uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Honorata Zurakowski

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biochemistry)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Expression of Discoidin Domain Receptors in 3T3-L1 Preadipocytes and Adipocytes

TITRE DE LA THÈSE / TITLE OF THESIS

Alexander Sorisky

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Mary-Ellen Harper

Johné Liu

Gary W. Slater

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES /
DEAN OF THE FACULTY OF GRADUATE AND POSTDOCORAL STUDIES

EXPRESSION OF DISCOIDIN DOMAIN RECEPTORS IN 3T3-L1 PREADIPOCYTES AND ADIPOCYTES

By

Honorata Zurakowski

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa
Ottawa, Ontario, CANADA

© HONORATA ZURAKOWSKI, Ottawa, Ontario, Canada, 2006

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-494-14981-7
Our file Notre référence
ISBN: 0-494-14981-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Discoidin domain receptors (DDRs) are receptor tyrosine kinases, of which there are two members, DDR1 and DDR2, which are activated by collagen and are involved in cellular proliferation. Therefore, given their role in extracellular matrix interactions and proliferation, both of which affect adipogenesis, we hypothesized that the DDRs influence preadipocyte biology, adipogenesis and adipocyte biology. DDR1 protein expression was decreased in 3T3-L1 adipocytes versus preadipocytes, whereas, DDR1 mRNA expression was decreased at day 4 of 3T3-L1 adipogenesis compared to day 0. DDR2 mRNA expression was reduced at day 2 compared to day 0 of adipogenesis. DDR2-overexpressing preadipocytes display reduced subconfluent 3T3-L1 proliferation and reduced mitotic clonal expansion. DDR2-overexpressing adipocytes were larger and had a greater triacylglycerol mass compared to empty-vector control adipocytes. DDR2-overexpressing adipocytes exhibited a decrease in insulin-stimulated phophorylation of IRS-1 and an increase in Akt and ERK1/2 phosphorylation. Overexpression of DDR2 in preadipocytes also led to decreased phophorylation of IRS-1 upon stimulation with insulin, without an effect on Akt and ERK1/2 phosphorylation. These results suggest that DDR2 is pertinent to 3T3-L1 adipogenesis.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Alexander Sorisky for his support, encouragement and advice during my entire stay in his laboratory and throughout the writing of this thesis. I owe many thanks to Dr. AnneMarie Gagnon for her daily help in the lab and for being so patient with my endless stream of questions. I would also like to thank my thesis advisory committee members, Dr. Kursad Turksen and Dr. Fraser Scott for their helpful advice. Many thanks go out to my colleagues in the Sorisky Lab who provided a fun and friendly work environment and who changed my cells over many weekends. I owe many thanks to past and present technicians of the Sorisky Lab, including Anne, Denise and Jeanique, for their technical assistance. I would especially like to acknowledge the help and friendship of Yulia and Tayze who began their studies with me and would like to wish them good luck with their future experiments.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACLP	Aortic carboxypeptidase-like protein
ATCC	American Type Culture Collection
BMI	Body mass index
Cdk	Cyclin-dependent kinase
C/EBP	CCAAT/enhancer-binding protein
CS	Calf serum
DDR	Discoidin domain receptor
DMEM	Dulbecco's modified Eagle's medium
DN	Dominant-negative
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
*gadd*45	Growth arrest and DNA damage-inducible gene 45
GDF-3	Growth differentiation factor 3
GLUT4	Glucose transporter 4
G6PD	Glucose-6-phosphate dehydrogenase
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IL-6	Interleukin-6
IOD	Integrated optical density
IP	Immunoprecipitation
IRS	Insulin receptor substrate
KRH	Krebs-Ringer-Hepes
LB	Luria broth
MAPK	Mitogen activated protein kinase
MDCK	Madine-Darby canine kidney
Mest	Mesoderm-specific transcript
MMP	Matrix metalloproteinase
MCE	Mitotic clonal expansion
MIX	methylisobutylxanthine
mRNA	Messenger ribonucleic acid
NaF	Sodium fluoride
NaPPi	Sodium pyrophosphate
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK	Phosphoinositide-dependent protein kinase
PI3K	Phosphoinositide 3-kinase
PI(4,5)P2	Phosphoinositide-4,5-biphosphate
PI(3,4,5)P3	Phosphoinositide-3,4,5-triphosphate
PKB	Protein kinase B
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor

Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src homology 2
SMC	Smooth muscle cell
SOS	Son-of-sevenless
SPARC	Secreted protein acidic and rich in cysteine
SREBP1	Sterol regulatory element binding protein 1
TAB1	TGF-β-activated protein kinase 1
TG	Triacylglycerol
TNF-α	Tumor necrosis factor-alpha
TRAF6	TNF-receptor-associated factor 6

LIST OF FIGURES AND ILLUSTRATIONS

INTRODUCTION

Obesity and Overweight

Obesity and overweight are conditions of excess adipose tissue that have reached pandemic proportions worldwide. Obesity and overweight are typically assessed by using the body mass index (BMI), which is defined as the weight in kilograms divided by the square of the height in meters. According to guidelines set by the World Health Organization, adults with a BMI of 18.5-24.9 kg/m^2 are classified as normal weight, overweight with a BMI of 25.0-29.9 kg/m^2 and obese with a BMI$\geq$30 kg/m^2 (Katzmarzyk, 2002). The BMI distribution is shifting upwards in many countries. In the United States, more than half of all adults are considered overweight or obese (Must et al., 1999). In the last two decades, a large increase in the prevalence of overweight Canadians has been observed. The overall national prevalence rate of obesity in adults more than doubled: 5.6% and 14.8% for the years 1985 and 1998 respectively (Katzmarzyk, 2002). The prevalence rates of obesity and overweight for Canadians aged 18 years and older in 2003 were 15 % and 33 % respectively (Belanger-Ducharme and Tremblay, 2005). An estimate that included direct and indirect costs attributed to obesity showed that the total impact of obesity on the Canadian economy was $4.3 billion in 2001 (Katzmarzyk and Janssen, 2004). This study also showed that the total economic cost of obesity represented 2.2 % of the total health care costs in Canada. Obesity is so prevalent throughout the world that it is beginning to replace undernutrition and infectious diseases as the most significant contributor to ill health (Kopelman, 2000). Excess weight is associated with the development of type 2 diabetes, cardiovascular disease, hypertension, stroke, dyslipidemia, osteoarthritis, and some cancers (Must et al., 1999). Current approaches to the treatment and prevention of obesity are not

very effective (Haslam and James, 2005). Thorough understanding of the molecular and cellular events that control adipose tissue growth will help in developing better strategies to combat the current global epidemic.

Adipose Tissue

1. Function of adipose tissue in health

In addition to being a mechanical and thermal insulator, adipose tissue is the largest energy store of the human body (Drevon, 2005). Adipose tissue is distributed throughout the body and it can markedly change its mass in response to energy imbalances. The cellular components of adipose tissue are adipocytes and preadipocytes at various stages of development (Hauner, 2004). Fibroblasts, macrophages, nerve fibers, endothelial cells, vascular smooth muscle cells and blood cells are also present in adipose tissue (Gimeno and Klaman, 2005). Adipocytes, however, are the cells that represent the majority of the adipose tissue population, constituting roughly two thirds of all cells present (Hauner, 2004). They allow excess energy to be stored as triacylglycerol (TG) during caloric abundance and permit the liberation of this energy during periods of caloric restriction (Lafontan, 2005). Lipolysis of TG stores in periods of caloric restriction releases fatty acids into the circulation where they reach skeletal muscle and the liver and provide energy via oxidation (Lafontan, 2005). This process occurs under tight hormonal control. Insulin, which promotes TG synthesis, and glucagon, which promotes lipolysis are two of a number of different hormones and growth factors that control this process (Cornelius et al., 1994). However, energy storage is not the sole function of the adipocyte. Currently, adipose tissue is also considered an endocrine organ that produces and secretes numerous proteins, termed adipokines, with broad biological activity. The physiology of adipose tissue is altered in the obese state.

2. Altered function of adipose tissue in obesity

Obesity is a risk factor for the development of insulin resistance and it is now recognized that derangement of adipose tissue function in obesity contributes to this process. Insulin resistance is defined as the inability of insulin to act efficiently on its target tissues including muscle, liver and adipose tissue. Adipose tissue of obese individuals who develop insulin resistance displays a different adipokine profile than adipose tissue from healthy people and is less able to trap fatty acids (Heilbronn et al., 2004). The increase in adipose tissue that exists in obesity is accompanied by a change in the secretion of various adipokines, some of which have profound effects on metabolism (Sharma and Chetty, 2005). Some examples of these compounds include leptin, adiponectin, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Leptin is secreted by adipocytes in proportion to adipose tissue mass, and in addition to its function in the control of appetite, is also known to increase insulin sensitivity (Considine et al., 1996; Pittas et al., 2004). Obese individuals have leptin levels that correlate with their percentage of body fat and display severe leptin resistance (Gimeno and Klaman, 2005). Adiponectin is another adipokine that has been shown to have insulin sensitizing effects in the body (Yamauchi et al., 2003). Circulating levels of adiponectin are inversely proportional to the amount of adipose tissue present in the body; therefore, levels of this adipokine are decreased in obesity. TNF-α is a proinflammatory cytokine that may act in an autocrine or paracrine fashion to impair insulin action in the adipocyte and whose production is increased in adipose tissue of obese individuals (Kern et al., 1995). Another cytokine, IL-6, is produced by adipocytes and is associated with the development of insulin resistance (Pittas et al., 2004). Its circulating levels are increased in obesity (Vozarova et al., 2001). These adipokines affect insulin sensitivity through complex mechanisms that have

only been partially elucidated. Adipose tissue of obese individuals is known to upregulate the secretion of adipokines, like TNF-α and IL-6, that are known to be inflammatory markers. Thus, obesity is now recognized as a state of low-grade systemic inflammation (Gimeno and Klaman, 2005). Additionally, obesity has recently been shown to be associated with macrophage infiltration of adipose tissue (Curat et al., 2004). These studies have shown that the number of macrophages present in adipose tissue is directly correlated with adipocyte size and adipose tissue mass.

A decrease in insulin sensitivity precedes the development of type 2 diabetes and the majority of people with type 2 diabetes are overweight or obese (Bays et al., 2004). In addition, obese individuals have large insulin-resistant adipocytes whose capacity to store triglycerides is compromised. Insulin action in the adipocyte promotes lipogenesis and suppresses lipolysis (Bays et al., 2004). It has been shown that insulin-mediated suppression of lipolysis is defective in obese, insulin-resistant individuals (Faraj et al., 2004). In addition, these individuals display insufficient lipogenesis. Together, this leads to decreased storage capacity for fatty acids in adipose tissue and a subsequent increased plasma free fatty acid concentration. Inappropriate deposition of these fatty acids in nonadipose tissues, such as the muscle and liver, decreases the insulin sensitivity of those tissues (McGarry, 2002) (Bays et al., 2004), whereas build-up in the pancreas may reduce insulin secretion (Boden and Shulman, 2002). Therefore, adipose tissue of obese, insulin resistant individuals displays disordered fatty acid and adipokine secretion and inadequate energy storage.

3. Expansion of adipose tissue

Adipose tissue formation begins before birth with the determination of pluripotent mesenchymal stem cells to the adipocyte lineage (Pittenger et al., 1999). A series of poorly

understood molecular events then leads to the formation of preadipocytes from these mesenchymal stem cells (MacDougald and Mandrup, 2002). Although preadipocytes appear during embryogenesis, major expansion of adipose tissue is delayed until after birth when an energy reserve becomes necessary (Cornelius et al., 1994). Shortly after birth, adipose tissue expansion occurs primarily through an increase in adipocyte number, also known as adipocyte hyperplasia (Harp, 2004). New adipocytes are formed through the differentiation of preadipocytes to adipocytes, a process termed adipogenesis. Expansion of adipose tissue occurs throughout life whenever energy consumption exceeds the energy demand of the body (Hirsch et al., 1989). Adipose tissue can then expand through adipocyte hyperplasia due to adipogenesis and through an increase in adipocyte size, also known as adipocyte hypertrophy. Changes in adipocyte number are generally considered to occur through changes in adipogenesis, but the proliferation of preadipocytes also has an effect on adipocyte cell number (Hausman et al., 2001). Adipocyte hypertrophy occurs when mature adipocytes accommodate excess energy through increased TG storage (Harp, 2004). In summary, adipose tissue expands through a coordinated response due to enlargement of existing adipocytes and due to the proliferation and subsequent adipogenesis of preadipocytes (Cinti, 2005; Hirsch et al., 1989). Interestingly, adipogenesis occurs in clusters *in vivo* and *in vitro*, suggesting that mature adipocytes release factors that trigger adipogenesis in surrounding cells (Rosen and Spiegelman, 2000). Although much work using cell culture models of adipogenesis has allowed for the elucidation of *in vitro* inducers of adipogenesis, the *in vivo* triggers have not yet been entirely elucidated (Hausman et al., 2001).

If adipogenesis is impaired, for reasons that have not yet been discovered, or if preadipocytes are few due to decreased preadipocyte proliferation, then adipose tissue will expand primarily due to enlargement of existing adipocytes (Heilbronn et al., 2004). Enlarged adipocytes have been shown to correlate strongly with whole body insulin resistance (Ravussin and Smith, 2002). For example, Weyer *et al.* (2002) demonstrated that subcutaneous abdominal adipocyte size is inversely proportional to insulin sensitivity. Large adipocytes can lead to metabolic disturbances. This is thought to occur because enlarged adipocytes have an unfavourable adipokine profile compared to small adipocytes and are also unable to efficiently store fat, both of which lead to insulin resistance (Ravussin and Smith, 2002). Therefore, it has been proposed that with the positive energy balance that occurs in obesity, insufficient adipogenesis increases the proportion of hypertrophied adipocytes and the risk of insulin resistance (Danforth, 2000).

Adipogenesis

1. Overview of adipogenesis

Adipogenesis is the process whereby flat, fibroblast-like preadipocytes differentiate into spherical, lipid-filled adipocytes. Before adipogenesis can occur, the committed preadipocyte must withdraw from the cell cycle (Gregoire et al., 1998). In the presence of appropriate adipogenic signals, such as insulin and insulin-like growth factor (IGF)-1, preadipocytes begin the process of differentiation and progressively acquire the adipocyte phenotype. The quiescent preadipocytes first re-enter the cell cycle and undergo several rounds of mitosis before exiting the cell cycle once more. Numerous adipocyte genes are then activated at the transcriptional level and expression of adipocyte-specific genes is accompanied by dramatic physiological and morphological changes (MacDougald and Lane,

1995). This process is characterized by the presence of early, intermediate and late markers of differentiation and by triacylglycerol accumulation (Gregoire et al., 1998). An extensive study of the cellular and molecular events that take place during adipogenesis has been made possible through the use of various cell culture models.

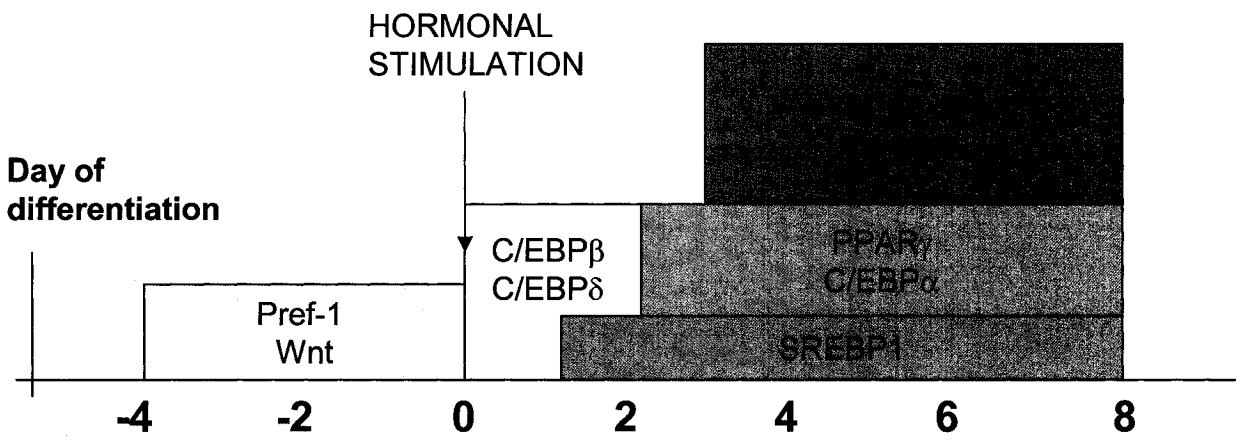
2. 3T3-L1 *in vitro* model of adipogenesis

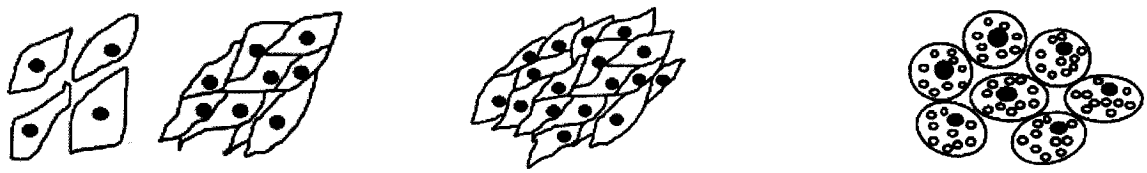
Cell culture models of adipogenesis, including primary culture of preadipocytes and immortalized preadipocyte cell lines, have been developed. Primary culture of preadipocytes occurs following the isolation of preadipocytes from adipose tissue; however, this is time-intensive and genetic manipulation of these cells is difficult (Sorisky et al., 2002). Therefore, immortalized preadipocyte cell lines are widely used. Of the preadipocyte cell lines, the 3T3-F442A and 3T3-L1 cell lines are the ones most frequently used (Gregoire et al., 1998). Green and Meuth clonally isolated the 3T3-L1 cell line from disaggregated mouse embryos from which cells were selected for their ability to accumulate lipid droplets (Green and Meuth, 1974). Much investigation has been done into the events that take place during 3T3-L1 adipogenesis, some of which are displayed in Figure 1.

The prerequisite for 3T3-L1 differentiation to occur is growth arrest and this is achieved by cell-cell contact. Once preadipocytes reach confluence, differentiation is induced with the addition of the synthetic glucocorticoid agonist, dexamethasone, supraphysiological concentrations of insulin, the cAMP phosphodiesterase inhibitor, methylisobutylxanthine (MIX) and fetal bovine serum (FBS) (Cornelius et al., 1994). This collection of hormones and growth factors induce the expression of various transcription factors. Addition of adipogenic inducers also causes the preadipocytes to undergo one round or more of cellular division known as mitotic clonal expansion (MCE) (MacDougald and Mandrup, 2002).

Figure 1: Events of 3T3-L1 adipogenesis

Temporal stages of 3T3-L1 adipogenesis including subconfluent proliferation, confluence, mitotic clonal expansion / growth arrest and terminal differentiation are shown along with temporal changes in gene expression. The major changes in the expression of the transcription factors, including C/EBPβ, C/EBPδ, SREBP1, PPARγ, C/EBPα and SREBP1, expression of pref-1 and Wnt and the expression of adipocyte-specific genes are presented chronologically. Also presented as schematic illustrations are the morphological changes that occur with 3T3-L1 adipogenesis. Modified from Cowherd *et al.* (1999) and Rangwala *et al.* (2000).





Subconfluent proliferation | Confluence | Mitotic clonal expansion / Growth arrest | Terminal differentiation

MCE has been shown to be required for 3T3-L1 adipogenesis (Tang et al., 2003). The genetic events that occur during adipogenesis are often portrayed as a cascade of transcription factor activation. These transcription factors belong to three families and include CCAAT / enhancer-binding protein (C/EBP) α, β, and δ, peroxisome proliferator-activated receptor (PPAR) γ and sterol regulatory element binding protein 1 (SREBP1). The first transcription factors to be induced after exposure of confluent preadipocytes to the adipogenic cocktail are CEBPβ and CEBPδ and their expression is transient (Gregoire, 2001). CEBPβ and CEBPδ are involved in activating the master adipogenic factors, CEBPα and PPARγ, and the expression of these transcription factors coincides with the end of the MCE phase (Rosen and Spiegelman, 2000). A final stage of growth arrest follows clonal expansion and the adipocyte phenotype begins to develop. C/EBPα and PPARγ maintain each other's expression, and along with SREBP1, transactivate adipocyte-specific genes. Certain signaling molecules, such as pref-1 and Wnt, are inhibitors of adipogenesis, and their expression must be reduced if adipogenesis is to occur (Ross et al., 2000).

A study that used microarray technology showed that the progressive acquisition of the adipocyte phenotype is associated with a change in the expression of over 2000 genes, some of which are uncharacterized (Guo and Liao, 2000). The earliest change of adipogenesis is a dramatic change in cell shape as the preadipocyte loses its fibroblastic shape and adopts a more spherical one. This is accompanied by a decrease in the cytoskeletal proteins, actin and tubulin, and a change in the composition of the extracellular matrix (ECM) (Gregoire et al., 1998). There is also a progressive accumulation of TG droplets in the cell cytoplasm at this time. Therefore, during the middle and late stages of adipogenesis, there is an increase in the activity of enzymes and other proteins involved in TG synthesis and metabolism, such

as fatty acid synthase and fatty acid binding protein (aP2) (Gregoire, 2001). The insulin receptor gradually increases in number, as do glucose transporters, such as glucose transporter 4 (GLUT4), thus increasing the insulin sensitivity of the cell. Adipogenesis also involves a change in the production of various adipokines. For example, the expression of adiponectin increases with 3T3-L1 differentiation. Eight days after the induction of differentiation, 3T3-L1 cells are terminally differentiated and have acquired the characteristics of adipocytes *in vivo*.

A great deal of evidence shows that the 3T3-L1 cell model is a good model of *in vivo* adipogenesis. Firstly, when 3T3-L1 preadipocytes are induced to differentiate, they acquire the morphological characteristics of *in vivo* adipocytes. An early examination using electron microscopy revealed that mature 3T3-L1 adipocytes possess all of the ultrastructural features of adipocytes obtained from animal tissue (Novikoff et al., 1980). Secondly, the process of 3T3-L1 adipogenesis involves the progressive acquisition of the biochemical and functional characteristics of adipocytes found in adipose tissue. These characteristics include expression of lipogenic enzymes, lipid accumulation, expression of enzymes necessary for lipolysis of accumulated TG stores, uptake of fatty acids, production of many adipokines and an established sensitivity to all of the hormones that impact on adipocytes (Rosen, 2002). Finally, transplantation studies provide some of the most convincing evidence that 3T3-L1 adipogenesis is a good representation of the process *in vivo*. Subcutaneous injection of 3T3-F442A preadipocytes into nude mice leads to the development of mature fat pads (Green and Kehinde, 1979). More recently it was demonstrated through labeling with beta-galactosidase that it is the 3T3-F442A preadipocytes that are implanted into nude mice, rather than endogenous preadipose cells that give rise to the mature fat pads in vivo (Mandrup et al.,

1997). Unlike 3T3-F442A preadipocytes, 3T3-L1 preadipocytes do not undergo adipogenesis when injected into nude mice (Mandrup et al., 1997). Ross and associates demonstrated that the inability of 3T3-L1 preadipocytes to differentiate *in vivo* is due to endogenous Wnt signaling that inhibits adipogenesis (Ross et al., 2000). Consequently, implantation of 3T3-L1 preadipocytes differentiated *in vitro* does lead to the development of fat pads *in vivo*. It was shown that subcutaneous implantation of 3T3-L1 adipocytes that had been differentiated for 35 days on 3-D scaffolds into nude mice resulted in the formation of vascularized fat pads *in vivo* (Fischbach et al., 2004). These experiments show that the 3T3-L1 cell line is a reliable model for studying the conversion of preadipocytes into adipocytes.

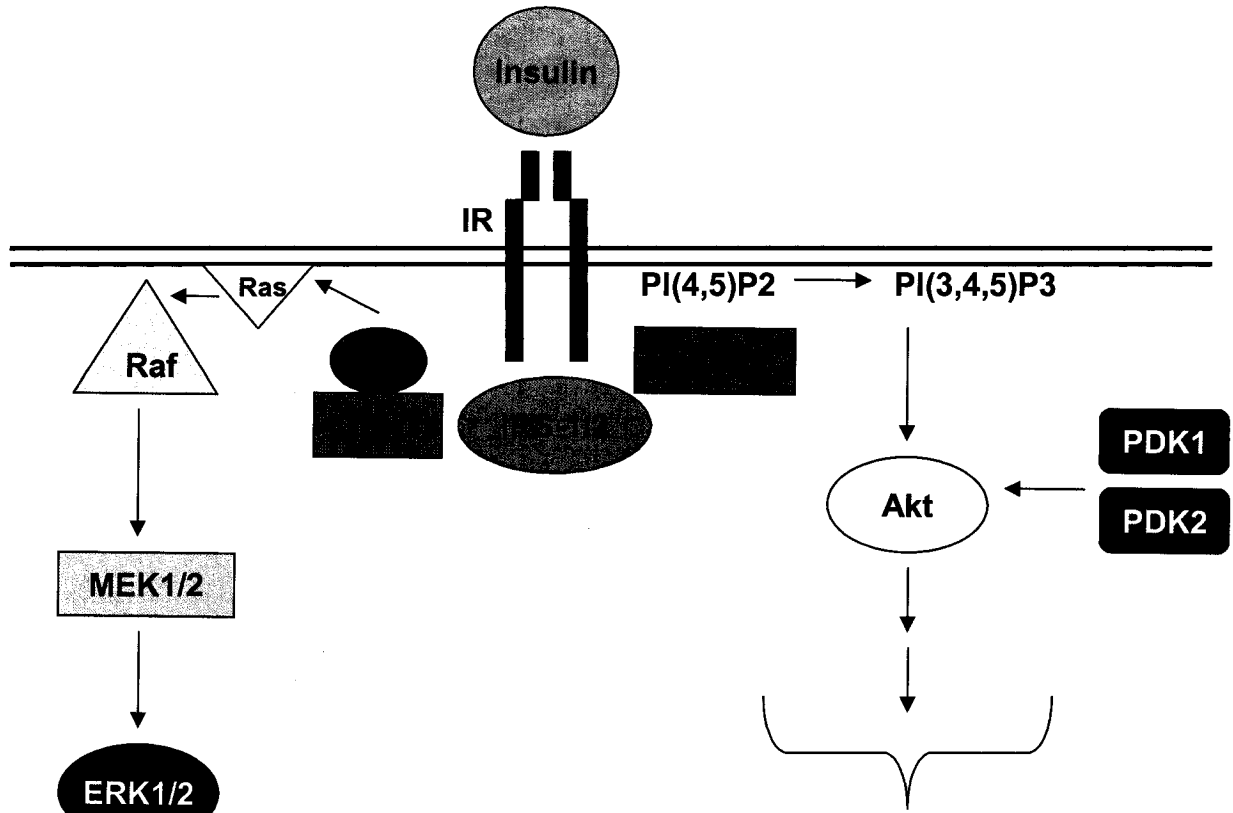
3. Insulin signaling in preadipocytes and adipocytes

Insulin plays a major role in the metabolic function of adipose tissue and also controls its development. It should be noted that the insulin-mediated cellular responses in preadipocytes are very different from those observed in adipocytes. *In vitro* insulin signaling in preadipocytes promotes adipogenesis; whereas, insulin signaling in adipocytes both *in vivo* and *in vitro* promotes lipogenesis, glucose transport and inhibits lipolysis. It has not yet been established whether insulin stimulation is required for *in vivo* adipogenesis. Insulin exerts its effects in preadipocytes and adipocytes through various intracellular signaling cascades (Figure 2). Binding of insulin to its receptor causes tyrosine phosphorylation of the receptor and downstream substrates and this signals to a series of phosphorylation and dephosphorylation events of tyrosine and serine / threonine kinases (Bjornholm and Zierath, 2005).

Insulin signal transduction begins when insulin binds to the insulin receptor. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that also includes insulin-like

Figure 2: Insulin signal transduction in preadipocytes and adipocytes

Insulin signaling begins when insulin binds to the insulin receptor. This induces autophosphorylation of the receptor and the phosphorylated receptor can then bind to IRS-1/2. Phosphorylated IRS-1/2 binds PI3K, which activates PI3K to convert PI(4,5)P2 into PI(3,4,5)P3 at the plasma membrane. PI(3,4,5)P3 recruits Akt to the plasma membrane where it is phosphorylated by PDK1/2. Activated Akt dissociates from the plasma membrane, phosphorylates many downstream targets in order to affect a wide range of cellular process. Signaling downstream of Akt has different effects in adipocytes and preadipocytes. In addition, binding of IRS-1/2 to Grb-2 recruits SOS to the plasma membrane where it activates Ras. Activated Ras stimulates the step-wise activation of Raf, MEK1/2 and ERK1/2. Activation of ERK1/2 has different cellular consequences in adipocytes and preadipocytes.



ADIPOCYTES:
- Lipogenesis
- Glucose uptake
- Lipolysis
- Fatty acid uptake

PREADIPOCYTES:
- Adipogenic transcription factors
- Adipogenesis

ADIPOCYTES:
- Fatty acid uptake

PREADIPOCYTES:
- Early on:
- MCE, adipogenic transcription factors
- Adipogenesis
- Later on:
- Inhibits adipogenesis

growth factor (IGF)-I receptor (Saltiel and Kahn, 2001). These receptors are heterotetrameric membrane proteins that consist of two α-subunits and two β-subunits. Insulin binds to the extracellular α-subunit of the insulin receptor and induces a conformational change that brings the α-subunits together and this causes rapid autophosphorylation of the β-subunits of the receptor (Bjornholm and Zierath, 2005). Once activated, the receptor binds and phosphorylates several proximal substrates, including members of the insulin-receptor-substrate (IRS) family (Bryant et al., 2002). IRS-1 and IRS-2 are the two most ubiquitously expressed members of the IRS family of proteins (Miki et al., 2001). IRS-1 is widely expressed in insulin-sensitive tissues, including adipose tissue, and is known to be involved in the metabolic actions of insulin in adipocytes, as well as in skeletal muscle (Bjornholm and Zierath, 2005). IRS-2 is involved in the metabolic actions of insulin in the liver (Yamauchi et al., 1996). In preadipocytes, IRS-1 and IRS-2 are expressed and are involved in insulin-mediated differentiation; however, IRS-1 was shown to be much more important in preadipocyte differentiation *in vitro* (Miki et al., 2001). This study also showed that mice lacking both IRS-1 and IRS-2 had a severe reduction in adipose tissue, suggesting that the essential role of IRS-1 and IRS-2 in adipogenesis *in vitro* can be extended to the *in vivo* process as well.

Phosphorylated IRS-1/2 binds Src homology 2 (SH2) domain-containing proteins such as phosphoinositide 3-kinase (PI3K) and Grb-2 (Withers and White, 2000). Binding of IRS-1/2 to the lipid kinase PI3K results in activation of PI3K and the subsequent conversion of phosphoinositide-4,5-bisphosphate (PI(4,5)P2) into phosphoinositide-3,4,5-trisphosphate (PI(3,4,5)P3) at the plasma membrane (Schinner et al., 2005). PI(3,4,5)P3 is then able to bind to Akt, also known as protein kinase B (PKB), and thus recruits it to the plasma

membrane. This recruitment facilitates the phosphorylation of Akt on specific serine/threonine residues by upstream Akt kinases. Akt is phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) at Thr 308 and by PDK2 at Ser 473 (Zdychova and Komers, 2005). Activation of Akt causes it to dissociate from the plasma membrane. Akt is then able to phosphorylate its downstream targets in various subcellular localizations, in order to affect a wide range of cellular processes (Zdychova and Komers, 2005). In mature adipocytes, insulin signaling via Akt regulates lipogenesis, glucose uptake, lipolysis and fatty acid uptake (Lafontan, 2005) (Zdychova and Komers, 2005). Insulin is a known inducer of preadipocyte differentiation in preadipocyte cell culture. Signaling downstream of Akt activation leads to the upregulation of various adipogenic transcription factors and adipogenesis results. The expression of constitutively activated forms of Akt is sufficient to induce 3T3-L1 preadipocyte differentiation (Magun et al., 1996) (Kohn et al., 1996). More recently, mice deficient in the most ubiquitously expressed Akt isoforms, Akt1 and Akt2, were reported to have a deficit in adipogenesis (Peng et al., 2003). This same report showed that Akt1 and Akt2 are required for the induced expression of PPAR γ, a transcription factor considered to be the master regulator of adipogenesis. Therefore, it is evident that signaling downstream of Akt is required for adipocyte differentiation.

Activated IRS-1 also binds the SH2 domain-containing protein, Grb-2. Grb-2 links IRS-1 to the extracellular signal-regulated kinase (ERK) 1 and 2 signaling pathway. ERK1 and ERK2 constitute one of three groups of mitogen activated protein kinases (MAPKs). The MAPKs are serine/threonine kinases regulated by phosphorylation cascades (Bost et al., 2005). IRS-1 binding to Grb-2 recruits the Son-of-sevenless (SOS) exchange protein to the plasma membrane where it is able to activate Ras (Saltiel and Kahn, 2001). Once Ras is

activated, it acts as a molecular switch that stimulates the step-wise activation of Raf, MEK1/2 and finally ERK1 and ERK2 (Bost et al., 2005). The activated ERKs can then translocate into the nucleus where they activate various transcription factors and thus initiate transcription programs that lead to cellular proliferation or differentiation (Saltiel and Kahn, 2001).

The role of ERK signaling pathway in the metabolic effects of insulin stimulation in adipocytes has been investigated. It was shown by two groups that inhibition of the ERK signaling pathway using the MEK inhibitor PD98059 does not affect Glut4 translocation (Kayali et al., 2000) (Fujishiro et al., 2001). However, Stahl and associates recently showed that insulin-induced increases in fatty acid uptake in adipocytes were partially blocked by pretreatment with PD98059 (Stahl et al., 2002). Therefore, in adipocytes, ERK signaling is implicated in insulin-mediated fatty acid uptake but is not implicated in insulin stimulated glucose uptake.

In adipogenesis, the ERK pathway has multiple and conflicting effects. An early study done by Sale and associates used an antisense strategy against ERK1 and ERK2 and showed that ERK1 and ERK2 are necessary for 3T3-L1 adipogenesis (Sale et al., 1995). These results were then contradicted by the finding that ERKs phosphorylate and deactivate PPAR-γ, which inhibits adipogenesis (Hu et al., 1996) (Camp and Tafuri, 1997). It is now believed that ERK signaling has a dual role in adipogenesis; its effects depend on when it is activated during adipogenesis (Bost et al., 2005). ERK signaling is necessary during the MCE phase of adipogenesis. This was demonstrated by Tang and colleagues who showed that the potent MEK inhibitor U0126, when administered during MCE, blocks adipogenesis (Tang et al., 2003). Many other studies also showed that ERK signaling is necessary for adipogenesis

and for the expression of adipogenic transcription factors (Aubert et al., 1999) (Prusty et al., 2002). Therefore, it appears that ERK activation through insulin stimulation is needed during the initial proliferative step of differentiation; whereas, ERK signaling is down regulated during the later stages of adipogenesis (Bost et al., 2005). This is in agreement with the fact that PPARγ expression is not detected during MCE and increases progressively during the middle and later stages of differentiation, when ERK activity is returned to a low level (Bost et al., 2005). Thus, in preadipocytes insulin-mediated activation of ERK leads to proliferation and subsequent differentiation, whereas, in later stages of adipogenesis, ERK signaling needs to be down-regulated in order for adipogenesis to occur. Interestingly, whereas, ERK2 knockout mice died *in utero* (Saba-El-Leil et al., 2003), those with a knockout in ERK1 were viable but had decreased adiposity and fewer adipocytes than wild type mice (Bost et al., 2005). Additionally, preadipocytes isolated from ERK1 knockout mice exhibited impaired adipogenesis (Bost et al., 2005). This suggests that the two ERK isoforms may have different functions in adipogenesis.

4. Changes in the ECM during adipogenesis

The ECM consists of a complex network of polysaccharides and proteins secreted by cells. The ECM functions to organize cells into more complex units such as tissues and to provide texture, strength and integrity to these tissues (Vu, 2001). Interestingly, the diversity of a tissue depends not only on the type of cells present but also on the composition of the ECM (Vu, 2001). It is now apparent that the ECM can influence cellular physiology and development. The interaction of a cell and its ECM regulates many processes such as growth, survival, motility, change in cell shape and differentiation (Werb, 1997). The process of adipogenesis involves a dramatic change in cell shape as the preadipocyte loses

its fibroblastic shape and adopts a more spherical one. Accompanying this is a change in the composition of the ECM. Many of the studies examining the change in the composition of the ECM during 3T3-L1 adipogenesis were performed very early on in the characterization of this cell type and the exact molecular mechanisms by which the ECM affects this process remain to be elucidated (Gregoire et al., 1998).

During adipogenesis, ECM remodeling defines the onset of differentiation and is characterized by the conversion from the fibronectin-rich stromal matrix of the preadipocyte to the basement membrane of the adipocyte (Lilla et al., 2002). Upon 3T3-L1 preadipocyte differentiation, collagen types I and III and fibronectin are down-regulated, whereas collagen type IV, laminin and entactin are up-regulated (Weiner et al., 1989) (Aratani and Kitagawa, 1988) (Bortell et al., 1994). The relative concentrations of type I and III collagen mRNAs decline by 80-90 % upon differentiation of 3T3-L1 preadipocytes; whereas, the transcription of collagen IV mRNA is increased 2.6 fold upon differentiation (Weiner et al., 1989). Therefore, it appears that a switch in collagen secretion may be a key element in adipocyte differentiation. Remodeling of the ECM is a critical event in the process of 3T3-L1 adipogenesis as evidenced by the fact that alterations of the ECM strongly inhibit differentiation. For example, growth of preadipocytes on a fibronectin matrix inhibits differentiation (Selvarajan et al., 2001). Treatment of preadipocytes with transforming growth factor-β, which induces fibronectin as well as collagen type I production, also inhibits adipogenesis (Bortell et al., 1994).

Proteinases have been implicated in the reorganization of the ECM that occurs during adipogenesis. The matrix metalloproteinases (MMPs) constitute a large, multigene family that process or degrade pericellular substrates and thus are able to regulate many biological

processes, one of which is adipogenesis (Sternlicht and Werb, 2001). Recently, it was demonstrated that MMP-2 and MMP-9 are required for the early steps of 3T3-L1 differentiation into adipocytes (Bouloumie et al., 2001). Serine proteases have also been implicated in the process of adipogenesis. Plasmin is a serine protease that directly cleaves various ECM molecules, one of which is fibronectin. Plasminogen is activated to plasmin by various other serine proteases, including plasma kallikrein (Lilla et al., 2002). A plasma kallikrein-dependent plasminogen cascade of serine proteases is required for the differentiation of 3T3-L1 cells because it degrades the fibronectin-rich stromal ECM of preadipocytes (Selvarajan et al., 2001).

Remodeling of the ECM has also been shown to be necessary *in vivo*. Selvarajan and colleagues found that the plasminogen system is necessary for degradation of fibronectin in the ECM during the process of adipogenesis that occurs in mammary gland involution (Selvarajan et al., 2001). A recent study examining gene expression in human preadipocytes and adipocytes using microarray technology implicated MMPs the human *in vivo* process of adipogenesis (Urs et al., 2004). They found that in addition to fibronectin and type III collagen, human preadipocytes overexpress MMP2 and MMP9 when compared to adipocytes. This same report identified secreted protein acidic and rich in cysteine (SPARC) / osteonectin / BM-40 as another gene upregulated in preadipocytes. SPARC is a protein that associates with the ECM but does not contribute significantly to its structural integrity (Bradshaw et al., 2003). A report by Bradshaw and colleagues showed that the absence of SPARC in mice resulted in increased adipose tissue mass (Bradshaw et al., 2003). It is evident that the ECM affects adipocytes and adipogenesis *in vitro* and *in vivo*; however, the exact molecular mechanisms involved in this process still need to be elucidated.

As mentioned earlier, a switch in collagen type secretion is a key element in the process of *in vitro* 3T3-L1 adipogenesis. Integrins are receptors known to bind extracellular collagen, and the expression of collagen binding integrins has been shown to be down-regulated with 3T3-L1 adipogenesis (Rodriguez Fernandez and Ben-Ze'ev, 1989). Integrins are a large family of heterodimeric transmembrane glycoproteins composed of α and β subunits that link cells to the ECM (Franco et al., 2002). Integrins are receptors for various ECM proteins, such as fibronectin, laminin, tenascin, thrombospondin and collagens (Heino, 2000). The integrins that are receptors for collagen are structurally distinct when compared to integrins that bind other ECM proteins (Heino, 2000). They have an inserted domain as part of their α subunit that is responsible for collagen recognition. To date, four collagen binding integrins have been described, α1β1, α2β1, α10β1 and α11β1 (Heino, 2000). The α1β1 and α2β1 integrins are the ones that are most commonly expressed (Franco et al., 2002). The binding of an integrin to its ECM ligand activates intracellular signaling pathways by recruiting cytoplasmic tyrosine kinases and adapter proteins to focal adhesion sites at the cell membrane (Swartz et al., 2001). Signaling via integrin α1β1 has been shown to be necessary to induce cell proliferation and reduce collagen synthesis; whereas, α2β1 integrin increases matrix remodeling by regulating type 1 collagen and collagenase gene expression (Heino, 2000). Until recently, integrins were thought to be the only cell surface receptors for collagens in the ECM. However, it is now known that discoidin domain receptors (DDRs), DDR1 and DDR2 also bind, and are activated by, extracellular collagen.

Discoidin Domain Receptors

1. **Features of DDRs**

DDR1 and DDR2 are members of the DDR subfamily of receptor tyrosine kinases (RTKs). RTKs allow for cells to communicate with their extracellular environment by transducing signals from the outside to the inside of a cell. In general, RTKs are activated by soluble proteins that are present in the blood or other body fluids (Leitinger, 2003). DDR1 and DDR2 are unusual in that they are activated by triple-helical collagen. The two closely related tyrosine kinases that contain discoidin domains, DDR1 and DDR2, were originally cloned by several different groups and were given several names. DDR1 was previously termed DDR, MCK-10, EDDR1, NEP, Cak, trkE, Ptk-3, NTRK4 and RTK6 (Cutuli et al., 1993) (Johnson et al., 1993) (Zerlin et al., 1993) (Laval et al., 1994) (Perez et al., 1994) (Sanchez et al., 1994) (Alves et al., 1995) (Shelling et al., 1995) (Shelling et al., 1995). DDR2 was previously known as Tyro 10, TKT and CCK-2 (Lai and Lemke, 1994) (Lai and Lemke, 1994) (Alves et al., 1995). In 1997, two groups (Shrivastava et al., 1997) (Vogel et al., 1997) reported that a variety of different collagen types serve as ligands for the previously recognized orphan receptors. Since then, these two receptors have been known exclusively as DDR1 and DDR2.

DDRs are characterized by a region in their extracellular domain that is homologous to discoidin-1, a lectin that mediates intercellular adhesion in the slime mold *Dictyostelium discoideum* (Franco et al., 2002). Lectins are proteins that bind tightly to carbohydrates. Both DDR1 and DDR2 have an approximately 160-amino-acid-long N-terminal discoidin homology domain followed by a single transmembrane region, an extended proline-rich juxtamembrane region and a C-terminal catalytic tyrosine kinase domain (Vogel, 1999). Discoidin domains are found in approximately 20 other proteins that are located at the cell surface or are secreted (Franco et al., 2002). DDR1 and DDR2 are encoded by distinct

genes; however, DDR1 appears in several variants produced by alternate splicing. Five DDR1 isoforms (a, b, c, d and e) have been reported (Matsuyama et al., 2003a). DDR1a and DDR1b differ in that DDR1b has an additional 37 amino acid insertion in the juxtamembrane region of the receptor. DDR1c has a 6 amino acid insertion in its kinase domain, as well as the same 37 amino acid insertion as DDR1b in the juxtamembrane region (Alves et al., 2001). The recently identified DDR1d and DDR1e do not have a kinase domain and are thus kinase-dead (Alves et al., 2001). It should be noted that many studies of DDR1 do not differentiate between the various isoforms. Also, later studies seem to have focused more on the DDR1a and DDR1b isoforms. At present, only one form of DDR2 has been reported.

2. Expression of DDRs

DDR1 and DDR2 are broadly expressed in human and mouse tissues, as shown through early studies that utilized Northern blot analysis and *in situ* hybridization. DDR1 is widely expressed during development and in adult tissues such as kidney, lung, stomach, gallbladder, small intestine, colon, brain, breast, pancreas, thyroid, cornea, skin and in tissue-infiltrating macrophages (Barker et al., 1995) (Franco et al., 2002) (Yoshimura et al., 2005). DDR1 expression was also detected in breast adipose tissue (Barker et al., 1995). However, Barker and associates evaluated adipose tissue that had developed due to manipulation of breast tissue and therefore, might not reflect the expression of DDR1 in all adipose tissue depots (Barker et al., 1995). Therefore, studies that examine the expression of both DDRs in different depots of adipose tissue need to be undertaken.

The expression of DDR1 is also increased in human tumors such as primary breast cancer, ovarian, esophageal, lung and in pediatric brain cancer (Vogel, 1999). It should be

noted that early expression studies did not differentiate between the different DDR1 isoforms. Thus, not much is known about the expression of the DDR1 isoforms. It should be noted that DDR1d and DDR1e were shown to exist in human colon cancer lines; however, their expression has not been examined in normal tissues (Alves et al., 2001).

Little investigation has been done into the expression of DDR2 but its expression has been demonstrated in the heart, skeletal muscle, lung, brain, kidney, synovial tissue and connective tissue (Alves et al., 1995) (Vogel, 1999) (Wang et al., 2002). In addition, DDR2 is expressed in ovarian and lung tumors (Alves et al., 1995). A recent study that examined differences in gene expression in the proliferative human endometrium found that DDR2 is expressed eight times more in stromal compared to epithelial areas (Yanaihara et al., 2005).

From the information available thus far, it appears that DDR1 and DDR2 are expressed in a variety of different cancerous and non-cancerous tissues and are mutually expressed in some tissues such as lung, brain and kidney. It is evident that both receptors are widely expressed physiologically.

3. Ligand specificity and signaling of DDRs

DDR1 and DDR2 are receptors for native, triple-helical collagen and to this date, there appear to be no other ligands for these receptors. To date, 27 genetically different collagen types have been identified (Eyre, 2004) (Kim et al., 2005). Collagen types I, II, III and V form fibers and are thus termed fibrillar collagens; whereas, collagen type IV is the collagen type that allows for the formation of network-like structures and is the main component of the basement membrane that surrounds various tissues and organs, including adipose tissue. Interestingly, DDR1 and DDR2 differ in their ligand specificities and are activated by

distinct collagen types. Whereas, both DDR1 and DDR2 are activated by fibrillar collagens (types I-III and type V), only DDR1 can be activated by basement membrane constituting, type IV collagen (Vogel et al., 1997) (Shrivastava et al., 1997). DDR2 is activated most strongly with type I and type III collagen; whereas, DDR1 is activated evenly by collagen types I-V. Another intriguing feature of DDRs is that once they are activated, it takes an unusually long time for autophosphorylation to occur. Whereas autophosphorylation with most RTKs takes seconds or minutes to occur after ligand stimulation, it takes approximately 90 minutes for maximal DDR autophosphorylation to occur and it is sustained for up to 18 hours following activation with soluble collagen (Shrivastava et al., 1997) (Vogel et al., 1997). Interestingly, it was observed that DDR1 was activated much sooner with collagen I stimulation in cancer cell line cells that were in suspension versus those that were attached to a cell culture plate, indicating that cell adhesion has an effect on DDR1 activation (L'Hote et al., 2002).

Signaling in RTKs begins with the binding of a ligand to the extracellular domain of the receptor, which induces receptor dimerization, and subsequent transphosphorylation of cytoplasmic kinase domains. Evidence that shows that DDR activation occurs in a way that is different from that of the classical model of RTK activation. A detailed study of the binding and activation of the DDRs revealed that the DDR-collagen interaction requires the receptors to be dimerized (Leitinger, 2003). This suggests that DDR dimerization precedes and is necessary for collagen binding to occur. This study also found that the discoidin domain of DDR2, but not DDR1, is sufficient for collagen-induced receptor autophosphorylation. DDR1 activation requires both the discoidin domain and the extracellular region that follows it. This result is in agreement with data by Curat and

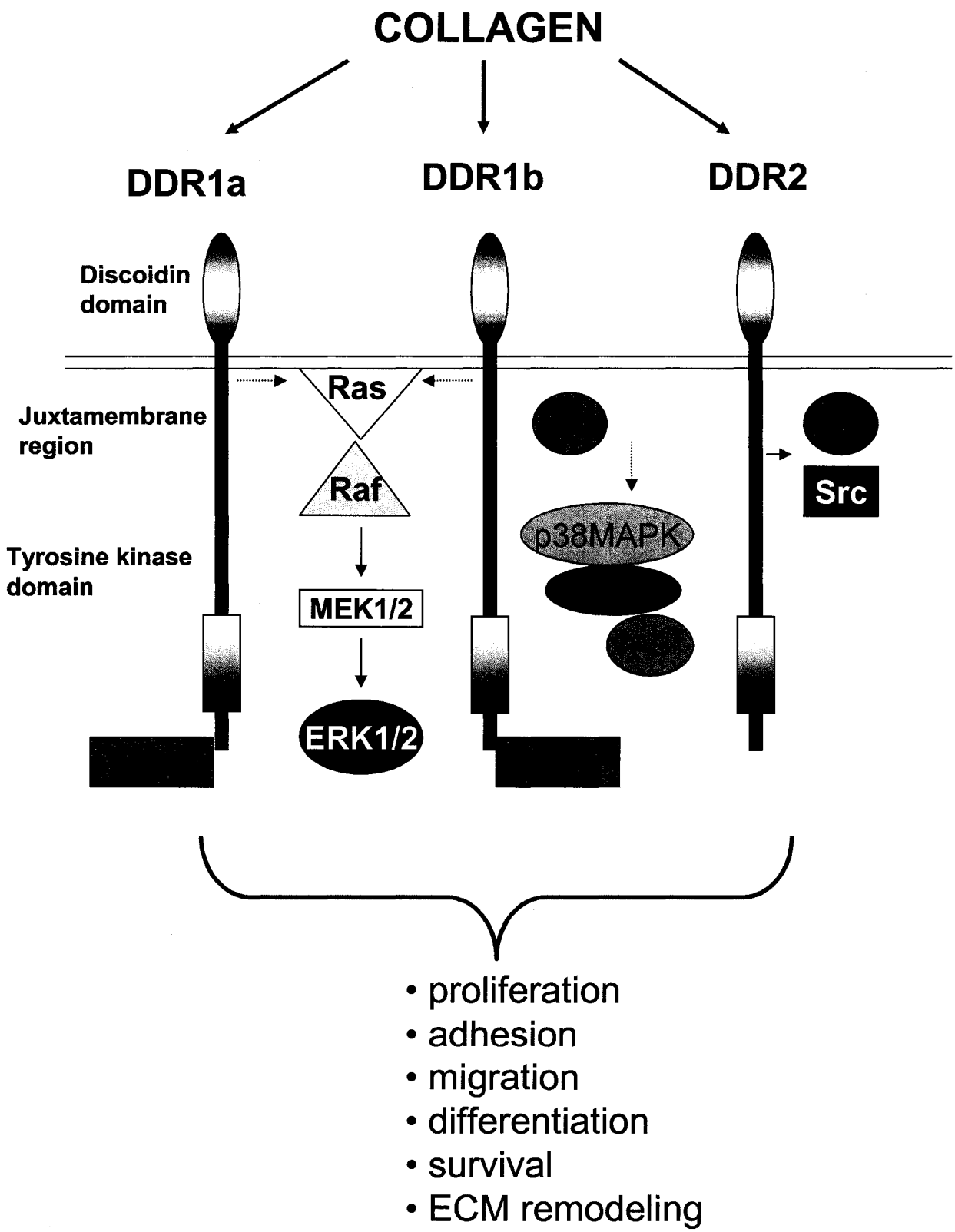
associates who demonstrated that the extracellular discoidin domain of DDR1 is necessary and sufficient for collagen binding, whereas the entire extracellular domain is essential for transmembrane signaling (Curat et al., 2001).

The intracellular signaling events that take place after DDR1 activation have not yet been completely elucidated. Some of the intracellular signaling events that occur following DDR activation are represented in Figure 3. DDR1a and DDR1b differ in that DDR1b has an additional 37 amino acid insertion in the juxtamembrane region of the receptor, and this leads to the association of different signaling molecules with the two DDR1 isoforms. The extra insert in the DDR1b isoform contains the LXNPXY motif, which upon DDR1b activation becomes phosphorylated and associates with the Shc adaptor protein (Vogel et al., 1997). In a more recent analysis of the intracellular signaling pathways downstream of DDR1, activation of DDR1b in THP-1 cells in response to collagen lead to the association of the receptor with Shc and phosphorylation of p38 MAPK (Matsuyama et al., 2003b). It was later demonstrated by the same group that p38 MAPK activation was dependent on TNF receptor-associated factor 6 (TRAF6) and TGF-β-activated protein kinase 1 (TAB1), indicating that the TRAF/TAB/p38MAPK is involved in DDR1b signaling (Matsuyama et al., 2003a). A very recent study implicated p38 MAPK and NF-κB in DDR1b signaling in human macrophages (Matsuyama et al., 2004).

Whereas signaling molecules downstream of DDR1b have been identified, less is known about the signaling molecules downstream of DDR1a and DDR2. Some of the tyrosines that are conserved in both DDR1 and DDR2 display the consensus sequence for the SH2 domains of Nck, GAP and the p85 subunit of PI3-kinase (Vogel, 1999). Consequently,

Figure 3: Structural features and signaling of DDRs

DDRs are composed of an N-terminal discoidin domain, followed by a single transmembrane region, an extended juxtamembrane region and a C-terminal catalytic tyrosine kinase domain. Please note that only one DDR is shown for simplicity; however, dimerization of the DDRs is necessary for downstream signaling to occur. Both DDR1 and DDR2 are activated by collagen found in the ECM. Following activation, DDR1b has been shown to associate with Shc and this leads to the phosphorylation of p38 MAPK. The activation of p38 MAPK is dependent on TRAF6 and TAB1. Activation of DDR1a/b leads to activation of ERK1/2, through the step-wise acitivation of Ras, Raf and MEK1/2. Phosphorylated PI3K also binds DDR1a/b. DDR2 signaling is propagated through interaction with both the Src non-receptor tyrosine kinase and the adaptor molecule Shc. Dotted arrows indicate that other signaling molecules may be involved in leading to the recruitment/activation of the indicated signaling molecule.


COLLAGEN
DDR1a
DDR1b
DDR2
Discoidin domain
Juxtamembrane region
Tyrosine kinase domain
Ras
Raf
MEK1/2
ERK1/2
p38MAPK
Src
• proliferation
• adhesion
• migration
• differentiation
• survival
• ECM remodeling

L'Hote and colleages did find that upon type I collagen stimulation of DDR1, the p85α subunit of PI3K binds DDR1, most likely through its SH2 domain to the YELM motif present in the carboxy-terminal tail of DDR1 (L'Hote et al., 2002). This study did not show any activation of ERK1/2 following DDR1 activation with type I collagen. Another study demonstrated that stimulation of DDR1-transfected 293T cells with type IV collagen led to the activation of DDR1 and subsequent phosphorylation of ERK1/2 in a Ras-dependent manner (Ongusaha et al., 2003). This same study demonstrated that overexpression of DDR1 in various cell types, in the presence of collagen IV, led to high levels of exogenous DDR1 expression and tyrosine phosphorylation along with increased levels of phosphorylated Akt. A more recent study also showed that tyrosine phosphorylated PI3K binds activated DDR1 and this binding is inhibited by pertussis toxin (Dejmek et al., 2003). L'Hote and associates (2001) also found that treatment of a cancer cell line with the phosphatase inhibitor pervanadate resulted in strong DDR1 phosphorylation even in the absence of exogenous collagen, suggesting that a phosphatase may act to limit DDR1 phosphorylation.

The Wnt signaling pathway has also been implicated in DDR1 signaling. Jonsson and Anderson showed that Wnt-5a is a cofactor necessary for collagen-induced activation of DDR1 receptors in mammary epithelial cells (Jonsson and Andersson, 2001). More recently, this same group showed that a $G_{i/o}$-protein signaling pathway mediates the effect of Wnt-5a expression and thus enables collagen-induced activation of DDR1 of mammary tumor cells (Dejmek et al., 2003).

Only one study has examined the intracellular signaling pathways mediating DDR2 signaling. In this study it was seen that DDR2 signaling is propagated through interaction

with both the Src non-receptor tyrosine kinase and the adaptor molecule Shc (Ikeda et al., 2002). Furthermore, these signals were shown to mediate the transactivation of the MMP-2 promoter. Microarray technology was used in one study in order to investigate the target genes downstream of DDRs (Faraci et al., 2003). It was observed that a common event downstream of DDR1 and DDR2 signaling in human and mouse cells is the up-regulation of P-selectin glycoprotein ligand and the repression of agrin, syndecan and α3 integrin.

4. Function of DDRs

There has been much investigation into the function of DDRs. Evidence from *in vitro* studies and from the generation of DDR1 and DDR2-null mice, as discussed further below, suggests that the receptors are implicated in the regulation of cell proliferation, adhesion, migration, differentiation and survival. In addition, DDR1/2 signaling has been implicated in the expression and activity of MMPs, indicating that these receptors are implicated in ECM remodeling. The fact that DDRs serve as receptors for collagen suggests that these receptors monitor the composition of the ECM and regulate the synthesis of enzymes that degrade it. The observation that the activation of DDRs after collagen stimulation is very slow provides more evidence for the idea that DDRs monitor the relationship of cells to the ECM rather than mediating an acute signaling response (Vogel et al., 1997).

DDR1-null mice were viable but smaller in size than control littermates (Vogel, 2001). The authors could not provide an explanation as to why the DDR1-null mice had a reduced size, because DDR1 did not have an influence on bone formation or mineralization and chondrocyte proliferation or apoptosis. Female DDR1-null mice also displayed severe reproductive defects. The majority of them were unable to bear offspring due to a lack of proper blastocyst implantation into the uterine wall and when implantation did occur, the

mutant females were unable to lactate. Upon histological examination, this defect in lactation was attributed to the hyperproliferation and abnormal branching of mammary ducts. A more recent study examined the kidney phenotype of DDR-1 knockout mice (Gross et al., 2004). Gross and colleagues found that mice lacking DDR1 develop proteinuria due to an altered glomerular basement membrane. This suggests that the interaction between type IV collagen and DDR1 plays an important role in maintaining the structural integrity of the glomerular basement membrane.

DDR2-null mice exhibited dwarfism and shortening of long bones (Labrador et al., 2001). This phenotype was caused by reduced chondrocyte proliferation. In a skin wound healing model, epidermal fibroblasts and keratinocytes of DDR2-deficient mice had a reduced proliferative response. Lastly, when in vitro studies were performed, it was seen that fibroblasts derived from DDR2 -/- mutants proliferate more slowly than wild-type fibroblasts. Taken together, the results from the DDR1-null and DDR2-null mouse experiments suggest that both receptors modulate cell proliferation. None of the studies mentioned above reported seeing any gross differences in adipose tissue of the DDR-null mice; however, careful examination of adipose tissue in these mice was not performed.

After the creation of DDR-null animals, many of the *in vitro* studies that followed used cells derived from these mutant animals. For example, smooth muscle cells (SMCs) isolated from the aortas of DDR1-null mice exhibit decreased attachment to collagen, reduced proliferation on collagen, reduced migration towards collagen and reduced expression of MMP-2 and MMP-9 (Hou et al., 2001). Another study done by this group using SMCs taken from the aortas of DDR1-null mice corroborated the above findings and further showed that transfection of DDR1b rescues the deficits in attachment but not migration (Hou

et al., 2002). An examination of mesangial cells derived from the kidneys of DDR1-null mice found that these cells exhibit reduced adhesion and increased proliferation (Curat and Vogel, 2002). When skin fibroblasts were taken from DDR2-null mice, it was observed that they proliferate more slowly and have a markedly impaired migration capacity (Olaso et al., 2002). Concomitantly, the cells display diminished MMP-2 transcription and a diminished MMP-2 activity; however, these defects were ameliorated with the retroviral infection of DDR2.

The ECM function of DDR1 in leukocytes, including neutrophils, monocytes, and lymphocytes has been extensively studied *in vitro*. Overexpression of DDR1a, but not DDR1b, in human leukocytes promotes their migration through three-dimensional collagen lattices; however, overexpression of both isoforms increased their adherence to collagen-coated plates (Kamohara et al., 2001). A dramatic morphologic difference was seen between cells expressing the two DDR1 isoforms in this study. Whereas, DDR1a-overexpressing cells extended long pseudopods after being plated on collagen-coated plates, DDR1b-overexpressing and control cells did not. The results of two subsequent studies revealed that the DDR1b-collagen interaction facilitates the differentiation of monocytes into macrophages and also aids in the maturation of dendritic cells (Matsuyama et al., 2003b) (Matsuyama et al., 2003a). Recently, activation of DDR1b was also shown to upregulate chemokine production in human macrophages (Matsuyama et al., 2004). Based on the findings mentioned above, it appears that the DDR1-collagen interaction facilitates the adhesion, migration, differentiation/maturation and cytokine/chemokine production of leukocytes (Yoshimura et al., 2005).

The matrix-related function of both DDR1 and DDR2 has been studied in other cell types as well. Using a dominant negative (DN) form of DDR1, it was found that DDR1-collagen signaling is necessary for granule neuron axon formation (Bhatt et al., 2000). A very recent study done by Wang and colleagues examined the function of DDR1 in Madine-Darby canine kidney (MDCK) cells by overexpressing DDR1a, DDR1b and DN DDR1 in MDCK cells grown on collagen gels (Wang et al., 2005). They found that cells overexpressing DDR1a and DDR1b, grow more slowly, have a reduced migration capacity and develop shorter tubules and fewer branches in collagen gel. The overexpression of DN DDR1 enhances cell death on collagen gel, suggesting that DDR1 is involved in cell survival. Mohan and associates found that collagen-stimulated mitosis of corneal fibroblasts in culture is mediated by DDR1 and DDR2, implicating both receptors in proliferation of yet another cell type (Mohan and Wilson, 2001). Furthermore, the overexpression of DDR2 in hepatic stellate cells results in enhanced proliferation and invasion through Matrigel and these activities are attributed to increased expression of MMP-2 in the overexpressors (Olaso et al., 2002).

The expression of DDRs in various types of tumors was investigated early in the characterization of this receptor subtype and suggests that the DDRs play a role in tumor progression. For example, a recent study found that DDR1 is overexpressed in epithelial ovarian cancer and can thus be used as a biomarker for this type of cancer (Heinzelmann-Schwarz et al., 2004). Many recent studies have investigated the role of DDRs in other disease processes, especially in those where an aberrant ECM or aberrant ECM signaling is known to exist. Ferri and colleagues showed that DDRs play a role in the regulation of collagen turnover mediated by SMCs in the obstructive diseases of the lung and blood

vessels, lymphangioleimyomatosis and atherosclerosis (Ferri et al., 2004). The role of DDR2 in arthritis has also been investigated. Wang and colleagues demonstrated that increased signaling through DDR2 is responsible for the increase in MMP-1 production normally observed in the synovial fibroblasts of people suffering from rheumatoid arthritis (Wang et al., 2002).

OBJECTIVES

Adipogenesis involves considerable remodeling of the ECM. Specifically, a switch in collagen type composition of the ECM may be necessary for 3T3-L1 adipogenesis to occur. Additionally, MCE is an early and required step of adipogenesis. The DDRs are involved in cell-ECM interaction and preferentially bind and are activated by different collagen types. As well, DDR1 and DDR2 are involved in cellular proliferation. Therefore, given their role in ECM interactions and in proliferation, both of which affect adipogenesis, we were interested in elucidating the role of DDR1 and DDR2 in 3T3-L1 preadipocytes, in adipogenesis and in adipocytes. We hypothesized that DDR1 and DDR2 influence preadipocyte biology, adipogenesis and adipocyte biology.

Objective #1: To evaluate the endogenous expression of DDR1 and DDR2 during the process of adipogenesis.

DDR1 expression will be analyzed during 3T3-L1 adipogenesis at the protein level. The mRNA expression of DDR1 and DDR2 will be analyzed during 3T3-L1 adipogenesis.

Objective #2: To generate preadipocytes overexpressing DDR1a, DDR1b and DDR2.

Retroviral infection will be used to generate 3T3-L1 preadipocytes overexpressing DDR1a, DDR1b or DDR2. Overexpression will be verified at the mRNA level for DDR1a-, DDR1b- and DDR2-expressing preadipocytes. Overexpression will be verified at the protein level for DDR1a- and DDR1b-overexpressing preadipocytes. The functionality of DDR1a and DDR1b with respect to their ability to be activated upon collagen stimulation will also be examined.

Objective #3: To investigate the effect of DDR1a, DDR1b and DDR2 sustained overexpression on subconfluent preadipocyte proliferation, the MCE phase of adipogenesis, and the adipocyte phenotype.

Because DDR1 and DDR2 have been shown to be involved in cellular proliferation, we will evaluate whether overexpression of DDR1a, DDR1b and DDR2 would have an effect on subconfluent 3T3-L1 preadipocyte proliferation. The proliferation rate of DDR-overexpressing preadipocytes will be evaluated using cell enumeration and ^{3}H-thymidine incorporation. Using cell enumeration, we will also evaluate the effect of DDR-overexpression on the MCE phase of adipogenesis. Finally, the morphology of DDR1a-, DDR1b- and DDR2-overexpressing adipocytes will be assessed visually using phase-contrast microscopy and the size of adipocytes will be quantified using image processing and analysis software.

Objective #4: To discern the effect that DDR2 overexpression has on insulin signaling in preadipocytes and adipocytes.

The effect that DDR2 overexpression has on insulin signaling in 3T3-L1 preadipocytes and adipocytes will be evaluated by examining the phosphorylation of key molecules involved in the insulin signaling cascade: IRS-1, Akt and ERK1/2.

MATERIALS AND METHODS

Cell culture

Murine 3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC), maintained at a low passage and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10 % calf serum (CS; Invitrogen), 100 U/mL penicillin (Invitrogen) and 0.1 mg/mL streptomycin (Invitrogen). Culture medium was changed every 2-3 days. When necessary, preadipocytes were briefly exposed to trypsin-EDTA (Invitrogen) for subpassaging. Cells were kept in a humidified incubator at 37°C and at an atmosphere of 10 % CO_2.

Differentiation of 3T3-L1 preadipocytes into adipocytes

In order to induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes were placed in DMEM supplemented with 10 % CS or FBS (Invitrogen) and antibiotics at the concentrations mentioned above, for 8 days. Thereafter, the medium was replaced every 2 days. For the first 4 days of differentiation, 1 µM insulin (Roche) was also added to the medium and for the first 2 days, the medium contained 0.5 mM MIX (Sigma) and 0.25 µM dexamethasone (Steraloids). Differentiation was assessed morphologically with the light microscope. Control preadipocytes were maintained in DMEM supplemented with 10 % CS and antibiotics for 8 days and medium was replaced every 2 days.

Retroviral infection

1. Generation, transformation and isolation of plasmids

Full-length cDNAs encoding DDR1a, DDR1b or DDR2 were contained separately in pcDNA3 plasmids. DDR1a-pcDNA3, DDR1b-pcDNA3 and DDR2-pcDNA3 plasmids were kindly provided by M.D. Layne (Harvard University, Boston, MA). These cDNAs were excised from pcDNA3 by EcoR1 restriction endonuclease digestion, isolated by agarose gel electrophoresis and purified using the QiaEx II gel extraction kit (Qiagen). The purified cDNAs were ligated into pLXSN that had been cut with EcoR1 and treated with shrimp intestinal phosphatase (Invitrogen) according to manufacturer's instructions in order to de-phosphorylate 5^1-phosphate DNA ends to prevent self-religation. Ligations were performed using T4 DNA Ligase (Invitrogen) according to manufacturer's instructions. DH5α competent E. coli cells (Stratagene) were transformed with pLXSN, pLXSN-DDR1a, pLXSN-DDR1b or pLXSN-DDR2 in order to obtain suitable quantities of plasmid DNA required for future transfections. E. coli cells were transformed by the heat-shock method. Briefly, 0.5 μg of plasmid DNA was added to 50 μL of competent cells and the mixture was incubated on ice for 60 minutes. Samples were heat-shocked at 42°C for 1 minute and immediately cooled on ice for 2 minutes. Warm Luria broth (LB) was added, and samples were placed in a shaking incubator at 37°C for 1 hour. 100 μL of each sample was plated onto 100 mm LB agar plates that contained 100 μg/mL ampicillin and plates were incubated at 37°C overnight.

Colonies were isolated and used to inoculate 5 mL of LB that contained 100 μg/mL ampicillin and bacteria were grown while shaking at 37°C overnight. The next day, glycerol stocks were made by adding 700 μL of the cell suspension to 300 μL of glycerol and were stored at –80 ^{0}C for future use. Plasmid DNA in the remaining cell suspension was isolated using Qiagen mini-prep kit (Qiagen) according to manufacturer's instructions. The plasmids

that had been purified were cut with appropriate restriction endonucleases and digests were electrophoresed on an agarose gel in order to verify that pLXSN, pLXSN-DDR1a, pLXSN-DDR1b and pLXSN-DDR2 were isolated. Once colonies that had yielded positive results for the DDR cDNA-containing plasmids were validated, the glycerol stocks mentioned above were used to inoculate a starter culture. 3 mL of LB containing 100 μg/mL ampicillin were inoculated with a colony stored as a glycerol stock and the E. coli cells were grown for 4-8 hours, shaking at 37°C. 500 μL of the starter culture was then used to inoculate 500 mL of LB that contained 100 μg/mL ampicillin and this was grown while shaking overnight at 37°C. The next day, absorbance of the culture at 600 nm was measured and cultures with values less than 1.3 were kept for isolation via maxi-prep. Plasmid DNA was then isolated from each culture using a Qiagen maxi-prep kit (Qiagen) according to manufacturer's instructions. The concentration and purity of plasmid DNA was measured spectrophometically (Pharmacia Biotech Ultraspec 3000) using the ratio of absorbancy readings at 260 and 280 nm. A ratio of 1.8-2.0 indicated that the sample contained DNA of a sufficient purity. Again, appropriate restriction digests were performed and electrophoresed to assess plasmid size and to verify that the required plasmids had been isolated.

2. Transient transfection of Pheonix-Eco packaging cells

The 293T/17-derived Pheonix-Eco packaging cells were obtained from ATCC and were grown in DMEM supplemented with 10 % FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. When the cells were approximately 80 % confluent, they were transiently transfected with pLXSN, pLXSN-DDR1a, pLXSN-DDR1b and pLXSN-DDR2 by the calcium phosphate method. 48 hours after transfection, viral supernatants were collected,

filtered, mixed with 4 μg/mL polybrene and applied to exponentially growing 3T3-L1 preadipocytes for 24 hours. Stable selected clones were made by splitting the cells into an appropriate number of plates, changing the media to DMEM supplemented with 10 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin for 24 hours. The preadipocytes were selected in DMEM supplemented with 10 % CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 400 μg/mL G418 (Rose Scientific). When the cells reached 80 % confluence, they were detached with trypsin/EDTA and frozen in DMEM supplemented with 50 % CS, 17 % dimethyl sulfoxide (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin at –80°C and then transferred to liquid nitrogen. When required, frozen preadipocytes were thawed and plated on a 100 mm dish and grown in DMEM supplemented with 10 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Two days post-thawing, cells were changed to G418 selection media. G418-resistant 3T3-L1 preadipocytes were assessed for DDR1a and DDR1b protein expression and DDR1a, DDR1b and DDR2 mRNA expression. It was expected that preadipocytes overexpressing-DDR1a and -DDR1b would express a transcript of approximately 6 kb in size. This is because endogenous DDR1 mRNA is approximately 3.7 kb and the transcript that was overexpressed contains coding sequences such as a viral promoter and packaging signal needed to generate viral particles. The DDR2-overexpressing preadipocytes were also expected to overexpress a transcript of approximately 6 kb.

Collagen stimulation of 3T3-L1 preadipocytes

For stimulation with collagen, 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes that had been infected by viral particles with no insert (pLXSN), were grown to confluence. One day post-confluence,

preadipocytes were placed in DMEM supplemented with 0.5 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin overnight for 16-20 hours. The next day, cells were stimulated with 100 μg/mL type I collagen solubilized in 0.01 M acetic acid (Sigma) or with vehicle (0.01 M acetic acid) both in DMEM supplemented with 0.5 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin for 2 hours. Preadipocytes were then processed for immunoprecipitation analysis as described below.

Insulin and PDGF stimulation of 3T3-L1 preadipocytes and adipocytes

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes were stimulated with insulin or with platelet-derived growth factor (PDGF). 3T3-L1 differentiated adipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes were also stimulated with insulin. One day post-confluence, preadipocytes were placed overnight for 16-20 hours in DMEM supplemented with 0.5 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. On day 7 of the differentiation protocol, adipocytes were placed overnight for 16-20 hours in DMEM supplemented with 0.5 % CS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Preadipocytes and adipocytes were then washed once with Krebs-Ringer-Hepes (KRH) buffer (Gagnon et al., 1999) and then placed in KRH buffer at 37°C for 15 minutes. Cells were stimulated with 100 nM insulin or 0.2 % bovine serum albumin (BSA) in KRH buffer (vehicle) at 37°C for 5 minutes. When indicated, preadipocytes were additionally stimulated with 10 ng/mL PDGF-BB (Calbiochem) at 37°C for 5 minutes. Cells were lysed in Laemmli buffer (Laemmli, 1970) that contained 50 mM sodium fluoride (NaF), 5 mM EGTA, 5 mM sodium pyrophosphate (NaPPi), 1mM sodium orthovanadate and β-mercaptoethanol.

Lysates were passed through a 26-gauge syringe and boiled for 5 minutes and analyzed by immunoblotting.

Immunoprecipitation analysis

Cells were washed twice in phosphate-buffered saline (PBS) at a pH of 7.4, and then lysed in immunoprecipitation (IP) lysis buffer that consisted of PBS at pH 7.4, 1 % Nonindet P-40, 50 mM NaF, 5 mM NaPPi, 0.1 mg/mL phenylmethylsulfonylfluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1mM β-glycerophosphate, 200 μM sodium orthovanadate and 4 μg/mL benzamidine. Following a 5 minute incubation with IP lysis buffer, cells were scraped and lysates were centrifuged at 16,000 g at 4°C to remove insoluble materials. The resulting supernatants were pre-cleared with protein A-sepharose for 1 hour at 4°C. Equal amounts of pre-cleared lysates (ranging from 400-1000 μg of protein) were then immunoprecipitated overnight at 4°C with 2 μL of DDR1 antibody (SantaCruz) pre-absorbed to protein A-sepharose (Sigma). Immunoprecipitated proteins were washed 5 times with IP lysis buffer, resuspended in Laemmli buffer that contained 1mM sodium orthovanadate, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5 % acrylamide) and analyzed via immunoblot as described below.

Immunoblot analysis

Following acute stimulation with insulin, cells were lysed in Laemmli buffer that contained 1mM sodium orthovanadate, 5mM EGTA at pH 8.0, 50 mM sodium fluoride and 5 mM sodium pyrophosphate. The protein concentrations of cell lysates were determined using the modified Lowry reaction (Bio-Rad Laboratories), using BSA as a standard. Equal amounts of cellular proteins (25-100 μg) were resolved by SDS-PAGE (7.5 % acrylamide),

transferred to a Trans-blot nitrocellulose membrane (Bio-Rad Laboratories) at 70 V for 90 minutes and placed in 5 % skim milk in PBS/0.1 % Tween-20 for 1 hour at room temperature in order to block non-specific binding sites. The membrane was incubated overnight at 4°C with primary antibody diluted in 5 % skim milk in PBS. The primary antibodies used were: DDR1 (Santa Cruz) at 1:2000, phosphotyrosine (PY100; New England Biolabs) at 1:2000, phosphotyrosine (PY20; BD Biosciences) at 1:2000, IRS-1 (Upstate Cell Signaling Solutions) at 1:1000, phospho-Akt Ser473 (pAkt; Cell Signaling Technology) at 1:1000, Akt (Santa Cruz) at 1:9000, phospho-ERK1/ERK2 (pERK1/ERK2; Thr202/Tyr204; Cell Signaling Technology) at 1:1000, ERK1/ERK2 (Upstate Cell Signaling Solutions) at 1 μg/mL and PDGFR-β (Santa Cruz) at 1 μg/mL. Following incubation with primary antibody, the membrane was washed once for 15 minutes and 3 times for 5 minutes in PBS/0.1 % Tween-20 to remove unbound primary antibody. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The membrane was washed once for 15 minutes and 3 times for 5 minutes in PBS/0.1 % Tween-20 to remove unbound secondary antibody, after which a chemiluminescent substrate (Amersham Biosciences) was applied to the membrane for 5 minutes. Immunoreactivity was detected following exposure to Bioflex scientific imaging film (Clonex). The relative intensity of the immunoreactive bands was assessed using Molecular Analyst imaging software (version 1.4; Bio-Rad, Mississauga, ON) and expressed as integrated optical density (IOD) units.

Northern blot analysis

1. Isolation of RNA

At designated time points during the differentiation protocol, total RNA from cultured cells was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Firstly, cells were rinsed twice in PBS and then placed in 600 μL GTC solution consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, at pH 7.0, 0.05 % sarcosyl and 0.1 M β-mercaptoethanol on ice for 5 minutes. Cells were scraped and lysates were placed in microfuge tubes that were snap frozen in liquid nitrogen and subsequently stored at -80°C. Cell lysates were thawed on ice and sheared first through a 23 gauge needle 5 times and next through a 26 gauge needle 5 times, on ice. Sequentially, 60 μL of 2M sodium acetate, at pH 4, 600 μL water-saturated phenol and 120 μL of chloroform were added to each sample and samples were mixed thoroughly after each addition. The mixture sat on ice for 10 minutes after which it was spun at 9,000 g for 30 minutes at 4°C in order to separate the lower organic phase that contained DNA, the interphase that contained proteins and the upper aqueous phase that contained RNA. After centrifugation, the upper aqueous phase was carefully removed, transferred to a new microfuge tube and mixed with an approximately equal volume of isopropanol. RNA was precipitated overnight at –20°C and the next day was centrifuged for 30 minutes at 4°C at 16,000 g. The supernatant was discarded and the resulting pellet was resuspended in 100 μL GTC solution and thoroughly mixed with 300 μL anhydrous ethanol and RNA was precipitated overnight at –20°C. The next day, the mixture was centrifuged at 16,000 g for 30 minutes at 4°C. The supernatant was discarded and the resulting pellet was air-dried for approximately 5 minutes after which it was dissolved in 50 μL of diethyl pyrocarbonate (DEPC) treated water. The sample was then treated with DNAse 1 (Ambion) according to manufacturer's instructions in order to remove any residual DNA. The RNA in the samples was quantitated using the Pharmacia

Biotech Ultraspec 3000 spectrophotometer and samples with an A260/A280 ratio of 1.5 – 1.8 were analyzed further.

2. Electrophoresis of RNA and transfer onto membrane support

Equal amounts of purified RNA samples (5-10 μg) were electrophoresed initially at 90 V for 10 minutes and then at 55 V for approximately 90 minutes on a 1% agarose gel containing 3 % formaldehyde and 1x MOPS. The RNA samples had previously been mixed with DEPC water, 8 % formaldehyde, 1x MOPS, 60 % deionized formamide and 60 ng/mL ethidium bromide and denatured by heating to 55°C for 15 minutes. To verify equal loading, the RNA on the gel was visualized by ethidium bromide staining of 28S and 18S rRNAs. The RNA was transferred onto a Hybond-N nylon membrane (Amersham Biosciences) overnight by capillary blotting, in 3M sodium chloride and 0.3 M trisodium citrate, pH 7. The membrane was then washed in 6x SSC and baked for 2 hours at 80°C. Adequate transfer was verified visually by lack of ethidium bromide staining of 28S and 18S rRNAs on the gel.

3. Probe labeling and hybridization onto membrane

The approximate 1000 bp cDNA fragments for DDR1, DDR2, aP2 and 36B4 were isolated by restriction endonuclease digestion and agarose gel electrophoresis of full size cDNAs contained in pcDNA3 plasmids. DDR1a-pcDNA3 and DDR1b-pcDNA3 were cut with Bgl II and DDR2-pcDNA3 was cut with BamHI. aP2-pcDNA3 and 36B4-pcDNA3 plasmids were generously provided by H. Green (Harvard University, Boston, MA) and B.M. Spiegelman (Harvard University, Boston, MA), respectively. After purification of fragments from the agarose gel using the QiaEx II gel extraction kit (Qiagen), according to

manufacturer's instructions, 50 ng of the cDNA fragment was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by the random priming method using Ready-To-Go DNA Labeled Beads (Amersham).

To block non-specific binding of the cDNA probe, the membrane was pre-hybridized in 5x SSC, pH 7.0, 1 mg/mL boiled salmon sperm DNA, 5x Denhardt's solution and 0.5 % SDS at 60°C for 4 hours. The ^{32}P-labeled probe was added to the pre-hybridization solution and was hybridized to the membrane at 60°C overnight. The next day, the membrane was washed numerous times at progressively decreasing stringencies of 2x SSC /0.1%SDS, 1x SSC /0.1%SDS and 0.1x SSC /0.1%SDS, after which it was exposed to Kodak BioMax MS scientific imaging film.

Assessment of subconfluent preadipocyte proliferation

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes, were plated in duplicate at a density of 2.5 x 10^3 cells/cm^2 and cell enumeration was performed at days 1-3 after seeding. Cells were trypsinized in trypsin-EDTA, placed in DMEM supplemented with 20 % FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin, and enumerated in duplicate with a Neubauer hemacytometer. Alternatively, 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes, were plated in triplicate or quadruplicate at a density of 2.5 x 10^3 cells/cm^2 and ^{3}H-thymidine incorporation was measured three days after seeding. Preadipocytes were placed in DMEM supplemented with 10 % CS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 1.0 μCi/mL 3H-thymidine at 37°C for one hour. Medium was aspirated and cells were washed twice with PBS and lysed with 0.5 % SDS. Lysates were transferred to microfuge tubes after scraping, 500 μL of 10 %

trichloroacetic acid (TCA) was added and 400 μL was aliquoted and placed onto GF/C filters. After the filters had air-dried, they were washed separately 3 x for 5 minutes in 5 % TCA and then in 70 % ethanol for 5 minutes. After the filters had air-dried, they were placed in scintillation vials that contained 5 mL of Biodegradable Counting Scintillant (Amersham) and radioactivity was quantified with a Beckman L36500 scintillation counter.

Assessment of MCE

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes were grown to confluence and differentiated, as described above. At days 1-5 and at day 8 of the differentiation protocol, cells were trypsinized in trypsin-EDTA, placed in DMEM supplemented with 20 % FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin and enumerated in duplicate with a Neubauer hemacytometer.

Assessment of adipocyte cell size

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes, were induced to differentiate as described above. At day 8 of the differentiation protocol, adipocytes were photographed with a Nikon Coolpix 995 digital camera mounted on a Nikon Eclipse TS-100 microscope. For quantification of cell surface area of photographed cells, the ImageJ public domain Java image processing and analysis program was used. The cell surface area of 35-60 cells in an equal area of the photograph for each cell type was analyzed and a mean cell surface area calculated.

Determination of TG mass

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes were induced to differentiate as described above. At day 8 of the differentiation protocol, adipocytes were washed twice with PBS. TG was extracted with isopropanol/heptane (2:3). The extract was dried in a Savant SpeedVac Plus SC110A. The TG pellet was resuspended in 300 µL isopropanol. TG was saponified with saponification reagent, mixed thoroughly and incubated for 10 minutes. Next, 300 µL of 3 mM sodium metaperiodate and 300 µL of 1:250 acetyl acetone were added, mixed thoroughly and incubated at 65°C for 15 minutes. Samples were cooled for 15 minutes and the colour product was quantified spectrophotometrically at 410 nm using the Pharmacia Biotech Ultraspec 3000. Triolein was used to obtain a standard curve and total TG was calculated. Cellular protein was assayed using a modified Lowry method (Bio-Rad Laboratories), using BSA as a standard. Total TG amounts (µg) per well were divided by total protein (mg) amounts per well, in order to obtain a final value of TG/protein.

Statistical analysis

To assess differences between two means, Student's t test (two-tailed) was used with p values <0.05 considered significant. To determine differences between multiple means, ANOVA (GraphPad Instat, version 3.05) was used followed by Tukey's test with p values <0.05 considered significant.

RESULTS

Endogenous expression of DDR1 and DDR2 during adipogenesis

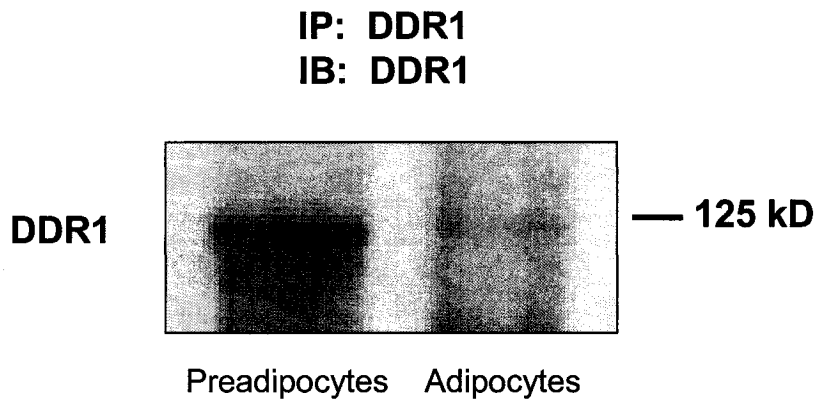
The expression of the DDRs during the 8-day process of 3T3-L1 preadipocyte differentiation was analyzed. Firstly, the protein expression of DDR1 was analyzed in preadipocytes and in adipocytes. 3T3-L1 preadipocytes were grown to confluence and were either differentiated or kept in control medium for 8 days, as described in 'Methods'. Assessed visually using phase-contrast microscopy, approximately 95 % differentiation was observed. At day 8, preadipocytes and adipocytes were lysed and equal amounts of cell proteins were immunoprecipitated and then analyzed using immunoblot. DDR1 expression was markedly reduced in adipocytes versus preadipocytes (Figure 4A). DDR1 protein was decreased by 85 % (mean; n=3; $p<0.05$) in adipocytes versus preadipocytes (Figure 4B).

In order to gain further insight into the mechanisms underlying the decrease in DDR1 protein expression, the mRNA expression of DDR1 during 3T3-L1 adipogenesis was investigated. Northern blot analysis was performed on RNA isolated from cells at day 0, day 4 and day 8 of the differentiation protocol. DDR1 mRNA was decreased at day 4 of preadipocyte differentiation (Figure 5A). Figure 5A demonstrates that loading of RNA was equal for all lanes, as assessed by 36B4 levels. As well, differentiation was demonstrated by increased mRNA expression of aP2 at day 4 and day 8 of the differentiation protocol. There was a 39 % decrease (mean; n=3; $p<0.05$) in DDR1 mRNA at day 4 of differentiation compared to day 0 (Figure 5B). DDR1 mRNA levels increased again at day 8, but they remained lower than day 0 levels; however, they were not significantly lower than day 0 levels (Figure 5A,B). Therefore, a decrease in both the protein and mRNA expression of DDR1 was observed with adipogenesis. However, DDR1 protein expression was decreased

Figure 4: DDR1 protein expression in 3T3-L1 adipocytes versus preadipocytes.

3T3-L1 preadipocytes were grown to confluence and induced to differentiate. At day 8, those cells that had been induced to differentiate, as well as control preadipocytes that had been left in culture for 8 days, were lysed. Equal amounts of cell protein (400-1000μg of protein) was then immunoprecipitated with anti-DDR1 antibody. Immunoprecipitated proteins were separated by SDS-PAGE, followed by immunoblot analysis with anti-DDR1 antibody. **A.** The immunoblot shown is from a single representative experiment **B.** Densitometry results are expressed as integrated optical density units (IOD) of DDR1 protein and are the mean ± SEM of three independent experiments. Student's t test, *$p<0.05$

A.


IP: DDR1
IB: DDR1
DDR1
125 kD
Preadipocytes
Adipocytes

B.

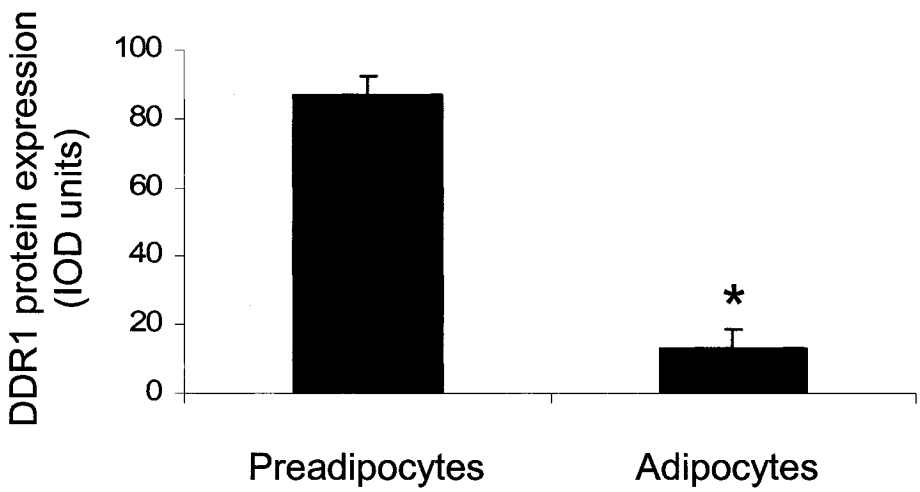
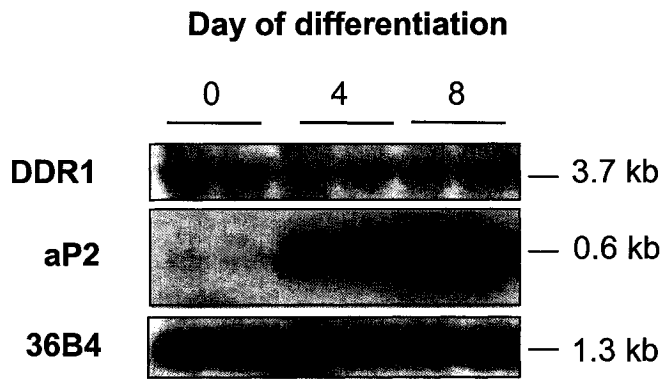

DDR1 protein expression
(IOD units)
100
80
60
40
20
0
*
Preadipocytes
Adipocytes

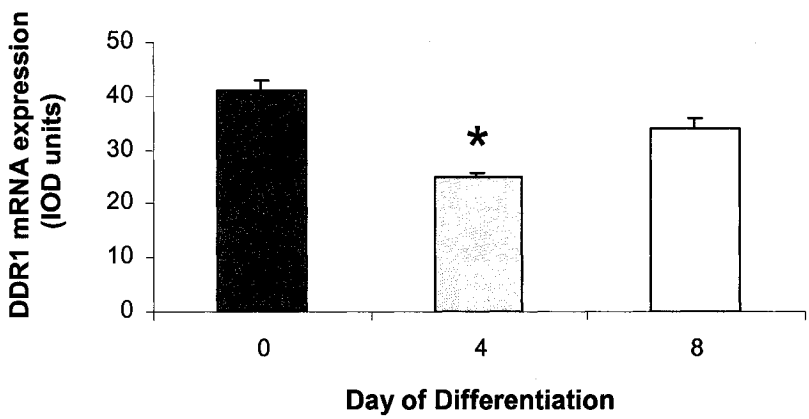
Figure 5: DDR1 mRNA expression during 3T3-L1 adipogenesis.

A. 3T3-L1 cells were grown to confluence and induced to differentiate. At days 0, 4 and 8 of the differentiation protocol, total RNA from cultured cells was isolated and subjected to Northern blot analysis (10 μg/lane). The blots were hybridized with ^{32}P-labeled DDR1, aP2 and 36B4 cDNA probes. The 36B4 probe was used as a loading control, whereas the aP2 probe was used as a marker of differentiation. The Northern blot shown is from a single representative experiment. **B.** Densitometry results are expressed as integrated optical density units (IOD) of DDR1 mRNA and are the mean ± SEM of three independent experiments. ANOVA followed by Tukey's test, *$p<0.05$ compared to day 0 of differentiation.

A.


Day of differentiation
0
4
8
DDR1
aP2
36B4
3.7 kb
0.6 kb
1.3 kb

B.


DDR1 mRNA expression
(IOD units)
50
40
30
20
10
0
*
0
4
8
Day of Differentiation

significantly in day 8 adipocytes versus preadipocytes. DDR1 mRNA expression was decreased significantly at day 4 compared to day 0 of adipogenesis.

Lastly, the mRNA expression of DDR2 during 3T3-L1 adipogenesis was analyzed. An analysis of DDR2 protein expression was precluded due to a lack of functioning DDR2 antibody. RNA was isolated from cells at day 0, day 2, day 4 and day 8 of preadipocyte differentiation and Northern blot analysis was performed. Figure 6A reveals that DDR2 mRNA was reduced at day 2 of adipogenesis. DDR2 mRNA levels then rose again on day 4 and at day 8 had reached levels initially observed at day 0. Robust expression of aP2 at day 4 and day 8 of the differentiation protocol confirmed that differentiation was achieved. DDR2 mRNA was reduced by 66 % (mean; n=3; $p<0.05$) at day 2 compared to day 0 of adipogenesis (Figure 6B).

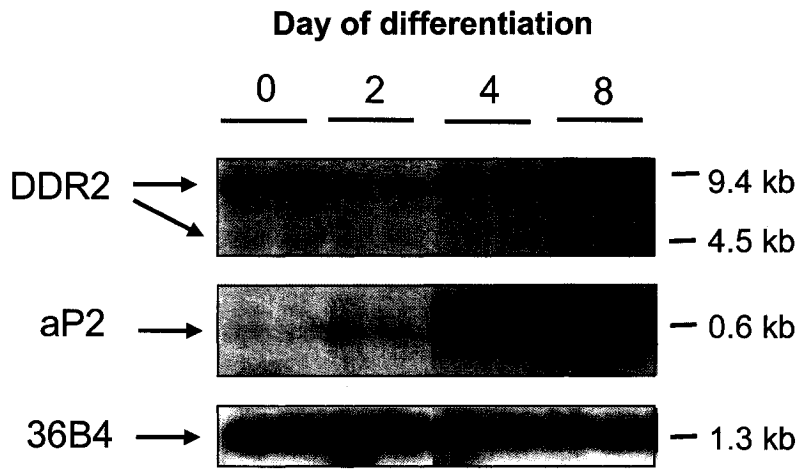
Generation of DDR1a-, DDR1b- or DDR2-overexpressing preadipocytes

The expression of DDR1 and DDR2 decreased with 3T3-L1 adipogenesis. Therefore, we generated DDR1- or DDR2-overexpressing preadipocytes in order to examine the effects of sustained overexpression on the process of adipogenesis. 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2 were created by retroviral infection as discussed in 'Methods'. It was decided that overexpression of the a and b isoforms of DDR1 would be examined because these are the isoforms that are most widely expressed. Preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty vector control cells (pLXSN), were grown to confluence. Northern blot analysis was then performed on RNA isolated from preadipocytes that were two days post-confluent. Figure 7A reveals that successful overexpression of DDR1a and DDR1b was achieved. Preadipocytes overexpressing the DDR1a or DDR1b constructs displayed robust expression

Figure 6: DDR2 mRNA expression during 3T3-L1 adipogenesis.

A. 3T3-L1 preadipocytes were grown to confluence and induced to differentiate. At days 0, 2, 4 and 8 of the differentiation protocol, total RNA from cultured cells was isolated and subjected to Northern blot analysis (10 μg/lane). The blots were hybridized with ^{32}P-labeled DDR2, aP2 and 36B4 cDNA probes. The 36B4 probe was used as a loading control, whereas the aP2 probe was used as a marker of differentiation. The Northern blot shown is from a single representative experiment. **B.** Densitometry results are expressed as relative units of DDR2 mRNA / 36B4 mRNA and are the mean ± SEM of three independent experiments. ANOVA followed by Tukey's test, *$p<0.05$ compared to day 0 of differentiation.

A.


Day of differentiation
0
2
4
8
DDR2
9.4 kb
4.5 kb
aP2
0.6 kb
36B4
1.3 kb

B.

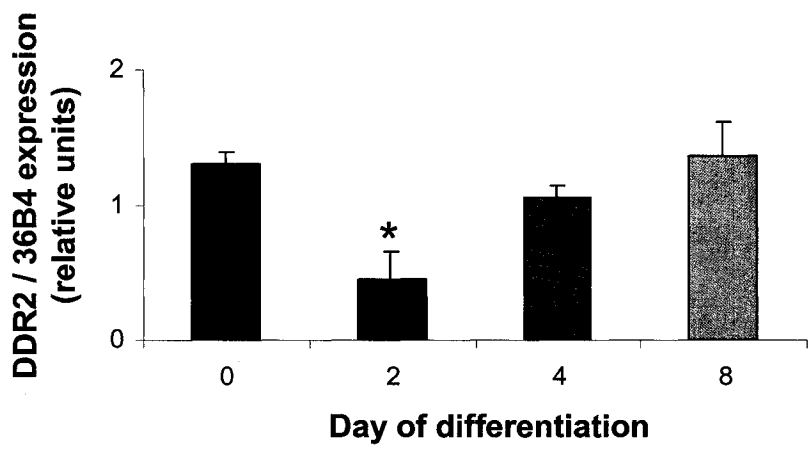
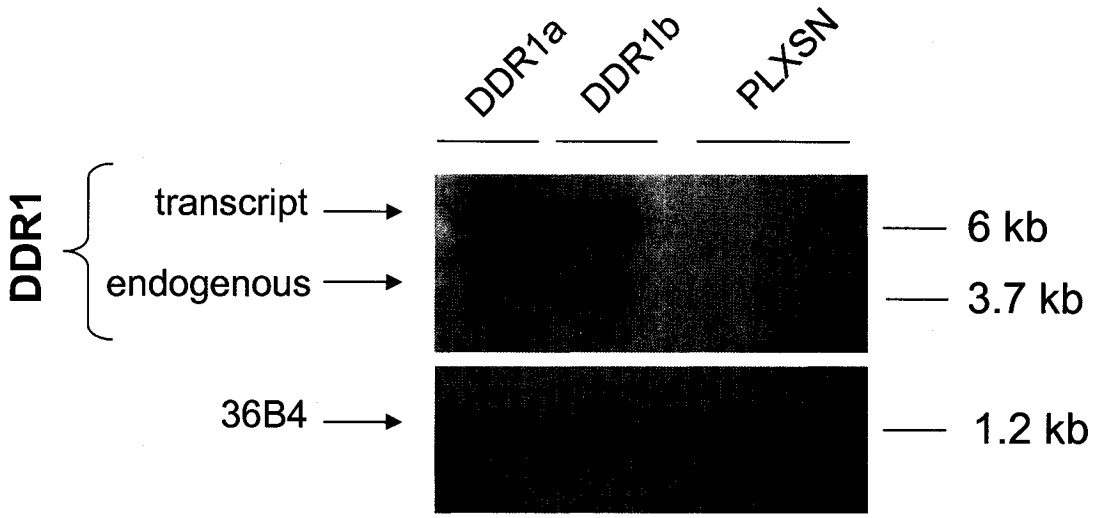

DDR2 / 36B4 expression
(relative units)
2
1
0
*
0
2
4
8
Day of differentiation

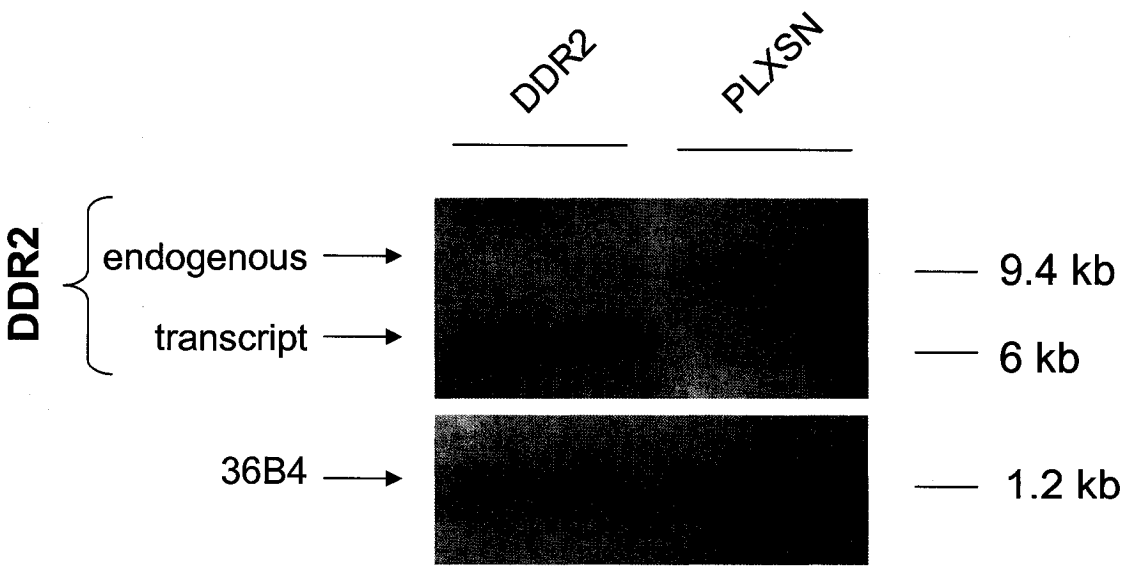
Figure 7: Examination of stable DDR overexpression at the mRNA level in DDR1a-, DDR1b- or DDR2-overexpressing 3T3-L1 preadipocytes.

3T3-L1 preadipocytes stably expressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes (pLXSN) were grown to confluence. Two days post-confluence, total RNA from cultured cells was isolated and subjected to Northern blot analysis (10 μg/lane). Preadipocytes expressing DDR2 and empty vector control preadipocytes were analyzed in duplicate. The blots were hybridized with ^{32}P-labeled DDR1 (**A**), DDR2 (**B**) and 36B4 cDNA probes. The 36B4 probe was used as a loading control.

A


DDR1a
DDR1b
PLXSN
DDR1
transcript
endogenous
36B4
6 kb
3.7 kb
1.2 kb

B


DDR2
PLXSN
DDR2
endogenous
transcript
36B4
9.4 kb
6 kb
1.2 kb

of transcripts of 6 kb in size. Figure 7B reveals that DDR2-overexpressing preadipocytes also expressed a transcript of 6 kb in size. The expression of these transcripts was not observed in empty-vector control preadipocytes. Also seen in cells overexpressing DDR1a, DDR1b or DDR2, as well as in empty-vector control cells, was the expression of endogenous DDR1a, DDR1b or DDR2 mRNA that ran at the 3.7 kb, 3.7 kb and 9.4 kb level, respectively.

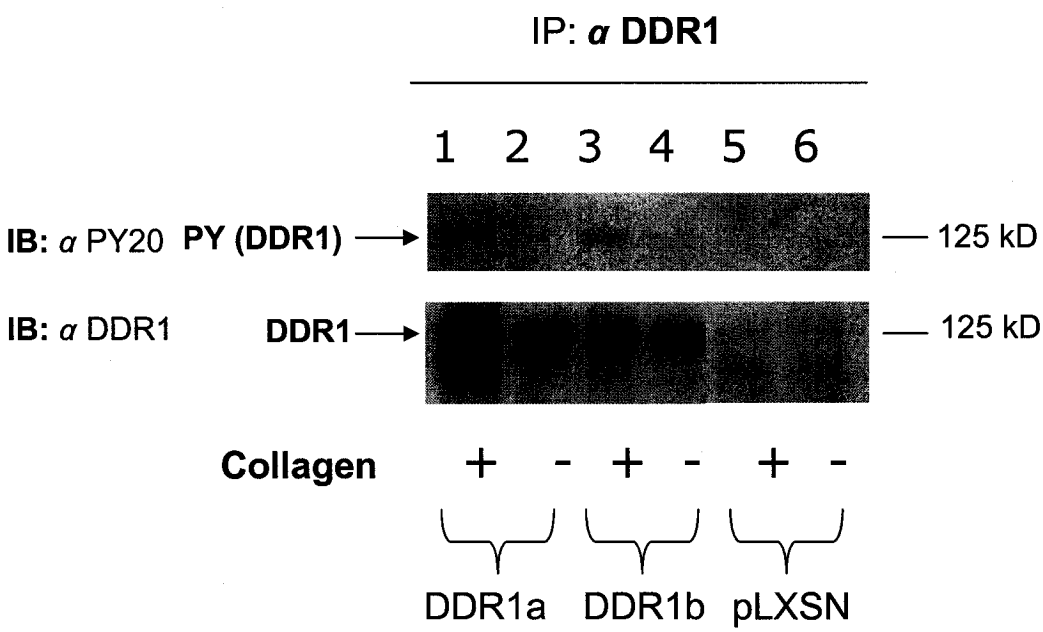
We next examined whether the DDR1a and DDR1b receptors that we overexpressed in 3T3-L1 preadipocytes were functional with respect to stimulation by collagen. To this end, two days post-confluence, preadipocytes overexpressing DDR1a, DDR1b or empty-vector controls were stimulated with 100 μg/mL soluble type I collagen or vehicle for 2 hours.

DDR1 protein overexpression in preadipocytes overexpressing DDR1a or DDR1b was ascertained from the immunoblot presented in Figure 8. The DDR1a- and DDR1b-overexpressing preadipocytes express approximately 15 fold more DDR1 protein than empty-vector control cells. This is consistent with the mRNA data seen in Figure 7. Analysis of DDR2 activation upon collagen stimulation in preadipocytes expressing DDR2 was precluded due to a lack of functioning DDR2 antibody.

Immunoblot analysis revealed that DDR1 in preadipocytes overexpressing DDR1a or DDR1b was weakly tyrosine phosphorylated even in the absence of collagen stimulation (Figure 8). With collagen stimulation, there was an increase in the tyrosine phosphorylation of DDR1 compared to levels observed in vehicle-stimulated preadipocytes. This indicates that preadipocytes overexpressing DDR1a or DDR1b possess functional receptor constructs. No tyrosine phosphorylation of DDR1 was observed in the collagen-stimulated or in the

Figure 8: Assessment of DDR1 tyrosine phosphorylation upon collagen stimulation in DDR1a- or DDR1b-overexpressing preadipocytes

3T3-L1 preadipocytes stably overexpressing DDR1a or DDR1b, as well as empty-vector control preadipocytes (pLXSN) were grown to confluence. Two days post-confluence, preadipocytes were stimulated with 100 μg/mL soluble type I collagen or vehicle for 2 hours. Equal amounts of cell proteins were then immunoprecipitated with anti-DDR1 antibody. Immunoprecipitated proteins were then separated by SDS-PAGE, followed by immunoblot analysis with PY20 antibody and anti-DDR1 antibody. Two independent experiments were performed.


IP: α DDR1
1 2 3 4 5 6
IB: α PY20
PY (DDR1)
125 kD
IB: α DDR1
DDR1
125 kD
Collagen
+ - + - + -
DDR1a
DDR1b
pLXSN

vehicle-stimulated empty-vector control preadipocytes. This is likely due to the fact that endogenous DDR1 is expressed at a very reduced level in empty-vector control preadipocytes compared to that in preadipocytes overexpressing DDR1a or DDR1b.

Effect of DDR1a, DDR1b or DDR2 overexpression on preadipocyte proliferation

DDRs have been implicated in the proliferation of various cell types. Therefore, the subconfluent proliferation of 3T3-L1 preadipocytes overexpressing DDR1a, DDR1b or DDR2 was examined. Preadipocytes were seeded at a density of 2.5 x 10^3 cells/cm^2 and cell enumeration was performed on days 1-3 after seeding. The subconfluent proliferation rate of DDR2-overexpressing preadipocytes was reduced compared to empty-vector controls (Figure 9). The cell number of DDR2-overexpressing preadipocytes was decreased by 52 % (mean; n=3; $p<0.05$) at day 3 when compared to empty-vector control cells. DDR1a- and DDR1b-overexpressing preadipocytes also had growth curves that tended to be lower than that of empty-vector control cells, although these results were not significant.

In order to confirm that the decreased proliferation rate of DDR2-overexpressing preadipocytes was due to a direct effect on DNA synthesis, the ^{3}H-thymidine incorporation of these cells was measured. 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control cells, were seeded at a density of 2.5 x 10^3 cells/cm^2 and ^{3}H-thymidine was measured three days after seeding. DDR2-overexpressing preadipocytes had a lower ^{3}H-thymidine incorporation compared to empty-vector controls (Figure 10). The ^{3}H-thymidine incorporation of DDR2-overexpressing preadipocytes three days after plating was 28 % lower (mean; n=3; $p<0.05$) when compared to empty-vector control cells. DDR1a- and DDR1b-overexpressing preadipocytes both had slightly lower ^{3}H-thymidine incorporation than empty-vector controls but these results were not

Figure 9: Effect of DDR1a, DDR1b or DDR2 overexpression on subconfluent preadipocyte proliferation as assessed by cell enumeration

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes (pLXSN) were plated at a density of 2.5×10^3 cells/cm^2. Cell enumeration was performed on days 1-3 after seeding. Results are the mean ± SEM of three independent experiments, each done in duplicate. ANOVA followed by Tukey's test, *$p<0.05$ compared to pLXSN three days after seeding.

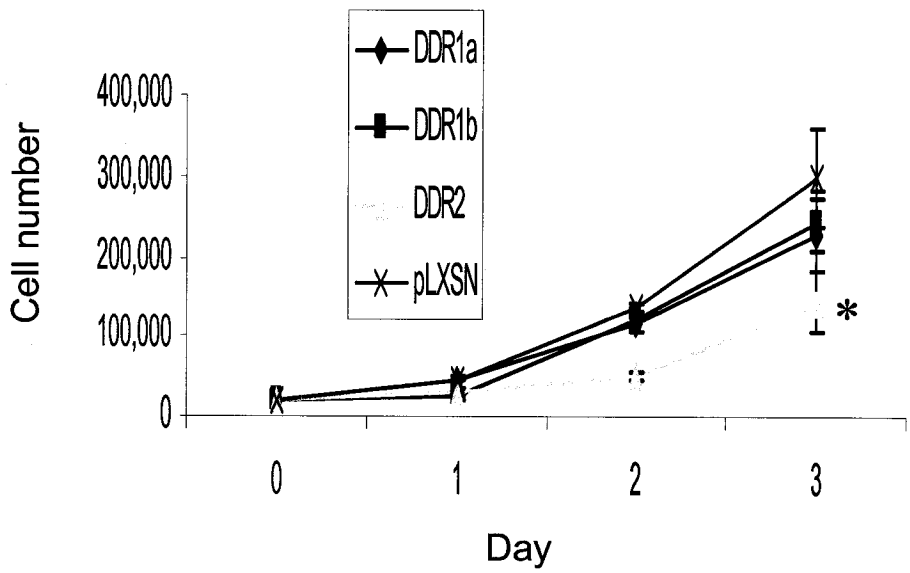
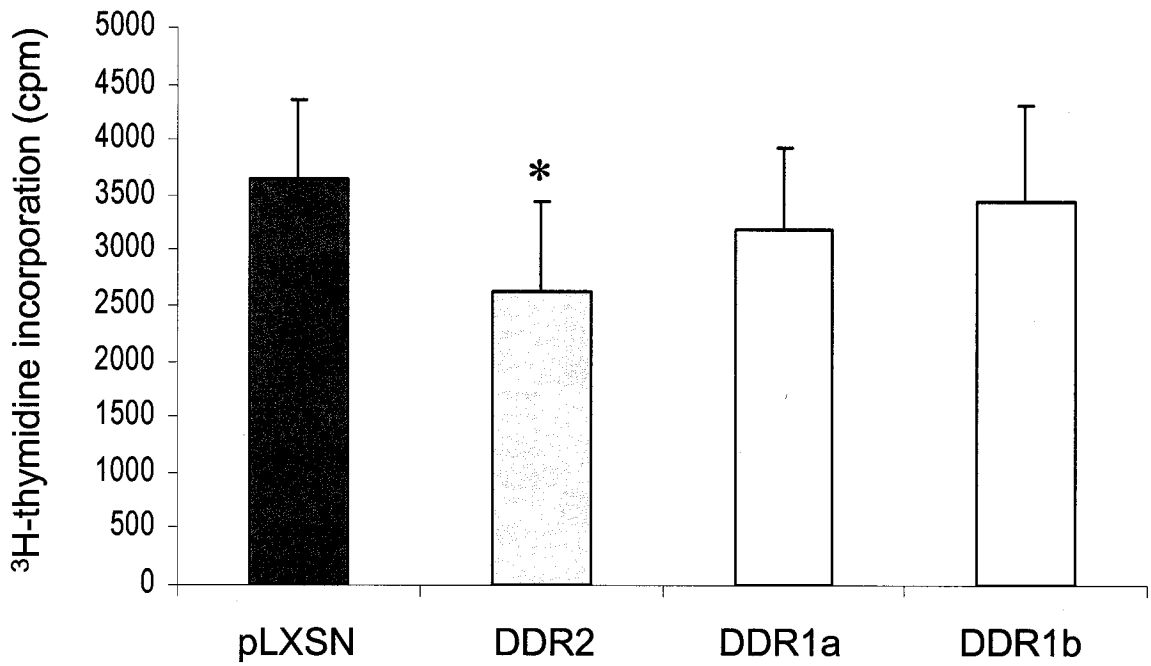

DDR1a
DDR1b
DDR2
pLXSN
Cell number
400,000
300,000
200,000
100,000
0
0
1
2
3
Day
*

Figure 10: Effect of DDR1a, DDR1b or DDR2 overexpression on subconfluent preadipocyte proliferation as assessed by ^{3}H-thymidine incorporation

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes (pLXSN) were plated at a density of 2.5 x 10^3 cells/cm^2. ^{3}H-thymidine was then measured three days after seeding. Results are the mean ± SEM of three independent experiments, each done in duplicate. ANOVA followed by Tukey's test, *$p<0.05$ compared to pLXSN.


^{3}H-thymidine incorporation (cpm)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
pLXSN
DDR2
DDR1a
DDR1b

statistically significant. Therefore, the results from the ^{3}H-thymidine experiments were consistent with the cell enumeration data and suggest that DDR2 overexpression decreases 3T3-L1 subconfluent preadipocyte proliferation.

Effect of DDR1a, DDR1b or DDR2 overexpression on adipogenic MCE

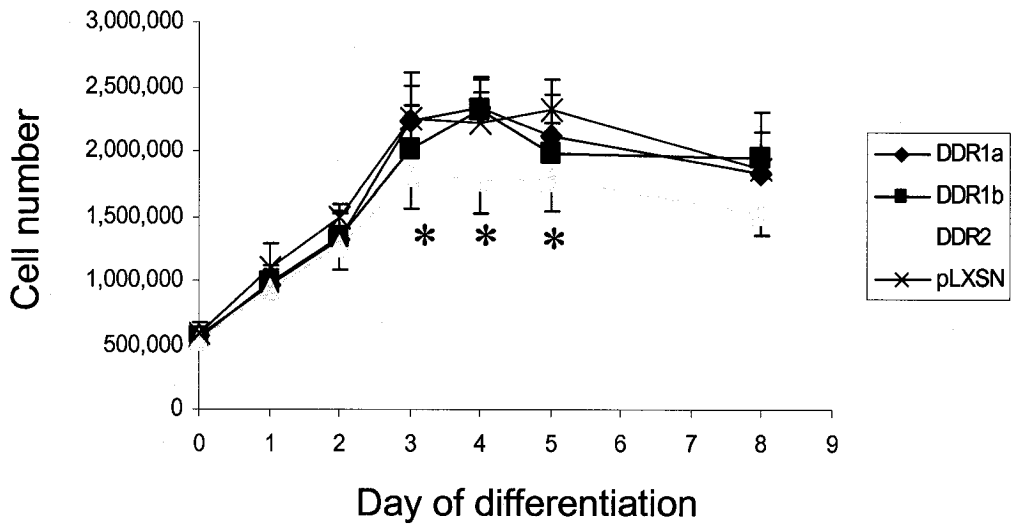
Because the expression of DDR1 and DDR2 was decreased during the early phase of adipogenesis and because the preconfluent proliferation rate of DDR2-overexpressors was reduced, we examined the MCE phase of the DDR-overexpressing preadipocytes. 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector controls, were grown to confluence and differentiated. Cell enumeration was performed daily from day 0 through day 5 and on day 8 of adipogenesis. The rate of MCE of DDR2-overexpressing preadipocytes was lower compared to empty-vector controls (Figure 11). Specifically, cell numbers for DDR2-overexpressing preadipocytes were 18 %, 21 % and 24 % lower (mean; $n=3$; $p<0.05$) 3, 4 and 5 days after the induction of differentiation, respectively, when compared to empty-vector control cells. DDR1a- and DDR1b-overexpressing preadipocytes had a similar rate of MCE as empty-vector control cells. These results indicate that DDR2 overexpression reduces 3T3-L1 MCE.

Effect of DDR1a, DDR1b or DDR2 overexpression on adipocyte morphology

Since the adipogenic MCE phase was reduced when DDR2 was overexpressed in preadipocytes, we investigated whether there was an effect on mature adipocyte morphology. 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector controls, were differentiated. Assessed visually, day 8 DDR2- and DDR1b-overexpressing adipocytes were larger in size than empty-vector controls

Figure 11: Effect of DDR1a, DDR1b or DDR2 overexpression on MCE

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector controls preadipocytes (pLXSN), were induced to differentiate. Cell enumeration was performed daily from day 0 through day 5 and on day 8 after the induction of differentiation. Results are the mean ± SEM of three independent experiments, each done in duplicate. ANOVA followed by Tukey's test, *$p<0.05$ compared to pLXSN at days 3, 4 or 5 of differentiation.


3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
Cell number
0
1
2
3
4
5
6
7
8
9
Day of differentiation
DDR1a
DDR1b
DDR2
pLXSN

(Figure12). DDR1a-overexpressing adipocytes, on the other hand, were similar in size to empty-vector control cells.

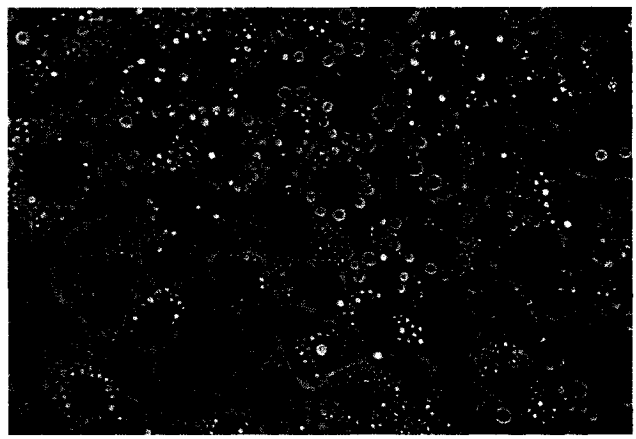
The changes that were observed visually by phase-contrast microscopy were then quantified using software that assesses surface area of photographed cells. The ImageJ image analysis program was used in order to assess cell surface area of the photographed cells. The DDR2-overexpressing and DDR1b-overexpressing adipocytes were significantly larger than empty-vector control cells (Figure 13). Specifically, the DDR2-overexpressing adipocytes had a 55 % larger cell surface area and the DDR1b-overexpressing adipocytes had a 34 % larger (mean; n=3; $p<0.05$) cell surface area compared to empty-vector controls. The cell surface area of DDR1a-overexpressing adipocytes was similar to that of empty-vector controls. Therefore, overexpression of either DDR2 or DDR1b led to a larger adipocyte. Thus, despite a reduction in the MCE phase of adipogenesis, adipocytes overexpressing DDR2 were larger than empty-vector control adipocytes.

Because overexpression of DDR2 or DDR1b increased adipocyte size, we examinined whether this was accompanied by an increase in TG mass. 3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes, were differentiated. Eight days after the induction of differentiation, TG mass and cellular protein were measured (Figure 14). DDR2-overexpressing adipocytes had 35 % greater (mean; n=3; $p<0.05$) TG mass compared to empty-vector control adipocytes. DDR1a- and DDR1b-overexpressing adipocytes had slightly more TG than empty-vector controls but these differences were not statistically significant. Therefore, DDR2-overexpressing adipocytes contain more TG, consistent with their larger cell size.

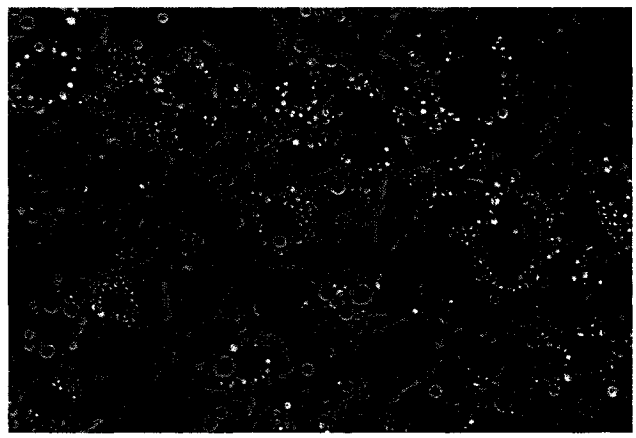
Figure 12: Cell size of DDR1a-, DDR1b- or DDR2-overexpressing adipocytes

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes (pLXSN), were differentiated. Eight days after the induction of differentiation, adipocytes were photographed with a Nikon Coolpix 995 digital camera mounted on a Nikon Eclipse TS-100 microscope at 400x magnification.

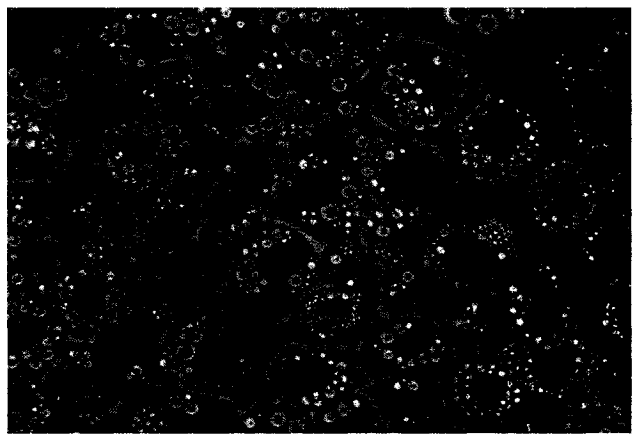
pLXSN



DDR1a



DDR1b



DDR2

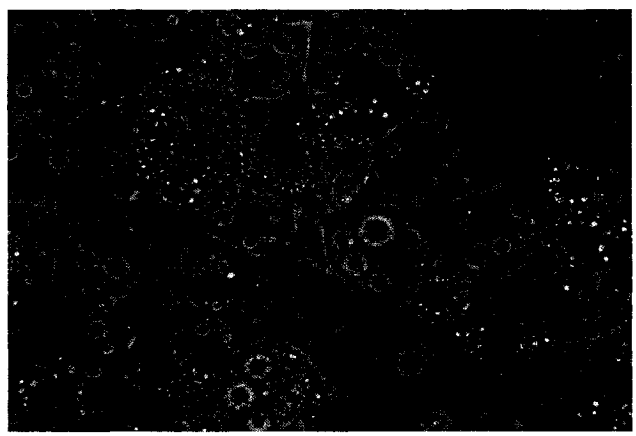


Figure 13: Cell surface area quantification of adipocytes overexpressing DDR1a, DDR1b or DDR2.

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b or DDR2, as well as empty-vector control preadipocytes (pLXSN), were differentiated. ImageJ software was used in order to measure the cell surface area of the photographed cells. Results are the mean ± SEM of three independent experiments. ANOVA followed by Tukey's test, *$p<0.05$ compared to pLXSN.

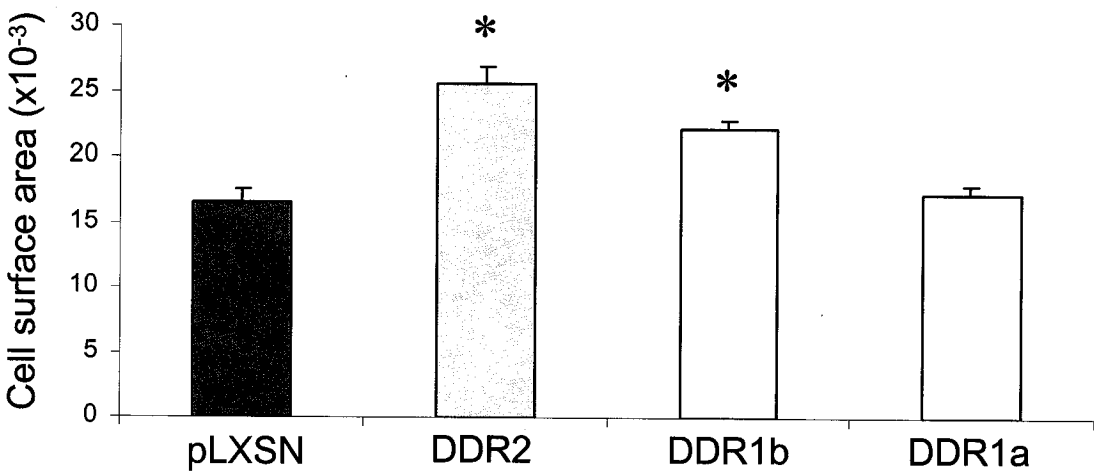
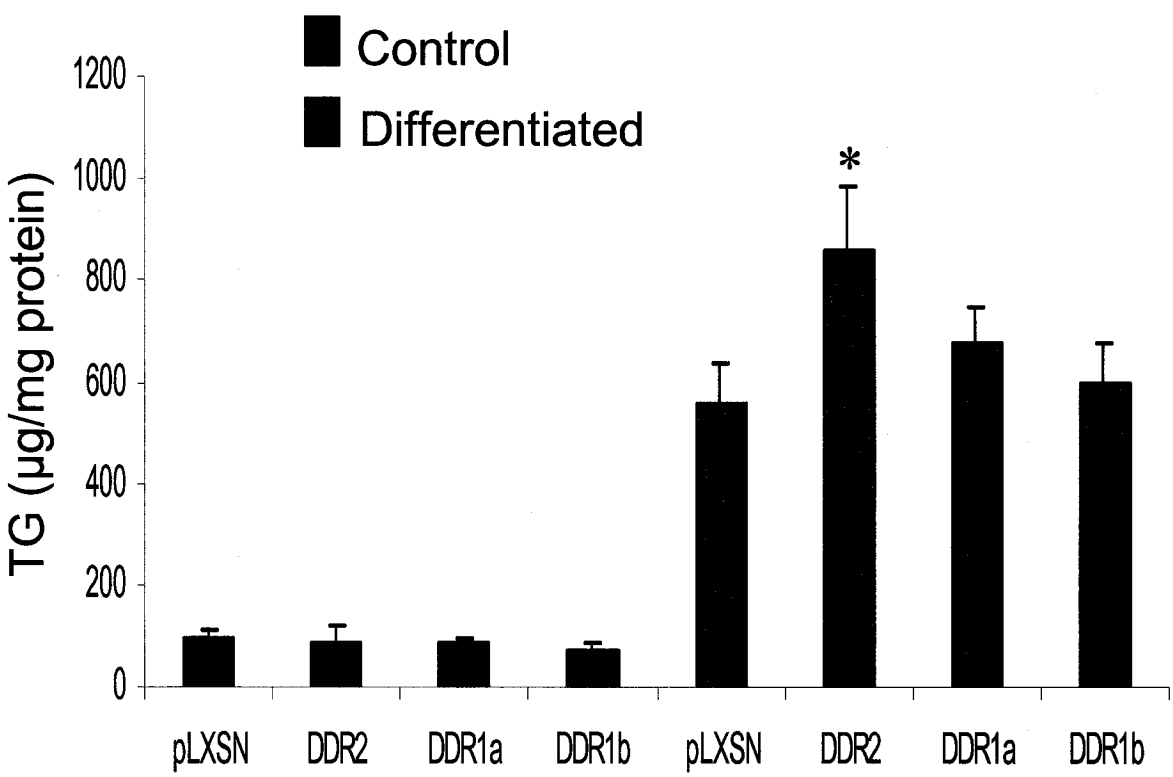

Cell surface area (x10-3)
30
25
20
15
10
5
0
*
*
pLXSN
DDR2
DDR1b
DDR1a

Figure 14: TG mass of adipocytes overexpressing DDR1a, DDR1b or DDR2.

3T3-L1 preadipocytes stably overexpressing DDR1a, DDR1b and DDR2, as well as empty-vector control preadipocytes, were differentiated. Eight days after the induction of differentiation, TG mass and cellular protein were measured. Results are the mean ± SEM of three independent experiments. ANOVA followed by Tukey's test, *$p<0.05$ compared to differentiated pLXSN.


Control
Differentiated
TG (µg/mg protein)
1200
1000
800
600
400
200
0
*
pLXSN
DDR2
DDR1a
DDR1b
pLXSN
DDR2
DDR1a
DDR1b

Insulin signaling in DDR2-overexpressing adipocytes and preadipocytes

Sensitivity to insulin is an important aspect of the mature adipocyte, and adipocyte hypertrophy is known to decrease insulin sensitivity. DDR2-overexpressing adipocytes appear to be hypertrophied and have more TG than empty-vector controls. Therefore, we stimulated DDR2-overexpressing adipocytes with insulin and examined whether insulin signal transduction was altered. 3T3-L1 preadipocytes overexpressing DDR2 and empty-vector control preadipocytes, were differentiated. Eight days after the induction of differentiation, adipocytes overexpressing DDR2, as well as empty-vector control adipocytes, were stimulated with 100 nM insulin or vehicle for 5 minutes.

Treatment with insulin strongly increased the tyrosine phosphorylation of IRS-1, which ran at the 170 kDa level, in empty-vector control adipocytes adipocytes (Figure 15A). There was less phosphorylation of IRS-1 in insulin-stimulated DDR2-overexpressing adipocytes compared to that observed for empty-vector control adipocytes. IRS-1 protein levels were similar in all lanes. There was a 34 % decrease (mean; $n=3$; $p<0.05$) in the tyrosine phosphorylation of IRS-1 in DDR2-overexpressing adipocytes stimulated with insulin when compared to that observed for empty-vector control adipocytes (Figure 15B).

Despite this, DDR2-overexpressing adipocytes exhibited greater insulin-stimulated phosphorylation of Akt at serine 473 compared to empty-vector control adipocytes (Figure 15A). Akt protein levels were similar in all lanes. Specifically, Akt phosphorylation at serine 473 was increased by 122 % (mean; $n=3$; $p<0.05$) in DDR2 overexpressing adipocytes when compared to that observed for empty-vector control adipocytes, in response to insulin (Figure 15B). The phosphorylation of ERK1/2 was also increased in insulin-stimulated DDR2 overexpressing adipocytes compared to empty-vector control adipocytes. Protein

Figure 15: Insulin signaling in DDR2-overexpressing adipocytes

3T3-L1 preadipocytes stably overexpressing DDR2, as well as empty-vector controls, were differentiated. Eight days after the induction of differentiation, adipocytes were stimulated with 100 nM insulin or vehicle (0.2 % BSA/KRH) for 5 minutes after which the cells were lysed. **A.** Equal amounts of cell proteins were then separated by SDS-PAGE, followed by immunoblot analysis with anti-phosphotyrosine antibody (PY100), anti-IRS-1 antibody, anti-phosho-Akt (Ser 473) antibody, anti-Akt antibody, anti-phospho-Erk1/Erk2 antibody (Thr 202/Tyr 204) and anti-Erk1/Erk2 antibody. A single representative blot of three experiments (PY100, IRS-1, P-Akt, Akt, ERK1/2) or two experiments (P-ERK1/2) is shown. **B.** Densitometry was performed on IRS-1, Akt and ERK1/2 phosphorylation of insulin-stimulated cells. Densitometric data are expressed as integrated optical density units (IOD). Results shown are the mean ± SEM of three independent experiments, *$p<0.05$, for IRS-phosphorylation and Akt phosphorylation (Student's t test) and the mean ± range for two independent experiments for ERK1/2 phosphorylation.

A.

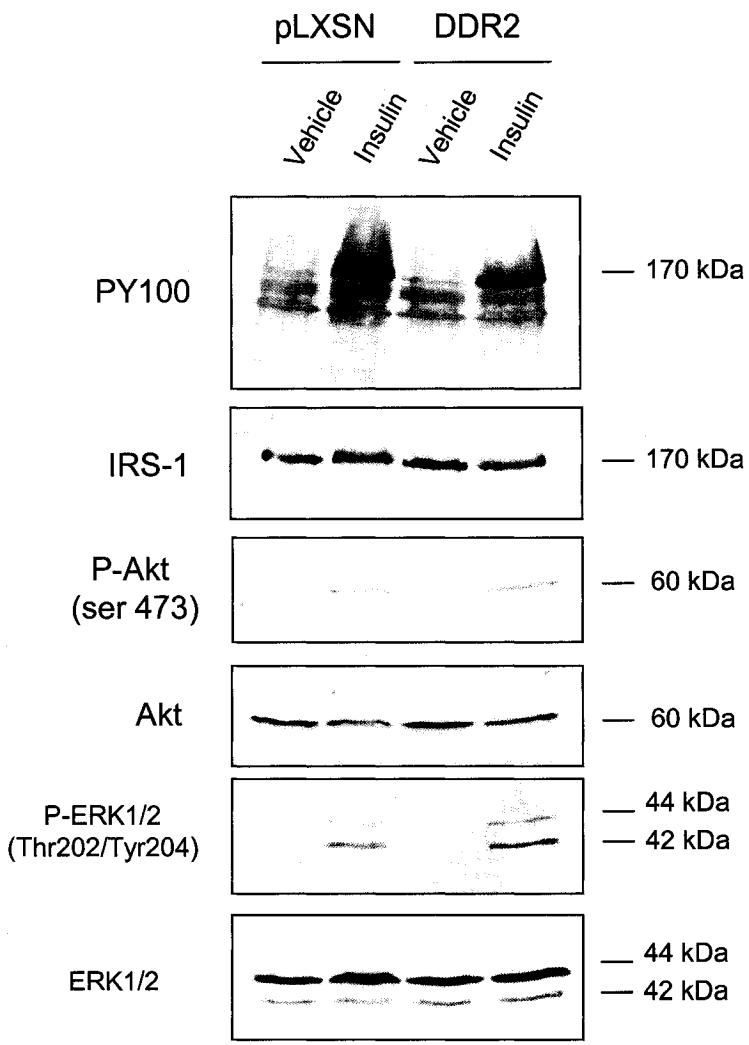
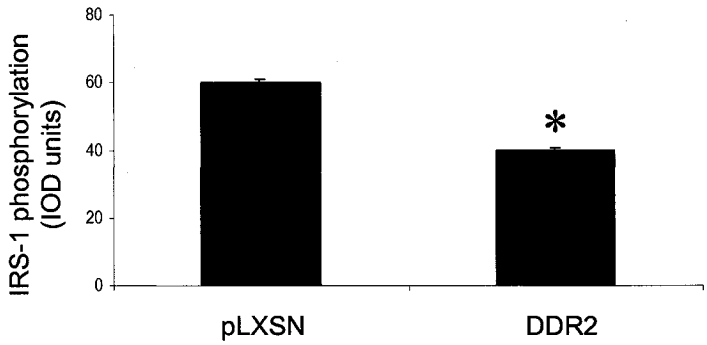
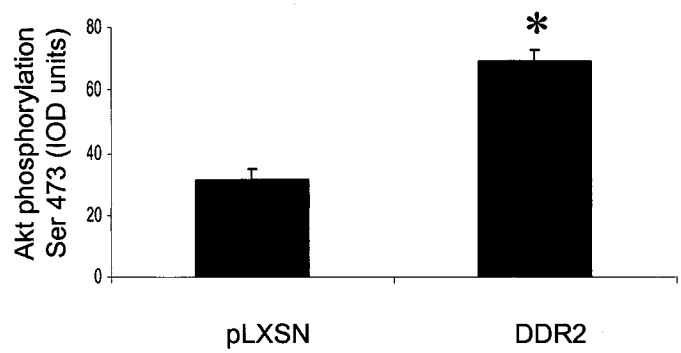
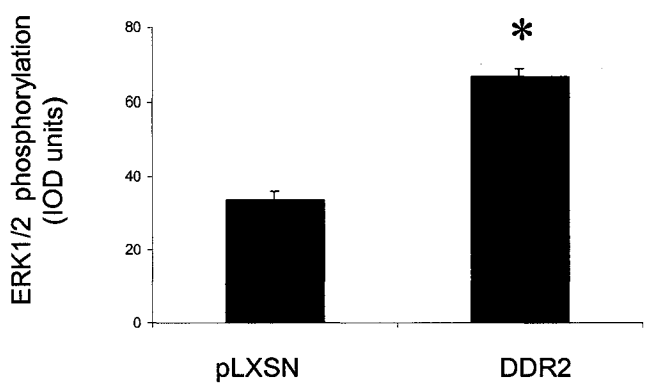

pLXSN
DDR2
Vehicle
Insulin
Vehicle
Insulin
PY100
170 kDa
IRS-1
170 kDa
P-Akt
(ser 473)
60 kDa
Akt
60 kDa
P-ERK1/2
(Thr202/Tyr204)
44 kDa
42 kDa
ERK1/2
44 kDa
42 kDa

Figure 15 (Continued)

B.


IRS-1 phosphorylation (IOD units)
80
60
40
20
0
*
pLXSN
DDR2


Akt phosphorylation Ser 473 (IOD units)
80
60
40
20
0
*
pLXSN
DDR2


ERK1/2 phosphorylation (IOD units)
80
60
40
20
0
*
pLXSN
DDR2

levels of Erk1/2 were similar in all lanes. Specifically, ERK1/2 phosphorylation at threonine 202 and tyrosine 204, increased in insulin-stimulated DDR2-overexpressing adipocytes by 100 % (mean; n=2) compared to empty-vector control adipocytes. Therefore, despite a decrease in insulin-stimulated phosphorylation of IRS-1, DDR2-overexpressing adipocytes exhibited an increase in Akt and ERK1/2 phosphorylation with insulin stimulation.

We next investigated whether the altered signaling response of DDR2-overexpressing adipocytes was due solely to adipocyte hypertrophy or whether it was due to DDR2 overexpression itself. Therefore, we next examined the signaling response to insulin in preadipocytes overexpressing DDR2. Two days post-confluence, preadipocytes were stimulated with 100 nM insulin or vehicle for 5 minutes.

Treatment with insulin strongly increased the tyrosine phosphorylation of IRS-1 in empty-vector control preadipocytes (Figure 16A). However, insulin-stimulated IRS-1 phosphorylation was reduced in DDR2-overexpressing preadipocytes compared to that observed for empty-vector control preadipocytes. There was a 68 % decrease (mean; n=3; $p<0.05$) in the tyrosine phosphorylation of IRS-1 in DDR2-overexpressing preadipocytes when compared to that observed for empty-vector control preadipocytes, in response to insulin (Figure 16B).

Despite this, there was no significant decrease in phosphorylation of Akt at serine 473 in DDR2-overexpressing preadipocytes after stimulation with insulin (Figure 16A,B). Insulin-stimulated phosphorylation of ERK1/2 was also the same for DDR2-overexpressing preadipocytes and empty-vector controls (Figure 16A,B). Protein levels of ERK1/2 were similar in all lanes (Figure 16A). Therefore, as was observed in adipocytes, overexpression of DDR2 in preadipocytes also led to decreased phosphorylation of IRS-1 upon stimulation

Figure 16 : Insulin signaling in DDR2-overexpressing preadipocytes

3T3-L1 preadipocytes stably overexpressing DDR2, as well as empty-vector controls, were grown to confluence. Two days post-confluence, preadipocytes were stimulated with PDGF, 100 nM insulin or vehicle (0.2 % BSA/KRH) for 5 minutes after which the cells were lysed. **A.** Equal amounts of cell lysates were separated by SDS-PAGE, followed by immunoblot analysis with anti-phosphotyrosine antibody (PY100), anti-IRS-1 antibody, anti-PDGFR antibody, anti-phosho-Akt (Ser 473), anti-Akt antibody, anti-phospho-ERK1/2 antibody and anti-ERK1/2 antibody. A single representative blot of three independent experiments is shown. **B.** Densitometry was performed on IRS-1, Akt and ERK1/2 phosphorylation, as well as IRS-1 expression of insulin-stimulated cells, and phosphorylation of PDGFR, as well as PDGFR expression of PDGF-stimulated cells. Densitometric data are expressed as integrated optical density units (IOD). Results shown are the mean ± SEM of three independent experiments. Student's t test, *$p<0.05$

A.

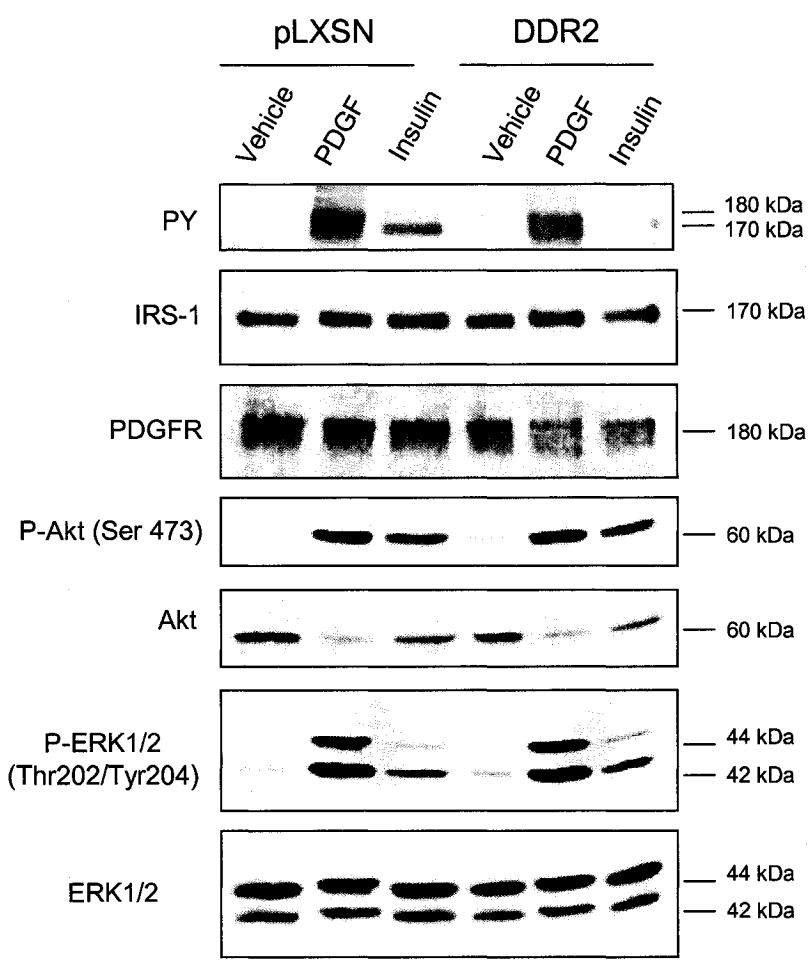
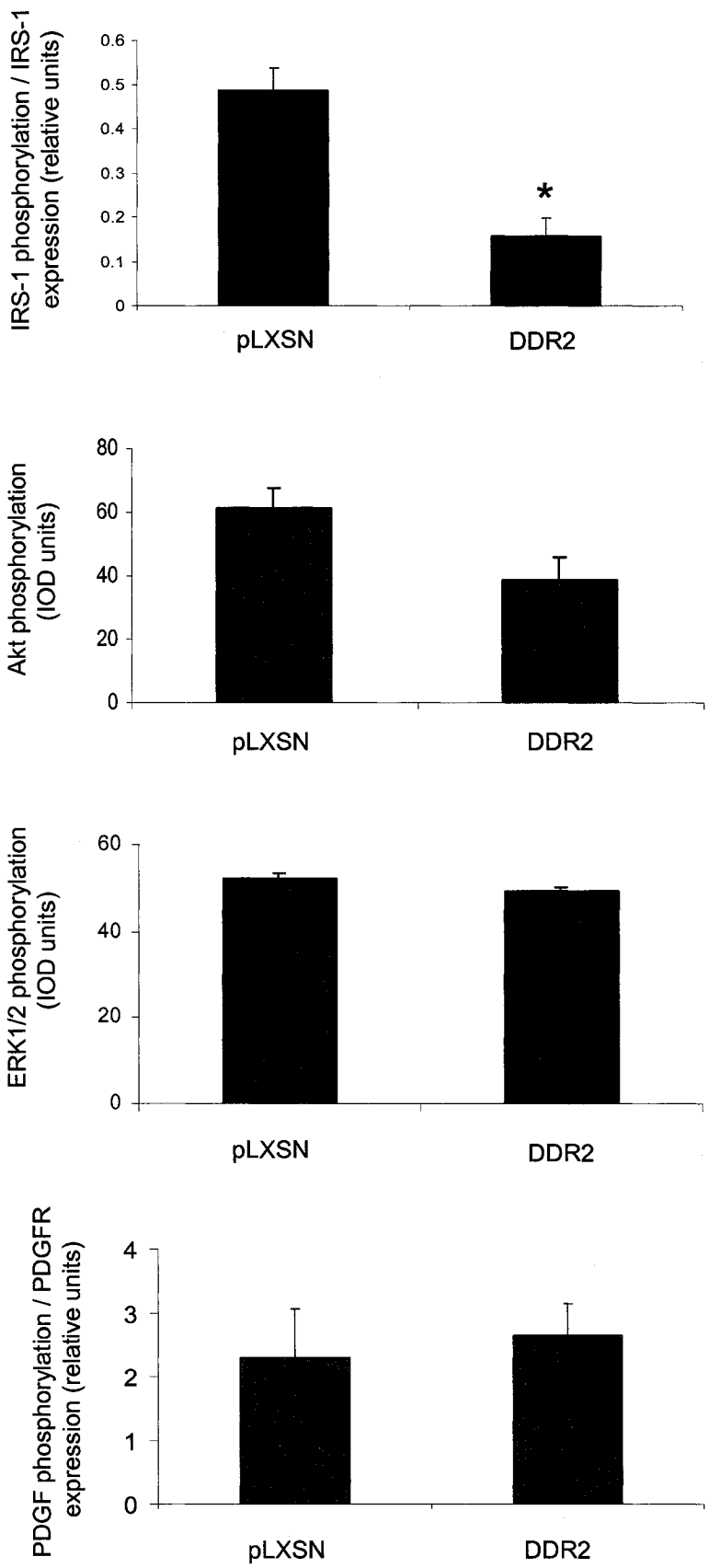

pLXSN
DDR2
Vehicle
PDGF
Insulin
Vehicle
PDGF
Insulin
PY
180 kDa
170 kDa
IRS-1
170 kDa
PDGFR
180 kDa
P-Akt (Ser 473)
60 kDa
Akt
60 kDa
P-ERK1/2
(Thr202/Tyr204)
44 kDa
42 kDa
ERK1/2
44 kDa
42 kDa

Figure 16 (Continued)

B.


IRS-1 phosphorylation / IRS-1 expression (relative units)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
pLXSN
DDR2
Akt phosphorylation (IOD units)
80
60
40
20
0
pLXSN
DDR2
ERK1/2 phosphorylation (IOD units)
60
40
20
0
pLXSN
DDR2
PDGF phosphorylation / PDGFR expression (relative units)
4
3
2
1
0
pLXSN
DDR2

with insulin compared to the response observed in empty-vector control preadipocytes. Despite this, there were no significant decreases in phosphorylation of the downstream signaling molecules, Akt and ERK1/2.

To determine if overexpression of DDR2 had an impact on other tyrosine kinase receptors in the preadipocyte, we examined platelet-derived growth factor receptor (PDGFR). 3T3-L1 preadipocytes were grown to confluence and stimulated with 10 ng/mL PDGF or vehicle for 5 minutes, as described above for insulin. Regardless of treatment, DDR2-overexpressing preadipocytes expressed less PDGFR than empty-vector control preadipocytes. Therefore, this was taken into account when the phosphorylation of PDGFR was analyzed upon PDGF stimulation, in DDR2 overexpressing preadipocytes compared to empty-vector controls. PDGFR, which ran at the 180 kDa level, was phosphorylated upon stimulation with PDGF in both DDR2-overexpressing preadipocytes and empty-vector control preadipocytes (Figure 16A). When PDGFR phosphorylation was normalized to PDGF expression, no difference in PDGFR phosphorylation was observed between DDR2-overexpressing preadipocytes and empty-vector controls (Figure 16B). Overall, although PDGFR expression is altered by DDR2-overexpression, PDGF-stimulated tyrosine phosphorylation of PDGFR appears to be intact.

DISCUSSION

As a first step to evaluate whether DDRs are pertinent to adipogenesis, we examined their expression during this process. During adipogenesis, a decrease in both the protein and mRNA expression of DDR1 was observed. However, a discordance between DDR1 protein and mRNA data exists. Whereas DDR1 protein expression was decreased in day 8 adipocytes versus preadipocytes, DDR1 mRNA was reduced at day 4 of adipogenesis.

There are several possibilities that may account for this discordance. Firstly, DDR1 protein in day 8 adipocytes was compared to day 8 preadipocytes, whereas DDR1 mRNA levels were compared to day 0 preadipocytes. It is possible that DDR1 protein expression increases with increased culture of preadipocytes. Therefore, day 8 preadipocytes may express more DDR1 than day 0 preadipocytes. Thus, preadipocytes left in culture for differing amounts of time may differ in their protein and mRNA expression. An analysis of the protein and mRNA expression of DDRs in preadipocytes and in differentiating adipocytes, every two days, would determine if this is true. Secondly, a slight, although not significant decrease was still observed in DDR1 mRNA levels in adipocytes compared to day 8 preadipocytes. Therefore, additional experiments might provide enough statistical power to demonstrate that DDR1 mRNA is consistently reduced in adipocytes compared to preadipocytes. Thirdly, it is recognized that mRNA expression does not always parallel protein expression. Therefore, it is possible that adipogenesis has a greater effect on the expression of DDR1 protein versus DDR1 mRNA.

DDR2 mRNA levels were reduced only during the early phase of 3T3-L1 preadipocyte differentiation. The early phase of 3T3-L1 adipogenesis is characterized by a change in the composition of the ECM. This allows the preadipocytes to adopt a more

spherical shape and accumulate triglycerides as they differentiate. Upon preadipocyte differentiation, collagen types I and III, fibronectin, and β1-integrins are down-regulated, whereas collagen type IV, laminin and entactin are up-regulated (Gregoire et al., 1998). Another ECM component, aortic carboxypeptidase-like protein (ACLP), is also decreased during 3T3-L1 preadipocyte differentiation (Gagnon et al., 2002). ACLP is found in collagen-rich tissues, including subcutaneous adipose tissue (Ith et al., 2005). ACLP contains a discoidin domain, a signal peptide and is secreted to possibly associate with the ECM (Layne et al., 1998). Gagnon and associates (2002) showed that ACLP protein and mRNA were severely down-regulated at day 2 of adipogenesis and increased during the later stages of adipogenesis. This is similar to the expression pattern observed for DDR1 and DDR2. Therefore, ACLP and the DDRs are both discoidin domain-containing proteins that interact with the ECM and are decreased in 3T3-L1 adipogenesis. Thus, DDRs have a pattern of expression that is similar to many proteins that are part of the ECM such as collagen type I/III and fibronectin, and to ones that interact with the ECM, such as β1-integrins and ACLP.

Both DDR1 and DDR2 have been implicated in the proliferation of various cell types. Therefore, we were interested in examining whether the DDRs are involved in the proliferation of 3T3-L1 preadipocytes. The subconfluent proliferation rate of DDR2-overexpressing preadipocytes was reduced compared to empty-vector controls, as assessed by cell enumeration and ^{3}H-thymidine incorporation. These results suggest that a decrease in DDR2 expression may be necessary for optimal cell division. In the future, a comparison between DDR2 expression in proliferating subconfluent 3T3-L1 preadipocytes versus growth-arrested confluent preadipocytes could be made. This would determine whether

subconfluent preadipocytes that are rapidly proliferating express lower levels of DDR2. Changes in cell number and ^{3}H-thymidine incorporation can also occur as a result of increased apoptosis. However, DDR2-overexpressing preadipocytes did not display increased apoptosis, as determined by Hoechst staining (data not shown). Therefore, the decrease in the number of DDR2-overexpressing preadipocytes was not due to an increase in programmed cell death.

DDR2 has been shown to necessary for *in vivo* proliferation. DDR2-null mice exhibit dwarfism and shortening of long bones and this phenotype is caused by reduced chondrocyte proliferation (Labrador et al., 2001). In this same study, DDR2 was also shown to be required in the proliferative response during skin wound healing. Numerous *in vitro* studies have also shown that DDR2 regulates cellular proliferation. Fibroblasts derived from DDR2-null mice display reduced proliferation (Labrador et al., 2001) (Olaso et al., 2002). Furthermore, overexpression of DDR2 leads to enhanced proliferation in hepatic stellate cells (Olaso et al., 2002). Taken together, the results of these studies suggest that DDR2 is necessary for proliferation in many different cell types. However, our data show that overexpression of DDR2 in 3T3-L1 preadipocytes leads to decreased proliferation and suggests that DDR2 is a negative regulator of proliferation in this cell type. This suggests that the function of DDR2 on cellular proliferation may depend on cell type.

In a similar fashion, DDR1 also has a varied effect on proliferation, which depends on the cell type tested. For example, Vogel and associates (2001) found that the defect in lactation seen in DDR1-null mice was partially attributed to hyperproliferation of mammary epithelium. Similarly, mesangial cells derived from the kidney of DDR1-null mice showed increased proliferation. However, SMCs isolated from the aorta of DDR1-null mice

exhibited reduced proliferation when plated on type I collagen (Hou et al., 2001) (Hou et al., 2002). Overexpression of DDR1 in MDCD cells grown in collagen gel reduced the proliferation capacity of the cells (Wang et al., 2005). This is similar to the decrease in proliferation that we observed in DDR2-overexpressing 3T3-L1 preadipocytes. The results of these studies show that DDR1 has both positive and negative effects on cell growth. DDR1, like DDR2, appears to function differently in regulating proliferation in various cell types.

Factors that inhibit or promote preadipocyte proliferation are essential to the study of adipose tissue function since the number of new adipocytes formed depends on the number of preadipocytes able to be recruited (Harmon and Harp, 2001). A reduction in the proliferation of preadipocytes decreases the preadipocyte pool from which adipocytes are generated and could constrain adipogenesis. This would favour the predominance of insulin-resistant hypertrophied adipocytes within adipose tissue, which can lead to whole-body insulin resistance (Danforth, 2000). Therefore, any factor that inhibits preadipocyte proliferation may ultimately decrease the number of adipocytes to be generated, and lead to pathological consequences. A thorough examination of factors that affect preadipocyte proliferation *in vitro* will aid in the elucidation of factors that affect the *in vivo* process.

Several growth factors have been shown to stimulate 3T3-L1 preadipocyte proliferation. These include PDGF-BB, fibroblast growth factor, epidermal growth factor, insulin and IGF-1 (Schmidt et al., 1990). Among them, PDGF-BB is the most potent stimulator of 3T3-L1 preadipocyte proliferation. Interestingly, it was observed that DDR2-overexpressing preadipocytes express less PDGFR. Therefore, it is possible that one way that DDR2 overexpression might affect subconfluent preadipocyte proliferation is by its

reduced expression of the growth factor receptor, PDGFR. However, from the results of our experiments, we cannot give any explanation as to why the PDGFR is reduced in DDR2-overexpressing preadipocytes.

Other factors, such as catecholamines, also regulate preadipocyte proliferation. For example, norepinephrine was shown to inhibit rat preadipocyte proliferation (Jones et al., 1992). Recently, studies have examined the effects of other factors on 3T3-L1 preadipocyte proliferation. Overexpression of adiponectin in preadipocytes promoted subconfluent cell proliferation, as well as adipogenesis (Fu et al., 2005). Conversely, interleukin-4 was shown to inhibit PDGF-BB-induced preconfluent preadipocyte proliferation (Hua et al., 2004). The green tea catechin, epigallocathechin gallate, had an ERK- and Cdk2-dependent anti-mitogenic effect on preadipocyte proliferation (Hung et al., 2005). Finally, Harmon and Harp demonstrated that the flavanoids, genistein and naringenin inhibited proliferation of preconfluent 3T3-L1 preadipocytes in a time- and dose-dependent manner (Harmon and Harp, 2001). However, genistein, but not naringenin, inhibited MCE and markers of adipogenesis, such as TG accumulation and PPAR-γ expression. This last study suggests that a factor that inhibits the mitoses involved in subconfluent preadipocyte proliferation does not always inhibit the cell divisions that constitute MCE.

The differentiation-associated decrease in DDR2 mRNA levels coincides temporally to the MCE phase of 3T3-L1 adipogenesis and this suggests that a decrease in DDR2 expression may be necessary for MCE. Our overexpression data supports this idea. DDR2-overexpressing 3T3-L1 preadipocytes have a reduced MCE phase, in addition to having reduced subconfluent proliferation. The reduction in the rate of MCE may have occurred

because the usual decrease in DDR2 was prevented in the preadipocytes that were overexpressing DDR2.

After the addition of differentiation-inducing agents to growth-arrested 3T3-L1 preadipocytes, preadipocytes that had previously been arrested at the G1-phase of the cell cycle re-enter the cell cycle and undergo one or two rounds of mitosis termed MCE (Camp et al., 2002). The MCE phase of adipogenesis occurs through days 0-4 of the differentiation of 3T3-L1 preadipocytes: before the expression of genes that give rise to the adipocyte phenotype (Tang et al., 2003). The progression from the G1-phase to the S-phase is characterized by the activation of cyclin-dependent kinase 2 (Cdk2) by cyclins A and E (Reichert and Eick, 1999). Retinoblastoma protein (Rb) is a substrate of Cdk2 and becomes phosphorylated once Cdk2 is activated (Cole et al., 2004). Phosphorylation of Rb causes it to release C/EBPβ, which is then able to bind to DNA (Cole et al., 2004). It is believed that MCE is necessary to unwind DNA, thus allowing transcription factors access to regulatory response elements present in genes involved in acquisition of the mature adipocyte phenotype (Ntambi and Kim, 2000). After the cells complete MCE, they enter a unique state of growth arrest, G_D that is permissive for subsequent differentiation (Otto and Lane, 2005). This final stage of growth arrest is characterized by the induction of several genes including the growth arrest and DNA damage-inducible gene 45 and cyclin-dependent inhibitors (p21 and p27) (Reichert and Eick, 1999).

Cdks are key regulators of the cell cycle in vertebrate cells. Cdk2 in particular, appears to also be one of the main controllers of the MCE phase of adipogenesis in 3T3-L1 preadipocytes. Therefore, because DDR2-overexpressing preadipocytes have a reduced subconfluent proliferation rate and undergo a reduced MCE phase of adipogenesis, it is

possible that overexpression of DDR2 may somehow affect Cdk2 activation. As a first step to elucidating this, future studies of DDR2-overexpressing preadipocytes could examine whether these cells have a reduced activity of any of Cdk2. Using a Cdk2 activity assay, Hung and associates showed that the anti-mitogenic effect of EGCG on subconfluent 3T3-L1 preadipocyte proliferation is dependent on the Cdk2 pathway and requires inactivation of the Cdk2 protein (Hung et al., 2005). DDR2-overexpression might act in a similar way to EGCG in mediating its antimitogenic effect on the subconfluent proliferation and MCE of 3T3-L1 preadipocytes.

There has been much evidence to demonstrate that MCE is a prerequisite for the terminal differentiation of 3T3-L1 preadipocyte undergoing adipogenesis. Numerous studies have shown that inhibition of MCE prevents adipogenesis. For example, MCE and adipogenesis are both blocked by the various DNA synthesis inhibitors, including aphidicolin (Schmidt et al., 1990) and the antiproliferative reagent rapamycin (Yeh et al., 1995). More recently, as mentioned above, genistein was seen to inhibit both MCE and adipogenesis (Harmon and Harp, 2001). Zhang and associates showed that disruption of signaling from C/EBPβ prevents MCE and subsequent adipogenesis (Zhang et al., 2004). Qui and colleagues showed that treatment of 3T3-L1 preadipocytes with PD98059, a MEK inhibitor, prevented both DNA replication and MCE without affecting differentiation (Qiu et al., 2001). However, this finding was refuted by Tang and associates (2003). They found that PD98059 delays, but does not block, MCE, expression of cell cycle and adipocyte markers, or cytoplasmic TG accumulation. Instead they showed that blocking MCE with the more potent and specific MEK inhibitor, U0126, prevented the expression of cell cycle markers, adipocyte gene markers and the accumulation of cytoplasmic TG. Therefore,

evidence in the literature to date clearly states that MCE is required for 3T3-L1 adipogenesis.

Surprisingly, overexpression of DDR2 in preadipocytes led to a reduced MCE phase but did not inhibit 3T3-L1 adipogenesis. Instead, adipocytes overexpressing DDR2 have an increased cell size, as well an increased TG mass. Therefore, at first glance, our findings would appear to be in disagreement with previous findings that demonstrate that blockage of MCE prevents adipogenesis. However, we did not completely block the increase in cell number that normally occurs in MCE. Therefore, the reduction in MCE that we observed in DDR2-overexpressing preadipocytes might not have been large enough to induce antiadipogenic effects seen with complete blockage of MCE.

Tang and associates (2003) showed that high doses of the MEK inhibitor, PD98059, used to treat preadipocytes undergoing differentiation, partially reduces and delays differentiation. Also, they showed that the extent to which MCE is reduced by PD98059 corresponds closely to the reduction observed in the expression of adipocyte markers and accumulation of TG. However, the reduction in MCE we observed was not accompanied by the expected inhibition of adipogenesis. Instead, the DDR2-overexpressing adipocytes had an increased TG content, although adipogenic markers were not measured. It is possible that preadipocytes overexpressing DDR2 receive alternate adipogenic signals through this receptor that compensate for the reduction in MCE that normally inhibits/reduces adipogenesis.

Recently, Park and associates overexpressed glucose-6-phosphate dehydrogenase (G6PD), an enzyme necessary for the production of cellular NADPH that is required for the biosynthesis of FAs and cholesterol, in 3T3-L1 adipocytes (Park et al., 2005). This

overexpression was associated with increased accumulation of cellular TG and larger droplets of TG in the adipocyte. This is similar to what we observed when DDR2 was overexpressed in adipocytes. Presumably, G6PD-overexpressing adipocytes also had a larger size, in addition to increased TG stores; however, Park *et al.* (2005) did not measure adipocyte cell size directly. Park and associates (2005) were able to demonstrate that the increase in cytoplasmic TG was associated with an increase in the expression of most adipocyte and lipogenic marker genes. Therefore, they were able to show that the overexpression of G6PD in preadipocytes undergoing differentiation to adipocytes stimulates adipogenesis as well as lipogenesis.

It is not clear whether the increased size and increased TG content of DDR2-overexpressing adipocytes was caused by increased adipogenesis or due to an increase in lipogenesis. Because lipogenesis is tightly linked to adipogenesis, it is often difficult to distinguish the two processes. However, measurement of adipogenic and lipogenic markers would have allowed us to see if the increase in size of DDR2-overexpressing adipocytes was due to increased adipogenesis, lipogenesis, or both. This is something that can be examined in the future in order to elucidate the molecular mechanisms by which DDR2-overexpression leads to an increased adipocyte size with increased TG. In addition, increased TG content could be due to an increase in lipid synthesis, an increase in lipid influx, a decrease in lipolysis or an increase in lipid efflux (Wang et al., 2004).

Many factors have been shown to modulate adipocyte cell size. For example, mice with a knock-out in diacylglycerol transferase, an enzyme that catalyzes the final step in mammalian TG synthesis, are resistant to diet-induced obesity and have smaller adipocytes that wild-type control mice both on chow and high-fat diets (Chen et al., 2002). On the other

hand, mice with a fat-specific insulin receptor knockout exhibit a polarization of white adipose tissue into two populations of cells, one small and one large, accompanied by changes in insulin-stimulated glucose uptake, triglyceride synthesis, and lypolysis (Bluher et al., 2004). A subsequent investigation using a proteomics approach by Bluher et al. (2004) identified multiple differentially expressed proteins that are involved in the regulation of adipocyte differentiation, TG storage and adipocyte metabolism in the small versus large adipocytes.

Several recent reports have examined the role of other factors in modulating adipocyte cell size. One recent report overexpressed growth differentiation factor 3 (GDF-3), which is structurally a bone morphogenetic protein subfamily member of the transforming growth factor-β superfamily, in mice by adenovirus-mediated gene transfer (Wang et al., 2004). The exact function of GDF-3 in unknown, however, mice overexpressing GDF-3 display a profound weight gain when fed a high fat diet and have highly hypertrophic adipocytes. Takahashi and colleagues showed that overexpression of mesoderm-specific transcript (Mest), the function of which is not entirely clear, in mice markedly enlarged their adipocytes (Takahashi et al., 2005). However, the authors were unable to detect a change in adipocyte size when Mest was overexpressed in 3T3-L1 adipocytes. To the best of our knowledge, our study is the first to report a change in size of 3T3-L1 adipocytes.

Lipid storage within the adipocyte *in vivo* varies considerably in response to dysequilibria in energy balance. This is particularly obvious in the obese state where a long-term positive energy balance produces massive fat cell hypertrophy. It has been suggested that failure of preadipocyte differentiation may lead to expansion of adipose tissue primarily

due to enlargement of existing adipocytes (Danforth, 2000). However, the exact mechanisms by which adipocytes become hypertrophied have not yet been elucidated. Therefore, an examination of factors that lead to enlargement of adipocytes is necessary because adipocyte size correlates to adipocyte insulin resistance and whole body insulin resistance (Ravussin and Smith, 2002). In fact, subcutaneous abdominal adipocyte size, but not obesity itself, was seen to predict type 2 diabetes (Weyer et al., 2000). Yang and colleagues also demonstrated that non-diabetic insulin-resistant first-degree relatives of type 2 diabetic patients have adipose cells that are significantly enlarged (Yang et al., 2004). The metabolic disturbances caused by large adipocytes are believed to be the result of a disordered adipokine profile and an inability of hypertrophied adipocytes to efficiently store fat, both of which lead to insulin resistance. Enlarged adipocytes are themselves resistant to insulin.

Park and colleagues (2005) showed that their 3T3-L1 adipocytes overexpressing G6PD expressed more TNF-α and resistin, less adiponectin and increased the release of free fatty acids, all of which are key players in the development of insulin resistance. Therefore, they examined whether G6PD overexpression affects insulin signaling in the adipocyte. Overexpression of G6PD inhibited insulin signaling in these adipocytes, as assessed by phosphorylation of key insulin signaling molecules, such as IRS-1 and Akt. We also stimulated our enlarged DDR2-overexpressing adipocytes with insulin and examined whether insulin signal transduction was altered. Similarly to the results obtained by Park et al. (2005), IRS-1 was phosphorylated to a lesser degree in insulin-stimulated DDR2-overexpressing adipocytes. However, contrary to the results of Park et al. (2005), our signaling studies on DDR2-overexpressing adipocytes in response to insulin revealed a

complex effect. Whereas insulin-stimulated IRS-1 tyrosine phosphorylation was inhibited, the DDR2-overexpressing adipocytes exhibited greater phosphorylation of Akt and ERK1/2. Akt and ERK1/2 are downstream of IRS-1 in the insulin-signaling pathway. Therefore, it would be expected that decreased phosphorylation of IRS-1 would lead to decreased phosphorylation of Akt and ERK1/2; however, this was not the case. One possible explanation for why DDR2-overexpression does not lead to decreased phosphorylation of Akt and ERK1/2 is because overexpression somehow leads to decreased activity or expression of the serine/threonine phosphatase, protein phosphatase 2A (PP2A), a phosphatase that regulates both Akt and ERK1/2.

PP2A is one of the major serine/threonine phosphatases in the cell and is constitutively expressed (Lechward et al., 2001). The PP2A core structure consists of a catalytic subunit ($PP2A_c$) and a regulatory A subunit (Van Hoof and Goris, 2004). The core dimer can recruit a third regulatory B subunit, of which three major classes have been described. Each of the subunits of PP2A exists in at least two isoforms leading to a multitude of possible combinations of holoenzymes, which partially explains the multiple and diverse functions of PP2A (Janssens et al., 2005). Regulation is accomplished mainly by the A and B regulatory subunits, which determine the substrate specificity, cellular localization and catalytic activity of the PP2A holoenzymes (Janssens and Goris, 2001). Furthermore, the catalytic subunit can be affected by post-translational modification, such as phosphorylation and methylation, which are also though to be important regulatory mechanisms. This regulation can be achieved with the association of PP2A subunits with a number of different cellular and viral proteins (Janssens et al., 2005). PP2A has been implicated in the regulation of many processes including regulation of different signal

transduction pathways, cell cycle progression, DNA replication, gene transcription and protein translation (Janssens et al., 2005).

PP2A has been shown to be a negative regulator of insulin-signaling pathways. PP2A is known to be involved in the downregulation of the ERK pathway by dephosphorylating and inactivating MEK1 and ERK1/2 (Lechward et al., 2001). In adipocytes, PP2A has been shown to directly dephosphorylate ERK1/2 (Milward et al., 1999) (Ugi et al., 2002). PP2A is also involved in the regulation of the metabolic action of insulin. Ugi and associates recently showed that PP2A directly dephosphorylates and inactivates Akt, leading attenuated glucose transport in 3T3-L1 adipocytes (Ugi et al., 2004). In this study, PP2A was not involved in regulating the activation of PI3K. PP2A was shown to directly modulate Akt by decreasing its phosphorylation, which in turn, decreased glucose transport. It appears that PP2A is a physiologically relevant Akt and ERK1/2 phosphatase and it is possible that overexpression of DDR2 in 3T3-L1 adipocytes inhibits PP2A. This would eliminate the negative regulatory effect that PP2A normally has on the phosphorylation of Akt and ERK1/2. Consequently, the phosphorylation of Akt and ERK1/2 would be increased with insulin stimulation, which is what was observed in DDR2-overexpressing 3T3-L1 adipocytes. Therefore, in the future, it would be useful to determine whether DDR2-overexpression in 3T3-L1 adipocytes decreases PP2A activity.

PP2A activity could be assessed using a PP2A activity assay, as performed previously by Ugi *et al.* (2004). Simian virus 40 small t antigen and okadaic acid, have both been shown to inhibit PP2A (Ugi et al., 2004). Simian virus 40 small t antigen associates with and inhibits the core dimer of PP2A and inhibits the association of PP2A with its cellular substrates, and because of this, has been shown to enhance glucose transport and

GLUT4 translocation (Mateer et al., 1998) (Ugi et al., 2004). Okadaic acid also activates glucose transport and GLUT4 translocation by inhibiting PP2A by binding to its catalytic subunit and enhancing its tyrosine phosphorylation, which in turn, inactivates the enzyme (Janssens and Goris, 2001; Standaert et al., 1999). DDR2-overexpressing 3T3-L1 adipocytes may lead to the negative regulation of PP2A and thus, might act in a similar manner to simian virus 40 small t antigen and okadaic acid and decrease PP2A activity.

In the future, it will be essential to examine how the altered insulin signaling response that we observed in DDR2-overexpressing adipocytes relates to the functional aspect of the adipocyte. Insulin signaling in 3T3-L1 adipocytes promotes lipogenesis, glucose transport and inhibits lipolysis. Future measurement of glucose uptake, as assessed by 2-deoxyglucose uptake, would be necessary to see whether increased insulin-induced phosphorylation of Akt in DDR2-overexpressing adipocytes leads to increased glucose transport. Rates of lipogenesis and lipolysis of DDR2-overexpressing adipocytes should also be measured in response to insulin.

In order to determine whether the altered insulin-stimulated signaling response of DDR2-overexpressing adipocytes could be due to DDR2 overexpression itself, we examined the insulin-stimulated signaling response of 3T3-L1 preadipocytes. IRS-1 was phosphorylated to a lesser degree in insulin-stimulated DDR2-overexpressing preadipocytes. This is similar to what we had observed in DDR2-overexpressing adipocytes. This suggests that the observed decrease in insulin-stimulated IRS-1 phosphorylation in DDR2-overexpressing adipocytes was not due solely to the enlargement of DDR2-overexpressing adipocytes. Instead, decreased phosphorylation of IRS-1 might be directly due to overexpression of DDR2 in adipocytes and preadipocytes.

Decreased activation of PP2A, in DDR2-overexpressing preadipocytes and adipocytes, might explain the reduction observed in the tyrosine phosphorylation of IRS-1 upon stimulation with insulin. In addition to acting as a negative regulator of the phosphorylation of Akt and ERK1/2, PP2A is also involved in regulating the serine phosphorylation of IRS-1. While the tyrosine phosphorylation of IRS-1 is required for insulin-stimulated responses, the serine phosphorylation of IRS-1 has a dual role, either to enhance or terminate the insulin-induced effects (Gual et al., 2005). Additionally, while the role of IRS-1 tyrosine phosphorylation has been largely elucidated, the role of serine/threonine phosphorylation is not fully understood.

There has been some investigation into the role of PP2A in the serine phosphorylation of IRS-1. For example, Tanti and colleagues demonstrated that the potent PP2A inhibitor, okadaic acid, severely decreases the effect of insulin on glucose transport and on Glut 4 translocation in adipocytes and skeletal muscle cells (Tanti et al., 1994). This alteration correlated to a decrease in IRS-1 tyrosine phosphorylation. In addition, they found that okadaic acid markedly increased the serine phosphorylation of IRS-1, which made it a poor substrate for the activated insulin receptor. This suggests that inhibitors of PP2A, such as okadaic acid, may lead to increased serine phosphorylation of IRS-1 and this may cause IRS-1 to interact less efficiently with the insulin receptor following insulin stimulation and thus decrease the tyrosine phosphorylation of IRS-1. Therefore, the inhibition of IRS-1 that we observed both in DDR2-overexpressing preadipocytes and adipocytes following stimulation with insulin might be a result of increased serine phosphorylation of IRS-1. This increase in serine phosphorylation may have been due to an inhibition in the activity of PP2A in cells overexpressing DDR2.

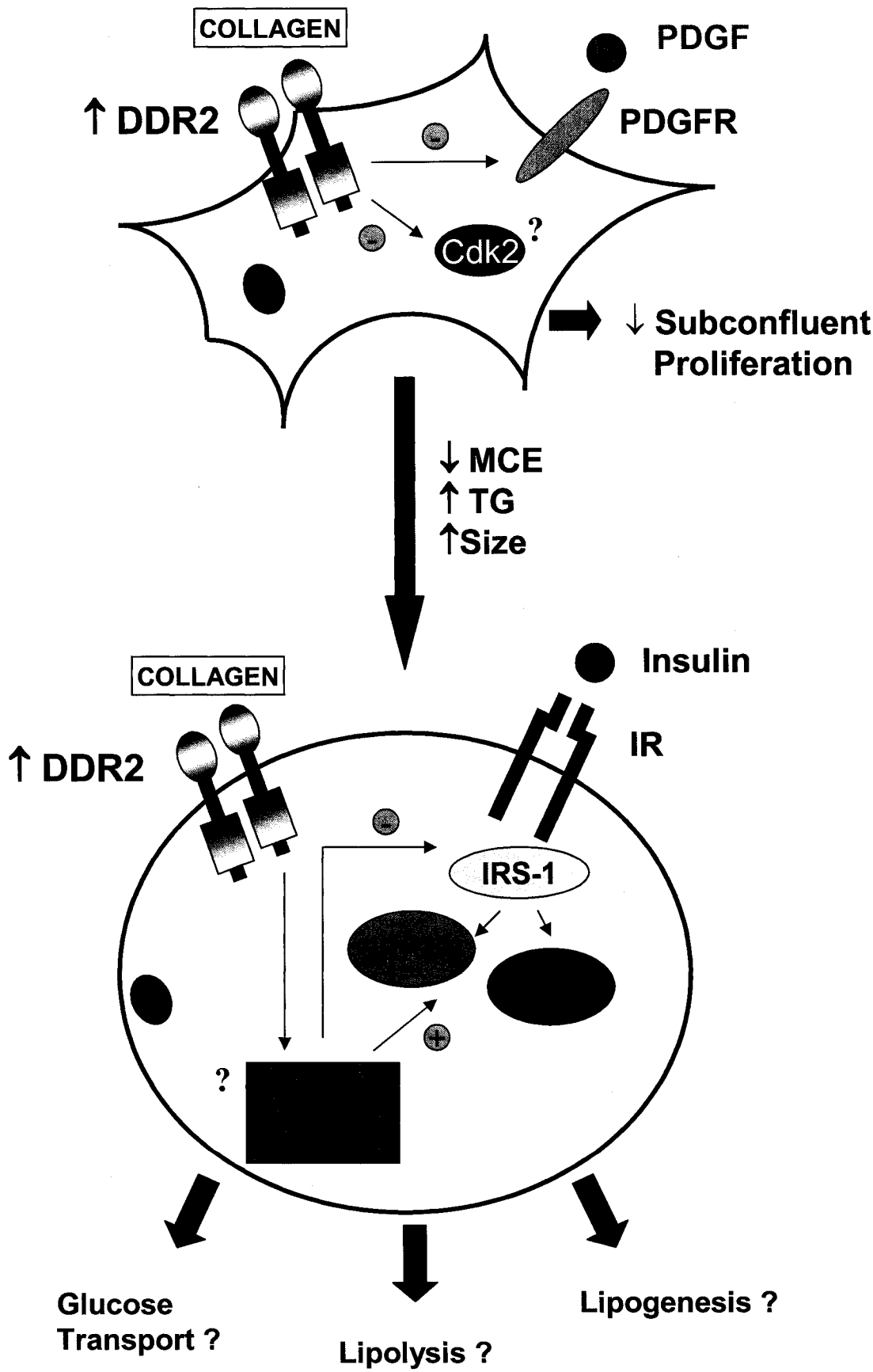
preadipocytes. As well, DDR1b-overexpressing adipocytes had an increased cell size; whereas DDR1a-overexpressing adipocytes did not display a change in cellular size. However, DDR1b- and DDR1a-overexpressing adipocytes both appeared to contain slightly increased amounts of cytosolic TG; however, a significant increase was not observed. Therefore, the effect of DDR1 overexrpession in preadipocytes and adipocytes was weak and variable. Hence, these effects have not been emphasized in this discussion.

A summary of the effects of DDR2 overexpression on 3T3-L1 preadipocytes and adipocytes is shown in Figure 17. Taken together, the data in this thesis suggest that DDR1 and DDR2 are downregulated during 3T3-L1 adipogenesis. Overexpression of DDR2 decreases the subconfluent and post-confluent proliferation rate of 3T3-L1 preadipocytes. DDR2 overexpression also increases the cell size, as well as the TG content of 3T3-L1 adipocytes. Insulin-stimulation of 3T3-L1 adipocytes overexpressing DDR2 reveals that IRS-1 phosphorylation is downregulated, whereas Akt and ERK1/2 phosphorylation is upregulated. This altered signaling response appears to be as a result of DDR2 overexpression, and not due solely to the enlarged cell size of DDR2 overexpressing adipocytes. This is because insulin-stimulation of 3T3-L1 preadipocytes overexpressing DDR2 again showed downregulation of IRS-1 phosphorylation, with no apparent change in the phosphorylation of Akt or ERK1/2.

Some suggestions as to what other future experiments can be done in order to elucidate the role of DDR2 in 3T3-L1 preadipocytes and adipocytes have already been mentioned above. In addition, DDR1-overexpressing and DDR2-overexpressing 3T3-L1 preadipocytes can be plated on a collagen-coated cell culture dish. Then, the effect that this ECM protein has on subconfluent preadipocyte proliferation, postconfluent MCE, adipocyte

Figure 17: Summary of the effects of DDR2 overexpression in 3T3-L1 preadipocytes and adipocytes and proposed model of how DDR2 may lead to these effects

Overexpression of DDR2 decreases the subconfluent proliferation rate and post-confluent MCE rate of 3T3-L1 preadipocytes. Overexpression of DDR2 in preadipocytes leads to decreased expression of PDGFR. It is possible that one way that DDR2 overexpression might affect subconfluent preadipocyte proliferation is by its reduced expression of PDGFR, which binds the growth factor, PDGF. DDR2-overexpression might also affect Cdk2 activation, which could be the cause of the decrease in subconfluent and postconfluent proliferation of these cells. Effects of DDR2 overexpression on insulin signaling in preadipocytes were omitted in this diagram. DDR2 overexpression also increases the cell size and the TG content of 3T3-L1 adipocytes. DDR2 overexpression also leads to an altered signaling response of 3T3-L1 adipocytes to insulin. Insulin-stimulation of 3T3-L1 adipocytes overexpressing DDR2 reveals that IRS-1 phosphorylation is downregulated, whereas Akt and ERK1/2 phosphorylation is upregulated. These changes in insulin-signaling may be due to the possibility that DDR2-overexpressing adipocytes have a reduced activity of PP2A. Reduced activity of PP2A in these cells may be what causes decreased phosphorylation of IRS-1 and increased phosphorylation of Akt and ERK1/2 following insulin stimulation.


COLLAGEN
PDGF
↑ DDR2
PDGFR
Cdk2
?
↓ Subconfluent
Proliferation
↓ MCE
↑ TG
↑Size
COLLAGEN
Insulin
↑ DDR2
IR
IRS-1
?
Glucose
Transport ?
Lipolysis ?
Lipogenesis ?

cell size, TG content and insulin-signaling in both preadipocytes and adipocytes could be examined. It is possible that plating the DDR2-overexpressing preadipocytes might exaggerate the effects of overexpression that we observed in the experiments presented in this thesis. Similarly, it might expose some effects of DDR1-overexpression in preadipocytes that could not be observed in this present thesis. As well, the effect of DDR2-overexpression on adipokine release could also be examined. Finally, the experiments presented in this thesis based on the 3T3-L1 model could be extended to primary rodent and human adipose cells.

REFERENCES

Alves, F., Saupe, S., Ledwon, M., Schaub, F., Hiddemann, W., and Vogel, W. F. (2001). Identification of two novel, kinase-deficient variants of discoidin domain receptor 1: differential expression in human colon cancer cell lines, FASEB Journal *15*, 1321-3.

Alves, F., Vogel, W., Mossie, K., Millauer, B., Hofler, H., and Ullrich, A. (1995). Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer, Oncogene *10*, 609-18.

Aratani, Y., and Kitagawa, Y. (1988). Enhanced synthesis and secretion of type IV collagen and entactin during adipose conversion of 3T3-L1 cells and production of unorthodox laminin complex, Journal of Biological Chemistry *263*, 16163-16169.

Aubert, J., Dessolin, S., Belmonte, N., Li, M., McKenzie, F. R., Staccini, L., Villageois, P., Barhanin, B., Vernallis, A., Smith, A. G., *et al.* (1999). Leukemia inhibitory factor and its receptor promote adipocyte differentiation via the mitogen-activated protein kinase cascade, Journal of Biological Chemistry *274*, 24965-72.

Barker, K. T., Martindale, J. E., Mitchell, P. J., Kamalati, T., Page, M. J., Phippard, D. J., Dale, T. C., Gusterson, B. A., and Crompton, M. R. (1995). Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours, Oncogene *10*, 569-75.

Bays, H., Mandarino, L., and DeFronzo, R. A. (2004). Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach, Journal of Clinical Endocrinology & Metabolism *89*, 463-78.

Belanger-Ducharme, F., and Tremblay, A. (2005). Prevalence of obesity in Canada, Obesity Reviews *6*, 183-186.

Bhatt, R. S., Tomoda, T., Fang, Y., and Hatten, M. E. (2000). Discoidin domain receptor 1 functions in axon extension of cerebellar granule neurons, Genes & Development *14*, 2216-28.

Bjornholm, M., and Zierath, J. R. (2005). Insulin signal transduction in human skeletal muscle: identifying defects in Type II diabetes, Biochemical Society Transactions *33*, 354-357.

Bluher, M., Wilson-Fritch, L., Leszyk, J., Laustsen, P. G., Corvera, S., and Kahn, C. R. (2004). Role of insulin action and cell size on protein expression patterns in adipocytes, Journal of Biological Chemistry *279*, 31902-9.

Boden, G., and Shulman, G. I. (2002). Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction, European Journal of Clinical Investigation *32 Suppl 3*, 14-23.

Bortell, R., Jackson, M. E., Marks, S. C., Jr., Stein, J. L., Lian, J. B., and Stein, G. S. (1994). TGF beta 1 prevents the down-regulation of type I procollagen, fibronectin, and TGF beta 1 gene expression associated with 3T3-L1 pre-adipocyte differentiation, Journal of Cellular Biochemistry *55*, 182-9.

Bost, F., Aouadi, M., and Binetruy, B. (2005). The role of MAPKs in adipocyte differentiation and obesity, Biochimie *87*, 51-56.

Bouloumie, A., Sengenes, C., Portolan, G., Galitzky, J., and Lafontan, M. (2001). Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation, Diabetes *50*, 2080-2086.

Bradshaw, A. D., Graves, D. C., Motamed, K., Sage, E. H., and Taneda, S. (2003). SPARC-null mice exhibit increased adiposity without significant differences in overall body weight Amelioration of diabetic nephropathy in SPARC-null mice, Proceedings of the National Academy of Sciences of the United States of America *100*, 6045-50.

Bryant, N., Govers, R., and James, D. (2002). Regulated transport of the glucose transporter GLUT4, Molecular Cell Biology *3*, 267-277.

Camp, H. S., Ren, D., and Leff, T. (2002). Adipogenesis and fat-cell function in obesity and diabetes, Trends in molecular medicine *8*, 442-447.

Camp, H. S., and Tafuri, S. R. (1997). Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase, Journal of Biological Chemistry *272*, 10811-6.

Chen, H. C., Smith, S. J., Ladha, Z., Jensen, D. R., Ferreira, L. D., Pulawa, L. K., McGuire, J. G., Pitas, R. E., Eckel, R. H., and Farese, R. V., Jr. (2002). Increased insulin and leptin sensitivity in mice lacking acyl CoA:diacylglycerol acyltransferase 1, Journal of Clinical Investigation *109*, 1049-55.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Annals of Biochemistry *162*, 156-159.

Cinti, S. (2005). The adipose organ, Prostaglandins Leukotrienes & Essential Fatty Acids *73*, 9-15.

Clark, S., Molero, J., and James, D. (2000). Release of insulin receptor substrate proteins from an intracellular complex coincides with the development of insulin resistance, The Journal of Biological Chemistry *275*, 3819-3826.

Cole, K. A., Harmon, A. W., Harp, J. B., Patel, Y. M., and Shao, J. (2004). Rb regulates C/EBPbeta-DNA-binding activity during 3T3-L1 adipogenesis, American Journal of Physiology - Cell Physiology *286*, C349-54.

Considine, E. L., Sinha, M., Heiman, M., Kriauciunas, A., Stephens, T., Nyce, M. R., Ohannesian, J. P., Marco, C., McKee, L., Bauer, T. L., and Caro, J. F. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans, The New England Journal of Medicine *334*, 292-295.

Cornelius, P., MacDougald, O., and Lane, M. D. (1994). Regulation of adipocyte development, Annual Reviews in Nutrition *14*, 99-129.

Curat, C. A., Eck, M., Dervillez, X., and Vogel, W. F. (2001). Mapping of epitopes in discoidin domain receptor 1 critical for collagen binding, Journal of Biological Chemistry *276*, 45952-8.

Curat, C. A., Miranville, A., Sengenes, C., Diehl, M., Tonus, C., Busse, R., and Bouloumie, A. (2004). From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes, Diabetes *53*, 1285-92.

Curat, C. A., and Vogel, W. F. (2002). Discoidin domain receptor 1 controls growth and adhesion of mesangial cells, Journal of the American Society of Nephrology *13*, 2648-56.

Cutuli, N., Guerra, L., Cancedda, R., De Luca, M., and Di Marco, E. (1993). Molecular cloning of trkE, a novel trk-related putative tyrosine kinase receptor isolated from normal human keratinocytes and widely expressed by normal human tissues, Journal of Biological Chemistry *268*, 24290-5.

Danforth, E. (2000). Failure of adipocyte differentiation causes type II diabetes mellitus?, Nature Genetics *26*, 13.

Dejmek, J., Dib, K., Jonsson, M., and Andersson, T. (2003). Wnt-5a and G-protein signaling are required for collagen-induced DDR1 receptor activation and normal mammary cell adhesion, Journal of Biological Chemistry *278*, 16761-9.

Drevon, C. A. (2005). Fatty acids and expression of adipokines, Biochimica et Biophysica Acta *1740*, 287-92.

Eyre, D. R. (2004). Collagens and cartilage matrix homeostasis, Clinical Orthopaedics & Related Research, S118-22.

Faraci, E., Eck, M., Gerstmayer, B., Bosio, A., and Vogel, W. F. (2003). An extracellular matrix-specific microarray allowed the identification of target genes downstream of discoidin domain receptors, Matrix Biology *22*, 373-81.

Faraj, M., Lu, H. L., and Cianflone, K. (2004). Diabetes, lipids, and adipocyte secretagogues, Biochemistry & Cell Biology *82*, 170-90.

Ferri, N., Carragher, N. O., and Raines, E. W. (2004). Role of discoidin domain receptors 1 and 2 in human smooth muscle cell-mediated collagen remodeling: potential implications in atherosclerosis and lymphangioleiomyomatosis, American Journal of Pathology *164*, 1575-85.

Fischbach, C., Spruss, T., Weiser, B., Neubauer, M., Becker, C., Hacker, M., Gopferich, A., and Blunk, T. (2004). Generation of mature fat pads in vitro and in vivo utilizing 3-D long-term culture of 3T3-L1 preadipocytes, Experimental Cell Research *300*, 54-64.

Franco, C. D., Hou, G., and Bendeck, M. P. (2002). Collagens, integrins, and the discoidin domain receptors in arterial occlusive disease, Trends in Cardiovascular Medicine *12*, 143-8.

Fu, Y., Luo, N., Klein, R. L., and Garvey, W. T. (2005). Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation, Journal of Lipid Research *46*, 1369-79.

Fujishiro, M., Gotoh, Y., Katagiri, H., Sakoda, H., Ogihara, T., Anai, M., Onishi, Y., Ono, H., Funaki, M., Inukai, K., *et al.* (2001). MKK6/3 and p38 MAPK pathway activation is not necessary for insulin-induced glucose uptake but regulates glucose transporter expression, Journal of Biological Chemistry *276*, 19800-6.

Gagnon, A., Abaiian, K. J., Crapper, T., Layne, M. D., and Sorisky, A. (2002). Down-regulation of aortic carboxypeptidase-like protein during the early phase of 3T3-L1 adipogenesis, Endocrinology *143*, 2478-85.

Gagnon, A., Chen, C. S., and Sorisky, A. (1999). Activation of protein kinase B and induction of adipogenesis by insulin in 3T3-L1 preadipocytes: contribution of phosphoinositide-3,4,5-trisphosphate versus phosphoinositide-3,4-bisphosphate, Diabetes *48*, 691-8.

Gimeno, R. E., and Klaman, L. D. (2005). Adipose tissue as an active endocrine organ: recent advances, Current Opinion in Pharmacology *5*, 122-8.

Green, H., and Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line, Journal of Cellular Physiology *101*, 169-171.

Green, H., and Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture., Cell *3*, 127-33.

Gregoire, F. M. (2001). Adipocyte differentiation: from fibroblast to endocrine cell, Experiment Biology Medicine *226*, 997-1002.

Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998). Understanding adipocyte differentiation., Physiological Reviews *78*, 783-809.

Gross, O., Beirowski, B., Harvey, S. J., McFadden, C., Chen, D., Tam, S., Thorner, P. S., Smyth, N., Addicks, K., Bloch, W., *et al.* (2004). DDR1-deficient mice show localized subepithelial GBM thickening with focal loss of slit diaphragms and proteinuria, Kidney International *66*, 102-11.

Gual, P., Le Marchand-Brustel, Y., and Tanti, J. (2005). Positve and negative regulation of insulin signaling through IRS-1 phosphorylation, Biochimie *87*, 99-109.

Guo, X., and Liao, K. (2000). Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation, Gene *251*, 45-53.

Harmon, A. W., and Harp, J. B. (2001). Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis, American Journal of Cellular Physiology *280*, C807-C813.

Harp, J. B. (2004). New Insights into inhibitors of adipogenesis, Current Opinion in Lipidology *15*, 303-307.

Hartley, D., and Cooper, G. (2002). Role of mTOR in the degradation of IRS-1: regulation of PP2A activity, Journal of Cellular Biochemistry *85*, 304-314.

Haslam, D., and James, P. (2005). Obesity, Lancet *366*, 1197-1209.

Hauner, H. (2004). The new concept of adipose tissue function, Physiology & Behavior *83*, 653-8.

Hausman, D. B., DiGirolamo, M., Bartness, T. J., Hausman, G. J., and Martin, R. J. (2001). The biology of white adipocyte proliferation, Obesity Reviews *2*, 239-54.

Heilbronn, L., Smith, S. R., and Ravussin, E. (2004). Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type II diabetes mellitus, International Journal of Obesity *28*, S12-S21.

Heino, J. (2000). The collagen receptor integrins have distinct ligand recognition and signaling functions, Matrix Biology *19*, 319-23.

Heinzelmann-Schwarz, V. A., Gardiner-Garden, M., Henshall, S. M., Scurry, J., Scolyer, R. A., Davies, M. J., Heinzelmann, M., Kalish, L. H., Bali, A., Kench, J. G., *et al.* (2004). Overexpression of the cell adhesion molecules DDR1, Claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer, Clinical Cancer Research *10*, 4427-36.

Hirsch, J., Fried, S. K., Edens, N. K., and Leibel, R. L. (1989). The fat cell, Medical Clinics of North America *73*, 83-96.

Hou, G., Vogel, W., and Bendeck, M. P. (2001). The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair, Journal of Clinical Investigation *107*, 727-35.

Hou, G., Vogel, W. F., and Bendeck, M. P. (2002). Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression, Circulation Research *90*, 1147-9.

Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma, Science *274*, 2100-3.

Hua, K., Deng, J., and Harp, J. B. (2004). Interleukin-4 inhibits platelet-derived growth factor-induced preadipocyte proliferation, Cytokine *25*, 61-7.

Hung, P. F., Wu, B. T., Chen, H. C., Chen, Y. H., Chen, C. L., Wu, M. H., Liu, H. C., Lee, M. J., and Kao, Y. H. (2005). Antimitogenic effect of green tea (-)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways, American Journal of Physiology - Cell Physiology *288*, C1094-108.

Ikeda, K., Wang, L. H., Torres, R., Zhao, H., Olaso, E., Eng, F. J., Labrador, P., Klein, R., Lovett, D., Yancopoulos, G. D., *et al.* (2002). Discoidin domain receptor 2 interacts with Src and Shc following its activation by type I collagen, Journal of Biological Chemistry *277*, 19206-12.

Ith, B., Wei, J., Yet, C., Perella, M., and Layne, M. D. (2005). Aortic carboxypeptidase-like protein is expressed in collagen-rich tissues during mouse embryonic development, Gene Expression Patterns *5*, 533-537.

Janssens, V., and Goris, J. (2001). Protein phophatase 2A: a highly regulated family of serine/threonine phophatases implicated in cell growth and signaling, Biochemistry Journal *353*, 417-439.

Janssens, V., Goris, J., and Van Hoof, C. (2005). PP2A: the expected tumor suppressor, Current Opinion in Genetics and Development *15*, 34-41.

Johnson, J. D., Edman, J. C., and Rutter, W. J. (1993). A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain.[erratum appears in Proc Natl Acad Sci U S A. 1993 Nov 15;90(22):10891; PMID: 8248187], Proceedings of the National Academy of Sciences of the United States of America *90*, 5677-81.

Jones, D. D., Ramsay, T. G., Hausman, G. J., and Martin, R. J. (1992). Norepinephrine inhibits rat pre-adipocyte proliferation, International Journal of Obesity & Related Metabolic Disorders: Journal of the International Association for the Study of Obesity *16*, 349-54.

Jonsson, M., and Andersson, T. (2001). Repression of Wnt-5a impairs DDR1 phosphorylation and modifies adhesion and migration of mammary cells, Journal of Cell Science *114*, 2043-53.

Kamohara, H., Yamashiro, S., Galligan, C., and Yoshimura, T. (2001). Discoidin domain receptor 1 isoform-a (DDR1alpha) promotes migration of leukocytes in three-dimensional collagen lattices, FASEB Journal *15*, 2724-6.

Katzmarzyk, P. (2002). The Canadian obesity epidemic, 1985-1998, Canadian Medical Association Journal *166*, 1039-40.

Katzmarzyk, P., and Janssen, I. (2004). The economic costs associated with physical inactivity and obesity in Canada: an update, Canadian Journal of Applied Physiology *29*, 90-115.

Kayali, A. G., Austin, D. A., and Webster, N. J. (2000). Stimulation of MAPK cascades by insulin and osmotic shocks lack of involvement of p-38 mitogen-activated protein kinase in glucose transport in 3T3-L1 adipocytes, Diabetes *49*, 1783-1793.

Kern, P., Saghizadeh, M., Ong, J., Bosch, R., Deem, R., and Simsolo, R. (1995). The expression of tunor necrosis factor in human adipose tissue, Journal of Clinical Investigation *95*, 2111-2119.

Kim, J. K., Xu, Y., Xu, X., Keene, D. R., Gurusiddappa, S., Liang, X., Wary, K. K., and Hook, M. (2005). A novel binding site in collagen type III for integrins alpha1beta1 and alpha2beta1, Journal of Biological Chemistry *280*, 32512-20.

Kohn, A., Summers, S., Birnbaum, M., and Roth, R. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation, The Journal of Biological Chemistry *271*, 31372-31378.

Kopelman, P. (2000). Obesity as a medical problem, Nature *404*, 635-43.

Labrador, J. P., Azcoitia, V., Tuckermann, J., Lin, C., Olaso, E., Manes, S., Bruckner, K., Goergen, J. L., Lemke, G., Yancopoulos, G., *et al.* (2001). The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism, EMBO Reports *2*, 446-52.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature *227*, 680-5.

Lafontan, M. (2005). Fat cells: afferent and efferent messages define new approaches to treat obesity, Ann Rev Pharmacol Toxicol *45*, 119-146.

Lai, C., and Lemke, G. (1994). Structure and expression of the Tyro 10 receptor tyrosine kinase, Oncogene *9*, 877-83.

Laval, S., Butler, R., Shelling, A. N., Hanby, A. M., Poulsom, R., and Ganesan, T. S. (1994). Isolation and characterization of an epithelial-specific receptor tyrosine kinase from an ovarian cancer cell line, Cell Growth & Differentiation *5*, 1173-83.

Layne, M. D., Endege, W., Jain, M., Yet, C., Hsieh, C., Chin, M. T., Perella, M., Blanar, M., Haber, E., and Lee, M. E. (1998). Aortic carboxypeptidase-like protein, a novel protein with discoidin and carboxypeptidase-like domains, is up-regulated during vascular smooth muscle cell differentiation, The Journal of Biological Chemistry *273*, 15654-15660.

Lechward, K., Awotunde, O. S., Swiatek, W., and Muszynska, G. (2001). Protein phosphatase 2A: variety of forms and diversity of functions
Eukaryotic translation termination factor 1 associates with protein phosphatase 2A and targets it to ribosomes, Acta Biochimica Polonica *48*, 921-33.

Leitinger, B. (2003). Molecular analysis of collagen binding by the human discoidin domain receptors, DDR1 and DDR2. Identification of collagen binding sites in DDR2, Journal of Biological Chemistry *278*, 16761-9.

L'Hote, C. G., Thomas, P. H., and Ganesan, T. S. (2002). Functional analysis of discoidin domain receptor 1: effect of adhesion on DDR1 phosphorylation, FASEB Journal *16*, 234-6.

Lilla, J., Stickens, D., and Werb, Z. (2002). Metalloproteases and adipogenesis: a weighty subject, American Journal of Pathology *160*, 1551-4.

MacDougald, O., and Lane, M. D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation, Annu Rev Biochem *64*, 345-373.

MacDougald, O., and Mandrup, S. (2002). Adipogenesis: forces that tip the scales, Trends Endocrinol Metab *13*, 5-11.

Magun, R., Burgering, B. M., Coffer, P. J., Pardasani, D., Lin, Y., Chabot, J., and Sorisky, A. (1996). Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation, Endocrinology *137*, 3590-3.

Mandrup, S., Loftus, T., MacDougald, O., Kuhajda, F., and Lane, M. D. (1997). Obese gene expression at in vivo levels in fat pads derived from s.c. implanted 3T3-F442A preadipocytes, Proceedings of the National Academy of Sciences of the United States of America *94*, 4300-4305.

Mateer, S. C., Fedorov, S. A., and Mumby, M. C. (1998). Identification of structural elements involved in the interaction of simian virus 40 small tumor antigen with protein phosphatase 2A, Journal of Biological Chemistry *273*, 35339-46.

Matsuyama, W., Faure, M., and Yoshimura, T. (2003a). Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-beta-activated protein kinase 1 binding protein 1 beta/p38 alpha mitogen-activated protein kinase signaling cascade, Journal of Immunology *171*, 3520-32.

Matsuyama, W., Kamohara, H., Galligan, C., Faure, M., and Yoshimura, T. (2003b). Interaction of discoidin domain receptor 1 isoform b (DDR1b) with collagen activates p38 mitogen-activated protein kinase and promotes differentiation of macrophages, FASEB Journal *17*, 1286-8.

Matsuyama, W., Wang, L., Farrar, W. L., Faure, M., and Yoshimura, T. (2004). Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-kappa B, Journal of Immunology *172*, 2332-40.

McGarry, J. (2002). Banting Lecture: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes, Diabetes *51*, 7-18.

Miki, H., Yamauchi, T., Suzuki, R., Komeda, K., Tsuchida, A., Kubota, N., Terauchi, Y., Kamon, J., Kaburagi, Y., Matsui, J., *et al.* (2001). Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation, Molecular and Cellular Biology *21*, 2521-2532.

Milward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999). Regulation of protein kinase cascades by protein phophatase 2A, Trends in Biochemical Sciences *24*, 186-191.

Mohan, R. R., and Wilson, S. E. (2001). Discoidin domain receptor (DDR) 1 and 2: collagen-activated tyrosine kinase receptors in the cornea, Experimental Eye Research *72*, 87-92.

Must, A., Spadano, J., Coakley, E., Field, A., Colditz, G., and Dietz, W. (1999). The disease burden associated with overweight and obesity, Journal of the American Medical Association *282*, 1523-1529.

Novikoff, A. B., Novikoff, P. M., Rosen, O. M., and Rubin, C. S. (1980). Organelle relationships in cultured 3T3-L1 preadipocytes, Journal of Cell Biology *87*, 180-96.

Ntambi, J., and Kim, Y. (2000). Adipocyte differentiation and gene expression, journal of Nutrition *130*, 3122S-3126S.

Olaso, E., Labrador, J. P., Wang, L., Ikeda, K., Eng, F. J., Klein, R., Lovett, D. H., Lin, H. C., and Friedman, S. L. (2002). Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2, Journal of Biological Chemistry *277*, 3606-13.

Ongusaha, P. P., Kim, J. I., Fang, L., Wong, T. W., Yancopoulos, G. D., Aaronson, S. A., and Lee, S. W. (2003). p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop, EMBO Journal *22*, 1289-301.

Otto, T. C., and Lane, M. D. (2005). Adipose development: from stem cell to adipocyte, Critical Reviews in Biochemistry and Molecular Biology *40*, 229-242.

Park, J., Rho, H., Kim, K., Choe, S., Lee, Y., and Kim, J. (2005). Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity, Molecular & Cellular Biology *25*, 5146-5157.

Peng, X., Xu, P., Chen, M., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararjan, D., Chen, W., Crawford, S., Coleman, K., and Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2, Genes and Development *17*, 1352-1365.

Perez, J. L., Shen, X., Finkernagel, S., Sciorra, L., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Wong, T. W. (1994). Identification and chromosomal mapping of a receptor tyrosine kinase with a putative phospholipid binding sequence in its ectodomain, Oncogene *9*, 211-9.

Pittas, A., Joseph, N., and Greenberg, A. (2004). Adipocytokines and insulin resistance, The Journal of Clinical Endocrinology and Metabolism *89*, 447-452.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells, Science *284*, 143-7.

Prusty, D., Park, B. H., Davis, K. E., and Farmer, S. R. (2002). Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes, Journal of Biological Chemistry *277*, 46226-32.

Qiu, Z., Wei, Y., Chen, N., Jiang, M., Wu, J., and Liao, K. (2001). DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes, The Journal of Biological Chemistry *276*, 11988-11995.

Ravussin, E., and Smith, S. R. (2002). Increased fat intake, impaired fat oxidation, and failure of fat cell proliferation result in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus, Annals of the New York Academy of Sciences *967*, 363-78.

Reichert, M., and Eick, D. (1999). Analysis of cell cycle arrest in adipocyte differentiation, Oncogene *18*, 459-66.

Rodriguez Fernandez, J. L., and Ben-Ze'ev, A. (1989). Regulation of fibronectin, integrin and cytoskeleton expression in differentiating adipocytes: inhibition by extracellular matrix and polylysine, Differentiation *42*, 65-74.

Rosen, E. (2002). The molecular control of adipogenesis, with special reference to lymphatic pathology, Annals of the New York Academy of Sciences *979*, 143-158.

Rosen, E., and Spiegelman, B. M. (2000). Molecular regulation of adipogenesis, Annual Reviews in Cellular and Developmental Biology *16*, 145-171.

Ross, S., Hemati, N., Longo, K., Bennett, C., Lucas, P., Erickson, R., and MacDougald, O. (2000). Inhibition of adipogenesis by Wnt signaling, Science *289*, 950-953.

Saba-El-Leil, M. K., Vella, F. D., Vernay, B., Voisin, L., Chen, L., Labrecque, N., Ang, S. L., and Meloche, S. (2003). An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development, EMBO Reports *4*, 964-8.

Sale, E. M., Atkinson, P. G., and Sale, G. J. (1995). Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis, EMBO Journal *14*, 674-84.

Saltiel, A., and Kahn, C. R. (2001). Insulin signaling and the regulation of glucose and lipid metabolism, Nature *414*, 799-806.

Sanchez, M. P., Tapley, P., Saini, S. S., He, B., Pulido, D., and Barbacid, M. (1994). Multiple tyrosine protein kinases in rat hippocampal neurons: isolation of Ptk-3, a receptor expressed in proliferative zones of the developing brain, Proceedings of the National Academy of Sciences of the United States of America *91*, 1819-23.

Schinner, S., Scherbaum, W. A., Bornstein, S. R., and Barthel, A. (2005). Molecular mechanisms of insulin resistance, Diabetic Medicine *22*, 674-682.

Schmidt, W., Poll-Jordan, G., and Loffler, G. (1990). Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP, Journal of Biological Chemistry *265*, 15489-95.

Selvarajan, S., Lund, L. R., Takeuchi, T., Craik, C. S., and Werb, Z. (2001). A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation, Nature Cell Biology *3*, 267-75.

Sharma, A. M., and Chetty, V. T. (2005). Obesity, hypertension and insulin resistance, Acta Diabetologia *42*, S3-S8.

Shelling, A. N., Butler, R., Jones, T., Laval, S., Boyle, J. M., and Ganesan, T. S. (1995). Localization of an epithelial-specific receptor kinase (EDDR1) to chromosome 6q16, Genomics *25*, 584-7.

Shrivastava, A., Radziejewski, C., Campbell, E., Kovac, L., McGlynn, M., Ryan, T. E., Davis, S., Goldfarb, M. P., Glass, D. J., Lemke, G., and Yancopoulos, G. D. (1997). An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors, Molecular Cell *1*, 25-34.

Sorisky, A., Gagnon, A., and Abaiian, K. (2002). Aortic carboxypeptidase-like protein (ACLP): what is a protein with a name like that doing in adipose tissue?
Molecular links between obesity and cardiovascular disease, Hormone & Metabolic Research *34*, 764-6.

Stahl, A., Evans, J. G., Pattel, S., Hirsch, D., and Lodish, H. F. (2002). Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes, Developmental Cell *2*, 477-88.

Standaert, M. L., Bandyopadhyay, M., Sajan, L., Cong, M., Quon, M., and Farese, R. (1999). Okadaic acid activates atypical protein kinase C (zeta/lambda) in rat and 3T3-L1 adipocytes. An apparent requirement for activation of Glut4 translocation and glucose transport, Journal of Biological Chemistry *274*, 14074-14078.

Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior, Annual Review of Cell and Developmental Biology *17*, 463-516.

Swartz, M. A., Tschumperlin, D. J., Kamm, R. D., and Drazen, J. M. (2001). Mechanical stress is communicated between different cell types to elicit matrix remodeling, Proceedings of the National Academy of Sciences of the United States of America *98*, 6180-5.

Takahashi, M., Kamei, Y., and Ezaki, O. (2005). Mest/Peg1 imprinted gene enlarges adipocytes and is a marker of adipocyte size, American Journal of Physiology - Endocrinology & Metabolism *288*, E117-24.

Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003). Mitotic clonal expansion: a synchronous process required for adipogenesis, Proceedings of the National Academy of Sciences of the United States of America *100*, 44-9.

Tanti, J., Gremeaux, T., Obberghen, V., and Le Marchand-Brustel, Y. (1994). Serine/threonine p hophorylation o f i nsulin r eceptor s ubstrate 1 m odulates i nsulin r eceptor signaling, The Journal of Biological Chemistry *269*, 6051-6957.

Ugi, S., Imamura, T., Maegawa, H., Egawa, K., Yoshizaki, T., Shi, K., Obata, T., Ebina, Y., Kashiwagi, A., Olefsky, J. M., and Wang, Y. (2004). Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes, Molecular & Cellular Biology *24*, 8778-89.

Ugi, S., UImamura, T., Ricketts, W., and Olefsky, J. M. (2002). Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling, Molecular & Cellular Biology *22*, 2375-2387.

Urs, S., Smith, C., Campbell, B., Saxton, A. M., Taylor, J., Zhang, B., S noddy, J., Jones Voy, B., and Moustaid-Moussa, N. (2004). Gene expression profiling in human preadipocytes and adipocytes by microarray analysis, Journal of Nutrition *134*, 762-70.

Van Hoof, C., and Goris, J. (2004). PP2A fulfills its promises as a tumor suppressor: Which subunits are important?, Cancer Cell *5*, 105-106.

Vogel, W. (1999). Discoidin domain receptors: structural relations and functional implications, FASEB Journal *13 Suppl*, S77-82.

Vogel, W., Gish, G. D., Alves, F., and Pawson, T. (1997). The discoidin domain receptor tyrosine kinases are activated by collagen, Molecular Cell *1*, 13-23.

Vogel, W. F. (2001). Collagen-receptor signaling in health and disease, European Journal of Dermatology *11*, 506-14.

Vozarova, B., Weyer, C., Hanson, K., Tataranni, P., Bogardus, C., and Pratley, R. (2001). Circulating interleukin-6 in relation of adiposity, insulin action, and insulin resistance, Obesity Research *9*, 414-447.

Vu, T. H. (2001). Don't mess with the matrix., Nature Genetics *28*, 202-203.

Wang, C. Z., Hsu, Y. M., Tang, M. J., and Leitinger, B. (2005). Function of discoidin domain receptor I in HGF-induced branching tubulogenesis of MDCK cells in collagen gel The D2 period of collagen II contains a specific binding site for the human discoidin domain receptor, DDR2.[erratum appears in J Mol Biol. 2005 Feb 18;346(2):661], Journal of Cellular Physiology *203*, 295-304.

Wang, J., Lu, H., Liu, X., Deng, Y., Sun, T., Li, F., Ji, S., Nie, X., and Yao, L. (2002). Functional analysis of discoidin domain receptor 2 in synovial fibroblasts in rheumatoid arthritis, Journal of Autoimmunity *19*, 161-8.

Wang, W., Yang, Y., Meng, Y., and Shi, Y. (2004). GDF-3 is an adipogenic cytokine under high fat dietary condition, Biochemical & Biophysical Research Communications *321*, 1024-31.

Weiner, F. R., Shah, A., Smith, P. J., Rubin, C. S., and Zern, M. A. (1989). Regulation of collagen gene expression in 3T3-L1 cells. Effects of adipocyte differentiation and tumor necrosis factor alpha, Biochemistry *28*, 4094-9.

Werb, Z. (1997). ECM and cell surface proteolysis: regulating cellular ecology, Cell *91*, 439-42.

Weyer, C., Foley, J. E., and Bogardus, C. (2000). Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance, Diabetologia *43*, 1498-1506.

Withers, D. J., and White, M. (2000). Perspective: The insulin signaling system - a common link in the pathogenesis of type 2 diabetes, Endocrinology *141*, 1917-1921.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., *et al.* (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects.[erratum appears in Nature. 2004 Oct 28;431(7012):1123], Nature *423*, 762-9.

Yamauchi, T., Tobe, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto-Honda, R., Takahashi, Y., Yoshizawa, F., Aizawa, S., Akanuma, Y., *et al.* (1996). Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice, Molecular & Cellular Biology *16*, 3074-84.

Yanaihara, A., Otsuka, Y., Iwasaki, S., Aida, T., Tachikawa, T., Irie, T., Okai, T., and Dejmek, J. (2005). Differences in gene expression in the proliferative human endometrium Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival, Fertility & Sterility *83 Suppl 1*, 1206-15.

Yang, X., Jansson, P. A., Nagaev, I., Jack, M. M., Carvalho, E., Sunnerhagen, K. S., Cam, M. C., Cushman, S. W., and Smith, U. (2004). Evidence of impaired adipogenesis in insulin resistance, Biochemical & Biophysical Research Communications *317*, 1045-51.

Yeh, W. C., Li, T. K., Bierer, B. E., and McKnight, S. L. (1995). Identification and characterization of an immunophilin expressed during the clonal expansion phase of adipocyte differentiation, Proceedings of the National Academy of Sciences of the United States of America *92*, 11081-5.

Yoshimura, T., Matsuyama, W., and Kamohara, H. (2005). Discoidin domain receptor 1: a new class of receptor regulating leukocyte-collagen interaction, Immunologic Research *31*, 219-229.

Zdychova, J., and Komers, R. (2005). Emerging role of the Akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications, Physiology Research *54*, 1-16.

Zerlin, M., Julius, M. A., and Goldfarb, M. (1993). NEP: a novel receptor-like tyrosine kinase expressed in proliferating neuroepithelia, Oncogene *8*, 2731-9.

Zhang, J. W., Tang, Q. Q., Vinson, C., and Lane, M. D. (2004). Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes, Proceedings of the National Academy of Sciences of the United States of America *101*, 43-7.

CURRICULUM VITAE

HONORATA ZURAKOWSKI

Master of Science Candidate – Biochemistry

6256 Ravine Way, Ottawa, Ontario, K1C 7G2, CANADA
Phone: (613) 824-1765
Email: honnie@gmail.com

EDUCATION:

09/2003–present	**University of Ottawa** Ottawa, Ontario **Supervisor:** Dr. Alexander Sorisky	**M.Sc. in Biochemistry** . **will defend *12/2005*** . **Title:** *Expression of discoidin domain receptors in 3T3-L1 preadipocytes and adipocytes*
09/1999-04/2003	**Queen's University** Kingston, Ontario **Supervisor:** Dr. Richard Oko	**B.Sc. in Life Sciences (Honours)** . **Title:** *Localization and expression of somatic histones in mature rat sperm perinuclear theca*

ACADEMICALLY RELATED WORK EXPERIENCE:

05/2002-09/2002	**Carleton University** Ottawa, Ontario **Supervisor:** Dr. David Miller	**Research Assistant** . laboratory work involving the extraction of a mycotoxin from corn and preparation for HPLC

AWARDS:

09/2004-09/2005 . Department of Biochemistry Stipend Scholarship (6000$)

09/2002-04/2003 . Queen's University Dean's Award

PUBLICATIONS:

Zurakowski, H, Gagnon, A, Layne, M and A Sorisky. (2005) Expression of discoidin domain receptors in 3T3-L1 preadipocyte differentiation (*Manuscript in preparation*)

MEMBERSHIP IN PROFESSIONAL ASSOCIATIONS:

Endocrine Society

CONFERENCE PRESENTATIONS:

Zurakowski, H, Gagnon, A, Layne, M and A Sorisky. (2005) *Discoidin domain receptor 2 expression during the differentiation of 3T3-L1 preadipocytes to adipocytes*. The Endocrine Society's Annual Meeting (June 4-7, 2005; San Diego, California, USA)

Zurakowski, H, Gagnon, A and A Sorisky. (2004) *Expression of discoidin domain receptors during the differentiation of 3T3-L1 preadipocytes to adipocytes*. Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism (CDA/CSEM) Professional Conference and Annual Meetings (October 27-30, 2004; Quebec City, Quebec, Canada)

CONFERENCES ATTENDED:

CDA/CSEM Professional Conference and Annual Meetings (October 15-18, 2003; Ottawa, Ontario, Canada)

OTHER WORK EXPERIENCE:

05/2003-08/2003	**Poplar Grove Golf Club** Ottawa, Ontario	Client Services Representative
05/2001-09/2001	**Meadows Golf Club** Ottawa, Ontario	Client Services Representative
05/2000-09/2000	**Region of Ottawa** Ottawa, Ontario	Assistant Waste Management Inspector
12/1995-01/2000	**Mont Cascades** Ottawa, Ontario	Ski Instructor
07/1997-09/1998	**Ottawa Athletic Club** Ottawa, Ontario	Sports Instructor

LANGUAGES:

Fluent in English (written, read, spoken)
Competent in French (some spoken, written and read)
Competent in Polish (spoken, some read)