

**Exploring Novel Anti-Obesity Strategies:
Nutritional and Pharmacological Interventions to Induce
Browning and Brown Adipogenesis**

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Doctor of Philosophy
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ABSTRACT

Obesity is a global pandemic and its comorbidities are the top causes of preventable death in America. Brown and inducible beige fat can significantly contribute to energy expenditure, possibly leading to weight loss, and is considered a novel approach to combat obesity. Browning of white adipocytes can be achieved using cold exposure, β [beta]-adrenergic receptor (**β -AR**) and peroxisome proliferator-activated receptor gamma (**PPAR γ**)-agonists, and some nutritional and pharmaceutical agents. *Trans*-resveratrol (**Res**) and indomethacin (**Indo**) have shown promising abilities to promote browning.

Res has been shown to induce browning *in vitro* and *in vivo*; however, its rapid metabolism and poor aqueous solubility reduce bioavailability, limiting its clinical utility. Therefore, two forms of nanocarriers were synthesized to encapsulate Res (**RNano** and **RLipo**). Both forms led to enhanced aqueous solubility, chemical stability, and sustained release patterns (**Chapter III**). Both RNano and RLipo significantly enhanced browning of murine 3T3-L1 adipocytes as shown by increased uncoupling protein 1 (**Ucp1**) and PPAR γ coactivator-1 alpha (**Pgc1 α** [alpha]) mRNA expression when stimulated by β -AR-agonist, isoproterenol.

To further enhance Res effectiveness, a ligand for delta-decorin (Δ [delta]**DCN**), a receptor specific to adipose stromal cells (**ASCs**) within white adipose tissue (**WAT**), was incorporated into nanocarriers (**L-NR**) (**Chapter IV**). Freshly isolated ASCs from the inguinal WAT of C57Bl/6J mice were used to confirm Δ DCN protein expression, enhanced uptake of the ligand-bound nanocarrier, and augmented browning using two different differentiation models.

Finally, the browning and brown adipogenic capacity of the non-steroidal anti-inflammatory drug, Indo, was tested using murine 3T3-L1, human primary subcutaneous (**HPsubQ**), and human brown (**HBr**) adipocytes. Indo is shown to have strong partial-PPAR γ -agonist activities (**Chapter V**). It was found that Indo dose-dependently promoted browning in 3T3-L1 and HPsubQ adipocytes as well as enhanced brown adipogenesis in HBr adipocytes via its PPAR γ -agonist activity as demonstrated by increased UCP1 expression, mitochondrial biogenesis, proton leak, and respiratory capacity.

In conclusion, we have shown enhanced browning capacity of Res using nanocarriers, which is augmented by the addition of ASC-targeting peptides as well as browning and brown adipogenesis induced by Indo. Given these results, these compounds can be considered novel browning agents to help combat obesity.

TABLE OF CONTENTS

CHAPTER I: INTRODUCTION.....	1
1.1 REFERENCES.....	4
1.2 APPENDIX.....	6
CHAPTER II: LITERATURE REVIEW.....	7
2.1 DEFINING OBESITY AND RECOGNIZING ITS PREVALENCE.....	8
2.2 ADIPOSE TISSUE BIOLOGY AND FUNCTION.....	9
2.3 RESVERATROL AS A NUTRITIONAL INTERVENTION TO COMBAT OBESITY.....	24
2.4 NANOPARTICLES CONTAINING RESVERATROL AS A NUTRACEUTICAL INTERVENTION FOR OBESITY.....	42
2.5 THE ANTI-OBESITY POTENTIAL OF INDOMETHACIN.....	46
2.6 REFERENCES.....	52
2.7 APPENDIX.....	80
CHAPTER III: RESVERATROL LIPOSOMES AND LIPID NANOCARRIERS: COMPARISON OF CHARACTERISTICS AND INDUCING BROWNING OF WHITE ADIPOCYTES	85
3.1 ABSTRACT.....	88
3.2 INTRODUCTION.....	89
3.3 MATERIALS AND METHODS.....	90
3.4 RESULTS AND DISCUSSION.....	95
3.5 CONCLUSIONS.....	102
3.6 REFERENCES.....	104
3.7 APPENDIX.....	107
CHAPTER IV: TARGETING NANOPARTICLES ENCAPSULATED WITH RESVERATROL INDUCE BROWNING OF MURINE PRIMARY SUBCUTANEOUS WHITE ADIPOCYTES.....	117
4.1 ABSTRACT.....	118
4.2 INTRODUCTION.....	119
4.3 MATERIALS AND METHODS.....	121
4.4 RESULTS	125
4.5 DISCUSSION.....	129
4.6 CONCLUSIONS AND LIMITATIONS.....	134
4.7 REFERENCES.....	135

4.8 APPENDIX.....	140
CHAPTER V: INDOMETHACIN PROMOTES BROWNING AND BROWN	
ADIPOGENESIS IN BOTH MURINE AND HUMAN FAT CELLS.....	146
5.1 ABSTRACT.....	147
5.2 INTRODUCTION.....	148
5.3 MATERIALS AND METHODS.....	149
5.4 RESULTS	154
5.5 DISCUSSION.....	158
5.7 REFERENCES.....	164
5.8 APPENDIX.....	167
CHAPTER VI: CONCLUSIONS AND FUTURE DIRECTIONS.....	
6.1 CONCLUSIONS.....	195
6.2 SIGNIFICANCE OF STUDY.....	196
6.3 LIMITATIONS AND FUTURE DIRECTIONS.....	197
6.4 REFERENCES.....	199
VITA.....	200

LIST OF TABLES

CHAPTER III

Table 3.1. Primer sequences for qRT-PCR using SYBR green.....	116
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CHAPTER V

Table 5.1. Murine primer sequences for qRT-PCR using SYBR green	192
Table 5.2. Human primer sequences for qRT-PCR using SYBR green	193
Table 5.3. Murine mitochondrial DNA primer sequences for qRT-PCR using SYBR green	193

LIST OF FIGURES

CHAPTER I

Figure 1.1. Dissertation project flow diagram.....	6
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CHAPTER II

Figure 2.1. Regulators of adipogenesis: a cascade of signaling events.....	80
Figure 2.2. Anatomical locations of brown and white adipose tissue in humans and mice.....	81
Figure 2.3. Uncoupling of oxidative phosphorylation.....	82
Figure 2.4. Morphological and transcriptional differences between WAT, BAT, and beige adipocytes.....	83
Figure 2.5. Resveratrol effect on lipid and glucose clearance.....	84

CHAPTER III

Figure 3.1. Characteristics of Rnano and Rlipo.....	107
Figure 3.2. Chemical stability of native Res, Rnano, and Rlipo under the light (A) or the dark (B) at different temperatures.....	108
Figure 3.3. Physiochemical characterization.....	109
Figure 3.4. <i>In vitro</i> release profiles.....	110
Figure 3.5. Cytotoxicity and Res content in 3T3-L1 cells.....	111
Figure 3.6. Activation of PPAR response reporter by various forms of Res.....	112
Figure 3.7. Browning activities.....	113
Figure 3.8. Gene expression of white and beige adipocyte markers.....	114
Figure S.3.1. Gene expression of mitochondrial biogenesis markers.....	115

CHAPTER IV

Figure 4.1. Greater rhodamine uptake of ligand-bound nanoparticles by Δ DCN cells....	140
Figure 4.2. Enhanced resveratrol-induced PPARE and PPAR γ transactivation by	

ligand-bound nanoparticles in Δ DCN cells.....	141
Figure 4.3. Detection of decorin protein expression in murine stromal cells from subcutaneous fat.....	142
Figure 4.4. Enhanced uptake of ligand-bound nanoparticles by murine stromal cells.....	143
Figure 4.5. ASC-targeting nanoparticles enhance resveratrol's efficiency to promote browning at lower, physiologically relevant doses.....	144
Figure 4.6. Ligand-bound nanoparticles encapsulated with resveratrol promote browning at higher doses when treating fully differentiated murine stromal cells.....	145
CHAPTER V	
Figure 5.1. Indomethacin does not cause toxicity and enhances adipogenesis and lipid accumulation in 3T3-L1 cells.....	167
Figure 5.2. Indomethacin promotes browning as well as mitochondrial biogenesis in 3T3-L1 adipocytes.....	170
Figure 5.3. Suppression of PPAR γ and ADRB3 by Rosi and Indo in differentiating 3T3-L1 adipocytes.....	174
Figure 5.4. Timely removal of Rosi and Indo rescues browning phenotype in 3T3-L1 adipocytes.....	177
Figure 5.5. Indomethacin significantly enhances oxygen consumption in 3T3-L1 adipocytes.....	180
Figure 5.6. Indomethacin dose-dependently activates PPAR γ , deficiency of which attenuates browning.....	183
Figure 5.7. Indomethacin induces browning in primary stromal cells derived from human subcutaneous fat.....	186
Figure 5.8. Indomethacin promotes brown adipogenesis in a human brown cell line.....	189

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ACC	Acetyl CoA Carboxylase
ADP	Adenosine Diphosphate
ADRB3	Adrenergic Receptor Beta-3 (gene encoding for β 3-AR)
AF-1/-2	Activation Function-1/-2
AMPK	5' Adenosine Monophosphate (AMP) Activated Protein Kinase
ANOVA	Analysis of Variance
ASCs	Adipose Stromal Cells
AT	Adipose Tissue
α TA	Alpha-Tocopherol Acetate
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine Triphosphate
BA	Bioavailability
β -AR / β 3-AR	Beta-Adrenergic Receptor / Type 3
BAT	Brown Adipose Tissue
BAX	B-Cell Lymphoma Associated X
BBB	Blood Brain Barrier
BCL	B-Cell Lymphoma
BF%	Body Fat Percentage
β -gal	Beta-Galactosidase
BMI	Body Mass Index
BMP7	Bone Morphogenic Protein 7
BP	Blood Pressure
BSA	Body Surface Area
BTM-0512	3,5,4'-Trimethoxystilbene
BW	Body Weight
cAMP	Cyclic Adenosine Monophosphate
CD137 or TNFRRS9	Tumor Necrosis Factor Receptor Superfamily, Member 9
CDC	Center for Disease Control
CDK	Cyclin Dependent Kinase
CE	Cold Exposure
C/EBPs	CCAT Enhancer Binding Proteins

C _{free}	Concentration of Free-Res
CITED1	Cbp/p300-Interacting Transactivator with Glu/Asp-rich Carboxy-Terminal Domain 1
CLS	Crown-Like Structure
C _{max}	Max Concentration (Peak Plasma Levels)
CNP	Cardiac Natriuretic Peptide
COX-1/-2	Cyclooxygenase Type 1 / 2
COX4A	Cytochrome C Oxidase Subunit IV Isoform 1
CPT1	Carnitine Palmitoyltransferase 1
CR	Calorie Restriction
CREB	cAMP Response Element Binding Protein
CS	Calf Serum
C _{total}	Total Res Concentration
CVD	Cardiovascular Disease
CYP1A1	Cytochrome P450 family 1 type A1
CYTC	Cytochrome Complex
DAPI	4', 6-Diamindine-2'Phenylindole Dihydrochloride
DBD	DNA Binding Domain
DCN	Decorin
ΔDCN	Delta-Decorin
Dex	Dexamethasone
DHAP	Dihydroxyacetone Phosphate
DH-RES	Dihydroxy-Resveratrol
DI	Deionized Water
DIO	Diet Induced Obesity
DIO2	Deiodinase Iodothyronine Type 2
DMEM	Dulbecco's Modified Eagle's Medium
DSC	Differential Scanning Calorimetry
EBF2	Early B-cell Factor 2
EC ₅₀	Half-Maximal Effective Concentration
ECM	Extra-cellular Matrix
EE	Energy Expenditure
ER	Estrogen Receptor
ETC	Electron Transport Chain

FABP4	Fatty Acid Binding Protein 4
FAO	Fatty Acid Beta-Oxidation
FASN	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FDA	Federal Drug Administration
FDG	Fluorodeoxyglucose
FFA	Free Fatty Acid
FR	Free-Resveratrol
G3P	Glycerol-3-Phosphate
GI	Gastrointestinal
GLUT4	Glucose Transporter Type 4
GPDH	Glycerol-3-Phosphate Dehydrogenase
GR	Glucocorticoid Receptor
GSK3 β	Glycogen Synthase Kinase 3 beta
GYK	Glycerol Kinase
HATs	Histone Acetyl Transferase
HBr	Human Brown Cell Line
HDACs	Histone Deacetylase
HEDs	Human Equivalent Dose
HFD	High Fat Diet
HPLC	High-Performance Liquid Chromatography
HPSubQ	Human Primary Subcutaneous Adipocytes
HRP	Horse Radish Peroxidase
HSL	Hormone Sensitive Lipase
IBMX	3-Isobutyl-1-Methylxanthine
IC	Intracellular
IC ₅₀	Half-Maximal Inhibitory Concentration
IGF1	Insulin Growth Factor 1
IGFBP3	Insulin Growth Factor Binding Protein 3
IL	Interleukin
IMM	Inner-Mitochondrial Matrix
Indo	Indomethacin
IR	Insulin Receptor
IRF4	Interferon Regulatory Factor 4

IRS	Insulin Receptor Substrate
ISO	Isoproterenol
iwAT	Inguinal White Adipose Tissue
KC	Kolliphor Control
KD	Knockdown
KLF	Kruppel-Like Factor
LBD	Ligand Binding Domain
LBP	Ligand Binding Pocket
LD	Lipid Droplet
LDL	Low-Density Lipoprotein
L-NR	Ligand-Bound Nanoparticle encapsulated with Resveratrol
L-NV	Ligand-Bound Nanoparticle-Void (without Resveratrol)
L-Rhod-Nano	Ligand-Bound Rhodamine-Labeled Nanoparticle
M1	Classically Activated Macrophage
M2	Alternatively Activated Macrophage
MAPK	Mitogen Activated Protein Kinase
MCE	Mitotic Clonal Expansion
MCP1	Monocyte Chemoattractant Protein 1
MEF	Mouse Embryonic Fibroblast
MEOX2	Mesenchyme Homeobox 2
METRNL	Meteorin-Like Protein
MFN2	Mitofusin-2
mLDs	Micro-Lipid Droplets
MMP	Matrix Metalloproteinase
MNSOD	Manganese-Containing Superoxide Dismutase
MS	Metabolic Syndrome
MSC	Mesenchymal Stem Cells
mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Myf5	Myogenic Factor 5
NAFLD	Non-Alcoholic Fatty Liver Disease
N-CoR	Nuclear Receptor Co-Repressor
NE	Nor-Epinephrine
NHANES	National Health and Nutrition Examination Survey

NIH	National Institute of Health
NLC	Nanostructured Lipid Carriers
NO	Nitric Oxide
NP	Nanoparticle
NR	Nanoparticle encapsulated with Res (Chapter IV)
NRF1	Nuclear Respiratory Factor 1
NS	Non-Significant
NSAID	Non-Steroidal Anti-Inflammatory Drug
NST	Non-Shivering Thermogenesis
NV	Nanoparticle-Void (without Resveratrol)
Ob-Rb	Obesity Receptor (Leptin Receptor)
OCR	Oxygen Consumption Rate
ORO	Oil Red O
oxLDL	Oxidized Low-Density Lipoprotein
PAD	Pharmacologically Active Doses
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCL	Polycaprolactone
PDA	Photo Diode Array
PDE	Phosphodiesterase
PDH	Pyruvate Dehydrogenase
PEG	Poly-Ethylene Glycol
PET-CT	Positron-Emission Tomography–Computed Tomography
PG	Prostaglandin
PGC1 α	Peroxisome Proliferator-Activated Receptor gamma Coactivator 1 alpha
PI	Polydispersity Index
PI3K	Phosphoinositol 3 Kinase
PKA	Protein Kinase A
PLA2	Phospholipase A2
PLGA	Poly-Lactic-co-glycolic Acid
PLIN	Perilipin
PPAR	Peroxisome Proliferator-Activated Receptor
PPI	Proton Pump Inhibitor

PPRE	Peroxisome Proliferator-Activated Receptor Response Element
PRDM16	Positive Regulatory Domain Containing Protein 16
PTM	Post Translational Modifications
Q	Quercetin
RBP4	Retinol Binding Protein 4
Res	Resveratrol
Rhod-Nano	Rhodamine-Labeled Nanoparticle
Rlipo	Liposome encapsulated with Resveratrol
Rnano	Nanoparticle encapsulated with Resveratrol (Chapter III)
Rosi	Rosiglitazone
RSVA	Resveratrol Analogues
RXR α	Retinoid X Receptor alpha
SAT	Subcutaneous Adipose Tissue
SCR	Scrambled (Non-Targeting Control)
Sir2	Silent Information Regulator 2
SIRT1	Sirtuin 1
SLN	Solid Lipid Nanoparticles
SMRT	Silencing Mediator of Retinoid and Thyroid Receptors
SNS	Sympathetic Nervous System
SRC	Steroid Receptor Coactivator
SREBP	Sterol Response Element Binding Protein
SVF	Stromal Vascular Fraction
T2DM	Type 2 Diabetes Mellitus
T ₃	Triiodothyronine
T ₄	Thyroxin
TBST	Tris-Buffered Saline with Tween 20
TCF21	Transcription Factor 21
TEM	Transmission Electron Microscope
TFAM	Mitochondrial Transcription Factor A
TG	Triglyceride
TH	Thyroid Hormone
Th1	T-helper cell Type 1
TH-R β	Thyroid Hormone Receptor beta
TMEM26	Transmembrane Protein 26

TNF α	Tumor Necrosis Factor alpha
TRE	Thyroid Response Element
TSH-R	Thyroid Stimulating Hormone Receptor
TZD	Thiazolidinedione
UCP1	Uncoupling Protein 1
U.S.	United States
VAT	Visceral Adipose Tissue
VDAC1	Voltage-Dependent Anion Channel 1
Vlipo	Liposome-Void (without resveratrol)
Vnano	Nanoparticle-Void (without resveratrol)
WAT	White Adipose Tissue
WC	Waist Circumference
WT	Wild Type
XRD	X-Ray Diffractometry
ZFP423	Zinc-Finger Protein 423
ZIC1	Zinc Fingers in the Cerebellum 1

CHAPTER I:

INTRODUCTION

Obesity affects millions of people worldwide and its associated metabolic complications lead to illness and many unfortunate fatalities.¹ Obesity can be attributed to a complex interplay of modifiable (lifestyle, behavioral) and non-modifiable (age, gender, race, genetics) factors, and even some interplay between these factors (i.e., epigenetics).² However, in the simplest form, weight gain occurs when energy intake exceeds energy expenditure (**EE**), leading to excess energy being stored in the form of fat in the adipose tissue.³ A common attribute of obesity is excess intrabdominal fat storage, which is characteristic of metabolic syndrome and the ensuing dyslipidemia and insulin resistance.⁴ However, normal adipose tissue has many crucial functions as fundamental as fat storage and insulation, and as critical as playing roles in whole-body metabolism and energy balance.⁵ Two primary depots of fat are defined as white adipose tissue (**WAT**) and brown adipose tissue (**BAT**). WAT provides the body's largest storage site for energy in the form of triglycerides (**TGs**), but also functions to release adipokines which can self-signal (autocrine), locally (paracrine), and systemically (endocrine).⁶ BAT is shown to play a pivotal role in EE by rapidly metabolizing lipids and glucose to produce heat instead of energy.⁷ This heat-generating process is known as non-shivering thermogenesis and can be primarily attributed to BAT's very high expression of uncoupling protein 1 (**UCP1**) which functions to dissociate the electron chemical gradient in oxidative phosphorylation; the substrates normally used to generate ATP are released as heat instead.⁷ Brown-like or beige adipocytes are said to have functions and thermogenic capacity comparable to BAT but arise within or are induced from WAT. Just like BAT, UCP1 also aids in the thermogenic function of beige adipocytes, therefore, serving as a marker for these cells.⁸ Due to its manifestation (*de novo*) or transformation (*transdifferentiation*) within WAT, but having comparable metabolic and genetic attributes to BAT, beige adipocytes have emerged as a desirable therapeutic target for metabolic research.⁹ Creating a more energy insufficient WAT and increasing EE has made beige adipocytes a sought after means to combat obesity.^{8, 9}

Adipose stromal cells (**ASCs**) within WAT can be differentiated into beige adipocytes in response to various stimuli, such as peroxisome proliferator-activated receptor (**PPAR γ**)-agonists, beta-adrenergic receptor (**β -AR**)-agonists, cold exposure, as well as some nutritional and pharmaceutical compounds.¹⁰⁻¹² Agents which can mimic these agonists or work through these pathways could also activate the brown fat program in WAT.¹⁰ Indomethacin (**Indo**) is a non-steroidal anti-inflammatory drug (**NSAID**) which has been shown to work as a partial PPAR γ agonist, similar to that of the full agonist, rosiglitazone (**Rosi**).¹³ Rosi is a type of thiazolidinedione (**TZD**) which are commonly used as insulin sensitizing drugs in the treatment

of type 2 diabetes mellitus.¹⁴ Rosi has been shown to induce the browning program in WAT, suggesting that Indo could also induce browning through similar mechanisms.¹²

Trans-resveratrol (**Res**) is another compound which has been shown to induce browning *in vitro*,¹⁵⁻¹⁸ animal studies,¹⁹⁻²¹ as well as in human clinical trials.^{22, 23} Unfortunately, there are many claims from *in vitro* and animal studies which remain unsubstantiated or inconsistent as compared to clinical trials.^{24, 25} This could primarily be pinpointed to Res poor aqueous solubility and low bioavailability due to its rapid intestinal and hepatic metabolism.^{26, 27} Nanotechnology has been used previously to encapsulate bioactive compound to improve their chemical stability, solubility, and bioavailability.²⁸⁻³¹ Moreover, the addition of ligands or peptides on the surface of nanocarriers can be used to target specific cells or tissues, further reducing excess metabolism and removal.^{32, 33} Recently, an extracellular matrix protein and isoform of decorin lacking glycanation (**ΔDCN**) was found to be isolated to WAT-ASCs.³⁴ The interaction of ΔDCN and the peptide which can home this receptor provides a technique which can be exploited to develop a drug-delivery system for treating obesity.

In this dissertation, we will use biocompatible and biodegradable nanocarriers encapsulated with Res (**Rnano**) in order to overcome critical limitations previously discussed. To enhance targeting specificity, we will incorporate an ASC-targeting ligand on the surface of Rnano (**L-NR**). We hypothesize that (1) nanoparticle encapsulation and ligand-coating will increase aqueous solubility and chemical stability of Res (**Chapter III and IV**); (2) As compared to Rnano, L-NR will have a higher binding affinity and uptake by ΔDCN-transduced 3T3-L1 cells as well as murine stromal cells isolated from inguinal fat pads of C57Bl/6J mice (**Chapter IV**); (3) L-NR greater than Rnano greater than free-Res will have ability to brown 3T3-L1 adipocytes as well as murine primary stromal cells *in vitro* (**Chapter III and IV**); and (4) Indo will have the ability to promote browning of murine 3T3-L1 and human primary subcutaneous white adipocytes as well as human brown adipocytes in a manner similar to Rosi. The structure of this dissertation is outlined in **Figure 1.1**.

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1.2 APPENDIX

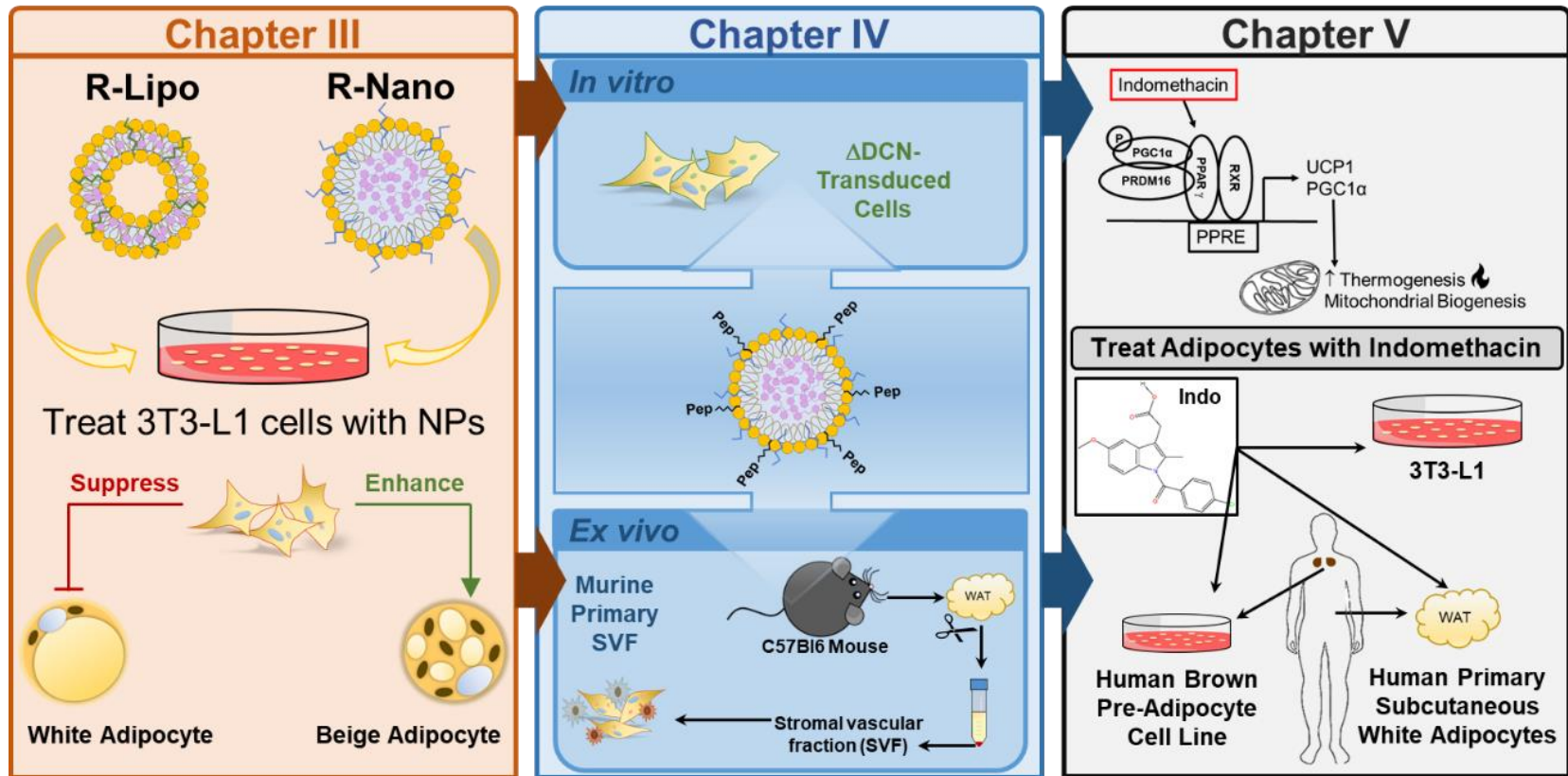


Figure 1.1. Dissertation project flow diagram.

CHAPTER II:

LITERATURE REVIEW

2.1 DEFINING OBESITY AND RECOGNIZING ITS PREVALENCE

As a consequence of accessibility and excess consumption of energy-dense foods compounded by a lack of physical exertion, obesity, and its comorbidities continue to be among the leading causes of death and illness in the United States (**U.S.**) and is becoming a major issue globally.¹⁻³ Obesity in America is considered to be at pandemic levels, and despite continued efforts, it is still growing.⁴ A 2017 report from the Center for Health and Disease (**CDC**) estimated that 71.6% of U.S. adults were overweight or obese and among these, 39.8% were obese.⁵ This number is up from the most recent National Health and Nutrition Examination Survey (**NHANES**) data brief which states that in 2015-2016, 69% of U.S. adults were overweight or obese and 34.9% were obese.³

Obesity is technically defined by having a body mass index (**BMI**) of greater than 30, which is calculated using height and body weight (**BW**) alone ($BMI = kg/m^2$).⁶ However, many factors weigh into the variances of BMI, including race, sex, age, hydration and disease status, etc.^{6,7} Although useful in most adults, without other anthropometric measures, BMI has several limitations. For example, BMI can overestimate body fat percent (**BF%**) in very lean and muscular individuals as well as underestimate BF% in elderly individuals that have severely lost lean body mass.⁸ Moreover, individuals could be categorized as having a “normal BMI”, but carry more abdominal fat, known as visceral adipose tissue, which is highly associated with insulin resistance and other metabolic diseases.^{9,10} Therefore, waist circumference (**WC**) is used in conjunction with BMI to provide a better assessment of metabolic health than BMI alone.¹¹ A WC of >35 inches for women and >40 inches for men indicate abdominal obesity (apple/android distributed fat) and is therefore associated with obesity-related metabolic diseases.¹²

Obesity itself is not a fatal disease; rather, its comorbidities top the list of causes of preventable deaths.⁵ Obesity’s associated diseases include type 2 diabetes mellitus (**T2DM**), cardiovascular disease (**CVD**), metabolic disease, and some types of cancer.¹³ Creating a negative energy-balanced state by way of consuming an energy-restrictive, well-balanced diet with an emphasis on plant-based foods, along with including regular physical activity, although the foremost and best-characterized means to treat and prevent obesity, is not always feasible.⁶ Extreme and even moderate weight loss can be difficult to achieve and even more difficult to maintain.¹⁴ Other weight loss methods, such as bariatric surgeries, can be extreme and invasive, and not all individuals may meet the requirements for these surgeries, but still need

help.¹⁴ Certain disease states, disability, and aging are among many obstacles which may further hinder progress.¹⁴

There is no one single cause for obesity; rather, it is a collection of multiple factors including genetics, behavior, lifestyle, environment, and even the interplay of genetics and environment (epigenetics) which work in concert.^{15, 16} To better understand obesity pathology, it is critical to understand adipose tissue biology and function, as well as its dysfunction.

2.2 ADIPOSE TISSUE BIOLOGY AND FUNCTION

Adipose tissue (**AT**) is an integral organ with specialized physical, metabolic, and endocrine functions. Physically, AT helps provide insulation and cushion to internal organs, providing another layer to the body's 'physical barrier' from the outside world.¹⁷ Previously thought to be an inert tissue, AT is now recognized as a high-rolling player in whole-body metabolism, storing and releasing lipids based on energy needs, helping to control appetite, as well as helping to regulate angiogenesis, blood pressure, immune function, and energy expenditure (**EE**).¹⁸ Its activity as an endocrine/secretory organ can be considered a relatively recent discovery, in the history of AT biology.^{19, 20}

While the normal function of AT acts as a critical energy buffer, extreme excess of AT (obesity) leads to dysfunction (insulin resistance and the ensuing inflammatory response).²¹ AT is primarily composed of lipid-laden adipocytes with remarkable, but limited expandability.²² Based on energy demands, pluripotent stem cells from mesenchymal origin can differentiate into adipocytes with the proper stimulation.²³ Although adipocytes comprise most of the total volume of AT, they only make up about 25% of the total cell population.²⁴ The stromal vascular fraction (**SVF**) of AT is also composed of a variety of cell types, including immune cells such as macrophages, lymphocytes, and pericytes as well as preadipocytes (or adipose stromal cells (**ASCs**)), fibroblasts, endothelial cells, and blood cells all of which highly impact its metabolic function²⁴ and may be the primary source of AT secretory factors.²⁵

Two primary types of fat which are widely recognized can be classified as white adipose tissue (**WAT**) and brown adipose tissue (**BAT**). One of WAT's primary functions which is considered the 'classically characterized AT role', is to store fat for future use, whereas BAT generates heat via non-shivering thermogenesis (**NST**).²⁶ The relatively recent discovery of a third type of fat, called beige fat, has emerged and is shown to have similar functions as BAT, but found within WAT.^{26, 27} With a relatively greater abundance of mitochondria and enhanced thermogenic capacity, the inducibility of beige adipocytes from WAT by using various

nutraceutical and pharmaceutical compounds has become a promising approach to use as an anti-obesity therapy.²⁶ Adipose biology, physiology, function, and dysfunction in the context of obesity, as well as regulation of white, brown, and beige fat, is reviewed here.

2.2.1 White adipose tissue and adipocytes

As previously discussed, the primary functions of WAT include insulation, mechanical cushion, and storage.²⁸ Although WAT does not conduct heat well, the extra layer provides internal organs insulation and protection from trauma.²⁸ WAT also provides the largest reservoir of energy in the human body.²⁸ AT is not found in discrete compartments, rather it is distributed throughout the body and can be found in, on, and around most organs.²⁹ With that said, there are major depots of fat, including under the skin, or subcutaneous adipose tissue (**SAT**), as well as around the organs, or visceral adipose tissue (**VAT**).²⁹ SAT typically comprises about 70-90% of total body fat, whereas VAT comprises about 10-30%.^{24, 29} The over-expansion of VAT is highly associated with dyslipidemia and insulin resistance.³⁰

WAT controls energy balance primarily by managing the storage and release of free fatty acids (**FFA**).³¹ FFA are stored in adipocytes as triglycerides (**TG**) into lipid droplets (**LD**) which then accumulate and merge to form large unilocular LDs.³¹ This process of lipogenesis, lipid accumulation, differentiation, and maturation is known as adipogenesis.^{18, 32} LDs are coated in various types of LD-associated proteins including adipophilin, TIP47, OXPAT/MLDP, S3-12, and the most abundant of these are isoforms of perilipin (**PLIN**).³³ These LD-associated proteins stabilize LDs, incorporate new TGs, and prevent TG release in energy-replete states while maximizing hormonally stimulated lipolysis with the help of hormone-sensitive lipase (**HSL**) and adipose TG lipase (**ATGL**) during energy-depleted states.³³ Mature adipocytes in WAT are said to originate from mesenchymal stem cells (**MSCs**) not expressing myogenic factor 5 [(**Myf5**)-negative], which then undergoes two phases during adipogenesis: determination and differentiation.^{18, 34, 35} A pluripotent stem cell which has committed to adipocyte lineage loses the ability to differentiate into other cell types (i.e., chondrocytes, osteocytes, myocytes, etc).^{36, 37} Once cells are committed, a series of morphological and biochemical changes marks the inception of adipocyte differentiation, during which the cell acquires the equipment necessary to synthesize and store lipids, respond to insulin for nutrient uptake and storage, and to synthesize and secrete adipocyte-specific factors.^{18, 38}

During adipogenesis, peroxisome proliferator-activated receptor gamma (**PPAR γ**) and CCAT-enhancer-binding proteins (**C/EBPs**) play critical roles in the adipogenic transcription

cascade.^{39, 40} PPAR γ is known as the master regulator of adipogenesis and has two protein isoforms; PPAR γ 1 (55 kDa), which is constitutively expressed and PPAR γ 2 (57 kDa), an AT-specific isoform.^{41, 42} Interestingly, PPAR γ 2 expression is inversely correlated with BMI and weight, whereas higher expression of PPAR γ 1 in SAT is associated with hyperinsulinemia and insulin resistance.⁴³ At any rate, it is known that PPAR γ and C/EBPs display critical cross-talk with each other as well as other transcription factors such as kruppel-like factor (**KLF**)-5 and KLF-15 and sterol response element binding protein (**SREBP**) to promote adipogenesis.⁴⁴⁻⁴⁶

Figure 2.1. demonstrates positive and negative regulators of adipogenesis (all figures for Chapter II are located in 2.7 Appendix).

From experience with cultured preadipocytes, it is understood that a combination of hormones is required to promote terminal differentiation.^{38, 50} When murine preadipocyte cell line, 3T3-L1 cells, are exposed to signals such as insulin, insulin-like growth factor 1 (**IGF-1**), and cyclic AMP (**cAMP**), they undergo mitotic clonal expansion (**MCE**) after having reached confluence prior to succumbing to growth arrest and subsequent differentiation.^{38, 50} Interestingly, there is cAMP-dependent ligand activity during the first two days of differentiation in 3T3-L1 cells, thereby also requiring cAMP Response Element Binding protein (**CREB**).^{18, 46} This is said to be needed to induce differentiation, but elevated levels of cAMP are not needed to maintain PPAR γ -dependent differentiation.⁵¹ For this reason, 3-isobutyl-1-methylxanthine (**IBMX**) is used, among other differentiation-stimulating agents, in the induction media of 3T3-L1 cells.⁵² IBMX is a phosphodiesterase (**PDE**)-inhibitor and is therefore called the “cAMP elevating agent”.⁵³ This cAMP-dependent differentiation is only present within the first 48 h, making the timing of reagent exposure critical.⁵⁴ The differentiation cocktail of IBMX, insulin, and dexamethasone (a corticosteroid which activates glucocorticoid receptor (**GR**)) causes a transient but dramatic increase in C/EBP β/δ which further commits the cells to differentiate and causes a cell cycle switch (G1 $\rightarrow$ S phase).⁵⁴⁻⁵⁶ Glycogen synthase kinase 3 beta (**GSK3 β**) and mitogen-activated protein kinase (**MAPK**) then further activate C/EBP β by hyperphosphorylation, leading to induction of PPAR γ and C/EBP α expression and activation.^{57, 58} PPAR γ and C/EBP α then activate their downstream differentiation targets, including a positive feedback loop further activating themselves.⁵⁹ Cyclin D3 then phosphorylates and further activates C/EBP α which inhibits proliferation and the cells enter the final steps of differentiation.⁵⁹

2.2.2 Adipose tissue as an endocrine organ

The relatively recent discovery of adipocyte-specific secretory proteins has led to the conclusion that AT is much more than a storage site for fuel, rather, it is a specialized organ with critical endocrine functions.⁶⁰ WAT produces a number of secretory factors such as adipokines and chemokines, among others, which are shown to have both local and systemic effects.⁶⁰ Synthesis and secretion of adipokines such as leptin, adiponectin, visfatin, resistin, omentin, among others acts as signals to convey the status of body fat depots and plays roles in whole-body energy regulation.^{61, 62} These adipokines have a multitude of downstream effects ranging from affecting WAT insulin sensitivity, angiogenesis, inflammation, adipocyte differentiation, and energy balance.^{62, 63} Other biologically active peptides and inflammatory cytokines are released from both the adipocytes and other cells within the AT microenvironment including monocyte chemoattractant protein 1 (**MCP1**), lipocalin 2, fibroblast growth factor 21, retinol-binding protein 4 (**RBP4**), tumor necrosis factor-alpha (**TNF α**).^{64, 65} However, among these adipose-secreted factors, leptin is highly recognized to both be preventative and a contributing factor in the context of obesity and is therefore discussed further.⁶⁶

Leptin is primarily synthesized and secreted by AT, although it can be synthesized by some other tissues, and secretion typically occurs in proportion to AT abundance, but its secretory patterns can be altered based on food intake.⁶⁶ Leptin is highly intertwined in whole-body metabolism by regulating food intake and increasing EE.⁶³ Regulation of EE by leptin is caused by stimulated secretion of norepinephrine (**NE**) which leads to thermogenesis.^{67, 68} Leptin helps to regulate food intake by suppressing appetite by signaling the leptin receptor (or obesity receptor (**Ob-Rb**)); which is a member of the class I cytokine receptor family located in the hypothalamus.⁶⁹ This signaling relays an energy replete state to the hypothalamus, reducing the hunger-response.⁶⁶ Leptin resistance occurs in obesity in response to excess leptin secretion overwhelming the Ob-Rb, which effectively diminishes response to the anorexigenic hormone, leading to hyperphagia.⁷⁰ In fact, mutations in or forced genetic deficiency of leptin production (*ob/ob*) in mice leads to obesity and T2DM development in mice.⁷¹

2.2.3 Adipose tissue dysfunction

Inflated and dysfunctional AT is a key player in metabolic disturbances, specifically, enlarged VAT, or central adiposity, is indicative of metabolic syndrome.⁷² Metabolic syndrome (**MS**) is a disorder characterized by a culmination of insulin resistance, atherogenic dyslipidemia, elevated blood pressure (**BP**), and a proinflammatory and prothrombotic state.⁷³ Individuals with MS are at greater risks for developing fatty liver, asthma, sleep apnea and other

sleep disorders, polycystic ovarian syndrome, gallstones, and some forms of cancer.⁷³ Excessive accumulation of TGs and adipocyte hypertrophy leads to a hypoxic environment with increased oxidative stress.⁷⁴ The differences in SAT and VAT and their mediated inflammatory response is substantial, VAT being the primary offender. Visceral adipocytes have a critical death size that is much less than that of their SAT counterparts, leading to greater macrophage infiltration.^{75, 76} Adipocyte apoptosis leads to elevated production of inflammatory mediators such as leptin, resistin, and RBP4.^{77, 78} These signals initiate a type 1 pro-inflammatory immune response which is characterized by aggregating CD4⁺ T helper type 1 (**Th1**) cells, cytotoxic CD8⁺ cells, and pro-inflammatory classically-activated macrophages (**M1**).⁶⁴ Visceral adipocytes are quick to release FFA from stored TGs, leading to rapid increases FFA concentrations in the liver.⁷⁹ This rapid influx of FFA in the liver stimulates gluconeogenesis and TG synthesis without clearance of insulin.⁷⁹ The ensuing development of dyslipidemia, hyperglycemia, and hyperinsulinemia are all contributing factors to the increased risk of metabolic diseases associated with inflated VAT.⁷⁹

Although there is some debate of whether it is the cause or consequence of, it is understood that insulin resistance is a key underlying factor in the pathology and physiology of AT dysfunction.⁸⁰ AT synthesizes and secretes several cytokines which are shown to work both as a result and a cause of insulin resistance including TNF α , interleukin (**IL**)-6, IL-1 β , and transforming growth factor-beta.^{64, 79} Of these, TNF α , which is primarily secreted from macrophages in these inflammatory conditions, is a key modulator of the pro-inflammatory immune response in diet-induced obesity (**DIO**) and contributes to decreased glucose uptake and insulin resistance.⁶⁴ It does this by inhibiting both the insulin receptor (**IR**) and IR substrate (**IRS**) proteins, which through the phosphoinositol-3-kinase (**PI3K**) signaling pathway, reduces translocation of glucose transporter (**GLUT4**) to the cell surface.⁷⁹ By inhibiting the response to the anti-lipolytic hormone, insulin, the net effect of TNF α on adipocytes is an increase in lipolysis and a decrease in lipogenesis, thereby increasing the release of FFA into plasma.^{64, 79} Increased non-esterified fatty acids in plasma leads to the hyperlipidemic metabolic dysfunction, which is also highly associated with insulin resistance.^{25, 81}

Adipocyte hypertrophy and over-accumulation of TGs together with decreased vasculature and hypoxic environment due to both a lack of oxygen availability and increased need triggers cell death inducible factors and pro-inflammatory adipokines such as RBP4, leptin, and resistin.⁶⁴ From this, a pro-inflammatory cascade is set into motion triggering a type 1 (pro-inflammatory) immune response from WAT-residing immune cells.⁶⁴ Leptin causes T-helper

cells polarization to type 1 (Th0 → Th1), which causes the release of $\text{TNF}\alpha$ and interferon- γ , contributing to insulin resistance. Other adipokines recruit and activate pro-inflammatory classically-activated macrophages (M1), cytotoxic CD8+ T cells, and natural killer T cells, which further release pro-inflammatory cytokines and collectively form crown-like structures (**CLS**).⁶⁴ The vicious cycle is established with CLS-mediated phagocytosis of apoptotic adipocytes, which leads to antigen presentation and further mediates the release of pro-inflammatory cytokines, impairing insulin action.⁶⁴ When anti-inflammatory mediators are brought into this dysregulated environment, they are also transformed and polarized into type-1 immune cell mediators. For example, anti-inflammatory alternatively activated macrophages (**M2**) in this setting will lose their ability to store iron which empties into AT, leading to increased production of reactive oxygen species caused by iron-mediated lipid peroxidation.⁶⁴ M2s in this environment will also lead to collagen synthesis and deposition in AT, leading to fibrosis and exacerbating already critical circumstances.⁶⁴

Contrary to popular belief, inhibiting differentiation or inducing cell death as a means to treat or prevent obesity, in an already hypoxic and inflamed AT environment would only serve to augment already metabolically dysfunctional circumstances.⁸² The AT expandability hypothesis claims that it is the failure of AT expansion that leads to the metabolic detriments associated with obesity, rather than obesity and fat tissue itself.²² This is further supported by the existence of 'metabolically healthy obese' individuals, who have excessive fat stores and BMI that categorize them as obese but do not have the associated metabolic diseases.^{83, 84} Furthermore, it is suggested that the majority of individuals with T2DM are obese; however, many obese individuals do not have T2DM.⁸²⁻⁸⁴ Again, this evidence suggests that the inflammation induced by insulin resistance is the primary contributor to the metabolic disturbances associated with obesity, rather than AT itself.

Therefore, treating AT with insulin-sensitizing compounds without inhibiting differentiation may improve metabolic outcomes.^{85, 86} Pharmaceutical agents such as thiazolidinediones (**TZDs**) are associated with this very outcome; although there are some drawbacks to its use, including some weight gain due to edema and increase in adiposity.⁸⁵⁻⁸⁸ Their pleiotropic nature has led to many therapeutic outcomes that cannot be directly accounted for by any one single mechanism.⁸⁵⁻⁸⁸ However, it is partly due to both the insulin-sensitizing effect and the increase in adiposity which leads to improved metabolic outcomes.^{86, 87} In fact, individuals taking TZDs show improvements in blood lipid profiles, blood pressure, glycemic control, endothelial function, and decreased inflammation markers.^{86, 87} Some TZDs have also been shown to

induce a more thermogenic, brown-fat-like profile in WAT, which has led to increased energy utilization and weight loss.⁸⁹

2.2.4 Brown adipose tissue and adipocytes

BAT was previously thought to be present, but limited to small mammals and infant-humans only.⁹⁰ However, the use of positron-emission-tomography and computed tomography (**PET-CT**) in combination with 18F-fluorodeoxyglucose (**18F-FDG**) led to the discovery of functional BAT in adult humans as well.⁹⁰⁻⁹² PET-CT scan after administration of 18F-FDG revealed the anatomical locations of BAT in humans (cervical, supraclavicular, axillary, paravertebral, periadrenal, subscapular)⁸⁹ and mice (interscapular, axillary, perirenal).^{93, 94} **Figure 2.2.** demonstrates anatomical locations of WAT and BAT in both human and mice. Due to its relatively small volume in the human adult, it was assumed this tissue could not have an impact on overall EE.⁹⁵ Quite contrarily, it was shown to utilize more glucose per gram than any other tissue and significantly contribute to whole-body EE.^{92, 94} Some studies have shown that BAT preferably utilizes intracellular fuel sources in response to short term cold exposure (**CE**), having minimal plasma-substrate utilization.⁹⁶ However, other studies have shown thermogenically active BAT in mice had increased uptake of FFA from TG-rich lipoproteins which led to greater hepatic clearance of cholesterol remnants.^{97, 98} This was shown to improve blood lipid profiles, reducing the rate of dyslipidemia and atherosclerosis development.⁹⁸⁻¹⁰⁰ Clinical observations have shown that the amount of BAT has an inverse relationship with BMI and is positively correlated with leanness and euglycemia, pointing towards its potential therapeutic use in obesity and diabetes.¹⁰¹⁻¹⁰⁴

The means by which BAT can elicit these beneficial effects is through its specialized function of producing heat via adaptive NST.^{26, 27, 105, 106} The inner mitochondrial membrane (**IMM**) of BAT has very high expression of uncoupling protein 1 (**UCP1**), which drives increased proton leak across the mitochondrial membrane, dissociating the chemical gradient from ATP synthesis during oxidative phosphorylation.¹⁰⁶ Therefore, instead of generating ATP, these electron transport chain (**ETC**) substrates are released in vain as heat, helping to maintain thermoneutrality in times of acute and chronic CE without having to rely on muscle-shivering-generated heat alone.^{96, 106} Uncoupling of the ETC is visually demonstrated in **Figure 2.3**. Due to its very high expression and association with the thermogenic capacity of BAT, UCP1 is used as a hallmark of BAT and thermogenesis.¹⁰⁷ BAT has been shown to help maintain body temperature in thermoneutrality at rest (obligatory thermogenesis), when acutely exposed to cold during long-term ambient temperature exposure (facultative thermogenesis), and in

response to long-term CE (adaptive thermogenesis).^{108, 109}

BAT is a highly vascularized and sympathetically innervated tissue which contains brown adipocytes with multilocular LDs and numerous mitochondria.⁹⁴ The high vascularization of BAT allows increased access and delivery of plasma substrates such as glucose, lipids, and oxygen for rapid consumption and oxidation, as well as providing efficient means for heat distribution to the whole body.^{26, 106} BAT is also surrounded by a dense network of sympathetic nerves, which secretes NE when prompted, inducing lipolysis and fatty acid beta-oxidation (**FAO**) via beta-3-adrenergic receptor (**β3-AR**) stimulation.¹¹⁰ cAMP is a second messenger that is generated in response to β3-AR stimulation which activates protein kinase A (**PKA**).¹¹⁰ Activated PKA phosphorylates and activates lipolytic enzymes, such as HSL and ATGL, leading to TG hydrolysis and release of glycerol and FFA which will be further broken down in FAO.¹¹⁰ Surface area is a critical limitation for lipolysis; therefore, having small multilocular LDs increases the surface area and accessibility for lipase interaction, promoting more efficient lipolysis.^{31, 111} Increased FFA release from lipolysis is then shuttled to BAT's enriched network of mitochondria for FAO. Mitochondrial biogenesis in BAT is regulated by a transcriptional cascade primarily controlled by PPAR_γ coactivator 1 alpha (**PGC1α**), which can work in conjunction with nuclear respiratory factor 1 (**NRF1**) to further promote the thermogenic response in BAT.^{112, 113}

Adipocytes in BAT originate from the somatic multipotent Myf5-positive precursor population which can also give rise to myocytes.²⁶ Due to this, BAT and myocytes have similar gene expression profiles. Several transcription factors are shown to help regulate brown fat cell fate. Bone morphogenic protein 7 (**BMP7**) is shown to trigger the commitment of the precursor cells into brown-fat lineage as well as activate the full-blown brown-fat adipogenic program, including activation of positive regulatory domain-containing protein 16 (**PRDM16**) and PGC1α, and is required for expression of UCP1.¹¹⁴ Early B-cell factor-2 (**EBF2**) is an embryonic brown preadipocyte specific marker and can recruit PPAR_γ to promote brown-fat selective genes such as PRDM16.^{115, 116} PRDM16 is a zinc-finger protein which binds to and coregulates PPAR_γ, PPARα, C/EBPβ, and PGC1α to further promote brown-fat-specific gene expression.¹¹⁷⁻¹²²

Differentiation of brown-fat committed precursor cells can be induced by a variety of factors.¹²³⁻¹²⁵ As previously discussed, CE can induce NST, or adaptive thermogenesis, causing NE-stimulated increase in cAMP levels.¹⁰⁸ cAMP then works through a P38-MAPK-mediated signaling cascade leading to lipolysis and the resulting heat generation from UCP1 expressing mitochondria leaking protons during FAO.^{126, 127} Through a similar pathway, leptin was shown to

mediate UCP1 expression in BAT through sympathetic nervous system (**SNS**) stimulation.¹²⁸ The thyroid hormone (**TH**) is another example of an internal regulator of the brown-fat program. Pituitary stimulation of the thyroid gland causes synthesis and secretion of the inactive form of TH, thyroxine (**T₄**).¹²⁹ T₄ traverses the bloodstream to target tissues expressing deiodinase iodothyronine type 2 (**DIO2**) which converts T₄ to the biologically active form, triiodothyronine (**T₃**).¹²⁹ BAT has a very high expression of DIO2¹³⁰ which is downregulated by T₄ and upregulated by SNS activation (i.e., cold stimulation).¹³¹ The TH can upregulate UCP1 via two mechanisms: (1) the UCP1 promoter contains a TH receptor β (**TH-R β**) regulatory region, which directly upregulates UCP1 expression,^{132, 133} and (2) the TH-R α helps to regulate β -AR expression, which sensitizes BAT to SNS activation.¹³⁴ It has also been shown that thyroid-stimulating hormone receptor (**TSH-R**) also induces the brown-fat program, enhances thermogenesis and UCP1 expression, and protects from hypothermia in hypothyroid conditions.¹³⁵ Transcriptional regulation of the TH is primarily achieved through activation of thyroid response elements (**TREs**) as well as other genes, which can heterodimerize with PPAR in the PPRE and further induce thermogenesis.¹³⁶

Exogenous treatment with β -AR agonists, such as isoproterenol (**ISO**), forskolin, and other more-specific agonists can induce similar UCP1 driven thermogenesis programs in BAT.¹³⁷ High-calorie diets have also been shown to activate the thermogenic program in BAT as a compensatory mechanism, pointing towards its protective capacity against the harmful metabolic effects of DIO.¹⁰⁶ Studies suggest β -AR signaling is required for diet-induced thermogenesis.¹³⁸ Exercise-induced activation of the PGC1 α pathway leads to the production of myokines such as irisin and meteorin-like protein (**METRNL**), which activate UCP1-thermogenic program in BAT, leading to an increase in EE.^{105, 139, 140} PPAR γ agonists, such as TZDs, are also shown to further promote the expansion of BAT depots.^{89, 141} Several bioactive compounds have demonstrated abilities to target or work through similar pathways as described above, further promoting the thermogenic program in BAT.¹⁴²

2.2.5 Beige and brite adipocytes

Recently, a third type of fat cell has been characterized which has similar characteristics as brown adipocytes but is primarily induced and arises within WAT.^{26, 27, 105, 106, 143} These cells have accumulated many names over the years, including “**brite**” (**brown-in-white**), beige, inducible or recruitable brown, as well as brown adipocyte-like cells.¹³⁸ Due to its inducibility, plasticity, and distinct characteristics, the terminology used throughout the rest of this review is

“beige adipocytes”. Similar to brown adipocytes, beige adipocytes have a smaller cell size, multilocular LDs, and higher cellular respiration as a result of their greater number and function of mitochondria expressing UCP1.¹³⁸

As previously discussed, brown adipocytes have many small (multilocular) LDs which are important to facilitate expedited lipolysis by increasing the surface area accessible to lipases.³¹ In response to CE or β -AR stimulation, FFAs are released and used to generate heat via UCP1-dependent uncoupling of oxidative phosphorylation in the mitochondria.³¹ This is associated with increased expression of LD-associated proteins such as Cidea, Perilipin, ATGL, HSL, and adipose differentiation related protein, which are highly expressed in BAT.³¹ Interestingly, expression of these proteins in addition to an increase in expression of both lipogenesis and lipolysis proteins are also critical during the browning process of WAT. This is a process described as “the futile cycle of TAG hydrolysis and re-synthesis”.³¹ In this process, lipolysis induced by CE and β -AR stimulation breaks down the unilocular LD; however, lipogenesis proteins, such as fatty acid synthase (**FASN**) and glycerol kinase (**GYK**), act to recycle the FFA to form TGs which are stored within newly formed microLDs (**mLDs**).³¹ This process helps to transform the unilocular LD morphology of white adipocytes into a more brown-like multilocular LD morphology so that UCP1-positive beige adipocytes have many smaller LDs.³¹ Similar to BAT, these small LDs formed within beige adipocytes have greater surface area allowing for a more efficient lipolysis and downstream heat production.³¹ Interestingly, PPAR γ -agonists are shown to promote this TAG-cycling process further by inducing expression and activity of GYK.^{31, 144} Reducing FFA release into serum by recycling them within adipocytes is one of the many theories of how PPAR γ -agonists elicit its therapeutic effects in the context of insulin resistance and T2DM.¹⁴⁴

Beige adipocytes share characteristics and thermogenic capacity similar to brown adipocytes; however, there are several distinguishing features that make them separate cell types. The first of these distinctions being that beige adipocytes are shown to originate from Myf5-negative MSCs similar to and within WAT, whereas it has been long accepted that brown adipocytes are from Myf5-positive precursor cells in BAT.¹⁴⁵ Interestingly, this has recently been challenged by a study by using lineage-tracing which demonstrated that Myf5-positive precursors could give rise to both white and brown fat cells.^{34, 146, 147} In fact, it is suggested that the distribution of adipocytes from Myf5-lineage can be modified based on genetic, behavioral, environmental, dietary, and other factors, and it should not be considered a discerning factor for BAT.^{34, 146} Another distinguishing characteristic of BAT is mitochondrial abundance; whereas

classical brown adipocytes have abundant mitochondria with high constitutive expression of UCP1 with or without various stimulants, beige adipocytes require β 3-AR stimulation in order to induce and maintain expression of brown-like gene markers.¹⁴⁸⁻¹⁵⁰ Beige adipocytes are also shown to have genetic markers that are distinct from both WAT and BAT.¹⁴⁵ **Figure 2.4.** demonstrates WAT, BAT, and beige differences visually.

The process of inducing beige adipocyte formation in WAT is known as “browning” and was first reported in 1984 when a study utilizing CE in mice resulted in the development of brown-fat characteristics in the parametrial fat pad.¹⁵⁴ Since then, a number of compounds that can induce the browning program have been reported, many of which can also induce brown adipogenesis.^{27, 155, 156} These include, but are not limited to, CE, β -AR (i.e., NE, ISO) and PPAR γ agonists (i.e., TZDs), as well as some dietary bioactive compounds and pharmaceutical agents.^{27, 155, 156} Inguinal WAT (or SAT) is especially susceptible to browning, even with mild stimulation of these compounds.¹⁰⁶ As previously discussed, EBF2 is a brown-fat specific marker and also controls the browning program in WAT.¹¹⁶ BMP7 is another agent that can induce the thermogenic program in BAT as well as a subpopulation of SVF isolated from WAT expressing Sca1⁺, CD45⁻, and Mac1⁻.¹⁴⁷ Interestingly, there are subpopulations of both white and beige adipogenic precursor cells within WAT having distinct gene expression patterns.¹⁴⁵ However, there is also evidence suggesting that beige adipocytes arise from the browning of mature white adipocytes.^{143, 157} For this reason, there is much debate on whether beige adipocytes arise from *de novo* synthesis from beige adipogenic precursor cells or transdifferentiation from mature white adipocytes.

2.2.5.1 *De novo* synthesis or transdifferentiation?

De novo synthesis or transdifferentiation of beige adipocytes has been a topic of discussion for decades. However, it seems that groups that support inter-conversion also support and acknowledge the existence of beige-fat precursor cells with a distinct genetic lineage, whereas, groups that support *de novo* differentiation may oppose the concept of transdifferentiation.¹⁰⁶ The evidence for both sides is briefly reviewed here.

As previously discussed, there is solid evidence of competent beige adipogenic precursor cells present in both human and murine WAT. These beige precursor cells express their own set of beige-fat-specific markers including CITED1, CD137, TMEM26, TBX1, among others.^{106, 145} Pulse-chase fate-mapping is a popular experiment used to label pre-existing mature adipocytes to better understand adipocyte dynamics. Utilizing this method of experimentation, a

study labeled mature adipocytes in WAT of mice with β -galactosidase (**β -gal**) and measured β -gal staining following one to three days of CE or β 3-AR agonist treatment.¹⁵⁸ The results of this study revealed that newly formed beige adipocytes were β -gal-negative, pointing towards beige-adipocyte *de novo* synthesis rather than recruitment from mature adipocytes, although the developmental defects of these triple transgenic mice and consumption of a high-fat diet (**HFD**) were possible confounders of the study outcomes. In another study, transgenic mice having WAT-specific overexpression of cyclooxygenase 2 (**COX-2**) induced *de novo* BAT recruitment in WAT and β 3-AR-agonist-stimulated-thermogenesis was diminished in COX-2-deficient mice.¹⁵⁹ That said, this study did not differentiate whether the thermogenesis seen was from UCP1-positive precursor cells ("*de novo*" synthesis) or mature adipocytes undergoing browning and may have been using the term interchangeably.

There is ample evidence for *de novo* differentiation as the mechanism for beige adipocyte formation; however, it does not completely rule out the possibility of *transdifferentiation*. Evidence suggests the inter-conversion of white-to-beige may be the dominant, but not the only, mechanism when it comes to browning of WAT.¹⁰⁶ A study treating mice with CL-316,243, a β 3-specific-AR agonist, showed increased thermogenesis and browning, but with little change in DNA content or adipocyte number, pointing towards transdifferentiation.¹⁶⁰ Another study demonstrated that cold acclimation of wild-type (**WT**) C57Bl/6J mice led to an increase in browning and a concomitant decrease in white adipocytes with no significant changes in total adipocyte number, and no signs of apoptosis or mitosis, again, pointing towards transdifferentiation.¹⁶¹ In fact, several studies have shown most beige adipocytes arise from non-dividing cells, suggesting mature adipocytes may be the most feasible source.¹⁶¹⁻¹⁶³ Using lineage tracing, it was reported that CE led to *de novo* differentiation of brown adipocytes from resident PDGFR α precursor cells in *BAT*, whereas essentially all beige adipocytes were derived from mature adipocytes in WAT.¹⁶⁴ Interestingly, it was found that a tissue's microenvironment may play a critical role in an adipocyte's ability to transdifferentiate. This includes vascular and neural density, SVF cell types in the area, as well as the extra-cellular matrix (**ECM**) composition.¹⁴³

Arguments for both sides are compelling yet still controversial; however, what is typically agreed upon is WAT's metabolic plasticity, or the ability to rapidly transform to meet metabolic needs, which is especially notable in SAT. A lineage-tracing study found that mature adipocytes in WAT undergoing CE and subsequent rewarming can inter-convert between fully mature white

and brown adipocyte phenotypes.¹⁶⁵ This bi-directional switch demonstrates that regardless of the mechanism, beige adipocytes formed from CE or other stimulation can undergo warm adaptation and lose expression of UCP1 but are retained in WAT and can be induced again to regain the thermogenic profile. This further supports the well-accepted notion that beige adipocytes are inducible but require constant adrenergic stimulation to support the brown-like phenotype.

There are also substantial age and species-specific differences in the amount of BAT and beige adipocytes. Analogous to what was previously assumed, infants and young mammals have a greater amount of BAT for heat-generation until sufficient insulation is accumulated.¹⁶⁶ Furthermore, BAT found in infant humans and rodents is shown to take on a more beige-adipocyte phenotype in the older age.^{94, 166-168} In fact, this “*bona fide*” classical BAT has not yet been found to exist in adult humans, which further demonstrates both the species and age differences in the context of BAT.¹⁶⁹ Additionally, young mammals have a larger pool of stem cells which can be prompted to *de novo* differentiate into beige adipocytes or white adipocytes, depending on the environment raised.^{106, 145} This may be an important point in the debate of *de novo* vs transdifferentiation of beige adipocytes; *de novo* differentiation may be more relevant to the young mammals which have greater number of precursor cells, whereas transdifferentiation may be critical to older mammals and in obesity, where there is already a substantial number of mature adipocytes.

2.2.5.2 Regulation of beige adipocyte formation

Transcription factors and regulatory pathways that control brown adipocyte formation also play critical roles in beige adipocyte formation, including UCP1, PPAR γ , PGC1 α , and PRDM16 which are further reviewed here.

The nuclear receptor **PPAR γ** is known as the master regulator of differentiation due to its critical actions as a transcription factor for white, brown, and beige adipocyte differentiation (especially during the late-stages), maintenance, and survival.²⁰⁰⁻²⁰² C/EBP α is shown to be required to maintain PPAR γ expression, and although PPAR γ is shown to promote differentiation in both BAT and WAT, studies have shown there may be depot-specific binding sites on PPAR γ .²⁰¹ PPAR γ involvement in adipogenesis was further demonstrated using genome-wide mapping techniques, where it was shown to bind to the promoters of adipogenic genes in both mice²⁰³⁻²⁰⁵ and humans.^{206, 207} PPAR γ -agonists, such as TZDs, have been shown to have potent browning effects.⁸⁹ PPAR γ -agonists/TZDs, such as Rosi, was shown to activate

the PPAR γ -receptor while also promoting association with DNA leading to upregulation or downregulation of target gene transcription.²⁰⁸ This has been shown by several groups using Rosi and other TZDs analogs which revealed suppressed expression of β 3-AR,²⁰⁹ resistin,²¹⁰ leptin,^{211, 212} as well as PPAR γ itself, without affecting adipogenesis.^{208, 213, 214}

PPAR γ has six functional domains, termed “A to F”; N-terminal A/B domain is responsible for transcriptional activation and is called the “activation-function 1” (**AF1**) domain, the C domain is known as the DNA-binding domain (**DBD**), followed by the hinge D domain, and the C-terminal E/F domain is the ligand-binding domain (**LBD**).²¹⁵ The E/F domain also contains the ligand-binding pocket (**LBP**), an AF-2 domain, and also contains regions important for dimerization.²¹⁵ For PPARs to bind to DNA, they must first form a heterodimeric complex with retinoid X receptor alpha (**RXR α**) primarily located in PPAR response element (**PPRE**) regions.²¹⁵ Regulation of PPARs occurs through ligand binding and post-translational modifications (**PTMs**).²¹⁵ Binding of LBD causes conformational changes which can affect downstream interactions with co-repressors and coactivators.²¹⁵ Without a ligand-bound, PPAR γ is bound to co-repressors (i.e., nuclear receptor corepressor (**N-CoR**) and silencing mediator of retinoid and thyroid receptor (**SMRT**)) which acts to silence the transcription of target genes.¹⁹⁵ Whereas ligand binding leads to conformational change and recruitment of coactivators (i.e., steroid receptor coactivator (**SRC**) family and PGC1 α).¹⁹⁵ Histone-modifying enzymes (i.e., histone acetyltransferases (**HATs**) and histone deacetylases (**HDACs**)) form multiprotein complexes with PPAR γ and its coactivators and corepressors, respectively, which modify chromatin structure, thereby regulating transcription.^{195, 216} Both the type and location of PTMs can affect PPAR activity. In general, phosphorylation and acetylation of PPAR γ has metabolically detrimental downstream effects, seeing as studies showing dephosphorylation and deacetylation is associated with recruitment of browning co-factors and coactivators, promoting insulin sensitivity, as well as increased oxidative capacity, EE, and BAT-associated genes in WAT.^{173, 217-220}

Specifically, and of interest to this review, TZDs and NAD-dependent deacetylase sirtuin-1 (**SIRT1**) have elicited beneficial effects via PTMs of **PPAR γ** . Rosi was shown to inhibit cyclin-dependent kinase (**CDK**)-5-dependent phosphorylation of serine 273 in PPAR γ which promoted insulin sensitivity in adipocytes.²¹⁸ Rosi dose-dependently deacetylated PPAR γ and increased the interaction of SIRT1 and PPAR γ which further led to decreased PPAR γ acetylation.¹⁷³ TZDs and SIRT1 mediated deacetylation of lysine 293 and 268 were required to recruit PRDM16 to

PPAR γ , which induced induction of BAT genes.¹⁷³ In fact, one study revealed that treating adipocytes with TZDs led to the accumulation of PRDM16 protein via stabilization and prolonging the half-life, which allowed induction of browning and brown-fat development.¹⁷¹ It seems likely that the alterations in PTMs led to increased activity of PPAR γ and reduced receptor turnover, resulting in PRDM16-stabilization and subsequent browning.

PRDM16 is an essential determining factor for inducing the brown fat program¹⁷⁴ and has also been shown to induce browning in WAT.²²¹ In fact, PRDM16 deficient WAT has reduced thermogenic gene expression and diminished beige adipocyte formation in response to β 3-AR and PPAR γ agonists, whereas overexpression leads to beige adipocyte formation.²²¹ Increased stabilization by deacetylated PPAR γ allows PRDM16 to partner with PGC1 α to coactivate the PPAR γ -RXR α heterodimerized complex bound to the PPRE which induces mitochondrial biogenesis and the expression of thermogenic genes, such as UCP1.¹⁷⁴

PGC1 α is known as a master regulator of mitochondrial biogenesis and coactivation of PPAR α and PPAR γ , which are bound to the UCP1 promoter region, and therefore, induces thermogenesis, oxidative metabolism, and energy-expenditure related genes.^{126, 222-224} Although PPAR γ activation can induce the browning program, many of the factors that induce browning actually modulate PGC1 α activity.²²⁵ PGC1 α is recognized as the central regulator of β 3-AR-mediated thermogenesis via activation of the P38-MAPK signaling pathway.¹²⁶ In addition to PPAR γ , PGC1 α also coactivates interferon regulatory factor 4 (**IRF4**), which leads to enhanced thermogenic gene expression, cold tolerance, and EE.²²⁶ PGC1 α plays major roles in whole-body energy-expenditure by helping to regulate glucose and lipid metabolism in both skeletal muscle,²²⁷ and adipose tissue.^{172, 228} Interestingly, adipocyte-specific loss of PGC1 α reduces the browning capacity of WAT and leads to insulin resistance.²²⁸ Whereas adenovirally-induced PGC1 α expression in primary human white adipocytes led to enhanced thermogenesis as evidenced by increased UCP1 and ETC-component expression as well as enhanced FAO.¹⁷² In adipocytes, PGC1 α regulates the expression of respiratory chain components such as cytochrome complex (**CYTC**)¹⁷² and mitochondrial structural components such as mitofusin 2 (**MFN2**).^{172, 229, 230} PGC1 α -mediated mitochondrial biogenesis was also shown to be potently activated by direct interaction with NRF1, further mediating the thermogenic program in BAT and beige adipocytes.^{113, 172, 230}

2.2.6 Nutritional, pharmacological, and nutraceutical interventions for browning and brown adipogenesis in humans

Due to its targeting of the pertinent tissue and strong evidential support, browning of WAT and activation of BAT is a promising strategy to prevent and treat obesity in humans. The sheer volume and sensitivity to browning agents make SAT a desirable target for many nutrient and pharmaceutical-based drugs.²³¹ However, many of the studies mentioned so far were primarily performed in rodents and/or *in vitro* using cell culture, which may have limitations in translatability or efficacy in humans. Large variations in metabolism, environment, and genetics as well as determining human-relevant doses make clinical applications of experimental findings difficult.²³² Contrary to popular belief, the current standard scale used for rodent-to-human conversion, body surface area (**BSA**), is actually not recommended by the Federal Drug Administration (**FDA**) to determine therapeutic dosage range.²³² Using pharmacologically active doses (**PADs**) as well as other scientifically validated approaches which use physiologic and pharmacokinetic data to more accurately estimate human-equivalent doses (**HED**) is now recommended instead of BSA.²³² With that said, there are many single-target drugs and multi-target functional foods that have been used as anti-obesity therapies in human clinical trials. Unfortunately, aside from issues of maintaining compliance, many currently used pharmaceutical interventions have several undesirable off-target effects, including hypertension, sleep disorders, depression, and even heart disease, while other phytotherapeutic agents may not show immediate efficacy.²³³ Nevertheless, human trials utilizing bioactive food ingredients have shown increases in daily EE as much as 2-5%.²³⁴ Furthermore, promising future compounds are awaiting clinical trial testing which could, alone or synergistically with other compounds, have a thermogenic effect upwards of 10-15% increased EE.²³⁵⁻²³⁷ An increase in the total daily EE of 10-15% would be sufficient to facilitate weight loss, especially in the context of obesity.⁶ There are many studies that demonstrate dietary, nutritional, polyphenolic, and/or bioactive compounds to have an impressive capacity to induce browning, resveratrol is one of particular interest and will be discussed further in 2.3.

2.3 RESVERATROL AS A NUTRITIONAL INTERVENTION TO COMBAT OBESITY

2.3.1 Resveratrol overview

Resveratrol (*trans*-3,4',5-trihydroxystilbene, **Res**) is a well-studied naturally occurring polyphenolic compound, which is typically found in the skins and seeds of a number of dietary sources including grapes, berries, peanuts, and wine, as well as the inedible portions of Japanese giant knotweed.²³⁸⁻²⁴⁰ Chocolate and cocoa-containing products are lesser-known

sources of Res, but are actually one of the most concentrated, second only to red wine and grape juices, and end up comprising the most total Res in the diet due to the sheer quantity of consumption.²⁴¹ Polyphenols are organic chemicals containing multiple phenol structural units which can be further broadly categorized into flavonoids, such as anthocyanins and tannins, and non-flavonoids, such as stilbenes.²⁴² Res is categorized as the latter.²⁴² Interestingly, Res and other flavonoids are also known as “phytoalexins” which are compounds formed as an innate immune defense mechanism by the plant in response to microbial infections as well as UV radiation, giving Res its antibiotic and antioxidant properties.²⁴³

Wine is a popular dietary source of Res; the bitter compounds present in grape-skins give what is referred to as “structure” to the flavor and mouth-feel of wine.^{244, 245} Because of this, grape-growers encourage the formation of these by intentionally stressing the vines.²⁴⁶ Red wine, in particular, is a concentrated source of Res, due to maceration, fermentation, and incorporation of the whole-grape.²⁴⁷ On the other hand, white wines do not incorporate the skins and grapes used for juices are cultured so that the skins are sweet, therefore having less Res content.²⁴⁷ For example, concentrations of free-Res and piceid (the 3-beta-glucoside metabolite of Res) found in Concord grape or cranberry juices were found to be within the 1.5 – 1000 nM/g range, whereas Italian red wine was found to be within the 8 – 24 μ M.²⁴⁷ Interestingly, the alcohol that develops during the fermentation of wine acts as a solvent which promotes the extraction and chemical stability of Res, while aging wine in oak and acacia wooden barrels may lead to additional phenols present.^{248, 249} Observational studies have shown a J-shaped curve associated with the relative risk of disease incidence and wine consumption; with moderate consumption being at lower risk than both non-consumption and high-consumption.²⁵⁰⁻²⁵³ One of the first of these correlations was seen in an epidemiologic observational study of a population in southern France.²⁵⁴ The results of this study revealed a very low incidence of CVD despite having high-CVD-risk lifestyle behaviors (i.e., high intake of saturated fat, smoking, and alcohol intake).²⁵⁵ The now well-accepted conundrum was called “The French Paradox” coined by the television show, *60 Minutes* in 1991, which was later attributed to their low-stress lifestyle and relatively frequent but moderate consumption of red wine.²⁵⁶⁻²⁵⁹ A similar scenario is seen in the Mediterranean population, which in addition to having a diet rich in omega-3 from fish and nutrient-dense fruits and vegetables, wine is also a central component of.^{250, 260} The low-incidence of CVD and general health of the population made following the “Mediterranean Diet” a popular disease-fighting and health-promoting trend in the mid-to-early 1990s and is still followed today.²⁶⁰⁻²⁶²

2.3.2 Resveratrol structure and function

There are two structural conformations known of Res, *cis*- and *trans*- form.²⁶³ *Trans*-Res is the biologically active form, but can easily be converted to *cis*- when exposed to UV light, making storage conditions important.²⁶³ Due to its abundance and biological relevance, *trans*-Res will be used in this review and will be simply referred to as Res.

The beneficial correlational observations, its abundance in nature, and accessibility in the diet led to increased interest in Res especially in the early 1990s and became a popular target used for research. Since then, Res has emerged as a robust therapeutic compound conferring beneficial effects and protection against a wide-spread set of conditions and diseases ranging from anti-aging,^{264, 265} neuroprotective,²⁶⁶⁻²⁶⁹ cardioprotective,^{270, 271} anti-tumorigenic,²⁷²⁻²⁷⁴ and anti-obesogenic.²⁷⁵

2.3.3 The many beneficial effects of resveratrol

The structure of Res lends itself to the many beneficial aspects; the most dominant of which is most likely Res' potent anti-inflammatory actions. As previously mentioned, Res has many subcategories and alias', one of these being a phytoalexin, which due to it being a compound made by the plant-immune-defense mechanism, by nature, has anti-inflammatory, antimicrobial, and antioxidant properties.^{243, 276} Res is also a non-flavonoid stilbene; the tetrameric stilbene oligomers are known inhibitors of matrix metalloproteinases (**MMPs**), which are responsible for the breakdown of collagen which is highly implicated in aging and cancer.²⁷⁷⁻²⁷⁹

In fact, Res is shown to work through many pathways to inhibit cancer; inhibition of cytochrome P450 family 1 member A1 (**CYP1A1**),²⁸⁰ promoted DNA stability, and anti-inflammatory actions suppress tumor initiation, inhibition of COX-2 and induction of apoptosis helps when tumor is already present but prior to metastasis, and suppression of angiogenesis and MMPs when tumor becomes invasive.^{281, 282} There are many other known ways which Res is shown to be beneficial in cancer, but it is not the focus of this review. In similar pathways, Res is also shown to be anti-atherogenic; upregulation of nitric oxide (**NO**) leads to vasodilation and lowers BP,²⁸³ and reduced production of adhesion molecules and endothelial-cell cytokines,^{283, 284} COX inhibition, and decreased levels of oxidized low-density lipoproteins (**oxLDLs**)²⁸⁵ leads to retarded plaque progression.²⁸⁶ The anti-inflammatory effects of Res in obesity is discussed in its own section below.

Res wears many hats, one of them includes actions as a phytoestrogen, with its primary

target and action being an estrogen receptor (**ER**)-agonist.²⁸⁷ In general, there are mixed reviews on phytoestrogens, due to their potential beneficial effects on bone health and chemoprotective capacity, and nearly equivalent adverse effects on certain types of cancer.²⁸⁸ Although the consensus on phytoestrogen's effects on health and disease often fluctuates, the phytoestrogen effect of Res has primarily yielded positive results. An epidemiological observational study revealed a dose-dependent relationship of bone-density and consumption of wine,^{289, 290} which could be attributed to increased BMP expression in bone stimulated by Res.^{291, 292} Although ER activation in the context of breast cancer can be deleterious, Res actually acts as an anti-tumorigenic compound via actions as an ER-antagonist in the breast tissue, which is attributed to its pleiotropic, dose, timing, and tissue-dependent effects.²⁹³⁻²⁹⁵ It is through these pathways, among others, that Res is shown to elicit its beneficial effects on metabolic diseases, which is discussed in greater detail here.

2.3.4 The anti-obesity effects of resveratrol

2.3.4.1 The link between resveratrol and calorie restriction

Due to obesity being a disease of excess energy, an obvious lifestyle intervention for weight loss is calorie restriction (**CR**). Reducing intake at any point below EE should lead to weight loss. The more technical definition for CR used as a nutritional intervention for weight loss is the reduction of energy intake of 10-40% of *ad libitum* levels without causing malnutrition to occur.²⁹⁶ Studies using budding yeast *S. cerevisiae*,²⁶⁴ worms *Caenorhabditis elegans* and fruit flies *Drosophila melanogaster*,²⁶⁵ rodents, and rhesus monkeys have shown CR to enhance longevity, improve stress resistance, and delay the onset of aging-related diseases.^{297, 298} Although CR showed some benefit in human studies, such as lowered risk for CVD, metabolic diseases, and some cancers, long-term CR in human studies is difficult to achieve and maintain, primarily due to lack of compliance.²⁹⁹

Although not fully understood, the molecular mechanisms through which CR confers metabolic benefits are mainly attributed to its relief of oxidative damage to cells and tissues, which is otherwise hypothesized to be a primary mechanism for the inevitable deterioration of cells and tissues in aging.³⁰⁰ The relief of oxidative damage has been shown to work in several tissues in rodents³⁰¹⁻³⁰³ and overall reduced markers were found in humans in a 20% kcal deficit.^{304, 305} One of the first breakthrough discoveries of the molecular targets of aging and CR was made in 1999 when the silent information regulator 2 (**Sir2**) gene in yeast, was found to be a pivotal regulator in the aging process.^{264, 306, 307} Later, the mammalian ortholog of Sir2, NAD-

dependent deacetylase SIRT1, was found to elicit the same aging-related effects.²⁶⁴ Although somewhat controversial, SIRT1 is suggested to be a critical mediator in the CR-induced signaling pathways.^{264, 307, 308-310} The activation of SIRT1 by CR was shown to help regulate EE by modulating glucose and fat utilization tissues with metabolic functions.³¹¹ Using fluorescent-labeled substrates in an *in vitro* screening, several small molecules were identified as activators of SIRT1, Res being the most potent.²⁶⁴ After this discovery, all of the beneficial effects seen in previous CR studies were then shown to be induced by Res treatment as well. Res treatment in lower-level organisms (i.e., yeast, worms, flies) was shown to extend lifespan,^{264, 265} and Res treatment in rodents proved beneficial effects on both lifespan and metabolic health.^{275, 299} Mice fed a HFD and then supplemented with Res were protected against DIO and also showed improved blood-lipid profile as well as enhanced glycemic control.²⁷⁵ The results of these studies left the door wide open for utilizing Res as a therapeutic agent for a wide variety of metabolic diseases, as well as mechanism of action exploration.

2.3.4.2 The molecular targets and effects of resveratrol in obesity

Due to the many beneficial effects of Res, great efforts have been made to identify the molecular targets of Res within biological systems.³¹² Whether a molecule simply binds to the cell surface, is taken up via endocytosis, or some other receptor-mediated pathway critically alters its downstream effects and molecular targets.^{312, 313} Res has been shown to interact with both intracellular (**IC**) and extracellular molecules, therefore, most likely in a dose- and tissue-dependent manner, Res could trigger signaling pathways by binding to cell-surface receptors, activating IC factors, or even mediating effects directly in/on the nucleus.^{312, 313} In the context of obesity and adipose tissue, Res has been shown to have multiple downstream molecular targets and effects. Many studies have implicated Res to work via regulating pathways involved in adipogenesis and apoptosis,³¹⁴ lipogenesis and lipolysis,³¹⁵⁻³¹⁷ as well as EE, plasma substrate utilization, and FAO.^{318, 319} Recent evidence suggests Res can promote browning and brown adipogenesis leading to enhanced insulin sensitivity and weight loss.³²⁰ Res has been shown to work through many of the same pathways implicated in the BAT activation and beige adipocyte formation, leading to upregulated UCP1 expression via signaling pathways including SIRT1, PPAR γ , PGC1 α , and 5'AMP-activated protein kinase (**AMPK**).³²¹ The pathways Res is implicated in WAT, BAT, and beige adipocytes, specifically, are further discussed below.

2.3.4.2.1 Resveratrol and WAT

Many studies demonstrate Res to be beneficial in the context of obesity by inhibiting differentiation and promoting apoptosis in adipocytes via downregulation of critical adipogenesis regulatory factors such as PPAR γ , C/EBP α , and C/EBP β .³⁹ Res was shown to inhibit 3T3-L1 preadipocyte proliferation and initiation of differentiation through cell-cycle arrest during the S-phase, preventing DNA synthesis.³¹⁴ Similarly, another study demonstrated that treating 3T3-L1 preadipocytes within the first 24 h of adipogenesis led to proliferation arrest and impaired MCE by suppressing entry of the G2/M phase of the cell cycle and suppression of expression of cyclin-A and CDK-2, which are critical cell-cycle regulators.³²² Through activation of the major cellular-energy sensing network, AMPK, Res can sufficiently shut down differentiation via activation of cell-signaling cascades which shifts to energy-saving metabolic processes.^{323, 324} A study utilizing potent Res analogs (**RSVA**) also demonstrated inhibition in 3T3-L1 preadipocyte differentiation via interfering with MCE.³²⁵ RSVA successfully attenuated adipogenesis-related transcription factors such as PPAR γ and C/EBP α as well as adipocyte-specific genes such as FASN, fatty acid-binding protein (**FABP4**), RANTES, and resistin.³²⁵ A study utilizing porcine preadipocytes showed Res may mediate suppression of proliferation and differentiation via SIRT1-mediated suppression of FOXO, PPAR γ 2, and other adipocyte-specific genes.³²⁶

Studies have also shown Res to have “beneficial” effects on obesity by inducing apoptosis in adipocytes as mediated by caspases and other cell-death inducible factors.³²⁷ Whereas intermediate-level concentrations (i.e., 20-50 μ M) are sufficient to inhibit adipogenesis, higher concentrations (i.e., 40-200 μ M) are required to induce apoptosis in adipocytes.³²⁸ A study utilizing 3T3-L1 preadipocytes demonstrated that 40-80 μ M of Res led to the induction of apoptosis via a SIRT1-dependent mechanism.³¹⁴ SIRT1 upregulated AMPK α which downregulated cell-survival protein expression, AKT and Survivin, and induced cell-death mediators, caspase-3 and caspase-9.³¹⁴ Primary porcine preadipocytes treated with 200 μ M of Res led to preadipocyte apoptosis as shown by cell viability assays, which were accompanied by increased apoptotic gene expression including caspase-3, and b-cell lymphoma (**BCL**) associated x (**BAX**), as well as downregulation of anti-apoptotic genes such as BCL-2, P53, and NF- κ B.³²⁹

Res is also shown to elicit beneficial metabolic effects by promoting insulin sensitivity and plasma substrate utilization in WAT via a variety of mechanisms. As previously mentioned, Res has potent phytoestrogenic properties. It is through this capacity, Res activates ER α which then

works through a PI3K-AKT signaling pathway to induce GLUT4 expression, translocation, and activity.³³⁰ Res is also shown to work through AMPK to enhance translocation of GLUT4, which would then lead to increased IC uptake and clearance of blood-glucose.³³¹⁻³³³ Res activation of the SIRT1-AMPK axis led to increased protein expression of SIRT1, increased phosphorylation and activation of AMPK causing phosphorylation of AKT (**p-AKT**).³³² Downstream consequences of elevated p-AKT led to translocation and degradation of transcription factor, FOXO1, causing reduced expression of adipokine, resistin, and enhanced insulin sensitivity.³³² Res can also help to regulate blood sugar by inducing glycogen synthase expression and activity with simultaneous suppression of glycogen phosphorylase.³³⁴ This results in an accumulation of glycogen in the liver, promoting clearance of plasma glucose.³³⁴

Working as a PDE-inhibitor, Res increases cAMP levels, which induces lipolytic and inhibits anti-lipolytic processes, further reducing lipid accretion.^{335, 336} Res also works directly on inhibiting lipogenesis; thereby indirectly (through SIRT1) inhibiting adipogenesis.^{328, 337, 338} PGC1 α is induced via SIRT1 activation by Res, leading to increased mitochondrial biogenesis and oxidative phosphorylation.^{275, 339, 340} Res was also shown to activate lipolysis via SIRT1-mediated suppression of PPAR γ .³⁴¹ Res was shown to significantly induce AMPK activity in both HFD-induced obese rats³⁴²⁻³⁴⁴ and obese Zucker rats,³⁴⁵ further demonstrating beneficial metabolic outcomes.

Res has also been shown to work through regulating critical enzymes in lipogenesis and lipolysis pathways. Through AMPK activation, Res successfully reduces acetyl-CoA carboxylase (**ACC**) activity via inhibitory phosphorylation by AMPK, increasing FAO and inhibiting FA synthesis.³⁴⁶ It has also been shown that Res downregulates expression of critical lipogenesis enzymes ACC and FASN, as well as decreased lipoprotein lipase (**LPL**) expression in Sprague Dawley rats fed a HFD supplemented with Res.³⁴⁷ Several studies have also shown the ability of Res to increase lipolytic enzymes expression, including ATGL and HSL in murine, porcine, and human cultured adipocytes.^{317, 348} A study utilizing 3T3-L1 cells as well as mouse embryonic fibroblasts (**MEF**) cells with increasing doses of Res (5, 10, 20 μ M) throughout differentiation also observed lowered TG accumulation and reduced lipogenesis-associated-genes in both cell types.³⁴⁹ More specifically, 20 μ M of Res led to enhanced flux through the FAO pathway by increasing mRNA expression of carnitine palmitoyltransferase 1 (**CPT1**), the rate-limiting enzyme in mitochondrial FAO, and reducing mRNA expression of receptor-interacting protein 140, which is an inhibitor of oxidative metabolism.³⁴⁹

It was also demonstrated that Res elicits beneficial effects on fat mass, inflammation, and adipogenesis *in vivo*.³⁵⁰ This study utilized C57Bl/6J mice fed a HFD supplemented with 0.4% Res and showed significantly reduced body and VAT weight gain and also showed decreased inflammatory markers associated with VAT, such as TNF α and MCP1.³⁵⁰ In this study, Res supplemented mice had lower levels of blood glucose, TG, FFA, and cholesterol, as well as suppressed markers of adipogenesis, including PPAR γ 2, C/EBP α , SREBP-1c, FASN, LPL, FABP4, and leptin.³⁵⁰ Another study also demonstrated that Res treatment led to decreased inflammation markers in adipocytes; Res dose-dependently decreased MCP1 and IL-6, as well as a dose-dependent increase in adiponectin when TNF α -challenged (10 ng/mL) 3T3-L1 adipocytes were treated with 10, 25, and 50 μ M of Res.³⁵¹ Res was shown to dose-dependently inhibit adipogenesis by suppressing C/EBP β and PPAR γ expression as well as elicit anti-inflammatory benefits by reducing MMP-2 and MMP-9 activity in 3T3-L1 preadipocytes.²⁷⁷ Res has also been shown to work to reduce weight gain and appetite control in non-human primates (NHP).³⁵²

All of the studies mentioned in this section demonstrate Res' protective metabolic effects in WAT by decreasing inflammation, preventing lipid accumulation, and enhancing lipid clearance. The lipid and glucose clearing mechanisms and pathways are summarized in **Figure 2.5**.

2.3.4.2.2 Resveratrol and BAT

Not only has Res shown to elicit beneficial effects on metabolic parameters by reducing WAT mass and inflammation and improving insulin sensitivity, but it is also shown to enhance the thermogenic program in BAT. Although primarily focused on the anti-aging capacity of Res, Lagouge et al reported that supplementing 4 g/kg of Res for 15 weeks in C57Bl/6J mice fed a HFD led to weight loss, increased aerobic capacity, and increased tolerance to CE when mice were housed in 4°C for 6 h.²⁷⁵ This was coupled with the observation of induced oxidative phosphorylation and mitochondrial biogenesis genes, including CYTC, SIRT1, and PGC1 α , and also demonstrated protection from DIO and insulin resistance. Later, another study using rats fed a HFD supplemented with Res for 6 weeks demonstrated increased whole-body EE and increased gene expression of brown-fat enriched markers including UCP3, SIRT1, COX-2, PPAR β/δ , PGC1 α , and mitochondrial-transcription-factor-A (**TFAM**) as compared to rats fed the HFD alone.³⁵⁹ Rats fed a standard chow diet supplemented with Res for two months demonstrated improved blood glucose and lipid profiles, increased oxygen consumption rate, enhanced EE, and increased expression of BAT-enriched markers including UCP1, SIRT1,

PTEN, and BMP7.³⁶⁰ As previously discussed, BMP7 is a critical mediator of the brown-fat program and induces the early regulators of BAT including PRDM16,¹⁷⁴ PGC1 α ,²²⁴ as well as increased PPAR γ and P38-MAPK and PGC1 α -dependent mitochondrial biogenesis and activation of UCP1.^{114, 360} BMP7 is also shown to be responsible for triggering the brown-fat lineage in MSCs.¹¹⁴ Therefore, the ability for Res to further increase BMP7 demonstrates its ability to promote BAT activity and explains its ability to enhance EE and thermogenesis.

Many of the same pathways by which Res leads to beneficial effects and reduce WAT are also shown to induce BAT. Although it has been questioned whether Res and other small molecules work as direct SIRT1 activators, it has been shown time and time again that Res at least works through this pathway to elicit its effects on BAT.^{361, 362} Res either works to directly or indirectly activate SIRT1 activity, which functions to de-acylate many downstream targets including PGC1 α and PPAR γ , leading to enhanced BAT thermogenesis.^{340, 363, 364} Evidence from a study utilizing mice with whole-body deletion of SIRT1 led to a complete reversal of the thermogenic effect of Res, which further serves to substantiate that Res mediates its beneficial effects by working through SIRT1.³⁶⁵ Interestingly, many studies suggest Res acts as a potent PDE-inhibitor which increases IC-cAMP levels, activating energy-sensing networks in the cell.^{335, 336, 366, 367} Elevated cAMP activates the cAMP-effector protein, EPAC1, leading to increased IC levels of Ca²⁺.³⁶⁷ Increased IC levels of Ca²⁺ causes phospholipase C and ryanodine receptor Ca²⁺-release-channel to activate CamKK β and AMPK signaling pathway.³⁶⁷ The primary effect of this signaling cascade is to increase NAD⁺ causing activation of SIRT1 and its downstream activities. Several studies suggested PDE4 to be a potential specific target of Res, as the administration of PDE4-specific pharmacological inhibitors caused the same downstream effects of Res.³⁶⁵⁻³⁶⁷ Another study used both *in vitro* and *in vivo* approaches; utilizing CD1 mice on a 45% HFD supplemented with 0.1% Res for 4 weeks as well as SVF isolated from the iBAT of CD1 mice.³⁶⁸ The results from the *in vivo* study demonstrated that the BAT from Res-treated mice had enhanced brown-fat markers, including PRDM16, UCP1, and CYTC as well as greater oxygen consumption and Res-treated brown adipocytes had smaller multilocular LD compared to the controls. The results from the *in vitro* study demonstrated that 10 μ M of Res was sufficient to enhance oxygen consumption and increase expression of PGC1 α and pyruvate dehydrogenase (**PDH**) in addition to increased UCP1, PRDM16, and CYTC. The beneficial effects of Res seen in the *in vivo* and *in vitro* studies were attributed to AMPK1 α , as evidenced by inhibitor and knock out studies reducing Res' beneficial effects. In conclusion, many studies confirm the ability of Res to enhance BAT activity.

2.3.4.2.3 Resveratrol and beige adipocytes

In addition to the effects on WAT and BAT, Res has been reported to be beneficial in promoting the formation of beige adipocytes within WAT, which were thought to contribute to the effects of Res on weight and body fat loss, and increased thermogenesis.

A study reported that 25 μ M Res in maturing 3T3-L1 cells decreased lipid accumulation by 43% and decreased cell viability by 25%, which were accompanied by decreased mRNA levels of PPAR γ , C/EBP α , SREBP1c, FASN, HSL, LPL, and upregulated the mRNA expression of genes regulating mitochondrial activity, such as SIRT3, UCP1, and MFN2.³⁵⁷ The author's conclusion from the results of this study suggested a browning effect of Res; however, it is important to pair the observations with mitochondrial functionality and cellular bioenergetic assays to ensure cell health in addition to confirming thermogenic induction. Another study examining MEF cells with increasing doses of Res (5, 10, 20 μ M) during differentiation observed increased mitochondrial DNA (**mtDNA**), increased CYTC and UCP1 expression, as well as reduced expression of resistin and RBP4 compared to controls.³⁴⁹ The results of this MEF study suggest that Res enhances oxidative capacity and insulin sensitivity. Res has been shown to induce the development of beige adipocyte formation *in vitro*³⁷¹ and *in vivo*³⁷² mostly demonstrating Res beneficial effects are mediated through the AMPK-SIRT1-PGC1 α axis and PPAR γ activation.

One of the first studies completed *in vivo* showing Res to promote beige adipocyte formation was shown by the Du group.³⁷² CD1 female mice fed a 45% HFD supplemented with 0.1% Res for 4 weeks had increased beige adipocyte number within inguinal WAT (**iWAT**), which was accompanied by significant weight loss. The adipocytes formed within this "beiged" iWAT were smaller and had multilocular LDs with many mitochondria expressing UCP1 and showed evidence for enhanced FAO, demonstrating Res' capacity to induce brown-like fat cell formation within WAT. Furthermore, they also isolated SVF from the iWAT and demonstrated enhanced oxygen consumption, increased phosphorylation of AMPK1 α , and increased brown markers such as UCP1, PRDM16, CIDEA, EVOLVE3, PGC1 α , and PDH, when these SVF cells were treated with 10 μ M Res. To further explore and confirm the mechanism by which Res was able to promote browning, they used AMPK inhibitor, compound C, as well as AMPK1 α knock out, both of which were sufficiently abolished Res' browning ability. Consistently, the enhanced metabolic rate and decrease in fat mass seen in WT C57BL/6J mice fed a HFD supplemented with Res (400 mg/k/d) for 12 weeks were abolished in AMPK1 α -/- mice, but not in AMPK2 α -/-

mice.³⁷³ Both of these studies demonstrate the importance of AMPK1 α in mediating Res browning effects.

Not all studies were able to show that Res caused browning of WAT. A study using rats fed an obesogenic diet with high-sucrose (20%) and fat (24%) supplemented with Res (15 mg/kg/d) was able to show increased CIDEA and UCP1 expression in BAT but failed to see beige adipocyte formation in WAT or overall weight loss.³⁷⁴ Interestingly, when 30 mg/kg/d of another well-known polyphenolic compound, Quercetin, was added to the same diet, it showed a synergistic effect with Res in promoting the formation of adipocytes with multilocular LD and UCP1 expression within WAT. Compared to similar *in vivo* experiments supplementing HFD or chow diets with Res, 15 mg/kg/d seems to be a somewhat low dose. This may suggest moderate doses of Res to be most beneficial to preventing HFD-associated outcomes, rather than high-sucrose containing diets, further potentiating that the effects of Res could be species, dose, and diet-specific.

Interestingly, the effects of Res are shown to be transgenerational.³⁷⁵ It has been established that maternal obesity leads to increased risk of obesity, insulin resistance, and other detrimental metabolic diseases in human offspring.³⁷⁶ Similarly, maternal HFD impairs BAT function and leads to obesity in the offspring of mice.³⁷⁷ However, WT C57Bl/6J mothers fed a HFD supplemented with Res (0.2%) during gestation and lactation led to BAT metabolic recovery, promotion of beige adipocyte formation within WAT, and protection from obesity in offspring.³⁷⁷ When the offspring pups from Res-treated mothers were later challenged with a HFD, they exhibited increased brown-fat markers, such as UCP1, PRDM16, CIDEA, and PGC1 α as well as increased beige-fat specific markers, such as CD137 and TMEM26. Furthermore, these offspring showed overall increased whole-body EE, lowered adiposity, and increased insulin sensitivity in adulthood.³⁷⁷

2.3.4.2.4 Resveratrol and human clinical trials

As previously discussed, the presence of both brown fat^{95, 379} and beige fat¹⁴⁵ which have both been found to be inducible and functional, significantly promote thermogenesis, EE, as well as other beneficial effects on metabolic health in humans.³⁸⁰ The promising effects seen *in vitro* and animal studies have led researchers to try to find ways to promote and induce browning and brown adipogenesis in humans. There are many different agents which have been shown to induce browning and brown adipogenesis in humans, many of which have ended up being unsustainable or have potentially harmful side effects, for example, CE and sympathomimetics,

respectively.^{380, 381} Both strategies were shown to promote BAT activity and induce beige-fat formation in WAT in the neck³⁸² and SAT.^{383, 384} The effects of Res on inducing BAT activity and beige adipocyte formation, as well as promoting metabolic health *in vitro* and *in vivo* have been promising; however, the inconsistent results in human clinical trials makes determining if the results will be translatable for treating human obesity and metabolic conditions questionable.³⁸⁵
³⁸⁶ The use of Res in human clinical trials is further discussed here.

The following studies showed some beneficial effects of Res treatment on human health, in the context of metabolic diseases. A two-part randomized double-blind placebo-controlled clinical trial utilizing 11 obese, but otherwise healthy men were given 150 mg Res daily for 30 days.³⁸⁷ Although no changes in overall body weight or temperature were seen, the Res-group demonstrated CR mimetic effects with enhanced lipid clearance, better blood-lipid profiles, reduced blood pressure, lower inflammatory markers, higher muscle-mitochondrial respiration, as well as activation of AMPK-SIRT1-PGC1 α axis in muscle tissue.³⁸⁷ In another publication, the same group further analyzed additional outcomes in this same cohort of Res-supplemented men and reported enhanced adipogenesis as well as adipocytes with overall smaller diameter in WAT.³⁸⁸ Unfortunately, this study did not measure brown or beige-fat specific markers in WAT; however, the smaller size of the WAT adipocytes points towards a potentially healthier and more metabolically active tissue.³⁸⁸ When considered in conjunction with the activated muscle tissue, may explain the beneficial results seen in lipid clearance, blood pressure, and inflammation.^{387, 388} Another randomized double-blind placebo-controlled clinical trial using 24 patients with MS supplemented with 500 mg of Res three times daily for 90 days reported a significant decrease in overall BW, BMI, WC, fat mass, as well as AUC of total circulating insulin and total insulin secretion.³⁸⁹ This study demonstrated the beneficial effects of higher and multiple doses of Res on metabolically ill patients. Similarly, another study utilizing older adults (avg. 72 $\pm$ 3 years) with previously determined impaired glucose tolerance were given 1-2 g Res daily for 4 weeks reported enhanced insulin sensitivity and increased glucose clearance.³⁹⁰ Furthermore, a randomized double-blind placebo-controlled clinical trial using 10 patients with T2DM given 3 g of Res daily for 12 weeks reported increased SIRT1 and pAMPK/AMPK ratio expression in muscle as well as increased EE.³⁹¹ Although the study did not take AT biopsy samples or measure AT gene expression profiles, the author's conclusion of this study was that Res may have exercise mimetic beneficial effects in patients with insulin resistance.

Unfortunately, there are almost just as many clinical trials using Res to treat obesity and other metabolic diseases in humans with either negative or inconsequential results. A very

thorough randomized double-blind placebo-controlled clinical trial using 24 obese healthy male patients who were given 500 mg Res daily for 4 weeks showed no significant effects on blood pressure, insulin sensitivity, resting EE, VAT accumulation, nor inflammatory markers.³⁹² A randomized double-blind placebo-controlled clinical trial using non-obese postmenopausal women without glucose intolerance supplemented with 75 mg of Res daily for 12 weeks showed no differences in insulin sensitivity and no difference in expression of the "typical" Res molecular targets (i.e., AMPK, SIRT1, NAMPT, or PGC1 α) in either AT nor muscle.³⁹³ Finally, a randomized clinical trial using overweight and obese male patients with non-alcoholic fatty liver disease (**NAFLD**) were given 3 g of Res daily for 12 weeks.³⁹⁴ This study not only concluded no beneficial effects of Res but reported increased liver stress in the Res group as shown by elevated liver enzymes.³⁹⁴ It seems the majority of human clinical trials use Res supplementation with doses ranges anywhere from 75 mg – 5 g, the majority of which fall in the 500 mg – 1 g range. As discussed later in this review, much of oral-doses of Res is highly metabolized and excreted by the liver and kidneys, possibly making larger doses more hepatotoxic or at least increase stress on the liver. This study which utilizes a somewhat large dose of Res (3 g) in individuals with malfunctioning liver and already experiencing liver stress, seems like the study was already set up for failure.³⁹⁴

Discrepancies in outcomes can be both confusing and frustrating for researchers and consumers alike. There may be many reasons the disparities between *in vitro*, animal studies, and human clinical trials exist; however, the following is what the literature determines to be among the most likely reasons:

First, there are large differences in the concentration of Res used among cell culture, animal studies, and human clinical trials. Not only is the dose itself different, but, as previously discussed, the formulas used for human dose-equivalency are often outdated or still controversial.²³² Rodent studies are shown to use Res in ranges from 1-400 mg/kg of body weight per day, the most effective dose showing beneficial effects on metabolic health is in the higher range (0.4 g/kg*BW/d). Using direct conversion, without considering BSA or PAD, this would be equivalent to treating a 70 kg adult with 28 g Res per day. Although using BSA-based formulas are not recommended,²³² a BSA-based formula, the Raegan Shaw formula,³⁹⁵ determines the rodent's dose of 400 mg/kg*BW/d would be equivalent to 10.8 mg/kg*BW/d which turns out to be approximately 756 mg/d for a 70 kg adult.³⁹⁶ This ends up being within an average range of what many studies use. This considered, in both normal weight and obese individuals supplemented with up to 5 g/d were shown to be well-tolerated without significant

negative side effects or toxicity. With that said, well-tolerated and safe are not synonymous and higher doses do not always equvalate to greater outcomes.³⁹⁷ Its ability to interfere with medication has also not been established, so very high Res intake should be approached with caution.³⁹⁷

Second, both the age differences and time duration of studies are verifiably difficult to compare between rodents and humans, with the lifespan difference of rodents and humans being the primary obstacle. Although there are many formulas and methods which can determine equivalence factors for matching rodent and human age,³⁹⁸ many studies do not always take this into account. One very simple method uses direct conversion of life expectancy; the lifespan of rodents, lab rats, for example, is approximately 3 years, whereas worldwide human life expectancy is approximately 80 years, and a simple calculation provides a conversion factor of approximately 27.6 human days is equivalent to 1 rat day.³⁹⁸ So far, the human clinical trials using Res have shown to range from 4 to 12 weeks, whereas rodent studies have shown to range from 4 weeks and up to 6 months. This would be equivalent to approximately 2 – 13 years for humans, meaning the human study lengths would be considered relatively short. Similarly, many studies and much of the target population for humans would be adults and older adults (i.e., 40-70 years), whereas many studies utilize mice and rats which are young,³⁹⁹ further making it difficult to translate *in vivo* results.

Lastly, the low aqueous solubility and bioavailability of orally-administered Res are likely among the greatest constraining factors. Res solubility in water is very low at approximately 0.03 mg/mL,⁴⁰⁰ which would be equivalent to any physiological fluid, whereas in other solvents such as ethanol it is shown to be very high at 50 mg/mL.⁴⁰¹ Although it has been shown that humans can have at least 70% absorption and uptake of Res in the gut, post-absorption first-pass hepatic metabolism is extensive, leaving little free-Res in the serum, regardless of dose.⁴⁰² This metabolism and bioavailability are further discussed in 2.3.4.2.5.

2.3.4.2.5 Metabolism, pharmacokinetics, and bioavailability of resveratrol

Bioavailability (**BA**) is technically defined as the ability, degree, and rate in which the administered drug is absorbed systemically.⁴⁰³ In the case of Res, the term refers to the ability for the free form of *trans*-Res to be absorbed and circulate systemically. What will be discussed further here, is Res very high absorption rate, but very low BA due to its rapid hepatic and intestinal metabolism by phase II enzymes into water-soluble conjugates.^{313, 402, 404, 405} As discussed later, the majority of these derivatized products are seen in the urine and feces within

a short time after administration, making the rapid metabolism of Res a critical rate-limiting step in the BA of Res.⁴⁰⁶

One of the first evaluations of Res absorption and BA completed in humans was done by Goldberg et al,⁴⁰⁷ which evaluated if Res BA was altered by various food matrices in healthy subjects. The results of this study determined that there was little to no difference in absorption of different types of Res from liquids (whether Res was natural or added) and that food, especially foods high in fat, leads to a short delay in absorption, whereas Res from capsules delays absorption and causes Res to remain in the gastrointestinal (**GI**) system longer.⁴⁰⁷ Interestingly, another study showed that Res BA in humans shows greater interindividual variability, whereas gender and age were not shown to have a direct effect on BA.⁴⁰⁸

To better understand Res BA, understanding its digestion, absorption, and circulation patterns are important. There are two proposed mechanisms for absorption in the human GI which is shown to vary based on concentration. At very high concentrations, Res is primarily absorbed via simple/passive diffusion across brush-border membrane in the small intestines,⁴⁰⁹ with the possible formation of a complex with membrane transporters such as integrins.³¹³ However, at lower concentrations, Res uptake occurs via mediated apical transport into the enterocyte (i.e., ATP-binding cassette transporters).^{404, 409, 410} When inside enterocytes, Res is conjugated into metabolites to allow for IC aqueous solubility.^{404, 409, 410} In the plasma, Res can be found in 3 forms, including the free form as well as Res-conjugated with sulfate and glucuronide,³¹³ of these, sulfate conjugates comprise the majority of metabolites found in the plasma.⁴¹¹ In addition to the sulfate and glucuronide conjugated Res, several other Res metabolites have been recovered in the urine, including the *cis*- form of Res and *cis*-Res-metabolites, with sulfate conjugates comprising the majority of metabolites in urine as well.^{411, 412} The BA of the *cis*- form of Res is shown to be lower than the *trans*- form due to it undergoing glucuronidation 5-10 times faster.⁴⁰⁵ It is theorized the majority of the conjugates formed after being absorbed in GI and into circulation is the glucuronide form;⁴¹³ however, it has also been shown the primary metabolite formed within intestinal cells is the sulfate-conjugated Res.⁴⁰⁴ The addition and removal of sulfates, glucuronides, and glycosyl groups by phase II enzymes help to promote the aqueous solubility of Res as well as acting as a marker for removal by the liver and kidneys.⁴⁰⁶ However, when glycosyl groups are removed and replaced by hydrogen atoms,⁴¹⁴ the resulting aglycone form of Res is shown to be BA and its presence in the serum is highly accredited to enterohepatic recirculation.⁴¹⁵

Free-Res has been shown to circulate unbound; however, it is primarily found bound to albumin, so much so it has been hypothesized to be a natural reservoir for Res.^{313, 416} Res is also shown to be bound to LDLs, meaning cells expressing receptors for these plasma-proteins can dissociate Res, while also meaning another vehicle for transport to the liver.⁴¹⁶ It has also been shown that Res binding affinity to LDLs may mediate its beneficial antioxidant effects on LDLs, endothelial cells, and cells which take up LDLs.^{417, 418}

Many pharmacokinetic studies have been completed in an attempt to determine the optimal dose and timing based on its metabolism. A dose-escalating study giving 10 healthy volunteers a single dose of 0.5, 1, 2.5, and 5 g of oral Res tested both urine and plasma free-Res and metabolite levels.⁴¹⁹ The results of this study showed that 77% of both free- and metabolite forms of Res were detected in urine at 4 h after the lowest dose administered, and that the peak levels of free-Res at the highest dose were 539 ± 384 ng/mL which is approximately $2.4 \mu\text{M/L}$ after 1.5 h.⁴¹⁹ When metabolites were included in this peak plasma levels, it was much higher (3-8 fold higher). Another study using up to 150 mg 6 times a day only reported a peak plasma level (C_{max}) 63.8 ng/mL with a half-life of approximately 1-3 h for a single dose and 2-5 h with repeat dosing.⁴²⁰ Using a more “dietary relevant dose” of 25 mg oral and intravenous (**IV**) administration of ¹⁴C-labeled-Res showed peak plasma of Res and metabolites to be 491 ± 90 ng/mL ($\sim 2 \mu\text{M}$) with a half-life of approximately 9.2 ± 0.6 h.⁴⁰² One of the highest BA forms of Res used in many of these kinetic studies was seen when using 5 g of orally administered micronized Res in 40 healthy volunteers.⁴²¹ The results of this study revealed a plasma C_{max} of free-Res alone to be 967 ng/mL ($\sim 4.2 \mu\text{M}$), whereas, with free-Res and metabolites, the plasma C_{max} was up to $18 \mu\text{M}$.

Interestingly, there have been some studies to reveal there were some gender and species differences with Res-metabolite formation and excretion. A dose-escalation study in rats given high-doses of Res (300, 1000, and 3000 mg/kg*BW) revealed male rats had a peak plasma of 576, 991, and 2728 ng/mL respectively, whereas female rats had peak plasma of 333, 704, and 1137 ng/mL respectively ($1.1 \mu\text{g/mL} \cong 5 \mu\text{M}$).⁴²² Results of one study revealed the free-Res and metabolites in the urine of rodents was only 1.5% of what was initially administered intra-gastrically,⁴²³ whereas the free-Res metabolites found in human urine was at least 24.6% of what was originally administered orally.⁴²⁴ There were five different types of Res-metabolites recovered in human urine: Res-mono-sulfate, mono-sulfate dihydro-Res, mono-glucuronide dihydro-Res, as well as two isomeric forms of Res mono-glucuronide.⁴²⁵ Interestingly, some

studies have shown the synergistic relationship of Res in combination with other polyphenols, such as enhanced browning with the addition of quercetin³⁷⁴ and increased free-Res plasma-concentration with the addition of piperine.⁴²⁶ In the latter mentioned study, it was demonstrated that the addition of orally administered piperine (10 mg/kg) with Res (100 mg/kg) in C57Bl/6J mice led to more than 200% increased exposure to Res and over 1500% greater concentrations of free-Res in plasma when compared to Res administered alone.⁴²⁶ The results here and in other synergistic-relationship studies may at least partially be due to what one study reveals as reduced sulfation and glucuronidation of Res when administered in combination with other polyphenols, improving its BA and possibly its effectiveness.⁴²⁵

A comprehensive review of over 25 different pharmacokinetic studies revealed doses given to healthy subjects ranged from as little as 1 mg and up to 5 g Res per day which was given either as single doses or over a period of a month.²⁶³ Given the results of these studies, it seems like the highest concentration seen in human plasma (without including metabolites), was approximately 4.2 μ M after a mega-dose of 5 g of micronized Res; however, most studies showed within the nanomolar range to around 2 μ M. This considered, the doses given *in vitro* or in animal studies are typically much higher.²⁹⁹ Even though there are such great discrepancies and variances in dose between cell/animal studies and human clinical trials, there are still many health benefits associated with Res use in humans.^{299, 427} Although there is limited evidence to prove Res promotes BAT activation and beige adipocyte formation in humans, there is still a large body of evidence to support the beneficial effects of Res on other diseases and health parameters.^{299, 427} This effect has now been coined as the "Resveratrol Paradox", which describes Res high activity and beneficial effects despite this rapid metabolism and low BA.^{397, 428} This has led some researchers to determine whether Res-metabolites themselves may have some beneficial function.

2.3.4.2.6 Can resveratrol metabolites have a beneficial effect?

Identifying different types of metabolites and using Res-metabolites as treatments in studies have been shown in both animals (pigs)⁴²⁸ as well as humans.^{429, 430} However, the use of metabolites in research is limited due to the lack of suitable standards.^{431, 432} With that said, some studies are showing beneficial effects of Res-metabolites including a study by Lasa et al which showed that Res and Res-metabolites produced an anti-adipogenic effect in 3T3-L1 cells at 25 μ M.⁴³³ This study showed that while Res, trans-Res-4'-O-glucuronide, and trans-Res-3-O-sulfate reduced C/EBP β mRNA expression in mature 3T3-L1 adipocytes, only trans-Res-3-O-

sulfate alone also reduced C/EBP α , PPAR γ , and LPL expression.⁴³³ Res was able to induce SIRT1, ATGL, and PGC1 α expression in mature 3T3-L1 adipocytes, whereas trans-Res-3-O-glucuronide reduced FASN and increased SIRT1 expression, and trans-Res-4'-O-glucuronide increased HSL and SIRT1 expression as well.⁴³³ More recently, this same group attempted to identify potential microRNAs (**miRNAs**) which were responsible for mediating Res and Res-metabolites' anti-adipogenic effect.⁴³⁴ This study used 25 μ M of free-form of Res as well as 3 Res-metabolites: trans-res-3-O-sulfate (3S), trans-Res-3'-O-glucuronide (3G), and trans-Res-4'-O-glucuronide (4G) to treat differentiating 3T3-L1 preadipocytes.⁴³⁴ The results of their study revealed that free-Res and the glucuronide metabolites upregulated miR-155, which then led to downregulation of C/EBP β and its downstream adipogenesis targets.⁴³⁴ Interestingly, sulfate-metabolites did not mediate their anti-adipogenic effect through any of the 23 miRNAs tested (all of which were previously shown to have predicted or validated downstream adipogenesis targets), but did so through other pathways.⁴³⁴ Therefore, it seems that both Res and its metabolites are responsible for some of the anti-adipogenic and anti-obesity effects previously discussed.

Other studies utilizing Res metabolites were primarily focused on cancer, especially colon cancer. A study done by Aires et al demonstrated that sulfate and glucuronide conjugates are likely to play a role in the Res-mediated anti-carcinogenic effect on human metastatic colon cancer cells.⁴³⁵ Similarly, another study using Res and Res-metabolites showed beneficial effects on colon cancer patients.⁴³⁶ These effects are suggested to work via a synergistic interplay between Res and its metabolites to have overall greater effects than Res itself.⁴³² It is suggested the extensive metabolism and subsequent accumulation of Res and Res-metabolites in human intestinal epithelial cell line, Caco-2, was a primary mechanism for its chemoprotective effect in these cells.⁴⁰⁴ Another Res derivative has recently been described, called 3,5,4'-trimethoxystilbene (**BTM-0512**), was shown to have greater stability and oral bioavailability than free-Res which may have led to the enhanced browning and weight loss observed.^{378, 437}

Pharmacometabonomic studies have shown significant interactions of the gut microbiome and drug metabolism in humans.⁴³⁸ Specifically, the beneficial effects of microbiome metabolic byproducts of Res have been investigated and some of these studies have demonstrated these to have beneficial effects on health *in vitro*, in animal studies, and in humans.⁴³⁹⁻⁴⁴² The gut-microbiota derived dihydro-Res (**DH-RES**) along with similar analogs have been studied in humans.⁴⁴³ One study demonstrated that using as little as 100 nM of Res or DH-RES, but not Res-metabolites, led to decreased FABP4 expression in macrophages in response to oxLDL

exposure.⁴⁴⁴ Interestingly, this study demonstrated that Res and its metabolites have a hormetic-like response,⁴⁴⁴ which is used to describe nutritional, pharmacological, or environmental agents that have a dose-dependent-biphasic response.⁴⁴⁵ This hormetic response of Res demonstrates that a low-dose stimulation may have a beneficial effect, whereas the high-dose may have an inhibitory or toxic effect.⁴⁴⁵ This further demonstrates Res's pleiotropic downstream effects, which are tissue, dose, and time-dependent.

Overall, it seems the results observed using Res treatment *in vitro* and *in vivo* are not always translatable to human clinical trials, primarily due to its rapid metabolism and therefore low BA. That said, if low BA of Res is the primary limitation for its use in human clinical trials, it is imperative to explore other avenues to optimize BA. As previously discussed, there have been several attempts at improving the BA of Res, including alterations in the food matrix in which Res is administered,⁴⁰⁷ utilizing micronized powders,⁴²¹ as well as combining with other bioactive compounds and polyphenols in hopes for a synergistic effect.³⁷⁴ Nanoparticles and the use of nanotechnology is also a promising strategy to improve BA and possibly targeting capacity in humans.⁴⁴⁶

2.4 NANOPARTICLES CONTAINING RESVERATROL AS A NUTRACEUTICAL INTERVENTION FOR OBESITY

2.4.1 What is nanotechnology?

Nanotechnology can be described as the study and control of microscopic material (usually 100 nm or smaller).⁴⁴⁷ The use of nanotechnology as therapeutic agents is a practice known as 'nanomedicine', and its use in pharmaceutical applications has been exponential and so far, very successful.^{448, 449} There have been a countless number of compounds loaded into the nanoparticles for various disease applications including several types of cancer, diabetes, liver fibrosis, neurodegenerative and bone diseases, atherosclerosis, obesity, and many more.^{448, 449} These nano-scale molecules are generated to be biodegradable and biocompatible for enhanced BA *in vitro* and *in vivo* as well as for therapeutic use in humans.⁴⁴⁷ Nanocarriers can be generated using several different bio-materials and formulations, giving each of them advantages for various applications.⁴⁴⁷ Included in the various forms of nanocarriers are liposomes, solid lipid nanoparticles, micelles, emulsions, and polymeric nanoparticles which will briefly be discussed further here.

2.4.2 Types of nanocarriers

Liposomes, or nanoliposomes (the nanometric size of liposomes),⁴⁵⁰ are primarily composed of phospholipids whose hydrophilic heads and hydrophobic tails form a bilayered lipid membrane.⁴⁴⁷ The structure of liposomes allows it to carry both hydrophilic and hydrophobic compounds; hydrophobic compounds can be loaded between the lipid bilayers, whereas hydrophilic compounds can be loaded within the aqueous central compartment.⁴⁵¹ Since its initial formulation and use in the mid-1960s, liposomes have been used in several arenas such as the food and cosmetic industry, drug delivery, and diagnostic testing, and has also been FDA approved to treat several diseases.⁴⁵²⁻⁴⁵⁶ Smaller and uniform-sized nanocarriers are optimal for use in clinical and laboratory settings; however, liposome size can be highly variable.^{455, 457} Microfluidization is a common process that can create the smaller sized nanoliposomes which have advantages over larger liposomes by creating a larger area for nanoliposomes to interact with the target, therefore enhancing its BA.⁴⁵⁰ Although smaller sized nanoliposomes enhance the BA by increasing surface area interaction, it also reduces the loading capacity of hydrophobic molecules due to limited space between the bilayered lipid membranes.⁴⁵⁸ Coating with inert polymers, such as poly(ethylene glycol) (**PEG**)⁴⁵⁹ can help to stabilize nanoliposomes and reduce clearance by the reticuloendothelial system⁴⁶⁰ or disintegration by electrostatic interactions.^{461, 462} PEG is widely used in nanoparticle preparation for the hydrophilic shell portion due to its hydrophobicity, low toxicity and immunogenicity, and biocompatibility.⁴⁶³⁻⁴⁶⁶

Solid lipid nanoparticles (**SLNs**) are a popular type of nanocarrier also having a hydrophilic shell and hydrophobic lipid core which has a high melting temperature and is solid at room temperature.⁴⁶⁷ The lipid core is usually composed of solid FFA, TGs, and waxes, or some combination,^{468, 469} stabilization and dispersion of the lipids is assisted by the use of surfactants such as phospholipids and bile salts, among others.⁴⁶⁸ The advantage of using SLNs is that it has very high biocompatibility and has been shown to provide reproducible results without the use of organic solvents.⁴⁶⁷ Unfortunately, however, it has relatively low loading capacity and is shown to leak its contents during storage.⁴⁶⁹ A similar compound known as nanostructured lipid carriers (**NLCs**) was synthesized to overcome the limitations of SLNs.⁴⁶⁷ NLCs have similar beneficial characteristics as SLNs, including being small, stable, biodegradable, and biocompatible, but have the advantage of increased loading capacity.⁴⁶⁷ Imperfect crystals are formed by varying the types of solid-lipid particles (i.e., use of mono-, di-, and tri-glycerides instead of just TGs) used for synthesizing the NLCs, reducing leakage and enhancing compatibility with insoluble compounds.⁴⁷⁰⁻⁴⁷³ Interestingly, SLNs and NLCs are used to help

improve BA of bioactive components which are hydrophobic by ways of absorption, digestion, and circulation within the body.^{474, 475} Due to the composition of these nanocarriers, when orally administered, enteral digestion takes up the SLNs and NLCs into lacteal-lymphatic transportation which allows entry into systemic circulation via the jugular and left subclavian vein, thereby bypassing the first-pass through the liver.⁴⁷⁴⁻⁴⁷⁶ This effectively enhances the BA of the nanocarriers as well as the components they are carrying.

Similar to SLN and NLCs, **emulsions** have a hydrophilic shell and hydrophobic lipid core.⁴⁷⁷ However, whereas the SLN and NLCs have a solid lipid core, emulsions have a liquid lipid core which is a result of the dispersion and formation of a solution between two otherwise immiscible liquids.⁴⁷⁷ Emulsions are also stabilized using surfactants and emulsifiers, which are then able to reduce surface tension and increase medium viscosity.^{478, 479} To remain small and thermodynamically stable, smaller emulsions require more surfactants and higher energy input to lower interfacial tension.⁴⁸⁰ Oral administration of the small emulsions is shown to increase the BA of bioactive compounds,^{481, 482} which disperse in the GI and have large size-to-surface area equivalency and therefore improve aqueous solubility.⁴⁸³

Similar to SLNs, NLCs, and emulsions, but without having a solid nor liquid lipid core, **micelles** are formed using amphiphilic colloids such as phospholipids when critical micelle concentrations and temperatures are met.^{484, 485} The advantages of using micelles to encapsulate and transport hydrophobic compounds include an increase of aqueous solubility, extended circulation time, and lower toxicity.^{486, 487}

Although there are several other formulations of nanocarriers, the last reviewed here are **polymeric nanocarriers**, which are also biocompatible and biodegradable polymers, which have been FDA approved and have low toxicity.⁴⁸⁸ Polymeric nanocarriers include poly(lactic-co-glycolic acid) (**PLGA**), **PEG**, and polycaprolactone (**PCL**), and can be made by several different processes, including of solvent evaporation, emulsification-diffusion, and nanoprecipitation.^{489, 490}

2.4.3 Nanocarriers enhance resveratrol solubility, bioavailability, and targeting capacity

Nanotechnology is a novel method of delivery which can enhance Res aqueous solubility and BA and can also be used to enhance targeting capacity, further enhancing its BA and anti-obesity therapeutic activity. Nanoparticle encapsulated Res has been used in several studies to demonstrate these improvements in various disease states, which are further discussed here.

Liposomes have been commercially used in the food and supplement industry to help promote solubility and BA of proteins, enzymes, vitamins, and even flavors.⁴⁵⁵ Liposomes are shown to enhance Res chemical and photostability, as evidenced by preventing isomerization from *trans*- to *cis*- when exposed to 16 min of UV radiation by seven to one when compared to free-Res.⁴⁹¹ Liposomes were also shown to have 95% encapsulation efficiency and enhanced physical stability of Res, which was shown by no significant change in particle size, polydispersity (measures molecular weight distribution), nor zeta potential (electrokinetic potential of the nanoparticle versus the medium) after 2 months in 4°C storage conditions.⁵⁰¹ As previously discussed, however, liposomes have limited loading capacity for hydrophobic molecules such as Res.⁴⁵⁸ This presents a major disadvantage for using liposome as a vehicle for Res delivery *in vitro*, in animal studies, and in human studies.

SLNs have been used to encapsulate Res and was also shown to enhance solubility, chemical and physical stability, and BA by providing protection from enzymatic degradation and metabolism *in vivo*.⁵⁰² SLN-encapsulated with Res has also been shown to extend and provide sustained release over time, which was attributed to a diffusion-controlled mechanism.⁴⁹⁵ A study using SLN composed of glyceryl-behenate encapsulated with Res demonstrated a sustained release pattern as well as an increased concentration of the SLN-Res as compared to free-Res in the brain tissue of adult Wistar rats.⁴⁹⁵ The primary implications of this study demonstrate SLN help drugs to cross the blood-brain barrier (**BBB**), which is a primary obstacle for the delivery of cytotoxic drugs for the treatment of neoplastic tissues.⁴⁹⁵ Similar to liposomes, SLN was also shown to help prevent Res isomerization when exposed to UV light.⁵⁰³ SLN also enhances IC delivery and reduces cytotoxicity; SLN-Res less than 180 nm in diameter were quickly internalized and distributed throughout IC compartments without inducing cytotoxicity, unlike free-Res, which was shown to be slightly cytotoxic when in solution.⁴⁹⁶

Similar to SLNs, polymeric nanoparticles have been shown to enhance Res solubility and BA and provide a controlled and steady release pattern via diffusion.⁴⁶⁵ A combination of PCL: PLGA-PEG-based nanoparticles was shown to have high encapsulation capacity (73-98%) and released Res continuously over the course of 24 h which led to a greater chemoprotective effect in prostate cancer cell lines.⁴⁶⁶

Many of these nanocarriers can be modified via the addition of specific formulations of lipids as well as additions of peptides and ligands to enhance their targeting specificity, thereby augmenting treatment effect and BA.⁵⁰⁴ As previously mentioned, alteration of the lipid-

formulation of the SLN (i.e., addition of Compritol as the lipid core) allowed SLNs to cross BBB and target brain tissue in rats.⁴⁹⁵ On the other hand, there are some types of lipid-based formulations which can effectively bypass the BBB, therefore avoiding the brain and reach the desired destination without being targeted and removed by the reticuloendothelial system.⁵⁰⁵ Incorporation or conjugation of these targeting molecules on the surface of liposomes, SLNs, and polymeric nanoparticles can help to target cell-surface receptors or ECM proteins which are only expressed in a certain tissue or cell type.^{504, 506}

In conclusion, nanocarriers can help to improve BA and solubility, extend release time, and enhance Res targeting capacity and effectiveness. These show promise for the therapeutic use to treat obesity and other metabolic disorders. However, there are several limitations in the wide-spread use of nanoparticles due to the fact their synthesis is time-consuming and expensive, and future studies are still needed to determine their longitudinal safety and effectiveness.^{507, 508}

2.5 THE ANTI-OBESITY POTENTIAL OF INDOMETHACIN

2.5.1 Indomethacin introduction: Structure, history, and function

Indomethacin (**Indo**) is a type of nonsteroidal anti-inflammatory drug (**NSAID**) which primarily functions as a prostaglandin G/H synthase 1 and 2, also known as COX-1 and -2, inhibitor.^{509, 510} COX-1/-2 functions to catalyze the conversion of membrane phospholipid-derived fatty acids into bioactive lipid cell-signaling molecules known as prostaglandins (**PGs**) or eicosanoids.^{510, 511} These fatty acids are cleaved from sn-2 positions of the phospholipids via enzymatic hydrolysis by phospholipase A2 (PLA2), primarily releasing arachidonic acid (**AA**) but can also include polyunsaturated fatty acids.⁵⁰⁹ PGs mediate a number of autocrine and paracrine activities in which the downstream effects can be substrate-, isoform-, and tissue-specific.⁵¹² Some of these activities and downstream effects include, but are not limited to, vasodilation and constriction, blood clotting, cell growth and differentiation, GI secretions (i.e., stomach acid, mucus, bicarbonate, etc), as well as pro- and anti-inflammatory and pain sensory signals.⁵¹² COX inhibitors primarily function by binding and antagonizing COX, thereby preventing innate ligands and substrates from entering the active catalytic site, effectively diminishing the production of PGs.⁵¹⁰

Both isoforms of COX are widely expressed throughout the body; however, COX-1 is constitutively expressed, whereas COX-2 is strictly inducible in response to stress, injury, and inflammation.^{513, 514} Therefore, anucleate cells (i.e., erythrocytes such as red blood cells) cannot

induce the expression of COX-2.⁵¹³ Although both isoforms are involved, COX-1 more-so than COX-2 is involved in production PGs which regulate GI secretions helping to maintain the protective mucosal barrier, as well as promoting epithelial cell and tight-junction health and function.⁵¹⁵⁻⁵¹⁹ It is for this reason that the use of dual COX inhibitors has been highly implicated in toxicities including excessive bleeding, ulceration, and mucosal permeability and inflammation.^{513, 515} Although Indo is a non-selective COX inhibitor, it has a predilection for inhibiting COX-1 over COX-2,⁵¹⁰ therefore, having a higher risk for toxicities as compared to other NSAIDs.⁵¹³

The chemical name for Indo is 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid and its chemical classification is as an indole-acetic acid derivative.⁵¹⁴ Indoles are aromatic heterocyclic organic compounds having a bicyclic structure composed of a 5-carbon pyrrole ring fused with a 6-carbon benzene ring.⁵²⁰ Indo was one of the first NSAIDs synthesized after acetyl-salicylic acid (aspirin) in the mid-1960s by the director of Merck Sharp & Dohme, Carl Stevenson, and was shown to have potent analgesic, antipyretic, and anti-inflammatory properties.^{513, 514} Indo was generated in response to the observation that serotonin has potent anti-inflammatory properties, and tryptophan, which is a precursor to serotonin, has an indole structure.^{513, 521} Indo has since become a well-studied FDA-approved drug that is used to treat and relieve inflammatory states of the body such as fever, pain, swelling, stiffness, in addition to more serious disease states including pleurisy, pericarditis, and gout.⁵²² Typical recommendations for intake ranges from 25-50 mg two to three times daily, depending on the severity of the condition, as prescribed by a physician.⁵²³

2.5.2 Indomethacin pharmacology: absorption, metabolism, and pharmacokinetics

Indo is shown to work through several transporters including multidrug-resistant protein^{524, 525} and solute carrier organic anion transporter family member 1A2.^{526, 527} Absorption of Indo in the GI is rapid and has a very high BA, with approximately 100% absorbed after oral administration.⁵¹⁴ Whereas plasma concentrations of Indo are linear and dose-proportional, the half-life and systemic clearance is dose-dependent.^{514, 528} A dose-escalation study using single doses of Indo at 25, 50, and 75 mg resulted in a peak plasma concentration of 1.54, 2.65, and 4.92 µg/mL, respectively, in fasting subjects after 1.5 h.⁵²⁹ Due to Indo's high BA, it is suggested to be unlikely to undergo first-pass hepatic metabolism.⁵³⁰ However, Indo-bound to albumin, when transported to the liver, is subjected to phase II enzymatic hepatic metabolism which creates inactive metabolites.⁵³⁰ Indo-metabolites are primarily excreted in the urine (~60%),

followed by feces (33%), as well as bile.^{523, 530} Although the half-life of Indo is variable based on dose and enterohepatic recirculation, the estimated average is approximately 4.5 h.^{523, 530}

2.5.3 Indomethacin non-COX-inhibitor mechanisms

Although the FDA-approved indication for Indo is its action as a dual-COX inhibitor, it also has other mechanisms for reducing the inflammatory response and other off-target effects.⁵¹⁴ Indo's anti-inflammatory and analgesic effects are primarily contributed to its inhibition of PG production, whereas its antipyretic effects have been attributed to actions in the hypothalamus which override PG signals, resulting in vasodilation and increased blood flow, allowing heat to be distributed and dissipated.⁵²² Unlike other NSAIDs, Indo has been shown to not only block COX from producing PGs, but it is also shown to inhibit the action of PLAs, thereby reducing substrate for COX enzymatic activity.⁵³¹ Although the American Society of Health-System Pharmacists 2015, which is cited by both National Institute of Health (NIH) supported drug-databases PubChem and ToxNet, suggest that Indo may work as a PDE-inhibitor, increasing IC concentrations of cAMP, potentially playing a role in the anti-inflammatory response, there was no other supporting literature.^{510, 532, 533} In fact, most literature suggests that treatment with pharmacologically-designed PDE-inhibitors helps to mitigate the effects of Indo, thereby having opposing mechanisms.⁵³⁴⁻⁵³⁶ The majority of early Indo literature focused on its COX inhibitory functions;⁵⁰⁹ however, relatively recent studies have shown Indo as well as other NSAIDs to have off-target effects, such as activation of PPARs.⁵³⁷⁻⁵⁴²

2.5.4 Indomethacin activates PPARs

Several studies have shown NSAIDs, including Indo, to bind to and activate PPARs. By using a PPAR γ competitive binding assay, Lehmann et al revealed Indo successfully displaced a TZD with a half-maximal inhibitory concentration (IC₅₀) of 100 μ M.⁵⁴⁰ This study also demonstrated that Indo is able to activate PPAR γ greater than other NSAIDs and to a lesser extent PPAR α at 100 μ M in NHP kidney cells (i.e., CV-1).⁵⁴⁰ Similarly, Puhl et al demonstrated that Indo significantly binds to and activates PPAR γ , as shown by displacement assays (Indo IC₅₀ = 21.3 μ M) and a PPAR γ reporter gene assay (Indo half-maximal effective concentration (EC₅₀) = 21 μ M) done in human cervical cancer cells (i.e., HeLa), respectively.⁵⁴¹ However, it was also shown that Indo did not activate and bind to PPAR γ greater than other tested NSAIDs (binding affinity = sulindac > diclofenac > Indo > ibuprofen ; activation = diclofenac > Indo > ibuprofen).⁵⁴¹ Although diclofenac was shown to elicit the half-maximal response at a lower dose

than Indo (diclofenac $EC_{50} = 1.69 \mu M$), Indo $\geq 10 \mu M$ was more efficient in activating PPAR γ than any other NSAID tested at the same concentration.⁵⁴¹ Furthermore, this study revealed that Indo displayed binding modes similar to that of *strong partial* agonists, whereas diclofenac acted as a *weak partial* agonist and sulindac displayed toxicity preventing completion of the assay.⁵⁴¹ For example, diclofenac was shown to work as a weak partial agonist by binding to PPAR γ in the LBP near the β -sheets and helix 3, whereas Indo was shown to bind similar to that of a full agonist by occupying the C-terminal helix H12 as well as partial agonist site, H3 of PPAR γ .⁵⁴¹ Although Indo is not able to replicate the “U-shape” conformation of TZDs which occupies and bridges two sub-pockets of the LBP, Indo was still shown to closely interact with the “strong” H12 site with a higher affinity than the “partial” H3 site separately, stabilizing H12 and likely accounting for its “strong” partial agonist capacity.^{541, 543-546}

A *partial* agonist can be described as a substance that binds to and activates its target receptor but does not reach the maximal response capable of a full agonist and can even act as an antagonist or not function if the receptor is already bound.⁵⁴⁷ The latter description of a partial agonist was demonstrated in a study done by Adamson et al which demonstrated that although diclofenac had an affinity to PPAR γ 50-times greater than other NSAIDs, it antagonized beneficial effects seen by Rosi in adipocytes, prostate cancer cells, and NHP kidney cells.⁵⁴⁸ This study also demonstrated that Indo significantly activated PPAR γ at 500 μM in NHP kidney fibroblasts (i.e., COS-1).⁵⁴⁸ Cho et al also found that Indo promoted PPAR γ binding to coactivators in a ligand- and dose-dependent manner,⁵³⁸ and later used Indo in another study as a positive control for testing other PPAR γ ligands.⁵³⁷ Lastly, Jaradat et al showed that Indo significantly activated PPAR γ in CV-1 cells greater than the two ibuprofen isomers.⁵³⁹ Interestingly, ibuprofen currently on the market for therapeutic use is actually a racemic equal mixture of the two isomers, R(-)-ibuprofen and S(+)-ibuprofen; however, S(+)-enantiomer is able to inhibit COX whereas R(-)-ibuprofen is not.⁵⁴⁹

Several studies suggest that Indo elicits this off-target PPAR γ -activating effect at higher concentrations, whereas it only has COX inhibiting effects at lower concentrations.^{540, 541} Several observations demonstrated that the concentration required to inhibit PG synthesis did not activate PPAR γ in reporter gene assays.⁵⁴⁰ On the other hand, it was observed that higher concentrations of Indo not only inhibited PG synthesis but also activated PPAR γ and enhanced adipocyte differentiation, which PPAR γ is a critical mediator for.^{540, 550}

2.5.5 Indomethacin promotes adipocyte differentiation

Indo has been shown to enhance adipocyte differentiation in many studies and is now a common compound used in adipocyte differentiation cocktails. One of the first of these observations was made by Williams et al, which demonstrated Indo to stimulate differentiation of 3T3-L1 adipocytes at concentrations ranging from 2.7 – 130 μ M in an insulin-concentration-dependent manner.⁵⁵¹ Using a cell line that will spontaneously differentiate after being confluent for some time even without hormonal or chemical stimulation, Knight et al demonstrated that Indo (at 125 μ M) enhanced TA1 adipocyte differentiation greater than dexamethasone (at 1 μ M) alone, in an undetermined COX-independent mechanism.⁵⁵⁰ Indo was also shown to enhance mesenchymal stem cell line (C3H10T1/2) adipocyte differentiation in a manner that reflected its ability to activate PPAR γ (~100 μ M).⁵⁴⁰ Another study demonstrated Indo at as little as 10 μ M was able to promote adipogenesis, lipid accumulation, and adipocyte-related genes almost to the extent of Rosi at 1 μ M in 3T3-L1 adipocytes.⁵⁴¹ A recent study by Hao et al in collaboration with our lab was the first to demonstrate the possibility of Indo to promote brown adipogenesis *in vitro* and *in vivo*.⁵⁵² Using a dose-escalation of Indo (2, 5, 10, 20, 50 μ M), this study demonstrated a dose-dependent increase in brown adipogenesis in a murine brown cell line as shown by enhanced ORO lipid accumulation, PPARE activation, mtDNA, UCP1 and PGC1 α mRNA and protein expression, as well as *Ppar γ* , *Prdm16*, and *Nrf1* mRNA expression.⁵⁵² Indo is also a current staple used in the induction media for adipocyte differentiation, usually at 125 μ M, along with insulin, IBMX, dexamethasone, Rosi, T3, and sometimes other compounds.^{553, 554} Indo is used in the media which is used to induce beige adipocyte formation in ASCs isolated from SVF in mice.⁵⁵⁵

There have been only a few instances where Indo was shown to inhibit adipogenesis. In one case, Ob1771 (cell line from *ob/ob* C57Bl/6J mice), were differentiated in serum-free media in the presence of exogenous AA and 10 μ M of Indo.⁵⁵⁶ The decrease in adipogenesis was solely measured by reduced glycerol-3-phosphate dehydrogenase (**GPDH**) activity, which is responsible for catalyzing the reversible conversion of glycerol-3-phosphate (**G3P**) to dihydroxyacetone phosphate (**DHAP**). Some of the results of this study were not well understood or explained; however, there are a few confounding factors that may influence these contradictory outcomes. First, the use of Ob1771, the cell line from genetically transformed obese mice, may have altered outcomes as compared to wild-type adipocytes due to some obese WAT having altered PPAR γ expression.⁴³ Second, the use of serum-free media with

exogenous AA may have forced the cells to utilize AA for energy rather than convert to PG. However, none of these explained the findings that cells treated with both Indo and AA were not able to differentiate. The authors of this study claimed it was due to Indo inhibition of COX-production of PGs which would stimulate the cAMP rise necessary for serum-free, hormone-supplemented differentiation. Seeing as Indo did not affect GPDH activity when AA was not added to the media, it seems the effect may be due to AA rather than Indo. Another study reported that Indo limited insulin signaling and lipogenesis via suppressing autophosphorylation of the IR and IRS in rat adipocytes when using 1 mM of Indo.⁵⁵⁷ Although there is no direct explanation of these effects, there may be cytotoxic effects associated with using very high concentrations of Indo. Indo's recommended therapeutic dose ranges from taking 25 mg 1-3 times daily and up to 75 mg once daily, depending on the severity of the disease or ailment.⁵²³ Pharmacokinetic studies have shown peak plasma levels in humans after a single 75 mg dose to be just over 10 μ M ($C_{\max} = \sim 14 \mu$ M)⁵²⁹ which is 70 times less than the 1 mM of Indo used in this study which demonstrated reduced insulin signaling and lipogenesis.⁵⁵⁷ Therefore, the dose used in this study may not be physiologically relevant, and cytotoxicity should be evaluated.

In conclusion, Indo has shown strong partial PPAR γ agonist binding and activity with the closest ability similar to TZDs among any other NSAID that was not toxic. Indo has been shown to enhance adipogenesis, including brown adipocytes; however, its effects on browning of white adipocytes have not been studied.

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2.7 APPENDIX

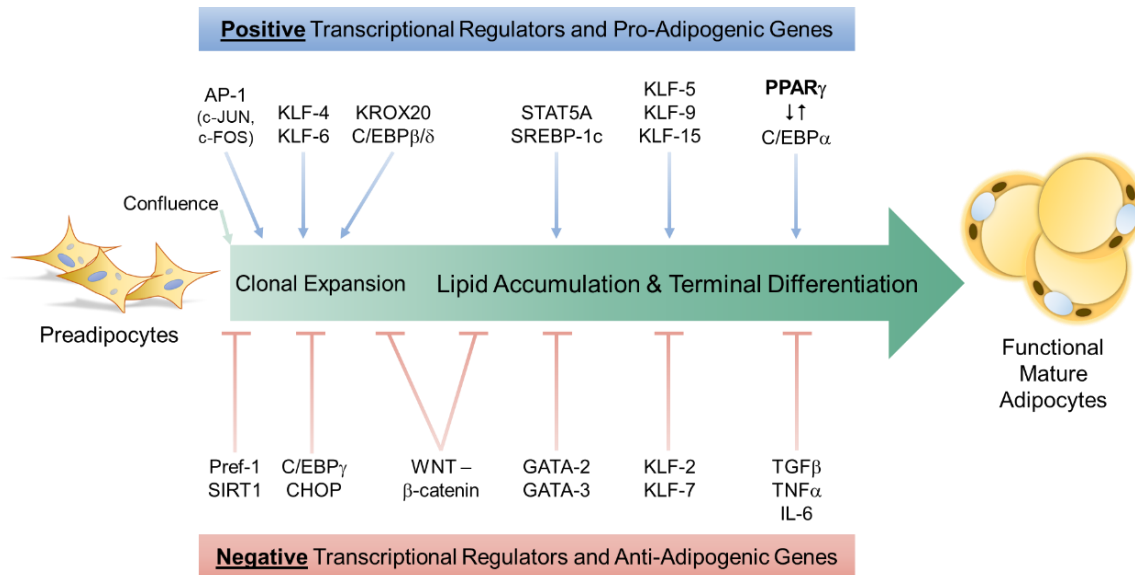


Figure 2.1. Positive and negative regulators of adipogenesis. Adapted from: Sarjeant K, Stephens JM. Adipogenesis. *Cold Spring Harb Perspect Biol.* 2012;4(9):a008417. (Additional References: ^{18, 46-49}). **Figure Key:** Blue-colored arrows represent pro-adipogenic regulatory effectors. Red-colored bar-headed lines represent anti-adipogenic regulatory effectors. **Abbreviations:** AP-1: Activating Protein-1 Family; CHOP: C/EBP Homologous Protein; GATA: Zinc-Finger DNA-binding proteins; KLF: Kruppel-Like Factor; KROX20: Early Growth Response Protein 2 (aka EGR2); WNT: Wingless-Integrated.

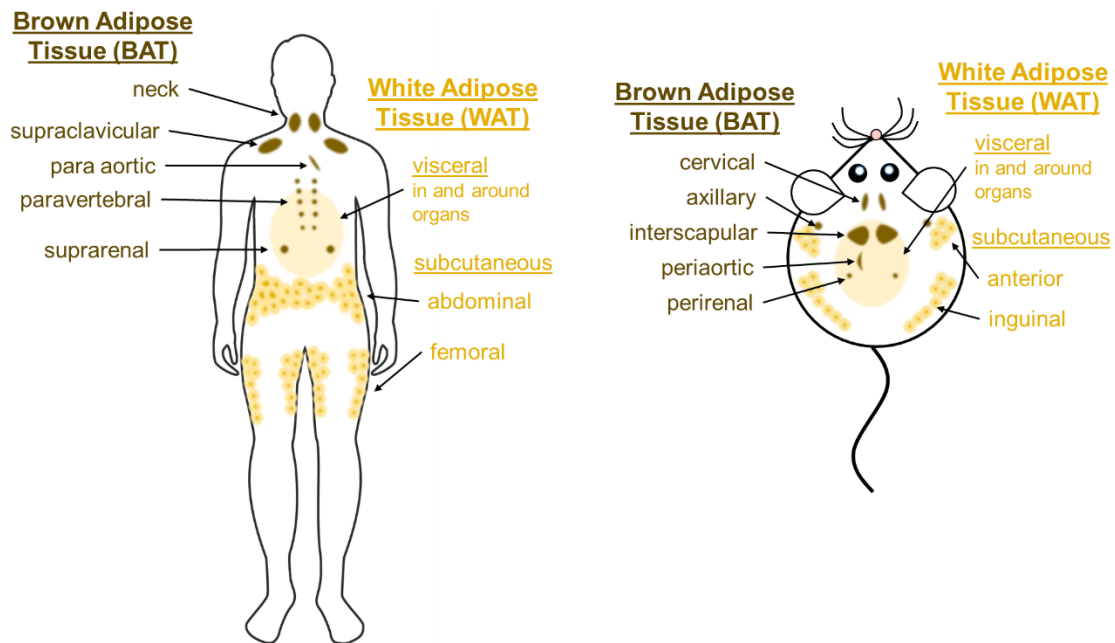


Figure 2.2. Anatomical locations of brown and white adipose tissue in humans and mice.

Adapted from: van den Berg SM, van Dam AD, Rensen PCN, de Winther MPJ, Lutgens E.

Immune modulation of browning adipose tissue in obesity. *Endocr Rev.* 2017;38(1):46-68.

(Additional References: ^{89, 93, 94}). Figure Key: Yellow letters and shapes represent white adipose tissue. Brown letters and shapes represent brown adipose tissue.

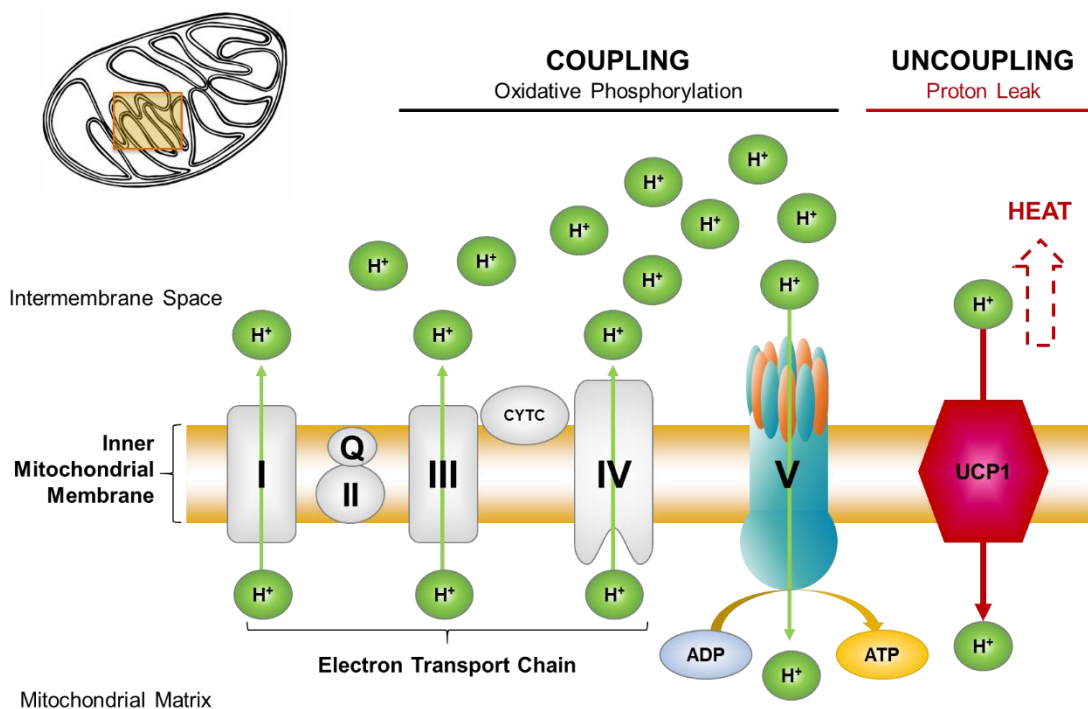


Figure 2.3. Uncoupling of oxidative phosphorylation. Adapted from: Brondani LA, Assmann TS, Coutinho G, Duarte K, Gross JL, Canani LH, Crispim D. The role of the uncoupling protein 1 (UCP1) on the development of obesity and type 2 diabetes mellitus. *Arq Bras Endocrinol Metab.* 2012;56(4):215-225. (Additional References: ^{26, 27, 105, 106}). **Figure Key:** Green arrows represent normal function of oxidative phosphorylation and the electron transport chain. The red arrow represents uncoupling of oxidative phosphorylation, and the production of heat. **Abbreviations:** ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CYTC: Cytochrome Complex; H^+ : hydrogen atoms (protons); I-IV: Complex I, II, III, and IV of the ETC; Q: Ubiquinone; UCP1: Uncoupling protein 1; V: Complex five or ATP Synthase.

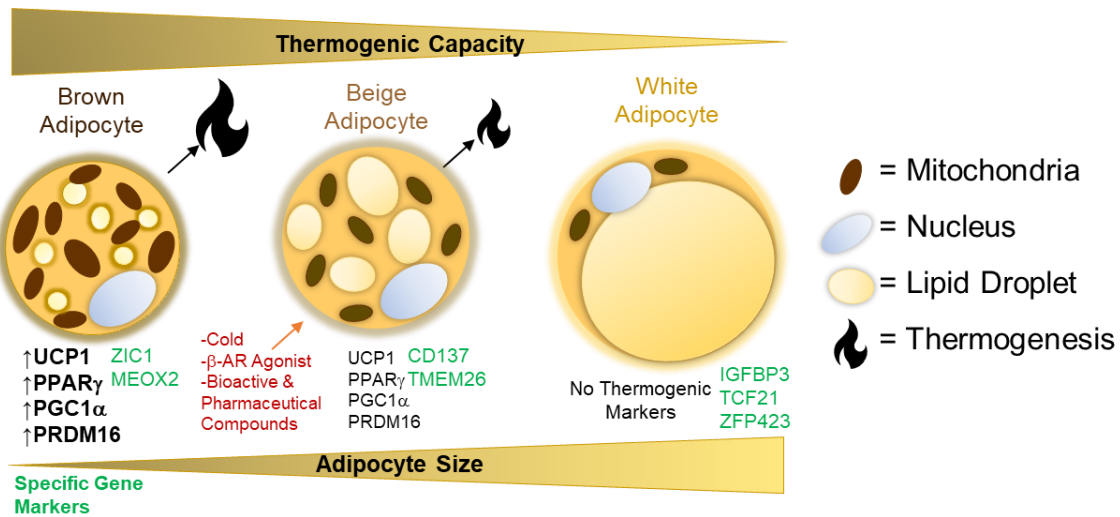


Figure 2.4. Morphological and transcriptional differences between brown, beige, and white adipocytes. Adapted from: Contreras C, Nogueiras R, Dieguez C, Medina-Gomez G, Lopez M. Hypothalamus and thermogenesis: Heating the BAT, browning the WAT. *Molec Cell Endocrinol.* 2016;438:107-115. (Additional References: ^{145, 151-153}). **Figure key:** Larger-bold font represents gene markers that are highly expressed in BAT. Green lettering represents gene markers which are specific for each respective cell type. **Abbreviations:** CD137(TNFRRS9): tumor necrosis factor receptor superfamily, member 9; IGFBP3: insulin growth factor binding protein 3; MEOX2: mesenchyme homeobox 2; PGC1 α : peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPAR γ : peroxisome proliferator-activated receptor gamma; PRDM16: positive regulatory domain containing protein 16; TCF21: transcription factor 21; TMEM26: transmembrane protein 26; UCP1: uncoupling protein 1 ; ZIC1: zinc fingers in the cerebellum 1; ZFP423: zinc-finger protein 423.

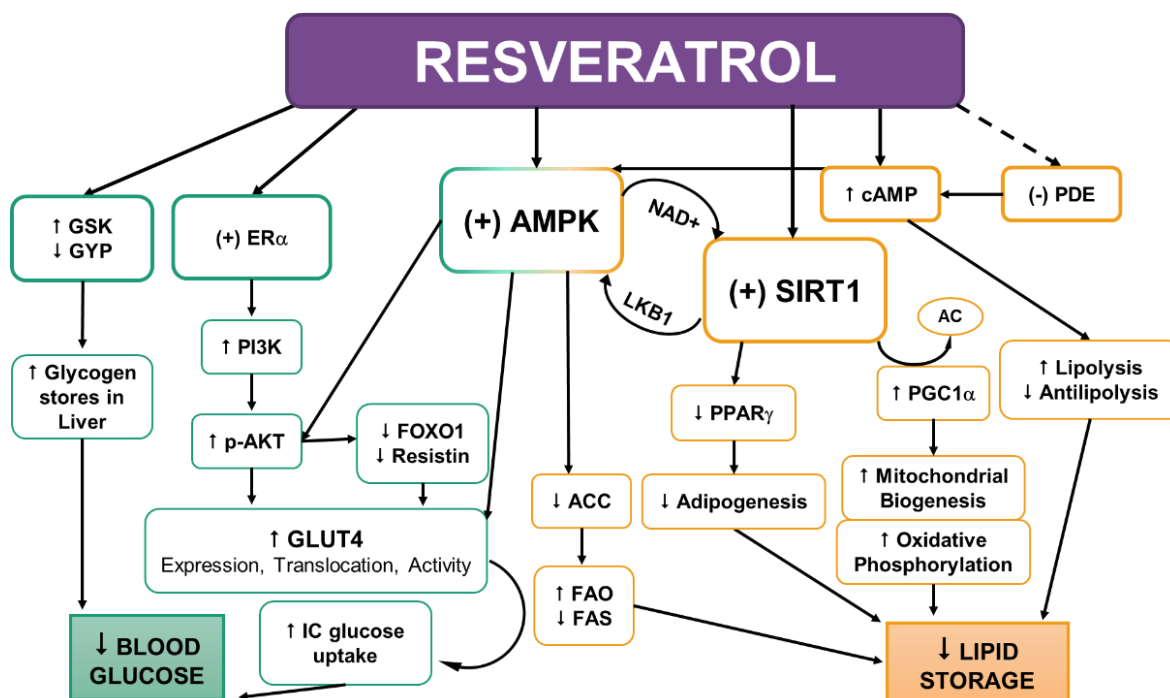


Figure 2.5. Various signaling pathways Res works to lower plasma glucose and lipid accumulation. Adapted from: Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes.

Eur J Pharmacol. 2010;635(1-3):1-8. (Additional References: 275, 330, 331, 333-336, 341, 346, 353, 354).

Figure Key: Thicker lines and outlines represent direct targets of Res. Teal-colored outlined boxes represent pathways leading to decreased blood glucose. Peach-colored outlined boxes represent pathways leading to decreased lipid storage. (-) and (+) represent negative and positive actions by Res on targets, respectively. **Abbreviations:** AC: Acetyl (SIRT1 de-acetylates PGC1α); ACC: Acetyl CoA Carboxylase; FAO: Fatty Acid Oxidation; FAS: Fatty Acid Synthesis; GSK: glycogen synthase kinase; GYP: glycogen phosphorylase; IC: Intracellular; LKB1: Liver Kinase B1; NAD⁺: Nicotinamide adenine dinucleotide (+ = oxidized); PDE: Phosphodiesterase.

CHAPTER III:

RESVERATROL LIPOSOMES AND LIPID NANOCARRIERS: COMPARISON OF CHARACTERISTICS AND INDUCING BROWNING OF WHITE ADIPOCYTES

A version of this chapter was originally published by Haley Overby, Yujiao Zu, Guofeng Ren, Zhaoyang Fan, Ling Zhao, and Shu Wang:

Zu Y*, Overby H*, Ren G, Fan Z, Zhao L, Wang S. Resveratrol liposomes and lipid nanocarriers: Comparison of characteristics and inducing browning of white adipocytes. *Colloids Surf B Biointerfaces*. 2018;164:414-423.

*Denotes both authors contributed equally to this work and shared the first authorship.

This article has been briefly revised from the published article in the following ways: font changes, abbreviation for resveratrol altered to match the use of it in this dissertation, figure and table numbers altered to fit with this dissertation format, and slight elaboration on materials and methods (some supplemental materials and methods were incorporated into manuscript).

Haley Overby has contributed the following to this chapter: experiments and data reported in Figures 3.5A, 3.6-3.8, Table 3.1, and Figure S.3.1 in addition to the study designs, results, discussion, and other written portions of the manuscript regarding this data.

Yujiao Zu contributed to the data presented in Figures 3.1-3.4 and 3.5.B. Zhaoyang Fan and Guofeng Ren contributed to Figure 3.3. Ling Zhao and Shu Wang are the corresponding authors and contributed to the study design and data analysis. All persons mentioned here contributed to the manuscript preparation and revisions.

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3.1 ABSTRACT

Trans-resveratrol (**Res**) has a potential to increase energy expenditure via inducing browning in white adipose tissue. However, its low levels of aqueous solubility, stability, and poor bioavailability limit its application. We have successfully synthesized biocompatible, and biodegradable Res encapsulated lipid nanocarriers (**Rnano**), and Res encapsulated liposomes (**Rlipo**). The mean particle size of Rnano and Rlipo were 140 nm and 110 nm, respectively, and their polydispersity index values were less than 0.2. Nanoencapsulation significantly increased aqueous solubility and enhanced chemical stability of Res, especially at 37°C. Rlipo had higher physical and chemical stability than Rnano while Rnano had more prolonged release than Rlipo. Both Rnano and Rlipo increased cellular Res content in 3T3-L1 cells. Both Rnano and Rlipo dose-dependently induced uncoupling protein 1 (**Ucp1**) mRNA expression and decreased white specific marker insulin growth factor binding protein 3 expression under isoproterenol (**ISO**)-stimulated conditions. At the low dose (5 µM), nanoencapsulated compared to native Res enhanced *Ucp1* and beige marker *Cd137* expression under ISO-stimulated conditions. Compared to Rnano, Rlipo had better biological activity, possibly due to its higher physical and chemical stability at the room and body temperature. Taken together, our study demonstrates that nanoencapsulation increased Res' aqueous solubility and stability, which led to enhanced browning of white adipocytes. Even though both Rlipo and Rnano increased Res' browning activities, their differential characteristics need to be considered in obesity treatment.

3.2 INTRODUCTION

Obesity remains to be the major public health issue in the United States and worldwide, paralleled by rising rates of co-morbidities such as metabolic syndrome, diabetes, coronary heart disease, and certain types of cancer.¹

Two different adipose tissues are found in mammals: white adipose tissue (**WAT**), which is responsible for energy storage; and brown adipose tissue (**BAT**), which is responsible for thermogenic energy expenditure.² BAT has been positively associated with energy expenditure and negatively associated with adiposity in animal models and humans. Uncoupling protein 1 (**UCP1**) found in the inner mitochondrial membrane of brown adipocytes in the BAT can dissipate the proton electrochemical gradient generated by oxidative phosphorylation in the form of heat.³ Emerging data have demonstrated that UCP1 is expressed not only in classical brown adipocytes but also in “brown-like” or beige adipocytes within WAT upon stimulations, such as a chronic cold challenge or pharmacological or bioactive compounds.³ To increase adipocyte UCP1 expression and induce WAT “browning” might result in enhanced thermogenic and fat-burning activities, which subsequently lead to weight loss.

Trans-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, **Res**) is a polyphenolic compound, abundant in the skin of grapes and red wine. Many *in vitro* studies have demonstrated that Res at concentrations between 10 to 100 μ M exhibits anti-obesity activities by modulating adipocyte differentiation, lipolysis, fatty acid oxidation, and mitochondria biogenesis and activities.⁴ Res activates NAD-dependent deacetylase sirtuin 1 (**SIRT1**), which can deacetylate peroxisome-proliferator-activated receptor γ (**PPAR γ**). This modification is essential for enhancing PPAR γ -binding activity, recruiting transcription factor PR-domain-containing 16 (**PRDM16**) to PPAR γ , and activating PPAR γ coactivator 1 α (**PGC1 α**), which subsequently enhance UCP1 expression and initiate browning of WAT.⁴ Native Res (at 10 μ M) enhanced mRNA expression of UCP1 and beige marker CD137 and TMEM26 during brown-like differentiation of primary stromal cells derived from inguinal WAT (**iWAT**) of CD1 mice.⁵ Consistently, *in vivo* feeding of Res promoted the appearance of multilocular adipocytes and increased UCP1 expression in iWAT in the mice fed with a high-fat diet.⁵ These results suggest that browning WAT may be a new anti-obesity target for Res.

Human studies indicate that Res can maintain metabolic health, but the evidence is inconclusive.⁴ The major problems are its low aqueous solubility and bioavailability, and high metabolism in humans.⁶ The solubility of Res in water and physiological fluid is very low, less than 0.1 mg/mL.⁷ When orally administering around 0.3 mg/kg body weight of Res to healthy adult

males, blood peak concentrations of Res appeared at 0.5 h, and the peak plasma concentrations were less than 1 μM .⁸ Even when Res was given a single dose of 5 g, the peak plasma concentrations were still less than 10 μM .⁹ Moreover, Res stability is further reduced by various metabolic transformations, including methylation, glucuronidation and others, primarily in the liver *in vivo*.⁴

Nanoencapsulation has been proven effective in increasing aqueous solubility, chemical stability, and bioavailability of many bioactive compounds in combating obesity and associated metabolic disorders.⁷ Recent animal and human studies indicate that encapsulating Res into nanocarriers can increase Res's aqueous solubility, protect Res from metabolic degradation, and enhance its transport across the plasma membrane, with ultimately augmented absorption and bioavailability.¹⁰

In this study, we synthesized Res encapsulated lipid nanocarriers (**Rnano**) and Res encapsulated liposomes (**Rlipo**), two biodegradable and biocompatible nanocarrier delivery systems, and compared their physical and physicochemical characteristics and browning activities with native Res in 3T3-L1 white adipocytes. This study provides a foundation for developing innovative anti-obesity approaches using natural bioactive compounds and nanocarrier delivery systems.

3.3 MATERIALS AND METHODS

3.3.1. Chemicals and reagents

Res was purchased from Cayman Chemical Co. (+)-Alpha (α)-tocopherol acetate (**α TA**), cholesterol, dexamethasone (**Dex**), 3-isobutyl-L-methylxanthine (**IBMX**), insulin, isoproterenol (**ISO**) and rosiglitazone (**Rosi**) were purchased from Sigma-Aldrich Chemical Co. Soy L- α -phosphatidylcholine (**PC**) was purchased from Avanti Polar Lipids Inc. Kolliphor[®] HS15 was given as a gift from BASF Chemical. All organic solvents were the high-performance liquid chromatography (**HPLC**) grade.

3.3.2. Preparation of Rnano and Rlipo

Rnano was prepared using a mixture containing 1 mg of Res, 70 mg of soy PC, 17.6 mg of Kolliphor[®] HS15 and 18 mg of α TA. The mixture was dissolved in ethanol and completely dried under nitrogen gas. After suspending the mixture with 76°C deionized water, the suspension was homogenized for 1 min followed by sonication for 1 min. The Rnano tube was put on ice immediately. After ultrafiltration to remove free-Res, the Rnano was resuspended in phosphate-

buffered saline (**PBS**). Rlipo was prepared using 1 mg of Res, 20 mg of soy PC and 2 mg of cholesterol by a film dispersion method followed by a membrane extrusion method. The void nanocarriers (**Vnano**) and void liposomes (**Vlipo**) were prepared using the above methods without adding Res.

3.3.3. Particle size, zeta potential, and morphology

The particle size and polydispersity index (**PI**) values of Rnano, Rlipo and their void counterparts were measured using a Brookhaven BI-MAS particle size analyzer, and the zeta potential was measured using a Zeta PALS analyzer. The morphology and size of nanocarriers and liposomes were determined using a 200kV Hitachi H-8100 transmission electron microscope (**TEM**) as described.¹¹

3.3.4. Encapsulation efficiency and loading capacity

One volume of Rnano or Rlipo was dissolved in 9 volumes of methanol, and the total Res concentrations (**Ctotal**) in the Rnano or Rlipo solution were measured using a Shimadzu HPLC system equipped with two LC-20AD solvent delivery units, a SIL-20AC HT autosampler, and a SPD-M20A photo diode array (**PDA**) detector (Shimadzu scientific instruments, Inc., Japan) with a C18 reverse-phase column (Symmetry® C18, 3.5 µm, 4.6×75 mm). The mobile phase was composed of methanol/water/acetic acid (50/50/0.5, v/v/v) with a flow rate of 1 mL/min. The detection wavelength was 310 nm. Non-encapsulated Res of Rnano or Rlipo was separated from nanoencapsulated Res using an ultrafiltration method (Millipore Amicon Ultra-15) and measured by the HPLC system (**Cfree**). To calculate the loading capacity, a certain volume of Rnano or Rlipo (V) was dried using a vacuum freeze-drying system (Labconco, Kansas City, MO). The weight of dried Rnano or Rlipo was expressed as WNANO. The encapsulation efficiency and loading capacity of Res in the Rnano or Rlipo were calculated according to the following equations, respectively:

$$\text{Encapsulation efficiency} = (\text{Ctotal} - \text{Cfree})/\text{Ctotal} \times 100\%$$

$$\text{Loading capacity} = (\text{CtotalV} - \text{CfreeV})/\text{WNANO} \times 100\%$$

3.3.5. Physical and chemical stability

The freshly made Rnano and Rlipo were aliquoted into transparent or black tubes and stored at 4°C, 22°C, and 37°C for 7 days, and their physical and chemical stability was measured during this period. The mean particle size, PI and zeta potential were measured every 2 h for the first 10

h, and every 24 h for 7 days. The chemical stability of Rnano, Rlipo and native Res was measured using the HPLC system every day for 7 days. Three independent experiments were conducted for each measure.

3.3.6. Physicochemical characterization

3.3.6.1. *In vitro* release study

Before the *in vitro* release study, the stability of Rnano, Rlipo and native Res in the dissolution medium were measured at 37°C for 24 h. The dissolution medium was composed of PBS and methanol (80:20, v/v). The *in vitro* release behaviors of Rnano, Rlipo and native Res containing 0.5 mg of Res were performed in the dissolution medium using a dialysis method.¹¹ Samples equivalent to 0.5 mg of Res were dispersed in 1 mL of PBS and then placed in the dialysis bags with Molecular Weight Cut Off (MWCO) 6,000-8,000. The dialysis bags were dipped with the help of a thread in a conical flask containing 20 mL of dissolution medium and stirred at 200 rpm at 37°C for 24 h. The dissolution medium was totally replaced by the fresh pre-warmed dissolution medium every 2 h to protect the released Res from degradation in the medium during a long period. The Res released into the medium at each time point was determined using the Shimadzu HPLC system.

3.3.6.2. Raman spectroscopy analysis

All samples were measured using a Bruker Optics Senterra dispersive Raman microscopy at room temperature. The native Res, Rnano, Rlipo, Vnano, and Vlipo were frozen and then lyophilized using a vacuum freeze-drying system before taking Raman spectra. The spectral resolution is 0.5 cm⁻¹, and the measured spectrum range covers 500-1800 cm⁻¹. The excitation laser has a wavelength of 532 nm and a tunable power for better signal intensity when measuring different samples.

3.3.6.3 X-ray diffractometry (XRD)

The XRD pattern of lyophilized samples was measured with Siemens/Bruker D5005 diffractometer (Bruker Corporation, Germany) at room temperature. The instrument is equipped with 2.2 kW Cu X-ray source, and the measurement was conducted with a step size of 0.02° and a step duration of 1 s. An aluminum sample holder was used, and the measured XRD patterns were aligned to the aluminum diffraction angle.

3.3.6.4 Differential scanning calorimetry (DSC) analysis

The DSC analysis of all the samples was carried out on the METTLER Toledo DSC822e thermal analyzer (Mettler-Toledo, LLC, Columbus, OH) with its STARE software. The measurements were conducted from 25°C to 400°C with a heating rate of 10°C/min under nitrogen flow.

3.3.7. Cytotoxicity, cellular Res content and browning activities in 3T3-L1 fibroblasts

3.3.7.1. Cell culture and treatments

Murine 3T3-L1 fibroblasts purchased from ATCC were grown in Dulbecco's modified Eagle's medium (**DMEM**) containing 10% calf serum in a 5% CO₂, 37°C environment until they reached confluence. Cells were differentiated with DMEM containing 10% fetal bovine serum (**FBS**, Atlas Biologicals), 1 µM Dex, 0.5 mM IBMX, and 10 µg/mL insulin for 3 days followed by DMEM containing 10% FBS and 10 µg/mL insulin for additional two days until day 5. The media was then changed to DMEM containing 10% FBS until day 7. On day 7 the cells were stimulated with ISO (1 µM) or the vehicle control (**H₂O**) for 6 h before the cells were subjected to total RNA isolation. All versions of nanocarriers and the controls were added as indicated during each change of the media. Rosi (1 µM) was used as the positive control.

3.3.7.2. Cell viability

Cell viability was measured by the colorimetric 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (**MTT**) assays. 3T3-L1 cells grown into confluency were treated with Rnano, Rlipo, and the controls for 24 or 72 h. MTT reagent was added to the cells in the dark and incubated for additional 2-4 h. The formazan formed was eluted, and OD absorbance at 570 nm was measured with the Promega GloMax-Multi Detection System. These results were normalized with protein concentrations of the cell lysate.

3.3.7.3. Cellular Res content studies

3T3-L1 cells were seeded in 6-well plates at a density of 5×10⁴ cells/well. After 24 h, cells were treated with 10 or 20 µM of Rnano, Rlipo or native Res at 37°C for 4 h. After washing cells with ice cold PBS twice, the attached cells were scraped and collected in 0.6 M acetic acid in a glass tube, and 10 µL of quercetin (**Q**, internal standard, 30 µM) dissolved in methanol was added to each sample. After mixing, 4 mL of acetone was added into the tube, followed by sonication for 1 min and centrifugation at 5,500×g for 15 min at 4°C. Supernatant volume was accurately

measured and dried using a nitrogen evaporator at room temperature. After drying, 100 μ L methanol was added into the tube to dissolve Res and internal standard Q. The tubes were centrifuged at 5,500 $\times$ g for 10 min at 4°C, and 70 μ L clear supernatant samples were transferred and directly injected into the HPLC system for measuring Res and internal standard (Q) concentrations. The protein precipitates were dried in the chemical hood, then digested using 0.5 N NaOH overnight. Cellular protein concentrations were measured using a Pierce BCA assay kit. Total cellular Res content was expressed as μ g of Res per mg of protein.

3.3.7.4. Real time-PCR

Total RNA was prepared using the TRI reagent from Molecular Research Center, according to the manufacturer's instructions. Total RNA abundance was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription kit. Expression of target genes was measured semi-quantitatively by PCR reactions using Power Up SYBR master mix with an ABI 7300HT instrument (Applied Biosystems, Austin, TX). Relative gene expression was calculated using the $2(-\Delta\Delta C_t)$ method, which normalizes against the house keeping gene *36b4*. Primer sequences of target genes are listed in **Table 3.1**. (all tables and figures for Chapter III are located in 3.6. Appendix).

3.3.7.5. Transfection and reporter gene assays

3T3-L1 cells were transiently transfected with a peroxisome proliferator response element (PPRE)-driven luciferase reporter (PPRE-Luc) and β -galactosidase (β -gal) control plasmid with Lipofectamine 3000 and PLUS Reagent for 24 h. The cells were then treated with nanoparticles and the controls for an additional 15-18 h. The luciferase activities were measured by a Promega GloMax-Multi Detection System and normalized by the β -gal activities.

3.3.8. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences and SigmaPlot 13 (Systat Software, Inc). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method or Holm-Sidak method to compare with controls. Each experiment was conducted independently at least three times; each measurement was performed in triplicates within each experiment. The level of significance was set at $p < 0.05$.

3.4 RESULTS AND DISCUSSION

3.4.1. Characteristics of Rnano and Rlipo

Rnano and Rlipo were successfully synthesized. Traditional nanostructured lipid carriers (NLCs) as drug carriers usually contain a large amount of triglyceride.¹² In our laboratory, we have successfully replaced triglyceride with α TA, consequently eliminating exogenous triglyceride and increasing the anti-oxidative capacity of nanocarriers,^{11, 13} making them more functional and beneficial. Fig 3.1.A shows that 1 mg of native Res was hardly dissolved in 1 mL of PBS and precipitated from the suspension immediately; however, 1 mg of nanoencapsulated Res in both Rnano and Rlipo was dissolved in the same volume of PBS, which had 25-fold higher aqueous solubility than native Res. Both Rnano and Rlipo were translucent and opalescent (Fig 3.1.A). Several studies have demonstrated that entrapment of Res in nanoparticles increases Res' aqueous solubility and further bioavailability.¹² TEM images indicated that both Rnano and Rlipo were spherical (Fig 3.1.A). The average particle size of Rnano and Rlipo was around 140 nm and 110 nm, respectively (Fig 3.1.A). Recently research data indicate that tissue distribution of nanocarriers was size-dependent,¹⁴ and nanocarriers close to 100 nm was found to be delivered effectively into adipose tissues of obese mice. The PI values of Rnano and Rlipo were 0.084 and 0.140, respectively. The low PI values indicated a high level of size homogeneity of Rnano and Rlipo. While higher PI values (> 0.3) indicate higher levels of heterogeneity.¹² Surface charges of nanocarriers play vital roles in cellular uptake, biodistribution, and bioavailability of nanocarriers.¹² The Zeta potentials of freshly made Rnano and Rlipo were around -19 and -28 mV, respectively. Soy PC, the major surface component of Rnano and Rlipo, rendered negative charges on the surface of particles.¹² In general, they can be dispersed stably due to the electric repulsion of negative charges among particles.

The encapsulation efficiency of Rnano and Rlipo was 96.5% and 96.0%, respectively; and Res' loading capacity in Rnano and Rlipo was 28.5% and 25.3%, respectively. Since a high proportion of hydrophobic α TA on Rnano, we predict that Rnano might have a monolayer of PC and Kolliphor® HS15 on the surface and a hydrophobic α TA core (Fig 3.1.A). The high encapsulation efficiency and loading capacity of Rnano were partially due to the hydrophobicity of α TA, which accommodates more Res in the hydrophobic core. Rlipo might have multiple PC bilayers and a hydrophilic core. Due to the biphasic characteristics of PC, Res can be embedded into the sections of hydrophobic fatty acid tails of PC bilayer.¹⁵ Multiple PC bilayers on liposomes also accommodate a good amount of Res.

3.4.2. Physical and chemical stability

Storage of both Rnano and Rlipo at 4°C for 4 days, their diameters and zeta potentials did not change significantly, and the PI values remained under 0.2, indicating relatively good homogeneity and physical stability (Fig 3.1.B). Storage of Rnano at room temperature (22°C) for 4 days, its diameters, and PI values were slightly increased (Fig 3.1.B). At 37°C, Rnano can only maintain its original size for 8 h, and its PI values were gradually increased (Fig 3.1.B). There could be two reasons contributing to the poor physical stability of Rnano at 37°C. First, the hydrophobic lipid core of Rnano was composed of α TA. The melting point of α TA was around 25°C (Sigma T3001) indicated on the product sheet, and the solid core would turn to liquid resulting in the fragile and unstable structure of Rnano. Second, a high temperature may break the hydrogen bonds of Kolliphor® HS15,¹⁶ the surfactant incorporated on the surface of Rnano, leading to a reduced stability of Rnano at 37°C. The diameter and zeta potential of Rlipo did not change significantly at 22°C and 37°C for 7 days, and the PI values remained under 0.2 (Fig 3.1.B), indicating its higher levels of physical stability and homogeneity than Rnano. It should be emphasized that the cholesterol was incorporated into Rlipo to increase its physical stability. Even though Rlipo is more stable than Rnano, other characteristics and anti-obesity bioactivities should be considered and measured.

Rnano and Rlipo also enhanced the chemical stability of Res that is sensitive to light. In a neutral pH 7.4 condition, native Res was degraded by 40% at 4°C, 60% at 22°C and 96% at 37°C under the light after 7 days (Fig 3.2.A). Storage of native Res in the dark decreased the Res degradation rates to 17% at 4°C, 48% at 22°C, and 52% at 37°C after 7 days (Fig 3.2.B). After incubation at 37°C for 6 days under the light, Res degradation rates were 90%, 80% and 20% in native Res, Rnano, and Rlipo, respectively (Fig 3.2.A). Both lipo and nanostructures protected Res from degradation no matter under dark or light, and their protective capability was similar at 4°C and 22°C (Fig 3.2.). Rnano might be solid at the room temperature because the melting temperatures of α TA (Sigma T3001), soy PC (Avanti 441601) and Kolliphor® HS15 are 25°C and above. The hydrophobic Res can be encapsulated into the solid core, which can enhance its stability and prolong its release.¹⁷ Rlipo had better protective capacity than Rnano at 37°C. The higher Res degradation rate in Rnano could be partially due to lack of a solid phase of Rnano at 37°C. Additionally, many research studies have indicated the nanoencapsulation can increase Res chemical stability via protecting it from light degradation.^{18, 19}

3.4.3. Physicochemical characterization

3.4.3.1. Raman spectroscopy analysis

Raman spectroscopy is a proved method to quickly and effectively identify the encapsulated molecules.²⁰ Raman spectra of native Res, Rnano, Vnano, Rlipo, Vlipo, were presented in Fig 3.3. The spectrum of native Res shows three major characteristic bands, including olefinic bands at 980–1022 cm^{-1} , C-O stretching at 1132–1188 cm^{-1} and C-C aromatic double-bond stretching at 1570–1655 cm^{-1} , in addition to several other weak features, which are consistent to the reference.²¹ The Raman spectra of Vlipo and Vnano are very similar, dominated by characteristic peaks of PC, the common component of these two nanocarriers. Since the signatures for native Res and Vnano/Vlipo have no overlap, it becomes unambiguous to differentiate the compositions in the encapsulated particles and void nanocarriers. As observed from the spectra, the major characteristic peaks of native Res were present in Rnano and Rlipo, but absent in Vnano and Vlipo, while those peaks of Vnano and Vlipo were present in their corresponding Rnano and Rlipo, but not in native Res. These clearly indicate the encapsulation of Res into Rnano and Rlipo.

3.4.3.2. XRD analysis

The XRD analysis can effectively distinguish between the crystalline and amorphous phases of Rnano and Rlipo. The crystallinity of nanocarriers is critical because the solubility and dissolution rate of Rnano and Rlipo in the Res delivery process can be significantly affected by the degree of crystallinity.²² The XRD patterns of native Res, Rnano, Rlipo and the corresponding control forms and physical mixtures were shown in (Fig 3.3.). The diffractogram of native Res exhibited intense peaks between 5° and 35°, indicating the native Res in a highly crystalline form. In the lyophilized nanocarriers, the sharp peaks from the crystalline native Res were absent, suggesting less crystallinity or more amorphous state after Res molecules were loaded in the soy PC shell to form an amorphous complex. More interestingly, although the Raman spectra of Vlipo and Vnano are very similar, their XRD patterns are dramatically different, suggesting their different composition and structures, as two different nanocarriers. With the absence of Res crystalline peaks, XRD patterns of Rlipo and Rnano present the features of Vlipo and Vnano, respectively.

3.4.3.3. DSC analysis

The DSC curves of native Res, Rnano, Rlipo and the corresponding control forms were presented in Fig 3.3. as a second method to examine the crystallinity. The difference between Vlipo and Vnano again was notified. The DSC thermogram of native Res showed the

characteristic endothermic peak at 267°C, corresponding to its melting temperature. However, Res' endothermic peak was disappeared in the DSC scans of Rnano and Rlipo, suggesting its amorphous phase in these nanocarriers.²³ Interestingly, the DSC curves of Rlipo and Vlipo and those of Rnano and Vnano exhibit different features. This difference strongly suggests that Res molecules were dispersed into the two nanocarriers, resulting in the amorphous phase. This conclusion is reinforced by the XRD measurements in which only the characteristic crystalline peaks of the Vnano or Vlipo were observed. All three physicochemical analyses confirm that Res drug amorphization occurred in both Rnano and Rlipo nanocarriers.

3.4.3.4. *In vitro* release

Hydrophobic native Res has a low level of aqueous solubility. Methanol was added to make the dissolution medium to dissolve released native Res. Different methanol contents had been tested before conducting the release study to ensure to use the minimal amount of methanol. The dissolution medium containing 20% methanol was the optimal formula, which could dissolve almost all released native Res without destroying nanostructures (data not shown). The release study was conducted in the dark to prevent light-induced Res degradation. To minimize the effect of Res stability, the dissolution medium was changed completely each h for the first 2 h, and every 2 h for the rest 8 h. Native Res showed a burst release phenomenon, while Rnano and Rlipo exhibited a sustained release behavior (Fig 3.4.). After 1 h, about 0.17 mg, 0.05 mg, and 0.02 mg of Res was released from dialysis bags containing native Res, Rlipo, and Rnano, respectively (Fig 3.4.A). At the 2-4 h period, about 0.02 mg, 0.05 mg and 0.04 mg of Res was released from dialysis bags containing native Res, Rlipo, and Rnano, respectively (Fig 3.4.A). After dialysis for 2 h, the accumulative released Res mass from the native Res dialysis bag reached a plateau (Fig 3.4.B), while for Rlipo and Rnano, Res was continuously released in the 10 h period. Furthermore, Rlipo released more Res than Rnano (Fig 3.4.B). Hydrophobic compounds like Res might release faster from the membrane PC bilayers of liposomes than from the hydrophobic core of nanocarriers.¹² The data indicate Res is probably distributed in the PC bilayers of Rlipo, and in the hydrophobic core of Rnano.

3.4.4. Cytotoxicity

None of the forms of Res at tested doses negatively affected cell viability in 3T3-L1 cells after 24 or 72 h (Fig 3.5.A). The results suggest that two biocompatible and biodegradable nanostructures are safe to use, at least in 3T3-L1 cells.

3.4.5. Cellular Res content

To investigate cellular bioavailability, we measured the Res content in 3T3-L1 cells (Fig 3.5.B). The cellular Res content was dose-dependently increased by all treatments. As compared to native Res, Rnano and Rlipo only slightly increased Res content at 10 μ M; but increased Res content by more than 20% and 25% at 20 μ M, respectively. Due to the instability of native Res at 37°C, 3T3-L1 cells were only treated for 4 h in this experiment. Considering only 4 h treatment, the increase in cellular Res content by Rnano and Rlipo is significant.

3.4.6. Activation of PPAR responsive reporter

It has been reported that Res (native form) activates PPAR γ ,²⁴ which heterodimerizes with retinoid X receptor (**RXR**) to bind to PPRE sites in the promoter regions to transactivate target genes, including UCP1²⁵. All forms of Res significantly activated PPRE-Luc compared to their controls ($p < 0.05$). No significant differences were detected among the various forms of Res (Fig 3.6.). Rlipo and Rnano had similar activation abilities compared to native Res (Fig 3.6.), a property that is associated with browning activities of Rosi.²⁶ The results suggest that encapsulation did not change the biological capability of Res in activating PPAR γ .

3.4.7. Browning activities

We further studied browning effects of native Res, Rnano, and Rlipo in differentiating 3T3-L1 white adipocytes, a commonly used cell model to study browning.^{27, 28} The hallmark of beige adipocytes is induced thermogenesis in response to stimuli, such as β -adrenergic agonist ISO. We investigated the effects of native Res, Rlipo, and Rnano on the gene expression of markers of brown and white adipocytes, and mitochondrial biogenesis under either basal (non-stimulated) or ISO-stimulated conditions.

3.4.7.1. Effects on mRNA expression of brown adipocyte markers

Neither Rosi (positive control) nor any form of Res significantly induced *Ucp1* mRNA expression at basal conditions. However, upon ISO stimulation, all forms of Res significantly and dose-dependently induced *Ucp1* mRNA expression compared to their controls ($p < 0.05$), similar to Rosi ($p < 0.05$). When compared to various forms of Res, there were no significant differences between Rlipo and Rnano. However, at 5 μ M (the low dose), Rlipo induced a higher *Ucp1* expression than native Res under ISO stimulated conditions ($p < 0.05$) (Fig 3.7.).

Ppar γ , *Pgc1 α* , and *Prdm16* are known core regulators of browning and *Ucp1* mRNA expression (Reviewed in ^{29, 30}). No forms of Res affect *Ppar γ* mRNA under the basal condition, in contrast to Rosi, which suppressed *Ppar γ* mRNA ²⁶; Rlipo induced higher *Ppar γ* mRNA levels than Rnano and native Res ($p < 0.05$) (Fig 3.7.).

Under basal conditions, Rosi significantly induced *Pgc1 α* mRNA expression ($p < 0.05$); however, various forms of Res did not induce significant changes in *Pgc1 α* expression at all tested doses. Under ISO stimulated conditions, Rlipo significantly induced *Pgc1 α* mRNA expression at 20 μ M compared to its control ($p < 0.05$) and to a level that is higher than Rnano and native Res at the same dose ($p < 0.05$). At 5 μ M, both Rlipo and Rnano induced higher *Pgc1 α* mRNA than native Res ($p < 0.05$) (Fig 3.7.).

Rosi significantly induced *Prdm16* mRNA expression under both conditions ($p < 0.05$). Under the basal conditions, native Res did not change *Prdm16* mRNA expression. In contrast, both Rlipo and Rnano dose-dependently increased *Prdm16* mRNA expression, reaching significance at 20 μ M ($p < 0.05$). Under ISO stimulated conditions, all forms of Res similarly and dose-dependently increased *Prdm16* mRNA expression, reaching significance at 20 μ M compared to the controls ($p < 0.05$) (Fig 3.7.).

3.4.7.2. Effects on mRNA expression of white and beige adipocyte markers

Next, we examined various forms of Res on white and beige marker mRNA expression during the browning of 3T3-L1 adipocytes. Insulin-like growth factor-binding protein 3 (*Igfbp3*) was identified as a white adipocyte specific marker by a transcriptome analysis of brown versus white adipocyte gene expression.³¹ Rosi significantly decreased *Igfbp3* mRNA expression ($p < 0.05$) under both conditions, consistent with a previous report ²⁶. Native Res also dose-dependently decreased *Igfbp3* expression compared to the controls under both conditions ($p < 0.05$ at 20 μ M under basal and $p < 0.05$ at all tested doses under ISO stimulated conditions). Comparing to native Res, both Rlipo and Rnano further decreased *Igfbp3* mRNA expression at 20 μ M at both conditions ($p < 0.05$) (Fig 3.8.A).

Cd137 and *Tmem26* have been identified as beige-fat specific markers.³² Rosi significantly increased *Cd137* mRNA expression under both conditions ($p < 0.05$). Various forms of Res did not affect *Cd137* mRNA expression compared to the control under basal conditions. However, all forms of Res dose-dependently increased *Cd137* mRNA expression under ISO stimulated

conditions ($p < 0.05$ for native Res and Rlipo at 20 μM and $p < 0.05$ for all tested doses for Rnano) (Fig 3.8.B).

Rosi significantly decreased *Tmem26* mRNA expression under both conditions ($p < 0.05$). Native Res decreased *Tmem26* mRNA expression under basal conditions ($p < 0.05$ at 5 and 10 μM). In contrast, native Res increased *Tmem26* expression under ISO stimulation ($p < 0.05$ at all tested doses). There were no differences in *Tmem26* mRNA expression between Rlipo and Rnano and their controls under both conditions (Fig 3.8.B)

Using differentiating 3T3-L1 white adipocytes coupled with ISO-induced thermogenic activation we demonstrate, for the first time, that various forms of Res enhanced ISO-induced mRNA expression of *Ucp1* and other browning markers, such as *Prdm16* and *Pgc1 α* . In addition, various forms of Res enhanced beige marker *Cd137* mRNA expression but suppressed white specific marker *Igfbp3* mRNA expression. Our results are consistent with a previous study⁵. Of note, for the first time, we demonstrated that various Res suppressed *Igfbp3* and Rnano and Rlipo had better suppression than native Res at 20 μM under ISO-stimulated conditions. Taken together, Res-induced browning may contribute to the beneficial effects of Res for obesity and associated metabolic dysfunction. Whether nanoencapsulated Res can enhance the browning over native Res *in vivo* warrant further investigation.

Compared to Rnano, Rlipo induced significantly higher levels of *Ucp1* mRNA than native Res when both used at 5 μM (Fig 3.7.). Moreover, Rlipo induced higher levels of other browning markers *Ppar γ* and *Pgc1 α* than Rnano and native Res under either basal and/or ISO stimulated conditions (Fig 3.7.). The better browning activities of Rlipo may be due to its higher physical and chemical stability compared to Rnano and native Res. Moreover, the better biological activities demonstrated by Rlipo at 5 μM is more physiological relevant since this dose is within the physiologically achievable dose range of Res for human consumption.^{8,9} Compared to native Res, both Rlipo and Rnano had better suppression of *Igfbp3*, possibly due to improved overall bioavailability by nanoencapsulation.

3.4.7.3. Effects on mRNA expression of mitochondrial biogenesis markers

Rosi significantly increased *Tfam*, *Nrf*, *Cox4a*, and *Uqcrrh* under either basal and/or ISO stimulated conditions ($p < 0.05$). There were minimal differences among the three forms of Res in any of the mitochondrial biogenesis markers in 3T3-L1 cells except for native Res, which increased *Cox4a* mRNA at 20 μM under ISO stimulated conditions ($p < 0.05$) (Fig S.3.1.).

In contrast to Rosi, all three forms of Res had minimal effects on mitochondrial biogenesis genes under both basal and ISO stimulated conditions, suggesting that Rosi and Res may induce browning of 3T3-L1 adipocytes via different molecular mechanisms. Lack of changes in mitochondrial biogenesis suggests that Res may induce browning of 3T3-L1 by directly upregulating *Ucp1* expression whereas Rosi may increase mitochondrial biogenesis.

Our results suggest that various forms of Res may promote browning by activating PPAR γ . Currently, we cannot rule out the possibilities that in addition to activating PPAR γ responsive promoters Rlipo and Rnano may activate other signaling pathways, such as SIRT1,^{33, 34} AMPK,⁵ or PDE³⁵ to induce browning. Future studies are needed to characterize potential differences in molecular mechanisms by which Rlipo and Rnano induce enhanced browning. Additionally, animal studies are required to determine the anti-obesity activity of Rlipo and Rnano *in vivo*. Oral administration is the most convenient route, but Rlipo and Rnano might be degraded in the gastrointestinal tract. Intravenous injection will ensure intact Rlipo and Rnano into the circulation system, but frequent intravenous injection faces compliance and safety issues, and clinical visits will be costly. Since animals have subcutaneous WAT, microneedles, skin patches and other transdermal approaches can be considered. Rosi increased all the mitochondrial biogenesis markers under both basal and ISO-stimulated conditions compared with the controls ($p < 0.05$). There were minimal differences in any of the mitochondrial biogenesis markers among the treatments in differentiated 3T3-L1 cells with the exception of native Res increasing *Cox4a* mRNA under both basal and ISO stimulated conditions ($p < 0.05$ at 20 μ M). The results are shown in the Figure S.3.1.

3.5 CONCLUSIONS

We have successfully encapsulated Res into nanocarriers and liposomes, which increased Res aqueous solubility and stability. Rlipo showed higher physical and chemical stability but with a less sustained release than Rnano. Both Rnano and Rlipo increased cellular Res content in 3T3-L1 cells, which led to higher expression *Ucp1*, beige marker *Cd137* and other browning markers, and lower expression of white marker *Igfbp3*. Our study demonstrates a novel strategy of using nanoencapsulation of Res to achieve improved browning efficacy with minimal side effects.

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3.7 APPENDIX

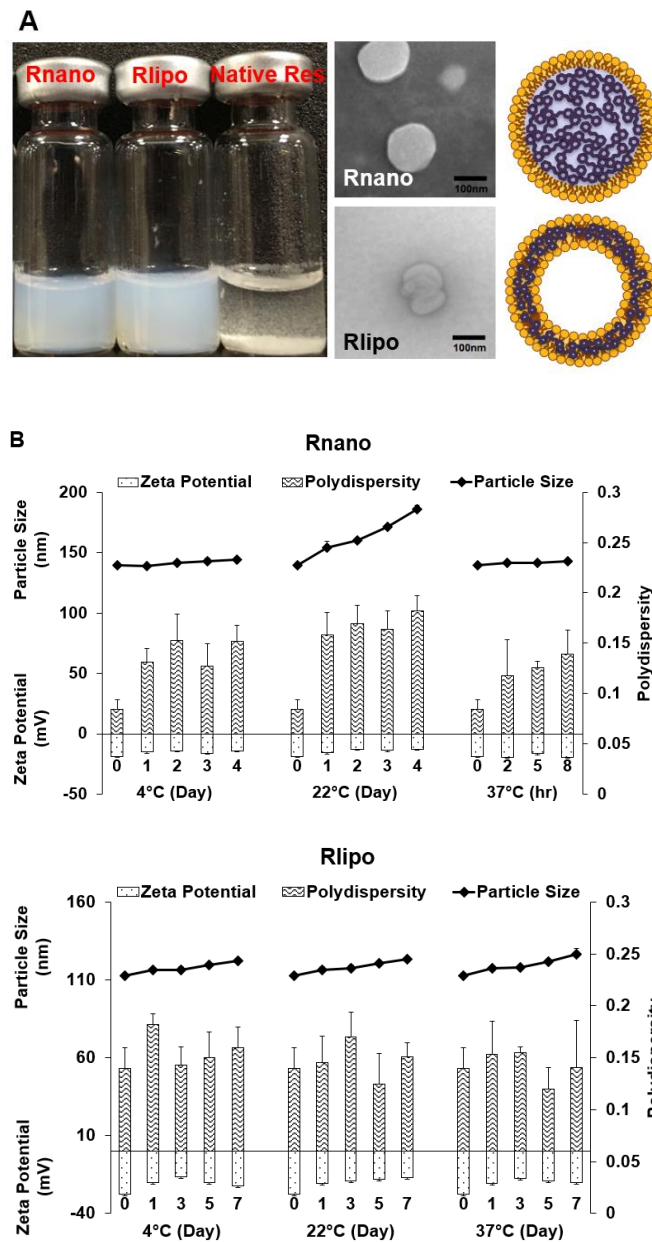


Figure 3.1. Characteristics of Rnano and Rlipo. Visual observation of Rnano, Rlipo, and native Res containing 1 mg of Res suspended in 1 mL of PBS, transmission electron microscope (TEM) images of Rnano and Rlipo, and predicted structures of Rnano and Rlipo (A); changes of particle size, zeta potential, and polydispersity index of Rnano and Rlipo (B) at different temperatures.

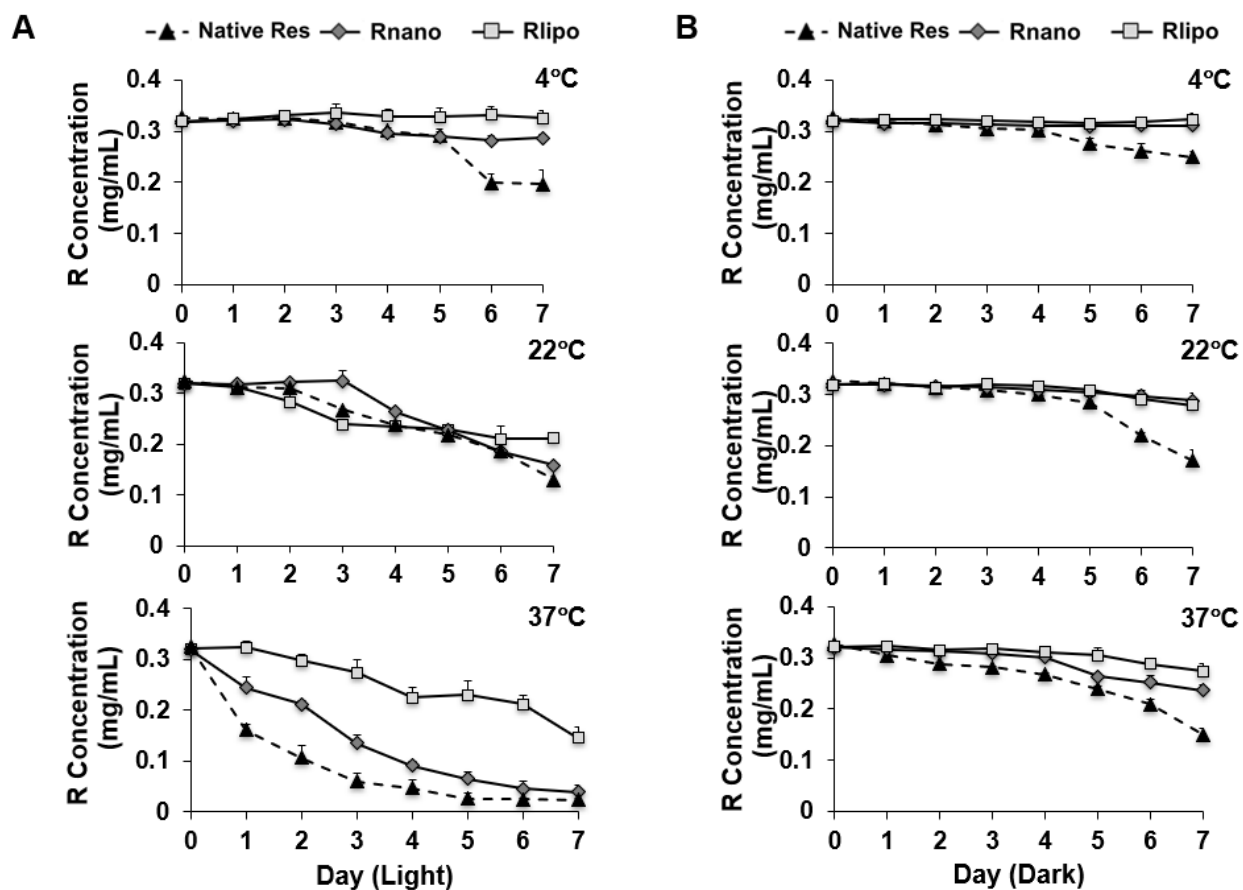


Figure 3.2. Chemical stability of native Res, Rnano, and Rlipo under the light (A) or the dark (B) at different temperatures.

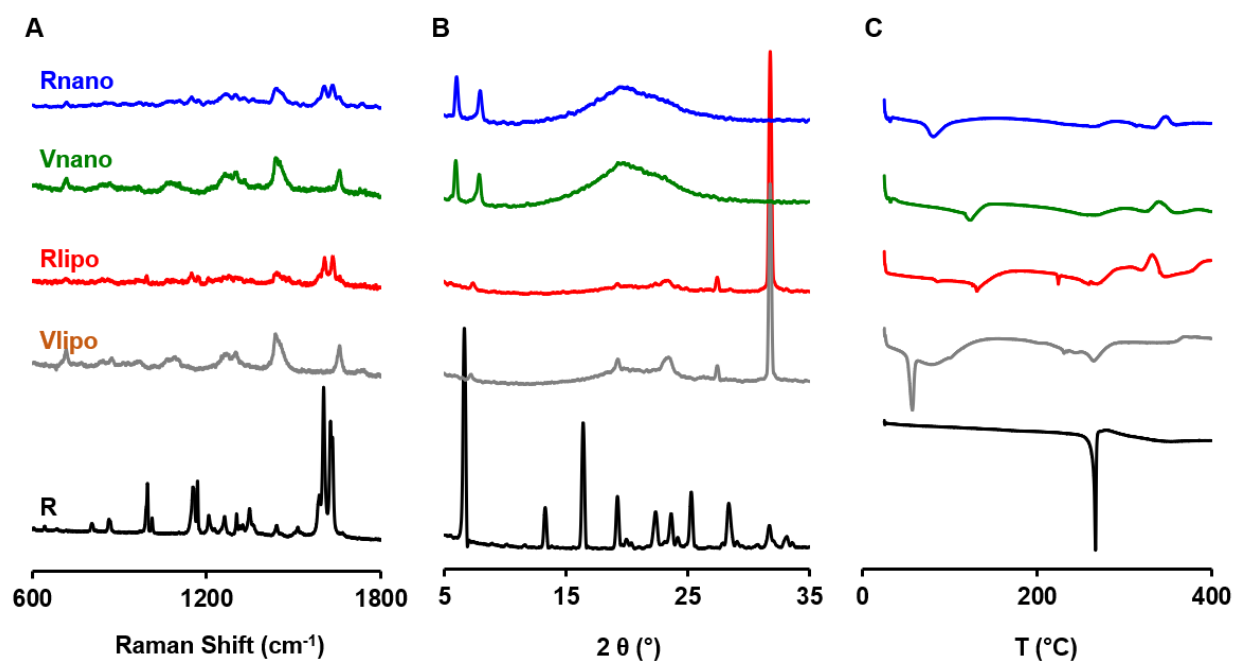


Figure 3.3. Physicochemical characterization. Raman spectra, X-ray diffraction patterns, DSC thermograms of lyophilized Rnano or Rlipo; lyophilized Vnano or Vlipo; native Res.

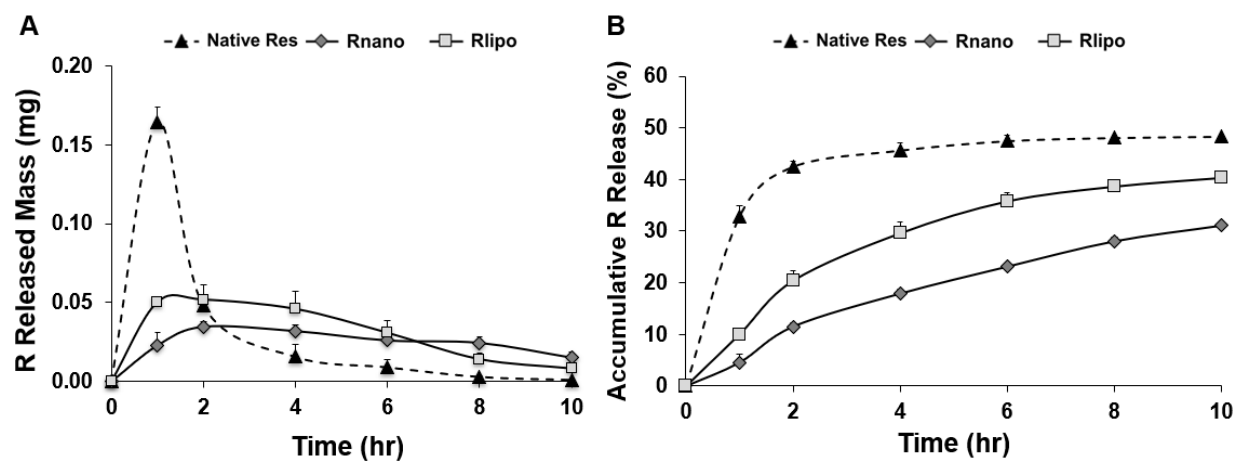


Figure 3.4. *In vitro* release profiles. Hourly (A) and accumulative (B) Res release for native Res, Rnano, and Rlipo.

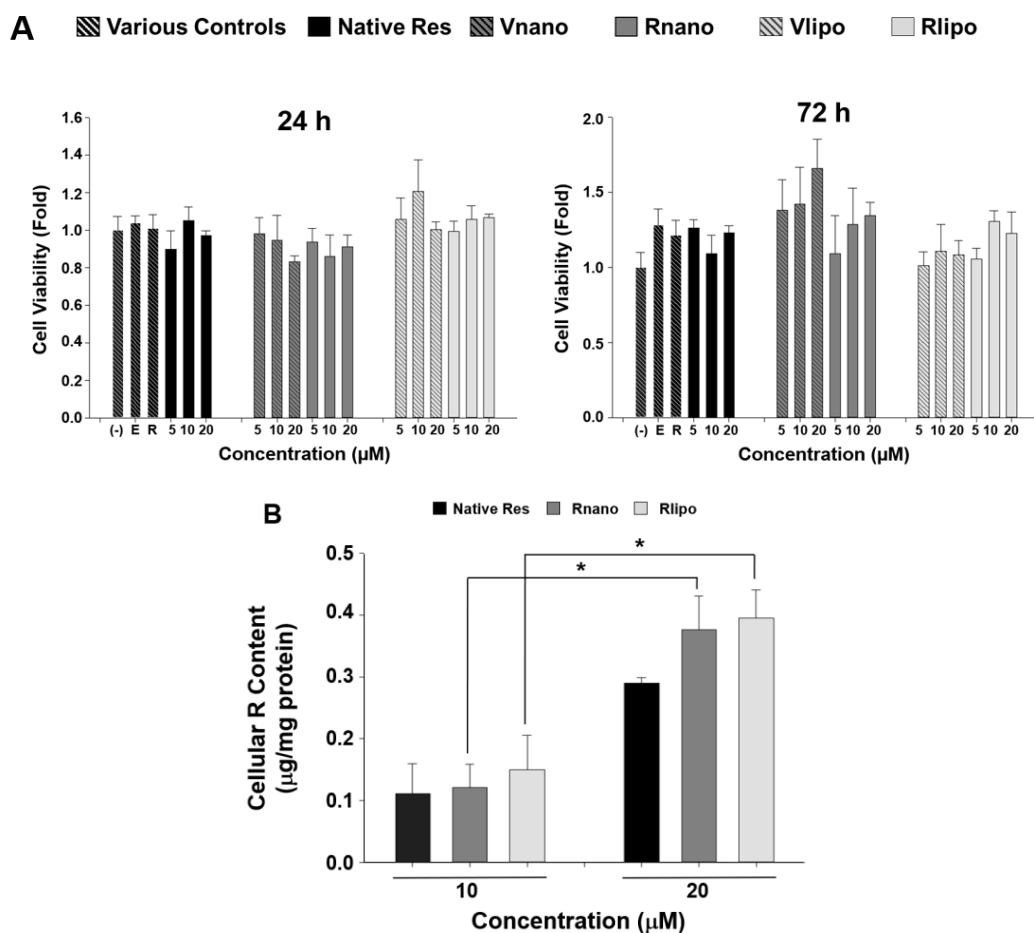


Figure 3.5. Cytotoxicity and Res content in 3T3-L1 cells. Cytotoxicity was measured by MTT assays after treating 3T3-L1 cells with native Res, Rlipo, and Rnano (5, 10, 20 µM) or their respective controls for 24 and 72 h (**A**); Res content in 3T3-L1 cells after treating them with 10 and 20 µM of native Res, Rnano and Rlipo at 37°C for 4 h (**B**). Data = means ± SEM (n=3). *, p<0.05. E, Ethanol containing vehicle control for native Res. R, rosiglitazone, a positive control.

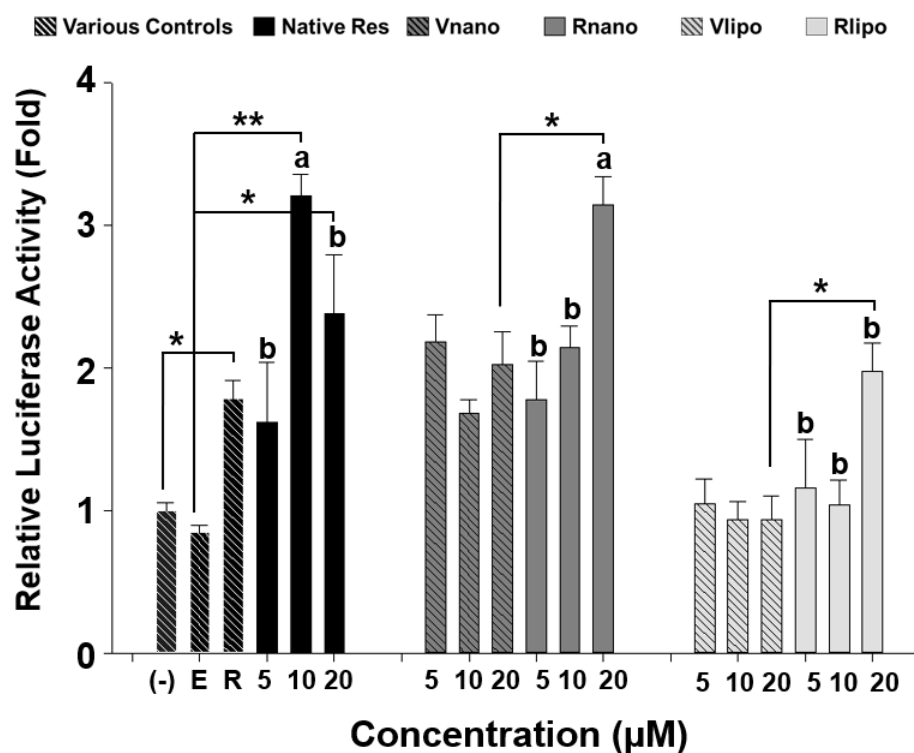


Figure 3.6. Activation of PPAR responsive reporter by various forms of Res. 3T3-L1 cells were seeded and transiently transfected with PPRE-Luc and transfection control plasmid β -gal for 24 h. The cells were then treated with native Res, Rlipo, and Rnano (5, 10, 20 μ M) and their controls for 15 h. Relative luciferase activities were normalized by β -gal activities. Data = means + SEM (n=3). E, Ethanol containing vehicle control for native Res. R, rosiglitazone, a positive control. Different letters on top of the bars indicate significant differences among various forms of Res. *, p<0.05; **, p<0.01 compared to their controls.

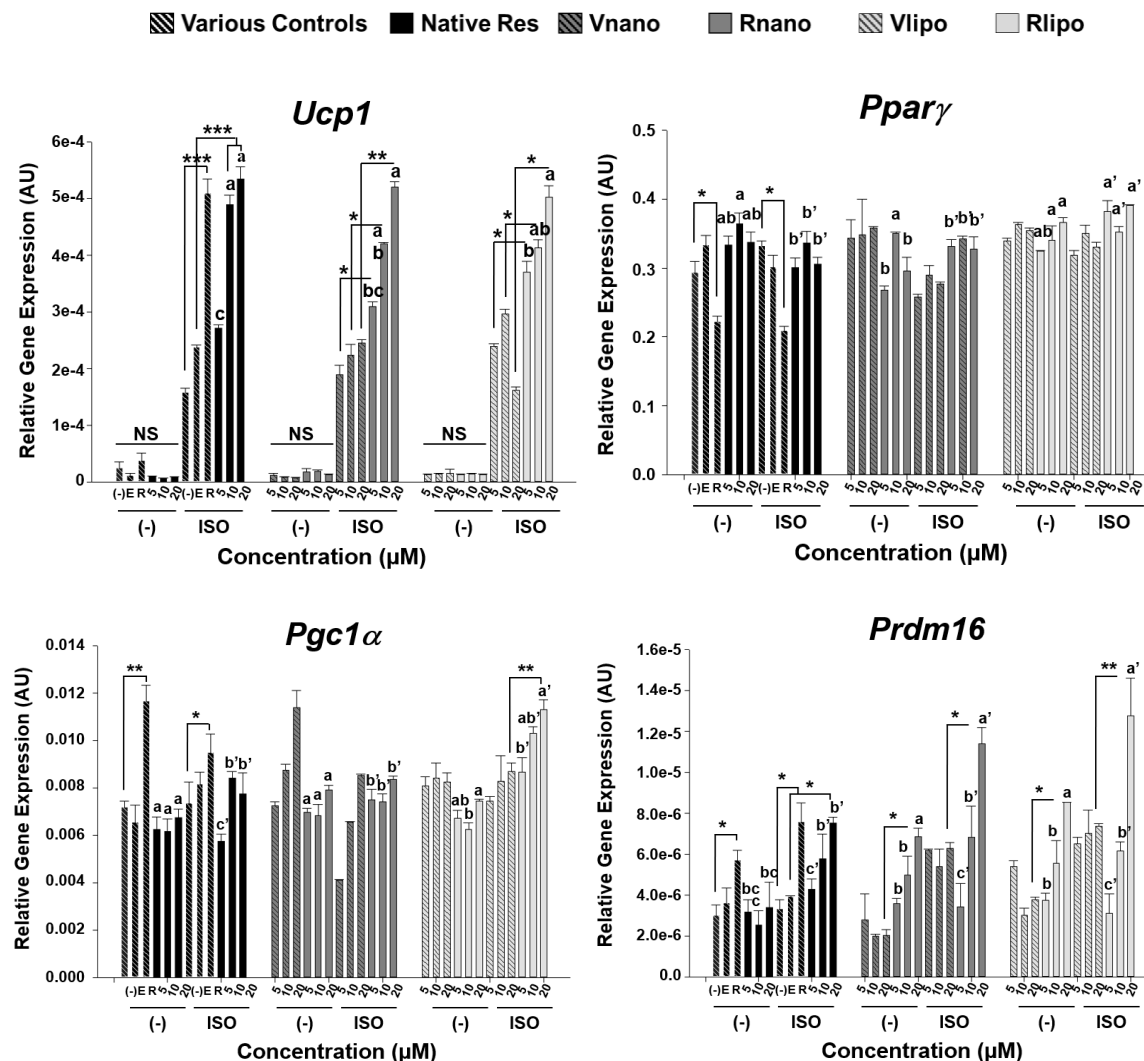


Figure 3.7. Browning activities. 3T3-L1 cells were induced to undergo white adipocyte differentiation in the presence of native Res, Rlipo, and Rnano (5, 10, 20 μ M) and their controls for 7 days. Cells were then stimulated with ISO for 6 h. Relative mRNA expression of *Ucp1*, *Pparγ*, *Pgc1α*, and *Prdm16* were analyzed by semi-quantitative Real Time-PCR. Data = means + SEM (n=3). E, Ethanol containing vehicle control for native Res. R, rosiglitazone, a positive control. Letters of a-c or a'-c' on top of the bars define differences among various forms of Res under either basal or ISO-stimulated conditions, respectively. Different letters indicate significant differences among various Res. *, p<0.05; **, p<0.01; ***, p<0.001 compared to their controls.

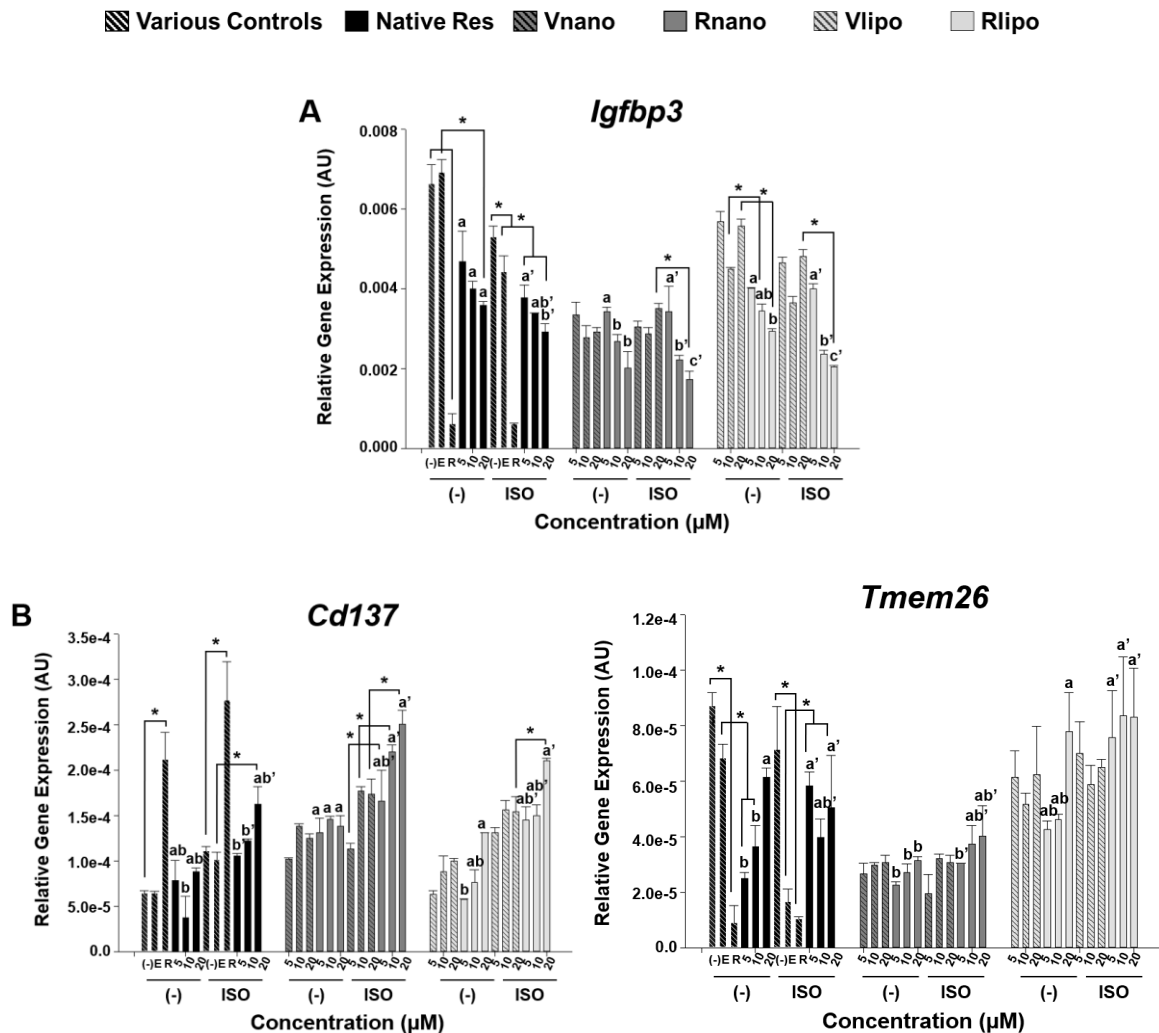


Figure 3.8. Gene expression of white and beige adipocyte markers. 3T3-L1 cells were induced to undergo white adipocyte differentiation in the presence of native Res, Rlipo, and Rnano (5, 10, 20 μ M) and their controls for 7 days. Cells were then stimulated with ISO for 6 h. Relative mRNA expression of white marker *Igfbp3* (A) and beige markers *Cd137* and *Tmem26* (B) were analyzed by semi-quantitative RT-PCR. Data = means +SEM (n=3). E, Ethanol containing vehicle control for native Res. R, rosiglitazone, a positive control. Letters of a-c or a'-c' on top of the bars define differences among various forms of Res under both basal and ISO-stimulated conditions, respectively. Different letters indicate significant differences among various R. * $p < 0.05$ compared to the controls.

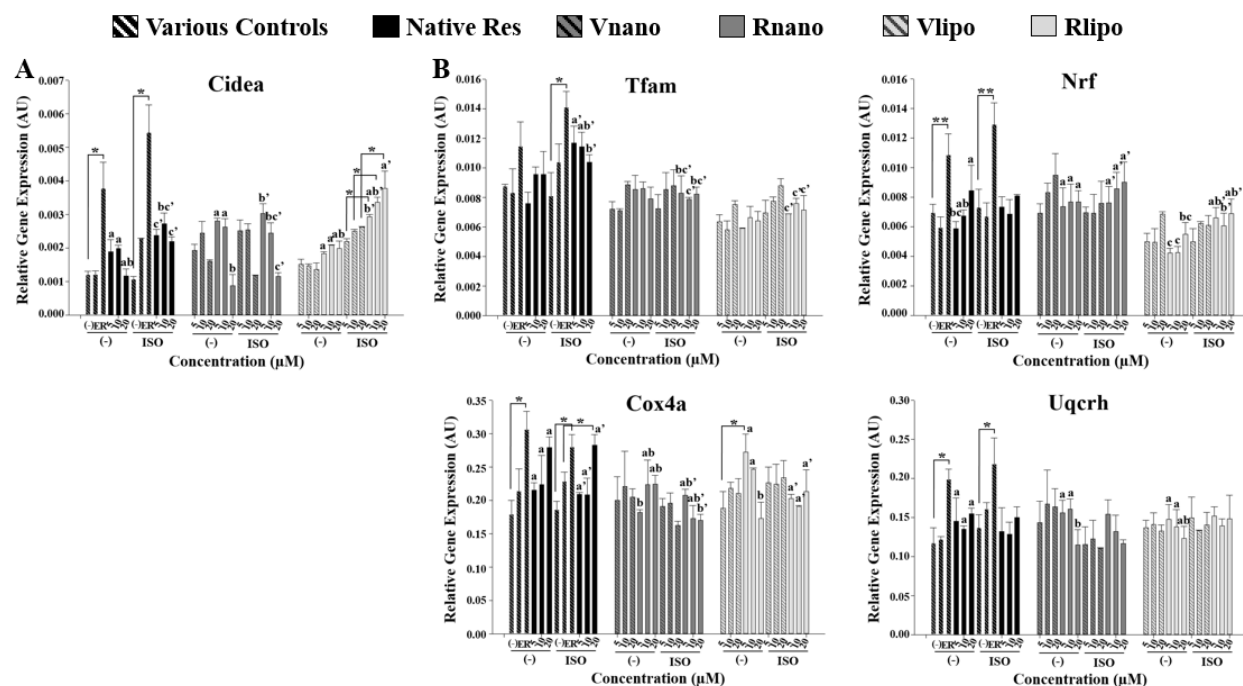


Figure S.3.1. Gene expression of mitochondrial biogenesis markers. 3T3-L1 cells were induced to undergo white adipocyte differentiation in the presence of native Res, Rlipo, and Rnano (5, 10, 20 μ M) and their controls for 7 days. The cells were then stimulated with ISO for 6 h. Relative mRNA expression of (A) *Cidea* (brown-fat marker) or (B) *Tfam*, *Nrf*, *Cox4a*, and *Uqcrrh* (mitochondrial makers) were analyzed by semi-quantitative Real Time-PCR. Data = means + SEM (n=3). E, Ethanol containing vehicle control for native Res. R, rosiglitazone, a positive control. Letters of a-c or a'-c' on top of the bars define differences among various forms of Res under both basal and ISO-stimulated conditions, respectively. Different letters indicate significant differences among various Res. *, $p < 0.05$; **, $p < 0.01$ compared to the respective controls.

Table 3.1. Primer sequences for qRT-PCR using SYBR green.

Gene name	Primer sequences	
<i>36b4</i>	Forward	GCTTCGTGTTACCAAGGAGGA
	Reverse	GTCCTAGACCAGTGTTCTGAGC
<i>Cd137</i>	Forward	CGTGCAGAACTCCTGTGATAAC
	Reverse	GTCCACCTATGCTGGAGAAGG
<i>Cidea</i>	Forward	GGTGGACACAGAGGAGTTCTTTC
	Reverse	CGAAGGTGACTCTGGCTATTCC
<i>Cox4a</i>	Forward	TCATTGGCTTCACTGCGCTCGT
	Reverse	TCCAGCATTCGCTTGGTCTGCA
<i>Igfbp3</i>	Forward	GTCGCAAATAATGGTGCGCT
	Reverse	GAAAACTGCCTTCCCGCAG
<i>Nrf1</i>	Forward	GGCAACAGTAGCCACATTGGCT
	Reverse	GTCTGGATGGTCATTTACCGC
<i>Pgc1α</i>	Forward	GAATCAAGCCACTACAGACACCG
	Reverse	CATCCCTCTTGAGCCTTTCGTG
<i>Pparγ</i>	Forward	GTA CTGT CGGTTTCAGAAAGTGCC
	Reverse	ATCTCCGCCAACAGCTTCTCCT
<i>Prdm16</i>	Forward	ATCCACAGCACGGTGAAGCCAT
	Reverse	ACATCTGCCCACAGTCCTTGCA
<i>Tfam</i>	Forward	GAGGCAAAGGATGATTGGGCTC
	Reverse	CGAATCCTATCATCTTTAGCAAGC
<i>Tmem26</i>	Forward	ACCCTGTCATCCCACAGAG
	Reverse	TGTTTGGTGGAGTCCTAAGGTC
<i>Ucp1</i>	Forward	GCTTTGCCTCACTCAGGATTGG
	Reverse	CCAATGAACACTGCCACACCTC
<i>Uqcrh</i>	Forward	GTGAGAGAGCACTGTGAACAGC
	Reverse	CAATCCTCTTCTGTCTGTGACCG

CHAPTER IV:

TARGETING NANOPARTICLES ENCAPSULATED WITH RESVERATROL INDUCE BROWNING OF MURINE PRIMARY SUBCUTANEOUS WHITE ADIPOCYTES

4.1. ABSTRACT

The plant-derived polyphenolic compound, *trans*-resveratrol (**Res**), is shown to possess a plethora of health-promoting and disease-fighting benefits ranging from anti-inflammatory, anti-bacterial, and anti-aging, to anti-carcinogenic, anti-atherogenic, and anti-obesogenic. However, poor aqueous solubility and rapid metabolism presents a critical limitation in clinical utility for human research. Encapsulating Res in nanocarriers has shown to overcome some of these limitations and demonstrate promising therapeutic potential *in vitro* and *in vivo*; however, there still seems to be non-specific binding and excessive elimination. Targeting the adipose stromal cells (**ASC**) found in white adipose tissue (**WAT**) would help to focus therapeutic potential of Res to promote beige adipocyte formation, leading to increased energy expenditure and weight loss. An extra-cellular matrix-associated post-translationally cleaved fragment of decorin lacking the glycanation site (**ΔDCN**) is specifically found on the surface of WAT-ASCs. An ASC-targeting peptide and ligand for ΔDCN, GSWKYWFGEGGC, was successfully incorporated in nanoparticles encapsulated with Res (**L-NR**). The primary objectives of this study were to assess the targeting capacity and efficiency as well as the biological effect of L-NR in ΔDCN-transduced 3T3-L1 cells (**ΔDCN cells**) and freshly isolated murine primary cells from the stromal vascular fraction (**mSVF**) of inguinal WAT. Cellular uptake was assessed using Rhodamine-labeled nanocarriers with or without the targeting ligand bound (**L-Rhod-nano** and **Rhod-nano**, respectively). L-Rhod-nano had significantly greater uptake in ΔDCN cells as shown by confocal microscopy. There was no further activation of peroxisome proliferator response element and peroxisome proliferator-activated receptor γ by L-NR compared to free-Res (**FR**) in ΔDCN or the control cells, likely due to the short treatment time. Western analysis confirmed ΔDCN expression in freshly isolated mSVF, during confluence, and at the beginning of differentiation, which was reflected by significantly enhanced uptake of L-Rhod-nano in mSVF cells at these two time points. When mSVF were treated with various forms of Res throughout differentiation, L-NR promoted browning to a greater extent than the non-ligand bound nanoparticle encapsulated with Res (**NR**) and FR at doses as low as 1 μ M, as shown by enhanced *Ucp1*, *Pgc1 α* , and *Cd137* as well as suppressed *Igf1bp3* mRNA expression. When fully differentiated mSVF were treated with various forms of Res, L-NR enhanced browning greater than NR and FR at 10 μ M as shown by the same above markers. Taken together, our results suggest that the targeting nanocarriers encapsulated with Res significantly enhanced delivery and browning in mouse primary subcutaneous stromal cells and adipocytes. The targeting nanocarrier delivery system can be a novel and beneficial strategy to combat obesity.

4.2. INTRODUCTION

Obesity is a still-growing health burden in developed countries and is becoming more prevalent in developing countries.^{1, 2} Obesity can be characterized by individuals having high body mass index (**BMI**) combined with a large waist circumference, which is indicative of greater central adiposity.³⁻⁵ Increases in visceral adipose tissue (**VAT**) is highly associated with obesity's comorbidities such as metabolic syndrome, cardiovascular disease, and some types of cancers,⁶ which are leaders in causes of preventable morbidity and mortality.⁷ Therefore, discovery of novel therapies to treat and prevent obesity is of vital importance and targeting the culpable tissue is a sensible start.

Although adipose tissue has many functions, including insulation and cushion, its primary function is as a metabolic and endocrine organ, storing and exchanging energy and nutrients in response to internal stimuli as well as secreting hormones, cytokines, and chemokines.⁸⁻¹⁰ The two main types that are primarily recognized are white adipose tissue (**WAT**) and brown adipose tissue (**BAT**). WAT is the most predominant type of fat in the adult human, with adipocytes having a large unilocular lipid droplet and is primarily associated with energy storage.¹¹ BAT is a highly thermogenic tissue whose adipocytes have smaller multilocular lipid droplets and many mitochondria which express uncoupling protein 1 (**UCP1**).¹² UCP1 disrupts the electron transport chain (**ETC**) chemiosmotic gradient, causing substrates which are normally used for synthesizing ATP to be released as heat instead.^{12, 13} Due to this characteristic, BAT can act as a “metabolic sink” for excess glucose and fat, and therefore significantly contribute to energy expenditure and lead to weight loss.^{14, 15} A type of intermediate of brown and white adipocytes, called beige or brite adipocytes, is now recognized to have origins in WAT but function similar to BAT.^{16, 17} It is thought that beige adipocytes can either “transdifferentiate” from fully mature adipocytes,^{18, 19} or be induced in the adipose stromal cells (**ASCs**) found in the stromal vascular fraction (**SVF**) of WAT in response to metabolic cues and stimuli.²⁰⁻²² Activation of the brown-fat program (i.e., **UCP1** expression) in these cells has been shown to occur upon cold stimulation and treatment with β -adrenergic receptor (**β -AR**) agonists, peroxisome proliferator-activated receptor- γ (**PPAR γ**) activators, and some nutritional and pharmacological agents.^{12, 13, 17, 23-25} Studies have shown beige adipocytes from these sources have led to increased energy expenditure causing weight loss.^{17, 26} The thermogenicity and inducibility of beige adipocytes differentiated from ASCs from the ample WAT has made it a desirable target in the hunt for obesity therapies.^{27, 28}

Among the nutritional compounds with a variety of health benefits, *trans*-resveratrol (**Res**) stands out as a naturally occurring polyphenol with potent antioxidant properties^{29, 30} and several anti-obesity effects³¹⁻³³ including promoting beige adipocyte formation within WAT.^{34, 35} Res has been shown to mediate its beneficial effects via interaction with a variety of transcriptional factors implicated in signaling pathways responsible for regulating beige adipocyte formation.^{33, 36} These include, but are not limited to, direct or indirect activation of AMP-activated protein kinase (**AMPK**),³⁷⁻⁴⁰ NAD-dependent deacetylase sirtuin-1 (**SIRT1**),^{37, 41-43} PPAR γ ,⁴⁴⁻⁴⁶ as well as PPAR γ coactivator 1 α (**PGC1 α**),⁴⁷ among many others.³⁶ Although there are many studies to support Res' beneficial effects *in vitro* and *in vivo*, the evidence to support anti-obesity effects in human clinical trials is limited and controversial.^{48, 49} The extensive hepatic and intestinal metabolism in addition to the poor aqueous solubility could possibly be the primary limiting factor in respect to bioavailability, which may provide some explanation for the discrepancies between human and animal Res studies.^{30, 35, 49, 50} Nanotechnology has been explored in an effort to improve bioavailability of several polyphenolic compounds and has shown effective in the delivery of a variety of disease-fighting therapeutic agents.⁵⁰⁻⁵⁵ Using biodegradable and biocompatible nanocarriers to encapsulate Res could help to overcome these critical limitations to enhance efficacy and allow for more targeted delivery, strengthening its clinical utility in the fight against obesity.^{50, 52, 56, 57}

In a previously published study, we provided evidence to support that nanoparticles encapsulated with Res (**NR**) improved chemical stability, aqueous solubility, as well as promoted browning in 3T3-L1 cells (**Chapter III**). The results of this study demonstrated that the encapsulation enhanced its overall bioavailability and further enhanced browning activity in 3T3-L1 adipocytes. Recently, a strategy to target ASCs has been reported.⁵⁸ The targeting system involves a peptide, GSWKYWFGEGGC, which acts as a ligand for delta-decorin (**Δ DCN**), an extracellular matrix (**ECM**)-associated protein and an isoform of decorin (**DCN**) lacking the glycanation site.⁵⁸ Δ DCN is shown to solely be expressed on the surface of ASCs in WAT.⁵⁸ We have successfully inserted this peptide on the nanocarrier surface, thus developing an ASC-targeting ligand-coated NR (**L-NR**).

The primary objectives of this study were to determine the uptake capacity and biological response to L-NR using 3T3-L1 cells over-expressing Δ DCN (**Δ DCN cells**) as well as freshly isolated murine SVF. In using this novel nanocarrier delivery system with ASC-WAT-targeting capacity, we expect that we will see greater uptake which will translate into enhanced browning of mouse primary stromal cells.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals and reagents

Dexamethasone (**Dex**), 3-Isobutyl-L-methylxanthine (**IBMX**), insulin, triiodothyronine (**T₃**), and rosiglitazone (**Rosi**) were purchased from Sigma-Aldrich (St. Louis, MO). Indomethacin (**Indo**) was purchased from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle's medium (**DMEM**) and Calf serum (**CS**) was purchased from Hyclone (Logan, UT) and fetal bovine serum (**FBS**) from Atlanta Biologicals (Flowery Branch, GA). Antibiotics, penicillin and streptomycin (**P/S**) were purchased from Gibco by Life Technologies (Burlington, ONT). Mouse decorin antibody was purchased from R&D Systems, Inc (Minneapolis, MN). Tubulin and anti-goat antibodies were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX), and anti-rabbit horse-radish peroxidase (**HRP**)-conjugated antibody were from Cell Signaling Technology (Danvers, MA).

4.3.2. Nanoparticle preparation

Nanoparticles (**NP**) encapsulated with Res, with or without the targeting peptide-ligand (**L-NR** and **NR**, respectively) as well as free-Res (**FR**), were prepared fresh for each change of the media by collaborators at Texas Tech University. NP's loading capacity and Res release at various temperatures have been previously described.⁴⁶ Res concentration was determined for L-NR, NR, and FR for each batch made using High Performance Liquid Chromatography (**HPLC**) as previously described.⁴⁶

4.3.3. Cell models

4.3.3.1. Delta-DCN over-expressed cells

3T3-L1 cells lentivirally transduced to over-express delta-decorin-GFP (**ΔDCN cells**) or GFP vector alone (**CON cells**) were a gift from the Mikhail Kolonin group at the University of Texas Health Science Center at Houston. ΔDCN and CON cells were grown and maintained in DMEM containing 10% CS with 1% P/S and 5 μg/mL puromycin (selection maintenance) in a 5% CO₂, 37°C environment.

4.3.3.2. Murine stromal cells

4.3.3.2 (1) Isolation procedure

Isolation of SVF from male C57Bl/6J mice was performed as previously described with minor modifications.^{59, 60} Briefly, inguinal subcutaneous white fat pads were excised, weighed, and temporarily stored in ice-cold PBS. Fat pads were minced with razors and added to

isolation buffer (120 mM NaCl, 0.5 mM KCl, 1.2 mM KH₂PO₄, 0.6 mM MgSO₄·7H₂O, 0.9 mM CaCl₂·6H₂O, 20 mM HEPES, and 2.5% BSA) in a ratio of 1 g tissue : 3 mL buffer. Type I collagenase was freshly added to the tissue-buffer mixture 60 µL of collagenase per 1 mL buffer. Digestion of the mixture took place in a 37°C shaking water bath at 150 rpm for 30 min (with periodic gentle vortexing and pipette-mixing every 10 min). Digested tissues went through two filtration steps followed by centrifugation at 500 g for 5 min at 4°C, first through 100 µm nylon mesh sterile-cell filter, then through 70 µm nylon mesh sterile-cell filter. After filtering and centrifugation, supernatant was removed, and the cell pellet was washed and resuspended in isolation buffer. After the two washing and filter steps, the cell pellet was resuspended in DMEM containing 15% FBS with 1% P/S and counted prior to plating into 60 mm dishes in a 5% CO₂, 37°C environment. After 6 h to overnight incubation, non-adherent cells were removed with gentle washing with culture media. After 48 h, fully adherent cells were maintained in DMEM with 10% FBS and 1% P/S until induced to differentiate. Where indicated, cell-culture plates were coated with 5% sterile gelatin solution (Sigma-Aldrich, St. Louis, MO) to help with cell attachment. Cells were passaged up to two times prior to differentiation, all experiments shown here were on the same passage, and isolated from mice of matching age, gender (male), and genetic background.

4.3.3.2 (2) Differentiation protocol

Murine primary stromal cells were differentiated as previously described,²⁸ with minor modifications. Briefly, confluent cells (designated as D0) were induced to differentiate with DMEM containing 10% FBS and 1% P/S with 1 µM Dex, 0.5 mM IBMX, 5 µg/mL insulin, 0.5 µM Rosi, 125 µM Indo, and 1 nM T₃ for three days. Differentiating cells were maintained in DMEM containing 10% FBS and 1% P/S with 5 µg/mL insulin, 0.5 µM Rosi, and 1 nM T₃ for the remainder of cell differentiation. Medium was replenished every two to three days until fully differentiated at day 10. “Complete Differentiation” (Complete) refers to cells that received treatment with every change of media. “Transdifferentiation” refers to cells that only received treatment on day 7 of media change.

4.3.4. Quantifying uptake of nanoparticles using confocal microscopy

Rhodamine-labeled NPs (**Rhod-nano**) were diluted and fluorescence quantified using a BioTek Microplate Reader to establish equal incorporation of Rhodamine between the targeting and non-targeting NP. ΔDCN and freshly isolated murine primary stromal cells were seeded in 24-well plate (1x10⁵ cells/well) onto sterile Corning BioCoat Poly-D-Lysine Coverslips (Thomas

Scientific, Swedesboro, NJ) overnight and treated with Rhod-labeled nanocarriers without or with the targeting-ligand (Rhod-nano and **L-Rhod-nano**, respectively) at 37°C for 2 h. Ice-cold sterile PBS was used to wash cells three times prior to termination and fixation with 3.7% formaldehyde in PBS for 15 min at room temperature. After fixing followed an additional three-step washing process with ice-cold PBS. Cells were then stained with 4', 6-diamindine-2'-phenylindole dihydrochloride (**DAPI**) at room temperature for 5 min, followed by one PBS rinse. Fixed and stained cells' cover slips were mounted on disposable microscope slides using ProlongTM Gold Antifade mounting solution (both from ThermoFisher Scientific, Waltham, MA). Cells were imaged using Leica SP8 White Light Laser Confocal System (Leica Microsystems Inc, Buffalo Grove, IL). Rhodamine uptake, shown in red, was detected using the white-light laser (λ_{exc} : 560 nm, λ_{em} : 583 nm). DAPI-stained nuclei, shown in blue, was detected using the "405 laser" (λ_{exc} : 358 nm, λ_{em} : 461 nm). All exposure and imaging settings are identical for comparable analysis. Rhodamine uptake in florescent images were quantified using Image J, graphs made to represent at least 3 separate experiments each having at least 3 different areas of cells imaged and quantified, areas were chosen at random.

4.3.5. Transfection and reporter gene assays

Transfection and reporter gene assays were performed as previously described.⁴⁵⁻⁴⁶ PPAR transactivation reporters were a gift from Dr. Susanne Mandrup (University of Southern Denmark, Denmark) which consisted of murine PPAR γ ligand binding domain (**LBD**) fused with a Gal4 DNA binding domain (**DBD**) (mPPAR γ -Gal4) and a reporter construct comprised of an upstream activating sequence (**UAS**) thymidine kinase-linked luciferase, 4xUAS-TK-Luc (hereon referred to as PPAR γ -Gal4-Luc or simply PPAR γ (trans)activation). Another transactivation reporter used included peroxisome proliferator response element (**PPRE**)-driven luciferase reporter (PPRE-Luc). Δ DCN or CON preadipocytes were seeded one day prior to transfection in a manner so that cells reached approximately 50-70% confluence the day of treatment (1×10^4 per well in 48-well format). The preadipocytes were then transiently transfected with PPRE-Luc or PPAR γ -Gal4-Luc, and β -galactosidase (**β -gal**) control plasmid using Lipofectamine 3000 and PLUS Reagent (ThermoFisher Scientific, Waltham, MA) for 24 h. The cells were then subjected to treatment with the various forms of Res or the controls for an additional 15-18 h. Cell lysate was prepared using Reporter Lysis Buffer (Promega, Madison, WI) with Luciferase substrate and β -gal substrate. Luciferase activity and β -gal was quantified using luminescence or absorbance, respectively by GloMax-Multi Detection System (Promega, Madison, WI).

4.3.6. Western blot analysis

Western blot analysis was performed as previously described.⁴⁵ Briefly, protein extraction was completed using ice-cold PBS with phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA) to rinse the cells while cell lysate was prepared using 1x RIPA buffer (Cell Signaling, Danvers, MA) with freshly added PMSF (Sigma). Protein concentrations were determined by BCA Protein Assay kit (ThermoFisher Scientific, Waltham, MA). Total cell lysates were subjected to electrophoresis on 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (TBST at pH 7.4) containing 5% nonfat milk. The membrane incubated with diluted primary antibodies with varying times, followed by incubation with diluted secondary antibody. Washing steps between antibody incubations included TBST on rocker four times for 10 min. Each membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA) for 5 min. Excess substrate was removed, and protein expression was detected using Odyssey[®] FC imaging system (Licor Biosciences, Lincoln, NE) or a x-ray film processor (Konica Corporation, Taiwan) utilizing HyBlot CL Autoradiography Film (Denville, Holliston, MA) which was exposed to the substrate-incubated membrane for various times. The membrane was stripped in buffer (62.5 mM Tris-HCl, 2 % SDS, and 100 mM 2-mercaptoethanol) at 50°C for 5-10 min. The signal was quantified by densitometry using Odyssey[®] FC imaging system.

4.3.7. mRNA isolation and semi-quantitative RT-PCR analysis

RNA isolation was performed as previously described.⁴⁵⁻⁴⁶ Briefly, total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify total RNA. After RNA isolation, High Capacity cDNA Reverse Transcription kit (Thermo Scientific, Pittsburgh, PA) was used to carry out reverse transcription following the manufacturer's instructions. Target genes and the housekeeping gene, *36b4*, mRNA expression was determined quantitatively using Power Up SYBR Green Master Mix following manufacturer's instructions (Applied Biosystems, Austin, TX). An ABI 7300HT instrument was used to run PCR reactions using a 96-well format. The cycle number (C_t) was obtained for each reaction, the $2(-\Delta\Delta C_t)$ method was used to calculate the relative gene expression, normalizing against the house keeping genes which is then converted to fold.

4.3.8. Statistical analysis

Data in figures presented here is shown as means $\pm$ SEM. Experiments were performed at least three times with triplicates. SigmaPlot 14 (Systat Software, Inc) was used to perform statistical analysis. To determine the differences within and between treatment groups, t-tests, one-way ANOVA, or mixed model ANOVA was performed followed by post-hoc comparison tests with Student-Newman-Keuls method. The level of significance was set at $p < 0.05$.

4.4. RESULTS

4.4.1. Enhanced uptake of rhodamine by ligand-bound nanoparticles in Δ DCN cells

Rhodamine-labeled nanocarriers without or with the targeting-ligand bound (Rhod-nano and L-Rhod-nano, respectively) were used to treat Δ DCN preadipocytes. This uptake was visualized by fluorescence and confocal microscopy. L-Rhod-nano had significantly greater uptake of the Rhodamine-labeled nanocarriers in Δ DCN cells (Fig 4.1.A and B) (all tables and figures for Chapter IV are located in 4.8. Appendix). There were no differences in uptake of Rhodamine-labeled NPs in 3T3-L1 cells, which were used as a control for the Δ DCN cells (data not shown). Z-stack confocal 3D-projections with zoomed in image, demonstrates NPs getting into the cells (Fig 4.1.C).

4.4.2. Enhanced resveratrol-induced PPRE and PPAR γ transactivation by ligand-bound nanoparticles in Δ DCN cells

Previous studies have shown FR binds^{36, 61} to and activates⁴⁴⁻⁴⁶ PPAR γ , which can transactivate brown-fat target genes including UCP1 when PPAR γ heterodimerizes with retinoid X receptor (**RXR**) to bind to PPRES sites.⁶² We have previously determined that FR in addition to nanoparticle-encapsulated Res in two forms (lipid nanocarriers = Rnano, and liposomes = Rlipo) significantly activated PPRES compared to their controls.⁴⁶ Similarly, we showed here that all forms of Res significantly activated PPRES greater than their controls in Δ DCN cells, and L-NR produced the greatest PPRES activation of all forms of Res in Δ DCN cells (Fig 4.2.A). FR significantly activated PPRES in the CON cells, whereas there were no differences between the nano-encapsulated Res forms and their void controls (Fig 4.2.A). Moreover, the Nano-encapsulated forms of Res also significantly activated PPAR γ greater than controls in Δ DCN cells; however, L-NR yielded the greatest PPAR γ activation among all forms of Res in Δ DCN cells (Fig 4.2.B). In contrast, there were no significant differences in any form of Res compared to controls nor between the forms of Res seen in the CON cells (Fig 4.2.B). These results

suggest that the Δ DCN-targeting nanocarrier is able to deliver and promote Res' capacity to bind and activate PPAR γ greater than the free and non-ligand-bound NP forms of Res, and significantly activate PPAR γ downstream target, PPRE, greater than the NR.

4.4.3. Detection of decorin protein expression in murine stromal cells from subcutaneous fat

Murine primary SVF (**mSVF**) isolated from subQ iWAT samples were collected at various time points including the mSVF pellet, when cells reached 70-80% and 100% confluence, as well as 3 days into differentiation (3-Day Diff) in either gelatin-coated or non-coated plastic-culture plating conditions. Our results show that, indeed, Δ DCN expression is high when mSVF are pelleted, and the expression significantly falls off when cells are plated in culture (i.e., at 70-80% confluence) (Fig 4.3.A-B). However, this expression begins to return when cells are 100% confluent, and this increase is augmented in the beginning stages of differentiation (after post-mitotic cell expansion) (Fig 4.3.A-B). These results suggest cells may need cell-cell contact for the ECM-associated protein to begin to be secreted.

4.4.4. Enhanced uptake of rhodamine by ligand-bound nanoparticles in murine stromal cells

Rhodamine-labeled nanocarriers without or with the targeting ligand bound (Rhod-nano and L-Rhod-nano, respectively) were used to treat mSVF at various cell states, including 70-80% and 100% confluence, and three days into differentiation (3-Day Diff). This uptake was visualized by fluorescence and confocal microscopy. L-Rhod-nano treated cells had significantly greater uptake of the Rhodamine-labeled nanocarriers than those of Rhod-nano when the cells were at 100% confluence and 3-Day Diff, shown visually (Fig 4.4.A) and quantitatively (Fig 4.4.B). There were no significant differences in uptake of Rhodamine-labeled NPs between the L-Rhod-nano and Rhod-nano when the cells were at 70-80% confluence (Fig 4.4.A-B). Z-stack confocal 3D-projections with zoomed in image, demonstrate diffused Rhodamine uptake around the nuclei, suggesting that NPs were taken in by the mSVF cells at 3-Day Diff (Fig 4.4.C).

4.4.5. ASC-targeting nanoparticles enhances resveratrol's efficiency to promote browning at lower, physiologically relevant doses in murine stromal cells

When murine primary stromal cells isolated from iWAT were subjected to differentiation in the presence of various forms of Res and their controls at 1, 5, or 10 μ M throughout the entire differentiation program, there was a significant induction in BAT and beige marker mRNA

expression with a simultaneous suppression of white-fat specific marker mRNA expression. At 1 μ M, ligand-bound L-NR significantly induced select browning markers and suppressed white-fat specific marker, *Igfbp3*, to a greater extent than its vehicle control and other forms of Res. When cells are treated throughout the differentiation process, as Res concentrations increased (to 5 and 10 μ M), the differences among the various forms of Res become less apparent. The gene specific changes are described as follows.

Ppar γ mRNA expression (Fig 4.5.A)

PPAR γ is a master adipocyte differentiation regulator, is highly enriched in BAT, and activation of which has been shown to lead to browning in WAT.²⁵ At 1 and 5 μ M, only L-NR significantly induced *Ppar γ* mRNA expression greater than its vehicle control and L-NR induced the highest *Ppar γ* mRNA expression among the various forms of Res at 1 μ M (Fig 4.5.A). There were no significant differences in *Ppar γ* mRNA expression between the forms of Res and their vehicle controls at 10 μ M (Fig 4.5.A).

Pgc1 α mRNA expression (Fig 4.5.B)

PGC1 α is a transcriptional coactivator for energy-metabolism genes and is well known for its role as a master mitochondrial biogenesis regulator.^{63, 64} PGC1 α is highly expressed in muscle and BAT and can be induced in the browning of WAT.⁶⁵ At 1 μ M, only L-NR significantly induced *Pgc1 α* mRNA expression greater than its vehicle control and the other forms of Res (Fig 4.5.B). At 5 μ M, both NR and L-NR significantly induced *Pgc1 α* mRNA expression greater than their vehicle controls; however, L-NR induced the highest *Pgc1 α* mRNA expression among various forms of Res at 5 μ M (Fig 4.5.B). At 10 μ M, only NR significantly induced *Pgc1 α* mRNA expression greater than its vehicle control; however, there were no significant differences among the various forms of Res (Fig 4.5.B).

Ucp1 mRNA expression (Fig 4.5.C)

UCP1 is the hallmark gene for BAT and beige fat cells, playing a key role in the non-shivering thermogenic program in fat cells.⁶⁶ At 1 μ M, both FR and L-NR significantly induced *Ucp1* mRNA expression greater than their vehicle controls; however, only L-NR induced the highest *Ucp1* mRNA expression among the various forms of Res at 1 μ M (Fig 4.5.C). At 5 μ M, all forms of Res significantly induced *Ucp1* mRNA expression greater than their vehicle controls; however, both NR and L-NR were greater than FR (Fig 4.5.C). At 10 μ M, there were no

significant differences between the forms of Res and their vehicle controls nor among the various forms of Res (Fig 4.5.C).

Cd137 mRNA expression (Fig 4.5.D)

CD137 is identified as a specific marker for beige fat cells.^{17, 67} At 1 μ M, both FR and L-NR significantly induced *Cd137* mRNA expression greater than their vehicle controls; however, L-NR induced the highest *Cd137* mRNA expression among the various forms of Res at 1 μ M (Fig 4.5.D). At 5 μ M, both NR and L-NR significantly induced *Cd137* mRNA expression greater than their vehicle controls and both were significantly greater than FR (Fig 4.5.D). At 10 μ M, both FR and NR were significantly lower than their vehicle controls. In contrast, L-NR not only induced higher *Cd137* mRNA expression than the vehicle control, but also had the highest *Cd137* mRNA expression among the various forms of Res (Fig 4.5.D).

Igfbp3 mRNA expression (Fig 4.5.E)

IGFBP3 is a marker highly expressed and associated with WAT, and therefore, is used here as a white-fat specific gene marker.⁶⁸ At 1 μ M, only L-NR significantly suppressed *Igfbp3* mRNA expression than the vehicle control and induced the lowest *Igfbp3* mRNA expression among the various forms of Res (Fig 4.5.E). At 5 μ M, only FR significantly suppressed *Igfbp3* mRNA expression compared with its vehicle control. There were no significant differences between NR and L-NR and their respective controls or between each other in *Igfbp3* mRNA expression at 5 μ M (Fig 4.5.E). At 10 μ M, NR significantly induced *Igfbp3* mRNA expression greater than its vehicle control and greater than the other forms of Res (Fig 4.5.E).

4.4.6. Ligand-bound nanoparticles encapsulated with resveratrol promote browning at higher doses when treating fully differentiated murine stromal cells

When murine primary stromal cells isolated from iWAT were subjected to differentiation into fully mature adipocytes prior to being treated with various forms of Res and their controls at 1, 5, or 10 μ M for three days, there was a significant induction in BAT and beige mRNA expression with a simultaneous suppression of white-fat specific marker, *Igfbp3*, mRNA expression by higher doses of the targeting ligand-bound L-NR, but not other forms of Res. In this “transdifferentiation” program setup, the various forms of Res at 1 and 5 μ M had less browning effects when compared to these same treatments given throughout the entire differentiation process.

Ppar γ mRNA expression (Fig 4.6.A)

Only L-NR at 10 μ M significantly induced *Ppar* γ mRNA expression greater than its vehicle control and greater than the other forms of Res (Fig 4.6.A). Although NR and L-NR at 1 μ M were greater than FR in inducing *Ppar* γ mRNA expression, they were not greater than their vehicle controls (Fig 4.6.A).

Pgc1 α mRNA expression (Fig 4.6.B)

Similar to the *Ppar* γ mRNA expression results, only L-NR at 10 μ M significantly induced *Pgc1* α mRNA expression greater than its vehicle control and the other forms of Res (Fig 4.6.B).

Ucp1 mRNA expression (Fig 4.6.C)

Consistently, L-NR at 10 μ M significantly induced *Ucp1* mRNA expression greater than its vehicle control and the other forms of Res (Fig 4.6.C). Interestingly, only NR at 1 μ M significantly induced *Ucp1* mRNA expression greater than its vehicle control and the other forms of Res (Fig 4.6.C).

Cd137 mRNA expression (Fig 4.6.D)

Unlike *Ppar* γ , *Pgc1* α , and *Ucp1* mRNA expression results, there was a greater response at lower doses of Res in *Cd137* mRNA gene expression. At 1 μ M, all tested forms of Res significantly induced *Cd137* mRNA expression greater than their vehicle controls; however, the induction by NR and L-NR were greater than that of FR (Fig 4.6.D). At 5 μ M, FR and NR significantly induced *Cd137* mRNA expression greater than their vehicle controls; however, the induction by NR and L-NR were greater than that of FR (Fig 4.6.D). At 10 μ M, NR and L-NR significantly induced *Cd137* mRNA expression greater than their respective vehicle controls and that of FR (Fig 4.6.D).

Igfbp3 mRNA expression (Fig 4.6.E)

At 1 μ M, FR and NR significantly suppressed *Igfbp3* mRNA expression compared to their vehicle controls; however, NR and L-NR suppressed *Igfbp3* mRNA expression to a greater extent than that of FR (Fig 4.6.E). At 5 μ M, all tested forms of Res significantly suppressed *Igfbp3* mRNA expression, with NR and L-NR being greater at suppressing than FR (Fig 4.6.E). At 10 μ M, only L-NR significantly suppressed *Igfbp3* mRNA expression compared to its vehicle control, with NR and L-NR being greater at suppressing than FR (Fig 4.6.E).

4.5. DISCUSSION

Res is a stilbene phytoalexin and a popular natural compound often used experimentally in the context of inflammation, cancer, and obesity and its comorbidities.²⁹⁻³⁵ Due to its rapid

metabolism to lesser compounds which have been conjugated and marked for removal from the body, studies utilizing the compound and measuring its effectiveness have been inconsistent, making translating the results for human therapeutic use controversial.^{30, 35, 48-50} Nanoparticles encapsulated with Res, which was shown to slow the release of Res which could help protect it from quick metabolism and excretion, have been used to successfully induce browning in 3T3-L1 cells.⁴⁶ In this study, we have expanded upon this line of research by inserting a peptide whose receptor has been found to be specific for SAT-ASCs onto the surface of the nanoparticle encapsulated with Res in hopes to further enhance its targeting specificity and effectiveness.

The results of this study have demonstrated that there is enhanced uptake of rhodamine by using ligand-bound nanoparticles in Δ DCN cells. The ligand-bound nanoparticles also enhanced resveratrol-induced PPRE responsive reporter and PPAR γ transactivation in Δ DCN cells. We have confirmed protein expression of decorin in murine stromal cells from subcutaneous fat at various stages of cell growth. Consistently, we show enhanced uptake of rhodamine by ligand-bound nanoparticles in murine stromal cells. Lastly, our results indicate that the ASC-targeting nanoparticles enhances Res's efficiency to promote browning at lower, physiologically achievable doses in murine stromal cells when treated throughout differentiation and also promoted browning at higher doses when treating fully differentiated murine stromal cells.

Targeting nanoparticles does not further enhance resveratrol's PPAR γ or PPRE activity

One of the many advantages of nanoparticles is to promote extended release of its' contents.⁶⁹ Among the various disadvantages of resveratrol, is its quick metabolism *in vitro* and *in vivo*.^{30, 49, 70, 71} Although there is some evidence to suggest Res metabolites can have their own beneficial effects, measuring outcomes can be difficult to assess, making the literature controversial and difficult to translate.^{72, 73} Encapsulating resveratrol in nanoparticles to extend the release, and therefore extending its stability and time of effectiveness, provides benefits over longer periods of treatment time. As shown in Chapter III, FR is dramatically released within the first 2 h of incubation and falls thereafter, whereas Res encapsulated in nanocarriers displayed an extended and stable release over the course of a 10 h measurement, showing no dramatic decrease as compared to FR.⁴⁶ Therefore, when treatment times are shorter (1-24 h), the advantages nanoparticles have over the free form of Res is limited.

In the case of reporter gene assays, whose time under treatment is within this limited time-range (15-18 h treatment), this diminished advantage of encapsulation or targeting of Res is

observed. Results from a previous study showed that the various forms of Res were all better than their vehicle control when treating 3T3-L1 cells which were transiently transfected with PPRE-Luc; however, there were no differences among the forms of Res, showing no benefit of the nanoparticle encapsulation within this brief treatment time period.⁴⁶ It was also concluded from that study that the formulation of the nanoparticles did not seem to affect the biological capacity for Res to activate PPAR γ .⁴⁶

The results of our study show that, similar to the previous results, neither forms of NPs (ligand bound or not) displayed an advantage over FR in transactivating either PPRE-Luc nor PPAR γ -Gal4-Luc in either the Δ DCN nor CON cells (Fig 4.2.A-B). Although L-NR had greater PPRE activation than both NR and FR, it was only found to be significant when compared to NR in the Δ DCN cells. There were no significant differences or benefits of NP or targeting seen in the CON cells, which makes sense since this cell does not contain the receptor the ligand is designed to target.

The literature on Res interaction with PPAR γ is controversial. There are many reports showing that Res binds to and activates,⁴⁴⁻⁴⁶ binds to and inhibits,^{36, 39, 40, 43, 61, 74} or indirectly inhibiting PPAR γ (i.e., via SIRT1).⁴³ It is also suggested that the inhibition of PPAR γ transcriptional activity by Res leads to suppression in adipogenesis.^{36, 39, 40, 43} Considering that some PPAR γ -agonists can not only promote differentiation, but also lead to suppression of its own expression.⁷⁵⁻⁷⁷ Therefore, it is critical to investigate PPAR γ activation by Res in addition to PPAR γ and adipogenic gene expression. Furthermore, the literature demonstrating the inhibition of adipogenesis by Res typically utilizes supraphysiological doses (i.e., 20-100 μ M) which are unachievable *in vivo*.^{78, 79} This may again point towards the pleiotropic and biphasic effects of Res; behaving like an agonist or activator at lower doses and an antagonist at higher doses.⁸⁰ These discrepancies and misunderstandings make result-translatibility and clinical utility of Res controversial.

Decorin is expressed in murine primary stromal cells at various stages of confluence, which is reflected in enhanced uptake of targeting-ligand-bound nanoparticle

The term ' Δ DCN' was first coined and discovered by the Kolonin group, which described the membrane-bound N-terminally truncated isoform of DCN, Δ DCN, to be specifically and solely expressed by WAT ASCs.⁵⁸ The authors also suggested that there was a loss of Δ DCN cell surface expression when ASCs are plated in plastic culture-conditions as quickly as over-

night.⁵⁸ However, there remained the question of whether the secreted protein isoform expression returns at various stages of confluence or how its expression might change during differentiation of stromal cells.

Our data suggests that, although there is a loss of Δ DCN expression in freshly isolated cells plated in plastic conditions for about 2 days (samples collected when 70-80% confluent), the expression comes back when cell-to-cell contact is re-established (100% confluent) and in the beginning of differentiation (3-Day Diff). Recently, it has been established that human ASCs also express Δ DCN.⁸¹ We also found that, similar to the murine ASCs, human primary cells also have an increase in Δ DCN expression upon 100% confluence and in the beginning of differentiation, but that this expression begins to fall off further into differentiation (data not shown). Therefore, our data does not contradict the Kolonin group's data, rather, it further corroborates that this ECM-associated protein is specific to ASC's. These results may suggest that Δ DCN expression requires cell-to-cell contact and reaches a maximal expression after post-mitotic expansion, whereas end-of-term differentiating and fully mature stromal cells begin to lose Δ DCN expression.

After expression was confirmed, the targeting capacity and uptake of nanoparticles which have the targeting ligand bound to the surface was tested using 3T3-L1 over-expressing this receptor (Δ DCN cells) as well as murine primary stromal cells at various stages of confluence. Labeling the NPs with Rhodamine, a tracer dye that can be visualized using fluorescence confocal microscopy, is used to represent cellular uptake of nanocarrier contents. Fluorescence confocal microscopy determined significantly enhanced uptake of Rhoda-labeled NPs with the ligand bound (L-Rhod-nano) by the Δ DCN cells and mSVF at 100% confluence and even more-so in the 3-Day Diff sets of cells. Interestingly, the L-Rhod-nano uptake reflected the Δ DCN expression patterns in mSVF cells. The 3D z-stack images further demonstrate that Rhodamine, which is representative of Res, is being taken up into the cells and are not just attached to the cell-surface.

Ligand-bound nanoparticles encapsulated with resveratrol promote browning of murine primary adipocytes

After targeting capacity, efficiency, and uptake of ligand-bound NPs was confirmed, murine primary stromal cells were differentiated using two different experimental approaches: complete and transdifferentiation programs. The literature suggests differential effects of Res on adipogenesis based on dose and timing.⁷⁰ These effects range from promotion or suppression

of adipogenesis when treated throughout the differentiation process (i.e., complete program) as well as promote lipolysis, apoptosis, or possibly browning when treating fully mature adipocytes (i.e., transdifferentiation program). Therefore, we tested the effects of timing and dose using free and targeting or non-targeting NP-Res using murine primary stromal cells.

Via several mechanisms, Res is shown to increase cAMP levels in cells (i.e., as a phosphodiesterase inhibitor,^{31, 82} and through SIRT1-AMPK-PGC1 α axis⁸³⁻⁸⁶). *In vitro* differentiation of several adipocyte models, especially 3T3-L1 cells, have a cAMP-dependent induction of differentiation, therefore, Res *should* lead to enhanced differentiation when treatments start during confluent preadipocyte stage.⁸⁷ However, these effects may be time and dose-dependent. It has been previously shown that 10 μ M of Res can promote differentiation and browning of 3T3-L1 adipocytes^{45, 46} as well as induce browning of mSVF.^{39, 88} However, much of the literature utilizing higher, supraphysiological concentrations of Res have shown inhibition of adipogenesis.^{33, 39, 40, 43, 61, 74} Unfortunately, many groups have not utilized lower, physiologically relevant doses, perhaps due to the endorsement that “more is better” and for fear of negative results.

The results of our study demonstrate that the ligand-bound NP enhances the effectiveness of Res to induce browning, as shown by *Ucp1*, *Pgc1 α* , and *Cd137* mRNA expression, of mSVF using concentrations as low as 1 μ M as compared to FR and NR at the same concentration. However, the advantage of the ASC targeting peptide is diminished at the higher dose of Res at 5 μ M, when NR is just as successful as the L-NR in inducing *Ucp1*, and *Cd137* mRNA expression, but both forms of NP are more successful than FR.

Interestingly, the cell's health status strongly influences the response to Res when treated throughout differentiation. 3T3-L1 cells treated with as much as 20 μ M of free-Res and two other nanoparticle forms encapsulated with Res did not inhibit differentiation or adipogenesis.⁴⁶ However, when freshly isolated murine primary stromal cells displayed signs of stress by not responding to a well-characterized differentiation program, Res treated throughout the differentiation process (5-10 μ M) led to severe hinderance in differentiation and adipogenesis (data not shown). On the other hand, when freshly isolated murine primary stromal cells are healthier and responding to the differentiation program, Res treated throughout the same differentiation process (5-10 μ M) did not hinder differentiation or adipogenesis (data not shown). Therefore, it is imperative to ensure proper health and differentiation when using bioactive molecules and phytochemicals, as to not lead false conclusions.

Studies using Res to treat fully mature adipocytes have shown increased lipolysis⁴³ as well as inducing apoptosis.^{78, 79} There still seems to be a debate of whether there is the possibility that fully differentiated white adipocytes could “transdifferentiate” into brown-like adipocytes or whether beige adipocyte formation is limited to *de novo* synthesis.^{89, 90} However, our results demonstrate that there is a possibility for fully mature adipocytes to become more “brown-like” using some-what higher doses of Res (10 μ M) when encapsulated in NPs with the targeting ligand bound. It remains to be determined whether lower doses of L-NR with longer treatment time would be effective in inducing browning in these fully mature adipocytes

4.6. CONCLUSIONS AND LIMITATIONS

In conclusion, the results of this study reveal that freshly isolated murine primary stromal cells regain Δ DCN expression when confluent and in the beginning of differentiation, and that attaching a peptide specific for Δ DCN to the surface of NPs significantly enhances uptake of NPs and their contents. The targeting ligand proved especially beneficial in the biological response in mSVF, when lower, physiological doses of Res successfully induced browning in these cells as shown by *Ppar γ* , *Ucp1*, *Pgc1 α* , *Cd137*, and *Igfbp3* mRNA expression when treated throughout the differentiation process.

Although compelling, the results of this study are preliminary, and require further experimentation to substantiate enhanced browning phenotype and functionality of L-NR treated mSVF adipocytes. This can be done by using experiments such as western blotting to confirm translation of browning genes into protein and Seahorse assay to measure oxygen consumption rate and proton leak, among other functional mitochondrial measures. Nonetheless, the results of this study lay solid foundation on which the convincing argument can be made for the therapeutic potential of ASC-targeting NPs encapsulated with Res used for the treatment and prevention of obesity.

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4.8. APPENDIX

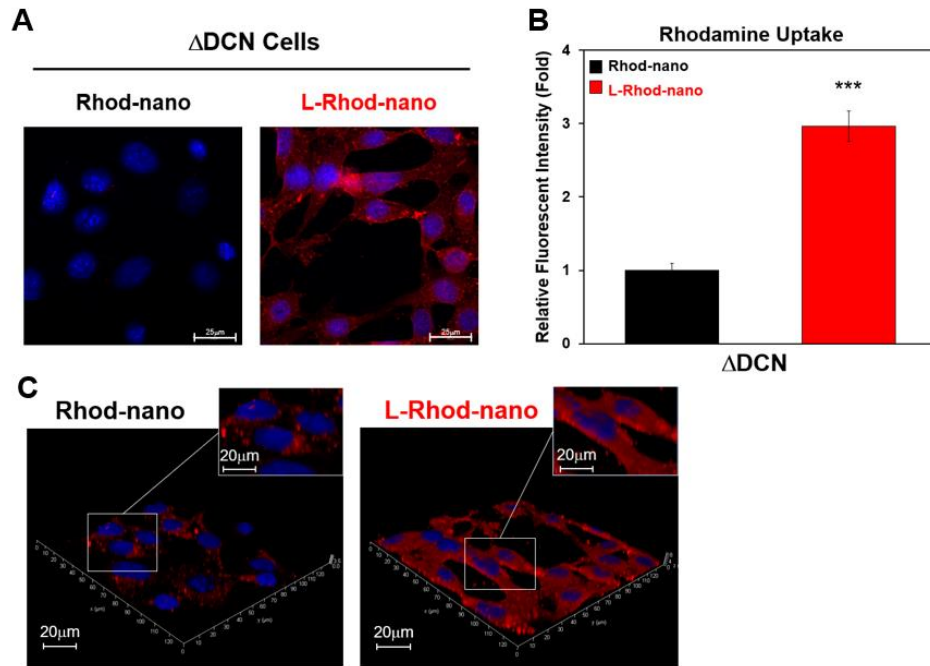


Figure 4.1. Greater rhodamine uptake of ligand-bound nanoparticles by Δ DCN cells.

Δ DCN preadipocytes were evenly seeded in 24-well plate (1×10^5 cells/well) onto sterile coverslips overnight and treated with Rhod-labeled nano-carriers without or with the targeting-ligand (Rhod-nano and L-Rhod-nano, respectively) at 37°C for 2 h. Cells were then rinsed, fixed, rinsed, and stained with DAPI for 5 min. After another rinsing step, cells were mounted on disposable microscope slides and imaged using Leica SP8 White Light Laser Confocal System. Rhodamine uptake, shown in red, was detected using the white-light laser set at λ 561nm. DAPI-stained nuclei, shown in blue, was detected using the “405 laser”. **(A)** Fluorescent images of Δ DCN cells treated without (image to left) or with (image to right) the targeting-ligand-bound NP. DAPI and Rhod filters are merged for this view; all settings for images were the same for equal comparison. The scale bars represent 25 μm . **(B)** Rhodamine uptake from fluorescent images quantified. Data shown here represent at least 3 separate experiments each having at least 3 different areas of cells imaged and quantified, areas were chosen at random. Δ DCN cells treated with Rhod-nano is set at fold-1. **(C)** Z-stack confocal 3D-projections with zoomed in image, demonstrating NP getting into the cell. The scale bars represent 20 μm . Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

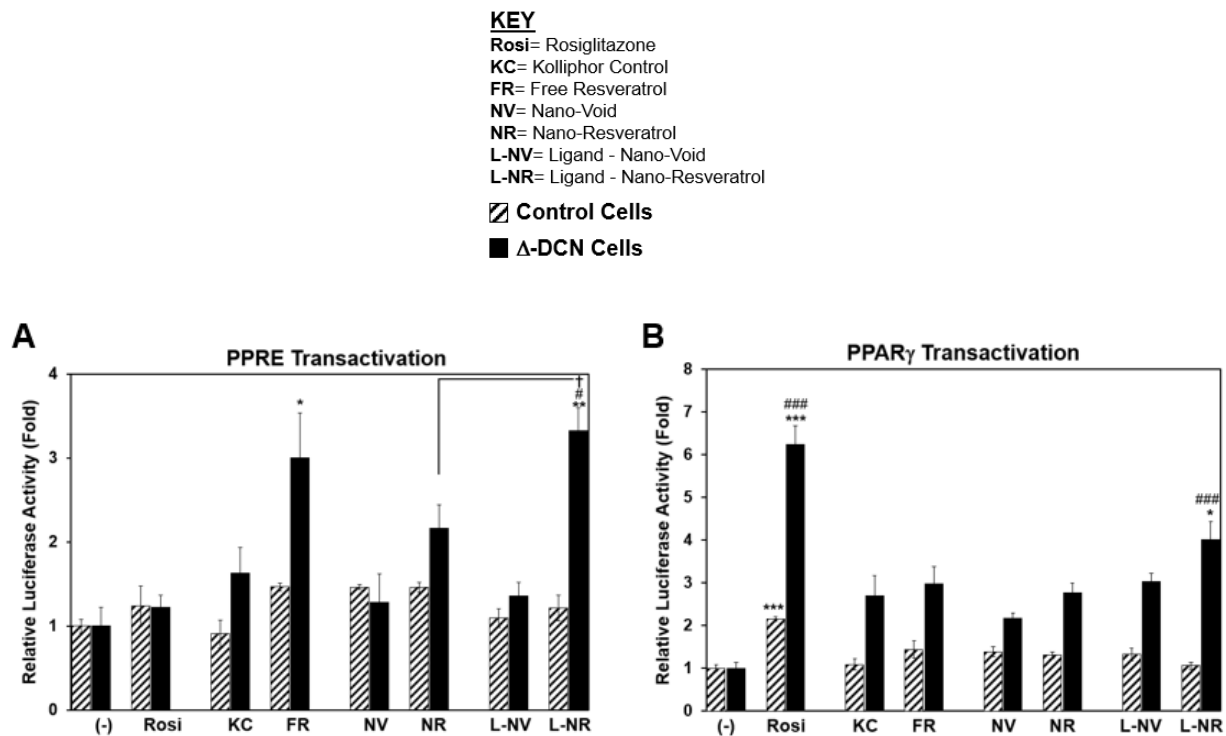


Figure 4.2. Enhanced resveratrol-induced PPARE and PPAR γ transactivation by ligand-bound nanoparticles in Δ DCN cells.

Δ DCN and its scrambled control cell line (Control) preadipocytes were transiently transfected with (A) PPAR γ -Gal4-Luc or (B) PPAR α -Gal4-Luc and β -gal for 24 h, then treated with free-Res (FR) or its Kolliphor vehicle Control (KC), Nanoparticle encapsulated with Res (NR) or its Void control (NV), or Ligand-bound Nanoparticle encapsulated with Res (L-NR) or its Ligand-bound Void control (L-NV) at 10 μ M, or Rosi (1 μ M) or nothing (-) for an additional 18 h. Luciferase activity was normalized to β -gal activity, the non-treated (-) “Control Cells” sample was set at fold-1. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) indicates significant difference between the treatment and its vehicle control within the same cell line. (#: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$) indicates significant difference of the same treatment between Control and Δ DCN cells. (†: $p < 0.05$) indicates significant difference between types of Res within the same cell line.

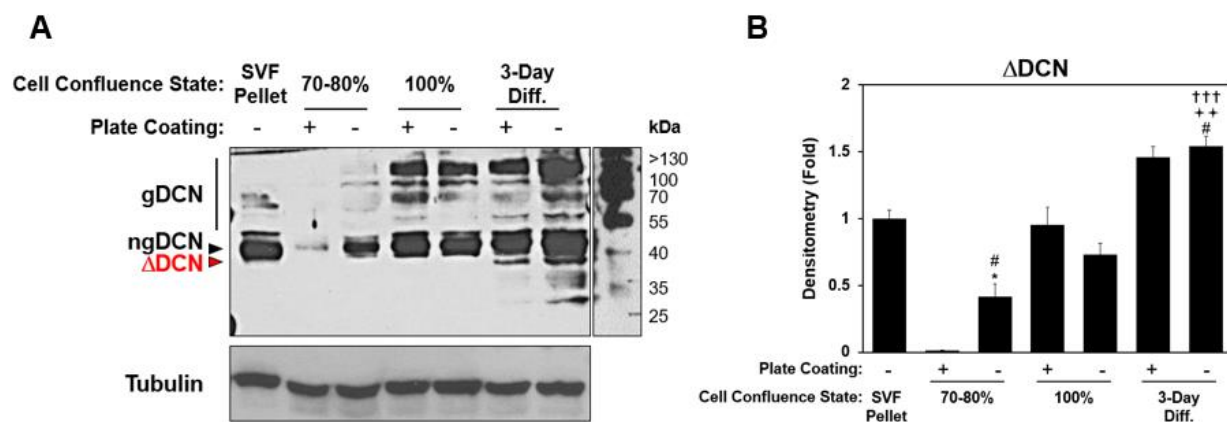


Figure 4.3. Detection of decorin protein expression in murine stromal cells from subcutaneous fat.

Murine primary stromal cells from 10-12 week old male C57Bl/J6 mice were isolated from the inguinal subcutaneous fat tissue, and the stromal vascular fraction (**SVF**) was pelleted, collected, and plated on 60 mm dishes. In addition to the SVF pellet, samples were collected when cells reached 70-80% confluence, 100% confluence, and three days into differentiation (3-Day Diff). (**A**) Immunoblot of total-cell-lysate of various states of murine stromal cells probed with DCN antibody, showing expression of decorin protein at various states of glycanation (gDcn = glycanated DCN, ngDcn = nonglycanated DCN, ΔDCN = 40 kDa protein lacking the dermatan/chondroitin sulfate glycosaminoglycan chain) and their densitometry (**B**). Densitometry of each protein was normalized to the loading control, Tubulin, with the SVF Pellet sample set as fold-1. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for confluence-state-dependent expression differences. (*: $p < 0.05$) indicates significant difference of coated vs non-coated within the same cell-confluence group. (#: $p < 0.05$) indicates significant difference of the non-coated (-) from each cell-confluence group compared to SVF Pellet. (†††: $p < 0.001$) indicates significant difference between the 3-Day Diff (-) (non-coated sample) compared to the 70-80% confluent group's (-) (non-coated sample). (+ +: $p < 0.01$; + + +: $p < 0.001$) indicates significant difference between the 3-Day Diff (-) (non-coated sample) compared to the 100% confluent group's (-) (non-coated sample).

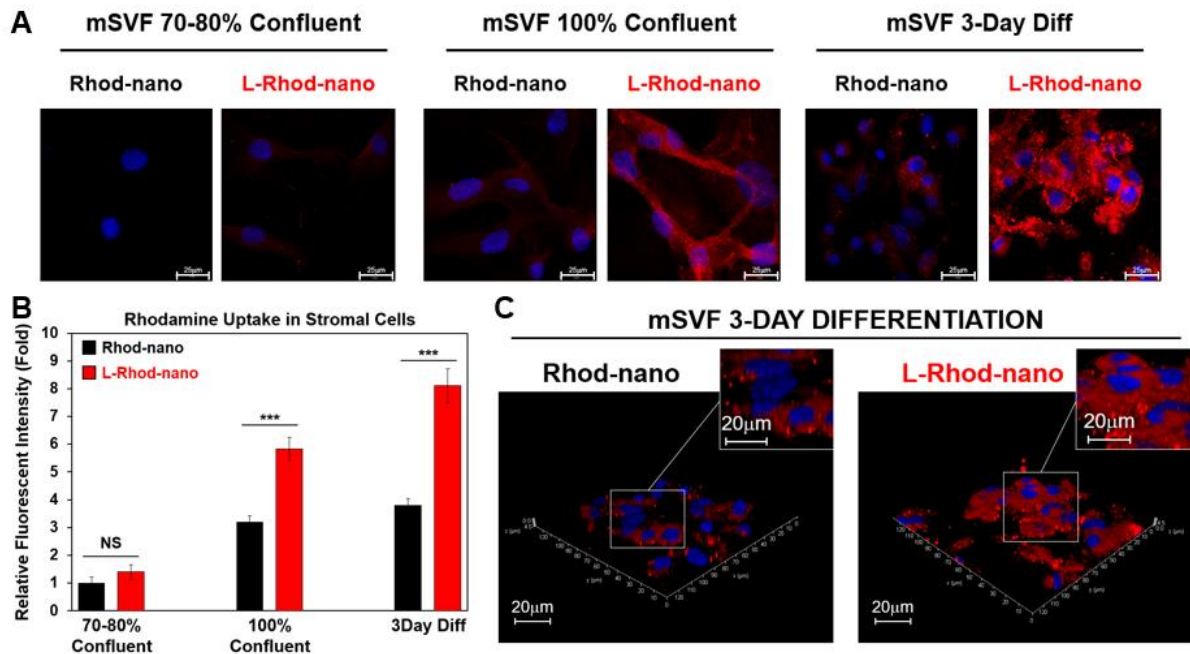
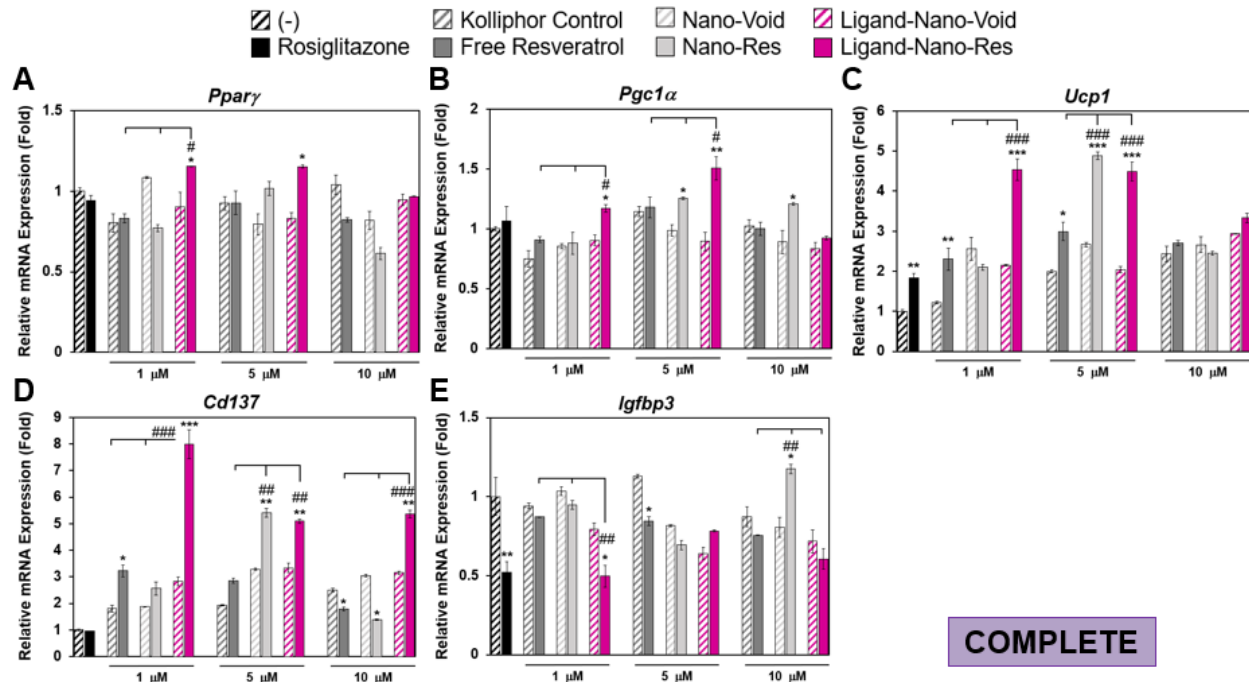


Figure 4.4. Enhanced uptake of ligand-bound nanoparticles by murine stromal cells.

mSVF from 10-12 week old male C57Bl/J6 mice were isolated from the inguinal subcutaneous fat tissue, and the SVF was pelleted, collected, and plated on 24-well plates onto sterile coverslips. Cells were seeded so that they reached 70-80% confluence, 100% confluence, and three days into differentiation (3-Day Diff) at time of treatment. Cells were treated with Rhod-labeled nano-carriers without or with the targeting-ligand (Rhod-nano and L-Rhod-nano, respectively) at 37°C for 2 h. Cells were then rinsed, fixed, and stained with DAPI for 5 min. Imaging was completed using Leica SP8 White Light Laser Confocal System. Rhodamine uptake, shown in red, was detected using the white-light laser set at λ 561nm. DAPI-stained nuclei, shown in blue, was detected using the “405 laser”. **(A)** Fluorescent images of murine primary cells at various states of confluence, cells treated without (image to left) or with (image to right) the targeting-ligand-bound NP. DAPI and Rhod filters are merged for this view; all settings for images were the same for equal comparison. The scale bars represent 25 μ m. **(B)** Rhodamine uptake from fluorescent images quantified; data shown represents at least 3 separate experiments each having at least 3 different areas of cells imaged and quantified, areas were chosen at random. mSVF at 70-80% confluence treated with Rhod-nano is set at fold-1. **(C)** Z-stack confocal 3D-projections with zoomed in image, demonstrating NP getting into the cell. The scale bars represent 20 μ m. Statistical significance is indicated by * $p < 0.05$.



COMPLETE

Figure 4.5. ASC-targeting nanoparticles enhance resveratrol's efficiency to promote browning at lower, physiologically relevant doses.

Murine primary stromal cells from 10-12 week old male C57Bl/J6 mice were isolated from the inguinal subcutaneous fat tissue, and the SVF was pelleted, collected, and plated in 6-well plates. Confluent mSVF were induced to differentiate in the presence free-Res (**FR**) or its Kolliphor vehicle Control (**KC**), Nanoparticle encapsulated with Res (**NR**) or its Void control (**NV**), or Ligand-bound Nanoparticle encapsulated with Res (**L-NR**) or its Ligand-bound Void control (**L-NV**) at 1, 5, or 10 μ M, or Rosi (1 μ M) or nothing (-) for 10 days. Media was changed with freshly prepared Res and NPs every 2-3 days, on day 10 cells were terminated for further analysis. Relative mRNA gene expression (**A-C**) of brown (*Ppar γ* , *Pgc1 α* , *Ucp1*), (**D**) beige (*Cd137*), and (**E**) white (*Igfbp3*), were normalized to the housekeeping gene, *36b4*. Each graph is expressed as fold-1 of that of the (-) sample for each gene. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment vs its vehicle (statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively), as well as types of Res within its concentration group (statistical significance is indicated with a bar adorned by #, ##, ###, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; longer vertical lines assign significance compared to the shorter vertical lines).

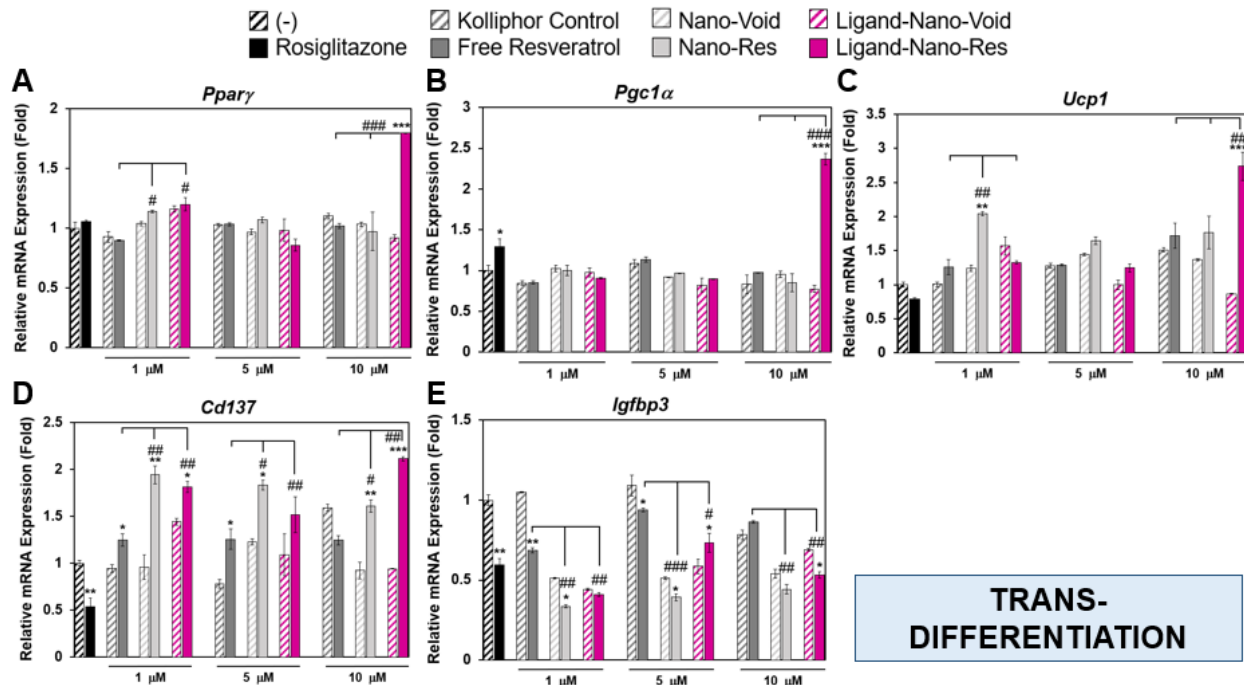


Figure 4.6. Ligand-bound nanoparticles encapsulated with resveratrol promote browning at higher doses when treating fully differentiated murine stromal cells.

Murine primary stromal cells from 10-12 week old male C57Bl/J6 mice were isolated from the inguinal subcutaneous fat tissue, and the SVF was pelleted, collected, and plated in 6-well plates. Confluent mSVF were induced to differentiate as described for 7 days, media was changed every 2-3 days without treatment. On day 7, fully differentiated cells were treated with freshly prepared free-Res (**FR**) or its Kolliphor vehicle Control (**KC**), Nanoparticle encapsulated with Res (**NR**) or its Void control (**NV**), or Ligand-bound Nanoparticle encapsulated with Res (**L-NR**) or its Ligand-bound Void control (**L-NV**) at 1, 5, or 10 μ M, or Rosi (1 μ M) or nothing (-) for 3 days. On day 10 cells were terminated for further analysis. Relative mRNA gene expression (**A-C**) of brown (*Ppar γ* , *Pgc1 α* , *Ucp1*), (**D**) beige (*Cd137*), and (**E**) white (*Igfbp3*), were normalized to the housekeeping gene, *36b4*. Each graph is expressed as fold-1 of that of the (-) sample for each gene. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment vs its vehicle (statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively), as well as types of Res within its concentration group (statistical significance is indicated with a bar adorned by #, ##, ###, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; longer vertical lines assign significance compared to the shorter vertical lines).

CHAPTER V:

INDOMETHACIN PROMOTES BROWNING AND BROWN ADIPOGENESIS IN BOTH MURINE AND HUMAN FAT CELLS

5.1 ABSTRACT

Indomethacin (**Indo**), a non-steroidal anti-inflammatory drug, has been shown to promote brown adipogenesis in murine brown fat cells and in brown adipose tissue in mice; however, it is unclear whether Indo can induce browning of white adipocytes in murine or human cell models. To bridge the gap, this study investigated the effects of increasing concentrations of Indo on murine 3T3-L1, and human primary subcutaneous and brown adipocytes. Indo dose-dependently enhanced 3T3-L1 adipocyte differentiation and upregulated both mRNA and protein expression of brown and beige adipocyte markers, while simultaneously suppressing white-adipocyte-specific marker mRNA gene expression. mRNA and protein expression of mitochondrial biogenesis and structural genes were dose-dependently enhanced in Indo treated 3T3-L1 adipocytes; which was accompanied by augmented mitochondrial DNA, enhanced oxygen consumption rate, proton leak, and maximal and spare respiratory capacity. Dose-dependent transactivation of peroxisome proliferator-activated receptor gamma (**PPAR γ**) confirmed Indo's PPAR γ -agonist activity in 3T3-L1 cells. Knockdown of PPAR γ significantly attenuated Indo activities in selective browning genes, confirming a receptor dependence of these effects. Moreover, Indo enhanced mRNA and protein expression of brown markers in primary stromal cells derived from human subcutaneous white adipose tissue. Interestingly, Indo induced differential effects on individual PPAR γ isoforms with significant dose-dependent induction of PPAR γ 2 and suppression of PPAR γ 1. Finally, Indo also promoted brown adipogenesis in a human brown cell line. Taken together, these results demonstrate Indo to be a potent thermogenic compound in both murine and human fat cells, showing a potential to be used as a therapeutic agent for obesity treatment and prevention.

5.2 INTRODUCTION

Energy-dense diets and sedentary lifestyles are strong contributing factors to the still-growing obesity epidemic faced in the United States and worldwide.¹⁻³ Obesity-related conditions such as diabetes, heart disease, and cancer are among the leading causes of preventable deaths in America.³ Advocating for improved diet and lifestyle factors while also increasing physical activity is always the first line of treatment. However, these heavily funded pursuits are difficult to maintain and may not be feasible for ill or disabled individuals. Thus, it is imperative to develop novel strategies for obesity treatment and prevention, while also promoting increased energy utilization via enhanced thermogenesis is an alluring therapeutic avenue.

Promoting brown adipogenesis of brown adipose tissue (**BAT**) and browning of white adipose tissue (**WAT**) are considered novel approaches to combat obesity and associated conditions. BAT is a highly thermogenic tissue, with the potential to significantly contribute to total energy expenditure.^{4, 5} Targeting treatments to enhance BAT activity, mass, and function can be an effective attempt to restore energy balance in obesity, promote fat loss, and improve the metabolic profile.³⁻⁶ BAT is a highly vascularized, mitochondria-enriched tissue that rapidly burns fat, producing heat in lieu of fuel via uncoupling protein 1 (**UCP1**)-mediated dissociation of the proton gradient.^{4, 6} Peroxisome proliferator-activated receptor gamma (**PPAR γ**) is upregulated in brown fat and its activation triggers a series of downstream signaling events causing increased UCP1 and PPAR γ -coactivator 1-alpha (**PGC1 α**) expression, resulting in increased thermogenesis and mitochondrial biogenesis which ultimately leads to increased energy expenditure.⁷

Similar to brown adipocytes, beige or brite (“brown in white”) adipocytes generated by browning are characterized by their multilocular lipid droplet morphology, larger volume of functional mitochondria, and expression of brown-fat marker genes such as UCP1, PPAR γ , PGC1 α , PR Domain-containing 16 (**PRDM16**), and cell death inducing DFFA like effector a (**CIDEA**).⁴⁻⁶ These UCP1 expressing adipocytes are formed within WAT in response to cold exposure, β -adrenergic receptor (**β -AR**) agonists (e.g., norepinephrine, isoproterenol), and some nutritional and pharmacological agents.^{4, 8, 9} Initial screening of various pharmacological agents for their potential to induce browning and brown adipogenic effects revealed that a variety of non-steroidal anti-inflammatory drugs (**NSAIDs**) may be promising, of these, however, indomethacin stuck out as a strong contender.

Indomethacin (**Indo**) is a non-selective cyclooxygenase (**COX**)-1/COX-2 inhibitor which blocks arachidonic acid from entering the catalytic site, thus suppressing the production of pro-inflammatory metabolites.¹⁰ Indo is a well-studied FDA approved drug used to treat and relieve inflammatory states of the body such as fever, pain, swelling, and stiffness, in addition to more serious disease states including pleurisy and pericarditis.^{10, 11} Several NSAIDs, including Indo, have been shown to have off-target effects, such as activation of PPAR γ .¹²⁻¹⁴ However, Indo is distinct from other NSAIDs in that it acts as a strong *partial* PPAR γ agonist, having one of the greatest abilities to bind and activate PPAR γ similar to that of the well-known PPAR γ -agonist, Rosiglitazone (**Rosi**).^{12, 13} Rosi and other thiazolidinedione (**TZD**) analogs have been and still are used as insulin-sensitizing drugs in the treatment of Type 2 Diabetes Mellitus (**T2DM**).¹⁵ Rosi is commonly used in cell culture to promote adipogenesis as well as induce browning of white adipocytes.¹⁶⁻¹⁹ It is postulated that this browning effect occurs as a result of this PPAR γ -agonist action.^{19, 20} Therefore, having ability to act as a strong *partial* PPAR γ agonist makes Indo of particular interest in the hunt for browning agents.

It has been previously demonstrated that Indo promotes brown adipogenesis in murine brown adipocytes *in vitro* as well as *in vivo* in the BAT of C57Bl/6J mice when Indo was delivered via osmotic-pumps.²¹ However, it is not clear whether Indo induces browning of white adipocytes or whether it exhibits similar effects in human fat cells. Therefore, to bridge the gap, the effects of Indo on murine white fat cells and human white and brown fat cells are investigated here.

5.3 MATERIALS AND METHODS

5.3.1 Reagents

Dexamethasone (**Dex**), 3-Isobutyl-L-methylxanthine (**IBMX**), insulin, triiodothyronine (**T₃**), rosiglitazone (**Rosi**), and isoproterenol (**ISO**) were purchased from Sigma-Aldrich (St. Louis, MO). Indo was purchased from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle's medium (**DMEM**) and Calf serum (**CS**) was purchased from Hyclone (Logan, UT) and fetal bovine serum (**FBS**) from Atlanta Biologicals (Flowery Branch, GA). Penicillin and streptomycin (P/S) were purchased from Gibco by Life Technologies (Burlington, ONT). UCP1 and β -Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), PGC1 α from Millipore Sigma (Darmstadt, Germany), Perilipin, VDAC, CYTC, and MNSOD from Santa Cruz (Dallas, TX), and ERK1/2, PPAR γ , β -AR3, and anti-rabbit horse-radish peroxidase (**HRP**)-conjugated antibody were from Cell Signaling Technology (Danvers, MA).

5.3.2 Cell culture, induction of adipocyte differentiation, and treatments

5.3.2.1 3T3-L1 preadipocyte maintenance and differentiation

3T3-L1 differentiation was performed as previously described.²² Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in DMEM containing 10% CS and 1% P/S in a 5% CO₂, 37°C environment until they reached confluence. Fully confluent cells were induced to differentiate with DMEM containing 10% FBS, 1 µM Dex, 0.5 mM IBMX, and 10 µg/mL insulin for 3 days. The differentiating cells were then maintained with DMEM containing 10% FBS and 10 µg/mL insulin for additional two days until day 5 when the media was changed to DMEM containing only 10% FBS until day 7. On day 7 the cells were stimulated with β-AR-agonist, ISO (1 µM), or the vehicle control (**DMSO**) for 6 h (for mRNA and mitochondrial DNA (**mtDNA**) analysis) or 24 h (for western blot). Indo was added fresh during each change of the media at concentrations of 0, 2, 5, 10, 20, and 50 µM. PPAR_γ-agonist, Rosi (1 µM), was used as the positive control.

5.3.2.2 Human primary subcutaneous adipocytes

Human primary subcutaneous cell pools (**HPsubQ**) originating from 6 female obese, non-diabetic donors (Lot: SL0031) and their media were obtained from ZenBio (Research Triangle Park, NC). Cells were maintained and differentiated for 14 days, according to the manufacturer's instructions. Indo was added fresh during each change of the media at concentrations of 0, 2, 5, 10, and 20 µM. Rosi (1 µM) was used as the positive control.

5.3.2.3 Human brown cell line differentiation

The human brown cell line (**HBr**) was a gift from Dr. Yu-hua Tseng at Joslin Diabetes Center. HBr preadipocytes were grown in DMEM containing 10% FBS and 1% P/S in a 5% CO₂, 37°C environment until they reached confluence. Fully confluent cells were induced to differentiate with DMEM containing 10% FBS, 0.5 mM IBMX, 33 µM biotin, 17 µM pantothenate, 0.5 µM insulin, 0.1 µM Dex, and 2 nM T3 for 4 weeks. Treatments were added fresh to each change of media, which was changed every 2-3 days for a total of 21 days prior to lysis for further analysis.

5.3.3 Cell viability

Cell viability was measured via the colorimetric MTT (yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) metabolic activity assay (RPI, Mt Prospect, IL). 3T3-L1 cells were seeded in 48-well plates so they would be 50-70% confluent

the following day. The cells were then treated with varying concentrations of Indo (0, 2, 5, 10, 20, and 50 μ M) for 24 h. At 24 h MTT reagent was added to cell media in a 1:5 ratio in dark conditions for a 3 h incubation at 37°C. To quantify the insoluble formazan, the media was removed, and the formazan was eluted in 60% isopropanol. After rocking the cells with isopropanol for 5 min, a portion of the formazan-solution was added to a clear 96-well plate where absorbance was measured at 570 nm using GloMax-Multi Detection System (Promega, Madison, WI). These results were normalized with cell lysate total protein concentration using BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA).

5.3.4 ORO staining and quantification

Lipid accumulation in differentiated 3T3-L1 cells was assessed using Oil Red O (**ORO**) staining as previously described.²² Briefly, on day 7 of differentiation, mature 3T3-L1 adipocytes were imaged prior to fixing with 4% paraformaldehyde overnight. Fixed cells were then rinsed with deionized (**DI**) water and subsequently stained with 60% ORO solution diluted with isopropanol for 20 min, which was then followed by solution removal and further rinsing with DI water. Images of stained cells were obtained via an integrated digital camera linked to a Micromaster inverted phase contrast microscope (Thermo Fisher Scientific). The stain uptake by lipids was quantified by eluting ORO stain with 100% isopropanol for 5 min, and absorbance was measured at 500 nm using GloMax-Multi Detection System (Promega, Madison, WI). These results were normalized with cell lysate total protein concentration using BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA).

5.3.5 RNA and DNA isolation and semi-quantitative RT-PCR analysis

RNA and DNA isolation and quantification were performed as previously described.^{21, 22} Briefly, total RNA and DNA was prepared and isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify total RNA and DNA abundance. After RNA isolation, reverse transcription was carried out using High Capacity cDNA Reverse Transcription kit (Thermo Scientific, Pittsburgh, PA) following the manufacturer's instructions. Target genes and the housekeeping gene, *36b4*, mRNA expression was then measured quantitatively using Power Up SYBR Green Master Mix following manufacturers protocol (Applied Biosystems, Austin, TX). Similarly, mtDNA abundance was measured via PCR amplification of mtDNA marker CoxIIa and subsequently normalized to the mtDNA housekeeping gene, 18S. Primer sequences are shown in **Table 5.1-5.3** (all tables and

figures for Chapter V are located in 5.7. Appendix). PCR reactions were run in a 96-well format using an ABI 7300HT instrument and the cycle number (C_t) was obtained for each reaction. Relative gene expression was calculated using the $2(-\Delta\Delta C_t)$ method, which normalizes against the housekeeping genes and then converted to fold.

5.3.6 Western blot analysis

Western blot analysis was performed as previously described.²¹ Briefly, protein extraction was completed using ice-cold PBS with phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA) to rinse the cells and cell lysate was prepared using 1x RIPA buffer (Cell Signaling, Danvers, MA) with freshly added PMSF (Sigma). Protein concentrations were determined by BCA Protein Assay kit (ThermoFisher Scientific, Waltham, MA). Total cell lysates were subjected to electrophoresis on 10-12% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (tris-buffered saline with tween 20; **TBST** at pH 7.4) containing 5% nonfat milk. The membrane incubated with diluted primary antibodies with varying times, followed by incubation with diluted secondary antibody. Washing steps between antibody incubations included TBST on rocker four times for 10 min. Each membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA) for 5 min. Excess substrate was removed, and protein expression was detected using Odyssey[®] FC imaging system (Licor Biosciences, Lincoln, NE) or a x-ray film processor (Konica Corporation, Taiwan) utilizing HyBlot CL Autoradiography Film (Denville, Holliston, MA) which was exposed to the membrane for varying times. Membranes were re-probed using stripping buffer (62.5 mM Tris-HCl, 2 % SDS, and 100 mM 2-mercaptoethanol) at 50°C for 5-10 min. The signal was quantified by densitometry using Odyssey[®] FC imaging system.

5.3.7 Flux experiment

Seahorse XF24 Analyzer (Agilent Technologies, Santa Clara, CA) was used to measure changes in oxygen consumption rate (**OCR**) in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were seeded in a 6-well format and differentiated in the presence of indicated treatments. On day 6, cells were re-seeded (at an identical cell number per well) into XF24 Microplates (Agilent Technologies, Santa Clara, CA) in maintenance media only (no treatment was added) for 24 h. On day 7, cells were treated with ISO (1 μ M) without Indo or Rosi treatment for an additional 24 h. In preparation for cells to undergo mitochondrial stress tests, cells were washed 3 times and incubated with sterile-filtered 37°C XF-Assay DMEM media with 1 mM sodium pyruvate, 2 mM

Glutamax, 10 mM D-glucose, and 2% BSA (pH 7.4 +/- 0.1) in 37°C non-CO₂ incubator for 1 h. During this incubation, mitochondrial stress test chemical compound injections were prepared using XF assay buffer in the following manner [final concentration]: Port A = Oligomycin [1.25 µM], Port B = Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) [5 µM], and Port C = Rotenone and Antimycin [1 µM]. The diluted compounds were loaded into their respective ports in a pre-hydrated sensor cartridge. Following sensor cartridge calibration, the calibration plate was replaced with the experimental cell-plate and began the assay using company recommended template design. Respiration calculations were performed as previously described.^{23, 24}

5.3.8 Transfection and reporter gene assays

Reporter gene assays were performed as previously described.^{21, 22, 25} Briefly, PPAR transactivation reporters consist of murine PPAR γ or PPAR α ligand binding domain (**LBD**) fused with a Gal4 DNA binding domain (**DBD**) (mPPAR γ -Gal4 or mPPAR α -Gal4) and a reporter construct comprised of an upstream activating sequence (UAS) thymidine kinase-linked luciferase, 4xUAS-TK-luc. 3T3-L1 preadipocytes were seeded one day prior to transfection in a manner so that cells were approximately 50-70% confluence the day of treatment (1x10⁴ per well in 48-well format). The preadipocytes were then transiently transfected with mPPAR α -Gal4 or mPPAR γ -Gal4, 4xUAS-TK-Luc (hereon referred to as PPAR α or PPAR γ -Gal4-Luc), and β -galactosidase (**β -gal**) control plasmid using Lipofectamine 3000 and PLUS Reagent (ThermoFisher Scientific, Waltham, MA) for 24 h. The cells were then subjected to Indo or the controls for an additional 15-18 h. Cell lysate was prepared using Reporter Lysis Buffer (Promega, Madison, WI) with Luciferase substrate and β -gal substrate. Luciferase activity and β -gal were quantified using luminescence or absorbance respectively by GloMax-Multi Detection System (Promega, Madison, WI).

5.3.9 Lentiviral shRNA particle infection generating stably-infected cell pools

PPAR γ knockdown (**PPAR γ -KD**) and scrambled non-targeting control (**SCR**) in 3T3-L1 stable-cell lines were generated as previously described.²⁵ Briefly, 3T3-L1 preadipocytes were seeded at 50% confluence in 6-well plates, and subsequently infected with MISSION shRNA lentiviral transduction particles for PPAR γ or control (Sigma Aldrich) according to manufacturer's instructions. Puromycin (2 µg/mL) was used to select stably infected cells.

5.3.10 Statistical analysis

Data in figures were presented as mean $\pm$ SEM. Experiments were performed at least three times with triplicates. SigmaPlot 14 (Systat Software, Inc) was used to perform statistical analysis. T-tests, one-way ANOVA, mixed model ANOVA, or ANOVA with repeated measures was performed followed by post hoc comparison tests with Student-Newman-Keuls method or Tukey method to determine the differences within and between treatment groups. The level of significance was set at $p < 0.05$.

5.4 RESULTS

5.4.1 Indomethacin is not cytotoxic to 3T3-L1 preadipocytes

To eliminate the possibility of potentially harmful effects concerning the multi-target chemical compound, Indo (Fig 5.1.A), 3T3-L1 preadipocytes were treated with increasing concentrations (2, 5, 10, 20, and 50 μ M) of Indo for 24 h as described above. Utilizing MTT assays, it was determined that there were no significant changes in cell viability, indicating that the range of concentrations of Indo used is not cytotoxic to 3T3-L1 cells, justifying further experimentation (Fig 5.1.B).

5.4.2 Differentiation of 3T3-L1 adipocytes is augmented by indomethacin treatment

Treating 3T3-L1 cells with Indo throughout the differentiation process led to a significant increase in differentiation, as demonstrated by increased lipid accumulation and induction of differentiation markers. Indo dose-dependently promoted lipid accumulation as shown by ORO staining, which is shown both visually (Fig 5.1.C) and quantitatively (Fig 5.1.D). In addition to lipid accumulation, adipocyte-differentiation markers were assessed in mature 3T3-L1 cells, which also showed significant induction in CCAAT enhancer binding protein alpha (*C/ebp α*) and fatty acid binding protein-4 (*Fabp4*) mRNA expression, while fatty acid synthase (*Fasn*) mRNA expression was downregulated (Fig 5.1.E). Differentiation markers, Perilipin-1 (PLIN1) and FABP4, protein expression were also significantly induced (Fig 5.1.F-G).

5.4.3 Induction of browning markers mRNA expression in 3T3-L1 adipocytes by indomethacin

In addition to augmented differentiation, Indo-treated mature 3T3-L1 adipocytes also had increased expression of brown-fat, beige-fat, and mitochondrial markers and decreased expression of white adipocyte-specific markers. Master adipocyte differentiation marker, *Ppar γ* mRNA expression was not changed by Indo treatment; however, under ISO stimulated

conditions, Rosi downregulated *Ppar γ* mRNA expression (Fig 5.2.A). mRNA expression of *Ucp1*, the keystone marker for browning and brown adipogenesis, was dose-dependently induced by Indo treatment, which was further augmented by ISO stimulation (Fig 5.2.B). Brown-enriched marker, *Cidea*, also showed significant induction by both Indo and Rosi treatment (Fig 5.2.C). Indo treatment also led to a dose-dependent suppression of white-adipocyte specific marker, Insulin Growth Factor Binding Protein 3 (*Igfbp3*), both in basal and ISO stimulated conditions, with no additional suppression by ISO (Fig 5.2.D). Beige specific marker, Tumor Necrosis Factor Receptor Superfamily, Member 9 (*Cd137*), also was induced by Indo treatment but did not have similar dose-dependent relationships as seen with other markers (Fig 5.2.E).

Mitochondrial biogenesis marker, *Pgc1 α* mRNA is significantly induced by Indo treatment (Fig 5.2.F). mRNA expression of nuclear respiratory factor-1 (***Nrf1***), a gene encoding for a transcription factor to activate genes required for mitochondrial DNA transcription and replication, was also significantly induced by Indo treatment (Fig 5.2.G). Indo also led to a significant dose-dependent increase in cytochrome c oxidase subunit IV isoform 1 (***Cox4a***) mRNA expression, which as an important structural component of the electron transport chain, further demonstrating enhanced mitochondrial biogenesis (Fig 5.2.H). These results were reinforced by quantifying the relative abundance of mtDNA, which was also significantly increased by Indo treatment (Fig 5.2.I).

5.4.4 PPAR γ -agonist suppresses PPAR γ expression and diminishes response to β -adrenergic receptor agonist in 3T3-L1 adipocytes

Indo treated 3T3-L1 adipocytes also significantly induced UCP1 protein expression under basal conditions; however, the augmentation seen in mRNA expression by ISO stimulation (Fig 5.2.B) was not reflected in the protein expression (Fig 5.3.A-B). Treatment of 3T3-L1 adipocytes with Indo and Rosi throughout the entire differentiation process revealed a significant suppression of PPAR γ protein expression (Fig 5.3.A-B). This also led to a dose-dependent decrease in the β -3 adrenergic receptor (***Adrb3***) mRNA expression (Fig 5.3.C) which was further suppressed upon ISO stimulation (Fig 5.3.C). Although not in such a uniform manner, ADRB3 protein expression was also suppressed (Fig 5.3.D-E). Removal of Indo and Rosi 24 h prior to ISO stimulation successfully recovers the expression of both PPAR γ and ADRB3 (Fig 5.4.A-B).

Moreover, removal of Indo and Rosi 24 h prior to the ISO stimulation also led to increased protein expression of brown markers which were more consistent with their corresponding

mRNA expression. As shown in Fig 5.4., Indo significantly induced UCP1 and PGC1 α protein expression in both basal and ISO stimulated conditions (Fig 5.4.A-B). In addition, protein expression of other mitochondrial biogenesis markers was assessed, including voltage-dependent anion channel 1 (**VDAC1**), cytochrome complex (**CYTC**), and manganese-containing superoxide dismutase (**MNSOD**), which were all dose-dependently increased in response to Indo in both basal and ISO stimulated conditions (Fig 5.4.A-B). To further confirm the enhanced mitochondrial functionality, cellular bioenergetics assays were used to evaluate respiratory capacity.

5.4.5 Enhanced flux capacity of indomethacin treated 3T3-L1 adipocytes

To assess the competency of these brown-like adipocytes, fully differentiated 3T3-L1 adipocytes treated with or without increasing concentrations of Indo underwent flux measurements using the Seahorse XF Analyzer. Mitochondrial stress tests revealed that Indo-treated 3T3-L1 adipocytes not only had greater basal OCR, but also had increased proton leak, maximal respiration, and spare respiratory capacity (Fig 5.5.A-B). Furthermore, ISO stimulation led to significantly enhanced spare respiratory capacity when Indo and Rosi were removed 24 h prior to ISO stimulation (Fig 5.5.C-D).

5.4.6 Indomethacin activates PPAR γ in 3T3-L1 cells

Indo has previously been reported to act as a PPAR γ -agonist in C3H10T1/2¹⁴ and HeLa cells¹³. Similarly, Indo significantly activated PPAR γ in a dose-dependent manner in 3T3-L1 preadipocytes using PPAR γ transactivation assays (Fig 5.6.A). This activity was not mirrored when testing PPAR α transactivation, further demonstrating its specificity for PPAR γ (Fig 5.6.B).

5.4.7 Browning genotype induced by indomethacin is attenuated by PPAR γ -knockdown

To confirm Indo's ability to operate as a browning agent via its action as a PPAR γ -agonist, PPAR γ knockdown (**PPAR γ -KD**) was generated in 3T3-L1 cells. Ppar γ protein expression analysis confirmed an 86% knockdown efficiency in the selected KD cell pools compared to the parental cells (Fig 5.6.C). PPAR γ -KD cells and non-targeting control (**SCR**) cells were differentiated in the presence of increasing concentrations of Indo, Rosi, or vehicle control, DMSO. *Ucp1* mRNA expression was significantly suppressed in the PPAR γ -KD as compared to the SCR cells (Fig 5.6.E). *Pgc1 α* mRNA expression was not significantly reduced in PPAR γ -KD cells as compared to the control cells. In fact, in some cases, PPAR γ -KD cells displayed an

ability to enhance *Pgc1α* mRNA expression (Fig 5.6.F). There was over-all lower *Pparγ* mRNA expression in PPAR γ -KD cells as compared to SCR cells; however, in the basal, non-ISO stimulated condition there was a dose-dependent increase in *Pparγ* mRNA expression in PPAR γ -KD cells, whereas there was no difference in response to increasing concentrations of Indo in SCR cells (Fig 5.6.G). Interestingly, in ISO-stimulated conditions, both PPAR γ -KD cells and SCR cells dose-dependently induced *Pparγ* mRNA expression in response to increasing concentrations of Indo (Fig 5.6.G). Expression of beige-fat-specific marker, *Cd137*, was significantly attenuated in PPAR γ -KD cells compared to the SCR cells (Fig 5.6.H). Suppression of white-fat-specific marker, *Igfbp3*, was significantly hindered in PPAR γ -KD cells compared to the SCR cells (Fig 5.6.I).

5.4.8 Indomethacin also induces browning in primary stromal cells derived from human subcutaneous fat

The browning phenomena demonstrated in 3T3-L1 adipocytes is similarly apparent in HPsubQ cells treated with Indo. Indo dose-dependently increased brown-fat marker, *UCP1* mRNA (Fig 5.7.A), with simultaneous suppression of white-fat-specific marker, *IGFBP3* mRNA (Fig 5.7.A). Similar to what was previously shown here in 3T3-L1 cells, there is no significant change in *PPARγ* mRNA expression (Fig 5.7.A). In contrast, there was no significant increase, but rather a decrease, in *PGC1α* mRNA expression (Fig 5.7.A).

Additionally, Indo dose-dependently increased UCP1 and VDAC1 protein expression in response to increasing concentrations of Indo (Fig 5.7.B-C). However, PGC1 α and PLIN1 protein expression did not show a significant response to Indo treatment (Fig 5.7.B-C). There is no significant change in total PPAR γ protein expression (Fig 5.7.B-C); however, independent analysis of PPAR γ isoforms reveals a significant dose-dependent suppression of PPAR γ 1 and a reciprocal dose-dependent increase in PPAR γ 2 protein expression (Fig 5.7.B,D).

5.4.9 Indomethacin promotes brown adipogenesis in human brown cell line

Previous studies demonstrated that Indo promotes brown adipogenesis in murine brown cell line and C57Bl/6J mice when Indo was delivered via an osmotic pump directly onto BAT.²¹ In our study, a HBr cell line was used to further investigate the potential for Indo to promote brown adipogenesis in human brown fat cells. Indo dose-dependently induced UCP1 mRNA and protein expression in HBr adipocytes (Fig 5.8.C, E-F). Moreover, Indo at 20 μ M significantly increased mRNA expression of BAT enriched markers, *PPARγ*, *PGC1α*, and Deiodinase

Iodothyronine Type II (**DIO2**) (Fig 5.8.A-E). PGC1 α protein expression was also dose-dependently increased by Indo (Fig 5.8.E-F).

5.5 DISCUSSION

In this study, we investigated the dose-dependent effects of Indo on murine and human white adipocytes and human brown adipocytes. The results of this study showed that Indo is not cytotoxic to 3T3-L1 preadipocytes at concentrations as high as 50 μ M. Indo augmented differentiation of 3T3-L1 cells as shown by ORO lipid-staining and absorbance in addition to mRNA and protein expression of critical differentiation markers. Treating 3T3-L1 adipocytes with Indo led to a significant induction in mRNA and protein gene expression of brown and beige markers. Browning of the 3T3-L1 adipocytes was accompanied by enhanced mitochondrial biogenesis and functionality as shown by desirable cellular bioenergetics profiles. Indo was shown to activate PPAR γ in 3T3-L1 cells, knock-down of which led to an attenuation of the browning effect. Indo was also shown to induce browning in human subcutaneous primary adipocytes as well as promote brown adipogenesis in a human brown cell line.

Although some literature suggests inhibiting adipogenesis and promoting adipocyte apoptosis to be an effective means to combat obesity, it is actually contraindicated. Limiting adipose tissue expansion without alteration in energy intake can lead to ectopic lipid depositions, increased levels of free fatty acid (**FFA**) in the circulation, and exacerbated release of pro-inflammatory signals from hypertrophied and dying adipocytes. All the aforementioned are pathologies which are directly associated with metabolic disturbances including hyperlipidemia and insulin resistance.^{26, 27} Evidence showing existence of 'metabolically healthy obese' describes individuals who have BMIs that categorize them as obese, but do not have the associated comorbidities, suggesting that sustained insulin sensitivity is more critical than quantity of fat-mass itself.^{28, 29} On the other hand, allowing slight expansion to accommodate excess energy while inducing browning in WAT may promote normal organ function, reduce hyperlipidemic and inflammatory circumstances, enhance insulin sensitivity, and increase energy expenditure, possibly leading to weight loss.^{27, 30, 31} TZDs are common pharmacological drugs used in the treatment of T2DM which elicit outcomes that demonstrate the beneficial effects of the expansion hypothesis.^{15, 32-34}

Although there is some hesitation in the clinical utility of TZDs due to some adverse side-effects including weight gain due to fluid retention and some increase in adiposity, patients still see favorable outcomes.^{15, 32-34} In addition to its tremendous insulin-sensitizing effect, TZDs

have also been shown to exhibit anti-inflammatory, antioxidant, and antiproliferative beneficial effects.³³ Due to its pleiotropic nature, not one single mechanism can be attributed to TZDs therapeutic benefits; however, it is partly due to both the insulin-sensitizing effect *and* the increase in adiposity that these individuals show improvements in glycemic control, blood-lipid-profiles, blood pressure, endothelial function, as well as decreased inflammation markers, thereby reducing the comorbidities typically associated with inflated AT.^{15, 32-34} These beneficial metabolic effects were also attributed to TZDs ability to promote browning of WAT and brown adipogenesis in BAT.^{16, 17, 19, 31, 35, 36} Several studies suggest partial agonists or ligands with similar high-affinity binding with PPAR γ may have the ability to retain desired insulin sensitizing function without the unwanted side effects.^{37, 38} Therefore, compounds with *partial* PPAR γ -agonist activities could offer viable alternative strategies.

Consistent with previous reports demonstrating that TZDs and Indo promote white adipocyte differentiation *in vitro* and *in vivo*,^{13, 20, 39, 40} our results similarly demonstrate that Indo dose-dependently promoted differentiation in 3T3-L1 adipocytes. This enhanced differentiation is demonstrated by ORO lipid accumulation and absorbance, as well as mRNA and protein expression of differentiation markers (Fig 5.1.C-G).

In addition to its ability to augment differentiation, Indo also significantly induced browning in 3T3-L1 adipocytes as shown by enhanced UCP1 mRNA and protein expression as well as beige and brown fat specific markers, *Cd137* and *Cidea*, respectively, with simultaneous suppression of white-fat-specific marker, *Igfbp3*. It is worth noting that continuous treatment of Indo increased *Ucp1* mRNA expression under basal and ISO stimulated conditions; however, removal of Rosi and Indo for 24 h prior to ISO stimulation is required for Indo to augment UCP1 protein expression. This is likely due to the observation that continuous PPAR γ activation by Rosi and Indo suppressed protein expression of PPAR γ and ADRB3, which is the receptor for ISO (Fig 5.3.A and D). The downregulation of PPAR γ protein expression by Rosi and Indo may be explained by self-regulation, feedback inhibition, and/or protection of the receptors from over-utilization.⁴¹ Taking into consideration that adipogenesis was not inhibited, but rather enhanced by continuous treatment with Rosi or Indo, may suggest that there is a threshold of receptor expression required for sufficient differentiation of adipocytes.⁴² Furthermore, Indo dose-dependently suppressed *Adrb3* mRNA expression in 3T3-L1 adipocytes, which was further suppressed by ISO stimulation. These results are consistent with a previous report which demonstrated HIB-1B cells treated with TZD, troglitazone, led to acute suppression of *Adrb3* mRNA levels, which was accompanied by decreased responses to various β -AR agonists.⁴³

Slight adjustment in the differentiation program by removal of Rosi or Indo for 24 h prior to ISO stimulation led to recovery and subsequent increase of both PPAR γ and ADRB3 protein expression (Fig 5.4.A-B). Furthermore, there was no longer a suppression in UCP1 protein expression in response to ISO stimulation, which was accompanied by sustained protein expression of other mitochondrial markers (PGC1 α , VDAC1, CYTC, MNSOD) (Fig 5.4.A-B).

Among the critical differences between brown and white adipocytes, is BAT's relatively large abundance of functional mitochondria expressing UCP1.⁴⁻⁷ The results of our study reveal that treating 3T3-L1 cells with Indo led to enhanced synthesis, quantity, and function of adipocyte mitochondria, as shown by increased mRNA and/or protein expression of mitochondrial marker genes, mtDNA, and mitochondrial respiration. Regulation of quality and abundance of mitochondria is a tightly controlled and highly coordinated process involving hyperplasia and hypertrophy of pre-existing mitochondria.⁴⁴⁻⁴⁶ Master regulator of mitochondrial biogenesis, PGC1 α , interacts with the mitochondrial-gene transcription factor, NRF1, to further promote mitochondrial biogenesis and regulate genes involved in energy expenditure and metabolism.^{31, 45} The increased demand for mitochondria is accompanied by increased need for mitochondrial enzymes and structural components, such as COX4A.⁴⁴ mRNA expression of BAT-enriched mitochondrial markers *Pgc1 α* , *Nrf1*, and *Cox4a* were all dose-dependently increased in response to Indo treatment in 3T3-L1 cells. Together with the elevated mtDNA abundance, these results suggest an overall amassed total mitochondrial quantity, which have greater UCP1 expression. However, increase in mitochondria abundance alone is not generally a sufficient marker for browning; rather, abundance, quality, *and* functionality are critical for proper assessment.⁴⁶ Therefore, upon confirmation of heightened mitochondrial quantity, cellular bioenergetic assays were used to substantiate the functionality and uncoupling capacity of 3T3-L1 adipocytes treated with Indo.

Results of mitochondrial stress tests showed that Indo dose-dependently increased basal OCR, proton leak, and maximal and spare respiratory capacity in 3T3-L1 adipocytes under basal (non-ISO-stimulated) conditions (Fig 5.5.A-B). Under ISO stimulated conditions, Indo treated 3T3-L1 adipocytes still showed increases in all of the respiratory calculations; however, only spare respiration was significantly augmented by ISO stimulation compared to the basal (unstimulated) conditions. Enhanced basal OCR demonstrate that the mitochondria of Indo treated cells to have remarkably greater functionality.⁴⁶ Pharmacological interrogation with FCCP confirmed the UCP1 enhanced phenotype, which was shown by increases in oligomycin-insensitive respiration, otherwise known as proton leak.^{46, 47} By itself, increased basal OCR and

proton leak could merely indicate physiological adaptation or a response to stress.⁴⁶ However, these values in combination with increased maximal and spare respiratory capacity indicates cells to have a larger and healthier network of mitochondria having a heightened ability to withstand oxidative damage and stress.⁴⁶ Therefore, together with gene expression data, our results demonstrate that Indo has a significant capacity to promote browning and give way to a larger population of healthy and functional mitochondria. However, the manner in which Indo is able to elicit these browning effects has yet to be determined until now.

Although primarily recognized as an NSAID, which inhibits COX-1 and COX-2, Indo has also been shown to act as a PPAR γ -agonist in several cell types, including C3H10T1/2¹⁴ and HeLa¹³ cells. Similarly, our results show that Indo dose-dependently activates PPAR γ , but not PPAR α , in 3T3-L1 preadipocytes as shown by PPAR transactivation assays (Fig 5.6.A-B). Several studies have suggested that Indo effects may vary based on concentration. It was shown that Indo acts as a PPAR γ agonist at higher concentrations (10 – 100 μ M),^{13, 14} whereas it only has COX inhibiting effects at lower concentrations (0.1 – 1.5 μ M).⁴⁸ Studies have demonstrated that COX-2 expression/activity is required for UCP1 expression and browning of white adipocytes.⁴⁹ Therefore, it is sensible to assume that Indo's browning effects seen here are not due to its COX inhibition capacity.

As previously mentioned, many reports demonstrate Rosi as a robust adipocyte “browning agent” via its action as a potent full PPAR γ -agonist,^{31, 36} at least in part by induction of PGC1 α expression.⁹ Therefore, it is conceivable that Indo's browning effects may work through the PPAR γ -PGC1 α axis as well. To investigate the role of PPAR γ in Indo's browning effects, PPAR γ knockdown (**KD**) in 3T3-L1 cells was generated and differentiated with or without Indo. These results revealed upregulated mRNA expression of *Ucp1* and *Cd137* by Indo which were significantly attenuated by PPAR γ -KD as compared to the control (**SCR**) cells (Fig 5.6.E-F). In contrast, although similar in pattern, there was a significantly hindered downregulation of *Igf1bp3* mRNA expression by Indo in PPAR γ -KD cells (Fig 5.6.G). There were minimal differences in *Pgc1 α* mRNA expression between PPAR γ -KD and SCR cells, suggesting that *Pgc1 α* mRNA expression is independent of PPAR γ regulation (Fig 5.6.H). As shown in the wild-type mRNA results, continuously treating 3T3-L1 cells with Indo and Rosi led to either no change or a suppression of Ppar γ expression. However, treating PPAR γ -KD cells with Indo led to a dose-dependent increase in *Ppar γ* mRNA expression, albeit the overall expression was understandably lower than SCR (Fig 5.6.I). These results demonstrate the PPAR γ receptor-

dependence for the browning effects and provide strong evidence to suggest that Indo's capacity to induce browning in 3T3-L1 cells is due to its PPAR γ -agonist activity.

Differences on PPAR γ -agonist capacity of Rosi and Indo have previously been characterized revealing that Rosi is a *full* PPAR γ -agonist whereas Indo is a strong *partial* PPAR γ -agonist.¹²⁻¹⁴ Although it has been reported that *partial* PPAR γ -agonists did not induce the browning program in murine primary subcutaneous WAT,³⁵ the results of our study reveal that Indo's strong *partial* PPAR γ -agonist activity is, in fact, able to significantly induce browning in both murine and human white adipocytes. This browning is demonstrated by dose-dependent increases in UCP1 mRNA and protein expression as well as dose-dependent suppression of mRNA expression of white-fat-specific marker, *IGFBP3*, in HPsubQ adipocytes. Moreover, Indo also led to increased protein expression of VDAC1, a major mitochondrial structural component. However, there was little to no change or rather a slight decrease in PGC1 α mRNA and protein expression in HPsubQ adipocytes as compared to 3T3-L1 adipocytes, further suggesting that Indo's browning effects in HPsubQ adipocytes may be PGC1 α -independent.

Interestingly, whereas 3T3-L1 adipocytes continuously treated with Indo led to dose-dependent suppression of total PPAR γ , HPsubQ cells had differential expression of the PPAR γ isoforms. When both PPAR γ 1 (55kDa) and PPAR γ 2 (57kDa) are taken together, there were seemingly no differences among the treatments; however, when assessed separately, it becomes evident that Indo led to a significant dose-dependent increase in PPAR γ 2 and reciprocal dose-dependent decrease of PPAR γ 1 protein expression in HPsubQ adipocytes. Although the literature on the differential expression of PPAR γ isoforms is dearth and controversial, a large study of 50 obese female patients demonstrated that PPAR γ 2, an adipose-specific isoform, has overall higher expression compared to PPAR γ 1, in both visceral adipose tissue (**VAT**) and subcutaneous adipose tissue (**SAT**).⁵⁰ Moreover, it was found that PPAR γ 2 is inversely correlated with BMI and weight, and that higher expression of PPAR γ 1 in SAT is associated with higher circulating insulin levels.⁵⁰ Therefore, our results demonstrating an inverse relationship of PPAR γ 1 and PPAR γ 2 suggest that Indo treatment induces beneficial profiles of PPAR γ isoforms in HPsubQ adipocytes which may be associated with enhanced insulin sensitivity.⁵⁰ Further studies are required to confirm Indo-induced insulin sensitivity in HPsubQ adipocytes.

Lastly, Indo significantly enhanced brown adipogenesis in the HBr cell line, as demonstrated by augmented UCP1 and PGC1 α mRNA and protein expression as well as increased *PPAR* γ and *DIO2* mRNA expression when using 20 μ M of Indo. Taken together, the results of HPsubQ and human brown cells treated with Indo show similar increases of UCP1 expression as Indo-treated 3T3-L1 adipocytes and murine brown adipocytes. Future studies to examine the UCP1 mediated functionality of these cells, such as cellular flux measurements would help to further confirm the metabolic benefit of Indo treatment in human adipocytes.

In conclusion, for the first time, the results presented here provide evidence that Indo, through its *PPAR* γ -agonist activity, possesses the capacity to dose-dependently promote browning in murine and human white adipocytes as well as enhance brown adipogenesis in human brown adipocytes. Indo's browning effect on 3T3-L1 adipocytes is further demonstrated by increased UCP1 expression, mitochondrial biogenesis, proton leak, as well as maximal and spare respiratory capacity. In light of Indo's potential to induce browning and brown adipogenesis, and taking into consideration possible contraindications, it would be interesting to explore the possibilities of local delivery of Indo to be used as a therapeutic agent for obesity treatment and prevention.

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5.7 APPENDIX

Figure 5.1. Indomethacin does not cause toxicity and enhances adipogenesis and lipid accumulation in 3T3-L1 cells.

(A) Indo chemical structure. (B) MTT cell proliferation assay in 3T3-L1 preadipocytes seeded in 48-well format were treated with increasing concentrations of Indo and the control for 24 h followed by 3 h incubation with MTT reagent. Confluent 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 8 days prior to lysis and further analysis. (C) Oil Red O (ORO) stained cell morphology of 3T3-L1 adipocytes at day 8 of differentiation. (D) 3T3-L1 adipocyte lipid accumulation at day 8 measured by ORO stain absorbance. (E) Differentiation marker mRNA gene expression of *C/ebp α* , *Fabp4*, and *Fasn*. The relative mRNA expression was normalized to *36b4* and expressed as fold-1 of the (-) sample for each gene. (F) Western blotting of PLIN1 and FABP4. (G) Densitometry of PLIN1 and FABP4 protein expression normalized to loading control, total ERK1/2 with the (-) sample set at fold-1 for each gene. A second loading control β -Actin is provided. Statistics were computed using one-way ANOVA followed by multiple comparison tests with Student-Newman-Kewls method, significant differences between control and treatment, unless otherwise noted, are indicated with *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

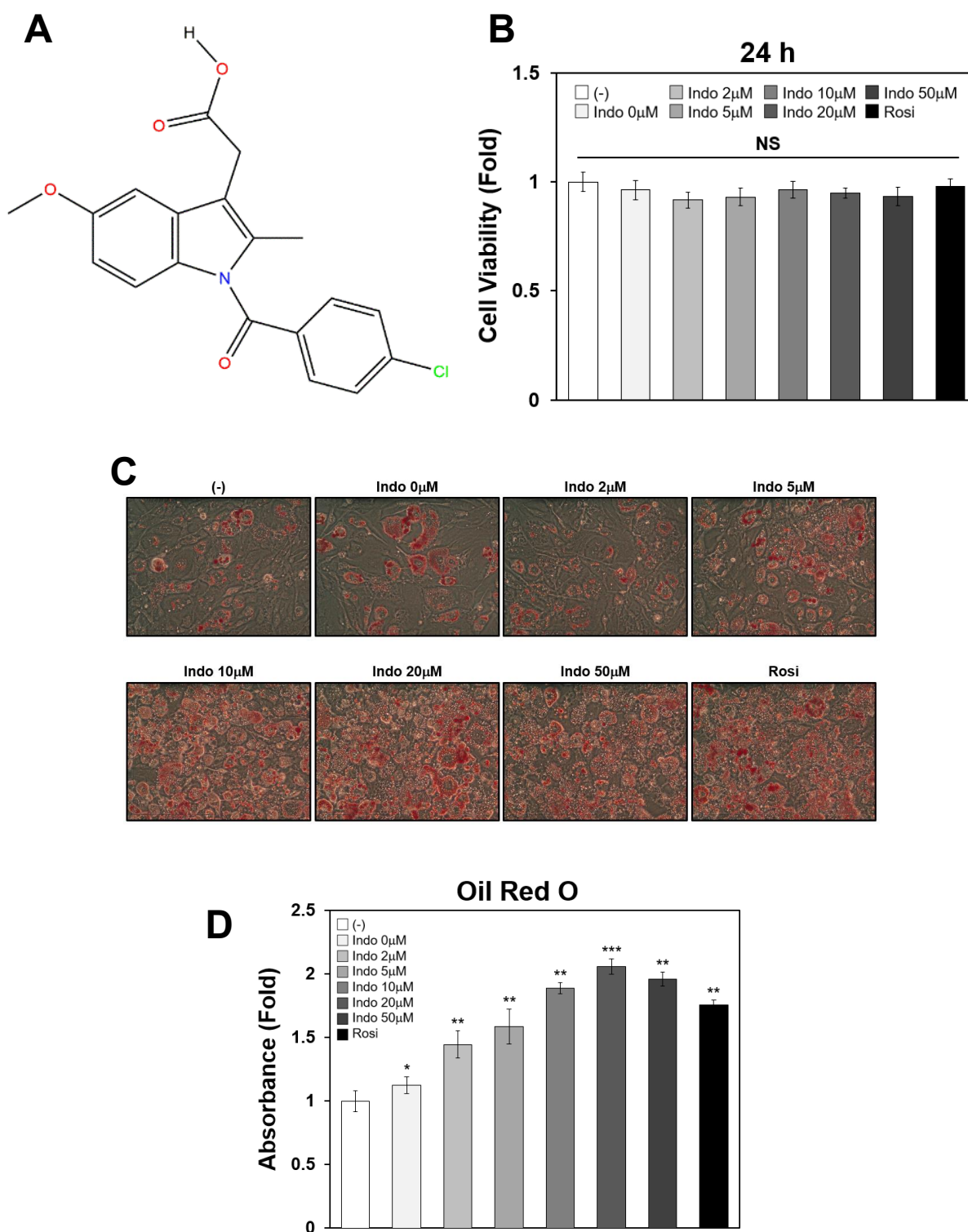


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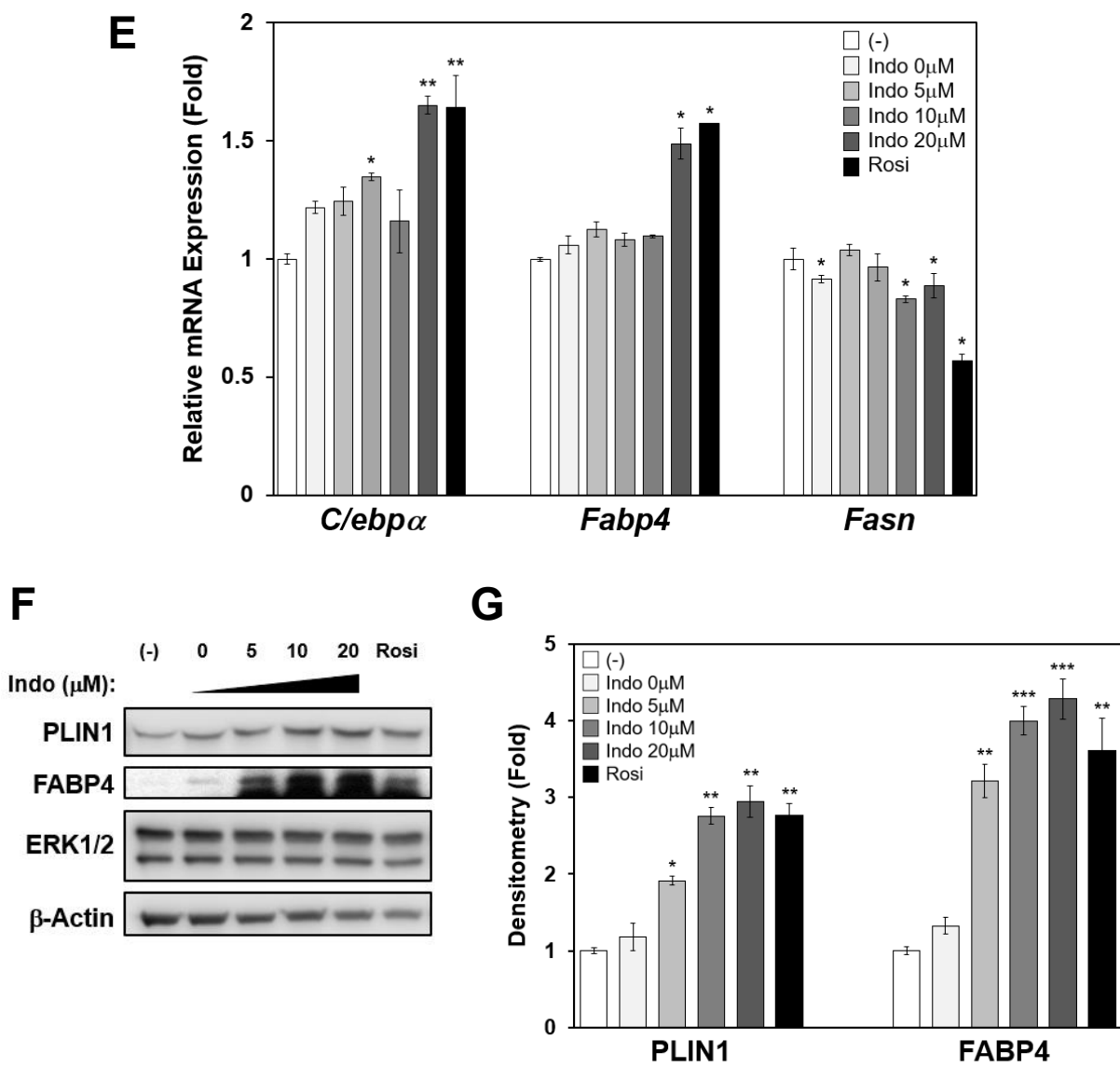


Figure 5.1. Continued.

Figure 5.2. Indomethacin promotes browning as well as mitochondrial biogenesis in 3T3-L1 adipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 7 days. On day 7, in addition to treatments, cells were stimulated for 6 h with ISO (1 μ M) prior to lysis and further analysis. Relative mRNA gene expression (**A-H**) of brown (*Ppar γ* , *Ucp1*, *Cidea*), beige (*Cd137*), white (*Igf1bp3*), and mitochondrial markers (*Pgc1 α* , *Nrf1*, *Cox4a*), normalized to *36b4*. (**I**) Mitochondrial DNA, *CoxIIa* normalized to 18S. Each graph is expressed as fold of that of the (-), non-ISO stimulated sample (set at 1). One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment and ISO-responsive relationship. The bar indicates the statistical significance of the non-ISO group compared to ISO group. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

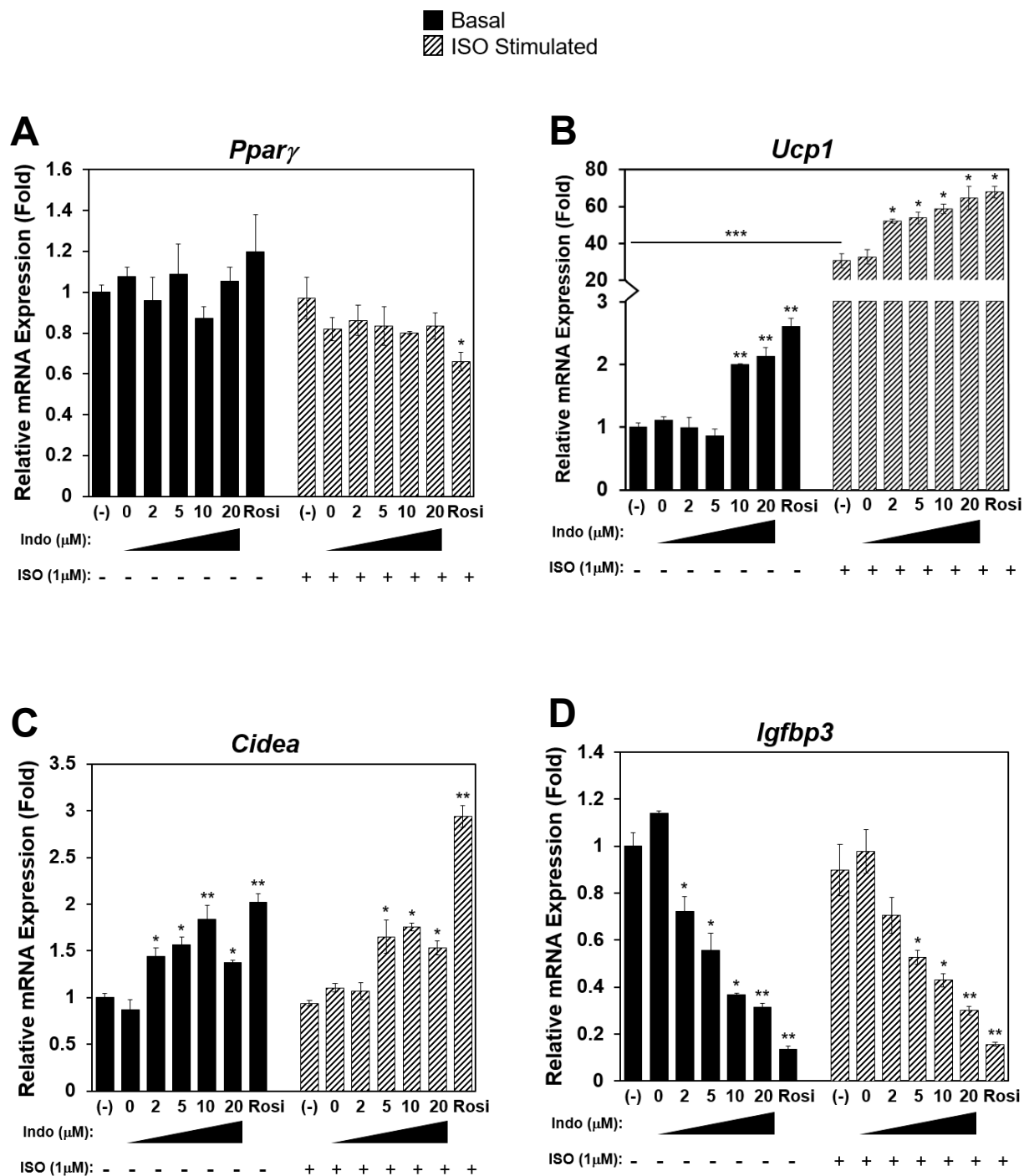


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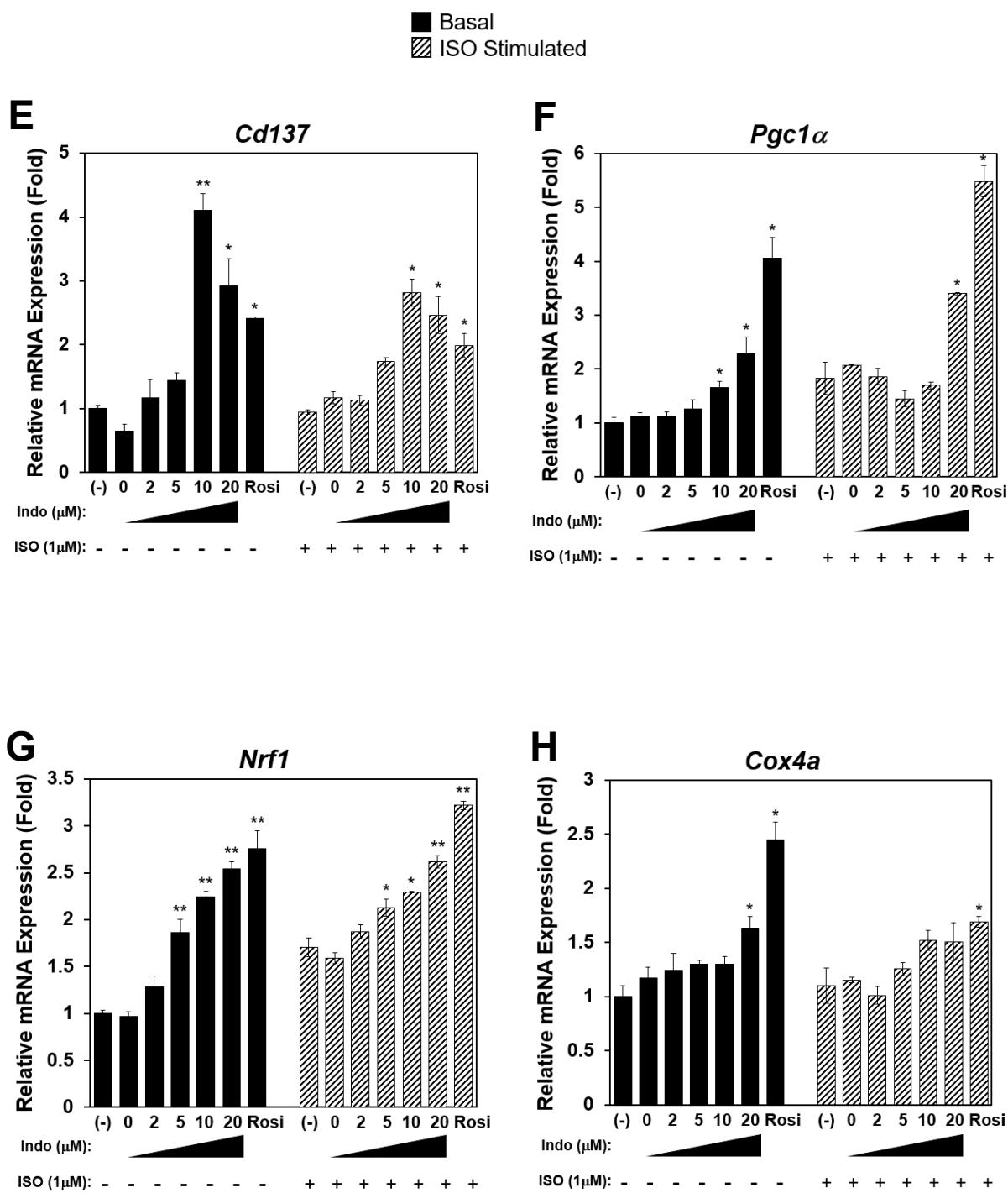


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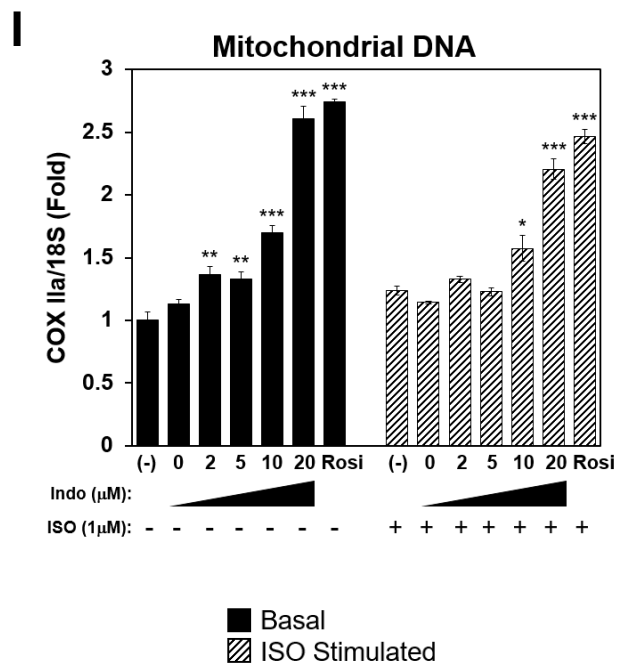


Figure 5.2. Continued.

Figure 5.3. Suppression of PPAR γ and ADRB3 by Rosi and Indo in differentiating 3T3-L1 adipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 7 days. On day 7, in addition to treatments, cells were stimulated for 6 h (**C**) or 24 h (**A,D**) with ISO (1 μ M) prior to lysis and further analysis. (**A**) PPAR γ and UCP1 protein expression and their densitometry (**B**), as well as *Adbr3* mRNA (**C**) and ADRB3 protein expression (**D**) and its densitometry (**E**). Densitometry of PPAR γ and UCP1 (**B**) and ADRB3 (**D**) protein expression were normalized to loading control, total ERK1/2 with the (-), non-ISO stimulated sample, set at fold-1 for each gene. (**C**) Relative mRNA expression of *Adbr3* normalized to *36b4* and expressed as fold-1 to the (-), non-ISO stimulated sample. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment and ISO-responsive relationship. The bar indicates the statistical significance of the non-ISO group compared to ISO group. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

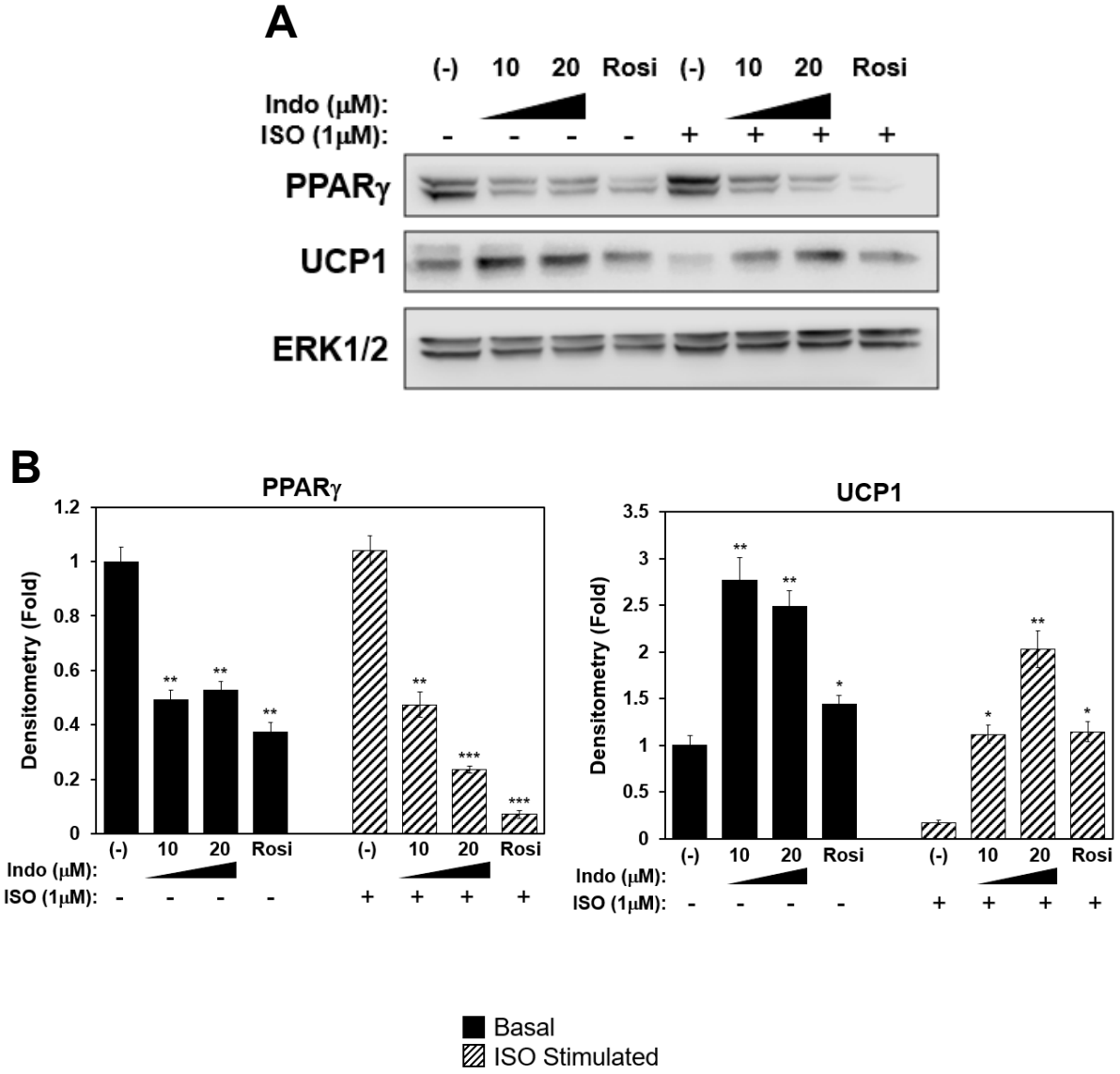


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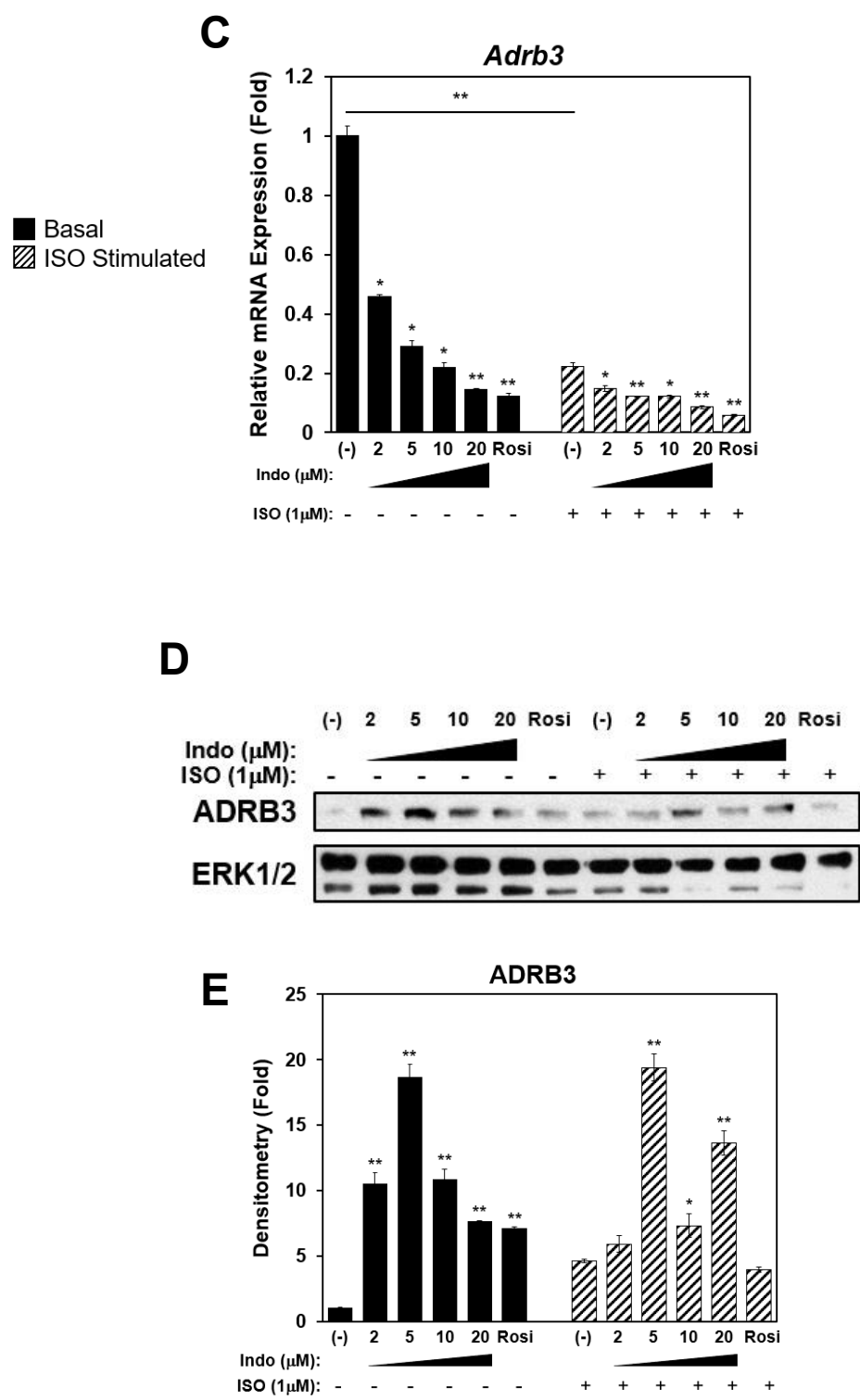
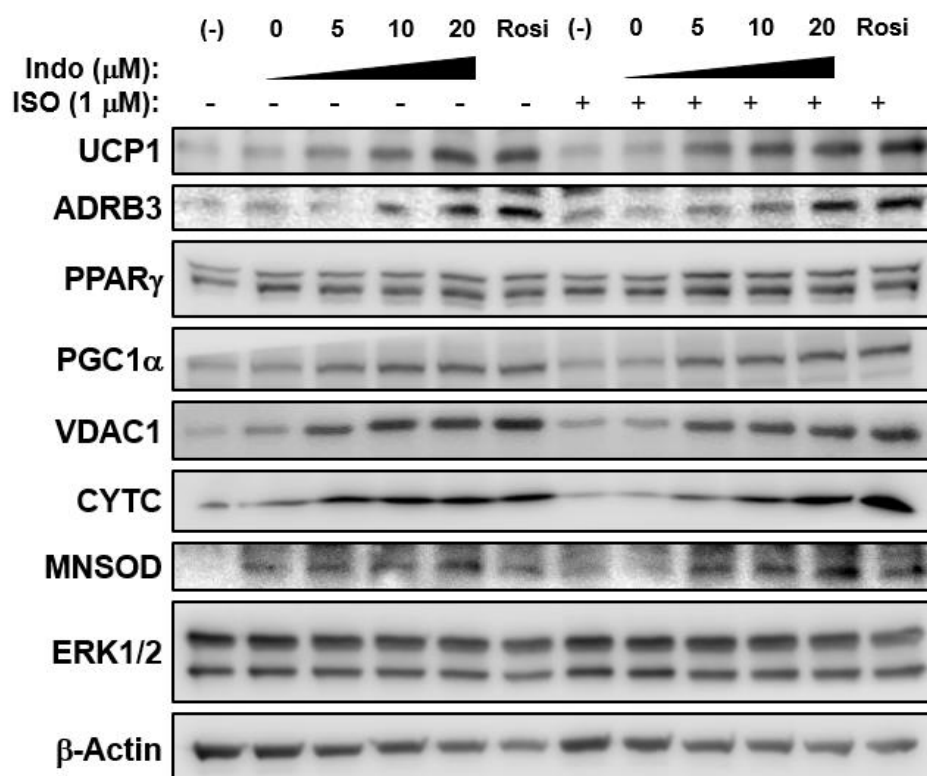


Figure 5.3. Continued.

Figure 5.4. Timely removal of Rosi and Indo rescues browning phenotype in 3T3-L1 adipocytes.

Confluent 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 6 days. On day 6, the Indo or Rosi treatment were removed for 24 h, after which the cells were stimulated with ISO (1 μ M) or vehicle for 24 h prior to lysis and further analysis. **(A)** Protein expression of brown-marker (**UCP1**), rescued adipocyte genes (**ADRB3** and **PPAR γ**), mitochondrial biogenesis (**PGC1 α**), and mitochondrial structural (**VDAC**, **CYTC**, **MNSOD**), as well as their densitometry **(B)**. Densitometry of each protein was normalized to loading control, total ERK1/2 with the (-), non-ISO stimulated sample, set at fold-1 for each gene. A second loading control β -Actin is provided. Mixed model ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment and ISO-responsive relationship. The bar indicates the statistical significance of the non-ISO group compared to ISO group. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

A



■ Basal
▨ ISO Stimulated

B

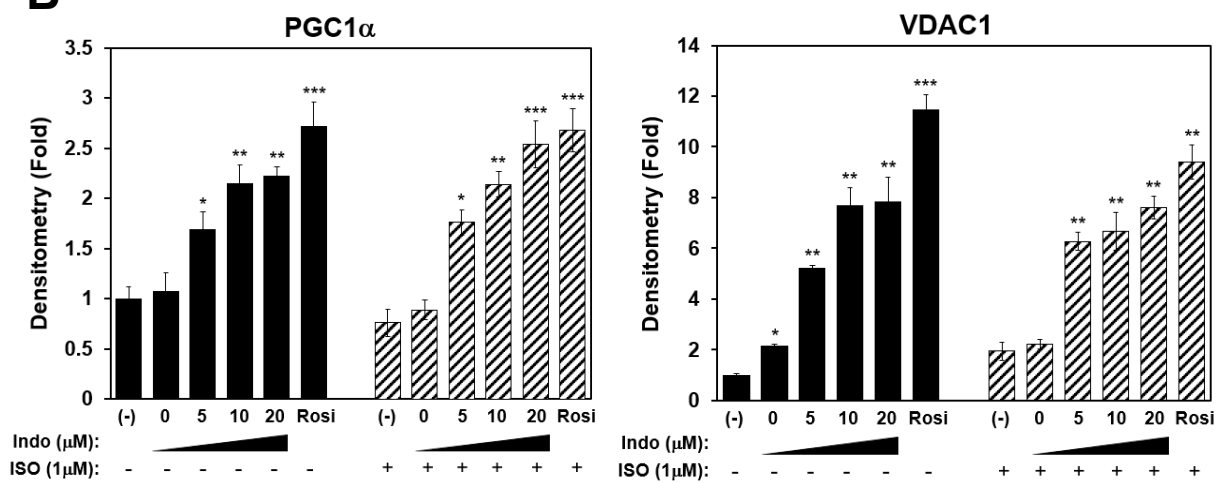


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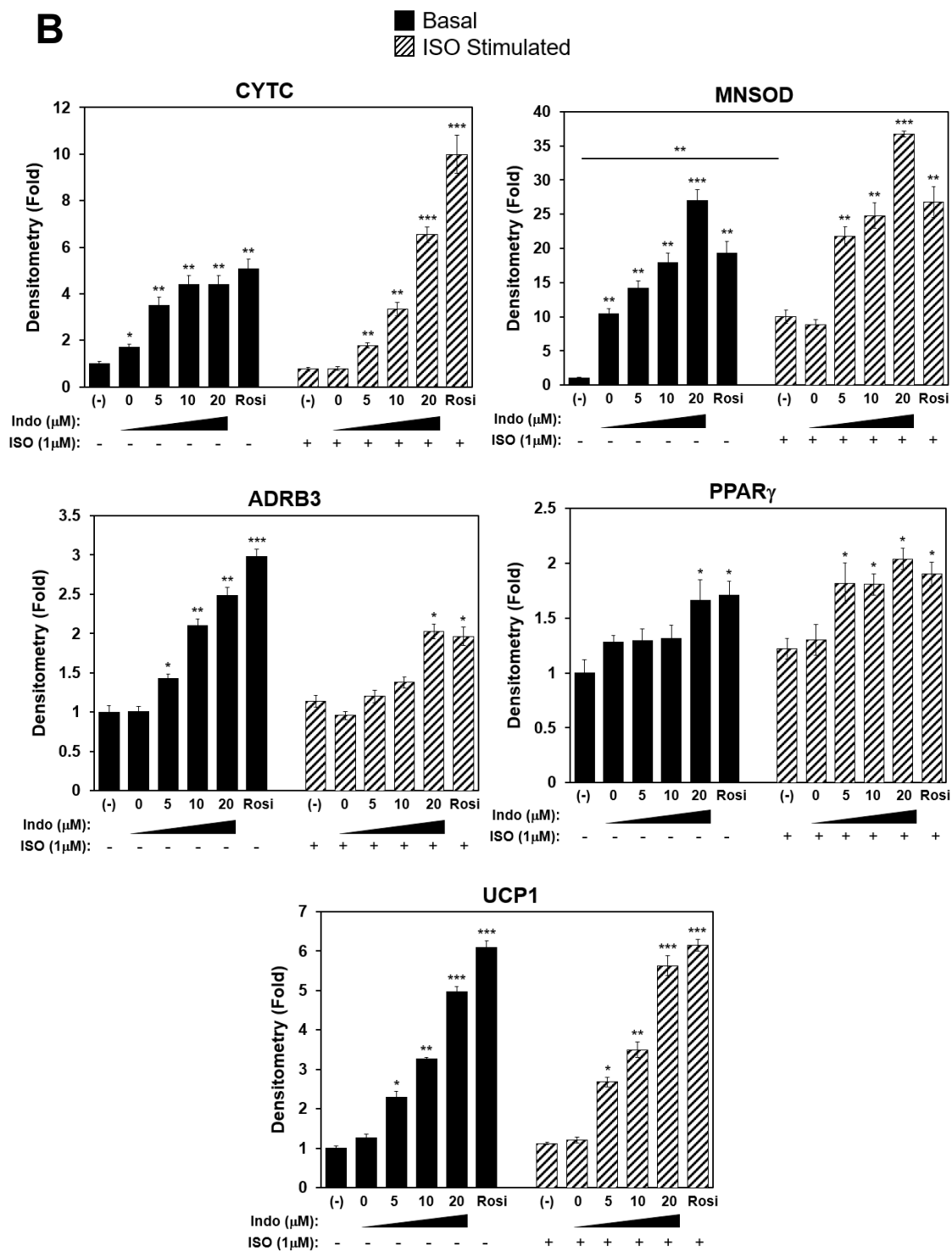


Figure 5.4. Continued.

Figure 5.5. Indomethacin significantly enhances oxygen consumption in 3T3-L1 adipocytes.

Confluent 3T3-L1 preadipocytes in a 6-well format were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 6 days. On day 6, cells were evenly re-seeded into XF-24 well plate. **(A-B)** Re-seeded cells were maintained Indo and Rosi treatment until flux-experiment initiation on day 8. **(C-D)** Re-seeded cells were maintained in maintenance media without Indo or Rosi for 24 h. On day 7, cells were treated with ISO (1 μ M) or vehicle for 24 h prior to flux-experiment. Mitochondrial stress tests were performed as described in the materials and methods. Respiration calculations **(B,D)** were performed, and are displayed so that the (-), non-ISO sample average is set at fold-1 for each type of respiration calculation group. Statistical differences in **(D)** are treatment compared to its (-) control. The bar indicates the statistical significance of the non-ISO group compared to ISO group. ANOVA with repeated measures and one-way ANOVA was performed to show statistical differences between treatment and controls indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

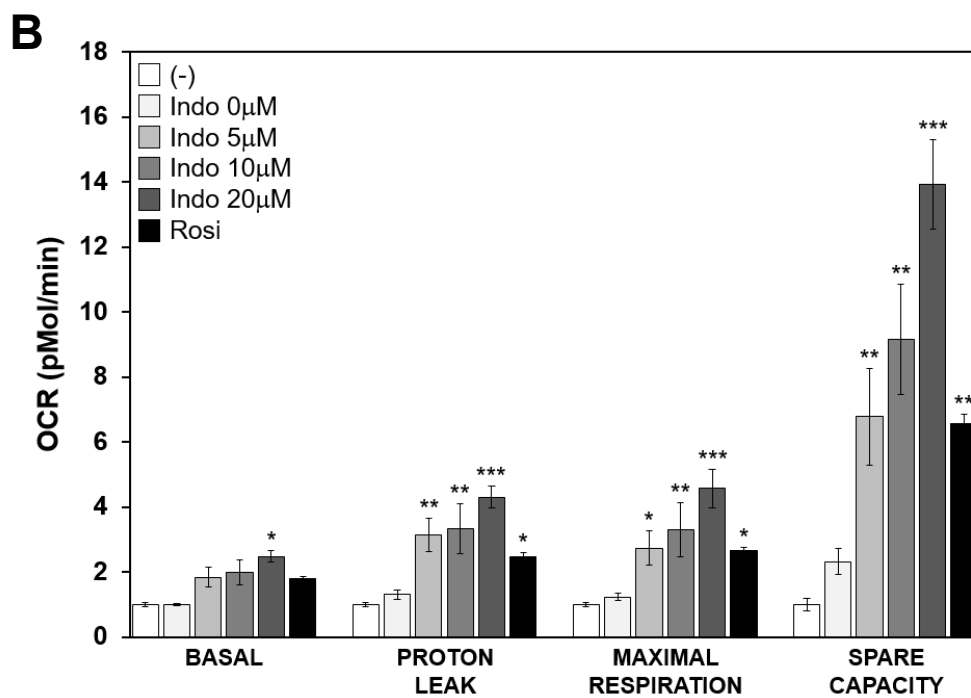
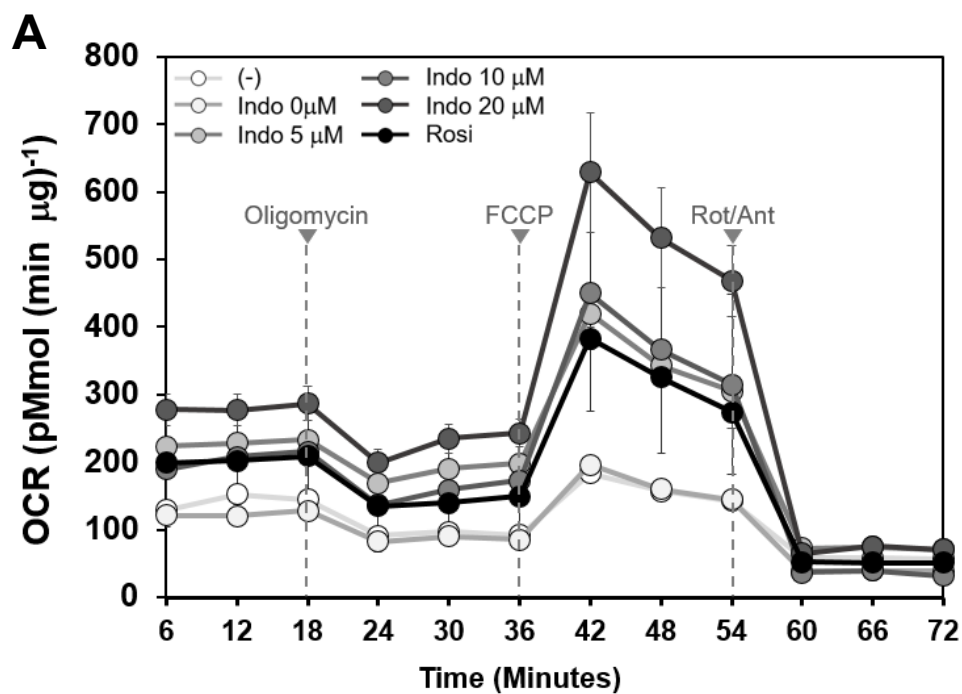


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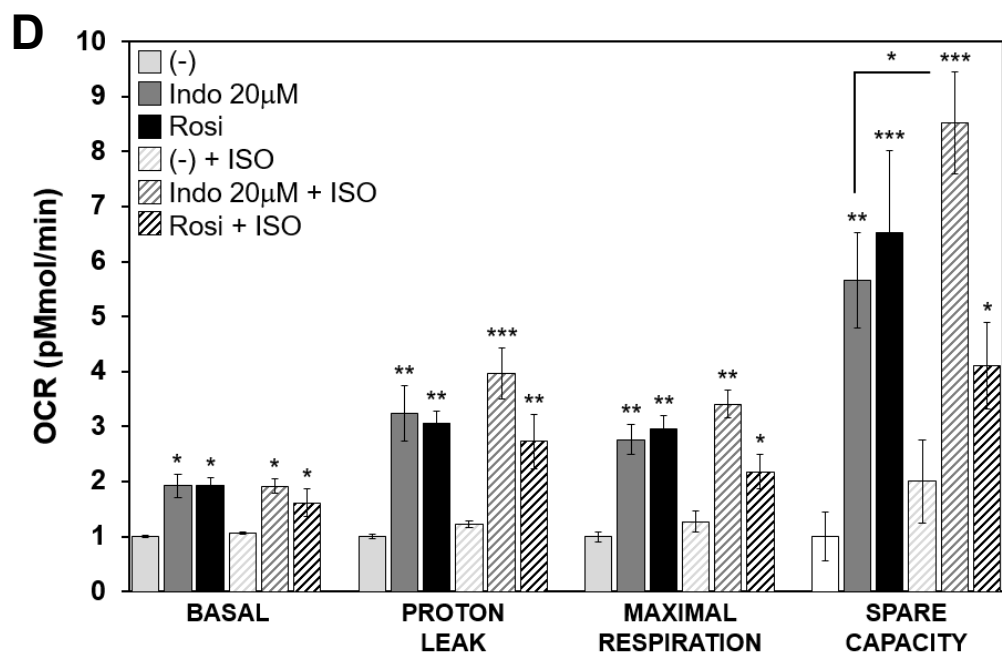
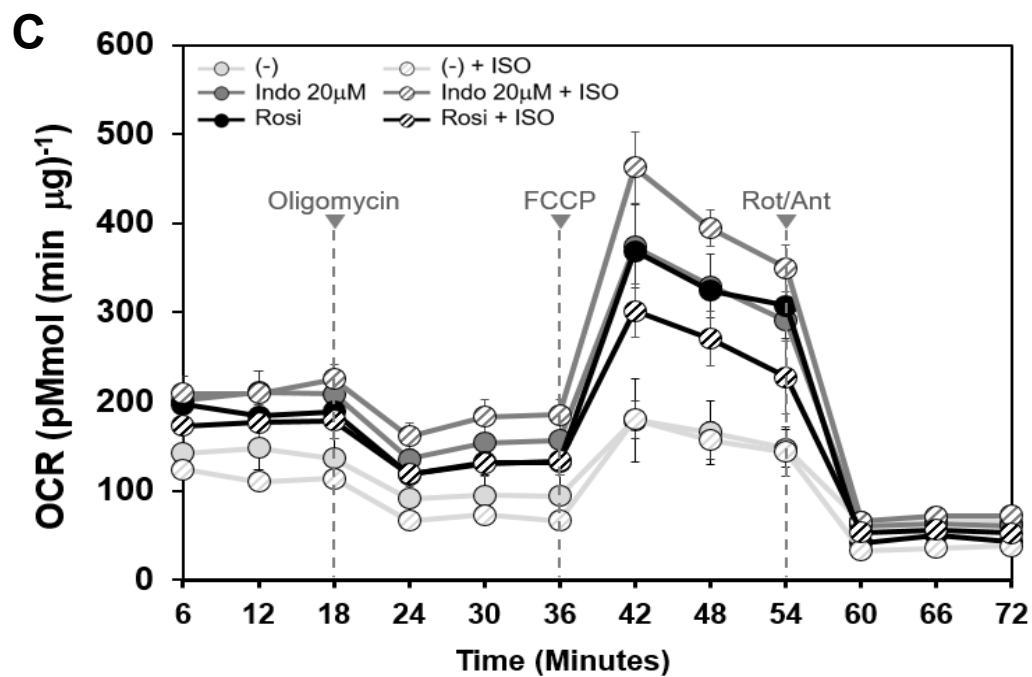


Figure 5.5. Continued.

Figure 5.6. Indomethacin dose-dependently activates PPAR γ , deficiency of which attenuates browning.

(A-B) 3T3-L1 preadipocytes were transiently transfected with PPAR γ or PPAR α -Gal4-Luc and β -gal for 24 h, then treated with increasing concentrations of Indo for an additional 18 h. Luciferase activity was normalized to β -gal activity, the non-treated (-) sample was set at fold-1. (C) 3T3-L1 preadipocytes were stably infected with lentiviral shRNA particles targeting PPAR γ or a scrambled control (**SCR**). Knockdown (**KD**) efficiency was determined by western blot analysis. (D) Densitometry of Ppar γ average protein expression was normalized to the loading control, β -Actin, with the parental 3T3-L1 cells set as fold-1. (E-I) Confluent 3T3-L1 cells with stable knockdown of PPAR γ (**PPAR γ -KD**) or SCR were induced to differentiate in the presence of absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 7 days. Relative mRNA expression (E-I) of *Ucp1*, *Pgc1 α* , *Ppar γ* , *Cd137*, and *Igfbbp3* normalized to *36b4* are shown, the (-)-non-ISO-stimulated SCR sample and (-)-ISO-stimulated SCR sample was set at fold-1 for each group, independently. Mixed model ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for treatment, KD, and ISO-responsive relationship. The bar indicates the statistical significance between PPAR γ -KD and SCR for each treatment. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

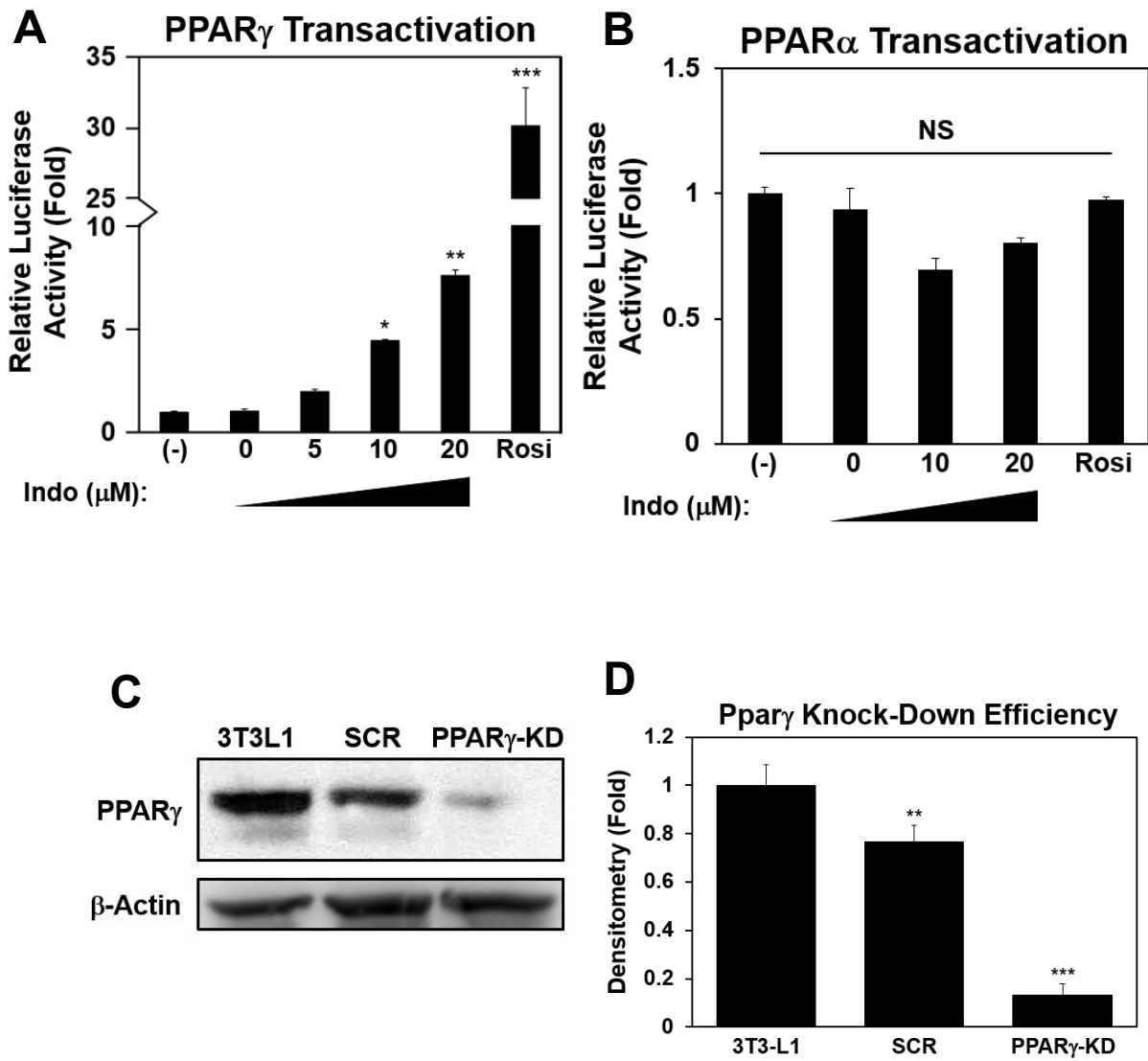


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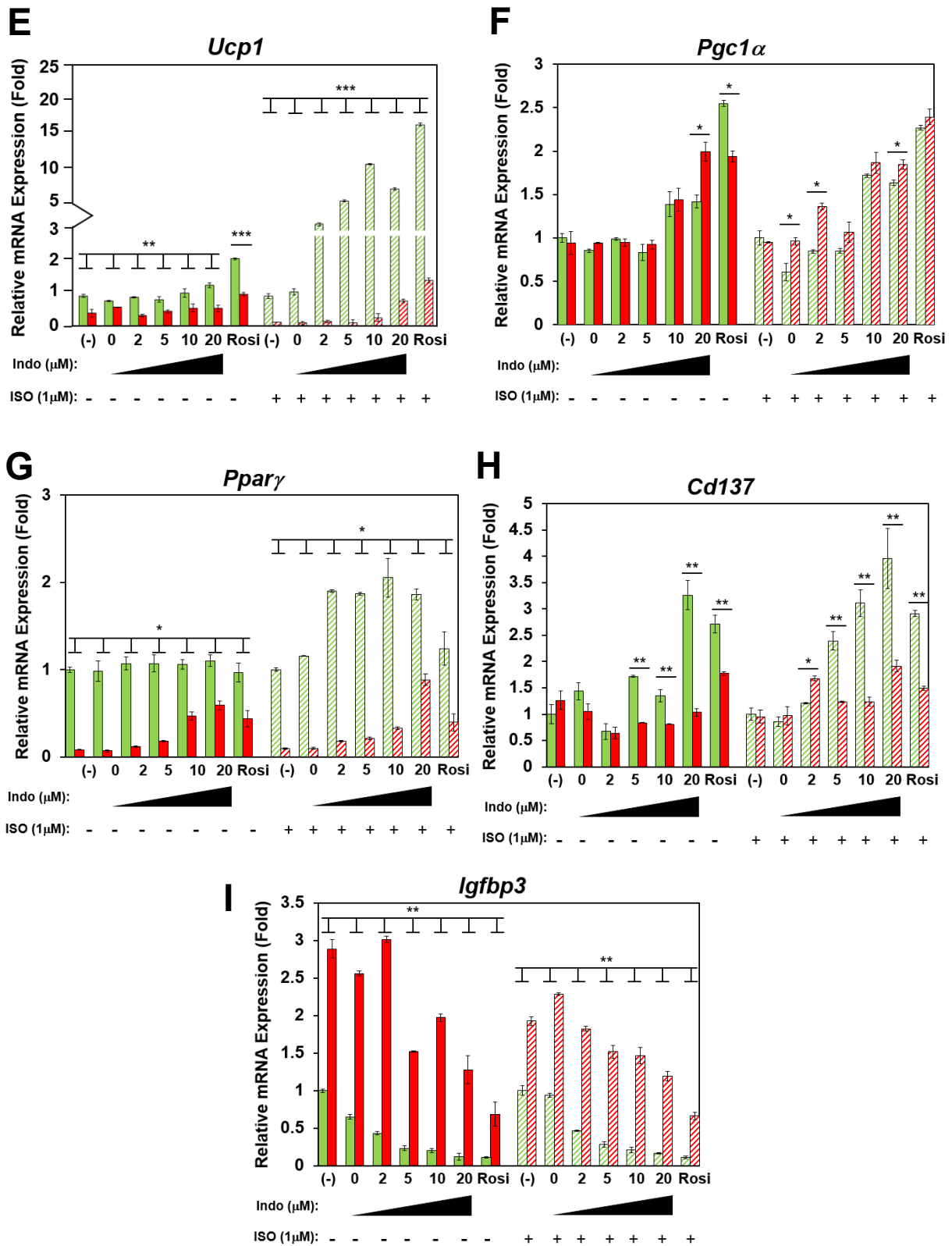


Figure 5.6. Continued.

Figure 5.7. Indomethacin induces browning in primary stromal cells derived from human subcutaneous fat.

Human primary stromal cells from subcutaneous fat were induced to differentiate in the presence or absence of increasing concentrations of Indo, vehicle control DMSO (Indo 0 μ M), or Rosi (1 μ M) for 14 days. **(A)** mRNA gene expression of *PPAR γ* , *PGC1 α* , *UCP1*, and *IGFBP3*, normalized to human *36B4*. **(B)** PGC1 α , UCP1, PLIN1, total PPAR γ , and VDAC protein expression and their densitometry **(C)**. **(D)** PPAR γ 1 and PPAR γ 2 quantified separately, graphing raw data to demonstrate relative expression levels of each gene. Unless otherwise noted, all densitometric comparison of the average expression normalized to loading control, total ERK1/2. A second loading control β -Actin is provided. Each graph is expressed as fold-1 of that of the 0 μ M (mRNA) or (-) (protein) sample for each gene. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for concentration-responsive relationships. The bar indicates the significant or non-significant (**NS**) state for individual samples compared to their control within each gene group. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

A

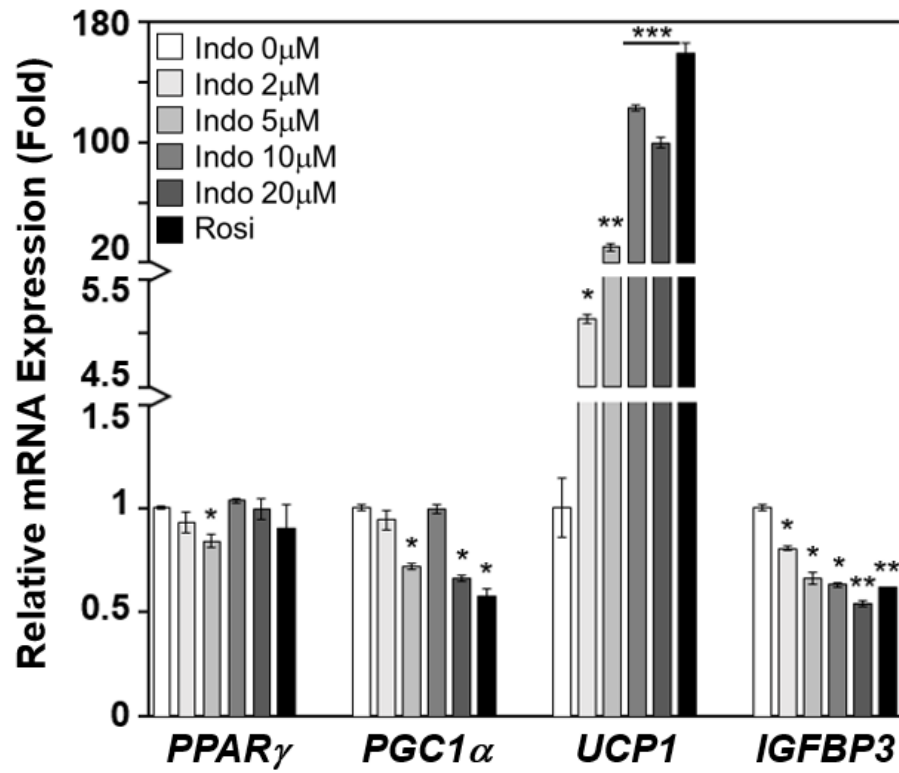


Figure 5.7. Continued.

Figure 5.8. Indomethacin promotes brown adipogenesis in human brown cell line.

Human brown cells were induced to differentiate in the presence or absence of increasing concentrations of Indo and vehicle control DMSO (Indo 0 μ M) for 21 days. Relative mRNA gene expression (**A-D**) of *PPAR γ* , *PGC1 α* , *UCP1*, and *DIO2*, normalized to human 36B4. (**E**) *PGC1 α* and *UCP1* protein expression and their densitometry (**F**). Densitometry of *PGC1 α* and *UCP1* protein was normalized to loading control, total ERK1/2. Each graph is expressed as fold-1 of that of the 0 μ M sample. One-Way ANOVA was performed followed by multiple comparison tests with Student-Newman-Kewls method for concentration-responsive relationship. Statistical significance is indicated by *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

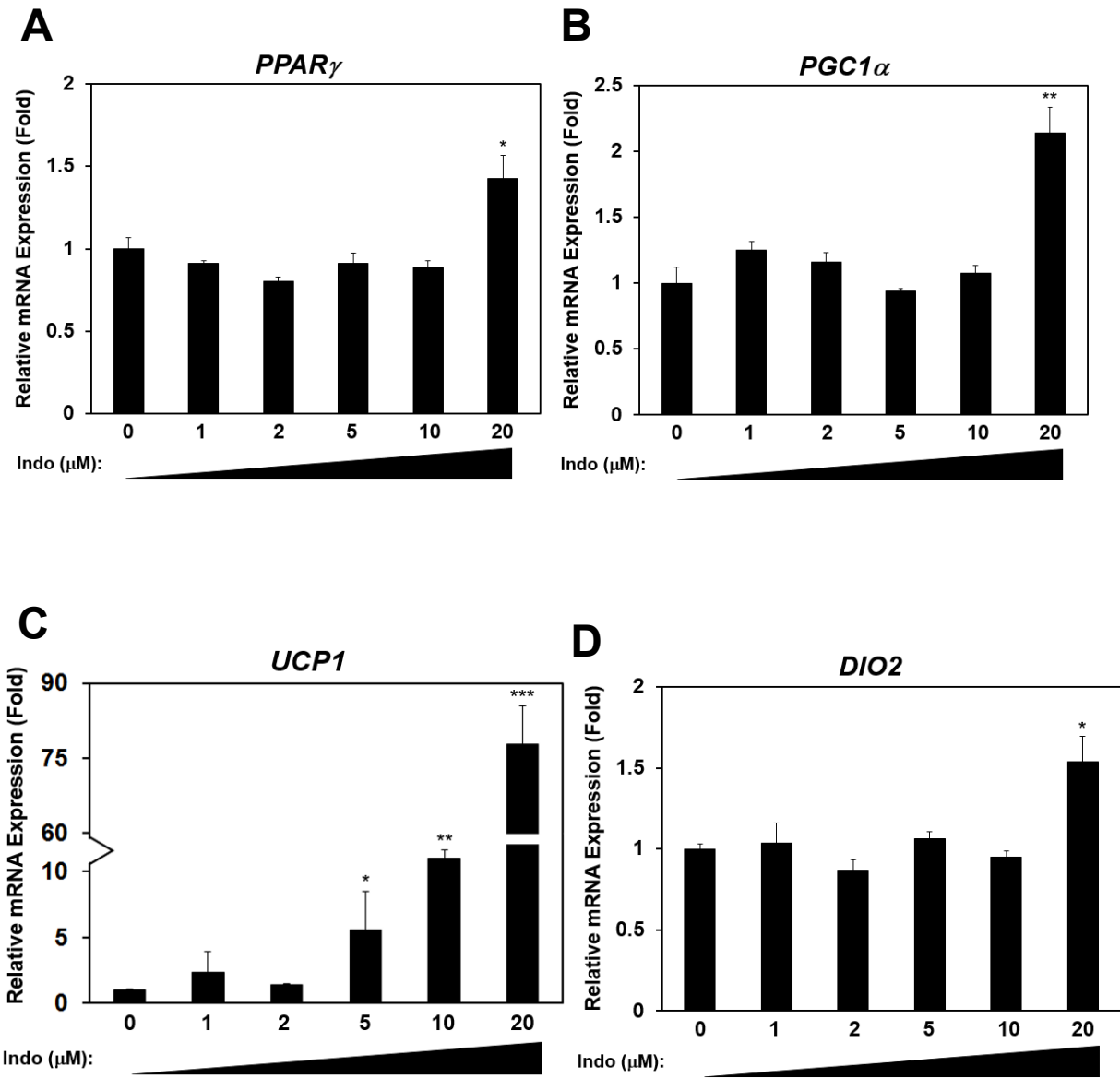


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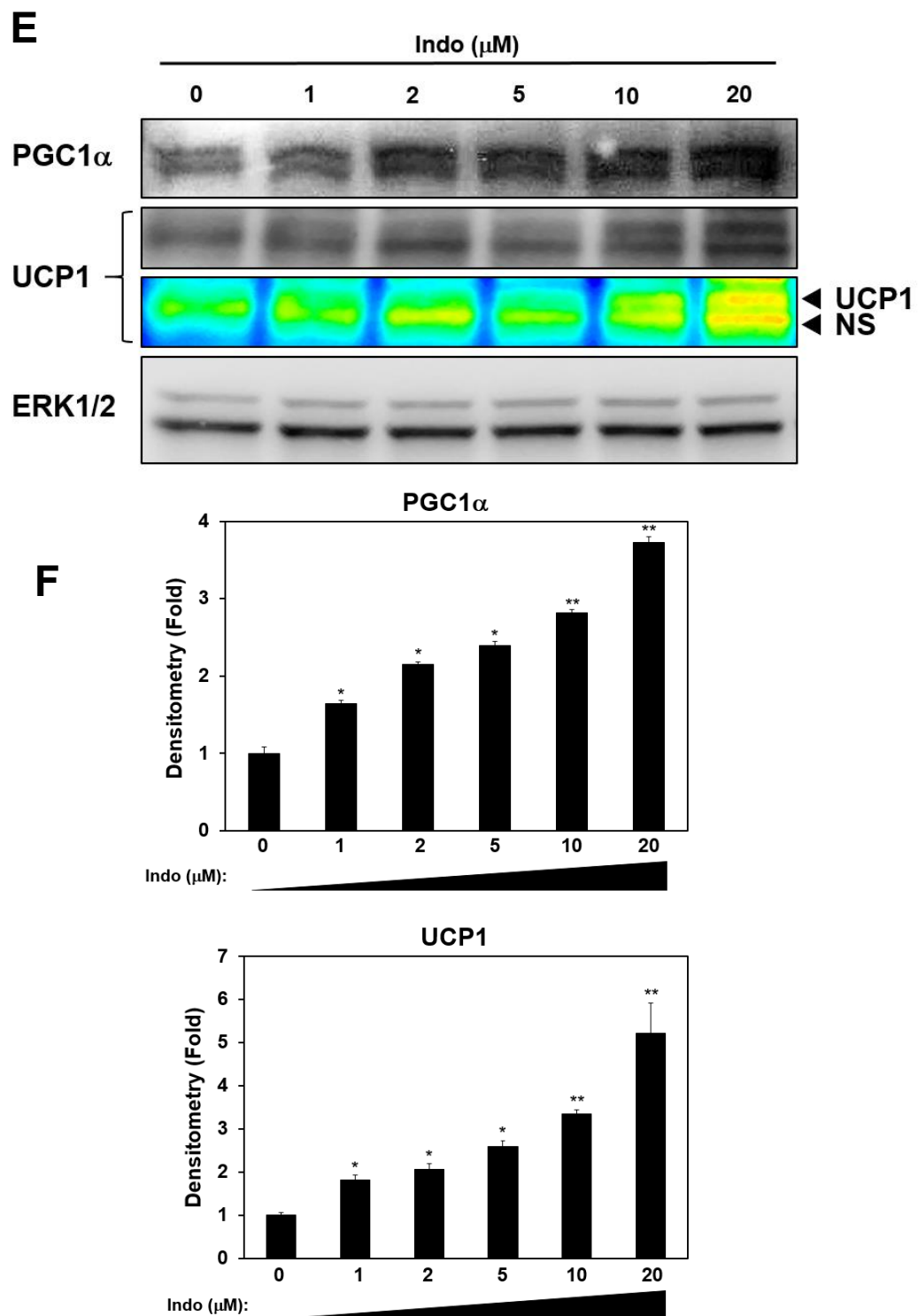


Figure 5.8. Continued.

Table 5.1. Murine primer sequences for qRT-PCR using SYBR green.

Gene name	Primer sequences	
36B4	Forward	GCTTCGTGTTACCAAGGAGGA
	Reverse	GTCCTAGACCAGTGTTCTGAGC
β -AR3	Forward	AGGCACAGGAATGCCACTCCAA
	Reverse	GCTTAGCCACAACGAACACTCG
CD137	Forward	CGTGCAGAACTCCTGTGATAAC
	Reverse	GTCCACCTATGCTGGAGAAGG
C/EBP α	Forward	GCAAAGCCAAGAAGTCGGTGGA
	Reverse	CCTTCTGTTGCGTCTCCACGTT
CIDEA	Forward	GGTGGACACAGAGGAGTTCTTTC
	Reverse	CGAAGGTGACTCTGGCTATTCC
COX4a	Forward	TCATTGGCTTCACTGCGCTCGT
	Reverse	TCCAGCATTGCTTGGTCTGCA
FABP4	Forward	TGAAATCACCGCAGACGACAGG
	Reverse	GCTTGTCACCATCTCGTTTTCTC
FASN	Forward	CACAGTGCTCAAAGGACATGCC
	Reverse	CACCAGGTGTAGTGCCTTCCTC
IGFBP3	Forward	GTCGCAAATAATGGTGCGCT
	Reverse	GAAAACTGCCTTCCCGCAG
NRF1	Forward	GGCAACAGTAGCCACATTGGCT
	Reverse	GTCTGGATGGTCATTTACCCGC
PGC1 α	Forward	GAATCAAGCCACTACAGACACCG
	Reverse	CATCCCTCTTGAGCCTTTCGTG
PPAR γ	Forward	GTA CTGTCGGTTTCAGAAGTGCC
	Reverse	ATCTCCGCCAACAGCTTCTCCT
UCP1	Forward	GCTTTGCCTCACTCAGGATTGG
	Reverse	CCAATGAACACTGCCACACCTC

Table 5.2. Human primer sequences for qRT-PCR using SYBR green.

Gene name	Primer sequences	
36B4	Forward	GCTTCGTGTTACCAAGGAGGA
	Reverse	GTCCTAGACCAGTGTCTGAGC
β -AR3	Forward	AGCCCAGGCTTTGCCAACGGC
	Reverse	GGGACTCATTGAGAACAGAGGC
DIO2	Forward	AGTGCAGAAGGAGGTGACAACAGT
	Reverse	AAAGTCAAGAAGGTGGCATGTGGC
IGFBP3	Forward	CGCTACAAAGTTGACTACGAGTC
	Reverse	GTCTTCCATTTCTCTACGGCAGG
PGC1 α	Forward	AGTGGTGCAGTGACCAATCA
	Reverse	CTGCTAGCAAGTTTGCCTCA
PPAR γ	Forward	AGCCTGCGAAAGCCTTTTGGTG
	Reverse	GGCTTCACATTGAGCAACCTGG
PRDM16	Forward	CAGCCAATCTCACCAGACACCT
	Reverse	GTGGCACTTGAAAGGCTTCTCC
UCP1	Forward	AGTTCCTCACCGCAGGGAAAGA
	Reverse	GTAGCGAGGTTTGATTCCGTGG

Table 5.3. Murine mitochondrial DNA primer sequences for qRT-PCR using SYBR green.

Gene name	Primer sequences	
Mouse mtCox-II	Forward	GCTCTCCCCTCTCTACGCAT
	Reverse	AGCAGTCGTAGTTCACCAGG

CHAPTER VI:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1. CONCLUSIONS

The contents of this dissertation included three primary experiments: Chapter III, IV, and V. Chapter III demonstrates successful encapsulation of *trans*-resveratrol (**Res**) within two forms of nanocarriers, nanoparticle (**Rnano**) and liposome (**Rlipo**). Nanotechnology in this case led to greater aqueous solubility, chemical stability, and cellular uptake, which led to greater biological effects in browning 3T3-L1 murine adipocytes. Both Rnano and Rlipo enhanced PPRE-responsive luciferase reporter gene activity and increased mRNA expression of browning markers including uncoupling protein-1 (**Ucp1**), peroxisome proliferator-activated receptor coactivator 1 alpha (**Pgc1 α**), beige maker *Cd137*, and mitochondrial markers, while markedly decreasing white-fat specific marker, *Igfbp3*.

Chapter IV added to these novel findings by demonstrating the effects of the addition of an adipose stromal cell (**ASC**)-targeting peptide (GSWKYWFGEGGC) onto the surface of nanoparticles encapsulated with Res. The resulting ligand-bound nanoparticle encapsulated with Res was referred to as (**L-NR**), and was shown to have high-binding affinity to ASCs due to its specificity for the extra-cellular matrix protein, delta-decorin (**Δ DCN**). Δ DCN is an isoform of decorin (**DCN**) that lacks a glycanation site and is only expressed in ASCs of the stromal cell population in subcutaneous white adipose tissue (**WAT**). As compared to the non-ligand bound nanocarrier (**NR**), L-NR had increased uptake in 3T3-L1 preadipocytes overexpressing Δ DCN and in murine primary stromal cells isolated from 10-12 week old male C57Bl/6J mice. L-NR induced browning in murine primary stromal cells when treated throughout differentiation at 1 μ M as shown by brown and beige-fat marker mRNA expression (i.e., *Ucp1*, *Pgc1 α* , *Cd137*). When treating fully mature adipocytes differentiated from the primary stromal cells from murine inguinal WAT, only L-NR, but not NR or FR, was able to induce the brown-like phenotype using 10 μ M.

Finally, in Chapter V, the effects of non-steroidal anti-inflammatory drug (**NSAID**), indomethacin (**Indo**), were tested using 3T3-L1 murine white fat cell line, human primary subcutaneous WAT cells (**HPsubQ**), and human brown fat cell line. Indo dose-dependently induced browning in 3T3-L1 adipocytes, as demonstrated by increased brown- and beige-fat marker mRNA and protein expression as well as decreased white-fat-specific marker mRNA expression. Indo led to increased mitochondrial biogenesis as shown by enhanced mRNA and protein expression of mitochondrial biogenesis and structural markers, as well as increased mitochondrial DNA and functionality in 3T3-L1 adipocytes. Indo also led to enhanced basal oxygen consumption rate, proton leak, and maximal and spare respiratory capacity in 3T3-L1

adipocytes. Indo dose-dependently enhanced transactivation of PPAR γ , knockout of which attenuated the browning effects. Moreover, Indo led to browning in HPsubQ adipocytes as shown by mRNA and protein expression of brown-fat markers. Indo also enhanced brown adipogenesis in a human brown fat cell line.

In summary, nanoparticle encapsulation increased solubility, extended the release, and enhanced the bioavailability and biological activity of Res. The targeting peptide further augmented the capacity of Res nanoparticles to induce browning in white adipocytes using lower, physiological-relevant doses. Finally, providing an alternate mechanism and beneficial effect for an FDA approved drug, Indo is now shown to work in both murine and human white and brown fat cells to induce browning and brown adipogenesis.

6.2. SIGNIFICANCE OF STUDY

Obesity is a multi-factorial disease, and therefore, requires a multi-factorial approach. The use of both pharmaceutical and nutraceutical compounds should be investigated for the treatment and prevention of obesity.

Res has been shown to promote beige adipocyte formation within WAT; however, the poor bioavailability has been a critical limitation for clinical utility. In this dissertation, the ASC-targeting, ligand-bound nanoparticles encapsulated with Res significantly increased the solubility, stability, and bioavailability of Res while simultaneously increasing its targeting capacity for the subcutaneous tissue within WAT. This resulted in enhanced white adipocyte browning and improved thermogenic capacity using lower, physiologically-relevant doses of Res (i.e., 1-10 μ M). Enhanced targeting specificity is expected to minimize side-effects by being able to use lower-doses of Res with reduced interaction or filtration with off-target organs. Therefore, we have shown that the use of targeting-nanotechnology in this case has overcome the previously recognized limitations of Res and provides proof for a potential breakthrough for its therapeutic use in preventing and treating obesity.

Indo has previously been used for the treatment of inflammatory disorders and diseases based on its non-specific cyclooxygenase (**COX**)-inhibitory actions. Until now, Indo has never been used to treat or prevent obesity by inducing browning of white adipocytes. The results of our study demonstrate a great potential of Indo to promote browning, comparable to that of the insulin sensitizing drug, rosiglitazone. Our results suggest that Indo could be used as a therapeutic adjunct in obese patients to possibly promote energy expenditure and weight loss and improve insulin sensitivity. Many drugs must go through years of costly human clinical trial testing to confirm efficacy or obtain approval. However, Indo is an already FDA approved drug,

and would therefore not have to undergo expensive and time-consuming testing.

Considering obesity is still burgeoning disease rising to pandemic levels, finding adjuncts to lifestyle changes is critical to prevent and treat obesity. Both targeting nanoparticles encapsulated with Res and Indo show promise as novel nutritional and pharmaceutical strategies to combat obesity, which could not only help to reduce medical costs but also promote health and quality of life.

6.3 LIMITATIONS AND FUTURE DIRECTIONS

There are several limitations to this project which should be acknowledged. The time and cost associated with nanocarrier production as well as the therapeutic delivery route of nanocarriers are of concern when considering use in human clinical trials. Generating nanoparticles is a time-consuming, technically difficult, and expensive, which may not be feasible or cost-efficient for wide-spread use. However, over time, the cost and production methods should reciprocally progress in parallel with technology progression. The current methods of delivery for nanoparticles are oral or intravenous injection. Oral administration would not be effective in enhancing the delivery of Res or reducing the metabolism, since the outer-shells are composed of inert lipids which the body would recognize and digest as any other dietary lipid. Intravenous (IV) injection is routine in animal studies; however, IV injections may not be feasible for human testing. Depending on injections site, there is a considerable amount of nanocarriers filtered out and trapped by the liver.¹ One strategy to overcome the issue with IV injection, is the use of injectable hydrogels primarily composed of biodegradable hyaluronic acid and alginate.² Using this, the nanocarrier-hydrogel mixture can be injected directly into the subcutaneous WAT through the skin. Therefore, generation and testing for the targeting-delivery and the anti-obesity biological effect of L-NR using IV compared to subcutaneous injection in animal studies would be the logical next step.

Although the results of Chapter IV were compelling and novel, they are preliminary and require more in-depth analysis of the gene expression, mechanism, and function elicited in murine stromal cells by L-NR using various doses and timing. This analysis would include protein expression of brown and beige fat markers, inhibitor and/or knock-down studies, as well as glucose/insulin-tolerance and mitochondrial respiration assays to confirm beige-phenotype and function.

Finally, a primary limitation for the use of Indo is its unwanted toxic side effects seen after oral administration. Similar to other NSAID long-term use, Indo has been shown to have toxic side effects, which primarily includes gastrointestinal (GI) distress and ulceration. Therefore, it

would be pertinent to find alternative routes to oral administration to reduce GI exposure and therefore, reduce toxic side effects. Fortunately, there have been many attempts made in this therapeutic-field for other drug compounds which could possibly be applied to Indo.

Surgically implanted pumps have been used in rodent studies to locally deliver Indo to brown adipose tissue (**BAT**) fat pads.³ A similar approach is used in some human-based therapies, including internal insulin pumps,⁴ some types of birth control,⁵ as well as newer vaccine-based implant approaches.⁶⁻⁹ Topical ointments and patches are also shown to help with local joint, bone, and muscle delivery.^{6, 10-13} Small, pre-measured sterile injection-pens are used to deliver a wide-variety of drug-compounds.^{14, 15} Therefore, the use of any of these already established drug-delivery methods would be viable options for helping with local and/or sustained release of Indo. It would be interesting to establish an Indo-based compound possibly in combination with a proton-pump inhibitor (**PPI**) (to help reduce ulceration and GI distress),¹⁶ with one of the aforementioned drug-delivery methods to further reduce side effects and enhance tissue-targeting.

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VITA

Haley Beth Overby is the daughter of Laura Michelle Murakami and Kevin Kazukuni Murakami from Nashville, Tennessee where she completed high school. She then obtained a Bachelor of Science degree in Nutrition and Food Sciences with a concentration in Dietetics from Middle Tennessee State University (**MTSU**) with honors as *Magna Cum Laude*. During her time at MTSU, she received many awards and honors, including the Rita Davenport, Diversity and Enrichment, Academic Service, and HOPE scholarships. During this time, Haley worked at a local food service establishment, where she worked as manager of at least two store locations simultaneously, and improved stores sales significantly. She also worked as a dietary aide at a long-term care facility. After graduating from MTSU in 2015, Haley moved to Knoxville to obtain a Doctor of Philosophy degree in Cellular and Molecular Nutrition at the University of Tennessee at Knoxville (**UTK**). While at UTK, she received many awards and honors, including the Chancellor's Award (a 4-year prestigious scholarship), Shipley-Swann Graduate Fellowship, and was also the president of the UTK Graduate Nutrition Student Association in 2016. She began the pursuit of this degree in August 2015, defended her dissertation on August 2nd, 2019, and plans to walk for the graduate-hooding ceremony in December 2019.

During her time at UTK, she completed her dissertation research in Dr. Ling Zhao's lab, and gained considerably developed analytical, research, reporting, professional, and administrative skills. She was employed by UTK as a graduate research assistant (**GRA**) and graduate teaching assistant (**GTA**) for at least 3 years.

As a GRA, Haley was able to hone-in research, problem-solving, and time management skills through leadership of multiple laboratory bench studies. She performed and mastered wet lab (cell culture) and molecular biological experimentation techniques while also training some faculty and students in laboratory safety, molecular biology experimentation, equipment use, and study design. Haley was acting lab manager for Dr. Zhao's lab, assisting in inventory use and reordering, as well as implementing Bio-Safety, radiation, and IACUC protocols in order to maintain compliance. She also obtained many skills in the following areas: cell culture, genetic manipulation, cell functionality assays, rodent handling and surgical experience, advanced microscopy and imaging, protein and nucleotide concentration measures, as well as microbial, immunological, and biological micro-technique. She also became proficient in the following computer techniques: Microsoft Word, Power Point, Excel, Adobe Photoshop, EndNote citing manager, Sigma Plot and SAS statistics software (experience with programming and coding in SAS), navigating various Learning Management System platforms (i.e., Blackboard, Canvas,

MacMillan, Great River Learning, etc), as well as navigating and utilizing NCBI's various research tools and databases.

As GTA for Nutrition 100 courses, Haley helped to teach introductory nutrition and metabolism in a standard class setting and online. In these courses, she taught application-based lessons designed to improve student skill set and metacognition. Outside of the classroom, Haley also helped to conceive and develop nutrition analysis software programs and related assignments as well as design and generate diagrams, figures, & instructional technology for online-integrated nutrition textbook.

While at UTK, Haley has authored or co-authored the following oral or poster presentations at Experimental Biology or American Society of Nutrition:

- *Nanoparticles encapsulated with resveratrol induce browning of white adipocytes.*
- *Pharmacological inhibition of soluble epoxide hydrolase promotes brown adipogenesis.*
- *Indomethacin induces browning and brown adipogenesis in murine 3T3-L1 white adipocytes and human brown fat cells.*
- *Soluble epoxide hydrolase inhibitor t-AUCB promotes murine brown adipogenesis: Role of PPAR gamma and PPAR alpha (P21-069-19).*

Haley has also been first or second author on 3 publications:

- *Parabens inhibit fatty acid amide hydrolase: A potential role in paraben-enhanced 3T3-L1 adipocyte differentiation.*
- *Methylparaben and butylparaben alter multipotent mesenchymal stem cell fates towards adipocyte lineage.*
- *Resveratrol liposomes and lipid nanocarriers: comparison of characteristics and inducing browning of white adipocytes.*

She has submitted another manuscript earlier this year, which is under review for publication, and she plans to submit another manuscript after her dissertation is finalized, both of which she will be first author of.

Haley is a proactive and hardworking team player able to work on own initiative with proven ability to manage multiple projects in a fast-paced work environment. She is a skilled communicator with professional and organized obtaining and reporting skills able to make well thought out decisions and suggest ideas. Haley hopes to utilize the skills obtained during her time at UTK in her career and is currently seeking full time research and development career opportunities with an innovative, challenging, and responsible company.