INCREASED FAT OXIDATION IN 3T3-L1 ADIPOCYTES THROUGH FORCED EXPRESSION OF UCP1

A Thesis

by

SANTHOSH PALANI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Arul Jayaraman
Committee Members,	Juergen Hahn
	Edward Harris
	Mariappan Muthuchamy
Head of Department,	Kenneth Hall

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ABSTRACT

Increased Fat Oxidation in 3T3-L1 Adipocytes through

Forced Expression of UCP1. (August 2005)

Santhosh Palani, B.Tech., University of Madras

Chair of Advisory Committee: Dr. Arul Jayaraman

Obesity is a chronic condition that primarily develops from an increase in body fat in the form of white adipose tissue (WAT) mass. The resulting adiposity is a risk factor for many diseases, including type 2 diabetes (T2D), cardiovascular diseases, and some forms of cancer. Reducing WAT mass by targeted modulation of metabolic enzymes in fat cell metabolism is an attractive molecular therapeutic alternative to dietary approaches. In the present study, we exogenously up-regulate a novel respiratory uncoupling protein to increase substrate oxidation, and thereby control adipocyte fatty acid content.

Increasing molecular evidence points to a family of uncoupling proteins (UCPs) playing an important role in adipocyte fat metabolism. Of specific interest is UCP1, which in brown adipocytes mediates energy dissipation as heat by de-coupling respiration and ATP synthesis. UCP1 is minimally expressed in white adipose tissue (WAT). We hypothesize that controlled expression of UCP1 in WAT will result in enhanced fatty acid

oxidation to compensate for reduced ATP synthesis. We used a Tet-Off retroviral transfection system to express UCP1, with doxycycline being used to control the extent of expression. UCP1 cDNA was cloned into pRevTRE and was stably transfected into 3T3-L1 preadipocytes prior to differentiating them into adipocytes. A reporter gene (EGFP) was also transfected in parallel to optimize the transfection and preadipocyte differentiation conditions as well as to demonstrate regulated expression. Metabolite measurements showed that the UCP1-expressing adipocytes accumulated 83% less triglyceride and 85 % free fatty acids while maintaining constant ATP levels. These results suggest UCP1 and other metabolic enzymes as potential targets for development of pharmacological agents for the treatment of obesity and related disorders.

To Dhivya

for her love and support

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I thank Dr. Juergen Hahn and Dr. Mariappan Muthuchamy for their suggestions and constructive criticisms.

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INTRODUCTION

Obesity is a chronic condition that develops due to excess accumulation of fats in adipose tissue and skeletal muscle. It increases the risk of many diseases including Type II diabetes and cardiovascular diseases. In the US, one out of three adults is obese and among African Americans and Hispanics one out of two adults is obese (1). Current dietary therapies are mostly reversible and only few of the patients maintain their weight loss. Obesity drug development has mainly concentrated on reducing food intake and altering whole body metabolism. As of 2001, only 2 drugs (siburtramine and orlisat) have been approved for obesity treatment, but even these drugs suffer from numerous side effects (2).

Adipose tissue is a connective tissue that functions as a storage site for triglycerides. It is found in two forms: BAT and WAT. Obesity develops due to the expansion and increase in the number of fat cells in WAT. Adipose tissue stores fats in the form of large lipid droplets. When the body needs stored energy, adipocytes respond by undergoing lipolysis, breaking of lipids in to free fatty acids and transporting it to the necessary tissues through the plasma;

Dissipation of the proton gradient, also called the proton leak in the

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This thesis follows the style and format of *The Journal of Biological Chemistry*.

inter membrane space of mitochondria has been attributed to the family of uncoupling proteins UCP1, UCP2 and UCP3 (3). Cold bearing animals tend to produce heat in brown adipose tissue (BAT) by dissipating their proton gradient and Uncoupling Protein 1 (UCP1) is the protein which has been implicated in this process (4). Adults have very less percentage of BAT compared to white adipose tissue (WAT). Discovery of uncoupling proteins has led to a significant interest in approaches to treat obesity by increasing the overall metabolic rate of the body (5). Proteins analogous in DNA sequence to UCP1 have been found in WAT, UCP2 and UCP3. While UCP2 is expressed in numerous tissues, UCP3 is largely found in skeletal muscles (6). However, the mechanism by which these UCPs dissipate the proton gradient is not fully understood.

Uncoupling proteins are inactive in their native state as they are bound by purine nucleotides (GTP, GDP, ATP, and ADP). They get activated when their channel thickness is constricted by fatty acids, which also act as a proton carrier through the UCP channel. This contraction of the UCP channel dislocates the purine nucleotides from the UCP, and initiates uncoupling (7). Dissipation of the proton gradient causes a reduction in the synthesis of ATP which leads to an increase in whole cell metabolic rate which induces the cell to break down macromolecules to produce more energy substrates to meet up with ATP demands.

The goal of this study is to develop an efficient transfection system to modulate a gene of interest for desired expression levels, and to stably transfect UCP1 and a reporter gene (EGFP) into preadipocytes using the developed transfection system for expression. An uncontrolled uncoupling activity inside the cell can lead to scarcity of ATP molecules, resulting in various physiological disorders including insulin resistance.

We propose to use a retroviral vector for transfection which will provide a unique way to modulate gene expression using a chemical inducer such as doxycycline in a dose dependent manner (8).

To develop a fully functional retroviral vector system, a gene-carrier plasmid (pRevTRE-Gene) and a Tet Response Element (TRE) binding regulatory plasmid was transfected into 3T3-L1 preadipocytes. Developing double stable cells expressing both plasmids is a complex and tedious process, but the transfection is highly efficient in making stable cells compared to other plasmids having constitutive promoters (9).

Uncoupling mechanism of UCP1 has been vastly studied in BAT, but less information is available regarding its activity in WAT. Experimental evidence for UCP2 uncoupling is yet to be revealed. Hence this study will help understand the uncoupling behavior of these UCPs, as well as will provide insights into treating genetic disorders like obesity and insulin resistance by gene therapy.

Metabolism of Adipocytes- Till the 1980's, WAT was considered only as a storage depot. But recent findings show that WAT secretes and responds to a variety of endocrine, paracrine and autocrine factors which affect whole body and WAT metabolism (10). WAT fat metabolism comprises of both anabolic and catabolic reactions (Fig. 1). The anabolic reactions in adipocytes, lipogenesis, consists of synthesis of long chain fatty acids (LCFA), and their esterification into triglycerides, stored in the form of lipid droplets. The complementary catabolic reactions, lipolysis, involves the breaking down of triglycerides into free fatty acids and their subsequent oxidation into Acetyl-CoA to act as an energy substrate for the cell. Studies indicate that this process of lipolysis can be stimulated by cytokine signaling. During injury, TNF-α has been shown to stimulate lipolysis in adipose tissue. This process increases the plasma free fatty acid concentration to supply energy during inflammation to the injured tissues (11). Induced TNF-α stimulation of lipolysis, to treat obesity increases the free fatty acid concentration in blood, thereby causing insulin resistance in various tissues. Also, TNF-α serine phosphorylates IRS1&2, and aggravates insulin signaling.

Expansion of adipose mass is carried out in two steps, hypertrophy, an increase in cells size followed by hyperplasia, an increase in cell number. It's shown that both in human and non-human, hypertrophy always precede hyperplasia in a cyclic manner. This concept is supported by the "critical fat

cell size" hypothesis (12), which claims that adipocytes grow to a critical size and then recruit other precursor cells (preadipocytes) to proliferate and differentiate into mature adipocytes.

Mitochondria provide the main source of energy for the adipocytes, through oxidation of three classes of energy substrates, amino acids, fatty acids and monosaccharide. These substrates branch into the glycolysis pathway for oxidation into CO_2 and H_2O through the Krebs cycle and electron transport chain (ETC) (13).

Complexes in the ETC pump H+ from the matrix to the intermemberane space of the mitochondria, thereby creating a proton gradient. The energy derived from this gradient is used by the F1-F0 ATP synthase to produce ATP by a process known as oxidative phosphorylation. The backflow of protons into the matrix dissipates the gradient and results in lesser production of ATP. It is observed that not all the energy supplied by the substrate is converted to ATP, as few non-protein membrane pores at the protein-lipid interfaces mediate the transfer of protons across the inner membrane. Protein mediated dissipation of the proton gradient is also called uncoupling and was first observed in BAT in 1964 (14). Cold bearing animals tend to dissipate their proton gradient to produce heat in BAT and the protein which creates the leak was called thermogenin, currently termed as Uncoupling Protein 1 (UCP1).

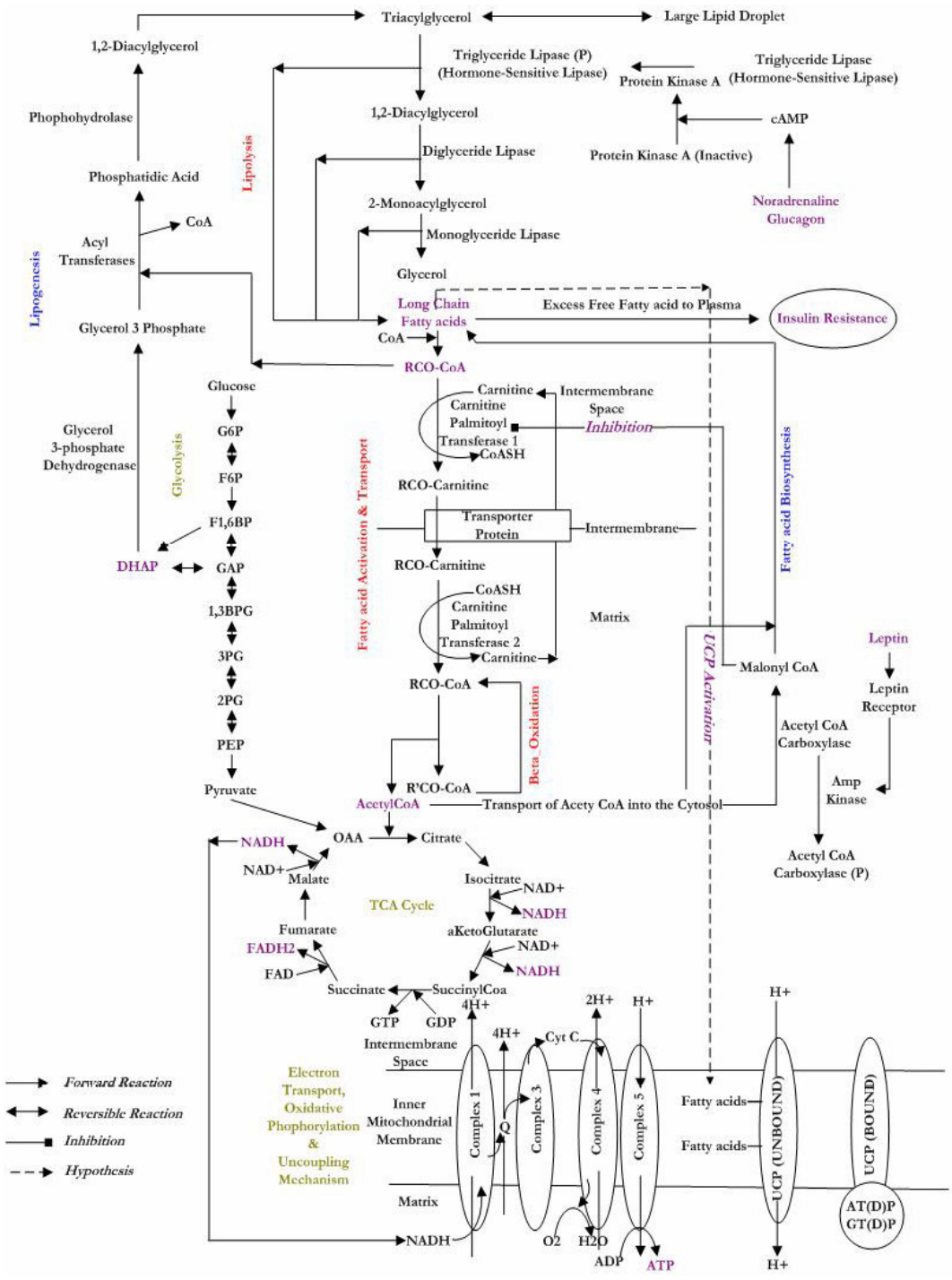


Fig. 1. **Metabolic pathway chart of adipocytes showing glycolysis, TCA cycle, lipolysis, lipogenesis, UCP activation and ATP synthesis**

Adipocyte Differentiation- The early molecular events engaged in the conversion of primitive mesenchymal precursor cells to committed cell lines are completely unknown. Due to this reason, most of the research on adipocytes is done with predetermined clonal cell lines or preadipocytes. 3T3-L1 preadipocytes are isolated from non-clonal Swiss 3T3 cells, and have morphology similar to fibroblasts. The differentiation steps (15) for the formation of mature adipocytes from preadipocytes are clearly defined (Fig. 2). The first step in differentiation is the proliferation of the preadipocytes to reach confluence. The next step is the growth arrest induced by contact inhibition between neighboring preadipocytes.

This step also involves the activation of the transcription factors (TF) CCAAT/ enhancer binding protein ß (C/EBPß) and C/EBPδ (16). These transcription factors activate other transcription factors like (Peroxisome Proliferator activated receptor γ (PPAR γ) and C/EBPα, which are the main TF regulating fat cell differentiation. Following growth arrest, preadipocytes undergo two rounds of mitotic division known as clonal expansion, which concurs with the activation of PPAR γ and C/EBPα. After clonal expansion, preadipocytes undergo a second growth arrest, followed by differentiation into young adipocytes, which grow in size to become mature adipocytes.

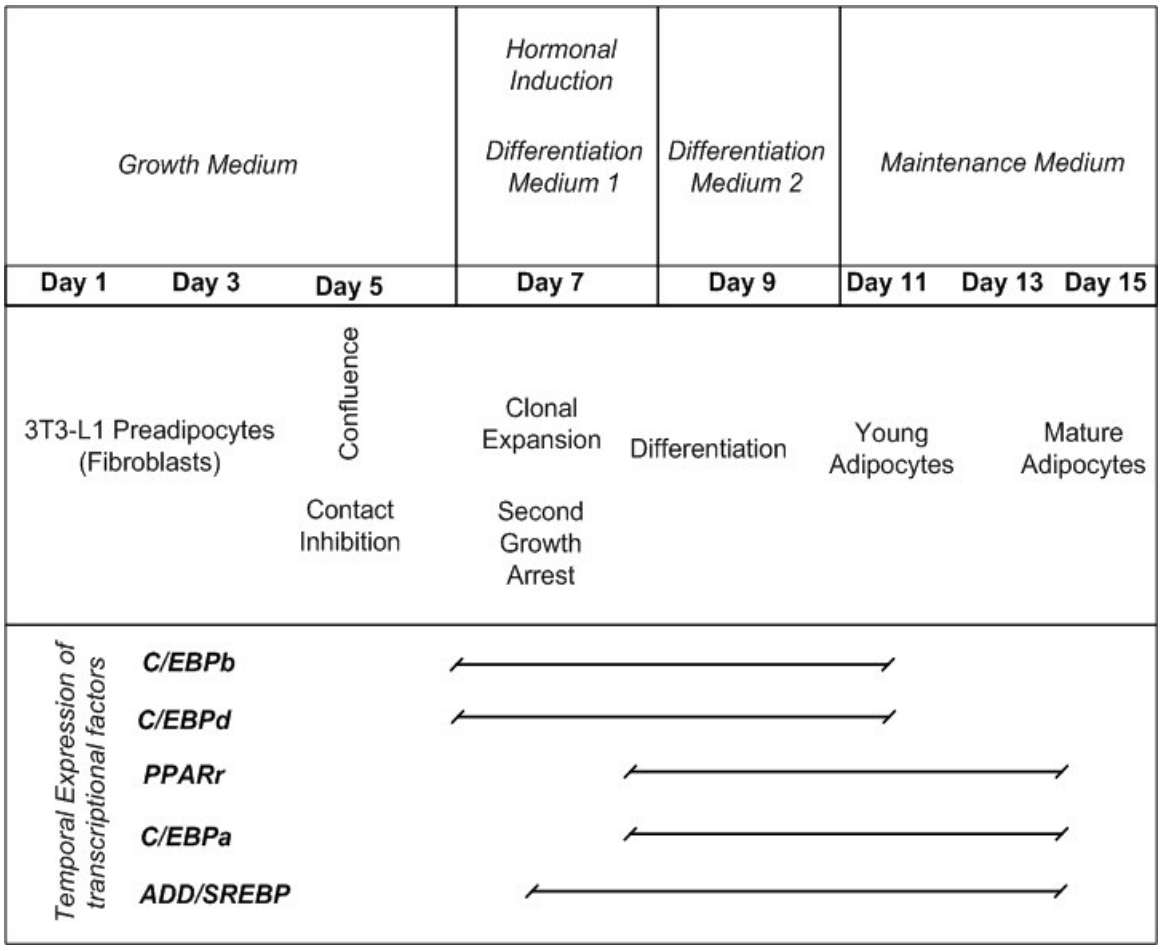


Fig. 2. **Features of adipocyte differentiation**

The process of adipocyte differentiation is highly controlled several transcription factors (17). These transcription factors are expressed in a cascade in which C/EBPβ and C/EBPδ are the first ones to get activated. They induce the expression of PPARγ, which further activates C/EBPα. C/EBPα in turn activates PPARγ through a feedback pathway to maintain the differentiated state of the adipocytes. It is observed that PPARγ is also activated by another transcription factor Adipocyte Determination and

Differentiation dependent factor 1(ADD1) or Sterol regulatory element binding protein 1(SREBP1) (18).

Inhibitors and Inducers of Adipogenesis- It has been observed by several empirical methods that hormones including, Insulin, Insulin like growth factor 1(IGF-1), Glucocorticoid, Triiodothyronine and cAMP facilitate the differentiation of preadipocytes in vitro (19). Insulin/IGF-1 activates the Insulin like growth factor receptor (IGFR) and signals through the RAS and Protein Kinase B (PKB) pathways to induce adipogenesis (Fig. 3) (20).

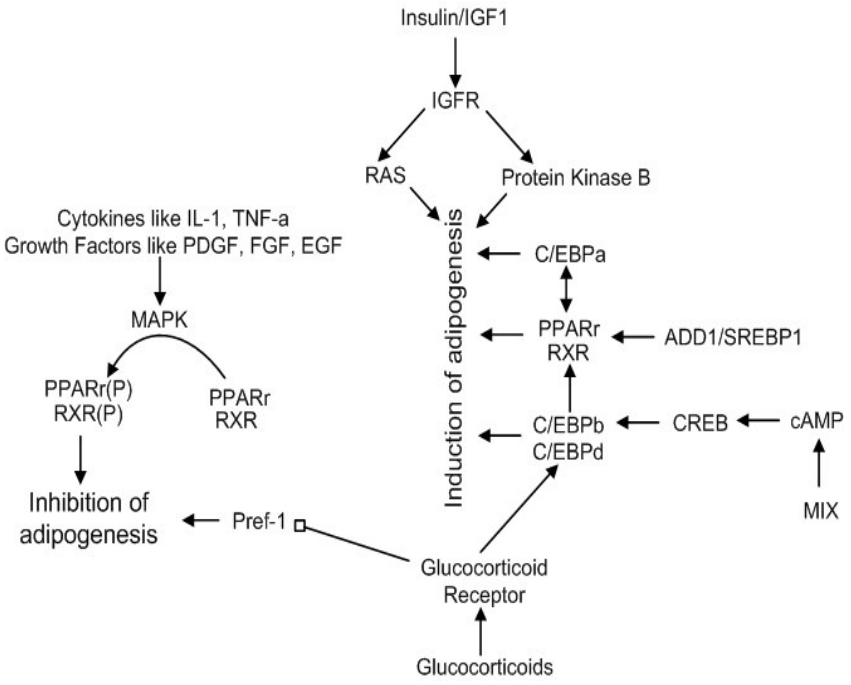


Fig. 3. **Inhibitors and inducers of adipogenesis**

Glucocorticoid acts on the Glucocorticoid receptor to induce the expression of C/EBPδ and also to inhibit the expression of Preadipocyte factor 1 (pref-1) (21). Methylisobutylxanthine (MIX) activates cAMP and induces expression of C/EBPβ through the cAMP element binding protein (CREB) pathway (22). Combination of Glucocorticoid, MIX and insulin is considered to be the optimum mixture for efficient differentiation of preadipocytes.

A variety of cytokines like TNF-α, IL-1 and growth factors like PDGF, FGF, EGF (5) have found to inhibit adipocyte differentiation (23, 24). These growth factors and cytokines activate the Mitogen Activated Protein Kinase (MAPK) pathway, which phosphorylates PPARγ and its hetrodimerization nuclear receptor retinoid X receptor (RXR), leading to inhibition of adipogenesis. Pref-1 is a surface glycoprotein shown to inhibit preadipocyte differentiation. Pref-1 can also be released as an autocrine or a paracrine factor to regulate adipogenesis. The receptor for Pref-1 is completely unknown.

UCP1 Signaling Pathway in BAT- BAT dissipates its energy by uncoupling to regulate the body temperature when exposed to cold. The pathway regulating UCP activity is well understood (Fig. 4). When cold is sensed by the brain, it activates series of sympathetic nerves to produce noradrenaline

(25). Noradrenaline activates the ß-adrenergenic receptor, which in turn activates CREB through cAMP and Protein Kinase A. CREB phosphorylation induces the expression of PPARγ coactivator 1α (PGC-1α) and Type II thyroxine deiodinase (DII). PGC-1 activates the transcription factors PPARγ and RXR, RXR and RAR located on the UCP1 enhancer. DII in turn converts tetraiodothronine (T4) to triiodothyronine (T3), the agonist for a thyroid hormone receptor, which further increases expression levels of UCP1.

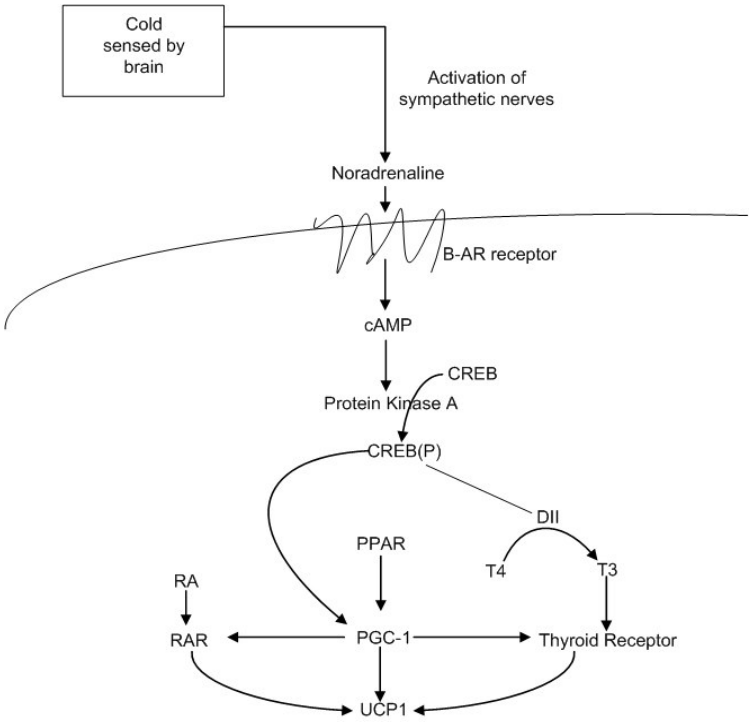


Fig. 4. **Adaptive thermogenesis**

Protein kinase A also activates hormone sensitive lipase which increases the concentration of fatty acids and leads to UCP1 activation (Fig. 1). PGC-1 also activates nuclear respiratory factors (NRF1, NRF2) which lead to the activation of genes for mitochondrial biogenesis and function (26).

Uncoupling Mechanism of UCP1- While the activation of UCP1 is well studied, UCP1 uncoupling mechanism is poorly understood. UCP1 is inactive in its native state as it is bound by purine nucleotides (GTP, ATP, GDP, and GTP). Threshold concentrations of free fatty acids allosterically displace purine nucleotides and activate UCP1 uncoupling. There are two mechanisms currently in literature which explains UCP1 uncoupling (27).

In one model (28), UCP1 is considered as a true H+ carrier across the mitochondrial inner membrane (Fig. 5A). FFA binds to UCP1 in a way that their carboxylic groups act as H+ donors/acceptors to resident carboxylic groups. Histidine residues are speculated to be the final H+ carrier before the proton is released into the matrix. In the other model, UCP1 is considered as a fatty acid anion carrier (Fig. 5B). UCP1 carries the deprotonated fatty acid across the membrane to the intermembrane space (29). In the intermembrane space, FFA gets protonated and sent back to the matrix through a flip-flop mechanism. It has been recently shown that the mechanism of uncoupling can change depending on the fatty acid

concentration. In the presence of small amount of FFA, UCP1 acts mainly as an H+ carrier. With high FFA concentration, UCP1 performs as a FFA carrier (30).

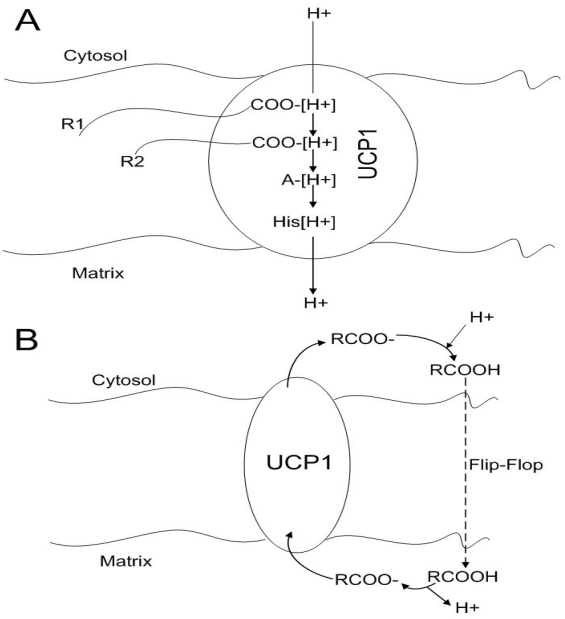


Fig. 5(A). **UCP1 as a proton carrier**; (B). **UCP1 as a FFA carrier-** (adapted from (28))

Functional Roles of UCPs- UCP2 and UCP3 are homologues of UCP1 found in WAT and share 56% and 57% DNA sequence similarity to UCP1 respectively (31). In addition, UCP2 and UCP3 have 71% homology as well. The question whether UCP2 and UCP3 are novel uncouplers is still unanswered. Studies of UCP1, UCP2 and UCP3 activity in liposomes (32)

and yeast confirmed their abilities to dissipate the proton gradient (Table 1). Studies of UCP1 activity in transgenic mice showing their respiration uncoupling behavior; interruption of the murine UCP1 gene demonstrating their role in adaptive thermogenesis confirmed UCP1 as a novel uncoupler.

Unlike UCP1, presence of UCP2 and UCP3 in humans has been attributed to the suppression of reactive oxygen species in the mitochondria rather than playing a role as uncouplers. Active electron transport chain produces lots of free electrons which react with oxygen to form reactive oxygen species. Respiration is connected with the production of reactive oxygen species (ROS).

TABLE 1

Different roles of uncoupling proteins (UCPs) (adapted from (33))

Method	UCP1	UCP2	UCP3
Liposome	Proton leak	Proton leak	Proton leak
Yeast	Proton leak	Proton leak	Proton leak
Transgenic mice	Respiration uncoupling	Not determined	Proton leak
-/- mice	Cold-induced Thermogenesis,	No ROS scavenging	No ROS scavenging
Normal mice	Cold-induced thermogenesis	ROS scavenging, Fatty acid oxidation	ROS scavenging, Fatty acid oxidation
Human genetics	Body fat content	Resting metabolic rate	Resting metabolic rate

It is known that mild uncoupling of the proton gradient by UCP2 and UCP3 helps in suppressing the production of ROS (34). Interruption of the UCP2 and UCP3 gene by knockout studies stimulated the production of ROS validating their function (34). Recently, UCP2 and UCP3 have been also related to the maintenance of resting metabolic rate (33).

The RevTET-On/Off Transfection System- The commercially available Tet On/Off system is a regulated, high level gene expression system (35). In the Tet-Off system gene expression is turned on when tetracycline or doxycycline is removed from the culture medium, whereas in the Tet-On system gene expression is turned on when doxycycline is added to the system (Fig. 6). Both systems provide variation in gene expression with varying concentrations of doxycycline (36) and provide extremely tight on/off regulation without any background or leaky expression. The binding between the Tet transcription factor and the response element is very specific (37). The system can provide high induction levels and fast response times. Expression levels obtained by the Tet system is more than expression levels obtained from CMV promoter or other constitutive promoters (37). The inducer doxycycline is well characterized, inexpensive and yields reproducible results. In both systems the antibiotics concentration used is far below cytotoxic levels. The Tet on/off vector system when fused with a

retroviral cassette yields a RevTet on/off system. This system provides stable transfection of gene into any dividing cell line. The transfection efficiency of the RevTet on/off is very high (36).

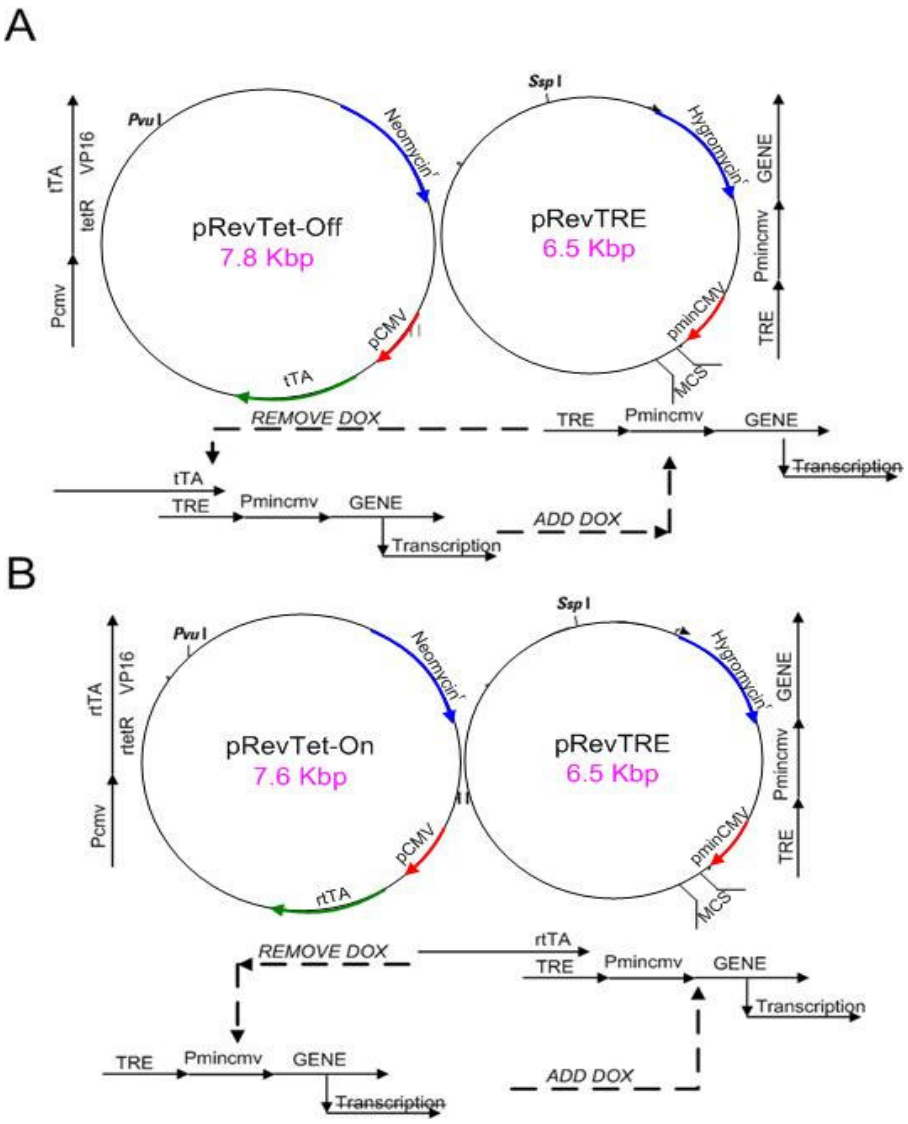


Fig. 6(A) **Tet Off gene expression system;** (B) **Tet On gene expression system** (38)

PROBLEM STATEMENT AND SPECIFIC AIMS

Hypothesis- Forced expression of uncoupling protein 1 in white adipose tissue will decrease the levels of ATP synthesis, causing an increase in the whole cell metabolic rate of the cell through increased fat oxidation.

The purpose of this study are to develop and characterize a defined experimental system for studying adipocyte metabolism, to develop an efficient transfection for modulating gene of interest; and stably transfect UCP1 and a reporter gene (EGFP) into preadipocytes using the developed transfection technique. The various steps involved in the study are briefly stated below:

Specific Aim 1- To develop and characterize a defined experimental system for transfection of a gene of interest into adipocytes, in order to obtain regulated, high expression.

1. Optimization of cell culture protocol for preadipocytes.
2. Develop expression construct of pRevTRE-EGFP from pRevTRE and pEGFP.
3. Optimization of retroviral vector transfection conditions.
4. Transfection of pRevTET-On into preadipocytes.
5. Obtain stably expressing cell lines by G418 selection

6. Transfection of pRevTRE-EGFP into preadipocytes using the optimized conditions.
7. Obtaining double stable cell lines expressing EGFP by hygromycin selection.
8. Optimize differentiation conditions for preadipocytes expressing EGFP.
9. Perform doxycycline assay to regulate the gene expression levels of EGFP.
10. Perform TG, FFA and ATP assay to characterize adipocyte behavior.
11. Repeat the assay on EGFP cells and compare with the control.
12. Perform lipolysis assay on EGFP cells.

Specific Aim 2- To use the developed experimental system to transfect UCP1 into preadipocytes and to characterize their behavior

1. Develop expression construct of pRevTRE-UCP1 from pRevTRE and pCmv-Sport6-UCP1
2. Transfection of pRevTRE-UCP1 into preadipocytes using the optimized conditions
3. Obtain double stable cell lines expressing UCP1 by hygromycin selection.

4. Optimize differentiation conditions for preadipocytes expressing UCP1
5. Perform TG, FFA and ATP assay and compare it with the control.

MATERIALS AND METHODS

Optimization of Cell Culture Protocol for Increased Differentiation of Preadipocytes-3T3-L1 preadipocytes (Passage 2) were seeded on T-25 flasks with different seeding densities (1*10^4 to 2.5*10^5 cells/T-25flask). Growth medium (DMEM, Bovine Serum [10%]) was replenished every 48 hours till the cells reach confluence (Day 0). The cells were kept in growth medium for an additional 2 days to initiate natural differentiation. On day 2, cells were exposed to differentiation medium 1(DMEM containing insulin, Fetal Bovine Serum (FBS) [10%], isobutylmethylxanthine (IBMX) [1uM] and dexamethasone) [0.5 mM] to supply hormonal inducers for differentiation. The medium was changed to differentiation medium 2(DMEM, insulin [1μg/ml], FBS [10%]) on day 4 and incubated for two days to maintain their differentiation state. Typically, at the end of this exposure, young adipocytes were observed. Adipocytes were maintained in maintenance medium (DMEM, FBS [10%]) for up to 3 weeks.

Construction of RevTRE-EGFP (Reporter Gene) and RevTRE-UCP1 Vectors-The pRevTET-Off, pEGFP, pRevTRE vectors was purchased from BD Biosciences (Mountain View, CA). The vector was linearized with Sca1 for transfection into 3T3-L1 preadipocytes. Plasmid pEGFP was cut with Not1 and then blunted with T7 DNA polymerase (Fig. 7). The vector was then

digested with BamH1. EGFP gene cut from pEGFP was then ligated with pRevTRE, which was digested with BamH1 and Hpa1. PRevTRE-EGFP was linearized by BsaA1 for transfection in to pRevTET-Off selected 3T3-L1 cells.

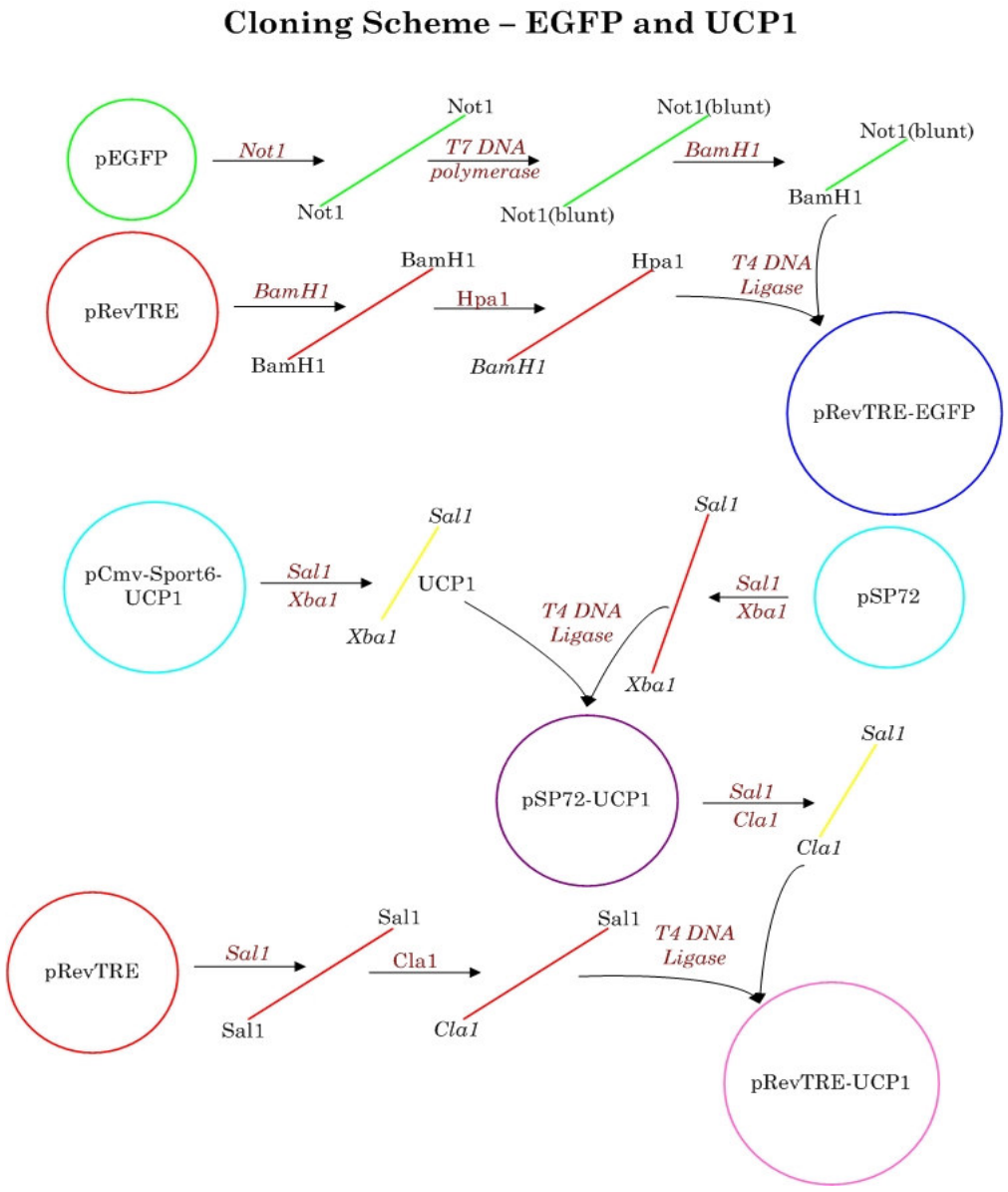


Fig 7. **Construction of pRevTRE-EGFP and pRevTRE-UCP1 vectors**

Plasmid pCMV-Sport6-UCP1 containing the mus musculus full length cDNA for UCP1 was purchased from Invitrogen (Carlsbad, CA). It was digested with Sal1 and Xba1 to remove the UCP1 gene from the plasmid. pSP72, a cloning vector was purchased from Promega (Madison, WI). It was digested with Sal1 and Xba1 and then ligated to the UCP1 gene cut from pCMV-Sport6-UCP1 (Fig. 7). pSP72 was then digested with Sal1 and Cla1 to remove UCP1 which was further ligated into pRevTRE, digested with the same set of restriction enzymes. pRevTRE-UCP1 was linearized with BsaA1 for transfection into pRevTET-Off selected 3T3-L1 preadipocytes.

Retroviral Vector Transfection and Optimization- All plasmids were transfected by electroporation using Gene Pulsar II from Bio-Rad (Hercules, CA) at 240 volts and 960 micro-farads (39). Transfection protocol was optimized during the pRevTET-Off electroporation. Two different cells/DNA ratio (10^5 cells/10μg DNA and 10^5 cells/20μg DNA) were used. Cells were kept on ice prior to electroporation and were kept at room temperature after electroporation. The cells were then seeded in a T-25 flask and selected with G418 for neomycin resistance. pRevTRE-EGFP and pRevTRE-UCP1 cells were transfected into the pRevTET-Off selected 3T3-L1 preadipocytes as described above and selected with hygromycin to obtain 3T3-L1 cells that had either pRevTRE-EGFP or pRevTRE-UCP1 stably integrated into the

genome. These cells were grown up and frozen down in aliquots (15*10^4 cells/ml) for subsequent differentiation.

Detection of EGFP Expression- EGFP expression in the double stable 3T3-L1 preadipocytes was detected using phase contrast and fluorescence microscopy. These cells were then allowed to differentiate into adipocytes by the optimized cell culture protocol and their EGFP expression was again confirmed using fluorescence microscopy.

Regulation of Expression Using the Tet-Off System-3T3-L1-EGFP preadipocytes were plated on 6 well plates (2.5*10^5 cells/well) and differentiated as described above. Cells were treated with doxycycline (8, 1, 0.1 μg/ml) for 72 hrs and the EGFP fluorescence levels were observed at specific time intervals 0hrs, 24hrs, 48hrs and 72 hrs post doxycycline addition. Fluorescence was detected using a SpectraMax GeminiEM from Molecular Devices. After 72 hrs, the cells were fed with normal maintenance medium and their fluorescence levels were observed after 24 hrs.

Adipocytes Functional Assays- Triglyceride Assay- Mature adipocytes (control or expressing either EGFP or UCP1) was lysed with RIPA lysis buffer(Nacl, SDS, Deoxycholate, Tris-Hcl, NaF, EDTA, glycerol, Nonidet-

P40) and their intracellular TG was measured using the triglyceride and free glycerol reagent purchased from Sigma (St. Louis, MO) and compared with the control adipocytes. The absorbance levels were measured at 540nm using SpectraMax 340PC plate reader purchased from Molecular Devices (Sunnyvale, CA). Free Fatty Acids (FFA) Assay- Culture medium from the mature adipocytes expressing EGFP was collected for analyzing the levels of free fatty acids expelled out from the cells. FFA levels were quantified using the Half Micro Kit purchased from Roche Applied Sciences (Indianapolis, IN) as per the manufacturer's protocol using a SpectraMax 340PC plate reader purchased from Molecular Devices. ATP Assay- Mature adipocytes expressing EGFP or UCP1 were lysed with the lysis buffer provided in the ATP Bioluminescence HSII kit from Roche applied sciences. ATP levels were measured as per the manufacturer's protocol and luminescence values were measured using the SpectraMax GeminiEM from Molecular Devices and compared with the control adipocytes. Lipolysis Assay- 3T3-L1 mature adipocytes were exposed to 15ng/ml of TNF-α for 24 hrs. The supernatant was then collected and the amount of free fatty acids and free glycerol present were analyzed as described above. The cells were then lysed by RIPA buffer and their TG levels were measured as explained above.

RESULTS AND DISCUSSION

Optimization of Cell Culture Protocol for Increased Differentiation of Preadipocytes- 3T3-L1 preadipocytes were seeded at different densities and allowed to reach confluence. Confluent cells were differentiated into mature adipocytes as described in materials and methods. As expected, cells seeded with lesser densities took more time to reach confluence, i.e., more mitotic divisions before the cells reach confluence. Surprisingly, the degree of differentiation observed also varied depending on the time to reach confluence (Table 2). Results show that the more the number of days for the cells to reach confluence, the lesser is its efficiency to differentiate. Through these results, we were able to determine an optimum seeding density to attain enhanced differentiation. It is still unclear why the number of mitotic divisions before confluence affects the percentage of differentiation.

TABLE 2
Optimization of preadipocytes differentiation to adipocytes

Seeding Density Cells/T25 flask	Days to confluence	Percentage of differentiation
1x10^4	9	<10%
5x10^4	7	10-20%
1x10^5	6	~50%
2.5x10^5	5	>90%

Retroviral Vector Transfection and Optimization- Plasmid pRevTET-Off was transfected into 3T3-L1 preadipocytes by electroporation and was stably integrated into the cells genome. pRevTET-Off expresses the neomycin resistant gene. So, the cells after electroporation were selected for G418 resistance. There was a massive cell death after a week from electroporation killing all the cells which don't have the plasmid. Table 3 shows that only the cells having the plasmid survived. The percentage of survival was more for condition 1 with lower DNA compared to condition 2.

TABLE 3
Optimization of pRevTET-Off transfection (Cells:DNA ratio)

Condition No:	Amount of DNA (μg)	Percentage of survival upon selection with neomycin
1	10	15%
2	20	10%
3	0	None
4	No electroporation	None

Cells were then allowed to grow to reach ~70% confluence for the second electroporation. Cells that survived from conditions 1 and conditions 2 were used for the pRevTRE-EGFP electroporation. pRevTRE-EGFP expresses the gene for hygromycin resistance; Table 4 shows the percentage of survival upon selection with hygromycin.

TABLE 4

Optimization of pRevTRE-EGFP transfection

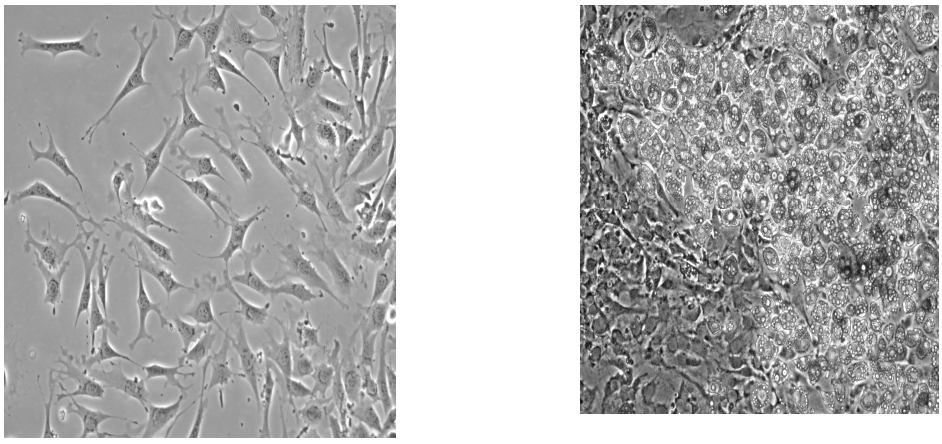
Sample No:	Cells	Amount of DNA	Percentage of survival upon selection with hygromycin
1	From condition 1	10ug of pRevTRE-EGFP	~10%
2	From condition 2	10ug of pRevTRE-EGFP	~10%
3	From condition 1	10ug of pRevTRE	None
4	From condition 1	No Vector	None

Cells survived from both the electroporation have both plasmids pRevTET-Off and pRevTRE-EGFP. The cells from the first electroporation were used for transfection with pRevTRE-UCP1. The control cells died during this electroporation showing again the survival of only those cells with the gene. Again, approximately 10% of the cells survived the electroporation. The double stable EGFP and UCP1 cells were then allowed to differentiate into mature adipocytes.

Detection of EGFP Expression in 3T3-L1 Adipocytes- EGFP was chosen as the reporter gene as its expression can be easily detected using a a fluorescence microscope. EGFP expressing double stable 3T3-L1 preadipocytes are shown using phase contrast and fluorescence microscopy (Fig 8A). These cells were then allowed to differentiate into mature

adipocytes and their EGFP expression was again confirmed using fluorescence microscopy (Fig 8B).

A



B

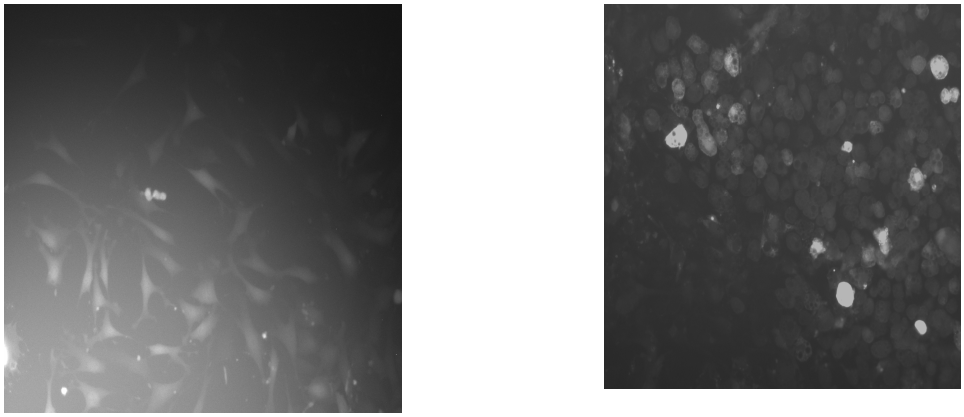
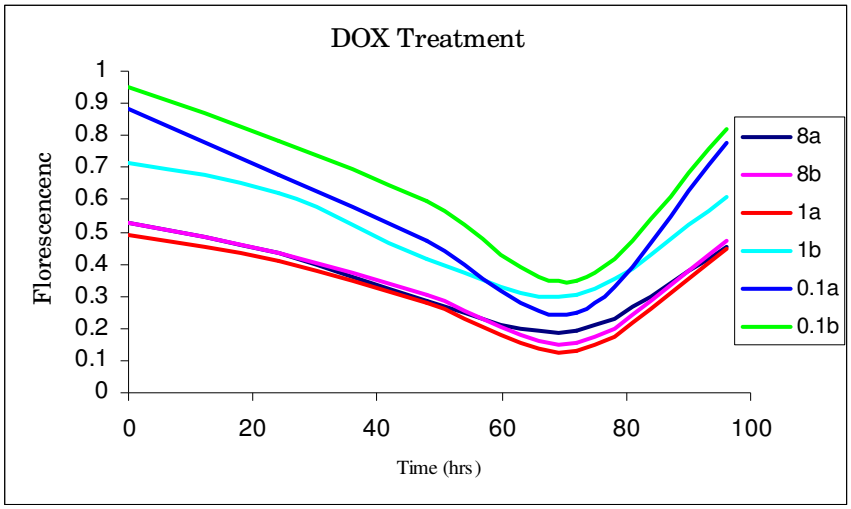


Fig 8(A) **Phase contrast images of preadipocytes and mature adipocytes;** (B) **Fluorescence images of preadipocytes and mature adipocytes.**

*Regulation of EGFP Expression with DOX- E*xpression of EGFP was modulated using the chemical inducer doxycycline. Addition of doxycycline in a Tet-Off system decreases the binding affinity between the regulatory protein (transcription factor-tTA) and its binding site (response element) in a dose dependent manner. Since binding of the TF to the response element directly relates to the level of transcription of the gene, the expression of EGFP (or any gene) can be regulated. Doxycycline assay was performed over a four day period. It should be noted that the half-life of EGFP is around 24 hours. Hence a detectable decrease in fluorescence levels was observed only after 24 hours. Since half-life of doxycycline in serum is 24 hours, doxycycline containing media was added to the cells every 24 hours. Cells were exposed to DOX medium for 72 hours and replaced with normal maintenance medium for the last 24 hours of the experiment. Three different concentrations of doxycycline were used covering an 80 fold range in concentration. Figure 9A shows that fluorescence values in all the wells decreased significantly during the 72 hour period of the assay. When doxycycline was removed at the end of 72 hours, fluorescence levels again started to increase to the original levels. Figure 9B shows the percentage reduction in fluorescence during the 3 day period for different concentrations of doxycycline. In addition, the percentage reduction in fluorescence for different time points and time taken for a specific reduction in fluorescence

A



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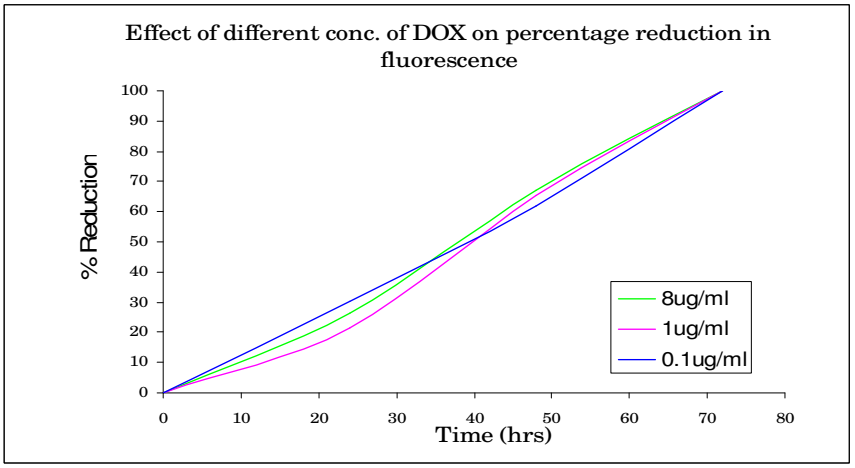


Fig.9 (A) **A time course plot on the effect of different concentrations of doxycycline on EGFP expression levels;** (B) **Effect of different concentrations of doxycycline on percentage reduction in fluorescence**

for different concentrations of doxycycline remained almost constant (Figure 9B & Table 6). This assay clearly shows that gene expression in adipocytes can be regulated by doxycycline with the RevTET-Off system.

TABLE 5
Analysis of fluorescence values obtained from the doxycycline assay

Time taken for 20% reduction in fluorescence		**Time taken for 40% reduction in fluorescence**		**Time taken for 60% reduction in fluorescence**	
Conc(µg/ml)	Time (hrs)	Conc(µg/ml)	Time (hrs)	Conc(µg/ml)	Time (hrs)
8	19	8	32	8	44
1	23.5	1	35	1	44
0.1	17	0.1	32	0.1	46
% reduction in fluorescence at 20 hrs		**% reduction in fluorescence at 40 hrs**		**% reduction in fluorescence at 60 hrs**	
Conc(µg/ml)	% reduction	Conc(µg/ml)	% reduction	Conc(µg/ml)	% reduction
8	21	8	55	8	83
1	16	1	52	1	83
0.1	25	0.1	51	0.1	81

Adipocytes Metabolic Assays- Triglyceride Assay- Since the plasmids pRevTet-off and pRevTRE-EGFP are randomly integrated in the adipocyte genome, it is important to determine if the site of integration impacted the

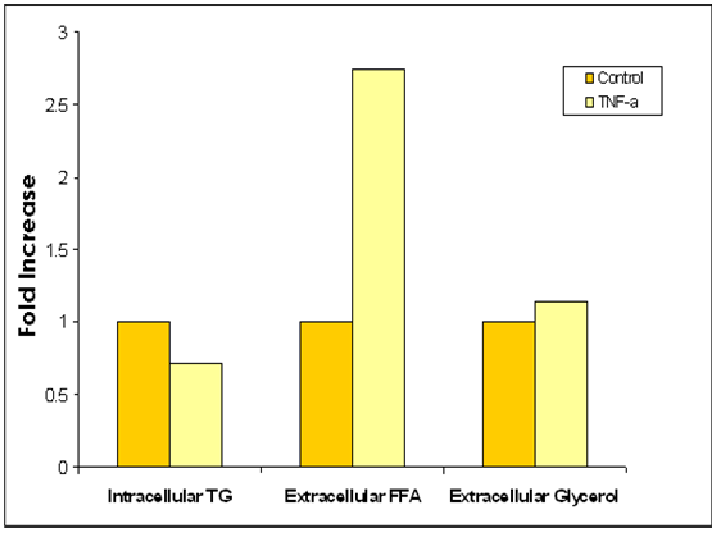
metabolic functions of the adipocytes. Since adipocytes, store fat in the form of triglycerides, determining the levels of TG is an extremely pertinent assay for adipocyte function. Therefore, we quantified the triglyceride levels in adipocytes expressing EGFP and compared it to that seen in control (untransfected) adipocytes. Table 6 shows that there is no statistically significant difference between the TG values of the control adipocytes and EGFP expressing adipocytes.

TABLE 6
Triglyceride assay on EGFP cells

TG levels (mol glycerol/g-DNA)	
Control Adipocytes	0.232 +/- 0.067
EGFP expressing adipocytes	0.248 +/- 0.072

Lipolysis Assay- When TG are broken down in adipocytes, they are converted to glycerol and FFA. Both glycerol and FFA, when in excess are expelled from the cells. Lipolysis or Adipolysis is another important functional assay for the mature adipocytes. To show the RevTet system has no effect on lipolysis, we induced TG break down in adipocytes by exposing with TNF-α, a well known cytokine to induce adipolysis. Intracellular TG, extracellular glycerol and extracellular FFA assays were performed on the

EGFP cells and their levels were compared with the control adipocytes. Fig 10 shows a strong increase in the extracellular FFA and a 29% reduction the intracellular TG levels and a 14% increase in the extracellular glycerol levels. This assay clearly shows that the EGFP expressing cells respond to TNF-α stimulation in a manner similar to that reported in the literature (11).

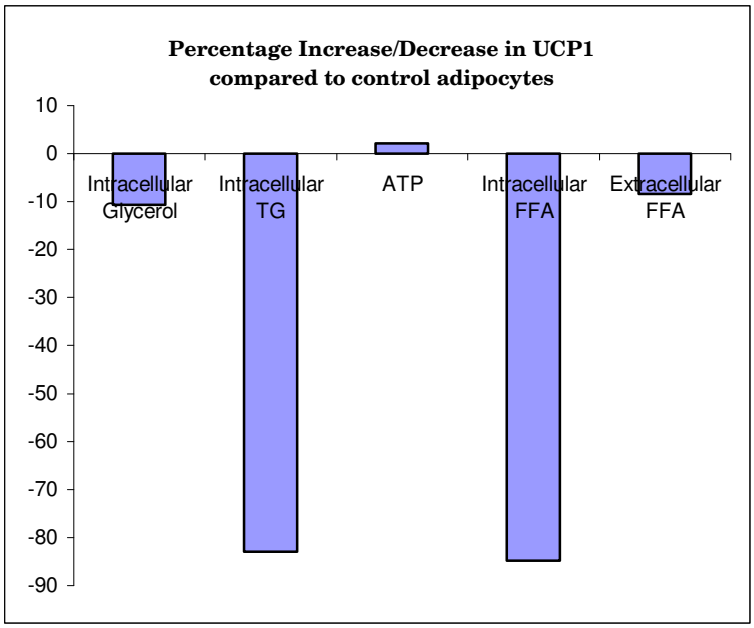


Assays	%Change
Intracellular TG	-28.87+/-1.5
Extracellular FFA	175+/-2.1
Extracellular Glycerol	13.95+/-1.1

Fig.10. **Lipolysis assay showing the difference in intracellular TG, extracellular FFA and extracellular glycerol between EGFP expressing cells and EGFP cells induced with TNF-α**

Functional Evaluation of UCP1 Expressing Adipocytes- Dissipation of the proton gradient in the adipocytes expressing UCP1 can result in changes in the levels of the metabolites such as triglycerides, glycerol and free fatty

acids in the cell. To characterize these changes, the levels of these functional markers in the UCP1 expressing cells were determined and compared with the control adipocytes. Figure 11 shows a 83% decrease in the intracellular TG in UCP1 cells (compared to the control) which suggests an increase in lipolysis. The intracellular FFA levels also decreased by 85%, showing an increase in fatty acid oxidation.



% change in UCP1 cells compared to control adipocytes	
Intracellular Triglyceride	-82.89+/-2.33
ATP	2.1 +/- 0.05
Intracellular Glycerol	-10.63+/-1.12
Intracellular FFA	-84.85+/-3.02
Extracellular FFA	-8.33+/-0.92

Fig.11. **Percentage change in intracellular glycerol, intracellular TG, ATP, intracellular FFA and extracellular FFA in UCP1 compared to the control adipocytes**

The levels of intracellular glycerol and extracellular FFA changes were also determined and showed decrease when compared with the control.

As discussed previously, a reduction in the proton gradient will cause a decline in ATP synthesis. It is important to find whether a reduction in ATP synthesis caused by UCP1 will decrease the TG content in the adipocytes to maintain the ATP levels inside the cell through increased fat oxidation. Figure 11 shows that ATP levels remain almost constant for UCP1 expressing cells when compared to the control showing the cell has the capacity to recover from ATP depletion by breaking down TG and increasing fatty acid oxidation.

CONCLUSIONS

The RevTet-Off system was shown as an efficient method to transfect genes of interest (EGFP, UCP1) into 3T3-L1 preadipocytes. Metabolic assays – Triglyceride, free fatty acid, and lipolysis assays performed on EGFP expressing cells clearly showed that RevTet-Off transfection has no effect on preadipocytes differentiation or on adipocytes function. Through the doxycycline assay, RevTet-Off system was shown to act as an on/off switch for gene expression of the transfected vectors in 3T3-L1 adipocytes.

Metabolic assays – triglyceride, free fatty acid, ATP, and glycerol assays performed on UCP1 expressing cells showed reduced TG and FFA levels but showed no change in ATP levels. We conclude that UCP1 expressing cells have the capacity to recover from ATP depletion by breaking down their triglycerides and by increasing fatty acid oxidation.

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