From the Department of Cell and Molecular Biology
Karolinska Institutet, Stockholm, Sweden

Novel properties of mature adipocytes in obesity and hyperinsulinemia

Qian Li

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Cover picture shows the representative images of human adipocyte nuclei from lean and obese individuals stained with a DNA dye. The morphological variation of adipocyte nuclei caught our attention, and this is where this thesis work started.

Novel properties of mature adipocytes in obesity and hyperinsulinemia

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By

Qian Li

Principal Supervisor:

Assistant Professor Kirsty Spalding
Karolinska Institutet
Department of Cell and Molecular Biology

Co-supervisor(s):

Professor Henrik Druid
Karolinska Institutet
Department of Oncology-Pathology

Assistant Professor Carolina Hagberg
Karolinska Institutet
Department of Medicine Solna
Division of Cardiovascular Medicine

Opponent:

Associate Professor Patrick Seale
University of Pennsylvania
Department of Cell and Developmental Biology

Examination Board:

Associate Professor Jorge Ruas
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Professor Arne Lindqvist
Karolinska Institutet
Department of Cell and Molecular Biology

Associate Professor Martin Jastroch
Stockholm University
Department of Molecular Biosciences

To my beloved family!

ABSTRACT

Adipose tissue expansion and dysfunction, which leads to obesity and related metabolic diseases (e.g., diabetes and hypertension) are currently the most costly challenges for public health, yet the mechanisms underlying adipocyte functional dysregulation have not been fully elucidated[1]. Investigation is largely hindered by the unique technical limitations associated with handling these large, lipid-filled fat cells. New techniques need to be developed in order to better understand the physiology and pathology of adipocytes.

In **Paper I**, by using an adipocyte-specific reporter mouse, we proved that previously reported adipocyte flow cytometry techniques missed the major adipocyte population. Therefore, we defined several crucial cytometer settings required for large cell types that allow us to analyze and sort both white and brown mature adipocytes. This improved strategy is applicable to sort adipocytes based on size without fixation, which greatly facilitates subsequent downstream analyses. In combination with immunostaining, the presented approach can effectively sort UCP1 positive adipocytes from mouse white and brown adipose tissue. Furthermore, we demonstrated a heterogeneous ADRB2 expression pattern in human adipocytes, which confirmed the applicability of our newly developed technique to further explore other aspects of adipocyte identity.

In **Paper II**, we developed a MAAC (membrane mature adipocyte aggregate culture) system as a novel, high-viability model for human mature adipocytes. With a permeable membrane insert sitting on top to facilitate cell aggregation and nutrition access, adipocytes cultured in the MAAC system maintained adipogenic properties, did not dedifferentiate and had reduced hypoxia. This newly developed *in vitro* system allows us to compare depot-specific adipocyte gene expression, and analyze the crosstalk between adipocytes and macrophages. In particular, we demonstrated that human adipocytes can be transdifferentiated to brown-like adipocytes under the conditions of rosiglitazone stimulation or PGC-1α overexpression. Taken together, we provided a versatile tool for modulating primary adipocytes, opening up numerous downstream applications.

In **Paper III**, we revealed that a large group of mature human adipocytes express an array of cell cycle-specific markers indicative of a cell cycle re-entry profile, and that this is associated with whole-body insulin resistance. We demonstrated that insulin is a critical driver of adipocyte cell cycle re-entry, subsequently making them vulnerable to undergo cellular

senescence. Our data showed that hyperinsulinemia in obese patients is associated with increased p16 and senescence associated β-galactosidase activity in mature adipocytes. Furthermore, we showed that senescent adipocytes are hypertrophic and develop a senescence-associated secretory phenotype (SASP), defined by the secretion of IL-6, IL-8, and MCP1. These findings challenge the dogma that adipocytes permanently exit the cell cycle upon differentiation and reveals cellular senescence as a new mechanism associated with inflammation-related adipocyte pathology.

In conclusion, the research within this thesis has provided important techniques for both *in vitro* adipocyte modulation and high throughput flowcytometric adipocyte analysis, supporting multiple downstream research applications to investigate mechanisms regulating adipocyte physiology and pathology. Furthermore, we demonstrated the phenomenon of cell cycle re-entry and senescence in human mature adipocytes, thereby introducing novel insights into obesity and hyperinsulinemia-induced adipocyte dysfunction, suggesting potential targets for treating obesity-related metabolic diseases.

LIST OF SCIENTIFIC PAPERS

I. Hagberg CE, **Li Q**, Kutschke M, Bhowmick D, Kiss E, Shabalina IG, Harms MJ, Shilkova O, Kozina V, Nedergaard J, Boucher J, Thorell A, Spalding KL (2018). Flow cytometry of mouse and human adipocytes for the analysis of browning and cellular heterogeneity. Cell Rep. 24(10):2746-2756.

II. Harms MJ, **Li Q**, Lee S, Zhang C, Kull B, Hallen S, Thorell A, Alexandersson I, Hagberg CE, Spalding KL, Boucher J (2019). Mature human white adipocytes cultured under membranes maintain identity, function, and can transdifferentiate into brown-like adipocytes. Cell Rep. 27(1):213-225

III. **Li Q***, Hagberg CE*, Silva Cascales H, Salehzadeh F, Chen P, Hyvönen M, Terezaki E, Harms MJ, Kutschke M, Willebrords J, Sárvári A, Aouadi M, Boucher J, Thorell A, Spalding KL. Hyperinsulinemia drives human adipocytes to re-enter cell cycle and senesce. Manuscript.

* Equal contribution

Other publication not included in the thesis:

Petrus P, Mejhert N, Corrales P, Lecoutre S, **Li Q**, Maldonado E, Kulyté A, Lopez Y, Campbell M, Acosta JR, Laurencikiene J, Douagi I, Gao H, Martínez-Álvarez C, Hedén P, Spalding KL, Vidal-Puig A, Medina-Gomez G, Arner P, Rydén M (2018). Transforming growth factor-β3 regulates adipocyte number in subcutaneous white adipose tissue. Cell Rep, 25(3):551-560.

CONTENTS

LIST OF ABBREVIATIONS

4EBP	eIF4E binding protein
53BP1	p53-binding protein 1
8-OHdG	8-hydroxy-2-deoxyguanosine
ADRA2	α2 adrenergic receptor
ADRB	β-adrenergic receptor
AMPK	Adenosine monophosphate-activated protein kinase
AKT	also known as protein kinase B, PKB
APC/C^{CDH1}	Anaphase-promoting complex/cyclosome
AS160	AKT substrate of 160 kDa
ATGL	Adipose triglyceride lipase
ATM	Ataxia telangiectasia-mutated kinase
BAT	Brown adipose tissue
BMI	Body mass index
BrdU	5-bromo-2-deoxyuridine
c/EBPα	CCAAT/enhancer-binding protein α
CDK	Cyclin dependent kinase
CHK1/2	Checkpoint kinase 1/2
DDF	DNA damage foci
DDR	DNA damage response
DNL	*De novo* lipogenesis
DSBs	DNA double-strand breaks
EdU	5-ethynyl-20-deoxyuridine
ERK	Extracellular-regulated protein kinase
FAs	Fatty acids
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FGF2	Fibroblast growth factor 2
FSC	Forward scatter
γ-H2AX	Phosphorylated form of histone H2AX at Ser139
GLUT4	Glucose transporter 4
GSK3	Glycogen synthase kinase-3
HFD	High fat diet
HMGB1	High mobility group box protein 1
HSL	Hormone-sensitive lipase
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
INSR	Insulin receptor
IR	Insulin resistance
IRS1	Insulin receptor substrate 1

LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein 1
MDC1	Mediator of DNA-damage checkpoint 1
MEK	MAPK/ERK kinase
MGL	Monoacylglycerol lipase
MAAC	Membrane mature adipocyte aggregate culture
MMP	Matrix metalloproteinase
mTORC1/2	Mechanistic target of rapamycin complex 1/2
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NAFLD	Non-alcoholic fatty liver disease
NEFAs	Non-esterified fatty acids
NF-κB	Nuclear factor-κB
NPs	Natriuretic peptides
PCNA	Proliferating cell nuclear antigen
PDK1	Phosphoinositide-dependent kinase 1
PGC-1α	PPARγ coactivator 1α
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphorylate phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PPARγ	Peroxisome proliferator-activated receptor γ
RB	Retinoblastoma protein
SABG	Senescence associated β-galactosidase assay
SASP	Senescence-associated secretory phenotype
SSBs	DNA single-strand breaks
SSC	Side scatter
SVF	Stromal vascular fraction
TG	Triglyceride
TNF-α	Tumour necrosis factor α
TSC2	Tuberous sclerosis complex 2
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue

1 INTRODUCTION

1.1 OBESITY AND ADIPOSE TISSUE

Obesity is described as excessive fat tissue expansion that may have adverse health effects. Body mass index (BMI), calculated by weight-to-squared height, is the most commonly used parameter to determine the severity of obesity. A BMI in the range of 25 to 25,9, is defined as overweight, with a BMI of 30 or above classified as obese.

More than being of mere cosmetic concern, obesity is a high risk factor associated with numerous metabolic problems, including diabetes, cardiovascular disease, and cancer [2]. Adipose tissue, and several other organs including the liver, skeletal muscle and pancreas, are known to be actively involved in obesity and obesity-related metabolic abnormalities. Still, the fact that moderate weight loss (5%) can already improve insulin sensitivity in metabolic tissues and reduce plasma levels of some risk factors (such as, circulating glucose, triglyceride, and alanine transaminase levels) for cardiometabolic disease suggests that adipose tissue is one of the central organs controlling metabolic balance [3]. Thus, preventing or reversing unhealthy obesity becomes an efficient way to counter multiple chronic metabolic disorders at the same time.

Currently, therapeutic methods for reducing body weight in morbid obesity include exercise, caloric restriction, and bariatric surgery. The first two demand long term persistent efforts and considerable willpower, and are therefore often unsuccessful in assisting obese individuals in achieving significant and sustained weight loss. Bariatric surgery reconstructs the digestive system and reduces food intake, and in most cases induce remarkable weight loss. However, side effects like chronic nausea and hernias, coupled with the chance of weight regain, make this operation not always be the best option for lots of obese individuals. It is therefore in urgent need to develop new treatment strategy. As such, further studies on adipocyte biology will bring new insights to the mechanisms regulating metabolic homeostasis in adipose tissue, and eventually shed light on the crucial targets to reverse obesity-related metabolic abnormalities.

Adipose tissue is made up of mature adipocytes and other types of cells including adipocyte progenitors (preadipocytes), stem cells, immune cells, endothelial cells, and fibroblasts. Except for mature adipocytes, all the other cells are collectively termed the stromal vascular fraction (SVF). Classically, there are two types of adipose tissue: white adipose tissue (WAT) and

brown adipose tissue (BAT). WAT is located in distinct regions of the body, with the most abundant human depots being subcutaneous abdominal and visceral omental. In humans, only small pockets of BAT persist into adulthood, and are mainly located in the supraclavicular region, the thoracic paravertebral region, along the great vessels and around solid organs like the adrenal gland and kidney [4-6].

White adipocytes (also known as mature adipocytes or fat cells) are the primary cell type that makes up 90% of the volume of WAT [7]. Mature adipocytes are regarded as terminally differentiated cells, derived initially from committed progenitors (preadipocytes). Preadipocytes proliferate to increase cell number. Once committed to differentiation they begin to express mature adipocyte-associated genes and proteins, start to accumulate lipid and eventually grow to become large mature fat cells [8]. White mature adipocytes are characterized by the presence of a single large lipid droplet, with the nucleus located to the side of the cell, close to the plasma membrane (**Fig. 1**). Between the lipid and plasma membrane, there is a thin layer of cytoplasm containing the cell organelles. Mature adipocytes are essential for keeping the whole-body energy balance by storing and releasing fat. However, they also function as an endocrine cell, secreting cytokines into the circulation to regulate a variety of functions in other organs (**Chapter 1.2**).

Brown adipocyte   Beige adipocyte   White adipocyte
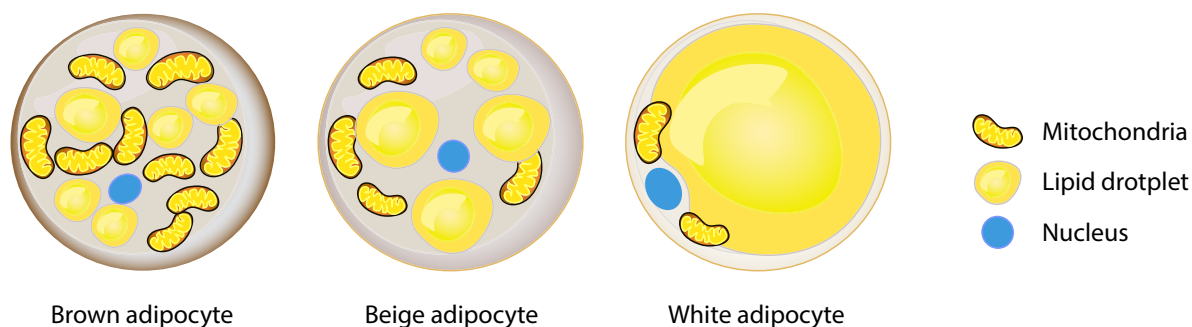
Figure 1. Morphology and characteristics of three types of adipocytes: brown, beige and white adipocytes.

Brown adipocytes are specialized in energy expenditure and thermogenesis [9, 10]. Brown adipocytes are smaller than white adipocytes, they contain multiple lipid droplets and have abundant of mitochondria, which allow them to convert chemical energy directly into heat. Brown adipocytes express high levels of uncoupling protein 1 (UCP1) (**Fig. 1**).

More recently, a third type of fat cell has been identified. Beige [11-13] or brite [14] adipocytes **(Fig. 1)** are functionally similar to brown adipocytes, however, compared to the developmentally-derived brown adipocytes, beige (brite) adipocytes are inducible, they can be reprogrammed and transdifferentiate from white adipocytes [12, 14, 15]. Beige adipocytes have drawn a lot of attention as a potentially promising future therapeutic tool to increase energy expenditure and combat obesity.

This thesis work is mainly focused on understanding mature white adipocytes and their physiological and pathological mechanisms that contribute to obesity.

1.2   THE MUTIPLE FUNCTIONS OF WHITE ADIPOCYTES

The primary function of white adipocytes is to serve as an energy bank to store and release fatty acids (FAs), as needed for maintaining whole body metabolic balance [16]. Fat storage is mediated by a process called lipogenesis, which utilizes FAs to efficiently synthesize triglycerides (TGs) and store them in the lipid droplet. Fatty acid release is regulated by lipolysis, a process through which TGs are broken down to glycerol and FAs. The FAs released from adipocytes are then oxidized in the muscle and other organs for energy expenditure. Both lipogenesis and lipolysis are sensitive to hormones and precisely regulated by multiple enzymes to maintain the balance between lipid flux in and out of the adipocytes. If the lipid storage capacity exceeds lipid removal capacity, the consequence is increased lipid droplet size and adipocyte size, leading to whole body obesity [17].

1.2.1   Lipogenesis

Adipocyte lipogenesis mainly utilizes the fatty acids derived from circulating TGs. During the period of energy excess, circulating TGs that are originally synthesized in the liver and intestine are packaged in the form of lipoproteins and travel to WAT. In adipose tissue, TGs are first hydrolyzed to non-esterified fatty acids (NEFA) at the surface of capillary endothelial cells via the lipoprotein lipase (LPL) [18, 19]. Next, NEFAs move through the endothelial lumen and are taken up by adipocytes through fatty acid transporters such as scavenger receptor CD36, fatty acid transport proteins (FATPs), fatty acid binding protein (FABPs) and caveolins [20]. Aside from exogenous fatty acids, adipocytes also make use of glucose transported into fat cells in response to insulin to synthesize endogenous fatty acids through *de novo* lipogenesis (DNL).

Although not the major contributor to lipogenesis in adipocytes, DNL produces adipocyte-specific fatty acids that are essential for regulating insulin sensitivity and energy homeostasis [21-23]. Finally, fatty acids that originate exogenously and endogenously are esterified to form TGs via sequential actions of multiple enzymes, and eventually join in the lipid droplet. The detailed steps of lipogenesis are shown in **Figure 2**. Insulin is one of the most influential hormones stimulating lipogenesis. Apart from stimulating glucose uptake to facilitate DNL, insulin enhances both the expression of LPL in adipocytes and the LPL activity at the endothelial lumen to promote lipid transfer to adipocytes [24, 25]. The translocation of FATP1 and CD36-containing vesicles to cytoplasm membranes are also stimulated by insulin to facilitate fatty acid uptake [26, 27].
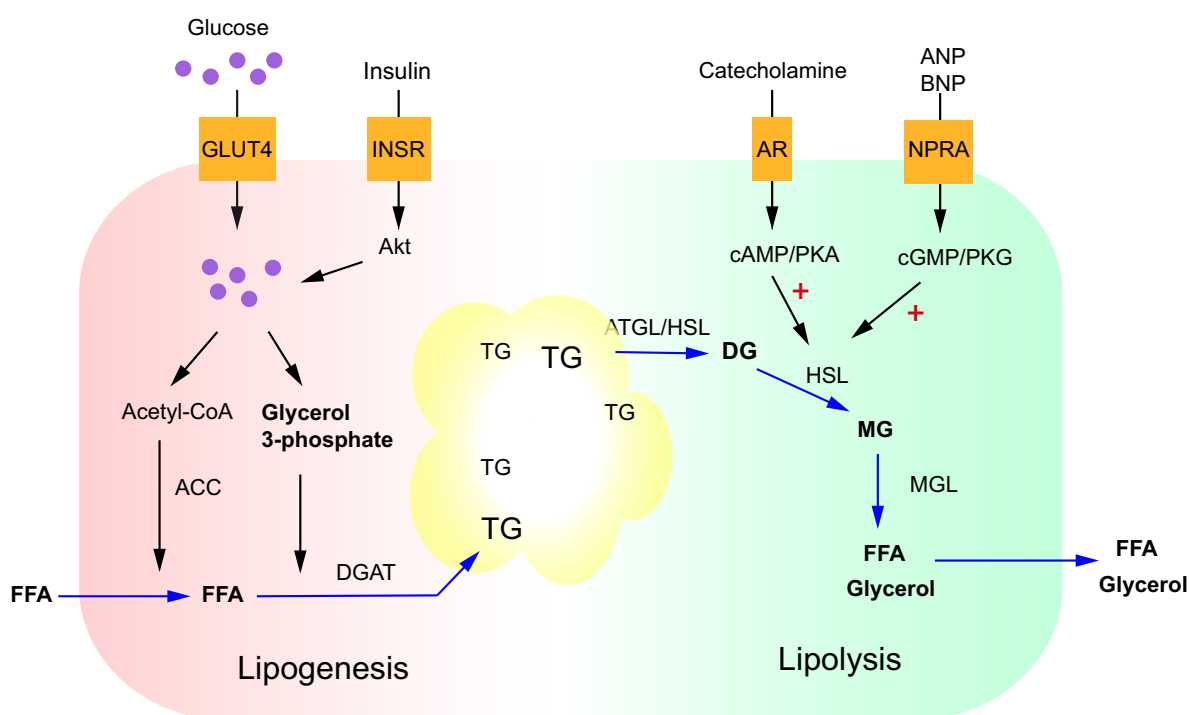
Figure 2: Schematic representation of the lipogenesis and lipolysis processes in adipocytes. In lipogenesis, FFA (free fatty acids) from circulation or generated through DNL are used for synthesizing TGs, which are stored as lipid in adipocytes. In lipolysis, TGs are broken down to FAs and glycerol that are released out to circulation and oxidized in organs. ACC, acetyl-CoA carboxylase; DGAT, diacylglycerol acyltransferase; cAMP, cyclic adenosine monophosphate.

1.2.2 Lipolysis

Lipolysis is the biochemical pathway in which TGs in the lipid droplet are hydrolyzed to liberate glycerol and three molecules of FAs. This is controlled via the action of three lipases:

adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) (**Fig. 2**). FAs released into the circulation can be taken up by skeletal muscle, brown adipocytes, liver, and kidney for mitochondrial β-oxidation to fulfill energy demands. Released FAs can also travel back to the liver to be used as a major source of substrate for producing very-low-density lipoproteins (VLDLs) [16].

Adipocytes experience a basal level of lipolysis. Yet, to a large extent, lipolysis is triggered by hormones, such as catecholamines, cortisol and glucagon [28]. Catecholamines bind β-adrenergic receptors (ADRB) on the adipocyte plasma membrane to stimulate cAMP production and activate protein kinase A (PKA), resulting in the phosphorylation of the rate-limiting enzyme HSL that initiates the lipolysis process (**Fig. 2** and ref. [29]). The density of ADRB on the adipocyte membrane is one of the essential factors that determine lipolysis rate [30]. In rodents, white and brown adipocytes express all three ADRBs (ADRB1, ADRB2, ADRB3) which are responsible for lipolysis and non-shivering thermogenesis [31]. In human adipocytes, ADRB1, ADRB2 and α2 adrenergic receptor (ADRA2) are the major subtypes which respond to catecholamines. ADRB1 and ADRB2 promote lipolysis while ADRA2 is an anti-lipolytic receptor. Therefore, the balance between β and α adrenergic receptor determines the lipolysis rate [32, 33]. More recently, the cardiac-derived natriuretic peptides (NPs), including atrial NP (ANP) and the B-type NP (BNP), have also been shown to be robust stimulators of adipocyte lipolysis in humans [34]. ANP and BNP bind to the membrane receptor NP receptor A (NPRA) promote HSL mediated lipolysis through cGMP-dependent protein kinase pathway [35]. Compared to catecholamine and NPs pathways, insulin acts as the major anti-lipolysis hormone suppressing both ATGL and HSL activation to limit lipid digestion [36, 37].

1.2.3 Lipolysis in obesity

As measured by lipid ^{14}C dating, during the average 10 year life span of a human adipocyte the lipid contained within is replaced approximately 6 times, with an average age of 1.6 years [38, 39]. This indicates that adipocyte lipogenesis and lipolysis are dynamic processes [39]. A follow-up study using the same technique on a large human cohort revealed that lipid age in obese individuals is significantly higher than that in non-obese ones, suggesting that lipid removal capacity is blunted, causing increased lipid deposition and body weight gain [17].

Reduced energy expenditure and, more importantly dysregulated lipolytic response, are the major factors limiting lipid removal rate [29, 40].

Basal lipolysis. Studies on lipolysis have demonstrated that overall basal lipolysis in obese subjects is increased, while stimulated lipolysis is decreased [41, 42]. Studies using microdialysis to measure the release of the lipolytic substrate glycerol reveal a higher lipolytic rate in obese individuals compared to lean. However, when the amount of glycerol released was normalized to the amount of tissue, no difference between lean and obese was observed, thus suggesting that it is the total fat mass that contributes to increased lipolysis in obesity and not intrinsic changes in the adipocytes themselves [30, 41]. Furthermore, Tchernof *et al*. analyzed basal lipolytic rate in relation to adipocyte number and showed that large adipocytes release more glycerol than smaller ones, indicating that the observed increase in lipolysis in obese individuals could also be due to enlarged adipocyte size [43].

Stimulated lipolysis. Although basal lipolysis is enhanced, obesity is found to be associated with a low rate of lipid removal, which is significantly attributed to impaired stimulated lipolysis [42]. Catecholamine-stimulated lipolysis is largely determined by the abundance of ADRBs on the plasma membrane. It has been observed that β-adrenergic lipolytic responsiveness is impaired in obese subcutaneous adipose tissue [30]. As mentioned above, both ADRB1 and ADRB2 are involved in the human lipolytic response. The administration of selective agonists of either the β1 or β2 adrenergic receptors (dobutamine and salbutamol) reveal no difference in ADRB1 stimulation, glycerol or FFA release in lean and obese animals [44]. However, ADRB2-induced lipolysis significantly decreased in obese mice, implying that blunted ADRB2 activity is responsible for decreased adipose lipolysis in obesity [44]. Similar results were observed in human studies, where obese individuals exhibited an almost 70% reduction in ADRB2 sensitivity, as measured by saturated ^{125}I-cyanopindolol binding. However, ADRB1 and ADRA2 sensitivity was unchanged compared to lean individuals [30]. This compelling data, again, emphasizes the importance of ADRB2 in regulating lipolysis.

The studies measuring ADRB2 expression levels mentioned above were mostly performed at a bulk cell level, which lacks information from individual cells and is difficult to calibrate with adipocyte size [30]. This is particularly relevant because adipocytes are known to be heterogenous in both size and function [45]. In response to the lack of tools to efficiently analyze large numbers of individual adipocytes, we have developed a new flow cytometry-based

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method to analyze adipocytes in a high throughput way and used it to detect the ADRB2 expression in human adipocytes (**Paper I**). Our results demonstrated a heterogeneous expression of ADRB2 on human adipocytes and that decreased ADRB2 expression in obesity was explained by ADRB2 receptor loss in a subpopulation of adipocytes. The unexpected finding of an ADRB2 negative population warrants further investigation.

1.2.4 Adipocyte endocrine functions

Apart from serving as the lipid storage pool, adipocytes also interact with the surrounding environment by secreting multiple adipokines and peptides that are physiologically important for maintaining the metabolic balance. Collective evidence has revealed that adipocytes secrete more than 50 peptides involved in metabolism, immune response and extracellular matrix remodeling [46]. The secretory list is composed of not only the dominant adipokines such as leptin and adiponectin, but also includes proinflammatory cytokines such as tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP1) and interleukin 6 (IL-6), which are expressed by both adipocytes and macrophages [47].

Leptin. Adipocytes are the major source of circulating leptin, which acts on leptin receptors present in neuron to suppress appetite and increase energy expenditure [48, 49]. Through sympathetic nerve fibers that innervate adipocytes, leptin has been shown to suppress adipocyte lipogenesis and enhance lipolysis [50, 51]. Mouse models of genetic disruption of leptin or the leptin receptor (ob/ob, db/db) result in profound body weight gain and increased food intake [52, 53]. Furthermore, the administration of recombinant leptin to humans or animals with leptin gene mutations induced a rapid depletion of fat mass and corrected multiple obesity related abnormalities [54]. However, administering additional leptin to obese individuals does not have the same impact on body weight, as leptin resistance develops during obesity [54-57]. In obesity, hypertrophic adipocytes secrete more leptin to compensate for the loss of sensitivity, resulting in increased serum leptin levels, positively correlating with the percentage of body fat in obesity [58, 59]. In turn, excessive serum leptin can induce the expression of fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF), which stimulates adipose tissue angiogenesis and increases vascular permeability, potentially alleviating hypertrophy-induced hypoxia [60].

Adiponectin is crucial for maintaining healthy adipose tissue expansion and improving insulin sensitivity while enhancing lipogenesis and adipogenesis [61, 62]. Administration of adiponectin

to mice increases fat mass but prevents ectopic lipid accumulation, thus lowering the risk of insulin resistance [63]. Serum adiponectin levels are negatively associated with insulin resistance, but not with BMI, indicating an insulin-related effect on adiponectin secretion [64].

Inflammatory factors. Adipocytes can secrete TNF-α, IL-6, and MCP-1, with a low expression in lean subjects that increases in obesity. These inflammatory factors have been shown to contribute to the chronic inflammation occurring in obese adipose tissue and are associated with impaired insulin sensitivity [65]. TNF-α is a proinflammatory cytokine, influencing the immune response by activating the release of other proinflammatory cytokines, such as IL-6 and IL-1β [66]. TNF-α and IL-6 downregulate the expression of the adipogenic genes peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα), thus impeding preadipocyte proliferation and differentiation, which in turn inhibits adipose tissue hyperplastic expansion [67, 68]. Both TNF-α and IL-6 suppress adiponectin secretion and inhibit expression of several mediators of the insulin signaling pathway, including insulin receptor substrate 1 (IRS1) and glucose transporter 4 (GLUT4) [68, 69]. MCP-1 recruits macrophages which further secrete TNF-α, MCP-1, and other chemokines, consolidating the chronic inflammatory environment, in turn, driving whole body insulin resistance [70].

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## 1.3   INSULIN AND INSULIN RESISTANCE IN ADIPOCYTES

Insulin is one of the essential hormones for cell survival, growth and proliferation [71]. Insulin is synthesized and secreted by β-cells of the pancreas in response to increases in the serum glucose level. The secreted insulin is delivered to target tissues and recognized by insulin receptors (INSR) expressed on cell plasma membranes in organs including the liver, adipose tissue, skeletal muscle and brain [72].

1.3.1   Insulin signaling

Insulin binding to the INSR triggers the recruitment and phosphorylation of intracellular insulin receptor substrate 1 (IRS1), which can further assemble phosphoinositide 3-kinase (PI3K) and phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) [73]. Through PIP3, the key downstream effector AKT (also known as protein kinase B; PKB) is translocated to the plasma membrane and phosphorylated at two sites for full function: Thr-308 by phosphoinositide-dependent kinase 1 (PDK1); or at Ser-473 by mechanistic target of rapamycin complex 2 (mTORC2) [74, 75]. The phosphorylation of AKT activates several downstream effectors to regulate a complex metabolic program (**Fig. 3**). For example, AKT activates AS160 (also called TBC1D4) to stimulate GLUT4-mediated glucose uptake, AKT inactivates GSK3 (glycogen synthase kinase-3) to promote glucose storage as glycogen, and AKT inhibits TSC2 (tuberous sclerosis complex 2) to activate mTORC1 to enhance protein and lipid synthesis and stimulate cell growth [76, 77]. Aside from the PI3K/AKT pathway, the mitogenic effect of insulin is mediated by extracellular-regulated protein kinase (ERK) [78], which is another insulin receptor downstream pathway responsible for cell growth, proliferation, and differentiation [79].

Insulin in adipocytes. The crucial role of insulin in adipocytes is to promote nutrient storage, including inducing glucose uptake and lipid uptake, as well as stimulating DNL while inhibiting lipolysis (**Chapter 1.2**). Glucose uptake is an important action of insulin for carbon energy deposition, in which GLUT4 is the major transporter mediating glucose uptake. When serum insulin levels are low, GLUT4-containing vesicles are maintained in the cytoplasm. When insulin levels increase, in an immediate response, insulin-activated AKT triggers GLUT4 translocation to the plasma membrane to facilitate the transportation of glucose derived from dietary carbohydrates into adipocytes [80]. The majority of glucose in adipocytes is transformed to glycerol, used as the backbone for TGs synthesis. Whereas, a small fraction of

glucose can be stored as glycogen for later energy demands, or be broken down through glycolysis and the tricarboxylic acid cycle to eventually be used in DNL [81]. Furthermore, insulin is essential for adipose tissue expansion. Insulin can efficiently stimulate fatty acid uptake through activation of LPL, and the redistribution of fatty acid transporters CD36 and FATP to the cellular membrane, while inhibiting lipolysis through dephosphorylation of HSL. Therefore, insulin promotes lipid storage in adipocytes, which is one essential reason inducing adipocyte size enlargement. Fat specific insulin receptor knockout mice rapidly displayed lipodystrophy with profound loss of subcutaneous and visceral adipose tissue, hyperlipidemia, insulin resistance, and severe fatty liver disease [82]. This study, supported by numerous other studies demonstrates the important role of insulin in adipogenesis and adipocyte growth [83-85].
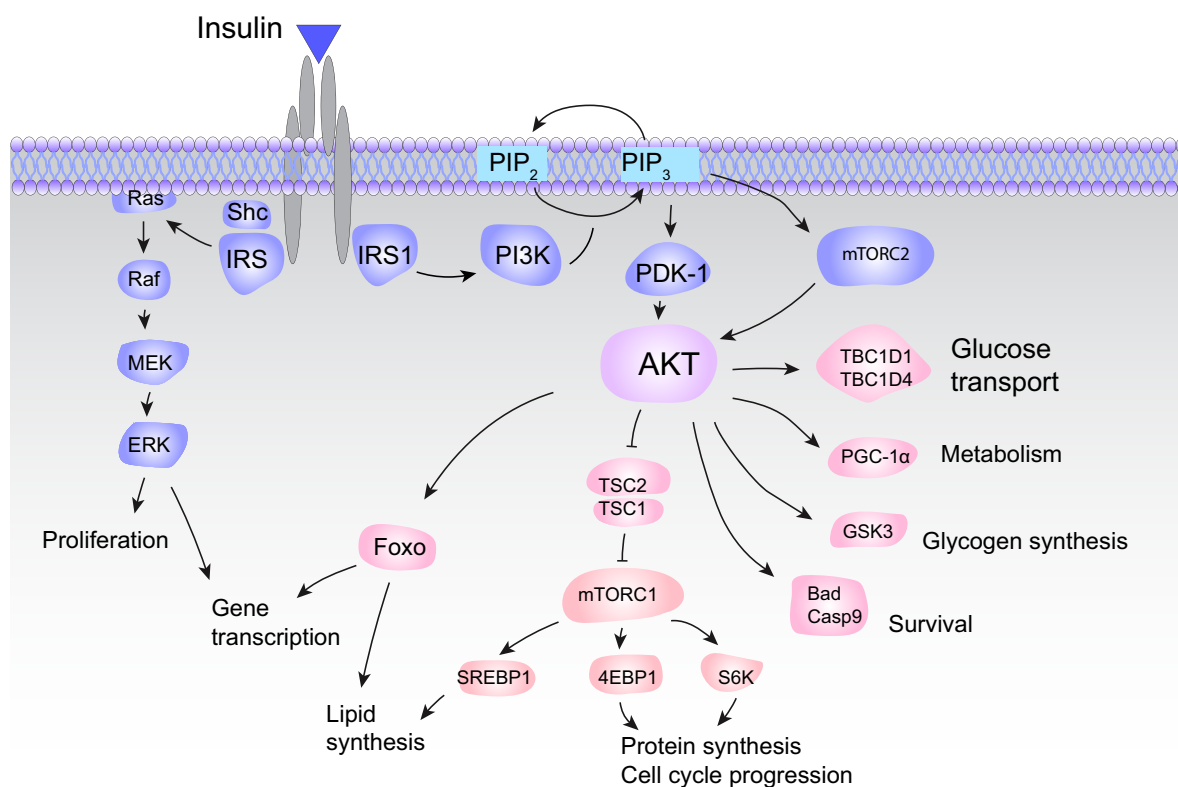
Figure 3. Schematic representation of insulin signaling pathway. The binding of insulin on the insulin receptor (INSR) initiates a cascade of phosphorylation events, with the two major downstream pathways being PI3K/AKT and Ras-Raf-MEK-ERK (MAPK/ERK) pathway. Activation of AKT regulates multiple metabolic effects including glucose transport, lipid and glycogen synthesis, as well as cell cycle progression. The MAPK/ERK pathway plays a critical role in regulation of cell proliferation.

1.3.2 Insulin resistance, hyperinsulinemia, and obesity

Insulin resistance. The above-mentioned multiple insulin functions are dysregulated during the development of obesity, as fat cells gradually lose their insulin sensitivity and become insulin resistant (IR) [86]. It has been shown that almost 50% of diabetic obese individuals express reduced INSR tyrosine kinase activity in the adipocytes [87]. The AKT downstream GLUT4 expression in adipocytes is reduced in insulin resistant obese and type 2 diabetic individuals [88]. As such, reduced glucose uptake is considered as the most significant consequences of insulin resistance. The blunted insulin signaling and glucose uptake ability are attributed to high serum glucose. Therefore, measurement of insulin-stimulated glucose uptake is a widely used method to assess insulin sensitivity.

Hyperinsulinemia develops in parallel with insulin resistance. Due to adipose tissue insulin resistance, glucose and lipid cannot be stored in adipocytes efficiently. In situations of excessive caloric intake, ectopic lipid accumulate in liver and muscle. This fat deposition in non-adipose organs is detrimental to organ health (known as lipotoxicity) and can lead to the loss of insulin sensitivity in these tissues and increased serum glucose. To maintain a normal glucose level the pancreas secretes a higher amount of insulin, referred to as hyperinsulinemia. If insulin resistance persists, the high production of insulin eventually results in pancreatic β-cell exhaustion and functional failure. At this point, endogenous insulin secretion decreases. When the level of insulin is unable to maintain glucose homeostasis, hyperglycemia occurs and type-2 diabetes is diagnosed [89].

Hyperinsulinemia induced obesity. It remains debated whether hyperinsulinemia drives obesity, or whether obesity induces hyperinsulinemia [90]. Most obese individuals tend to present with various degrees of hyperinsulinemia, whereas others can still maintain a healthy level of fasting insulin, controvercially called "healthy-obese" [91]. This suggests that obesity may develop before hyperinsulinemia. However, there is abundant evidence that supports initial hyperinsulinemia as the driver of obesity: meal-induced transient hyperinsulinemia already occur in individuals with mild glucose intolerance but who are not yet obese similar to what happens during a glucose tolerance test [92]. This indicates the possibility that adipose tissue in moderately obese individuals may have already been stimulated by high levels of insulin occasionally. High levels of insulin facilitate lipogenesis and stimulate adipogenesis [81], leading to adipocyte hypertrophy and tissue expansion under conditions of nutrient excess. It has been observed that type 1 diabetic individuals gain significant body weight after insulin treatment,

which also occurs in type 2 diabetics, indicating a remarkable effect of insulin on adipose expansion even in insulin resistant adipose tissue [93, 94]. Furthermore, a short stimulation of hyperinsulinemia was shown to promote inflammation by inducing the secretion of TNF-α, IL-6 and MCP-1, in turn further aggravating tissue insulin resistance [95-98]. Taken together, the evidence suggests that hyperinsulinemia, induced by nutritional overload, can occur at an early phase of obesity, trigger adipocyte hypertrophy and induce tissue inflammation which further exacerbates adipose tissue insulin resistance.

Paradox of selective insulin resistance. It is well established that insulin plays a vital role in promoting adipocyte growth in insulin-sensitive adipocytes [81]. So what happens in insulin resistant adipocytes, and how does hyperinsulinemia affect adipocyte function? It has been suggested that the insulin pathway is selectively blocked in insulin resistant adipocytes, mainly impairing the glucose uptake arm of insulin signaling, leaving the other side effectors of AKT intact [99]. This data also indicates a possibility that in the presence of hyperinsulinemia, part of the insulin signaling pathway could even be overstimulated and potentially trigger hyperactivation of downstream insulin effectors such as ERK or mTORC1. This hypothesis, which has been explored in the liver, vasculature and brain, is referred to as the 'paradox of selective insulin resistance [100-102]. However, whether it also occurs in human adipocytes is still unclear.

1.4 ADIPOSE FAT MASS REGULATION IN OBESITY

Fat mass expansion is an adaptive response of adipocytes to increase their storage capacity. Despite being linked to obesity, fat mass expansion is crucial for keeping metabolic balance. Healthy adipocytes enlarge, increasing their storage capacity, to keep excess lipids in a 'safe' manner while maintaining metabolic homeostasis. When the adipose tissue fails to expand, for example in medical conditions like lipodystrophy, the consequences for the individual are strikingly similar as those of severe obesity, namely metabolic disorders including insulin resistance, hepatic steatosis, hyperlipidemia and ectopic lipid accumulation in non-adipose organs [103, 104]. Ectopic lipid accumulation is deleterious, as it can cause insulin resistance, cardiovascular problems and other lipotoxic outcomes [103, 105]. Therefore, it is crucial to preserve adipose tissue for maintaining a safe lipid storage capacity.

Excessive calorie intake induces adipose tissue expansion mainly through two processes: increasing adipocytes cell size (hypertrophy), and/or recruiting new adipocytes to increase cell number (hyperplasia) (**Fig. 4**). During weight gain, the lipid accumulation is greater than release and adipocytes enlarge their size to store more fat [40, 106, 107]. If the calorie-overloading situation is not compensated for by increased energy expenditure, adipogenesis is triggered to expand fat cell number in response to increased storage needs [37, 108].

1.4.1 Hyperplastic adipose expansion

Adipogenesis is the process through which new adipocytes arise from committed preadipocytes (**Fig. 4**). The development of hypertrophic expansion activates adipogenesis, which thus relates to severe body weight gain [109, 110]. Generally, adipogenesis is considered to be a protective and 'safe' way to expand adipose tissue, as this process recruits more insulin sensitive small adipocytes [111-113]. Obese individuals with hyperplastic adipocytes are associated with a better metabolic profile, as demonstrated by Arner *et al*. in a large cross-sectional study [114]. Individuals who regain body weight after bariatric surgery are more insulin sensitive compared to body-weight matched never-obese controls. This is associated with a significantly increased fat cell number during body weight regain, while cell size is not changed [115]. Moreover, rosiglitazone treatment which paradoxically increases adipogenesis and thus adipocyte number, significantly improves the metabolic profile of individuals. On a tissue level, hypertrophic adipose tissue is associated with increased levels of hypoxia, fibrosis and macrophage infiltration, whereas hyperplastic adipose tissue contains relatively high blood

vessel density and lower hypoxia and inflammation [116, 117]. If adipogenesis is impaired, the existing adipocytes continue to uptake the lipid present in the circulation leading to adipocyte hypertrophy, insulin resistance and eventually ectopic fat storage [118]. Therefore, at the state of energetic imbalance, adipose tissue hyperplasic response is healthier than a hypertrophic one.
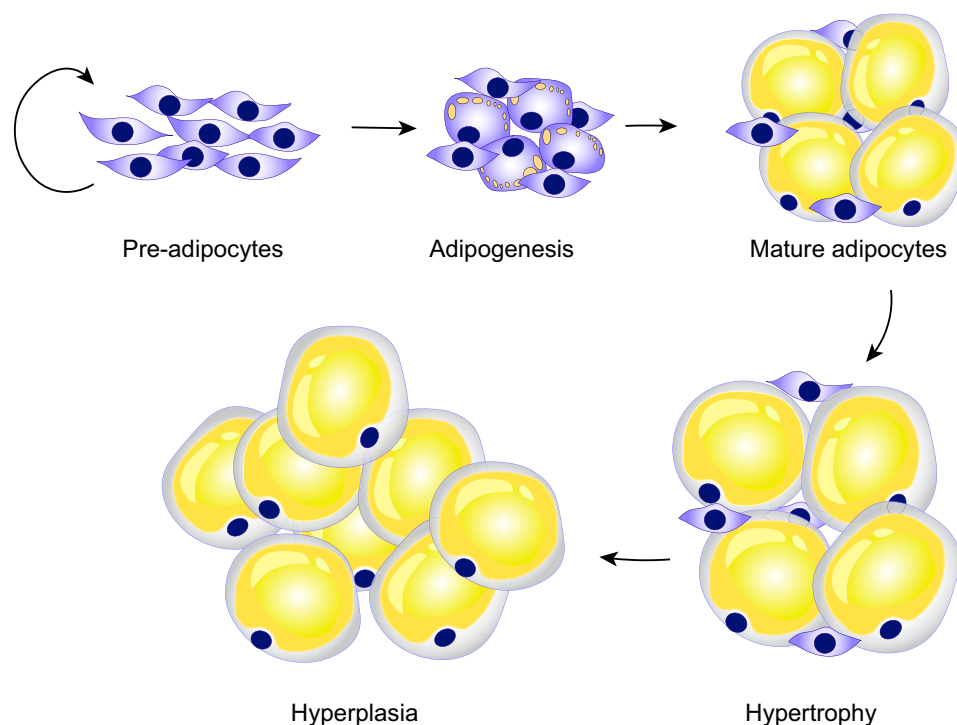
Figure 4. Schematic representation of adipose tissue expansion. Adipocyte number is determined by preadipocyte proliferation and adipogenesis, whereby terminally differentiated preadipocytes accumulate lipid and increase cell size to become mature adipocytes. Adipose tissue expansion takes place through hypertrophy, an increase in the cell size of existing adipocytes, with or without subsequent hyperplasia.

Adipogenic regulation. A stable adipocyte cell number is maintained dynamically, with approximately 10% of human adipocytes replaced every year [38, 114]. This means adipogenesis is continually ongoing with new adipocytes constantly being lost and generated. Despite the crucial importance of adipogenesis and hyperplasia, the mechanisms controlling cell number amplification are not fully understood. The process of adipogenesis includes two aspects: preadipocyte proliferation to increase their number, and the differentiation of preadipocytes to become fully mature fat cells. Precisely controlled adipogenesis involves the cooperation of a complex array of transcriptional signals, endocrine factors and hormones. Growth hormone, glucocorticoids and thyroid hormone promote adipogenesis. Insulin and IGF-1 are known to have a robust pro-adipogenic effect also.

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A large body of cell culture and mouse model studies has broadened our knowledge of adipogenesis mechanisms. C/EBPα [119] and PPARγ are two master adipogenic transcription factors coordinating the expression of a myriad of proteins to establish the characteristics of mature adipocytes [118, 120]. In mice, C/EBPα is essential for adipocyte terminal differentiation after birth, while PPARγ is required for developmental adipogenesis, both in embryogenesis and adulthood [121]. Other transcription factors, including SREBP1c (sterol regulatory element-binding protein), STATs (signal transducers and activators of transcription), AKT2 [122] and PDGFRβ (platelet-derived growth factor receptor β)[123], have also been shown to play a significant role in controlling adipogenesis, however the list is still expanding.

Despite this, more human studies are needed to evaluate these mechanisms proposed in animal models. It would be informative to understand how cell number is strictly controlled in humans, and in turn elucidate new aspects of fat mass control and metabolic homeostatic maintenance.

1.4.2   Adipocyte size and hypertrophic adipose expansion

When overnutrition exceeds adipose storage capacity, the lipid deposited in non-adipose organs is considered a significant driving force causing insulin resistance and obesity related metabolic disorders [103]. This "limited expandability hypothesis" emphasizes that both adipocyte size and number can reach saturation points of maintained healthy expansion. Adipocyte size is particularly crucial for determining adipose tissue homeostasis, as hypertrophic adipocytes have been widely recognized as the primary component of obesity-related metabolic syndrome [111].

1.4.2.1   Hypertrophic adipocytes

The association between adipose tissue hypertrophy and metabolic dysregulation has been intensively explored. A short-term overfeeding study revealed that a 3kg increase in body weight in moderately obese individuals resulted in a 10% adipocyte size enlargement [106]. Adipocyte hypertrophy is a high risk factor predicting impeded insulin sensitivity [106]. A large cohort study of 764 non-obese and obese subjects demonstrated that adipocyte hypertrophy is an independent factor correlating with circulating insulin levels. In this study, female subjects with a large fat cell volume relative to BMI matched individuals exhibited multiple adverse

metabolic profiles, such as decreased insulin sensitivity and increased circulating cholesterol and triglycerides levels [114].

Human mature adipocyte size varies greatly, with a broad range from 20 μm to 300 μm in diameter. Generally, an adipocyte with a diameter less than 70 μm is considered a small adipocyte, while 70 μm-120 μm is large, and adipocytes over 120 μm are considered very large [124]. Compared to small adipocytes, large fat cells display distinct transcription profiles, in which the major alteration is an overactivated immune and inflammatory response [125], demonstrated by a large body of transcriptomic studies (reviewed in ref.[126]). Skurk *et al*. have separated adipocytes based on a similar size category mentioned above and revealed that the larger adipocytes secreted increased levels of IL-6, IL-8, MCP-1 and leptin, supporting the role of hypertrophic adipocytes in their contribution to adipose tissue inflammation [127]. Moreover, hypertrophic adipocytes show reduced insulin sensitivity [128]. In a study that compared small versus large adipocytes from matched individuals, Western blot analysis from an equal volume of cells revealed no difference in insulin receptor or GLUT4 expression [129]. However, insulin-stimulated GLUT4 translocation to the plasma membrane was increased two fold in small cells versus large cells, implying that insulin-GLUT4 signaling is impaired in hypertrophic adipocytes [129, 130]. Together, these data illustrate multiple functional alterations in large fat cells which are crucial contributors to adipose tissue metabolic dysfunction.

1.4.2.2 Mechanisms regulating adipocyte size

Despite the importance of cell size alterations, the mechanisms regulating adipocyte size are still under investigation. Several hypotheses have been proposed to explain adipocyte hypertrophy. Firstly, adipocyte size expansion is determined by lipogenic capacity, whereby the lipid availability and adipocyte lipid uptake capacity are vital components [131-133]. Moreover, recent studies in mice reported that adipocytes utilize ion channels to create a hypotonic environment to facilitate cell swelling. Two proteins, transient receptor potential cation channel subfamily V member 4 (TRPV4) [134] and SWELL1 (VRAC, voltage-regulated anion channel)[135] were recognized as crucial regulators controlling hypotonic or hypertonic pressure, inducing cell size alteration and mediating GLUT4 membrane translocation. Furthermore, caveolae, the bulb-shaped surface pits located on the adipocyte plasma membrane, are also important for regulating GLUT4 translocation [136]. Caveolae can also function as a sensor of mechanical and osmotic stress, and therefore coordinate the alteration of cell size [137].

So far, the consequences of altering cellular mechanical morphology have largely focused on glucose uptake, though this likely does not explain all the functional changes in hypertrophic fat cells. Notably, although cell enlargement is significantly associated with increased lipid droplet size, it also translates into higher cellular demands, in that cells require a larger cytoplasm with more organelles to produce enough transcripts and proteins to support regular cellular function [138, 139]. For example the production of perilipin, a lipid droplet-associated protein, needs to increase to maintain the integrity of the lipid droplet membrane. Similarly, large adipocytes need more cytoskeleton to keep efficient intracellular trafficking, preserve cellular integrity and avoid cell breakage [140]. Indeed, the total number of transcripts produced in large adipocytes has been shown to be increased compared to small cells [138]. However, it is still not clear how adipocytes adapt to this growth demand. It is also not clear whether this can be driven by overloaded growth stimuli such as insulin, IGF-1 (insulin-like growth factor 1) and others.

1.4.2.3 The mTOR pathway in cell growth

The mechanistic target of rapamycin (mTOR), and especially mTOR complex 1 (mTORC1), is a highly conserved effector regulating cell growth in all eukaryotes and is suggested to be involved in adipocyte size regulation [141, 142]. Insulin and IGF-1 activate mTORC1 through the phosphorylation of AKT to inhibit the mTORC1 negative regulator, TSC (**Fig. 3**). Feeding-induced amino acid levels can also activate mTORC1, which is sensed by the heterodimeric Rag GTPases [143]. As a result, mTORC1 activates downstream substrates p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP) to promote protein synthesis, *de novo* lipid synthesis for plasma membrane expansion, and nucleotide synthesis required for DNA replication [142].

The mTOR pathway is activated in obese adipose tissue [144, 145]. Hyperinsulinemic rats, maintained by chronic insulin-containing pumps, developed insulin resistance [146]. Interestingly, the liver and muscle from these rats showed impaired insulin signaling, while the adipose tissue was unaffected, showing preserved pAKT and mTORC1/S6K expression [146]. Additionally, mTORC1 deactivation via adipocyte-specific Raptor knockout resulted in lipodystrophy and ectopic lipid deposition in the liver, suggesting a crucial role of mTORC1 in regulating adipose expansion via affecting both size and adipogenesis [147]. Furthermore, metformin, as the first-line anti-diabetic drug, inhibits mTORC1 via activation of adenosine monophosphate-activated protein kinase (AMPK). Metformin significantly promotes insulin

sensitivity and reduces fat mass in obese insulin-resistant children [148]. Moreover, treatment with metformin and the mTORC1 inhibitor rapamycin, revealed a profound anti-inflammatory profile through inhibition of NF-κB (nuclear factor-kappa beta) [149, 150], suggesting that the mTORC1 pathway could potentially regulate both adipocyte growth and inflammation, in line with our results (**Paper III**).

1.4.3 Cell cycle related growth

High levels of insulin, growth factors and some hormones can be strong growth stimulators, known to induce cell enlargement and proliferation [151-155]. Terminally differentiated mature adipocytes, with their large lipid droplet, are traditionally considered as postmitotic cells. How adipocytes react to such strong growth stimuli remains poorly examined and not fully understood.

Interestingly, it has been demonstrated that apparently postmitotic neurons can also adopt a cell cycle-like profile, expressing multiple cell cycle related cyclins and proteins [156]. Neuron cell cycle re-entry seems to be dependent on the activation of the mTOR pathway but has been linked to cell death in Alzheimer's disease and other neuronal degeneration disorders [157-160]. Whether adipocytes can also be stimulated to re-enter a cell cycle program in the presence of a mitogenic stimuli is in investigated here (**Paper III**), and we demonstrated that adipocytes exhibited an endoreplication profile, rather than a mitotic cell cycle (**Fig. 5**).

1.4.3.1   Endoreplication

Endoreplication, where cells proceed through the cell cycle without division, has recently been proposed to be an important mechanism coordinating cellular proliferation and differentiation [161]. Endoreplicating cells move through the G1, S, and G2 phases, increasing cell size and replicating their DNA (**Fig. 5**). In the absence of cytokinesis, the cell either skips mitosis resulting in a polyploid mononuclear cell, or proceeds through mitosis with nuclei division giving rise to multi-nucleated cells [162]. Increased DNA content allows cells to enhance their capacity to produce transcripts and proteins to permit cell growth, and facilitates nutritional storage and transportation [161]. Therefore, endoreplication usually occurs in terminally differentiated cells, and is considered an advantageous strategy for cells adapting to continuous growth. In the absence of cytokinesis, endoreplication does not involve cytoskeletal

rearrangement, and unlike mitotic division, causes less disruption to highly structured tissues [163].
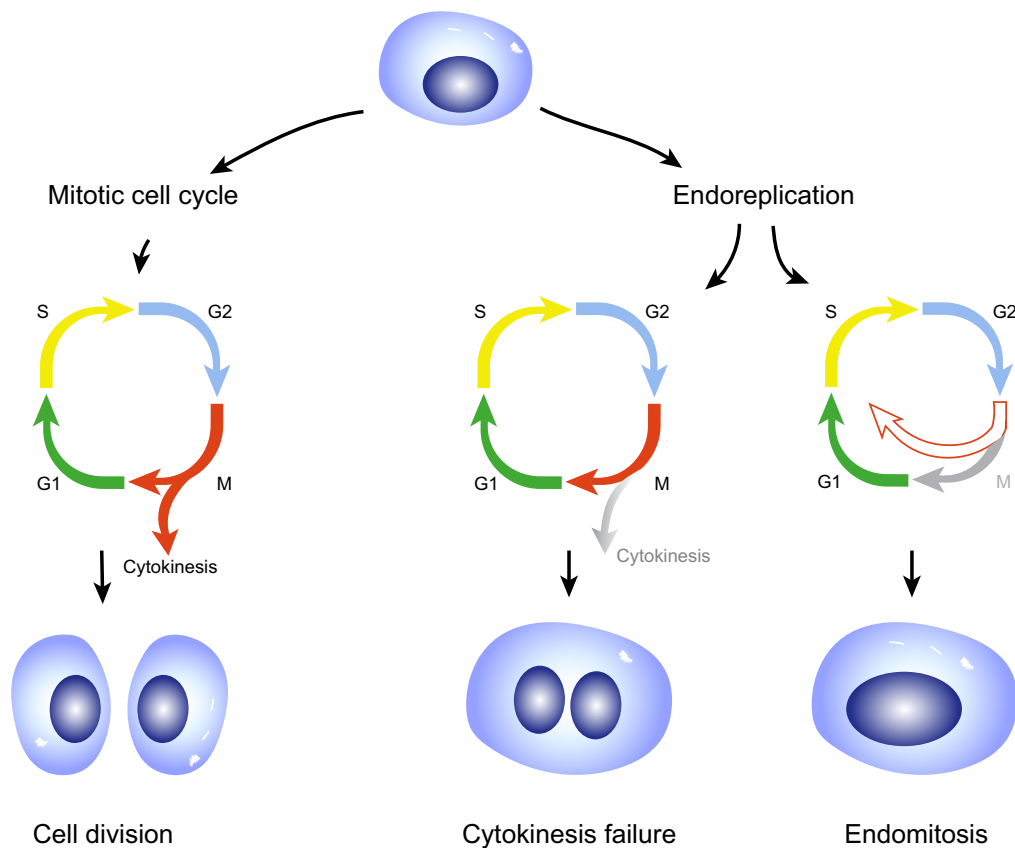
Figure 5. Schematic representation of mitotic cell cycle and endoreplication. A mitotic cell goes through 4 phases of cell cycle, passing through M-phase, undergoing cytokinesis and becoming two daughter cells. Endoreplicating cells replicate DNA, although they do not pass through M-phase (endomitosis) or pass through M-phase but fail to undergo cytokinesis.

While endoreplication has been investigated extensively in worms and flies [164], its role in human cells is attracting increasing attention. Megakaryocytes are known for endoreplicating before fragmenting into platelets. Furthermore, hepatocytes, trophoblast giant cells, and mammary epithelial cells are also reported to undergo endoreplication to support their specialized functions [165-167]. In particular, hepatocyte endoreplication was found to be induced by insulin through PI3K/AKT pathway, implying an important role of insulin in regulating cell growth via endoreplication [154]. Whether this mechanism also occurs in human adipocytes was still not clear. Addressing this question will potentially provide new insights into adipocyte hypertrophy and related functional regulation.

1.4.3.2 Cell cycle data in adipocytes

Several studies have observed the expression of cell cycle related proteins in mature adipocytes. For example, the expression of cyclin proteins (D1, E1, A2, and B1) are well known to drive cell cycle progression, through the formation of complexes with their corresponding cyclin dependent kinases (CDKs) to regulate a myriad of substrates. CDK4/Cyclin D complex, which phosphorylates retinoblastoma protein (RB) to initiate cell cycle entry and support progression of G1 to DNA synthesis [168], has been observed in adipocytes. Lagarrigue *et al.* show the expression of cyclin D3 and CDK4 in mature adipocytes in both mice and humans, but suggest that these perform cell cycle independent roles. Their study found that CDK4 phosphorylates IRS-2 to activate the insulin pathway. Secondary to promoting insulin sensitivity, CDK4 activity induced lipogenesis and inhibited lipolysis, leading to increased fat mass and adipocyte hypertrophy [169]. This evidence suggests an insulin-induced adipocyte growth mechanism that is associated with cell cycle-related proteins. Consistently, cyclin D1 expression has also been shown to be upregulated in adipocytes that differentiated *in vitro* [170]. However, examination of *in vivo* expression by analyzing adipose tissue sections has not revealed cyclin D1 expression in mature adipocytes [170].

Ki-67 is a well-established proliferation marker expressed in all phases of an active cell cycle, except for the G0 phase or cell-cycle arrested cells [171]. Two different studies used mouse models to investigate the expression of Ki-67 in white and brown adipocytes, supporting the hypothesis of adipocyte proliferation [172, 173]. However, these studies examined only adipose tissue sections, where distinguishing adipocytes and cells from the SVF is not straightforward and raises questions about the specificity of the observed Ki-67 signal.

In order to investigate these fundamental questions of adipocyte biology, in **Paper III**, we used sensitive quantification methods that specifically focus on a pure population of mature adipocytes. By showing the expression of multiple cyclins (D1, E1, and A2), and cell cycle related proteins, including pHH3, PCNA (proliferating cell nuclear antigen), Ki-67, and Anillin, we demonstrated a cell cycle profile occurred in human mature adipocytes, indicated a potential link between adipocyte cell cycle re-entry and hypertrophy.

1.5 CELLULAR SENESCENCE

Hypertrophic adipocytes display an augmented secretion profile in obesity, which is one of the detrimental driving forces in adipose tissue inflammation, insulin resistance and obesity-related pathologies. However, the mechanisms underlying adipocyte size enlargement and proinflammatory secretion are still elusive [127]. Senescence, a cell fate that is defined by an irreversible proliferative arrest, is known to induce a profound pro-inflammatory secretory pattern which alters the local microenvironment. Whether this cell cycle-related program also regulates the secretion pattern in terminally differentiated adipocytes is still poorly understood.

1.5.1 Cellular senescence

Senescent cells are in irreversible cell cycle arrest, accompanied by an active DNA damage response (DDR), high metabolic activity, and exhibit a senescence-associated secretory phenotype (SASP). Cellular senescence was first reported by Hayflick who described the phenomenon in proliferating cells in culture that gradually lost their capacity for division after 50 serial passages, but still stayed alive for weeks in this a non-dividing state [174]. This phenomenon is now known as 'replicative senescence', induced by constant telomere shortening through each round of cell division [175], and is an essential aspect of cellular aging. Meanwhile, a large body of recent studies has revealed several other mechanisms by which cells can become prematurely senescent. These include activation of oncogenes, oxidative stress, and DNA damaging agents, which lead to stress-induced premature senescence [176].

Cellular senescence is implicated in both physiological and pathological processes [177]. The most widely-studied role of senescence is in the context of cancer development, where senescence irreversibly blocks tumor cell proliferation and is viewed as an onco-suppressive safeguarding mechanism against tumorigenesis [178, 179]. In mammalian embryonic development, senescence is essential for remodeling tissue and shaping organogenesis, such as in the development of the mesonephros and inner ear [180, 181]. In wound healing, accumulation of senescent endothelial cells accelerates wound closure and reduces the size of the fibrotic scar [182, 183].

Depending on the biological context however, a senescent cell and its associated secretory phenotype can also be detrimental. In stress-induced premature senescence, the altered cellular function and secretory profile of cells has been associated with several aging-related diseases including osteoarthritis, atherosclerosis and Alzheimer's disease [184-187].

Nevertheless, independent of aging, an increasing number of pathologies are being recognized as associated with premature cellular senescence, therefore, placing senescence as a central player in the cellular pathology of a variety of disorders, across many cell types and tissues [188].

1.5.2 Characteristics of senescence

Senescent cells are largely heterogeneous. The functional alterations that senescent cells exhibit, like cell size alteration or secretion profile, can partially also be present in other cellular states, and may diverge depending on the cell types or the stress triggers. Because of this complex phenotype there is still a lack of consensus on a senescent-specific profile of signature genes and proteins. Therefore, in order to precisely distinguish senescent cells, several hallmarks need to be considered together, including a DDR, cell cycle arrest, and the senescence associated secretion phenotype (SASP).
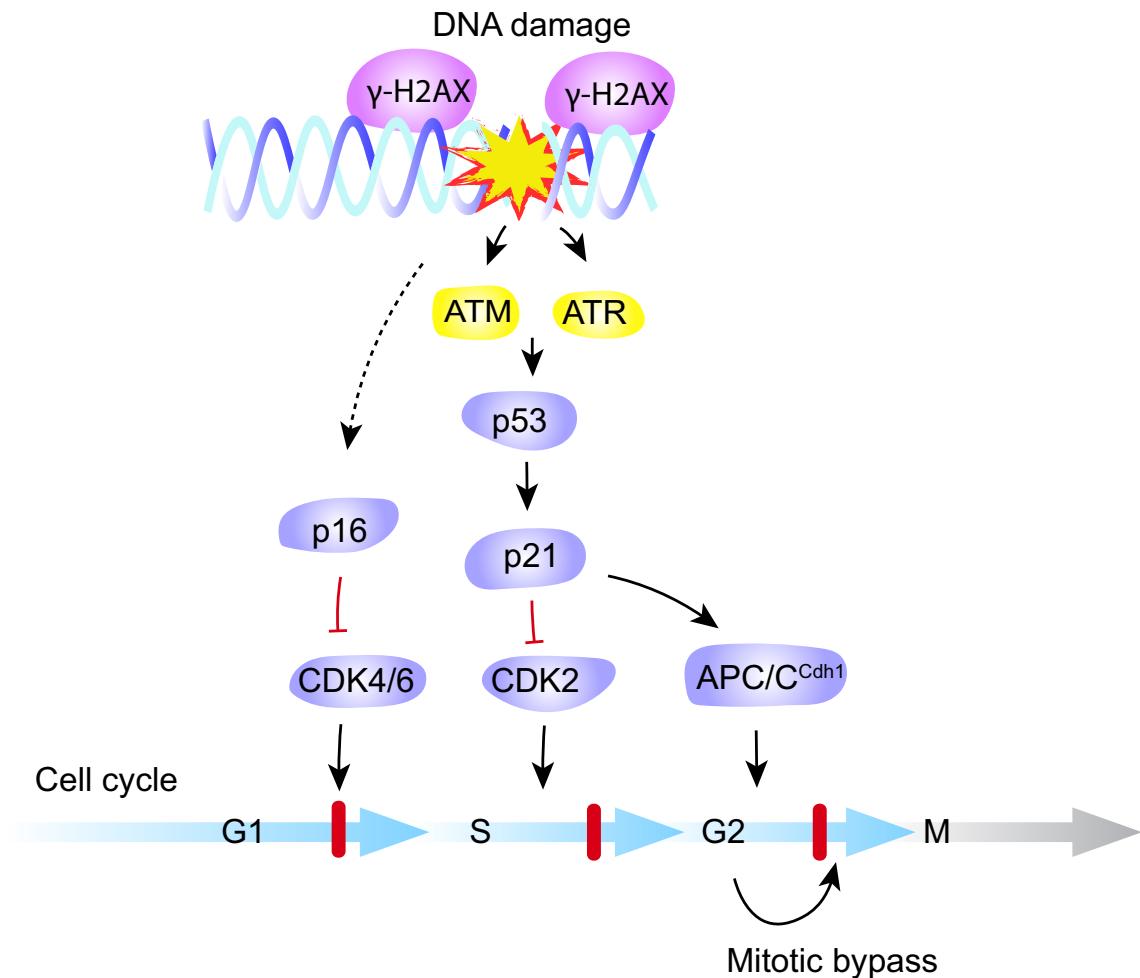
1.5.2.1 DNA damage response

It is widely believed that premature senescence is triggered by a persistent active DDR (**Fig. 6**). DNA is sensitive to different types of stress such as telomere erosion and oxidative stress. The main consequence of stress is the accumulation of DNA single- and double-strand breaks (SSBs, DSBs) which, if left unrepaired, can lead to the accumulation of mutations and genome instability. To preserve genome integrity, cells respond to DNA damage by engaging a signal amplification cascade called DDR to block cell cycle progression and induce repair of the DNA lesion [189]. If the damaged sites in proliferating cells are properly fixed, cell cycle will be quickly resumed. Otherwise, unrepaired damage can lead to a prolonged activation of the DDR which eventually translates into an irreversible cell cycle arrest (senescence) or apoptosis [190].

After DSBs, the DDR is initiated by the recognition of the damage by a large protein complex, composed by Mre11, Rad50 and Nbs1, called the MRN complex. MRN recruits and activates the essential kinase ataxia telangiectasia-mutated (ATM) kinase, which efficiently phosphorylates the histone H2AX at Ser139 (called γ-H2AX) [191]. The loading of active ATM onto the damage site leads to the recruitment of additional ATM molecules, mediated by p53-binding protein 1 (53BP1) and mediator of DNA-damage checkpoint 1 (MDC1), to strengthen the repair cascade and amplify the chromatin modification signals in a positive feedback loop.

The accumulation of repair factors (MRN complex, ATM, 53BP1 and MDC1) at the damage site generates a cytologically detectable structure called DNA damage foci (DDF) [192, 193]. After the initial phosphorylation of γ-H2AX, ATM phosphorylates the checkpoint kinases CHK1 and CHK2 within the foci, quickly followed by a distribution throughout the nucleus that helps spread the DDR signaling and further activate their downstream substrates, including p53 to block cell cycle progression [194, 195].

Figure 6. Schematic representation of a DNA damage response (DDR) induced cell cycle arrest. ATM/ATR mediated DDR activates p53 pathway. The expression of two major CDK inhibitors, p16 and p21, blocks cell cycle progression, leading to senescent cells arrest either at G1/S phase, or at G2 phase that, via mitotic bypass, resulting in polyploidy in endoreplicated cells.

The detection of DDF together with the expression of γ-H2AX and p53 are often used as markers for the DDR and senescence (**Fig. 6**). However, it has been noted that the DDR is not always present in senescent cells [196]. Moreover, oxidative stress-induced single-strand DNA

damage, which involves the activation of repair protein X-ray repair cross-complementing protein 1 and p16 expression, are also able to induce cellular senescence [197, 198].

1.5.2.2 Cell cycle arrest

Irreversible cell cycle arrest is one of the hallmarks of senescence. *In vitro*, a reduction in serial colony proliferation or decreased DNA synthesis measured by reduced EdU (5-ethynyl-20-deoxyuridine) or BrdU (5-bromo-2-deoxyuridine) incorporation, are considered as signs of cell cycle arrest. While being technically easy *in vitro*, defining cell cycle arrest *in vivo* is not as straightforward.

The DDR activates p53 which induces the transcription of the CDK inhibitor p21 (CDKN1A). p21 in turn blocks the interphase activity of CDKs, including CDK2, CDK4, and CDK6, thus preventing the phosphorylation of Rb to promote cell cycle arrest (**Fig. 6** and ref. [199]). Moreover, it was recently shown that the expression level of p21 is crucial to determine cell fate after DNA damage. Cancer cells with moderate DNA damage in G1 had a moderate p21 state that could either evolve to low p21 expression and result in a proliferative phenotype or progress to high p21 expression and become senescent [200]. Although p21 is suggested to be essential for initiating senescence, this study indicates that only using p21 as a cell cycle arrest marker is not sufficient.

P16 is another Rb regulator that selectively inhibits the activity of CDK4 and CDK6, and blocks cell cycle progression. While p53/p21 are essential for initiating cell cycle arrest, p16 is recognized as important for maintaining the arrest, and thus enforcing the maintenance of senescence [201, 202]. Independent of the p53 pathway, it has also been suggested that reactive oxygen species (ROS) trigger SSBs that upregulate the expression of p16 via p38 MAPK (mitogen-activated protein kinase) pathway, leading to cellular senescence [198, 202]. More importantly, specifically targeting p16 expression in mice can dramatically reduce senescent cell burden and result in marked improvements in health and extended lifespan [203-205]. Taken together, numerous pieces of evidence emphasize the importance of p16, which is now widely used as a reliable senescence biomarker.

G1 or G2 arrest? Previously, it was believed that senescence only arises from p21 or p16-induced G0/G1 arrested cells. Such a conclusion was however challenged by evidence that there is an accumulation of polyploid mononuclear senescent cells after multiple rounds of

division-induced proliferation exhaustion, which is accounted for by G2-arrested cells [206, 207]. Not only limited to replicative senescence, others also described stress-induced p53 activation in G2 phase leading to senescence through mitosis bypass (**Fig. 6** and **ref.** [208]). This process is mediated by p53-induced p21 activity, where p21 activates the anaphase-promoting complex/cyclosome (APC/C^{CDH1}) to degrade mitotic cyclin B1, thus leading to either cell cycle exit or mitotic skipping, resulting in tetraploid (4N) mononucleated cells [208]. Since the G1/S checkpoint is stronger than the G2/M transition, polypoid cells seem to further readapt to a G1-like state, characterized by the accumulation of G1 cyclins (cyclin D1) [177, 209].

1.5.2.3   *Senescence associated secretory phenotype*

The senescence associated secretory phenotype (SASP) is one of the key hallmarks of senescence, and is composed of an array of cytokines, chemokines, pro-inflammatory factors and extracellular matrix remodeling factors. SASP-related factors are upregulated at a transcriptional level and dynamically released into the microenvironment, influencing multiple processes such as tissue remodeling, recruitment of immune response factors, induction of angiogenesis and fibrosis [210]. Also, SASP transmitted signals can cause peripheral cells to senesce [203]. Pro-inflammatory cytokines (interleukin (IL)-1α, IL-1β, IL-6, and IL-8) and chemokines (MCP-1) are common SASP factors. However, most SASP factors have cell type-specific characteristics. Even in the same cell type, different stress stimuli or time-course of senesce progression can induce a different secretory profile [211, 212], making the SASP very hard to define in a standard way across all cell types.

The mechanism regulating the SASP is still not fully elucidated. Coppé *et al*. [213] suggested that ectopic expression of p16 and p21 induces cell cycle arrest and cellular senescence without a secretory phenotype, indicating that the two processes, cell cycle arrest and the SASP, can be separated in senescent cells. The main triggers inducing a SASP are more related to the presence of an active/ongoing DDR, followed by the activation of NF-κB [214]. More recently, signaling pathways such as P38 MAPK, NOTCH, mTOR, as well as the mitochondrial NAD+/NADH ratio were suggested to participate in the regulation of the SASP [196, 215-217]. Different mechanisms proposed to induce a SASP response might be triggered by different stimuli, which may also be linked to each other. Defining the target and SASP components in a specific cell type is necessary for understanding SASP related consequences.

1.5.2.4 Senescent cells morphology alteration

Senescent cells exhibit multiple morphological alterations. Cell size is often increased and in some cases cells flatten when becoming senescent [218]. Senescent cells are associated with increased mitochondrial biogenesis, however they are dysfunctional and generate excessive amounts of ROS [219]. Senescent cells contain a large amount of lysosomes, which can be detected by high β-galactosidase activity, through a senescence associated β-galactosidase assay (SABG). Although the β-galactosidase enzyme *per se* may not play a direct role in senescence, the SABG positivity has been established as a reliable biomarker of senescent cells [215]. Blagosklonny and coworkers summarize these morphological alterations as a 'hypertrophic' phenotype, primarily induced by growth stimuli. Such growth stimuli are essential to push arrested cells to senesce, and is one of the predominant characteristics distinguishing senescence from quiescence, which is instead a transient type of cell cycle arrest [220, 221].

MTOR activation associates to a hypertrophic phenotype. It is well accepted that growth stimuli derived from excessive nutrition, hormones or growth factors can efficiently activate the AMPK and mTOR pathways. The major mTOR protein complex, mTORC1, is an essential element regulating cell growth and proliferation [222, 223]. So far, the connection between mTOR activation and a senescence-related hypertrophic phenotype is still controversial [224], and the role of mTOR in senescence also needs clarification. However, it has been shown that mTOR activity is constitutively enhanced in senescent fibroblasts and other cell types [143, 225, 226]. Overexpression of the mTORC1 substrate eIF-4E drives cellular senescence both *in vitro* and *in vivo* [227]. Furthermore, rapamycin or metformin that inhibited the mTOR activation has been shown to extend life span, which is also associated with lower levels of senescence [228]. Wang R *et al.* report that a low dose of rapamycin can significantly reduce SABG activity, as well as eliminate pro-inflammatory cytokine secretion in senescent fat tissue, without alteration of p16 expression [228].

In senescent cells, proliferative stimuli activate mTORC1, which stimulates S6K1 expression, and induces the expression of cyclin D1 [226]. Since cell cycle progression is blocked by the DDR, cyclin D1 expression accumulates and has been consistently observed in senescent cells [229-231]. Therefore, as a sign of high proliferative potential, high cyclin D1 has become a key factor in distinguishing senescence from quiescence [221]. Still, more studies are needed to test whether reduced S6K1 activity can lead to a decrease in senescent cell burden. It is thus vital

to understand the mechanisms by which mTOR triggers senescence, to potentially identify valid targets to ameliorate senescence-associated tissue injury.
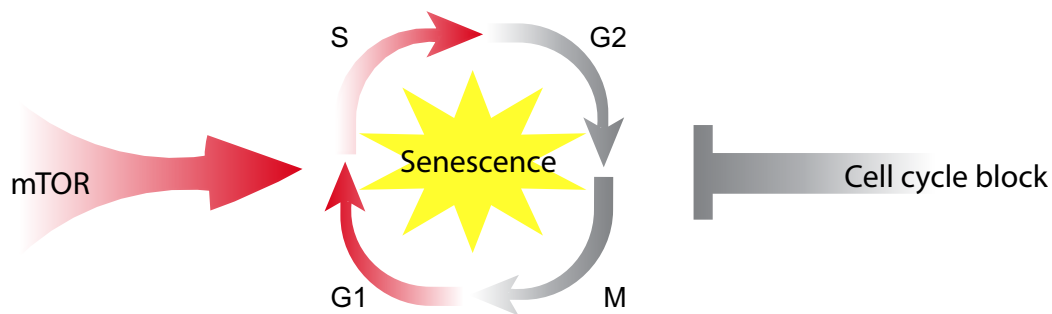
Figure 7. Schematic of mechanisms inducing senescence. On one side, mitotic stimuli induce mTOR activation, pushing cell cycle entry. On the other side, DDR activated by oxidative stress, oncogenic signaling, etc, blocks the cell cycle progression. Forces from both sides are essential for establishing a senescent state.

1.5.3 Adipose tissue senescence

Although the exact cell type(s) contributing to WAT senescence are debated, it has been shown that senescent cells accumulate in adipose tissue and that such accumulation is related to aging, obesity and diabetes [232]. Studies in murine models have shown that increased ROS are one of the main causes of senescence. In response to increased ROS, the DDR activates the p53/p21 pathway, promoting a senescent phenotype including high β-galactosidase activity and secretion of TNF-α and IL-6 [233]. The importance of DNA damage in the establishment of adipose tissue senescence was further investigated by the ablation of DNA polymerase η in mice, which led to extensive DNA damage followed by upregulated expression of ATM, p53 and p21 [234]. Interestingly, with a normal chow diet, the poly η $^{-/-}$ mice already showed marked body fat gain, accompanied by accumulation of senescent cells and an increase in the SASP profile [234]. These studies highlight the contribution of DNA damage to the development of adipose tissue senescence, obesity, tissue inflammation, and metabolic dysfunction in mice.

In contrast, exercise or using antioxidant N-acetylcysteine (NAC) to decrease the amount of DNA damage has been shown to efficiently reduce the senescent cell burden in adipose tissue [234, 235]. In addition, the clearance of either p16-expressing cells or treating senescent apoptosis-resistant cells with two senolytic drugs dasatinib (D) and quercetin (Q) increased adipogenic

potential, reduced adipocyte hypertrophy and macrophage infiltration, and largely improved glucose homeostasis and insulin sensitivity [204]. These studies highlighted that cellular senescence is involved in adipose tissue regeneration, inflammation, and immune response, and is in turn associated with obesity and the complications of diabetes.

1.5.3.1   Preadipocyte senescence

Given the cellular heterogeneity of adipose tissue, it would be important to characterize the senescent phenotype at the cellular level, to understand the cell type specific senescence associated consequences. Amongst the various cell types in adipose tissue, it has been previously reported that preadipocytes can senesce, either via proliferative exhaustion in the aging process or stimulated by increased oxidative stress in obesity [203, 236, 237].

The phenotype of senescent preadipocytes has been well characterized. Firstly, senescent preadipocytes, induced either by irradiation or serial rounds of replication, display a complex SASP profile. The array of cytokines shared by these two inducers include IL-1α, IL-1β, IL-6, IL-8, MCP-1, matrix metalloproteinase (MMP)-3, MMP-12, PAI-1, and TNF-α. These secreted SASP factors contribute to local inflammation and induce macrophage infiltration, in turn amplifying the immune response and consolidating inflammation at a whole adipose tissue level [238]. Secondly, both the proliferation and differentiation potentials of preadipocytes are reduced in the senescent state [236-238]. Moreover, through the pervasive effect of the SASP, senescent preadipocytes further impair the adipogenic capacity of surrounding progenitors and induce them to senesce [204, 238]. Specific clearance of p16 positive senescent cells in obese mice restores adipogenesis, as demonstrated by upregulation of PPARγ or C/EBPα, subcutaneous adipose tissue expansion and improved insulin sensitivity [204]. Interestingly, this study also showed that visceral adipocyte size decreased dramatically, indicating that p16-specific cell clearance may affect mature adipocytes as well [204].

1.5.3.2   Mature adipocyte senescence

Increased oxidative stress, inflammation, and the growth factor expression that occur in obesity are recognized as high risk factors causing cellular senescence [239]. However, it is still poorly understood how mature adipocytes deal with accumulating environmental stress, or as non-proliferating cells whether they can even senesce.

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Previous studies have shown the upregulation of senescent markers at the whole adipose tissue level. Nevertheless, it is difficult to distinguish potentially senescent mature adipocytes from SVF cells by analyzing tissue sections. Two studies focused on isolated adipocytes: the first in obese mice induced by ectopic expression of agouti peptide and overeating [233]; and the second in DNA polymerase η knockout mice [234]. Both studies demonstrated that a DDR can be activated in mature adipocytes, evidenced by upregulation of ATM, p53, p21 and an increased inflammatory secretion profile including TNF-a, IL-6 and MCP-1. This therefore suggests senescent-like changes in adipocytes [233, 234].

More recently, Vergoni *et al*. tracked the development of obesity in mice fed a high fat diet (HFD) and observed that the DDR was induced very early [240]. Two weeks after initiating HFD, mature adipocytes exhibited high level of ROS, significant upregulation of the oxidative DNA damage marker 8-OHdG (8-hydroxy-2-deoxyguanosine), increased γ-H2AX positive foci, and upregulated p53 and p21. Interestingly, despite the activation of the DDR at such an early stage of obesity, adipocyte insulin sensitivity and expression of GLUT mRNA was not altered after the 2 weeks of HFD stimulation. Compared to chow diet fed mice, HFD did not induce the upregulation of the inflammatory markers TNF-a, IL-6, IL1β and CCL2 after two weeks, as suggested previously by other studies [233, 234]. Furthermore, the number of immune cells, including macrophages, neutrophils and lymphocytes, were also unchanged, and changes in these were only detected after 18 weeks of HFD. Unfortunately, analysis of the senescence-related effects of long term HFD were missing in this study [240]. The dissociation between the rapid DDR and delayed inflammatory secretion profile may be attributed to an insufficient induction of DNA damage, which is needed to be active for a long and persistent time to induce a final senescent state [214]. Alternatively (and not exclusively), other factors may be involved that promote senescence in the late stages of obesity.

Blaskolonny and others have demonstrated that merely a persistent DDR or the activation of p53 is not enough to induce cellular senescence, especially in cells with low proliferation potential such as quiescent cells (**Chapter 1.5.2.4**, and ref. [241, 242]). To test whether this theory is also true in adipocytes and whether adipocytes acquire the proliferation potential that leads them to senesce, in **Paper III**, we demonstrated that mature adipocytes exhibited a cell cycle profile driven by hyperinsulinemia, which is an essential reason causing adipocyte to senesce. The data we provided hopefully can bring new insights into the mechanisms involved in adipocyte dysfunction in obesity and related diseases.

2 AIMS OF THE THESIS

The general aim of this thesis is to understand the mechanisms underlying adipose tissue dysfunction in obesity. Particularly, I have focused my thesis study on human mature adipocytes to understand how these cells adapt to cellular hypertrophy and why they alter their function from being a safe fat storage pool to a risk factor for type 2 diabetes. Given the challenging nature of adipose tissue, I aimed to develop and optimize methods and techniques to be able to better study human mature adipocytes. Lastly, I also wished to identify any essential mechanism behind adipocyte dysfunction.

Specific aims:

1. To develop a flow cytometry-based method to analyze mature adipocytes in a high throughput manner and answer the question about how human adipocyte ADRB2 expression levels are affected by obesity (**Paper I**).

2. To establish an *in vitro* system for culturing human primary adipocytes without losing cellular specificity and investigate whether it is possible to transdifferentiate human adipocytes into brown-like adipocytes (**Paper II**).

3. To characterize the effects of obesity and hyperinsulinemia on adipocyte phenotypic alterations in relation to cell cycle entry and in relation to cellular senescence (**Paper III**).

3 RESULTS AND DISCUSSION

3.1 PAPER I

Adipocytes exhibit large cellular variations in response to high nutrient overloading and are, as such, recognized as a complex heterogeneous cell type [243, 244]. Even from a single depot, based on preadipocyte lineages, adipocytes can be divided into several subgroups, which exhibit distinct metabolic properties and responses to stimuli such as insulin [45]. Future treatments against obesity may greatly benefit from a precision medicine approach that specifically targets dysfunctional adipocytes. However, the molecular heterogeneity of adipocytes remains unclear. Flow cytometry is a powerful tool that can analyze and sort single cells in a high throughput manner and is widely used for understanding the molecular mechanisms of cellular heterogeneity. Unfortunately, the morphological characteristics of mature adipocytes (lipid-filled, large, and fragile) make the use of flow cytometry very challenging.

Although several adipocyte flow cytometric methods have previously been described [245-247], we observed that mouse adipocytes, genetically labelled with tdTomato, were undetectable using these reported methods, which instead mainly captured tdTomato negative SVF cells (**Fig. 1 in Paper I**). We therefore developed a flow cytometric strategy that allowed us to analyze and sort mature adipocytes with high sensitivity and throughput (**Fig. 2**). The new flow cytometric approach was found by modifying several critical settings: inserting a neutral density (ND) filter to reduce the general forward scatter (FSC) intensity and bring the high FSC adipocytes into a detectable range; increasing the FSC threshold to reduce the noise caused by small debris and bringing large adipocytes into focus; and the use of an in-tube stirring system to adequately mix the cell suspension and deliver buoyant adipocytes into the sampler. With the newly developed strategy, we were able to show that tdTomato positive adipocytes appear as a large, easily detected cluster, allowing for subsequent analysis and sorting (**Fig. 3**). The described critical settings are not only feasible for adipocytes, but may also be used in the detection of other large or fragile particles by flow cytometry.

We further observed that side scatter (SSC), not FSC, provided an approximation of adipocyte size (**Fig. 4**). This allowed us to efficiently sort small and large adipocytes based on their SSC characteristics, without involving any steps of fixation or staining with fluorescent dyes, which constituted a significant advantage for subsequent downstream molecular applications. The size-based sorting approach can greatly facilitate studies which investigate adipocyte

hypertrophy and heterogeneity. Furthermore, the improved flow cytometry strategy can be used in combination with an antibody staining step for adipocytes. As a proof of concept, we tested UCP1 immunostaining on mouse adipocytes, and specifically sorted UCP1+ adipocytes from brown adipose tissue and β3-adrenergic agonist (CL 316,243) stimulated subcutaneous white adipose tissue (**Fig. 6**). Thus, we demonstrated the rapid and straightforward detection of adipocyte subpopulations, which in addition to investigating the browning potential of various treatments on white adipocytes, can be equally useful for any study that requires high-throughput single cell adipocyte information.

The expression density of ADRB2 on human adipocytes is essential for inducing adipocyte lipolysis, however, its expression pattern on human adipocytes remains not fully clear. Therefore, we sought to investigate the expression of ADRB2 in human adipocytes using our newly established flow cytometry method, testing two different antibodies against ADRB2. We observed a decrease in ADRB2 expression in adipocytes from obese insulin-resistant individuals (**Fig. 7A-D**), a result that was consistent with previous reports [30]. However, the flow cytometry data revealed that the reduced ADRB2 level is not due to a general decrease of protein level in every adipocyte, but instead can be explained by a heterogeneous distribution of ADRB2 expression in different subgroups of adipocytes, namely, we observed two distinct adipocyte clusters: an ADRB2 negative cluster and an ADRB2 positive one (**Fig. 7E**). The percentage of ADRB2 negative adipocytes increased dramatically in the insulin resistant individuals and revealed a heterogeneous loss of ADRB2 signals. These results allowed us to speculate that the reduced rate of stimulated lipolysis in obesity may be due to an increase in a subpopulation of dysfunctional adipocytes (lacking the main lipolytic receptor, ADRB2), while the rest of the adipocytes remain healthy [107]. This evidence also suggested that specifically targeting unhealthy adipocytes may be considered a future therapeutic strategy to treat insulin resistance.

In conclusion, our study confers new technological possibilities for high-throughput analysis and sorting of human and mouse adipocytes. Our flow cytometry approach represents a significant improvement in the toolbox available to explore the biological mechanisms underlying adipocyte hypertrophy, heterogeneity, and browning. Given the relative simplicity of the method, we are positive that it can be further applied to the study of other questions in the field of adipose tissue biology and contribute to the understanding of obesity-related pathological mechanisms.

3.2 PAPER II

Brown and beige adipocytes consume stored lipids and glucose through thermogenic energy expenditure. Inducing white adipocytes to express UCP1 or transform to a thermogenic brown-like phenotype has been shown to be able to decrease body weight, promote insulin sensitivity and reverse metabolic disorders in a large body of animal studies (reviewed in ref. [248]). White adipocyte browning is therefore becoming an attractive therapeutic avenue for obesity and type 2 diabetes treatment. However, translating the studies from animal models to human adipocytes has been obstructed, partly due to the limitations of *in vivo* human studies, but also because of the lack of a reliable *in vitro* model for testing browning mechanisms in human adipocytes.

Most studies on adipocytes have relied on the *in vitro* differentiation of pre-adipocytes into mature adipocytes, however this presents a series of limitations. Most importantly, *in vitro* differentiated adipocytes are small and multilocular, missing the crucial unilocular and spherical characteristics of mature adipocytes.

In **Paper II** we developed a method to culture mature adipocytes that keep buoyant adipocytes floating while aggregating underneath a transwell membrane, termed "Membrane Mature Adipocyte Aggregate Cultures", MAAC (**Fig. 1A in Paper II**). This setup provides ample cell-cell contact and mimics the *in vivo* environment. We showed that the MAAC successfully maintained primary adipocyte properties and metabolic functions. Both mouse and human MAAC-cultured adipocytes, compared to non-cultured controls, preserved the expression levels of a number of adipogenic transcripts, including *PPARG, FABP4, ADIPOQ, LIPE,* and LPL (**Fig. 1B, C**). Further, by using unbiased mRNA sequencing, we compared transcriptional differences of adipocytes cultured using MAAC, floating cultures, traditional progenitor differentiation-based cultures, and transplanted tissue piece cultures (**Fig. 2**). The sequencing results revealed that adipocytes in MAAC displayed the highest similarity to non-cultured *in vivo* controls. We observed that cells from currently used methods of *in vitro* differentiated adipocytes also showed a transcript profile similar to the non-cultured controls, despite the obvious differences in cell size and multilocular lipid droplets. Furthermore, we also showed that MAAC can successfully preserve fat depot specific genetic expression patterns even after 14 days of culture (**Fig. 3**). Moreover, mature adipocyte morphology, adipocyte functions such as lipogenesis and lipolysis, as well as the response to insulin and several pharmacological stimuli were also maintained after culturing adipocytes for two weeks using MAAC (**Fig. 4**).

Altogether, our data demonstrates that using MAACs to study primary adipocytes is highly advantageous compared to existing methods in the field.

It is known that cold stimuli can induce the generation of brown-like adipocytes in rodent white adipose tissue. *In vitro* differentiated human pre-adipocytes stimulated by rosiglitazone or other PPARγ enhancers have also been shown to acquire a brown adipocyte-like phenotype. However, differentiated adipocyte model involves using a differentiation cocktail that contains non-physiological levels of hormones which may interfere with the induction of browning. Even in mice, it is still under debate whether beige adipocytes arise from the differentiation of a separate population of pre-adipocytes, or through the transdifferentiation of mature cells [249, 250]. Furthermore, it remains unknown whether transdifferentiating primary human mature adipocytes into brown-like cells is possible. To answer this question, we first transduced human mature adipocytes with adenovirus-containing PGC-1α (PPARγ coactivator 1α), the master mitochondria biogenesis regulator. Using MAAC, we showed that both overexpression of PGC-1α and rosiglitazone could successfully induce the expression of UCP1 in human subcutaneous white adipocytes (**Fig. 6**). Therefore, we confirmed that human adipocytes are also able to acquire a brown-like phenotype which argues against previous studies claiming that subcutaneous white adipocytes do not have the capacity to brown [5]. Whether different adipose depots in humans harbour varying potentials for browning needs to be investigated, likely providing new insight as to the mechanisms of functional transition in human adipocytes.

Taken together, in **Paper II**, we established a mature adipocyte culture system, MAAC, that faithfully maintains adipocyte identity and function for a prolonged period of time. The MAAC can be widely exploited in a variety of *in vitro* studies of mature adipocytes. We demonstrated its potential by investigating a fundamental question of adipocyte biology, namely the capacity of white adipocytes to brown; however, MAAC can also be used in more direct applications like the development of high-throughput drug screens to improve obesity treatments. Co-culturing and looking at the interplay between various cell types and adipocyte is also another powerful application of the MAAC system.

3.3 PAPER III

During the establishment of obesity, nutrient excess remodels adipocytes, causing adipocyte hypertrophy. In addition to the enlarged capacity for storing fat, hypertrophic adipocytes also secrete various pro-inflammatory cytokines that attract immune cells and impair the insulin signaling pathway. Despite the important metabolic role of adipocytes, the mechanism underlying adipocyte dysfunction in obesity/metabolic disease has not been fully elucidated.

In order to better understand the regulation of adipocyte dysfunction, we performed mRNA sequencing on human adipocytes collected from non-obese, obese normo-insulinemic, and obese hyperinsulinemic individuals. The enriched pathway analysis surprisingly highlighted differences in cell cycle and senescence-related transcripts between the patient groups, including expression of cyclins, replication proteins, and replication-dependent histones in adipocytes (**Fig. 1b, c in Paper III**). Furthermore, immunocytochemistry staining of freshly isolated adipocytes revealed that human adipocytes express multiple canonical cell cycle markers such as Ki-67, Anillin, PCNA (proliferating cell nuclear antigen), and pHH3, but also express cyclins, including D1, E1, and A2 (**Fig. 1d, e and extended data Fig. 1**). This data strongly indicated a cell cycle re-entry profile in mature adipocytes, which was confirmed by *in vitro* data showing that cultured human primary adipocytes actively incorporate EdU, demonstrating *de novo* DNA synthesis and progression through S-phase (**Fig. 3**).

Despite the solid evidence supporting cell cycle re-entry, human mature adipocytes do not go through mitosis or cell division as was previously proposed in mouse brown fat tissue [173, 251]. Firstly, M phase-related transcripts such as *FOXM1*, *AURKB*, *CDK1* were not expressed in adipocytes(**Fig. 1c**). Secondly, despite the expression of mitotic cyclin B1, its uniquely cytoplasmic location did not support progression into mitosis. Similarly, Anillin and pHH3 were also expressed in adipocytes in a G2-phase fashion and did not show a mitotic pattern (**Extended data Fig. 1f-h**). This led us to propose that human adipocytes can re-enter cell cycle, but are unable to undergo mitosis or cell division. This phenomenon, referred as endoreplication, is known to happen in large cells, but has not been reported for mammalian adipocytes. Cells that have undergone endoreplication are usually polyploid and contain large nuclei with increased DNA content, allowing cells to enhance their protein production, enabling them to adapt to an increased cell size [161].

We also observed that hypertrophic adipocytes contained larger nuclei than small adipocytes. The volume of a large adipocyte nucleus was two-fold that of a small adipocyte nucleus from the same individual (**data not shown**). It is established that increased nuclear size often associates with increased genomic content [162]. Interestingly, in our *in vitro* studies we observed that EdU positive adipocytes, namely, cells that had undergone a new round of DNA synthesis, clearly possessed larger nuclei. *In vivo*, we found that Cyclin D or cyclin A positive adipocytes tended to have larger nuclei than their negative counterparts (**Fig. 2d**). Altogether, the data indicated that a proportion of nuclei may contain high amounts of DNA. Still, further studies are needed to determine the exact ploidy in adipocytes to better understand the cell cycle re-entry program relating to adipocyte hypertrophy.

Importantly, adipocyte cell cycle entry was not an accidental phenomenon. We discovered that the expression levels of cyclin D1, A2, and pHH3, as well as nuclear size, displayed significant positive correlations with the levels of C-peptide (insulin secretion) and insulin resistance (**Fig. 2c**). This suggests that hyperinsulinemic obese individuals have more adipocytes committed to re-enter the cell cycle than subjects with normal insulin levels or non-obese individuals. Consistently, the addition of insulin to cultured adipocytes significantly increased EdU incorporation. On the other hand, depletion of insulin by using charcoal treated serum lowered the number of adipocytes undergoing DNA synthesis (**Fig. 3**). Together, this data suggests that adipocyte cell cycle re-entry is driven by serum insulin.

We also quantified the expression of the cell cycle marker Ki-67 in adipocytes. Contrary to those of cyclins, Ki-67 levels were lower in adipocytes from hyperinsulinemic individuals, indicating a pattern of cell cycle arrest (**Fig. 4a**). We found that this cell cycle arrest was associated with cellular senescence, a phenomenon not commonly described for postmitotic cells. Adipocytes displayed several senescence markers, including increased β-galactosidase activity, reduced nuclear expression of HMGB1 (high mobility group box protein 1), increased p16, p21 and γ-H2AX, as well as upregulated transcripts that associate with a senescence related secretion phenotype, including *IL6* and *CXCL8* (IL-8) (**Fig. 5**). Interestingly, adipocyte senescence was not correlated with aging, but rather mostly occurred in hyperinsulinemic obese individuals.

The finding that insulin can induce cell cycle entry, but also associates with adipocyte senescence may seem paradoxical. As the mTOR pathway has been suggested to be involved in both of these processes, we used the AMPK-activator metformin, which is currently the most

prescribed treatment for type 2 diabetes, to inhibit mTOR activation in human adipocytes. Given together with insulin, metformin completely blocked adipocyte cell cycle re-entry, as well as prevented adipocyte senescence (**Fig. 6g-i**). These results suggest that the mTOR pathway mediates adipocyte cell cycle entry, which is necessary for the establishment of senescence. Next, we introduced a CDK4/6 inhibitor, palbociclib, which is used in the clinic to treat amongst other cancers, breast cancer, to insulin-stimulated adipocytes. Palbocilib induced a distinctive cell cycle arrest but, in contrast to metformin, dramatically increased adipocyte senescence, indicating that blocking cell cycle downstream of mTOR signalling led to senescence. As expected, metformin prevented adipocyte senescence, resulting in a profound decrease of the SASP, including IL-6, IL-8, IL-1β, MCP-1 and TNF-α. Interestingly, Palbociclib-induced adipocyte senescence did not affect adipocyte secretion factors, which may represent a treatment time not long enough to induce changes. Taken together, the data demonstrate that hyperinsulinemia can drive adipocyte cell cycle entry through activation of mTOR signaling, which along with cellular stress can facilitate the development of cellular senescence.

Although adipocytes isolated from hyperinsulinemic individuals develop insulin resistance with impaired glucose uptake, the insulin signaling in the cells is not entirely blocked, as previously suggested in animal studies [99, 146]. In human adipocytes, we observed that the insulin-induced AKT phosphorylation was preserved in hyperinsulinemic individuals, supporting the existence of an intact insulin signaling pathway in these subjects (**Fig. 3i**). During the establishment of obesity, chronic high levels of insulin may thus activate the mTOR pathway through the selectively preserved insulin signaling pathways and stimulate adipocyte cell cycle re-entry.

Finally, although the presence of a mitotic stimuli such as insulin is a prerequisite for stimulating adipocyte cell cycle re-entry, the presence of a cellular block is also required for the induction of senescence. Whilst metformin prevented cell cycle entry, palbociclib only affected cell cycle progression, further supporting the notion that cell cycle blockers such as p16 and p21 activate a senescent program in mature human adipocytes. In obesity, it has been shown that the DDR can be stimulated by a high amounts of ROS, which are also associated with high adipocyte senescence [233]. It is therefore tempting to speculate that the persistent DDR in adipocytes that may occur in obese individuals can block the hyperinsulinemia-induced cell cycle re-entry, eventually resulting in senescence. Still, further experiments are required to support this hypothesis.

In conclusion, in **Paper III**, we discovered that human mature adipocytes display a cell cycle re-entry program in response to hyperinsulinemia, characterized by enlarged nuclear and cell size. Cell cycle re-entry is an essential step leading to cellular senescence, which then induces the secretion of inflammatory factors that exacerbates adipose tissue inflammation.

SASP

Senescent adipocyte
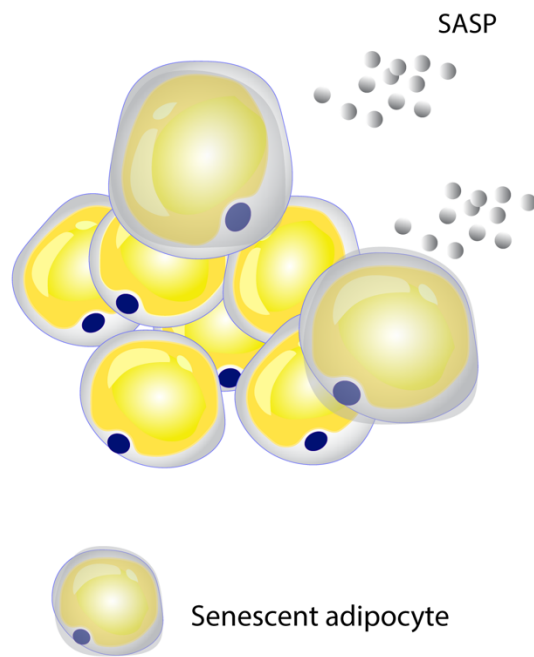
4 CONCLUSIONS AND FUTURE PERSPECTIVES

Adipocyte dysfunction is associated with metabolic disorders like insulin resistance and hyperlipidemia, are direct causes for type 2 diabetes and cardiovascular disease. These considerations bring adipocytes to the center of the mechanisms regulating whole-body metabolic homeostasis. Studies in this thesis provide new methods and aspects in understanding adipocyte biology, but also open many new questions to be answered.

In **Paper I**, the flow cytometry method we developed offers a new option to analyze adipocytes in a highly effective way, which could greatly benefit investigations seeking to understand adipocyte heterogeneity. As we found the heterogeneous expression pattern of ADRB2 expression, it would be interesting to further investigate other aspects related to the ADRB2-negative adipocyte population. Do ADRB2-negative cells display enlarged cell size, reduced insulin sensitivity or other dysregulated characteristics? What are the mechanisms that mediate the downregulation of ADRB2 in a specific subgroup of cells? Is there a heterogeneous expression pattern of other molecules regulating lipolysis or insulin sensitivity in adipocytes as well? Is there functional heterogeneity between adipocytes or does one particular cluster of adipocytes contain all the dysregulated functions? Answers to these questions will hopefully provide new insights into adipocyte biology.

In **Paper II**, we present a novel cell culture method for adipocytes, allowing us to directly modulate human or mouse mature adipocytes *in vitro,* whilst maintaining improved *ex vivo* adipocyte characteristics. This new model can hopefully help to translate a large amount of animal study results into human adipocytes, including future studies on inducing human adipocyte browning, for instance, comparing the adipose depot differences in the ability of adipocyte browning. Investigating adipocyte crosstalk with other cells types, such as macrophages or endothelial cells could also greatly benefit from the MAAC co-culture possibilities. Furthermore, it would be interesting to use the MAAC for high throughput compound screening experiments and discover targets that regulate adipocyte function.

In **Paper III**, our finding of cell cycle re-entry and senescence in human mature adipocytes opens up a whole new perspective in adipocyte biology. Considering adipocytes as post-mitotic cells, unable to re-activate a cell cycle program has been a cornerstone of adipocyte biology. We provide data that challenges this and prompts one to reconsider current views of adipocyte physiology. Additional experiments investigating the ploidy of adipocytes and how adipocytes

respond to obesity and hyperinsulinemia at a cell size/DNA level are important next steps. Additionally, it is still not clear which parts of the insulin signaling pathway are preserved in hyperinsulinemic adipose tissue, and which ones may be overactivated. Further studies are required to investigate the role of the mTOR pathway in adipocyte cell cycle re-entry and senescence. Lastly, research needs to continue on adipocyte senescence. How is senescence initiated, is it also in human adipocytes due to a DDR? We still do not know the complete extent of the functional alterations in senescent adipocytes. For example, does senescence affect normal adipocyte lipid handling, such as lipolysis or lipogenesis? How do senescent adipocytes communicate with immune cells? Are senescent cells more resistant to apoptosis? Sorting senescent adipocytes to investigate these functional changes could be one way forward to better understand the consequences of senescence in mature, human adipocytes.

In conclusion, this thesis work provides a novel primary adipocyte culture model and a flow cytometric analysis approach that are useful tools for the study of mature adipocytes. We discovered and characterized human adipocyte cell cycle re-entry and senescence, shedding new light on the mechanisms inducing adipocyte dysfunction, and thus contributing to finding future therapeutic strategies for combating obesity-related metabolic disorders.

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