, inhibiting fat absorption, increasing energy consumption and thermogenesis, and affecting neurotransmitter levels such as serotonin, norepinephrine, and dopamine (Mohamed et al., 2014). Although effective, these medications may pose adverse effects, particularly gastrointestinal discomfort, which can outweigh their benefits. Several obesity medications that were previously approved by the United States Food and Drug Administration (FDA), such as amphetamine, rimonabant, and sibutramine, have been withdrawn from the market due to serious side effects, including an increased risk of psychiatric disorders and non-fatal myocardial infarction or stroke (Kang & Park

2012). Weight loss surgeries such as Roux-en-Y gastric bypass, gastric banding, and sleeve gastrectomy are recommended for individuals with obesity and significant medical complications. However, these surgeries carry risks, including bowel obstruction, dumping syndrome, nausea, and other severe complications (Pandolfino et al., 2004; Ma & Madura, 2015). In addition to their undesirable side effects, the high costs of medications and surgical procedures often limit access to underserved patients. Considering the risks associated with obesity and the inadequacies of current treatments, it is crucial for researchers to explore alternative and effective approaches to obesity prevention and management.

Obesity is characterised by the excessive expansion of adipose tissue, resulting from an increase in the size (hypertrophy) and number (hyperplasia) of adipocytes (Jo & Gavrilova, 2009). Two main types of adipose tissue are found in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT, the predominant type, is composed of white adipocytes located under the skin (subcutaneous fat) and around internal organs (visceral fat) (Choe et al., 2016). It serves as an energy storage site, and dysregulation of its function may contribute to obesity and type 2 diabetes. BAT, primarily composed of brown adipocytes, is mainly concentrated in the interscapular and perirenal regions (Frontini & Cinti, 2010). It specialises in energy expenditure via adaptive thermogenesis, which makes brown adipocytes a promising therapeutic target for combating obesity. The therapeutic potential of brown adipocytes has been shown where higher brown adipocyte levels correlate with reduced adiposity in people (Saito et al., 2009). Activating brown adipocytes to expend energy is one approach, alongside remodelling white adipocytes into beige adipocytes, which are thermogenic brown-like adipocytes (Spiegelman et al., 2009). However, a major challenge is that adults and obese patients have a low presence of metabolically active brown adipocytes,

accounting for less than 1% of total body weight (Pilkington et al., 2021). Exposure to a cold environment can stimulate brown adipocyte activation. Nonetheless, it is not feasible since long-term subjection to cold is required, and even when activated, the number of brown adipocytes remains small compared to white adipocytes (Lee et al., 2014). Therefore, any substances or compounds that effectively stimulate and activate brown adipocyte differentiation may offer potential solutions to address the obesity epidemic and its associated metabolic disorders.

Several naturally occurring compounds and dietary extracts have shown promise in regulating brown adipocyte differentiation and increasing energy expenditure. These include chilli peppers (Yoneshiro et al., 2013), capsaicin (Saito 2015), cinnamon (Kwan et al., 2017), green tea (Neyrinck et al., 2017), raspberry (Leu et al., 2018), and black ginseng (Park et al., 2019). In concomitance with this, studies on *Averrhoa bilimbi,* commonly known as bilimbi, have recently been initiated for the ability of their ethanolic leaves extract to stimulate brown adipocyte differentiation (Lau et al., 2019). Subsequent studies revealed that hexane fractions of bilimbi leaves exhibited more significant adipocyte browning activity compared to the crude extract or any other solvent tested (Hamzah et al., 2021). However, the mechanism by which bilimbi fractions stimulate brown adipocyte differentiation remains obscure. Gas chromatography-mass spectrometry (GC-MS) analysis identified squalene and phytol as major natural compounds in hexane bilimbi leaves extract and fractions, suggesting their involvement in adipocyte browning. Phytol has been demonstrated to induce adipocyte browning in previous experiments with 3T3-L1 preadipocytes and mice fed a high-fat diet (Zhang et al., 2018). *In vitro* studies on squalene have been limited to its adipogenic effect on adipose-derived stem cells, which may contribute to resolving insulin resistance and obesity-related metabolic disorders (Ganbold et al., 2020).

Therefore, the present study aims to elucidate the mechanism by which bilimbi fractions, squalene, and phytol stimulate brown adipocyte differentiation at transcriptional and translational level. The study reports that bilimbi fractions of F7, F8, F9, squalene, and phytol stimulate brown adipocyte differentiation, as evidenced by the co-development of myotubes and brown-like adipocytes in C2C12 myoblasts, as well as the upregulation of brown-related gene and protein markers at both the transcriptional and translational basis. Interestingly, PRDM16, initially believed to serve a functional role in adipocyte browning activity, was found to be dispensable with the presence of UCP-1 and PGC1-α upon treatment.

1.2 Scope of Research

Over the years, reports have shown bilimbi's pharmacological properties, including anti-diabetic, anti-hypertensive, anti-cancer, and antimicrobial properties. It was recently discovered that hexane bilimbi leaves fractions induce brown adipocyte differentiation, a targeted mechanism to combat obesity and its associated diseases. Phytol and squalene are the two most abundant natural compounds in the bilimbi leaves extract and fractions, and they may contribute to the induction of browning activity. However, the mechanism underlying their browning activity has yet to be investigated. Therefore, this study is important to elucidate the mechanisms involved in the induction of brown adipocyte differentiation by bilimbi fractions, phytol, and squalene. This study incorporates various aspects of experimental approaches, including bioassay-guided fractionation, which aims to identify several fractions responsible for stimulating the browning of adipocytes. Another analytical technique involves GC-MS, which identifies specific compounds and correlates them with the adipocyte browning effects of bilimbi fractions. Cell-based assays using C2C12

myoblasts were conducted to assess the phenotypic changes following treatment by bilimbi fractions and natural compounds. These cells are chosen due to their ability to differentiate into brown adipocytes, which is evident through the formation of lipid droplets. Another cell types, 3T3-L1 preadipocytes were used to validate adipogenesis induction and confirm the white-to-brown differentiation via gene expression studies on adipogenesis- and brown adipocyte-related genes using qRT-PCR, which provides insights into the activity of specific genes. Additionally, immunofluorescence studies are used to visualise the protein expression of brown adipocyte-related proteins and allow for the localisation and quantification of proteins within the treated 3T3-L1 preadipocytes. The gene knockdown experiments involving silencing the expression of a specific gene are conducted to investigate the specific targets regulated by the treatments and contribute to the stimulation of brown adipocyte differentiation. By employing these experimental approaches, the study aimed to shed light on the transcriptional and translational mechanisms underlying the stimulation of brown adipocyte differentiation by bilimbi fractions, phytol, and squalene. Understanding these mechanisms can provide insights into potential strategies for manipulating adipocyte browning, which has implications for metabolic health and obesity research.

1.3 Objectives of Research

In general, this study aims to elucidate the mechanism of action of bilimbi fractions and its natural compounds in stimulating brown adipocyte differentiation. Meanwhile, there are three specific objectives, which include:

1. To determine the effect of bilimbi fractions and its natural compounds on gene expression related to brown adipocyte differentiation.

2. To evaluate the translational effects of bilimbi fractions and natural compounds on the expression of adipocyte surface protein markers.
3. To identify the regulatory target of bilimbi fractions and its natural compounds in stimulating brown adipocyte differentiation.

Chapter 2

LITERATURE REVIEW

2.1 The Plant *Averrhoa bilimbi*

2.1.1 Description of Plant

Averrhoa bilimbi, also known as bilimbi or locally called belimbing asam, belimbing buluh, b'ling, or billing-billing, is a tropical fruit native to Indonesia, India, Malaysia, and the Philippines (Morton, 1987). It belongs to the Oxalidaceae family. The bilimbi tree is a perennial with a long lifespan, reaching a height of 5 to 10 m. It has a small trunk and a few upright branches, as depicted in Figure 2.1.

Figure 2.1: Bilimbi plant
Illustrations represent the bilimbi tree, its flowers, leaves, and fruits.
Adapted from: (Orwa, 2009)

The compound leaves are pale green, with each leaf consisting of 20–40 leaflets measuring 5–12 cm in length. The leaflets are alternately arranged and tend to cluster near the ends of the branches. The flowers are small and fragrant, displaying five petals ranging from yellowish green to reddish-purple. They emerge directly on the branches and trunk. Bilimbi fruits are ellipsoidal, slightly lobed, and can grow up to 10 cm in length. They are covered by a thin, star-shaped calyx at the stem end, while the apex is adorned with five hair-like floral remnants. When unripe, the fruit has a crisp texture. As it ripens, the colour changes from bright green to yellowish green, with shiny, thin, and smooth outer skin. The fruit contains a jelly-like, juicy flesh with

a sour flavour. The seeds are few, typically 6 to 7, and are flat, measuring about 0.25 inch (0.635 cm) in length and 4-6 mm in width. They have a smooth, brown surface. The wood of the bilimbi tree is white, soft, yet tough.

2.1.2 Ethnomedicinal Uses of Bilimbi

Ethnopharmacologically, the leaves, flowers, and fruits of bilimbi have been used for many years by Malaysians and other Southeast Asians to treat a variety of human maladies and diseases (Roy et al., 2011). The infusion of its flowers is used to treat thrush and coughing. The syrup made from its fruits is consumed to alleviate fever, inflammation, beri-beri, and biliousness, as well as to stop rectal bleeding and treat internal haemorrhoids (Kumar et al., 2013). Fruit juices from bilimbi are used to treat scurvy, hypertension, obesity, and diabetes (Alsarhan et al., 2012). The leaves are applied as a paste or directly to relieve itching and swellings due to mumps, rheumatism, stings, pimples, skin eruptions, and snake bites (Roy et al., 2011). The concoction of the leaves and fruits has been used to treat venereal diseases like syphilis (Ali et al., 2013). Leaf infusions are used as a remedy for coughs and as a tonic after childbirth (Samuel et al., 2010).

2.1.3 Studies on Bilimbi Leaves Extract and Their Pharmacological Activities

Bilimbi is one of the medicinal plants in the Ayurvedic system of medicine with established pharmacological activities. Over the years, many *in vitro* and *in vivo* studies have been conducted, mainly on bilimbi leaves with different extractive solvents, which led to the discovery and scientific validation of the pharmacological activities of bilimbi leaves. Testing for cytotoxicity is crucial to ensure the safety of substances, including plant extracts or biologically active compounds from plants before they are consumed for public health (McGraw et al, 2014). Several

investigations have examined the toxicology of bilimbi leaves extracts with various solvents. Using brine shrimp lethality assays, ethanolic extract of bilimbi leaves demonstrated moderate cytotoxic activity, with a lethal concentration (LC_{50}) and LC_{90} values of 5.81 µg/L and 10.28 µg/mL, respectively (Karon et al., 2011). Decoction and methanol extracts were found to be safe even at the highest concentration tested, 400 µg/mL, as determined using the XTT (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) methods on Vero cell lines (Seebaluck-Sandoram et al., 2019). Employing a resazurin-based assay, ethanol leaves extracts displayed no cytotoxicity at a 200 µg/mL concentration in both 3T3-L1 preadipocytes and C2C12 myoblast cell lines (Lau et al., 2019).

Bilimbi leaves extracts have shown antimicrobial properties against various pathogenic microorganisms and fungi. Specifically, the ethanolic bilimbi leaves extract exhibited antimicrobial activity against both gram-positive bacteria such as *Bacillus cereus* and *Bacillus megaterium*, gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa,* and fungi like *Aspergillus ochraceous* and *Cryptococcus neoformans* (Mackeen et al., 1997). In other studies, both aqueous and chloroform extracts of bilimbi leaves displayed positive antimicrobial activity against *Staphylococcus epidermidis, Staphylococcus aureus, Salmonella typhi, Aeromonas hydrophila,* and a few others (Zakaria et al., 2007). Recently, it was discovered that aqueous and methanol extracts of bilimbi leaves are effective against *E. coli.* Phytochemicals present in the extracts, such as alkaloids, saponins, tannins, flavonoids, steroids, carbohydrates, and glycosides, are believed to contribute to their antimicrobial effects (Mohammed Atiyah et al., 2022). These phytochemicals may inhibit the growth of bacteria by destroying the bacterial cell wall, as suggested in other studies (Abuga et al., 2020).

Several *in vivo* studies conducted using streptozotocin (STZ)-induced diabetic rats revealed the antidiabetic activities of ethanolic bilimbi leaves extracts. The ethanol leaves extract, at 125 mg/kg of body weight, has lowered the rats' blood glucose and triglyceride levels (Pushparaj et al., 2000). In subsequent studies, the mechanism of hypoglycaemic effects was investigated using oral administration, demonstrating that the aqueous fraction significantly impacted glucose tolerance and insulin secretion, which increased blood insulin levels (Pushparaj et al., 2001). In another study, it was observed that butanol fractions exhibited significant lipid-lowering effects, akin to the aqueous fractions, in mice induced with a high-fat diets and streptozotocin (STZ) (Tan et al., 2005). Nevertheless, more recent research is required to elucidate the hypoglycaemic properties of bilimbi leaves extracts or fractions.

The antioxidant activity of ethanolic bilimbi leaves extracts protected mice from oxidative damage caused by ultraviolet radiation. Malondialdehyde levels have decreased by up to 50% in mice treated with 4% topical ethanol extract, lessening the effects of ultraviolet (UV)-light-induced photoaging (Precious et al., 2012). The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) scavenging activity also showed that the antioxidant activity in the ethanol extract (82.82 mg GAE/g) was much higher than in the water extract (42.01 mg GAE/g) (Iwansyah et al., 2021). Seebaluck-Sandoram et al. (2019) found that decoction of bilimbi leaves showed significant DPPH radical scavenging activity at a half-maximal inhibitory concentration (IC_{50}) of 5.30 µg/mL, equivalent to the positive control, ascorbic acid, which had an IC_{50} of 5.89 µg/mL. Employing different methods of ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid (ABTS) assays, methanol, and decoction extracts of bilimbi leaf demonstrated a higher FRAP value (Trolox equivalent, (TE) range: 87.04–109.86 µM). At the same time, moderate activity was

shown against ABTS cation radical (IC_{50}) range: 31.68–39.26 μg/mL) (Seebaluck-Sandoram et al., 2019). Several antioxidant compounds, such as flavonoids, tannins, and coumarins, are thought to be responsible for the reducing effects. These compounds exert their activity by donating a hydrogen atom to break the free radical chain.

Recent preliminary research has revealed the potential anti-obesity effects of bilimbi, with ethanolic leaves extract stimulating brown adipocyte differentiation *in vitro*, comparable to the established browning agent, Rosiglitazone (Lau et al., 2019). Induction of the browning activity was supported by the up-regulation of PRDM16 and UCP-1 and increased basal metabolic rate and mitochondrial respiratory capacity. Several hexane bilimbi fractions exhibited the co-development of brown adipocytes and myotubes on C2C12 myoblasts in subsequent investigations employing bioassay-guided fractionation, and the effect was significant compared to the hexane extract (Hamzah et al., 2021). However, further investigations are needed to understand the mechanisms underlying the adipocyte browning activity of bilimbi fractions.

2.1.4 Studies on Bilimbi Natural Compounds and Their Pharmacological Activities

Gas chromatography-mass spectrometry (GC-MS) analysis on the hexane bilimbi leaves extracts revealed several major natural compounds. These include hexadecanoic acid, phytol, 9-octadecanoic acid (Z), and squalene (Hamzah et al., 2021). Gunawan et al. (2013) identified phytol and squalene as natural compounds in bilimbi earlier. Thus far, the most remarkable pharmacological properties of squalene are antioxidant properties. Squalene acts as an effective quencher of singlet oxygen upon oxidative stress, such as exposure to sunlight, and prevents lipid peroxidation at

the surface of human skin (Shimizu et al., 2018). With regards to anti-obesity, squalene supplementation in obese or diabetic mice has significantly increased high-density lipoprotein (HDL) cholesterol, an important anti-atherosclerotic factor (Liu et al., 2018). A novel amphiphilic squalene derivative has recently been synthesized in which it effectively prevents excessive lipogenesis by suppressing early adipogenic markers and key lipogenic genes such as Peroxisome Proliferator-Activated Receptors (PPAR), sterol regulatory element-binding transcription factor-1 (SREBF-1), and CCAAT/enhancer-binding protein-alpha (C/EBP-α) (Ganbold et al., 2020). However, the mechanism by which squalene may induce the browning of adipocytes has not yet been determined. Phytol has been widely employed as a food additive and a common aromatic constituent in many scent compounds of cosmetic and non-cosmetic items due to its antinociceptive and antioxidant properties (Kubo et al., 2018). Regarding its anti-obesity properties, phytol has been shown to induce brown adipocyte differentiation on 3T3-L1 adipocytes by increasing the expression of brown adipocyte marker genes such as Uncoupling Protein-1 (UCP-1), PR domain-containing protein 16 (PRDM16), Peroxisome proliferator-activated receptor-gamma coactivator (PGC1-α), Pyruvate dehydrogenase (PDH), Cytochrome C (Cyto C), mitochondrial content, and oxygen demand (Zhang et al., 2018). Activation of lipid metabolism regulators, such as the PPAR and retinoid-X receptor (RXR), was thought to be a possible mechanism (Roca-Saavedra et al., 2017). However, the transcriptional effect of phytol on several essential adipogenic genes, such as C/EBP-α and C/EBP-β, the translational effect of its browning activity, and other potential regulatory pathways involved in stimulating adipocyte differentiation remain uncovered.

2.2 Obesity

Obesity is a persistent and pervasive global health issue. Generally, obesity occurs when energy intake exceeds energy expenditure, which leads to an excessive accumulation of human body fat mainly stored in adipose tissue (Wen et al., 2022). Obesity is a multifaceted issue with far-reaching health consequences and metabolic co-morbidities, including type 2 diabetes, osteoarthritis, cancer, hypertension, hyperlipidaemia, and cardiovascular disease (Lin and Li, 2021). This is a major concern, as these conditions significantly increase the risk of morbidity and mortality. According to the World Obesity Atlas 2022, 1 billion people worldwide, including 1 in every 5 women and 1 in every 7 males, will be obese by 2030 (Lobstein et al., 2022). In addition, the current prevalence of obesity among Malaysian adults is already high, with nearly 20% of the population affected (Mohd-Siddik et al., 2021). This underscores the urgency of addressing obesity as a public health priority in the country. The Body Mass Index (BMI) is an effective epidemiological tool for measuring obesity in humans. BMI is calculated by dividing an individual's weight in kilogrammes by the square of their height in metres. An individual is considered overweight when their BMI lies between 25 and 29.9 kg/m^2 and obese when it exceeds 30 kg/m^2.

Numerous risk factors contribute to the development of obesity, and these factors can also exacerbate the health consequences of obesity itself. Some key risk factors include dietary habits. Diets high in calories, saturated fats, sugars, and processed foods are associated with weight gain and obesity. Also, sedentary lifestyles, characterised by limited physical activity and extended periods of sitting, increase the risk of obesity. Genetic predisposition is another crucial factor, as it can make certain individuals more prone to gaining weight. Furthermore, other factors such as

emotional eating, stress, depression, and other psychological factors often contribute to overeating, thus leading to increased weight gain.

The management of obesity involves a multifaceted approach. Lifestyle modification aimed at moderate caloric restriction and increasing physical activity while monitoring dietary intake are essential components of non-pharmaceutical obesity management. However, these interventions may not always be effective, leading to the need for pharmaceutical treatments in some cases. Current anti-obesity medications are targeted at suppressing appetite or decreasing cravings and have become a popular means to overcome weight gain. While these drugs are generally effective, their high costs, severe adverse toxicities, and side effects may limit their overall efficacy, which is critical to consider. The burden of obesity-related diseases places significant strain on healthcare systems. Treating obesity-related conditions requires substantial resources, and prevention and management strategies need to be a priority to alleviate this burden.

In understanding obesity, it is crucial to consider the role of different types of adipose tissue. White adipose tissue (WAT) and brown adipose tissue (BAT) play distinct roles in the regulation of energy balance and can be associated with the development of obesity in different ways (Gomez-Hernandez et al., 2016). WAT is primarily involved in energy storage and can contribute to obesity when individuals consistently consume more calories than they expend. WAT also secretes adipokines, which are bioactive molecules involved in metabolic regulation (Clemente-Suarez et al., 2023). Dysregulation of adipokines in obesity can contribute to insulin resistance, inflammation, and other metabolic disturbances (Ahmed et al., 2021). On the other hand, BAT is specialised in energy expenditure and heat dissipation, a process known as thermogenesis which can counteract obesity by burning excess calories rather than

storing them (Shinde et al., 2021). Recent research has shown that WAT can undergo a process called 'browning' or 'beiging', where it acquires thermogenic ability of BAT (Chu et al., 2017). The browning of WAT and activation of BAT represents a promising therapeutic target to counteract the caloric surplus underlying obesity.

2.3 Adipose tissue and Adipocytes

Adipose tissue is a specialised form of connective tissue mostly composed of adipocytes. They serve as an endocrine organ, synthesising and secreting several hormones and regulating whole-body metabolism (Coelho et al., 2013). Two main types of adipose tissue have been identified: brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is morphologically different from WAT, composed of brown adipocytes with numerous lipid droplets and a distinct brown colour owing to higher mitochondrial content, as shown in Figure 2.2.

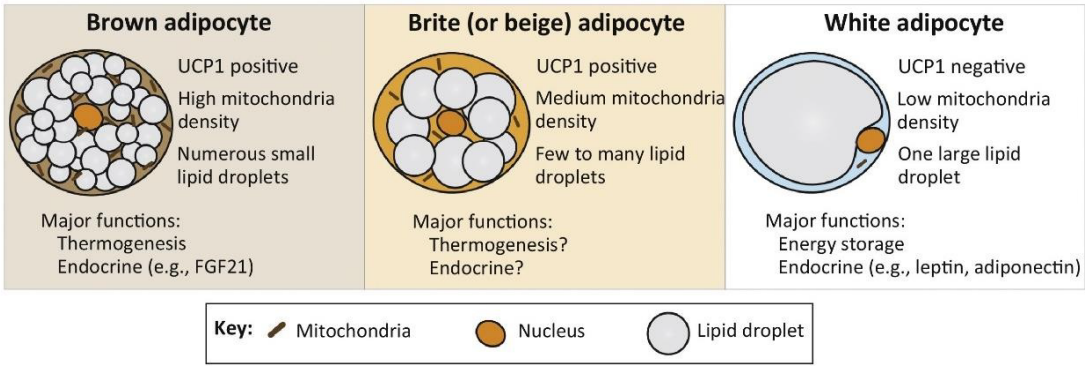


Figure 2.2: Morphological characteristics of brown, beige, and white adipocytes Brown adipocytes have single nuclei, multilocular lipid droplets, and high mitochondrial content. Beige adipocytes have fewer lipid droplets and a lower number of mitochondria. UCP-1 is found in both brown and beige adipocytes. White adipocytes are UCP-1 negative and feature unilocular, large lipid droplets, single nuclei, and few mitochondria.

Adapted from: (Sanchez-Gurmaches et al., 2016)

Indeed, brown adipocytes are restricted to neonatal and hibernating mammals and are believed to be extinct after infancy (Zhang et al., 2018). WAT is the most dominant type of adipose tissue in adults and is mostly made up of white adipocytes.

White adipocytes are distinguished morphologically by a single lipid droplet of triglycerides that occupies 90% of the cell space (Torres et al., 2016). Mitochondria in white adipocytes are thin and elongated, yet few. A third, novel brown-like adipocyte known as beige was discovered in the past decade. Beige adipocytes appear in WAT depots and share many morphological and functional features in common with brown adipocytes. Beige adipocytes are rich in mitochondria and express UCP-1, yet their UCP-1 content is only 10% of that of brown adipocytes (Wu et al., 2013).

Adipose tissue depots are distinguished by their different anatomical locations. WAT is the most abundant type, appearing throughout the human body as subcutaneous (sWAT) and visceral (vWAT) adipose tissue (Vishvanath & Gupta, 2019). The sWAT is subclassified as anterior and posterior in rodents, anatomically corresponding to the upper and lower sWAT in humans. The location of sWAT differs from those of vWAT: sWAT is located inside the abdominal cavity and beneath the skin, typically around the hips (gluteal) and thighs (femoral), and provides insulation from heat or cold, as shown in Figure 2.3.

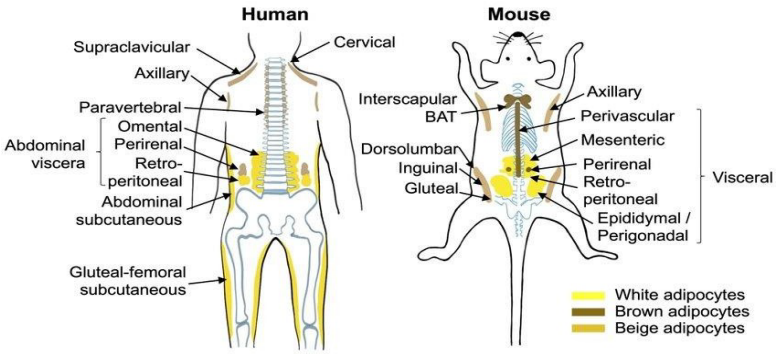


Figure 2.3: Distribution of Adipose Tissues in Humans and Mice
In humans, BAT is found in the supraclavicular, axillary, cervical, paravertebral, and perirenal abdominal regions, while in mice, BAT is distributed into anterior (interscapular and axillary), visceral (perivascular and perirenal), and posterior (dorsolumbar, inguinal, and gluteal). Human WAT is divided into subcutaneous WAT (sWAT), located in abdominal and gluteal-femoral depots, and visceral WAT

(vWAT), found in omental and retroperitoneal. In mice, WAT is mainly in the visceral mesenteric (intestines), perigonadal and retroperitoneal.

Adapted from: (Cheong & Xu., 2021)

Major vWAT or abdominal depots is distributed around internal organs (stomach, liver, intestines, and kidneys) and provides protective padding (Park, 2014). One distinction between human and rodent VAT is that humans have prominent omental WAT (oWAT), whereas rodents have large perigonadal WAT (pgWAT) (Luong et al., 2019). In contrast to WAT, BAT was found in the supraclavicular (upper neck), cervical (neck region beneath the head), axillary (under the shoulder joint), and spinal depots of mice and humans, while interscapular and posterior depots (dorsolumbar, inguinal, and gluteal) are exclusive to rodents (Leitner et al., 2017).

Brown adipocytes differ from white adipocytes since they specialise in thermogenesis, a physiological process in which chemical energy is lost as heat in response to environmental changes such as cold and nutrition (Cypess & Kahn, 2010). Central to this unique thermogenic ability of brown adipocytes is the high density of mitochondria and the expression of mitochondrial UCP-1 proton transfer protein (Cannon & Nedergaard, 2004). Upon stimulation of brown adipocytes, UCP-1 increases the permeability of the mitochondrial membrane, resulting in the uncoupling of the electron transport chain and the dissipation of electrochemical energy as heat rather than adenosine triphosphates (ATPs) (Sudhakar et al., 2020). Although its role in thermogenesis is irrelevant, the WAT has considerably more extensive functional capabilities. White adipocytes secrete various hormones, including insulin and ghrelin, as well as growth factors, enzymes, adipocytokines (leptin), complement factors, and matrix proteins (Coelho et al., 2013). These secretory products regulate adipose mass, food intake, energy expenditure, metabolism, immunity, and blood pressure homeostasis. However, epigenetic changes and changes in the concentrations of

circulating factors such as hormones, adipocytokines, and nutrients in response to environmental changes might induce dysregulation of WAT and set the stage for obesity (Kwok et al., 2020). Cold exposure (Wang et al., 2013), diet (Okla et al., 2017), exercise (Mika et al., 2019), pre-and probiotics (Reynes et al., 2019), plant-based bioactives such as curcumin (Azhar et al., 2016), pharmacological treatments such as PPAR agonists, sirtuin-1 (SIRT-1), β- adrenergic receptor stimulation, thyroid hormone, fibroblast growth factor-21 (Fgf-21), and irisin (Lee et al., 2014), and, even adipokines (Fisher et al., 2012; Kaisanlahti & Glumoff, 2019) have been reported to promote the development of beige adipocytes in mouse models. Nevertheless, further study remains required to ascertain the efficiency of these interventions in stimulating the beiging effect and their clinical implications. Beige adipocytes, like brown adipocytes, are important in maintaining energy balance; hence, they are currently considered a potential therapeutic target to improve metabolic health and thwart excessive weight gain.

2.3.1 Molecular Mechanisms of Adipogenesis

Mesenchymal stem cells (MSCs) are multipotent stem cells that have a high ability to self-renew and differentiate into different types of cells, such as adipocytes (fat cells), osteoblasts (bone cells), chondrocytes (cartilage cells), and myocytes (muscle cells). Focussing on adipogenesis, adipocytes arise from MSCs in two main steps: lineage commitment from MSCs to committed progenitors (preadipocytes) and maturation from progenitors (preadipocytes) to mature phenotypes (adipocytes) (Chen et al., 2016). Preadipocytes undergo multiple cycles of mitosis until they reach the G_1 phase of the cell cycle, where growth arrest occurs. At this stage, the preadipocytes must re-enter the cell cycle, undergo mitotic clonal expansion until they exit the cell

cycle, acquire the metabolic characteristics of mature adipocytes, change their morphology, and accumulate cytoplasmic triglycerides (Otto & Lane, 2005). Mature adipocytes are believed to have lost their ability to divide following the completion of terminal differentiation (Tang & Lane, 2012). 3T3-L1 has been extensively used cell lines in obesity and diabetes research areas owing to their high adipogenic potential. Differentiation of 3T3-L1 was accomplished using a standard 'hormonal differentiation cocktail' containing insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX). Insulin is a hormone that induces adipogenesis and promotes synchronous cell division. Treatment with DEX is essential for inducing differentiation because it activates the transcription factor C/EBP-β. IBMX is a phosphodiesterase inhibitor that elevates intracellular cyclic adenosine monophosphate (cAMP) levels, activating the transcription factor C/EBP-δ (Park, 2014).

MSCs can be committed to either an adipogenic lineage of Myf5 negative (-) cells or a myogenic lineage of Myf5 positive (+) cells, as shown in Figure 2.4.

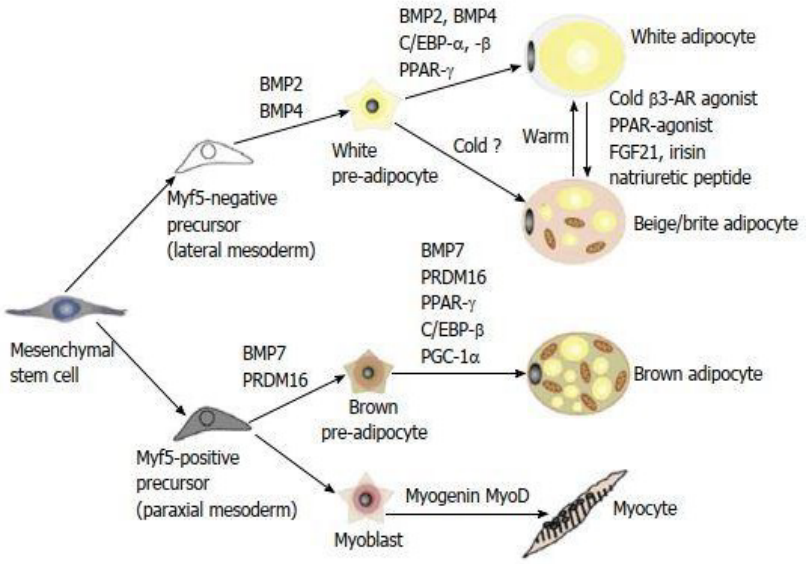


Figure 2.4: Transcriptional control of adipogenesis and myogenesis

Both Myf5+ and Myf- were derived from MSCs. Myf5- is transformed into a white preadipocyte by BMP2 and BMP4, which, when combined with C/EBPs and PPAR-γ, will differentiate into a white, mature adipocyte. White adipocytes may transform into beige ones in response to cold, β-adrenergic, or other stimulants. BMP7 and PRDM16, PPAR-γ, C/EBP-β, and PGC1-α, transform Myf5+ brown preadipocytes into mature brown adipocytes, while MyOD converts Myf5+ to myocytes.

(Adapted from Rossi et al., 2018)

Genetic lineage studies have shown that brown adipocytes and skeletal muscle share the same Myf5+ precursor cells. In contrast, white adipocytes, and inducible beige or brite adipocytes are derived from Myf5- adipogenic lineage cells. Even though adipocytes originate from different lineages, the subsequent adipogenic differentiation shares a common transcriptional cascade dominated by PPAR and C/EBPs (Rosen & MacDougald, 2006; Tang & Lane, 2012).

2.3.2 Differentiation into White Adipocytes

C/EBPs, such as C/EBP-β, members of the leucine zipper family, are important early transcriptional factors in white adipocyte differentiation. C/EBP-β forms a heterodimer with C/EBP-δ to induce the expression of PPAR-γ and C/EBP-α as shown in Figure 2.5.

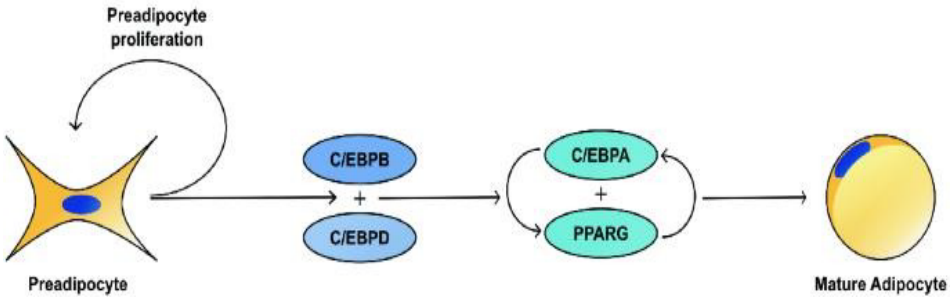


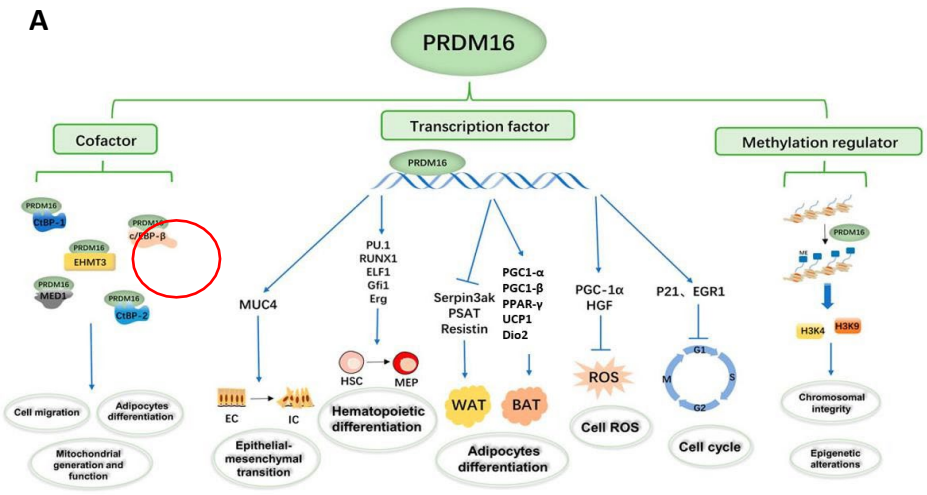
Figure 2.5: Transcriptional control in white adipocyte differentiation.
CEBP/β and CEBP/δ are expressed in early adipogenesis and regulate the expression of master adipogenic transcription factors of PPAR-γ and C/EBP-α in the last stages of adipogenic differentiation, which directly activate adipogenesis.

Adapted from: (Carrageta et al., 2020)

PPAR-γ regulates gene transcription to promote and maintain adipocyte differentiation, whereas C/EBP-α maintains PPAR-γ expression and induces mature white adipocyte development (Kajimura et al., 2009). PPAR-γ and C/EBP-α exert positive feedback on each other upon expression, and this stage is considered crucial in white adipocyte differentiation. Interestingly, the lack of C/EBP-α affects WAT development in mice but does not affect BAT development. Previously, it has been demonstrated that C/EBP-β knockout in mice accumulated minimal adipose tissue, whereas C/EBP-β and C/EBP-δ double knockout mice accumulate significantly less, suggesting their function overlap (Tanaka, 1997). The gene expression level of C/EBP-α and C/EBP-β from each level cascade will be analyzed in the current study to determine the adipogenesis effect of bilimbi fractions, phytol, and squalene in the 3T3-L1 preadipocytes.

2.3.3 Differentiation into Brown Adipocytes

PRDM16 is notably abundant in adipose tissue (Seale et al., 2011; Ohno et al., 2013). It plays several critical roles in gene expression regulation, functioning as a cofactor, a transcription factor, and a methylation regulator as illustrated in Figure 2.6(A) from Jiang et al. (2022).



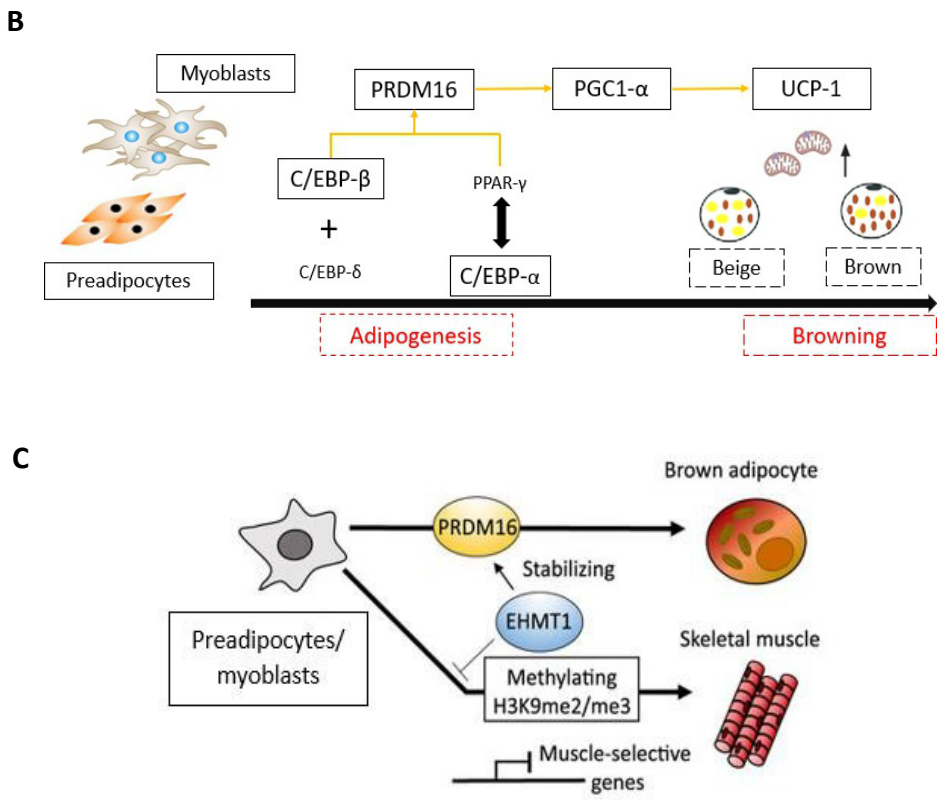


Figure 2.6: PRDM16 in brown adipocyte dtifferentiation.
PRDM16, as a cofactor, transcription factor, and methylation regulator in many biological processes (A). In brown adipocyte differentiation, PRDM16 forms a complex with C/EBP-β, stimulating the brown adipocyte differentiation. As a transcription factor, PRDM16 activates essential, brown-related genes of PGC1-α, PGC1-β, PPARy, and UCP-1 (B). PRDM16 serves as methyl regulator on H3K9, facilitating epigenetic alterations (C). The EHMT1 enzyme suppresses muscle-selective genes, contributing to brown adipocytes differentiation.

Adapted from: (Tanimura et al., 2019; Jiang et al., 2022)

Focusing on brown adipocyte differentiation, PRDM16 forms a transcriptional complex with the active form of C/EBP-β. This complex facilitates the expression of key brown adipocyte-related genes including PGC1-α (Seale et al., 2007), PPAR-y (Seale et al., 2008) and uncoupling protein 1 (UCP-1) (Seale et al., 2007; Iida et al., 2015) as shown in Figure 2.6(B). PGC1-α has been demonstrated to coordinate the expression of thermogenic genes in UCP-1, making it an important regulator of BAT thermogenesis. UCP-1 decouples electron transport from producing chemical energy in ATP (Fedorenko et al., 2012). Changes in the equilibrium of electrons and protons