PATEL, AMIT P., M.S. The Role of Increased Reactive Oxygen Species on 3T3-L1 Preadipocyte Differentiation. (2012)
Directed by Dr. Yashomati M. Patel. 44 pp.

Differentiation of preadipocytes plays an important role in human physiology by accommodating adipocyte expansion and maintaining energy homeostasis. Any process that interferes with preadipocytes differentiation could alter energy homeostasis. In our study, we investigated the effect of increased levels of ROS, on preadipocytes differentiation. Our study shows that increased levels of ROS can inhibit 3T3-L1 preadipocytes differentiation. The mechanism by which increased levels of ROS impaired differentiation was by decreasing and delaying Cyclin D1 expression. This would affect the phosphorylation status of retinoblastoma protein which is required for cell cycle progression into the S-phase. Since clonal expansion was halted, the activity and expression of essential transcription factors needed for differentiation C/EBPβ and PPARγ were affected, leading to impaired differentiation.

Furthermore, we showed that once the increased ROS levels were restored to normal, the 3T3-L1 preadipocytes were able to differentiate into mature adipocytes when exposed to MDI. The amount of differentiation observed was slightly diminished compared to the normally differentiating 3T3-L1 preadipocytes, but was significantly greater than that observed in cells induced to differentiate in the presence of increased ROS. Overall, we have outlined the timeframe during which oxidative stress impairs 3T3-L1 preadipocyte differentiation. We have shown that, once that increased ROS

levels are lowered, differentiation can be restored, but not to the same degree as untreated differentiating 3T3-L1 preadipocytes.

THE ROLE OF INCREASED REACTIVE OXYGEN SPECIES ON 3T3-L1 PREADIPOCYTE DIFFERENTIATION

by
Amit P. Patel

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2012

Approved by

_____
Committee Chair

Dedicated to Praful and Urmila Patel

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to acknowledge the following for their help and provision: My advisor Dr. Yashomati M. Patel and committee members Dr. Dennis Lajeunesse and Dr. Zhenquan Jia for all of their help and support throughout my research. I also would like to thank Faculty and Staff of the University of North Carolina at Greensboro Biology Department.

TABLE OF CONTENTS

Page

LIST OF FIGURES vii

CHAPTER

I. INTRODUCTION 1

Obesity 1
Adipose tissue 1
Adipogenesis 2
Clonal expansion 3
Oxidative stress 5
Hypothesis 7

II. METHODS 8

Cell culture 8
Cholormethyl - 2', 7 – dichlorofluorescein diacetate (CM-H_2DCFDA) assay 8
Cell Count 9
Oil Red O Staining 9
Protein Extraction 9
SDS-PAGE and Western Blot Analysis 10

III. RESULTS 11

ROS production decreases during 3T3-L1 preadipocyte differentiation 11
Differentiating 3T3-L1 preadipocytes become resistant to increased levels of exogenous ROS 13
Increase in ROS impairs 3T3-L1 preadipocytes differentiation 14
Increased ROS levels delay expression of PPARγ and decrease expression of C/EBPβ 16
Increased ROS halts cell cycle progression during 3T3-L1 preadipocytes differentiation 19
Impaired differentiation induced by increased levels of ROS is reversible 22

IV. DISCUSSION 24

LITERATURE CITED 30

LIST OF FIGURES

Page

Figure 1. The initiation of adipogenesis by MDI....3

Figure 2. MDI treatment leads to re-entry into the cell cycle....4

Figure 3. Expression of essential transcription factors needed for adipocyte differentiation....5

Figure 4. ROS production during 3T3-L1 preadipocyte differentiation....12

Figure 5. Effect of PQ on ROS production during differentiation....14

Figure 6. Dose dependent effect of ROS on 3T3-L1 preadipocyte differentiation....15

Figure 7. Increased levels of ROS decrease PPARγ expression....17

Figure 8. C/EBPβ expression decreases in presence of increased ROS....19

Figure 9. Increased levels of ROS impair Cyclin D1 expression during differentiation....21

Figure 10. Normalization of ROS levels rescues differentiation....23

CHAPTER I

INTRODUCTION

Obesity

In our society adipose tissue carries a negative stigma because of its association with obesity and diabetes. The rate of obesity is steadily increasing worldwide and currently over one-third of the US adult population is classified as clinically obese (1). By 2015, it is predicted that 75% of U.S. adults will be overweight while 41% will be obese (2). The Centers for Disease Control and Prevention (CDC) predicts that by 2050, 100% of U.S. adults will be clinically overweight. More alarming is the current increase in the incidence of childhood obesity. Today, one in three children in the U.S. is considered either overweight or obese, a statistic that has tripled since 1963 (3). According to the American Heart Association, childhood obesity has become the primary health issue for adolescents due to associated long term health problems carried into adulthood (4). Childhood obesity correlates with adult diseases, such as type 2 diabetes and cardiovascular diseases. According to the CDC, the medical cost of obesity in the U.S. in 2008 was roughly $147 billion making it both a public health and economic issue.

Adipose tissue

Adipose tissue functions as a storehouse of triacylglycerol, which can be utilized during times of food deprivation (5). Storage of triacylglycerol allows adipose tissue to

maintain metabolic and energy homeostasis (6). This important function of adipose tissue is often overlooked. As caloric intake increases, adipocytes increase in size and number to accommodate the need for excess energy storage (6, 7). To increase in number, preadipocytes are induced to differentiate into mature adipocytes and thus result in the expansion of adipose tissue (7- 9). This process, called adipogenesis, maintains energy balance and prevents lipid storage in peripheral tissues, which can negatively affect their function.

Adipogenesis

Most of our understanding of adipogenesis comes primarily from *in vitro* models. The 3T3-L1 murine fibroblast cell line is one of the most characterized models for studying adipogenesis and has advanced our knowledge of adipogenesis by allowing the identification of different molecular markers of differentiation (10, 11). During differentiation, 3T3-L1 preadipocytes exhibit distinct stages which are similar to endogenous adipocyte differentiation (10, 11). Initially, 3T3-L1 preadipocytes proliferate until reaching confluency. Once confluent, the preadipocytes enter contact inhibited cell cycle arrest (10). Exposure to a combination of isobutylmethylxanthine (MIX), dexamethasone (DEX) and insulin (I) allows the preadipocytes to reenter the cell cycle and differentiate. MIX is a phosphodiesterase inhibitor that increases intracellular cAMP levels and DEX is a synthetic glucocorticoid that binds to the glucocorticoid receptor affecting gene transcription. Insulin binds to the insulin-like growth factor receptor 1 (IGF-1) stimulating various signaling pathways needed to initiate differentiation (Fig. 1). The combination of MIX, DEX, and I is known as MDI treatment.

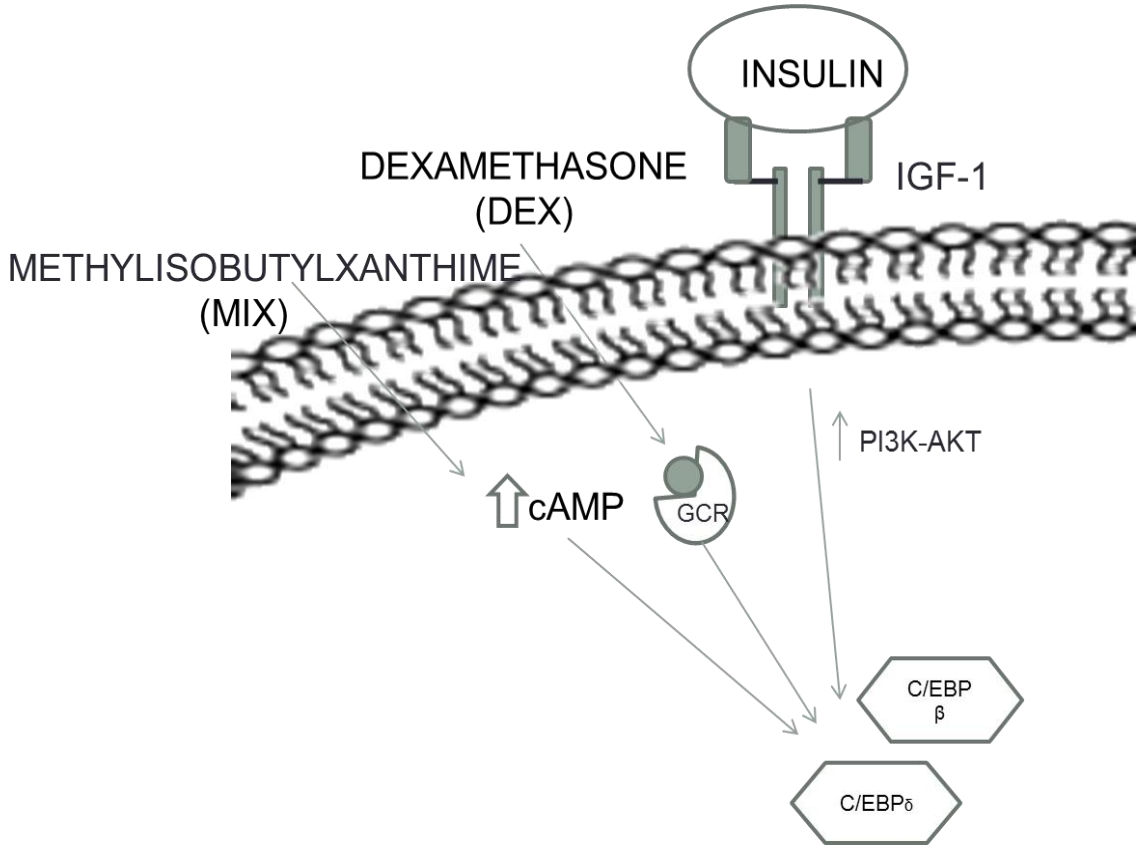


Figure 1. The initiation of adipogenesis by MDI

Clonal expansion

Once cells are stimulated by MDI, the fibroblast re-enter the cell cycle; a stage referred to as clonal expansion (11). Clonal expansion requires phosphorylation of the retinoblastoma protein (Rb) (10, 11). Rb is a cell cycle checkpoint protein that prevents cell cycle progression from the G_1 to S- phase (14). Once Rb is phosphorylated, 3T3-L1 preadipocytes synchronously undergo two rounds of cell division before re-entering growth arrest (10, 15). Cyclin D-dependent kinases (CDK) play a major role in the phosphorylation of Rb (12, 13). Cyclin D forms a complex with CDK4/CDK6 which

phosphorylates Rb, thus deactivating it (12 -14). Any impairment of cyclin D would affect the phosphorylation of Rb and cell cycle progression (Fig. 2).

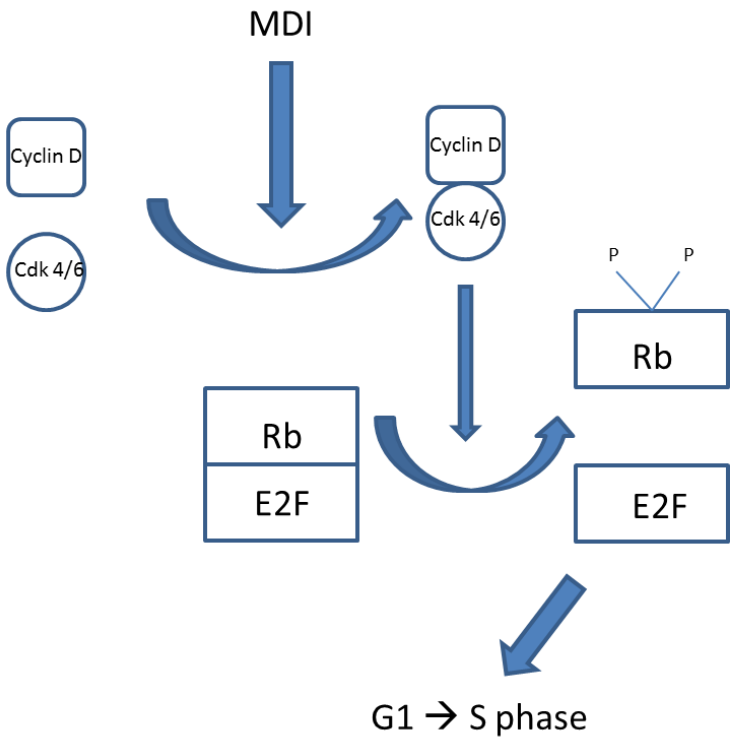


Figure 2. MDI treatment leads to re-entry into the cell cycle.

Upon reentering the cell cycle, transcription factors such as CCAAT/enhancer binding protein β and δ are expressed which induce expression of the key adipogenic transcription factors, peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα (10). C/EBPβ specifically is required for the activation of PPARγ and C/EBPα (16, 17 and 18). Expression of C/EBPα and PPARγ induce the expression of adipocyte-specific genes that lead to the adipocyte phenotype (10, 19). Differentiation of 3T3-L1 preadipocytes is achieved within 7days and is defined by lipid accumulation (Fig. 3).

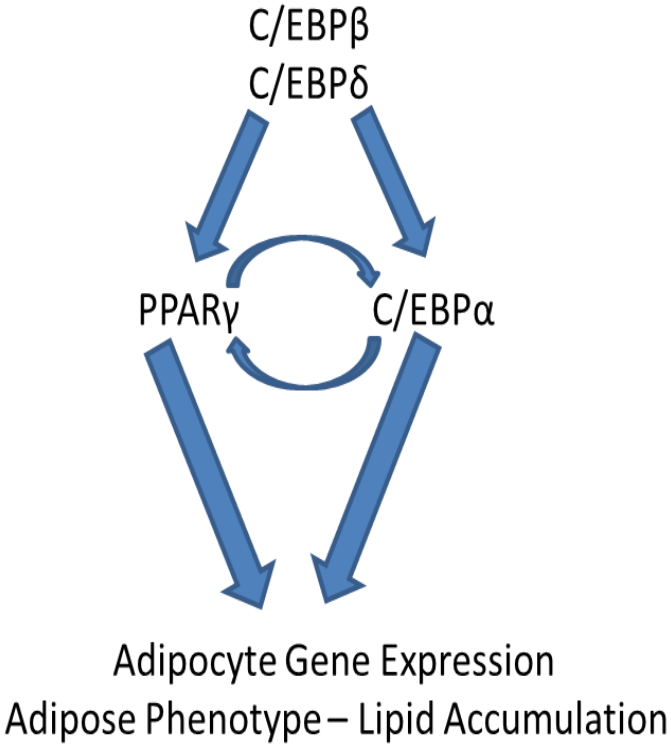


Figure 3. Expression of essential transcription factors needed for adipocyte differentiation.

Oxidative stress

One aspect of adipose tissue that is critical in maintaining energy homeostasis is adipogenesis. Alterations in adipogenesis can lead to health complications such as obesity and type 2 diabetes (10). One process that is known to influence adipocyte biology and energy homeostasis is oxidative stress. Prolonged oxidative stress has been shown to impair glucose uptake leading to insulin resistance in 3T3-L1 adipocytes (20). Oxidative stress also alters activities of various adipocytokines which play a major role in the inflammatory response and metabolic regulation of adipocytes which affects insulin

signaling (21). While oxidative stress is known to impact adipocyte biology, its role in adipogenesis remains unclear.

While the role of oxidative stress on adipogenesis remains uncertain, there is evidence that it influences differentiation in various other cell types. In myoblasts, oxidative stress inhibits differentiation by hindering peptidyl-prolyl-*cis*-*trans*-isomerase activity (PPIase) (22). PPIase facilitates protein folding during myosin differentiation. Oxidative stress has also been shown to inhibit osteoblast differentiation by stimulating NF-(kappa) B activity. NF-(kappa)B is a transcription factor that responds to inflammatory stimuli. Activation of NF-(kappa) B leads to a decrease in the differentiation markers, alkaline phosphatase (AP) and type I collagen (23). Along with its ability to impair differentiation, oxidative stress can also induce differentiation in certain cell types. In L6 myoblasts, exposure to reactive oxygen species (ROS) during differentiation accelerates cell growth (24). In nueroblastoma cells, hydroxyl free radicals have been shown to induce differentiation making it a vital part of differentiation (25).

While some studies have examined the role of oxidative stress during preadipocyte differentiation, the results remain unclear (26). ROS have been shown to interfere with the transcription factor, hypoxia-inducible factor 1 (HIF-1), which inhibits $PPAR\gamma_2$ gene expression and thus inhibits differentiation of preadipocytes (27). Carriere et al show that increased levels of hydrogen peroxide (H_2O_2) lead to the overexpression of the transcription factor CHOP-10/GADD153, which results in an inhibition of preadipocyte differentiation (28). In contrast, other studies have shown that ROS

accelerates early mitotic clonal expansion during adipogenesis leading to increased differentiation (29). Moreover, cells treated with diallyl disulfide, which increases intracellular H_2O_2 production, enhanced differentiation of 3T3-L1 preadipocytes (30). What remains unknown is whether increased ROS accelerates or impairs differentiation.

Hypothesis

Since the role of increased ROS on differentiation remains unclear, we were interested in elucidating the response of increased ROS on differentiation. We also want to examine the timeframe during which increased ROS impacts differentiation and determine whether the effects of increased ROS on differentiation are reversible.

CHAPTER II

METHODS

Cell culture

Murine 3T3-L1 preadipocytes were grown to confluency in high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum (BCS). Forty-eight hours post confluency (day 0), the 3T3-L1 preadipocytes were induced to differentiate in high glucose DMEM containing 10% fetal bovine serum (FBS) with 0.52 mM 3-isobutyl-1-methylxanthine, (M), 1 µM dexamethasone, (D) and 1.7 µM insulin, (I). On day 2, cells were incubated in high glucose DMEM containing 10% FBS and 0.425 µM insulin. On day 4, cells were incubated in high glucose DMEM with 10% FBS, which was replaced every two days until analysis.

Choloromethyl - 2', 7 – dichlorofluorescein diacetate (CM-H_2DCFDA) assay

In order to assay for ROS, 3T3-L1 preadipocytes during different days of differentiation were washed twice in 1X phosphate buffered saline (PBS) (2 mL/per plate) and incubated with 10 µM of CM-H_2DCFDA in 1x PBS for 30 min at 37°C in the dark (34). Following the 30 min, the cells were washed twice in 1x PBS (2 mL/per plate) and cells were again incubated in high-glucose DMEM phenol red free media. The oxidation yields a fluorescence that is trapped inside the cell. Fluorescence (for 1 to 3

days) was measured using a Microplate Spectofluorometer (Emission: 492/495 – excitation: 517/527 nm) (34).

Cell Count

3T3-L1 preadipocytes were differentiated as described above. Once the cells were confluent, Cells were trypsinized at 37°C for 5 min. Nine µl of the cell-trypsin solution was loaded onto a hemocytometer, and counted in four squares of the hemocytometer and the total cells per milliliter (ml) was determined by dividing the total number of cells in the four squares by 4 and then multiplying by 10^4 (cells/ml).

Oil Red O Staining

3T3-L1 preadipocytes were differentiated as described above. On Day 8, plates were washed twice in 1X PBS (2 mL/per plate) and fixed in 3.7% formaldehyde (4 mL/ per plate) in a fume hood for 4 min. Then, each plate was washed twice with 1X PBS (2 mL/ per plate). Cells were incubated with one mL of 0.5% ORO at room temperature for an hour. ORO was removed and cells were washed twice with 1X PBS (2 mL/ 6cm plate). The prepared ORO samples were imaged for lipid accumulation using the EVOSxl transmitted light microscope. The samples were imaged at 20x and 40x magnification.

Protein Extraction

On various days of differentiation, a 10 cm plate was washed twice with 1X PBS (2mL/plate) and cells were lysed with 200 µL of lysis buffer containing of 25 mM Hepes, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 2% glycerol, 5 mM NaF, 1 mM EDTA, 1 mM

Na3VO4, 1 mM NaPPi, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg / ml aprotinin, 5 mg / ml leupeptin, and 5 mg/ml pepstatin. The protein samples were rocked for 20 min at 4°^{C} and then were centrifuged for 20 min at 6000 rpm at 4°C.

SDS-PAGE and Western Blot Analysis

Approximately 80-100 µg of protein was subjected to 10% SDS-PAGE. The proteins were transferred to an Immobilon filter. Filters were incubated in a 4% BSA / 1x Tris-Buffered Saline – Tween 20 (1X TTBS) solution for one hour. The primary antibody was applied to the Immobilon filter and incubated overnight at 4°C. The filter was washed three times in 1X TTBS then a secondary antibody was used at 1:2000 dilutions in 1X TTBS for 45 minutes and then washed, three more times in 1X TTBS.

Super Signal West Pico Chemiluminescent Substrate kit was used to detect proteins on the Immobilon filter. The Immobilon filter was imaged on the Bio-Rad imaging station and proteins were quantified using the Quantity One densitometry program.

CHAPTER III

RESULTS

ROS production decreases during 3T3-L1 preadipocyte differentiation

Previous studies have shown that alterations in ROS levels can modify adipocyte biology by affecting glucose uptake and altering activities of various adipocytokines (20, 21). We were interested in observing what effect elevated levels of ROS would have on adipogenesis which is a key part of adipocyte physiology. Initially, we were interested in determining the endogenous levels of ROS produced during differentiation to establish a baseline. In order to measure ROS production, we used the CM-H_2DCFDA assay. CM-H_2DCFDA is a useful indicator of ROS in live cells because it passively diffuses into cells where intracellular exterases cleave its acetate groups and the thiol-reactive chloromethyl group reacts with intracellular glutathione or other thiols to fluoresce (34). Differentiating 3T3-L1 preadipocytes were incubated with CM-H_2DCFDA for 30 min in the dark at 37°C and ROS production was measured on day 1, 2, 3, 4, and 7.

As shown in Fig. 4, ROS levels are high in confluent growth arrested preadipocytes, but upon MDI induction of differentiation ROS levels decrease dramatically during days 1-3 and then remain constant at a reduced level. The measured ROS production was adjusted to reflect ROS levels per cell during each day of

differentiation and normalized to day 0 levels. ROS production was adjusted per cell because clonal expansion, which occurs during the first 3 days of differentiation, would result in an increase in the number of cells during differentiation. The highest ROS production during differentiation was observed during day 0. Our study is the first to show that ROS production decreases over the course of differentiation. One of the reasons ROS production could have decreased during differentiation is because ROS production could interfere with cellular processes involved in differentiation. These findings suggest that there is a steady decrease of ROS during the course of differentiation which leads to a new constant steady state lower level in adipocytes.

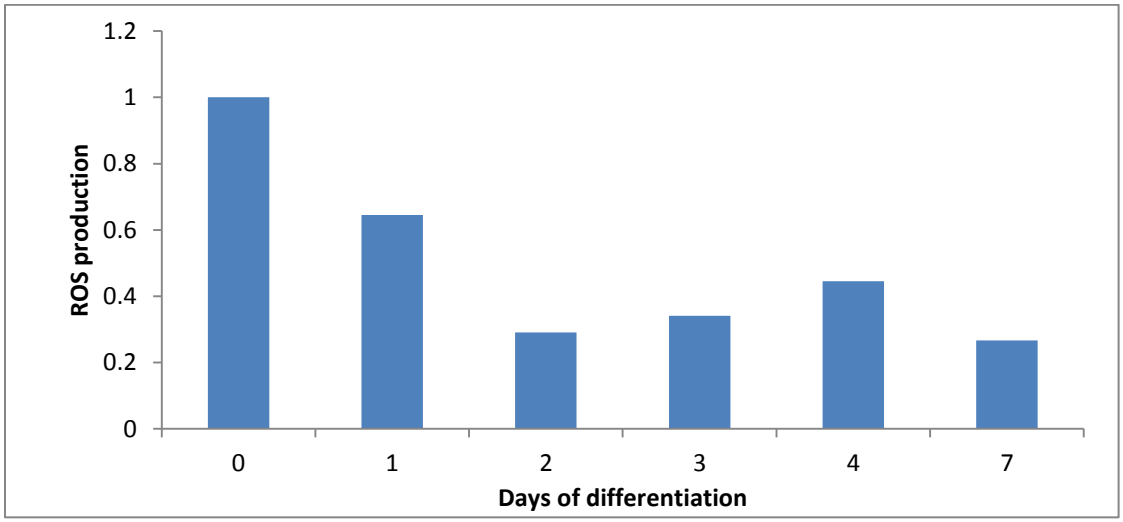


Figure 4. ROS production during 3T3-L1 preadipocytes differentiation. 3T3-L1 preadipocytes were differentiated as described in Methods. ROS levels in differentiating 3T3-L1 preadipocytes were determined using 10 μM of CM-H_2DCFDA and measured using a Microplate Spectrofluorometer (Emission: 492/495 – excitation: 517/527 nm) during different days of differentiation (day 1, 2, 3, 4, and 7). The amount of ROS production was normalized to cell number.

Differentiating 3T3-L1 preadipocytes become resistant to increased levels of exogenous ROS

To determine if increased ROS production alters 3T3-L1 preadipocytes differentiation, we treated induced preadipocytes with a compound that increases ROS levels, paraquat (PQ), and measured ROS levels using a CM-H_2DCFDA assay. Differentiating 3T3-L1 preadipocytes were incubated in the presence or absence of PQ and ROS production was measured during days 1, 2, 3, 4, and 7.

As shown in Fig. 5, PQ causes a significant increase in ROS levels compared to normally differentiating 3T3-L1 preadipocytes throughout differentiation. During the initial three days of differentiation there was a significant increase in ROS production in the presence of PQ. PQ caused a two fold increase in ROS production during day 1 compared to normally differentiating 3T3-L1 preadipocytes. The ROS levels remained higher in presence of PQ until day 3 of differentiation. ROS levels could not be increased in treated cells on day 4 or 7, this could be due to the activation of intracellular stress response pathways that would initiate antioxidant production and thus decrease excess ROS production for cell survival. These adaptive stress response pathways are vital for cell survival because they protect against cellular damage which would increase reactive oxygen species (48). Our findings suggest that PQ does elicit a substantial increase in ROS only during the early stages of 3T3-L1 preadipocytes differentiation. Our studies further show that at later time points ROS levels are refractory to treatments. The dramatic increase in ROS production during the initial days of differentiation could

potentially be during the timeframe when increased levels of ROS impair 3T3-L1 preadipocytes differentiation.

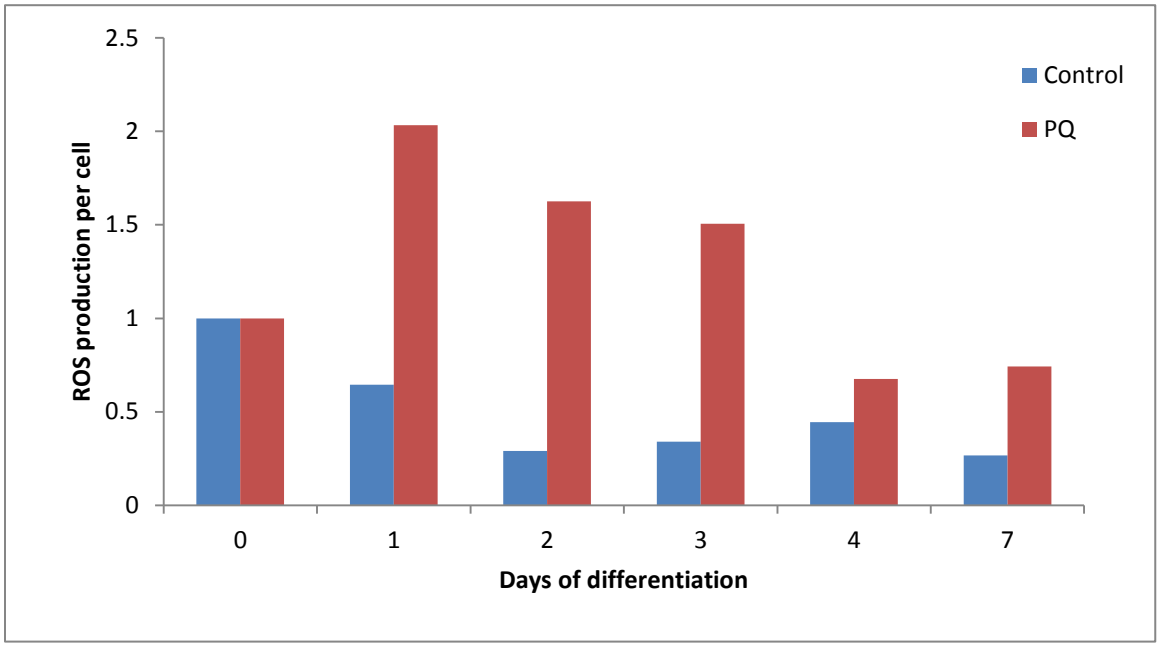


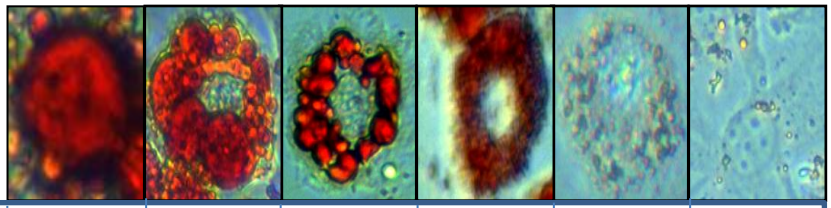
Figure 5. Effect of PQ on ROS production during differentiation. 3T3-L1 preadipocytes were differentiated in presence or absence of 40 μM PQ throughout differentiation as indicated in Methods. CM-H_2DCFDA dye was used to detect ROS production in cells and assayed using a Microplate Spectofluorometer (Emission: 492/495 – excitation: 517/527 nm). The amount of ROS production was normalized to cell number.

Increase in ROS impairs 3T3-L1 preadipocytes differentiation

Next we wanted to determine the effect of increased levels of ROS on 3T3-L1 preadipocytes differentiation. Since differentiation was impaired in the presence of increased ROS, we wanted to determine the precise threshold of ROS that impairs 3T3-L1 preadipocytes differentiation. To determine this, 3T3-L1 preadipocytes were differentiated in the presence of different levels of ROS via treatments of increasing

concentrations of PQ throughout differentiation (day 0 – 7). Differentiation was quantified on Day 8 by Oil Red O staining.

We observed that as ROS levels increased, differentiation decreased. The extent of differentiation decreased inversely up to a threshold level of ROS (40 µM PQ) at which point the levels of lipid accumulation detected by ORO staining were minimal (Fig. 6). There is a slight difference in the amount of differentiation at the low concentration of PQ (10 – 20 µM); however as the concentrations of PQ increased past 20 µM, the extent of differentiation decreased in a dose-dependent manner with increasing levels of ROS. The greatest impairment of differentiation was seen at a concentration of 40 µM PQ. This finding indicates that, lipid accumulation is completely impaired during differentiation at a specific concentration of ROS.



PQ (µM)	-	10	20	30	40	50

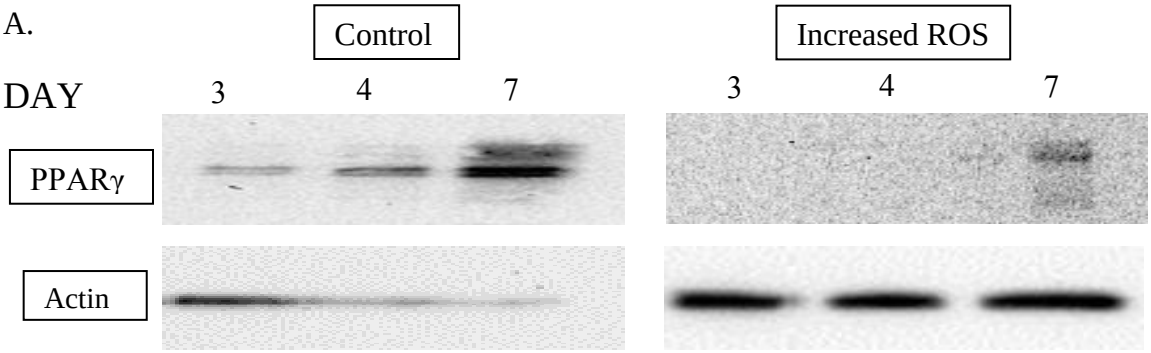
Figure 6. Dose dependent effect of ROS on 3T3-L1 preadipocytes differentiation. 3T3-L1 preadipocytes were differentiated in the presence of 10 – 50 µM PQ for 7 days. On day 8, the cells were fixed and stained with ORO. The cells were imaged using an EVOSxl transmitted light microscope at 40x magnification.

Increased ROS levels delay expression of PPARγ and decrease expression of C/EBPβ

Next, we were interested in determining the target of elevated levels of ROS during differentiation. Since increased ROS impairs lipid accumulation, we wanted to determine the mechanism by which ROS exerts its affects. To determine this, we examined the expression of the essential transcription factors PPARγ and C/EBPβ under increased ROS concentrations. C/EBPβ and PPARγ play a critical role in adipogenesis. C/EBPβ is required for the expression of PPARγ, which is required for the expression of adipocyte specific genes needed for the adipocyte phenotype (10). Inhibition of either PPARγ or C/EBPβ expression can impair adipocyte differentiation (10, 19).

As shown in Fig. 7A and B, the expression as PPARγ was significantly delayed with increased ROS levels when compared to control preadipocytes. Since the expression of PPARγ was drastically delayed, the signal needed for the expression of adipocyte specific genes was also delayed, leading to impaired differentiation. Since the expression of PPARγ was delayed but not completely silenced, minimal differentiation was observed in the presence of increased ROS. Actin was used as a marker to evaluate differentiation. It is hypothesized that as the lipid droplets increase in size, they aid in supporting the characteristic structure of the adipocyte, thus reducing the need for actin for structural support. In control cells, we observed a decrease in actin expression as the cells proceeded through differentiation, supporting previous findings, however in cells with increased ROS levels the actin levels did not decrease (10).

A.



B.

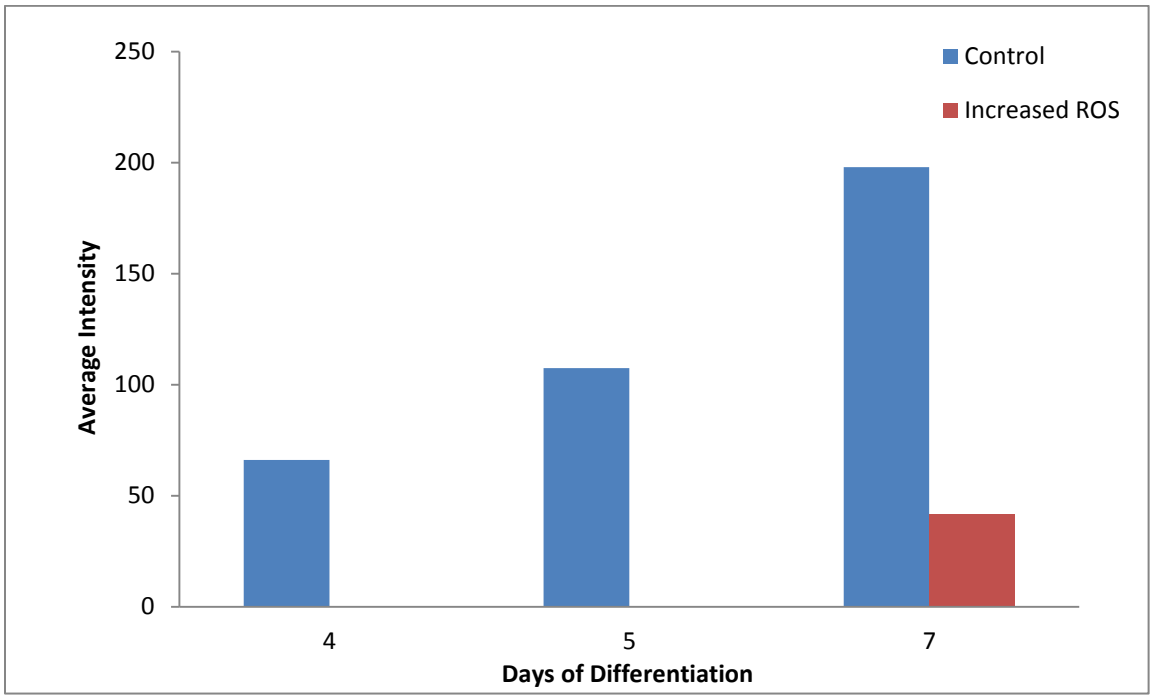
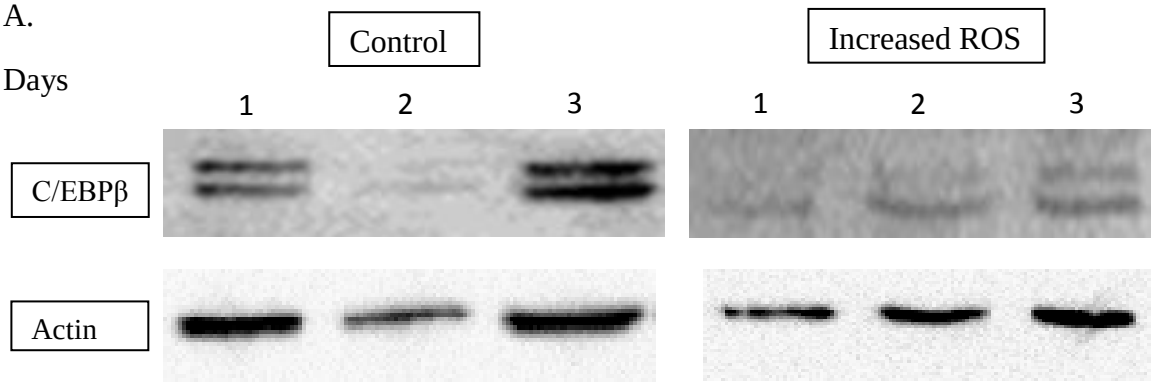


Figure 7. Increased levels of ROS decrease PPARγ expression. Cells were incubated in presence or absence of increased ROS levels throughout differentiation (day 0 – 7). A.) During different days of differentiation cells were lysed and protein lysates were subjected to 10% SDS-PAGE, transferred to an Immobilon filter and then probed with antibodies against PPARγ. B.) PPARγ was visualized using the Bio-Rad gel documentation station and quantified by densitometry using Quality One software.

Since PPARγ expression was delayed, we wanted to determine if transcription factors regulating the expression of PPARγ were affected by increased levels of ROS. C/EBPβ induces the expression of PPARγ during adipocyte differentiation. The effect of increased ROS on C/EBPβ was evident during the early-stages of differentiation. The expression of C/EBPβ was reduced during differentiation in the presence of increased ROS compared to untreated cells (Fig. 8A and B). While C/EBPβ is still expressed, its function in adipogenesis could be altered through increased ROS. One possible way increased ROS could affect C/EBPβ is by affecting its DNA-binding activity. Previous studies have shown that while C/EBPβ is expressed on day 0, its DNA-binding activity is not seen until 8 h after the induction of differentiation and corresponds with entry into S-phase of the cell cycle (35). This would suggest that while C/EBPβ is expressed in both groups, its role in differentiation might not be relevant until the preadipocytes re-enter the cell cycle.

A.



B.

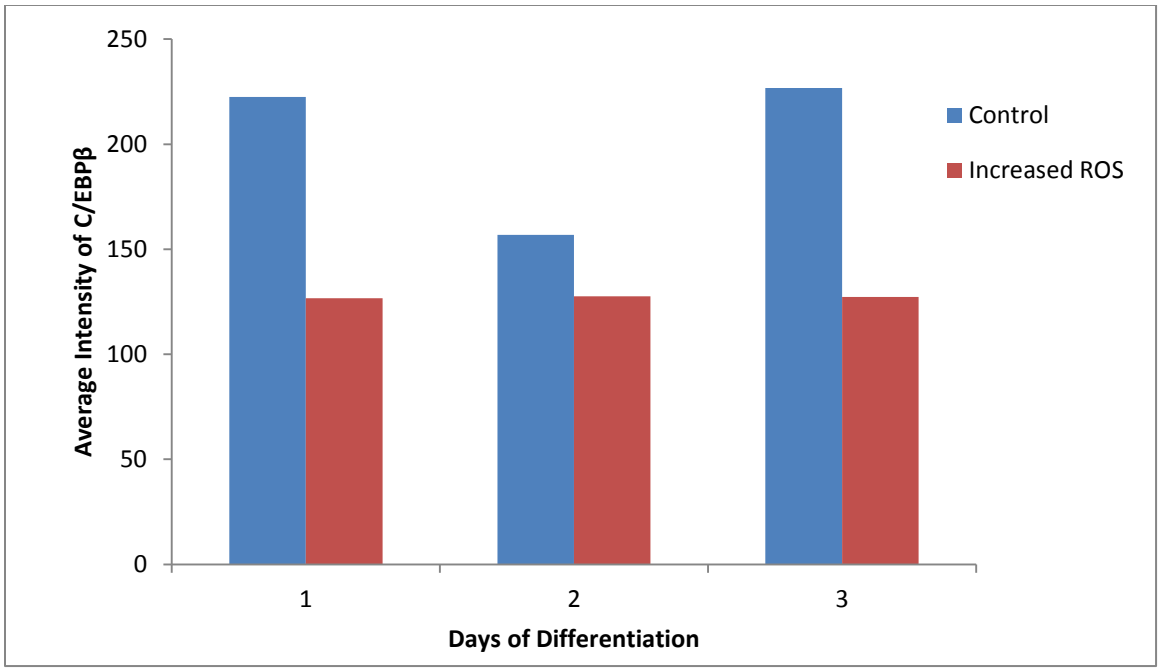


Figure 8. C/EBPβ expression decreases in presence of increased ROS. Cells were incubated in presence or absence of increased ROS levels from day 0 – 3 of differentiation. A. During different days of differentiation cells were lysed and lysates were subjected to 10% SDS-PAGE and then probed with an antibody against C/EBPβ. B.) C/EBPβ was visualized using the Bio-Rad gel documentation station and quantified by densitometry using Quality One software.

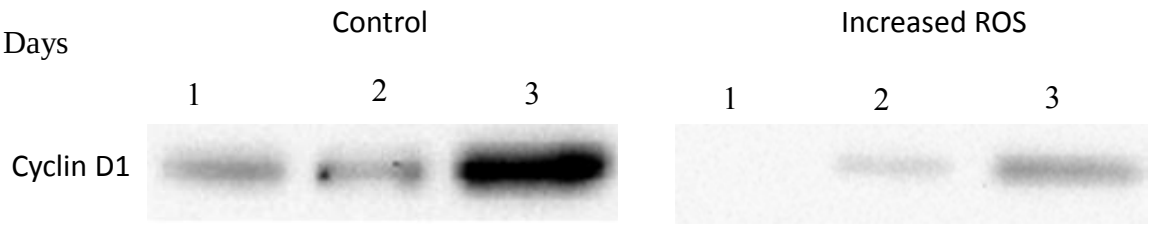
Increased ROS halts cell cycle progression during 3T3-L1 preadipocytes differentiation

Our previous study showed that increased ROS affects the expression of the essential transcription factors PPARγ and C/EBPβ. Because the effect of increased ROS on later stages of differentiation was evident, we wanted to further investigate if that was due to its effects on mitotic clonal expansion during the early stages of 3T3-L1 differentiation. To determine whether increased ROS levels affected re-entry of growth arrested preadipocytes into the cell cycle, we examined Cyclin D1 expression. Cyclin D

is one of the early markers of cell cycle progression. In order for the 3T3-L1 preadipocytes to differentiate, they must re-enter the cell cycle upon MDI induction. Re-entry into the cell cycle requires the phosphorylation of Rb protein through formation of the Cyclin D- CDK4/CDK6 complex (12, 13). When activated by the Cyclin D – CDK4/CDK6 complex, Rb becomes phosphorylated and inactivated allowing for progression into S-phase and cell cycle progression. If increased ROS diminishes Cyclin D expression then that would preclude the formation of the Cyclin D- CDK4/CDK6 complex, which is essential for entry into S-phase of the cell cycle (43, 44). This would impair later phases of differentiation and lead to the impairment of differentiation.

Cyclin D expression increases immediately after MDI treatment. Increased ROS delayed and decreased the expression of Cyclin D1 when compared to normally differentiating preadipocytes (Fig. 9 A and B). In cells that differentiated in the presence of increased levels of ROS, Cyclin D1 was not detected until day 2 of differentiation and the levels were significantly reduced when compared to control cells. This delay in Cyclin D1 would affect Rb phosphorylation and halt or delay cell cycle progression. This could impact C/EBPβ's DNA-binding activity because its role in differentiation is associated with cell cycle progress through S-phase (35). This would then delay PPARγ expression and lead to impaired differentiation. We have identified that increased ROS does impact early stages of differentiation by affecting Cyclin D1 expression which is involved in cell cycle progression.

A.



B.

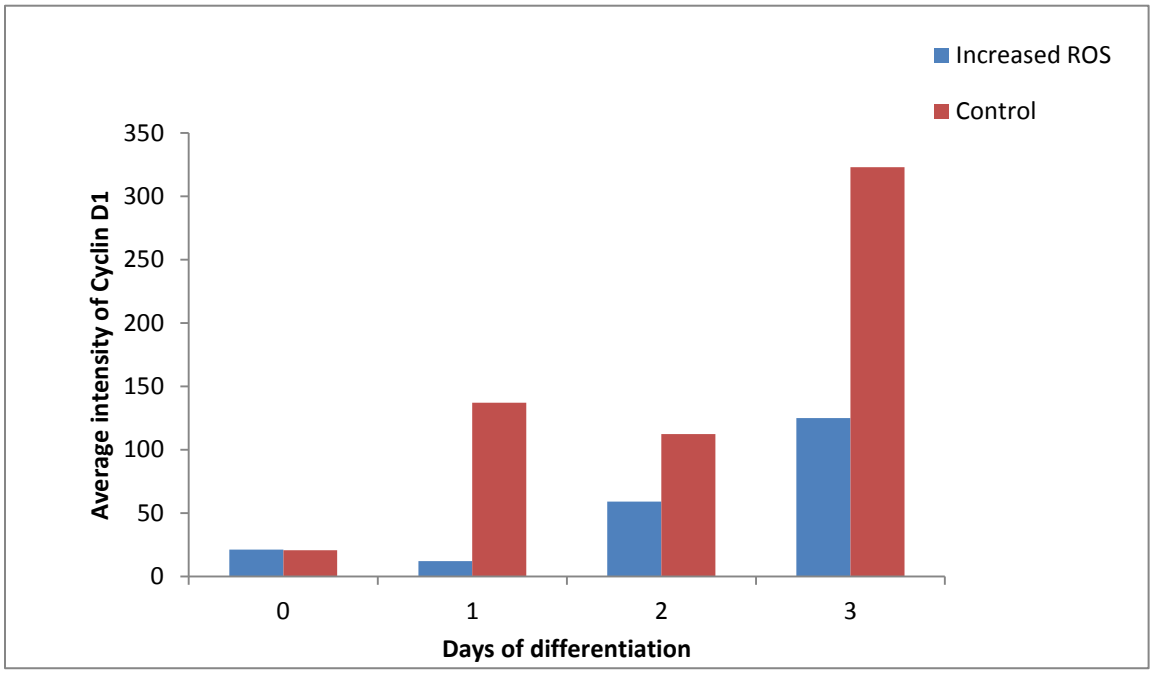


Figure 9. Increased levels of ROS impair Cyclin D1 expression during differentiation. A. Day 0 preadipocytes were incubated in presence or absence of increased levels of ROS through day 3 of differentiation. Cells were lysed and protein lysates were analyzed by 10% SDS-PAGE, blotted to an Immobilon membrane, and then probed with an antibody against Cyclin D1. B. Cyclin D1 was visualized using the Bio-Rad gel documentation station and quantified by densitometry using Quality One software.

Impaired differentiation induced by increased levels of ROS is reversible

Since we show that increased ROS impairs 3T3-L1 preadipocytes differentiation by disrupting cell cycle progression, we wanted to investigate whether 3T3-L1 preadipocytes would be able to re-enter the cell cycle once ROS levels are returned to normal. This is important because if differentiation is achievable once ROS levels are returned to normal, then we know that the effect of increased ROS on differentiation is not permanent but reversible. If differentiation is permanently impaired, then this could cause long term ramifications and lead to serious health complications.

For this study, cells were differentiated in the presence of increased ROS for day 7, at which point no differentiation was observed. On day 7, one group of cells was continued in media containing high levels of ROS, while the other group was induced to differentiate in the absence of increased ROS for another 7 days, until day 14. As shown in Fig. 10, 3T3-L1 preadipocytes were able to differentiate when induced to differentiate in the absence of high levels of ROS. The progression of differentiation was compared with the control group, which differentiated in the absence of increased ROS for 14 days. While differentiation was seen once ROS levels were returned to normal, the level of differentiation was reduced (Fig. 10) but was considerably greater than that observed in cells that were differentiated in the presence of high levels of ROS. This finding illustrates that increased ROS does not permanently alter differentiation and that once the ROS levels are normalized, differentiation can be induce by MDI treatment. This would allow for the expansion of adipose tissue and maintenance of energy homeostasis.

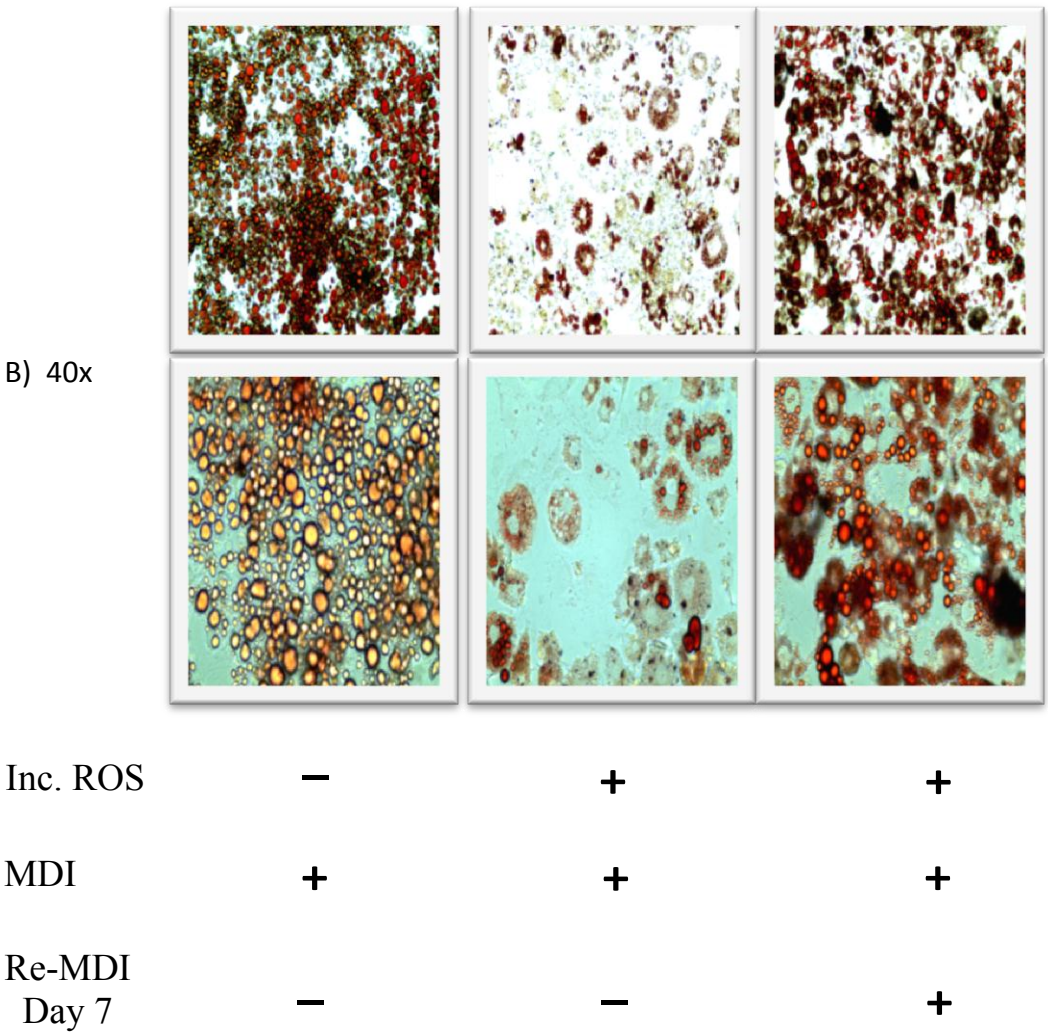


Figure 10. Normalization of ROS levels rescues differentiation. 3T3-L1 preadipocytes were differentiated in the presence or absence of increased ROS levels. On day 7, one of the treated samples was subjected to MDI again in the absence of increased ROS. Another sample was differentiated in presence of increased ROS for 14 days in the absence of MDI at day 7. On day 14, cells were fixed and stained with ORO. The cells were imaged using a light microscope at 20x and 40x magnification.

CHAPTER IV

DISCUSSION

In our study, we examined the role of increased ROS levels on 3T3-L1 preadipocyte differentiation. First, we determined the level of ROS produced normally during adipogenesis and then established how increased ROS would impact differentiation of 3T3-L1 preadipocytes (Fig. 4, 5).We have shown that increased ROS can impair the lipid accumulation of 3T3-L1 preadipocytes (Fig. 6). As ROS production increased, the amount of lipid accumulation we observed decreased to levels seen in cells that were not induced to differentiate. Our study showed a threshold in ROS production after which differentiation was completely impaired. This is significant because many factors can cause an increase in ROS production, such as exposure to pesticides, UV radiation and obesity. All these factors which cause an increase in ROS could play a key role in adipose biology by negatively affecting preadipocyte differentiation, which would also affect adipose tissue expansion.

Initially, the cells were sensitive to increased levels of ROS, but ultimate they became resistant. Our study is the first to show that during the later stages of differentiation, exogenous ROS is unable to raise ROS levels within the cell. One possible explanation for this is that cells activate adaptive stress response pathways for survival. The initial response to stressful stimuli for cells is to eliminate the stress to

ensure cell survival and avoid apoptosis (48). In the experimental group, this mechanism could be the reason ROS production decreased during differentiation even though the amount of increased ROS exposed to the cells remained constant throughout differentiation. For future studies, the effect of increased ROS on ROS-metabolizing enzymes such as catalase and superoxide dismutase during differentiation could be determined.

Once it was established that increased ROS levels impaired 3T3-L1 preadipocytes differentiation, we determined the timeframe during which it exerted its anti-adipogenic effects. First, we examined the effect of increased ROS on the essential transcription factor; PPARγ. We found was that PPARγ expression was delayed in the presence of increased ROS (Fig. 7 A and B). During differentiation, PPARγ is expressed on day 2 (10), however in the presence of increased ROS, PPARγ expression was delayed until day 7 of differentiation. This delay in PPARγ expression explains the reduced lipid accumulation we saw since PPARγ is required for the expression of genes responsible for lipogenesis, lipid droplet formation, and the development of the adipocyte phenotype. Since PPARγ is still expressed although later than normal in the presence of increased ROS, future studies could focus on the role of increased ROS on 3T3-L1 preadipocytes differentiation past day 7. Since PPARγ expression was observed at day 7, we wonder if elevated levels of ROS just delayed differentiation instead of blocking it.

Similarly, the expression of C/EBPβ was decreased in the presence of increased ROS compared to normally differentiating 3T3-L1 preadipocytes (Fig.8 A and B). This is

noteworthy because C/EBPβ is required for the expression of PPARγ which induces the expression of genes responsible for the adipose phenotype (11). While C/EBPβ was detected in the presence of increased ROS, its activity during differentiation is dependent on cell cycle progression which could have been impaired by increased ROS (35).

Since cell cycle progression is required for C/EBPβ DNA binding activity, we further examined the effect of increased ROS on clonal expansion by monitoring a marker of cell cycle progression, Cyclin D1. We found that the expression of Cyclin D1 was significantly delayed and the levels decreased in the presence of increased ROS (Fig. 9 A and B). The expression of Cyclin D1 increases immediately after exposure to MDI in untreated differentiating cells, but in the presence of increased ROS, Cyclin D1 expression was not observed until day 2 of differentiation. This is an important finding because phosphorylation of Rb is dependent on the Cyclin D-CDK4/6 complex (43 - 45). If Cyclin D1 expression is delayed, that would lead to decreased Cyclin D-CDK4/6 complex formation and decreased phosphorylation of Rb. The phosphorylation of Rb is required for cell cycle progression from G1- to S-phase and required for the DNA-binding activity of C/EBPβ (35). Our results reveal that the expression of Cyclin D1 was delayed in the presence of increased ROS; Future studies should focus on how the increased ROS affected Cyclin D1 expression. Does increase in ROS directly affect Cyclin D1 expression or indirectly through signaling components such as phosphatidylinositol 3- kinase (PI3K) and Mitogen-activated protein kinase? Both kinases play a role in cell proliferation. A previous study had shown that PQ induced oxidative stress impaired glucose uptake in 3T3-L1 adipocytes by interfering with the

PI3K signaling pathway (36). Elevated ROS levels could act by a similar mechanism to affect 3T3-L1 preadipocytes differentiation.

Lastly, we investigated if differentiation was achievable once the ROS levels were returned to normal. We found that differentiation was achieved once the stress of increased ROS was removed (Fig. 10). While the amount of differentiation observed was not to the same degree as the control, it was significantly greater than that observed in cells differentiated under constant exposure to increased ROS. This is an important finding because it reveals that the effects of increased ROS on differentiation are not permanent, and that differentiation is achievable once increased ROS are removed.

Future studies could determine whether differentiation could still occur after day 7 if induced by MDI in the presence of increased ROS. It would be noteworthy to examine because in our study, differentiation was achievable once increased ROS was cleared after day 7, but the amount of ROS produced in the presence of increased ROS was dramatically reduced by day 7. Thus would differentiation occur once the ROS levels were reduced even in presence of agents that increase ROS with exposure to MDI?

While previous studies have shown that ROS levels increase during the early time points of differentiation, these studies measured total ROS levels, which do increase, but did not normalize to the number of cells present, which also increase due to clonal expansion. (37, 38). In our study we normalized the levels of ROS per cell and found that there was a decrease in the levels of ROS during all time points of differentiation. Our study shows that while ROS are involved in differentiation, there is a threshold of ROS

production at which differentiation is impaired specifically by inhibiting re-entry of cells into the cell cycle. This information can be applied to future studies to determine whether increased ROS production has the same effect on 3T3-L1 preadipocyte differentiation when applied after clonal expansion.

Previous studies on adipocytes differentiation contradict our findings. One study showed that introducing H_2O_2 during differentiation can facilitate cell cycle progression through clonal expansion during 3T3-L1 preadipocytes differentiation (29). In that study, H_2O_2 increased cell cycle progression from the S- to the G_2/M-phase. However, in our study, we showed that increased ROS kept cells from entering the S-phase of the cell cycle. The difference between the two studies is the amount of ROS introduced during differentiation which may impact the role it has on differentiation.

Independent of preadipocytes differentiation, ROS production has been shown to facilitate differentiation of numerous cell types (39, 40,41and 42) but has also been shown to hinder differentiation in other cell types (22, 23). Our study can be used as a cornerstone for future studies to determine if the threshold of ROS that impairs 3T3-L1 preadipocytes differentiation can also be applied to the differentiation status of other cell types. It can also be used to determine if differentiation is possible once ROS levels are restored to normal amounts in other cell types.

The impairment of differentiation via increased ROS production could have a major impact on adipocyte biology by affecting the ability of preadipocytes to differentiate which could ultimately affect the efficiency of insulin signaling and glucose

uptake. If differentiation is impaired, adipocytes may still be able to recruit preadipocytes but the recruited cells would not differentiate due to increased ROS levels. A decrease in adipocyte differentiation could result in fewer mature adipocytes leading to a reduced number of adipocytes capable of maintaining glucose homeostasis. This would have a profound effect on basic physiology since excess glucose would remain in circulation. Also excess glucose would be alternatively stored in peripheral tissues such as the liver and muscle. Displaced lipid accumulation due to excess glucose would affect the target organs' function and could lead to other serious health complications.

Our study has shown that while ROS is present during differentiation, there is a threshold of ROS that completely impairs 3T3-L1 preadipocytes differentiation. We have also shown that increased ROS can be detrimental to 3T3-L1 preadipocytes differentiation by directly affecting clonal expansion. Finally, our study shows that once the increased ROS is removed, the cells are able to re-enter the cell cycle and differentiate, albeit not to the same degree as normally differentiating cells.

LITERATURE CITED

1) Centers for Disease Control and Prevention. 2012. Overweight and Obesity. <http://www.cdc.gov/obesity/data/trends.HTML> Jan. 2012.

2) Johns Hopkins Bloomberg School of Public Health. 2012. Obesity Rates Continues to Climb in the United States. Jan. 2012. <http://www.jhsph.edu/publichealthnews/press_releases/2007/wang_adult_obesity.html>

3) Caprio, S., Daniels, S., Drewnowski, A., et al. Influence of Race, Ethnicity, and culture on Childhood Obesity: Implications for Prevention and Treatment. *Diabetes Care*. Vol. 31: no.11, Nov. 2008.

4) American Heart Association. 2011. Overweight in Children. Jan. 2012. < http://www.heart.org/HEARTORG/GettingHealthy/Overweight-in-Children_UCM_304054_Article.jsp>.

5) Trayhurn, P. Endocrine and signaling role of adipose tissue: new perspectives on fat. *Acta Physiologica Scandinavic*. Vol. 184: Issue 4, pp.285-293, Jul. 2005.

6) Greenberg, A., Obin, M. Obesity and the role of adipose tissue in inflammation and metabolism. *The American Journal of Clinical Nutrition*. vol. 83 no. 2, Feb2006.

7) Venuto, T. "Why Fat Cells Multiply and What To Do About It." 7 Nov. 2010. <http://www.blog.holygrailbodytransformation.com/?p=206>. Nov. 2011.

8) Sumithra, U., Smith, C., Campbell, B., et al. Gene Expression Profiling in Human Preadipocytes and Adipocytes by Microarray Analysis. *The Journal of Nutrition*. 2004; Vol. 134: pp.762-770.

9) Halvorsen, Y., Pingerelli, P., Petre, M. et al. Human Primary Preadipocytes and Differentiated Adipocytes. *Agilent Technologies.* 2002.

10) Francine, G., Cynthia, S., Hei, S., et al. Understanding Adipocyte Differentiation. *American Physiology Society (Physiological Reviews).* 1998; Vol. 78: no.3 pp.783-809.

11) Gregoire, F. Adipocyte Differentiation: From Fibroblast to Endocrine Cell. *Experimental Biology and Medicine*. Vol. 226: no.11 pp.997-1002, Dec. 2001.

12) Kato, J., Matsushime, H., et al. Direct binding of cyclin D-dependent kinase CDK4. *Genes Development*. 1993; 7(3):331-342.

13) Lundberg, S., Weinberg, RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Molecular Cell Biology*. 1998; 18(2):753-761.

14) Weinberg, RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995; 81(3):323-330.

15) Sadowski, H., Wheeler, T., Young, D. Gene expression during 3T3-L1 adipocyte differentiation Characterization of initial responses to the inducing agents and changes during commitment to differentiation. *The Journal of Biological Chemistry.* 1991; Vol. 266: no. 7 pp.4722-4731,

16) Cao, Z., Umek, RM., McKnight, SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes & Development*. 1991; Vol. 5: pp.1538-1552.

17) Wu, Z., Bucher, N.L., Farmer, SR. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ and glucocorticoids. *Molecular Cellular Biology*. 1996; pp.4128–4136.

18) Tanaka, T., Yoshida, N., Kishimoto, T., et al. Defective adipocyte differentiation in mice lacking the *C/EBP$_\beta$* and/or *C/EBP$_\delta$* gene. *The EMBO Journal*. 1997; Vol. 16: pp.7432–7443.

19) Zhidan, W., Rosen, E., et al. Cross-Regulation of C/EBPα and PPARγ Controls the Transcriptional Pathway of Adipogenesis and Insulin Sensitivity. *Molecular Cell*. 1999; Vol. 3: Issue 2 pp. 151-158.

20) Rudich, A., Tirosh, A., et al. Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes*. 1998; Vol. 47: no.10 pp.1562-1569.

21) Furukawa, S., et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of Clinical Investigation.* 2004; Vol. 114: pp.1752–1761.

22) Hong, F., Lee, J., et al. Cyclosporine A blocks muscle differentiation by inducing oxidative stress and inhibiting the peptidylprolyl-cis-trans isomerase activity of

cyclophilin A: cyclophilin A protects myoblasts from cyclosporine A-induced cytotoxicity. *The FASEB Journal*. 2002; Vol. 16: no.12 pp.1633-1635.

23) Bai, X., Lu, D., et al. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kB. *Biochemical and Biophysical Research Communications*. 2004; Vol. 314: Issue 1, pp.197-207.

24) Qiu, XZ., Yu, L., et al. Oxidative stress-induced differentiation of L6 myoblasts. *Institute of Clinical Anatomy, Southern Medical University, Guangzhou*. 2005; pp. 1384-1386.

25) Oravecz, K., Kalka, D., et al. Hydroxyl free radicals induce cell differentiation in SK-N-MC neuroblastoma cells. *Tissue and Cell*. 2002; Vol. 34: issue 1, pp.33-38.

26) Gummersbach, C., Hemmrich, K., et al. New aspects of adipogenesis: radicals and oxidative stress. *Differentiation.* 2008; Vol. 77: pp.115-120.

27) Yun, Z., Maecker, H., et al. Inhibition of *PPARγ2* Gene Expression by the HIF-1-Regulated Gene *DEC1/Stra13*: A Mechanism for Regulation of Adipogenesis by Hypoxia. *Developmental Cell*. 2002; Vol. 2: Issue 3, pp.331-341.

28) Carriere, A., Carmona, M., et al. Mitochondrial Reactive Oxygen Species Control the Transcription Factor CHOP-10/GADD153 and Adipocyte Differentiation. *The Journal of Biological Chemistry*. 2004; Vol. 279.

29) Lee, H., Lee, Y. J., Choi, H., et al. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *The Journal of Biological Chemistry.* 2009; Vol. 284.

30) Lee, J., Kim, K., et al. Diallyl disulfide accelerates adipogenesis in 3T3-L1 cells. *International Journal of Molecular Medicine*. 2007; Vol. 20: no.1 pp.59-64.

31) Shibata, M., Hakuno, F., Yamanaka, D., et al. Paraquat-induced oxidative stress represses PI 3-kinase activities leading to impaired glucose uptake in 3T3-L1 adipocytes. *The Journal of Biological Chemistry*. 2010; Vol. 285.

32) *"P17676 (C/EBPB_HUMAN)."*< http://www.uniprot.org/uniprot/P17676>. Oct. 2012.

33) "Digital Inverted Microscope for Transmitted Light Applications." http://www.amgmicro.com/en/media/evos_xl/user_guide_evos_xl.pdf. Jun. 2012.

34) "CM-H2DCFDA (General Oxidative Stress Indicator)." <http://products.invitrogen.com/ivgn/product/C6827>. 2012.

35) Cole, K. A., Harmon, A. W., Harp, J. B., Patel, Y. Rb regulates C/EBPβ-DNA-binding activity during 3T3-L1 adipogenesis. *American Journal of Physiology: Cell Physiology.* 2004; *55*.

36) Shibata M., Hakuno F., et al. Paraquat-induced oxidative stress represses phosphatidylinositol 3-kinase activities leading to impaired glucose uptake in 3T3-L1 adipocytes. The Journal of Biological Chemistry. 2010; 20915-25.

37) Kanda, Y., Hinata, T., Kang, S., Watanabe, Y. Reactive oxygen species mediate adipocyte differentiation in mesenchymal stem cells. *Life Sciences.* 2011; 89, 250-258.

38) Tormos, K. V., Anso, E., Hamanaka, R. B., et al. Mitochondrial Complex III ROS Regulate Adipocyte Differentiation. *Cell Metabolism.*2011; 14, 537-544.

39) Sauer H., Wartenberg, M., Hescheler, J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cell Physiology Biochemistry.* 2001; 11(4):173-86.

40) Owusu-Ansah, E., Banerjee, U. Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. *Nature.* 2009; 461, 537.

41) Cucoranu, I., Clempus, R., et al. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circulation Research*. 2005; 97(9): 900-7.

42) Chenais, B., Andriollo, M., et al. Oxidative stress involvement in chemically induced differentiation of K562 cells. *Free Radical Biology & Medicine*. 2000; 28(1): 18-27.

43) Sherr CJ. G1 phase progression: Cycling on cue. *Cell.* 1994; 79: 551–555.

44) Lukas J, Pagano M, Staskova Z, Draetta G, Bartek J. Cyclin D1protein oscillates and is essential for cell cycle progression in human tumour cell lines. *Oncogene*. 1994; 9:707–718.

45) Tam SW, Theodoras AM, Shay JW, Draetta GF, Pagano M. Differential expression and regulation of cyclin D1 protein in normal and tumoral human cells: Association with Cdk4 is required for cyclin D1 function in G1 progression. *Oncogene*. 1994; 9: 2663–2674.