The Interaction between Prohibitin-1 and Sex Steroid Hormones in Adipogenesis

By

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ABSTRACT

Prohibitin-1 (PHB1), an evolutionarily conserved mitochondrial chaperone, has an important role in adipocyte differentiation. Recently, our laboratory has developed a novel transgenic mouse named “Mito-Ob” by overexpressing PHB1 in adipocytes under the adipocyte protein-2 gene promoter. Mito-Ob mice develop obesity due to upregulation of adipocyte mitochondrial biogenesis in a sex-neutral manner, but only male mice develop impaired glucose homeostasis and insulin resistance. The female Mito-Ob mice are protected from these abnormalities. Sex steroid hormones, estradiol and testosterone, play pivotal roles in body metabolism including sex differences in energy balance and fat distribution. Based on the above information, the present study investigates the relationship between sex steroid hormones and PHB1 on sex differences in Mito-Ob phenotype and in adipocyte functions. Data from both *in vitro* and *in vivo* experiments suggest that PHB1 and sex steroid hormones regulate each other’s function in the growth of adipose tissue and subsequently in the regulation of adipose tissue function in a sex dimorphic manner. Overexpression of PHB1 in adipocytes further amplifies sex differences in adipose tissue function suggesting a role of PHB1 in mediating sex dimorphic effects of sex steroid hormones in adipose tissue biology. Furthermore, results suggest that intrinsic differences in adipose tissue exist between male and female independent of sex steroid hormones, which play a role in sex differences in adipose tissue function and consequently on whole body metabolic homeostasis.

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ABBREVIATIONS

%	percent
ACC	acetyl-CoA carboxylase
Akt OR PKB	Ak transforming (a serine/threonine specific kinase) OR protein kinase B
aP2 OR FABP4	adipocyte protein 2 OR fatty-acid-binding protein 4
AR	androgen receptor
ATGL	adipose triacylglycerol lipase
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
C	degree Celsius
C/EBP	cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT) enhancer-binding protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CM	chylomicron
CO_2	carbon dioxide
DAG	diacylglycerol
ddH_2O	double-distilled water
DMSO	dimethyl sufoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E2	estradiol

ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
EnR	endoplasmic reticulum
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
FBS	fetal bovine serum
FFA	free fatty acid
g	gram
GLUT	glucose transporter
GTP/GDP	guanosine triphosphate/diphosphate
GTPase	guanosine triphosphatase
HCl	hydrochloric acid
HRP	horseradish peroxidase
HSL	hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IL-6	interleukin 6
IRS	insulin receptor substrate
kg	kilogram
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MGL	monoglyceride lipase
min	minute(s)
mRNA	messenger ribonucleic acid

mtDNA	mitochondrial deoxyribonucleic acid
NEFA	non-esterified fatty acid
OD	optical density
PBS	phosphate buffered saline
PDH	pyruvate dehydrogenase
PHB1	prohibitin-1
PHB2 OR REA	prohibitin-2 OR repressor of estrogen activity
PI3K	phosphoinositide 3 kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PPAR-γ	peroxisome proliferator-activated receptor-γ
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
RT	room temperature
SAT	subcutaneous adipose tissue
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Shc	sarcoma homologous/collagen protein
siRNA	small interfering RNA
SOS	Son of Sevenless
T2DM	Type 2 Diabetes Mellitus
TAG	triacylglycerol
TBS	tris-buffered saline

TBS-T	tris-buffered saline, 0.1% Tween 20
TEMED	tetramethylethylenediamine
TNF-α	tumor necrosis factor alpha
TWEEN-20	polyoxyethylene 20 sorbitan monolaurate
µl	microlitre
VAT	visceral adipose tissue
VLDL	very low density lipoprotein
WAT	white adipose tissue
WHR	waist-to-hip ratio
WT	wild-type

I. INTRODUCTION

Research Rationale

Sex differences have profound effects on the susceptibility and pathophysiology of obesity-related diseases and their complications [1]. One of the sex-biasing factors is the effect of gonadal steroid hormones. Estradiol and testosterone play pivotal roles in metabolism including energy balance and fat distribution in the body. Obese men exhibit a progressive decline of testosterone level and increased body weight [2]. In women, epidemiological data suggests that obesity and metabolic dysregulation are more prevalent after menopause, and estrogen is used as a therapeutic agent to reverse these complications [3-5]. At the molecular level, sex steroid hormones regulate gene expression through binding to estrogen and androgen specific receptors expressed in adipose tissues. Research has found that testosterone treatment in murine 3T3-L1 cells inhibits lipoprotein lipase (LPL) activity and lipid uptake [6]. Estradiol acts to decrease the expression of adipogenic gene peroxisome proliferator-activated receptor-γ (PPARγ) [7-9]. With the overwhelming evidence of sex steroids effect on adipose tissue, the underlying mechanisms involved in the actions of sex steroid hormones in regulating adipogenesis and lipid metabolism are still unknown; key factors that contribute to sex differences in adipose tissue biology and pathobiology remain elusive.

Recent studies have highlighted the potential relevance of mitochondria in the cellular physiology of adipocytes and its impact on systemic metabolic regulation [10,11]. The adipocyte interprets nutritional and hormonal cues in its microenvironment, and then coordinates its mitochondrial response either to oxidize incoming fatty acid and carbohydrate, or to store them in the form of triacylglycerol (TAG) until signal for

release. Our laboratory discovered that prohibitin-1 (PHB1), which is known to function in mitochondrial biology, has an important role in adipocyte differentiation [12]. To explore the role of PHB1 in adipose tissue biology at the systemic level, we have developed a novel transgenic obese mouse model by overexpressing PHB1 in adipocytes under the adipocyte protein-2 (aP2) gene promoter [13]. We named these mice “Mito-Ob” because they display increased mitochondrial biogenesis in adipocytes and develop obesity independent of food intake [13]. Mito-Ob mice develop obesity in a sex-neutral manner, but only male mice develop impaired glucose homeostasis and insulin resistance, whereas female mice maintain normal glucose homeostasis and insulin sensitivity [13]. Furthermore, serum TAG and cholesterol levels are significantly lower in female Mito-Ob mice, whereas free fatty acid levels are significantly higher in male Mito-Ob mice in comparison with respective wild-type controls [13]. In addition, adiponectin levels were significantly higher in female Mito-Ob mice, while leptin levels were higher in male Mito-Ob mice [13]. Collectively, these data suggest a sex dimorphic role of PHB1 in adipocytes or adipose tissue biology. Of note, Mito-Ob mice start to gain weight during puberty indicating a relationship between gonadal sex steroids and PHB1 in Mito-Ob phenotype. Emerging evidence from other laboratories also supported the role of PHB1 in modulating sex hormone action in a variety of tissues as well as PHB1 as a downstream target protein for sex steroids [14-16] . Based on these findings, the present study investigates the relationship between sex steroids and PHB1 on Mito-Ob phenotype and sex differences in adipocyte functions. The first objective is to study the effect of gonadectomy on metabolic phenotype in Mito-Ob mice. The second objective is to study

the effect of sex steroid hormones on primary preadipocytes differentiation isolated from Mito-Ob mice.

Literature Review

1. White Adipose Tissue

1.1 Basic Physiology

White adipose tissue (WAT) has essential roles in the body. The primary function is to store excess nutrients in the form of triacylglycerol (TAG) and cholesteryl esters [17,18]. Compared to the other macromolecules, fatty acids release the most ATP per unit mass (9kcal/g), making it an excellent and efficient form of fuel storage. Adipocyte is the smallest unit of adipose tissue hierarchy with the remaining population made up of preadipocytes, fibroblasts, endothelial cells, and immune cells. Adipocyte is mono-vacuolar, and contains a large lipid droplet surrounded by a layer of cytoplasm, where the nucleus is flattened and located on the periphery. The lipid droplet can range from 20 to 200 micrometers in diameter, and they occupy about 95% of the cell volume, and thereby determine the cell size [19]. Within a specified anatomical site, all adipocytes achieve nearly uniform size. If weight is gained as an adult, adipocytes undergo hypertrophy about threefold in diameter before hyperplasia [20]. Researchers have found that adipocyte hypertrophy is strongly diet-induced and accompanied by changes in cellular function, whereas hyperplasia is a complex interaction dependent on both nutrition and individual's genetics [20,21]. Matured adipocytes stay in a dynamic state: they accumulate fat droplets when the energy intake is higher than expenditure and undergo mobilization by breaking down TAGs when the energy expenditure exceeds intake [17,22].

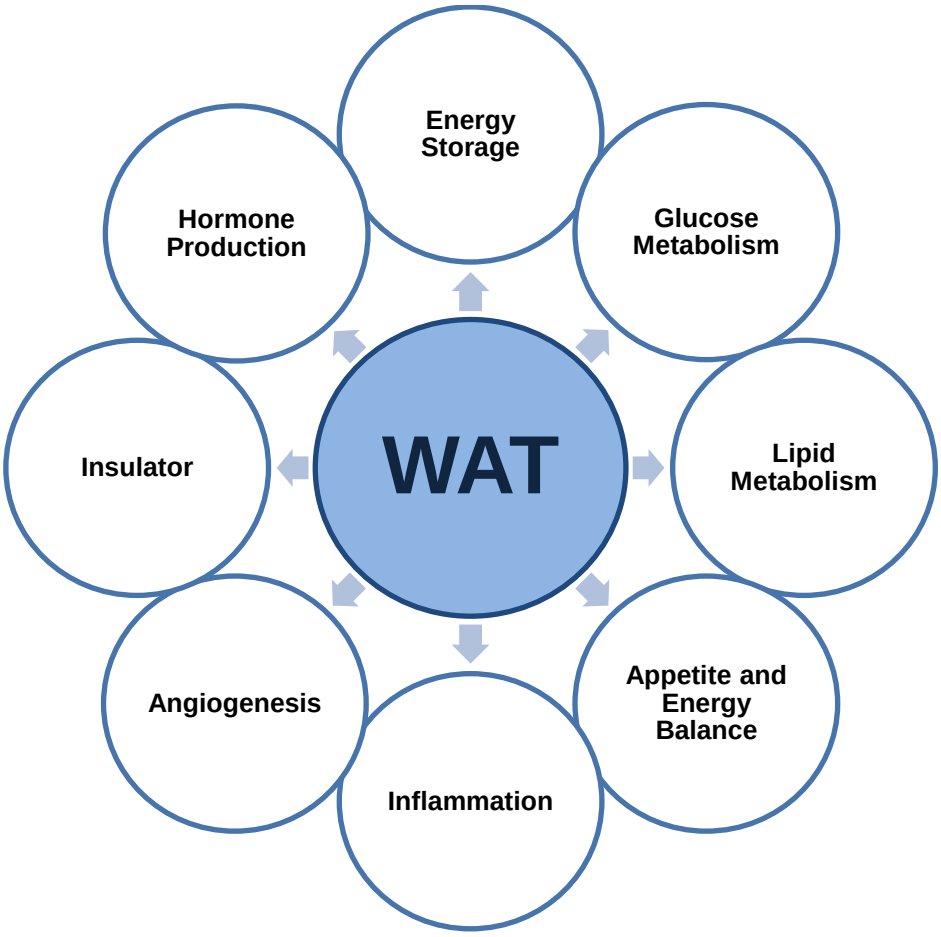

Figure 1. White Adipose Tissue (WAT) is Involved in A Variety of Physiological and Metabolic Processes. Other than the traditional roles of energy storage and as an insulator, WAT communicates systemically with other tissue types through secretion of various adipokines or metabolites and contributes to the regulation of whole body energy homeostasis. These interactions may be autocrine, paracrine, or endocrine. The figure is adapted and modified from Ref. 23.

In addition to fat storage, WAT performs a wide range of functions (Fig. 1). It acts as an insulator, protects against mechanical force, and plays a role in inflammatory process and energy balance [18,24]. More importantly, WAT is identified as the largest endocrine organ in the body performing in conjunction autocrine and paracrine functions. Its importance in these functions is brought forth by the adverse metabolic

consequences of adipose tissue both in excess and in deficiency. The discovery of anorexigenic hormone leptin was the first to bring attention to adipose tissue as an endocrine organ [25]. Leptin is encoded by the *ob* gene and acts on central receptors in the brain to regulate food intake and fat storage [25]. Its function was identified in the mutant phenotype of *ob/ob* mice showing hyperphagia and impaired satiety. Leptin secretion is proportional to total percent body fat, and remains higher in females compared with males [25]. Level of leptin rises several hours after food intake, which functions to inhibit appetite and food consumption and promote insulin sensitization [26]. However, there is a tendency to develop leptin resistance through chronic overfeeding as demonstrated by high leptin concentration in individuals with obesity [27]. After the discovery of leptin, a diversity of adipose-secreted molecules termed adipokines have been identified including adiponectin, resistin, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) among others with wide range of metabolic control [28]. Adiponectin, synthesized almost exclusively by WAT, is a key regulator of glucose and lipid homeostasis with anti-inflammatory property [29]. Unlike leptin, its serum level is inversely proportional to body fat percentage. The plasma concentration of adiponectin is much lower in individuals with obesity than their normal weight counterparts, and weight loss significantly elevates plasma adiponectin level [30]. Adiponectin functions as an insulin-sensitizing agent by reducing hepatic glucose production and enhancing insulin action in the liver [31]. As a result, it is thought that adiponectin deficiency is responsible for the metabolic consequences caused by visceral adiposity. Recent research has acknowledged that obesity is tightly associated with systemic chronic inflammation, and resident macrophages is a major player in pro-inflammatory TNF-α and IL-6 secretions

from expanded WAT [32]. TNF-α in circulation interferes with insulin-mediated glucose uptake in obese mouse models through repression of glucose transporter type 4 (GLUT4) gene transcription and decreased GLUT4 mRNA stability [33]. Notably, inflammatory gene expression and macrophage infiltration in early stages of obesity is selectively induced in adipose tissues, but not in other metabolic organs including liver and skeletal muscle [34]. It appears that WAT plays a role in the acute high-fat diet induced insulin resistance and in initiating inflammation in obesity.

On top of being an active contributor to the endocrine system, WAT is also heavily influenced by circulating hormones. Adipocytes express receptors and enzymes involved in the metabolic pathway of insulin, glucagon, growth hormone, catecholamines, glucocorticoids and sex hormones [35]. Through hormones and adipokines, WAT is able to communicate systemically with other cell types and thus contribute to the regulation of whole body energy homeostasis. Taken together, the intensity and complexity of these signal networks are highly regulated, differ in each fat depots, and are dramatically altered by various disease states.

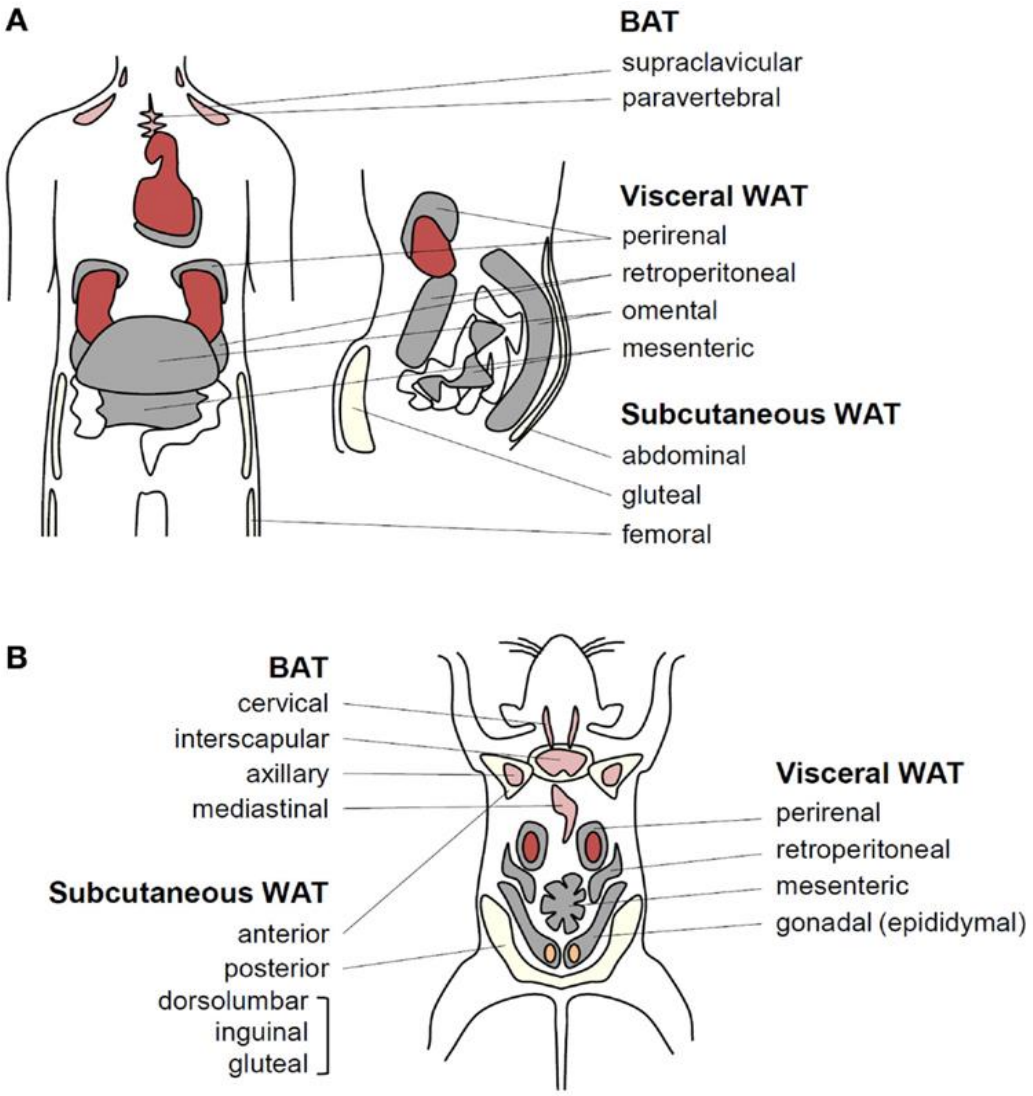

Figure 2. Anatomy of Major Fat Depots in Humans and Rodents. Visceral and subcutaneous white adipose tissue (WAT) depots and brown adipose tissue depots in humans (A) and rodents (B) are shown. In humans, subcutaneous WAT is divided into 3 categories: abdominal (superficial and deep), gluteal and femoral; visceral WAT is divided into 4 categories: intraperitoneal (mesenteric and omental), perirenal, retroperitoneal and pericardial. Rodents have different adipose tissue distributions, but posterior WAT and gonadal WAT are used as analogous models to study human subcutaneous and visceral depots, respectively. While brown adipose tissue depots are shown, they are not discussed in the thesis. The figure is adapted and modified from Ref. 36.

1.2 Subcutaneous vs. Visceral White Adipose Depots

The regional distribution of WAT has more effect than total adipose mass for the risk of developing obesity-related complications. Different anatomical locations of WAT exhibit different functional characteristics in terms of composition, blood supply, innervation, adipokines secretions and metabolic consequences [19,37-39]. There are two major white adipose depots: subcutaneous and visceral (Fig. 2). Subcutaneous adipose tissue (SAT) is found beneath the skin, and constitutes more than 80% of fat in the body [19]. Its largest sites of fat storage are located in the gluteal and femoral regions of lower body in humans and posterior inguinal region in rodents [40]. SAT are also found in the abdominal area, and are further sub-categorized into superficial and deep layers. Different locations of SAT are distinctively correlated with metabolic complications of obesity. Specifically, the fumerogluteal and superficial layer of abdominal SAT are thought to have a protective role in the body as they are more lipolytically inert and acts as a sink for excess dietary fat [41,42]. Although SAT receives relatively less blood flow and fatty acid flux, they are found to be the predominant contributor of two important adipokines, leptin and adiponectin in healthy individuals [43,44]. High secretions of the two hormones influence insulin sensitivity in a positive manner, which may explain the health-benefits of a pear-shaped body figure [45]. Another interesting distinction of SAT is the observation of its capacity to acquire thermogenic properties and transform into brown adipose tissue, which tilt the energy balance from storage to expenditure [46,47]. Transplantation of SAT into visceral compartments exhibited decreased body weight, total fat mass, and improved glucose metabolism in rodent models [48]. Clinical studies have also discovered obese people who gained weight preferentially in the femoral SAT

depot is associated with decreased risk of impaired glucose metabolism and dyslipidemia, independent of abdominal fat [42,49]. This protective mechanism however is only apparent in obese individuals when SAT provides an alternative path in sequestering excess fatty acids [50]. Yet, not all SAT are perceived as protective; several lines of evidence have suggested the danger of central adiposity may be associated with deep abdominal SAT in addition to VAT [51,52]. Deep abdominal SAT is observed to be more functionally similar to VAT with higher lipolytic activity and proinflammatory tendency [53,54].

Visceral adipose tissue (VAT) is found deep in the peritoneal cavity surrounding the internal organs with the majority located beneath the abdominal muscles [55]. It can be further divided into 4 categories in humans: intraperitoneal (mesenteric and omental), perirenal, retroperitoneal and pericardial [37] (Fig. 2). Rodents have slightly different VAT distributions, but given the gonadal depots represent the largest and most readily accessible, they are frequently used as the analogous model to study human intraperitoneal depot [40]. In humans, mesenteric fat is found wrapped around the intestine in a web-form, while the largest depot, omental, expands into the ventral abdomen from the stomach and spleen. Together, they are the major contributors to central obesity, and are well-documented to be associated with the risk of developing metabolic syndromes [56]. One of the preferred anthropometric indexes for Metabolic Syndrome is the measurement of waist circumference as a reflection of VAT amount and metabolic variables [38]. This factor holds true even for persons of overall normal body weight. While the beneficial effect of intra-abdominal VAT removal in humans is up for debate, a number of studies have demonstrated improvement in insulin sensitivity

upon VAT lipectomy in rodents [19]. Theories have been put forth to explain the depot specific cause of metabolic morbidity. For one, adipokines released from VAT drain directly to the liver via the hepatic portal system, giving them first-hand access as compared to SAT adipokines released into the systemic circulation [28]. On top of that, there is a distinct adipokine secretion profile due to depot-specific cell population. The abundance of milky spots and lymph nodes in VAT allow more macrophages and lymphocytes to accumulate, and participate in local immune responses [57]. Resident macrophages within VAT produce more proinflammatory cytokines, which contribute to low systemic inflammation responsible for insulin resistance [58]. Furthermore, obese mice exhibit markedly increased M1-proinflammatory macrophages that form crown-like structures around necrotic adipocytes, and further contribute to systemic inflammation and insulin resistance [59,60]. As compared to SAT, adipocytes in VAT are metabolically more active shown by experiments in rodents revealing higher mitochondrial content along with higher enzymatic activities and mtDNA, fueling more cellular metabolic activities [61]. In addition, VAT expresses higher density of β-adrenergic, glucocorticoid, and androgen receptors [38,62]. Specifically, in obesity, high expression levels of β3-adrenergic receptors make adipocytes more sensitive to catecholamine-induced lipolysis [63]. At the same time, VAT becomes less sensitive to the antilipolytic effect of insulin due to lower insulin receptor affinity, contributing in whole to elevating plasma non-esterified fatty acid (NEFA) levels [64]. Another characteristic of VAT is its lower abundance of adipogenic progenitors and proliferation ability evidently shown in primary preadipocyte cultures. Several studies have found that VAT grows predominantly through hypertrophy, while SAT has more adipogenic progenitors and undergoes

hyperplasia [65]. Hyperplasia is a less deleterious mechanism of fat expansion, producing more functional and organized adipocytes, whereas adipocyte hypertrophy is prone to inflammation, apoptosis, fibrosis and the release of NEFAs [66]. Altogether, VAT carries an overall greater risk for metabolic complications due to multiple effects that alter insulin sensitivity. The functional heterogeneity among the various adipose tissue depots prompts future studies to examine them individually.

1.3 Adipogenesis

The mass of WAT is reflected by the number and volume of adipocytes. While the volume of adipocyte varies by the amount of lipid stored, the number increases through new adipocyte differentiation from preadipocytes. In adipose tissue, 1-5% of adipocytes turn over each day, making it a highly dynamic tissue type [67]. Adipocytes are derived from pluripotent mesenchymal stem cells, and require key transcription factors along the way to guide maturation. Once committed, preadipocytes undergo terminal differentiation and become adipogenic through an up-regulation and interplay of numerous transcription factors including the CCAAT-enhancer-binding protein (C/EBP) gene families and PPARγ [68] (Fig. 3). Expression of C/EBPβ and C/EBPδ from the C/EBP family rises rapidly during the early stage of insulin-induced differentiation. In 3T3-L1 cells, both transcription factors reach a maximal level during the first 2 days of differentiation and decline sharply before the accumulation of two major downstream adipogenic genes: C/EBPα and PPARγ [69]. PPARγ activates the expression of C/EBPα that has a positive feedback action on PPARγ itself. Examination of PPARγ and C/EBPα co-localization on genes up-regulated in differentiation revealed 60%

similarities [70]. Together, they cooperatively orchestrate the terminal differentiation of preadipocytes and expression of downstream adipogenic factors involved in insulin sensitivity, adipokine secretion, glucose and lipid metabolisms [70,71]. Among these adipogenic factors are glucose transporter 4 (GLUT4), fatty-acid-binding protein 4 (FABP4, also known as adipocyte protein 2, aP2), lipoprotein lipase (LPL), acyl-CoA binding protein, and secreted adipokines such as adiponectin and leptin [72]. As the differentiation process proceeds, lipid droplets start to accumulate, and often coalesce into one or a few larger droplets visible through Oil Red O staining.

Experiments have confirmed the deficiency of any of the transcription factors mentioned above would cause defective preadipocyte development and energy metabolism [73-75]. C/EBPβ and -δ are already expressed in low levels in preadipocytes but are further induced and posttranslationally activated by adipogenic cocktail [76]. Mice lacking both C/EBPβ and -δ have impaired adipogenesis and significantly reduced epididymal fat pad despite normal expression of C/EBPα and PPARγ, suggesting the importance of C/EBPβ and -δ in complete adipocyte differentiation [73]. PPARγ remains the central player in adipocyte differentiation. It is sufficient to induce adipogenesis in C/EBPα deficient mouse embryonic fibroblasts *in vitro*, but not vice versa [77]. Diabetes treatment employs thiazolidinedione drugs, which act as agonists of PPARγ to promote storage of fatty acids in adipocytes and thereby decrease the amount present in circulation [78]. Following that, cells become more dependent on the oxidation of glucose, which effectively increase systemic glucose utilization and decrease glucose production.

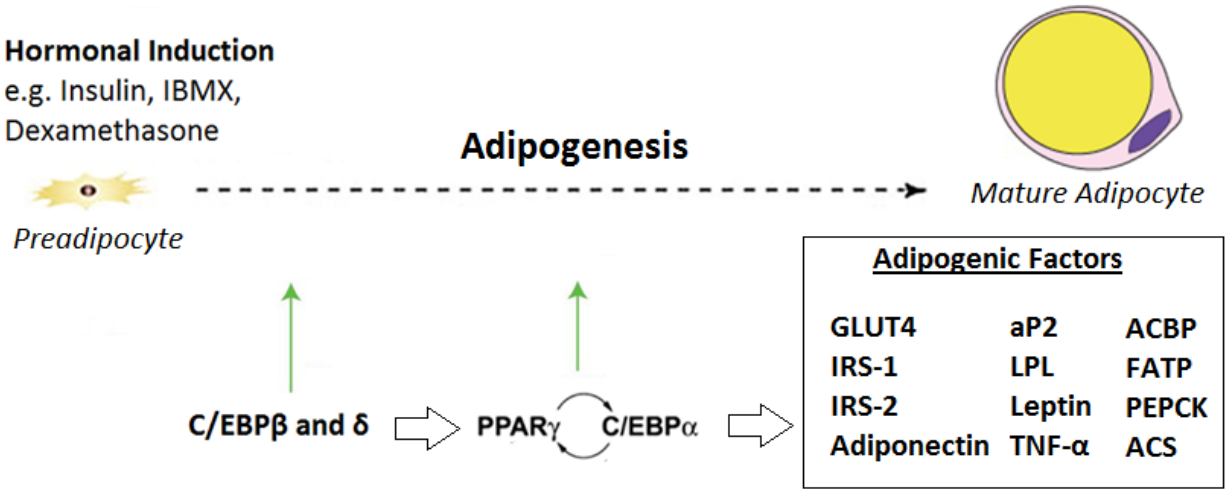

Figure 3. Adipogenesis. Adipogenesis refers to the differentiation of preadipocytes into mature adipocytes that are capable of making and storing fat. Preadipocytes are differentiated into mature adipocytes under the control of transcription factors: CCAAT-enhancer-binding protein (C/EBP) α, β, δ and peroxisome proliferator-activated receptor (PPAR) γ. *In vitro*, preadipocytes undergo adipogenesis upon stimulation from a cocktail of inducers: insulin, methylisobutylxanthine (IBMX) and dexamethasone. Early transcription factors C/EBPβ and -δ rise and upregulate two major adipogenic players: C/EBPα and PPARγ. The expression of these two transcription factors orchestrates the terminal differentiation and maintenance of adipocyte phenotype. Mature adipocytes express a range of adipogenic factors such as glucose transporter (GLUT4), insulin receptor substrates (IRSs), fatty-acid-binding protein (FABP4, also known as adipocyte protein 2, aP2), lipoprotein lipase (LPL), acyl-CoA binding protein (ACBP), fatty acid transport protein (FATP), phosphoenolpyruvate carboxykinase (PEPCK), acyl-CoA synthetase (ACS), tumor necrosis factor alpha (TNF-α), and secreted adipokines such as adiponectin and leptin. The figure is adapted and modified from Refs. 79 and 80.

Cellular model systems like murine 3T3-L1 are fibroblast-like preadipocytes that are committed to differentiating into adipocytes, which are used extensively to study the molecular pathways of adipogenesis *in vitro*. These studies commonly utilize 3 pharmacological agents: insulin, methylisobutylxanthine (IBMX) and dexamethasone to induce the adipogenic event [81]. Insulin, the most potent inducer of the three, controls adipogenesis through triggering the expression of major transcription factors mainly

through the PI3K/Akt pathway [81]. Studies have demonstrated the inhibition of PI3K blocked insulin-induced differentiation of 3T3-L1 preadipocytes [82]. Furthermore, insulin may also engage other branches downstream of its signaling pathway to induce adipogenesis, suggesting multilevel control over the event [83,84]. Both dexamethasone and IBMX induce C/EBPβ and C/EBPδ expressions and augment PPARγ activation [81]. IBMX, a phosphodiesterase inhibitor, acts to raise the intracellular cAMP and protein kinase A level, which is required for PPARγ activation [83]. Another inducer, indomethacin, is sometimes added to the induction cocktail, which also demonstrated strong evidence in increasing expression of C/EBPβ and PPARγ2 [85]. Overall, the abundance of pathways, transcription factors and modifications that regulate adipogenesis serves to illustrate the complexity of such programme [79]. It is important, therefore, for ongoing research to unravel the pathogenesis of obesity characterized by an increase in WAT by examining the process of adipogenesis and vital players involved.

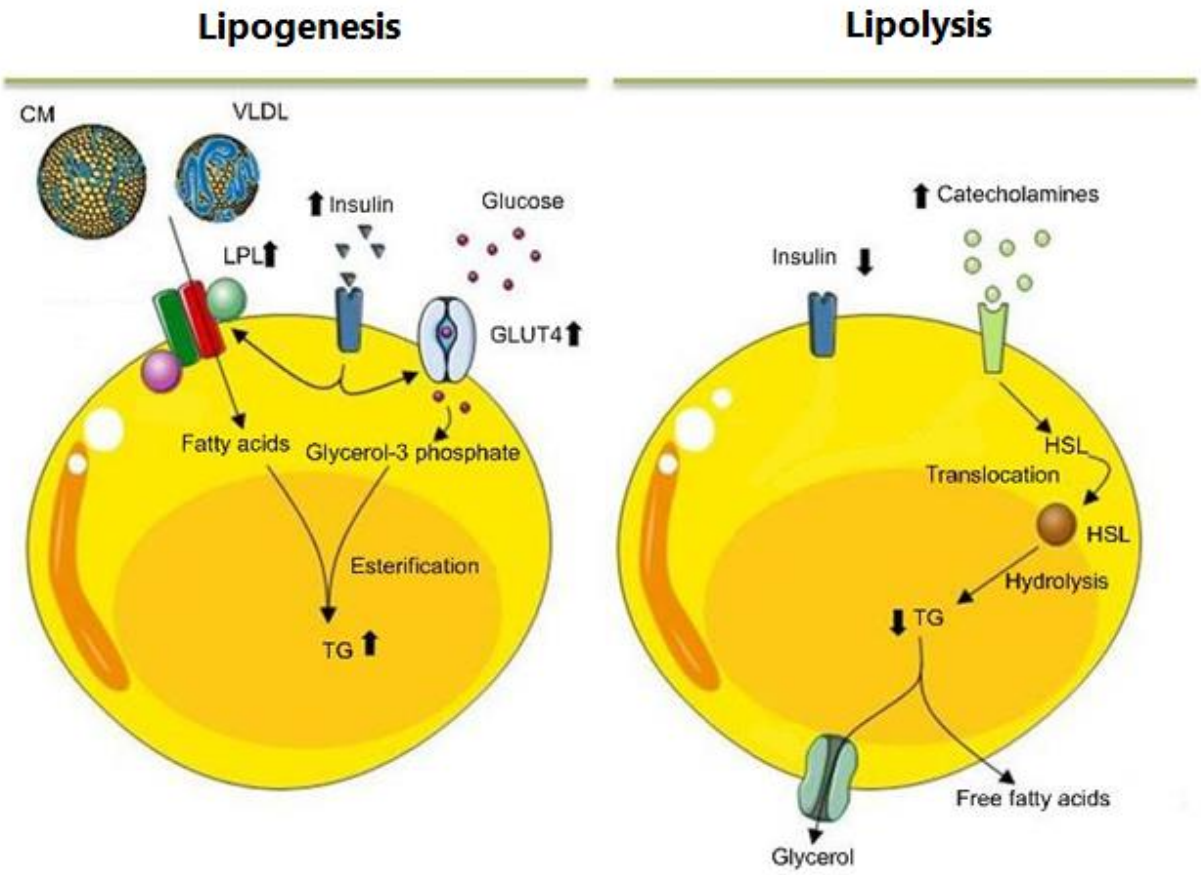

Figure 4. Lipogenesis and Lipolysis. Lipogenesis begins through the uptake of fatty acids (FAs) from circulating chylomicrons (CMs) and very low density lipoproteins (VLDL). Once inside the adipocyte, three FAs are esterified with glycerol-3 phosphate and synthesized into triacyglycerol (TAG) that are stored in the lipid droplets. Insulin stimulates the progress by upregulation of lipoprotein lipase (LPL) for FA release and GLUT4 transporters for the uptake and metabolism of glucose to glycerol-3 phosphate. On the other hand, lipolysis is activated when catabolic hormones such as catecholamines and glucagon are secreted in response to a low blood concentration of glucose. Upon stimulation, hormone-sensitive lipase (HSL) migrates from the cytosol to the surface of the lipid vacuole, where it hydrolyzes triglycerides to non-esterified fatty acids (NEFAs) and glycerol. They are then released into the general circulation where NEFAs bind to plasma albumin for transport and undergo beta-oxidation in target organs and glycerols are to be used by the liver as a source of carbon. The figure is adapted and modified from Ref. 86.

1.4 Lipogenesis

Lipogenesis is defined as the synthesis of TAG from three fatty acids and glycerol for the purpose of energy storage. Lipogenesis starts with fatty acids synthesis, which occurs mainly in the liver and adipose tissue, but is present in the brain and kidney [87]. Fatty acids can be made through endogenous (*de novo*) pathway or supplied exogenously [88]. In *de novo* biogenesis of fatty acids, excess sugar and amino acids are converted to TAGs by the liver. First, the glucose catabolism end-product, acetyl-CoA, is converted to malonyl-CoA in the cytoplasm of the cell catalyzed by acetyl-CoA carboxylase (ACC). From there, malonyl-CoA and acetyl-CoA are combined in a series of recurring steps with the help of fatty acid synthase (FAS) into the 16-carbon palmitic acid. Elongase and desaturase enzymes aid in the process of modifying palmitic acid to other variations of lipid species. In the end, three fatty acids are esterified with glycerol to form TAG that are packaged in very low density lipoproteins (VLDLs) and secreted to the circulation. VLDLs together with exogenous dietary fat in the form of chylomicrons travel to the predominant lipid storage site in the body: the adipose tissue (Fig. 4). Lipoprotein lipase (LPL) on the surface of adipose tissue recognizes apoproteins (B100 and B48, respectively) expressed by VLDLs and chylomicrons, and hydrolyzes the enclosed TAGs into free fatty acids (FFAs) and glycerol to enter the cells. Once inside the adipocytes, TAGs are then resynthesized and stored in lipid droplets.

1.4.1 Regulation of Lipogenesis

Lipogenesis is upregulated by increased insulin signaling and inhibited by growth hormone and leptin. Insulin is involved in major steps of lipogenesis such as glucose uptake, covalent modifications of key enzymes, and regulation of lipogenic gene

expressions [22]. Insulin stimulates the expression and translocation of GLUT4 transporters to the cell surface, aiding the uptake of glucose. It also stimulates the activation of pyruvate dehydrogenase (PDH) and ACC, which leads to respective increase of acetyl-CoA and malonyl-CoA for *de novo* synthesis of fatty acids [89,90]. In addition, insulin stimulates the production and secretion of LPL, thus increase the uptake of circulating lipids [91]. Lipogenesis is also responsive to a high carbohydrate diet, while inhibited by a high lipid diet and by fasting [22]. Nutrient sensing is translated into signals to change the expression levels of lipogenic genes. For example, glucose is a stimulant of insulin release, which indirectly helps to upregulate expression of lipogenic genes and promotes fatty acid synthesis. On the other hand, polyunsaturated fat decreases lipogenesis by suppressing gene expression in liver, including fatty acid synthase, spot14 and stearoyl-CoA desaturase [92]. Surprisingly, research has found that lipid species from *de novo* synthesis and those from the diet may possess distinctive bioactivities, and that an increase in *de novo* lipogenesis and lipokine secretion in mice prevent the deleterious systemic effects of dietary lipid exposure on adipose tissue [93]. This finding adds an additional layer of complexity in lipid signaling and regulation.

1.5 Lipolysis

Lipolysis, the reversal of lipogenesis, involves the hydrolysis of TAGs into NEFAs and glycerol, and marks the beginning of energy mobilization from lipid storage to meet the demands of other organs during energy deprivation. It is a biological process found in all cells of the body and not restricted to adipose tissue. TAG is hydrolyzed sequentially by three enzymes: adipose TAG lipase (ATGL), hormone sensitive lipase

(HSL) and monoglyceride lipase (MGL) [17]. ATGL performs the initial rate-limiting step of TAG breakdown producing diacylglycerol (DAG) and one NEFA. Then, DAG is cleaved into monoacylglycerol (MAG) and then glycerol by HSL and MGL respectively, releasing two more NEFAs [94] (Fig. 4). Once effluxed out of adipocytes, NEFAs bind to plasma albumin for transport and undergo beta-oxidation in target organs, and glycerol is used by the liver as precursor for gluconeogenesis [23]. Adipocytes primarily secrete NEFAs as a source of systemic energy substrate, while non-adipose cells secrete NEFAs for autonomous energy production or lipid synthesis [17]. Since the TAG storage capacity of non-adipose tissues is relatively minor, excessive ectopic lipid deposition in non-adipose tissues can lead to lipotoxicity and is associated with metabolic diseases [17].

1.5.1 Regulation of Lipolysis

Lipolysis is triggered by energy mobilizing hormones including glucagon and catecholamines. Glucagon binds its receptor and upregulate G-protein coupled cAMP level and subsequent PKA activation independent of the antagonistic effect of insulin [95]. Norepinephrine acts through beta-adrenergic receptors on adipocytes, and activates PKA similarly through elevating cAMP level [94]. PKA is responsible for an increased activity of ATGL and HSL, which increases lipolysis. Interestingly, in obese and insulin resistant people, the lipolytic effect of catecholamines is reduced in SAT but increased in VAT, adding to the detrimental excess of central adiposity [96]. Negative regulation of lipolysis primarily occurs during the postprandial state to allow storage of excess fuels. Not surprisingly, insulin is the main anti-lipolytic factor. Its PI3K/Akt signaling pathway activates phosphodiesterase 3B through PKB, which degrades cAMP in adipocytes leading to downregulation of HSL [97]. As well, insulin signaling leads to the activation of

protein phosphatase-1, which also dephosphorylates and deactivates HSL, causing a fall in the rate of lipolysis. Chronic exposure to abnormal nutritional statuses including obesity and starvation also allow metabolic adaptations which include modifications in lipolysis [94]. It was suspected that an increase in basal rate of lipolysis induces insulin resistance, as well as an impaired response to stimulated lipolysis [98].

2. Obesity

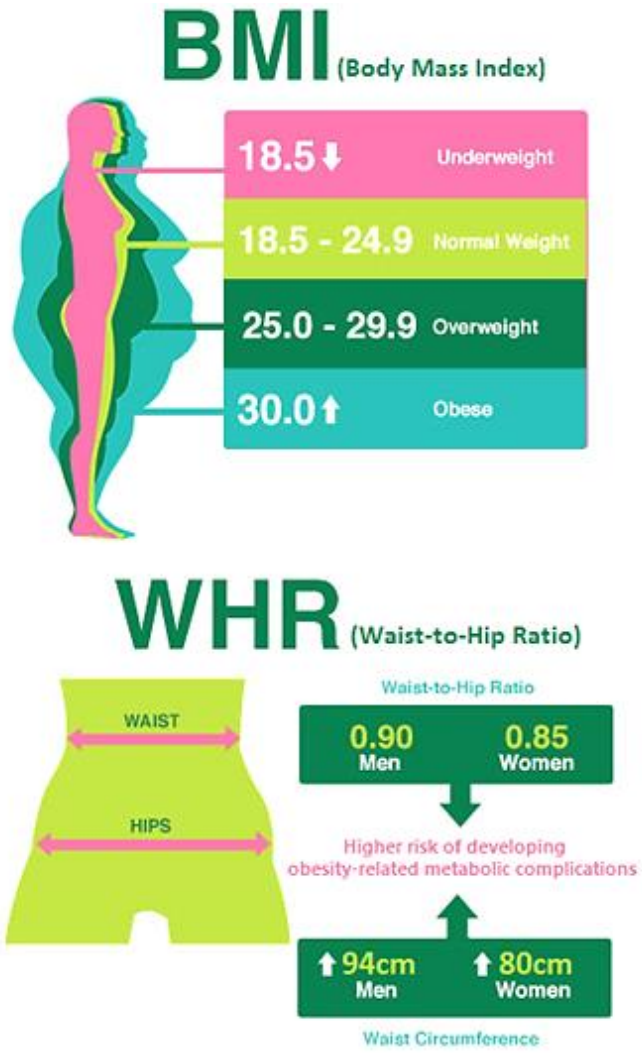

Figure 5. Classification of Obesity in Adults: Body Mass Index (BMI) and Waist-to-Hip Ratio (WHR) in Caucasians. BMI is defined as the body mass divided by the square of the body height, and expressed in units of kg/m^2. Individuals with BMI between 25 and 29.9 are considered overweight, and BMI higher than 30 are obese. WHR is a measurement of central adiposity. The waist circumference is measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest. The hip circumference is measured around the widest portion of the buttock. WHR above 0.90 for males and above 0.85 for females, as well as waist circumference of 94 cm for males and 80 cm for females are at increased risk of metabolic complications. Ethnic differences exist in these anthropometric indexes. Compared to Caucasians, Asian populations have higher percentage of body fat for a given waist circumference, and the opposite is observed in African and Pacific Islanders populations. Therefore, adjusted cut-offs are employed in reference to the European reference population guideline. The figure is adapted and modified from Refs. 99 and 100.

2.1 General

Obesity originated from a paradox between an evolutionary advantage to survive extended periods of famine and a morbid physiological process that threatens human health in the modern society. In 2013, obesity was officially classified as a disease by the American Medical Association [101]. It is defined as an excessive storage of body fat. This definition describes adults with a body mass index (BMI) of more than 30kg/m [102] (Fig.5), and child and adolescent above the 95th percentile in the same sex and age group [103]. While BMI is used as a standard diagnostic criterion, it does not directly measure fat mass, which is the hallmark of metabolic complications. Waist circumference, waist to hip ratio and skinfold thickness are methods used to determine central adiposity and body fat percentage. Obesity has been growing with alarming rate worldwide. Data released from the National Centre of Health Statistics in 2014 revealed that 68% of United States adults are overweight and half of them are obese [102]. In Manitoba Canada, 24.5% of the provincial population is overweight, which is 5% above the national average [104]. On the global scale, it is estimated that 1.5 billion adults are overweight or obese in 2015, and this number is expected to double in less than 15 years [105].

Obesity is a heterogeneous metabolic disorder, which results from a combination of excessive calorie intake, sedentary lifestyle, endocrine disruption, medication, and genetic susceptibility [106]. It occurs as a consequence of imbalance in energy metabolism. In today's sedentary environment, energy intake exceeds energy expenditure due to overconsumption of protein, carbohydrates, fats and alcohol but low levels of physical activity [106,107]. The human body can tolerate weight gain to a certain

extent owing to our evolutionary need to conserve energy. Macromolecules are catabolized and interconverted with each other in the liver, which is the major metabolizing centre for nutrients. Within each hepatocyte, glucose is first stored as glycogen through glycogenesis. Once the liver reaches full capacity of glycogen, glucose is converted into fatty acids and subsequently TAGs. TAGs are normally packaged into VLDLs for circulation to the periphery for long-term storage. However, excessive TAGs production can elevate its serum level and flood the liver causing steatosis, which contributes to the pathogenesis of non-alcoholic fatty liver disease and steatohepatitis.

Obesity itself is a risk factor for a distressing amount of problems with respect to endocrine, cardiovascular, respiratory, psychosocial complications and certain types of cancers [108,109]. Effective treatment of obesity is very limited, currently centered on behavioral interventions such as regular physical activities and healthy diet. More invasive options include medications and bariatric surgeries, but they are only available to limited candidates. Little success has been met on the pharmaceutical front in treating obesity due to its multifactorial nature. Therefore, ongoing research needs to focus on teasing out major molecular targets in the initial manifest of obesity to quench its development and complications along the way.

2.2 Insulin

2.2.1 Insulin Signaling Pathway

Insulin is arguably the most potent hormone influencing all aspects of adipose tissue homeostasis from cell differentiation to triglyceride synthesis. Its secretion from

beta cells coincides with an increase in blood glucose level. The insulin signaling pathway is a complex system involving many circuits, branches, and feedback loops, and is far from being fully understood. Here, two of the important pathways will be exemplified [110,111]. Upon binding to its receptor, a protein tyrosine kinase, insulin causes dimerization and autophosphorylation of the receptor cytoplasmic domains. The event leads to the recruitment and phosphorylation of receptor substrates including insulin receptor substrate (IRS) and sarcoma homologous/collagen protein (Shc). IRS and Shc proteins respectively serve as a docking and activation site for two major signaling cascades: 1) the **phosphoinositide 3 kinase (PI3K) / serine/threonine kinase (Akt) pathway** and 2) the **Ras/ mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) pathway**. In the PI3K/Akt pathway, activated PI3K promotes the phosphorylation of a regular plasma membrane component, phosphatidylinositol 4,5-bisphosphate (PIP2), converting it to phosphatidylinositol 3,4,5 trisphosphate (PIP3). PIP3 behaves like a second messenger and activates the kinase Akt. Akt (also known as protein kinase B, PKB) is a multifunctional kinase that phosphorylates various substrates and mediates a majority of insulin's metabolic effect. In particular, Akt isoform 2 has been shown to increase the expression of GLUT1 and translocation of GLUT4 receptors to the plasma membrane of adipocytes for the uptake of glucose [112,113]. Both mouse gene knockout studies and small-interfering RNA of Akt2 demonstrated a decrease in insulin sensitivity and reduced glucose disposal [114,115]. On the other hand, constitutively active PI3K and Akt both partially stimulated glucose uptake in the absence of insulin [112,116,117]. In addition, there is a positive correlation between Akt activities and rate of glycolysis, glycogen and lipid synthesis through an

upregulation of hexokinase, phosphofructokinase-1 and glycogen synthase kinase-3 expressions [118]. Interestingly, under insulin-resistant states where glucose uptake is impaired, the antilipolytic effect from insulin signaling is relatively preserved, suggesting differential threshold sensitivity to insulin stimulation [111].

The second essential Ras/MAPK/ERK pathway starts by the sequential binding of growth factor receptor-bound protein 2 (GRB2) and Son of Sevenless (SOS) to the Shc protein, activating them. SOS is then able to catalyze the exchange of GDP to GTP on the membrane bound GTPase, Ras. In its GTP-bound form, Ras initiates a signaling transduction between B-RAF, (MAPK/ERK Kinase) MEK, MAPK and ERK1/2, leading to the activation of ERK1/2 and its translocation to the nucleus. Once inside the nucleus, ERK1/2 activates several transcription factors, which mediate gene expression of those in proliferation and survival of the cells. While PI3K/Akt pathway is known as the metabolic branch, the Ras/MAPK/ERK pathway is involved in mitogenic effect. Analyses on the role of MAPK in regulating adipogenesis yield contradictory findings where studies have claimed either a stimulatory or inhibitory effect of MAPK phosphorylation on adipogenesis. This observation led to the proposed temporal effect of MAPK/ERK pathway activation that accounts for both observations [119]. For instance, in 3T3-L1 cell line, activation of MAPK/ERK pathway at early stage of adipogenesis may phosphorylate transcription factors that initiate PPARγ and C/EBPα expression, while activation of the pathway in later stage may block adipogenic gene expression due to a MAPK-dependent phosphorylation of PPARγ [119,120].

Signaling dynamics are influenced by positive and negative feedback loops, which are found in both pathways. The two pathways are by no means mutually

exclusive, and are involved in a number of cross-talking. Kinases such as Akt and ERK, which are core players in the signaling pathways are target sites of integration. They converge to phosphorylate the same substrate complex often to bring about the same phenotypic effects [121]. Other times, they regulate each other through cross-activation or cross-inhibition (Fig. 6).

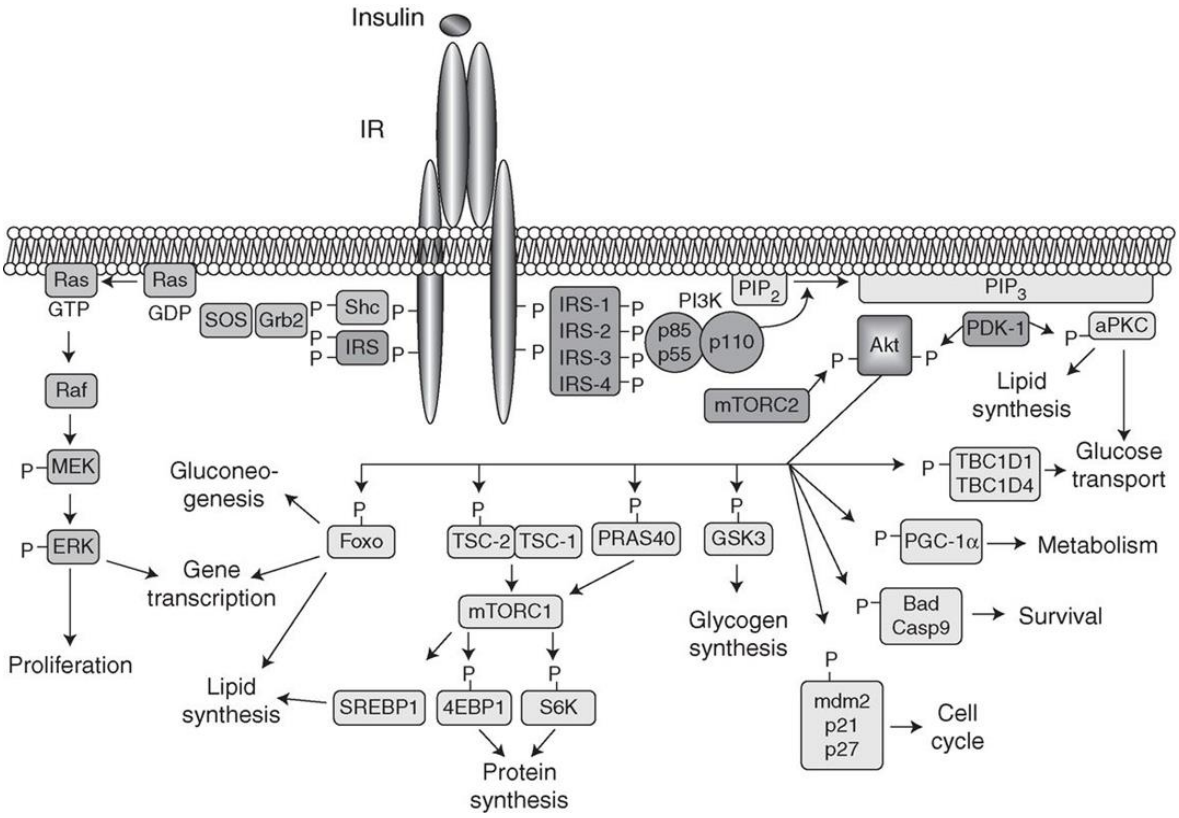

Figure 6. Insulin Signaling Pathway. Insulin binds and activates its tyrosine kinase insulin receptor (IR), which initiates a cascade of phosphorylation events. Upon binding, insulin receptor undergoes conformational change and autophosphorylation, leading to the recruitment and phosphorylation of receptor substrates including insulin receptor substrate (IRS) and Src homologous/collagen protein (Shc). IRS and Shc proteins respectively serve as a docking and activation site for two major signaling cascades: 1) the phosphoinositide 3 kinase (PI3K) / serine/threonine kinase (Akt) pathway and 2) the Ras/ MAPK/ERK Kinase (MEK)/ extracellular signal-regulated kinase (ERK) pathway. Akt is responsible for insulin's metabolic effects, regulating glucose transport, lipid synthesis, gluconeogenesis, and glycogen synthesis, while ERK pathway controls cellular proliferation and gene transcription. The figure is adapted and modified from Ref. 122.

2.2.2 Insulin Effect on the Liver

In a healthy individual, liver is the first site of clearance for endogenous insulin via the hepatic portal system, marking it an acute target for insulin action [123]. Liver expresses GLUT-2 transporters, which allow glucose uptake independent of insulin stimulation. However, insulin plays an important role in promoting fuel storage in the liver as glycogen through glycolysis and glycogenesis, while at the same time inhibits gluconeogenesis and glycogenolysis. These actions are carried out by insulin-directed activation of glucokinase and glycogen synthase concurrently with inhibition of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase [124]. In addition, insulin profoundly suppresses glucagon secretion by the alpha cells of the pancreas, and inhibits its effect on raising blood glucose [125]. Overall, insulin exhibits an inhibitory control on hepatic glucose production.

2.2.3 Insulin Effect on Adipose Tissue

Insulin is heavily involved in the pathway of fat metabolism. Research has shown that adipose tissue is responsible for about 10% of insulin stimulated glucose uptake [126]. Adipocytes express both the ubiquitous GLUT1 and the muscle and fat cell-specific GLUT4 transporters. Glucose is utilized to synthesize glycerol in adipocytes, which is then combined with fatty acids to form TAGs. Insulin stimulates fat storage in adipose tissue through an increase in levels of glycerol 3-phosphate, activation of LPL and inactivation of HSL [127]. The net effect of insulin is to promote lipogenesis while suppressing lipolysis; hence enhance storage and block mobilization and oxidation of fatty acids.

2.2.4 Insulin Resistance

Insulin resistance is the hallmark feature of T2DM, a chronic metabolic disorder paralleled by the pandemic of obesity [128]. The scientific community has recognized increasingly the importance of adipose tissue as a secretory organ, and molecules released such as fatty acids, adipokines and proinflammatory factors have paralleled the progressive worsening of insulin sensitivity. Due to its pleiotropic effect in multiple sites, insulin must be tightly controlled in intensity and duration. Disruption in any part of the signaling pathway can impair insulin sensitivity and make the hormone less effective. Initially, the pancreas can compensate for the drop in insulin sensitivity by an increase in insulin production, causing hyperinsulinemia. Over time, however, insulin resistance becomes symptomatic as the pancreatic beta cells start to wear out and eventually fail to produce enough insulin to overcome the cells' desensitization. Research interests accumulated around insulin resistance in three folds: 1) hyperglycemia and dyslipidemia, 2) endoplasmic reticulum stress and mitochondrial dysfunction, and 3) the release of cytokines and low-grade systemic inflammation [129].

Insulin resistance marks the beginning of a series of pathological sequelae that disrupts glucose and lipid metabolisms in the body. Chronic hyperglycemia and dyslipidemia can exert deleterious effects termed glucotoxicity and lipotoxicity [130]. It is well known that a sustained and chronic high level of plasma glucose causes structural and functional damage of beta cells and target tissues of insulin, which in turn causes further imbalance in glucose production and glucose utilization [131]. On the other hand, insulin resistance seen in obesity involves ectopic fat deposition in muscle and liver after adipose tissue reaches full capacity and loses its buffering ability on lipid influx [132].

As a result, individuals with insulin resistance have increased serum VLDL and LDL cholesterol and decreased high density lipoprotein (HDL) cholesterol, all of which points to defect in the production, conversion, and breakdown of lipid species in the *de novo* lipogenic pathway [133]. Some reviews have argued that hyperglycemia is a prerequisite for lipotoxicity to occur, while others suggest the opposite [130,134,135]. One of the puzzling pieces remaining is the cause and effect relationship between hyperglycemia, dyslipidemia and insulin resistance. It is apparent that a close relationship exists between the three factors, where abnormality of one is followed by abnormality of the others.

Glucose and lipid metabolism rely on mitochondria to generate energy in cells. The powerhouse organelle is responsible for energy homeostasis by metabolizing nutrients and producing ATP and heat. Excess intake of glucose and FFAs produce maladaptive consequences that increases reactive oxygen species (ROS) production and reduce mitochondrial biogenesis. ROS is produced from a low ratio of ATP production/oxygen consumption, and contribute to increased rate of mutagenesis and stimulate proinflammatory processes, while a diminished rate of mitochondrial biogenesis consequently reduces oxidative respiration, and further contributes to ROS production [136]. Recent findings provided evidence that high lipid diet induced mtDNA damage and increased oxidative stress in skeletal muscle and liver [137]. Moreover, mitochondrial dysfunction has been associated with insulin resistance in many other tissues such as adipose tissue, heart, and pancreas, which endorsed the idea that insulin resistance acts as the centre of a constellation of changes [138,139]. Another important organelle responsible for the biosynthesis of insulin is the endoplasmic

reticulum (EnR). It serves the function of folding, post-translational modification, and proper assembly of insulin in beta cells. As a result, beta cells have well-developed EnR, and remain one of the most susceptible cell type to EnR stress [140]. It has been demonstrated that obesity overloads the functional capacity of the EnR, causing impairment and stress-mediated apoptosis through transcriptional induction of cell degradation pathway, and act as a contributor to the development of insulin resistance [141,142].

Overproduction of proinflammatory cytokines such as TNF-α, IL-6 and C-reactive protein in adipose tissue is an important feature of obesity and contributes significantly to insulin resistance [143]. The autocrine and paracrine signaling of TNF-α is the first molecular link between obesity and inflammation [144]. Coordination between the inflammatory and metabolic pathways is demonstrated through overlapping biology and function of macrophages and adipocytes. Within the adipose tissue, resident macrophages express a majority of adipocyte-related gene products such as FABP4 and PPARγ, while adipocytes express many macrophage-related proteins such as TNF-α, IL-6, and matrix metalloproteinase-12 [129]. Under obese state, hypertrophic adipocytes produce proinflammatory cytokines, which leads to the recruitment of circulating monocytes through an increase in endothelium adhesion and vascular permeability [143]. An organized cross talk among endothelial cells, adipocytes, and resident macrophages induce a state of local inflammation in adipose tissue. Together, with the above-mentioned increase in oxidative stress from EnR and mitochondria induced by hyperglycemia and dyslipidemia, systemic inflammation takes over multiple tissues and further exacerbates the state of insulin resistance.

Researchers have uncovered potential genetic polymorphisms and down-regulated signaling pathways in the manifestation of insulin resistance [131]. Candidate genes with known polymorphisms include β3-adrenergic receptors, PPARγ, IRS-1, HSL, LPL and glycogen synthase, all of which may increase susceptibility to insulin dysregulation [145]. It was observed that both binding of insulin to receptor and its subsequent effector phosphorylations are reduced in insulin resistant state. Specifically, in adipocytes of T2DM patients, IRS-1 expression is reduced, resulting in decreased IRS-1 associated PI3K activity [127]. One of the major consequences is the impaired translocation, docking, and fusion of GLUT4 transport, which leads to impaired insulin-stimulated glucose transport [146]. In humans and rodents, an increased inhibitory serine/threonine phosphorylation along with decreased tyrosine phosphorylation of insulin receptor substrates further contribute to insulin-resistant states [147,148].

3. Prohibitins

3.1 General

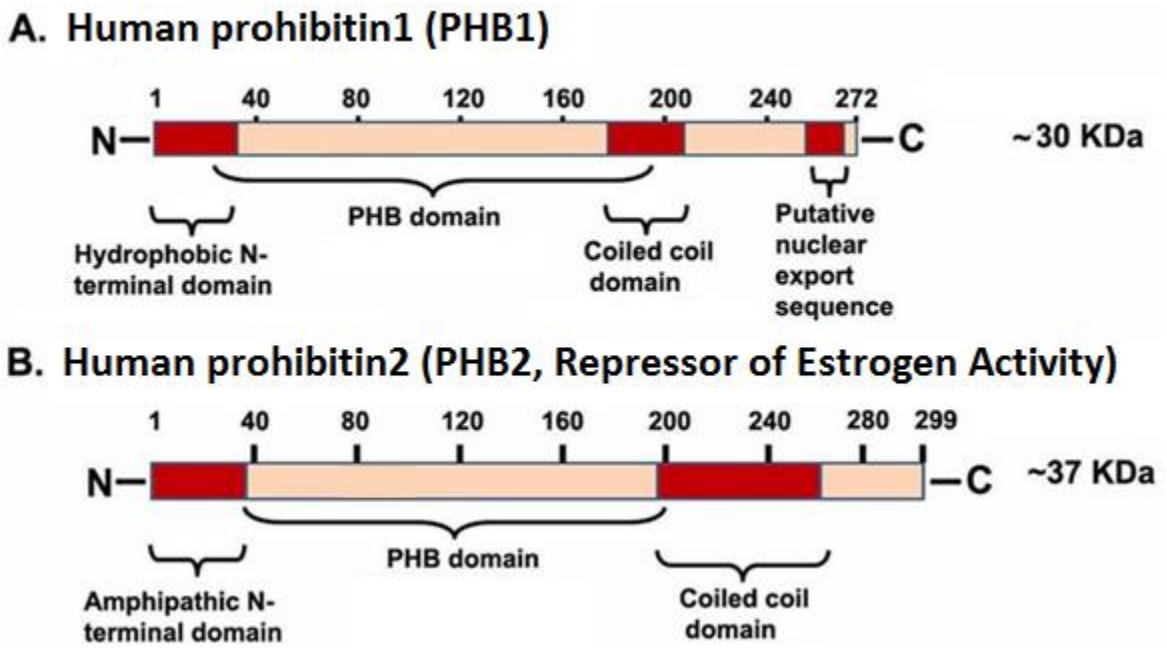

Figure 7. The Human Prohibitins (PHBs). Two homologs of PHBs termed prohibitin 1 (PHB1) and prohibitin 2 (PHB2, Repressor of Estrogen Activity, REA) have been discovered, and are found to be ubiquitously expressed in all eukaryotic cells with highly conserved functions. In the diagram, N represents the amino terminal, and C represents the carboxy terminal. There are 3 major domains shared between the PHB proteins. Starting from the N terminus, the hydrophobic stretch acts as an anchor to the cell membrane. The second domain termed PHB domain shows characteristic of the Stomatin, Prohibitin, Flotilin, HflC and HflK (SPFH) superfamily with an overall function in lipid rafts in the plasma membrane. The last coiled-coil region at the carboxy terminal end was found to play a role in assembly of PHB complexes in the mitochondria. The figure is adapted and modified from Ref. 149.

Prohibitin (PHB) was first identified as a potential anti-proliferative gene from cDNA differential hybridization to RNA in the rat liver, but this function was later attributed to the 3' untranslated region of PHB mRNA [150,151]. Two homologs of PHB termed prohibitin-1 (PHB-1) and prohibitin-2 (PHB-2) have since been discovered, and are found to be ubiquitously expressed in all eukaryotic cells with highly conserved

functions. At the same time, PHB1 and PHB2 were also shown to bind to the Immunoglobulin M receptor, and is thereby referred to as B-cell-receptor complex-associated proteins in humans (BAP32 and BAP37) [152]. There are 3 major domains within the protein sequence of PHB (Fig. 7). Starting from the N terminus, the hydrophobic stretch acts as an anchor to the cell membrane. The second domain termed PHB domain shows characteristic of the Stomatin, Prohibitin, Flotilin, HflC/K (SPFH) superfamily with an overall function in lipid rafts in the plasma membrane [149]. The last coiled-coil region at the carboxy terminal end was found to play a role in the assembly of PHB complexes in the mitochondria. PHB1, the protein of interest in this study, is located at the chromosome 17q21 locus, a region associated with propensity to visceral fat deposition in humans [153]. Mouse and rat PHB1 have identical sequences, and only differ from human PHB1 by one single conserved amino acid [154]. Since its discovery, PHB1 has been implicated in a number of diverse functions including scaffolding protein in mitochondria, gene modulator in the nucleus, ligand binding site on the plasma membrane, and a secreted signaling molecule in the general circulation [155] (Fig. 8).

In the mitochondria, PHB1 forms a ring-like complex with PHB2 in the inner mitochondrial membrane, and functions to stabilize subunits of mitochondrial respiratory enzymes and protect the functional integrity of mitochondria [156,157]. Elimination of either PHB1 or PHB2 results in the absence of the complex, implying interdependence of the two in complex formation. PHB complex acts as a chaperone that stabilizes both mitochondrial genome and newly synthesized proteins from the mitochondrial inheritance components, phosphatidylethanolamine biosynthetic pathway, and electron

transport chain, which protects them from degradation by the matrix-alanine-alanine-alanine (m-AAA) protease [158-161]. Consequently, knockdown of PHB1 in particular was shown to alter the mitochondrial nucleoid organization and mitochondrial DNA copy. Similarly, loss of PHB2 has severe consequences for the reticular mitochondrial network and aberrant cristae morphogenesis coupled to an impaired cellular proliferation [162]. In addition, PHB performs a role in the oxidative phosphorylation system. Our lab reported that PHB1 acts as an inhibitor of pyruvate carboxylase, and modulate insulin-stimulated glucose and fatty acid oxidation [163]. PHB overexpression in human epithelial cell line was shown to prevent oxidative stress and promote endogenous antioxidant expression including glutathione peroxidase, catalase, superoxide dismutase, and glutathione [164]. Of note, the PHB complex is required in *Caenorhabditis elegans* for the embryonic development of larval gonad, implying potential reproductive functions that have yet to be elucidated [165].

On top of the roles in the mitochondria, both PHB1 and PHB2 are prominent players in compartment specific cell signaling. Through a hybrid screening of protein–protein interactions, PHB2 was discovered to interact directly with the C terminus of Akt, which underlies a potential mechanism for Akt to partially reduce PHB2 function as a transcriptional repressor in cells [166]. Emerging studies have identified PHB1 as a player in both the PI3K/Akt and Raf/ERK pathways involved in insulin signaling [167]. It may be a key regulator of crosstalk between the two pathways by interacting with their signaling intermediates and modulating insulin action. In fact, PHB1 is phosphorylated directly at tyrosine 114 site by insulin receptor upon stimulation, and behaves as a negative regulator by recruiting SH2-domain containing phosphatase1/2 (Shp1/2) and

attenuating Akt phosphorylation [168]. On the contrary, Akt induced PHB1 phosphorylation at threonine 258 inhibits its interaction with PIP3 and Shp1, which facilitates PI3K/Akt signaling while competes with Raf1 phosphorylation on serine 259 and reduces Raf1 promotion on cell proliferation [169,170] (Fig. 8). The diverging cellular outcomes of PHB are further demonstrated through its role as a downstream functional converging point between two transforming growth factor β (TGFβ) signaling pathways in prostate cancer cells. Namely, the TGFβ activated MAPK/PKC pathway phosphorylates PHB1 and signal towards cell survival, whereas TGFβ upregulated 14-3-3 protein inhibits PHB1 phosphorylation, and induced mitochondrial permeability, resulting in apoptosis [171] (Fig.8).

PHB also interacts with a number of nuclear proteins involved in gene transcription. In breast cancer cell lines, PHB was found to co-localize with E2F1 and p53, two major players in cell proliferation, differentiation, and apoptosis. Specifically, PHB was found to enhance p53-mediated transcriptional activity while strongly repress E2F1-mediated transcription [172,173]. Furthermore, PHB translocates from the nucleus to mitochondria upon receiving apoptotic or stress signals, allowing it to integrate and crosstalk with multiple compartments [174]. PHB2 was first found as a transcriptional corepressor of nuclear estrogen receptor, hence given the name repressor of estrogen receptor action (REA) [175]. A range of signaling proteins that directly interact with and reduce REA level are found to be overexpressed in estrogen-dependent cancers (i.e. breast cancer, endometrial cancer) [176,177]. While homozygous deletion of PHB2 in mouse results in embryonic lethality, heterozygous animals exhibit phenotypes of overactivated estradiol (E2) signaling with a loss of down regulation in E2 repressed

genes [178]. As a result, female mice, but not male mice, exhibited increased body weight after puberty, greater uterine weight gain and epithelial hyperproliferation. More recently, PHB1 was also identified as a corepressor of estrogen receptor α in breast cancer cells, but its function was sequestered by heterodimer formation with PHB2 [15]. Elevated expression of PHB was found in WAT and the liver after treatment with 17-β-estradiol in diet-induced obese rats, and estrogen inhibitor markedly suppressed both PHB transcripts and protein levels, suggesting that PHB expression is regulated through estrogen receptor [16]. In prostate cancer cells, PHB1 is also identified as a target protein of androgen signaling in which it acts as a repressor for androgen receptor-mediated transcription and androgen-dependent cell growth [14]. Interesting, a serum screening of novel tumor antigens in colorectal patients revealed elevated levels of PHB1 and PHB2 in the circulation [179]. Its tumor suppressing effect is observed in hepatocyte-specific knockout of PHB1 in mouse, where there is an elevation of biochemical and histological markers of liver injury, oxidative stress and fibrosis. It is safe to conclude that PHB is a pleiotropic protein that regulates many aspects of cell metabolism. Its cellular localization is a determinant of function, and its responses vary in different tissues based on upstream signaling.

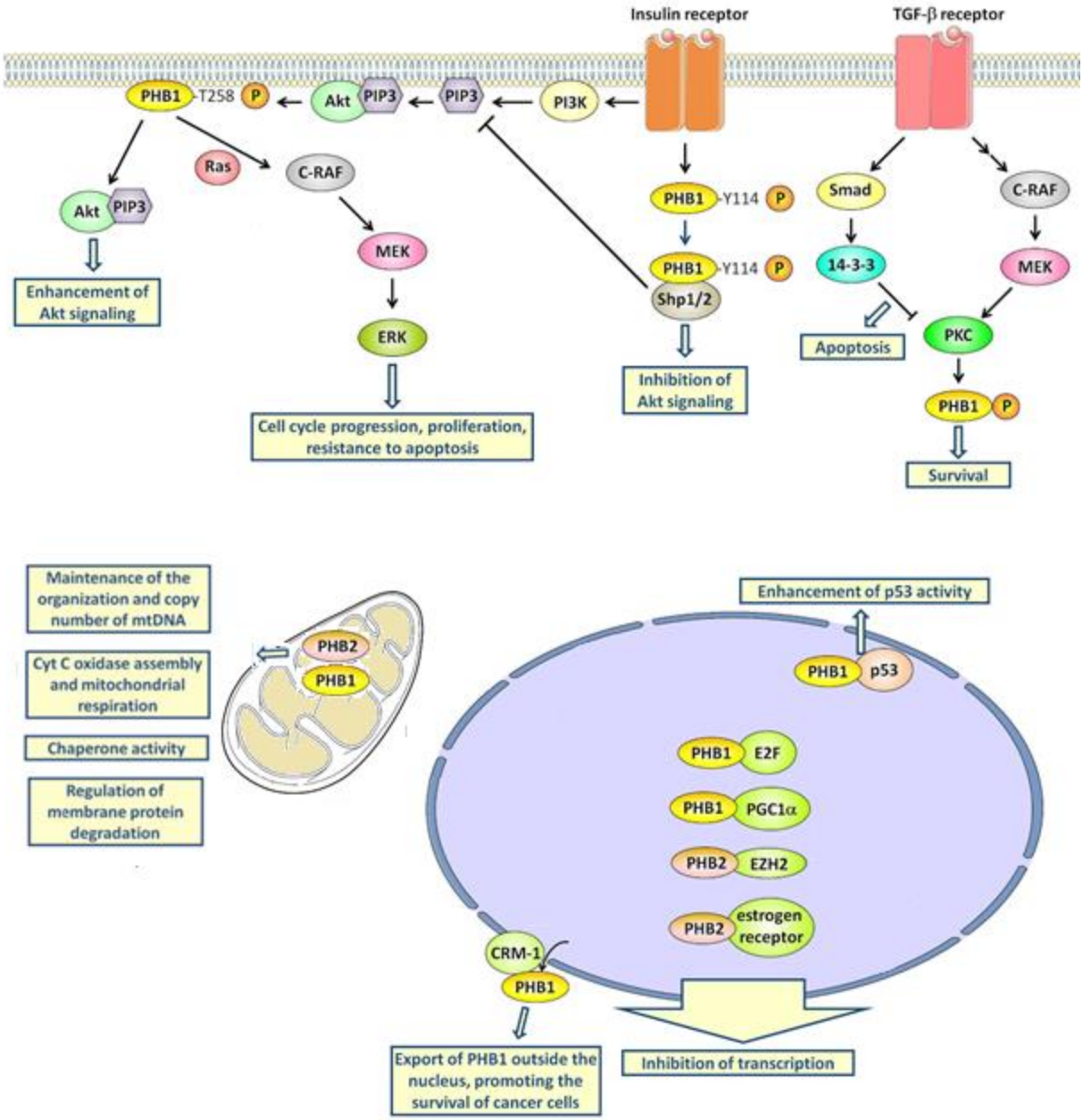

Figure 8. Proposed Prohibitins' (PHBs) Functions Within a Cell. PHBs are pleiotropic proteins that have been implicated in a variety of subcellular compartments. In the mitochondria, PHB1 and -2 form a complex that participates in scaffolding, maintenance of mtDNA, and performs a role in the oxidative phosphorylation system. In the nucleus, PHBs interact with different transcription factors to modulate gene expressions, and play an important role in cancer biology. On the plasma membrane, PHB1 integrates many signaling pathways including PI3K/Akt, C-RAF-MEK-ERK, PKC to orchestrate aspects of cell physiology. The figure is adapted and modified from Ref. 155.

3.2 Prohibitins and Adipose Tissue

Mitochondria are highly dynamic cellular organelles that adjust in shape and number depending on cell type and energy demands. They are the site of many biochemical pathways including the tricarboxylic acid cycle, oxidative phosphorylation, and fatty acid β-oxidation. In adipocytes, nutritional and hormonal cues are translated into mitochondrial response either to oxidize incoming fatty acids and carbohydrates, or to store them in the form of triacylglycerol (TAG) until signal for release. Although in lower abundance in WAT, mitochondria remain vital in energy regulation, and are intimately associated with adipogenesis in which there exists an intricate coordination between mitochondrial biogenesis and adipogenesis [180-182]. As a housekeeping protein in the mitochondria, accumulated research has point to the fact that PHB1 plays an important role in adipogenesis. Initial study in *C. elegans* found PHB1 complex promotes longevity by modulating mitochondrial ATP production and fat metabolism, and RNAi knockdown of PHB1 reduced intestinal fat and mitochondrial proliferation [183]. Subsequently, our lab discovered that PHB1, which is known to function in mitochondrial biology, plays an important role in adipocyte differentiation, in which PHB1 overexpression alone was shown to induce adipogenesis in 3T3-L1 fibroblasts [12]. Knockdown of PHB1 or PHB2 by oligonucleotide siRNA and microRNA significantly reduced the expression of adipogenic markers, lipid accumulation and impaired mitochondrial function, which further confirmed the importance of PHB1 in adipose tissue biology [184,185]. To explore the role of PHB1 in adipose tissue biology at the systemic level, our laboratory has developed a novel transgenic mouse model, “Mito-Ob”, by overexpressing PHB1 in adipocytes under the adipocyte protein-2 (aP2) gene

promoter [13]. Study results revealed that both sexes became obese as predicted through an upregulation of mitochondrial biogenesis, but have interesting sex dimorphic metabolic phenotypes [13]. Collectively, these evidences point towards a critical role of PHB in adipogenesis and adipose tissue homeostasis. However, the underlying mechanism involved in the role of PHB in these pathways is not well understood.

4. Sex Differences

4.1 General

Sex differences have profound effects on the susceptibility and pathogenesis of many diseases. Often, one sex is preferentially protected from a disease while other is more susceptible because of biological or environmental factors. Aside from disorders arise from the reproductive system that are specific to one sex, example of illness that show sex dimorphism includes cardiovascular diseases, autoimmune diseases and asthma [186]. In the past, a majority of biological researches have used male bodies as the default model, which assumed a uniform response in both sexes. As a result, data generated often ignored the importance sex difference plays in disease development and progression. Identifying the factors that protect one sex from a particular disease can shed light on treatments for both sexes.

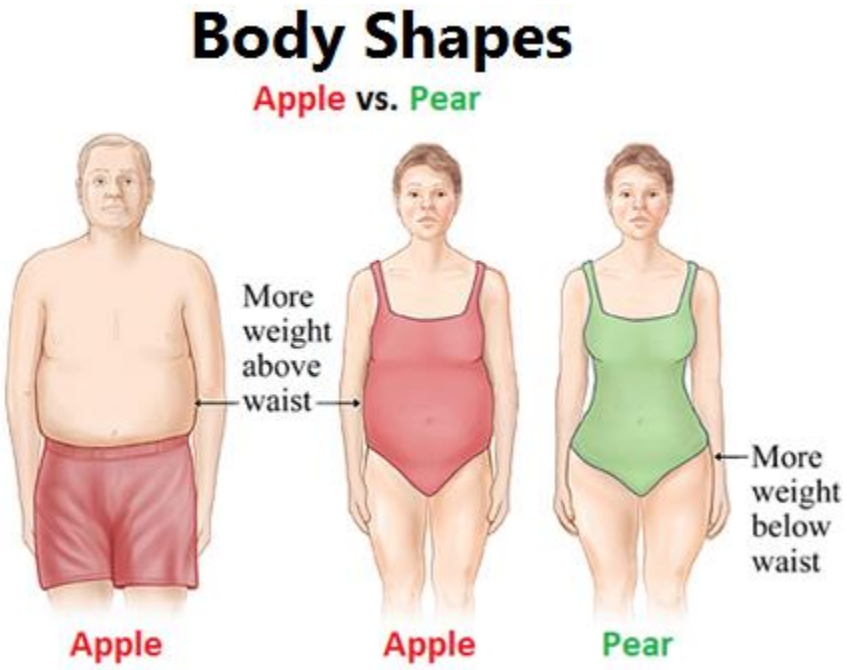

Figure 9. Gender Difference in Body Fat Distribution. Two body shapes, apple and pear, are frequently used to describe fat distribution. Apple-shaped individuals carry excess body fat around the abdominal region; pear-shaped individuals carry excess body fat around the femoral-gluteal regions (hips, buttocks, and thighs). Women tend to have more total body fat, and develop pear-shaped body figure (gynoid) during reproductive age. However, when the production of estradiol declines during menopause, deposition of fat migrates up to the abdominal area (apple-shaped). Men, on the other hand, have less total fat but are more likely to develop apple-shaped body figure (android). Apple-shaped body is associated with an increased chance of metabolic complications, which puts postmenopausal women and men at higher risk. The figure is adapted and modified from Ref. 187.

4.2 Sex Difference in Body Adiposity

Sex difference plays a profound role in fat distribution in the human body. Throughout evolution, survival pressures predisposed women to store fat for reproduction and men to burn stored fat in hunting and gathering [188]. In the modern world, as a result of living a sedentary lifestyle, men are not as well amenable for the excess weight gain as do women. Epidemiological data on obesity reveals sex difference in adiposity. The prevalence of obesity among men (21.8%) is higher than

women (18.7%) in Canada as indicated by the 2015 analysis released by Statistics Canada, and this gender difference is observed across most developed countries [104,189].

In general, women tend to have more total body fat, mostly stored in the femoral-gluteal regions during reproductive age. When the production of estradiol declines during menopause, deposition of fat migrates up to the abdominal area [55,190]. Men, on the other hand, have less total fat but are more likely to deposit fat around the waist and abdomen (Fig. 9). High levels of abdominal adiposity are associated with an increased chance of metabolic complications, which puts postmenopausal women and men at higher risk. In addition to location specific difference in fat accumulation, adipose sex dimorphism also extends to adipocyte secretion. The two major adipokines, leptin and adiponectin, are characterized by higher circulating levels in women than men [191,192]. Furthermore, men and women differ in the use of carbohydrates and lipids as fuel sources. For example, men utilize preferentially carbohydrates as the primary fuel during exercise, while women oxidize a greater proportion of lipids compared to carbohydrates [193]. In postmenopausal women that exhibit decreased estrogen level, there is an accompanied decrease in energy expenditure and lipid oxidation, suggesting a potential role of estrogen in regulating energy expenditure [194]. A closer look at the difference in food intake revealed that females have a greater resistance to energy lost during periods of food scarcity. When animals were subjected to complete starvation, females decreased energy expenditure, exhibited a greater ratio of lipid to protein loss, and were more likely to survive [195,196].

4.3 Sex Steroid Hormones

One of the sex-biasing factors is the effect of gonadal steroid hormones: androgens and estrogens. They are two families of hydrophobic hormones primarily produced from the gonads, and to a lesser extent, the adrenal cortex and adipose tissues. Enzymes such as aromatase, 17β-Hydroxysteroid dehydrogenase, and 5α-reductase are available at the point of action to convert androgens to estrogens, or to interconvert within its own family. In addition to their critical roles in reproductive tissues, estrogens and androgens take part in body metabolism evident by visible change in fat distribution during puberty, pregnancy and postmenopause [190]. Besides the gonads, WAT is the biggest peripheral contributor of sex steroid hormones [43]. Due to its relative mass in the body, WAT releases up to 100% of circulating estrogen in postmenopausal women and 50% of circulating testosterone in premenopausal women [28]. Data from idiopathic obese individuals supported the hypothesis that intra-adipose sex steroid metabolism is a determinant of gynoid vs. android body fat distribution [197]. While each sex steroid hormone is only predominant in one sex, both are present and crucial in the maintenance of energy homeostasis and sexual dimorphism. In women, estrogen brings about a protective effect against metabolic dysregulation as demonstrated using estrogen replacement therapy after menopause when estradiol production drops [198]. Androgens in women are mainly present as androstenedione, with other forms being synthesized from it as needed [199,200]. In men, testosterone is needed to maintain lean muscle mass. As men age, there is a natural decline in testosterone and increase in estrogen due in part to the aromatase action in adipose tissue. An elevated plasma estrogen level was also observed in obese men due to an increase in aromatase activity

in WAT that mediates peripheral conversion of androgens [201]. Consequently, the rise in estrogen level has been found to correlate with the increased incidents of high blood sugar and diabetes in men [202]. Based on these observations, it is clear that there lies a unique testosterone: estrogen ratio in both men and women, and deviation from this balance will result in metabolic consequences.

Sex steroid hormone can undertake one of two pathways to exert its action: the classical genomic signaling and the nonclassical cytoplasmic signal (Fig. 10). In the classical pathway, sex steroid hormone enters the cell through diffusion and binds with a specific cytosolic or nuclear receptor [203,204]. The steroid-receptor complex then binds to a response element on the DNA, thus controlling its expression. Leptin and lipoprotein lipase are among the many effector proteins regulated through transcriptional control [205]. Contrary to traditional belief, sex steroid hormones have been implicated in other nonclassical signaling pathways including the use of second-messenger and direct protein-protein interaction with other transcription factors in the nucleus [206,207].

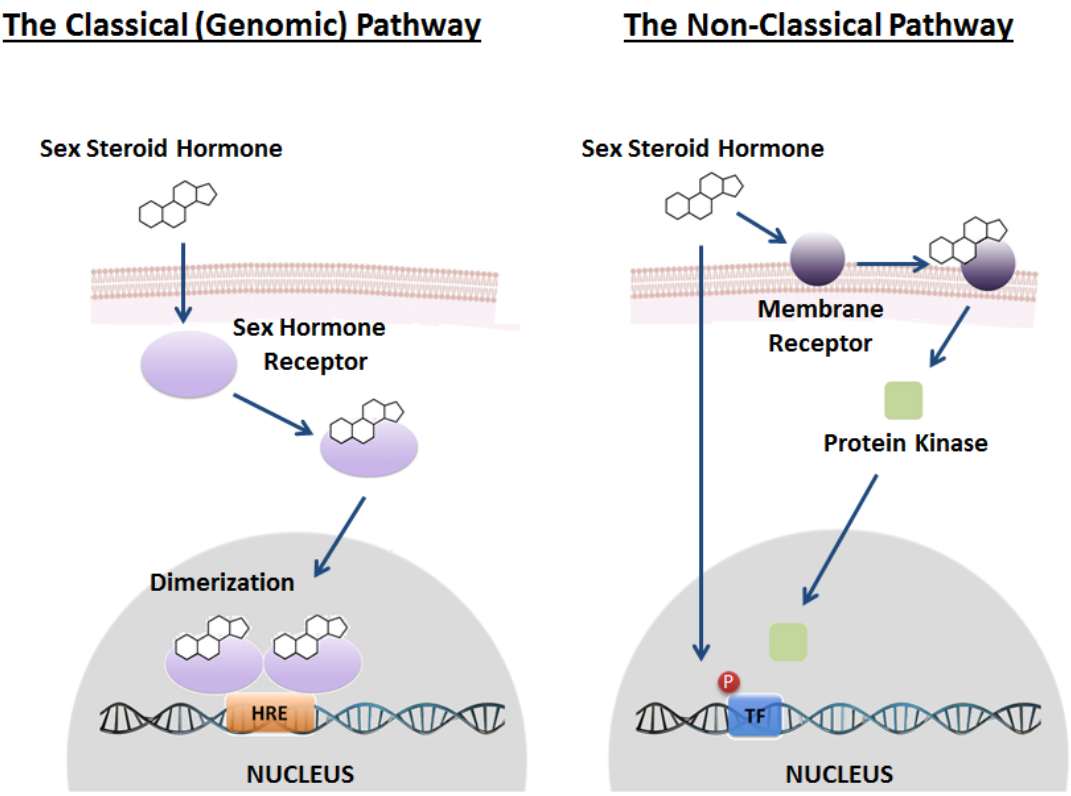

Figure 10: The Signaling Pathways of Sex Steroid Hormones. In the classical genomic pathway, sex steroid hormone diffuses across the cell membrane and binds with a specific cytosolic or nuclear receptor. The ligand/receptor complex then translocates into the nucleus, and binds to the genes with hormone responsive element (HRE), thus controlling gene expression. In the nonclassical cytoplasmic pathway, sex steroid hormones bind surface receptor, and exerts its action with the help of second-messenger or directs protein-protein interaction with other transcription factors in the nucleus. The figure is adapted and modified from Ref 208.

4.3.1 Estrogens

Estrogens (estrone (E1), estradiol (E2), and estriol (E3)) are a group of sex steroid hormones produced primarily in the ovaries, as well as in testes and adipocytes through the action of aromatase [209]. Of the three, E2 is the predominant and most potent estrogen in female during reproductive age. It targets multiple sites in the body that regulate key aspects of metabolism. Estrogen exerts its action through two estrogen receptors (ERs), ERα and ERβ. ERα in particular has been associated with

controlling energy expenditure and food intake. Rats with damaged ERα in the hypothalamus showed significant increase in body weight and food intake with decreased energy expenditure [210]. Specifically, ERα damage in the ventral lateral region of the ventral medial nucleus was associated with visceral weight gain and the whitening of brown adipose tissue in females. ERα knockout in the proopiomelanocortin (POMC) neurons also showed female specific increase in food intake and energy expenditure, indicating estrogen as an important regulator in the central nervous system with sex specific modulation for energy homeostasis [211].

Locally, ERs exert regulation on WAT homeostasis as observed by its expression in a variety of cell types including preadipocytes, mature adipocytes, and local macrophages [212,213]. *In vitro* studies on the effect of estrogen in adipocyte biology have been inconsistent. Several studies demonstrated estrogen as an inducer of adipocyte proliferation and differentiation in female rat and human preadipocytes [214,215]. At the same time, other studies found activation of ER receptors through E2 or phytoestrogen treatment interfered with DNA binding activity of PPARγ, and reduced adipogenic genes CEBPα, aP2, LPL and even PPARγ itself [7-9,216]. *In vivo* studies overall favor the notion that the lack of estrogen increases WAT accumulation and insulin resistance. ER knockout mice are more prone to obesity independent of sex. In particular, ERα disruption significantly increased visceral fat mass, macrophage infiltration, decreased insulin sensitivity and impaired glucose tolerance [217]. Furthermore, a subsequent study found that a lack of ERα specifically in adipocytes caused tissue inflammation and fibrosis, and they are seen more profoundly in males [217]. Ovariectomy in rodents led to weight gain, primarily as adipose tissue, and this can be reversed by estrogen

replacement treatment (ERT) [218]. Administration of E2 appears to downregulate adipose marker gene expressions through interferences with PPARγ action, and altogether led to reduced white adipose tissue mass, adipocyte size, and proteins involved in lipogenesis and fatty acid uptake including acetyl-coenzyme A carboxylate, lipoprotein lipase (LPL), and fatty acid synthase [4,5,219,220].

4.3.2 Androgens

Androgens make up a group of hormones responsible for male development and characteristics. Testosterone is the major circulating sex hormone in the body, along with dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione and androstenediol. Testosterone is predominantly synthesized in the testes, while other androgens are produced by the adrenal cortex or enzymatic conversion. It is well known that testosterone is an energy-mobilizing and fat-reducing hormone that contributes to the anabolic status of the body, and has an impact on the proliferation and location of WAT [2,214,215,221]. There is a strong inverse relationship between testosterone level and obesity with its associated metabolic complications [222]. Higher level of testosterone in the bloodstream of aging men was found to have better insulin sensitivity and a lowered risk of developing metabolic syndrome [223]. Reciprocally, obese men were shown to exhibit a progressive decrease level of testosterone and increased body weight especially in the abdomen [2,224]. Research has found 23% correlation between genes that regulate testosterone and those that control body fat composition, presenting a potential causal link to explain the testosterone-WAT relationship [225]. In addition, the high aromatase activity found in excess fat tissue converts testosterone to E2, further decreasing testosterone availability. Under a state of low testosterone, muscle biopsy in

men revealed impaired mitochondrial oxidative phosphorylation, while serum concentration of insulin, glucose, triglycerides and C-reactive proteins were increased [226,227]. At the molecular level, testosterone regulates adipogenic gene expressions through binding to androgen receptors (ARs) expressed in WAT. The presence of AR was found in sub-cultured human preadipocytes, and its density was higher in visceral preadipocytes than in subcutaneous preadipocytes [62]. Adult male AR knockout mice, which are testosterone resistant, developed late-onset obesity, and weight gained in both subcutaneous and visceral depots of WAT [228]. Further evidence revealed that testosterone dose-dependently inhibited adipogenic differentiation of 3T3-L1 cells, and down-regulated both C/EBPα and PPARγ mRNA and protein levels [6]. Research has also found that testosterone inhibited LPL activity and lipid uptake while at the same time upregulated adrenoreceptor for catecholamines, and thereby induce lipolysis [229,230]. The net effect of testosterone highlights that lipid accumulations were downregulated through increased lipolysis and decreased lipid uptake.

With the overwhelming evidence on the effect of sex steroid hormones on adiposity, there is much to explore in terms of sex difference in adipose tissue, and the exact molecular pathway of hormonal effects remains unclear. These differences can come from sex steroid hormones acting by receptor action directly in the white adipose tissue or from receptor actions elsewhere that indirectly affect adipose tissue homeostasis. There are still gaps to be filled in the underlying mechanisms involved in the actions of sex steroid hormones in regulating adipocyte physiology, and key factors that contribute to sex differences in metabolic homeostasis and disorders remain elusive.

II. HYPOTHESIS AND OBJECTIVES

Hypothesis

Sex differences exist in obesity and obesity related abnormalities. Using Mito-Ob mice, a novel mouse model developed through overexpression of PHB1 in adipose tissue, it was found that Mito-Ob female and male mice both developed obesity, but only males developed impaired glucose homeostasis and insulin sensitivity [13]. The observed sex difference raised speculations on the potential factors in play that generated the sex dimorphic phenotypes observed in Mito-Ob mice. Previous studies have identified interplay between PHB1 and sex steroid hormones in reproductive tissues, leading to the present investigation into the interaction between the two in adipose tissue homeostasis and metabolic regulation. It fits in the broader picture of how sex steroid hormones work in modulating lipid metabolism, adipose tissue homeostasis and the complications associated with obesity. The hypothesis of the thesis is that the **sex dimorphic metabolic phenotypes in Mito-Ob mice are produced by sex steroid hormones modulation of PHB1 functions in adipocytes**. Specifically, sex steroid hormones modulate the role of PHB1 in adipogenesis.

Objectives

The central objective of the thesis is to elucidate the interaction between prohibitin-1 (PHB1) and sex steroid hormones in adipogenesis and their effect on metabolic homeostasis. Two specific aims were formulated.

The **first aim** is to study the effect of gonadectomy on the metabolic phenotype of Mito-Ob mice.

The **second aim** is to study the effect of sex steroid hormones on adipogenesis from primary preadipocytes isolated from Mito-Ob mice.

III. MATERIALS & METHODS

A. Materials

Chemicals used in the study were purchased from the companies listed in Table1.

Table 1. Reagents used in the Experiments

Reagent	Company
0.25% Trypsin-EDTA, 1x	Life Technologies (Carlsbad CA, USA)
10% Buffered Formalin	Fisher Scientific (Kalamazo, MI, USA)
17-beta-estradiol (E2)	Sigma-Aldrich (Mississauga, ON, Canada)
2-Propanol	Sigma-Aldrich (Mississauga, ON, Canada)
30% (w/v) Acrylamide/Bis Solution (29:1)	Bio-Rad (Hercules, CA, USA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich (Mississauga, ON, Canada)
Ammonium Persulfate (APS)	Sigma-Aldrich (Mississauga, ON, Canada)
anti-Akt (pan), Rabbit mAb	Cell signaling (Danvers, MA, USA)
anti-MAPK, Rabbit mAb	Cell signaling (Danvers, MA, USA)
anti-PHB, Rabbit mAb	Cell signaling (Danvers, MA, USA)
anti-Phospho-Akt (S473), Rabbit mAb	Cell signaling (Danvers, MA, USA)
anti-Phospho-p44/p42 MAPK (Erk1/2), Rabbit mAb	Cell signaling (Danvers, MA, USA)

anti-rabbit HRP-linked	Cell signaling (Danvers, MA, USA)
Bromophenol Blue	Sigma-Aldrich (Mississauga, ON, Canada)
Chloroform	Fisher Scientific (Kalamazo, MI, USA)
Collagenase	Sigma-Aldrich (Mississauga, ON, Canada)
cOmplete ULTRA Tablets, Mini, EASYpack (Protease Inhibitor Cocktail Tablets)	Roche (Penzburg, Germany)
Dexamethasone, Water Soluble	Sigma-Aldrich (Mississauga, ON, Canada)
Dimethyl Sulfoxide (DMSO)	Fisher Scientific (Ottawa, CA)
Distilled Water DNAse, RNAse Free	Invitrogen (Burlington, CA)
Dithiothreitol (DTT)	Sigma-Aldrich (Mississauga, ON, Canada)
dNTP Mix	Invitrogen (Burlington, ON, CA)
Dulbecco's Modified Eagle Medium (DMEM) High Glucose with L-Glutamine, Sterile-filtered	ATCC (Manassas, VA, USA)
Enhanced Chemiluminescence Kit	Sigma-Aldrich (Mississauga, ON, Canada)
Ethanol, Denatured, Reagent Grade	Sigma-Aldrich (Mississauga, ON, Canada)
Fetal Bovine Serum (FBS)	Invitrogen (Burlington, CA)
Formalin	Fisher Scientific (Kalamazo, MI, USA)
Glycerol	Fisher Scientific (Kalamazo, MI, USA)
Glycine, Tissue Culture Grade	Fisher Scientific (Kalamazo, MI, USA)
Hematoxylin Stain Solution, Modified Harris	Ricca (Arlington, TX, USA)

Formulation, Mercury Free Nuclear Stain	
Hydrochloric Acid (HCl)	Fisher Scientific (Kalamazo, MI, USA)
Immunoblot Polyvinylidene Fluoride (PVDF) Membrane for Protein Blotting	Bio-Rad (Hercules, CA, USA)
Indomethacin	Sigma-Aldrich (Mississauga, CA, USA)
Insulin Solution-Human	Sigma-Aldrich (Mississauga, ON, Canada)
Isobutylmethylxanthine (IBMX)	Sigma-Aldrich (Mississauga, ON, Canada)
Luminata Crescendo Western HRP Substrate	Millipore (Billerica, MA, USA)
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific (Rackfard, USA)
Mammalian Cell Lysis Kit	Thermo Scientific (Rockford, USA)
Methanol, Sequencing Grade, Peroxide-free	Fisher Scientific (Kalamazo, MI, USA)
Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)	Invitrogen (Burlington, ON, CA)
Non-fat dry milk	Bio-Rad (Hercules, CA, USA)
Oil Red O	Sigma-Aldrich (Mississauga, ON, Canada)
Oligo(dT) 12-18 Primer	Invitrogen (Burlington, ON, CA)
Pan-Actin Rabbit mAb	Cell signaling (Danvers, MA, USA)
Penicillin/Streptomycin	Life Technologies (Carlsbad CA, USA)
Phosphate Buffered Saline (PBS) powder	Sigma-Aldrich (Mississauga, ON, Canada)

phosSTOP EASYpack (Phosphatase Inhibitor Cocktail Tablets)	Roche (Penzburg, Germany)
Ponceau S Solution	Sigma-Aldrich (Mississauga, ON, Canada)
Power SYBR Green PCR Master Mix	Thermo Scientific (Rockford, USA)
Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set	Thermo Scientific (Rockford, USA)
Precision Plus Protein Kaleidoscope Standards	Bio-Rad (Hercules, CA, USA)
Quick Start Bradford Dye Reagent, 1x	Bio-Rad (Hercules, CA, USA)
Restore PLUS Western Blot Stripping Buffer	Thermo Scientific (Rackfard, USA)
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich (Mississauga, ON, Canada)
Testosterone (T)	Sigma-Aldrich (Mississauga, ON, Canada
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich (Mississauga, ON, Canada)
Tris Base	Fisher Scientific (Kalamazo, MI, USA)
TRIzol Reagent	Life Technologies (Carlsbad CA, USA)
TWEEN-20	Sigma-Aldrich (Mississauga, ON, Canada)
Water, Sterile, Nuclease-free	Anresco (San Francisco, CA, USA)

B. Methods

1. Transgenic Mice

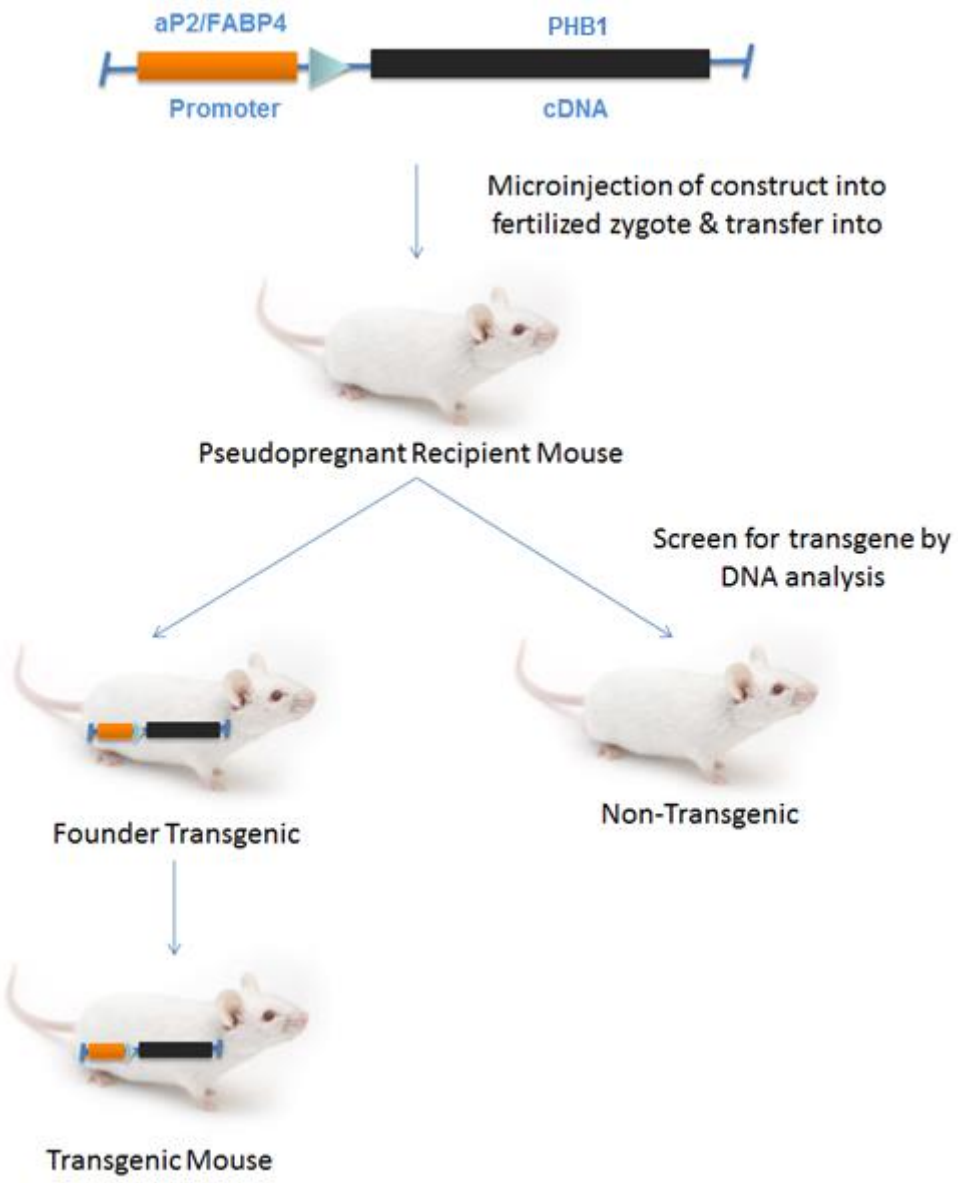

Figure 11. Schematic for the generation of Mito-Ob transgenic mouse obesity model. PHB1 clone was digested with *Not1*, and its full-length cDNA subcloned into the *Not1* site of the pBS-aP2 promoter vector. Then, the aP2-PHB1 construct was digested with *Sac1* and *Kpn1* restriction enzymes to release the fragment containing *aP2* promoter with full-length PHB1. This DNA was purified and used for pronuclear transfer into zygotes from CD1 mice. Founder animals were identified by PCR amplification of tail genomic DNA. Founder animals were then mated with CD1 female mice to establish PHB1 transgenic mouse lines.

The Mito-Ob transgenic mouse is a novel obesity model developed in our laboratory [13]. A transgenic gene construct was made by linking a PHB1 cDNA to the regulatory region of the *aP2* gene promoter to overexpress PHB1 in adipocytes (Fig. 11). CD1 mouse zygotes were microinjected with the transgene DNA construct. Genotyping of the founder Mito-Ob mice confirmed the overexpression of PHB1 in white adipose tissue. The Mito-Ob mouse is characterized by an increase in white adipose tissue mass due to PHB1-induced upregulation of mitochondrial biogenesis. Unlike previously existing models, which are based on appetite dysregulation or undefined genetic predisposal, Mito-Ob mouse allows spontaneous development of obesity and insulin resistance, diabetes and metabolic syndrome due to direct alterations in adipocytes/adipose tissue.

2. Animal Care and Body Weight

Male and female Mito-Ob mice and their wild-type counterparts were caged in groups of 4, and allowed normal chow (LabDiet, St. Louis, MO) and water *ad libitum*. Body weight was recorded weekly after weaning. Food intake during 3-6 months of age was determined. All experiments involving animals were carried out in accordance with the Animal Lab Requirements as per Animal Use Protocol #16-005 approved by the Animal Care and Use Committee, University of Manitoba.

3. Gonadectomy

Male and female Mito-Ob and wild-type mice were each distributed into four groups of six animals per group as followed: 1) Female sham operated; 2) Female ovariectomized; 3) Male sham operated; 4) Male orchidectomized as shown in Table 2.

Table 2. Number of animals in each treatment group of wild-type and Mito-Ob mice. Ox in female = ovariectomized; Ox in male = orchidectomized.

	Wild-type		Mito-Ob	
	Sham	**Ox**	**Sham**	**Ox**
Male	6	6	6	6
Female	6	6	6	6

Mice were orchidectomized at 12 weeks. Weights of animals were recorded before surgery. After giving isoflurane as anesthesia, gonadal area was cleaned with chlorohexanol and then with 70% ethanol, followed by metacam (2mg/kg) injection subcutaneously as analgesics.

3.1 Orchidectomy

Male mouse was placed in ventral recumbency, and a 1 cm median incision was made in the scrotum and the skin was retracted to expose the tunicia. The tunica was then pierced to extract testes one at a time. The testes were raised to expose the underlying spermatic cord. The spermatic cord was clamped and ligated at the confluence of blood vessels and epididymis. In sham-operated mice, the same procedures were performed except that testes were not removed. Skin incision was sutured up with adhesive wound clips. After the surgery body weight was taken every week for 12 weeks.

3.2 Ovariectomy

Female mouse was placed in ventral recumbency, and a 1-2 cm dorsal midline skin incision was made halfway between caudal end of the ribcage and the base of the tail. The facia was cleared away using blunt end dissection and underlying muscle wall was

pierced on both sides 1cm lateral to the spine. Ovary and oviduct were exteriorized through muscle wall. A hemostat was clamped around the uterine vasculature between oviduct and uterus. Each ovary and part of oviduct were removed with a single cut. Tissue adhesive wound clips were used to close the incision. In sham-operated mice, the same procedures were performed except that ovaries were not removed.

After the surgery, each mouse was given its own cage to recover from with food and water. Postoperative health card was used to record any abnormalities including signs of discomfort and pain. They were monitored every hour during the first 5 hours after waking up from anesthesia, and twice a day after for 3 days. 1mg/kg of metacam was given daily also for 3 days. After a week, would clips were removed. Body weight was taken thereafter every week for 12 weeks.

4. Immunohistochemistry

Liver and adipose tissues from 6 month-old Mito-Ob and wild-type mice were weighed and collected. Tissues were sent to histomorphological services, at the Department of Human Anatomy and Cell Science for paraffin embedding and sectioning. Samples were fixed in 10% formalin in PBS, dehydrated and embedded in paraffin according to routine histologic method for microscopy. Each slide contained 5 µm tissue section stained with hematoxylin-eosin.

5. Lipid Quantification

Histology sections were viewed under light microscope at 10x and 40x magnifications, and 5 images were randomly generated at each magnification using

Infinity 2 digital camera. All 40x images were analyzed with Adiposoft ImageJ. First, the image was split into red, green and blue channels, and the selected green channel with optimized colour balance was saved as a separate file. Then, MRI Adipocyte Tools [231] was added to the ImageJ launcher window. The image was analyzed under the 'S' button using a simple segmentation algorithm and binary watershed. The thresholding method was named 'triangle' with minimum and maximum sizes set between 4,000 and 400,000. Any cells not recognized by the program were added by freehand tracing. Finally, the number and mean cross-sectional area of adipocytes were calculated. A distribution graph was plotted using area as the parameter with 10 specified bins.

6. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

GTT and ITT were performed 12 weeks post-surgery at 6 months of age. Mice were fasted overnight for 16 hours, and their fasting blood glucose levels were taken the next morning right before both tests as time 0. GTT was performed with an intraperitoneal injection of glucose (1g/kg body weight) solution in saline. Blood glucose level was measured through saphenous vein puncture at 15, 30, 60, and 120 min after glucose injection. ITT was performed with an intraperitoneal injection of insulin (0.75 U/kg body weight). Blood glucose level was measured through saphenous vein puncture at 15, 30, 60, and 120 min after insulin injection. Blood glucose concentrations were measured using an ONETouch Ultra glucometer.

7. Cell Extraction and Primary Cell Culture

Visceral white adipose tissue from the peritoneal cavity and subcutaneous white adipose tissue from the femoral region were isolated from male and female Mito-Ob and wild-type mice between 3-4 months of age. Tissues were minced and homogenized through enzymatic digestion by collagenases I in HEPES buffer at 2-4 g tissue samples per 5 ml working solution. The cell mixture was incubated in a shaking water bath at 37^{o}C for 30 min with periodic vortexing until the mixture exhibited a creamy consistency. Cells were then passed through a 250 um nylon mesh filter, and centrifuged at 1,000 x g for 5 min. Pellets were washed with Dulbecco's Modified Eagle Medium (DMEM) and centrifuged at 1,050 x g for 5 min. Cell pellets were resuspended in DMEM and equal number of cells plated on 30 mm petri dishes. Primary adipocyte fibroblasts were attached to culture plate one day after plating. They were then monitored every other day for growth. Upon confluency, they were passaged into separate plates for subsequent treatments. Fibroblasts were only passaged once in the study.

Primary cultures were maintained in DMEM containing 10% FBS, and 5% penicillin-streptomycin in a humidified incubator at 37°C and 5% CO_2. Cells were seeded at a density of 3 x 10^5 measured by TC20 Automated Cell Counter (Bio-Rad, USA). One day after cells became confluent, they were incubated in differentiation media containing 40 µg/mL 3-isobutyl-1-methylxanthine (IBMX), 400 ng/mL dexamethasone, and 0.5 µg/mL insulin in DMEM. Two days after induction, cells were switched to maintenance medium containing 1.0 µg/mL insulin in DMEM for the rest of the differentiation process. Medium was changed every two days. 17β-estradiol and testosterone stocks were prepared by dissolving crude powder with absolute ethanol to

the desired concentrations (1.0 μM) before treatment. Sex steroids were added with differentiation media on day 0.

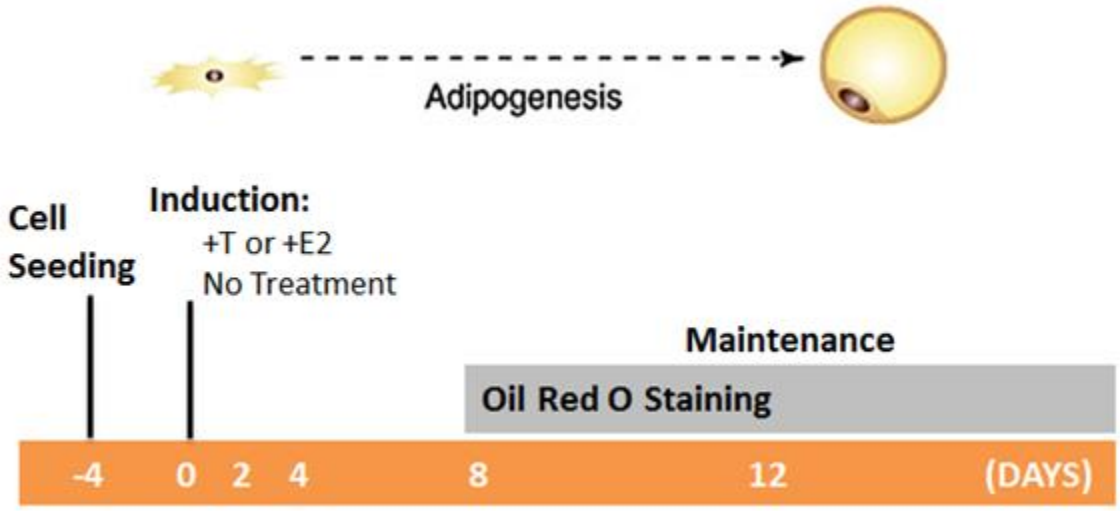

Figure 12. Schematic of Terminal Adipocyte Differentiation Protocol. Cells were seeded 4 days before induction (Day 0). 17β-estradiol (E2) or testosterone (T) was added on induction day to preadipocytes isolated from female or male mouse, respectively. Cell cultures were maintained for 12 days. Cells were collected on Day 8 and Day 12 for oil red O staining.

8. Oil Red O Staining

Primary adipocytes were fixed on Days 0, 4, 8, and 12 in 10% formalin in PBS at room temperature for 1 h. After washing thoroughly with ddH_2O and dehydrated in 60% isopropanol, cells were incubated with a working solution of Oil Red O dye for 2 h. Excess Oil Red O staining was removed and cells were washed immediately with sufficient amount of ddH_2O. Photomicrographs of Oil Red O stained lipid droplets in adipocytes were captured using Olympus BX40 microscope and Lumenera Infinity software (Lumenera Corporation, Ottawa, ON, Canada). The dye was then eluted by adding 100% isopropanol and quantified through absorbance measurement of OD at 500 nm using 100% isopropanol as a blank.

9. Statistical Analysis

All the data were analyzed using two-way ANOVA with Dunnett's multiple comparison or Student's t test. The results are expressed as mean ± SEM. A P value of < 0.05 was considered statistically significant in all cases. All testing and graphing was done using GraphPad Prism 6 software. Treatment results were compared with control. For primary pre-adipocytes, control is defined as cells isolated from CD1 mice with/without treatment of sex steroid hormones. For animal models, control is defined as sham-operated age and sex matched CD1 or Mito-Ob mouse.

IV. RESULTS

Effects of gonadectomy on the weight of Mito-Ob mice

PHB1 and sex steroid hormones are known to regulate each other's function in reproductive tissues and in their cell line derivatives [232]. However, it is not known whether this relationship also exist in non-reproductive tissues such as adipose tissue and its structural unit adipocytes. During phenotypic characterization of Mito-Ob, it was observed that the Mito-Ob mice start to gain weight after puberty indicating a potential relationship with pubertal surge of sex steroid hormones [13]. To find out if this is the case, growth of gonadectomized and sham-operated male and female Mito-Ob mice and their age matched wild type mice were followed for 3 months post gonadectomy.

In the male Mito-Ob mice, gonadectomy prevented their weight gain, while sham operated Mito-Ob mice showed continuous weight gain during the entire follow-up period of 3 months (Fig. 13A). Orchidectomized male Mito-Ob mice had significantly lower body weight at 2-3 months ($P < 0.05 - 0.01$) post surgery compared with sham-operated male Mito-Ob mice (Fig. 13A). In contrast to male Mito-Ob mice, orchidectomized wild type mice showed an increasing trend in body weight gain compared with sham-operated wild type mice, which became significantly different ($P < 0.05$) after two months post-surgery (Fig. 13A). This is in keeping with previous literature stating an inverse relationship between testosterone and adipose tissue content. This data suggests that male Mito-Ob mice indeed require testicular steroid hormones for the development of obesity and the absence of testicular steroid hormones attenuated weight gain induced by PHB1 overexpression in adipocytes.

Consistent with available information in the literature, ovariectomy resulted in an increase in the body weight of wild type mice, which became significantly higher than sham operated control mice ($P < 0.05$) after two months post-ovariectomy (Fig. 13B). Similar to male Mito-Ob mice, gonadectomy in female Mito-Ob mice prevented weight gain compared with sham-operated female Mito-Ob mice ($P < 0.05$-0.01; Fig. 13B). This data would imply that similar to male Mito-Ob mice, the female Mito-Ob mice also require ovarian steroid hormones for PHB1-induced obesity.

Collectively, these data suggest that sex steroid hormones modulate PHB1 functions in adipocytes; the absence of sex steroid hormones prevented weight gain observed in PHB1 overexpressed Mito-Ob mice.

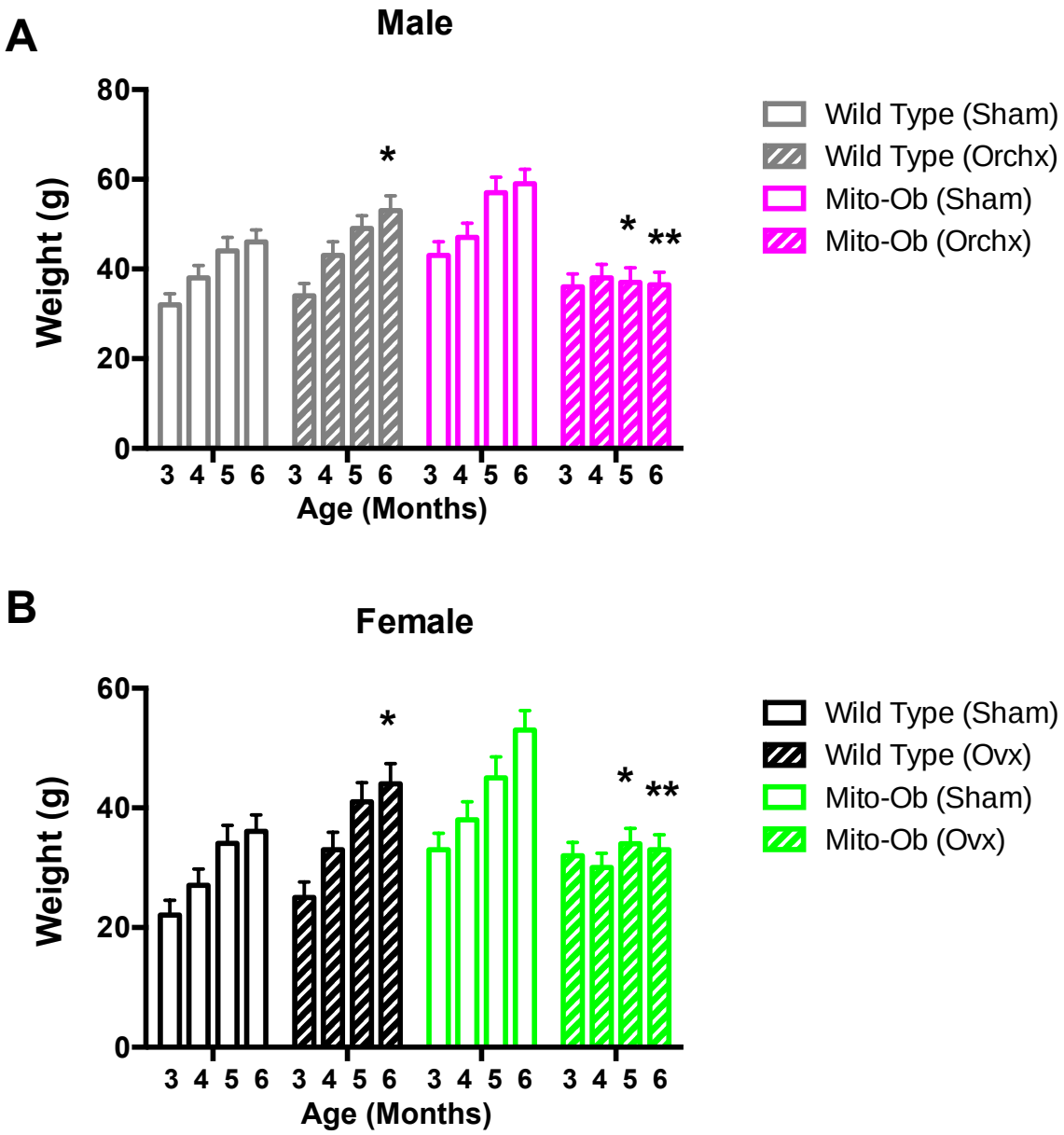

Figure 13. Histograms showing the effect of gonadectomy on the growth of (A) male Mito-Ob mice and (B) female Mito-Ob mice. Age and sex matched sham operated Mito-Ob mice and wild type mice are shown as controls. Data are presented as Mean ± SEM (n = 6). Ovx – ovariectomy; Orchx – orchidectomy. * $P < 0.05$ and ** $P < 0.01$ age and sex match gonadectomized mice vs sham operated control within each experimental group as determined by Student's *t*-test.

Effects of gonadectomy on food intake in Mito-Ob mice

To determine whether gonadectomy-induced changes in the weight gain of Mito-Ob and wild type mice are due to corresponding changes in the amount of food consumption, we measured food intake in the gonadectomized Mito-Ob and wild type mice and respective sham operated control mice. No significant difference in food intake was observed between wild type and Mito-Ob mice in sham control group, which is consistent with the previous finding that Mito-Ob mice develop obesity independent of food intake [13]. However, a deceasing trend in food intake in gonadectomized wild type and Mito-Ob mice was observed in comparison with respective sham operated control mice implying regulation on satiety may be altered due to lack of sex steroid hormones (Fig. 14). Together, these data suggest that gonadectomy-associated changes in the weight of male and female Mito-Ob mice their wild type counterparts are not affected by their food intake.

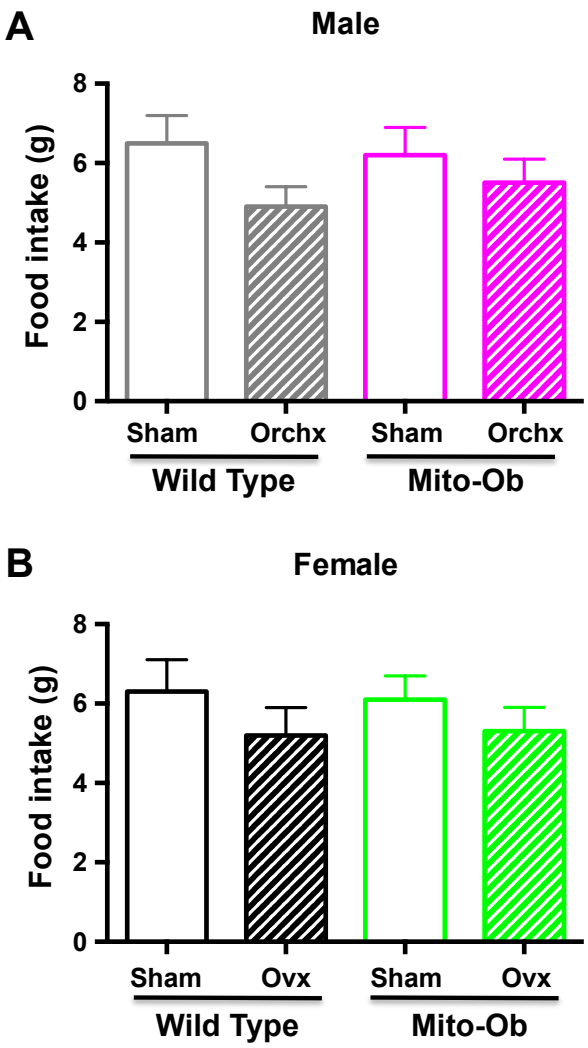

Figure 14. Histograms showing the effect of gonadectomy on the food intake in (A) male Mito-Ob mice and (B) female Mito-Ob mice. Age and sex matched sham operated Mito-Ob mice and wild type mice are shown as controls. Data are presented as Mean ± SEM (n = 6). Ovx – ovariectomy; Orchx – orchidectomy. No significant difference was found between sham operated control and gonadectomized mice within each experimental group.

Effects of gonadectomy on adipose tissue in Mito-Ob mice

To further explore the effect gonadectomy on adipose tissue growth in a sex-specific manner, visceral and subcutaneous fat pad depot weights were taken at the end of 3 months post gonadectomy. Orchidectomy resulted in significant decrease in subcutaneous and visceral adipose tissue weights ($P < 0.01$) in Mito-Ob mice compared with sham-operated male Mito-Ob mice (Fig. 15B). This difference in adipose tissue weight was not observed between orchidectomized and sham-operated wild type mice (Fig. 15A). Instead an increasing trend in visceral adipose tissue weight was observed in gonadectomized mice compared with sham control mice (Fig. 15A). Amongst adipose tissue depots in male Mito-Ob mice, relative decrease in visceral adipose tissue weight was higher than subcutaneous adipose weight (Fig. 15A, B).

In the case of female mice, wild type mice showed an increasing trend in weight of both adipose depots whereas significant reduction in visceral and subcutaneous tissue weight was found in Mito-Ob mice compared with respective sham operated control mice (Fig. 15C, D). Interestingly, amongst adipose tissue depots in female Mito-Ob mice, relative decrease in visceral adipose tissue weight was lower than subcutaneous adipose weight (Fig. 15). Collectively, these data suggest that in the absence of gonadal sex steroid hormones, PHB1 overexpression in adipocytes differentially reduced adipose tissue depot weight in male and female mice.

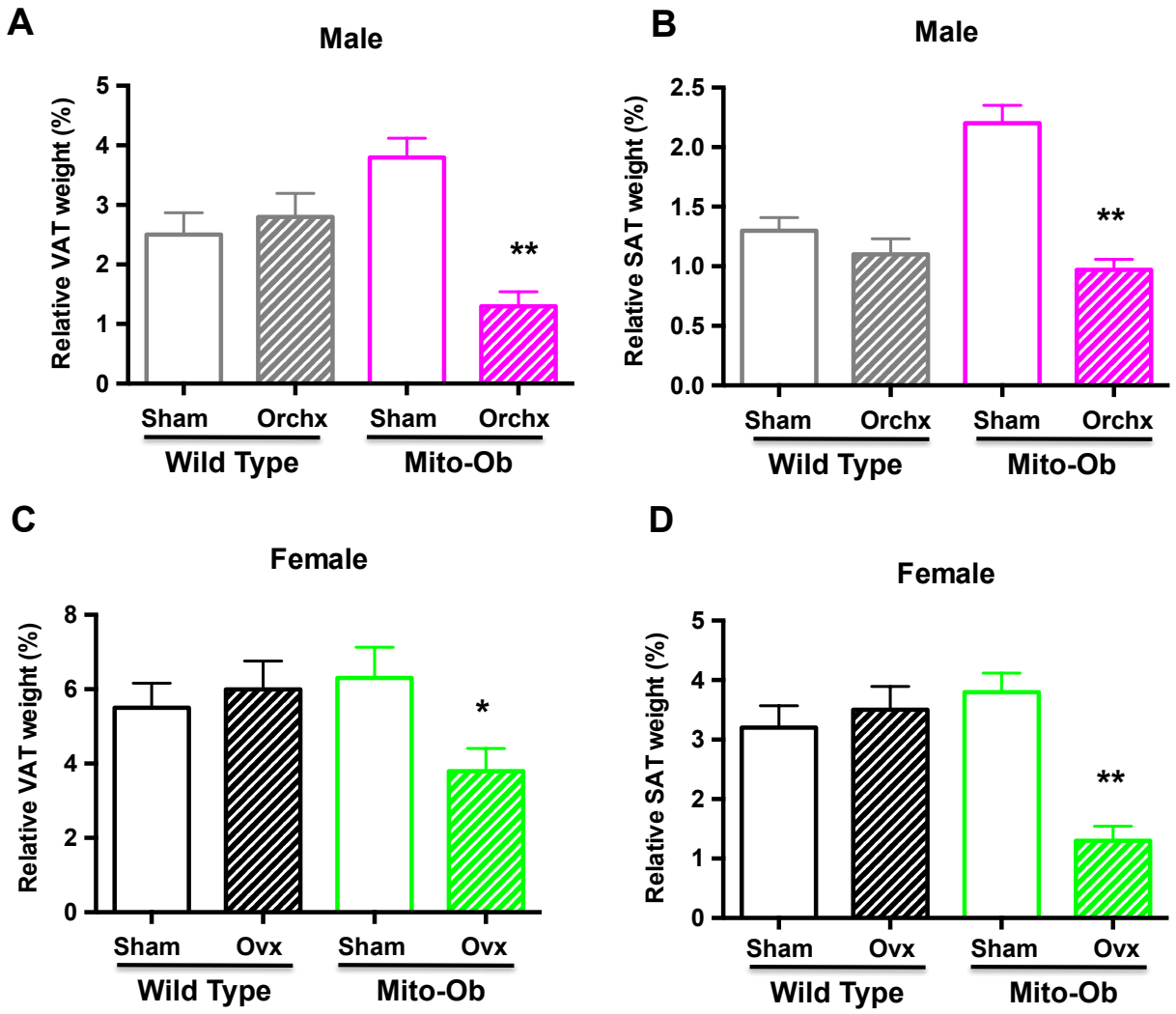

Figure 15. Histograms showing the effect of gonadectomy on (A, C) visceral adipose tissue weight and (B, D) subcutaneous adipose tissue weight in Mito-Ob mice. Age and sex matched sham-operated Mito-Ob mice and wild type mice are shown as controls. Data are presented as Mean ± SEM (n = 6). Ovx – ovariectomy; Orchx – orchidectomy; SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue. * $P < 0.05$ and ** $P < 0.01$ represent significant differences between age and sex match gonadectomized mice and sham operated control within each experimental group as determined by Student's *t*-test. No significant difference was found between sham operated and gonadectomized wild type mice.

Histological and morphometric analyses of adipose tissue from Mito-Ob mice

A first look at the histological analysis of adipose tissue from gonadectomized Mito-Ob and wild type mice revealed alterations in adipocyte sizes and displayed a distinct pattern between genotype and sexes. Gonadectomy-induced changes in adipocyte sizes may have contributed to changes in adipose tissue weight of Mito-Ob and wild type mice. To examine the structural basis of the differential effects of sex steroids on adipose tissue growth, size of adipocytes in subcutaneous and visceral adipose depot was determined by cross-cell area measurement. In male Mito-Ob mice, no significant difference was found in the area of subcutaneous and visceral adipocytes from the gonadectomized and sham-operated control group (Fig. 16B). In female Mito-Ob mice, gonadectomy resulted in a significant reduction in the size of adipocytes in both visceral and subcutaneous adipose tissue compared with sham-operated control groups ($P < 0.05$; Fig. 17B). However, in female wild type mice, gonadectomy resulted in an increasing trend in adipocyte size (Fig. 17B).

Taken together, these data suggest a sex dimorphic and depot specific effects of gonadectomy on the size of adipocytes compared with respective control groups. These results imply that sex steroid hormones and PHB1 together play a role in the regulation of cellular dynamics of adipocytes and triglycerides homeostasis within adipocytes.

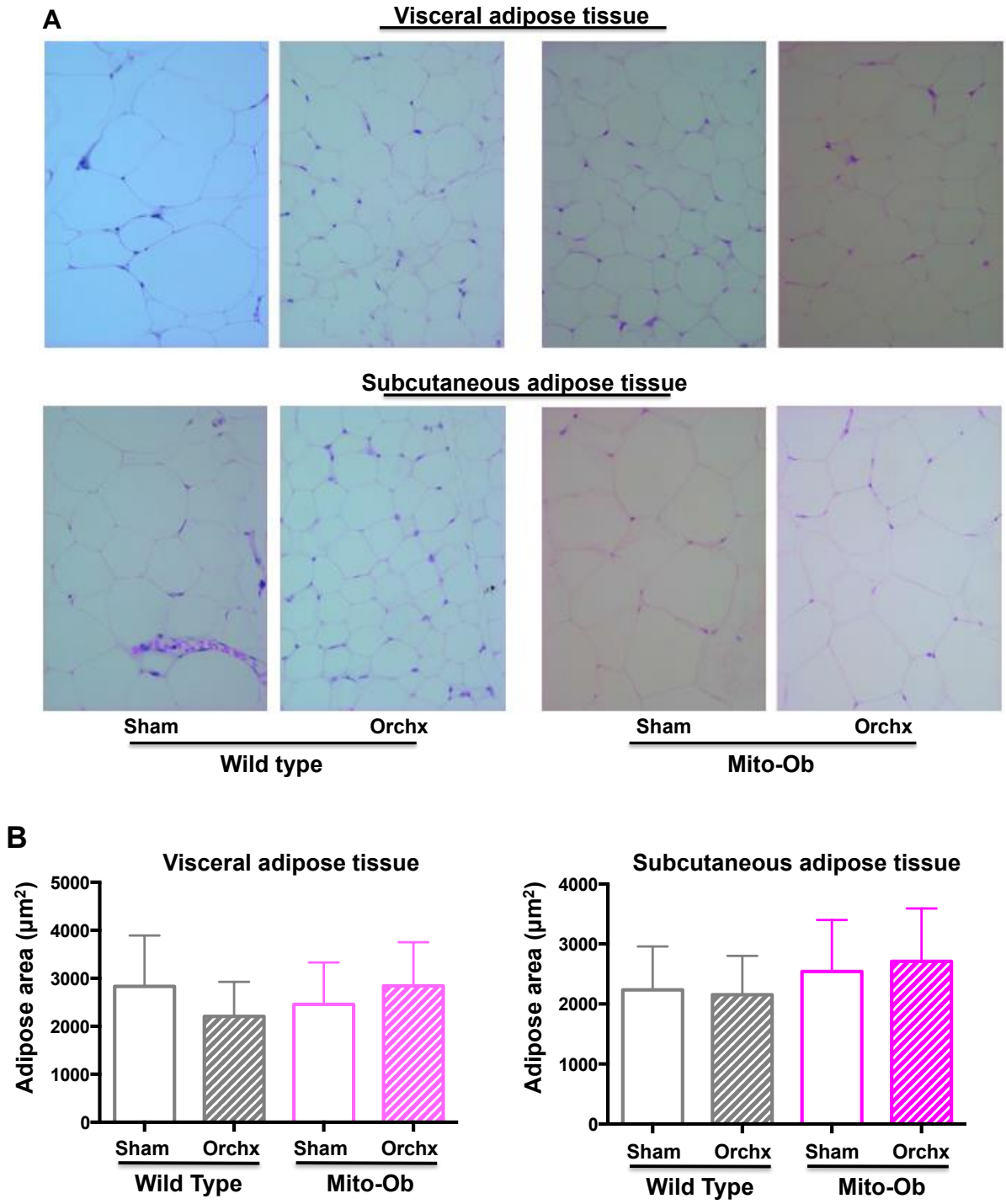

Figure 16. (**A**) Representative histomicrographs showing hematoxylin and eosin stained adipose tissue from orchidectomized (Orchx) and sham-operated male Mito-Ob and wild type mice (40x). (**B**) Histograms showing quantification of adipocyte area. Data are presented as Mean ± SEM (n = 6).

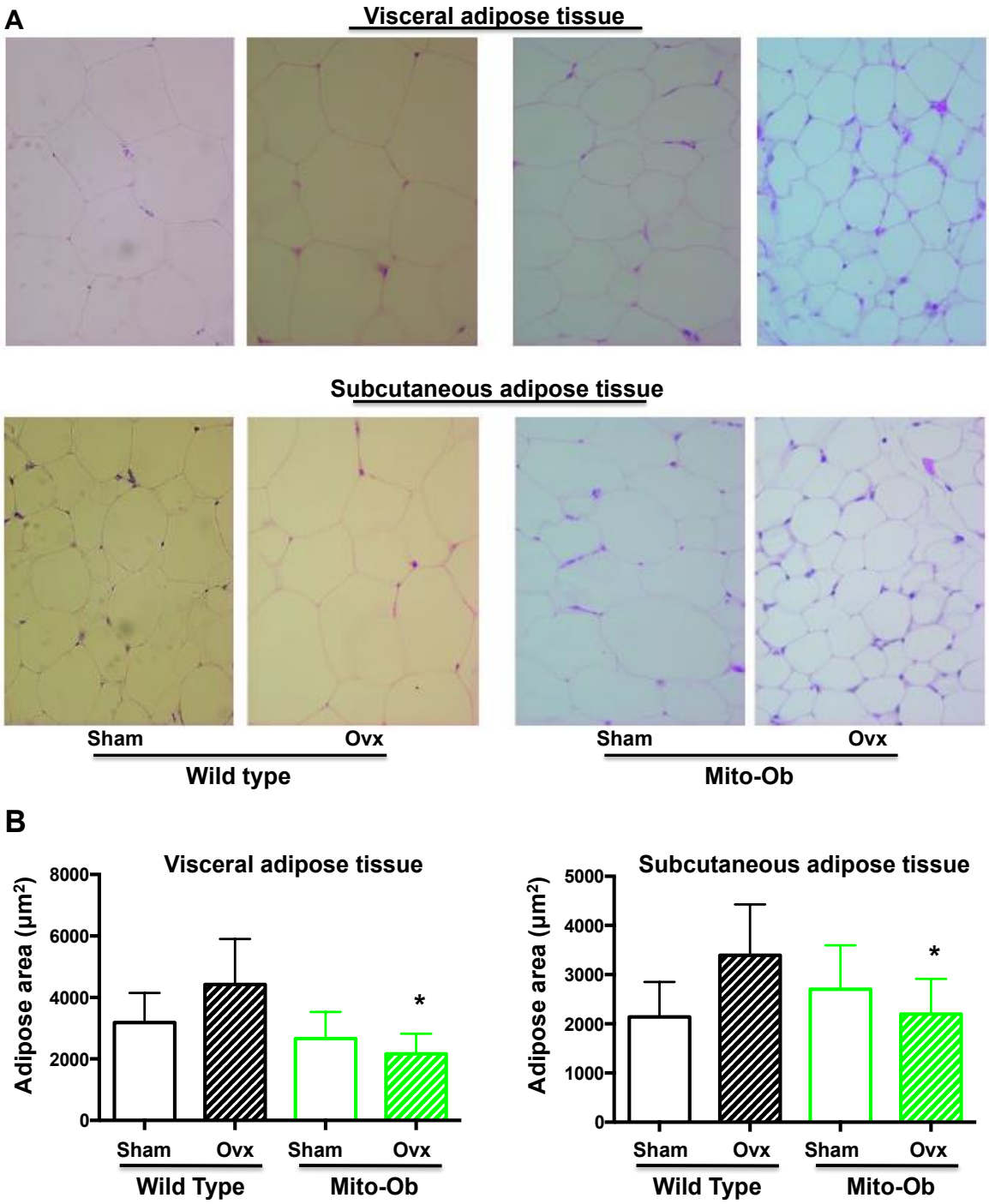

Figure 17. (**A**) Representative histomicrographs showing hematoxylin and eosin stained adipose tissue from the ovariectomized (Ovx) and sham-operated female Mito-Ob and wild type mice (40x). (**B**) Histograms showing quantification of adipocyte area. Data are presented as Mean ± SEM (n = 6). * $P < 0.05$ represents significant differences between Ovx mice and sham control as determined by Student's *t*-test.

Frequency distribution of different sizes of adipocytes in visceral adipose tissue

In male Mito-Ob mice, the frequency distribution of visceral adipocytes showed similar pattern between orchidectomized and sham control Mito-Ob mice (Fig. 18B, D). The cumulative numbers of larger adipocytes were higher in sham control Mito-Ob mice compared with orchidectomized mice. However, in the orchidectomized wild type mice the distribution frequency and pattern were distinct from Mito-Ob mice, and peaked toward smaller size (Fig. 18A, C).

In female Mito-Ob mice, ovariectomy resulted in a change in the frequency distribution of visceral adipocyte by sizes, which peaked towards smaller size compared with female sham Mito-Ob mice (Fig. 19B, D). This decrease in adipocyte size is correlated with the significant decrease in tissue weight observed in visceral depot (Fig. 17B). On the other hand, an opposite effect on distribution frequency was observed in the ovariectomized wild type mice compared with sham control mice, where ovariectomized mice had more adipocytes of larger size (Fig. 19C). Again, the increase in adipocyte size is correlated with the increased trend in visceral tissue weight found in ovariectomized wild type mice (Fig. 17B).

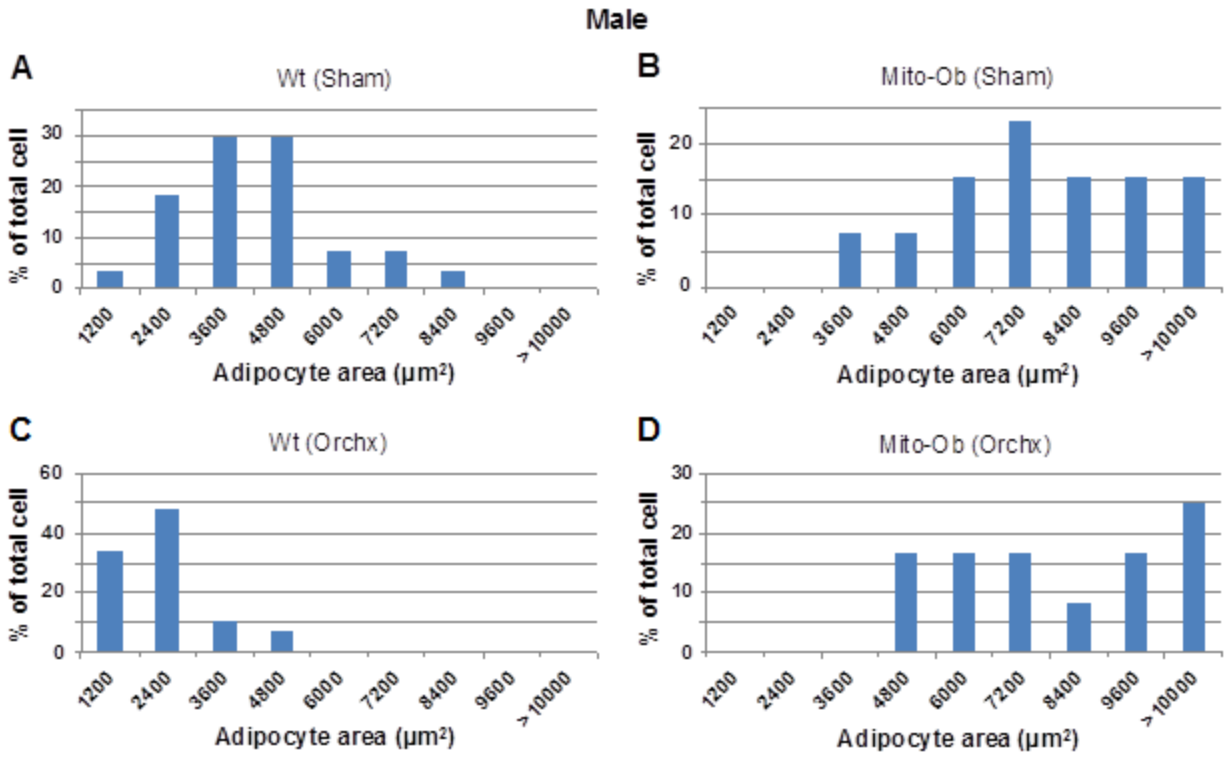

Figure 18. Histograms showing the effect of orchidectomy (Orchx) on the frequency distribution of visceral adipocyte sizes in male Mito-Ob mice compared with age and sex matched sham-operated control. Orchidectomized and sham-operated wild type mice are shown as control (A, C). Data are presented as percentage mean of total cell.

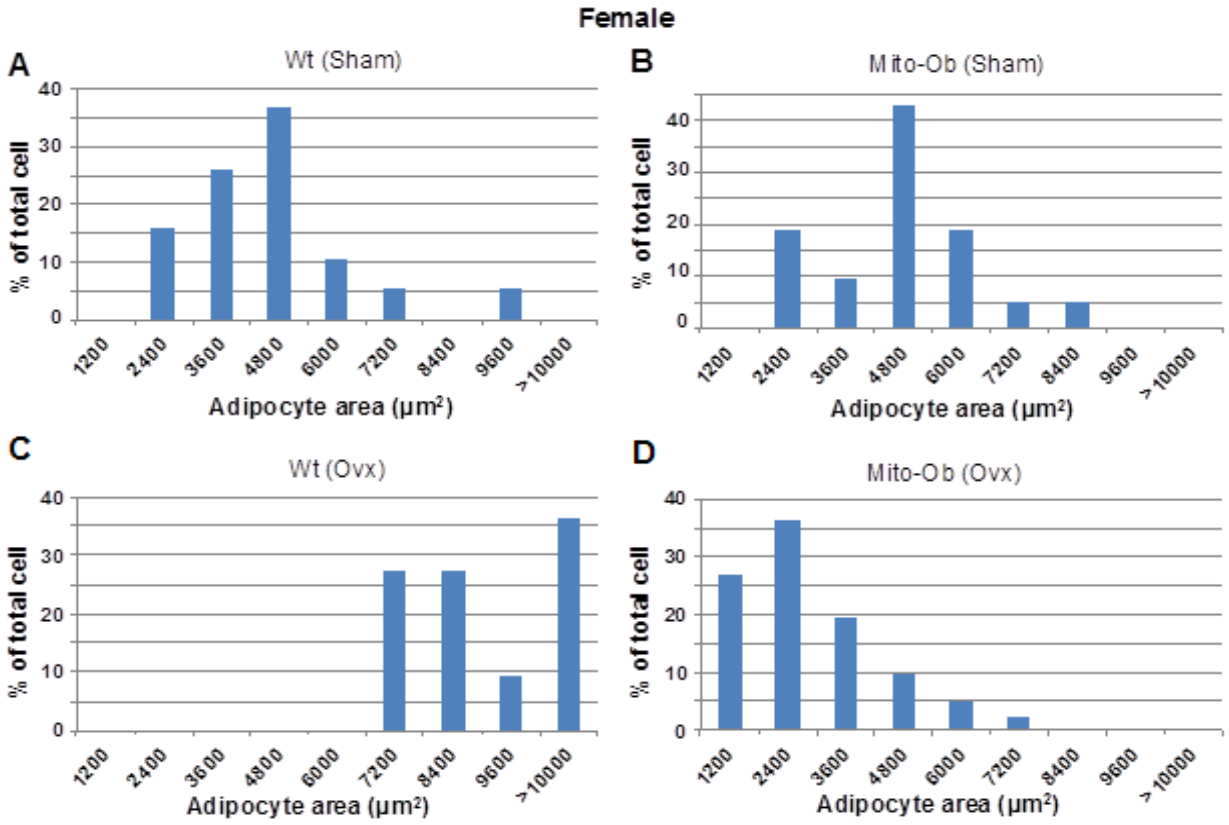

Figure 19. Histograms showing the effect of ovariectomy (Ovx) on the frequency distribution of visceral adipocytes sizes in female Mito-Ob mice compared with age and sex matched sham-operated control. Ovariectomized and sham-operated wild type mice are shown as control (A, C). Data are presented as percentage mean of total cell.

Effects of gonadectomy on the liver in Mito-Ob mice

Previous studies in our laboratory have shown that Mito-Ob mice develop obesity-related non-alcoholic fatty liver and steatohepatitis in a male sex-specific manner [13,232] suggesting a potential involvement of sex steroid hormones in sex dimorphic hepatic phenotype in Mito-Ob mice. To determine whether gonadectomy had any effect on liver phenotype in Mito-Ob mice, liver tissue samples were analyzed. Gonadectomy resulted in a significant reduction in liver weight in male Mito-Ob in comparison with sham control mice (Fig. 20A). A similar trend was found in gonadectomized male wild type mice, but the difference was not significant compared with sham control mice (Fig. 20A). This effect was not observed in gonadectomized female Mito-Ob mice or wild type mice (Fig. 20B). These results suggest that gonadectomy in Mito-Ob mice lead to reduction in liver weight specifically in male Mito-Ob mice.

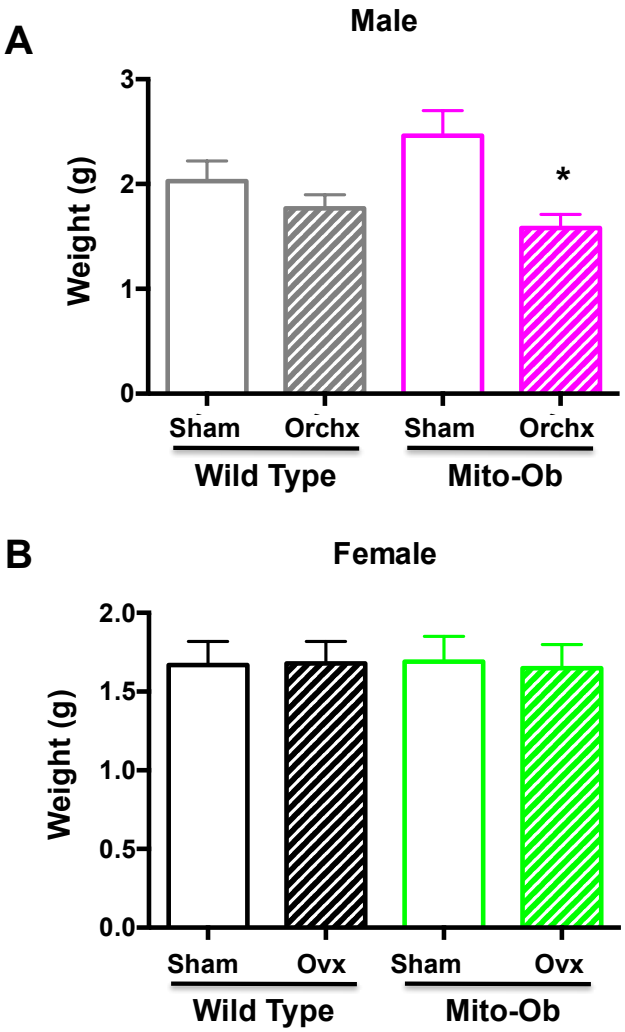

Figure 20. Histograms showing the effect of gonadectomy on the liver weight in male and female Mito-Ob mice in comparison with age and sex matched wild type mice. Sham operated mice are included as controls. Data are presented as Mean ± SEM (n = 6). Ovx – ovariectomy; Orchx – orchidectomy. * $P < 0.05$ represent significant differences between orchidectomized Mito-Ob mice and sham operated male Mito-Ob mice as determined by Student's *t*-test.

Structural differences in liver histology

Histological analyses of liver tissue from Mito-Ob mice showed sex-specific changes post gonadectomy. Male Mito-Ob mice showed an improvement in fatty liver compared with respective sham-operated control mice whereas immune cell infiltration was observed in female Mito-Ob mice (Fig. 21). Immune cell infiltration was not observed in the liver from gonadectomized wild type mice of either sex (Fig. 21). Taken together, these data imply that sex differences in the relationship between PHB1 and sex steroids in adipose tissue biology have a role in sex dimorphic hepatic phenotype in Mito-Ob mice.

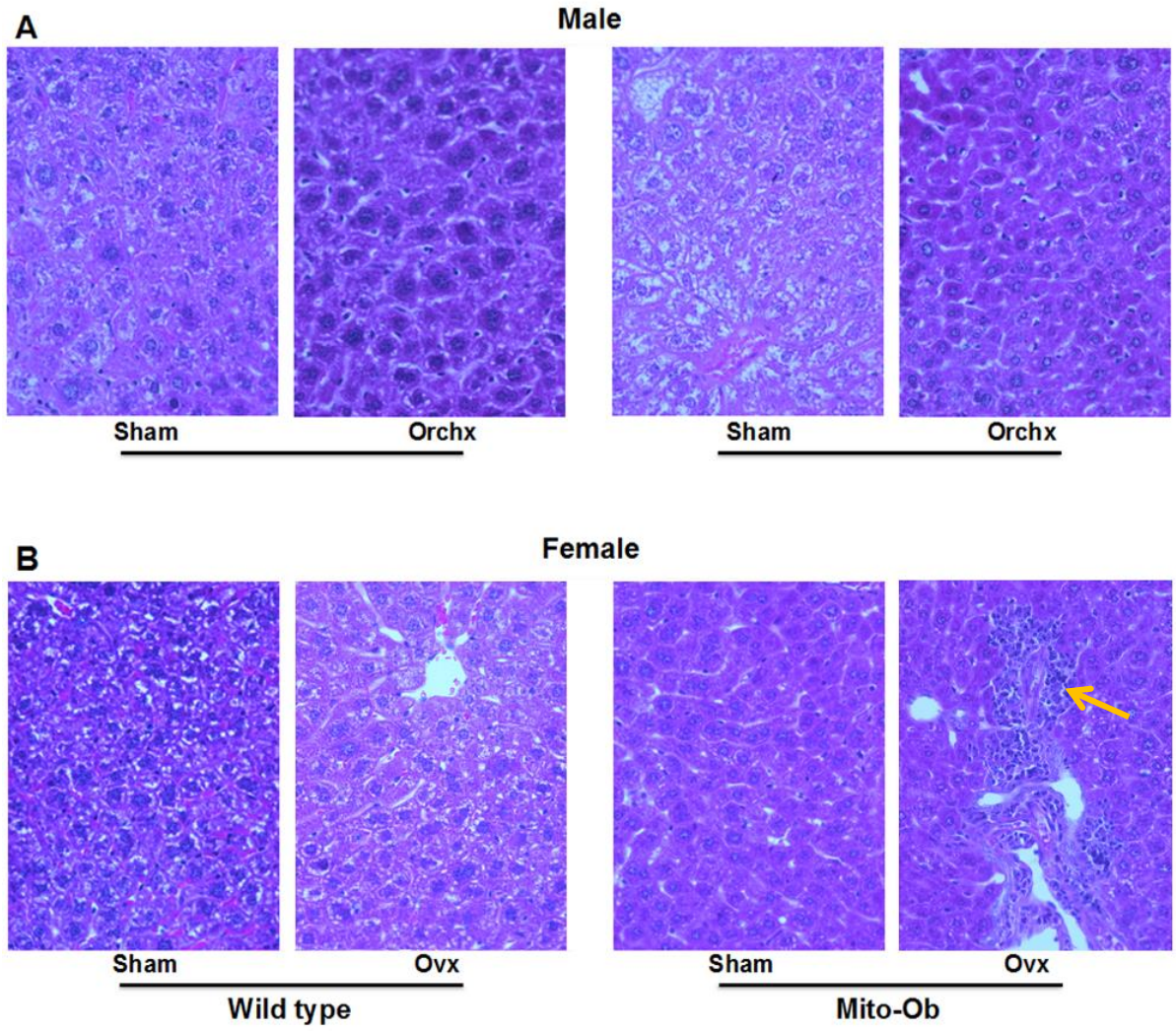

Figure 21. Representative photomicrographs of hematoxylin and eosin stained liver sections from male and female Mito-Ob mice and respective wild type mice. Liver from sham operated mice in each case are included as control. (n = 6 in each experimental groups). Immune cell infiltration in ovariectomized female Mito-Ob mice is shown with orange arrow.

Effect of gonadectomy on glucose homeostasis in Mito-Ob mice

Sex steroid hormones are known to influence metabolic homeostasis in rodents and humans, which appear to be mediated through their effects on major metabolic tissues including adipose tissue [1,190]. However, downstream intermediates that mediate such effects are largely unknown. Previous studies in our laboratory have shown that Mito-Ob mice develop obesity in a sex-neutral manner but obesity-associated impaired glucose homeostasis in a male sex-specific manner suggesting a potential relationship between sex steroid hormones and PHB1 in obesity-related metabolic differences in male and female Mito-Ob mice [13]. To further explore the relationship between sex steroid hormones and PHB1 in metabolic regulation in Mito-Ob mice, glucose homeostasis and insulin sensitivity in gonadectomized Mito-Ob mice were investigated. In male Mito-Ob mice, orchidectomy significantly improved glucose tolerance ($P < 0.05$) during first 15-30 minutes of the glucose tolerance test compared with sham operated control mice (Fig. 22A). However, this difference started to taper after 1 hour and eventually became insignificant at the 120 minutes' time point (Fig. 22A). An improvement in glucose tolerance was also observed in the orchidectomized wild type mice compared with sham operated control mice (Fig. 22A). There was a significant difference between the two groups at 30 and 60 minutes, which was eventually tapered by 120 minutes time point similar to that in Mito-Ob mice (Fig. 22A).

Interestingly, no difference in serum glucose concentration was found between the ovariectomized and sham operated female Mito-Ob mice and wild type mice (Fig. 22B). Of note, female Mito-Ob mice displayed relatively better glucose tolerance than

respective wild type control groups especially during first 30 minutes of glucose tolerance test (Fig. 22B).

Taken together, these data suggest that PHB1 overexpression in adipocytes leads to impaired glucose homeostasis in male sex-specific manner. This would imply that intrinsic sex differences exist in adipose tissue function, which may contribute to sex differences in metabolic status in health and disease process. In addition, our data suggests that overexpression of PHB1 in adipocytes further polarize sex differences in adipose tissue functions especially in the presence of gonadal steroid hormones.

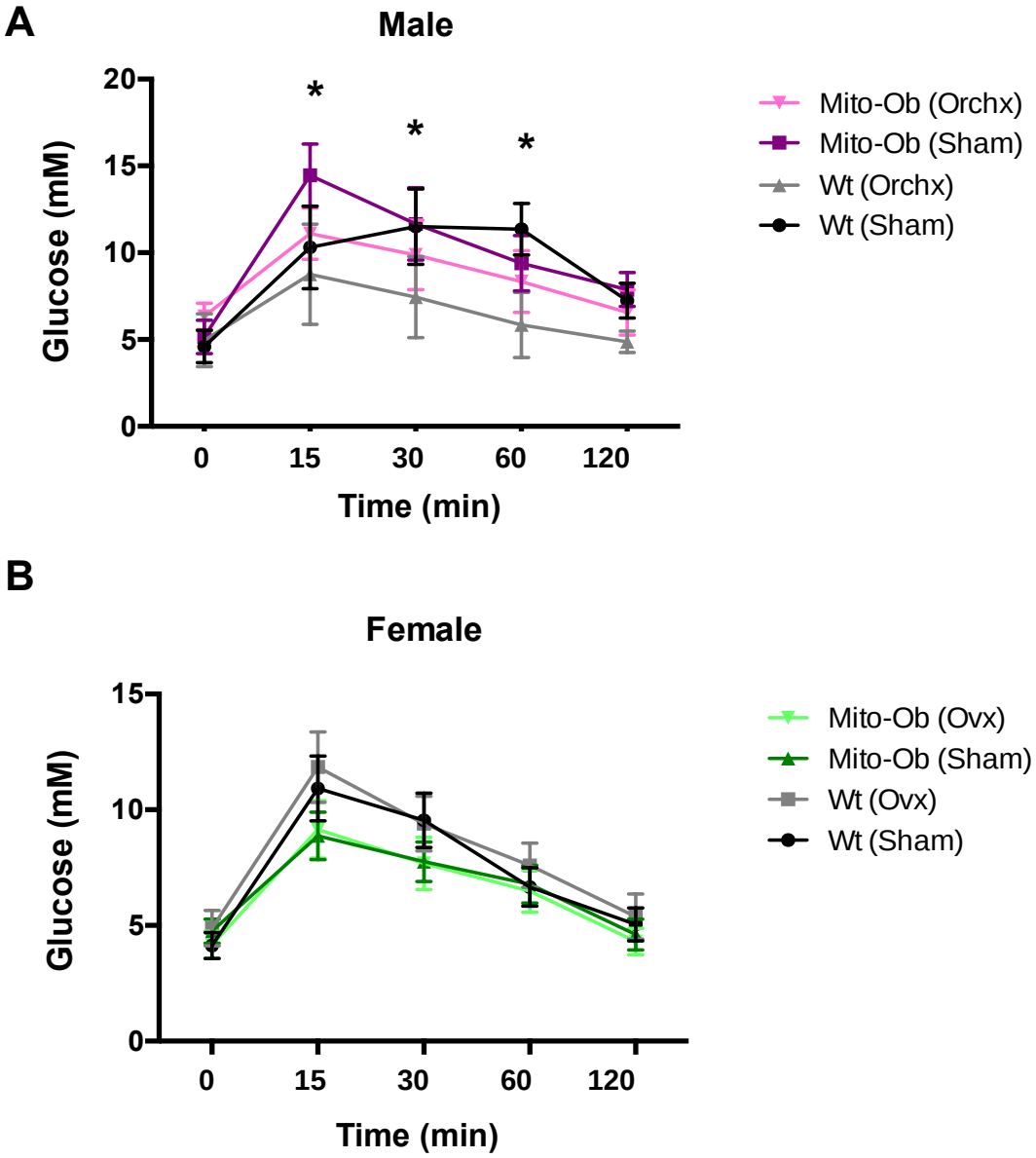

Figure 22. Line graphs showing the effect of gonadectomy in male and female Mito-Ob mice on glucose tolerance as determined by glucose tolerance test. Sham operated mice are included as control. Data are presented as Mean ± SEM (n = 6). Ovx–ovariectomy; Orchx–orchidectomy. * $P < 0.05$ represent significant differences between orchidectomized Mito-Ob mice and sham operated male Mito-Ob mice as determined by Student's *t*-test.

Effect of gonadectomy on insulin sensitivity in Mito-Ob mice

Previous studies in our laboratory have found that male Mito-Ob mice develop insulin resistance, whereas female Mito-Ob mice display normal insulin sensitivity similar to their wild type littermates [13]. To determine whether sex steroid hormones have a role in sex-specific insulin resistance in Mito-Ob mice, we investigated the effect of gonadectomy on insulin sensitivity by insulin tolerance test. No difference in insulin tolerance was found between orchidectomized male Mito-Ob mice compared with sham operated Mito-Ob mice during first 60 minutes of ITT (Fig. 23A). Subsequently, insulin tolerance curve started to bifurcate between the two groups but remain insignificant throughout the 120 minutes (Fig. 23A). In wild type male mice, orchidectomy resulted in significant increase in insulin sensitivity compared with sham control mice (Fig. 23A). The data suggests that male Mito-Ob mice respond differently to insulin compared with wild type mice in the absence of sex steroids.

In contrast to male Mito-Ob mice, female Mito-Ob mice displayed no difference in insulin sensitivity in comparison with sham control female Mito-Ob mice throughout the insulin tolerance test (Fig. 23B). Ovariectomized female wild type mice showed similar trend in insulin sensitivity as in the case of orchidectomized male wild type mice (Fig. 23B). The magnitude of difference between ovariectomized and sham control mice was relatively smaller in female wild type mice than in male wild type mice (Fig. 23B). Collectively, these data suggest that PHB1 overexpression in adipocytes nullifies the effects of sex steroid hormones on glucose tolerance and insulin sensitivity.

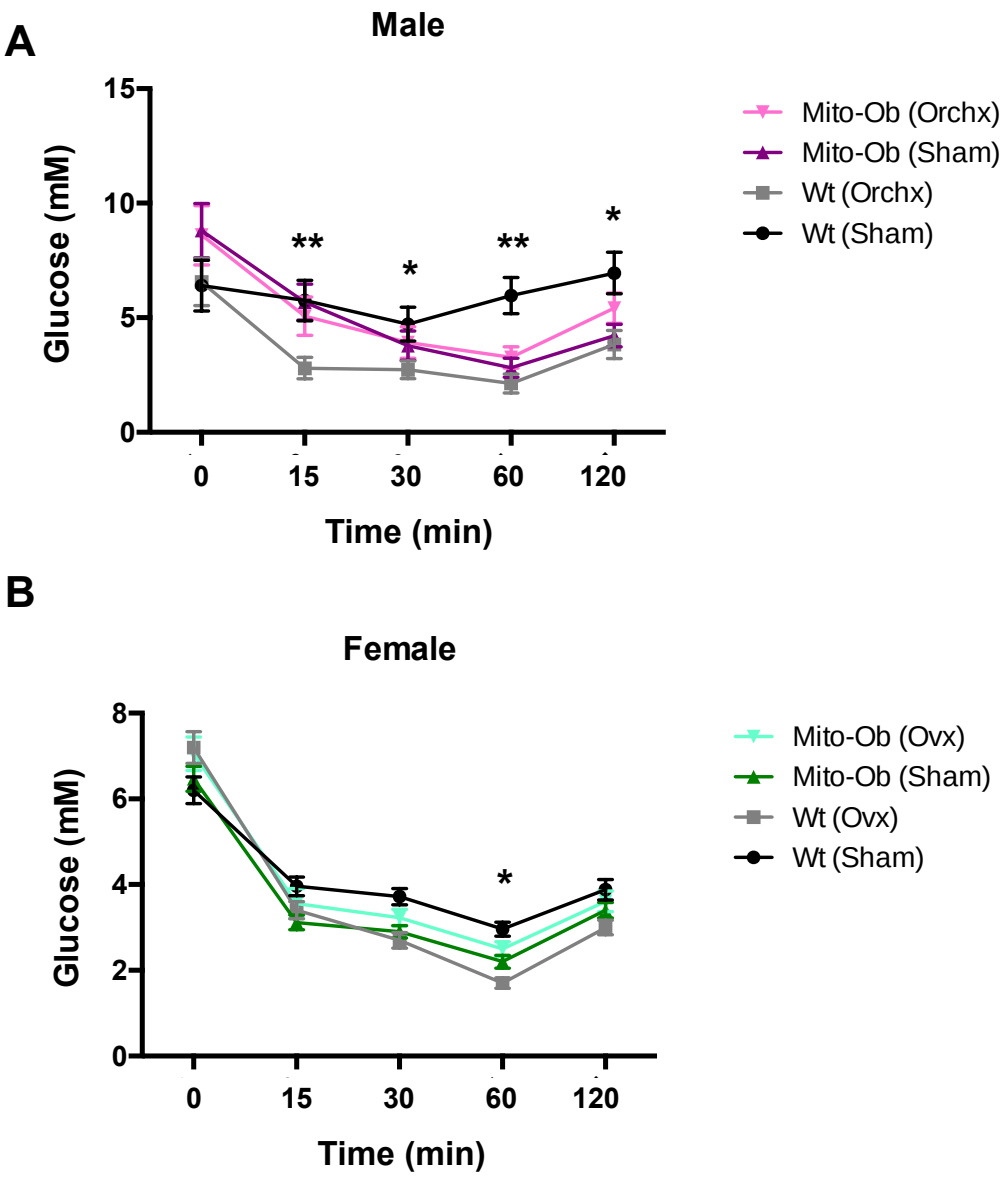

Figure 23. Line graphs showing the effect of gonadectomy in male and female Mito-Ob mice on insulin sensitivity as determined by insulin tolerance test. Sham operated mice are included as control. Data are presented as Mean ± SEM (n = 6). Ovx – ovariectomy; Orchx – orchidectomy. * $P < 0.05$ and ** $P < 0.01$ represent significant differences between gonadectomized wild type mice and sham operated male wild type mice as determined by Student's *t*-test.

Effect of sex steroid hormones on the differentiation of preadipocytes from Mito-Ob mice *in vitro*

To further define the relationship between PHB1 and sex steroid hormones in adipose tissue, differentiation of preadipocytes isolated from Mito-Ob mice was studied with and without treatment of sex steroid hormones. Oil Red O staining was employed to track lipid droplet accumulation. In Mito-Ob and wild type cell cultures, testosterone inhibited preadipocyte differentiation in comparison with respective control group without testosterone supplementation (Fig. 24). No significant difference in preadipocyte differentiation was found between wild type and Mito-Ob in the presence of testosterone. Although insignificant, it was observed that in the presence of testosterone treatment, preadipocytes from Mito-Ob mice showed increased differentiation potential compared with preadipocytes from wild type mice.

Degree of differentiation of subcutaneous preadipocytes from female wild type and Mito-Ob mice was again measured by Oil Red O staining. The result showed similar differentiation potential between wild type and Mito-Ob preadipocytes without estradiol (Fig. 25). However, in the presence of estradiol, an increase in preadipocytes differentiation was found from wild type but not from Mito-Ob mice (Fig. 25). This disparity between adipose tissue phenotype from wild type and Mito-Ob mice with their *in vitro* differentiation capacity would imply potential involvement of additional factors in the relationship between estradiol and PHB1 in adipose tissue biology.

Due to the relatively poor differentiation potential of visceral preadipocytes in comparison with subcutaneous preadipocytes *in vitro*, experiments were not pursued in

this adipose depot. Based on preliminary data, a similar trend in differentiation of visceral preadipocytes from wild type and Mito-Ob mice was observed with or without sex steroid treatment (data not shown).

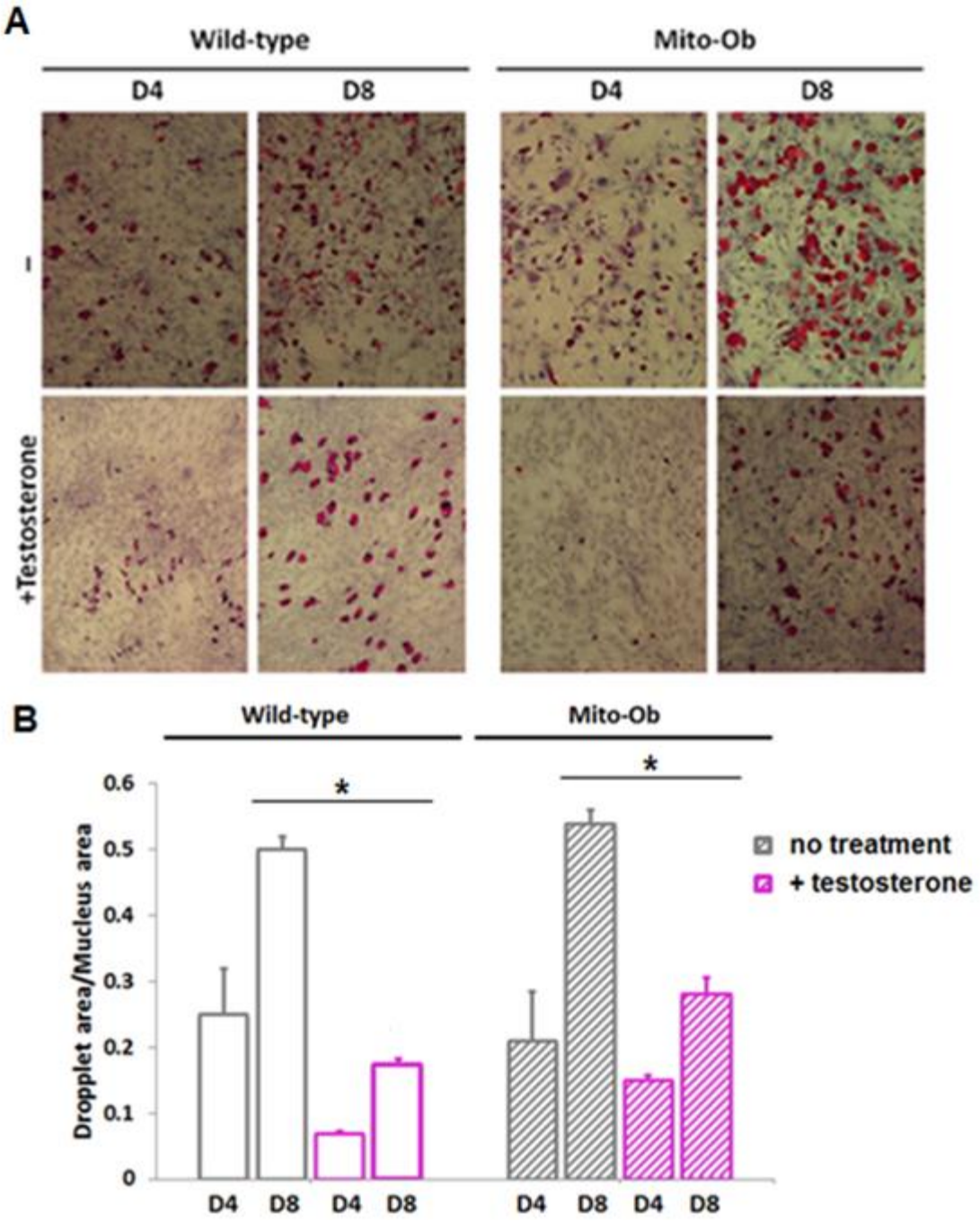

Figure 24. (A) Representative photomicrographs showing differentiation of subcutaneous preadipocytes from male Mito-Ob mice with and without testosterone supplementation as determined by Red Oil O staining (40x). (**B**) Histograms showing quantification of adipocyte differentiation. Preadipocytes from male wild type mice were included as control. Experiments were repeated 3-4 times. * $P < 0.05$ represents significant differences between wild-type and Mito-Ob cells from day 8 (D8) as determined by Student's *t*-test.

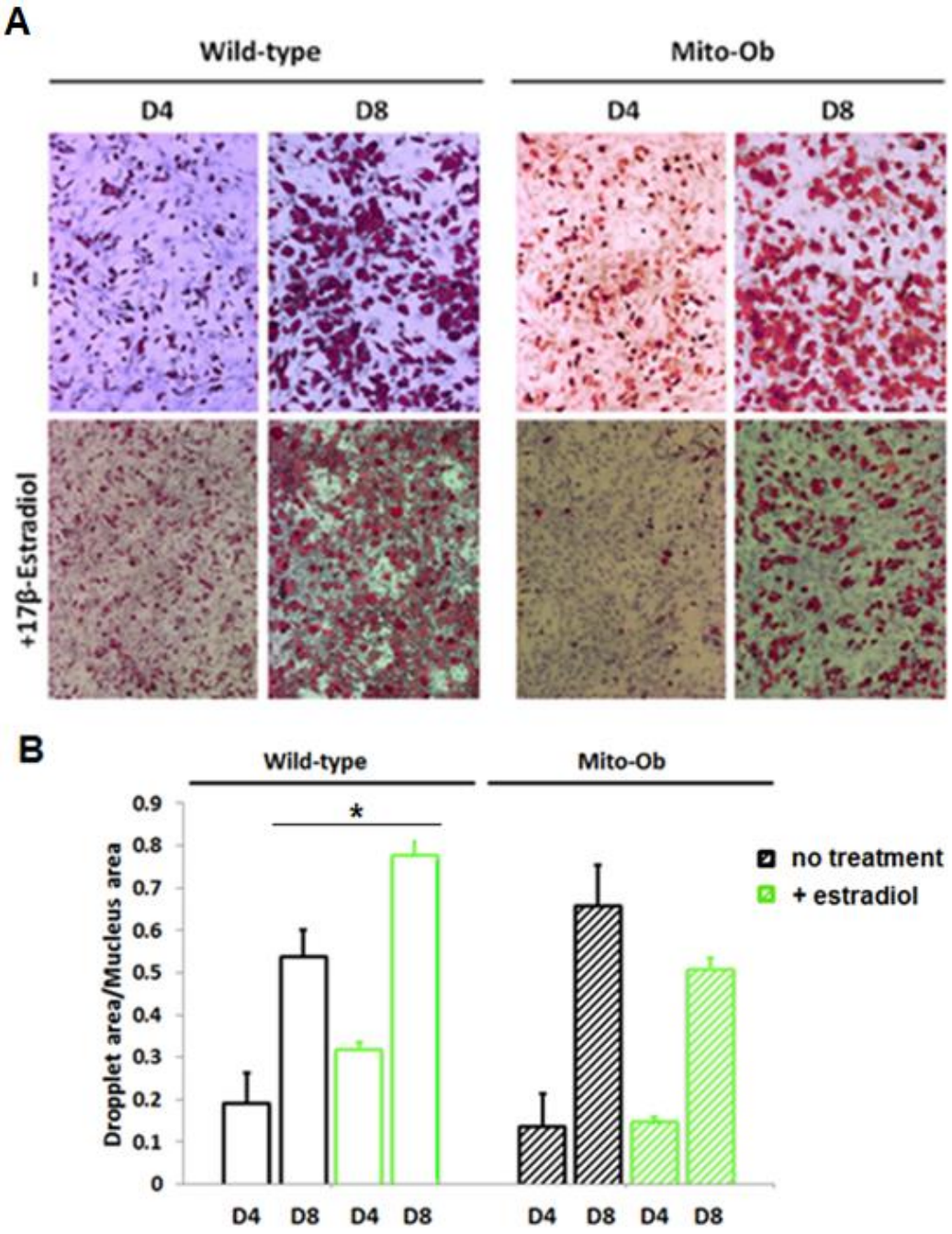

Figure 25. (A) Representative photomicrographs showing differentiation of subcutaneous preadipocytes from female Mito-Ob mice with and without estradiol supplementation as determined by Red Oil O staining (40x). (**B**) Histograms showing quantification of adipocyte differentiation. Preadipocytes from female wild type mice were included as control. Experiments were repeated 3-4 times. * $P < 0.05$ represents significant differences between wild-type and Mito-Ob cells from day 8 (D8) as determined by Student's *t*-test.

V. DISCUSSION

Adipose tissue is a complex, multicellular structure that profoundly influences the function of nearly all other organ systems in the body through its diverse metabolites and adipokines [233]. Adipocyte, the structural and functional unit of adipose tissue plays a key role in maintaining whole body energy and metabolic homeostasis, and sex steroid hormones have a role in both adipose tissue biology and in energy homeostasis [234]. Furthermore, sex differences are known to exist in the role of adipose tissue in energy and metabolic homeostasis in the body [1,205,214,235]. However, various factors that mediate such differences in adipose tissue functions and consequently on the body's metabolic homeostasis are largely unexplored. Deciphering sex differences in adipocyte biology is an important component of understanding sex differences in the role of adipose tissue in energy homeostasis and metabolic regulation in the body.

Previous work from our laboratory has shown that a protein, prohibitin-1 (PHB1), has an important role in adipocyte differentiation (adipogenesis) [12]. Subsequently, similar findings were also published from other laboratories, confirming our discovery of PHB1 as an important protein in adipocyte differentiation *in vitro* [184,236]. To further explore the role of PHB1 in adipose tissue biology at the systemic level, our laboratory developed a novel transgenic mouse by over-expressing PHB1 in adipocytes. We named the model, **"Mito-Ob",** because these mice exhibit increased mitochondrial biogenesis in adipocytes and develop obesity independent of diet or food intake [13]. Mito-Ob mice display an increase in adipose tissue mass in a sex-neutral manner, but they show male sex-specific metabolic dysregulation such as impaired glucose homeostasis and insulin sensitivity [13]. These findings from Mito-Ob mice suggested that PHB1 has an

important role in the maintenance of adipose tissue homeostasis in both sexes and pointed towards sex differences in its effect on adipocyte functions. Of note, Mito-Ob mice start to gain weight after the onset of puberty indicating a potential relationship between pubertal surge in sex steroid hormones and PHB1 in adipose tissue growth and functions [13]. This relationship is supported by the following evidences: (**a**) sex steroid hormones have roles in growth and distribution of adipose tissue in the body [205], (**b**) sex differences in adipose tissue structure and function become apparent post puberty [237-239], (**c**) PHB1 and its homologous protein PHB2 (also known as REA, **r**epressor of **e**strogen **a**ctivity) are known to modulate the effect of sex steroid hormones especially in reproductive tissues[175-178], and (**d**) PHB1 and PHB2 have been identified as downstream effector molecules for sex steroid hormones mainly in reproductive tissues [240]. To further explore the interaction between PHB1 and sex steroid hormones in adipose tissue biology at the systemic level, ***1)*** the effect of surgical gonadectomy on growth and metabolic homeostasis in male and female Mito-Ob mice was investigated. In addition, ***2)*** *in vitro* studies were undertaken to determine the direct effect of sex steroid hormones on the differentiation of preadipocytes from transgenic Mito-Ob mice in a sex-specific manner.

To study the effect of the loss of gonadal sex steroid hormones on the growth of Mito-Ob mice, animals were gonadectomized at 3 months of age instead of the pubertal age (2 months). This time point for gonadectomy was chosen in particular for the relative ease of discerning gonadectomy-induced changes in body weight from PHB1-induced changes in adipose tissue mass. According to previous findings, the loss of gonadal sex steroid hormones is known to cause weight gain, while Mito-Ob mice start

to gain weight post-puberty [13]. Surgical gonadectomy in Mito-Ob mice prevented weight gain in both male and female mice in comparison with age and sex matched respective sham control Mito-Ob mice. This data suggests that PHB1 indeed requires sex steroid hormones for the development of obesity in Mito-Ob mice. Thus, the effect of gonadectomy is consistent with sex neutral obesity development in Mito-Ob as observed previously during their phenotypic characterization [13]. In contrast, gonadectomized wild type mice started to gain weight compared with sham operated wild type mice. This is also consistent with previous literature showing that absence of gonadal estrogens or androgens leads to weight gain in both rodents and humans with aging [55,190,223]. The lack of weight gain in the gonadectomized Mito-Ob mice would imply that PHB1 overexpression in adipocytes prevents gonadectomy-induced weight gain. Alternatively, it is possible that PHB1 functions differently in adipocytes in the presence or absence of sex steroid hormones. Taken together, these data would imply that PHB1 and sex steroid hormones might regulate each other's function in the maintenance of adipose tissue homeostasis under normal condition. Alternatively, it is also possible that PHB1 overexpressing adipocytes respond differently to gonadal sex steroid hormones as they exhibit sex differences in obesity-related metabolic phenotype [13] and associated complications [232]. Collectively, these findings suggest that a diverse relationship exists between PHB1 and sex steroid hormones in the regulation of adipose tissue biology and reproductive tissues (Fig. 26).

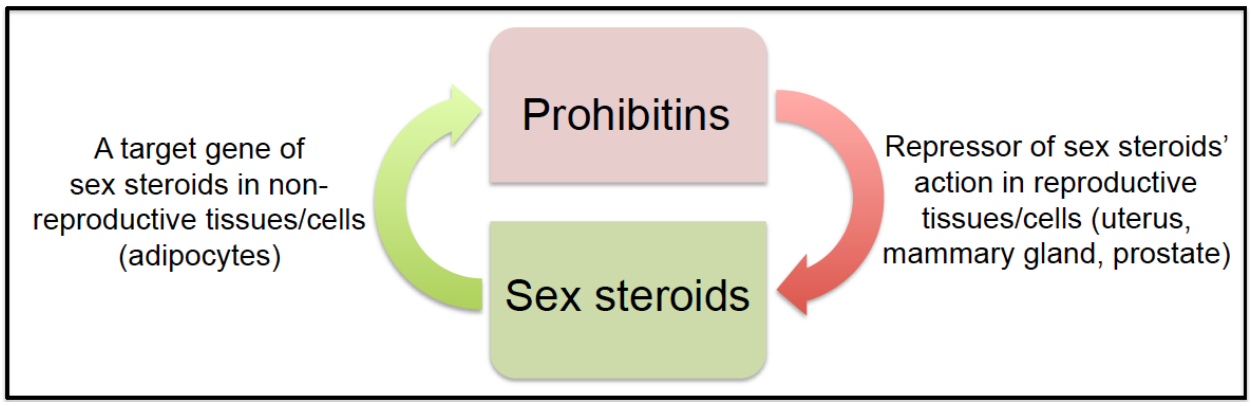

Figure 26. The relationship between PHB and sex steroids is complex. Schematic diagram showing the relationship between PHB and sex steroids in the regulation of each other's functions is different in reproductive and non-reproductive cells/tissues.

Differential effect of gonadectomy on the growth of wild type and Mito-Ob mice do not appear to be associated with their food intake because a similar trend in food intake was observed with opposite growth pattern between wild type and Mito-Ob mice. Similar food intake in sham operated wild type and Mito-Ob mice are consistent with previous findings that Mito-Ob mice develop obesity independent of food intake [13].

Consistent with the effect of gonadectomy on the growth of Mito-Ob mice, a decrease in adipose depot weight was observed in visceral and subcutaneous adipose tissues. However, an association between adipose tissue weight and adipocyte size was observed only in female mice. This would mean that there are differences in the relative contribution of various attributes of adipocyte dynamics in gonadectomy-related change in body and adipose tissue weight in male and female Mito-Ob mice. This may include a change in frequency distribution of adipocyte size. For example, the adipocyte size may vary widely depending on the amount of triglycerides stored. A change in

frequency distribution in adipocyte size may also account for gain or loss in adipose tissue and body weight. Analysis of frequency distribution of adipocyte size in adipose tissue also showed a close association in female Mito-Ob mice than in male mice. Thus, there may be a possibility that change in the weight of other metabolic target tissues of testosterone such as skeletal muscle and liver may also have contributed in change in the body weight. This would mean that gonadectomy induced changes in adipose tissue have contributed differently in corresponding changes in the weight of Mito-Ob mice.

However, unlike adipose depots, the effect of gonadectomy on the liver weight of Mito-Ob mice was male sex-specific. A closer look of the histological structure of liver revealed that gonadectomy improved fatty liver condition and histological architecture in male Mito-Ob mice. In contrast, a sign of immune cell infiltration was observed in the liver from gonadectomized female Mito-Ob mice. These findings suggest a role of sex steroid hormones in obesity-related susceptibility and resistance in the structural and functional regulation in metabolic tissues in Mito-Ob mice. In this context it is important to note that in relation to sex differences in the incidence of liver diseases including hepatocellular carcinoma, the focus has been on the direct effects of sex steroid hormones on hepatocytes [241]. However, new findings from Mito-Ob mice [13,232] and my own data suggest that sex differences in the dysregulation of adipose tissue function may also contribute to sex differences in liver diseases. This makes sense because adipose-hepatic crosstalk plays a crucial role in the regulation of energy and metabolic homeostasis in the body.

As mentioned previously, Mito-Ob mice develop obesity-related impaired glucose homeostasis and insulin sensitivity in a male sex-specific manner suggesting a potential

involvement of sex steroid hormones in sex differences in metabolic phenotype. Consistent with this notion, gonadectomy in male wild type significantly improved glucose disposal compared with sham control mice. Surprisingly, a differential effect of gonadectomy was observed on glucose disposal in male and female Mito-Ob mice despite a reduction adipose tissue weight and body weight. This discrepancy in the glucose homeostasis would mean that PHB1 overexpressing adipose tissue function differently in male and female in the presence and absence of gonadal sex steroid hormones, which may include differential secretion of various adipokines and metabolites. Alternatively, it may be due to changes in the insulin sensitivity in other insulin target tissue such as skeletal muscle and liver. It is anticipated that further analysis of various signaling molecules produced from adipose tissue will help to better define the relationship between sex steroid hormones and PHB1 in adipose tissue biology and in metabolic homeostasis. Interestingly, gonadectomized female Mito-Ob mice maintained glucose homeostasis and insulin sensitivity, which is unlike mutant Mito-Ob mice in which gonadectomy impairs glucose homeostasis and insulin sensitivity [232]. The mutant Mito-Ob mice overexpress Y114F-PHB in a manner that is similar to PHB in Mito-Ob mice, and both share the obesity and metabolic phenotypes. Taken together, these findings would mean that despite similarity in the development of obesity and metabolic phenotype between Mito-Ob and mutant Mito-Ob mice, there are differences in their adipose tissue response with and without sex steroid hormones. It is possible the relationship between PHB1 and sex steroid hormones in the regulation of adipose tissue biology involve phosphorylation of PHB1 at tyrosine-114. In addition, a potential role of phosphorylation of PHB1 at other sites may not be ruled out. For

example, phosphorylation of PHB1 at tyrosine-114 residue is known to have a relationship with phosphorylation at tyrosine-249, threonine-258 and tyrosine-259 residues with diverse functional consequences [168,169,242]. Furthermore, recent evidence suggests that phosphorylation of PHB1 has a role in cellular trafficking of PHB1 [243]. Thus, a possibility exists that different attributes of PHB1 are involved in sex-neutral adipogenic and but sex-dimorphic adipose tissue functions and metabolic dysregulation, which requires further investigations.

It would be interesting to know whether sex differences in adipocyte and adipose tissue function are intrinsic to sex steroids because they are two different hormones, or due to intrinsic differences in target cell/tissue response, or a combination of both. Irrespective of the underlying mechanisms involved, a crucial role of sex steroid hormones in adipose tissue functions raises an important question – why hormones, whose primary functions are to promote reproductive functions, have so much to do with metabolic functions in the body? Most importantly, what is the importance of this relationship during critical stages of development such as puberty on metabolic status later in life, especially overweight and obese condition? New findings from Mito-Ob mice suggest a crucial role of PHB1 in mediating the effects of sex steroid hormones in adipose tissue functions during the defining moment of puberty, which warrants further investigations. It is possible that dysregulation of the intricate relationship between sex steroid hormones and adipose tissue function may be a major driver in the development of diabetes and cancer later in life. In this context, it is important to note that recently the protective effects of estrogen on obesity-related metabolic phenotype in mutant-Mito-Ob have been investigated. Ovariectomy in mutant-Mito-Ob mice resulted in appearance of

metabolic dysregulation and tumor development similar to male mutant-Mito-Ob mice [232]. Most importantly, impairment of metabolic homeostasis in mutant-Mito-Ob mice was observed despite the reversal of obesity, suggesting a crucial role of PHB1 in sex differences in adipose tissue functions at the systemic level beyond triglyceride homeostasis.

VI. SUMMARY

In summary, data presented in this thesis suggests that PHB and sex steroid hormones regulate each other's function in the growth of adipose tissue, and subsequently in the regulation of adipose tissue function in a sex dimorphic manner. Overexpression of PHB1 in adipocytes further amplifies sex differences in adipose tissue function suggesting a role of PHB1 in mediating sex dimorphic effects of sex steroid hormones in adipose tissue biology. It appears that overexpression of PHB1 in adipose tissue alters adipose tissue response to sex steroids or sex differences in adipocyte functions. Furthermore, our data suggests that intrinsic differences in adipose tissue response exist between male and female independent of sex steroid hormones, which play a role in sex differences in adipose tissue function and consequently on whole body metabolic homeostasis. A discrepancy in the relationship between sex steroid hormones and PHB1 in their adipogenic function as observed in *in vivo* and *in vitro* experiments suggests a potential involvement of other factors. This may include local factor(s) at adipose tissue level or systemic factor(s). Alternatively, it is also possible that the discrepancy between *in vivo* and *in vitro* studies is due to the fact that *in vitro* cell culture system does not recapitulate adipogenic process *in vivo*, which involves interplay among various cell types within adipose tissue including mesenchymal precursor cells and immune cells. It is anticipated that a better understanding of the relationship between sex steroid hormones and PHB1 in adipose tissue biology will shed new light on the role of adipose tissue in sex differences in metabolic changes in health and metabolic diseases such as obesity, diabetes, and other related diseases.

VII. FUTURE DIRECTIONS

Since the molecular mechanisms of the relationship between sex steroid hormones and PHB1 in the regulation of sex differences and adipose depot specific functions remain to be determined, future experiments will include:

1. Study of lipogenic and lipolytic enzymes in visceral and subcutaneous adipose tissues from Mito-Ob mice in the presence and absence of gonadal sex steroid hormones.
2. Study of insulin signaling status in major metabolic tissues involved in systemic metabolic regulation including adipose tissue, liver, and skeletal muscle in Mito-Ob mice in the presence and absence of gonadal sex steroid hormones.
3. Study of expression levels of various adipogenic marker genes and proteins in adipose depot specific manner in male and female Mito-Ob with and without sex steroid hormones.
4. Study of glucose and fatty acid uptake and oxidation in major metabolic tissues from Mito-Ob mice with and without sex steroid hormones.
5. Study of the effects of steroid hormone replacement therapy on gonadectomy-induced changes in # 1-3 in a sex-specific manner. Furthermore, crossover experiments with sex steroid hormones may be pursued to explore adipose depot-specific intrinsic differences from the effects of sex steroid hormones.

VIII. CONCLUSION

In addition to a fundamental role in reproductive physiology, sex steroid hormones play a crucial role in controlling of energy homeostasis and metabolic regulation in the body. Sex steroid hormones are also important regulators of adipose tissue distribution in the body. In humans, adipose tissue distribution is different between males and females. The mechanism by which sex steroid hormones control the amount and distribution of adipose tissue in the body is not clear. The adipose sex dimorphism also extends to the function of adipocytes. However, various downstream intermediates that are involved in sex dimorphic functions of adipose tissue are not well explored. Our findings of PHB1 as a mediator of sex steroid hormones in adipose tissue functions as revealed by Mito-Ob mice is a step forward in this direction. However, the molecular mechanisms involved in the interplay between sex steroid hormones and PHB1 in adipose tissue biology including adipose depot specific functions remain to be determined. Future studies focusing on these issues will increase our understanding of the relationship between sex steroids and adipose tissue biology in sex differences in energy homeostasis and metabolic regulation at the systemic level, which is a fundamental aspect of whole body physiology in mammals. Furthermore, sex differences also exist in immune functions in the body. Many of these differences become apparent after pubertal surge in gonadal steroid hormones. However, factors that mediate sex differences in immune functions are largely unknown. Emerging evidence suggests that PHB1 has roles in immune cell functions. Because metabolic and immune functions are intimately associated with each other in normal physiology

and pathophysiology, it would be interesting to know whether PHB1 has a role in sex differences in immune function, which warrants further investigations.

IX. REFERENCES

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