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Role and regulation of microRNAs in adipose tissue during aging

Xiaohui Tong

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THÈSE

En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par l'Université Toulouse 3 - Paul Sabatier

Présentée et soutenue par

XIAOHUI TONG

Le 4 septembre 2018

**Rôle de la synthèse des miARN par le tissu adipeux dans
les pathologies de vieillissement.**

Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

Spécialité : **SCIENCES DU VIEILLISSEMENT**

Unité de recherche :

I2MC - Institut des Maladies Métaboliques et Cardiovasculaires

Thèse dirigée par

Philippe VALET

Jury

Mme Soazig LE LAY, Rapporteur

M. Miguel GODINHO FERREIRA, Rapporteur

M. Ez-zoubir AMRI, Rapporteur

Mme Laurence NIETO, Examineur

M. Philippe VALET, Directeur de thèse

M. Cedric DRAY, Co-directeur de thèse

RESUME

D'une part, le dicer, l'endoribonucléase RNase III responsable de la maturation des microARN, aurait été réduit chez les adipocytes au cours du vieillissement. Avec l'utilisation de souris dicerlox / lox / adiponectine-CreERT2 inductibles par le tamoxifène, nous avons constaté que le déficit en adipocytes favorisait l'apparition de certaines complications liées à l'âge, telles que la réduction de la taille des adipocytes et le dysfonctionnement du métabolisme. L'abrogation des marqueurs adipocytaires blancs, tels que Ppar γ , Glut4 ou Hsl, indique que le dicer est indispensable au maintien de l'identité des adipocytes blancs. De plus, les résultats montrant une accumulation de lipides et une fibrose hépatique chez des souris traitées par le dicerlox / lox / adiponectine-CreERT2 + traitées au tamoxifène ont indiqué que la carence en adipocyte pourrait contribuer au vieillissement du foie.

Mécaniquement, la fonction mitochondriale semblait être régulée à la hausse en raison d'une déficience en dicer adipocytaire, indiquée par une augmentation des taux de protéines des composants d'OXPPOS et de PGC1 α . En ligne, les répresseurs des mitochondries FOXO1 et FOXO3 ont été phosphorylés et inactivés, dont les cibles antioxydantes en aval, la *Catalase* et le *Sod*, ont également diminué. En outre, *P16*, un marqueur de la sénescence, a montré une tendance à augmenter en raison de la carence en dicer. Comme le surplus de ROS mitochondrial peut entraîner des dommages à l'ADN et la sénescence, nous avons assuré que la déficience en dicer adipocytaire pourrait induire une combinaison d'activation mitochondriale et une réduction de la réduction de la détoxification possiblement induite par l'inactivation de FOXO1 et FOXO3a. Enfin, le résultat selon lequel la restriction en éléments nutritifs a régulé positivement le taux de dicer dans les adipocytes a également confirmé qu'il existe une voie de vieillissement conservée dans les adipocytes impliquant le dicer.

Par ailleurs, grâce à la sélection de microréseaux et à la validation RT-qPCR, nous avons rapporté que le vieillissement augmentait le mir-149 dans les adipocytes chez les souris de type sauvage et favorisait probablement sa sécrétion à partir du tissu adipeux perigonadal in vitro. De manière correspondante, des résultats in vitro ont également suggéré que la sénescence augmentait la production de mir-149 et la sécrétion d'adipocytes. Sur le plan fonctionnel, la régulation positive du mir-149 dans les adipocytes

3T3-F442A régulait négativement le niveau du complexe mitochondrial II et la capacité de consommation d'oxygène, associées à l'accumulation de lipides. Fait intéressant, la régulation à la hausse soutenue du mir-1949 au cours de l'adipogenèse des préadipocytes 3T3-F442A avait tendance à augmenter les marqueurs adipocytaires blancs, tels que la leptine, Glut4 ou Hsl (N = 2). Enfin, combiné aux résultats indiquant que le jeûne de 24 heures a significativement augmenté le mir-1949 dans les tissus adipeux péri-radicaux et que la régulation positive du mir-1949 tendait à augmenter l'accumulation de lipides dans les adipocytes séniles 3T3-F442A (N = 1) augmente l'expression de mir-1949 dans les adipocytes, ce qui pourrait tenter de sauver des dysfonctionnements liés à l'âge dans les adipocytes, tels qu'une altération du stockage des lipides. Néanmoins, les actions précises de mir-1949 doivent être validées in vivo par injection de mimétique d'AAV-aP2-mir-1949 chez des souris âgées.

Parallèlement à mon projet de thèse, nous avons dû moduler les expressions géniques dans des adipocytes différenciés à partir de lignées cellulaires immortalisées avec des techniques de routine telles que les transfections d'ARNsi. Nous avons donc cherché à développer une technique permettant de conserver des adipocytes isolés pour des expériences. À l'aide d'inserts de culture cellulaire, nous avons réussi à séquencer des adipocytes matures flottants et à les exposer à l'air. En outre, en réduisant la tension de surface avec la supplémentation en co-solvants, le taux de survie des cellules adipeuses en culture a apparemment augmenté. Ensuite, les résultats de la qPCR ont démontré que les taux d'ARNm des marqueurs des adipocytes augmentaient après presque une semaine de culture, ce qui correspondait à la taille accrue des cellules. En outre, les adipocytes cultivés dans notre système de culture se sont avérés fonctionnels, pouvant répondre respectivement à la stimulation catécholamine / insuline pour la lipolyse et le transport du glucose. Par conséquent, la nouvelle façon dont nous avons invité est capable de rester des adipocytes matures pour une étude in vitro sans perdre ses caractéristiques de morphologie et de fonction.

Ensemble, ces deux aspects de ma thèse nous amèneraient à mieux comprendre le domaine des adipocytes et du vieillissement.

Mots-clés: Vieillissement, adipocytes, dicer, mir-1949

ABSTRACT

On the one part, *dicer*, the RNase III endoribonuclease responsible for microRNAs maturation, has been reported to be decreased in adipocytes during ageing. With the use of tamoxifen inducible *dicer*^{lox/lox}/ adiponectin-CreER^{T2} mice, we found that adipocyte *dicer* deficiency promoted the onset of some of the age-related complications, such as reduced adipocyte sizes and dysfunctions in systemic metabolism. The abrogation of white adipocyte markers such as *Pparγ*, *Glut4* or *Hsl*, indicated that *dicer* is indispensable for the maintenance of white adipocyte identity. In addition, the results that there were lipid accumulation and fibrosis in liver in tamoxifen treated *dicer*^{lox/lox}/ adiponectin-CreER^{T2+} mice, indicated that adipocyte *dicer* deficiency might contribute to liver aging.

Mechanistically, mitochondrial function seemed to be upregulated due to adipocyte *dicer* deficiency, indicated by increased protein levels of OXPHOS components and PGC1 α . In line, mitochondria repressors FOXO1 and FOXO3 were phosphorylated and inactivated, whose downstream antioxidant targets *Catalase* and *Sod* were also decreased. Moreover, *P16*, a marker of senescence, exhibited a trend to be increased due to adipocyte *dicer* deficiency. Since mitochondrial ROS surplus can lead to DNA damage and senescence, we assured that adipocyte *dicer* deficiency might induce a combination of mitochondrial activation and reduction in detoxification reduction possibly mediated by the inactivation of FOXO1 and FOXO3a. Finally, the result that nutrient restriction positively regulated *dicer* level in adipocytes further supported that there is a conserved aging pathway in adipocytes involving *dicer*.

On the other part, through microarray screen and RT-qPCR validation, we reported that aging increased mir-1949 in adipocytes in wild type mice and possibly promoted its secretion from perigonadal adipose tissue *in vitro*. Correspondingly, *in vitro* results also suggested that senescence increased mir-1949 production and secretion from adipocytes. Functionally, upregulation of mir-1949 in 3T3-F442A adipocytes negatively regulated mitochondrial complex II protein level and oxygen consumption capability, associated with lipid accumulation. Interestingly, sustained upregulation of mir-1949 during adipogenesis of 3T3-F442A preadipocytes, tended to increase white adipocyte markers, such as *Leptin*, *Glut4* or *Hsl* (N=2). Finally, combined with the results that 24h fasting significantly increased mir-

1949 in perigonadal adipose tissue as well as that upregulation of mir-1949 exhibited a trend to increase lipid accumulation in senescent 3T3-F442A adipocytes (N=1), we assured that aging increases mir-1949 expression in adipocytes which might try to rescue age-related dysfunctions in adipocytes, such as impaired lipid storage. Nevertheless, the precise actions of mir-1949 need to be validated *in vivo* by injection of AAV-aP2-mir-1949 mimic into aged mice.

In parallel of my PhD project, we faced the matter of modulating gene expressions in differentiated adipocytes from immortalized cell lines with routine techniques like siRNA transfection. Therefore, we aimed to develop a technique enabling us to conserve isolated adipocytes for experiments. With the help of cell culture inserts, we managed to sequence buoyant mature adipocytes from floating and exposing into air. Moreover, by reducing surface tension with supplementation of co-solvents, the survival rate of cultured fat cells was apparently increased. Then, RT-qPCR results demonstrated that mRNAs levels of adipocytes markers increased after nearly one week of culture, in consistent with the increased cell sizes. Besides, cultured adipocytes in our culture system were proved to be functional, being able to response to catecholamine/ insulin stimulation for lipolysis and glucose transport, respectively. Therefore, the new way that we invited is capable of remaining mature adipocytes for *in vitro* study without losing its characteristics of morphology and function.

Taken together, these two aspects of my PhD would lead us to understand more in the field of adipocytes and aging.

Key words: Aging, adipocytes, dicer, mir-1949

LISTS OF FIGURES AND TABLES

FIGURES

Introduction part

Figure 1: Growing interests in adipose biology over the last century.	10
Figure 2: Adipose tissue distribution associates with the incidence of diabetes development.	11
Figure 3: Representation and transmission electron microscopy of the morphology of white adipocytes.	14
Figure 4: Extracellular support of adipocytes <i>in vivo</i>	15
Figure 5: Schematic representation of FFA uptake mediated by CD36 in caveolin-1-FABP rich microdomain.	18
Figure 6: Insulin signaling regulates GLUT4 exocytosis by engaging the trafficking machinery.	20
Figure 7: Tight control of lipolysis process by IIS, β -adrenergic signaling and NP receptor A signaling pathways.	22
Figure 8 : Adipocytes crosstalk with various organs through adipokines.	26
Figure 9 : Simplified scheme of white adipocyte origins.	28
Figure 10: The classical pathway of microRNA biogenesis.	43
Figure 11: Hallmarks of senescent cells.	63
Figure 12 : The p53 and RB tumor-suppressor pathways.	64
Figure 13: Simple illustrations of microRNAs' participations in calorie restriction process in different species.	70
Figure 14: Examples of effects of microRNAs in sirtuins during aging or senescence.	73
Figure 15 : 3T3-F442A adipocytes differentiated from 3T3-F442A fibroblasts.	75
Figure 16: Isolation of SVF from adipose tissue with collagenase digestion and centrifugation for <i>in vitro</i> adipogenesis.	75
Figure 17: 3D adipogenesis of 3T3-L1 cells in electrospun polycaprolactone scaffolds.	77
Figure 18: Formation of spheroids of 3T3-L1 adipocyte aggregates on surface tethered with ELP-PEI.	78
Figure 19: Ceiling culture of isolated adipocytes.	79

Results part

Result 1: Role and regulation of microRNAs in adipose tissue during aging

Figure 1: Inducible reduction of dicer in adipocytes by tamoxifen in dicer ^{Lox/Lox} / adiponectin-CreER ^{T2+} mice.	85
Figure 2: Reduction in the average sizes of perigonadal adipocytes due to dicer deficiency.	87
Figure 3: Possible involved mechanisms underlying the effects of adipocyte dicer deficiency.	89
Figure 4: Adipocyte dicer deficiency promoted lipid accumulation and fibrosis in liver.	93
Figure 5: Regulation of dicer level in adipose tissue and in <i>in vitro</i> adipocytes.	96
Figure 6: Age-associated increase of mir-1949.	99
Figure 7: <i>In vitro</i> actions of mir-1949 on adipocytes.	101

Figure 8: Proposed molecular mechanisms underlying adipocyte dicer in aging.	105
Figure 9: GFP expression in adipocytes from ZsGreen ^{lx/lx} /adiponectin-CreER ^{T2+} mice by 4-hydroxytamoxifen.	108
Figure 10: Possible role of mir-1949 in the prevention of age-related dysfunctions.	110

Result 2: A novel method of culturing isolated adipocytes in vitro

Fig 1: Illustration of the working scheme.	119
Fig 2: Image of adipocytes in culture under different conditions.	122
Fig 3: Functional investigation of adipocyte <i>in vitro</i>	125
Fig 4: Characterizations of adipocytes during chronic culture <i>in vitro</i>	127

TABLES

Introduction part

Table 1: The average sizes of adipocytes in developing and adult human.	16
Table 2: List of miRNAs involved in adipogenesis.	46
Table 3 : MiRNAs involved in age-associated cardiovascular diseases.	59

Results part

Result 1: Role and regulation of microRNAs in adipose tissue during aging

Table 1: Plasma parameters (fed)	91
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ABBREVIATION

ACC	Acetyl CoA carboxylase
ACSL	Adipose acyl-CoA synthetase
ADSCs	Adipose tissue-derived stem cells
ADEV	Adipose tissue derived extracellular vesicles
ADRP	Adipose differentiation-related protein
ADRB3	B3-adrenergic receptor
AGO	Argonaute
AGRP	Agouti-related protein
ATGL	Adipose tissue lipase
ALDH3A2	Aldehyde dehydrogenase 3 family member A2
AMPK	AMP-activated protein kinase
ATF6	Activating transcription factor-6
ATMs	Adipose tissue macrophages
ATSC	Adipose tissue stromal cell
APAF1	Apoptotic protease-activating factor 1
APCs	Adipocyte progenitor cells
ARF6	ADP-ribosylation factor 6
BAT	Brown adipose tissue
BCL2	B-cell lymphoma 2
BMP	Bone morphogenetic protein
BMI	Body mass index
BMSCs	Bone marrow stromal cells
BSCL	Berardinelli-seipcongenital generalized lipodystrophy
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
CART	Cocaine and amphetamine regulated transcript
C/EBP α	CCAAT/enhancer binding protein α
C/EBP β	CCAAT/enhancer binding protein β
ChREBP	Carbohydrate response element binding protein
CR	Calorie restriction
CPT	Carnitine palmitoyl transferase
CLAs	Conjugated linoleic acids
DGAT1	Diacylglycerol acyltransferase 1
DDRs	DNA damage responses
DNL	De novo lipogenesis
DGCR8	DiGeorge critical region 8
ECM	Extracellular matrix
EPCs	Endothelial progenitor cells
ER	Endoplasmic reticulum

eNOS	Endothelial NO synthase
EVs	Extracellular vesicles
FABP4	Fatty acid binding protein 4
FABPpm	Plasma membrane fatty acid binding protein
FAT10	HLA-F adjacent transcript 10
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
FAS	Fatty acid synthesis
FFAs	Free fatty acids
FPLD	Familial partial lipodystrophic syndromes
FOXO	Forkhead box O
FGF21	Fibroblast growth factor 21
GCs	Glucocorticoids
GH	Growth hormone
GLUT4	Glucose transporter 4
GLP-1	Glucagon-like peptide-1
GO	Gene ontology
HDAC9	Histone deacetylase 9
HEK 293T	Human embryonic kidney cell line 293T
HK	Hexokinase
HDL	High density lipoprotein
HMGA2	High mobility group AT-hook 2
HOMA-IR	Homeostatic model assessment for insulin resistance
HSC	Hematopoietic stem cells
HSL	Hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IGF	Insulin like growth factor
IRS	Insulin receptor substrate
JAK	Janus Kinase
JNK	Jun-N-terminal kinase
KSRP	KH-type splicing regulatory protein
LCFA	Long chain fatty acid
LD	Lipid droplet
MGL	Monoglyceride Lipase
MiRNAs	MicroRNAs
MiT/TFF	Microphthalmia/ Transcription Factor E
MR	Mineralocorticoid Receptor

mTOR	Mammalian target of rapamycin
MUFA	Mono-unsaturated fatty acid
NAMPT	Nicotinamide phosphoribosyltransferase
NF-κB	Nuclear factor-κB
NGS	Next-generation sequencing
NPY	Neuropeptide-Y
NLRP3	Nod-like receptor 3
OPN	Osteopontin
PAI	Plasminogen activator inhibitor
PDK	Phosphoinositide-dependent kinase
PDE3b	Phosphodiesterase 3b
PEPCL	Phosphoenolpyruvate carboxykinase
PGC-1	Peroxisome proliferator-activated receptor γ coactivator 1
PKA	Protein kinase A
PLIN1	Perilipin 1
POMC	Proopiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor-γ
PRDM16	PR domain containing 16
Pri-miRNAs	Primary microRNAs
Pre-miRNAs	Precursor microRNAs
PTP1B	Protein tyrosine phosphatase
PTEN	Phosphatase and tension homologue
RB	Retinoblastoma
RGS	Rosiglitazone
RICTOR	RPTOR-independent companion of mtor complex 2
RIP140	Receptor-interacting protein 140
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
SA-β-gal	Senescence-associated β-galactosidase
SCD1	Stearoyl coenzyme A desaturase-1
S6k1	Ribosomal S6 protein kinase
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element binding transcription factor 1c
STAT	Signal transducers and activators of transcription
SVF	Stromal vascular fraction
TG	Triglyceride
TGFβ	Transforming growth factor β
TLR	Toll like receptor

TIP47	Tail interacting protein of 47 kda family
TRAF6	TNF receptor-associated factor 6
TNF α	Tumor necrosis factor A
TXNRD2	Thioredoxin reductase 2
UCP1	Uncoupling protein 1
UCP9	Ubiquitin carrier protein 9
3'-UTRs	3'-Untranslated regions
UV	Ultraviolet
WAT	White adipose tissue

Introduction	10
PART I: White adipose tissue and aging	10
1. The adipose tissue	10
1.1. Subcutaneous adipose tissue (sWAT)	11
1.2. Visceral adipose tissue (vWAT)	12
2. White Adipocytes	13
2.1. Architecture	13
2.2. Homeostasis of lipid droplets in adipocytes	15
2.2.1. Synthesis of lipid droplets (LDs)	16
2.2.2. Hydrolysis of lipid droplets	20
2.2.3. Lipid droplets “burning”	22
2.3. Endocrinal functions	23
2.4. Origins of white adipocytes	27
2.4.1. Adipocyte progenitor cells (APCs)	28
2.4.2. Adipogenesis	29
3. Alterations of WAT by aging	32
3.1. Senescence of adipocyte progenitors	33
3.2. Altered lipid turnover	34
4. Contribution of WAT to aging	36
4.1. Nutrition/age-associated obesity attenuates longevity	36
4.2. Removal or mobilization of vWAT increases longevity	38
4.3. Novel insights from microRNAs	40
PART II: MicroRNAs in white adipose tissue	42
1. Biogenesis and regulation of microRNAs	42
1.1. Biogenesis of microRNAs	42
1.2. Regulation of miRNAs expression in adipose tissue	44
2. MiRNAs in the formation of adipose tissue	45
2.1. MiRNAs in differentiation of adipocytes progenitors	45
2.2. Determination of adipocytes identities	48
2.3. Involvement in adipose tissue functions	50

PART III: MiRNAs in aging	56
1. Dysregulations of miRNAs in aging	56
1.1. MiRNAs involved in overall longevity	56
1.2.1. Age-associated cardiovascular diseases	57
1.2.2. Age-associated sarcopenia and osteoporosis	60
1.2.3. Age-related liver dysfunctions	61
1.2.4. Age-related immune defects	61
2. MiRNAs to senescence	62
2.1. Cellular senescence	62
2.2. miRNAs in p53/RB tumor-suppressor pathways	64
2.3. miRNAs in DNA damage pathways	66
3. MiRNAs in conserved aging	67
3.1. miRNAs affecting caloric restriction process	68
3.2. miRNAs affecting sirtuin families	71
PART IV: A novel method of culturing isolated adipocytes	74
1. Current in vitro models of adipocytes	74
1.1. Two-dimensional (2D) adipogenesis of preadipocytes	74
1.2. Three-dimensional (3D) adipogenesis of preadipocytes	76
2. First attempt to culture isolated adipocytes	78
Result 1: Role and regulation of microRNAs in adipose tissue during aging	84
Result 2: A novel method of culturing isolated adipocytes in vitro	117
Bibliography	136

GENERAL INTRODUCTION

Introduction

PART I: White adipose tissue and aging

1. The adipose tissue

The adipose tissue is a master regulator of energy balance and nutritional homeostasis. Viewed as an inert energy storage site and a form of connective tissue not long time ago, surging evidence now have revealed that adipose tissue is a remarkably complex organ with important endocrinal functions which enable its crosstalk with diverse organs. Moreover, with the very recent discovery of brown and beige adipocytes, interests have been fueled in the study of adipose biology (Figure 1).

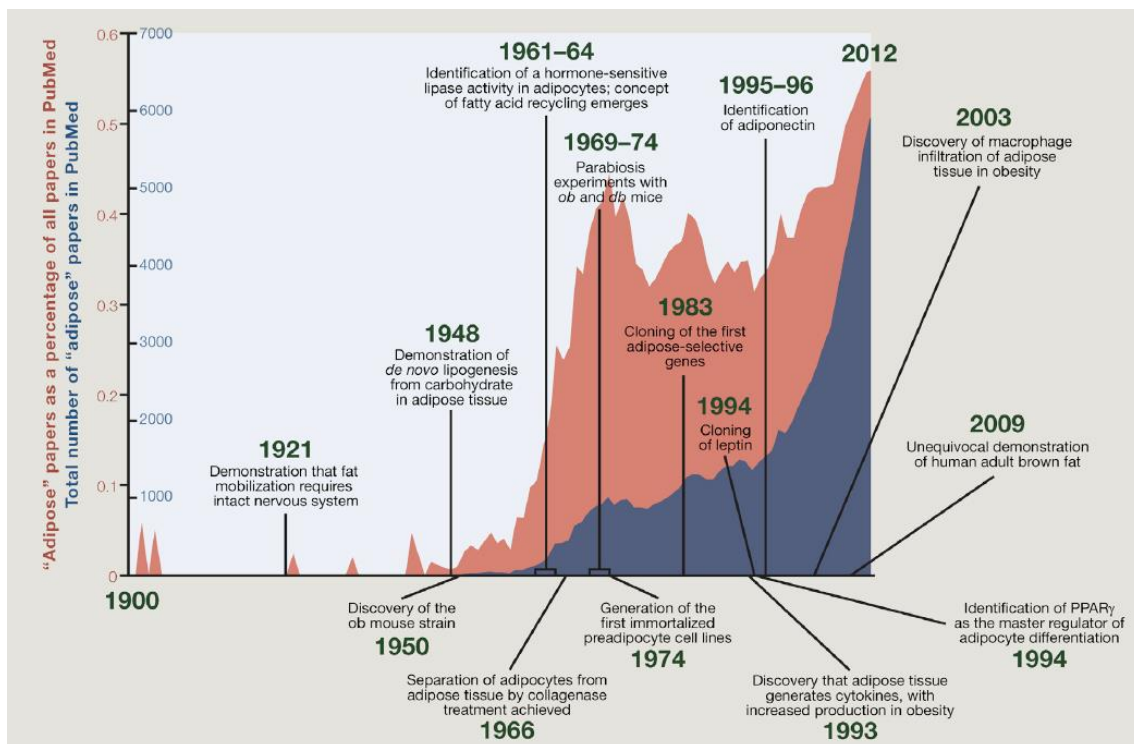


Figure 1: Growing interests in adipose biology over the last century.

Adopted from².

Adipose tissue is a dynamic organ which can range from 4% to over 40% of total body composition in adult humans and is dispersed throughout human body. Functionally,

adipose tissue could be categorized into white adipose tissue (WAT) and brown adipose tissue (BAT). BAT generates heat during cold exposure by adaptive thermogenesis to maintain normal body temperature and is more abundant in neonates and young children, and gradually declined in human adults³. Here in our topic, we are going to focus mainly on WAT.

Geographically, WAT is categorized into subcutaneous and visceral adipose tissue (sWAT and vWAT, respectively) (Figure 2a). Surprisingly, a pioneering clinical study in the 1950s firstly reported that location of fat depots has a great influence on the likelihood of an individual to develop many of the complications of obesity⁴ (Figure 2b). The data indicate that there is heterogeneity between sWAT and vWAT.

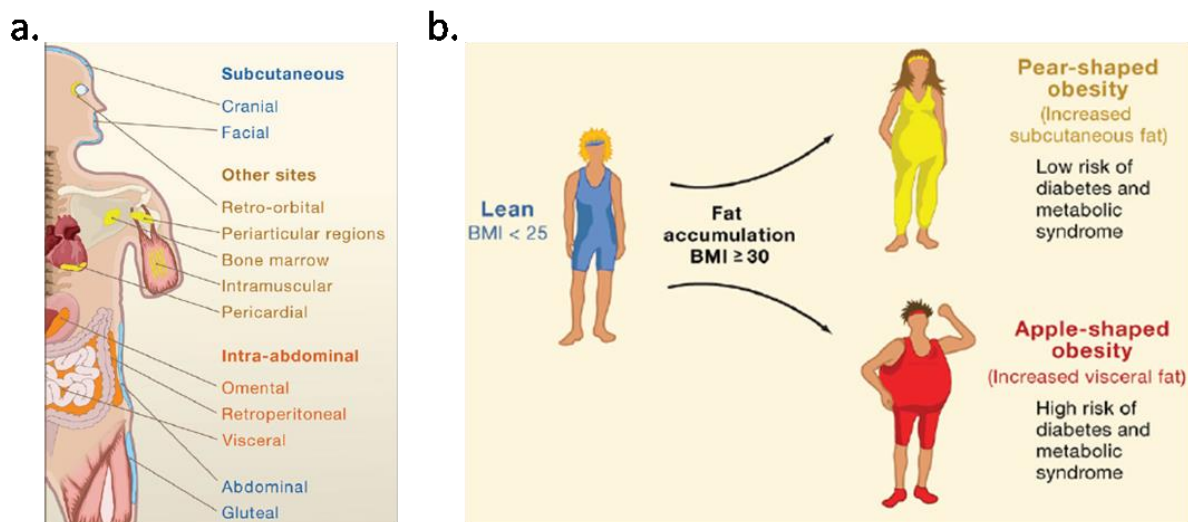


Figure 2: Adipose tissue distribution associates with the incidence of diabetes development.

(a) Distributions of WAT in human. (b) Incidence of development of diabetes associated with the fat distributions. Modified from⁵.

1.1. Subcutaneous adipose tissue (sWAT)

In human, sWAT forms just below the dermis, and includes periscapular and inguinal depots. Increased amounts of subcutaneous fat, especially in the gluteofemoral regions, are associated with improved insulin sensitivity and a lower risk of developing metabolic

syndromes⁶. In mice, transplantation of subcutaneous fat into the cavity of visceral fat significantly decreases body weight and total fat mass as well as improves systemic glucose tolerance, whereas transplantation of vWAT into the depot of sWAT has little effects⁷.

The metabolic benefits of sWAT might be associated with its unique property: sWAT is capable of 'browning' in response to cold exposure, β -agonists or other hormone-like stimuli⁸, which includes the induction of uncoupling protein 1 (UCP1) expression, a gene giving rise to uncoupled respiration and heat production. The UCP positive cells would emerge in white fat depots under certain stimuli and are termed beige or brite cells. Adipocyte-specific mutant of *PR domain containing 16* (PRDM16) markedly inhibits beige adipocyte function in sWAT following cold exposure or β 3-agonist treatment, acquiring numerous properties of vWAT including decreased thermogenic and increased inflammatory gene expression as well as macrophage infiltration. Moreover, transplantation of the sWAT derived from PRDM16 mutants into obese mice shows a loss of metabolic benefit⁹.

Recently, it has been reported that inhibitors of the Janus kinase/ signal transducers and activators of transcription 1/3 (JAK/ STAT1/3) signaling, like tofacitinib and R406 are strongly inducers of UCP1 expression in sWAT but not in vWAT¹⁰, indicating that inhibition of JAK/ STAT1/3 signaling might mediate the browning process in sWAT, whereas it is not employed in the futile energy cycle in vWAT.

1.2. Visceral adipose tissue (vWAT)

In human, there are 6 visceral fat depots: perirenal, gonadal, epicardial, retroperitoneal, omental and mesenteric depots. In contrast to sWAT, it is revealed that the size of abdominal adipocyte is a significant risk predictor for the development of type 2 diabetes, as revealed by a follow-up study of around 245 women from 1974 to 2001¹¹. Several plausible mechanisms may be associated with the properties of vWAT.

Firstly, Alike Kosteli et al, demonstrated that vWAT has a higher rate of basal lipolysis and therefore releases more free fatty acids (FFAs) compared to sWAT¹². Since vWAT is anatomically drained by the portal vein, the increased levels of portal FFAs might induce metabolic complications in liver, such as hepatic steatosis or hepatic insulin resistance in

obesity¹³. What's more, FFAs are also strong inducers of adipose tissue's recruitment of macrophages, whose chronic accumulation would lead to elevated local inflammatory state¹², which is negatively associated with insulin resistance. In addition, with the use of whole-body fluorodeoxyglucose positron emission tomography (FDG-PET) scans, it is found that vWAT has higher rates of glucose uptake compared to sWAT in both lean and obese humans. The isolated stromal vascular fractions (SVFs) from vWAT exhibit significant increase in both basal and cytokine stimulated glucose consumptions associated with a higher expression of hexokinase (HK) compared to that from sWAT¹⁴. These results indicate that vWAT has higher metabolic rates in comparison to sWAT.

Interestingly, although to a lesser extent, visceral fat is capable of responding to chronic β 3-adrenergic receptor stimulation demonstrated by the appearance of lipid droplet multilocularity and robust mitochondrial biogenesis, which might be explained by a prominent increase in futile creatine cycling in visceral fat under stimulation¹⁵. However, the physiological role of futile cycling of vWAT remains to be investigated.

Secondly, vWAT is also an endocrine organ that actively secretes lots of cytokines and bioactive mediators, which influence systemic inflammation, lipid metabolism and insulin sensitivity¹⁶. For example, B cells isolated from vWAT expressed higher levels of inflammatory immune activation markers and significantly higher nuclear factor- κ B (NF- κ B) activation and phospho-STAT3¹⁷. Expression of proinflammatory adipokines in vWAT could be elevated by obesity, which might contribute to their circulating elevation during obesity¹⁸.

2. White Adipocytes

Adipose tissue is composed of mature adipocytes and various other cell types in stromal vascular fractions (SVFs) that include leukocytes (e.g., macrophages and lymphocytes) and adipose tissue stromal cells (ATSCs) that include preadipocytes and fibroblasts. Adipocytes contribute mainly to metabolism and functions of adipose tissue¹⁹.

2.1. Architecture

White adipocytes contain a single large lipid droplet (LD) occupying approximately 90% of the cytoplasm, with the nucleus being squeezed to the cell periphery and the cytoplasm

forming a very thin rim. Mitochondria are scarce and internal cristae are poorly developed (Figure 3). Due to these characteristics, these cells are also termed unilocular adipocytes.

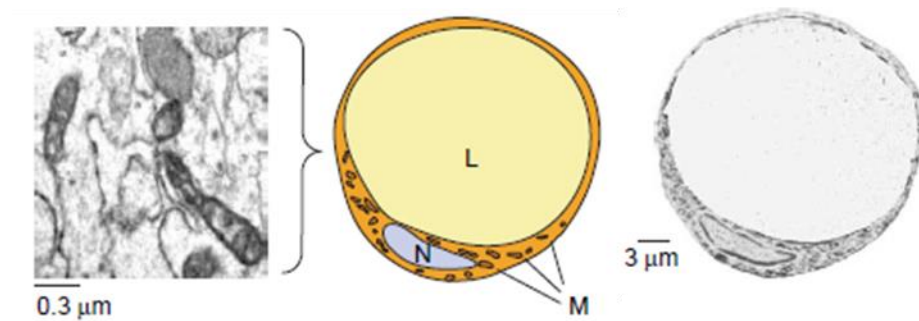


Figure 3: Representation and transmission electron microscopy of the morphology of white adipocytes.

Abbreviations: L, lipid; M, mitochondria; N, nucleus. Modified from²⁰.

At the tissue level, spherical adipocytes are typically arranged in an imperfect hexagonal packing architecture resembling honeycomb, with smaller preadipocytes and interweaving capillaries and nerves filling the interstitial space^{21,22}. Adipocytes themselves are interconnected *via* gap junctions and thereby share cytoplasm and respond in concert to electrical stimuli²³. Additionally, adipocytes are intensively interacted with the extracellular matrix (ECM), which provides both the flexibility needed for cell migration and hypertrophy and the stability needed for the structural and functional integrity of the tissue as a whole (Figure 4). For example, adipocytes are interacted with an isotropic network of collagen and elastic fibers, extracellular fibronectin and laminin²⁴, via attachment points for integrins anchored in the adipocyte membrane²⁵.

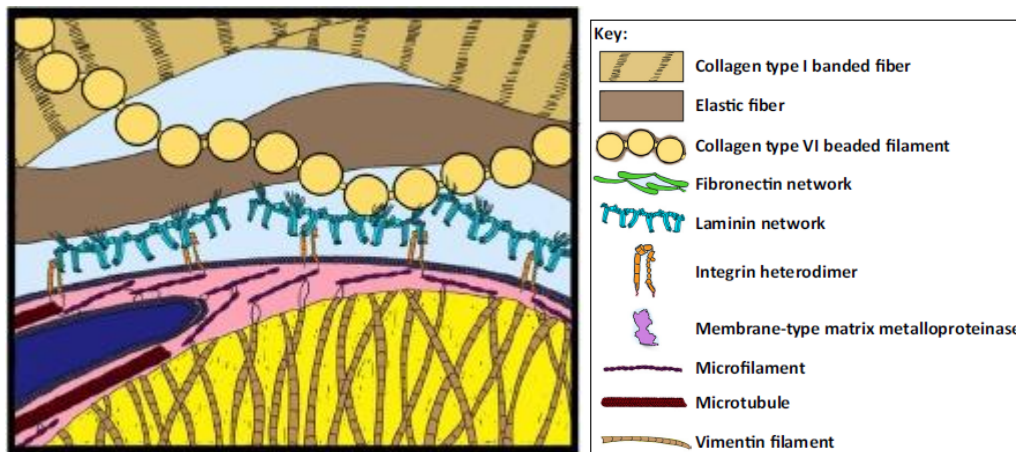


Figure 4: Extracellular support of adipocytes *in vivo*.

Structural support is provided by type I (100 nm to 100 μ m diameter) and type VI (50 nm diameter) collagen networks. The LD is caged in vimentin (10 nm, brown filament). The nucleus (blue) moves to the cell periphery and is deformed by the LD. Modified from²⁶.

The rounded LDs are surrounded by a single lipid layer (of endoplasmic reticulum membrane origin), which is in turn ensheathed by endoplasmic reticulum (ER). LDs are very often surrounded by one or more proteins of the PAT (perilipin, adipose differentiation-related protein (ADRP), and TIP47 (tail interacting protein of 47 kDa family)²⁷. The LDs of adipocytes are encased in a coating of perilipins, which have an important role in regulating triglyceride hydrolysis (breakdown of triglyceride into glycerol and free fatty acids), with perilipin A being the most abundant isoform in adipocytes. Perilipin null adipocytes exhibit elevated basal lipolysis but attenuated stimulated lipolytic activity, indicating that perilipin is required for maximal lipolytic activity²⁸.

2.2. Homeostasis of lipid droplets in adipocytes

Adipocytes are central in the regulation of triglyceride (TG) and FFAs homeostasis during energy expenditure and deprivation. The storage and removal of TG in adipocytes is essential to determine the sizes of adipocytes. *In vivo*, the sizes of adipocytes vary greatly according to the depots and the nutritional states (Table 1). The sizes of adipocytes correlate with the functions of adipocytes. It has been demonstrated that small white adipocytes have lower expression of enzymes involved in fatty acids use (β -oxidation) including acyl-CoA

dehydrogenase and carnitine palmitoyl transferase 2 (CPT2), as well as in fatty acid synthesis (FAS) compared to large white adipocytes, coinciding with that fact that small adipocytes have low storage of TG²⁹.

Cell Size	Subcutaneous and Visceral Fat			Subcutaneous Fat		Visceral Fat	
	Fetus ^a	Neonate	Infant ^b	Lean	Obese	Lean	Obese
Diameter range (µm)	40–50	50–80	90–130	50–130	90–270	45–110	90–200
Mean cell volume (µm ³)	48 000	144 000	697 000	382 000	3 054 000	244 000	1 596 000

Table 1: The average sizes of adipocytes in developing and adult human

^a25-30 weeks gestation, ^b1-3 months postpartum. Adopted from³⁰.

Several major functions of adipocytes determine the size of LDs in adipocytes: fatty acid uptake, *de novo* lipogenesis (DNL), lipolysis and possible burning of fat by being, which we will discuss in details in the following parts.

2.2.1. Synthesis of lipid droplets (LDs)

- Fatty acid uptake

Most of the lipids stored in adipocytes are transferred directly from diets. Several proteins are involved in fatty acid uptake in adipocytes: caveolin-1, fatty acid translocase (FAT/CD36), the 43-kDa plasma membrane fatty acid binding protein (FABPpm), and fatty acid transport protein (FATP), adipose acyl CoA synthetase (ACSL).

Caveolin-1

Caveolae are a subset of so-called membrane lipid raft domains that are often experimentally defined by their resistance to solubilization by nonionic detergents.

The surface of adipocytes is abundantly covered by caveolae (nearly 30%)³¹. Caveolin-1 is the primary protein driving the formation of caveolae in adipocytes³². Caveolin-1 is speculated to bind the fatty acids accumulated with the caveolar membrane, leading to membrane asymmetry and budding of caveolae from plasma membrane to form vesicles. These fatty

acid loaded caveolae vesicles could deliver fatty acids to subcellular compartments for further metabolism (Figure 5)³³.

FABPpm

Expression of FABPpm in 3T3-L1 preadipocytes that normally do not express FABPpm induces a significant increase in fatty acid uptake. Anti-FABPpm antibodies selectively inhibit uptake of oleate in 3T3-L1 adipocyte monolayer without affecting 2-deoxyglucose or octanoate (medium-chain fatty acid) uptake³⁴. Currently, the mechanism by which FABPpm used in LCFA uptake is unknown.

CD36

CD36 is an 88-kDa single chain membrane glycoprotein expressed in many tissues and cell types, including adipocytes, heart, skeletal muscle and macrophages. C/EBP α and C/EBP β would increase CD36 expression in different types of cells, with C/EBP α exhibits a significantly higher potency than C/EBP β . A C/EBP responsive element is identified in the CD36 promoter³⁵. It has been speculated that CD36 may bind and internalize long chain fatty acid (LCFA) by endocytosis or may bind LCFA and work in concert with FABP to provide a high concentration gradient of LCFA across the plasma membrane facilitating its uptake by other fatty acid transporters (Figure 5)³³.

FATP1 and ACSL

FATP1 and ACSL are two different classes of membrane bound enzymes catalyzing the ATP-dependent esterification of long-chain and very long-chain fatty acids into acyl-CoA derivatives^{36,37}. FATP1 and ACSL1 are enriched in adipose tissue.

FATP1 is induced during the differentiation of 3T3-L1 adipocytes, and increased LCFA uptake. Insulin could trigger FATP1 translocation from a perinuclear compartment to the plasma membrane, indicating that FATP1 is facilitated by insulin³⁸. Since it is reported that the conversion of incoming LCFA to TG occurs on/or around the plasma membrane in rat adipocytes³⁹, FATP1 might facilitate LCFA uptake by tightly coupling it to metabolism: by converting it into acyl-CoAs which is preferentially shunted into TG synthesis (Figure 5)³³.

ACSL1 is found to be involved in the reacylation of LCFA released from the LD during basal and hormone-stimulated lipolysis. Acylation of LCFA leads to reincorporation into the TG thereby preventing its cellular efflux⁴⁰.

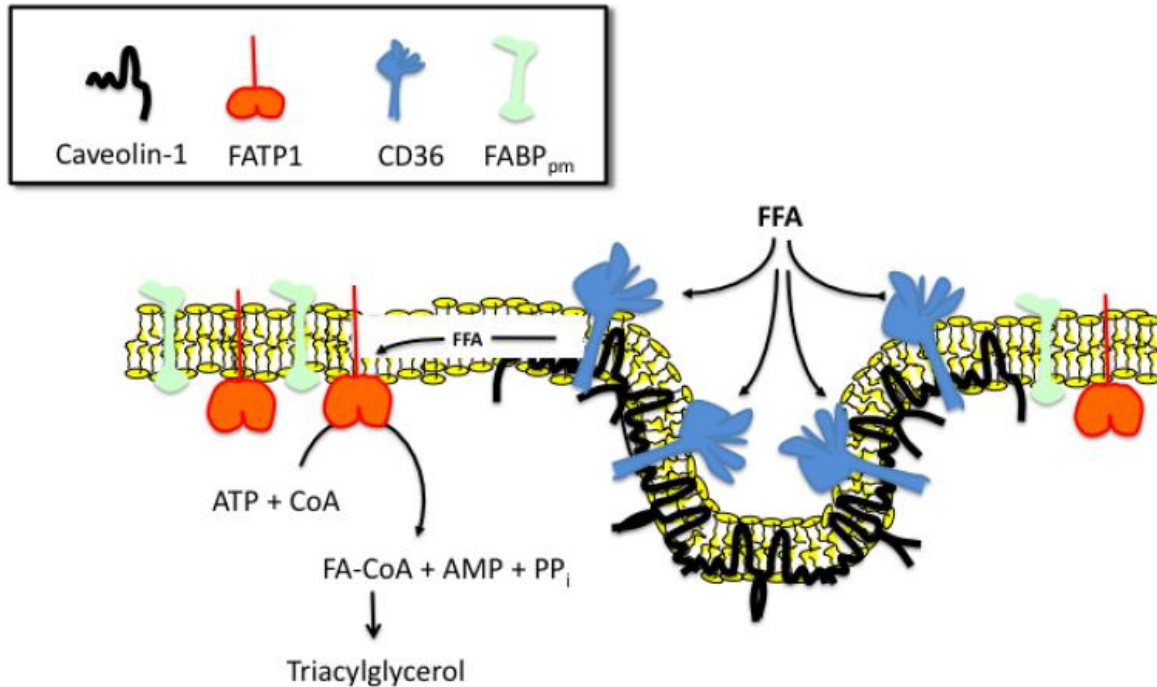


Figure 5: Schematic representation of FFA uptake mediated by CD36 in caveolin-1-FABP rich microdomain.

Adopted from³³.

- De novo lipogenesis (DNL)

On the other hand, adipocytes are capable of synthesizing new lipids from carbohydrates through DNL. The two major enzymes of DNL, fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), which are under the control of carbohydrate-response-element-binding protein (ChREBP) or sterol-response-element-binding protein 1c (SREBP1c), are abundantly expressed in fat cells⁴¹. Surprisingly, DNL contributes only a few percent of fatty acids to the TG that accumulates in the lipid droplets of adipocytes⁴². In addition, it was found that in vitro 3T3-L1 adipocytes preferentially use glycolysis to depose glucose into lactate even with the abundant oxygen availability, while only a small fraction of carbon from glucose would be converted into TG⁴³. However, DNL contributes to a number of

adipose-derived putative bioactive lipids, such as palmitoleate⁴⁴, the novel fatty acid esters of hydroxy-fatty acids⁴⁵ and PPAR γ ligand alkyl ether-lipid⁴⁶. In addition, studies have shown that up-regulation of DNL in adipose tissue mediated the improved insulin sensitivity and glycemic control of GLUT4 overexpression in adipocytes, whereas loss of ChREBP has the opposite effects^{41,47}. Besides, FAS was dramatically upregulated in WAT by caloric restriction (CR) process which is associated with increased insulin sensitivity and longevity, indicating that DNL might be important metabolic adaptations of caloric restriction benefits⁴⁸. Conversely, impairment of DNL, which is under rictor/mTORC2 signaling in adipose tissue, was found to be necessary for the loss of insulin sensitivity induced by high fat diet in wild type mice⁴⁹.

Glucose transport:

Insulin signaling plays an important role in lipid storage and the process of adipogenesis for adipocytes (Figure 6). The phosphatidylinositol-3-OH kinase (PI(3)K)-dependent signaling is initiated by binding of insulin onto its tyrosine kinase receptor on the cell surface, leading to the recruitment and tyrosine phosphorylation of the insulin receptor substrate (IRS). The phosphorylated IRS then serves as docking sites for the SH2 domain of the p85 regulatory subunit of class I PI3K, resulting the activation of PI3K, which promotes the synthesis of phosphatidylinositol-3,4,5-trisphosphate PtdIns(3,4,5) P₃ from PtdIns (4,5) P₂ at the plasma membrane. PtdIns(3,4,5) P₃ then serves as a docking site for several PH domain-containing Ser/Thr kinases that are implicated in glucose uptake, such as phosphoinositide-dependent kinase 1 (PDK 1) and AKT (also known as PKB)⁵⁰. AKT was then activated by PDK1 (phosphoinositide-dependent kinase 1) and mTORC2 through dual Ser/Thr phosphorylation. Downstream, AKT kinase affects the exocytic arm of the GLUT4 (glucose transporter 4) trafficking itinerary, probably by regulating the translocation, targeting and fusion of GLUT4-containing vesicles⁵¹.

On the other hand, insulin could also initiate a PI3K-independent signaling by recruiting the adaptor protein APS, which has high affinity to the activated insulin receptor⁵². Following insulin stimulation, APS recruits a complex that comprises the proto-oncogene c-CBL and c-CBL-associated protein (CAP), triggering tyrosine phosphorylation of CBL by insulin receptor⁵³. Upon phosphorylation, CBL is then translocated to lipid rafts, recruiting the CrkII-

C3G complex to lipid rafts, where C3G specifically activates the small GTP-binding protein TC10, which interacts with effector proteins that regulate GLUT4 vesicle exocytosis⁵⁴.

Adipocyte GLUT4 ablation resulted in systemic insulin resistance in liver and muscle⁵⁵, indicating that GLUT4 mediated glucose transport in adipocytes is indispensable for whole body glucose homeostasis.

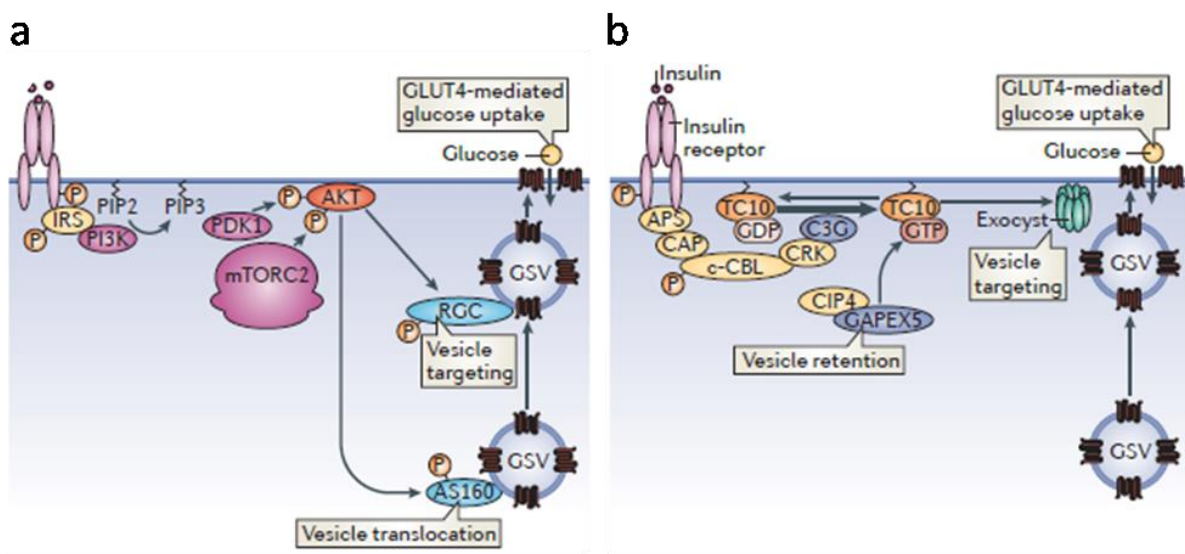


Figure 6: Insulin signaling regulates GLUT4 exocytosis by engaging the trafficking machinery.

Modified from⁵⁶.

2.2.2. Hydrolysis of lipid droplets

Lipolysis represents the process for FFAs to be liberated from TG in LDs. Total lipolysis is the sum of basal lipolysis and stimulated lipolysis. Basal lipolysis is the release of FFAs from adipocytes occurring in the absence of negative energy balance, which correlates positively with adipocyte size and is increased by obesity⁵⁷. Stimulated lipolysis refers to the hormonally regulated release of FFAs in response to nutritional demands. The lipolysis of TG in adipocytes is under tight control of various pathways in vivo (Figure 7).

Typically, the main regulating signaling pathway of stimulated lipolysis in adipocytes is β -adrenergic signaling. At least three major enzymes are responsible for the lipolytic process. The primary cleavage of TG to diacylglycerols is performed by the adipose tissue lipase (ATGL), which is predominantly expressed in adipose tissue. In ATGL^{-/-} mice, absence of

ATGL induced severe lipid accumulation in adipose tissues and heart, with dramatic inhibition of basal and isoproterenol stimulated lipolysis as well as premature death. On the contrary, the inability of fat catabolism due to ATGL deficiency led to increased glucose use, indicating that ATGL is rate limiting in the catabolism of cellular fat⁵⁸. Administration of Atglistain, a small molecule inhibitor of ATGL effectively improves high fat diet-induced obesity associated insulin resistance and liver steatosis in mice. Moreover, the drug predominantly targeted adipose tissue and the liver, and therefore did not induce cardiac lipid accumulation or cardiomyopathy even after long-term treatment⁵⁹. ATGL is highly regulated at both the transcriptional and post-transcriptional levels, including multiple phosphorylation events and translocation to the surface of LDs. It could be activated by its coactivator comparative gene identification-58 (CGI-58), which is normally bound in an inactive state by the LD protein perilipin 1 (PLIN1). And upon activation of β -adrenergic signaling, PKA induced phosphorylation of PLIN1 leading to the release of CGI-58, allowing its binding and activation of ATGL⁶⁰.

Subsequently, hormone-sensitive lipase (HSL) is dispensable for lipolysis. HSL is the major diglyceride lipase in adipocytes, with monoglyceride lipase (MGL) completes the process by generating glycerol and FFAs. Together, these three enzymes account for over 90% of the lipolytic activity in the adipocytes⁶¹.

Physiologically, insulin is the major suppressor of lipolysis, which functions through several different ways to block lipolysis: 1) It activates phosphodiesterase 3b (PDE3b) in a AKT dependent manner, which antagonize the intracellular levels of cAMP and thereby blocking protein kinase A (PKA) activation⁶². 2) Recently, a non-canonical pathway has been proposed in which insulin blocks activation of PKA selectively on PLIN1 through a PI3K-mediated, AKT-independent pathway⁶³. 3) Insulin could also repress lipolysis by transcriptionally silencing lipase genes via repressions of transcription factors forkhead box protein O1 (FOXO1) and interferon regulatory factor 4 (IRF4)⁶⁴ (Figure 7).

The size of fat cells *per se* also have an impact on both the spontaneous and hormone-stimulated rates of lipolysis, with big adipocytes exhibited higher rates of basal and hormone-stimulated hydrolysis, probably owing to the fact that hypertrophic adipocytes are enriched in the expressions of ATGL, HSL and PLIN1. However, the receptor sensitivities for

hormones like insulin, natriuretic peptides, dobutamine, terbutaline and clonidine, are comparable between small and large fat cells⁶⁵.

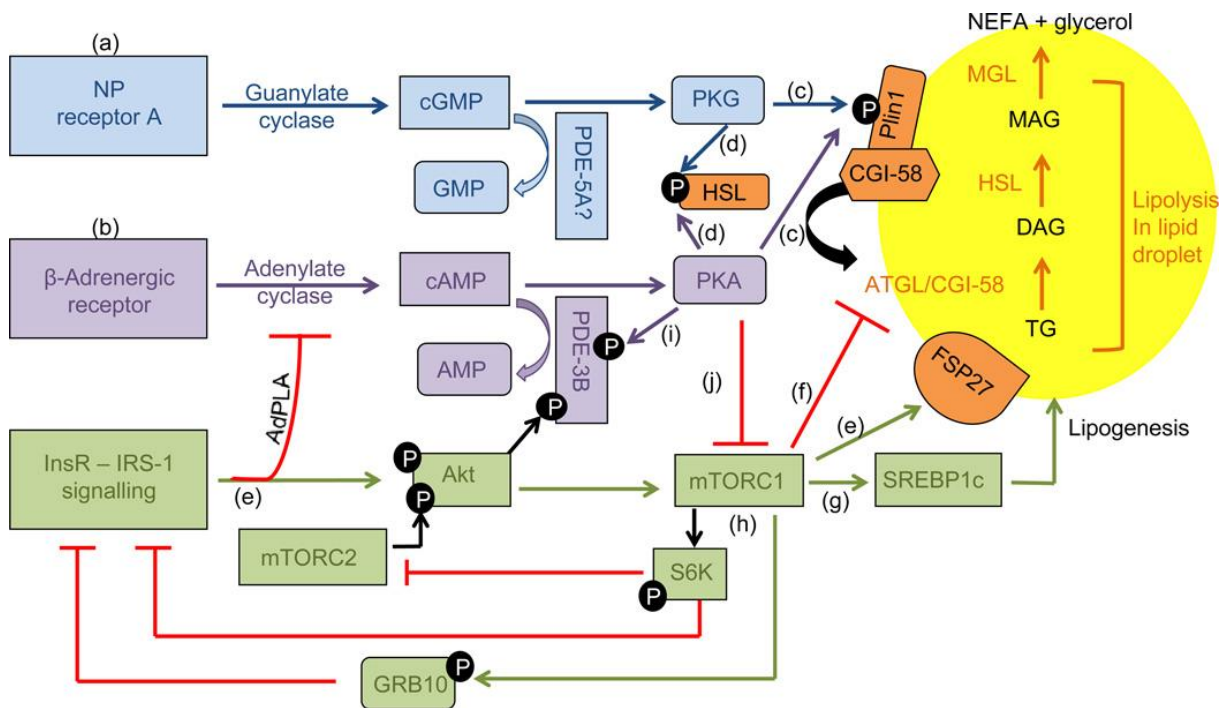


Figure 7: Tight control of lipolysis process by IIS, β -adrenergic signaling and NP receptor A signaling pathways.

Adopted from⁶².

2.2.3. Lipid droplets “burning”

Over the last three decades, it has been realized that mature adipocytes displayed high plasticity, especially those in the subcutaneous depots. In response to some external stimuli, including chronic cold exposure, exercise, long-term treatment with PPAR γ agonists or β 3-adrenergic receptor agonists, cancer cachexia, and tissue injury, a group of beige (also called brite) adipocytes are induced within WAT which are characterized by possessions of abundant cristae-dense mitochondria that express UCP1 and multilocular LDs⁶⁶. Fatty acids are rapidly consumed and deprived by mitochondria β -oxidation and UCP-mediated heat production in beige adipocytes, resulting in a smaller appearance of beige adipocytes compared to normal white adipocytes⁶⁷. It also has been demonstrated that beige-like adipocytes also exist in human adults⁶⁸.

Although some of beige adipocytes might directly come from adipogenesis of distinctive populations of progenitors within WAT^{69,70}, it has been reported that stimulation of β 3-adrenergic receptor (*Adrb3*) in mature adipocytes could directly lead the transformation into mitochondrial abundant beige adipocytes via activation of PRDM⁷¹.

Conversely, after withdrawal of external stimuli such as cold acclimation or β 3-adrenergic agonists, the produced beige adipocytes then possibly transform into normal white adipocytes, by upregulation of mitophagy (mitochondrial clearance via autophagy) activated by which is initiated by the activation of MiT/TFF (microphthalmia/ transcription factor E) transcription factor-mediated lysosome biogenesis⁶⁷.

2.3. Endocrinal functions

Adipocytes have been well acknowledged as a *bona fide* endocrine cell type that regulates systemic metabolic homeostasis in accordance to nutrient flux by equally matching the metabolic demands of positive or negative energy balance, via secreting a variety of bioactive peptides, known as adipokines (Figure 8). Secretion of adipokines, including leptin, adiponectin, bone morphogenetic protein (BMP)-4, dipeptidyl peptidase 4 (DPP-4), etc, is altered by adipose dysfunction and may contribute to obesity-associated diseases. Therefore, adipokines are promising candidates for pharmacological treatment and as diagnostic tools.

Leptin

Leptin was discovered more than 20 years ago⁷². Circulating levels of leptin are proportional to fat mass, and leptin receptors are abundantly expressed in various tissues, including on the adipocyte. Leptin negatively regulate adiposity. Indirectly, leptin negatively regulate satiety *via* inhibiting the orexigenic pathway comprising neuropeptide-Y (NPY) and agouti-related protein (AGRP)-containing neurons, while upregulation of anorexigenic pathway consisting of proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)-containing neurons in hypothalamus⁷³. Moreover, leptin would drive adiposity depletion by stimulation of lipolysis through activating sympathetic neurons innervating adipocytes in WAT *in vivo*⁷⁴. Also, *in vitro*, leptin was reported to decrease

lipogenesis, increase TG hydrolysis and oxidation of fatty acids in isolated rat white adipocytes⁷⁵.

Adiponectin

Adiponectin was firstly reported in 1995⁷⁶, which is almost exclusively synthesized by adipocytes. Adiponectin concentrations inversely correlate with fat mass and are down-regulated in obesity and type 2 diabetes⁷⁷. Overexpression of adiponectin in 3T3-L1 cells enhanced adipogenesis and lipid storage⁷⁸. Also, adiponectin has been shown to increase insulin sensitivity and maintains healthy adipose tissue expansion while rescuing ectopic lipid accumulation in mice⁷⁹.

The beneficial effects of adiponectin on insulin sensitivity seem to be mediated in part by its ability to activate AMP-activated protein kinase (AMPK) in skeletal muscle and liver⁸⁰. For example, adiponectin decreases hepatic lipogenesis and increases β -oxidation through activation of AMPK, a cellular energy sensor, inhibits lipogenesis by phosphorylating the rate-limiting enzyme of DNL, acetyl CoA carboxylase-1 (ACC-1). This decreases in ACC-1 activity which leads to decrease in malonyl CoA production, thereby relieves inhibition of carnitine palmitoyl transferase-1 (CPT-1) activity and enhances fatty acid transport into the mitochondria to undergo β -oxidation.

Adiponectin is also associated with cardio-protection. Administration of adenovirus-mediated adiponectin reduces atherosclerotic lesion size in apolipoprotein E knockout mice, accompanied by reduction in the expression of inflammatory cytokines⁸¹. Moreover, adiponectin is also reported to promote angiogenesis and inhibits endothelial apoptosis⁸².

Fatty acid binding protein 4 (FABP4/ aP2)

aP2 was elevated in circulation in obese humans and mice⁸³. Furthermore, aP2 was demonstrated to be an adipokine released from adipocytes under obesogenic conditions, such as hypoxia, to augment insulin secretion from pancreatic β -cells *in vivo*, while insulin could in turn inhibit aP2 secretion⁸⁴.

DPP-4

Dipeptidyl peptidase 4 (DPP4) is a 110 kDa glycoprotein, which is ubiquitously expressed on different cell types as well as in circulation. By proteomic profiling of the adipocyte secretome, soluble DPP4 is identified as a novel adipokine, for which mature adipocytes constitute are a major source⁸⁵. DPP-4 may not only reflect, but also contribute to adipose tissue dysfunction. Compared with healthy lean individuals, patients with obesity and insulin resistance have higher DPP-4 expression in vWAT, which may contribute to the higher circulating DPP-4 levels⁸⁶. In obesity, hypoxia-mediated upregulation of DPP-4 shedding from adipocytes by metalloprotease-9 might contribute to induced DPP-4 activity⁸⁷. Also, in mice, production of DPP-4 in adipocytes is shown to be negatively regulated by high glucose concentration under physiological conditions, such an effect is abolished in mouse models of diabetes⁸⁸.

Under physiological conditions, DPP-4 rapidly degrades the incretin hormones including glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1)⁸⁹, which have been shown to enhance glucose-dependent insulin secretion and inhibit glucagon secretion, which together may reduce hepatic gluconeogenesis⁹⁰. Moreover, under physiologic concentrations, DPP-4 directly induced insulin resistance in adipocytes and skeletal muscle⁸⁵

Nicotinamide phosphoribosyltransferase (NAMPT)

Very recently, NAMPT, a key NAD⁺ biosynthetic enzyme, which has two different forms, intra- and extracellular (iNAMPT and eNAMPT) in mammals, was found predisposed to secretion from adipocytes via deacetylation by the mammalian NAD⁺-dependent deacetylase SIRT1. Adipose tissue-specific NAMPT knockout and knockin mice show reciprocal changes in circulating eNAMPT, affecting the hypothalamic NAD⁺ production, SIRT activity and neural activation accordingly⁹¹. The story provides an elegant example in which adipocyte-derived molecules could be transferred and exert their function in other organs.

BMP-4

BMP-4 is secreted by differentiated preadipocytes and adipocytes, with higher expression in larger adipocytes⁹². It has been recently shown that differentiated human adipocytes can promote adipogenesis *via* endogenous BMP-4 activation, a process that may

be counter-balanced by the expression of the BMP-4 (and -7) inhibitor gremlin-1⁹². In hypertrophic obesity, adipose precursor cells are resistant to BMP-4, which may contribute to the limited expandability of adipose tissue and, subsequently, to obesity-related metabolic diseases⁹².

Interestingly, adipose tissue has been very recently demonstrated as a major source of circulating microRNAs, and adipocyte-derived microRNAs could regulate gene expressions in liver in mice⁹³, indicating that, aside the classical view that most of the adipokines are proteins, other factors like microRNAs might be considered as novel adipokines.

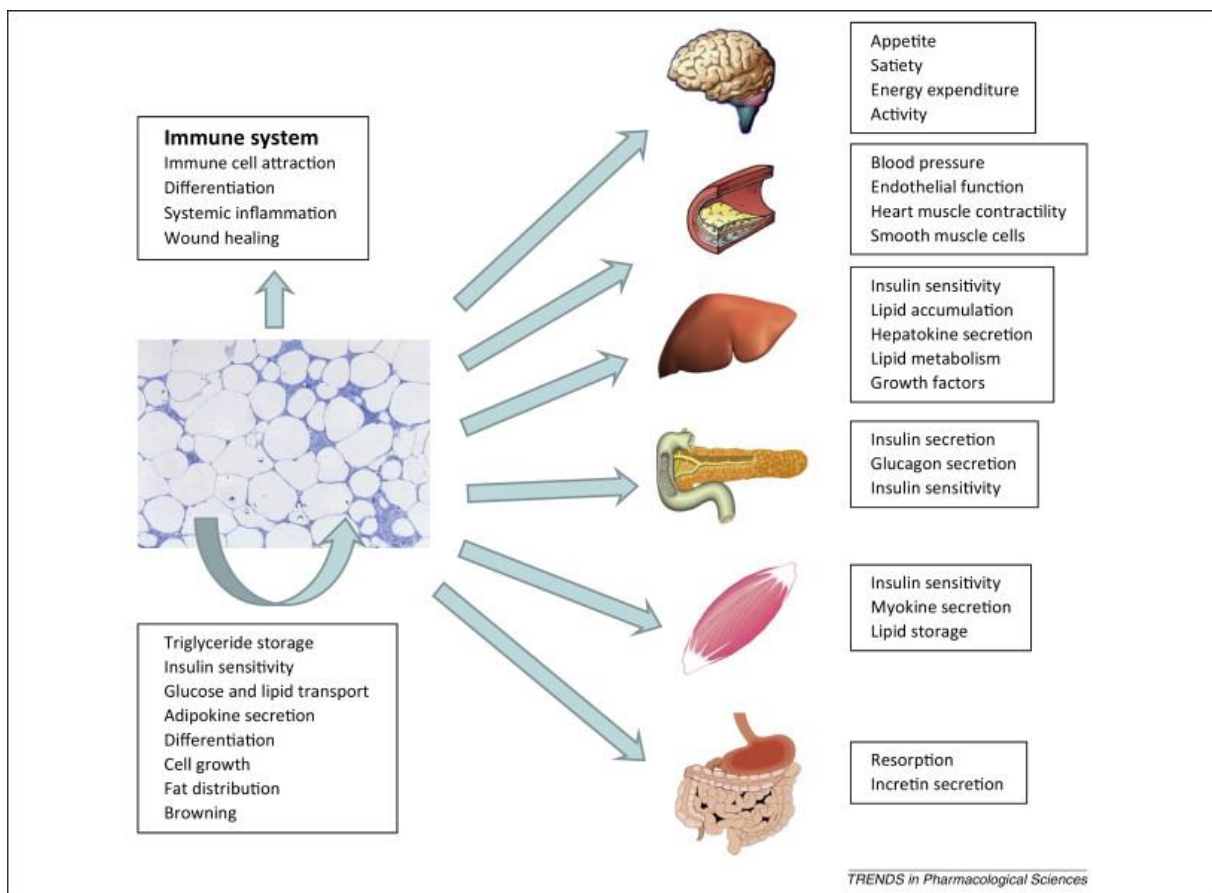


Figure 8 : Adipocytes crosstalk with various organs through adipokines.

Adopted from⁹⁴.

2.4. Origins of white adipocytes

Although adipose tissue development is poorly understood, the phenomenon that, in lipodystrophies, regionalized depot atrophy coexists with preservation and often expansion of other depots suggests that adipose tissue is an organ of multiple lineages⁹⁵. In mice, it has been demonstrated that sWAT and vWAT form in an ordered and timed manner throughout embryogenesis and within the first few weeks of birth. sWAT begins to develop during embryogenesis and the compartment of progenitors is established for all the depots of sWAT before the first few days of life. Whereas, vWAT principally form postnatally: the perigonadal lineage forms approximately between postnatal day 3 and the second week of life. The mesenteric WAT adipose compartment completes its establishment of lineage between the second and third weeks of life. The retroperitoneal WAT is formed in-between these pre- and postnatal stages, and has a morphogenesis, texture, and histology that also seem intermediate⁹⁶. Transcriptomic analysis of preadipocytes isolated from the SVF of various fat depots have revealed consistent differences in developmental gene profiles between vWAT and sWAT, indicating the possibility that sWAT and vWAT are of distinct origins⁹⁷.

Three critical events are necessary for the establishment and maintenance of functional adipocytes (Figure 9):

- 1) Determination of preadipocyte, commitment of multipotent progenitors into the adipocyte lineage.
- 2) Adipocyte differentiation, in which committed preadipocytes undergo a morphological and biochemical transition into mature adipocytes in response to appropriate stimuli.
- 3) Adipocyte maintenance, in which the cellular identity and functional properties of the terminally differentiated cells are maintained.

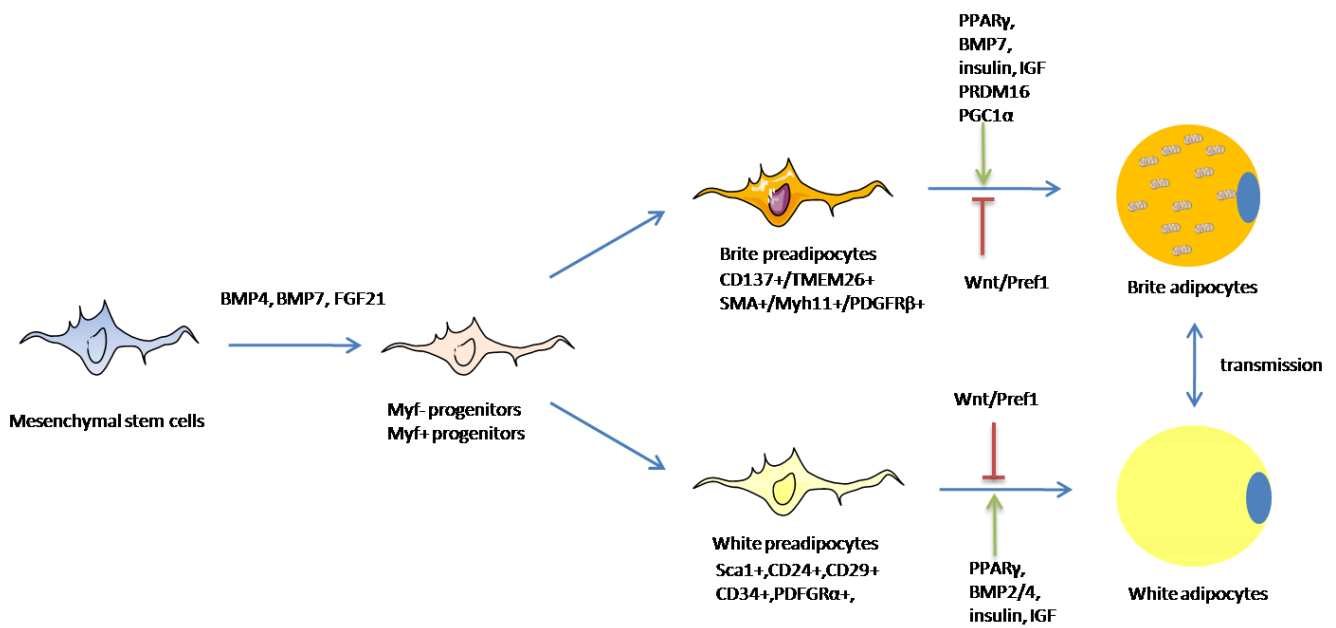


Figure 9 : Simplified scheme of white adipocyte origins.

2.4.1. Adipocyte progenitor cells (APCs)

Pluripotent stem cells, residing in the vascular stroma of adipose tissue, are able to be recruited into preadipocytes, which when appropriately induced, undergo mitotic clonal expansion and then differentiate into adipocytes (Figure 9). Adipocyte progenitor cells (APCs) reside in the SVF and could be separated from non-adipose precursors (e.g., endothelial progenitors, immune cell populations) using early adipocyte progenitor cells markers (Lin⁻: CD29⁺;CD34⁺;SCA1⁺:CD24⁺) with the technology of fluorescence-activated cell sorting (FACS), which could successfully proliferate and differentiate into WAT in A-Zip lipodystrophic transgenic mice⁹⁸.

To investigate the architecture of the stromal vascular (SV) compartment, Wei T., et al, developed an SV particulate (SVP) isolation procedure designed to partially maintain the native SV structure while removing adipocytes that obscure visualization of the precursor location. In the SVPs, the majority of peroxisome proliferator-activated receptor γ (PPAR γ)/GFP cells were arrayed in tubelike structures, which immunohistochemically

expressed platelet endothelial cell adhesion molecule (PECAM) and mural cell markers: SMA (endogenous smooth muscle actin), PDGFR β (platelet-derived growth factor receptor β), indicating that these adipocyte progenitors reside in the mural cell compartment of the adipose vasculature⁹⁹.

The large C2H2 zinc-finger transcription factor, Zfp423, is a factor associated with preadipocyte commitment. *Zfp423*^{GFP} reporter mice show that Zfp423 distinguished adipogenic from inflammatory-like mural cells. *Zfp423*^{GFP} expression was found not only in mature adipocytes but also in a distinct subset of perivascular cells expressing mural cell marker PDGFR β , supporting the hypothesis that preadipocytes resemble pericyte-like cells. Upon high fat diet feeding, the frequency and absolute number of cells positive for GFP and Pdgfr β significantly increase in gonadal WAT by comparison to inguinal WAT of male mice, consistent with the fact that gonadal WAT of adult mice expands by both adipocyte hypertrophy and adipocyte hyperplasia, while inguinal WAT expands predominantly through cellular hypertrophy⁷⁰.

Besides, adipocytes within different depots might have distinct progenitors. For example, the majority of the precursors and mature sWAT adipocytes in mice are labeled by paired related homeobox transcription factor 1 Cre (Prx1-Cre), while in contrast, few visceral adipocytes are marked by Prx1-cre¹⁰⁰. Conversely, the wilms tumor gene (*Wt1*) expressing cells give rise to adipocytes in vWAT but not adipocytes in sWAT, and are enriched in the mesothelium, supporting that the lateral plate mesoderm as a major source of vWAT¹⁰¹.

Multiple protein factors could increase the commitment of stem cells into preadipocytes. For example, following treatment of the C3H10T1/2 stem cells with bone morphogenic protein 4 (BMP4) during proliferation followed by differentiation inducers at growth arrest, the cells synchronously enter S phase and undergo mitotic clonal expansion, which is a hallmark of preadipocyte differentiation¹⁰².

2.4.2. Adipogenesis

During conversion of preadipocytes to adipocytes, preadipocytes employ a sequential induction of a series of transcription factors, including C/EBP families and PPAR γ to express genes necessary for adipocyte function. They also undergo change in morphology to become

rounded lipid-laden adipocytes. In the 1990s, the nuclear hormone receptor PPAR γ was identified as a master regulator of adipocyte differentiation. PPAR has three isoforms: PPAR α , PPAR δ , and PPAR γ . PPAR γ predominately cooperate with C/EBP α in the promotion of adipogenesis¹⁰³. C/EBP δ and C/EBP β are induced early during adipogenesis, peaking at day 2 of adipogenesis and after decreased¹⁰⁴. The early upregulation of C/EBP δ and C/EBP β facilitate the expression of PPAR γ , and then PPAR γ along with C/EBP β and C/EBP δ activates C/EBP α expression. Chromatin immunoprecipitation (ChIP) study revealed that most induced genes in adipogenesis were bound by both PPAR γ and C/EBP α . Thus, PPAR γ and C/EBP factors cooperatively orchestrate adipocyte biology by adjacent binding, which activate hundreds of genes responsible for terminal adipocyte differentiation¹⁰⁵. PPAR γ can promote adipogenesis in C/EBP α -deficient cells. However, conversely, ectopic expression of C/EBP α could not stimulate adipogenesis in PPAR γ -deficient fibroblast, demonstrating that C/EBP α and PPAR γ participate in a single pathway of fat cell development with PPAR γ being the proximal effect of adipogenesis¹⁰⁶. C/EBP α -deficient fibroblast underwent adipogenesis via activation of PPAR γ with several apparent defects, including impaired insulin stimulated glucose uptake secondary to reduced gene expression and tyrosine phosphorylation for the insulin receptor and IRS-1. Later it was demonstrated that insulin receptor promoter or enhancer was preferentially transactivated by C/EBP α ¹⁰⁷.

Signal transducer and activator of transcription 3 (STAT3) was reported to be activated by Janus kinase 2 (JAK2) within 2h of adipogenesis induction on 3T3-L1 adipocytes, translocating from cytoplasm to the nucleus and regulating the transcription of C/EBP β by binding the distal region of its promoter¹⁰⁸ (Figure 9).

PRDM16 is more enriched in sWAT compared to that in vWAT in mice, associated with its role in the development and function of beige cells. Ablation of PRDM16 caused a switch of sWAT to vWAT like phenotype, and impairment of thermogenic gene expression in response to cold exposure, indicating that PRDM16 pathway is necessary for the differentiation of brite adipocytes⁹. In human, the elevated levels of BMP4 in WAT correlated with a lean phenotype. Forced expression of BMP4 in mice induced a brown fat-like alterations in WAT through upregulation of mitochondrial biogenesis in association with increased energy expenditure and insulin sensitivity, which is mediated by the PGC1 α /

p38/MAPK/ATF2 pathway¹⁰⁹, indicating a potential role of BMP4 in the biogenesis of brite adipocytes (Figure 9).

Wnts are a family of paracrine and autocrine factors that regulate cell growth and cell fate. Signaling is initiated when Wnt ligands bind to transmembrane receptors of the Frizzled family, which signals through Dishevelled to inhibit the kinase activity of a complex containing glycogen synthase kinase 3 (GSK3), Axin, β -catenin, etc. This complex targets β -catenin for rapid degradation through phosphorylation. Thus, once hypophosphorylated due to Wnt signaling, β -catenin is stabilized and translocate to the nucleus where it binds the TCF/LEF family of transcription factors to regulate the expression of Wnt target genes. When Wnt signaling was activated in 3T3-L1 or 3T3-F442A, adipogenesis was significantly impaired, with no alterations in the expressions of C/EBP δ and C/EBP β , but decreased levels of C/EBP α and PPAR γ . Consistently, over-expression of C/EBP α and PPAR γ could partly rescue the impaired adipogenesis due to Wnt activation¹¹⁰ (Figure 9).

Pref-1 is a transmembrane protein encoded by the *pref-1/dlk1* gene and belongs to a family of EGF-like repeat-containing proteins that include Notch/Delta/Serrate, which are involved in cell fate determination. Interestingly, Pref-1 is highly expressed in 3T3-L1 preadipocytes, but almost abolished in adipocytes¹¹¹. The extracellular domain of Pref-1 undergoes two proteolytic cleavage events that generate 50 and 25 kDa soluble products. By fusing Pref-1 to human immunoglobulin- γ constant region (Pref-1/hFc), transgenic mice expressing the Pref-1/hFc transgene under the α 2 promoter, showed reduced expression of adipocyte markers and adipokines, whereas the preadipocyte markers Pref-1 was increased. Moreover, mice expressing the Pref-1/hFc transgene exclusively in liver under the control of the albumin promoter also showed a decrease in adipose mass and markers, suggesting an endocrine role of Pref-1 on adipogenesis¹¹² (Figure 9).

The seven Sirtuins in mammals (Sirt1-Sirt7), which are involved in the regulation of several essential cellular processes, also have important roles in adipogenesis. Sirtuin 1 (Sirt 1) is the ortholog of the yeast protein silent information regulator 2 (Sir2) and a nuclear NAD⁺-dependent class III histone deacetylase class III, and is a key component activated by CR. Sirt 1 partly mediated the inhibitory effects of CR on adiposity by attenuating adipogenesis through suppressing PPAR γ by docking with its cofactors NCoR (nuclear

receptor corepressor) and SMART (silencing mediator of retinoid and thyroid hormone receptors). Conversely, Sirt7 which is known to specifically deacetylate H3K18, is reported to promote adipogenesis by binding and repressing Sirt1 activity¹¹³. The forkhead transcription factor FOXO1 is an important downstream effector of insulin/PI3K/AKT signaling, which lead to its phosphorylation and thereby nuclear exclusion. Constitutively active FOXO1 attenuated adipogenesis, while dominant-negative FOXO1 restored adipogenesis of fibroblasts from insulin receptor deficient mice¹¹⁴. Interestingly, mammalian Sirt2 is localized mainly in the cytoplasm and is reported to be down regulated during adipogenesis, and its over-expression inhibits differentiation via enhancing insulin-stimulated phosphorylation of FOXO1¹¹⁵.

3. Alterations of WAT by aging

Over the last decades, the growing number of elderlies (aged 65 years and over) has become a considerable challenge to health and social care service provision and funding. Moreover, the numbers of the very old, those aged 85 years and over, are set to double over the next 20 years¹¹⁶. Aging is defined as a decline in survival and fecundity with advancing age, which is caused by damage to macromolecules and tissues.

What are the consequences of aging on WAT?

In human, age *per se* is a strong risk factor for fat accumulation¹¹⁷. Morphologically, computational tomography scans in men and women revealed that, as age increases, sWAT decreases while vWAT increases¹¹⁸. Rodents also demonstrate the same trend to develop age-associated central obesity¹¹⁹. Consequently, the dramatic alterations in fat distributions and functions occurring during life, are linked with the increasing risks of the developments of various aging-associated diseases, such as diabetes, cognitive dysfunction and atherosclerosis¹²⁰. Multi-isotope imaging mass spectrometry (MIMS) is an imaging method that enables multiplexed measurement of stable isotope tracers with subcellular resolution, and utility of stable isotope tracers of DNA synthesis and DNL to measure cell birth and lipid turnover. It was demonstrated that there's an age-dependent decline in the plasticity of adipose tissue which is characterized by impaired adipogenesis and adipocyte lipid turnover in subcutaneous fat, which might be mediated by a decline in insulin like growth factor-1 (IGF-1) in humans¹²¹. In addition, by measuring the 2nd World War nuclear bomb test-derived

^{14}C in lipids accumulated in adipocytes (in Hiroshima), it was reported that during the average ten-year-life span of human adipocytes, TG were renewed six times. Adipocyte lipid turnover is strongly related to conditions with disturbed lipid metabolism¹²².

Therefore, in the following part, we will detail effects of aging on adipogenesis and lipid turnover, respectively.

3.1. Senescence of adipocyte progenitors

Compromised adipogenesis during aging contributed to the impaired ability of adipose tissue to store lipids, leading to FFAs spillover and ectopic lipid accumulation in liver or muscle¹²³. Senescence of adipocyte progenitors mediates partly the impaired adipogenesis during aging.

Senescence of adipocytes progenitors is increased in aged adipose tissue, demonstrated with upregulation of senescence-associated markers: SA- β -galactosidase and p16/p53 expressions¹²⁴.

The senescence burden could further accelerate age-related dysfunctions in fat via both intrinsic and autocrine manners¹²⁵. For instance, senescent adipocyte progenitors would impede the adipogenesis of non-senescent cells, via the activation of the JAK/STAT and over production of the activin A protein¹²⁶.

Growth hormone (GH) plays a central role in regulating mammalian growth, metabolic homeostasis and adiposity. Both mice and human who have excessive GH production have increased mortality rates compared to age-matched counterparts¹²⁷. Ames and Snell dwarf mutant mice (*Prop1*^{df} and *Pit1*^{dw}, respectively) are the first GH-deficient genetic mutants to display extension of lifespan. Interestingly, deficiency of GH in Ames and Snell mice attenuated the age-related central accumulation of vWAT. Besides, the levels of senescent fat progenitors measured by p16 expressions or SA- β -galactosidase, were negatively associated with the levels of GH. Thus, attenuation of senescent burden in vWAT might mediate the life-extending effects of GH deficiency¹²⁸.

Clearance of senescent cells confers counteractions to age-related irregularities in adipocytes. For example, genetic clearance of p16 positive cells delayed or attenuated the

reduction of the volumes of adipocytes¹²⁹. In addition, depletion of p53 ameliorated senescence like changes in Ay obese mice, associated with improved insulin resistance¹³⁰.

3.2. Altered lipid turnover

As mentioned before, fat cells function to store cytotoxic lipids, for example FFAs into neutral LDs¹³¹. However, this lipid storage capacity of adipocytes is impaired by aging. In 26-month-old B6D2F1 mice, the average size of vWAT adipocytes was dramatically smaller compared with those in the counterparts of 6-month-old animals, indicating of a disability of lipid handling for adipocytes with aging¹³². The situation could be further demonstrated by the fact that middle-aged mice of around 14 months exhibited greater susceptibility to the toxicity of high fat diet, gaining more weight and fat content compared to their 3-month-old littermate¹³³. Furthermore, three days of high fat diet feeding specifically induced memory deficits and significant brain inflammation in the aged rats, possible owing the the spillover of FFAs in the brain in aged rats¹³⁴.

How could these dysregulations be explained?

Reduced sensitivity to nutrient stimuli

Age can drive insulin resistance in adipocytes. Both basal and insulin stimulated lipogenesis are reduced in isolated adipocytes from humans and rats by aging¹³⁵. In humans, glucose-mediated glucose disposal and glucose transport is decreased by 30-35% in the elderly (64 ± 4 years) compared to the nonelderly (32 ± 4 years)¹³⁶.

The exact mechanisms regarding to age-related insulin resistance is not well understand, but might be related to the alteraed immune-environments in adipose tissue in aging. For example, an increase in the portion of regulatory T (Treg) cells is observed in vWAT of 11-month-old mice compared to 1-month-old mice. Both genetic and pharmacological depletion of Treg cells in fat help to improve metabolic state and insulin stimulated glucose uptake¹³⁷. Besides, aging is associated with a chronic state of low-grade inflammation in WAT. This is likely due to the fact that vWAT is a major source of cytokines,

responsible mostly for the up-regulation of circulating levels of inflammatory and pro-coagulant factors such as, thrombospondin-1, plasminogen activator inhibitors-1 and -2, (PAI-1/-2), in comparison to liver or kidney. Furthermore, levels of these genes exhibited an age-associated increase in vWAT¹³⁸.

Impaired breakdown and oxidation of LDs

- Lipolysis

Fasting-induced levels of glycerol and FFAs release in visceral fat were significantly reduced in aged mice¹³⁹, indicating a decreased efficiency of hydrolysis of TG in adipocytes during aging. Aging is associated with reduced expression of lipolytic genes such as *HSL* and *ATGL* in adipose tissue¹⁴⁰. There is also a concerted decline in fatty acid reesterification (PEPCK: phosphoenolpyruvate carboxykinase) enzyme expression. This leads to reduced adipocyte's ability to recycle FFAs from extracellular pool, in 24-month-old mice compared to young mice¹⁴⁰. Age-related alterations in the functionality of macrophages also contribute to age-reduced lipolysis. Camell et al, demonstrated that aging up-regulate the Nod-like receptor 3 (NLRP3) inflammasome in adipose macrophages, which is a driving factor for the catabolism of noradrenaline by downregulating growth differentiation factor-3 and monoamine oxidase. Consequently, catecholamine-induced lipolysis is negatively influenced in aged vWAT and thereby leads to age-related central fat accumulation¹⁴¹.

- Mitochondrial oxidation

In humans, proteomic analysis of adipocyte mitochondria reveals global down-regulation of the oxidative phosphorylation system (OXPHOS) components by aging process¹⁴², indicating dysfunction of mitochondria. Dysfunction of mitochondria, through various aspects, contributes to reduced efficiency of lipid turnover in visceral adipocytes. Concomitant with this notion, oxygen influx stimulated by a combination of succinate and octanoyl carnitine during mitochondrial respiration is around 30% lower in older visceral WAT in relative to young vWAT¹³².

In the early stage of aging, mitochondrial abnormalities could be responsible for adipocyte enlargement in middle-aged mice. It has been reported that middle-age promote

hypoxia inducible factor 1 α (HIF 1 α) in adipocytes which, as a transcriptional factor, repressed cytochrome C oxidase 5b (COX5b) expression by binding to its proximal promoter. Reduced COX5b then inhibits complex IV assembly and impairs fatty acid oxidation then resulting in adipocyte enlargement¹⁴³. In addition, reduction in β -oxidation could also lead to increased rates of FFA efflux out of cells in order to avoid toxic FFA buildup in the cytoplasm¹⁴⁴. Moreover, adipocyte β -oxidation is also important in maintaining body temperature, since adipocyte specific CPT2 knockout leads to cold intolerance in mice¹⁴⁵. This might be one of the reasons to the cold intolerance by aging.

4. Contribution of WAT to aging

Adipose tissue is at the nexus of mechanisms and pathways involved in longevity and genesis of age-related diseases by general mechanisms such as inflammation or metabolic dysfunction. It has been proven that genetic profiles of WAT may affect lifespan¹⁴⁶. For instance, PPAR γ signaling pathway is revealed as the most significantly enriched biological pathway within the aging sub-network. In agreement, two independent PPAR γ knockout mice models (one lacks PPAR γ ½ exclusively in WAT and the other lacks PPAR γ 2 in all tissues), exhibit severe lipodystrophy and remain insulin-resistant, both having a significant reduction in lifespan¹⁴⁷. Under extreme conditions of adiposity depletion, e.g., lipodystrophy syndromes such as genetic Berardinelli-seipcongenital generalized lipodystrophy (BSCL) or familial partial lipodystrophic syndromes (FPLD), patients are markedly short-lived, suggesting that malfunctions of adipose tissue affect life quality and longevity.

4.1. Nutrition/age-associated obesity attenuates longevity

▪ The link between excessive fat development and aging

Obesity is defined by a body mass index over 30 (BMI, an indicator of body fat calculated by dividing a person's weight in kilograms by their height in meter squared). Pooled analysis data of 20 prospective studies from the United States, Sweden and Australia, reveal that people with Class III obesity (BMI > 40 kg/m²) have a dramatic reduction in life expectancy compared with people of normal weight, with most of the excess death due to

heart diseases, cancers and diabetes¹⁴⁸. Interestingly, a strong correlation between high BMI and the “epigenetic age” of liver in human exists, which might help to explain why obese people suffer from the early-onset of many age-related pathologies, such as liver cancer¹⁴⁹. Indeed, the group of Steve Horvath has recently developed a biomarker of aging (“epigenetic clock”) based on DNA methylation (DNAm) levels¹⁵⁰, which is defined as a prediction method of chronological age based on the DNAm levels of 353 CpGs, also referred to as “DNAm age”. The use of DNAm age could be seen as a proxy of epigenetic tissue age. Finally, obesity has been shown to induce DNA damage in adipose tissue *via* activation of p53 pathway¹⁵¹. Moreover, disorders derived from energy metabolism might also negatively influence age-related pathogenesis. For example, Alzheimer’s diseases patients are very often characterized by visceral fat accumulation, insulin resistance and abnormal secretion of leptin and adiponectin¹⁵².

- Age and adipose tissue

Besides the nutrition-induced obesity, age-related central obesity, which is accounted mainly by fat accumulation in visceral depot, is also negatively associated with insulin sensitivity. Different studies showed that aging *per se* does not drive this phenomenon, but it’s rather the augmented visceral fat during aging that is truly responsible for this age-related insulin resistance¹⁵³. Independently of other depots, vWAT plays an important role in modulating hepatic insulin action¹⁵⁴. One of the most distinguished features of vWAT is that it is believed to be a source of harmful peptides such as resistin, PAI-1 and angiotensinogen, which might contribute to various pathological states in peripheral organs¹⁵⁵. For instance, aging induces cardiac structural and functional changes associated with increased deposition of ECM proteins, such as osteopontin (OPN), resulting in progressive interstitial fibrosis. Visceral WAT strongly mediate this process¹⁵⁶. Indeed, it has been demonstrated that plasma OPN is increased in mice during aging, with vWAT showing the strongest OPN production in contrast to myocardium that does not express this protein. Moreover, vWAT removal or OPN inhibition greatly reverses senescence in cardiac fibroblasts¹⁵⁶.

Noticeably, not only the quantity but also the quality of fat mass matters. The epigenetic marker H3K4me3 is remodeled during aging and cellular senescence in mammals¹⁵⁷. In paradox, the depletion of H3K4me3 methyltransferase (the COMPASS chromatin

complex) in *Caenorhabditis elegans* (*C. elegans*) promotes lifespan as well as fat accumulation accounted by specific enrichment of mono-unsaturated fatty acids (MUFAs). Moreover, dietary MUFAs are sufficient to extend lifespan¹⁵⁸. These data suggest that not only the amount of fat but also the characteristics of the fat influence longevity. Indeed, the fatty acid composition of TG in visceral WAT is altered during aging in rats, with a marked decrease in shorter long-chain saturated fatty acids, whereas the levels of linoleic and oleic acids increase¹⁵⁹. In addition, there are increases in the levels of total ether-phosphatidylcholine, sphingomyelin, and free cholesterol in the lipid composition of adipocyte plasma membrane, which favor an enlarged cell size with aging¹⁵⁹. Therefore, it remains interesting to investigate how the composition of adipocyte fatty acids alters during aging in various fat depots and what are the effects of different fatty acids on longevity.

4.2. Removal or mobilization of vWAT increases longevity

Due to the notorious associations of vWAT with age-associated pathologies, it has been proposed that surgical removal or stimulation of lipid mobilization might exhibit positive actions on life extension-associated benefits. On one hand, Radhika M. et al demonstrated that removal of vWAT in 5-month-old mice increases the mean and maximum lifespan and reduces the incidence of severe renal diseases compared to sham control mice¹⁶⁰. On the other hand, in *C. elegans*, fat mobilization activation through the endocrine axis, which consists of nuclear translocation of the forkhead transcription factor DAF-16 (the equivalent of mammalian FOXO3a) and subsequent activation of the k04a8.5 lipase, mediates the prolonged longevity of germline stem cell arrest. Constitutive expression of this lipase in fat storage tissue generates lean and long-lived animals¹⁶¹. In a conserved manner, over-expression of dFOXO in the adult fruitfly (*D. melanogaster*) fat body, which is fly equivalent of mammalian liver and adipose tissue, increases longevity and reduces fecundity of females¹⁶².

Since the FOXO is downstream and inactivated by the insulin/insulin-like growth factor (IGF) signaling in diverse organisms, these data suggest that attenuation of certain signaling pathways in fat might be associated with life-span extension. In addition, in *D. melanogaster*, limited activation of dFOXO in the fat body increases lifespan, while

repressing the synthesis of insulin-like peptide *dilp-2* in neurons which then inhibites endogenous insulin-dependent signaling in peripheral fat body¹⁶³. These findings suggest that insulin/IGF pathway in the adipose tissue could contribute to the control of aging through both autonomous and non-autonomous manners.

This relationship has also been demonstrated in the mammals. Mouse animal models with reduced growth hormone (GH) and/or IGF-1 signaling have been shown to have an extended lifespan, the action of which is partly associated with a delayed increase in the ratio of visceral to subcutaneous fat, and most likely a reduced fat cell progenitor turnover¹⁶⁴. Likewise, reduced dietary methionine intake prolongs lifespan in male Fish 344 rats, with reduced serum levels of lipids and IGF-1 as well as reduction in vWAT and increased energy expenditure¹⁶⁵. Cre-Lox specific adipocyte depletion of insulin receptor protects mice from age-associated obesity and glucose response impairment, accounted mostly by an increase in small adipocyte populations less than 75 μ m^{166,167}. Moreover, inducible knockout of insulin receptor in mice of median life-span extends the life span of these animals by 3.5 months and is associated with dramatic reduction in adiposity without alterations in food intake¹⁶⁷. Interestingly, mice deficient for vasohibin-1, a protein originally isolated as an endothelium-derived angiogenesis inhibitor and a stress tolerance promoter in endothelial cells, have improved longevity. Although the exact molecular mechanisms remain elusive, the attenuated levels of IR and IRS-1 in WAT levels might contribute to the life extending effects of vasohibin-1 deficiency¹⁶⁸.

There are also other molecules/mechanisms in WAT that contribute to life span. Sirt1 (Sirtuin 1) is a member of the NAD⁺-dependent deacetylase family that catalyze the removal of acetyl groups from protein substrates. Mammalian Sirt1 acts as an energy and stress sensor modulating metabolic responses to nutrient availability. Selective over-expression of wild type human Sirt1 (AWSM) in adipose tissue in mice significantly prevents aging-associated insulin resistance and improves systemic energy homeostasis as well as enhanced spontaneous locomotory activity, which could be attributed to the promotion of lipid metabolism in adipose tissue and muscle¹⁶⁹. Increased mitochondrial oxidation in adipocytes also inhibits aging and diet associated-obesity in TFAM^{lx/lx}/ adiponectin-Cre mice. Indeed, TFAM (mitochondrial transcription factor A) depletion in adipocytes results in an increased

electron transport chain flux and a greater fat glucose oxidation due to complex I and IV deficiency¹⁷⁰. Conversely, in WAT, *in vivo* adipocyte specific knockout of acyl-CoA synthetase, which mediates conversion of FAs to acyl-CoAs, the limited step for FA metabolism, results in increased adipocyte volumes and cold intolerance in mice *via* an impaired β -oxidation in WAT, which is a hallmark of aging¹⁷¹.

Additionally, female mice deficient for diacylglycerol acyltransferase 1 (DGAT1), an enzyme that catalyzes the synthesis of TG, are protected from age-related fat accumulation, tissue TG accumulation as well as inflammation in WAT, which is accompanied with both increased mean and maximal lifespan. Moreover, these mice exhibit several longevity-associated features, such as decreased levels of circulating IGF1¹⁷².

Finally, in 2014, it was reported that FAT10 knockout mice had an extended lifespan in both female and male mice, associated with prevention of the age-associated obesity as well as metabolic dysfunctions¹⁷³. The HLA-F adjacent transcript 10 (FAT10) is a member of the ubiquitin-like gene family that alters protein function/stability through covalent ligation.

4.3. Novel insights from microRNAs

While previous studies mainly focus on the nutrient sensing pathways in fat to lifespan, it has recently been reported that the abundance of microRNAs in adipose tissue is an important determinant of longevity. The biogenetic enzyme for microRNAs: dicer is decreased in sWAT during aging. On the contrary, overexpression of this enzyme in the intestine of *C. elegans*, increases the lifespan as well as resistance to heat shock and oxidative stress¹⁷⁴. MicroRNAs from fat can play important roles in both cell autonomous and non-autonomous manners. For instance, mir-8 knockout drosophila exhibits a smaller body size compared to control drosophila, which could be rescued by its conditional overexpression in fat, which then leads to derepression of USH, an inhibitor of PI3K p85a subunit¹⁷⁵. This result then raises the question why PI3K inhibition in fat can lead to systemic effects, highlighting the endocrinal role of adipose tissue. For example, improved circulating profile of adipokines, e.g., reduced levels of leptin, TNF α , IL-6 or C-reactive protein might

mediate the cardio-protective effects of intermittent fasting and caloric restriction in human¹⁷⁶.

However, the precise molecular pathways that microRNAs in adipose tissue employ to achieve those benefits, remain to be largely unclear.

PART II: MicroRNAs in white adipose tissue

1. Biogenesis and regulation of microRNAs

1.1. Biogenesis of microRNAs

MicroRNAs (miRNAs) are a family of small non-coding RNAs that post-transcriptionally regulates mRNAs levels or stability by pairing to the 3'-untranslated regions (UTR) of their targets. In 1993, Ambros et al discovered the first miRNA, lin-4 in *C.elegans*, which was identified as a small non-coding RNA regulating the expression of the protein lin-14¹⁷⁷. Several years later, let-7, another famous miRNA was discovered in *C. elegans*, negatively regulating the expression of the gene lin-41 via RNA-RNA interactions with the 3'-UTR of lin-41¹⁷⁸. Subsequent reports further unveiled that miRNAs were abundant in both invertebrates and vertebrates, with some are highly conserved, suggesting that miRNA-mediated gene regulation is a general regulatory function across species^{179,180}. Currently, there are up to 1872 annotated human miRNA precursor genes that are processed into around 2578 mature miRNA sequences (<http://www.mirbase.org>) with functions of lots of them are waiting to be clarified.

Biogenesis of miRNAs starts with transcribing genes into large primary transcript (pri-miRNAs), which is 5' capped and 3' polyadenylated in structure. The transcription is typically mediated by RNA polymerase II^{181,182}. The pri-miRNAs are then cleaved by a microprocessor complex, consisting of the type III RNase Drosha and RNA-binding protein DGCR8, into an around 85 nucleotides stem-loop structure named precursor miRNA (pre-miRNA). Following transportation by Ran/GTP/Exportin 5 complex from nucleus to cytoplasm, the pre-miRNAs are processed by the RNase III enzyme Dicer to an around 20-22- nucleotide miRNA/miRNA* duplex (note: * indicates the passenger strand, while the other complementary strand is referred as the mature or guide strand).

After the duplex is unwound, mature miRNAs are incorporated into a protein complex termed RNA-induced silencing complex (RISC) and guide RISC to target mRNAs (Figure 10)^{177,178}. In many cases, miRNA-target interactions are mediated by the seed region, a 6- to 8-nucleotide-long fragment at the 5'-end of the miRNA that forms Watson-Crick pairs with the cognate target¹⁸³. Inside RISC, once binding to their target miRNAs, miRNAs would

result in translational repression if imperfect complementarity, or target mRNA degradation if perfect complementarity¹⁸⁴.

The biogenesis of miRNAs is under tight control at multiple levels, including the levels of miRNA transcription, processing by Drosha and Dicer, transportation, RISC binding and miRNA decay. For example, the KH-type splicing regulatory protein (KSRP), serves as a component of both Drosha and Dicer complexes to regulate the biogenesis of a subset of miRNAs in mammalian cells⁵⁵.

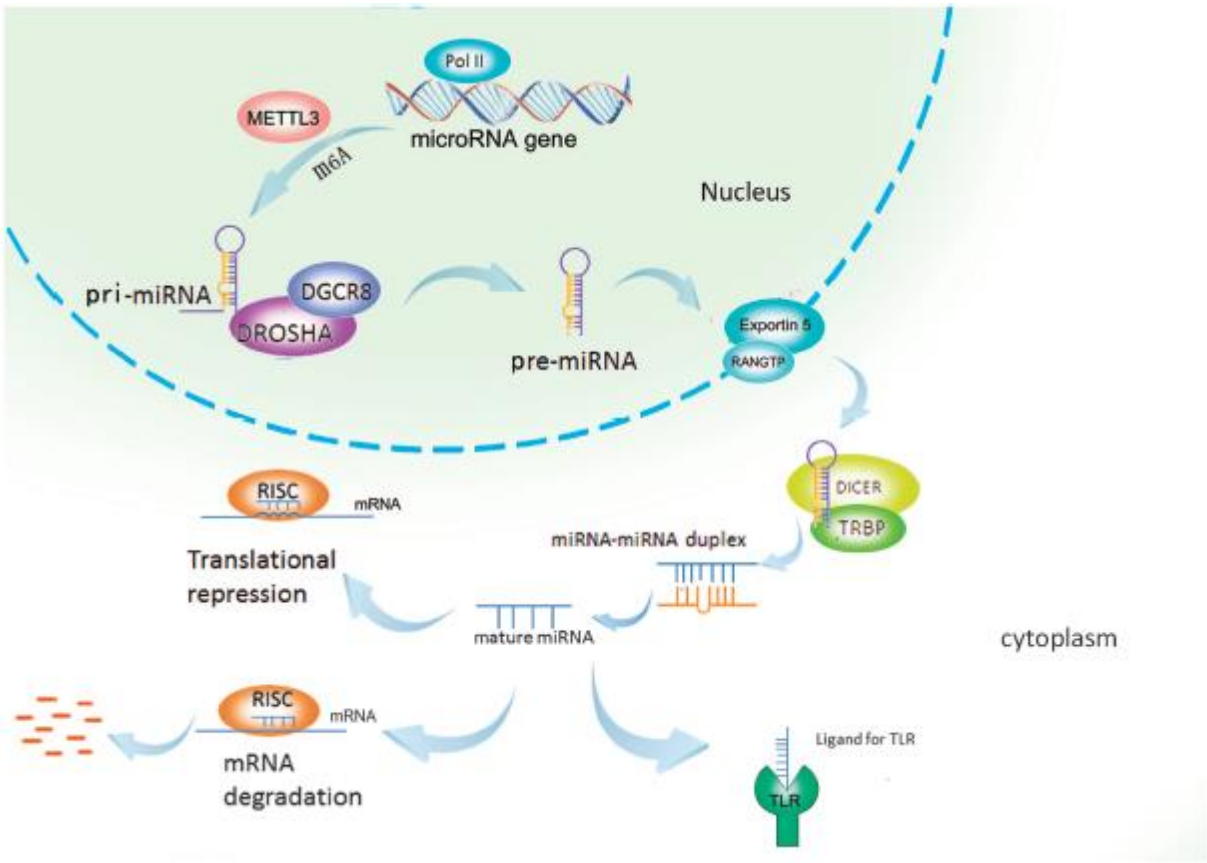


Figure 10: The classical pathway of microRNA biogenesis.

Adopted from¹⁸⁵.

1.2. Regulation of miRNAs expression in adipose tissue

Expression of miRNAs in adipose tissue could be regulated via several mechanisms. With the next generation sequencing technology, it has been reported that for a majority of miRNAs in adipose tissue, genetic regulation of expression was independent of the expression of the mRNA from which this miRNA is transcribed. Moreover, the degree of genetic variation of miRNA expression was substantially less than that of the mRNA¹⁸⁶. However, expression of some miRNAs is highly correlated with the mRNA from which the miRNAs are transcribed from. For instance, mir-378 resides in the sense strand of the first intron of PGC-1 β , which is a transcriptional co-activator that regulates mitochondrial biogenesis. Similar to PGC-1 β , mir-378 was also identified as key regulatory component underlying the biogenesis of mitochondria in adipose tissue¹⁸⁷.

On the other hand, expression of some miRNAs in adipose tissue could be regulated directly through alterations in their promoters. For example, NF- κ B stimulation in 3T3 adipocytes resulted in inhibition of PPAR γ transcription, with miR-130 participating in this process. Chromatin immunoprecipitation (ChIP) assay demonstrated that activation of NF- κ B pathway by TNF α induced increased binding of p65 to the promoters of both mir-130a and mir-130b. Deletion of these two miRNAs could partly block the inhibition of PPAR γ by TNF α ¹⁸⁸. Similarly, NF- κ B would directly bound to the promoter of mir-1275 and inhibited its transcription in human mature adipocytes¹⁸⁹. Glucocorticoids (GCs) could transcriptionally regulate the expression of miR-19b via a GC receptor-mediated binding to its promoter and significantly enhance its expression¹⁹⁰.

Still, for most of miRNAs, how their expressions are regulated in adipose tissue remain to be investigated. For example, the expression of mir-21 in human adipocytes was elevated following inflammatory stimulators such as TNF α , IL-6, leptin or FFAs¹⁹¹. Mir-224-5p was elevated in 3T3 adipocytes upon TNF α treatment. Knock down of mir-2224 suppressed NF- κ B activation and rendered adipocytes susceptible to apoptosis¹⁹². The exact mechanisms regarding these alterations remain to be investigated.

2. MiRNAs in the formation of adipose tissue

2.1. MiRNAs in differentiation of adipocytes progenitors

Adipogenesis is under tight regulations of various miRNAs in both mice and humans. Adipocyte dicer depletion resulted in extreme low adiposity due to depleted WAT¹⁹³. Since the fat mass is mainly determined by the number and the volume of adipocytes, it indicates that the loss of certain miRNAs due to dicer deficiency could have a role in adipogenesis or lipid homeostasis in adipocytes which lead to a loss fat mass. Subsequently, numerous evidence sare surging concerning various miRNAs affecting adipogenesis, via targeting classic components involved in adipogenic program. Here, we will review reported individual miRNA that exhibits either *pro-*or *anti-* effects on adipogenesis which are listed in Table 2.

Table 2: List of miRNAs involved in adipogenesis

miRNAs	Model of adipogenesis	of Targets/mechanisms	Pro/anti-adipogenesis	Reference
Mir-143-3p	ADSC	Repressing MAP2K5	Dual	194
Mir-15a/b	Porcine preadipocytes	Repressing FOXO1	Pro	195
Mir-17-92 cluster	3T3-L1	Down-regulating Rb2/p130	Pro	196
Mir-30a/d	hMADS	Inhibition of the osteogenesis TR RUNX2	Pro	197
Mir-30c	hMADS	Targeting PAI-1 and ALK2	Pro	198
Mir-103	3T3-L1	Targeting MEF2D and activation of AKT/mTOR	Pro	199
Mir-17-5p/mir-106a	hMADS	Suppressing osteogenesis via BMP2	Pro	200
Mir-146b	3T3-L1	Targeting the 3' UTR of SIRT1 and induced acetylation of FOXO1	Pro	201
Mir-342-3p	Human adipose-derived MSCs	Releasing C/EBP α from Binding with CtBP2	Pro	202
Mir-355	Ob/ob, db/db, KKAY mice	Levels in WAT correlated with body weight and adipocyte differentiation markers	Pro	203
Mir-455	3T3-L1	Targeting UCP1	Pro	204
Let-7	3T3-L1	Induction during differentiation, Unclear	Anti	205
Mir-27a	3T3-L1	Suppressing expression via 3' UTR	PPAR γ Anti	206
Mir-27b	Zebrafish/ 3T3-L1	Negatively correlated with the levels of as PPAR γ , C/EBP α and SREBP-1c.	Anti	207,208
Mir-29a/b/c	3T3-L1	Targeting DNA	Anti	209

methyltransferase DNMT3A							
Mir-33b	Human adipocytes	primary	Induced during adipocytes differentiation targeting HMGA2 and cyclin-dependent kinase 6	human	Anti		210
Mir-93	Lentivirus injection in visceral fat mice/ 3T3-L1/ mir-25-93-106b-/- mice		Negatively regulating and TBX3	Sirt7	Anti		211
Mir-125a-5p	3T3-L1		Negatively regulating	STAT3	Anti		212
Mir-130	Human adipocytes	primary	PPAR γ		Anti		213
Mir-155	3T3-L1		Induced by tnfa via the NF- κ B pathway and targeting C/EBP β and CREB		Anti		214
Mir-1275	Human visceral pre-adipocyte		Targeting and silencing of ELK1, an E-twenty-six-domain transcription factor		Anti		215

2.2. Determination of adipocytes identities

Different fat depots have distinctive characteristics and functions. Although precise mechanisms determining the fat distribution remain largely unknown, differential expressions of miRNAs amongst various fat depots might be involved. Interestingly, adipocytes dicer deficiency leads to partial lipodystrophy, with a dramatic decrease in epididymal fat but a significant increase in BAT, the situation of which would be further exacerbated by high fat diet exposure²¹⁶. Similarly, adipocyte -specific DGCR8 knockout mice display shrunked WAT with decreased specific gene markers and tolerance in response to cold exposure²¹⁷. The group of Steven R.S. finds that mir-196a is differentially expressed in gluteofemoral and in subcutaneous abdominal fat in humans, with its level more abundant in adipocytes of the gluteofemoral region²¹⁸. The favorably expressed mir-196a in gluteofemoral adipocyte might negatively regulate the homebox (HOX) family genes, which are involved in the transcriptional regulation of adipogenesis and less abundant in gluteofemoral fat²¹⁸. These results indicate that the abundance of mature miRNAs is vital in the determination and maintenance of identities of different adipocytes, and linked to their intrinsic properties and functions.

- The transcriptomes of miRNAs in sWAT and vWAT differ from each other²¹⁹.

MirRNAs enriched in sWAT are mainly related to adipogenesis and lipid metabolism. For example, mir-155-5p, mir-143-5p, mir-27b-3p or mir-193b-3p and mir-365, all of which are summarized in (Table 2) above, are amongst those miRNAs enriched in sWAT.

In contrast, the vast majority of miRNAs enriched in vWAT are associated with inflammation. For example, mir-145 is attenuated in the omental adipose tissue of obese patients and is proposed to improve macrophage-mediated inflammation by targeting ADP-ribosylation factor 6 (ARF6)²²⁰. In human adipose tissue, mir-99a expression is negatively correlated with inflammation, possibly regulating macrophage phenotype-switching by targeting TNF α ²²¹. Finally, let-7 family is strongly associated with fat mass and systemic glucose tolerance²²². Additionally, a group of vWAT-enriched miRNAs are found to be

involved in various pathologies. For instance, mir-125a is down regulated in adipose tissue by fat diet in mice or in obese patients and in *in vitro* insulin-resistant 3T3-L1 adipocytes²²³. Similarly, mir-103 is associated with insulin sensitivity in adipocytes²²⁴.

- MiRNAs mediate the metabolic flexibility of adipose tissue.

As mentioned before, human subcutaneous fat is capable of substantial metabolic flexibility, such as acquiring features of brown fat in response to stimuli of cold exposure, β -adrenergic receptor or PPAR γ agonists, associated with increased mitochondria biogenesis and UCP1 expression. MiRNAs are necessary for this identity switch.

Indeed, mir-30a-5p, which is less expressed in subcutaneous fat compared to its level in BAT and inversely associates with the level of ubiquitin carrier protein 9 (UCP9) between these two fat depots, would result in browning program in human white adipocytes: mitochondrial biogenesis and brown transcription factors expression *via* targeting and inhibiting UCP9²²⁵. *In vitro*, forced expression of mir-30b/c significantly increases thermogenic gene expressions and mitochondrial respiration in primary adipocytes from sWAT, by targeting the 3'UTR of the receptor-interacting protein 140 (RIP140)²²⁶. Conversely, let-7i-5p is decreased in brite adipocytes in sWAT and injection of let-7i-5p mimic into sWAT in mice partially inhibits the β 3-adrenergic activation of the browning process²²⁷. Similarly, mir-125b-5p level is reduced in sWAT upon β 3-adrenergic receptor stimulation, with injection of mir-125b-5p mimic directly into WAT inhibiting β 3-adrenoceptor-mediated induction of UCP1 and mitochondriogenesis²²⁸.

Further results demonstrate that the thermogenic gene program of PPAR γ /PRDM16 in sWAT is under the control of miRNAs: mir-30a-5p affecting PPAR γ /PRDM16 *via* targeting the E2 SUMO ligase UBC9 in sWAT, a factor repressing acquisition of brown fat features in human adipocytes²²⁵; mir-133a (mir-133a1/a2) directly targeting the 3' UTR of PRDM16, with mir-133 depletion in mice increasing UCP1 expression and browning of sWAT²²⁹.

Aside from being, subcutaneous fat could also acquire a visceral-like feature under certain conditions. For instance, 24-h fasting induces "visceralization" of sWAT in young mice, possibly accounted by a reduction in PRDM16 due to the upregulation of mir-149-3p in the

fat depot²³⁰. Therefore, it remains interesting to investigate whether mir-149-3p is a vWAT enriched miRNA.

2.3. Involvement in adipose tissue functions

MiRNAs expression in human adipose tissue is fat-depot specific and related to parameters of obesity, fat distribution, glucose metabolism and adipose tissue morphology²³¹.

- MiRNAs in lipolysis

In demand of nutrients deprivation, such as fasting or exercise, TG in adipocytes are hydrolyzed to release fatty acids to support other organs⁵⁷.

KSRP is a multifunctional RNA-binding protein involved in the posttranscriptional regulation of gene expressions, and is also important for pri-miRNA processing²³². KSRP deletion is associated with increased lipolysis in adipose tissue, accompanied with the impaired processing of pri-miR-145 into mature mir-145, which directly targets pro-lipolytic genes: CGI58 and FOXO1²³³. Mir-277 depletion results in severe impairment in lipid storage in female mosquitoes, associated with cytoplasmic retention of FOXO, arguing a role of mir-277 depletion in the promotion of lipolysis *via* FOXO²³⁴. Moreover, the expression of mir-124a is inversely correlated with the level of ATGL and its coactivator CGI-58 in various murine tissues such as adipose tissue and liver. In addition, ectopic expression of mir-124a leads to lipolysis inhibition which could be rescued by expressing mutant ATGL lacking its 3'UTR²³⁵.

In parallel, insulin signaling pathway is crucial for the various processes like glucose uptake and lipogenesis in adipocytes²³⁶. Abnormality of insulin signaling in obesity or over nutrition has been reported to be associated with irregularities of miRNAs levels²³⁷.

- MiRNAs in insulin-stimulated glucose uptake in adipose tissue

Some miRNAs are positively associated with insulin-stimulated glucose uptake.

For example, mir-26b is reduced in vWAT in obese rodents, human obesity and insulin resistant adipocytes, and is negatively correlated with BMI or HOMA-IR (homeostatic model assessment for insulin resistance). *In vitro* studies with differentiated human adipocytes suggest that mir-26b could promote insulin-stimulated glucose uptake *via* increasing GLUT4 translocation onto the plasma membrane *via* inhibition of the phosphatase and tensin homologue (PTEN), which is an antagonist of PI3K signaling²³⁸.

Some miRNAs are negatively associated with insulin-stimulated glucose uptake.

Mir-223 is increased in the adipose tissue of women with insulin resistance and is positively associated with HOMA-IR. Overexpression of mir-223 in human adipocytes directly reduces protein levels of GLUT4 and blocks insulin stimulated glucose uptake²³⁹. Likewise, mir-93 is up-regulated in the adipose tissue of women with insulin resistance as well as polycystic ovary syndrome patients (approximately 70% of who have intrinsic insulin resistance), correlating reversely with GLUT4 level and positively with HOMA-IR²⁴⁰. The mimic of mir-146b is directly transfected into porcine primary adipocytes, leading to impaired glucose uptake due to its inhibitory effect on IRS1 and GLUT4 expressions²⁴¹.

MiRNAs are found to be vital mediators of the obesity associated insulin resistance in adipose tissue. For example, in the context of obesity or diabetes in mice, mir-103 and mir-107 are elevated, while their silencing through systemic injection of its inhibitor significantly reduces adipocyte sizes and restores systemic glucose tolerance, arguing for a role of these two miRNAs in insulin resistance. Mechanical study reveals that caveolin-1, the principal protein of caveolae, a structure on the plasma membrane of adipocytes associated with insulin receptor stability, is a direct target of mir-103/mir-107²²⁴.

Mir-377 is increased in visceral fat in mice by high fat diet and impairs insulin stimulated AKT phosphorylation *via* suppression of SIRT1, which improves insulin sensitivity by repressing protein tyrosine phosphatase (PTP1B)²⁴². Similarly, mir-221 could also target SIRT1 and contribute to insulin resistance in WAT by high fat diet induced obesity²⁴³.

At the level of adipose tissue, adipose tissue macrophages (ATMs) infiltration in the context of obesity is a leading cause of the chronic inflammatory state in fat tissue, a strong contributor to insulin resistance. It has been reported that ATMs-derived mir-155 strongly

contributes to the impaired insulin stimulated glucose transport in adipocytes, possibly related to its direct suppression of the target gene *PPAR γ* ²⁴⁴.

- MiRNAs in lipogenesis

Various miRNAs could also modulate the sizes of adipocytes by targeting pathways related to lipogenesis. As an example, mir-192* is negatively correlated with serum TG and positively with high density lipoproteins (HDL) in morbidly obese subjects. *In vitro* experiments show that over expression of this miRNA in adipocytes, leads to a reduction in lipid storage accounted by inhibited expression of lipogenic enzymes such as stearoyl coenzyme A desaturase-1 (SCD-1) and aldehyde dehydrogenase 3 family member A2 (ALDH3A2)²⁴⁵.

Located in the first intron of PGC-1 β , miR-378/378* are coordinately expressed during adipogenesis and expressed in the same tissues as PGC-1 β . Overexpression of miR-378/378* in 3T3 adipocytes leads to lipid accumulation associated with increased expression of lipogenic genes, such as C/EBP α and C/EBP β ²⁴⁶.

In fat cells, mir-143-3p can increase insulin-stimulated lipogenesis, possibly related to the upregulation of protein kinase AMP-activated catalytic subunit α 2 (PRKAA), which encodes a catalytic subunit of AMPK²⁴⁷.

- MiRNAs in other aspects of adipocytes

MiRNAs also participate in other aspects of adipocytes, such as apoptosis, inflammation response and energy expenditure process, etc. For instance, natural conjugated linoleic acids (CLAs) show strong anti-obesity actions in humans and animals, which could reduce the TG content of mature adipocytes and induce apoptosis. The expression of mir-23a is inhibited in adipose tissue in mice administrated with CLAs and its inhibition mediates the apoptotic action of CLAs on adipocytes *via* its direct effect on apoptotic protease-activating factor 1 (APAF1)²⁴⁸. Very recently, mir-103/107 have been identified to promote ER stress-mediated apoptosis by targeting the Wnt3a/ β -catenin/ATF6 (activating transcription factor-6) pathway in preadipocytes²⁴⁹.

The obesity associated mir-146a is found to dampen the inflammatory response in human adipocytes demonstrated by reducing phosphorylation of p38 and c-Jun N-terminal kinase (JNK) *via* targeting TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), which act downstream of the IL-1 receptor and toll-like receptors²⁵⁰.

MiR-155-5p knockout prevents high fat diet induced adipocyte hypertrophy and weight gain, possibly owing to the increased energy expenditure²⁵¹. Likewise, elevation of mir-34a in adipocytes from obese individuals²⁵² inhibits the browning of adipose tissue partly by suppressing fibroblast growth factor (FGF21)/ SIRT1. In contrast, inhibition of mir-34a expression reduces high fat diet induced adiposity, associated with improved oxidative stress in mice²⁵³.

3. Release from adipose tissue

Besides being present and identified in tissues, miRNAs are also stable in the circulations with a large fraction of them found in exosomes²⁵⁴ (50-200 nm vesicles that are released from multivesicular bodies)²⁵⁵. Since adipocytes are now well appreciated as a type of cell with endocrine functions and could secrete hundreds of bio-molecules, it is tempting to investigate whether adipocytes are able to secrete miRNAs and these secreted miRNAs can crosstalk with other cells or organs. In this context, it has been clearly demonstrated that adipose tissue is able to release extracellular vesicles (EVs). Indeed, adipose tissue derived extracellular vesicles (ADEV) include exosomes that are released by multivesicular bodies fusing with the cell membrane and microvesicles (100-1000 nm vesicles originated by shedding of the plasma membrane)²⁵⁶⁻²⁵⁸. Surprisingly, adipose tissue-specific knockout of the miRNA-processing enzyme dicer (ADicer KO), results in a substantial decrease in most of the circulating exosomal miRNAs, which could be reversed by transplantation of adipose tissues from wild type mice, indicating that adipose tissue might even be a major source of systemic exosomal miRNAs⁹³.

- Adipocytes-derived miRNAs as biomarkers

Exosomal features of miRNAs could also be proposed as novel biomarkers. Treatment with cAMP substantially results in an approximately 5-fold increase in exosomes release from human brown adipocytes, containing more mir-92a insides, indicating that in response to different stimuli, fat could sort distinct individual or spectrum of miRNAs into exosomes for secretion²⁵⁹. Furthermore, with the study from a large cohort of humans, it has been demonstrated that exosomal mir-92a inversely correlates with human BAT activity measured by 18F-FDG PET/CT, suggesting that mir-92a represents a potential serum biomarker for BAT activity²⁵⁹.

- Adipocytes-derived miRNAs are functional

Then, aside from their presences in exosomes, are these miRNAs functional? Current literature supports this hypothesis.

Under physiological state, adipose tissue extracts are shown to be able to promote adipogenesis, suggesting that adipose tissue could produce bioactive factors to regulate adipogenesis via paracrine signals^{260,261}. *In vitro* studies have found that adipose tissue-derived exosomes-like vesicles could be internalized into adipose tissue-derived stem cells (ADSCs), and promote the adipogenesis of the cells *via* the pro-adipogenic miR-450a-5p which inhibits WSIP2 (WUS-interacting protein 2) expression²⁶².

In an endocrine view, FGF21 (fibroblast growth factor-21), produced by liver and other tissues, is elevated in blood and liver in ADicer KO mice, but could be reduced by injection of wild type exosomes. Moreover, *in vitro*, exosomes isolated from ADicer KO exhibit a less inhibitory effect on FGF21-3'UTR luciferase activity compared to those isolated from wild-type mice⁹³. These data indicate that adipocytes-derived miRNAs regulate FGF21 expression in liver. Pathologically, in response to high fat diet, adipocyte-derived mir-155 can be uptaken by macropahges, leading to M1 macrophage polarization²⁶³. Another study also elegantly demonstrates that miRNAs can mediate crosstalk between adipocytes and peripheral cells. One of the side effects of rosiglitazone (RSG) treatment is cardiac hypertrophy^{264,265}, which is also a same case in mice lacking PPAR γ specifically in cardiomyocytes^{264,265}. This phenomenon indicates that PPAR γ activation in noncardiac

tissues might contribute to the cardiac hypertrophy. Interestingly, Xi Feng et al reported that PPAR γ activation in adipocytes by RSG increases the content of mir-200a in adipocyte-derived exosomes, which then causes mTOR activation and hypertrophy in cardiomyocytes²⁶⁶. Therefore, adipocyte-derived mir-200a might mediate the hypertrophic effects of RSG in heart.

Although it has been reported that exosomes might be secreted in a ceramide-dependent manner²⁶⁷, the exact mechanisms underlying the regulation of exosomes release and miRNAs sorting into exosomes remain largely unknown and worthy of further investigation.

PART III: MiRNAs in aging

1. Dysregulations of miRNAs in aging

Molecular changes during the aging process are crucial to better understand the mechanisms of age-related diseases, such as cancer, cardiovascular, neurodegenerative and immunological diseases as well as diabetes, etc. Specific molecules such as telomerase, p16^{INK4A}, mTOR, NF-κB1 and SIRT1 have been found to be involved in the aging process, and have been used as predictors of longevity²⁶⁸⁻²⁷¹. Recently, mounting evidences have suggested that miRNAs are also crucial determinants of the longevity of the organisms. For instance, the preservation of biogenesis of miRNAs is shown to be vital for the life-extending effects of metformin. By activation of AMPK pathway, metformin alters the subcellular localization of the RBP AU-rich element-binding factor 1 (AUF1), disrupting its interaction with Dicer1 and protects it from being degraded by AUF1²⁷². Therefore, study of miRNAs' involvement in aging, might provide new insights and pharmaceutical targets for healthy aging.

Currently, plenty of miRNAs have identified to be involved in aging as well as age-related diseases.

1.1. MiRNAs involved in overall longevity

Evidence from *C. elegans* revealed that miRNA biogenesis genes are highly networked with transcription factors and aging-associated miRNAs. For instance, mir-71 is upregulated during aging, repressing ALG-1/AGO expression post-transcriptionally. Reversely, mir-71 loss of function leads to increased ALG-1 expression followed by a widespread mRNA expression dysregulations. Thus, the progressive molecular decline often thought to be the result of accumulated damage over an organism's lifespan, may be partially explained by a miRNA-directed mechanism of age-associated decline²⁷³. Additionally, Christopher et al investigated the liver transcriptome changes in mice model of dietary plus lifestyle interventions that modify aging-related phenotypes. By high-throughput deep sequencing, they found a significant larger proportion to have decreased expressions of miRNAs with longer lifespan. Furthermore, they experimentally validated mir-34a, mir-107 and mir-212-

3p targeted the chromatin remodeler CHD1, knockdown of which mimic high-fat diet and aging induced gene expression profiles²⁷⁴.

Currently, considerable attention has been devoted to age-associated changes in individual miRNA, which later is demonstrated to be critical in the regulation of mammalian lifespan. For example, depletion of mir-150 increased the survival rate from 7.7% to 28.1% in 30-month-old male mice. The suppression of renal insulin/IGF/AKT activity and upregulation of hepatic ACC activity might partially be responsible for the improved lifespan of mir-150 deficiency²⁷⁵. Conversely, over-expression of lin-4 increased the lifespan of *C.elegans*, with the insulin-like signaling pathway proposed to be the underlying targets²⁷⁶. Similarly, in drosophila, loss of mir-14 resulted in a reduced lifespan and semi-lethality, possibly owing to its effects of increasing the levels of TG and diacylglycerol as well as to the increase of its possible targets of apoptotic effectors²⁷⁷.

These results suggest that miRNAs might have strong impacts on various age-related disorders.

1.2. MiRNAs in age-related diseases

1.2.1. Age-associated cardiovascular diseases

Advancing age is an unmodifiable risk factor for the development of hypertension and cardiovascular diseases. MiRNAs associated with cardiovascular diseases during aging are briefly summed in Table 3.

Mice lacking mineralocorticoid receptors (MR) in smooth muscle cells (SMC-MR-KO) are protected from rising blood pressure during aging. In this context, mir-155 was identified as the most down-regulated miRNA with vascular aging in MR-intact but not in SMC-MR-KO mice. The age-associated decrease in mir-155 in mesenteric vessels was associated with increased mRNA abundance of MR and of predicted mir-155 targets such as CAV1.2 (L-type calcium channel subunit) and angiotensin type-1 receptor²⁷⁸. In HEK293 cells, MR repressed mir-155 promoter activity. Restoration of miR-155 specifically in SMCs of aged MR-intact mice decreased CAV1.2 and AGTR1 and attenuated LTCC-mediated and angiotensin II-induced vasoconstriction and oxidative stress²⁷⁸. Moreover, mir-155 levels in serum predicted the blood pressure treatment response in a clinical trial of MR blockade in elderly

humans²⁷⁸. Thus, the regulation of mir-155 by SMC-MR contributes to vasoconstriction during aging²⁷⁸.

On one hand, endothelial progenitor cells (EPCs) contribute to the regeneration of endothelium. Aging-associated senescence results in reduced number and function of EPCs, possibly contributing to increased cardiac risk and impaired cardiac repair effectiveness. Increased levels of mir-10a* and mir-21 were demonstrated to be responsible for the age-associated senescence in old EPCs, by suppressing HMGA2 (high mobility group AT-hook 2). In contrast, suppression of these two miRNAs in aged EPCs, rejuvenated EPCs and increased self-renewal potential and decreased senescence markers by rescuing the level of HMGA2²⁷⁹.

In the short-lived turquoise killifish *Nothobranchius furzeri* (Nfu), mir-29 family robustly increased in the heart with age. Mir-29 knockout in zebrafish with mir-29 sponges induced a significant cardiac spherization and hypertrophy, associated with increased deposition of collagen and global 5-methyl cytosine (5mC) levels. Moreover, in the context of hypoxia of human cardiac fibroblasts, mir-29 down modulation accounted for the accumulation of collagens and 5mC. These results indicated that mir-29 family upregulation might represent an endogenous mechanism aimed at ameliorating the age-dependent cardiac damage leading to hypertrophy and fibrosis²⁸⁰.

Table 3 : MiRNAs involved in age-associated cardiovascular diseases

miRNAs	Organ	Direction	Targets	Function	Reference
Mir-29 family (-29a,-29b,-29c)	Aortas/ WTmice	up	ECM components: Collagens/ elastin	Aortic aneurysm formation	²⁸¹
Mir-217	HUVECs	up	Sirt1/FoxO1	Pro-senescence	²⁸²
Mir-34a	Heart/ mice/ ku80-/- mice	wt up	PNUTS(PPP1R10)	Induction of cardiomyocyte apoptosis, telomere attrition and DDR.	²⁸³
Mir-22	Cardiac fibroblast/ C57/B16N	up	mimcan	Induction of senescence and chemotaxis	²⁸⁴
Mir-17-3p	Mouse cardiac fibroblasts	down	PAWR/CEBP and FAK signaling	Anti-senescence and apoptosis	²⁸⁵
Mir-125a-5p	Endothelial cells/ WT mice	up	Transcriptional enhancer factor-1/ eNOS/VEGF	Angiogenesis defects	²⁸⁶
Mir-146a	Endothelial cells/in vitro human	down	Possible targets: NOX4	unvalidated	²⁸⁷
Mir-217	Human HUVEC in vitro	up	Sirt1	Pro-senescence	²⁸²

1.2.2. Age-associated sarcopenia and osteoporosis

▪ Sarcopenia

Skeletal muscle is degenerative in mass and function by aging, which is termed sarcopenia. Study with muscle biopsy from man demonstrated that aging increased the expressions of let-7b and let-7e families associated with decreased their potential targets such as CDK6, CDC25A and CDC34 which are critical for cellular proliferation²⁸⁸. In addition, next-generation sequencing (NGS) technology permits the precise identification of mature and novel miRNAs. Sequencing of microRNAs and mRNAs from mouse gastrocnemius muscles from 6- and 24-month-old mice revealed sixteen novel deregulated microRNAs such as mir-206, mir-434, and mir-136, most of whose mRNAs targets were correspondingly modulated²⁸⁹. The decreased myogenic capacity is a major underlying cause of sarcopenia with age. However, the mechanisms remain less well understood. Mir-431 was found increased in old myoblasts. Ectopic injection of mir-431 greatly improved muscle regeneration through targeting SMAD4 levels and therefore transforming growth factor β (TGF β) signaling, indicating that age-associated mir-431 plays a key role in maintaining the myogenic capacity of skeletal muscle with age²⁹⁰.

▪ Osteoporosis

Age-related osteoporosis is characterized by reduced bone formation and increased marrow fat accumulation within marrow. The reduction in osteoblast activity is partly caused by the fact that bone marrow stromal cells (BMSCs) from elderly subjects have reduced capacity to differentiate into osteoblasts and increased capacity to differentiate into adipocytes. Mir-188 was found markedly higher in BMSCs from aged compared with young mice and humans. Intra-bone marrow injection of antagomiR-188 increased bone formation and decreased bone marrow fat accumulation in aged mice through targeting histone deacetylase9 (HDAC9) and RPTOR-independent companion of mTOR complex 2 (RICTOR), indicating that mir-188 is a potential therapeutic target for age-related bone loss²⁹¹. By contrast, aging specifically increased the mir-183 cluster in EVs inside bone marrow, and possibly drove senescence in BMSCs by endocytosis of EVs into BMSCs²⁹².

1.2.3. Age-related liver dysfunctions

Gradual increases in the levels of mir-669c and mir-709 were observed in aged liver in mice, with their potential targets involving in detoxification activity and regeneration capacity function declined in old liver²⁹³. The Ames dwarf mouse is one of the long-lived mammalian model with a point mutation in the PROP1 gene, which can live up to 70% longer than wild-type counterparts. In the liver of Ames dwarf mouse, mir-27a was markedly up-regulated compared to wild type counterparts, which was associated with a reciprocal down-regulation of its putative targets involved in polyamine biosynthesis (ODC1) and spermidine synthase (SRM). These data indicate that mir-27a was a key post-transcriptional regulator of intermediate metabolism in liver which might contribute to the long-lived phenotype in the Ames dwarf mouse²⁹⁴.

NGS analysis of miRNAs in the whole blood of mice of different ages helped to reveal several age-altered ones, amongst which, six were predicted to be involved in non-fatty liver disease: mir-16-5p, mir-17-5p, mir-21a-5p, mir-30c-5p, mir-103-3p and mir-130a-3p. Moreover, transfection of the six miRNAs into 18-month-old mice actually reduced the accumulation of age-related genes in liver, including mir-11a, p16 and mTOR, providing preliminary data that reversing age-associated miRNAs could be applied for treatments of age phenotype in the future²⁹⁵.

1.2.4. Age-related immune defects

Hematopoietic stem cells (HSC) homeostasis is disrupted under physiological stresses such as aging and periodic bacterial encounters, associated with declines in HSC number and quality. Chronic inflammation is demonstrated to be the major cause of the age-related decline in HSC functions. The absence of mir-146a triggered a chronic inflammatory state through pathways involving TRAF6/NF-KB/IL-6, which chronically could lead to severe pathologies, such as HSC exhaustion, bone marrow failure or myeloproliferative disease. These data indicate that the single mir-146a is crucial for guarding the quality and longevity of HSC in mice²⁹⁶.

Macrophages are critical effector cells of the innate immune system. Aging has been demonstrated to drive a functional drift in macrophages in mice, including epigenomic

alterations, impaired cholesterol metabolism and inability to inhibit pathological angiogenesis, which is proposed to contribute to the pathogenesis of various aging disorders, such as age-related macular degeneration (AMD). The group of Rajendra S. Apte has revealed that the initiation of mir-150 by aging in macrophages of diverse origins, such as spleen, bone marrow and peritoneal localization, induced a reprogramming of the macrophage transcriptome, with altered phospholipid and ceramide profiles. Moreover, mir-150 targeted stearoyl-CoA desaturase-2 (SCD2), dysregulating lipid metabolism and promoting pathologic angiogenesis. Finally, the upregulation of mir-150 in human peripheral blood mononuclear cells was associated with AMD²⁹⁷.

2. MiRNAs to senescence

2.1. Cellular senescence

How our cells are affected by aging? The answer might be that as our body turns aged, our cells turn senescent (Figure 11). Cellular senescence was formally described more than 40 years ago as a limited proliferation ability of normal human cells in culture²⁹⁸. The irreversibility of the senescence arrest is tightly associated with the senescent cell's special chromatin architecture, the so-called senescence-associated heterochromatin foci (SAHF). It constitutes a hallmark of senescent cells that contain several common markers of transcriptionally repressed heterochromatin and are hypothesized to silence genes important for cell proliferation, in particular those regulated by the E2F/RB1 repressor complex²⁹⁹. Despite an irreversible cell cycle, senescent cells actively secrete a spectrum of proinflammatory cytokines, chemokines, matrix metalloproteinases and growth factors, which is termed the senescence-associated secretory phenotype (SASP)³⁰⁰. SASP might be one of the manners that senescence employs to promote aging.

Nowadays, it has been demonstrated that senescent cells accumulate in various tissues and organs overtime demonstrated by accumulations of senescence markers such as senescence-associated β -galactosidase (SA- β -gal), p53 and cyclin-dependent kinase inhibitors p21 and p16. Senescence contributes to aging process. For example, Darren J.B. et al has shown that genetic or pharmacological removal of p^{16Ink4a}-positive cells (senescent cells)

would induce extended lifespan associated with reduced pathological alterations in various organs such as adipose tissue, heart and kidney³⁰¹.

It has been demonstrated that various stimuli could induce cellular senescence. On the one hand, the telomeric DNA loss during each S phase would result in telomeres erosion, which then generates a persistent DNA damage response (DDR). DDR then could further reinforce the senescence-related growth arrest³⁰². On the other hand, senescence could also occur without DDR development under various culture stressors such as serum deprivation or oxidative stress, etc³⁰³.

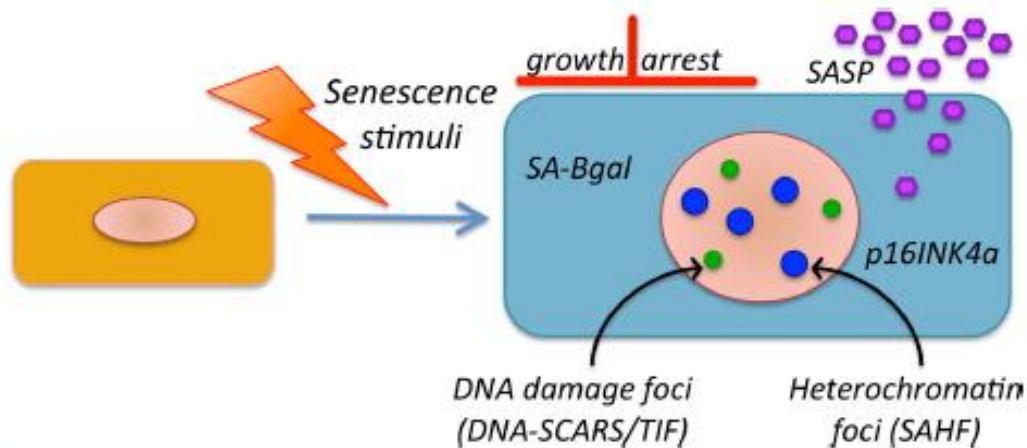


Figure 11: Hallmarks of senescent cells.

Hallmarks of senescent cells include an essentially irreversible growth arrest; expression of SA-Bgal and p16; robust secretion of SASP; nuclear foci containing DDR proteins (DNA-SCARS/TIF) or heterochromatin (SAHF). Adapted from³⁰⁴.

Interestingly, miRNAs have emerged as key regulators in the cellular senescence associated gene programs. Disruption of global miRNAs biogenesis by silencing of DGCR8, Dicer or Drosha could lead to a senescence-like state in human and mouse primary fibroblasts demonstrated by flattened morphology, increased SA- β -gal activity or formation of SAHF^{305,306}. So, how miRNAs could affect senescence? In the following part, we are going to focus on the effects of miRNAs on the two major pathways involved in senescence.

2.2. miRNAs in p53/RB tumor-suppressor pathways

Proliferative arrest, one of hallmarks of senescent cells, is induced by both endogenous and external influences that ultimately converge on either or both of the p16^{INK4a}/RB and p14^{ARF}/p53 pathways. The binding of p16 to the cyclin-dependent kinase 4-6/ cyclin D complex inhibits the phosphorylation of pRb family proteins and causes a G1 cell cycle arrest (Figure 12).

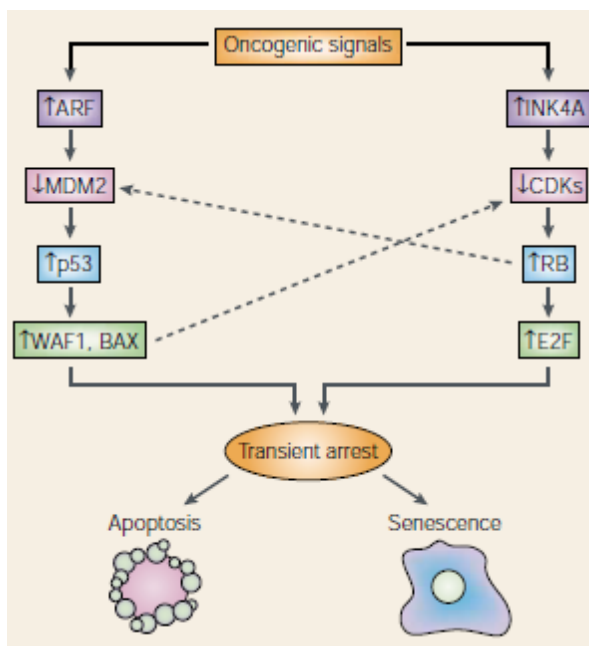


Figure 12 : The p53 and RB tumor-suppressor pathways.

In the p53 pathway, signals including the ARF (also known as p14 in human and p19 in mice), production of the *CDKN2A* locus, increase p53 levels by sequestering MDM2, which facilitates the degradation and inactivation of p53.

In the RB pathway, stress signals include INK4A, the product of the *CDKN2A* locus. INK4A inhibits CDKs that phosphorylate, and therefore inactivate, RB during the G1 phase of

▪ miRNAs upstream of p53/ RB pathways

In senescence, let-7/agronaute2 (AGO2) signaling induced implementation of silent-state chromatin modification at target promoters. Inhibition of the let-7/AGO2 effector

complex perturbed the timely execution of senescence³⁰⁷, suggesting that some miRNAs could potentially affect senescence *via* modifying the promoters of p16/p53 and thereby their expressions. Similarly, in human mammary epithelial cells, senescence-associated miRNAs (SA-miRs) such as mir-26b, mir-181a function in concert to repress expressions of polycomb group proteins, embryonic ectoderm development or enhancer of zeste homologue 2, thereby activating expression of p16 and senescence³⁰⁸. Likewise, amongst the human cancer-associated mir-17-92 cluster, mir-17/mir-20a seed family are necessary and sufficient for conferring resistance to oncogene-induced senescence, by directly targeting p21^{WAF1309}.

Senescence in the type II alveolar epithelial cells (AECs) contributes to the development of idiopathic pulmonary fibrosis (IPE). The upregulation of SA-miRs including mir-34 family and mir-20a, mir-29c and let-7f might participate in the induction of senescence in AECs, with its ectopic overexpression inducing senescence markers: p16, p53 and SA- β -gal³¹⁰. In addition, induction of mir-29 and mir-30 is necessary for the RB pathway driven-senescence through targeting and inhibition of the 3'UTR of B-Myb³¹¹, a transcription factor that negatively regulates senescence³¹². Moreover, mir-29 overexpression induced by Wnt-3a in muscle progenitor cells (MPC) during aging, induces senescence *via* targeting the 3' UTR of p85 α , IGF and B-Myb, suppressing the translation of these mediators of myoblast proliferation. Finally, electroporation of mir-29 into muscles of young mice recapitulates aging-induced responses³¹³.

Amongst these SA-miRs in human keratinocytes (NHKs), ectopic overexpression of mir-137 or mir-668 induced senescence in rapidly proliferating NHKs, demonstrated with increased SA- β gal activity and expression of p16 and p53³¹⁴.

P16 could improve and stabilize the levels of p53 through negatively regulated p53 suppressor MDM2 oncoprotein, which was mainly mediated by mir-141 and mir-146-5p dependent negative regulation of MDM2³¹⁵.

- miRNAs downstream of p53/RB pathways

On the other hand, some miRNAs are identified as down stream effectors of senescence marker genes. Recent evidence indicated that p16 is a modulator of gene expression. It has been demonstrated that p16 could contribute to senescence through modulating miRNAs expression. For instance, through the formation of the p16-CDK4-Sp1 heterocomplex which binds to the Sp1 consensus-binding motifs present in the promoters of mir-141 and mir-146b-5p, p16 enables the transcription of mir-141 and mir-146b-5p, which mediate p16's response to ultraviolet (UV) induced DNA damage³¹⁶.

2.3. miRNAs in DNA damage pathways

The most significant phenotype associated with mammalian cellular aging is telomere shortening which induces disruption of chromosome integrity by DNA damage and apoptosis³¹⁷. Telomerase lengthens telomeres in DNA strands by adding DNA sequence repeats (TTAGGG in all vertebrates) to the 3' end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes, thereby preventing senescent cells from postmitotic condition and apoptosis³¹⁸. However, aging is linked with reduced telomerase activity which reduces the regenerative capacity of proliferative organs and increases incidences of diseases onset and development³¹⁹. Interestingly, mir-195 was overexpressed in old mesenchymal stem cells (OMSCs) in mice, which targeted telomerase reverse transcriptase. Abrogation of mir-195 in OMSCs resulted in increased length of telomere in the nucleus, markedly improving their proliferative abilities. Moreover, transplantation of mir-195-knockout OMSCs reduced infarction size and improved heart function in mice³²⁰.

MiRNAs are also involved in the regulation of cellular reactive oxygen species (ROS) level which is major contributor to DDR. Surprisingly, in human HEK293 cells, induction of DDR by exposure to H₂O₂ resulted in phosphorylation of dicer (the miRNA producing enzyme) and subsequent nuclear recruitment of dicer to promote DNA repairment mediated by repair factors MDC1 and 53BP1³²¹, indicating that some miRNAs might be involved in this process.

Specifically, mir-31, a novel target of histone deacetylase inhibitor (HDACi) in breast cancer cells, induces cellular senescence *via* repression of the polycomb group (PcG) protein BMI1. Mir-31 overexpression leads to γ H2AX foci formation largely due to induction of mitochondrial ROS³²². Although in kidney the molecular basis for aging is not well understood, it was reported that mir-335 and mir-34a were increased in aged rat kidney, corresponding with down regulation of their targets superoxide dismutase 2 (SOD2) and thioredoxin reductase 2 (Txnrd2), respectively, which are critical antioxidants located in mitochondria. *In vitro* experiments demonstrated that overexpression of these two miRNAs led to senescence of young mesangial cells via suppression of SOD2 and Txnrd2 associated with a concomitant increase in ROS. Conversely, inhibition of these two actors in aged mesangial cells inhibited senescence via upregulation of SOD2 and Txnrd2 with decreased ROS³²³.

3. MiRNAs in conserved aging

Various conserved pathways have been unveiled to be essential for aging process. For example, age-related oxidative stress is associated with functional decline of cellular repairing mechanisms and contributes to the onset of age-associated pathologies, such as cardiovascular diseases. The accumulation of ROS could induce expression of TGF- β , activating fibroblasts and collagen deposition, which is the initial step of fibrotic diseases^{324,325}. In drosophila, Jun-N-terminal Kinase (JNK) pathway confers tolerance to oxidative stress and extends life span, which is mediated by the activation and nuclear translocation of FOXO, repressing insulin/IGF-signaling pathway³²⁶. In drosophila melanogaster, a heteroallelic, hypomorphic genotype of mutant *InR*, which is homologous to mammalian insulin receptor, could yield up to 85% of extension in longevity in females and reduced late age-specific mortality in males³²⁷. Ribosomal S6 protein kinase 1 (S6K1) is a downstream component of the nutrient-responsive mTOR (mammalian target of rapamycin) signaling pathway. Deletion of S6K1 leads to lifespan extension and resistance to age-related pathologies such as bone, immune, motor dysfunction and loss of insulin sensitivity³²⁸.

Evidence indicates that age-related miRNAs are intimately associated with age-conserved pathways. For example, bioinformatic analysis through gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) suggest the target genes of deregulated miRNAs in aged left ventricular (mir-132, mir-222 and mir-182, etc.) are enriched in conserved pathways such as mTOR signaling pathway, FOXO signaling pathway and adipocytokine signaling pathway³²⁹. Therefore these data suggest that miRNAs might impact aging process through affecting conserved aging pathways.

Currently, caloric restriction (CR) is one of the most robust interventions shown to delay aging in diverse species. Pharmaceutically, the polyphenol resveratrol, an activator of sirtuins, has been proposed to promote healthy aging and extend lifespan³³⁰. Therefore, in the following part, we mainly focus on relations between miRNAs with CR as well as sirtuin families to understand the participation of miRNAs in aging.

3.1. miRNAs affecting caloric restriction process

Similar to caloric restriction (CR), intermittent fasting extends lifespan of *C. elegans* and also mammals. Interestingly, the expressions of miRNAs machinery genes, such as Drosha, Argonaute and GW 182, are increased by this process. Conversely, the beneficial effects of fasting would be totally abrogated with Drosha depletion. These data, therefore, imply that miRNAs abundance might be upregulated by CR and be positively associated with CR-linked longevity. Microarray data further propose DAF-16, an effector of insulin/IGF-signaling, might be a downstream target of cellular miRNAs abundance's pro-longevity action³³¹. So, what are the miRNAs involved in CR in diverse organisms?

Firstly in *C.elegans*, it has been reported that mir-80 is a major regulator of the CR process. Mir-80 depletion conferred healthy aging, including maintained cardiac-like and skeletal muscle-like function at advanced age, and reduced accumulation of lipofuscin and extended lifespan, coincident with induction of physiological features of CR. Mechanistically, the expression of mir-80 is positively associated with the food availability. The acetyltransferase transcription co-factor CBP-1, interacting transcription factors daf-16/FOXO and heat shock factor-1 are essential for mir-80 deficiency's benefits³³² (Figure 10). Other miRNAs, such as mir-71 and mir-228 are also critical for the response to dietary

restriction in *C. elegans*, which are essentially and positively regulated by pHA-4 (a FOXA homolog) and SKN-1 (a NRF-related transcription factor)³³³ (Figure 13).

Secondly, in rodents, it was also reported that the inhibition of miRNAs biogenesis by dicer depletion in adipocytes mitigated the metabolic benefit of CR, which was due to impaired insulin signaling caused by mTORc1 hyperactivation. In addition, there was a shifted metabolism from oxidative metabolism to amino-acid utilization accounted by altered mitochondrial morphology³³⁴. In liver, expression of mir-125a-5p was down regulated while its targets STAT3, CASP2 and STARD13, were increased with age. 30% of CR would significantly reverse the expression of mir-125a-5p and its three targets, suggesting that mir-125a-5p contributes to the CR-mediated delay of aging liver³³⁵ (Figure 13). In brain, the decline in cognitive robustness with aging could be delayed by CR via upregulation of anti-apoptotic regulatory proteins that may prevent neuronal death. Interestingly, CR decreased the age-linked expressions of mir-30e and mir-34a, as well as reversed the decreased ratio of BCL2 to BAX in the brain. In vitro 3' UTR reporter assay demonstrated that BCL2 is a direct target of mir-30e and mir-34a. Therefore, these data indicates that down regulation of mir-34a and mir-30e could mediate neuroprotective roles of CR in the brain through targeting apoptosis³³⁶ (Figure 10). Although levels of certain miRNAs *per se* might not be modulated substantially by aging, their alterations in response to CR could mediate its pro-survival effects. For instance, down-regulation of mir-34a, -34e,-181a-1* delayed brain aging *via* upregulation of BCL-2 pro-survival pathway under CR³³⁶.

Thirdly, in primates, regression analysis revealed correlations between body weight, adiposity, and insulin sensitivity for about 10 of the CR-regulated known plasma miRNAs in rhesus monkeys. Sequence alignment and target identification for these 10 miRNAs identified a role in signaling downstream of the insulin receptor. Among them, the highly abundant mir-125a-5p in plasma correlated positively with adiposity and negatively with insulin sensitivity and was negatively regulated by CR³³⁷ (Figure 13).

In sum, the knowledge of the category of “aging-related miRNAs” as well as the precise mechanisms underlying the role of miRNAs in aging is still at the preliminary stages.

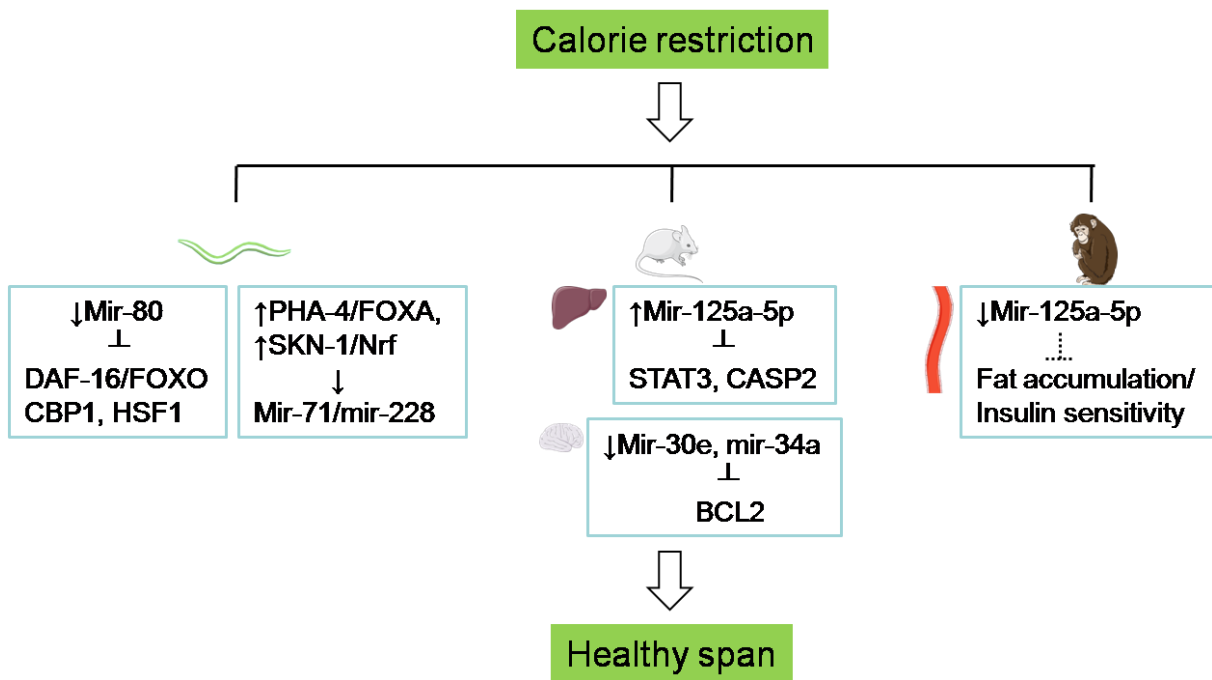


Figure 13: Simple illustrations of microRNAs' participations in calorie restriction process in different species.

3.2. miRNAs affecting sirtuin families

Mammalian sirtuins comprise a conserved protein family of seven members of NAD⁺-dependent deacetylases or, in case of SIRT4, ADP-ribosyltransferases. SIRT3, SIRT4 and SIRT5 are exclusively found in the mitochondrion. Increased expression of Sirtuins lowers the risk of age-related diseases. Various miRNAs have been demonstrated to affect aging via sirtuin family proteins.

▪ miRNAs with SIRT1

In peripheral blood mononuclear cells (PBMC), age-related decrease of SIRT1 was found negatively correlated with the expression of mir-34a and mir-9, with both of the two functionally interacted with the 3' UTR of SIRT1, indicating that mir-9/34a might impact the longevity through SIRT1³³⁸. The relation between mir-34a and SIRT1 was further confirmed in the case of kallistain, an endogenous protein protecting against vascular injury by inhibiting oxidative stress and inflammation in hypertensive rats and enhancing the mobility and function of endothelial progenitor cells (EPCs). *In vitro* data supported that kallistain could block the TNF α -induced senescence in EPCs via inhibiting the synthesis of mir-34a and mir-21, which negatively targeting SIRT1 and eNOS. Furthermore, mutation of mir-34 abrogated the life-extending effects of kallistain in wild-type *C.elegans* under oxidative or heat stress in a SIRT1-dependent manner³³⁹. Moreover, age-related hearing loss, also known as presbycusis, is thought to be the irreversible loss of cochlear hair cells in the inner ear. Upregulation of mir-34a was again demonstrated to contribute to the apoptosis of HEI-OCI cell and hair cell loss in mice through targeting SIRT1 which resulted in an increase in p53 acetylation, suggesting the mir-34a/SIRT1/p53 signaling might serve as a potential target for age-related hearing loss treatment³⁴⁰ (Figure 14A).

▪ miRNAs with SIRT4

Conversely, Sirt4 level is upregulated in senescence, inversely correlated with mir-15b in photoaged human skin. Inhibition of mir-15b promotes mitochondrial ROS generation and reduces mitochondrial membrane potential as well as impacts the expression of components of SASP in a SIRT4-dependent manner. These data indicate the mir-15b/SIRT4

axis mediated the stress-induced senescence associated with mitochondrial dysfunction and SASP regulation³⁴¹ (Figure 14B).

- miRNAs with SIRT6.

SIRT6 specifically deacetylates lysine 9 on histone H3 (H3K9Ac) or lysine 56 on histone H3 (H3K56Ac). In addition, SIRT6 is able to form a macromolecular complex with the DNA double-strand break repair factor DNA-dependent protein kinase (DNA-PK) to promote DNA double-strand break repair³⁴². Physically, it interacts with poly (adenosine diphosphate-ribose) polymerase 1 (PARP1) and mono-ADP-ribosylates PARP1 on lysine residue 521, thereby stimulating PARP1 poly-ADP-ribosylase activity and enhancing double-strand break repair under oxidative stress³⁴³. SIRT6 knockout mouse cells exhibit genomic instability and hypersensitivity to DNA damage. Moreover, SIRT6^{-/-} mice have been shown to develop a severe degenerative phenotype similar to that of premature aging. On the contrary, overexpression of SIRT6 in mice could increase the life span of male mice³⁴⁴. SIRT6 level was reduced in human dermal fibroblasts derived from older subjects compared to that from young subjects, coincided with an augmentation in the level of mir-766. *In vitro* experiments show that mir-766 post-transcriptionally regulated the expression of SIRT6, which could negatively regulate mir-766 transcription *via* a feedback regulatory loop through modulating acetylation levels at its promoter (Figure 14C). These data indicate that miRNAs have implications in the post-transcriptional regulation of SIRT6, which is vital for longevity³⁴⁵.

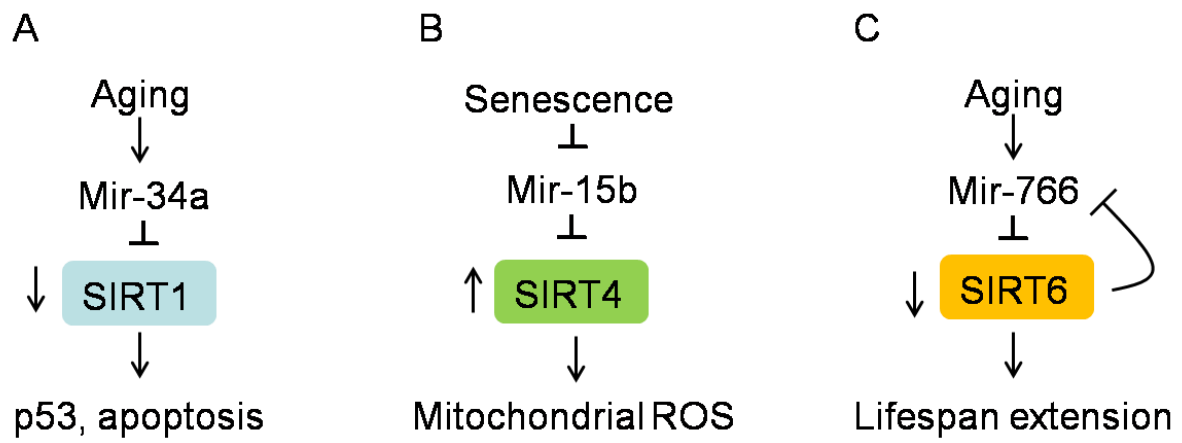


Figure 14: Examples of effects of microRNAs in sirtuins during aging or senescence.

(A) Aging-associated increase in mir-34a promotes p53 expression and apoptosis through inhibition of SIRT1. (B) Loss of mir-15b accounts for upregulation of mitochondrial ROS production by increased SIRT4 in cellular senescence. (C) Loop regulations of mir-766 and SIRT6 during aging.

PART IV: A novel method of culturing isolated adipocytes

1. Current *in vitro* models of adipocytes

The main functional unit inside adipose tissue are adipocytes, characterized by a unilocular lipid droplet and a peripherally located nucleus. Knowledge of adipocytes is fundamental to enhance our understanding of adipose tissue physiology/pathology. As early as in 1964, Rodbell M. firstly isolated mature adipocytes from adipose tissue with collagenase digestion³⁴⁶. However, turning to literature, it is surprising that currently there is no effective system to maintain mature adipocytes for *in vitro* culture, which greatly limits our understanding of the fat cells. Then, why adipocytes could not be cultured *in vitro* routinely? Because of engaging in lipid storage, adipocytes are highly buoyant and will float on top of cell culture media, which will then result in malnutrition and osmotic stress. So, currently what are the models for us to study adipocytes *in vitro*?

1.1. Two-dimensional (2D) adipogenesis of preadipocytes

Currently, *in vitro* culture of adipocytes is performed on generic 2D polystyrene. Cellular model systems including pluripotent fibroblasts such as 10T1/2, BALB/c-3T3 and RCJ3.1, as well as fibroblast-like preadipocytes that are committed to differentiate into adipocytes such as 3T3-L1, 3T3-F422A, 1246, and 30A5, are widely used to investigate adipogenesis and functions of adipocytes. Among them, 3T3-F442A and 3T3-L1 are the two most extensively characterized and used preadipocyte cell lines. Both of these cell lines are derived from disaggregated 17- to 19-day old Swiss 3T3 mouse embryos³⁴⁷. However, unlike mature adipocytes which contain a single lipid droplet, *in vitro* differentiated adipocytes are characterized by multiple lipid droplets and accompanied undifferentiated preadipocytes (Figure 13). Moreover, it has been reported that they could only produce 1-2 % of leptin of that of mature adipocytes³⁴⁸. Indeed, these cell lines are immortalized aneuploid cells whose genetic regulation may be distorted³⁴⁹.

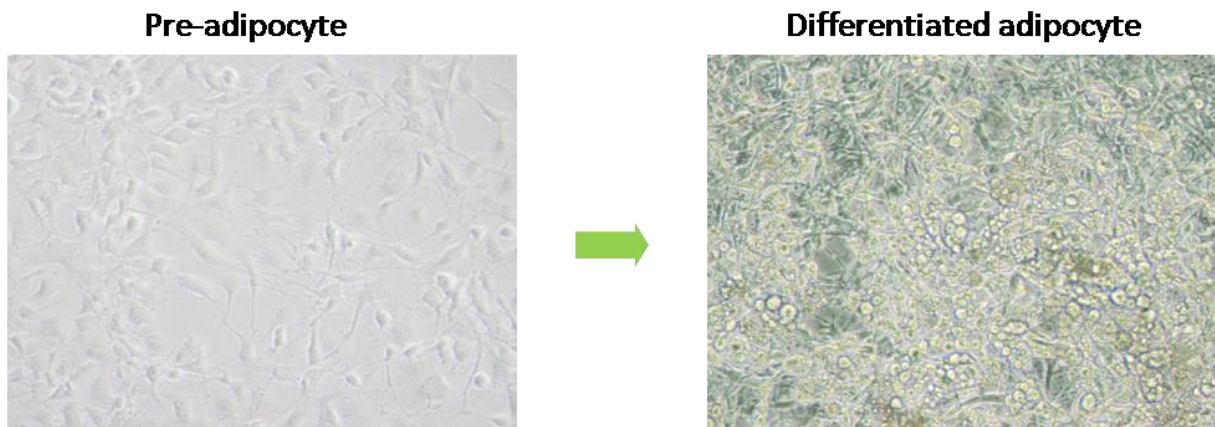


Figure 15 : 3T3-F442A adipocytes differentiated from 3T3-F442A fibroblasts.

Beside the use of preadipocytes from immortalized cell lines, primary culture of the isolated stromal vascular fractions (SVFs) from adipose tissue is also widely used to study adipocytes *in vitro* (Figure 14). Adipogenesis of SVFs is induced by hormone cocktails stimulation including insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, etc¹²⁴. Similarly, SVFs would differentiate into multiple lipid droplets.

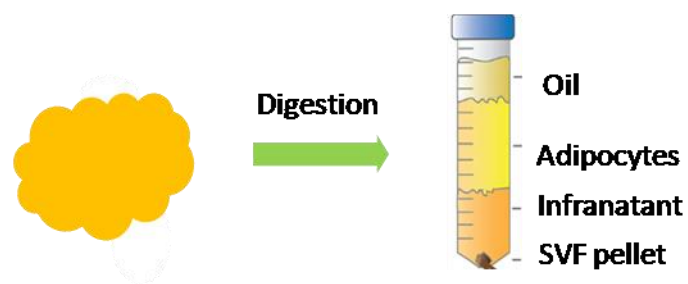


Figure 16: Isolation of SVF from adipose tissue with collagenase digestion and centrifugation for *in vitro* adipogenesis.

Finally, during differentiation and lipid accumulation, adipocyte buoyancy increases due to decreasing cell density and causes differentiated adipocytes to detach from planar culture surface and lyse.

1.2. Three-dimensional (3D) adipogenesis of preadipocytes

In parallel of the classical adipogenesis on 2D polystyrene, efforts have also been made to improve the physiological relevance of *in vitro* studies, and cell culture technologies are in development to mimic the *in vivo* accommodation of 3D adipocyte expansion. Several platforms have been developed to surmount the limitations of 2D culture on adipocyte morphology. For example, 3D structure of scaffolds for adipogenesis have been made from various materials such as particulate-leached polyglycolic acid³⁵⁰, silk fibroin³⁵¹, meshed microfibers³⁵², esterified hyaluronic acid sponges³⁵³, electrospun polycaprolactone³⁵⁴, poly L-lactic acid³⁵⁵, or freeze-dried mixtures of nanocellulose and alginate³⁵⁶. It has been reported that the 3D culture of mature adipocytes from the preadipocytes with scaffolds, allow the differentiated adipocytes acquired spheroid shapes and form multicellular aggregates. Moreover, compared to 2D culture, the sizes as well as the viability of mature adipocytes from 3D culture significantly are increased³⁵⁷ (Figure 15). Unfortunately, porous materials often exceed the acceptable scaffold rigidity and preadipocytes embedded directly in hydrogel may remain functionally impaired.

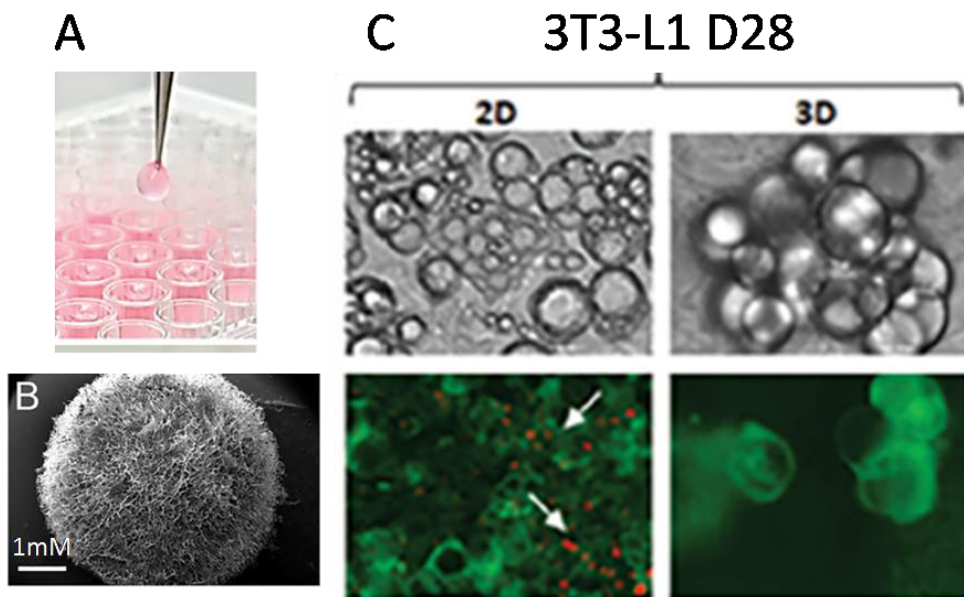


Figure 17: 3D adipogenesis of 3T3-L1 cells in electrospun polycaprolactone scaffolds.

(A) Once hydrated the scaffold can be easily handled with fine scissors. (B) SEM observation of the dehydrated scaffold. (C) Observations of growing cells and fluorescence images of the live/dead assay at day 28 (scale bar: 100 μ m). Data is from ³⁵⁷.

In another way, to mimic extracellular environment of adipocytes *in vivo*, which is composed of preadipocytes, adipocytes, interstitial cells and a microvascular system entwined with collagen-rich ECM, the conjugation of a genetically engineered elastin-like polypeptide (ELP) and a synthetic polymer, polyethyleneimine (PEI) (ELP-PEI) was coated on classical polystyrene plates, allowing the formation of spheroids of 3T3-L1 adipocyte aggregates with 72 hour after seeding³⁵⁸ (Figure 16). These spheroids are cellular aggregates allowing cell interactions through junctional complexes.

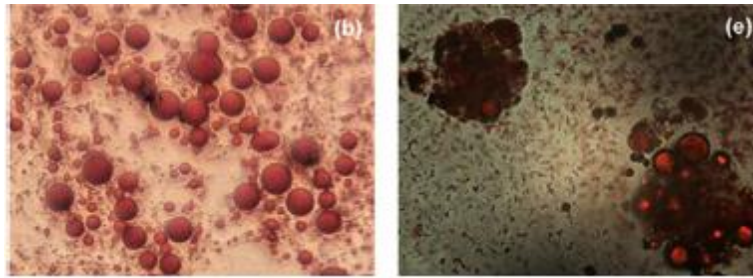


Figure 18: Formation of spheroids of 3T3-L1 adipocyte aggregates on surface tethered with ELP-PEI.

3T3-L1 cells were differentiated on surface tethered without (left) or with (right) ELP-PEI. Adopted from³⁵⁸.

2. First attempt to culture isolated adipocytes

Then, are there attempts having been made to culture isolated adipocytes directly? The answer is sure.

In 1986, Hajime Suihara et al firstly reported the so-called “ceiling culture” of fat cells. The authors took advantage of the buoyancy of these cells, allowing them to float and attach on the upper surface of a flask, which was previously fully-filled with medium and was air-free. After firm attachment, the flask was then placed upside down to allow regular observation. However, the author observed dedifferentiation of the cultured adipocyte on the 4th day, which would continue so that the previous unilocular cells were finally replaced by multilocular fat cells or fibroblast-like fat cells, which in turn proliferated extensively, indicating that cultured adipocyte gradually lose their identity³⁵⁹ (Figure 17).

Then, in 2000, HH Zhang et al tried to modify the “ceiling culture” by culturing adipocytes underneath a floating glass surface in wells of 6-well plate, so that their methods could be employed for fluorescence microscopy. However, there are still uncertainties since adipocytes would escape from the periphery of the glass slide, and it is quite unpractical because culture medium could not be changed within 10 to 14 days in order to ensure their attachment onto the covering glass. Similarly, the unilocular identity of fat cells could not be maintained and adipocytes were fragmented, which they mistakenly taken as budding of a daughter cells³⁶⁰.

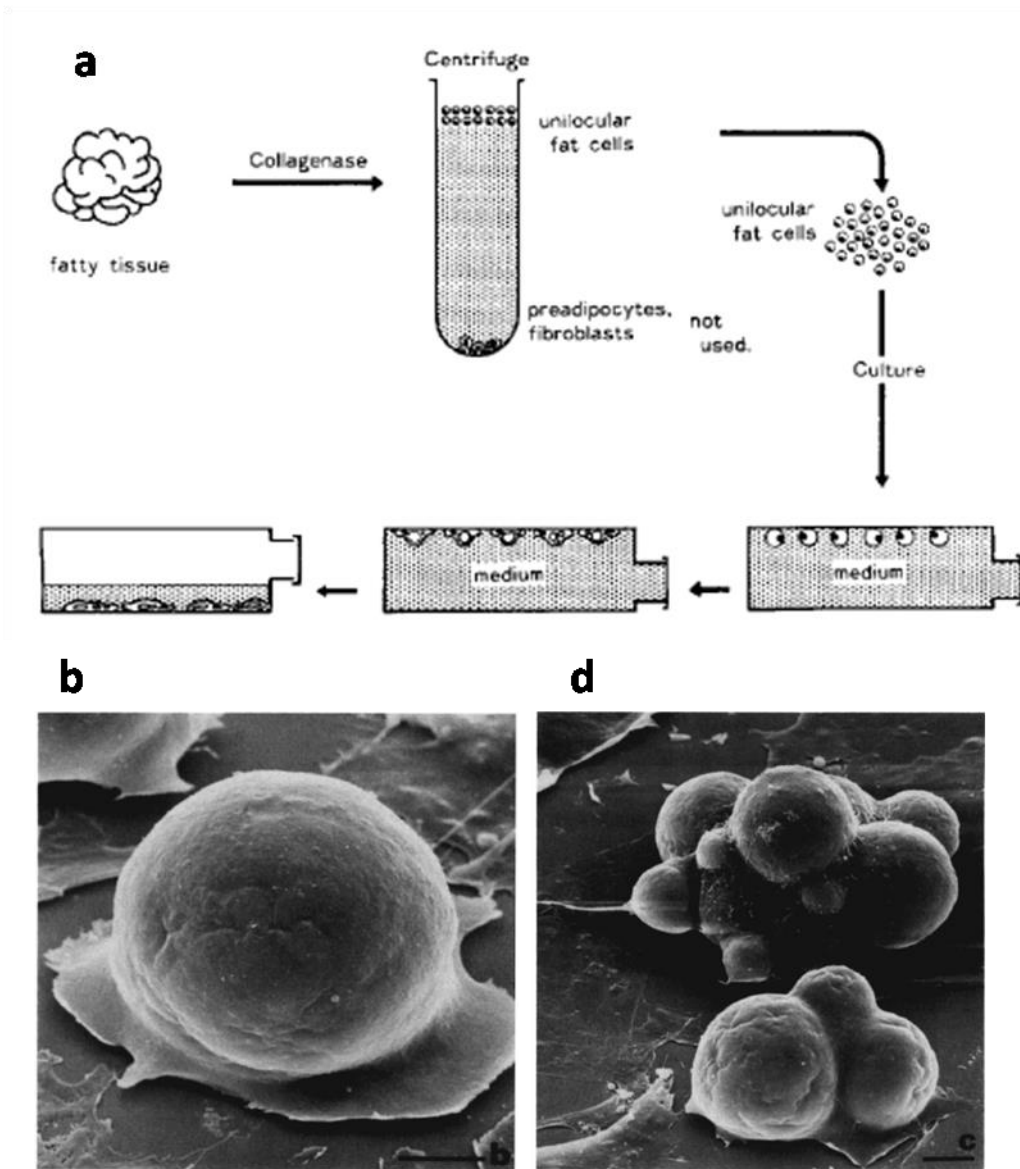


Figure 19: Ceiling culture of isolated adipocytes.

(a) Experimental scheme of ceiling culture. (b-d) SEM observations of adipocytes gradually change the unilocular LD into multiple LD, scale bar, 10µM. Adopted from³⁵⁹.

Besides 2D culture of isolated adipocytes, Hajime Sugihara et al, also tried to culture fat cells in 3D with collagen gel matrix, in which they embedded fat cells in 2mm in depth collagen bed to sequester the fat cell from floating. Unfortunately, the dedifferentiation process would still take place within one week of culture³⁶¹.

Due to the dedifferentiation of these cultured adipocytes, the method of “ceiling culture” or 3D culture with collagen could therefore not be prevailed. Then why attached adipocytes would undergo dramatic dedifferentiation? Unfortunately, there’s no clear answer to this phenomenon. However, it might be related to the factor that white adipocytes nowadays have been demonstrated with plenty of plasticity. Despite their committed status, mature adipocytes maintain a significant expression of genes involved in stem cell pathways. Very interestingly, it has been reported that cytoplasmic gap can form in adipocytes at the early stage of “ceiling culture” and large LDs would be released directly outside adipocytes, which is termed liposecretion³⁶².

EXPERIMENTAL RESULTS

Project I:

Role and regulation of microRNAs in adipose tissue during aging

Questions:

Although recent studies suggest that adipocyte dicer is decreased in aged mice and is involved in the determination of longevity in *C. elegans* and mice, the underlying mechanisms remain largely unknown³⁶³. Moreover, previous studies concerning the effects of adipocyte dicer are mostly obtained with aP2-dicer or adiponectin-dicer mice neglecting the influence of developmental defects in these models. In addition, more and more studies suggest that beside the classical drosha/dicer pathway, there are non-canonical pathways producing miRNAs *in vivo*, suggesting that the protein dicer *per se* might have a role in the control of longevity. In this way, the protein dicer *per se* in adipocytes might be proposed as a pharmaceutical target in the promotion of healthy aging. Based on these backgrounds, two aspects of age-associated dicer loss could be considered:

1) What are the consequences of adipocyte dicer deficiency in aging process?

2) Are there age-related microRNAs in adipocytes that might positively or negatively participate in age-related process?

Therefore, during my PhD, we develop different strategies to:

- I. confirm that adipocyte dicer (miRNAs) affects the physiology of adipose tissue or peripheral organs. To that aim, we used the inducible dicer knockout mice model in which the floxed dicer allele is specifically turn down with the adiponectin-Cre which is under the control of ERT2 receptors and could be activated by tamoxifen treatment.
- II. better understand the mechanism concerning dicer alterations in adipocytes.
- III. screen by microarray the potentially interesting age-related adipocyte miRNA and validate by qPCR.
- IV. assess the role of a selected age-related adipocyte miRNA in aging of adipose tissue.

Result 1: Role and regulation of microRNAs in adipose tissue during aging

A. Validation of the inducible adipocyte dicer knockdown mice model

The AdipoCreER^{T2}-Dicer^{Lox/lox} mice were obtained by crossing Adiponectin-CreER^{T2} mice and Dicer^{Lox/lox} mice. After breeding and backcrossing the strain over seven generations, we obtained a colony of AdipoCreER^{T2}-Dicer^{Lox/lox} mice. After genotyping, around six-month-old male mice bearing homozygotes of dicer flox alleles plus adiponectin-CreER^{T2} heterozygotes were selected and paired (**Figure 1A**). As this construction contains an ERT2 cassette rendering it inducible, mice were subjected to three times of tamoxifen (200mg/kg) gavage within approximately two months as already described³⁶⁴ (**Figure 1B**). Protein levels of dicer were demonstrated to be down regulated at the end of the protocol in Dicer^{Lox/lox} / adiponectin-CreER^{T2+} (thereafter termed iADicer KO) mice than that in Dicer^{Lox/lox} / adiponectin-CreER^{T2-} (thereafter termed control) mice (**Figure 1C** and **1D**). RT-qPCR results further demonstrated that dicer levels were significantly reduced by around 30% to 40% with tamoxifen treatment in adipocytes isolated from perigonadal and subcutaneous adipose tissue from the group of iADicer KO mice compared to that in control mice (**Figure 1E** and **1F**, respectively). Correspondingly, mir-143, one of the adipocytes enriched microRNAs, was significantly decreased by around 40 to 50% in perigonadal adipocytes in iADicer KO mice compared to that from control mice (**Figure 1G**).

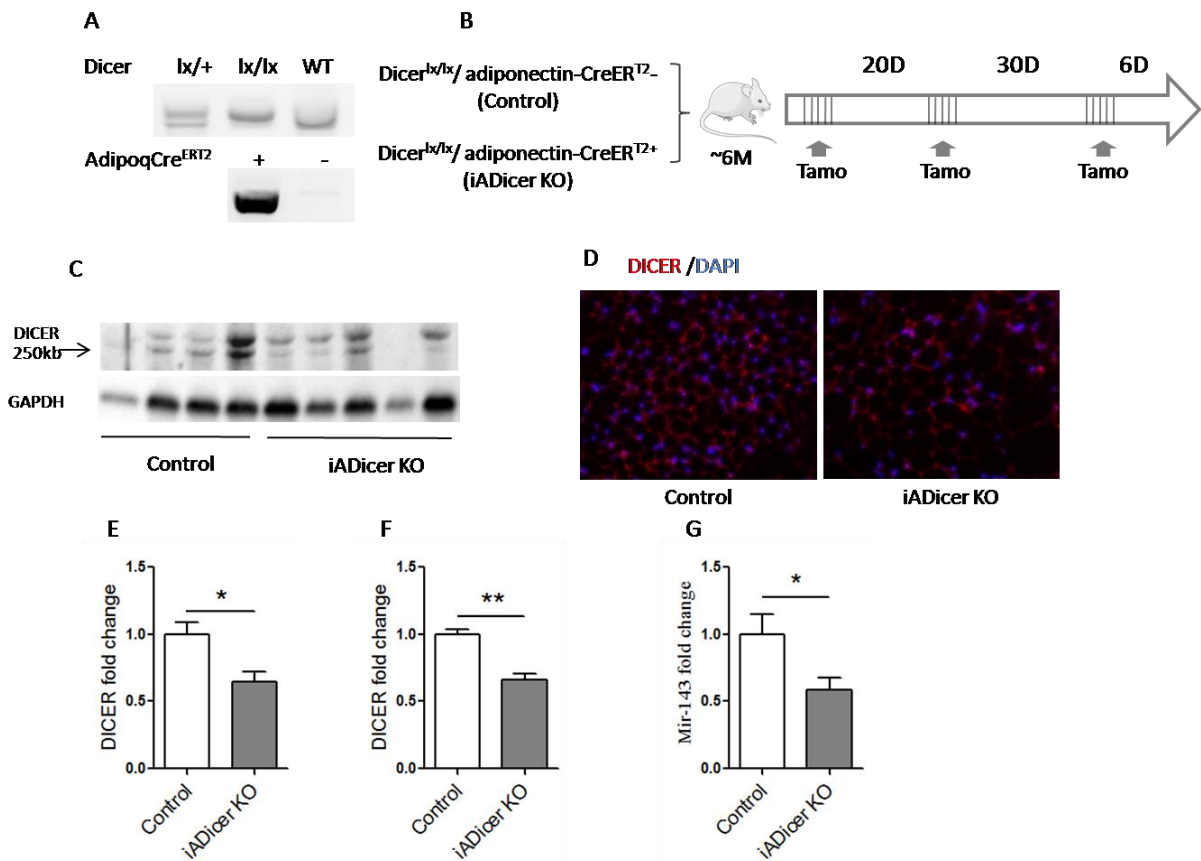


Figure 1: Inducible reduction of dicer in adipocytes by tamoxifen in $dicer^{Lox/Lox}/adiponectin-CreER^{T2+}$ mice.

(A) Genotyping results of $dicer^{Lox/Lox}/adiponectin-CreER^{T2}$ transgenic mice. (B) Experimental protocol: around six-month-old male mice ($dicer^{Lox/Lox}/adiponectin-CreER^{T2-}$ and $dicer^{Lox/Lox}/adiponectin-CreER^{T2+}$) were subjected to three times of orally given tamoxifen (200mg/kg) administration within 2 months. Each time of administration included 5 consecutive days of tamoxifen gavage. (C and D) Protein levels of dicer were successfully down regulated in perigonadal and subcutaneous adipose tissues at the end of the protocol. RNA levels of dicer were significantly reduced in isolated adipocytes from perigonadal (E) and subcutaneous (F) depots. (G) Mir-143 level was decreased in isolated adipocytes from perigonadal fat. Data represent the mean \pm SEM, * $P < 0.05$, ** $P < 0.01$. N=4-8.

B. Adipocyte dicer deficiency triggered a waste of perigonadal fat

To investigate the role of adipocyte dicer (miRNAs) in the physiology of adipose tissue *per se*, we characterized the body weight of the mice, the body fat proportion by EchoMRI as well as the sizes of adipocytes in subcutaneous and perigonadal adipose tissue by H&E staining at the end of the protocol.

Firstly, adipocyte dicer deficiency did not significantly alter the body weight of mice in this protocol (**Figure 2A**). However, adipocyte dicer deficiency significantly reduced the body fat proportion (**Figure 2B**). In agreement, the ratio of perigonadal fat to body weight was significantly reduced by over 50% in comparison to control group (**Figure 2C**), whereas the ratio of subcutaneous fat to body weight was unaltered (**Figure 2D**). Then, the result of H&E staining revealed that the average cell size of perigonadal adipocytes was reduced by near 50% by dicer deficiency, while deficiency of dicer had a trend to reduce the sizes of subcutaneous adipocytes (**Figure 2E, 2F and 2G**). Therefore, these data indicated that adipocyte dicer deficiency resulted in a loss of perigonadal fat mass accounted partly by reduced volumes of adipocytes.

Molecular studies were then performed to confirm these data as well as to investigate the underlying mechanisms. RT-qPCR results demonstrated that dicer deficiency led to dramatic reductions in *Leptin*, *Fsp27*, two genes known to be positively associated with adipocyte sizes (**Figure 2H**). Moreover, metabolic functional genes such as *Ppar γ _{1/2}*, *Glut4*, *Hsl*, *Atgl* and *Pepck* were also markedly decreased following dicer deficiency, indicating a possibility of attenuated adipocyte functions such as glucose transport and lipolysis (**Figure 2H and 2I**). The lipid droplet (LD) is stabilized by a surrounding monolayer of phospholipids with adherent proteins, in particular perilipin 1 (PLIN1), which could act dually to both suppress basal lipolysis and augment catecholamine stimulated lipolysis^{362,363}. The result of immunofluorescent staining exhibited that PLIN1 was markedly reduced with dicer loss in perigonadal adipocytes (**Figure 2G**).

Therefore, these results indicated that dicer abundance is indispensable for the maintenance of adipocytes identities in perigonadal adipose tissue. Therefore, in the following part, we mainly focused on the study of this adipose tissue.

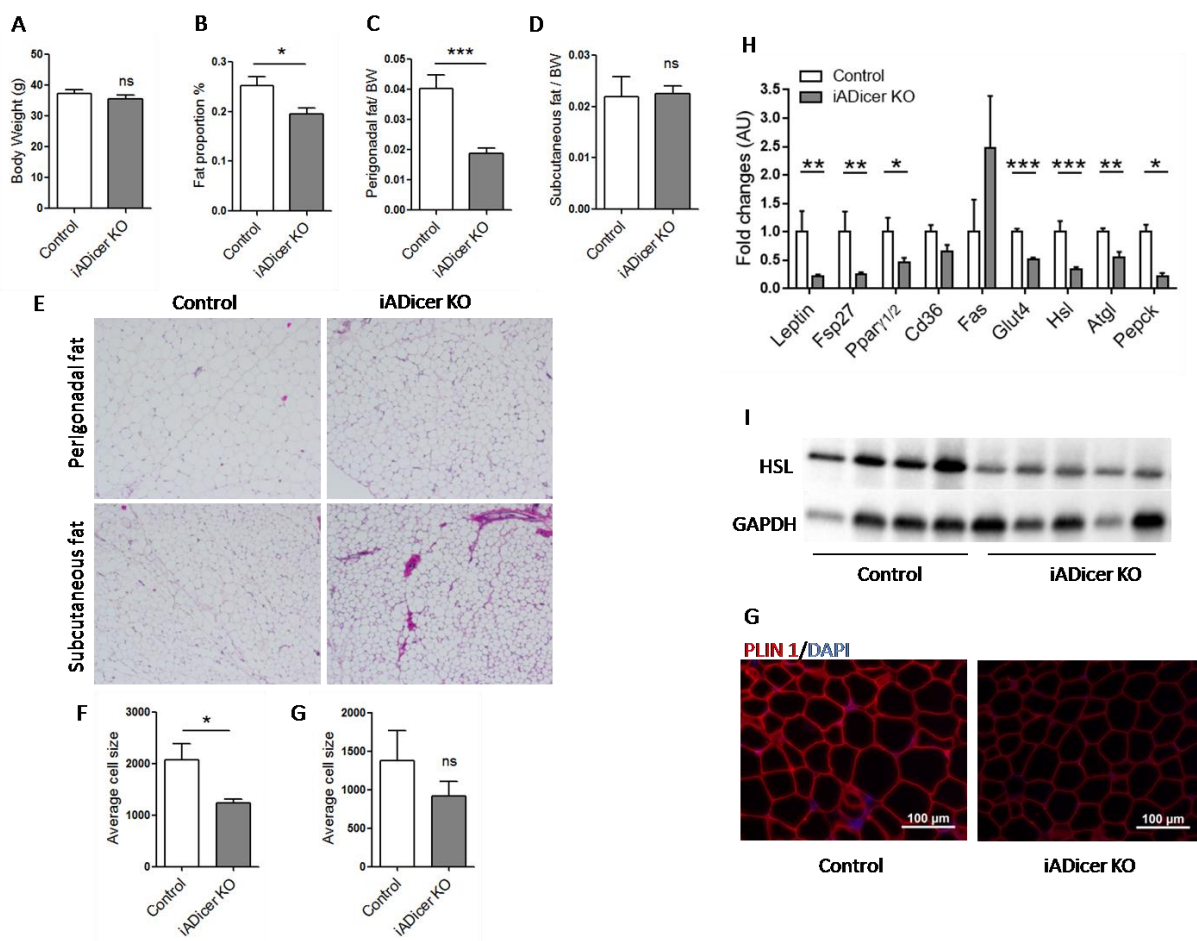


Figure 2: Reduction in the average sizes of perigonadal adipocytes due to dicer deficiency.

(A) Body weight between the two different genotypes at the end of the sacrifice. (B) Body fat proportion of the mice measured by EchoMRI at the end of the protocol. (C and D) Perigonadal and subcutaneous fat to body weight ratios. (E) H&E staining of the perigonadal and subcutaneous adipose tissue. (F and G) Average cell sizes of perigonadal and subcutaneous adipocytes. (H) Dramatic downregulations of white adipocyte gene markers associated with dicer decline in adipocytes by qPCR. (I) Reduced protein level of HSL in perigonadal fat revealed by immunoblotting. (J) Reduced protein level of PLIN1 in perigonadal adipocytes exhibited by IF staining. Data represent the mean \pm SEM, * $P < 0.05$, ** $P < 0.01$. $N = 4-8$.

C. Possible mechanisms underlying the effects of adipocyte dicer deficiency

As adipocyte size is mainly determined by its lipid contents, so we went on to investigate whether adipocyte lipid metabolism was affected by dicer deficiency, and we then turned our attention to mitochondria.

Surprisingly, PGC1 α , one of the transcriptional factors important in the transcription of genes involved in mitochondrial biogenesis and functions, was significantly upregulated in perigonadal adipocytes due to dicer deficiency at both the level of mRNA and protein (**Figure 3A** and **3B**). In addition, OXPHOS complex II/IV were significantly upregulated in perigonadal adipocytes due to dicer deficiency, and there was a strong trend for complex III to be increased ($P=0.606$) (**Figure 3C**). Although mitochondria recently have been suggested to be able to consume extra nutrients in subcutaneous adipose tissue³⁶⁵, it is also a major source of reactive oxidative species (ROS)³⁶⁶. Therefore, to better evaluate the state of mitochondria, antioxidant systems were checked. Interestingly, mRNA levels of antioxidants such as *Catalase* and *Sodium oxide dismutase (Sod)* were significantly reduced in perigonadal adipose tissue due to dicer deficiency (**Figure 3D**). Therefore, adipocyte dicer deficiency might induce a combination of mitochondrial activation and reduction in detoxification. According to literature, the transcriptional factor FOXO families are able to promote the transcription of antioxidant genes, like *Catalase* and *Sod*³⁶⁷, but also negatively regulate the transcription of genes important for mitochondrial functions³⁶⁸. The phosphorylation of FOXO families would lead to their relocation from nucleus to cytoplasm, thereby inhibiting their transcriptional activities³⁶⁹. Interestingly, the result of immunoblot showed that the phosphorylation of both FOXO1 and FOXO3 was increased in perigonadal adipose tissue due to dicer deficiency (**Figure 3F**), suggesting that adipocyte dicer deficiency might lead to inactivation of FOXO1 and FOXO3. Additionally, reduction in autophagy, one of downstream effector of FOXOs³⁷⁰, was also possibly inhibited by adipocyte dicer deficiency demonstrated with *Bnip3* reduction (**Figure 3G**), further in favor the possibility that adipocyte dicer deficiency reduced FOXOs activities. Therefore, with these preliminary data, we assumed that adipocyte dicer deficiency resulted in a combination of mitochondria activation and detoxification reduction possibly mediated by FOXO1 and FOXO3a inactivation.

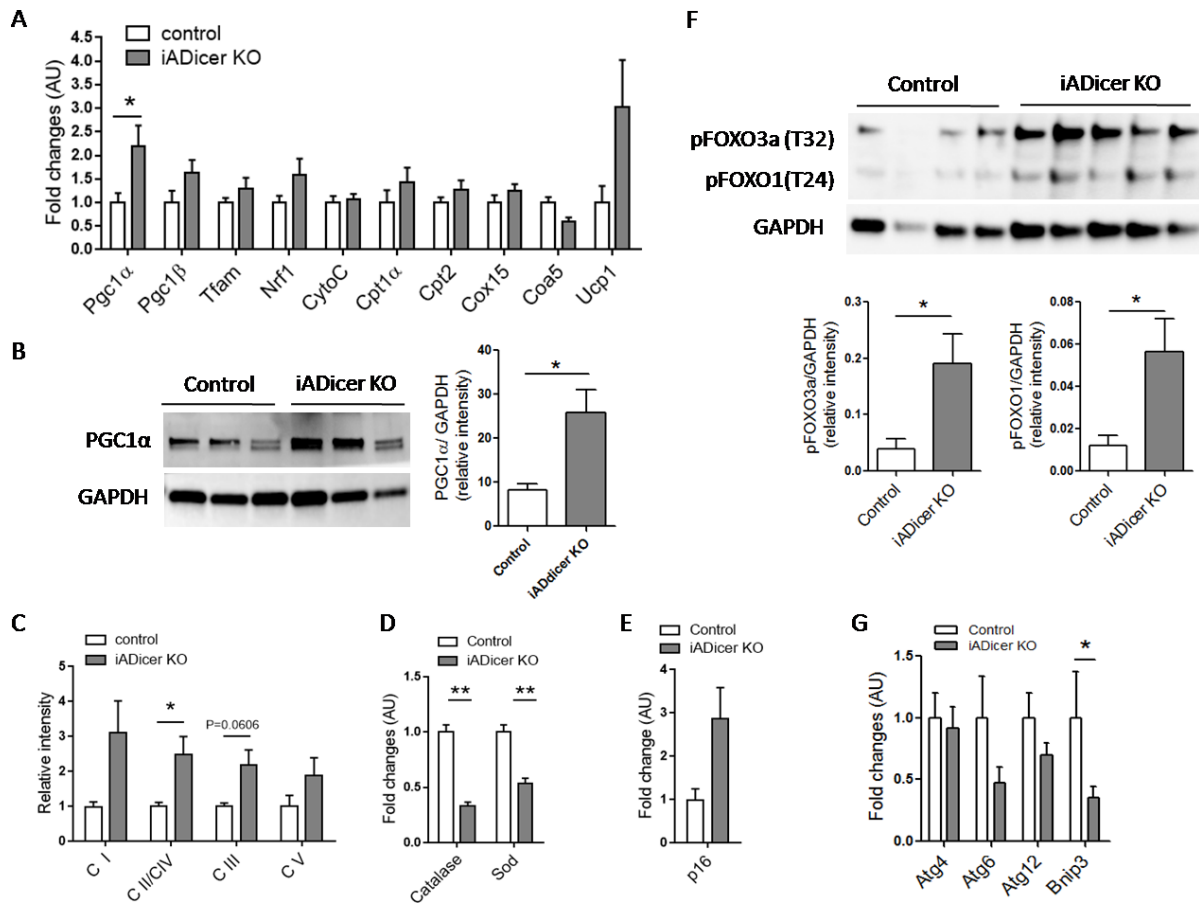


Figure 3: Possible involved mechanisms underlying the effects of adipocyte dicer deficiency.

(A) RT-qPCR of genes involved in mitochondrial functions in perigonadal adipocytes. (B) Protein level of PGC1 α in perigonadal adipocytes. (C) Quantification of protein levels of OXPHOS components in perigonadal adipocytes. (D) Downregulations of antioxidant genes of *Sod* and *Catalase* due to dicer deficiency in perigonadal adipocytes. (E) Increased phosphorylations of FOXO1 and FOXO3a in perigonadal adipose tissue due to reduced adipocyte dicer deficiency. (F) Increased phosphorylations of FOXO1 and FOXO3a in perigonadal adipose tissue due to reduced adipocyte dicer deficiency. (G) Autophagy gene *Bnip3* was negatively affected in perigonadal adipose tissue due to dicer deficiency. Data represent the mean \pm SEM, *P<0.05, **P<0.01. N=4-8.

D. Altered systemic energy homeostasis with adipocyte dicer deficiency

To better understand whether adipocyte dicer deficiency would lead to systemic effects, we characterized the plasma levels of various metabolites and hormones (**Table 1**). Although reaching no significance, there was a trend for plasma lipids such as TG, NEFAs and cholesterol to be decreased due to dicer deficiency in adipocytes. Of note, the plasma level of glycerol, a byproduct of lipolysis, was significantly increased because of dicer deficiency in adipocytes, indicating either increased basal lipolysis or inability of adipocytes to recycle glycerol for the synthesis of TG. The results correlated with the reduced *Pepck* and PLIN1 expressions in adipose tissue from iADicer KO mice compared to that in control mice (**Figure 2H** and **2G**). Surprisingly, plasma insulin exhibited a strong trend to increase to around 2.5 folds (3.82 ± 0.90 vs. 1.53 ± 0.54), indicating iADicer KO mice might have a higher risk of developing systemic insulin resistance (glycemia is not affected). The clinical variables for liver function: plasma ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels were comparable between the two groups of mice. In sum, adipocyte dicer deficiency led to alterations in systemic metabolism.

Table 1: Plasma parameters (fed)

	Control	iADicer KO
Glucose (mg/dl)	203.04 ± 11.59	192.1 ± 9.28
TG (mg/dl)	1.16 ± 0.16	0.90± 0.26
Glycerol (mM)	0.083 ±0.0089	0.114±0.0076*
NEFAs (mmol/L)	0.60± 0.086	0.34± 0.084
Cholesterol (mmol/L)	1.58 ±0.059	1.37±0.20
ALT (U/L)	22.03 ±0.033	23.25± 1.74
AST (U/L)	59.33 ±12.84	62.75± 3.48
Insulin (µg/ml)	1.53 ± 0.54	3.82± 0.90

Data represent the mean ± SEM, *P<0.05. N=4-8.

E. Abnormalities of liver associated with adipocyte dicer deficiency

To be more convincing that adipocyte dicer affected systemic metabolism, we went on to characterize liver, which is an important organ influencing systemic metabolism.

Interestingly, the result of H&E counter staining exhibited that white dots appeared in phase contrast microscope illustrating lipid overfilling in livers from iADicer KO mice treated with tamoxifen, suggesting that adipocyte dicer deficiency possibly result in lipid overload in liver (**Figure 4A**). To confirm this result and better understand the source of lipids in liver from iADicer KO mice, RT-qPCR was performed to detect the genes involved in lipid metabolism. Fat-specific protein 27 (FSP27), a lipid droplet-associated protein that promotes lipid droplet growth and TG storage was induced from iADicer KO mice compared to that from control mice (**Figure 3B**), supporting ectopic lipid development in liver with adipocyte dicer deficiency. Moreover, genes involved in DNL pathways such as *PAPRY_{1/2}*, *FAS*, and *CD36* also significantly increased in liver from iADicer KO mice compared to that from control mice (**Figure 3B**). In addition, immunoblot results of *PAPRY_{1/2}* and *FAS* further confirmed that DNL pathway was activated in liver consecutively to adipocyte dicer deficiency (**Figure 3C**). These results indicated that adipocyte dicer deficiency resulted in ectopic lipids in liver associated with upregulation of DNL pathway.

Since the accumulation of lipids is proposed to be crucial in the activation of hepatic stellate cells (HSCs) and the development of fibrosis in liver³⁷¹, we went on to investigate whether the ectopic lipids in liver resulted from adipocyte dicer deficiency was associated with fibrosis in the liver. Then, the result of semi-quantification of sirius red stained area suggested that fibrosis was indeed increased in liver from iADicer KO mice compared to control mice (**Figure 3D**). Furthermore, the results of gene markers involved in extracellular matrix (ECM) depositions had a trend to be increased by adipocyte dicer deficiency (**Figure 3E**). Moreover, genes involved in inflammatory pathways such as *MCP1* and *TGF β receptor 1* (*TGF β r1*) were significantly increased in mice from the group of iADicer KO mice (**Figure 3E**). Since *MCP1* is an important chemokine that triggers infiltrations of immune cells, in particular macrophages, it suggested that increased infiltrations of macrophages might lead to the activation of TGF β pathway and accumulation of ECM and thereby fibrosis in liver by dicer deficiency in adipocytes.

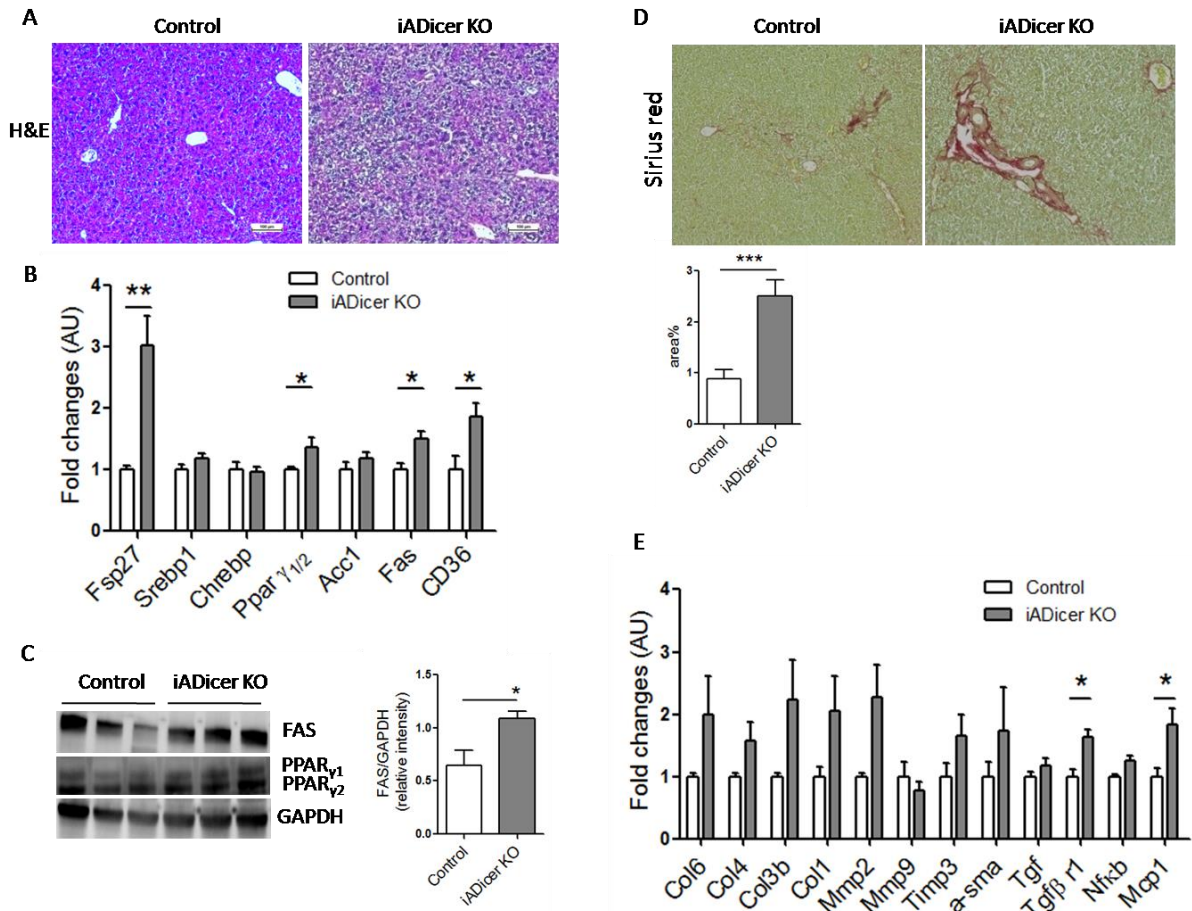


Figure 4: Adipocyte dicer deficiency promoted lipid accumulation and fibrosis in liver.

(A) The result of H&E staining suggested that adipocyte dicer deficiency resulted in lipid accumulation in liver. (B) Increased mRNA levels of genes involved in DNL pathway in liver as a result of dicer deficiency in adipocytes. (C) Increased levels of PPAR γ and FAS in liver due to adipocyte dicer deficiency by immunobot. (D) Sirius red staining of liver exhibited increased fibrosis as a result of adipocyte dicer deficiency. (E) mRNA levels of genes involved in fibrosis and inflammation in liver. Data represent the mean \pm SEM, *P<0.05, **P<0.01. N=4-8.

F. Regulation of dicer level in adipocytes

According to our previous data that adipocyte dicer deficiency reduced the lipid storage capability of adipocytes as well as induced abnormalities in systemic metabolism, like ectopic lipid overload and fibrosis in liver. Therefore, adipocyte dicer might have a positive role in the prevention of age-related complications, and adipocyte dicer might be proposed as a target to delay the onset of age-related complications. So, to know how this protein is regulated in adipocytes will be helpful to develop pharmaceutical compounds targeting it. Since adipocytes are sensitive to nutrient availability, we then wondered whether nutrient availability would affect dicer in adipocytes.

Interestingly, 24h fasting significantly increased *dicer* mRNA level in perigonadal adipose tissue in wildtype mice (**Figure 5A**). Then, *in vitro*, overnight deprivation of glucose in the culture media markedly increased dicer protein level in 3T3-F442A adipocytes (**Figure 5B**). Additionally, fetal bovine serum (FBS) contents in the culture media negatively regulated dicer protein level in 3T3-F442A adipocytes (**Figure 5C**). Therefore, negative energy state can positively regulate dicer level in adipose tissue and adipocytes.

Since nutrient deprivation is known to activate the lipolysis process in adipocytes by β -adrenergic signaling pathway⁵⁷. Therefore, we went on to see if dicer upregulation under nutrient deprivation is downstream of activation of β -adrenergic signaling pathway, by stimulation of adipocytes with 10^{-6} isoproterenol overnight. However, immunoblot result indicated that isoproterenol did not exhibit a similar upregulation effect on dicer as starvation (**Figure 5D**). Therefore, activation of β -adrenergic signaling pathway might not affect dicer level in adipocytes.

To continue to explore the underlying mechanisms how nutrient availability regulates dicer in adipocytes, we went on to see whether classical cellular nutrient sensor AMP-activated protein kinase (AMPK) is involved. Under nutrient deprivation, AMPK can be activated by the increased ratio of AMP/ATP, ultimately turning up ATP-generating catabolic processes³⁷². In line with the data that fasting or glucose/serum deprivation upregulated dicer, treatment of 1mM 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), the activator of AMPK, possibly induced dicer expression in 3T3-F442A adipocytes (N=2)

(Figure 5E). In contrast, mild inhibition of AMPK with 20 μ M Compound C (CC) seemed to reduce the basal level of dicer while not affecting the effect of serum deprivation on dicer level (N=2) **(Figure 5F)**. But strong inhibition of AMPK with 50 μ M CC exhibited no obvious effect on dicer expression in adipocytes (N=2) **(Figure 5F)**.

In sum, AMPK might positively regulate dicer expression in adipocytes. However, there might be other unknown factors participating in the regulation of adipocyte dicer expression.

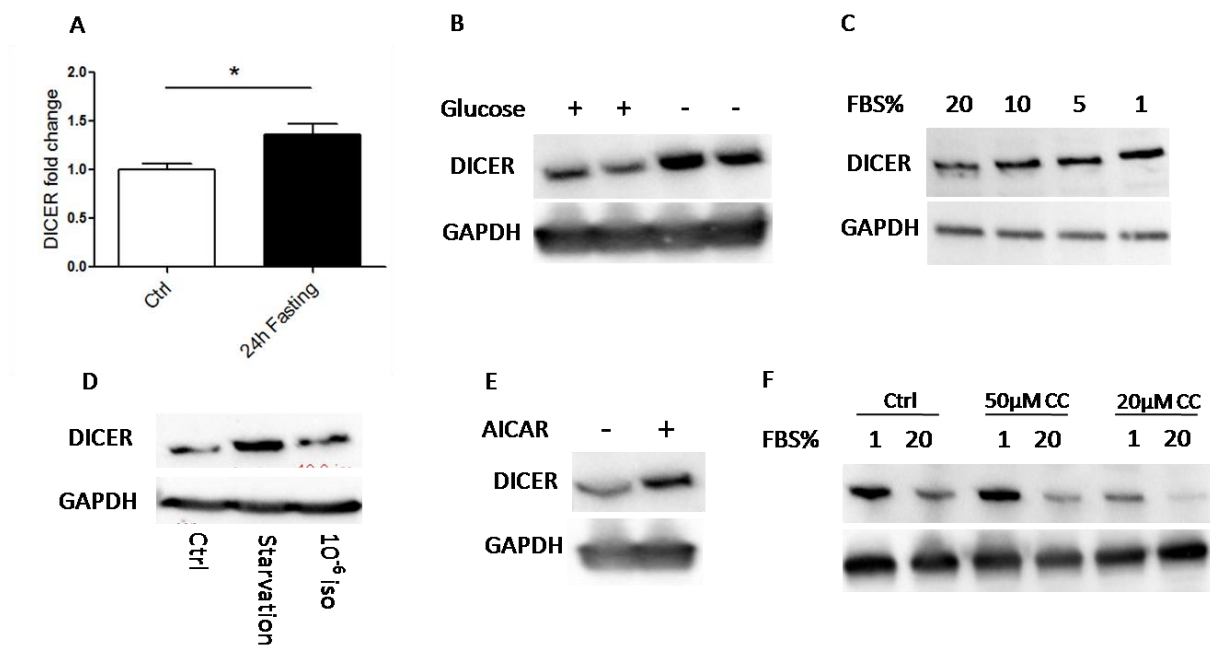


Figure 5: Regulation of dicer level in adipose tissue and in *in vitro* adipocytes.

(A) 24h fasting significantly increased mRNA level of *dicer* in perigonadal adipose tissue in wildtype mice (N=5). (B) Overnight glucose deprivation significantly increased dicer protein level in 3T3-F442A adipocytes (N=3). (C) Fetal bovine serum contents negatively affected dicer protein levels in 3T3-F442A adipocytes (N=3). (D) Overnight stimulation of 10⁻⁶ isoproterenol did not increase dicer protein expression as serum starvation (N=3). (E) Overnight treatment of 1mM AICAR might increase dicer protein level in 3T3-F442A adipocytes (N=2). (F) Overnight treatment of various concentrations of AMPK inhibitor compound C differentially affected dicer protein level in 3T3-F442A adipocytes (N=2). Data represent the mean \pm SEM, *P<0.05.

G. Selection of age-related microRNAs in adipocytes

In an effort to isolate potential aging-associated miRNA targets in adipocytes, previous microRNA-based microarray experiments in mouse visceral adipose tissue have been performed in the laboratory (by *By Karine Tréguer and Jason S. Iacovoni*). Wild type mice of 3-, 12- and 24-month-old were used. Through selection, eight potential candidates had been isolated including mir-1949, mir-672, mir-125b*, mir-805, mir-494, mir-143 and mir-133a. Therefore to continue, RT-qPCR was performed to further validate the 8 candidates from microarray.

1. Validation of miRNAs candidates from microarray

Surprisingly, among the candidates, only mir-1949 was found significantly upregulated in vWAT from 20-month-old mice compared young (6-month-old) control mice (**Figure 6A** and **6B**). Therefore, to be sure of the result, another set of experiment was performed. Similarly, mir-1949 was found dramatically upregulated in both whole vWAT and sWAT collected from 24-month-old mice and compared to 3-month-old mice (**Figure 6C** and **6D**). Moreover, the same result was obtained in isolated adipocytes from vWAT and sWAT, (**Figure 6C** and **6D**). Finally, its levels were relatively less modified in SVF isolated from vWAT and sWAT (**Figure 6C** and **6D**). Therefore, we concluded that mir-1949 might be proposed as an age-related miRNA in adipocytes.

Since miRNAs are also stable in the circulation and could positively be secreted by cells, we went on to investigate whether there was also an increased secretion of mir-1949 from adipocytes by aging. To do so, vWAT was isolated and cut into small pieces and then cultured *in vitro*. Conditioned medium (CM) was collected at the time points of 0.5h and 2h. Mir-1949 levels in CM were detected by qPCR. Interestingly, mir-1949 was elevated in CM collected from vWAT of 24-month-old mice compared to 3-month-old mice, indicating that aging might possibly increase the secretion of mir-1949 from adipose tissue (**Figure 6E**). To further confirm that aging promotes the upregulation and possibly the secretion of mir-1949 from adipocytes, we went on to investigate whether adipocyte senescence would also affect

this miRNA. To test this hypothesis, we set up an *in vitro* senescence model of adipocytes by long term exposure of 3T3 adipocytes to 0.2M glucose. Upregulations of senescence markers such as p16 expression (**Figure 6F**) and SA- β -galactosidase staining (**Figure 6G**) demonstrated the development of senescence in adipocytes treated by 0.2M glucose. Interestingly, mir-1949 was both upregulated in senescent adipocytes as well as in CM derived from these senescent adipocytes (**Figure 6H** and **6I**). Therefore, senescence of adipocytes also increases the production and secretion of mir-1949 *in vitro*.

In sum, we firstly reported that mir-1949 could be a potent age-associated miRNA in adipocytes.

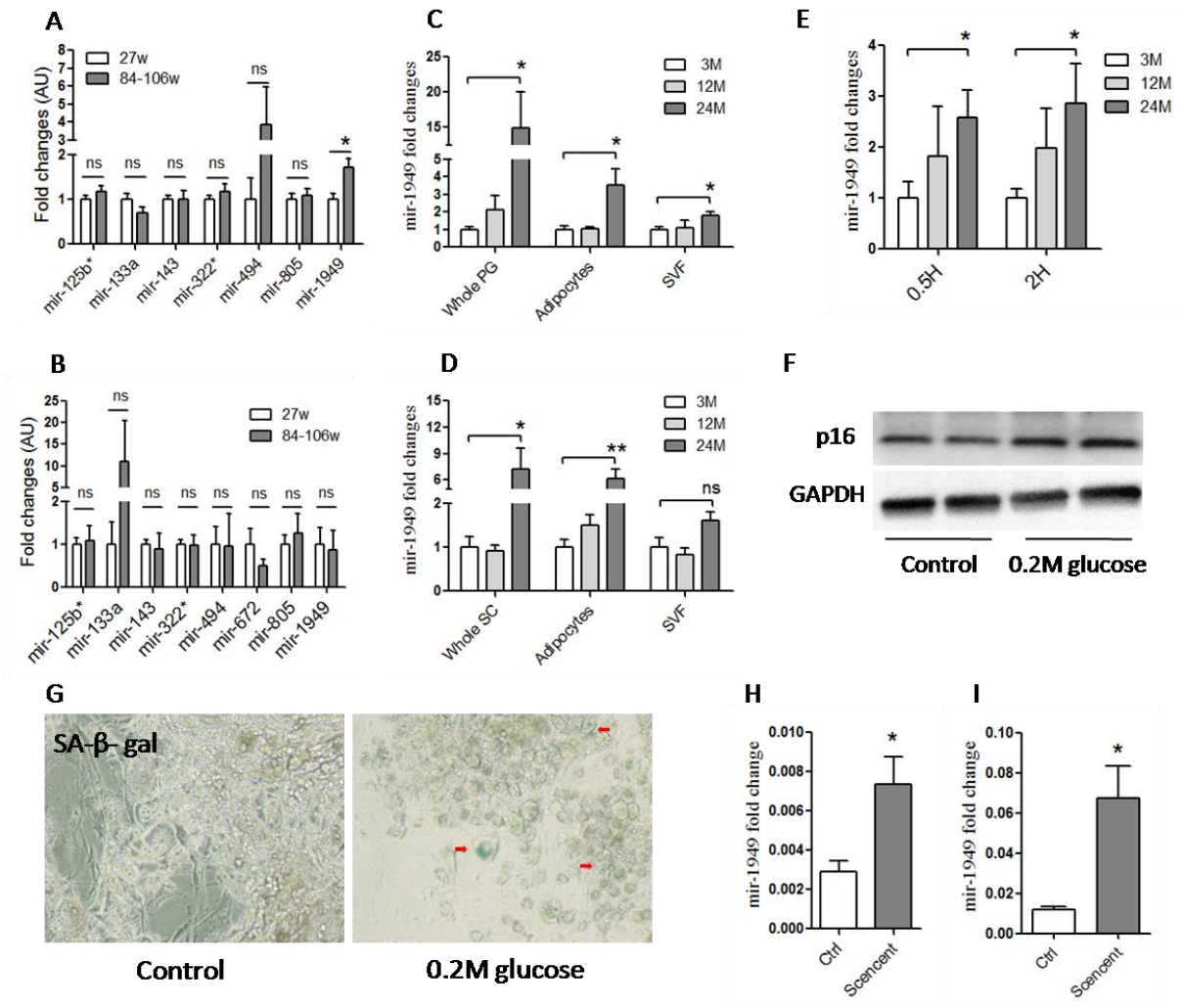


Figure 6: Age-associated increase of mir-1949.

(A and B) 8 candidates from microarray were further validated by qPCR in PG and SC from wild type mice of different ages (N=5-6 per group). (C and D) Further qPCR validation of mir-1949 expressions in whole PG, adipocytes and SVF from PG and in whole SC, adipocytes and SVF from SC, respectively (N=5-6). (E) Levels of mir-1949 in CM from ex vivo cultured PG isolated from mice of different ages was detected by qPCR (N=3-6 per group). (F and G) Senescence in 3T3 adipocytes induced with 0.2M glucose exposure for one week demonstrated with p16 elevation and SA-β staining (representative of 3 independent manipulations). (H and I) elevated levels of mir-1949 in both senescent 3T3 adipocytes as well as CM from senescent 3T3 adipocytes (N=3). Data represent the mean ± SEM, *P<0.05, **P<0.01.

2. Possible actions of age-related mir-1949 on adipocytes

To investigate the possible actions of this age-related miRNA on adipocytes, *in vitro* experiments with miRNA-mimic transfection was performed in 3T3-F442A adipocytes. RT-qPCR result indicated that with transfection of 10nM mir-1949 mimic, its level was dramatically upregulated (**Figure 7A**). Classical functions of adipocytes, like glucose transport and lipolysis were not significantly affected by upregulation of mir-1949 (data not shown). However, *in silico* analysis with public database like Target Scan 7.0, suggested that some of genes involved in mitochondria functions were proposed to be potential targets of mir-1949. Therefore, we went back to *in vitro* experiments to investigate whether this miRNA would affect mitochondria in adipocytes. Interestingly, RT-qPCR results suggested that most of the mRNA levels were only slightly modified by the elevation of mir-1949, with *Pgc1 α* was significantly decreased (**Figure 7B**). Then, the result of immunoblots of mitochondrial OXPHOS revealed that mir-1949 upregulation significantly decreased the level of complex II (**Figure 7C**). In agreement, functional analysis showed that the ability of oxygen consumption by complex II stimulated by its substrate succinate was significantly reduced by mir-1949 upregulation (**Figure 7D**) in 3T3-F442A adipocytes. Therefore, mir-1949 might negatively regulate mitochondrial complex II level and function in adipocytes *in vitro*. Since mitochondria activation participates in the determination of lipid contents in adipocytes, we went on to investigate whether lipid contents was affected by mir-1949. Interestingly, BODIPY staining suggested that upregulation of mir-1949 promoted lipid accumulation in 3T3-F442A adipocytes (**Figure 7E**). Moreover, upregulation of mir-1949 during adipogenesis by consecutively transfecting 3T3-F442A fibroblast every two days within 10 days, had a trend to increase classical white adipocyte gene markers such as *Leptin* and *Glut 4* (n=2) (**Figure 7F**).

In sum, with this preliminary *in vitro* results, we hypothesized that mir-1949 might be able to modulate the lipid storage capacity of adipocytes via negatively affecting mitochondrial complex II activities and positively affecting adipogenesis process. Nevertheless, this hypothesis absolutely needs to be further validated by *in vivo* experiments.

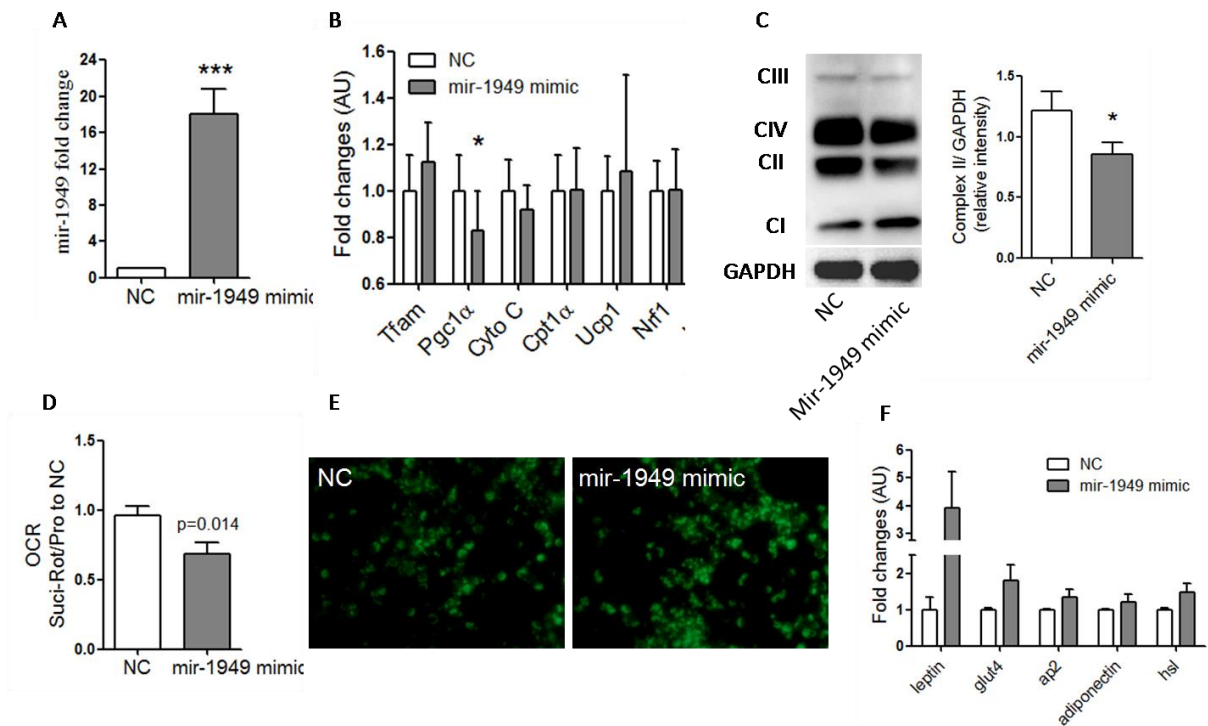


Figure 7: *In vitro* actions of mir-1949 on adipocytes.

(A) 3T3 differentiated adipocytes were transfected with 10nM of the mimic sequence of mir-1949 or a negative control sequence. The levels of mir-1949 in adipocytes were detected 72 hours post transfection (N=3). (B) Levels of mRNAs were measured in 3T3 adipocytes by qPCR 72 hours post transfection (N=3). (C) Protein levels of OXPHOS in 3T3 adipocytes were detected by immunoblots 72 hours post mir-1949 mimic transfection (N=3). (D) Succinate stimulated oxygen consumption by mitochondrial complex II was measured in 3T3 adipocytes 72 hours post transfection (N=3). (E) 3T3 adipocytes were stained by BODIPY 72 hours post transfection of mir-1949 mimic (representative of three independent manipulations). (F) 3T3 preadipocytes were consecutively transfected with 10nM mir-1949 mimic within differentiation. Then gene markers for adipocytes were detected by qPCR (N=2). Data represent the mean \pm SEM, *P<0.05, ***P<0.001.

CONCLUSION AND PERSPECTIVE

The fact that caloric restriction promotes healthy aging in diverse species reveals that systemic metabolism is crucial in longevity³⁷³. In following, several conserved nutrient-sensing pathways, such as, insulin-like growth factor-1 signaling, mTOR, AMPK and sirtuins have been identified as conserved molecules in aging³⁷⁴. Aging witnesses dysfunctions in metabolisms. Specifically, during glucose tolerance test, old people could not handle the glucose stimulation as well as the young, needing to produce significant more insulin to get rid of the exogenous glucose³⁷⁵, indicating that the peripheral metabolic tissues have a reduced efficiency to utilize the glucose. So, what kind of tissues responsible for this phenomenon? Currently, plenty of researches have indicated that aged liver and aged muscle make contributions to the insufficient insulin sensitivity in aging³⁷⁶⁻³⁷⁹. For example, senescence drives lipid accumulation and thereby steatosis in liver impairing its metabolic activities³⁷⁶.

However, relative less attention has been put on another important tissue in metabolism, which is white adipose tissue, one of the largest human organs¹²⁴. Adipose tissue is crucial in the regulation of systemic energy homeostasis. Despite the classic view that adipose tissue as an inert lipid tanker, elevating novel molecules in adipose tissue are revealed to process vital functions in recent years. In 2012, the work of Ronald Kahn's group demonstrated that dicer was reduced in adipose tissue as well as in isolated adipocytes in aging in mice¹⁷⁴, indicating that dicer/miRNAs might be novel molecules linking adipocytes and aging process.

With the use of the inducible adipocyte dicer knockout transgenic mice, we demonstrated that adipocyte dicer deficiency promoted the onset of some age-related complications. For example, adipocyte dicer deficiency significantly reduced perigonadal adipocytes' lipid storage capability, one of the age-associated dysfunctions in aging³⁸⁰. Moreover, the result that nutrient restriction, a process promoting healthy aging, positively regulates dicer level in adipocytes, further supports that there is a conserved aging pathway in adipocytes involving dicer. Mechanistically, molecules like FOXOs, mitochondrial ROS and detoxification system might be involved in the actions of dicer in adipocytes in aging (Figure 8B).

A. Adipocyte dicer abundance has a positive role in the prevention of age-associated complications.

The dramatic reductions in classical white adipocytes markers, like *Leptin* and *Glut 4*, HSL, as well as the reduction in LD coating proteins like *Fsp27* and PLIN1, suggesting that dicer is indispensable for the maintenance of white adipocyte identity as well as the homeostasis of LD inside white adipocytes.

Surprisingly, PGC1 α , one of the transcriptional factors important for the mitochondrial biogenesis and functions was increased due to dicer deficiency in perigonadal adipocytes. In addition, mitochondrial OXPHOS electron transport chain protein complexes were significantly increased or had a trend to be increased. These data indicated that there was an activation of mitochondria due to dicer loss in perigonadal adipocytes.

Activation of mitochondria has recently been proposed to have metabolic benefits in subcutaneous adipose tissue³⁸¹. However, the role of mitochondria in perigonadal adipocytes is relative less well understand. Since mitochondrial respiration is a major source of ROS in cells, therefore, to evaluate the state of mitochondria more comprehensively, it remains necessary to detect the ROS levels after dicer reduction in adipocytes. Surprisingly, there was a down regulation of antioxidants indicated by reduced mRNA levels of *Catalase* and *Sod*, indicating that there might be insufficiency in detoxification system. Moreover, the results that there was a trend for senescence markers like *p16* and pP53 (**Figure 8A**) to be increased due to adipocyte dicer deficiency, further supported the possibility that the combination of mitochondrial activation and detoxification reduction leads to DNA damage in adipocytes by dicer deficiency.

According to literature, the transcriptional factor FOXOs has been reported to be able to promote the transcription of antioxidant genes like *Sod* and *Catalase*³⁶⁷, whereas inhibiting the transcription of genes involved in mitochondrial biogenesis and functions³⁶⁸. The transcriptional activity of FOXOs can be regulated by acetylation and phosphorylation³⁸². For example, phosphorylation of FOXOs will lead its nuclear exclusion into cytoplasm,

thereby inhibiting their transcriptional activities³⁶⁷. Interestingly, the result that adipocyte dicer deficiency increased the phosphorylation of both FOXO1 and FOXO3a, indicating that adipocyte dicer deficiency might negatively affect the FOXOs activities. FOXOs are key regulators of cellular response to various stresses and have been identified as a conserved molecule in promoting healthy aging³⁸³. One of the mechanisms that FOXOs contribute to longevity is through regulation of autophagy³⁸⁴. For example, FOXO1 and FOXO3 active autophagy in various cell types to help to maintain protein homeostasis³⁸⁵. Then, the result that dicer deficiency decreased autophagy gene maker *Bnip3*, further supported the idea that adipocyte dicer deficiency might lead to FOXOs inactivation. Since autophagy is also an important process removing organelles inside cells, it remains to be solved whether the inhibition of autophagy by dicer deficiency in adipocytes might have a role in mitochondrial degradation in vWAT.

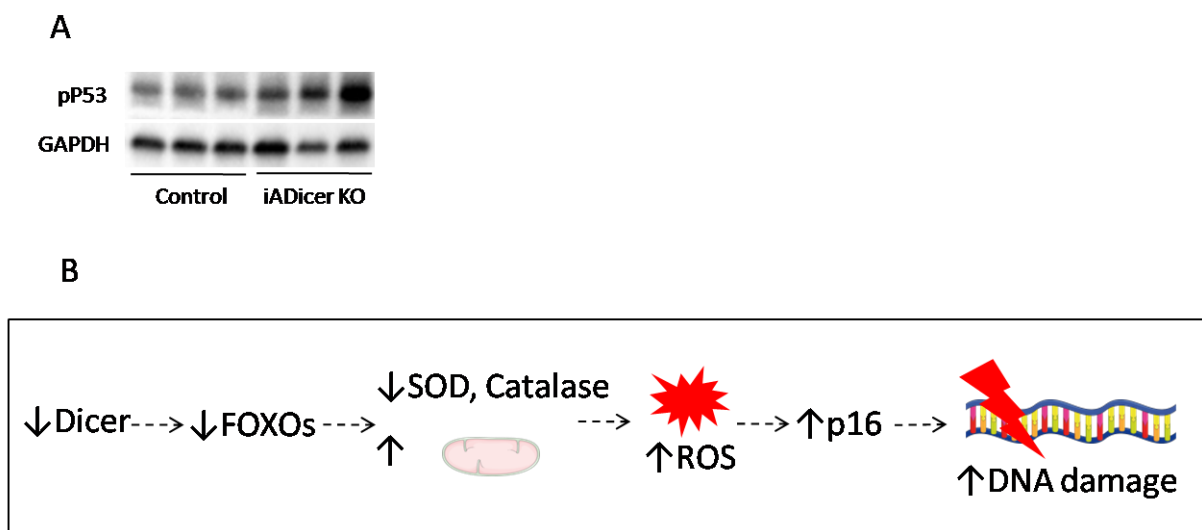


Figure 8: Proposed molecular mechanisms underlying adipocyte dicer in aging.

(A) The protein level of pP53 in perigonadal adipose tissue in control and iADicer KO mice. (B) Simple scheme of possible mechanisms underlying adipocyte dicer in aging.

Therefore, to illustrate well the molecular mechanisms of adipocyte dicer in aging, On one hand, to further confirm the involvement of detoxification system, several questions need to be addressed more clearly.

1. Firstly, adipocyte dicer level negatively associates with cellular ROS levels?

The answer to this question is probably crucial to determine whether adipocyte dicer will induce oxidative stress and thereby DNA damage. Therefore, to resolve this question, *in vivo*, we propose to measure cellular ROS levels in adipocytes in control and iADicer KO mice. *In vitro*, we propose to measure cellular ROS levels in adipocytes in normal 3T3-F442A adipocytes or in 3T3-FF442A adipocytes overexpressing dominant-negative dicer.

2. Secondly, adipocyte dicer loss reduces the enzymatic activities of SOD and CATALASE?

We've already demonstrated that the mRNA levels of antioxidant proteins: *Sod* and *Catalase* were dramatically reduced due to adipocyte dicer deficiency. Therefore, to be more convincing that adipocyte dicer deficiency induced detoxification reduction, the enzymatic activities of these antioxidants need to be well addressed in adipocytes in control and iADicer KO mice.

3. Thirdly, antioxidants will delay the age-related complications caused by adipocyte dicer deficiency?

To well support the hypothesis that oxidative stress partly contribute to the accelerated onset of age-related complications by adipocyte dicer deficiency, it's worthy of investigation whether the supplementation of antioxidants (vitamin C, *N*-acetylcysteine, and Trolox) to iADicer KO mice, would help to reduce the accelerated age-associated complications in iADicer KO mice.

On the other hand, the involvement of FOXO1/FOXO3a in the role of adipocyte dicer in aging process needs to be further clarified.

1. Firstly, our present data suggests that dicer deficiency induces increased phosphorylation FOXO1/FOXO3a, indicating their nuclear exclusion into

cytoplasm. Therefore, to better support this result, their cellular compartmental distributions need to be determined by immunohistochemistry or by protein extractions from nuclear and cytoplasm in adipocytes from control and iADicer KO mice.

2. Secondly, the transcriptional activities of FOXO1 and FOXO3a also should be determined in adipocytes from control and iADicer KO mice.
3. Finally, whether overexpression of FOXO1 or FOXO3a specifically in adipocytes (achieved by injection of control AAV sequence or AAV-adiponectin-dicer into iADicer mice) will prevent the onset of age-related complications in iADicer KO mice?

B. Our results suggested that adipocyte dicer deficiency promotes liver aging.

Aging is associated with the severity and poor prognosis of various liver diseases including nonalcoholic fatty liver disease (NAFLD) and fibrosis. For example, aged populations (those aged over 65 years), was reported to have a higher NAFLD prevalence rate than their younger counterparts (56 vs. 72%, $P=0.02$), and also displayed a higher rate of liver fibrosis³⁷⁸. Interestingly, by using iADicer KO mice, our results propose that adipocyte dicer deficiency might accelerate liver aging, through promoting lipid accumulation and fibrosis in liver.

There are 2 possible mechanisms might be involved.

Firstly, there's a crosstalk between adipocytes and liver in aging through adipocyte-derived miRNAs.

1. To test this possibility, we can make use of ZsGreen^{lx/lx}/ Adiponectin-CreER^{T2} mice, in which adipocyte-derived proteins will have GFP signal induced by tamoxifen treatment (**Figure 9**). By co-immunostaining of GFP and exosome markers like CD63 in liver, it can help to determine whether liver can uptake adipocyte-derived exosomes under physiological states.
2. In addition, through comparison of miRNA profile in exosomes extracted from circulations as well as miRNA profile in liver in control and iADicer KO mice by

microarray, it might help to determine if adipocyte dicer deficiency would lead to specific miRNA reduction in both circulating exosomes as well as in liver, which is needed for the maintenance of liver integrity and function in aging.

Secondly, inefficiency of adipocytes' capability of lipid storage leads to ectopic lipids in peripheral liver.

To test this possibility, the functions of adipose tissue like lipolysis, glucose transport and de novo lipogenesis need to be well studied in control and iADicer KO mice.

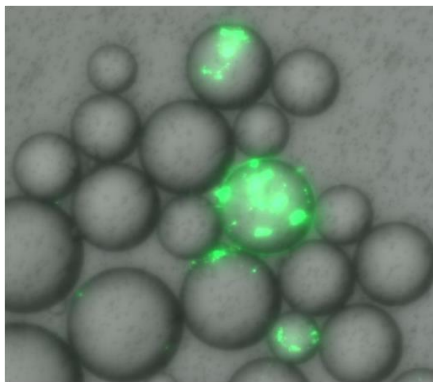


Figure 9: GFP expression in adipocytes from ZsGreen^{lx/lx}/adiponectin-CreER^{T2+} mice by 4-hydroxytamoxifen.

Perigonadal adipocytes were isolated from ZsGreen^{lx/lx}/adiponectin-CreER^{T2+} mice and cultured *in vitro*. GFP expression was induced by 4-hydroxytamoxifen treatment.

C. Age-related mir-1949 might have a positive role in the prevention of age-related dysfunctions in adipocytes.

In our above data, we've demonstrated that adipocyte dicer deficiency would promote the onset of some age-related complications, such as impaired lipid storage and systemic metabolic dysfunctions. However, in that part, we could not determine whether these are direct consequences of the dicer protein *per se*, or indirect consequence of some dicer produced miRNAs. Therefore, in my second part, we aimed at the selection of age-related miRNAs in adipocytes.

Interestingly, we firstly reported that aging increased mir-1949 expression in adipocytes in mice. Additionally and possibly, aging might also promote the secretion of

mir-1949 from adipocytes. Moreover, *in vitro* data indicated that adipocytes senescence also increased mir-1949 production and secretion from adipocytes.

Functionally, however, we did not observe significant effects of this miRNA in glucose transport and lipolysis in normal adipocytes *in vitro*. Interestingly, *PGC1 α* was significantly reduced by mir-1949 upregulation in 3T3-F442A adipocytes, indicating that mir-1949 might affect mitochondria in adipocytes. Subsequently, the results that mir-1949 upregulation led to a reduction in mitochondrial complex II protein level and oxygen consumption ability, further suggesting that mir-1949 might negative regulate mitochondria in adipocytes.

Interestingly, Bodipy staining suggested that upregulation of mir-1949 promoted lipid accumulation in adipocytes, coincide with that mir-1949 negatively regulates mitochondria and thereby a reduced lipid utility. Interestingly, at the same time, continuous upregulation of this miRNA during adipogenesis of 3T3-F442A preadipocytes, exhibited a trend to increase the expression of classical white adipocyte gene markers, indicating that mir-1949 might promote lipid storage in adipocytes. Moreover, this effect was only observed at the end of differentiation but not during differentiation (data not shown), suggesting that mature adipocytes are more sensitive to mir-1949's effects.

Then, interestingly, our preliminary results that upregulation of mir-1949 tended to increase lipid accumulation in senescent adipocytes as well as nutrient deprivation significantly increased its level in perigonadal adipose tissue in mice (**Figure 10A and 10B**), lead us to hypothesize that this age-related microRNA might try to rescue age-related dysfunctions in adipocytes by modulating lipid storage capacity as well mitochondrial activities.

Therefore, to further validate this hypothesis, *in vivo* experiments such as injection of AAV-aP2-mir-1949 into old mice (specific upregulation of mir-1949 in adipocytes), would be helpful to determine whether specific upregulation of this microRNA in aged adipocytes would help to rescue age-related complications in mice. *In vitro*, to better characterize the actions of mir-1949 on senescent adipocytes, such as, gene profiles, functions, etc.

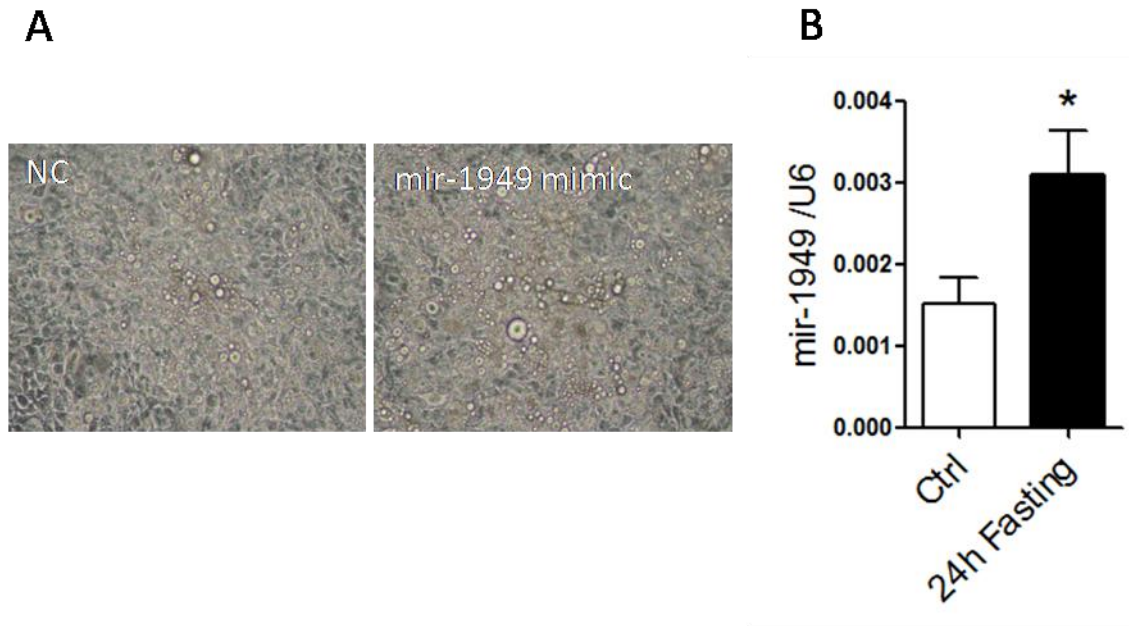


Figure 10: Possible role of mir-1949 in the prevention of age-related dysfunctions.

(A) Adipocyte senescence was induced by exposure to 0.2M glucose for one week. Then, senescent adipocytes were transfected with 10nM of the mimic sequence of mir-1949 or a negative control sequence (N=1). (B) Mir-1949 level in perigonadal adipose tissue in wildtype mice fasted for 24h (N=5). Data represent the mean \pm SEM, *P<0.05.

MATERIALS AND METHODS

Reagents and cell culture

The 3T3-F442A cell line (ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% of fetal bovine serum (FBS, Gibco®). Differentiation was induced by incubating confluent cells in differentiation medium (DMEM supplemented with 10% FBS plus 50nM insulin for up to 14 days. Cells were routinely cultured in 5% CO₂ at 37°C. SiRNA for *Dicer* were purchased from Santa Cruz.

AMPK activator AICAR and L-Ascorbic acid were obtained from sigma.

Animal studies and Genotyping

Mice used in this study were males of C57/BL6J background.

Dicer^{lx/lx}:adipoQ-Cre^{ERT2} mice were generated by crossing *Dicer*-floxed mice that possess *loxP* sites flanking exon 23 of the *Dicer1* locus³⁸⁶, with adipoq-Cre/ERT2 mice, which express a tamoxifen-inducible CRE recombinase in adipocytes³⁸⁷.

For genotyping, DNA was extracted from the tips of mice' tails with the REExtract-N-Amp Tissue PCR Kit (Sigma) and PCR was performed with the KAPA Mouse Genotyping Kit (KPAB BIOSYSTEMS).

Tamoxifen (sigma) was dissolved in coin oil at 40°C overnight. Mouse was orally administrated with tamoxifen (200mg/kg) for 5 consecutive days for 3 times with around 2 months.

Immunofluorescence

PLIN1 were performed with commercial kits (PROGEN and ThermoFisher Scientific, respectively) with slight modifications. Briefly, cells were rinsed with PBS, fixed with methanol for 5 mins on ice, and then rinsed with PBS twice. Primary antibody for PLIN1 was diluted in 1 to 200 inside 1 % BSA inside PBS, and laid on the surface of slides for incubation at 4°C overnight. Fluorescent second goat anti-Rabbit antibody (InvivoGen), which was diluted in 1 to 100 in 1 % BSA was incubated with cells for 30mins at RT in dark. Slides were rinsed with PBS and then mounted with fluorescent mounting medium (Dako). Pictures were

taken with microscopy. Lipid droplets were stained with BODIPYTM 493/503 (ThermoFisher) as described³⁸⁸.

Histological Analysis

Adipose tissues and liver were immediately excised and fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5µm were stained with hematoxylin and eosin (H&E), and imaged with Nikon microscope. Adipocyte sizes were quantified with *Adiposoft* software as described³⁸⁹.

Sirud red staining

The liver sections of 5µm were stained with 1% Sirius Red F3B (Sigma) in saturated picric acid (Sigma). The quantification of the red-stained collagen area was performed with Image J software.

Quantification of plasma parameters

Blood samples were taken from the portal vein of mouse, with EDTA, plasma were separated by centrifugation at 10000 rpm for 10 min at 4°C and were stored at -80°C. Plasma parameters were detected by *Centre Régional d'Exploration Fonctionnelle et de Ressources Expérimentales*, INSERM, Université Paul Sabatier (CREFRE).

Western Blot and antibodies

Cells were lysed in Laemmli buffer. Mouse tissues were homogenized in RIPA buffer supplemented with protease inhibitor, phosphatase inhibitor cocktail 1 and cocktail 2 (Sigma), and protein extracts were quantified using Bradford method (Bio-Rad). Proteins were electrophoresed on 15 % SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked and incubated with primary antibodies in 5 % BSA in PBST overnight at 4°C. Blots were the rinsed and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (Sigma). Immunoreactivity was detected by Amersham ECL prime Western Blotting Detection Reagent (GE Healthcare) and visualized with the ChemiDoc XRS+ system (Bio-Rad). Information about sources of antibodies was listed as following:

GAPDH(Ref: mAb2118, Cell Signaling TECHNOLOGY (CST)); HSL (Ref: 4107, CST); Dicer (Ref: sc-136979, Santa Cruz); Phospho-Akt(ser473) (Ref: 9271); AKT (Ref: 9272, CST); Phospho-AMPK α (Thr172) (Ref: 4188, CST); AMPK (Ref: 2532, CST); PPAR γ (Ref: 2430, CST); FAS (Ref: sc-20140, Santa Cruz); Total OXPHOS Rodent WB Antibody Cocktail (Ref: ab110413, Mitosciences); PGC1 α (Ref: ab54481, Abcam); LC3B (Ref: 2775, CST); Phospho-FoxO1 (Thr24)/FoxO3a (Thr32) (Ref: 9464, CST).

Lipolysis assay

3T3-L1 adipocytes were previously starved with DMEM for 4h and then treated with DMEM supplemented with 2.5% fatty acid free bovine serum albumin (sigma), containing 0 or 10⁻⁶ isoproterenol for 2 hours. Glycerol released in the medium was measured with the Glycerol-Free Reagent Kit (Sigma). Results were normalized to the levels of total proteins measured by Bradford methods (Bio-Rad).

Glucose transport

Cells were starved in serum-free DMEM for 6 h and then incubated in the absence or presence of 100nM insulin in Krebs-Ringer phosphate buffer (pH 7.4) supplemented with 0.2% bovine serum albumin, for 45minutes at 37°C. Transport was started by adding 4 μ Ci /1 ml [³H]2-deoxy-D-glucose (NEN, Boston, MA) for 10 minutes precisely and stopped by placing the cells on ice and rapidly washing them with ice cold 10mM glucose in PBS. Cells were lysed with 0.05N NaOH. Aliquots of the cell lysates were used for liquid scintillation counting and determination of protein contents by the Bradford method (Bio-Rod), respectively.

Senescence-associated β -Galactosidase staining (SA- β -gal)

B-gal activity was detected at pH 6, a known characteristic of senescent cells not found in quiescent or immortal cells with commercial kit from Cell signaling TECHNOLOGY (Ref: 9860).

Triglyceride content measurement

Cells were rinsed with PBS and lysed in PBS by brief sonication. TG was measured by determination of the concentration of glycerol released after lysing and enzymatically hydrolyzing the triglyceride molecules. Free glycerol reagent and Triglyceride reagent kit were obtained from sigma.

Measurement of mitochondria oxygen consumption

Oxygen consumption was determined at 37°C using the Oxygraph-2k (O2k, OROBOROS instruments). Cells were permeabilized with 0.1% saponin (Sigma) in BIOPS (2.77mM CaK₂EGTA, 7.23mM K₂EGTA, 5.77mM Na₂ATP, 6.56mM MgCl₂·6H₂O, 20mM Taurine, 15mM Na₂Phosphocreatine, 20mM Imidazole, 0.5mM Dithiothreitol, 50mM MES hydrate) with gentle ventilation at 4°C for 20 mins. Permeabilized cells were respired in MiRO5-Kit (110nM D-Sucrose, 60mM K-Lactobionic acid, 0.5mM EGTA, 3mM MgCl₂, 20mM Taurine, 10mM KH₂PO₄, 20mM HEPES). The following substrates L-Glutamic acid at 2000mM, L-Malic acid at 400mM and Succinate at 1000mM were applied.

RNA Extraction and Quantitative RT-PCR (qPCR)

For the analysis of mRNAs, cells or tissues were lysed with QiAzol lysis reagent (QIAGEN), and RNA was extracted with RNeasy Mini Kit (QIAGEN). The cDNA templates were prepared by reverse transcription of RNA (500ng) using High-Capacity cDNA Reverse Transcription kit (ThermoFisher) and gene expressions were measured using a MESA blue qPCRTM Mastermix plus for SYBR[®] assay (Eurogentec).

For the analysis of miRNAs, total RNA was extracted with MasterPureTM Complete DNA & RNA Purification kit 2.00 (EUROMEDEX) according to manufacturer's instructions. 10ng of total RNA were reverse transcribed using TaqManTM MicroRNA Reverse Transcription Kit (Applied Biosystem) and qPCR and data evaluation were performed as described previously²²⁸. The amplification data were analyzed using Vii7 (Applied Biosystems).

Statistical Analysis

All calculations were performed by using Prism 5 (GraphPad Software, USA). Statistical significance between two groups was determined by the Student's *t* test. Statistical significance was set at $p < 0.05$.

Project II:

A novel method of culturing isolated adipocytes *in vitro*

The striking rise of obesity-related metabolic disorders has focused attention on adipocytes as critical mediators of disease phenotypes. To better understand the role played by excess adipose in metabolic dysfunction, it is crucial to decipher the characteristics of adipocyte *per se*. However, during my PhD project, we found that it was very hard to successfully modulate gene expressions *in vitro* in differentiated adipocytes from immortalized cell lines, such as 3T3-F442A cells, with routine techniques like siRNA transfections. Therefore, a practical method for studying adipocytes *in vitro* is absolutely necessary for development of therapeutic targeting adipocyte.

Taking into considerations of the present situations of *in vitro* adipocytes models that are introduced in the introduction part, we are wondering why there is no established protocol for directly culturing isolated adipocytes. Two major questions might need to be uncovered as following:

- What is the main technical difficulty preventing the possibility of culturing adipocytes *in vitro* directly?
- Through what kind of interventions tha we could overcome these roadblocks?

Result 2: A novel method of culturing isolated adipocytes *in vitro*

1. Working scheme for culturing isolated adipocytes *in vitro*

The unique characteristics of adipocyte come from their structure: one big lipid droplet (LD) taking place of nearly all the cytoplasm (around 90%), which will result in extreme buoyancy forces to fat cells in aqueous system. Naturally, adipocyte would float on top of the culture medium which has a higher density than lipids. Consequently adipocytes will shortly die because of surface tension between atmosphere and the liquid culture medium, as well as poor nutrition. Therefore, it is necessary to solve this question in order to successfully culture mature adipocytes *in vitro*. Possibly, the solution is either to prevent the floating of buoyant adipocyte mechanistically or to modify the density of culture medium making it lower than that of fat. Herein, we tried to sequester adipocyte with the application of cell culture inserts by immersing them underneath the membrane of inserts (**Figure 1A**). However, since previous “ceiling culture”, where adipocyte were allowed floating on top of a glass flask or a cover slide on top of wells of culture plates which were fully filled with medium, resulted in dedifferentiation, deform or death of cells^{359,390}, we suggest that there are other factors affecting the lipid storage capability of adipocyte (LDs stability) or viability of adipocyte *in vitro*.

To resolve this question, it is crucial to understand the unique properties of LDs inside adipocyte. Structurally, LDs are composed of a neutral lipid core, consisting primarily of triglyceride and sterol esters, which are covered by a monolayer of phospholipids as well as other amphiphilic lipids, and proteins^{391,392}. For example, perilipin is the most abundant amphipathic protein on the surfaces of adipocyte LDs³⁹³, which is the major substrate for cAMP-dependent protein kinase (PKA) in lipolytically stimulated adipocytes. Perilipin serves important functions in the regulation of basal and hormonally stimulated lipolysis. Unlike the two leaflets of a bilayer, which are physically coupled, this monolayer can be stretched infinitely, resulting in an increasing surface tension. LDs with high surface tension are unstable and tend to fuse³⁹⁴.

Between the LDs and the bilayer phospholipid cell membrane is the scarce cytosol, a complex mixture of cellular organelles and substances dissolved in water. Due to the extreme limited proportion of cytosol in adipocyte, its buffer effects of preventing the LDs

from alterations in extracellular compartments, are dramatically weakened. For instance, it would be possible that immediate immersion of adipocytes into pure H₂O would lead to rapid osmosis of water into cytosol through cellular membrane, and then because of the extremely limited room of cytoplasm inside adipocyte, this swelling of cytosol might lead to the final destruction of cellular membrane and thereby cell death. On the other hand, it would be also possible that immersion of adipocytes into a solution which is higher concentrated than the cytosol of adipocytes would result in dehydration of adipocyte cytosol and thereby reduced cell viability (**Figure 1B**). Therefore, it is crucial to control the osmotic pressure of extracellular medium in order to maintain the homeostasis of adipocyte cytosol.

Glycerol is a well-known hyperosmolar agent and has a low toxicity. In addition, glycerol protects red cells against the trauma of freezing and thawing³⁹⁵. Therefore, in an attempt to modify the osmosis of culture medium, we tested whether the supplement of glycerol into culture medium would help to improve the survival rates of adipocytes (**Figure 1C**).

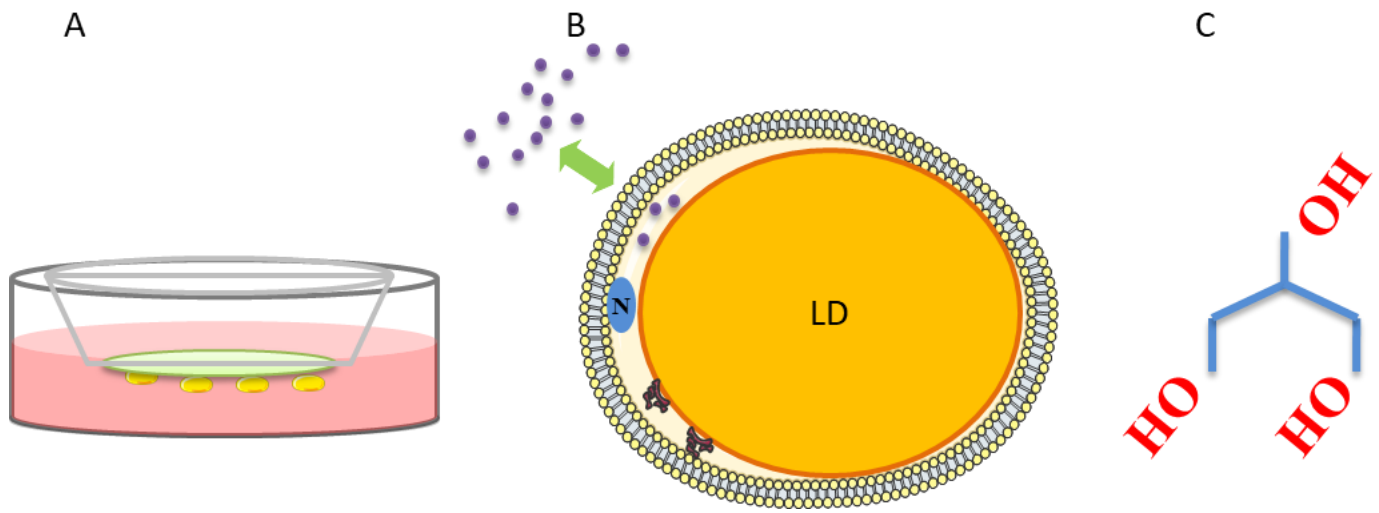


Fig 1: Illustration of the working scheme.

(A) Application of cell inserts to sequester buoyant adipocytes from floating for culturing *in vitro*. (B) Possible influence of the homeostasis of adipocyte cytosol by environmental tonicity. (C) The chemical structure of glycerol.

2. Modifications of culture medium

To investigate the morphology of cultured adipocyte, microscopy observation was applied and it was shown that fat cells exhibited a ball like spherical 3D structure (**Figure 2A** and **2B**), with a relatively smaller nucleus which is peripherally located (**Figure 2A** and **2C**), and the LDs nearly taking up of all the cytoplasm of adipocytes (**Figure 2D**). In addition, PHK26 which is a dye that identify the membrane structures, indicated that the cultured adipocytes maintained the integrity of cell membranes (**Figure 2A**). These data indicate that isolated adipocyte cultured with our working scheme maintained the spherical integrity at least for a short period (within 3 days). However, at the same time, it was also found that the survival rates of these adipocyte were very low with dead cells exhibiting an appearance of shrinking cell membranes (data not shown), confirming that other factors aside buoyancy are affecting the vitality of the *in vitro* cultured adipocytes. Therefore, we went on to investigate whether modification of the osmolarity of culture medium by supplement of glycerol would help to improve the survival rates of adipocytes.

Interestingly, glycerol supplement dose-dependently increased the survival rates of fat cells demonstrated with TUNEL staining (**Figure 2E**). The reason why there was the appearance of shrinking of cell membranes cultured with low concentration of glycerol is not well understand. Here we proposed that one of the factors leading to the crushes of the spherical 3D adipocytes might come from the osmosis of hypotonic culture medium into cell cytosol, resulting in imbalance of the homeostasis of cell cytoplasm and plasma membrane integrity and finally cytotoxicity.

On the one hand, to set up a better culture condition, we went on to select a suitable fetal bovine serum (FBS) concentration. Interestingly, we observed that when cultured without serum, fat cells started to exhibit strange alterations, which was “browning of the color” while still retaining the morphological integrity (**Figure 2F**). TUNEL assay helped to demonstrate that fat cells started apoptosis without serum, indicating that serum deprivation could also lead to apoptosis of terminal differentiated adipocytes. On the other hand, fat cells cultured with 20% FBS, exhibited “crushed” structures, which was demonstrated with TUNEL assay as dead cells. The reason that very high concentrations of FBS led to death of fat cells might due to the increased osmotic pressure that lead to

dehydration (drain) of the very limited aqueous cytosol of adipocyte and then death. Adipocyte cultured with 10 to 15% FBS remain relatively normal in term of structure. Therefore, the concentration of FBS is also vital for the viability of adipocytes in culture, with both low and high concentrations would lead to apoptosis.

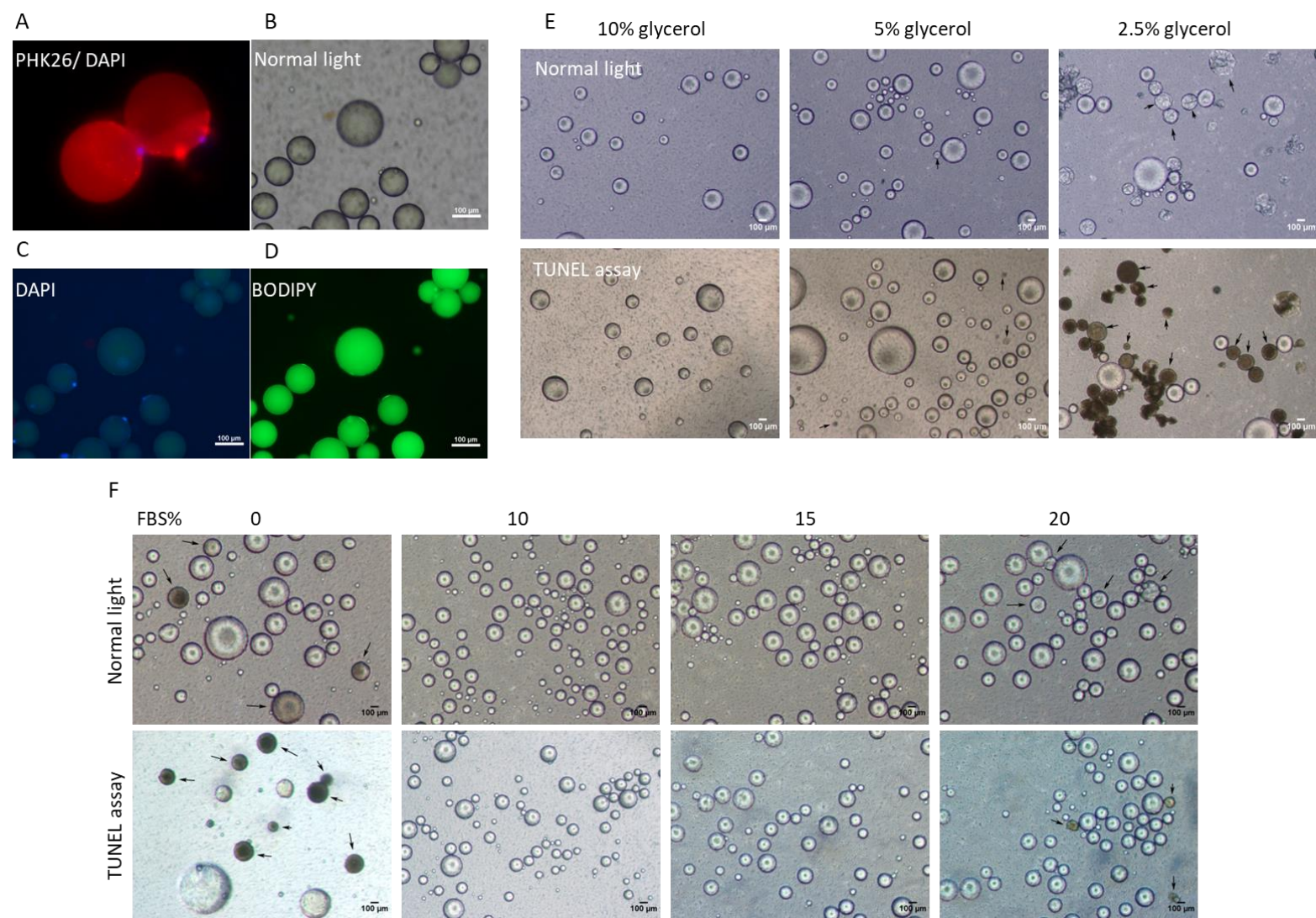


Fig 2: Image of adipocytes in culture under different condtions.

(A) PHK26 and DAPI staining demonstrated that cultured adipocytes maintained the integrity of structures with normal cell membrane and peripherally located nucleus at D10 of *in vitro* culture. (B, C and D) Microscopic observations show that cultured adipocytes exhibited ball-like 3D spherical structures, with DAPI identified an extreme small and peripherally located nucleus and bodipy staining demonstrated that a single LD took up nearly of all of the cytoplasm at D2 of *in vitro* culture. (E) Adipocytes were cultured with different concentrations of glycerol (2.5%, 5% and 10%) and were observed and imaged daily. Tunal assay were performed 2 weeks after to detect apoptosis. (F) Adipocytes were cultured with different concentrations of FBS (0, 10%, 15% and 20%) and were observed and imaged daily. Tunal assay were performed one week after to detect apoptosis. Representative of 3 independent manipulations.

3. Functional investigation of cultured adipocyte *in vitro*

To investigate whether cultured adipocyte are biologically functional, we analysed various functional analysis.

It is well known that insulin signal transduction triggers the phosphorylation of AKT at serine 473 through insulin substrate 1 (IRS-1) and phosphatidylinositol (PI) 3-kinase (PI3K), which is crucial for insulin stimulated glucose uptake into adipocyte. The result demonstrated that 100nM insulin stimulation increased the level of pAKT (s473) in cultured adipocytes (**Figure 3A**), indicating that sensitivity of insulin pathway is conserved. However, it was also noted that the basal level of AKT phosphorylation of AKT at serine 473 was high even after deprivation, which might due to the structure of culturing system, because it is impossible to change all the medium at one time (some medium have to be remained to cover the sequestered adipocytes), instead, dual dilutions of previous culture medium with serum and glucose with DMEM.

To investigate the glucose uptake ability of cultured adipocyte, we took advantage of a fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), which could directly measure glucose uptake in living cells³⁷⁹. The result shows that 100 or 1000nM insulin stimulation had a clear trend to increase the uptake of fluorescent 2-NBDG into cultured adipocyte (**Figure 3B**). Unfortunately, because of the hyper-sizes of adipocytes, flow cytometry could not be applied to detect the difference of fuorescence. However, the result obtained with [³H] 2-deoxy-D-glucosetracer further demonstrated that insulin would significantly stimulate the glucose uptake into culture adipocyte by around 50% at the day 5 of culture (**Figure 3C**). Therefore, *in vitro* cultured adipocytes are capable of extacellular glucose intake.

On the other hand, lipolysis was performed to evaluate the responsiveness of β 3-adrenergic signaling pathway of cultured adipocytes (**Figure 3D**). The result demonstrated that isoproterenol at the concentrations of 0.1 or 1 μ M successfully increased the glycerol release into medium by around 40 to 50% compared to basal release even at the day 8 of culture (0.39 ± 0.025 vs. 0.55 ± 0.037 and 0.60 ± 0.056 mM). In fact, it would apparently be observed that the size of adipocytes even reduced with isoproterenol stimulation (data not shown), indicating the dynamic of adipocytes. Technically, since adipocytes were cultured

with 10% of glycerol in medium, it required thorough refresh of the medium with glycerol free DMEM before the performance of lipolysis, which might lead to trauma to the adipocytes. Therefore, whether technical defects would result in effects of the results remain unknown. Nevertheless, these above results indicated that cultured fat cells were functional.

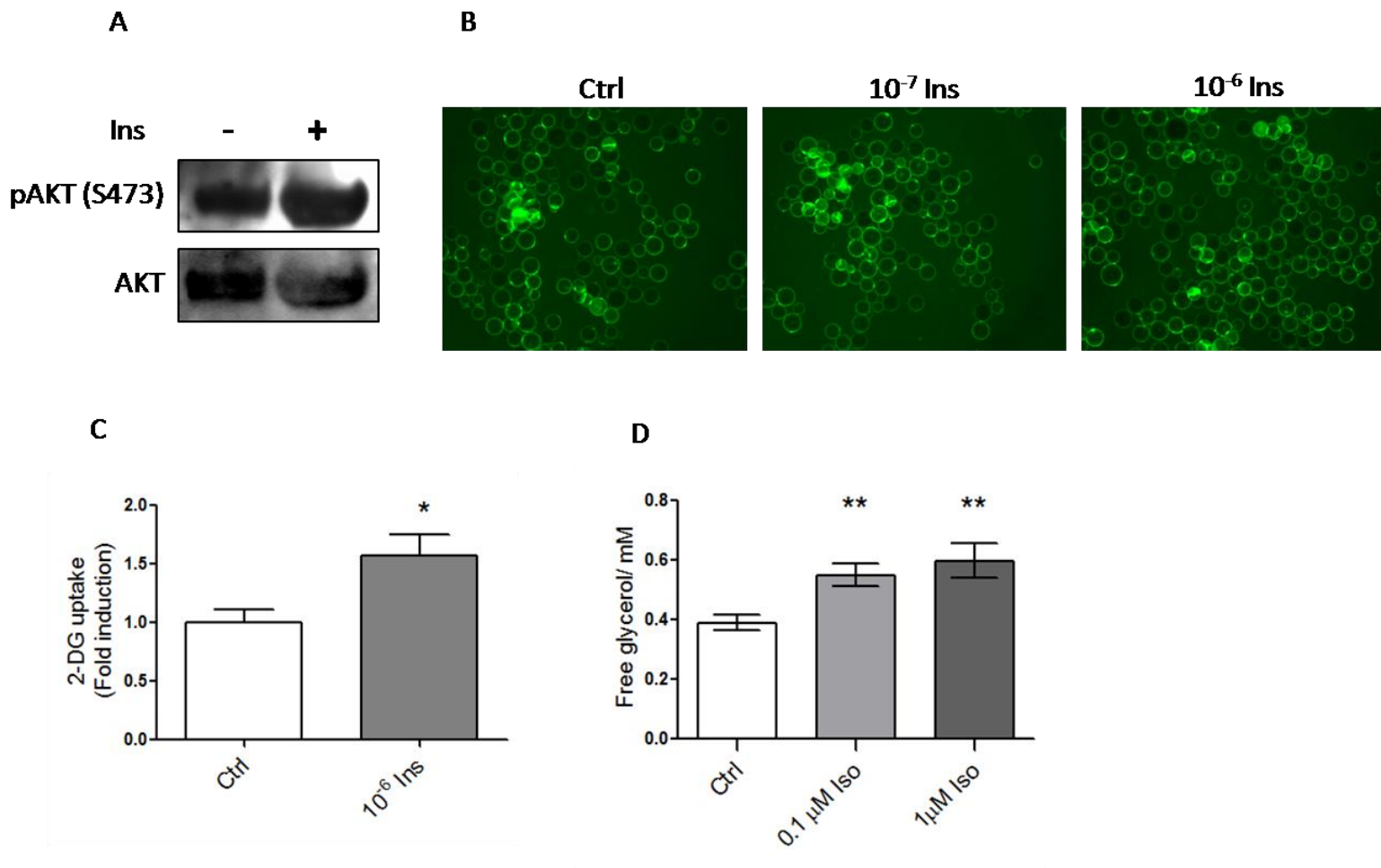


Fig 3: Functional investigation of adipocyte *in vitro*.

(A) Adipocytes at D5 of culture were previously starved in DMEM overnight followed by stimulation of 100nM insulin. Phosphorylation of Akt and total Akt were detected with immunoblot. (B) Adipocytes at D2 of culture were recovered into 1.5 ml tube, rinsed with DMEM, and then were stimulated with different concentration of insulin for 45 min, followed by incubation with 200mM 2-NBDG for 60 min. (C) Adipocytes at D5 of culture were previously starved in DMEM overnight followed by stimulation of 100 and 1000nM insulin. [³H] 2-deoxy-D-glucose was used to evaluate glucose uptake. (D) Adipocytes at D8 of culture were previously starved in DMEM for 4h followed by stimulation of 0.1 and 1 μ M isoproterenol. Glycerol release in the medium was determined. Data represent the mean \pm SEM, *P<0.05, **P<0.01.

4. Characterization of adipocytes during chronic culture *in vitro*

The results of PLIN1 and BODIPY double staining demonstrated that there was a preserved expression of PLIN1 on the surfaces of LDs inside adipocytes as long as at D13 of *in vitro* culture (**Figure 4A**), suggesting that the LDs might be stable during the chronic *in vitro* culture. Therefore, we then decided to further characterize the influence of chronic *in vitro* culture on adipocytes by studying their cell sizes and gene expressions.

Interestingly, characterization of the alterations in adipocyte size at D1, D3, D6 and D9, revealed that the average size of adipocytes had a trend to increase and peak at D6, after which it dropped again (**Figure 4B**) (N=1). Since the size of adipocytes reflects mainly the size of LDs inside adipocytes, which is mostly determined by the balance between lipolysis and lipogenesis. Thus, the possible alterations in adipocyte size might suggest that the cultured fat cells remain dynamic in terms of lipolysis and lipogenesis. To help find more clues about the effects of *in vitro* culture on adipocytes, RT-qPCR was performed to study the genetic profile of cultured adipocytes at D1, D3, D6 and D9. The adipocyte lipid binding protein *aP2* (*fatty acid binding protein (FABP) 4*) gradually increased and peaked at D6, after which it dropped back. It was also the same trend for other genes like *Adiponectin*, *Cebpa*, *Ppar γ ₂*, *Pgc1 β* , *CD36* and *Fatp* (*fatty acid transport protein*) (**Figure 4C**). These results might coincide with the morphological alterations of these fat cells (**Figure 4B**). However, genes like *Leptin* and *Glut4* surprisingly and strangely kept decreasing at the onset of culturing (**Figure 4C**). The reasons why these were abnormal alterations of genes are not fully understood.

Nevertheless, we then went on to see whether this system is applicable to human adipocytes. Preliminary results show that cultured human adipocytes had a much yellow appearance and could maintain their spherical morphology as long as at D11 (**Figure 4D**).

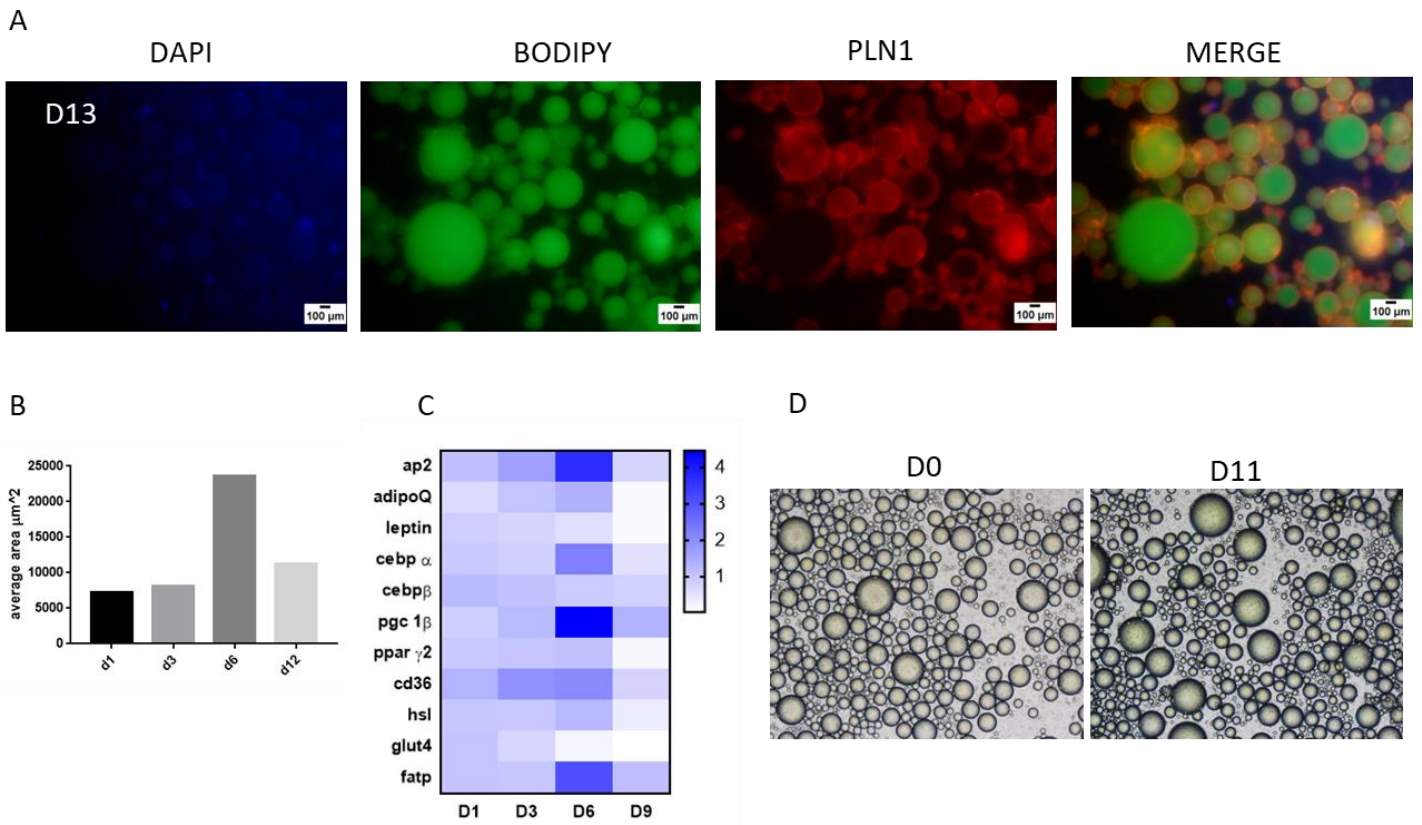


Fig 4: Characterizations of adipocytes during chronic culture *in vitro*.

(A) Triple stainings of adipocytes with BODIPY, PLIN1 and DAPI at the D13 of *in vitro* culture. (B) Alterations of cell sizes during culture. (C) Alterations of gene expressions in adipocyte during culture. (D) Applications of the method to culture human adipocytes.

CONCLUSION AND PERSPECTIVE

With the help of mechanical sequestering by cell culture inserts and supplement of glycerol into culture medium, we firstly reported that isolated adipocytes were able to remain spherical 3D structure for as long as nearly 2 weeks.

PHK26 dye staining demonstrated the integrity of plasma membrane of cultured adipocytes, with DAPI detecting a very small and peripherally located nucleus after chronic culture. Dual staining of BODIPY and PLIN1, demonstrated that the preserved expression of lipid coating protein on the surface of LDs inside cultured adipocytes as long as 2 weeks of *in vitro* culture, indicating of preserved stability of LDs inside adipocytes with our novel culture system.

The protection of glycerol supplement to adipocytes might due to the increased tonicity of hypertonic culture medium, which then modified the osmosis of water into the limited cytosol of adipocytes and thereby maintained the homeostasis of adipocytes cytoplasm and then the survival rates of the adipocytes. Next, the pro-apoptotic effects of high concentrations of FBS (20 to 25%) on the other hand suggested that hypertonicity of medium by FBS might lead to dehydration of adipocyte cytosol and thereby cell death. All these data indicated that the balance of the tonicity of culture medium is vital from culture mature adipocytes *in vitro*.

Moreover, functional analysis revealed that cultured fat cells were responsive to insulin stimulated Akt phosphorylation and glucose uptake, as well as responsive to catecholamine stimulation demonstrated by increased lipolysis. Genomic analysis revealed that there were abnormalities in terms of gene expressions with present culture conditions. They might be related to the effects of collagen digestion and need to be further investigated. The reasons why these are abnormal alterations of genes were not fully understood. However, several factors might contribute to the above phenomenon:

1) Trauma from collagenase digestion.

The process of isolation of adipocytes from adipose tissue with collagenase A digestion was firstly set up by Rodbell M. in 1964³⁴⁵. Since then, this method was widely applied for the study of adipocyte biology with minor modifications. However, precise actions of the digestion process *per se* on adipocytes are largely unclear. We tried to evaluate the effects of digestion on the viability of

adipocytes by staining isolated adipocytes with trypan blue shortly after digestion process. The result showed that most of the isolated adipocytes were positively stained at only one point of cell surface by trypan blue (data not shown), a dye that is endocytosed only by cells with impaired plasma membranes, indicating that digestion process *per se* might lead to reduced viability of adipocytes and thereby affecting their genome integrity during the *in vitro* culture thereafter.

2) Toxicity from fatty acids from broken adipocytes.

One of the limits of the present culture system with cell insert is that it could not prevent certain adipocytes from escaping through the peripheral rim covered without cell insert, which later would float on top of medium and break due to surface tension between air and medium, releasing large amount of lipids onto the medium. Therefore, lipid toxicity on adipocytes from broken adipocytes could not be excluded. To resolve this possibility, supplementation of BSA (free fatty acid-free) into medium might be helpful to neutralize spilled lipids from dead adipocytes.

3) Some nutrients or hormones are lacking in the medium to help preserve the genomic integrity of fat cells.

In vivo, the physiology of adipocytes is elegantly maintained by various hormones like insulin, leptin, catecholamine, etc. Therefore, it is plausible that proper supplementation of a cocktail of hormones might be necessary to maintain the normal gene expressions of cultured adipocytes.

In sum, for the first time, we demonstrated the possibility to culture isolated adipocytes *in vitro* with preserved morphology and LDs coating protein expressions as long as 2 weeks by glycerol supplementation. Functional studies confirmed that the cultured adipocytes were responsive to insulin and catecholamine stimulation. However, intriguing genetic profiles at different stage of *in vitro* culture, indicated that this technique is still at a preliminary stage with uncertainties, thereby might not be recommended for formal experiments.

Nevertheless, we imagine that in the future with progress in our technique protocol, we could directly modulate gene expressions in cultured adipocytes by routine gene therapy techniques like siRNA or CRISPR, which will better mimic *in vivo* situations.

MATERIALS AND METHODS

Isolation and culture mature adipocytes

Cell culture insert transparent PET membrane 12well 1.0µm pore size (Falcon, corining) were previous covered with 50µg/ml type I collagen (Santa Cruz, sc-136157) dissolved in 0.02N acetic acid for 1h at room temperature. Inserts were rinsed well with PBS and air dried under laminar flow hood before usage. Perigonadal adipose tissue was dissected from C57BL/6J background mice in accordance with the guidelines of the local ethical committee. Adipocytes were isolated after digestion in 1 mg/mL collagenase A (Roche, USA) for around 40minutes at 37°C in a heat bath with brief shaking at intervals. The digested tissue was filtered through a 100µm nylon mesh. The filtered adipocyte were washed four to five times with DMEM/F12 containing 20% fetal bovine serum (FBS, Gibco®). The floating layer of adipocytes was isolated for culture. 300µl DMEM/F12 supplemented with 20 percent fetal bovine serum were seeded into each well of 12 well plates to cover the surface. Then, 30 µL of packed and floated fat cells were carefully seeded. After, 300ul of culture medium were carefully added into the upper side of the insert. Medium was changed every two days using 1ml syringe equipped with 23G needle. Cells were routinely cultured in 5%CO₂, 37°C. Cells were observed with light microscopy.

PLIN1/ BODIPY staining

PLIN1/ BODIPY staining were performed with commercial kits (PROGEN and ThermoFisher Scientific, respectively) with slight modifications. Briefly, cells were rinsed with PBS, fixed with 100 percent methanol for 5 mins on ice, and then removed the methanol and rinsed cells with PBS twice, and keep the cells on ice for 15 mins. Primary antibody of perilipin was diluted in 1 to 200 inside 1 percent of BSA inside PBS. Carefully lay the primary antibody on the membrane of MILLICELL insert and incubated at 4 degree for overnight. Removed the primary antibody from the upper or down of the membrane, rinsed 3 times with PBS. Fluorescent second goat anti-Rabbit antibody (InvivoGen), which was diluted at the ratio of 1 to 100 in 1 percent BSA in PBS was incubated with cells at RT for 30mins in dark. After, cells were rinsed with PBS for 3 times, and further incubated with Bodipy (10l/ml) and Hoechst (1 to 50000) solution for another 30 min. Pictures were taken with microscopy.

TUNEL assay

Apoptotic or necrotic cells were detected by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using TUNEL Assays kit from R&D Systems.

Western blot

Cells were lysed with Laemmli buffer containing 0.2 % SDS. Proteins were electrophoresed on 15 % SDS-polyacrylamine gel and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked and incubated with primary antibodies in 5 % BSA in PBST overnight at 4°C. Blots were rinsed and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (Sigma). Immunoreactivity was detected by Amersham ECL prime Western Blotting Detection Reagent (GE Healthcare) and visualized with the ChemiDoc XRS+ system (Bio-Rad). Information about sources of antibodies was listed as following: GAPDH (Ref: mAb #2118, Cell Signaling TECHNOLOGY (CST)); Phospho-Akt (ser473) (Ref: 9271); AKT (Ref: 9272, CST).

Quantitative real-time PCR

Total RNA was isolated from cultured adipocytes, using MasterPure™ RNA purification kit (Epicentre, USA) according to manufacturer's instructions. The cDNA templates were prepared by reverse transcription of RNA (500ng) using High-Capacity cDNA Reverse Transcription kit (ThermoFisher scientific, USA) and gene expressions were measured using a MESA blue qPCR™ Mastermix plus for SYBR® assay (Eurogentec, Belgium). The amplification data were analyzed using Viia7 (Applied Biosystems).

Lipolysis assay

10µl of packed adipocytes were seeded into each well of corning® HTS transwell® 96 well permeable supports, pore size of which was 3.0 µm (sigma, cat. No.:CLS3385). At the 8th day of culture, 100 µl lipolysis buffer (KRBS buffer plus 0.1% glucose and 3.5% fatty acid-free bovine serum albumin, sigma) containing 0 or 10⁻⁶ isoproterenol was added into the 96-well plate for 4h at 37°C. The glycerol assay (Sigma) was used to measure glycerol concentrations in the medium, as manufacturer's instructions.

Glucose transport

Cells were starved in serum-free DMEM without glucose for 6 h. Cells were then transferred into 1.5 mL eppendorf for incubation with or without insulin in Krebs-Ringer phosphate buffer (pH 7.4) supplemented with 3% fatty acid-free bovine serum albumin (Sigma) for 45 minutes at 37°C. Transport was started by adding 4 μ Ci / 1 ml [³H]2-deoxy-D-glucose (NEN, Boston, MA) for 10 minutes precisely at 37°C and stopped by addition of cytochalasin B (Sigma). After aliquots of adipocytes were then transferred into microcentrifuge tubes with 200 μ l and then separated from incubation buffer by brief centrifugation and were used for liquid scintillation counting. Resting aliquots of adipocytes in incubation buffer were used for DNA detection with Quanti-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher). Final results of DMP were then normalized to DNA levels.

2-NBDG uptake

Cultured adipocytes were recovered into 1.5 mL eppendorf, rinsed briefly with PBS, and then incubated in DMEM containing 0 or 100 nM insulin for 60 min at 37°C. Then, 250 nM 2-NBDG was incubated with cells for another 30 min at 37°C. Fluorescent uptake of 2-NBDG were imaged with microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, USA). Adipocyte sizes were analyzed with ImageJ software. $P < 0.05$ was considered statistically significant.

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