CD24 is required for gene expression, but not glucose uptake, during adipogenesis *in vitro*

by

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Abstract

CD24 is a cell surface receptor that actively regulates lipid accumulation in adipocytes *in vitro.* The goal of this project was to determine how CD24 regulates lipid accumulation. Glucose is a key substrate for *de novo* lipid synthesis but I found that the knockdown of CD24 did not alter glucose uptake during adipogenesis. Instead, it regulated the expression of 134 genes as determined by DNA microarray analysis. I validated the changes to four of these genes during adipogenesis in 3T3-L1 pre-adipocytes *in vitro*. I also found that these genes were dysregulated when primary cells from inguinal, but not epididymal white adipose tissue from CD24 knockout mice that were induced to undergo adipogenesis *ex vivo.* These data suggest that CD24 is necessary for select gene expression, but not glucose uptake, during adipogenesis. This new knowledge could help understand the regulation of lipid accumulation in adipocytes, and to develop alternative treatment strategies for obesity and lipodystophy.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-deoxyglucose
ACC	Acetyl-CoA carboxylase
ADSC	Adipose-derived stem cells
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CD24KO	CD24 knock-out
CEBP	CCAAT enhancer binding proteins
ChREBP	Carbohydrate-responsive element binding protein
CoA	Coenzyme A

CREB	cAMP-responsive element-binding protein
Dex	Dexamethasone
DGAT	Diacylglycerol-O-acyltransferase
DHAP	Dihydroxyacetone phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPAC -1 -2	Exchange protein directly activated by cAMP 1 and 2
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acids
FOXO1	Forkhead transcription protein
GADP	Glycerol-3-phosphate
GATA	Globin transcription factors 2 and 3
GLUT	Glucose transporter

GPAT	Glycerol-3-phosphate O-acyltransferase
GPI	Glycosylphosphatidylinositol
GTT	Glucose tolerance test
HFD	High fat diet
HMGB1	High mobility group box 1
HSA	Heat stable antigen
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
KD	Knockdown
KLF	Kruppel-like factors
L1CAM	L1 Cell Adhesion Molecule
Lin	Lineage marker
LPA	Lysophosphatidic acid
NBCS	New-born calf serum
NIH	National Institute of Health

PA	Phosphatidic acid
PAP	Phosphatidic acid phosphohydrolase
PBS	Phosphate-buffered saline
PDC	Pyruvate dehydrogenase complex
Pen-Strap	Penicillin-streptomycin
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PLIN1	Perilipin 1
Pparγ	Peroxisome proliferator-activated receptor γ
Pref-1	Pre-adipocyte factor-1
qRT-PCR	Quantitative real-time polymerase chain reaction
RQ	Relative quantity
Sca-1	Stem cells antigen 1
Siglec	Sialic acid-binding immunoglobin-like lectin
siRNA	Small interfering RNA

SLC2A	Solute carrier family 2A
SREBP1c	Sterol regulatory element binding protein
SVF	Stromal vascular fraction
sWAT	Subcutaneous white adipose tissue
TAG	Triacylglycerol
TNF-α	Tumor necrosis factor α
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WT	Wild type

1.0 Literature Review

1.1 Obesity and Lipodystrophy

Obesity is defined as a Body Mass Index (BMI) of 30 or above (Berrington de Gonzalez et al. 2010) and involves the excess accumulation of white adipose tissue (WAT) in the body. It is caused by the excess intake of food, as well as environmental and genetic factors (Chaput, Doucet, Tremblay 2012). Obesity has been shown to be one of the major risk factors for coronary heart diseases, ischemic heart diseases, ventricular dysfunction, stroke, type 2 diabetes and certain types of cancer (Rabkin, Mathewson, Hsu 1977; Lew 1985; Chan et al. 1994; Duflou et al. 1995; Jensen et al. 2014; Bhaskaran et al. 2014). The extreme opposite of obesity is lipodystrophy, which involves the wasting of WAT. Similar to obesity, lipodystrophy is also caused by a variety of genetic and external factors and can also lead to health consequences such as diabetes and insulin resistance (Bindlish, Presswala, Schwartz 2015). Taken together, obesity and lipodystrophy affect over 600 million people worldwide and represent a global economic burden of over 2 trillion US dollars, or 2.8%, of the global gross domestic product (Dobbs et al. 2014; World Health Organization 2015). Over 50% of adult Canadians are either obese or overweight and the treatment costs alone account for about $7.1 billion annually (Twells et al. 2014). Even with drastic interventions such as gastric bypass surgery, long-term weight loss is extremely difficult to achieve and maintain (Ebbert, Elrashidi, Jensen 2014). On the other hand, there are no specific or effective therapeutics to combat lipodystrophy. Therefore, it is important to understand the development of

WAT and fully understand the biology of these diseases to develop effective therapeutics that target at-risk populations.

1.2 Adipose tissue

Adipose tissue is found throughout the body in heterogeneous depots and functions by storing energy in the form of lipids. Apart from its role in storing energy, the adipose tissue also has endocrine and metabolic roles. The adipose tissue is primarily composed of mature adipocytes and the stromal vascular fraction (SVF). The SVF in turn consists of pre-adipocytes, fibroblasts, vascular cells, endothelial cells and immune cells. In the body, the adipose tissue is classified into three main types: brown adipose tissue (BAT) (Cannon and Nedergaard 2008), beige adipose tissue (Wu et al. 2012) and WAT (Adebonojo 1975). All the three adipose tissues are derived from distinct cellular lineages, except the white adipose tissue can become beige adipose tissue under certain conditions, and perform different functions (Cousin et al. 1992). Due to their differences in function, these adipose tissues are targets for studying obesity and obesity related diseases.

1.2.1 White adipose tissue (WAT)

WAT is an energy storing tissue that stores excess energy in the form of triacylglycerols (TAGs) and cholesterol esters. Apart from this role, WAT also has endocrine functions because it secretes important appetite regulating hormones such as leptin and adiponectin (Friedman and Halaas 1998; Meier and Gressner 2004). In addition to regulating appetite, these hormones also act as regulators of glucose uptake

(Minokoshi, Toda, Okamoto 2012; Coelho, Oliveira, Fernandes 2013). WAT also secretes cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Coelho, Oliveira, Fernandes 2013). Therefore, WAT plays an important role in regulating food intake, metabolism and long-term energy storage.

WAT consists primarily of adipocytes in the form of mature white adipocytes that are unilocular and consist of a large lipid droplet surrounded by a thin cytoplasm with a nucleus located on the periphery. Apart from the mature adipocytes, WAT also consists of the SVF (Cinti 2005; Ouchi et al. 2011). The SVF in turn consists of a number of cell types including pre-adipocytes, macrophages/monocytes (Bastard et al. 2006), adipose derived stem cells (ADSCs), fibroblasts, progenitor endothelial cells and T regulatory cells (Riordan et al. 2009). Although WAT contains numerous cell types, only cells from the perivascular region of the WAT are committed to adipocyte lineage (Tang et al. 2008). ADSCs isolated from WAT can also be induced to differentiate into mature adipocytes (Spalding et al. 2008; Cawthorn, Scheller, MacDougald 2012). ADSCs are important targets for stem cell research as they can also be induced to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic lineages (Dicker et al. 2005; Zuk 2010; Cawthorn, Scheller, MacDougald 2012).

WAT can grow in two ways: hypertrophy and hyperplasia (Jo et al. 2009). Hypertrophy is the increase in cell size that occurs due to the accumulation of lipid droplets in white adipocytes (Villena et al. 2004; Jo et al. 2009). Hyperplasia is the increase in cell number that occurs due to cell division (Adebonojo 1975). The primary

mechanism for the increase in WAT size in childhood and adolescence includes hyperplasia and hypertrophy (Adebonojo 1975), whereas the increase in WAT size in adulthood is due almost exclusively to hypertrophy (Tchoukalova et al. 2010). Both hypertrophy and hyperplasia are influenced by excessive caloric intake. In hypertrophy, the adipocytes accumulate lipids in the form of TAGs, which leads to an increase in their overall cell sizes (Villena et al. 2004). Lipid accumulation in adipocytes can be either by *de novo* lipid synthesis (lipogenesis) or by accumulating pre-synthesized lipids from their surroundings (Lane and Tang 2005; Arner et al. 2010). Lipogenesis takes place in response to the action of insulin on WAT that leads to the synthesis and storage of TAGs within the adipocytes (Kersten 2001). Lipolysis, which is the process by which fatty acids are oxidized to release free fatty acids (FFA), is also an important function of WAT (Large et al. 2004). Adipocytes that contain more TAGs are associated with insulin resistance, whereas smaller adipocytes are more responsive to insulin (Kahn and Flier 2000; Geer and Shen 2009). Therefore, the insulin-sensitivity of WAT and its ability to store TAG is influenced by hypertrophy.

WAT is dispersed throughout the body in distinct depots. The two main types of WAT are the subcutaneous WAT (sWAT) depots that are present below the skin and the visceral WAT (vWAT) depots that surround the internal organs (viscera) within the abdomen (Cinti 2005). The sWAT and the vWAT depots show numerous differences including differences in metabolic characteristics (Tran et al. 2008; Karastergiou and Fried 2013; Cohen et al. 2014), gene expression patterns (Grove et al. 2010; Karastergiou et al. 2013; Cohen et al. 2014), adipokine secretion profiles (Shi, Seeley, Clegg 2009) and

in their developmental origins (Chau et al. 2014; Krueger et al. 2014; Sanchez-Gurmaches and Guertin 2014).

In humans, the development of subcutaneous adipocytes occurs during the second trimester with the developed sWAT being present at birth (Poissonnet, Burdi, Bookstein 1983; Poissonnet, Burdi, Garn 1984). vWAT on the other hand, develops at an uneven pace with some vWAT depots developing after birth. In mice, the development of the inguinal sWAT occurs concomitantly with birth and becomes distinguishable one-day post birth (Birsoy et al. 2011), whereas, the development of vWAT in mice is a slow process and happens after fully functional sWAT is developed (Han et al. 2011). Different depots possess different degrees of differentiation potential (Berry et al. 2013) and different compositions of pre-and mature adipocytes. For example, the subcutaneous inguinal WAT contains a greater number of pre-adipocytes and ADSCs than the epididymal visceral WAT (Joe et al. 2009).

The accumulation of sWAT and vWAT depots affect the pathogenesis of obesity differently. In male humans, high accumulation of vWAT and deep abdominal sWAT have been shown to increase the risk of diabetes, cardiovascular disease and mortality (Carey et al. 1997; Goodpaster et al. 1997; Kelley et al. 2000; Wang et al. 2005; Nicklas et al. 2006). In females, however, the accumulation of gluteofemoral sWAT is associated with insulin sensitivity and decreased risk of diabetes and cardiovascular disease (Misra et al. 1997; Snijder et al. 2003; Tanko et al. 2003; Manolopoulos, Karpe, Frayn 2010). Interestingly, males tend to accumulate vWAT more than sWAT, whereas premenopausal

women tend to accumulate more sWAT than vWAT (Palmer and Clegg 2015). This distribution of WAT depots between the sexes tend to result in the female "pear shaped" and the male "apple shaped" obesity (Gesta, Tseng, Kahn 2007; Karastergiou et al. 2012). After menopause, however, the WAT distribution in females tend to resemble a male-like pattern, showing the role of the sex hormone estrogen in the distribution of sWAT and vWAT depots (Palmer and Clegg 2015). In mice and other rodents, inguinal sWAT has been associated with improved metabolic parameters (Tran et al. 2008) while the perigonadal vWAT is associated with decreased insulin sensitivity (Foster et al. 2010). High fat diet (HFD) studies in mice have been shown to promote adipogenesis differently in different depots between the sexes. HFD feeding in male mice has been shown to induce adipogenesis in vWAT but not in sWAT, while in female mice HFD was shown to induce adipogenesis in both vWAT and sWAT in an estrogen dependent manner (Jeffery et al. 2016).

1.2.2 Brown adipose tissue (BAT)

BAT is an adipose tissue that is made up of multilocular brown adipocytes. BAT is predominantly found in newborns and slowly disappears during adulthood (Cannon and Nedergaard 2004). Developmentally, BAT formation occurs during embryonic differentiation even before the other adipose tissues are formed (Houstek et al. 1993). BAT is located in discreet depots although the size and number of BAT depots are lower than the WAT. In mice, the majority of BAT is located in the intrascapular space, which consists of the intrascapular fatpads, the auxillary fatpads and the cervical fatpads

(Sheldon 1924). BAT mainly functions to produce heat in newborn mammals through non-shivering thermogenesis (Cannon and Nedergaard 2004). BAT also contains large numbers of mitochondria compared to the other adipose tissues, and high levels of uncoupling protein-1 (UCP-1) (Ricquier and Bouillaud 2000). Although previously thought to be absent in adult humans, recent studies have shown the presence of UCP-1 expressing BAT in human brown adipocytes (Frontini and Cinti 2010). UCP-1 mediated burning of triglycerides causes the generation of heat with very little usage of ATP. The heat generated by this process is circulated throughout the body through the blood stream.

1.2.3 Beige adipose tissue

"Browning" or "beiging" of white adipose tissue is the process of formation of beige adipose tissue from WAT. This process occurs when the sympathetic tone of WAT increases due to external stimuli such as the exposure to cold temperatures or β adrenergic response (Cousin et al. 1992). Unlike WAT and BAT, the beige adipose tissue is not found in discreet depots, but are rather found within the WAT depots, especially in the subcutaneous inguinal WAT depot (Lee et al. 2012). Although little is known about the origins of the beige adipocytes, they do have many similarities with BAT. Similar to BAT, they are multilocular and contain large numbers of mitochondria. Although they express some BAT genes such as *Ucp1*, PPARG Coactivator 1 Beta (*Pgc1β*) and Cell Death-Inducing DFFA-Like Effector A (*Cidea*), the beige progenitors have distinct developmental origins to that of BAT (Sharp et al. 2012). Beige and BAT have important functions in regulating thermogenesis as well as in regulating metabolism in animals.

Despite being present within the WAT; beige adipose precursors are different from WAT precursors (Wu, Cohen, Spiegelman 2013).

1.3 Regulation of Adipogenesis

1.3.1 Adipocyte Development

1.3.1.1 The various stages of adipocyte differentiation

One of the most commonly used *in vitro* models to study adipogenesis is the mouse 3T3-L1 pre-adipocyte cell line (Williams and Polakis 1977). 3T3-L1 pre-adipocytes undergo adipogenesis to differentiate into mature adipocytes through four main stages: growth arrest, clonal expansion, initial differentiation and terminal differentiation. A schematic diagram that represents *in vitro* adipogenesis is shown in Figure 1.1. Different genes are expressed during the different stages to mediate adipogenesis (MacDougald and Lane 1995). The first stage of adipogenesis, the growth arrest stage, occurs due to contact-dependent growth inhibition of pre-adipocytes. The cells must undergo growth arrest at the G0/G1 phase of the cell cycle to differentiate (Reichert and Eick 1999), because they become more responsive to differentiation signals than if they are freely proliferating (Pairault and Green 1979). After undergoing growth arrest, the cells undergo the clonal expansion stage where the cells re-enter the cell cycle. In this stage, the cells must undergo at least two rounds of cell division after receiving a combination of mitogenic and adipogenic stimuli. 3T3-L1 pre-adipocytes have been shown to undergo 2-3 rounds of mitotic clonal expansion during the first 48 hours

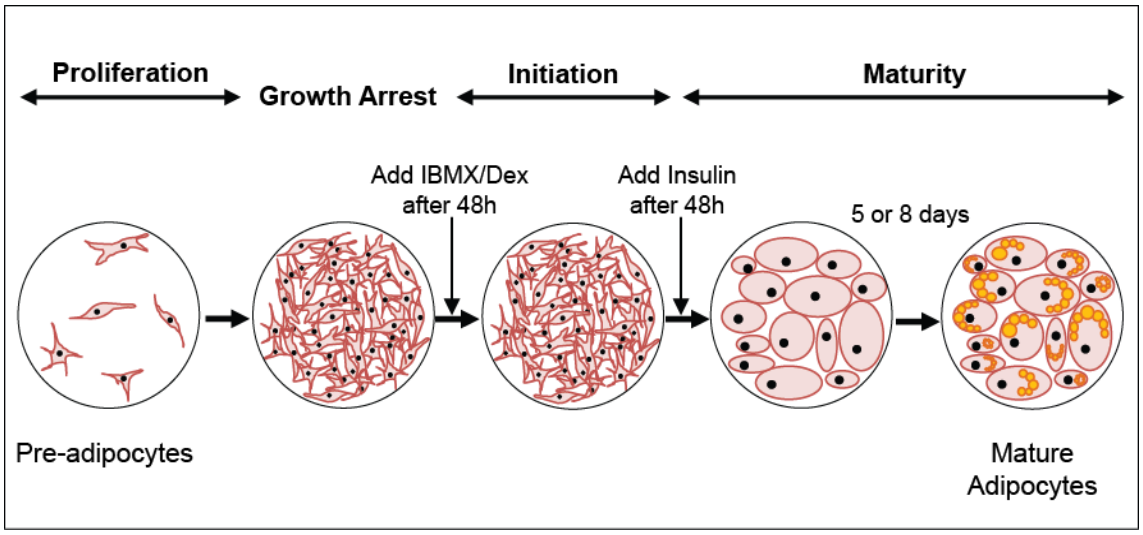

Figure 1.1: Schematic diagram of the *in vitro* adipogenesis assay. The fibroblastic pre-adipocytes are allowed to proliferate until they reach contact inhibition-mediated growth arrest. The cells are then treated with Isobutyl-1-methylxanthine (IBMX) and Dexamethasone (Dex), 48 h post growth arrest, to initiate differentiation. The cells are treated with Insulin after 48 h. Mature lipid-laden adipocytes are formed 5 days post insulin treatment, for 3T3-L1 pre-adipocytes, or 8 days post insulin treatment, for the stromal vascular fraction (SVF).

following the addition of the adipogenic cocktail (Bernlohr et al. 1985; Tang, Otto, Lane 2003). Blocking this mitotic clonal expansion with compounds like aphidicolin (Reichert and Eick 1999), rapamycin (Yeh, Bierer, McKnight 1995) or roscovitine (Tang, Otto, Lane 2003) blocks adipocyte differentiation. Aphidicolin, which is an inhibitor of cellular DNA synthesis, blocks cell cycle at the early S phase. 3T3-L1 pre-adipocytes treated with aphidnicolin have almost completely lost the ability to differentiate without any effect on cell viability (Reichert and Eick 1999). Disruption of one of the master regulators of adipogenesis, CCAAT enhancer binding protein α (*C/EBPα*), also prevents adipogenesis by blocking the mitotic clonal expansion. Once the cells undergo clonal expansion, they will exit the cell cycle and begin to differentiate. The clonal expansion stage is followed by the initial differentiation stage that involves the activation of the master regulators of adipogenesis, *C/EBPα* and peroxisome proliferator-activated receptor γ (*Pparγ*), that play important roles in further activating the downstream genes for adipogenesis (Lin and Lane 1992; Lin and Lane 1994; Rosen et al. 1999; Rosen and MacDougald 2006). After this stage, the cells undergo the terminal differentiation stage, where there is an increase in *de novo* lipogenesis followed by an increase in mRNA expression, protein expression and enzymatic activities that are required for lipogenesis. There is also increase in activities of enzymes such as acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyltransferase (GPAT), glycerol-3-phosphate dehydrogenase (GPDH), fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase (GADPH), all of which are important for lipogenesis (Spiegelman and Farmer 1982; Weiner et al. 1991). This is followed by an increase in glucose transporters,

insulin receptor, and insulin sensitivity during the later stages of adipogenic differentiation (Garcia de Herreros and Birnbaum 1989).

1.3.1.2 Morphological changes in adipocytes during adipogenesis

As the pre-adipocytes progress through the various stages of adipogenesis to become mature adipocytes, they also undergo changes in their morphology from a fibroblastic appearance to a spherical appearance. During this process, there is a decrease in the levels of actin and tubulin compounds (Spiegelman and Farmer 1982) as well as a decrease in type I and type III procollagen (Weiner et al. 1991). This is also accompanied by changes in their extracellular matrix (ECM) and cytoskeletal compounds. In pre-adipocytes, the proteolytic degradation of ECM by the plasminogen cascade is essential for the change in pre-adipocyte morphology, adipocyte gene expression and lipid droplet accumulation (Selvarajan et al. 2001).

1.3.2 Intracellular signaling during adipogenesis

1.3.2.1 Signal induction by the adipogenic cocktail

Adipogenesis is induced *in vitro* when the cells are treated with the adipogenic cocktail that consists of a mixture of 3-isobutyl-1-methylxanthine (IBMX), Dexamethasone (Dex) and insulin. This adipogenic cocktail increases the intracellular levels of cyclic adenosine monophosphate (cAMP), activates the glucocorticoid receptor and the insulin/Insulin-like growth factor-1 (IGF-1) signaling pathways. Although many researchers add the three components of the adipogenic cocktail together to induce

adipogenesis, some prefer to add IBMX and Dex together and then insulin (Smith et al. 2015; Hogan et al. 2016). IBMX is a cyclic nucleotide phosphodiesterase inhibitor that stimulates adenylyl cyclase activity, resulting in increased intracellular levels of the secondary messenger, cAMP (Parsons, Ramkumar, Stiles 1988). High levels of cAMP activate protein kinase A (PKA) (Taylor, Buechler, Yonemoto 1990) and an exchange protein directly activated by cAMP 1 and 2 (EPAC-1/-2) (de Rooij et al. 1998). Activated PKA promotes adipogenesis by activating the expression of the downstream transcription factor, cAMP-responsive element-binding protein (CREB) (Reusch, Colton, Klemm 2000). Activated CREB then activates the expression of *C/EBPβ*. EPAC -1/-2, which is activated by the high levels of cAMP, has two main functions; it can either promote adipogenesis by activating the Ras superfamily protein, Rap 1, or promote the IGF-1 dependent activation of protein kinase B (PKB) (Petersen et al. 2008). The activities of both PKA and EPAC -1, -2 together are essential for the cAMP mediated differentiation of pre-adipocytes (Petersen et al. 2008). Dex is a synthetic glucocorticoid that activates the glucocorticoid receptor by binding to the glucocorticoid receptor binding elements which then stimulates the transcription of *C/EBPβ* and *C/EBPδ* (Cao, Umek, McKnight 1991). This increases the expression of *C/EBPα* and *Pparγ* to promote adipogenesis (Wu, Bucher, Farmer 1996). Forty-eight hours post IBMX and Dex treatment, insulin is added to the cells to initiate a series of complex pathways. Insulin activates the insulin like growth factor-1 (IGF-1) pathway which leads to the activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The insulin mediated activation of the PI3K pathway can induce adipogenesis by activating various downstream events such as the protein kinase

B (PKB) (Magun et al. 1996). The inhibition of PI3K activity blocks overall adipogenesis. Activation of the PI3K pathway also induces the activity of the pro-adipogenic transcription factor CREB. Activated CREB then induces the activity of *C/EBPβ*, which then results in adipogenesis. The activation of the PI3K pathway also facilitates glucose uptake and *de novo* fatty acid synthesis (Satoh et al. 1993).

1.3.3 Transcriptional regulation of adipogenic genes during adipogenesis

1.3.3.1 The master regulators of adipogenesis

The two master regulators of adipogenesis that are involved in converting a pre-adipocyte to a mature adipocyte are *Pparγ* and *C/EBPα* (Schwarz et al. 1997; Rosen et al. 2002; Farmer 2006). Both *C/EBPα* and *Pparγ* are activated by *C/EBPβ* and *C/EBPδ,* that are expressed after IBMX/Dex treatment (Wu et al. 1995). Once activated, the master regulators regulate the expression of each other to promote adipogenesis (Schwarz et al. 1997; Shao and Lazar 1997). The overexpression of *Pparγ* in pre-adipocytes leads to growth arrest (Tontonoz et al. 1995) and adipogenesis (Sandouk, Reda, Hofmann 1993), whereas the *in vivo* knockout of *Pparγ* in mature adipocytes causes the death of adipose tissue (Rosen and Spiegelman 2006). Adipogenesis is also inhibited when 3T3-L1 pre-adipocytes are treated with a synthetic *Pparγ* antagonist that leads to the decreased expression of *C/EBPα* (Zuo, Qiang, Farmer 2006). In chimeric mice that have both wild type and *Pparγ* knockout embryonic stem cells, strong preference was shown towards the *Pparγ* expression (Rosen et al. 1999). On the other hand, overexpression of *C/EBPα* in 3T3-L1 pre-adipocytes has been shown to induce differentiation (Lin and Lane 1994),

whereas blocking its expression with siRNA inhibits adipogenesis (Lin and Lane 1992). Mice with a knockout of the *C/EBPα* gene were found to have lower WAT formation compared to control mice (Wang et al. 1995). The homozygous deletion of *C/EBPα* in primary pre-adipocytes has been shown to prevent *Pparγ* expression and adipogenesis, but when *C/EBPα* was re-expressed using plasmid transfection, *Pparγ* expression and adipogenesis was restored (Wu et al. 1999). This indicates that *C/EBPα* is important for adipogenesis and demonstrates that *C/EBPα* is upstream of *Pparγ*. Taken together all these results indicate that the expression of both the master regulators of adipogenesis is necessary and sufficient to promote adipogenesis.

1.3.3.2 The anti-adipogenic regulators of adipogenesis

Pre-adipocyte factor-1(*Pref-1)* is a marker of pre-adipocytes and an inhibitor of adipogenesis (Smas and Sul 1993). *Pref-1* inhibits adipogenesis by downregulating the expression of *C/EBPα* and *Pparγ2* (an isoform of *Pparγ*) (Kim et al. 2007). In addition to *Pref-1*, other anti-adipogenic regulators of adipogenesis include the kruppel like factor (KLF) family members KLF2 and KLF3. KLF2 and KLF3 function by inhibiting the expression of *Pparγ* and *C/EBPα* (Sue et al. 2008). The constitutive expression of KLF2 leads to the suppression of *Pparγ*, but has no effect on *C/EBPβ* and *C/EBPδ* expression (Banerjee et al. 2003) whereas overexpression of KLF3 blocks adipogenesis by suppressing the activity of *C/EBPα* (Sue et al. 2008). In addition, transcription factors such as the forkhead transcription protein FOXO1 (FOXO1) (Nakae et al. 2003) and globin transcription factors 2 and 3 (GATA2 and GATA3) (Jack and Crossley 2010) also

inhibit adipogenesis. The WNT/β-catenin pathway can also inhibit adipogenesis by blocking the expression of *Pparγ* and *C/EBPα* (Christodoulides et al. 2009).

1.3.3.3 The pro-adipogenic regulators of adipogenesis

There are several pro-adipogenic regulators that directly increase the expression of the master regulators of adipogenesis or decrease the expression of the anti-adipogenic regulators to induce adipogenesis. Some members of the KLF family such as the KLF4, KLF5, KLF6 and KLF15 are pro-adipogenic and work with the members of the C/EBP family to promote adipogenesis. KLF4 and Krox 20 function together to promote adipogenesis by directly binding to the *C/EBPβ* promoter region to increase the expression of *C/EBPβ* (Chen et al. 2005; Birsoy, Chen, Friedman 2008) . KLF5, expressed earlier during adipogenesis, is bound by *C/EBPβ* and *C/EBPδ* and together they increase the expression of *Pparγ* (Oishi et al. 2005). KLF6 increases adipogenesis by inhibiting the expression of *Pref-1* (Li et al. 2005). KLF15, which is also expressed earlier during adipogenesis, is induced by *C/EBPβ* and *C/EBPδ* and together they work synergistically with *C/EBPα* to increase *Pparγ* expression (Mori et al. 2005). In addition to these regulators, specific transcription factors can also act as pro-adipogenic regulators of adipogenesis. The early B cell factor-1 (EBF1) along with the sterol regulatory element binding transcription factor 1 (SREBP1c) promote the differentiation of pre-adipocytes (Kim et al. 1998; Jimenez et al. 2007). The expression of SREBP1c increases when insulin is added to pre-adipocytes and functions by suppressing the expression of the anti-adipogenic regulator FOXO1 through PKB (Kim et al. 1998; Nakae et al. 2003).

Taken together, these results indicate that the simultaneous decrease of anti-adipogenic genes and an increase in pro-adipogenic genes are necessary for adipogenesis to occur.

1.4 *In vitro* and *in vivo* models of adipogenesis

Adipogenesis is studied using both *in vitro* and *in vivo* models. The various stages of adipocyte differentiation and transcriptional regulation are commonly studied using *in vitro* cell culture systems, whereas *in vivo* models are used to study the etiology of obesity to develop effective therapeutics.

1.4.1 *In vitro* models of adipogenesis used in this thesis

1.4.1.1 3T3-L1 pre-adipocyte cell line

Mouse 3T3-L1 cell lines are pre-adipocyte cell lines that are isolated from Swiss 3T3 cells that are derived from 17-19-day old mouse embryos (Green and Kehinde 1975). 3T3-L1 pre-adipocytes are a well-established mouse cell line that are developed by clonal expansion. Therefore, a single cell type can homogenously respond to the differentiation cocktail mixture to differentiate. When treated with the adipogenic cocktail, 3T3-L1 pre-adipocytes undergo the four main stages of adipogenesis, growth arrest, clonal expansion, initial differentiation and terminal differentiation to differentiate into mature adipocytes. 3T3-L1 pre-adipocytes are a widely used *in vitro* model to study adipogenesis, as a result most of the available information regarding the transcriptional regulation of adipogenesis is due to work done in this cell line. 3T3-L1 pre-adipocytes are at an earlier stage of commitment to adipogenesis than other cell lines, such as the 3T3-F442A (Sarjeant and

Stephens 2012) and allow for the consistent production of adipocytes, although the differentiation ability of these adipocytes decreases with passage numbers above 15. 3T3-L1 cell lines have also been shown to develop into fully mature adipocytes *in vivo* (Rosen and Spiegelman 2006). When athymic mice, which have reduced immune system function due to the absence of thymus, were implanted with 3T3-L1 pre-adipocytes, they developed fat pads that are histologically and biochemically identical to that of their host (Green and Kehinde 1979). Due to these abilities, 3T3-L1 pre-adipocytes are widely used as *in vitro* models to study adipogenesis.

1.4.1.2 Stromal Vascular Fraction (SVF)

The SVF isolated from white adipose tissue predominantly contains pre-adipocytes that can undergo differentiation. The different cell types within the SVF can be identified using the different clusters of differentiation (CD) markers. The injection of cells positive for the markers CD29, CD34, Sca1, and CD24 from the SVF into transgenic mice that lack WAT (Reitman and Gavrilova 2000) has resulted in the growth of fully functional WAT (Rodeheffer, Birsoy, Friedman 2008). The differentiation of pre-adipocytes in the SVF is defined by several factors, including species, location of adipose depot, and donor age (Poulos, Dodson, Hausman 2010). ADSCs, isolated from the SVF, can also be induced to differentiate into adipocytes, chondrocytes, myocytes and osteoblasts under specific conditions (Zuk 2010). Since the various cell types within the SVF can be defined by specific phenotype markers such as ADSCs defined as CD31-CD34+CD45-CD90+CD105-CD145-, endothelial cells defined as CD34+CD31+ and

macrophages as CD14+CD31+, this allows for the selection of specific cell types in the SVF by magnetic bead sorting or flow cytometry (Sengenes et al. 2005; Armani et al. 2010). Similar to the 3T3-L1 pre-adipocytes, the SVFs are also widely used as *in vitro* models for studying adipogenesis as they represent one of the closest models to study human adipogenesis (Liu et al. 2017).

1.4.2 *In vivo* models for adipogenesis used in this thesis

1.4.2.1 The C57BL/6 mouse

Among the many different inbred strains that are used, the C57BL/6 strain is the most widely used strain of inbred mice (Claire 2003; Bryant et al. 2008), since they exhibit similar abnormalities to that of the human metabolic syndrome when fed a high fat diet (Collins et al. 2004). C57BL/6 strain is also one of the only strains to become obese when fed a HFD diet, whereas many other rodent strains can self-regulate their macronutrient intake. C57BL/6 mice have different responses to HFD induced obesity compared to other strains. They have similar weight gain to that of another mouse strain, the AKR strain, but only the C57BL/6 strain has been shown to exhibit glucose intolerance when fed on a HFD (Prpic et al. 2002; Gallou-Kabani et al. 2007). In C57BL/6 mice of the same litter, differences in phenotypic responses have been observed with some mice becoming 'high fat gainers' and others becoming 'low fat gainers'. This is due to the differential expression of adipogenic genes in the 'high fat gainers' which makes them susceptible to obesity, even before feeding on a HFD, compared to 'low fat gainers' (Koza et al. 2006; Pecoraro et al. 2006). Other mouse strains such as the sand

mice and the spiny mice respond to a HFD in a similar manner to that of the C57BL/6 mice and are also often used in diet induced obesity research (Hunt, Lindsey, Walkley 1976; Levin et al. 1997).

The C57BL/6 strain contains numerous substrains such as the C57BL/6J, and C57BL/6NCrlCrlj (C57BL/6N) substrains. The C57BL/6J substrain was obtained by crossing the female mouse N.57 with the male mouse N.52 (Staats 1980). The C57BL/6J substrain has been widely used in studies related to obesity, where HFD diet studies performed in male and female C57BL/6J mice showed significant mature adipocyte formation, from proliferative precursors, only after 7 weeks into the diet (Jeffery et al. 2015).

The C57BL/6N substrain, which was derived from the C57BL/6J substrain, was transferred from the Jackson Laboratories to the National Institute of Health (NIH) during the F32 generation in 1951 (Pettersson et al. 2012). The Charles River substrain, C57BL/6NCrl, has been maintained from the NIH since 1974. There are several SNP differences between the C57BL/6N and the C57BL/6J substrains (Bothe et al. 2004; Tsang et al. 2005; Mekada et al. 2009). Moreover, apart from the several phenotypic and behavioural differences (Blum et al. 1982; Radulovic, Kammermeier, Spiess 1998; Stiedl et al. 1999; Khisti et al. 2006; Mulligan et al. 2008; Matsuo et al. 2010) between these two substrains, studies on glucose metabolism between the substrains have shown that the C57BL/6J substrain exhibits the deletion of the *Nnt* gene (Simon et al. 2013), which

has been shown to be involved in regulating insulin response in pancreatic beta cells. These results indicate that the two substrains are distinct to each other.

Although both C57BL/6J and C57BL/6N are widely used *in vivo* models, based on their ability to respond to diet induced obesity and since the CD24KO mouse model was originally established on the C57BL/6N substrain, I used the C57BL/6N substrain as the *in vivo* model in this thesis.

1.5 Glucose uptake in adipocytes

Glucose is a six-carbon atom containing sugar that belongs to the class hexose, a sub-category of the monosaccharides. In adipocytes, 20-25% of all the glucose taken up by the cells is incorporated into TAGs and contributes to both the FFA and glycerol components of TAGs (Frayn and Humphreys 2012). Glucose acts as a lipogenic precursor and leads to the production of acetyl-Coenzyme A (CoA) (Figure 1.2). The acetyl-CoA produced can then be used to synthesize FFA, by the action of FAS (Rutkowski, Stern, Scherer 2015), and in TAG synthesis. The production of FFA from glucose activates *Pparγ,* which thereby increases adipogenesis. On the other hand, the TAGs produced by lipogenesis, are then surrounded by phospholipid monolayer and bind to Perilipin 1 to produce lipid droplets in mature adipocytes. The TAGs present in the lipid droplets act as energy reservoirs and are regularly hydrolyzed and oxidized for mitochondrial ATP synthesis (Berg, Tymoczko, Stryer 2002).

The converse of lipogenesis is lipolysis, which involves the liberation of glycerol from TAGs. Although the glycerols liberated from this process can be reused to

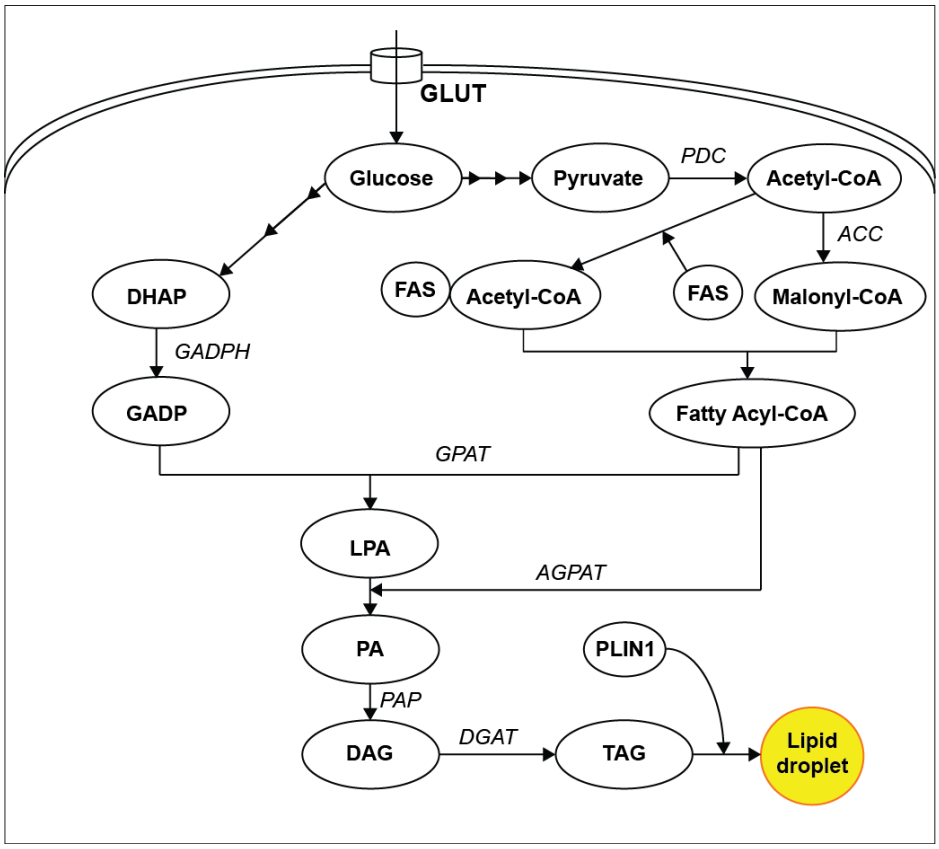

Figure 1.2: Schematic diagram of lipogenesis from glucose precursors in adipocytes. Glucose is transported into the adipocytes using either the GLUT1 or GLUT4 transporter proteins. Through glycolysis, glucose can produce dihydroxyacetone phosphate (DHAP) or pyruvate. Glyceraldehyde-3-phosphate (GADP) is synthesized from DHAP by glyceraldehyde-3-phosphate dehydrogenase. Pyruvate can undergo oxidative decarboxylation to produce acetyl coenzyme A (acetly-CoA) using pyruvate dehydrogenase complex (PDC). Acetyl-CoA binds to the fatty acid synthase (FAS) to form a complex. Acetyl-CoA can also be used to synthesize malonyl-CoA using Acetyl-CoA carboxylase (ACC), which then liberates fatty acyl-CoA by interacting with the acetyl-CoA/FAS complex. GADP then interacts with fatty acyl-CoA to liberate lysophosphatidic acid (LPA) using glycerol-3-phosphate acyltransferase (GPAT). Phosphatidic acid (PA) is produced due to the interaction of LPA with fatty acyl-CoA using acylglycerolphosphate acyltransferase (AGPAT). Phosphatidic acid phosphorylase (PAP) yields diacylglycerol (DAG) from PA, which is used to synthesize triacylglycerol (TAG) using diacylglycerol acyltransferase (DGAT). TAG is surrounded by phospholipid monolayer and binds to Perilipin 1 (PLIN1) to produce lipid droplets in mature adipocytes. Protein names are indicated in bold cases while the enzymes are in regular font italicized.

resynthesize TAGs (Coleman and Lee 2004), adipocytes express very low levels of glycerol kinase (Guan et al. 2002), and thus the liberated glycerol cannot contribute to TAG synthesis (Guan et al. 2002). Therefore, glucose must be catabolized to furnish new glycerol-3-phosphate for TAG synthesis (Guan et al. 2002; Temple et al. 2007). This indicates that adipocytes have a high glucose requirement during differentiation. In support of this, recent studies have shown that fully differentiated mature adipocytes have higher glucose uptake and lipid accumulation than undifferentiated pre-adipocytes (Hogan et al. 2016). Thus, there is a direct link between the adipocyte glucose uptake and lipid accumulation.

Glucose also plays important roles in both the full induction of adipogenic genes and the development of the mature adipocyte phenotype (Foretz et al. 1999; Hasty et al. 2000; Temple et al. 2007). Intracellular glucose in adipocytes promotes the transcriptional upregulation of the genes encoding lipogenic enzymes such as FAS, ACC, and GPAT (Ericsson et al. 1997; Horton et al. 2003; Gosmain et al. 2005). The induction of lipogenic enzymes take place through the transcription factor carbohydrate-responsive element binding protein (ChREBP) (Benhamed et al. 2012). ChREBP is activated when glucose metabolites are high, and this allows for the nuclear import and activity of ChREBP to occur (Iizuka and Horikawa 2008). The activation of ChREBP has also been linked with the activation of adipogenesis by activating *Pparγ* gene expression. On the other hand, when ChREBP activity is reduced due to reduced glucose concentrations, the adipogenic potential is decreased (Witte et al. 2015).

The transportation of glucose into the cells is an important process that is facilitated by a variety of proteins called the carrier proteins. GLUT, of the gene family SLC2A, belongs to a special class of twelve-member carrier proteins that specifically mediate glucose uptake into cells (Wood, Hunter, Trayhurn 2003; Zhao and Keating 2007). In human adipose tissue, glucose transport is mediated by the GLUT1, 3, 4, 8, 10, and 12 members of the GLUT family (Wood, Hunter, Trayhurn 2003). Whereas in mouse cell lines such as 3T3-L1 pre-adipocytes, glucose transport is mediated by GLUT4, GLUT1, GLUT8, and GLUT10 (Scheepers et al. 2001; Clarke et al. 1994; Wood, Hunter, Trayhurn 2003). GLUT1 expression is high in proliferating pre-adipocytes and decreases to slightly lower levels upon differentiation in mature adipocytes (El-Jack, Kandror, Pilch 1999). Also, GLUT1 levels increase drastically when there is less intracellular glucose (von der Crone et al. 2000). On the other hand, GLUT4 translocation to the plasma membrane occurs when insulin, secreted by the pancreatic β-cells in response to high plasma glucose levels, binds to the insulin receptor (Fu, R Gilbert, Liu 2013). Although GLUT8 and GLUT10 also function to transport glucose in adipocytes, less is known about their mechanism of action compared to the other GLUT members (Carayannopoulos et al. 2000; Dawson et al. 2001).

1.6 Cluster of Differentiation 24 (CD24)

Cluster of Differentiation 24 (CD24), also known as nectadrin or heat stable antigen (HSA), is a heavily glycosylated glycophosphatidylinositol (GPI)-linked cell surface receptor that was first identified in mice (Springer et al. 1978). The CD24 protein is 31-35 amino acids long and has a molecular mass of up to 70 kDa (Pirruccello and

LeBien 1986; Fischer et al. 1990; Hough et al. 1994). The serine, threonine and asparagine enriched CD24 protein has O- or N- linked glycosylation sites that form about half the residues in the core protein (Hough et al. 1994; Ayre et al. 2016). The O- or N-linked glycosylation patterns have been shown to vary considerably between species and between tissues of the same species (Ohl et al. 2003). CD24 has been shown to be evolved before the divergence of birds and reptiles, but is conserved across the mammalian species (Ayre et al. 2016).

CD24 is present on a number of different cell types including cancer cells, neurons, precursor B lymphocytes, epithelial cells, macrophages and pre-adipocytes (Fang et al. 2010). The CD24 gene and protein expression is dynamic and depends on the stimulation and cell type in which it is expressed. The expression of CD24 is higher in progenitor cells and decreases as the cells differentiate. In differentiated cells, the expression of CD24 is negligible or absent depending on the cell type. CD24 mainly functions by regulating cell survival or differentiation in a cell-type dependent manner (Fang et al. 2010). It regulates the survival of B lymphocytes, cancer cells, neurons, T cells, and dendritic cells. It has been shown to be pro-proliferative in some cancer cells, causing cancer cell growth, proliferation, and metastasis (Huang and Hsu 1995; Sagiv, Kazanov, Arber 2006; Agrawal et al. 2007) but anti-proliferative in other cancer cells depending on the cancer type (Kristiansen, Sammar, Altevogt 2004; Ju et al. 2011). Also, CD24-expressing exosomes are found to be present in fetal amniotic fluid (Keller et al. 2007). Recent studies in our lab have shown that CD24 regulates extracellular vesicle formation in B cells (Ayre et al. 2015). CD24 was also found to be expressed in all three

primitive embryonic germ layers as early as 9 days post fertilization (Shirasawa et al. 1993) and was found to be expressed in early tooth buds, developing intestinal epithelial cells, and the developing mid-, hind-, and forebrain during the later stages of development (Shirasawa et al. 1993). Moreover, CD24 is also known to be involved in a variety of other cellular processes including neurite outgrowth and adipogenesis (Chappel et al. 1996; Hough et al. 1996; Suzuki et al. 2001; Rodeheffer, Birsoy, Friedman 2008; Smith et al. 2015). CD24 also plays an important role in maintaining glucose and free fatty acid homeostasis in a diet independent manner (Fairbridge et al. 2015). CD24 has also been shown to interact with a variety of ligands in a cell type specific manner including, P-selectin, E-selectin, L-selectin, L1 cell adhesion molecule (L1CAM), High mobility group box 1 (HMGB1), Siglec-G and CD24 itself (Kadmon et al. 1992; Aigner et al. 1995; Myung et al. 2011; Ayre and Christian 2016; Ayre et al. 2016; Tan et al. 2016). Recently, it has been suggested that CD24 might not be directly interacting with these ligands, instead it might regulate the function of a signaling partner in a cell type dependent manner (Ayre and Christian 2016).

CD24 has also been shown to be a marker of pre-adipocytes *in vivo* (Rodeheffer, Birsoy, Friedman 2008; Berry and Rodeheffer 2013; Smith et al. 2015) as well as a specific regulator of adipogenesis *in vitro* (Smith et al. 2015). Although cell surface markers that discern pre-adipocyte populations *in vitro* are Lin-: CD29+: CD34+: Sca-1+: CD24+/-, only pre-adipocytes that are positive for CD24 are capable of reconstituting fully functional WAT depot *in vivo* in lipodystrophic mice (Rodeheffer, Birsoy, Friedman 2008). *In vivo* studies in mice fed a HFD have shown that the mice first rapidly gain and

then lose CD24-positive adipocyte progenitor cells (Jeffery et al. 2015). *In vitro* studies in primary cells and in 3T3-L1 pre-adipocyte cell lines have shown that both CD24 mRNA and surface protein expression dramatically increases during the initial stages of adipogenesis and remains high at the progression stage, but then decreases to near basal levels upon maturity (Smith et al. 2015). Moreover, in order for the adipocytes to differentiate optimally, CD24 expression is required during the initial stages of adipogenesis (Smith et al. 2015). Only cells that are positive for CD24 expression can undergo differentiation and become mature adipocytes *in vitro* but once they become mature adipocytes, they lose CD24 expression (Berry and Rodeheffer 2013; Smith et al. 2015). Blocking the expression of CD24 using siRNA-mediated knockdown significantly inhibits the expression of *C/EBPα* and *Pparγ*, and leads to the inhibition of overall adipogenesis and lipid accumulation (Smith et al. 2015). Taken together, these results indicate that both the upregulation of CD24 expression at the initiation of adipogenesis and its downregulation at maturity appear to be required for efficient adipogenesis (Smith et al. 2015).

The CD24KO mouse model was established in the C57BL/6N substrain by the homologous recombination-mediated replacement of the promoter and the first exon of the *CD24a* gene with a neomycin-resistance expression cassette in mouse embryonic stem cells (Nielsen et al. 1997). CD24KO mice have altered erythrocytes, that aggregate, and low numbers of late and immature B-cells in the bone marrow (Nielsen et al. 1997). They also exhibit increased susceptibility to danger associated molecular patterns but not to pathogen associated molecular patterns (Chen et al. 2009). CD24 knockout mice show

a generalized reduction in visceral and subcutaneous WAT weights and have high fasting blood glucose levels at 9 weeks of age, indicating that CD24 actively regulates WAT development up to 9 weeks of age (Fairbridge et al. 2015). CD24 regulates the development of WAT depots by regulating hypertrophy and not hyperplasia (Fairbridge et al. 2015). But the mechanism by which CD24 regulates hypertrophy is unknown. Answering this question would give us a better understanding of WAT depot development and would help develop better therapeutics to treat WAT-related diseases.

1.7 Preliminary DNA microarray analysis

To determine the genes that are regulated by CD24 during adipocyte development, a preliminary DNA microarray analysis was previously performed in our lab using the Affymetrix mouse Gene 2.0 DNA microarray (unpublished observations, Smith and Christian). In this analysis, global expression of over 41,000 genes was analyzed when CD24 was knocked down during adipogenesis. From these results, it was identified that siCD24 siRNA selectively prevented the upregulation of 134 genes (Figure 1.3) and the downregulation of 41 genes (not shown) during the initiation of adipogenesis (6h post IBMX/ Dex treatment). In this thesis, I focused on the genes that were upregulated and I hypothesized that some of the 134 genes from the preliminary transcriptomic data are regulated by CD24 and therefore contribute to the subsequent differentiation and lipid accumulation in mature adipocytes *in vitro*. If this were true, my study would provide new insights on the regulation of adipocyte development by these genes, which has never been shown previously.

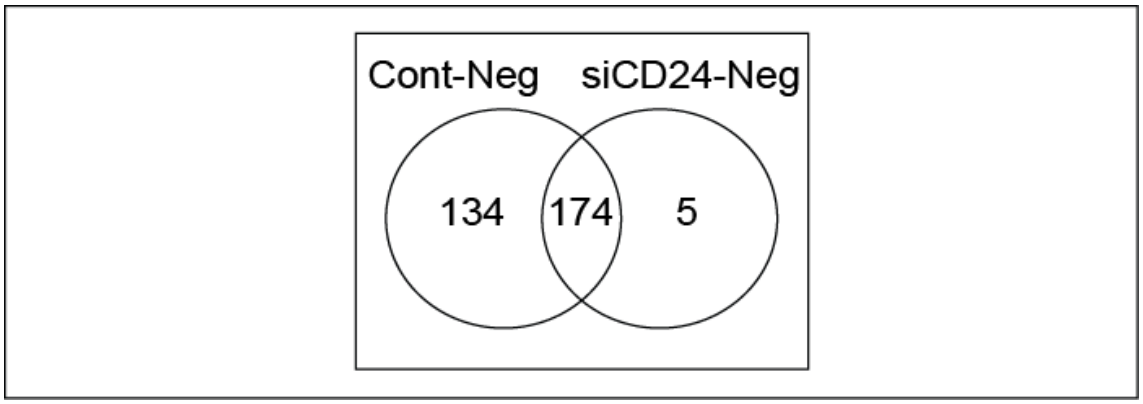

Figure 1.3: DNA microarray analysis in 3T3-L1 pre-adipocytes during adipogenesis (unpublished observations, Smith and Christian). Global expression of genes was analyzed when CD24 was knocked down during adipogenesis *in vitro.* Genes in the Cont-Neg circle were upregulated in the Scr siRNA treatment, whereas genes in the siCD24-Neg circle were upregulated when CD24 was knocked down using siCD24 siRNA. It was found that 134 genes were upregulated in the Scr vs untreated situation, 5 genes were upregulated in the siCD24 vs untreated situation and 174 genes were upregulated in both the situations, FDR<0.05.

1.7 Overall goal and hypotheses

1.7.1 Overall goal

CD24 actively regulates lipid accumulation in mature adipocytes *in vitro.* Therefore, the overall goal of my thesis is to determine how CD24 regulates lipid accumulation *in vitro.*

1.7.2 Primary hypothesis

CD24 regulates lipid accumulation by regulating the process of glucose uptake.

1.7.3 Secondary hypothesis

CD24 regulates lipid accumulation by regulating the expression of candidate genes from the preliminary transcriptomic analysis.

2.0 Materials and Methods

2.1 Animals and husbandry

All animal use was approved by the Institutional Animal Use and Care Committee at Memorial University of Newfoundland (protocol 14-05-SC). C57BL/6N mice wild type (WT, +/+) for CD24 and *Cd24*^{atm1Pjln} homozygous mice (CD24KO mice, -/-) (Nielsen et al. 1997; Chen et al. 2009) were used. Littermate WT and CD24KO mice that were congenic, 98% identical to the original C57BL/6NCrl strain, were bred at Memorial University. Congenic mice were first obtained by backcrossing the isogenic (95% identical to the original C57BL/6NCrl strain) CD24KO strain with WT mice from Charles River (C57BL/6NCrl) (Charles River Laboratories, Wilmington, MA, USA) for five generations. The resulting congenic male and female mice that were heterozygous (+/-) for CD24 were used as the parents for heterozygous (HetxHet) breeding. The HetxHet breeding produced pups that were homozygous for CD24 (WT), heterozygous for CD24 and that lack CD24 (CD24KO). Only littermate male WT and CD24KO mice born from the HetxHet breeding were used for the analyses. Mice were weaned onto the standard ProlabRMH 3000 rodent chow diet (chow, 4% sucrose, 60% carbohydrate, 14% fat [by kcal], Lab Diet, St, Louis, MO, USA) at 3 weeks of age and continued to be on the diet till they reached 15 weeks of age in order to ensure that all diet effects have been attained. Mice were housed one per cage in microisolator cages, on a 12 h: 12 h dark: light cycle, with *ad libitum* access to water. Prior to tissue harvest, the mice were anesthesized followed by cervical dislocation.

2.2 3T3-L1 and SVF adipogenesis assay and neutral lipid staining of SVF at maturity

3T3-L1 pre-adipocytes were purchased from ATCC (Manassas, VA, USA). To revive the cells, an individual vial (passage number 8 or 9) was taken and the contents were added to warmed growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NBCS), 1% penicillin-streptomycin solution (pen-strep), and 1% sodium pyruvate solution) (Life Technologies, Burlington, ON, Canada). The suspension was centrifuged for 5 minutes at 400 x g to pellet the cells. The pellet was resuspended in growth medium and the suspension was added to a 10-cm cell culture dish (Corning, New York, NY, USA) with growth medium. Cells were incubated at 37^{o}C and 5% CO_2. 3T3-L1 pre-adipocytes were grown to 70-80% confluency, at which time they were passaged. The growth medium was aspirated and the cells were incubated with 2 ml of trypsin dissociation solution (0.25% trypsin and 0.9 mM EDTA) (Life Technologies) for approximately 2-3 minutes to allow the cell monolayer to detach. Trypsin was inactivated by adding 5 ml growth medium and the resulting suspension was centrifuged for 5 minutes at 400 x g. The cell concentration was manually determined using a haemocytometer (Reichert, New York, NY, USA). An appropriate volume of cell suspension to yield 3.0 x 10^4 cells was added to 10 ml growth medium in a 10-cm culture dish.

3T3-L1 pre-adipocytes (up to passage 15) were used for differentiation into mature adipocytes. The cells were plated in growth media and were allowed to reach 100% confluency. Between 24 and 48 hours' post confluency the cells were initiated to

undergo adipogenesis in differentiation medium [DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% pen-strep, 1% sodium pyruvate, 0.5 mM IBMX (EMD Millipore, Billerica, MA, USA), and 0.1 μM Dex (EMD Millipore)]. The medium was changed 48 hours later with the progression medium [(DMEM supplemented with 10% FBS, 1% pen-strep, 1% sodium pyruvate, and 10 μg/ml recombinant human insulin (Sigma-Aldrich, St. Louis, MO, USA)] and every 48 hours after that to adipocyte maintenance medium (DMEM supplemented with 10% FBS, 1% pen-strep, and 1% sodium pyruvate) for 5 days.

For SVF isolation, inguinal and epididymal WAT were harvested from 15-week-old WT and CD24KO mice for the adipogenesis and the gene expression analyses. To isolate the SVF, about 100 – 200 mg of WAT was minced with a sterile razor blade and digested with 15mg/ml collagenase type 1 (Life Technologies) in Krebs-Ringer-HEPES bicarbonate buffer (pH 7.4) containing 200 nM adenosine and 1% bovine serum albumin (KRH) and incubated for 1 h at 37^{o}C (Joost and Schurmann 2001). Tissue debris was removed by filtering through a 100 μm nylon mesh cell strainer (ThermoScientific) and the SVF was collected by centrifuging for 5 minutes at 400 x g. The cells were washed three times in the adipogenesis maintenance media (see section 2.1) and 5.0 x 10^4 cells per well from each depot were plated in the adipogenesis maintenance media and were grown in 24-well plates for RNA isolation and Oil Red O staining. Adipogenesis was initiated as per the adipogenesis protocol. Unlike 3T3-L1 pre-adipocytes, SVFs reach maturity 8 days' post treatment with the adipogenesis progression media (Yang 2008). At maturity, the cells were stained with 0.36% Oil Red O in 60% isopropanol and visualized

using the Axio PrimoVert inverted microscope (Zeiss, Oberkochen, Germany). Images were taken after staining at 20X magnification using an Apple iPhone 4 (Apple Inc, Cupertino, CA, USA) primary camera (5 megapixel with autofocus) through the eyepiece (10X magnification). The images were cropped to the dimensions 2000x3000 pixels with ImageJ. After imaging, the excess Oil Red O stain was removed by washing the wells three times with 2 ml of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and pH 7.4). The dye was extracted with 500 µl of 100% isopropanol and quantified at 520 nm using the Agilent 8453 UV – visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.3 RNA Isolation, DNase treatment, cDNA synthesis and quantitative Reverse Transcriptase PCR

RNA was isolated from 5.0 x 10^4 cells using 500 µl of Trizol as per the Trizol protocol (Life Technologies). RNA concentrations, in ng/µl, were determined using the NanoDrop 2000c spectrophotometer (ThermoScientific, Waltham, MA, USA). An agarose gel was run with 200 ng of RNA for each sample, both before and after DNase treatment and visualized under the UV light of the ChemiImager 5500 (Alpha Innotech, San Leandro, CA, USA) to check for the RNA integrity. Contaminating genomic DNA was removed from a total of 2 µg of RNA from each sample using TURBO DNase (Life Technologies), following the instructions for highly concentrated RNA. cDNA was synthesized from 500 ng of Turbo DNase treated RNA using MMLV-RT (Life Technologies). Quantitative Reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out using the Fermentas Maxima SYBR Green qPCR mastermix (2X)

(ThermoScientific) with the Eppendorf Mastercycler RealPlex^2 qPCR instrument (Eppendorf, Hamburg, Germany). For the gene expression analysis in 3T3-L1 pre-adipocytes, cDNA was used at an optimized dilution based on standard curves (Table 2.1). For the gene expression analysis in SVF, the final cDNA concentration for all the samples used was 1 ng/µl. All primer sets have been validated and primer efficiencies determined according to the MIQE guidelines (Bustin et al. 2009) and can be found in Table 2.1. Ct values were analyzed after 40 cycles according to the Pfaffl equation (Pfaffl 2001), which is a modified version of ΔΔ cycle threshold (Ct) equation but takes into account the empirically determined efficiency of each primer set (Pfaffl 2001). *Rplp0* and *Gusb* were used as the normalizer genes for the gene expression analysis in 3T3-L1 pre-adipocytes, while *Actb* and *Rplp0* were used as the normalizer genes for the gene expression analysis in SVFs. The geometric mean of the normalizer genes was used to calculate the overall normalized relative quantities (RQs) of the target genes of interest (Table 2.1). The gene expression of *Pparγ* was only analyzed in the SVF samples as its expression in 3T3-L1 pre-adipocytes was determined previously (Smith et al. 2015). When performing multi-plate runs necessary for analysis of gene expression in SVF, a pooled inter-run calibrator that contained cDNA from all of the different samples (1ng/µl) was used on all the plates. The individual Ct values of the samples were normalized to the pooled calibrator Ct values.

The expression of *Cd24* and the normalizer genes *Rplp0* and *Actb* from cells plated in the 96-well format was determined using the Power SYBR Green qPCR mastermix for the 2 – step Cell to Ct kit (ThermoScientific). For RNA isolation,

Table 2.1: Validated qRT-PCR primers using Maxima SYBR green qPCR mastermix

Gene name and symbol	**Sequence**	**Efficiency**	**cDNA concentration used (ng/µl)**
Ribosomal Protein Lateral Stalk Subunit P0 (*Rplp0*)	F: 5'-TCA CTGTGC CAG CTC AGA AC-3' R: 5'-AAT TTC AAT GGT GCC TCT GG-3'	1.03	0.2
Glucuronidase Beta (*Gusb*)	F: 5'-CAG CGG CTG GGC TTT TTA G-3' R: 5'-CGC TTG CCC TCA ACC AAG TT -3'	0.91	5
Beta Actin (*Actb*)	F: 5'-CAC CCG CGA GCA CAG CTT CTT-3' R: 5'-TTT GCA CAT GCC GGA GCC GTT-3'	0.89	1
Peroxisome proliferator-	F: 5'-TGT TAT GGG TGA AAC TCT GGG -3'	1	1

activated receptor gamma (*Pparγ*)	R: 5'-AGA GCT GAT TCC GAA GTT GG -3'		
Cytotoxic T lymphocyte-associated protein 2 alpha Transcript variant 1 (*Ctla2a.v1*)	F: 5'-ACA GGC TGC TCT CCT CAA GT-3' R: 5'-GAT GAG CAG GAA GAC AGC ACT GAA-3'	1	5
Cytotoxic T lymphocyte-associated protein 2 alpha Transcript variant 2 (*Ctla2a.v2*)	F: 5'-TTG CTC TTC AGA GAC CGT GGA-3' R: 5'-TGC TTT TCT CTG CTC TCA CCT G-3'	1.04	0.2
RING1 and YY1 Binding protein (*Rybp*)	F: 5'-CCT TTA GGA ACA GCG CCG AA-3' R: 5'-ATT GAT GCG AGG TTT CCT GG-3'	0.9	1

Regulator of G-protein signaling 2 (*Rgs2*)	F: 5'-TGA AGC GGA CAC TCT TAA AGG A-3' R: 5'-CTT GCC AGT TTT GGG CTT CC-3'	0.83	0.2
Coiled-Coil Domain Containing 85B Transcript variant 1 (*Ccdc85b.v1*)	F: 5'-TCACCTGGGCGAGATCCGT-3' R: 5'-CTAGCTTCTGCCAACAGCCAC-3'	1.36	0.2
Coiled-Coil Domain Containing 85B Transcript variant 2 (*Ccdc85b.v2*)	F: 5'-CTGCCTCTTCCACTGCCTAC-3' R: 5'-GGTTCTCCGCCCAGCGCC-3'	0.98	25
Mesenteric Estrogen Dependent Adipogenesis (*Medag*)	F: 5'-GTGCCAATCAACCAGTGACA-3' R: 5'-GCCCTGATGTCCAGTGTACC-3'	1.03	1

Protein Phosphatase, Mg2+/Mn2+ Dependent 1D (*Ppm1d*)	F: 5'-GGGCAGATAACACAAGTGCC-3' R: 5'-CCCCTGTAGTACTTTGTACCTTGA-3'	1.08	1
Fibrinogen-like protein 2 (*Fgl2*)	F: 5'-GTCACAGCCGGTTCAACATC-3' R: 5'-AGGGGTAACTCTGTAGGCCC-3'	1.05	1
FOS Like 1 (*Fosl1*)	F: 5'-GCAAGTGGTTCAGCCCAAGA-3' R: 5'-CCGTAGTCTCGGTACATGCC-3'	0.95	1
Integrin Subunit Alpha 3 (*Itga3*)	F: 5'-TACCGATGGGCACAGACCTA-3' R: 5'-TCCACTTTGGCCCTTGACTC-3'	1.01	1
SH3 and PX Domains 2A (*Sh3pxd2a*)	F: 5'-TAGGGAAGCCTTTTCTGCCC-3' R: 5'-TTCCCCCACCAACCATCCTA-3'	1.05	1
TCDD Inducible Poly (ADP-Ribose)	F: 5'-AGGACTTCATCCAAGTGCCTG-3' R: 5'-AGTTTTCATACTTCTGACTCACCT-3'	1.14	1

Polymerase (*Tiparp*)			

1.0×10^4 cells/well were seeded in a 96-well plate and adipogenesis was induced. RNA isolation was carried out by adding 50 µl of the lysis mix (49.5 µl lysis solution and 0.5 µl DNase 1). The mixture was resuspended five times, by pipetting up and down, and incubated for five minutes at room temperature. After incubation, 5 µl of the stop solution was added to stop the lysis reaction. The cell lysate was then incubated at room temperature for two minutes followed by cDNA synthesis as per the Cell to Ct kit protocol. Standard curves were obtained by performing qPCR on serially diluted cDNA samples. Since the Cell to Ct kit does not provide a way to measure the RNA concentrations in ng/µl, cells/well was used as the concentration unit for serial dilutions instead. Efficiencies from the standard curves are shown in Table 2.2.

Table 2.2: Validated qRT-PCR primers using Power SYBR green qPCR mastermix

Gene symbol	**Sequence**	**Efficiency**
Rplp0	F: 5'-TCA CTGTGC CAG CTC AGA AC-3' R: 5'-AAT TTC AAT GGT GCC TCT GG-3'	1.29
Actb	F: 5'-CAC CCG CGA GCA CAG CTT CTT-3' R: 5'-TTT GCA CAT GCC GGA GCC GTT-3'	1.2
Cd24	F: 5'-ACT CAG GCC AGG AAA CGT CTC T-3' R: 5'-AAC AGC CAA TTC GAG GTG GAC-3'	1.01

2.4 Physiological assessments

Glucose tolerance tests (GTT) were performed by fasting the WT and CD24KO mice overnight for 14 h after which they were injected intraperitoneally with 2 g/kg D-glucose in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and pH 7.4). Blood glucose from the tail was determined at 0, 15, 30, 60 and 120 min post-injection, using a Freestyle Lite glucose meter (Abbott Diabetes Care, Mississauga, ON, Canada), which measures blood glucose concentrations up to 28 mM. Overall glucose load was determined by calculating total area under the curve from baseline, while the glucose response was calculated as area from the initial fasting blood glucose level. The mice were fasted overnight for 16 h before harvest.

2.5 Morphological assessments

Mice at 15 weeks of age were fasted overnight for 16h and then body weight, body length and body temperature were determined. The body length was measured under isoflurane anesthesia from the tip of the nose to the base of the tail, using a centimeter ruler. The body temperature of the mice was measured under isoflurane anesthesia by inserting the probe of the traceable total-range thermometer (ThermoScientific) into the rectum of the mice. Blood glucose levels after anaesthesia were measured for the WT and CD24KO mice as indicated above. Spleen and liver weights, subcutaneous inguinal and interscapular WAT, and visceral epididymal WAT depot weights of the mice were harvested and recorded. Percent (%) WAT and BAT depot weights were determined by calculating the ratio of the individual depot weights to the total body weight multiplied by 100. Body mass index (BMI), in g/cm^2 was

determined as the ratio of the body weight to the square of the body length for all the mice (He et al. 2010; Gargiulo et al. 2014; Mutiso, Rono, Bukachi 2014).

2.6 Glucose uptake and lipid accumulation in 3T3-L1 pre-adipocytes with CD24 siRNA knock-down

3T3-L1 pre-adipocytes (2.0 x 10^4 cells/well) were seeded in a flat-bottom μClearR black 96-well plate (Cat. No. 655090; Greiner Bio-One, Kremsmunster, Austria) as previously described (Hogan et al. 2016). In the empty wells surrounding the cells, 200 μl of 1X PBS was added to mitigate evaporation of the medium. After reaching 50% confluency, the cells were transfected with 100 nmol of one of the two siRNAs for CD24 (MM.RNAI.N009846.12.1, IDT, Coralville, Iowa, USA) or (CD24.13.1, IDT) or 100 nmol of scrambled (Scr) siRNA control (51-01-19-08, IDT) using a combination of 0.2 μl/well of Jetprime and 0.1 μl/well of Interferin (PolyPlus Transfection, New York, NY, USA) according to the manufacturer's protocol for Jetprime. The Scr and siCD24 siRNA transfection mixes were incubated at room temperature for 10 minutes and then 10 μl of the respective siRNA transfection mix was added dropwise to the cells. The media in the transfected wells were replaced with 100 μl of the growth medium after 6 h. After cells reached confluency, adipogenesis was induced as described in section 2.2.

To measure glucose uptake, cells were washed once with 40 μl of glucose-free serum-free (GS free media) DMEM (Life Technologies) and then 100 μl of GS free media added. The cells were stained for 45 minutes at 37ºC with 100 μg/ml 2-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) Amino)-2-deoxyglucose (2-NBDG) (Cayman

Chemical, Ann Arbor, MI, USA) in GS free media. After incubation with 2-NBDG, the wells were washed seven times with 1X PBS to remove excess 2-NBDG. Fluorescence was measured immediately using a Synergy Mx fluorescence plate reader (Biotek, Winooski, VT, USA) at an excitation and emission wavelength of 480 and 550 nm, respectively.

Cells were lysed after incubation for 6 h or 24 h, with or without IBMX/Dex or vehicle control, with the lysis buffer/DNase 1 mix from the 2 - step Cell to Ct kit (ThermoScientific) and the cell lysates were stored at -80^{o}C. cDNA was synthesized following the manufacturer's protocol. To determine the RQ of CD24 after the siRNA mediated knock down of CD24, qRT-PCR was performed for *Cd24*, *Rplp0* and *Actb* genes using the Power SYBR Green qPCR mastermix as mentioned above (section 2.3). The geometric mean of the normalizer genes was used to determine the final RQ of *Cd24*, using the Pfaffle's equation (Pfaffl 2001). For the mature adipocytes (5 days post insulin treatment) after 2-NBDG staining, the wells were stained with 10 μg/ml Nile red (Santa Cruz, Dallas, TX, USA) and fluorescence was measured on a Synergy Mx fluorescence plate reader at an excitation and emission wavelength of 520 and 580 nm respectively. Each experiment had five technical replicates, except for one which had three technical replicates, for each treatment. Data were analyzed from a minimum of three biological replicates.

2.7 Statistical analysis

Statistical analysis was performed in R v.3 (Team 2014). Pairwise comparison of parametric data was analyzed by Student's T-test. All other analyses were performed using a Two-way ANOVA followed by a Tukey post hoc test if significant, when comparing more than 2 samples. Differences were considered statistically significant at $p<0.05$.

3.0 Results

3.1 CD24 does not regulate glucose uptake in 3T3-L1 pre-adipocytes during adipogenesis *in vitro*

Previous *in vivo* analysis in our lab has shown that CD24KO mice have elevated blood glucose levels and hypotrophic adipocytes (Fairbridge et al. 2015). CD24 was also shown to regulate lipid accumulation and adipogenesis in 3T3-L1 pre-adipocytes *in vitro* (Smith et al. 2015). Since glucose is an important lipogenic precursor in adipocytes (Figure 1.2), I wanted to determine how CD24 regulates lipid accumulation by measuring glucose uptake in adipocytes. I hypothesized that CD24 might regulate lipid accumulation by directly regulating glucose uptake in adipocytes. To test this, CD24 expression was knocked down and glucose uptake was measured, at different stages of adipogenesis.

Since CD24 has been shown to be significantly upregulated during the initiation (6h to 24h post IBMX/Dex treatment) of adipogenesis (Smith et al. 2015), CD24 expression was blocked during the 6h and 24h post IBMX/Dex (ID) treated stages using siCD24 siRNAs. After knocking down the expression of CD24, the RQ of CD24 was determined by qRT-PCR. To verify that any effects that were observed were not due to off-target effects, two different siCD24 siRNA sequences (CD24 13.1 and N0098) were used to achieve the knockdown of CD24. While doing so, I initially observed that the knockdown of CD24 was variable in all the wells of the 96-well plate (data not shown). Therefore, to only analyze wells with an effective knockdown of CD24 for the glucose

uptake analysis, I used a selection criteria. When the Scr siRNA transfected cells had an RQ of CD24 greater than 70% of the average Scr control and the siCD24 siRNA transfected cells had an RQ of CD24 less than 50% of the average Scr control, they were selected for the glucose uptake analysis. Based on these criteria, a minimum of three technical replicates for the Scr siRNA transfected cells and a minimum of two technical replicates for the siCD24 siRNA transfected cells, per biological replicate, were used for the analysis. The average RQ of CD24 for Scr and siCD24 siRNA transfected cells were determined separately, for both the 6h and 24h post ID stages. It was found that the RQ of CD24 was significantly lower in the siCD24 siRNA transfected cells compared to the Scr transfected cells, for both the 6h (Figure 3.1A) and 24h post ID (Figure 3.1B) stages. This observation was consistent for both siRNA sequences used. These results indicate that the increase in CD24 expression was effectively reduced at both the stages examined.

After verifying the knockdown of CD24 at a gene expression level, I then verified the role of CD24 in regulating lipid accumulation at maturity, in a 96-well plate experimental layout. Previous studies in our lab determined that blocking the increase in CD24 expression significantly decreased lipid accumulation in 3T3-L1 pre-adipocytes *in vitro* (Smith et al. 2015). To first verify the differentiation of mature adipocytes, lipid accumulation between differentiated and undifferentiated adipocytes at maturity was compared and it was found that differentiated adipocytes had significantly higher lipid accumulation compared to the undifferentiated pre-adipocytes (Figure 3.1C & D). These results verify the differentiation of the mature adipocytes in the 96-well plate experimental layout. The expression of CD24 was then knocked down and it was found

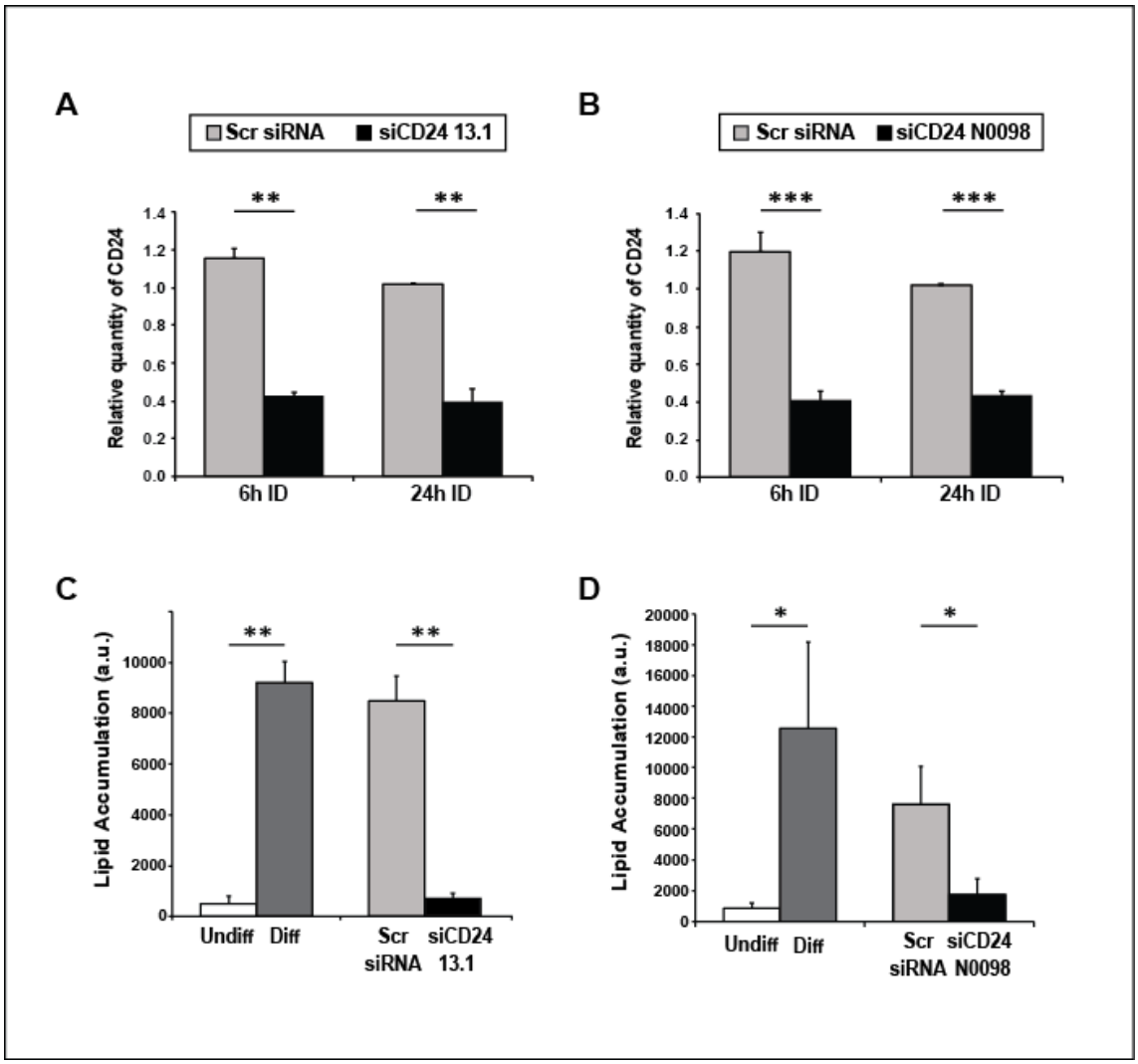

Figure 3.1: Validating the knockdown of CD24 at mRNA level and verifying lipid accumulation at maturity. The knockdown of CD24 was effective with the siCD24 siRNA 13.1 **(A)** and N0098 **(B)** at 6h post IBMX/Dex treatment (6h ID) and at 24h post IBMX/Dex treatment (24h ID) by qRT-PCR. Data shown as mean ± s.e.m., For siCD24 13.1, n = 3, 3 for scrambled (Scr) & siCD24 siRNA for both 6h & 24h ID respectively, For sequence siCD24 N0098, n = 4, 4 for Scr & siCD24 siRNA for 6h ID and n = 3, 3 Scr & siCD24 siRNA for 24h ID respectively. Verifying the role of CD24 in adipocyte lipid accumulation using siCD24 13.1 **(C)** and siCD24 N0098 **(D)**, at maturity. Data shown as mean ± s.e.m., For sequence siCD24 13.1, n = 3, 3 for controls and treatments respectively, For sequence siCD24 N0098, n = 4, 4 for controls and treatments respectively, a.u. = arbitrary unit. Statistical significance was determined using the student's T-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

that there was significant reduction in lipid accumulation when CD24 was knocked down compared to the Scr transfected cells, for both of the siRNA sequences used (Figure 3.1C & D). These results verify the role of CD24 in regulating lipid accumulation in 3T3-L1 pre-adipocytes.

Next, glucose uptake was measured using the fluorescent analog of glucose, 2-NBDG (Hogan et al. 2016). It was found that differentiated adipocytes had significantly higher glucose uptake compared to undifferentiated pre-adipocytes at maturity (Figure 3.2A & B). This result was in accordance with the previous results from our lab (Hogan et al. 2016). It was also found that there was no significant change in glucose uptake, when CD24 was knocked down, at any stage during adipogenesis, which was consistent for both siCD24 siRNA sequences (Figure 3.2A & B).

Since only a selected number of wells with cells that met the selection criteria, indicated above, was used, I then wanted to determine if there was a correlation between the RQ of CD24 and the glucose uptake of cells, irrespective of the selection criteria. No significant correlation between the overall glucose uptake and the overall RQ of CD24 for knockdown was observed with either siCD24 13.1 ($R^2 = 0.006$, $p = 0.857$) or siCD24 N0098 ($R^2 = 0.026$, $p = 0.168$) (Figure 3.2C). There was again no correlation between the expression of CD24 and glucose uptake at 6h ID for siCD24 13.1 ($R^2 = 0005$, $p = 0.704$) and for siCD24 N0098 ($R^2 = 0.046$, $p = 0.185$), respectively (Figure 3.2D). This was also the case between the expression of CD24 and glucose uptake at 24h ID for siCD24 13.1 ($R^2 = 0.021$, $p = 0.447$) and for siCD24 N0098 ($R^2 = 0.002$, $p = 0.787$), respectively

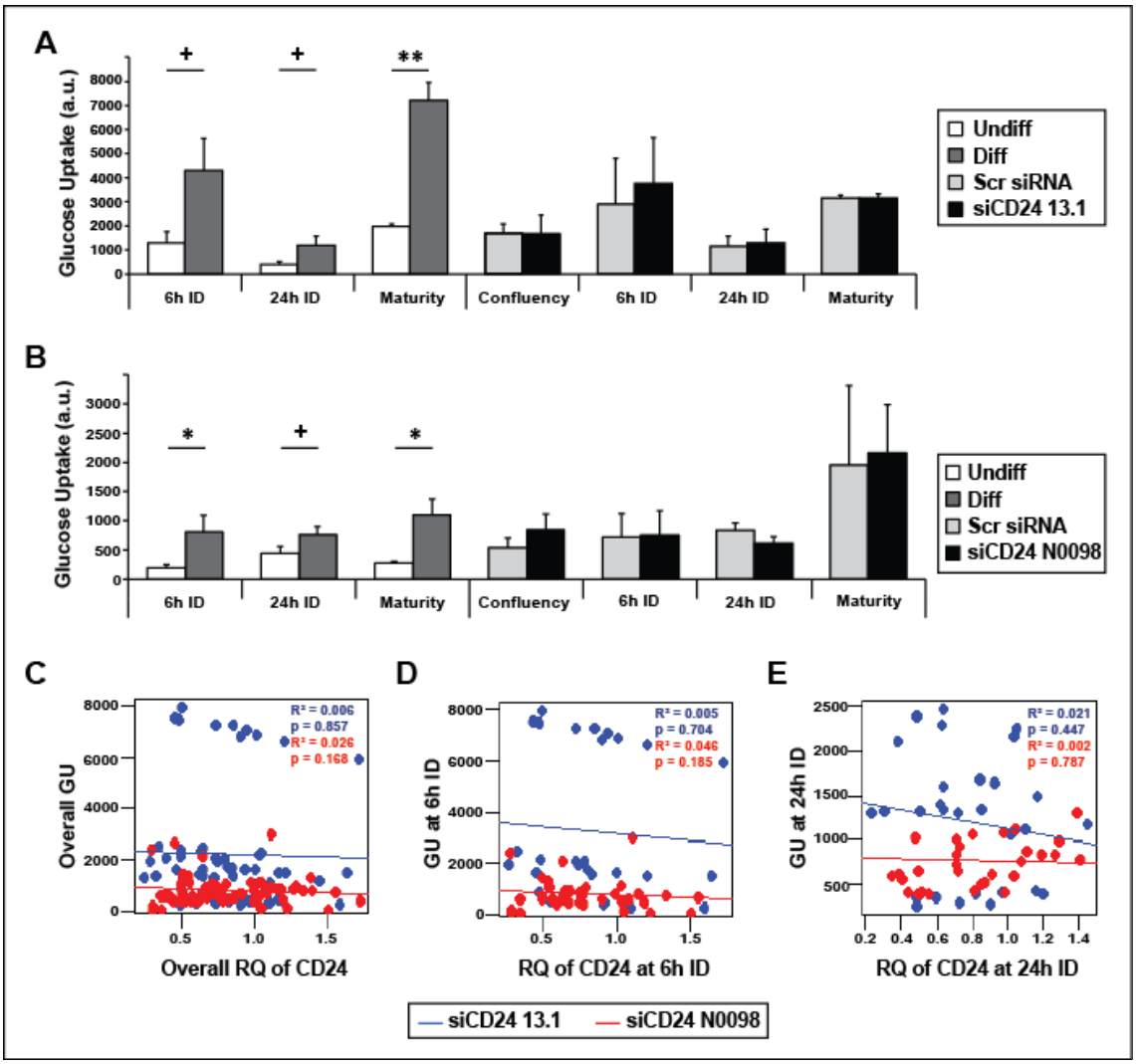

Figure 3.2: CD24 does not regulate glucose uptake in 3T3-L1 pre-adipocytes during adipogenesis. Knock down of CD24 does not affect adipocyte glucose uptake for siCD24 siRNA 13.1 **(A)** and N0098 **(B)** during adipogenesis. For sequence siCD24 13.1, n = 3 for controls and treatments and for sequence siCD24 N0098, n = 3 to 5 for controls and treatments, a.u. = arbitrary unit. **(C)** No correlation exists between the Relative Quantity (RQ) of CD24 and glucose uptake (GU) overall during adipogenesis, **(D)** at 6h post IBMX/Dex (6h ID) treatment and **(E)** at 24h post IBMX/Dex (24h ID) treatment respectively. n = 3, 6 for siCD24 sequences 13.1 & N0098 respectively. Correlation was determined using Pearson's product-moment correlation. Statistical significance was determined using the student's T-test. $^{+}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$.

(Figure 3.2E). Taken together, these results indicate that CD24 does not regulate lipid accumulation via the regulation of glucose uptake in 3T3-L1 pre-adipocytes.

3.2 CD24 regulates the expression of selected candidate genes in 3T3-L1 pre-adipocytes *in vitro* as determined by DNA microarray analysis and qRT-PCR

I next sought to determine if CD24 might regulate gene expression, which may then regulate lipid accumulation. Therefore, I focused on the results of a preliminary DNA microarray analysis using the Affymetrix mouse Gene 2.0 DNA microarray that was previously performed in our lab. In this analysis, global gene expression of over 41,000 genes was investigated when CD24 was knocked down, to identify genes that are regulated by CD24, early during adipogenesis. The results from the preliminary microarray analysis suggests that CD24 might regulate the expression of 134 genes during adipogenesis and thereby regulate lipid accumulation (unpublished observations, Smith and Christian; see Figure 1.3).

To confirm the changes in gene expression identified by the DNA microarray analysis, eleven candidate genes were selected from the 134 genes that were found to be differentially upregulated when CD24 expression was knocked down. The candidate genes were selected based on two selection criteria. Genes that were significantly changed in cells stimulated with ID for 6h after transfection with Scr siRNA compared to the untreated control but not upregulated in cells transfected with siCD24, using a False Discovery Rate (FDR) cut-off of 0.05, and based on the fold change in gene expression values in the Scr siRNA control were ranked (Table 3.1). Among the genes that were

Table 3.1: Candidate gene selection based on the LogFc values of the scrambled siRNA transfected samples with respect to the untreated samples.

Symbol	LogFc for Control-Negative	Transcript Variants
Rgs2	3.438	None
Ctla2a	2.706	2
Fgl2	2.643	None
Ccdc85b	1.704	2
Rybp	1.573	None
Medag	1.495	None
Tiparp	1.558	None
Ppm1d	1.060	None
Sh3pxd2a	1.115	None
Itga3	0.959	None
Fosl1	0.884	None

FDR<0.05. Control negative = scrambled transfected samples with respect to the untreated samples, Fc = Gene expression at IBMX/Dex treatment/ Gene expression at confluency.

selected, Cytotoxic T-lymphocyte antigen-2 alpha (*Ctla2a*) and Coiled-Coil Domain Containing 85B (*Ccdc85b*) had two mRNA transcript variants each. Transcript variants are the longer or shorter mRNA isoforms of the original gene, with the transcript variant 1 (v1) being the longer isoform compared to the transcript variant 2 (v2). Therefore, to determine if the transcript variants for these genes were regulated by CD24, both the transcript variants were analyzed independently.

The changes in the selected candidate genes were examined when CD24 was knocked down during confluency, initiation and maturity stages, by qRT-PCR. The knockdown of CD24 by the siCD24 siRNA sequence N0098, which was used in this study, was verified previously by qRT-PCR (Figure 7A of Smith et al. 2015). RQs of the candidate genes were determined relative to their respective Scr siRNA controls, and represented as ratios for analysis. Among the candidate genes selected, it was found that six candidate genes (*Rybp*, *Fgl2*, *Fosl1*, *Itga3*, *Sh3pxd2a* and *Tiparp*) were not significantly changed in the qRT-PCR analysis, relative to the Scr siRNA control, at any stage of adipogenesis analyzed (Figure 3.3A). This indicates that CD24 does not regulate the expression of these genes *in vitro* in 3T3-L1 pre-adipocytes. However, five candidate genes (*Ctla2a.v1 & v2, Rgs2, Ccdc85b.v1 & v2, Ppm1d* and *Medag*) were either significantly different ($p<0.05$) or trending towards significance ($p<0.1$) at initiation, maturity, or at both the stages, relative to the Scr siRNA control, during adipogenesis *in vitro* (Figure 3.3B). *Ccdc85b.v1, Ccdc85b.v2, Rgs2* and *Ctla2a.v2* were differentially decreased, whereas *Ppm1d* was significantly increased at maturity, relative to the Scr siRNA control. Since CD24 is significantly upregulated during the initiation of

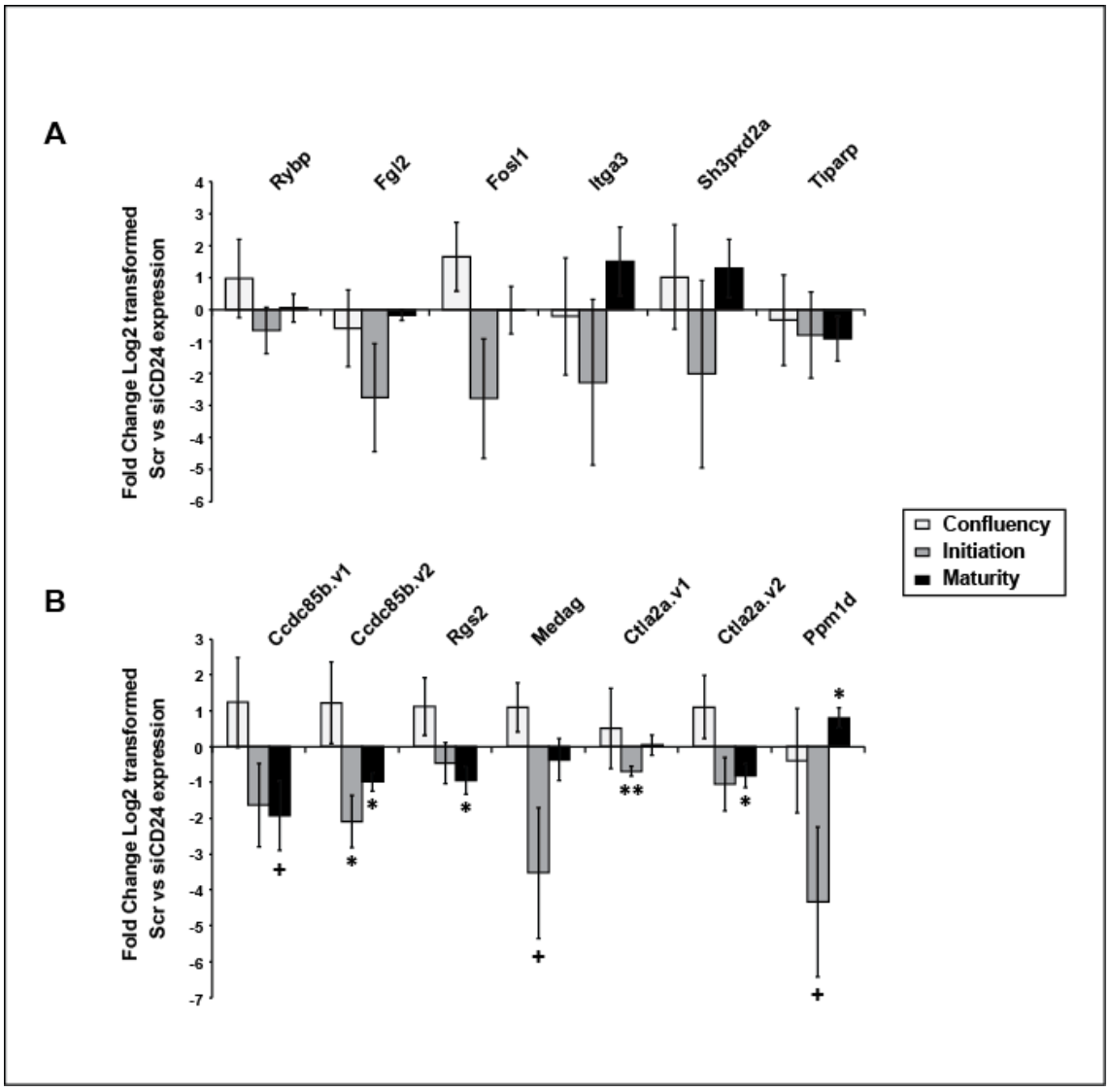

Figure 3.3: Candidate gene expression analysis in 3T3-L1 pre-adipocytes during adipogenesis. **(A)** Six candidate genes were not significantly changed when CD24 is knocked down during adipogenesis *in vitro*. Data shown as log2 transformed mean ± s.e.m., n = 4. Internal control genes used for normalization were *Rplp0* and *Gusb*. Relative quantities are shown as ratios with respect to the respective scrambled siRNA controls. **(B)** Seven genes were validated to have altered expression when CD24 is knocked down during adipogenesis. Data shown as log2 transformed mean ± s.e.m., n = 4. Internal control genes used for normalization were *Rplp0* and *Gusb*. Relative quantities are shown as ratios with respect to the respective scrambled siRNA controls. Statistical significance was determined using the student's T-test. $^{+}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$.

adipogenesis (Smith et al. 2015), I was particularly interested in the four genes that were differentially decreased, relative to the Scr siRNA control, during the initiation of adipogenesis when CD24 is knocked down: *Ccdc85b.v2, Ctla2a.v1,* Protein Phosphatase, Mg2+/Mn2+ Dependent 1D (*Ppm1d*) and Mesenteric estrogen-dependent adipogenesis protein (*Medag*).

3.3 CD24 regulates differentiation in the stromal vascular fraction *ex vivo*

Previous studies in our lab have shown that CD24 regulates hypertrophic adipocyte growth in both the visceral epididymal and subcutaneous inguinal WAT depots *in vivo* in 9-week-old male mice (Fairbridge et al. 2015). Although it is known that CD24 regulates differentiation in 3T3-L1 pre-adipocytes (Smith et al. 2015), the role of CD24 in adipocyte differentiation of SVF from the inguinal and epididymal depots is unknown. Therefore, epididymal and inguinal SVFs were isolated from 15-week-old littermate WT and CD24KO male mice and adipogenesis was induced as described above. Differentiation in treated SVF was measured by the neutral lipid stain, Oil Red O, and was quantified. The SVFs treated to differentiate, in both the epididymal and inguinal WAT depots of WT and CD24KO mice, had significantly higher lipid accumulation compared to the SVFs that were not treated to differentiate, at maturity (Figure 3.4A & B).

To account for the background staining of Oil Red O and for the spontaneous differentiation of the undifferentiated SVFs, the adipogenic potential of the SVFs was determined. The adipogenic potential is the ratio of the absorbance of the differentiated SVFs to that of the undifferentiated SVFs. The adipogenic potential was significantly

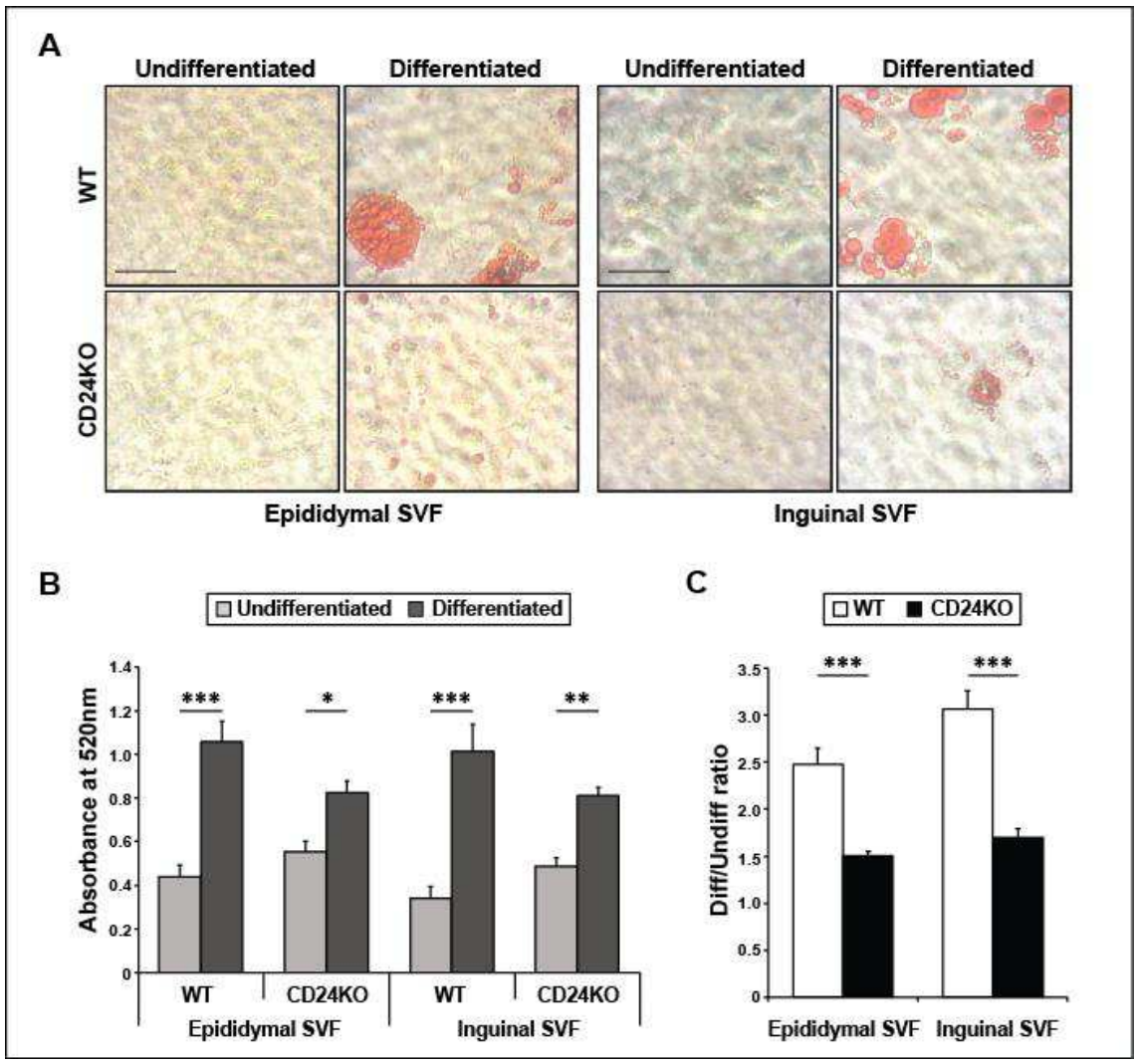

Figure 3.4: CD24 regulates differentiation in stromal vascular fraction (SVF) from the inguinal and epididymal depots, *ex vivo.* **(A)** Intracellular lipids of the SVF from the two WAT depots of WT and CD24KO mice, were stained with Oil Red O. Scale bar = 100 μm. Representative images are shown for both WT and CD24KO mice. **(B)** CD24 promotes maximal differentiation in SVF from both epididymal and inguinal depots. Data shown as mean ± s.e.m., For epididymal SVF, n = 6, 7 for WT and CD24KO mice respectively and for inguinal SVF, n = 5, 7 for WT and CD24KO mice respectively. Statistical significance was determined using a Two-way ANOVA **(C)** CD24 regulates the adipogenic potential in SVF from both the depots. Data shown as mean ± s.e.m., For epididymal SVF, n = 6, 7 for WT and CD24KO mice respectively and for inguinal SVF, n = 5, 7 for WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

lower in both the epididymal and inguinal SVFs of the CD24KO mice compared to the WT mice (Figure 3.4C).

3.4 CD24 regulates the expression of the selected genes in a depot specific manner *ex vivo*

CD24 was found to regulate the expression of four genes (*Ccdc85b.v2, Ctla2a.v1, Ppm1d* and *Medag*) during the initiation of adipogenesis *in vitro* in 3T3-L1 pre-adipocytes. The next question was whether CD24 also regulates these genes in SVF from WT and CD24KO mice. The expression patterns of these genes were measured in SVF of the epididymal and inguinal depots from littermate WT and CD24KO mice during adipogenesis *ex vivo*. Previous analysis in our lab measured the expression of *Pparγ* in 3T3-L1 pre-adipocytes and found the expression to be substantially and significantly decreased, but not abrogated, at maturity when CD24 was knocked down *in vitro* (Smith et al. 2015). From this analysis, it is known that CD24 is necessary for the expression of *Pparγ* in 3T3-L1 pre-adipocytes *in vitro,* however, it has not been determined whether CD24 is necessary for the expression of *Pparγ* in SVFs from the two WAT depots *ex vivo*. Therefore, the expression of *Pparγ* was determined in the epididymal and inguinal SVF from WT and CD24KO mice during adipogenesis by qRT-PCR. *Pparγ* expression was significantly lower at 6h post ID, 24h post ID and at maturity in both the epididymal and inguinal SVFs when CD24 was absent (Figure 3.5A & B).

Next the expression patterns of the four genes found to be differentially regulated by CD24 in 3T3-L1 pre-adipocytes was measured. The expression of *Medag* was significantly lower in the epididymal SVF at maturity in the CD24KO mice compared to

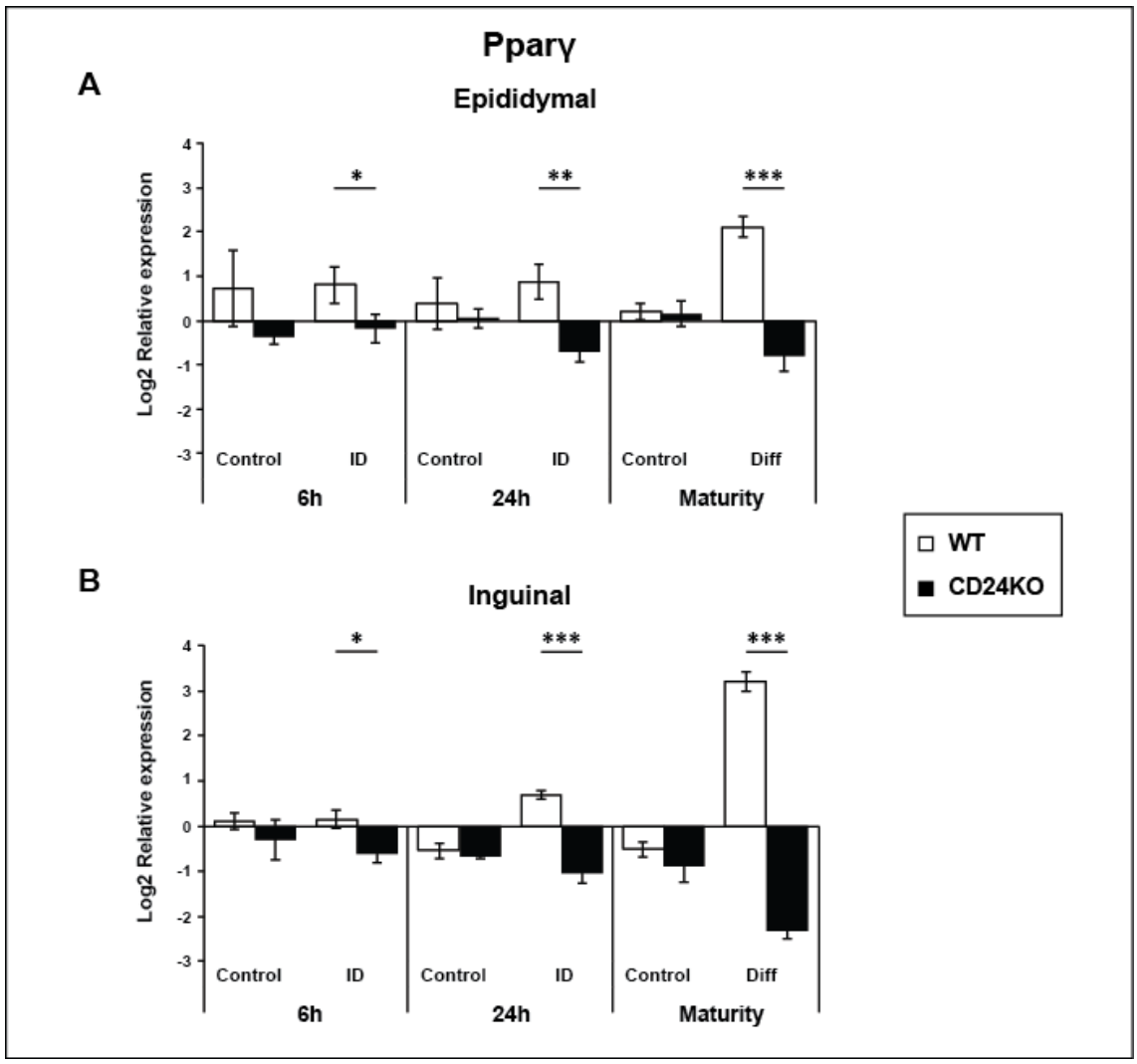

Figure 3.5: CD24 regulates the expression of *Pparγ* in the stromal vascular fraction (SVF) of both the epididymal and inguinal white adipose tissue (WAT) depots *ex vivo* during adipogenesis. The mRNA expression levels of *Pparγ* in SVF from epididymal **(A)** and inguinal **(B)** WAT depots were determined by qRT-PCR followed by normalization to the internal control genes *Rplp0* & *Actb.* Relative quantities are shown with respect to the levels in the pooled calibrator. Data is shown as log2 transformed mean ± s.e.m., n = 5, 5 for epididymal and inguinal WAT from both WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

WT mice, whereas there was no change in the expression of the other three genes (Figure 3.6A - D).

In the inguinal SVF, however, all four genes were significantly lower at one or more stages during adipogenesis in CD24KO mice, compared to the WT mice *ex vivo* (Figure 3.7). *Ccdc85b.v2* and *Ppm1d* were found to be significantly lower in the CD24KO mice compared to the WT mice at 6h post-confluency in control cells (Control 6h) and at maturity in differentiated cells (Figure 3.7A & D). *Ctla2a.v1* and *Medag* were found to be significantly lower at every stage of adipogenesis in the CD24KO mice compared to the WT mice, in all differentiating cells and in most of the control cells (Figure 3.7B & C). All the four genes were significantly higher in SVFs from the WT mice compared to the SVFs from the CD24KO mice, indicating that their expression in the control stage is necessary for the adipocytes to differentiate.

3.5 Cell autonomous effect of CD24 observed in the epididymal and inguinal SVF *ex vivo* wasn't maintained in 15-week old mice *in vivo*

Previous data from the lab showed that 9-week-old male mice that lack CD24 had significantly less inguinal and epididymal WAT weights than WT mice (Fairbridge et al. 2015). Since there was a decrease in adipogenesis in the SVFs from the CD24KO mice, it was important to verify whether the cell autonomous effect observed was maintained *in vivo* in the epididymal and inguinal WAT depots of the 15-week-old CD24KO mice. Therefore, WAT depot weights of the inguinal and epididymal depots, were compared between 15-week-old male WT and CD24KO mice, whose epididymal and inguinal

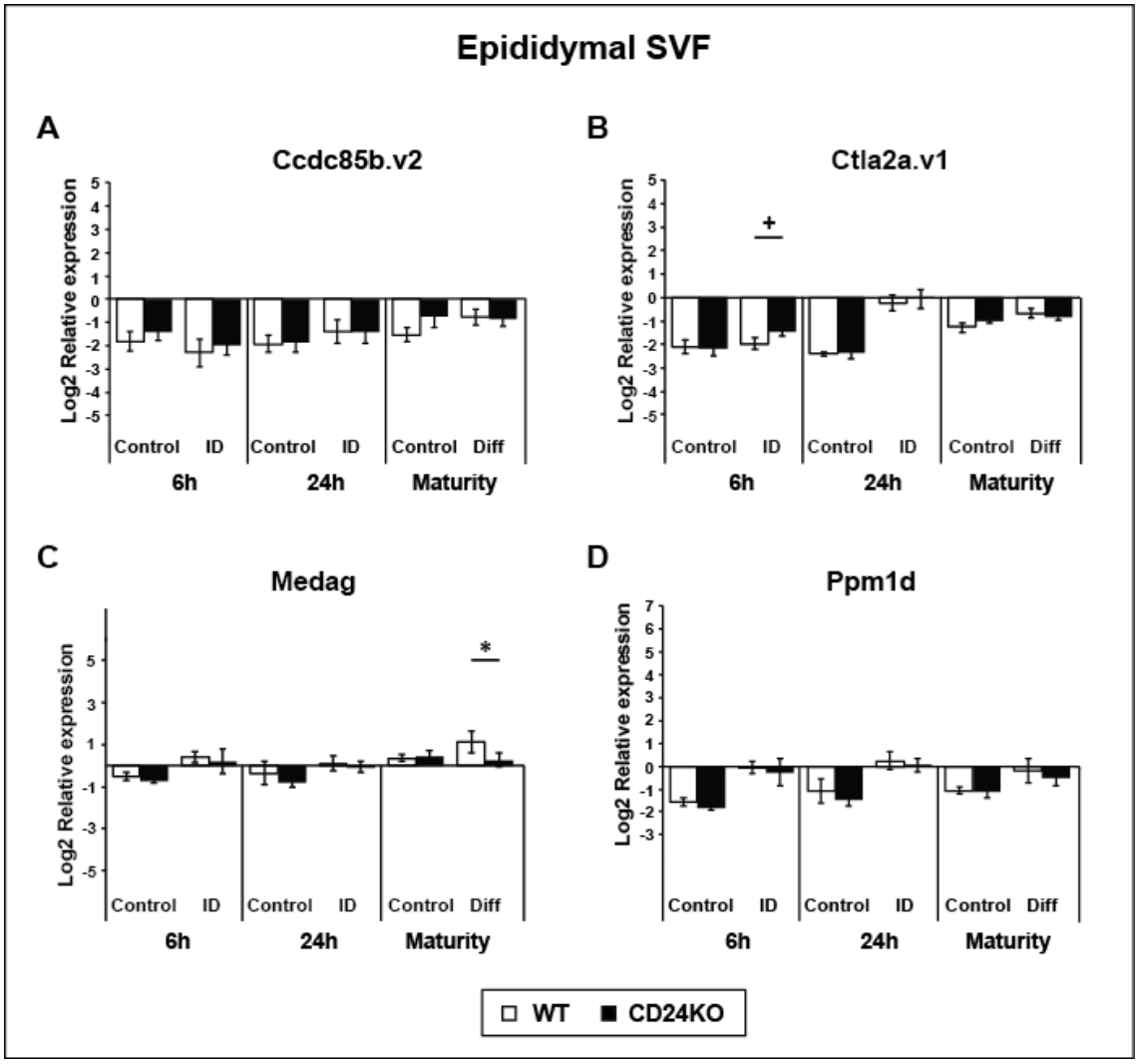

Figure 3.6: Three out of the four validated candidate genes were not significantly regulated by CD24 during adipogenesis *ex vivo* in stromal vascular fraction (SVF) from the epididymal white adipose tissue (WAT) depot. The mRNA expression levels of *Ccdc85b.v2* **(A)**, *Ctla2a.v1* **(B)**, *Medag* **(C)** and *Ppm1d* **(D)** in the SVF from the epididymal WAT depot were determined by qRT-PCR followed by normalization to the internal control genes *Rplp0* & *Actb.* Relative quantities are shown with respect to the levels in the pooled calibrator. Data is shown as log2 transformed mean ± s.e.m., n = 5, 5 for WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test. $^{+}p<0.10$, $^{*}p<0.05$.

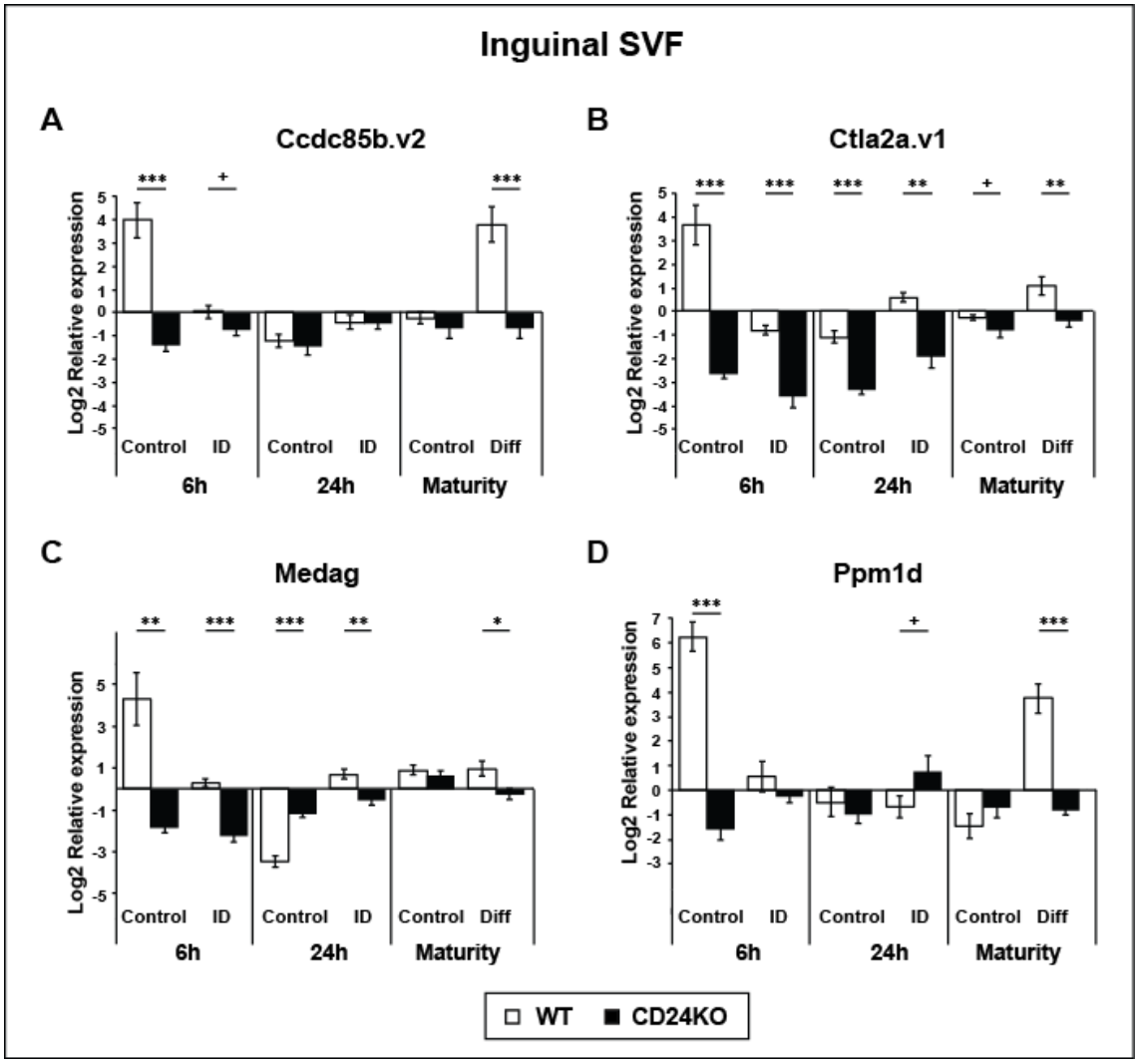

Figure 3.7: CD24 regulates the expression of all the four candidate genes, at different stages during adipogenesis *ex vivo* in stromal vascular fraction (SVF) from the inguinal white adipose tissue (WAT) depot. The mRNA expression levels of *Ccdc85b.v2* **(A)**, *Ctla2a.v1* **(B)**, *Medag* **(C)** and *Ppm1d* **(D)** in the SVF of the inguinal WAT depot were determined by qRT-PCR followed by normalization to the internal control genes *Rplp0* & *Actb.* Relative quantities are shown with respect to the levels in the pooled calibrator. Data is shown as log2 transformed mean ± s.e.m., n = 5, 5 for WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test. $^{+}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

SVFs were used for the differentiation and the adipogenic potential analyses. There was no significant difference in depot weights of intrascapular, inguinal, or epididymal WAT between the genotypes (Table 3.2). There were also no significant differences in % fat when compared to body weight, between the genotypes for these mice. No significant difference in body weight, body length, liver weight, interscapular BAT and % intrascapular BAT between the genotypes (Table 3.2 & 3.3) at 15 weeks of age. The Body Mass Index (BMI) and spleen weight, were also not significantly different between the genotypes (Table 3.2 & 3.3).

Previous data showed that male mice that lack CD24 had higher blood glucose levels compared to WT mice at 9 weeks of age (Fairbridge et al. 2015). To determine if 15-week-old CD24KO mice have alterations to glucose metabolism, fasting glucose levels in WT and CD24KO mice were measured. There was significantly higher blood glucose levels in the CD24KO mice, compared to the WT mice (Table 3.3), which was in accordance with the previous findings in 9-week-old mice (Fairbridge et al. 2015). The CD24KO mice also had higher body temperature in comparison to the WT mice (Table 3.3).

3.6 Absence of CD24 does not affect glucose tolerance in 15-week old mice

Since previous studies in our lab have shown that there was no significant difference in GTT between the CD24KO and WT mice (Fairbridge et al. 2015), I wanted to determine whether CD24KO mice have altered responsiveness to glucose as compared to WT mice at 15 weeks of age. Thus, glucose tolerance tests (GTT) was performed on

Table 3.2: Body weight, fat pad weights and % fat weights between WT and CD24KO male mice at 15 weeks of age.

	WT	**CD24KO**
Body weight (g)	26.42 ± 0.77	26.88 ± 0.79
Body Length (cm)	9.32 ± 0.08	9.46 ± 0.10
BMI (g/cm2)	0.31 ± 0.01	0.30 ± 0.007
Intrascapular WAT (g)	0.38 ± 0.04	0.37 ± 0.04
Inguinal WAT (g)	0.50 ± 0.06	0.55 ± 0.05
Epididymal WAT (g)	0.65 ± 0.08	0.73 ± 0.10
%fat Intrascapular WAT	1.44 ± 0.15	1.36 ± 0.11
%fat Inguinal WAT	1.86 ± 0.16	2.02 ± 0.15
%fat Epididymal WAT	2.42 ± 0.26	2.65 ± 0.26
Intrascapular BAT (g)	0.15 ± 0.02	0.18 ± 0.02
%fat Intrascapular BAT	0.58 ± 0.09	0.65 ± 0.06

Data is shown as mean ± s.e.m., n = 6, 8 for WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test.

Table 3.3: Fasting glucose level and body temperature of 15-week-old mice that lack CD24.

	WT	**CD24KO**
Fasting glucose (mM)	6.3 ± 0.41	7.59 ± 0.49*
Body temp (°C)	34.7 ± 0.36	35.98 ± 0.17*
Spleen (g)	0.05 ± 0.004	0.06 ± 0.003
Liver (g)	1.07 ± 0.09	1.12 ± 0.05

Data is shown as mean ± s.e.m., n = 6, 8 for WT and CD24KO mice respectively. Statistical significance was determined using the student's T-test. $^{*}p<0.05$.

both WT and CD24KO mice at 15 weeks of age. It was observed that CD24KO and WT mice showed no difference in total glucose load and glucose response to GTT (Figure 3.8A & B). No significant difference in adipocyte cell sizes was observed between the genotypes, on both the epididymal and inguinal depots, at 15 weeks of age (Figure 3.8C).

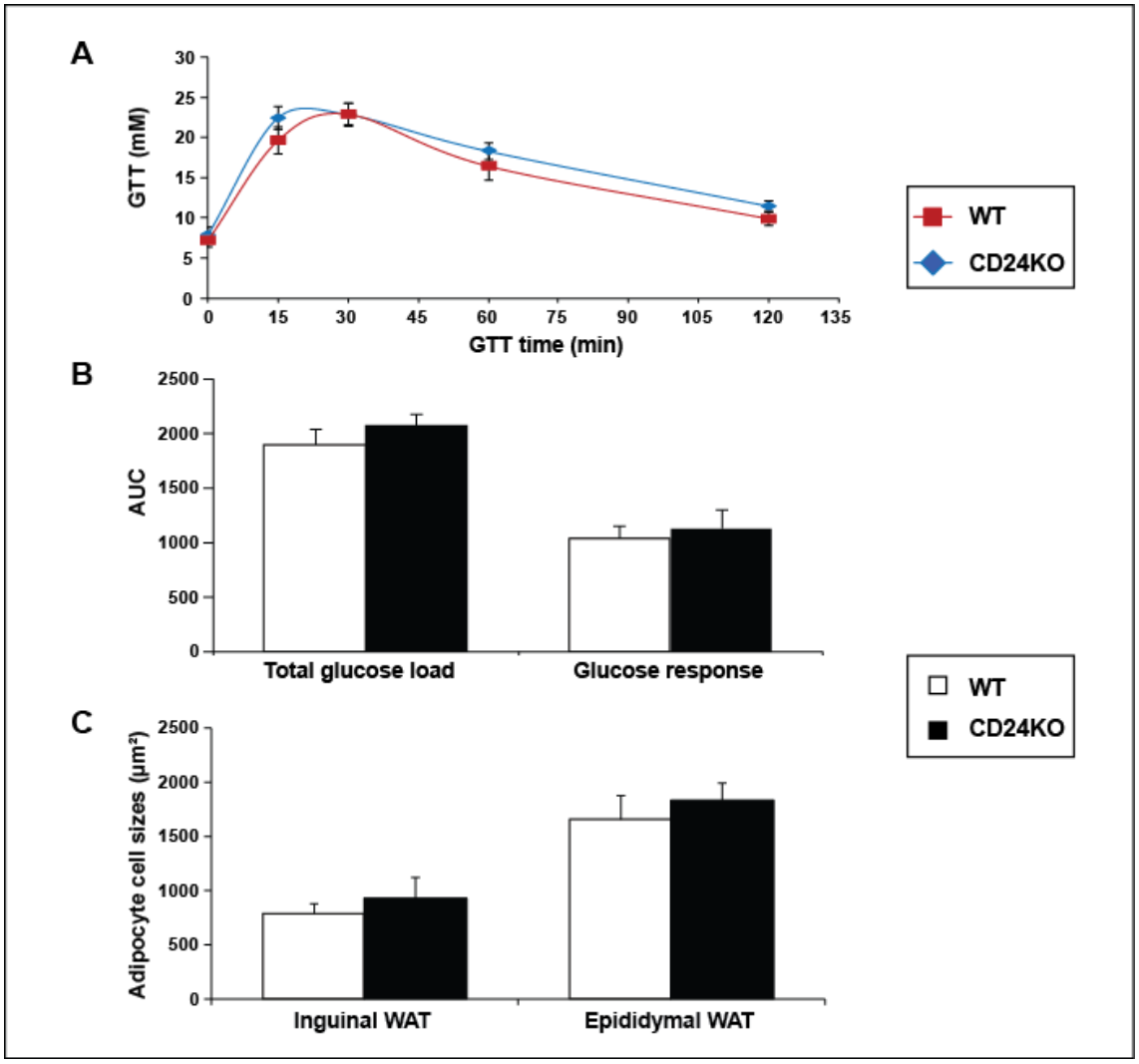

Figure 3.8: CD24KO male mice did not show altered glucose tolerance and adipocyte cell sizes on both the epididymal and inguinal WAT depots at 15-weeks of age. **(A)** Blood glucose was determined after 0 min, 15 min, 30 min, 60 min, and 120 min following intraperitoneal injection of 2 mg/g glucose for glucose tolerance test. **(B)** The total glucose load and the glucose response from control levels were determined for both WT and CD24KO mice. **(C)** Adipocyte cell sizes compared to the WT mice from the inguinal and the epididymal WAT depots are shown. Data is shown as mean ± s.e.m., n = 6, 8 for WT and CD24KO mice respectively. Statistical significance was determined using a student's T-test.

4.0 Discussion

The main goal of this thesis was to determine how CD24 regulates lipid accumulation in adipocytes. I first examined glucose uptake as glucose is an important precursor of lipid synthesis, however it was found that CD24 does not regulate glucose uptake in 3T3-L1 pre-adipocytes *in vitro.* Instead, CD24 knockdown dysregulated four genes during the initiation of adipogenesis in 3T3-L1 pre-adipocytes *in vitro.* Furthermore, these four genes were dysregulated when SVFs from the inguinal but not epididymal white adipose depot from CD24 knockout mice when induced to undergo adipogenesis *ex vivo.* Overall, these data demonstrate that CD24 is necessary for select gene expression but not glucose uptake during adipogenesis to affect lipid accumulation.

Previous *in vitro* studies in our lab using 3T3-L1 pre-adipocytes have shown that the upregulation of CD24, during the initiation of adipogenesis followed by its down regulation at maturity, is required for effective adipogenesis (Smith et al. 2015). Decreasing this initial increase in CD24 expression significantly decreases lipid accumulation in 3T3-L1 pre-adipocytes *in vitro* (Smith et al. 2015). To prevent the initial increase in CD24 expression, two independent siRNA sequences, siCD24 13.1 and siCD24 N0098 (Smith et al. 2015) were used to further verify that any effects observed were not due to off-target effects. Effective knockdown of CD24 was observed at two stages, 6h ID and 24h ID for both the sequences. It was also observed that the knockdown of CD24 significantly reduced lipid accumulation, thereby confirming previous findings that CD24 regulates lipid accumulation in 3T3-L1 pre-adipocytes *in vitro.*

Intracellular lipid accumulation is the distinct characteristic of mature adipocytes and is defined by triacylglycerol and cholesterol ester abundance in the lipid droplet (Frayn and Humphreys 2012). Triacylglycerol synthesis can occur from glucose precursors by *de novo* lipogenesis. For this, glucose from the extracellular space is transported into the adipocytes by the GLUT transporters. Glucose that is taken up by this method is used for the synthesis of both triacylglycerols and free fatty acids. The free fatty acids produced by this process activate *Pparγ.* The activated *Pparγ* further activates enzymes that regulate lipogenesis, thereby promoting overall adipogenesis (Rutkowski, Stern, Scherer 2015). Moreover, as the pre-adipocytes differentiate to mature adipocytes the demand for glucose increases which increases the expression of glucose transporters. To add to this, it was found that fully differentiated adipocytes have higher glucose uptake than undifferentiated pre-adipocytes, which was also in accordance with previous findings from our lab (Hogan et al. 2016). Suppressing the expression of CD24 with either siCD24 sequences did not affect the uptake of glucose during adipogenesis. These results indicate that glucose uptake in adipocytes is not regulated by CD24.

Previous studies from our lab have found that knocking down the increase in CD24 expression significantly decreases, but not completely inhibits, lipid accumulation in 3T3-L1 pre-adipocytes at maturity (Smith et al. 2015). The low levels of CD24 remaining after knockdown could have caused the low level of lipid accumulation. Therefore, I wanted to determine if the complete absence of CD24 in knockout mice would fully inhibit lipid accumulation during adipogenesis.

Therefore, I measured the differentiation of epididymal and inguinal SVF, from 15-week old male WT and CD24KO mice, *ex vivo*. I found that CD24 regulates the differentiation of both the epididymal and the inguinal SVFs. I also found that despite the absence of CD24, differentiation did occur in both the epididymal and inguinal SVFs, although it was not as prominent as in the SVFs from the WT mice. This supports the previous *in vitro* work showing that CD24 is a modulator but not a master regulator of adipogenesis (Smith et al. 2015).

Pparγ has been shown to play an important role in lipid accumulation of mature 3T3-L1 pre-adipocytes *in vitro* (Gregoire, Smas, Sul 1998; Tamori et al. 2002). Studies from our lab have identified that CD24 was necessary for the regulation of *Pparγ* in 3T3-L1 pre-adipocytes *in vitro* (Smith et al. 2015). My *ex vivo* lipid accumulation analysis in the epididymal and inguinal SVFs from WT and CD24KO mice showed that there was a decrease, but not absence, in lipid accumulation in the inguinal and the epididymal SVF, when CD24 was absent. Therefore, in order to confirm the effect on adipogenesis, at a gene expression level, and to determine if CD24 is necessary for *Pparγ* expression in the SVFs, the expression of *Pparγ* was analyzed in the epididymal and inguinal SVFs from WT and CD24KO mice. It was found that, in the absence of CD24, *Pparγ* was significantly lower throughout adipogenesis in both the epididymal and the inguinal SVF. Previous *in vitro* analysis in 3T3-L1 pre-adipocytes, when CD24 was knocked down, showed that *Pparγ* was significantly lower, compared to the Scr siRNA control, at maturity (Smith et al. 2015). Although this was the case at maturity, there was no significant change in *Pparγ* expression during the initiation of adipogenesis (Smith et al.

2015). Since the expression of CD24 was not completely abrogated by the siCD24 siRNA, low levels of CD24 might have caused the expression of *Pparγ* to be higher during the initiation stage (Smith et al. 2015). But from the current analysis, *Pparγ* was significantly lower, when CD24 was absent compared to the WT mice, in the SVFs from both the depots throughout adipogenesis. Moreover, I found that in the WT mice, *Pparγ* displayed a more substantial increase in the inguinal SVF than in the epididymal SVF, which was in accordance with previous reports (Tchkonia et al. 2002; Baglioni et al. 2012). These results indicate that CD24 is necessary for *Pparγ* expression in both the epididymal and the inguinal SVF *ex vivo*.

Since CD24 does not regulate lipid accumulation by regulating glucose uptake in adipocytes *in vitro,* the alternative hypothesis was that CD24 regulates lipid accumulation by regulating genes during adipogenesis *in vitro.* To identify genes regulated by CD24 during adipogenesis, one study that was done in our lab investigated the results of a preliminary DNA microarray data that showed that siCD24 selectively prevented the upregulation of 134 genes in response to 6h of ID treatment (unpublished observations, Smith and Christian). From these results, I identified genes that are regulated during the differentiation of adipocytes.

The expression patterns of selected candidate genes were then analyzed, which were selected based on the two selection criteria as indicated previously, by qRT-PCR analysis *in vitro* in 3T3-L1 pre-adipocytes. Although there was differential regulation in the selected genes in 3T3-L1 pre-adipocytes, when CD24 is knocked down, there was

high variability in gene expression patterns. This high variability might be due to the quality of the cDNA used or due to the variable knockdown of CD24. This might explain why *Medag,* which was shown to be involved in adipogenesis (Zhang et al. 2014), had a p value of 0.07 instead of being significantly decreased ($p<0.05$) during the initiation of adipogenesis, when CD24 was knocked down. The variability in CD24 knockdown might also explain why *Medag* and the other candidate genes were not significantly decreased at maturity or at other stages of adipogenesis. Another gene, *Ppm1d* was found to be significantly increased at maturity when CD24 was knocked down. *Rgs2* was significantly decreased, relative to the Scr siRNA control, at maturity. The second transcript variant of *Ccdc85b* was found to be significantly decreased at both initiation and maturity stages of adipogenesis. Apart from that, the first transcript variant of *Ctla2a* was also significantly decreased during the initiation stage, whereas the second transcript variant of *Ctla2a* was significantly decreased at maturity, relative to the Scr siRNA control, in 3T3-L1 pre-adipocytes *in vitro.*

Since CD24 is significantly upregulated during the initiation of adipogenesis (Smith et al. 2015), I was interested in four genes, *Ccdc85b.v2, Medag, Ctla2a.v1* and *Ppm1d,* as these genes were regulated by CD24 at initiation in 3T3-L1 pre-adipocytes *in vitro*. When the expression of these genes were analyzed in SVFs of 15-week-old WT and CD24KO mice, it was found that apart from *Medag,* none of the genes were regulated in the epididymal SVF. However, in the inguinal SVF it was observed that all the four genes were significantly regulated during adipogenesis *ex vivo.*

Medag is a proinflammatory cytokine that has been shown to regulate the expression of *Pparγ2*, *C/EBPα*, *aP2, C/EBPδ, Plin1, Pref-1* and *Fas* during adipogenesis (Anghel and Wahli 2007; Lefterova and Lazar 2009; Zhang, Chen, Sairam 2012). *Medag* expression was shown to increase during the early stages of adipogenesis and reach higher levels in differentiated cells (Zhang, Chen, Sairam 2012). This expression profile of *Medag,* early during adipogenesis, resembles that of the adipogenic genes *C/EBPβ* and *C/EBPδ* (Phan, Peterfy, Reue 2004). The overexpression of *Medag* in pre-adipocytes was shown to promote differentiation of adipocytes, whereas the knockdown of *Medag* decreases lipid accumulation in mature adipocytes (Zhang, Chen, Sairam 2012). These effects on lipid accumulation of *Medag* are similar to those observed when CD24 expression is blocked *in vitro* in 3T3-L1 pre-adipocytes (Smith et al. 2015). However, *Medag* has also been shown to promote glucose uptake in differentiating 3T3-L1 pre-adipocytes *in vitro* (Zhang, Chen, Sairam 2012). In this study, I have found that CD24 does not regulate glucose uptake during adipogenesis in 3T3-L1 pre-adipocytes *in vitro*. Moreover, the gene expression analysis of *Medag* in 3T3-L1 pre-adipocytes *in vitro* suggests that CD24 might be necessary for *Medag* expression to promote adipogenenic gene expression and subsequently lipid accumulation. This also suggests that CD24 acts as an upstream regulator of *Medag* in 3T3-L1 pre-adipocytes *in vitro*. On further analyzing the regulation of *Medag ex vivo* in SVFs from WT and CD24KO mice, I found that *Medag* expression was dependent on the CD24 expression in the inguinal but not the epididymal SVF, at every stage of adipogenesis analyzed. Since *Medag* was shown to promote the differentiation of adipocytes by promoting the expression of *Pparγ2* and

C/EBPα (Zhang, Chen, Sairam 2012), my analysis in the epididymal and inguinal SVF suggests that the CD24 dependent decrease in *Medag* expression may decrease the expression of *Pparγ*2 and *C/EBPα,* thereby reducing lipid accumulation, at maturity. Moreover, the decrease in *Medag* expression might have decreased glucose uptake in adipocytes (Zhang, Chen, Sairam 2012) thereby further decreasing lipid accumulation in mature adipocytes *in vitro.* But, further analyses are required to confirm these claims.

Apart from *Medag,* I also found that the second transcript variant of *Ccdc85b* to be differentially regulated in 3T3-L1 pre-adipocytes *in vitro. Ccdc85b* is a cellular homolog of the hepatitis delta virus antigen (HDAg) (Brazas and Ganem 1996) that acts as a transcriptional repressor. *Ccdc85b* has roles in negatively regulating cell growth, transcription and cellular differentiation. In pre-adipocytes, *Ccdc85b* has been reported to inhibit members of the C/EBP family, except *C/EBPα*, and thereby negatively regulates adipogenesis (Bezy et al. 2005). The overexpression of *Ccdc85b* led to the partial inhibition of adipogenesis, where only 45% of the cells produced lipid droplets compared to 80% of the cells in the control vector treatment (Bezy et al. 2005). This was due to the reduction of cells in the synthesis phase (S phase) of the cell cycle, where DNA replication occurs. This reduction in cell number thereby leads to the reduction in adipogenesis (Bezy et al. 2005). *Ccdc85b* has also been shown to inhibit the expression of important adipogenic markers like GPDH and lipoprotein lipase (LPL) to inhibit adipogenesis (Bezy et al. 2005). In my study, it was observed that the second transcript variant of *Ccdc85b* was decreased in 3T3-L1 pre-adipocytes during the initiation and maturity stages of adipogenesis. This suggests that the expression of *Ccdc85b*.v2 during

these two stages might be necessary for optimal mature adipogenic gene expression and lipid accumulation in 3T3-L1 pre-adipocytes *in vitro.* The decreased expression in *Ccdc85b.v2* observed at maturity, might have also caused partial expression of *C/EBPβ* and *C/EBPδ* thereby contributing to mild lipid droplet formation in mature adipocytes. Further *ex vivo* analysis showed that CD24 does not affect the expression of *Ccdc85b.v2* in the epididymal SVF. In the inguinal SVF, however, it was observed that high levels of *Ccdc85b.v2* expression during the Control 6h stage, which then decreased throughout adipogenesis and reached high levels at the maturity stage, in the WT mice compared to the CD24KO mice. These results suggest that CD24 is necessary for the expression of the negative regulator of adipogenesis, *Ccdc85b.v2,* in the inguinal but not the epididymal SVF. The high levels of *Ccdc85b.v2* in WT but not in the CD24KO mice, at Control 6h stage and at maturity, suggests that the CD24 dependent *Ccdc85b.v2* expression is necessary for the optimal differentiation of the inguinal adipocytes. Since *Ccdc85b* has been shown to inhibit the transcriptional activities of *C/EBPβ* and *C/EBPδ* (Bezy et al. 2005), CD24 might be indirectly controlling the expression of *C/EBPβ* and *C/EBPδ* via *Ccdc85b.v2*, thereby regulating lipid accumulation in mature adipocytes of the inguinal SVF. Since the expression of *C/EBPβ* is a prerequisite for mitotic clonal expansion, early during adipogenesis (Tang, Otto, Lane 2003; Zhang et al. 2004), the *Ccdc85b.v2* mediated decrease in *C/EBPβ* expression might have also decreased the mitotic clonal expansion in pre-adipocytes, thereby reducing adipogenesis and lipid accumulation overall. But further analyses are required to confirm these claims and to determine the cause of the depot specific regulation of *Ccdc85b.v2* by CD24 in SVFs.

It was also observed that the first transcript variant of *Ctla2a* to be differentially regulated during adipogenesis in 3T3-L1 pre-adipocytes *in vitro. Ctla2a* is a cysteine protease inhibitor protein that is found to be expressed in mouse activated T-cells, mast cells and dendritic cells (Luziga et al. 2008). Although many of its functions are not well understood, it has been shown to negatively regulate the inflammatory response and protein processing (Sugita et al. 2009) but positively regulate T cell differentiation (Sugita et al. 2011). Although this gene has not been previously shown to be involved in adipogenesis, my analysis in 3T3-L1 pre-adipocytes showed that *Ctla2a.v1* was significantly decreased during the initiation of adipogenesis, when CD24 is knocked down. This suggests that the CD24 dependent *Ctla2a.v1* expression, during the initiation of adipogenesis, might be necessary for downstream adipogenic gene expression to initiate lipid accumulation. On further analyzing the expression of *Ctla2a.v1* in the epididymal SVF *ex vivo,* similar expression patterns of this gene was observed in the CD24KO mice to that of the WT mice, throughout adipogenesis. However, the analysis in inguinal SVF showed a significant decrease of *Ctla2a.v1,* when CD24 is absent, throughout adipogenesis. The depot specific expression patterns of *Ctla2a.v1* along with its CD24-dependent decrease at every stage of adipogenesis suggests that *Ctla2a.v1* might be regulating other adipogenic genes, especially mature adipogenic genes, to promote lipid accumulation in the inguinal SVF. Further analyses of this gene during adipogenesis might provide new insights in its regulation of mature adipogenic genes and lipid accumulation in mature adipocytes, both in 3T3-L1 pre-adipocytes and in the inguinal SVF.

The fourth gene that was regulated in my analysis in 3T3-L1 pre-adipocytes *in vitro* was *Ppm1d. Ppm1d* belongs to the PP2C family of Ser/Thr protein phosphatases that are known to negatively regulate cell stress responses and are expressed in the cellular response to starvation (Takekawa et al. 2000). Similar to *Ctla2a.v1,* the involvement of *Ppm1d* in adipogenesis has not been reported previously. In my analysis, it was found that *Ppm1d* expression decreased during the initiation of adipogenesis and was increased at maturity, when CD24 is knocked down, in 3T3-L1 pre-adipocytes *in vitro.* Since pre-adipocytes must undergo clonal expansion after receiving the IBMX/Dex signal, the decrease in *Ppm1d,* during the initiation of adipogenesis, could potentially cause a reduction in cells that underwent the clonal expansion stage. Previous studies have shown that blocking clonal expansion with various compounds have disrupted adipocyte differentiation (Yeh, Bierer, McKnight 1995; Reichert and Eick 1999; Tang, Otto, Lane 2003). Therefore, the decrease in *Ppm1d* might have resulted in the overall decrease in adipogenesis leading to a decrease in lipid accumulation. On further analyzing *Ppm1d* expression in the epididymal SVF, it was found that there was no significant difference in *Ppm1d* expression between the genotypes during adipogenesis *ex vivo*. In the inguinal SVF, however, *Ppm1d* was significantly decreased in the CD24KO mice, compared to WT mice, at the Control 6h stage and at maturity. One of the key roles of *Ppm1d* is to mediate the G2/M transition of the mitotic cell cycle (Choi et al. 2002). The G2/M transition phase is one of the three checkpoints of the mitotic cell cycle, the other two being, the major G1 checkpoint and the metaphase checkpoint. The G2/M transition phase of the cell cycle ensures that cells do not initiate mitosis before repairing

their DNA after replication (Cuddihy and O'Connell 2003). The high levels of *Ppm1d* in the inguinal SVF of WT mice, at Control 6h stage, suggests that the CD24 dependent *Ppm1d* expression is necessary for the pre-adipocytes to undergo growth arrest, which allows them to receive the adipogenic signal mediated by IBMX/Dex, during the initiation stage. The high levels of *Ppm1d* in the inguinal SVF of WT mice, at maturity, suggest that the CD24 dependent *Ppm1d* expression might be required for the mature adipogenic gene expression and lipid accumulation in mature adipocytes. Further studies are required to confirm this claim and to determine the cause for the depot specific regulation of *Ppm1d,* by CD24.

Since the inguinal and the epididymal WAT depots display an array of differences including their gene expression patterns (Grove et al. 2010; Karastergiou et al. 2013; Cohen et al. 2014), it was not surprising that the select four genes showed differences in expression between the SVFs from the two depots. The differences observed in the biology of the two depots might possibly explain the difference in gene expression patterns of the select four genes in my study. For example, recent studies reveal that precursor cells from the epididymal depot display less differentiation capacity compared to the inguinal depot (Macotela et al. 2012). Moreover, studies have also shown the presence of more adipogenic progenitors in the inguinal WAT than the epididymal WAT depot (Tchkonia et al. 2002; Joe et al. 2009; Baglioni et al. 2012). It has also been demonstrated that pre-adipocytes from the epididymal depot highly express anti-adipogenic factors, as opposed to pre-adipocytes from the inguinal depot, which show higher expression of pro-adipogenic genes (Macotela et al. 2012). These results might

explain the large differences in the expression patterns of the four selected genes, between the two depots, in my gene expression analysis.

Moreover, studies performed on WT male C57BL/6J mice showed that the epididymal depot are heavier than the inguinal depot and that the mean adipocyte sizes of the inguinal depot were smaller than the epididymal depot (Sackmann-Sala et al. 2012). This could possibly be explained due to the simultaneous increase in the expression of the pro-adipogenic gene, *Medag,* and the decreased expression of the anti-adipogenic gene, *Ccdc85b.v2,* at maturity in the epididymal depot from my analysis. This simultaneous expression might have increased lipid accumulation thereby causing an increase in mean adipocyte sizes of the epididymal adipocytes further leading to the epididymal depot being heavier than the inguinal depot as claimed by that study (Sackmann-Sala et al. 2012). Similarly, the increased expression of *Ccdc85b.v2,* at maturity, in the inguinal depot of WT mice that was observed in my study might have led to reduced differentiation and lipid accumulation thereby causing a decrease in the mean adipocyte sizes of the inguinal adipocytes causing an overall decrease in inguinal depot as described before (Sackmann-Sala et al. 2012).

Apart from that, other studies have shown that there is increased expression of resistin, angiotensinogen, adiponectin, and *Pparγ* in the epididymal fat pad (Atzmon et al. 2002), while the inguinal fat showed greater expression of PAI-1 and leptin (Atzmon et al. 2002; Chusyd et al. 2016). Since *Medag* has previously been shown to regulate the expression of *Pparγ* (Zhang, Chen, Sairam 2012), it could be possible that the high levels

of *Medag* observed in the epididymal depot in my analysis could possibly be regulating *Pparγ* expression thereby leading to increased lipid accumulation in this depot. Studies have also shown that enzymes that are involved in glucose metabolism such as enolase (ENO) and triosephosphate isomerase (TIM) are highly expressed in the epididymal compared to the inguinal depot (Sackmann-Sala et al. 2012). Since *Medag* was shown to promote glucose uptake in adipocytes (Zhang, Chen, Sairam 2012), the high levels of *Medag,* at maturity, in the epididymal depot observed in my analysis might have promoted glucose uptake in this depot thereby facilitating glucose metabolism via ENO and TIM, whose expressions were shown to be high (Sackmann-Sala et al. 2012). Studies have also shown that the surgical removal of epididymal fat pad has restored insulin action and also normalized the levels of TNF-α, IL-6 and adiponectin levels (Pitombo et al. 2006). Moreover, it has also been shown that the oxidative damage is lower in the inguinal depot than the epididymal depot (Sackmann-Sala et al. 2012). Since *Ctla2a.v1* and *Ppm1d* play roles in negatively regulating inflammation and cell stress responses, it might be possible that their high expression levels, observed in my analysis, might have caused reduced oxidative damage in the inguinal depot. Since *Ctla2a.v1* and *Ppm1d* expression levels are low in epididymal depot, this might have led to high oxidative damage in the epididymal depot as observed by the previous studies (Sackmann-Sala et al. 2012). Apart from this, studies have also shown that HOXC8, a protein that has been shown to be involved in the differentiation of white and brown adipocytes (Nakagami 2013), is highly expressed in the epididymal depot (Tchkonia et al. 2002; Cantile et al. 2003; Vohl et al. 2004; Gesta, Tseng, Kahn 2007). Based on the expression levels of

Medag in my analysis, it could be possible that the CD24 dependent *Medag* expression could potentially be involved in the expression of HOXC8 and thereby leading to increased epididymal adipogenesis.

Apart from the expression of the select four genes, I also observed that the expression of CD24 to be different between the two WAT depots. CD24 increases rapidly and decreases in the 3T3-L1 pre-adipocytes and in the inguinal SVF, whereas in the epididymal SVF, there is a rapid increase but delayed, gradual decrease in expression (Smith et al. 2015). The expression patterns of CD24 and select four genes in 3T3-L1 pre-adipocytes and the inguinal SVF appeared to be similar. My analysis of the select four genes showed that CD24 was necessary for the expression of *Medag* in the epididymal SVF, whereas CD24 was necessary for the expression of all the four genes in the inguinal SVF *ex vivo*. In the inguinal SVF, it was also found that all the four genes were significantly higher in the WT mice compared to the CD24KO mice during the Control 6h stage. This might indicate that CD24 expression establishes a permissive environment for the expression of these genes to promote differentiation of the inguinal SVF. Moreover, all the four genes were significantly lower in the CD24KO mice compared to the WT mice at maturity, in the inguinal SVF, which suggests their role in the development of mature adipocytes in the inguinal WAT depot. Overall the results from the expression of *Pparγ* along with the expression of the four select genes in the inguinal SVF indicates that CD24 is a regulator of these genes during adipogenesis *ex vivo*.

Previous analyses on 9-week-old CD24KO male mice showed that CD24 regulates hypertrophic adipocyte growth *in vivo* (Fairbridge et al. 2015). I wanted to verify the role of CD24 in regulating hypertrophy in the epididymal and inguinal WAT depots in the 15-week-old WT and CD24KO male mice, which were used for the differentiation and the gene expression analyses. The study done in 9-week-old mice had a limitation in that CD24KO mice were not compared to littermate controls (Fairbridge et al. 2015). Therefore, sub-strain or *in utero* differences may have been a factor in some of the differences that were observed between CD24KO and WT mice, along with the absence of CD24. Therefore, singly housed WT and CD24KO male littermates, from the C57BL/6N background were used to better control for these effects. It was found that at 15 weeks of age, there was no difference in body weight between the genotypes, as expected (Fairbridge et al. 2015). Surprisingly, no difference in WAT depot weights or adipocyte cell sizes (Figure 3.8C) were observed between the genotypes on the epididymal and inguinal WAT depots. These results were not in accordance with the previous results that showed that CD24KO mice had significantly lower WAT depot weight, with the adipocyte cell sizes being approximately half the size to that of the WT mice at 9 weeks of age (Fairbridge et al. 2015). This suggests that the 15-week-old CD24KO mice were able to compensate for the CD24 mediated defect in WAT development and had similar WAT depot weights to the WT mice *in vivo. Ex vivo,* however, the CD24KO mice had significantly less differentiation potential compared to the WT mice on both the WAT depots. Therefore, at 15 weeks of age the small adipocytes in the CD24KO mice were able to effectively develop into large adipocytes.

This suggests that the SVFs from the epididymal and the inguinal WAT depots have a cell autonomous effect associated with them to behave differently *in vivo* and *ex vivo*. Further investigation is required to determine the cause of the cell autonomous effect in 15-week-old CD24KO mice. Since the 15-week-old WT and CD24KO littermates were born from the HetxHet breeding to parents that had at least one allele of CD24 present, I suspected that the maternal genotype would have somehow caused the unregulated expression of CD24 in the CD24KO pups, thereby causing the hypertrophic WAT growth of the CD24KO mice *in vivo.* CD24 has been shown to be present in exosomes of the fetal amniotic fluid in mice (Keller et al. 2007). Moreover, exosomes have also been shown to be present in various other body fluids such as blood plasma, urine and cerebrospinal fluid (Pisitkun, Shen, Knepper 2004; Caby et al. 2005; Gutwein et al. 2005; Street et al. 2012). Therefore, it would be interesting to determine if the CD24 expressing exosomes possibly rescued the CD24 mediated reduction in WAT depots in CD24KO offspring. Another possible explanation for the dysregulation of WAT depots in CD24KO mice includes the epigenetic modifications, such as DNA methylation or histone modifications, from the maternal programming that might have regulated the expression patterns of the adipogenic genes, such as *Pparγ,* in the SVFs of the pups differently, *in vivo* and *ex vivo.* Moreover, since the mice were singly housed, one other possible factor that could have influenced the cell autonomous effect in the CD24KO mice could be the stress of being singly housed. Therefore, identifying the possible factors that caused the cell autonomous effect might provide a better understanding of WAT development by CD24 *in vivo*.

I then compared the intrascapular BAT, liver and spleen weights and found no significant differences between the genotypes, as previously demonstrated (Fairbridge et al. 2015). But it was found that 15 weeks of age, CD24KO mice have significantly higher fasting glucose levels, which was in accordance with previous studies (Fairbridge et al. 2015). This observation might possibly be due to the effect of isofluorane anesthesia in blood glucose levels as previously reported (Pomplun et al. 2004). Although the differences in body temperatures between the genotypes were not analyzed in 9-week-old-mice, my study found that CD24KO mice have significantly higher body temperature than the WT mice at 15 weeks of age. Despite alterations in BAT metabolism of the CD24KO mice could have possibly caused an increase in body temperature, I was not able to find any information that supports this hypothesis from the literature. Therefore, further investigation is required to determine the cause of the increase in body temperatures of CD24KO mice at 15 weeks of age.

Similar to the 9-week-old mice data, the intrascapular BAT weight and the liver and the spleen weights were similar between the genotypes at 15 weeks of age(Fairbridge et al. 2015). Based on the GTT data, it was found that at 15 weeks of age, CD24KO mice are capable of clearing glucose in response to an insulin challenge, similar to the 9-week-old mice (Fairbridge et al. 2015). No significant difference in total glucose load and glucose response was observed after GTT between the genotypes, at 15 weeks of age, as observed in 9-week-old mice (Fairbridge et al. 2015). Therefore, the GTT response demonstrates that, in the CD24KO mice, glucose can be transported out of the blood in

response to insulin with comparable kinetics to WT at the level of the whole organism, similar to 9-week-old mice (Fairbridge et al. 2015).

4.1 Future directions

Further analyses are required to determine the mechanism of action of CD24 in regulating these genes. For example, subsequent targeted analysis on these genes will provide further evidence for their importance in lipid accumulation and adipocyte development. By silencing each of the genes using siRNA followed by lipid accumulation analysis and adipogenic gene expression analysis, the role of these genes in adipogenesis and lipid accumulation could be determined in future experiments. To determine if the increased gene expression of the four genes affect adipogenesis, the four genes should be overexpressed in 3T3-L1 pre-adipocytes and adipogenesis should be measured by both mRNA expressions of the adipogenic markers, as well as by Oil Red O staining. If these genes are identified to play a role in adipogenesis then they can be re-expressed, when CD24 is reduced, to see if this could potentially rescue the CD24-mediated defects in lipid accumulation. Future loss of function studies in CD24KO mice *in vivo* will be designed to knockout these genes and determine the role of CD24 in their regulation.

4.2 Conclusion

In conclusion, the major goal of this study was to determine how CD24 regulates lipid accumulation in adipocytes *in vitro.* It was found that CD24 does not regulate lipid

accumulation by regulating glucose uptake in 3T3-L1 pre-adipocytes *in vitro* (Figure 4.1A). Instead CD24 caused the dysregulation of the expression of 134 genes in 3T3-L1 pre-adipocytes as determined by DNA microarray analysis. Among the 134 genes, the expression of four genes by CD24 was validated in 3T3-L1 pre-adipocytes *in vitro.* Further analysis of these four genes in SVFs from WT and CD24KO mice showed that only *Medag* was dysregulated in the absence of CD24 in the epididymal SVF. Analysis of the four genes in the inguinal SVF revealed that all four genes were dysregulated in the absence of CD24 (Figure 4.1C). Moreover, it was also found that CD24 is necessary for *Pparγ* expression in both the epididymal and inguinal SVFs (Figure 4.1B & C). Taken together, these results suggest that *Medag* might play a role in lipid accumulation in epididymal SVF, whereas all four genes might play a role in lipid accumulation in the inguinal SVF. This study is also the first to show that the select four genes are regulated by CD24, at a gene expression level, during adipogenesis *ex vivo.* Future work that helps explain the role of these genes in lipid accumulation will provide a better understanding of the regulation of lipid accumulation in adipocytes and will be useful if targeted therapies aimed at obesity and lipodystrophy are to be produced.

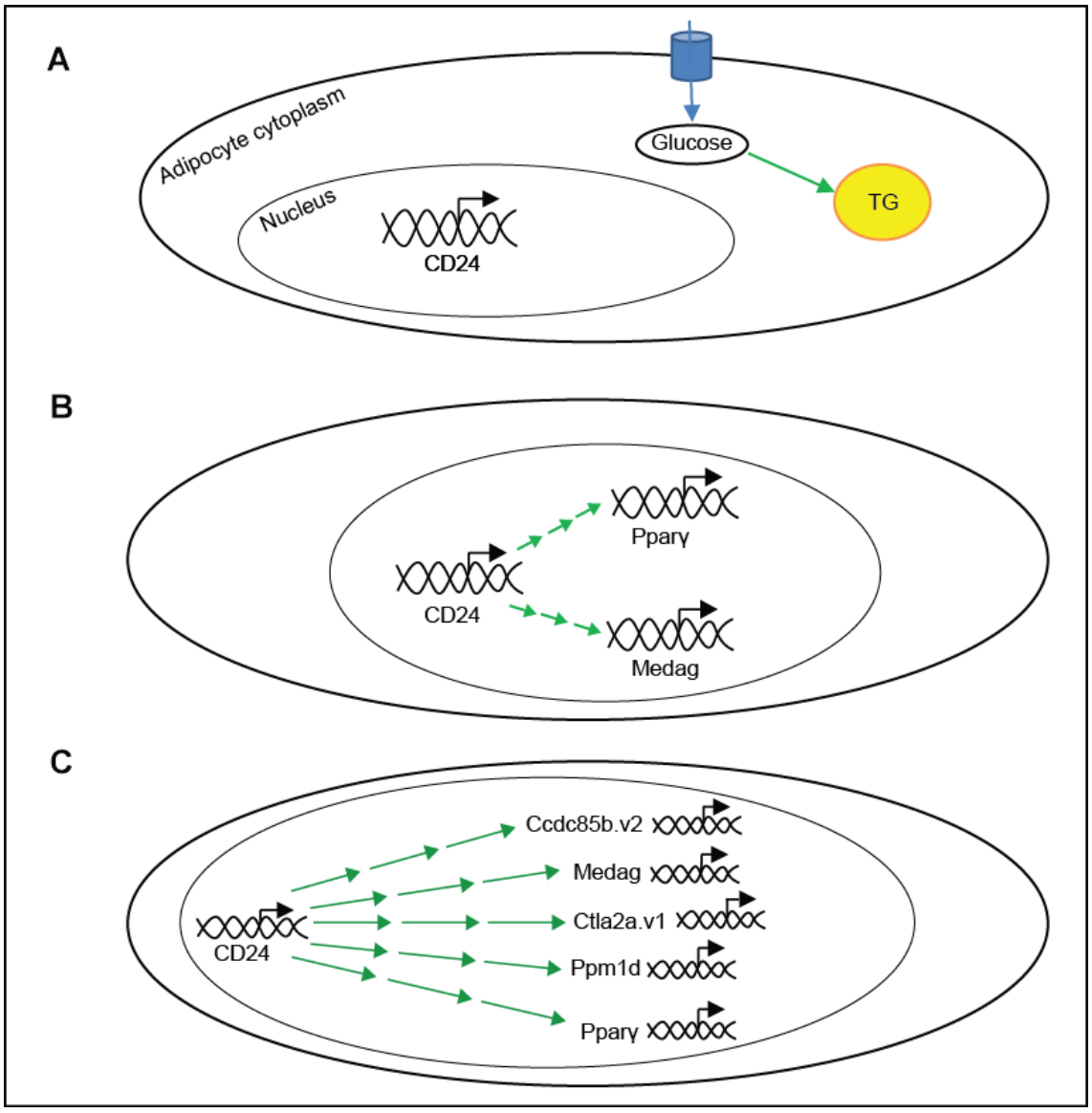

Figure 4.1: Schematic diagram of the role of CD24 in regulating key aspects of adipogenesis. **(A)** CD24 does not regulate glucose uptake in 3T3-L1 pre-adipocytes during adipogenesis **(B)** CD24 regulates the gene expression of *Pparγ* and *Medag* at maturity in the epididymal SVF. **(C)** CD24 regulates the gene expression of *Ccdc85b.v2, Medag, Ctla2a.v1, Ppm1d* and *Pparγ* at maturity in the inguinal SVF. Green arrows represent positive regulation across multiple stages of adipogenesis. TG = Triglycerides.

5.0 References

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