UNIVERSITAT ROVIRA i VIRGILI

Role of the nuclear receptor RORγ1 in the differentiation and function of brown adipocytes

FINAL DEGREE PROJECT

BIOCHEMISTRY AND MOLECULAR BIOLOGY

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Tarragona, 6 de juny de 2023

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ABBREVIATIONS

24S-OHC	4 S-hydroxycholesterol
25-OHC	25-hydroxycholesterol
β1AR	β_1-adrenergic receptor
β3AR	β_3-adrenergic receptor
ACC2	Acetyl-CoA carboxylase 2
ATF2	Activating Transcription Factor 2
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMAL1	Brain and muscle ARNT-like
BMI	Body Mass Index
C/EBPα	CCAAT/enhancer binding proteins alpha
C/EBPβ	CCAAT/enhancer binding proteins beta
C/EBPδ	CCAAT/enhancer binding proteins delta
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP Responsive Element Binding Protein
DBD	DNA-binding domain
DIO2	Type II thyroxine 5-deiodinase
DMEM F-12	Dulbecco's Modified Eagle Media/Nutrient Mixture F-12
En1	Engrailed Homeobox 1
FABP4	Fatty acid binding protein 4
FBS	Fetal bovine serum
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21
iBA	Immortalized brown adipocytes
IBMX	3-Isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin-1
IR	Insulin resistance
LBD	Ligand-binding domain
LTi	Lymphoid tissue inducer cells
miRNA	microRNA
MMP3	Matrix Metallopeptidase 3
MSC	Mesenchymal stem cell
Myf5	Myogenic factor 5
NE	Norepinephrine
NF-κB	Nuclear factor-κB
NR	Nuclear receptor
NRF1	Nuclear respiratory factor 1

PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PGC-1α	Peroxisome proliferator-activated receptor Gamma Coactivator 1 alpha
PGC-1β	Peroxisome proliferator-activated receptor Gamma Coactivator 1 beta
PKA	Protein Kinase A
PP	Peyer's patch
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator activated receptor γ
PRDM16	PRD1-BF1-RIZ1 homologous domain-containing 16
Rev-Erbα	Nuclear receptor subfamily 1 group D member 1
ROR	Retinoic acid-related orphan receptor
RORE	ROR response element
RORα	Retinoic acid-related orphan receptor alpha
RORβ	Retinoic acid-related orphan receptor beta
RORγ	Retinoic acid-related orphan receptor gamma
SAT	Subcutaneous adipose tissue
siRNA	Small interfering RNA
SREBP	Sterol response element binding protein
STAT3	Signal transducer and activator of transcription 3
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
T_3	Triiodothyronine
T_4	Thyroxine
TCA	Tricarboxylic acid cycle
TGF-β1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
UCP1	Uncoupling protein 1
VAT	Visceral adipose tissue
WAT	White adipose tissue
WHO	World Health Organization

ABSTRACT

Retinoic acid-related orphan receptor gamma 1 (RORγ1) is drawing interest as a therapeutic target for the treatment of obesity and diabetes. Preliminary results showed that RORγ1 is highly expressed in brown adipose tissue (BAT) and increases during differentiation of immortalized brown adipocytes (iBA). To elucidate its role in BAT, we silenced RORγ1 expression using small interfering RNAs and inhibited RORγ1 activity by RORγ inverse agonists in iBA preadipocytes. Although we did not obtain firm results regarding morphological differentiation changes and brown fat markers expression, we validated iBA preadipocytes transfection.

1. INTRODUCTION

1.1. Obesity and type 2 diabetes

Over the last few decades, obesity has become an increasing public health problem worldwide that affect millions of people. Obesity is defined as the excessive accumulation of body fat that may affect health, which is diagnosed at a Body Mass Index (BMI) ≥ 30 kg/m^2. The pathophysiology of this chronic metabolic disease is complex and multifactorial, involving interactions between genetic, environmental, psychological and lifestyle factors. The World Health Organization (WHO) reports that the prevalence of obesity and overweight in adults has nearly tripled globally since 1975, reaching 13% and 39%, respectively.[1] Thus, obesity presents a substantial burden, both at the individual and population levels, by affecting the quality and socio-economic of health and life.

The development of obesity represents a long-term energy imbalance where energy intake exceeds energy expenditure, leading to storage of the excess of energy as fat. That overabundance of energy is primarily deposited as triglycerides within the white adipose tissue (WAT) and ectopic fat in non-adipose tissue, including the skeletal muscle and endocrine pancreas. The sustained positive energy balance leads to the manifestation of insulin resistance and compromised metabolism. Consequently, promoting negative energy balance by restricting energy intake or increasing energy expenditure could serve as an efficacious strategy for treating obesity and associated pathological conditions.[2]

Obesity increases the risk of developing various health issues, including cardiovascular disease, hypertension, fatty liver disease, cancer, and type 2 diabetes (T2D). Then, it is not surprising to find out that, in parallel with the dramatic increase in obesity, T2D has become the most common metabolic disorder in the world, being recognized as one of the most deadly non-communicable diseases worldwide.[3] T2D is a chronic metabolic disorder that is characterized by insulin resistance (IR) and impaired glucose homeostasis, which increases blood glucose levels over a prolonged period of time. There is strong evidence that links obesity and T2D, being one of the leading risk factors for developing the disease.[4] Furthermore, in adipose tissue, IR produces an impaired inhibition of lipolysis, and consequently it results in elevated circulating levels of free fatty acids (FFA), which in turn contribute to worsen the insulin signaling pathway by inducing lipotoxicity.[5] Therefore, a defective insulin signaling results in an imbalanced metabolic function of the main insulin target tissues, and severely alters whole body glucose and lipid homeostasis.

1.2. Adipose tissues

The importance of adipose tissue in the regulation of energy balance and glucose homeostasis is widely acknowledged. In healthy individuals, adipose tissue comprises 20-28% of the total body mass, varying according to sex and energy status. Notably, in individuals with morbid obesity, the proportion of fat mass can constitute up to 80% of total body mass. The physiological function of adipose tissue is determined by its distribution and localization within the body. This complex tissue is principally composed by adipocytes, considered as the functional unit, and the stromal vascular fraction (SVF), containing a variety of cell types, including pre-adipocytes, macrophages, fibroblasts, and endothelial cells.[6] There are found two distinct types of adipose tissues in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT), each of which perform distinct physiological functions important for maintaining glucose homeostasis and energy balance regulation.

1.2.1. White adipose tissue

Moreover, white adipose tissue (WAT) is recognised for being a white or yellow tissue with less vascularization and innervation than BAT. At morphological level, white adipocytes, the functional units of WAT, are polyhedral and unilocular cells with a wide diameter and a single large lipid droplet that takes up most of their cytoplasm and store lipids for energetic demand. These cells also have lower content of mitochondria and other organelles than brown adipocytes (Figure 1). WAT is characterized by its lipid storage and endocrine functions.

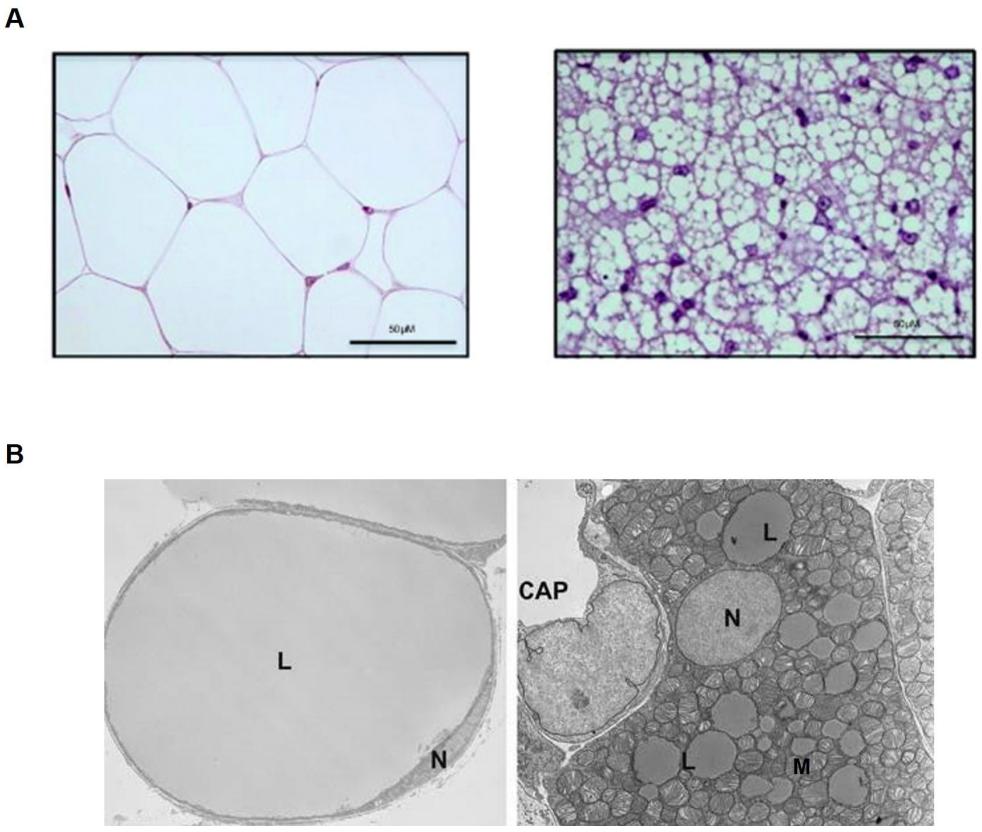

Figure 1. (A) Histological sections showing the morphology of WAT (left) and BAT (right). *Figure A extracted from* [7]. **(B) Transmission electron microscopy of WAT (left) and BAT (right).** WAT presents one big lipid droplet, whereas BAT presents numerous small lipid droplets and many mitochondria. L, lipid droplet; M, mitochondria; N, nucleus; CAP, capillary lumen. Figure B extracted from [8].

The store of excess calories by white adipocytes is in the form of triglycerides, which can be then released as free fatty acids through lipolysis and used as a source of energy in times of caloric need, for example during exercise or fasting. In addition to its primary function, WAT also serves as a thermal insulator and provides cushioning to the visceral organs, offering mechanical protection.[9]

One function of WAT, that is also recognized, is its endocrine activity. The adipose organ secretes more than one hundred human factors. Exosomes, microRNA (miRNA), lipids, peptide hormones and cytokines, termed "adipokines", are only a few of the many kinds of chemicals that WAT secretes.[10] Many of the adipokines produced by WAT are secreted by other cell types, such as the macrophages found in the SVF, even though some are released by adipocytes.

The most significant and well-characterized adipose secreted products are summarized in Figure 2. Resistin and adiponectin are adipokines that control the metabolism of glucose and lipids and leptin regulates the food intake. Tumor necrosis factor (TNF) and Interleukin-1 (IL-1) control inflammation, and PAI-1 is the plasminogen activator inhibitor

1 that regulate blood clotting. The blood pressure is regulated by angiotensinogen and angiotensin II. Nevertheless, many of these adipokines have an autocrine or paracrine effect. So, these hormones control immunological responses, insulin sensitivity, the reproductive axis, and food intake.

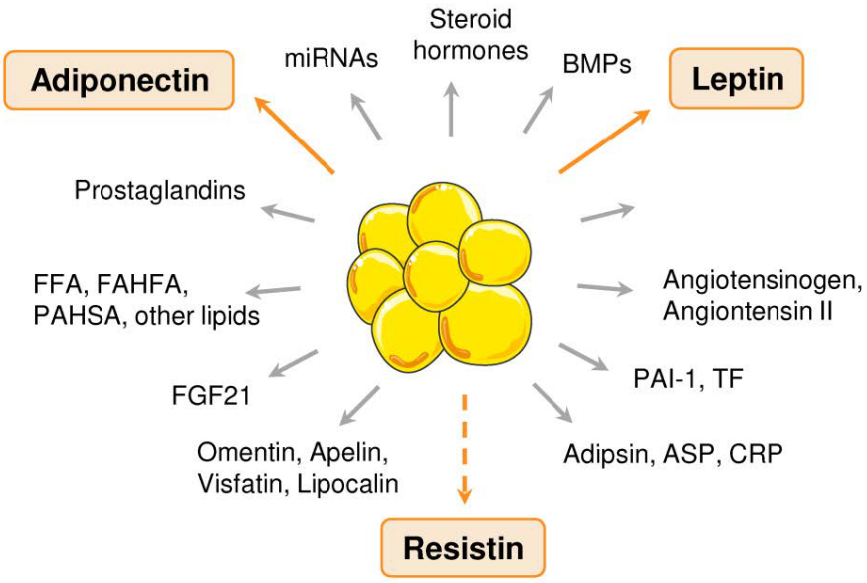

Figure 2. Factors released or secreted by WAT. *miRNA, microRNA; BMPs, bone morphogenetic proteins; PAI-1, plasminogen activator inhibitor 1, TF, tissue factor; ASP, acylation-stimulating protein; CRP, C reactive protein; FGF21, fibroblast growth factor 21; FFA, free fatty acid; FAHFA, fatty acid esters of hydroxyl fatty acids; PAHSA, palmitic acid hydroxy stearic acid. Figure adapted from* [11]

Based on where it is located anatomically, there are two main forms of WAT in humans. The first type is subcutaneous adipose tissue (SAT), which is located beneath the skin, whereas the second type is visceral adipose tissue (VAT), which is situated in the abdominal and thoracic cavities, surrounding internal organs. The metabolic and endocrine activities of SAT and VAT differ, and as a result, they each play a distinct role in the emergence of morbidities linked to obesity.

According to studies, VAT has a higher proportion of large adipocytes, a reduced capacity to differentiate preadipocytes, a higher number of immune and inflammatory cells, and a distinct profile of adipokine release that is more closely linked to inflammation and type 2 diabetes. Therefore, it seems logical that several studies have found a link between VAT and metabolic dysregulation, which encourages glucose intolerance and IR. In contrast, SAT is linked to enhanced insulin sensitivity and defense against metabolic disorders for its inverse association with glucose intolerance and decreased release of pro-inflammatory adipokines.[12]

1.2.2. Brown adipose tissue

Brown adipose tissue (BAT) plays a crucial role in regulating body temperature via a process known as non-shivering adaptive thermogenesis. This process is regulated by the sympathetic nervous system and is activated by norepinephrine (NE) released due to cold exposure. Like WAT, BAT is made up of stromal vascular fraction and brown adipocytes as the functional unit. Brown adipocytes, unlike white adipocytes, contain multiple small lipid droplets that are uniformly distributed throughout their cytoplasm instead of a single large vacuole (Figure 1). Brown adipose tissue is highly vascularized and innervated to ensure adequate oxygen and substrate supply and rapid distribution of heat produced during thermogenesis.[7] Mitochondria are abundant in brown adipocytes, which give them their reddish or brown colour and enable them to oxidize fatty acids more efficiently than white adipocytes.

The major depots found of BAT is in rodents, and in most mammals, include the interscapular, cervical and axillar localizations. However, minor depots are in the perirenal, intercostal and periaortic areas (Figure 3). The characteristic distribution of BAT along the body allows temperature preservation of vital organs.

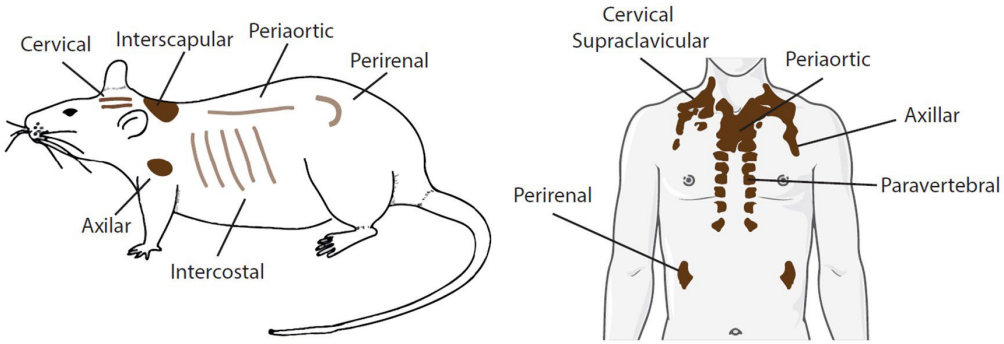

Figure 3. Distribution of major BAT depots in mice (left) and humans (right). *Figure adapted from* [13].

In addition to the previously mentioned discrete BAT depots, thermogenic adipocytes can also be found among white adipocytes from WAT under certain conditions. For instance, prolonged exposure to cold or treatment with β-adrenergic receptor agonists increases the development of brown adipocytes within WAT that are thermogenically competent, a process called “browning” of WAT. These adipocytes in WAT express the uncoupling protein 1 (UCP1) and other typical brown adipocyte markers (i. e. Dio2, β_3-adrenergic receptors) and are referred to as brite (brown in white) or beige adipocytes to distinguish them from the traditional brown adipocytes seen in BAT depots (Figure 5).[13]

1.2.2.1. Non-shivering adaptive thermogenesis

As previously established, the primary purpose of BAT is to produce heat through non-shivering thermogenesis. This distinctive molecular characteristic of brown adipocytes is conferred by UCP1 that is exclusively expressed in these cells. UCP1 is located in the inner membrane of the mitochondria and functions as a proton channel that permits proton flux from the intermembrane space to the mitochondrial matrix. As a result, it uncouples the production of ATP from the oxidation of reducing equivalents, bypassing the ATP synthase, and dissipating the proton gradient created by the respiratory chain as heat.[9]

The central nervous system plays a major role in controlling the process of thermogenesis. After exposure to cold (Figure 4), the hypothalamus senses the drop in body temperature, NE is released, and BAT is innervated through efferent sympathetic nerve fibers. In response to these signals, β_1- (β1AR) and β_3-adrenergic receptors (β3AR) present on the surface of brown adipocytes are activated that activates the production of heat. Moreover, if persists the thermogenic stimulation can also promote the growth and differentiation of brown preadipocytes.

Adrenergic receptor activation results in the activation of G stimulatory (Gs) proteins, which raises intracellular cAMP levels. The activation of Protein Kinase A (PKA), in turn, causes the phosphorylation of several proteins, resulting in the activation of different pathways required for a full thermogenic response. In particular, PKA, through cAMP Responsive Element Binding Protein (CREB), enhances *ucp1* expression. Therefore, PKA by inducing lipolysis leads to release of FFA which can activate UCP1, as FFA serve as substrates for β-oxidation in mitochondria and enhance thermogenesis. Furthermore, the activation of transcriptional regulators by norepinephrine signaling such as CREB, Activating Transcription Factor 2 (ATF2), and Peroxisome proliferator-activated receptor Gamma Coactivator 1 alpha (PGC-1α), increases UCP1 expression.[13]

NE is not the only hormone or factor that has been discovered as activating or recruiting BAT; other hormones and factors may also play a role[14]. Triiodothyronine (T_3), a well-known powerful transcriptional activator of the UCP1 gene, is one of them. Thyroxine (T_4), a hormone secreted by the thyroid gland, is converted into this hormone by the activity of type II thyroxine 5-deiodinase (DIO2), a particular enzyme for brown adipocytes that is activated in response to sympathetic stimulation. T_3 regulates several genes involved in lipid mobilization and storage and in thermogenesis. Although the thyroid hormone-controlled recruitment process of BAT is not fully understood, it was

shown that T_3 increases the recruitment of thermogenic capacity of BAT in mice through promoting brown adipocyte progenitor cells proliferation.

Even though NE is the primary inducer of non-shivering adaptive thermogenesis, T_3 is necessary for correct thermogenesis function since hypothyroid rats and *Dio2*-deficient mice with decreased levels of T_3 in BAT exhibited hypothermia as a result of BAT defective thermogenesis.[15]

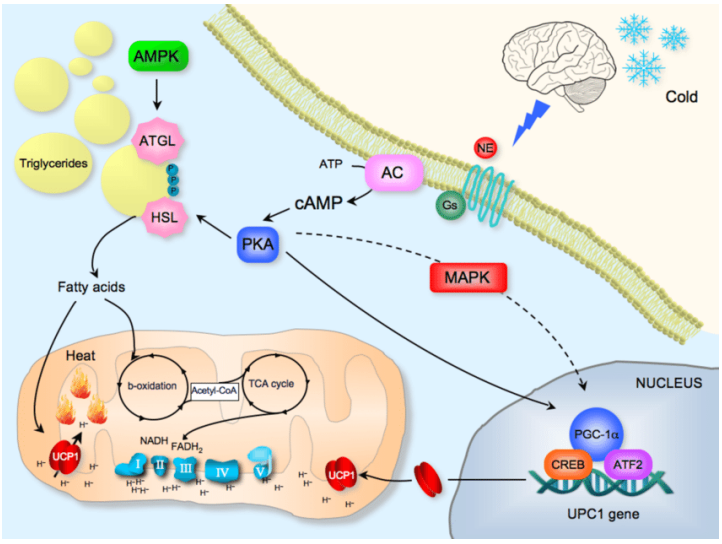

Figure 4. Overview of the regulatory mechanisms involved in cold-induced adaptive thermogenesis in BAT. *Figure extracted from* [13].

1.2.2.2. Origin and differentiation of brown adipocytes

It is known that adipocytes, skeletal muscle cells, chondrocytes, and bone cells originate from mesenchymal stem cells (MSCs). Adipogenesis is initiated when these progenitors cells undergo progressive fate restrictions to become committed preadipocytes.[16] In most species, including humans, BAT develops from a precursor cell distinct from that of WAT during late fetal life. The proliferation of precursor cells, their commitment to the brown adipose lineage, and their ultimate differentiation into functional brown and beige adipocytes are all parts of the natural process.

The differentiation of new brown adipocytes is regulated by various extracellular signals, including multiple hormones and growth factors such as norepinephrine (NE), thyroid hormones, insulin, and insulin-like growth factor 1 (IGF-1).[13] NE, which promotes brown adipocyte differentiation by activating a transcriptional cascade that controls the expression of genes necessary for the development of a brown adipocyte phenotype is possibly one of the most potent and well-studied adipogenesis signals. Although today is still controversial, these precursor cells would arise from MSCs, that express certain transcription factors such as Engrailed Homeobox 1 (En1)[17] and myogenic factor 5

(Myf5), which also give rise to skeletal muscle cells[18]. Therefore, this would indicate a close developmental relationship between brown adipocytes and skeletal muscle cells.

It is known that that both white and brown/brite adipocytes share a transcriptional cascade that controls the adipogenic process, although the differentiation of precursors cells into brown/brite adipocytes requires of specific factors for the development of the distinct phenotype and function of BAT.

The starting point of this specific network is the expression of the PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM16) protein, which is responsible for the developmental switch between brown adipocytes and myocytes but also functions as transcriptional co-regulator. In addition to these factors, it also includes CCAAT/enhancer binding proteins (C/EBPα and C/EBPβ) and peroxisome proliferator activated receptor γ (PPARγ). Forming a complex with C/EBPβ, PRDM16 induces the differentiation of precursor cells into brown/brite adipocytes inducing the thermogenic program by the interaction with several transcription factors such as C/EBPβ, PPARγ, PGC-1α and C-terminal binding proteins.[19] Moreover, PRDM16 seems to enhance nuclear receptor-dependent transcription of the brown fat-specific *Ucp1* gene.[20] PGC-1α plays as an important role of regulating the mitochondrial biogenesis and adaptive thermogenesis, and its homolog PGC-1β, which maintains the basal mitochondrial function.[21] PGC-1α was identified as a cold-inducible co-activator of PPARγ in brown adipocytes and drives the synthesis of UCP1 by stimulating its promoter during adipogenesis.[22] In contrast, depletion of PRDM16 in brown fat cells causes a near total loss of the brown features.[23]

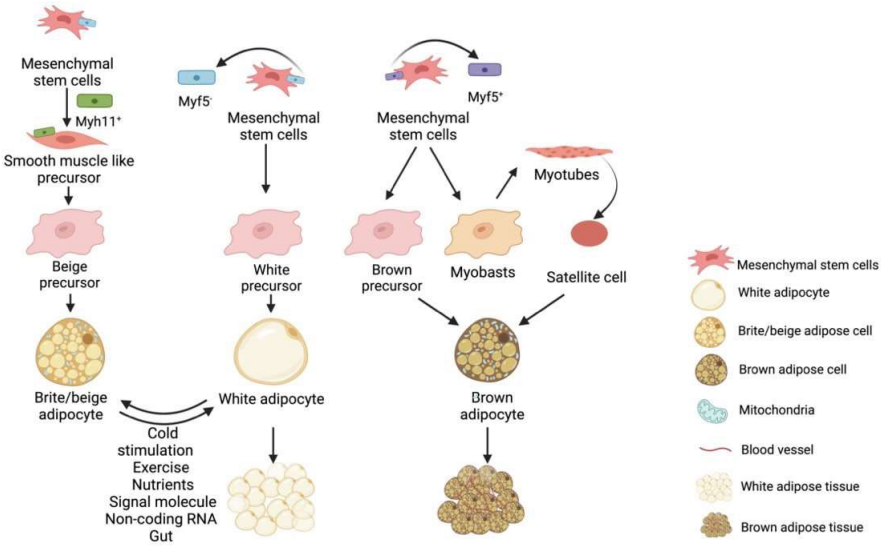

Figure 5. Ontogeny of adipose tissues in mammals. *Numerous hormones, growth factors and transcription factors participate in the determination and differentiation of the white, brite and brown adipocytes. Figure extracted from* [24].

1.2.2.3. Physiopathological implication of brown adipose tissue function

The attractive hypothesis that recruitment and activation of BAT and UCP1 could play an important role in the therapy of obesity and type 2 diabetes in humans is gaining acceptance.[25] It has been proven that mice that have UCP1 inactivated (*Ucp1*$^{-/-}$ mice) present a loss of non-shivering thermogenesis, which results in increase of the sensitivity and resistance to cold and increases susceptibility to obesity in response to high fat diet.[26] In addition, animals that lack the hormone leptin become obese due to the increase of food intake and the decrease of thermogenesis. Although the direct effects of leptin on BAT activity are still poorly explored and there is not much evidence, the action of leptin on thermogenesis appear to occur largely via an indirect central response.[27,28] Alterations in glucose and energy homeostasis have been also related to lower BAT mass and activity in humans. Furthermore, the activity of BAT is inversely correlated with body-mass index, suggesting a potential role of this tissue in adult human metabolism.[29]

Considering this knowledge, BAT activation or browning stimulation of WAT may help to reduce fat mass and enhance metabolic health since the beneficial effect of BAT activation is due to increased energy expenditure. BAT transplantation promotes fatty acid oxidation, which improves metabolic indices including IR and liver steatosis, according to several research carried out in rat models.[30] Additionally, when mice are treated with agonists of the β_3-adrenergic receptor, which induce BAT activity and increase thermogenesis and promote the differentiation of precursor cells into brown adipocytes, improve obesity and glucose homeostasis.[31] Treatment with mirabegron, a β_3-adrenergic receptor agonist, has also been shown to significantly improve multiple measures of glucose homeostasis in obese and insulin-resistant humans.[32]

These evidences indicate that increasing BAT activity or amount may be a potential strategy for the treatment of obesity and its associated conditions to restore the energy balance and reduce obesity. Despite these encouraging results and promising treatment, adrenergic stimulation in humans has significant negative effects on the cardiovascular system, although its seems that mirabegron is considered the most promising and safest β3AR agonist currently available.[33] Therefore, in order to create novel therapies for obesity and associated diseases, we require more insight and a profound understanding of the pathways and chemicals involved in BAT activation or recruitment.

1.2.2.4. Endocrine role of brown adipose tissue

Even though BAT has historically been thought that the only function was a heat-producing metabolic energy consumer, recent studies have demonstrated that BAT is a functional endocrine organ that can secrete a wide range of adipokines.[34,35] Interestingly, is noted that many of the adipokines found in WAT have been found in BAT as well, albeit in variable levels. As BAT is composed of both brown adipocytes and stromal vascular fraction (SVF), it is plausible that adipokines are secreted by both fractions. It is assumed that BAT-SVF contains a variety of cell types like WAT, including immune cells and preadipocytes, although the specific composition remains unclear. Therefore, the effects of adipokines may differ depending on their cellular source and their peripheral target tissues, that could be white adipose tissue, liver, pancreas, heart, and bone, as well as have an effect on the systemic metabolism by interacting with the central nervous system (CNS).[34]

Understanding the signalling pathways by which adipokines control metabolism is crucial for the development of new treatments for metabolic diseases like obesity and related illnesses because some adipokines could play a protective role in other organs as well as in the differentiation and function of BAT itself.

1.3. Retinoic acid-related orphan receptors

1.3.1. Structures and mechanisms of action

The retinoic acid-related orphan receptors (RORs) alpha, beta, and gamma (RORα-γ encoded by RORA-C) constitute a subfamily of nuclear receptors (NRs). NRs are ligand-dependent transcription factors, such as thyroid hormone and sterol receptors, that regulate the expression of genes related to a wide range of biological processes, including cell proliferation, differentiation, inflammation, metabolism, development, and homeostasis of the organism.[36] The natural ligands of about half of the NRs are known, however, the other receptors, including RORs, are categorized as orphan nuclear receptors since their corresponding ligands have not yet been discovered.[37]

RORs exhibit a typical nuclear receptor structure consisting of four functional domains (Figure 6). These domains include an N-terminal domain, which confers DNA binding specificity to the various ROR isoforms; a highly conserved DNA-binding domain (DBD) containing two zinc fingers (C domain); a ligand-binding domain (LBD), facilitating the binding of co-activator and/or co-repressor to the receptor; and a hinge region spacing the DBD and LBD. RORs regulate transcription by binding as monomers to ROR

response elements (ROREs) in the regulatory regions of target genes and the recruitment of co-activators or co-repressors. This ROREs region consists of the RGGTCA consensus sequence.[36]

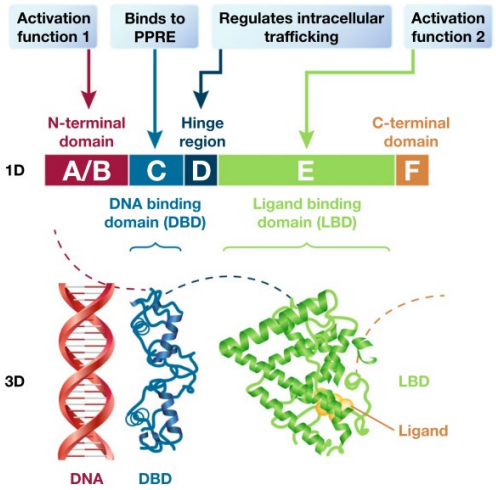

Figure 6. Structural organization of ROR functional domains. *Schematic diagram of the domain structure of RORs, similar to other NRs. Figure extracted from* [38].

The ROR family members are encoded by genes that uses alternative promoters and differential splicing to produce distinct isoforms, that regulate various biological processes and target genes. The *Rora* gene generates four isoforms, RORα1-4, while *Rorb* (RORβ1-2) and *Rorc* (RORγ1-2) each generate two isoforms. Still, the function of some of the mentioned isoforms are yet unknown.[39] Natural ligands or synthetic compounds may be able to modulate its transcriptional activity. Typically, ligands induce conformational changes in the receptor that cause co-repressors to dissociate and co-activators to bind. Nevertheless, RORs are constitutively active, which means they are in an active conformation even when not bound by a ligand (Figure 7). This raises the possibility that ligand interaction may repress their activity, acting as inverse agonist.[40]

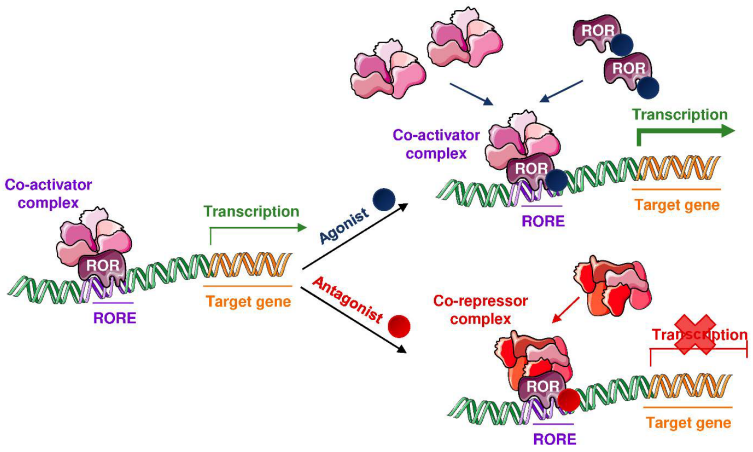

Figure 7. Regulation of ROR activity with synthetic ligands, acting as agonist or inverse agonist. *Figure adapted from* [40].

Multiples studies have found that natural oxysterols or intermediates of the cholesterol synthesis pathway are endogenous modulators that decrease ROR transcriptional activity, even though the identification of the endogenous ligands for RORs has been contentious. Indeed, 7-oxygenated sterols operate as inverse agonists by binding to both RORα and RORγ isoforms and inhibiting their transactivation capabilities. Many other endogenous RORα and RORγ ligands have been identified as agonists or inverse agonists, such as 25-hydroxycholesterol (25-OHC) and 24 S-hydroxycholesterol (24S-OHC), respectively.

Additionally, some synthetic drugs with inverse or agonistic effect on RORs activity have been reported. Understanding the crystal structure of RORγ allowed to develop new synthetic ligands. The selective modulator SR2211 (1,1,1,3,3,3-hexafluoro-2- (2-fluoro-4'- ((4-(pyridin-4-ylmethyl) piperazin-1-yl) methyl)-[1,1'-biphenyl]-4-yl) propan-2-ol) has been described as a potent and effective RORγ inverse agonist, which suppresses around 95% of its activity. This ligand has the potential utility for the treatment of autoimmune disorders, due to the inhibition of production of IL-17 in cells.[41] Moreover, it has been identified a novel RORγ-specific synthetic ligand, SR1555 (1-[4-[[4-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl) phenyl] phenyl] methyl] piperazin-1-yl] ethenone) as an inverse agonist of RORγ activity. Is reported that SR1555 can target both suppression of TH17 and stimulation of T regulatory cells.[42]

Otherwise, the synthetic drug SR1078 (N-[4-[2,2,2-trifluoro-1-hydroxy-1- (trifluoromethyl) ethyl] phenyl] -4-(trifluoromethyl)-benzamide) acts as a RORγ agonist, although it is less specific because it also activates RORα. Thus, SR1078 can be utilized as a chemical tool to probe the function of these receptors both *in vitro* and *in vivo*.[43]

Due to the known roles of RORs in metabolic and immune processes (see next section), there is significant interest in the identification of ligands that regulate the RORs because of their potential for clinical utilization as attractive new therapeutic targets.

1.3.2. Role of RORγ

The RORγ gene, *Rorc*, generates two different proteins, RORγ1 and RORγ2 (RORγt), through alternative promoter usage and differential splicing. Thus, these isoforms have distinctive expression patterns that are tissue specific. RORγt is only exclusively high expressed in the thymus and certain sub-populations of immune cells[39], whereas RORγ1 is extensively expressed in liver, adipose tissue, skeletal muscle tissue, kidney, and pancreatic β cells.[37,39]

On one hand, RORγt plays a critical role in regulating immune system processes, including lymph node organogenesis, thymopoiesis, and T cell lineage specification. RORγt is essential for the mentioned immunological functions and, for instance, the development of Peyer's patches (PPs) too by promoting the survival of lymphoid tissue inducer cells (LTi), as evidenced by the absence of lymph nodes and PPs in mice deficient in RORγt expression.[39] PPs are bundles of lymphatic cell nodules that develop only in the ileum, the lowest portion of the small intestine. Their function involves the complex interplay of the mechanisms that aim to recognize luminal antigens and induce an immunological response.[49]

Furthermore, RORγt also participates in CD4+ T cells differentiation into pro-inflammatory Th17 lineage by inducing genes like *Il17* or *Il22*, along with signal transducer and activator of transcription 3 (STAT3) and RORα. The fact that RORα also regulates the Th17 differentiation suggests a degree of functional redundancy between RORγt and RORα.[39] Interestingly, an increment of RORγt activity can be beneficial due to the enhance of antitumor immunity by increasing IL-17.[50]

On the other hand, the function of RORγ1, in contrast to RORγt, is not well understood. Studies have shown that RORγ1 plays a role in regulating genes related to lipid metabolism in the liver. The loss of RORγ1 in mice results in significantly reduced expression of several hepatic genes, including *insulin induced gene 2a* (*Insig2a*), *elongation of very long chain fatty acids 3* (*Elovl3*), *cytochrome P450 family 8 subfamily B member 1* (*Cyp8b1*) and *hepatic lipase C* (*Lipc*). The reduction of FFA, glycerol, and total ketone bodies in RORγ$^{-/-}$ mice suggests lower fatty acid consumption, even though triglycerides (TAG) in serum and liver were reduced. Instead, it is most likely the result of a slower rate of fatty acid/TAG synthesis. Additionally, it seems that RORγ1 promotes gluconeogenesis by regulating the expression of g*lucokinase* (*Gck*)*, glucose 6-phosphatase* (*G6Pase*)*, glycogen synthase 2* (*Gys2*)*, phosphoenolpyruvate carboxykinase* (*Pepck*)*, and peroxisome proliferator-activated receptor delta* (*Pparδ*), which regulates several genes involved in glucose and lipid metabolism.[51]

Many nuclear receptors, including RORs, display, at least in some tissues, rhythmic patterns of expression during the circadian cycle, indicating that their expression is controlled by the circadian clock.[39,51] At the molecular level, the clockwork consists of an integral network interconnected by negative feedback and positive feedforward loops.[39] The molecular circuitry of circadian clocks is similar across tissues. RORγ1 is involved in the circadian clock in several ways. In tissues including the liver, BAT, pancreatic β cells, and kidney, but not in WAT, it exhibits an oscillatory expression pattern that is controlled by circadian clock elements.

RORγ1 is recruited to the promoters of various clock genes containing ROREs, such as *Bmal1, nuclear receptor subfamily 1 group D member 1* (*Rev-Erbα*)*, cryptochrome 1* (*Cry1*)*, and circadian loco- motor output cycles kaput* (*Clock*), although its loss has no major impact on their expression or the circadian rhythms.[52] Overall, the role of RORγ1 in regulating lipid metabolism and the circadian clock requires further investigation.

As mentioned above, RORγ1 is found in both WAT and BAT. RORγ1 has been associated to metabolic disorders such as metabolic syndrome, IR, and glucose intolerance, according to several studies.[53] Thus, it was observed that RORγ1 expression in adipose tissue, particularly in the visceral depot, significantly increased in people with obesity and high degree of insulin resistance. In addition, it was also demonstrated that RORγ1 is an exclusive marker of the adipocytes, and not of the immune cells, which are known to accumulate in the SVF in the adipose tissue of the obese and obese-insulin resistant patients. This increase is linked to high levels of TAG, insulin, and leptin, but low levels of HDL and cholesterol.[53] According to this study, RORγ1 could contribute to adipocyte hyperplasia and hypertrophy, and that it is negatively associated with insulin sensitivity and good health status.

However, the specific role of RORγ1 in adipocyte function has been the subject of debate in studies using RORγ KO mice, yielding conflicting results. While some studies found no differences in WAT mass or adipocyte size[54], others discovered increased numbers of adipocytes, lower WAT mass, and improved insulin sensitivity[55]. In another study[56], it is shown that deletion of RORγ results in reduced body weight and a decrease in lipid biosynthesis genes expression, such as *sterol response element binding protein* (*Srebp*) and *peroxisome proliferator-activated receptor alpha* (*Ppara*), but a decrease in the expression of *acetyl-CoA carboxylase 2* (*Acc2*). These contradictory findings make it difficult to unravel the function of RORγ1 in adipose tissue and its potential implications for metabolic pathologies such as obesity and diabetes.

2. HYPOTHESIS

According to the laboratory's preliminary results and to investigate the potential contribution of RORγ1 to brown adipocyte function, the expression of *Rorc* in BAT was evaluated. Results showed that *Rorc* levels were 2-fold higher in BAT than WAT, even though the results were not statistically significant. Furthermore, results in our laboratory reported that *Rorc* expression is very low in pre-confluent and confluent preadipocytes and dramatically increases during differentiation of immortalized brown adipocytes cell line (iBA) (Figure 8). Taken together these findings, we hypothesize that:

The nuclear receptor RORγ1 could play an essential role in regulating the differentiation of brown adipocytes, contributing to the acquisition of their functional properties.

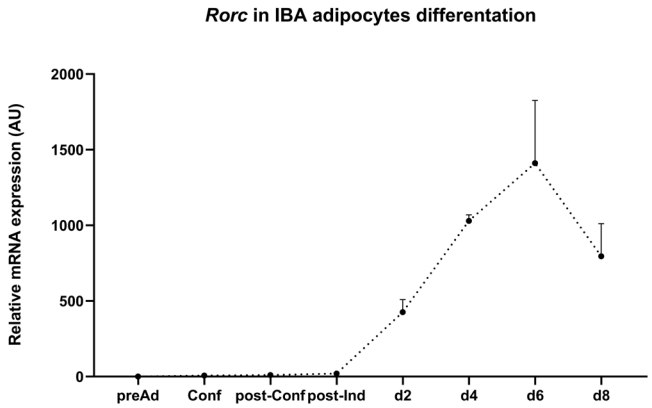

Figure 8. Gene expression analysis of RORγ1 during iBA cells adipogenesis. *mRNA expression of Rorc was assessed by real-time quantitative PCR. Pre, pre-confluent; C, confluent; Post, post-confluent; d, differentiation day. Results are expressed as mean ± SEM (n=4). Unpublished results from our laboratory.*

3. OBJECTIVES

Therefore, the main objective of the proposed study is to elucidate the role of RORγ1 in brown adipocytes. In order to prove our hypothesis, we propose three specific aims:

Aim 1. Set up the transfection technique of immortalized brown adipocyte cell line (iBA).

Aim 2. Carry out the silencing of *Rora* and *Rorc* expression using small interfering RNAs (siRNAs) and the inhibition of RORγ1 activity by RORγ inverse agonists in iBA preadipocytes.

Aim 3. Study the effect of silencing and inhibiting RORs on brown adipocyte differentiation and function, determining the morphological changes and the gene expression profiling of brown fat markers in both preadipocytes and adipocytes.

4. MATERIALS AND METHODS

4.1. iBA cell culture procedures

4.1.1. iBA cells subculture

Immortalized brown adipocytes (iBA) cells were a gift of Dr. S. Pedrotti from San Raffaele Scientific Institute (Milano, Italy). iBA cells were cultured in Dulbecco's Modified Eagle Media/Nutrient Mixture F-12 (DMEM F-12) supplemented with 10% Fetal Bovine Serum (FBS) and 1X antibiotic-antimycotic (10000 units/mL penicillin, 10000 µg/mL streptomycin, and 25 µg/mL amphotericin B). Cells were incubated at 37°C, 95% humidity and 5% CO_2. The culture media and supplements were purchased from Thermo Fisher Scientific.

Cells were passed every two days to avoid reaching confluence. Once they reach 60-70% confluence, the media was removed, and cells were rinsed with 5 mL of phosphate buffered saline (PBS), prepared in the laboratory. Next, the cells were incubated with 500 µL of TrypLE Express (Trypsin homolog from Invitrogen) for 3-4 minutes at 37ºC until cell layer was detached from the dish. Once the cells were detached, the trypsin was neutralized with 8 mL of DMEM-F12 media. 1 mL or 1.33 mL of this cells were seeded in a new 10cm culture dish with 10 mL of DMEM-F12 media to make a 1/8 and a 1/6 dilution, respectively.

4.1.2. iBA cells differentiation

The differentiation of iBA preadipocytes into brown adipocyte takes within 10 and 13 days (Figure 9). In first place, cells were grown in proliferation media and allowed to reach confluence before being differentiated. One day after confluence, fresh induction media (DMEM-F12 with 10% FBS, 1X Antibiotic-Antimycotic, 500 µM 3-Isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, 20 nM Insulin, and 1 nM T_3) was used in place of the proliferation media (Day -2 of differentiation). IBMX reagent was prepared in the laboratory, whereas T_3 hormone was obtained from Sigma-Aldrich.

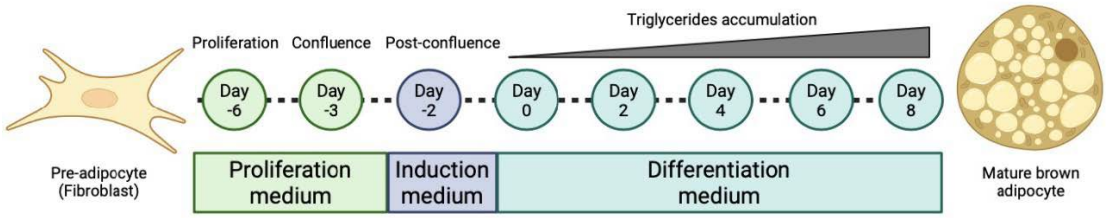

Figure 9. Scheme of protocol for brown adipocyte differentiation.

After two days on induction media (Day 0 of differentiation), media was replaced with differentiation media (DMEM with 10% FBS, 1X Antibiotic-Antimycotic, 20 nM insulin, and 1 nM T_3). Cells were then cultured for 6 to 8 days in differentiation media, which was changed every 48 hours. On Day 2 of differentiation, cells already began displaying a full brown adipocyte-like morphology, characterized by a cytoplasm containing multiple of lipid droplets of various sizes.

4.2. siRNA transfection of iBA preadipocytes

Since *in vivo* laboratory studies suggest that RORα could functionally compensate for the loss of RORγ1, the study was also performed by silencing RORα individually or together with RORγ1. In the present study, to knockdown *Rora* and *Rorc* genes in iBA cells we used specific chemically synthetized small interfering RNAs (siRNAs) that specifically target RORα and RORγ1 coding RNAs.

First, a positive control of the siRNA transfection was performed using siGLO Red Transfection Indicator (Horizon Discovery) that is labelled with a fluorochrome to monitor internalization of siRNA. To silence these genes, iBA preadipocytes were transfected using DharmaFECT 4 reagent (Horizon Discovery) with ON-TARGETplus SMART pool siRNAs specifically targeting *Rora* (siRORa), *Rorc* (siRORc) or both simultaneously (siRORa/c). These siRNAs consist in a pool of four different siRNA targeting *Rora* or *Rorc*, respectively, so that their specificity and potency are enhanced. ON-TARGETplus Non-Targeting siRNA#2 (siNT) was used as negative control and purchased from Horizon Discovery. A siRNA against NRF1, the nuclear respiratory factor 1, (siNRF1) was also used as a positive control. siRNAs targeting Rora, Rorc and NRF1 were designed and obtained from Thermo Fisher Scientific.

iBA preadipocytes were seeded in a 12-well dish with a confluency of 60-70%. One day after the seeding and about 6-7 hours before staring the transfection, the media was changed to 2 mL/well DMEM F-12 supplemented with 10% FBS, but no Antibiotic-Antimycotic.

In previous transfection optimization for white preadipocytes at the laboratory, the optimal concentration of siRNA to use was 50nM. Therefore, the final concentration of siRNA in each well was 100 nM to equilibrate the total RNA quantity: 100 nM siNT; 50 nM siRORa and 50 nM siNT; 50 nM siRORc and 50 nM siNT; 50 nM siRORa and 50 nM siRORc. DharmaFECT 4 mix (Tube 1) and siRNAs mixes (Tube 2) were prepared as indicated in the Table 1. Once mixes were done, they were incubated 5 min at RT. Then, the content of both tubes was mixed and were incubated 20 min at RT. The next step

was adding 160 µL of the total mix to each well containing iBA preadipocytes in 840 µL of DMEM F-12 supplemented with 10% FBS, but no Antibiotic-Antimycotic. Following an overnight incubation of 16 hours, the media was changed to complete DMEM F-12.

Table 1. Content of each tube to prepare the transfection mix. *The siRNA tube (Tube 1) and the DharmaFECT 4 tube (Tube 2) were diluted in OptiMEM media (Thermo Fisher Scientific). The volume of each reagent is indicated.*

		1.4 µL/cm² DharmaFECT – 12-well dish (4 cm²/well)							
		Control		**RORα**		**RORγ**		**RORα/γ**	
		siNT	**siNRF1**	**siRORa**	**siNT**	**siRORc**	**siNT**	**siRORa**	**siRORc**
Final concentration		100 nM		50 nM	50 nM	50 nM	50 nM	50 nM	50 nM
Tube 1	siRNA 5 µM	20 µL		10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
	OptiMEM	60 µL		60 µL		60 µL		60 µL	
Tube 2	DharmaFECT 4	5.6 µL							
	OptiMEM	74.4 µL							
Tube 1 and 2 volumes		160 µL/well							
Media without Antibiotic-Antimycotic		840 µL/well							
Total transfection volume		1000 µL/well							

4.3. Pharmacological treatment of iBA cells

To elucidate the function of RORγ1, iBA cells also were treated with synthetic ligands with RORs agonist or inverse agonist activity during the induction and differentiation phase. iBA preadipocytes were seeded in a 24-well dish with a confluency of 60-70% and allowed to achieve confluence before being differentiated. One day after confluence, the media was changed to fresh induction media supplemented with the synthetic drugs at 1 µM and 10 µM: RORα/γ agonist (SR1078) and RORγ inverse agonists (SR1555 and SR2211). After two days, the media was changed to differentiation media also supplemented with the synthetic drugs and changed every 48 hours. SR1078, SR1555 i SR2211 were acquired from Sigma-Aldrich.

4.4. Gene expression analysis

4.4.1. RNA isolation from cell culture

RNA isolation from iBA cells was performed by using GeneJet RNA purification Kit (ThermoFisher Scientific). To isolate RNA was used RNase-free solutions and materials to avoid RNA degradation by RNases and preserve its integrity. First, the media from the dish was discarded and the iBA cells were lysed by adding 0.5 mL NZYol reagent. The

following step was the cell harvest by scraping the cell monolayer and storing the samples on ice. Afterwards, 200 µL of chloroform for 1 mL of NZYol were added to each sample, followed by centrifugation at 12000 g for 15 minutes at 4 °C to separate the homogenate in three phases: an RNA-containing upper aqueous phase, an interphase composed by proteins and DNA, and a lower organic phase, composed by cell debris. The aqueous upper phase was transferred to a new tube and one volume of 70% ethanol was added to allow the precipitation of RNA. Then, samples were placed in RNeasy columns and were cleaned with Kit-containing alcohol buffers, discarding the flow-through between centrifugations. Finally, RNA was eluted in 30 µL of RNase-free water and stored at -80ºC for further analysis.

4.4.2. RNA quality control

After extraction, RNA concentration and purity were determined in a NanoDrop ND-2000 spectrophotometer. NanoDrop measures the absorbance of the sample at 260nm, where RNA shows maximum absorbance, and at 280nm and 230nm, to detect possible contaminants such as phenol or proteins, respectively. To assess RNA purity, we looked at A260/A280 ratio and A260/A230 ratio. In our study, only samples with an A260/A280 ratio ≥ 1.8 and A260/A230 ratio ≥ 2.2 were accepted.

To assess the integrity of previously quantified RNA samples, 200 ng of RNA were run on a 1.2% agarose electrophoresis gel in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) stained with ethidium bromide (Sigma-Aldrich). All the others reagents were prepared in the laboratory. If RNA's integrity is correct, two bands corresponding to the 28S rRNA and 18S rRNA should be observed, which is evidence of non-degraded RNA. Although, the band corresponding to 5S rRNA is lost when RNA extraction method is performed using silica gel columns.

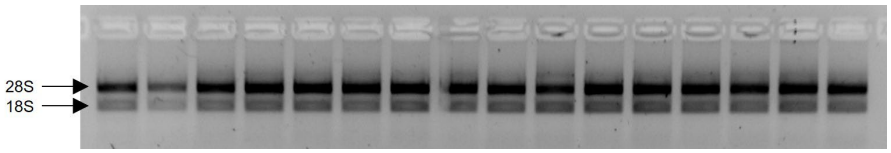

Figure 10. Results from electrophoresis to RNA quality control. *Presence of 28S and 18S bands prove that RNA is not degraded.*

4.4.3. Reverse transcription

In order to be amplified and analysed by real-time quantitative polymerase chain reaction (qPCR), RNA must be transcribed into complementary DNA (cDNA). This was performed by using a commercial kit for reverse transcription (SuperScript II Reverse Transcriptase, Invitrogen). For this, 400ng RNA were mixed with 0.5 µg Oligo(dT) primers and DEPC water. The mix was incubated at 65 °C for 10 minutes to denature RNA and chilled at 4ºC to allow its annealing with Oligo(dT) primers.

Then, another mix with a final concentration of 1x First-Strand Buffer, 0.01 M DTT, 1 µL 0.5 mM dNTPs and 12.5 U/µL SuperScript II RT were added into the samples and were incubated at 42°C for 50 minutes to allow cDNA extension followed by an incubation at 70ºC for 15 min to inactivate RT. Resulting samples were diluted in 20 µL of DEPC water and stored at -20°C.

4.4.4. Real-time quantitative PCR

Gene expression was determined by qPCR using SYBR Green (ThermoFisher Scientific) as a detection dye in LightCycler® 480 System (Diagnostics Roche). To perform the technique, mix SYBR green (2x) was mixed with Forward primer (5.5 µM), Reverse primer (5.5 µM), DEPC water and 2 µL of previously obtained cDNA. Each pair of primers was specific for each gene evaluated (Table 2). The program used for the qPCR was 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles with the following conditions: 20 seconds at 95°C, 20 seconds at 60°C and 34 seconds at 72°C. Finally, cDNA levels for the gene of interest were normalized by the comparative CT($2^{-\Delta\Delta CT}$) method, using *Cyclophilin A* (*Cyclo A*) as a reference gene.

Table 2. List of primes used for real-time quantitative PCR (RT-qPCR). *These primers were designed and obtained from Integrated DNA Technologies.*

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Rora	GTGGAGACAAATCGTCAGGAAT	GACATCCGACCAAACTTGACA
Rorc	CGACTGGAGGACCTTCTACG	AGAGCTCCATGAAGCCTGAA
Cyclo A	ATGGGGTAGGGACGCTCTCC	CAAGACTGAATGGCTGGATG

4.5. Statistical analysis

All results are presented in figures and tables as mean ± SEM. Data was represented with the statistics software GraphPad Prism v8.0. To determine whether there were significative differences between experimental groups, a Student's t test and ANOVA were used. Differences were considered significant when $P \leq 0.05$.

5. RESULTS AND DISCUSSION

To study the role of the nuclear receptor RORγ1 on the differentiation and functionality of BAT, it is necessary to have a good model of brown adipocyte cells that exhibit all molecular and functional features of brown adipocytes. In previous studies in the laboratory, the cell line of immortalized brown adipocytes (iBA) was characterized and validated. These cells, after differentiation, have the typical brown adipocyte morphology. So, to perform our study we use the iBA cell line as the cellular model.

5.1. Transfection validation of iBA preadipocytes

As the first aim is to set up the lipofection-based siRNA transfection protocol and demonstrate that iBA cells can be transfected without interfering with adipogenesis, iBA preadipocytes were transfected with siGLO, a fluorescent oligonucleotide duplex that allows visual assessment of siRNA uptake into the cells. Therefore, we use siGLO as an indicator of transfection success in our experiment requiring synthetic RNA delivery.

The siRNA transfection protocol in the iBA cells line is based on the use of a cationic lipid-based reagent called DharmaFECT 4. The method consists in introducing siRNA into iBA preadipocytes using an approach based on forming the siRNA/DharmaFECT complex with the preadipocytes attached to the culture dish. Previously in the laboratory, the maximal transfection efficiency in 3T3-L1 cells, a white adipocyte cell line, was achieved using a siGLO concentration of 100 nM. However, it was established that the optimal siRNA concentration was 50 nM because it was observed that 90% of cells were transfected by using that concentration. Thus, considering the relation between transfection efficiency and the amount of siRNA used, iBA preadipocytes were transfected with siGLO at 50 nM. In Figure 11, we observe the broad uptake of the fluorescent-labelled siGLO (red) by iBA preadipocytes, demonstrating that iBA preadipocytes can be transfected with a siRNA concentration of 50 nM.

When the iBA cells were transfected, we observed that siNT and siGLO cells showed different morphology. This might was due to the different day of the cell photo. siGLO cells photo was taken one day later than siNT cells photo, because we still did not see enough fluorescence in siGLO cells, and we left it one more day increasing the transfection time by 48 total hours. We understood that the difference probably came from that delay day that could have affected siGLO differently than siNT.

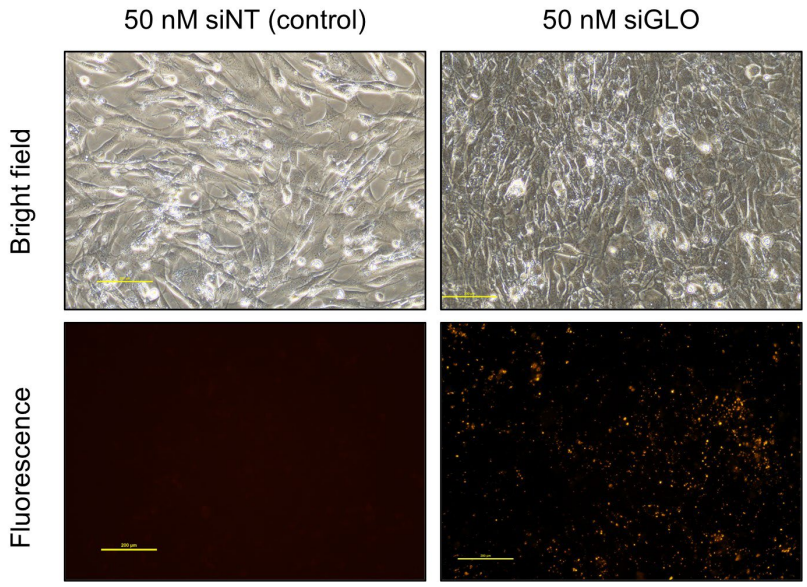

Figure 11. Assessment of iBA preadipocytes transfection with siRNAs. *Bright field and fluorescent images of adipocytes transfected with 1,4 µL/cm² of DharmaFECT 4 with 50 nM siGLO and 50 nM siNT, as control, are shown. siNT cells images were taken after 24 hours of transfection, whereas siGLO cells images were taken after 48 hours of transfection.*

5.2. Effects of *Rora* and *Rorc* knockdown in iBA preadipocytes

5.2.1. Evaluation of the gene knockdown efficiency

To determine the effect of gene knockdown on brown adipocyte differentiation, we first assessed the efficacy. Thus, in the Figure 12 we indicate whether gene knockdown was achieved efficiently or not when iBA pre-adipocytes were transfected using an siRNA targeting *Rora* (siRORa), *Rorc* (siRORc) and both simultaneously at a concentration of 50 nM. As shown in the figure, the results obtained from qPCR show that there are contradictions and many variations between duplicates. The source of this variability could have been because it was the first experiment and first qPCR that I conducted, and experimental errors such as pipetting could have heavily influenced these results.

When we determinate the relative expression of *Rora* mRNA in iBA preadipocytes, we see that *Rora* expression is reduced by half in the *Rora* silencing experiment (siRORa), We can also see that the reduction in *Rora* mRNA levels is more extreme when we silence *Rorc*, so this result does not make much sense unless RORa regulates expression of RORc. As for *Rorc* expression in preadipocytes, it seems that there was a mild silencing of *Rorc*, but we did not have a duplicate that was not an outlier to properly analyze this result.

If we now look at the mRNA levels in iBA adipocytes, we see that something similar happens in *Rora* expression as in preadipocytes. There is less *Rora* expression when

we silence *Rorc* than when we silence *Rora*. In the simultaneous silencing (siRORa/c), it appears that *Rora* expression is greatly reduced but again we have no duplicate.

In contrast, *Rorc* mRNA levels in adipocytes indicate that there has been effective silencing of Rorc expression, as relative mRNA levels in siRORc cells are reduced to 0.16, compared to *Rorc* expression in the negative control cells (siNT). In this case, simultaneous silencing appears to be effective as the levels of *Rorc* in adipocytes are also reduced.

In addition, a statistical test of these results was performed, but no statistically significant values were obtained. Although we can see a certain trend in these results, we cannot affirm that *Rora* and *Rorc* knockdown in iBA preadipocytes had been effectively achieved. This is because the qPCR results were too variable and we could not draw any conclusion. This variability may have been because the transfection of the cells did not go well, either because of the conditions of the technique itself or experimental errors such as the accumulation of mistakes during the RT-qPCR. Therefore, we decided to repeat the experiment again in order to obtain analysable results, but no new results could be obtained due to contamination in the culture room in this repeat.

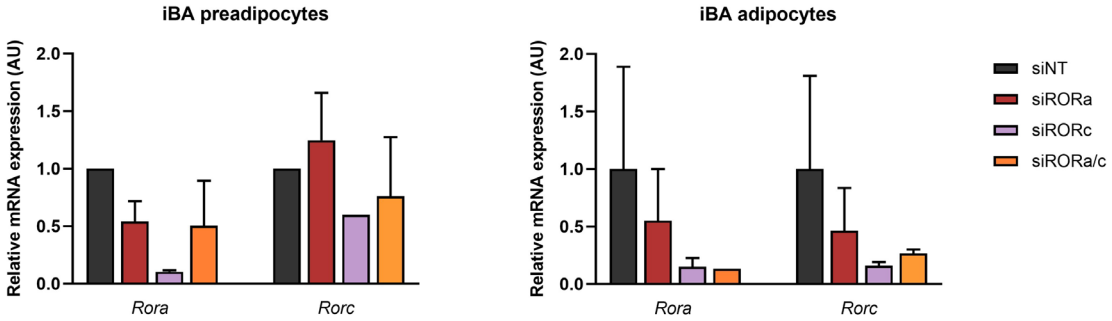

Figure 12. Assessment of efficient Rora and Rorc gene knockdown in iBA cells transfected with siRNAs targeting Rora, Rorc or both simultaneously. *mRNA levels of Rora and Rorc were assayed by qPCR in iBA preadipocytes and iBA adipocytes. Results are expressed as mean ± SEM of 3 experiments with duplicates.*

5.2.2. Morphological effects during adipocyte differentiation

Adipocytes exhibit different morphological and functional characteristics, depending on their anatomical location, developmental origin, and stimulus. The storage of triglycerides in numerous small droplets along the cytoplasm is a morphologically distinctive feature of brown adipocytes. An optical microscope was used to track morphological changes to see if iBA cells display this trait. Pictures in Figure 13 were taken before differentiation when iBA cells were still preadipocytes, and after differentiation. As seen in Figure 13, iBA preadipocytes exhibit a fibroblast-like

morphology without an accumulation of lipid droplets, whereas differentiated adipocytes take on a rounder shape and the brown adipocyte-specific lipid droplets can be seen dispersed throughout the cytoplasm.

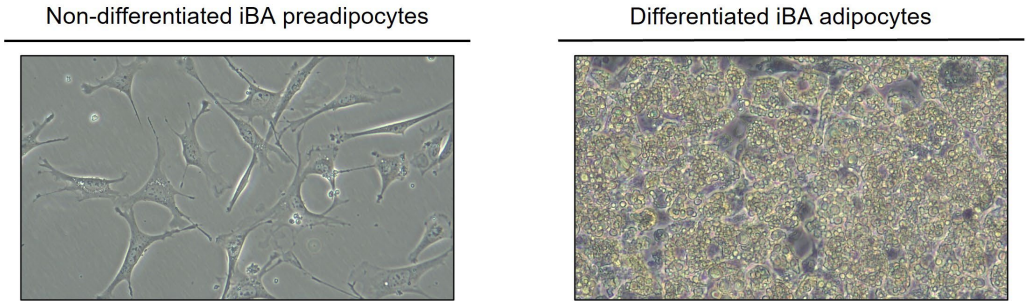

Figure 13. Non-differentiated and differentiated iBA morphology. *Non-stained images taken in a phase-contrast microscopy microscope.*

After describing the morphological differences between an iBA preadipocyte and its mature form, the following aim was to observe daily the morphological effect of *Rora* and *Rorc* knockdown on iBA preadipocytes during the adipocyte differentiation. Therefore, we leveraged that we had to repeat the previous silencing of the *Rora* and *Rorc* genes in iBA preadipocytes and perform qPCR again, to monitor daily the morphological differentiation of the cells.

In this second transfection of iBA cells, an additional well was seeded as a positive control using an siRNA against the transcription factor NRF1 (siNRF1), as it is known to be expressed in BAT both preadipocytes and adipocytes.[57]

In Figure 14 we can see the differences regarding the morphology of the cells after differentiation. If we first focus on iBA adipocytes that *Rora* gene have been silenced (siRORa cells), no significant morphological difference can be seen between these and the negative control cells (siNT cells). The results are compared only with the negative control because the transfection with siNRF1 (positive control) left a high cell mortality and cannot be analyzed.

On the other hand, we can observe that there are fewer siRORc cells that have differentiated into adipocytes compared to siNT cells. *Rorc* knockdown could be a cause of the lower acquisition of lipid droplets in the cytoplasm of these siRORc cells. It could also be since brown adipocytes can exhibit their morphological characteristics at different differentiation times from their immature form. Simultaneous *Rora* and *Rorc* silencing also did not obtain differential morphological changes in siRORa/c cells.

In order to analyse these results correctly, we have to take into account that the pictures taken of the adipocytes in Figure 14 were on Day 1 of differentiation, since on Day 2 of

differentiation the culture contaminated, and we had to stop the second experiment. Thus, the conclusions that we can draw from these results are biased by the impossibility of not being able to follow up until at least the end of differentiation, approximately Day 6. Unfortunately, we were also unable to extract RNA from the cells and analyse again the expression of *Rora* and *Rorc* by qPCR once differentiation was complete.

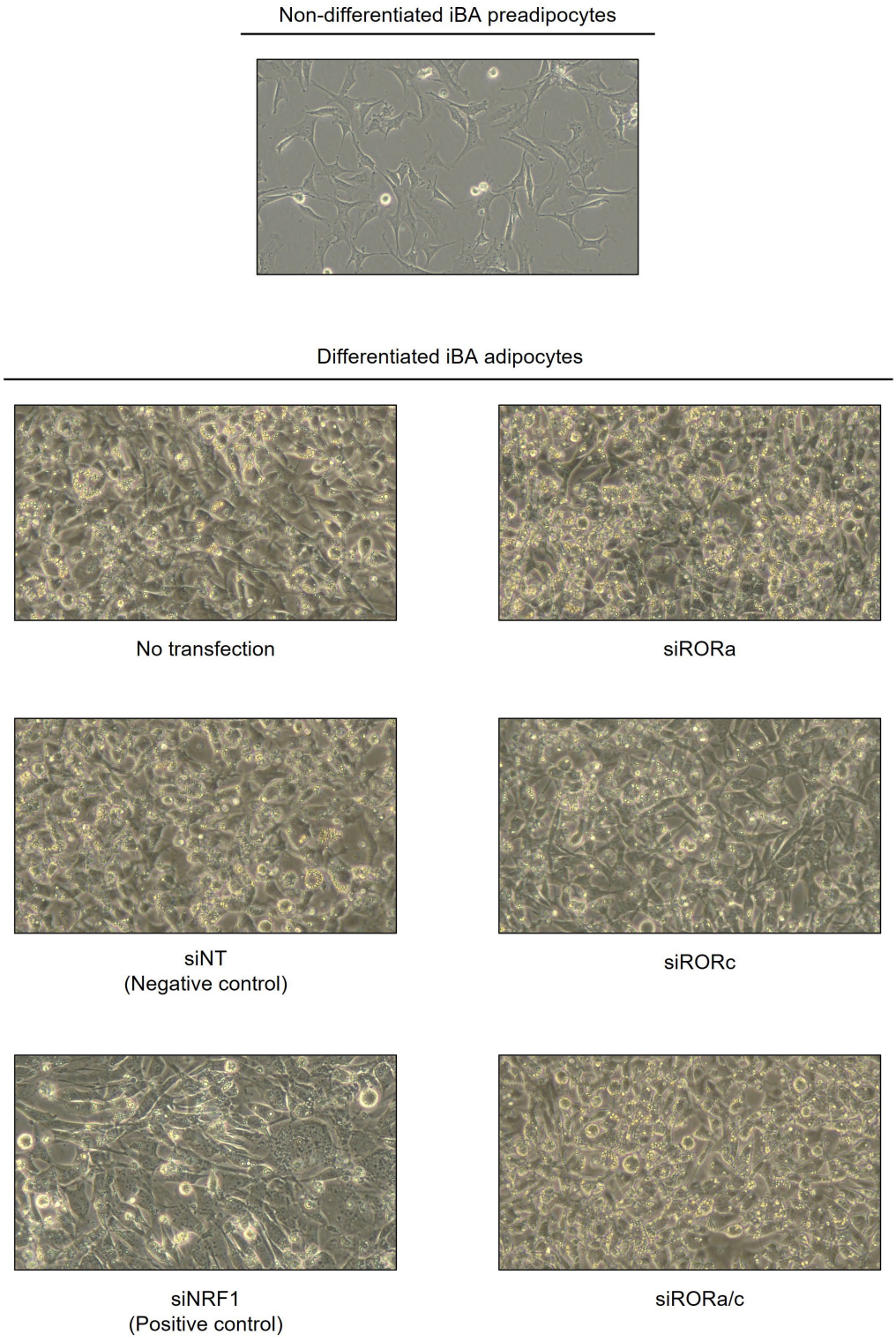

Figure 14. Rora and Rorc silencing in iBA preadipocytes and its effect in differentiation process. *Non-stained images taken in a phase-contrast microscopy microscope on Day 1 of differentiation process.*

5.3. Effects of the pharmacological treatment on RORα and RORγ function in iBA cells

5.3.1. Morphological effects during adipocyte differentiation

To elucidate the role of RORγ1 in brown adipocyte differentiation and function, we wanted to study the effect of several synthetic ligands with capacity to modulate (positively and negatively) this nuclear receptor. For this, we induced and differentiated iBA preadipocytes in presence of 3 different synthetic drugs at 1 μM and 10 μM: RORα/γ agonist (SR1078) and RORγ inverse agonists (SR1555 and SR2211, this last only at 1 μM). Drug treatment is given throughout the differentiation process, adding the drugs to the induction or differentiation media, respectively. According to bibliography and commercial properties, 10 μM of these synthetic drugs is sufficient to inhibit RORγ activity by 90%. Thus, we decided to treat iBA cells with RORγ agonist and invers agonists at 1 μM and 10 μM, to see if less concentration of drugs had the same inhibitory effect and less cytotoxic effect.

According to our results in Figure 15, the drug treatment resulted in high cell mortality in all conditions except in the untreated control. At the morphological level, we cannot analyze whether there are differences between agonist-treated and antagonist-treated cells. This is mostly caused by the fact that only some of the treated preadipocytes have survived and been able to start differentiating into adipocytes.

Furthermore, we have to take into account that our idea was to follow the cells daily to see the differentiation process in the two experiments, but due to contamination on day 2, we were not able to have more data, neither of the second silencing experiment nor of the pharmacological treatment, as the experiments were done in parallel.

The impossibility of not continuing the experiments is caused by contamination in the culture room, affecting also experiments of several groups. We carried out the contamination monitorization and containment. As happened in the previous *Rora* and *Rorc* knockdown experiment, the culture of iBA cells treated with drugs was also contaminated on Day 2 of differentiation and we had to stop the experiment too. We could not start another experiment again due to the lack of time in my laboratory internship. Therefore, the conclusions drawn here are not entirely sufficient as the iBA cells are not fully differentiated and we cannot observe and determine unequivocally the effect of the *Rora* and *Rorc* knockdown and the pharmacological treatment on RORα and RORγ function in iBA cells.

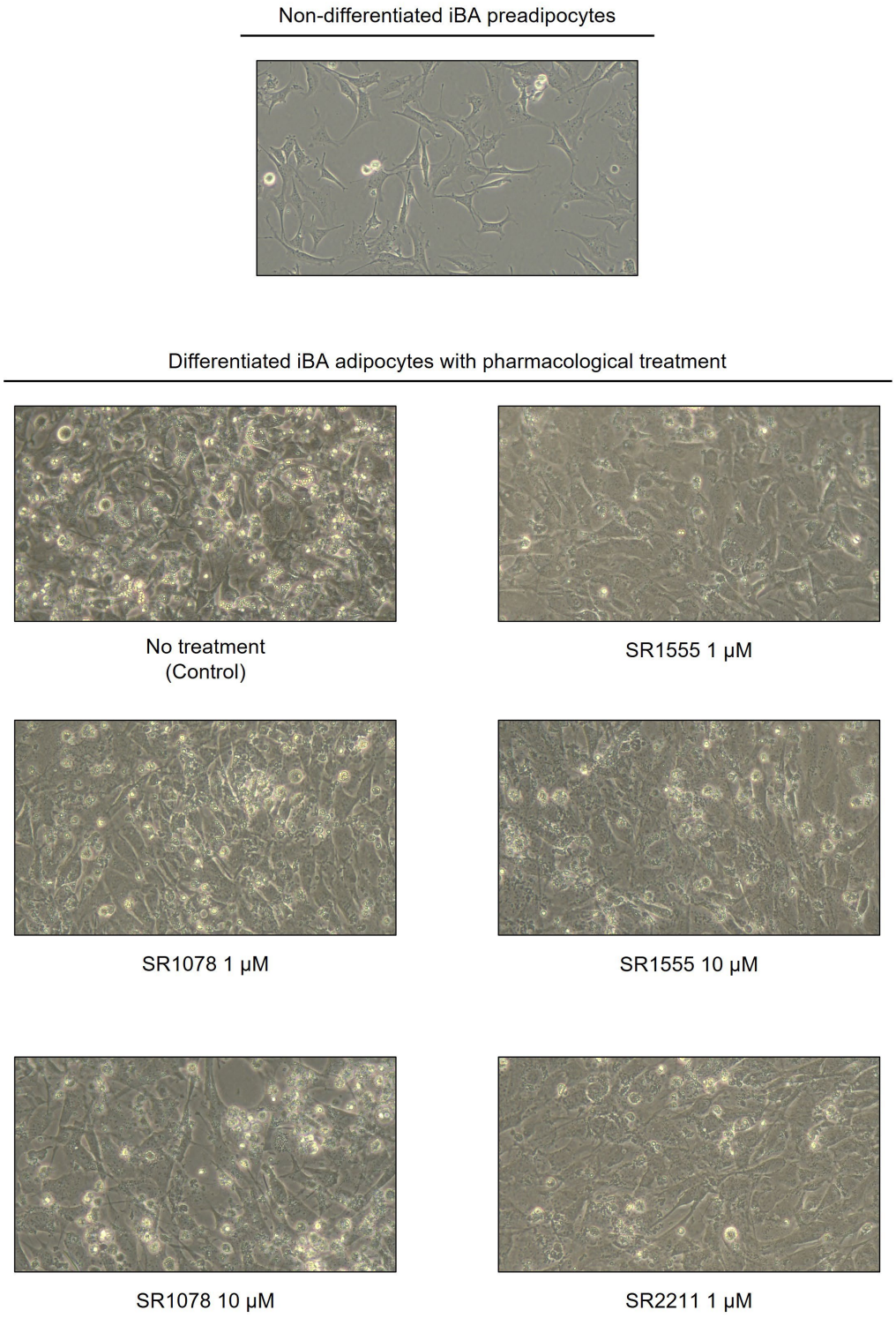

Figure 15. Pharmacological treatment in iBA cells differentiation process. *Non-stained images taken in a phase-contrast microscopy microscope on Day 1 of differentiation process.*

5.4. Gene expression profiling of brown fat markers

In this present study, the third aim was determining the expression profile of important genes in BAT, called brown fat markers, identifying the effect of inhibiting, or blocking the function of the nuclear receptor RORγ1, either by antagonists or gene silencing. Unfortunately, our experiments had to be interrupted due to contaminations and there was no time to produce more results.

Despite not having time to carry out the assessment, our idea was to focus on results obtained in previous studies in the laboratory. iBA cells were identified as expressing some specific markers characteristic of brown adipocytes during their differentiation, such as UCP1, the β3-adrenergic receptor (β3AR), DIO2 and PGC-1α coactivator. It was demonstrated that the expression level of these 4 genes increases as iBA cells differentiate in brown adipocytes. Several mitochondrial genes involved in thermogenesis followed the similar expression profile in iBA cells increasing during differentiation. These genes studied are related to fatty acid β-oxidation, the tricarboxylic acid cycle (TCA), and the oxidative phosphorylation system.

S. Austin et al.[58] demonstrated that RORα and RORγ1 mRNA were upregulated during adipocyte differentiation in preadipocyte D1 and 3T3-L1 cells, as well as our laboratory demonstrated in immortalized brown adipocytes (iBA) cells (Figure 8). They also observed that RORγ1 expression was inhibited by Tumor necrosis factor alpha (TNF-α) and Transforming growth factor beta 1 (TGF-β1), two known down-regulators of adipocyte differentiation. Furthermore, it was shown that after the expression of RORα and RORγ1 during the adipocyte differentiation process occurred an increase in PPARγ and C/EBPα expression, indicating a link between these factors and RORs. Moreover, overexpression of PPARγ resulted in an induction of RORα and RORγ1 in 3T3 cell line. Intend to continue in this way, it was also enhanced the expression of the fatty acid binding protein 4 (FABP4), also called aP2, a late marker for adipocyte differentiation. These results suggested that RORα and RORγ1 may play a role in the regulation of gene expression at a late stage of white adipocyte differentiation.

Another study instigated to study the mechanistic role of RORγ1 in adipocyte differentiation and it was shown that RORγ1 is an important negative regulator of adipogenesis through expression of its target gene MMP3 (Matrix Metallopeptidase 3).[59] This statement came from since adipocyte precursor cells from *Rorc* knockout mice showed a higher differentiation capacity in a physiological environment. Furthermore, in contrast to the above-mentioned study, it was observed that transient overexpression of RORγ1 inhibits expression of key adipogenic proteins such as C/EBPβ and C/EBPδ

leading to decreased expression of C/EBPα and PPARγ. These findings were corroborated by the fact that induction of adipogenic genes such as c-Jun, c-Fos and aP2 was repressed by RORγ1. Taken together, these data demonstrate that RORγ1 may exerts its inhibitory effect during the initial steps of adipogenesis repressing adipogenic genes.

Mi Ra Chang, et al.[60] asked whether SR1555, a selective RORγ inverse agonist, induces thermogenic genes in adipose depots in diet-induced obesity mice. The authors of this study observed that SR1555-treated WAT presented an ectopic induction of UCP1 protein. Moreover, when mice were treated with SR1555, gene expression of UCP1, PRDM16, FGF21 and aP2 was increased in BAT. This aP2 expression profile was similar to that observed in the above-mentioned study on RORγ1 overexpression that resulted in repression of aP2 expression[59]. Taken together, they concluded that inhibition of RORγ1 function by SR1555 induced the thermogenic gene program and altered mitochondrial potential in adipose tissue. Thus, it was attributed to RORγ1 regulatory functions of the oxidative phosphorylation process in mitochondria. Therefore, according to this study, it was suggested that SR1555 might be the start for the development of a therapy against diabetes and obesity.

Although the gene expression profile of important genes in BAT could not be determined in our work, we could speculate that if the nuclear receptor RORγ1 functions indeed by regulating the differentiation and function of brown adipocytes, deregulation of RORγ1 activity would alter the gene expression pattern of these brown fat markers. Thus, we would reveal the role of RORγ1 in brown adipose tissue.

6. CONCLUSIONS

1. We set up the transfection of immortalized brown adipocytes (iBA), demonstrating that iBA cells can be efficiently transfected with siRNAs in preadipocyte form.

2. We were unable to confirm that efficient and specific RORα and RORγ1 knockdown in iBA preadipocytes using siRNAs was successfully accomplished, therefore we could not draw any conclusion.

3. Due to lack of clear results, we were also unable to properly assess neither the effect of the knockdown and pharmacological treatment with RORα/γ agonists and RORγ inverse agonists on brown adipocyte differentiation and function.

4. The impossibility of not having more results in our work was due to experimental errors and the shutdown of the experiments caused by contamination in the culture room. Thus, the present study needs to be extended to reveal the role of RORγ1 in regulating the differentiation and function of brown adipocytes.

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