THE MOLECULAR REGULATION OF ADIPOGENESIS BY CD24

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by

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Abstract

Both an excess and a paucity of adipose tissue can result in major health complications such as heart disease, stroke and increased cancer risk. CD24 is a cell surface receptor involved in the regulation of cell survival. Previous research suggests that CD24 is expressed on cells that can become adipocytes, however the function of CD24 on these cells is unclear. The goal of this thesis was to determine if CD24 directly regulates adipogenesis by examining its expression and regulation on pre-adipocytes *in vitro* and *in vivo.* CD24 mRNA and protein expression was found to increase upon induction of adipogenesis *in vitro* in 3T3-L1 pre-adipocytes, followed by a decrease as the adipocyte matured. This increase in CD24 expression was mediated by cyclic adenosine monophosphate. Antibody-mediated clustering of CD24 resulted in decreased mRNA expression of late adipogenic markers and increased expression of CD24 itself, suggesting the presence of a positive feedback loop. Overall, the data presented here suggest that there is an inverse correlation between CD24 expression and adipocyte differentiation and/or function. This work has implications for improving the health of obese and lipodystrophic patients.

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List of Abbreviations

3' UTR	3' untranslated region
Abs	Antibodies
ACC	Acetyl-CoA carboxylase
ACT	Actinomycin-D
ADSC	Adipose-derived stem cells
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BCA	Bicinchoninic Acid
BMAL1	Brain and muscle arnt-like protein 1
cAMP	Cyclic adenosine monophosphate
CEBPα	CCAAT enhancer binding protein α
CEBPβ	CCAAT enhancer binding protein β
C/EBPδ	CCAAT enhancer binding protein δ
CREB	cAMP-responsive element-binding protein
CHX	Cyclohexamide
CD	Cluster of differentiation
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
EBF1	Early B cell factor-1
ECM	Extra cellular matrix
EPAC 1/2	Exchange protein directly activated by cAMP 1 and 2

FOXO1	Forkhead transcription protein
GATA 2/3	Globin transcription factors 2 and 3
GPI	Glycosylphosphatidylinositol
H&E	Hematoxylin and eosin
HFD	High fat diet
IBMX	3-isobutyl-1-methylxanthine
iFORSKOLIN	Inactive Forskolin
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
KLF	Krueppel-like factor 6
LFD	Low fat control diet
MSC	Mesechymal stem cells
NFAT5	Nuclear factor of activated T-cells 5
PI3-KINASE	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PPARγ	Peroxisome proliferator-activated receptor γ
PREF-1	Pre-adipocyte Factor-1
RIPA	Radioimmunoprecipitation assay
RT qPCR	Reverse transcription polymerase chain reaction
SREBP1c	Sterol regulatory element protein transcription Factor

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVF	Stromal vascular fraction
TBST	Tris-buffered saline + tween-20
TG	Triglycerides
TNF-α	Tumor necrosis factor α
WAT	White adipose tissue

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1.0 Literature Review

White adipose tissue (WAT) plays a critical role in energy balance, homeostasis and metabolism. It acts as a major energy reservoir, storing excess energy as triglycerides (TG) and mobilizing TG during energy deprivation. However, dysregulation of WAT production can cause major health problems. Obesity, the over production of adipose tissue, is associated with heart disease, stroke, type 2 diabetes, and certain types of cancer (Mokdad et al., 2003). Conversely, lipodystrophy, the wasting of adipose tissue, a condition commonly seen in cancer patients and those receiving anti-retroviral treatments, can result in insulin resistance and diabetes, and is a positive risk factor for death (Hadigan et al., 2001). Therefore, to develop better therapeutic strategies to combat the dysregulation of adipose tissue, it is important to fully understand the molecular regulation of adipose tissue production, known as adipogenesis.

1.1 White Adipose Tissue

There are two distinct types of adipose tissue in mammals, WAT and brown adipose tissue (BAT) (Cannon and Nedergaard, 2004). While both types of adipose tissue can store lipid, they have entirely different functions. BAT, found abundantly in infants, generates heat through non-shivering thermogenesis by uncoupling cellular respiration (Cannon and Negergaard, 2004). WAT is not only the main storage depot for energy, but it is also a metabolic and endocrine organ, secreting hormones such as leptin and adiponectin to control appetite and glucose levels, as well as several inflammatory cytokines such tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Frühbeck et al., 2001).

WAT is composed of multiple cells types including adipocytes, pre-adipocytes, macrophages, vascular, and epithelial cells (Cinti, 2005). The stromal vascular fraction (SVF) of adipose tissue, obtained through centrifugation of collagenase-digested WAT, contains pre-adipocytes and adipose-derived stem cells (ADSC) that can be induced to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic lineages (Zuk et al., 2001). In addition to pre-adipocytes and ADSCs, the SVF contains macrophages/monocytes, whose presence in adipose tissue is associated with the chronic low-grade inflammation that occurs during obesity (Bastard et al., 2006). Other cells found in the SVF include fibroblasts, progenitor endothelial cells and T regulatory cells (Riordan et al., 2009). The SVF is commonly used as an *in vitro* system to study adipocyte development (see section 1.2.2.3).

White adipocytes are unilocular, containing a large lipid droplet, surrounded by a thin cytoplasm, with the nucleus located on the periphery. WAT can respond to an increase in energy intake by storing the excess energy as lipid to increase adipocyte size (hyperplasia) and/or by generating new adipocytes to increase the adipocyte cell population (hypertrophy) (Lane and Tang, 2005). While mature adipocytes are post-mitotic, approximately 10% of adipocytes are replaced every year suggesting an adipocyte precursor population source is maintained throughout life (Spalding et al., 2008). However, the identity and localization of these precursors remains unknown.

1.2 Models of adipogenesis

There are several different models used to study adipogenesis. Insights to the etiology of obesity, as well as the development of therapeutics to combat obesity, have been accomplished from *in vivo* animal models, including diet-induced obesity in rodents,

and transgenic models such as in the ob/ob (obese) mouse, the agouti mouse, the Zuker fatty rat and the Wistar fatty rat. *In vitro* cell culture systems have been widely used to study adipocyte differentiation and have been a useful tool in determining the transcriptional regulation of adipogenesis.

1.2.1 In vivo models of adipogenesis

1.2.1.1 Diet induced obesity

Obesity induced with a high fat diet (HFD) is often used in obesity and adipocyte research. The C57BL/6 mouse is the most commonly used animal for diet induced obesity studies as they exhibit abnormalities similar to that of human metabolic syndrome when fed a HFD (Collins et al., 2004). Exposure to a high fat and high carbohydrate diet produces obesity in male C57BL/6J mice that had elevated concentrations of serum insulin, glucose and leptin, and low serum triglycerides (Collins et al., 2004; Guo et al., 2009; Petro et al., 2004). Female C57BL/6J will become obese when fed a HFD, however they will not exhibit glucose intolerance and hyperinsulinemia (Pettersson et al., 2012). Sand mice and spiny mice respond to a HFD in a similar manner as C57BL/6 mice and are also often used in diet induced obesity research, as well as Sprague Dawley and Long Evans rats (Hunt et al., 1976; Levin et al., 1997). Although diet induced obesity in rodents is commonly used, it must be noted that different strains can have differing responses to a HFD. When fed a HFD, C57BL/6 and AKR mice have similar weight gain, but only C57BL/6 will exhibit glucose intolerance, while the SWR/J and A/J mice are resistant to diet induced obesity (Prpic et al., 2002; Gallou-Kabani et al., 2007). Furthermore, even within the same strain, differences in phenotypical responses to a HFD have been observed. Differential expression of adipogenic genes has been found in

C57BL/6 mice before addition of a HFD suggesting that some mice are predisposed to be 'high gainers', gaining more weight when fed a HFD then 'low gainers' of the same litter (Koza et al., 2006; Pecoraro et al., 2006). In addition, adiposity during the first 3 weeks of a HFD does not correlate with difference in either food intake or feeding efficiency (Koza et al., 2006).

1.2.1.2 Ob/Ob mouse

The ob/ob mouse, or obese mouse, was discovered by chance in a colony at the Jackson Laboratory in 1949 (Ingalls et al., 1950). It took over 40 years to discover, through positional cloning, that a spontaneous single base deletion in the coding region of the leptin gene was responsible for the obese mutation. (Zhang et al, 1994). Leptin is produced in adipose tissue and plays a central role in appetite regulation. As a result, ob/ob mice have uncontrolled food intake and become massively obese (Friedman and Halaas, 1998). At birth, ob/ob mice are indistinguishable from their littermates, but after 2 weeks, they become heavier and develop hyperinsulinemia. While leptin deficiency is very rare in humans, ob/ob mice will exhibit many of the metabolic abnormalities of obesity and diabetes such as insulin resistance, non-fasting hyperglycemia, impaired glucose intolerance and adipose tissue abnormalities (Lindström, 2007).

1.2.1.3 Agouti mouse

The agouti gene is normally transiently expressed in the follicular melanocyte, where it functions to control the production of pigmentation (Millar et al., 1995). Its mutation however, gives rise to obese, yellow mice. There are five dominant agouti mutations, the most common and useful model of obesity being the lethal yellow mutant

mouse, which is characterized by the deletion of 120-170 kb genomic DNA, resulting in ubiquitous expression of the agouti gene (Duhl et al., 1994; Michaud et al., 1994). While the mechanism of agouti modulation of pigmentation is largely understood, the mechanism of agouti action in obesity is not. One proposal is that the agouti mutation results in increased expression of fatty acid synthase and stearoyl-CoA desaturase, two key enzymes in fatty acid synthesis (Jones et al., 1996), while another is that the agouti mutation results in disruption of the melanocortin-4 receptor, expressed in the brain, causing hyperphagia and hyperinsulinemia (Huszar et al., 1997). Yellow mutant mice develop obesity, hyperinsulinemia, hyperglycemia and increased likeliness to develop a variety of spontaneous and induced tumours (Klebig et al., 1995). The agouti mouse is highly relevant to human obesity as the agouti gene is overexpressed in the adipose tissue of obese patients with type II diabetes (Smith et al., 2003).

1.2.1.4 Zucker fatty rat

The Zucker fatty rat share many similarities with obese humans who have insulin-resistance and type II diabetes, as well as a strong genetic component in the transmission of obesity (Alonso-Galicia et al., 1996). The Zucker fatty rat contains a spontaneous recessive mutation in the 'fatty (fa) gene', shown to be the leptin receptor gene (Zucker, 1965; Ogawa et al., 1995). Like the ob/ob mouse, this mutation causes the Zucker fatty rat to be hyperphagic and therefore develop early onset obesity, hyperinsulinemia, hypercholesterolemia, hyperlipidemia and hypertension (Kurtz et al., 1989). The Zucker fatty rat exhibits insulin resistance, although showing normal blood sugar levels, and is commonly used as a model of obese metabolic syndrome.

1.2.1.5 Wistar fatty rat

The Wistar fatty rat was developed in 1981 by crossing the obese Zucker fatty rat and the Wistar-Kyoto rat, transferring the 'fattty (fa) gene' (Ikeda et al., 1981). The Wistar fatty rat develops obesity and obesity-related symptoms, such as hyperinsulinemia, hyperlipemia, and hypertension in a manner similar to the obese Zucker fatty rat. Male Wistar fatty rats display obesity 3 weeks after birth and develop severe hyperglycemia, glucosuria, and polyuria as early as 8 weeks of age. Female Wistar fatty rats, however, only demonstrate some insulin resistance and mild glucose intolerance (Kava et al., 1989).

1.2.2 *In vitro models of adipogenesis*

1.2.2.1 3T3-L1 and 3T3-F442A cell lines

The transcriptional cascade regulating adipogenesis has been extensively studied through the use of the mouse 3T3-L1 and 3T3-F442A pre-adipocyte cells lines. The 3T3-L1 and 3T3-F442A cell lines are well-established pre-adipocyte cell lines that reiterate the development of adipocytes *in vivo* (Rosen and Spiegelman, 2006). Both cells lines were isolated from Swiss 3T3 cells derived from disaggregated 17-19 day mouse embryos (Green and Kehinde, 1975). With addition of an adipogenic cocktail containing supraphysiological levels of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and insulin, 3T3-L1 cells will differentiate into mature, lipid filled adipocytes (Green and Kehinde, 1974). Compared to the 3T3-L1 cell line, the 3T3-F442A cell line is committed at a later stage in adipocyte development and requires only insulin to differentiate. When implanted into nude mice, both 3T3-L1 and 3T3-F442A cells will produce fat pads that are histologically and biochemically identical to that of the host (Green and Kehinde, 1979). Both cell lines were developed through clonal expansion and therefore contain one

single cell type that responds homogenously to treatments. In addition, these cells are immortalized, allowing for a consistent source of pre-adipocytes, although capacity to differentiate into adipocytes declines with increasing number of passages. For these reasons, the 3T3-L1 and 3T3-F442A cells line are the most frequently used *in vitro* cell lines in adipocyte research.

1.2.2.2 Ob17 cell line

The Ob17 cell line is derived from adipocyte precursors present in the epididmyal fat pads of the genetically obese ob/ob mouse (Négrel et al., 1978). With respect to the 3T3-L1 and 3T3-F442A cell lines, the Ob17 cell line is used less frequently in adipocyte research. The Ob17 cell line represents a much later pre-adipocyte commitment stage then the 3T3-L1 and 3T3-F442A cells lines and requires only insulin to differentiate into mature adipocytes (Négrel et al., 1978; Vannier et al., 1996). When implanted into nude mice, Ob17 cells will also develop into fat pads that are histologically and biochemically identical to that of the host (Green and Kehinde, 1979).

1.2.2.3 Stromal Vascular Fraction (SVF)

The SVF is isolated after the centrifugation of collagenase treated WAT, causing the mature white adipocytes to float, while the SVF forms a pellet. It is commonly used to study pre-adipocyte development and is one of the closest *in vitro* representations of human adipogenesis because instead of containing just one cell type, the SVF contains multiple different cells (see section 1.1). ADSCs from the SVF can be induced to differentiate into adipocytes, osteoblasts, chondrocytes, and myocytes under specific, optimized conditions (Zuk et al., 2001). Identification of the different cells types within

the SVF can be accomplished using clusters of differentiation (CD) markers. Adipose derived stem cells are defined by the phenotype CD31-. CD34+, CD45-, CD90+, CD105-, CD145-, while endothelial cells are defined by CD34+/CD31+ and macrophages by CD14 and CD31, allowing for the selection of specific cell types in the SVF by magnetic bead sorting or flow cytometry (Sengenès et al., 2005; Armani et al., 2010). Injection of CD29+, CD34+,Sca1+,CD24+ cells from the SVF into mice that genetically lack WAT, resulted in growth of WAT (Rodeheffer et al., 2008). Studies using the SVF have demonstrated that pre-adipocyte differentiation is defined by several factors including species, location of adipose depot, and donor age (Poulous et al., 2008).

1.2.3 Chosen Models of Adipogenesis used in this Thesis

For all *in vitro* experiments in this thesis, the 3T3-L1 pre-adipocyte cell line was used. The 3T3-L1 cell line is highly characterized and well established, making it an ideal cell line to study pre-adipocyte development. Diet induced obesity in C57BL/6 mice was the chosen model for the *in vivo* studies as these mice are highly susceptible to obesity when fed a HFD.

1.4 Regulation of Adipogenesis

1.4.1 Adipocyte development

The development from pre-adipocyte to mature adipocyte involves four stages – growth arrest, clonal expansion, early differentiation and terminal differentiation, each stage characterized by a unique pattern of gene expression (Macdougland and Lane, 1995). Pre-adipocytes, in cell culture, must undergo growth arrest at the G0/G1 cell cycle boundary before differentiating (Reichert and Eick, 1999). It is growth arrest, and not

cell-cell contact, that is required for adipocyte differentiation. The 3T3-F442A pre-adipocyte cell line can still undergo differentiation when shifted to a methylcellulose-stabilized suspension culture (Pairault and Green, 1979), and primary rat pre-adipocytes plated at low density in serum-free medium can also differentiate in absence of cell-cell contact (Gregoire et al., 1998).

Following growth arrest, pre-adipocytes must receive an appropriate combination of mitogenic and adipogenic signals before re-entering the cell cycle and undergoing at least 2 rounds of cell division, referred to as clonal expansion. Inhibiting clonal expansion will prevent differentiation. Blocking the cell cycle with aphidicolin (Reichert and Rick, 1999), rapamycin (Yeh et al., 1995) or roscovitine (Tang et al., 2003) disrupts pre-adipocyte differentiation, while disrupting the pro-adipogenic transcription factor CCAAT enhancer binding protein-β (C/EBPβ) prevents mitotic clonal expansion and consequently adipogenesis. Once clonal expansion is complete, cells will exit the cell cycle and begin to terminally differentiate.

Early and terminal differentiation is accompanied by changes in several transcription factors and proteins (see section 1.4.2). Dramatic modifications in adipocyte morphology occur where the cells change from a fibroblastic phenotype to spherical shape. There are also changes in the level and types of extra cellular matrix (ECM) as well as the levels of cytoskeleton components. A decrease in both actin and tubulin precede adipocyte morphological changes (Spiegelman and Farmer, 1982) as well as a decrease in type I and type III procollagen (Weiner et al., 1989) Proteolytic degradation of the ECM of pre-adipocytes by the plasminogen cascade is required for change in cell morphology, adipogenic gene expression and lipid accumulation (Selvarajan et al., 2001).

During terminal differentiation, adipocytes increase *de novo* lipogenesis, increasing the mRNA, protein and activity levels of several enzymes required for lipogenesis including acetyl-CoA carboxylase, stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase (Spiegelman et al., 1983; Weiner et al., 1991). Glucose transporters, insulin receptor number, and insulin sensitivity also increase during the later stage of adipogenesis (Garcia de Herreros and Birnbaum,1989).

1.4.2 Intracellular signalling

The addition of IBMX, DEX and insulin to induce 3T3-L1 differentiation, highlight the importance of the cyclic adenosine monophosphate (cAMP), glucocorticoid and insulin/Insulin-like growth factor-1 (IGF-1) signalling pathways in adipocyte development. 3T3-L1 pre-adipocytes must undergo growth arrest before differentiation, therefore, 48 hours post-confluency, adipogenesis is induced by adding a combination of IBMX and DEX. IBMX is a cyclic nucleotide phosphodiesterase inhibitor that can stimulate adenylyl cyclase activity, resulting in increased cAMP levels (Parsons et al., 1988). DEX is a synthetic glucocorticoid that activates the glucocorticoid receptor and can also act to increase cAMP levels by decreasing expression of cAMP-phosphodiesterase (Ahlström et al, 2005). Increased cAMP activates the downstream effectors, protein kinase A (PKA) (Taylor et al., 1990) and exchange protein directly activated by cAMP (EPAC-1, EPAC-2) (de Rooij et al., 1998). The cAMP/PKA pathway has been demonstrated to stimulate adipogenesis in 3T3-L1 cells by activating the downstream transcription factor cAMP-responsive element-binding protein (CREB)

(Reusch et al., 2000), which induces expression of the C/EBP-β (Otto and Lane, 2005). EPAC -1,-2 promotes adipogenesis via activation of the Ras superfamily protein Rap 1, as well as by enhancing IGF-1-dependent activation of protein kinase B (PKB) (Petersen et al., 2008). cAMP signalling, resulting in simultaneous activation of PKA and EPAC-1,-2, is necessary for the development of adipocytes from mesenchymal stem cells (Jia et al., 2012). Further, the synergistic activity of PKA and EPAC -1,-2 is required for cAMP mediated differentiation of 3T3-L1 cells (Petersen et al., 2008).

Forty-eight hours after IBMX and DEX addition, insulin is added to the cells, initiating a series of complex pathways. Addition of insulin activates the IGF-1 signalling pathway by recruiting and phosphorylating insulin receptor substrate proteins, leading to the activation of the phosphatidylinositol 3-kinase (PI3-Kinase) pathway. Activation of the PI3-Kinase pathway via insulin results in further stimulation of the pro-adipogenic transcription factor CREB, while inhibition of PI3-Kinase blocks insulin induced adipogenesis. Activation of PI3-Kinase stimulates downstream PKB. Constitutively active PKB induces spontaneous adipogenesis in 3T3-L1 pre-adipocytes (Magun et al., 1996). Further, activation of the PI3-Kinase pathway aids in the regulation of glucose metabolism, including glucose uptake and glycogenesis, promoting *de novo* fatty acid synthesis (Satoh, 1993).

1.4.3 Transcriptional regulation of adipogenesis

Two major transcription families, Peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPs, control the transition through each stage of adipocyte development. C/EBPβ and C/EBPδ are expressed early during adipogenesis, with increasing expression

upon addition of IBMX and DEX. The importance of C/EBPβ and C/EBPδ in adipogenesis is evidenced by the fact that over-expression of either protein in 3T3-L1 pre-adipocytes accelerates adipogenesis (Wu et al., 1995). Their expression is responsible for the regulation of C/EBPα and PPARγ, the 'master regulators' of adipogenesis (Fig. 1) (Cao et al., 1991; Lin and Lane, 1994; Clarke et al., 1997).

Both C/EBPα and PPARγ are transcriptionally activated by C/EBPβ and C/EBPδ and once expressed, PPARγ and C/EBPα positively regulate each others expression (Schwarz et al., 1997; Shao & Lazar, 1997). C/EBPα and PPARγ expression is both necessary and sufficient to promote adipogenesis. Overexpression of C/EBPα in 3T3-L1 pre-adipocytes induces differentiation into a mature adipocyte (Lin and Lane, 1994) while blocking its expression with antisense RNA inhibits adipogenesis (Lin and Lane, 1992). Further, mice with a homozygous deletion of the C/EBPα gene have dramatically reduced accumulation of WAT (Wang, 2005). Overexpression of PPARγ in fibroblastic cells induces growth arrest (Tontonoz et al., 1994) and adipogenesis (Sandouk et al., 1993) while knockout of PPARγ in mature adipocytes *in vivo* leads to death of adipose tissue (Rosen and Spiegelman, 2006). In addition, the development of white adipose tissue in chimeric mice composed of wild-type and PPARγ (-/-) embryonic stem cells, have a strong preference toward PPARγ expression (Rosen et al., 1999).

There are several other transcription factors and proteins that play a role in adipogenesis. Pre-adipocyte factor 1 (Pref-1) is an inhibitor of adipocyte differentiation that is highly expressed in pre-adipocytes, but not detectable in mature adipocytes (Smas, 1993). At the pre-adipocyte stage, expression of Pref-1 actively inhibits adipocyte maturation via downregulation of C/EBPα and PPARγ2 expression (Kim, 2007).

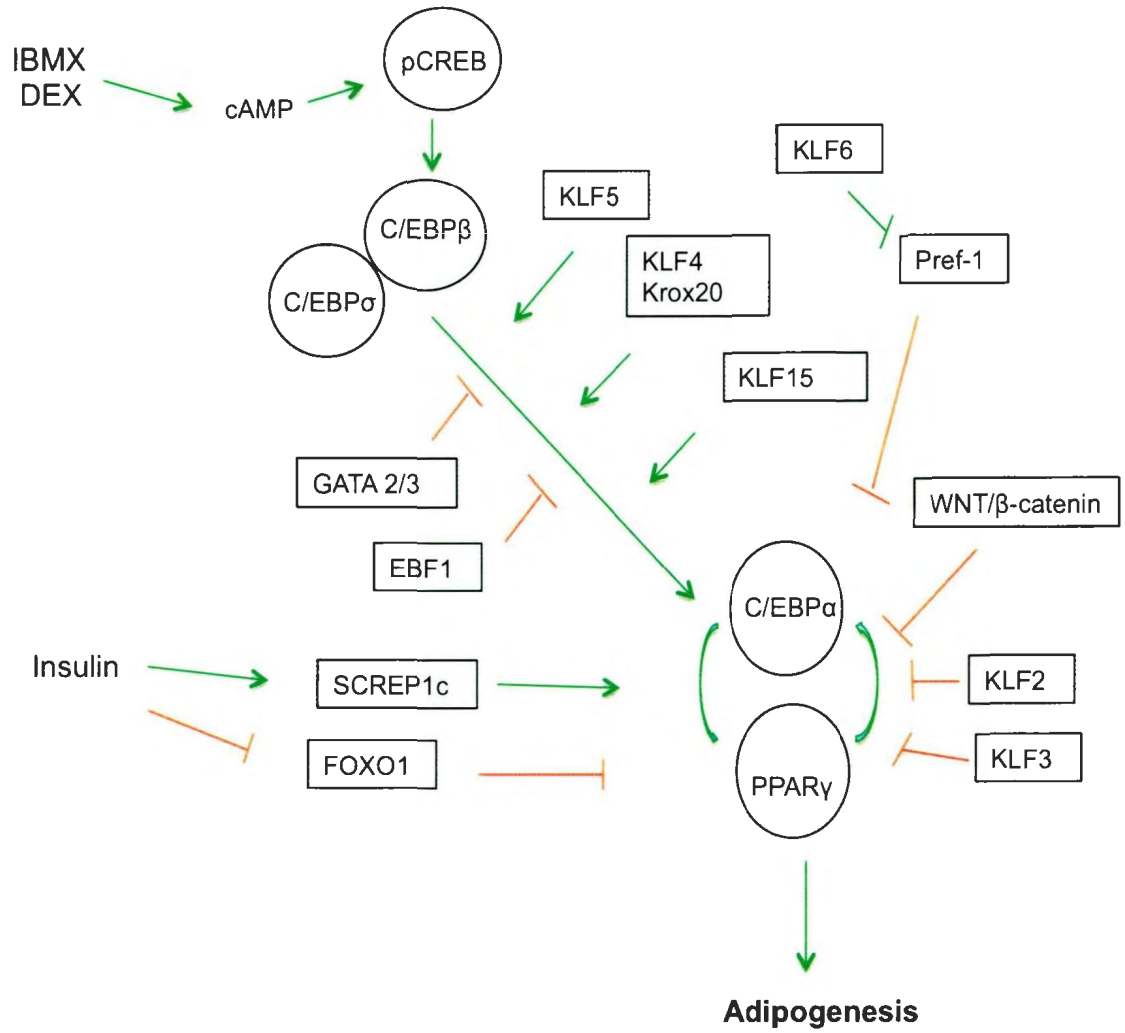

Fig. 1. **Transcriptional regulation of adipogenesis in 3T3-L1 cells.** IBMX and DEX and insulin induce a transcriptional cascade involving several proteins as described in section 1.4.2. IBMX and DEX increase levels of cyclic adenosine monophosphate (cAMP), resulting in phosphorylation (represented by a lower case p) of cAMP response element-binding protein (CREB) and activation of CCAAT/enhancer binding protein β and δ (C/EBPβ, C/EBPδ). Krupple like factor 5 (KLF5), KLF4 and Krox20, and KLF5 are pro-adipogenic, working together with C/EBPβ and C/EBPδ to induced C/EBPα and PPARγ expression, the master regulators of adipogenesis, while KLF2 and KLF3 are anti-adipogenic. KLF6 is pro-adipogenic, inhibiting pre-adipocyte factor 1 (Pref-1) expression, abolishing its inhibitory effects on C/EBPα and PPARγ . Early B cell factor-1 (EBF1) and sterol regulatory element binding transcription factor 1(SREBP1c) promote differentiation while forkhead transcription protein FOXO1 (FOXO1), globin transcription factors 2 and 3 (GATA2, GATA3), and WNT/β-catenin are inhibitory. Circles represent major transcription factors (TF), while squares represent other regulatory TF involved in adipogenesis. Green indicates pro-adipogenic factors, while red represents anti-adipogenic factors.

Increased expression of the pro-adipogenic transcription factor, kruppel-like factor 6 (KLF6), downregulates Pref-1 expression to promote adipogenesis (Li, 2005). Several other members of the KLF family also play a role in adipogenesis. KLF5, KLF4 and KLF15 are early pro-adipogenic transcription factors, working with C/EBPβ and C/EBPδ to induce adipogenesis. KLF5 is expressed early during adipogenesis, where C/EBPβ and C/EBPδ bind directly to the KLF5 promoter, and work together to facilitate the expression of PPARγ (Oishi et al., 2005). KLF4 binds to the C/EBPβ promoter, and along with Krox 20 (Chen et al., 2005), increases expression of C/EBPβ, promoting adipogenesis (Birsoy et al., 2008). Expression of KLF15 is induced by C/EBPβ and C/EBPδ at the initial stages of adipogenesis and KLF15 works synergistically with C/EBPα to increase PPARγ expresion (Mori et al., 2005). KLF2 and KLF3, however, are anti-adipogenic, and function directly on PPARγ and C/EBPα (Sue et al., 2008). Constitutive expression of KLF2 results in decreased expression of PPARγ, but has no effect on C/EBPβ and C/EBPδ expression (Banerjee et al., 2003), while the overexpression of KLF3 blocks adipocyte differentiation due to its direct association with the C/EBPα promoter (Sue et al., 2008).

In addition, early B cell factor-1 (EBF1) (Jimenez et al., 2007) and sterol regulatory element binding transcription factor 1 (SREBP1c) (Kim et al., 1998) promote differentiation, while forkhead transcription protein FOXO1 (FOXO1) (Nakae et al.,2003) and globin transcription factors 2 and 3 (GATA2 and GATA3) (Jack and Crossley, 2010) are inhibitory. Furthermore, WNT/β-catenin signalling inhibits adipogenesis by blocking induction of PPARγ and C/EBPα (Christodoulides et al., 2009). Addition of insulin to 3T3-L1 pre-adipocytes increases expression of pro-adipogenic SREBP1c, while it inhibits anti-adipogenic FOX01 via PKB (Kim et al., 1998; Nakae et al., 2003). Thus, both an

increase in pro-adipogenic gene expression and a decrease in anti-adipogenic gene expression are necessary for successful adipogenesis.

While the regulation of adipogenesis from pre-adipocyte to mature adipocyte is well established, little is known about the early molecular events defining pre-adipocyte development. The Friedman group (Rodeheffer et al., 2008) identified potential adipocyte progenitor cells by using fluorescence-activated cell sorting (FACS) to examine the SVF from WAT. They sorted the isolated SVF cells based on previously known stem cell markers and identified both a CD24+ and a CD24- subpopulation of early adipocyte progenitor cells that, with addition of an adipogenic cocktail, could differentiate *in vitro* into mature adipocytes. To determine if CD24+ and/or CD24- cells could differentiate *in vivo*, the Friedman group injected the cells into A-Zip lipodystrophic mice. A-Zip lipodystrophic mice lack WAT as they contain a dominant negative protein (A-Zip/F), which interferes with the C/EBPα transcription, thereby preventing adipocyte differentiation (Moitra, 1998). After injection, it was the CD24+ population that led to development of fat depots that showed normal morphology and function, suggesting CD24+ cells may be a source of white adipocyte precursors.

1.5 Cluster Of Differentiation 24 (CD24)

CD24 is a heavily glycosylated glycosylphosphatidylinositol (GPI) linked cell surface receptor found on a variety of cells types including cancer cells, precursor B-cells, neurons, epithelia cells, macrophages and pre-adipocytes, among others (Fang and Zhen, 2010). It is expressed highly on progenitor cells and to a lesser extent on terminally differentiated cells. CD24 expression can direct cell survival in a cell type dependent manner. It is overexpressed in many types of cancers, causing cancer cell growth,

proliferation and metastasis (Agrawal et al., 2007; Huang and Hsu, 1997; Sagiv et al., 2002), while it induces apoptosis in precursor B cells (Hough et al., 1996; Suzuki et al., 2001). The molecular role of CD24 in adipogenesis is not known.

CD24 mRNA levels can be controlled at the transcription level downstream of phospholipase C-β1 in erythroleukemia cells (Fiume, 2005), the Ral GTPase in bladder cancer cells (Smith, 2006) and the dysregulated Hedgehog signalling in breast cancer cells (Cao, 2012). The transcription factor nuclear factor of activated T-cells 5 (NFAT5) can upregulate CD24 expression in T lymphocytes by inhibiting hypermethylation of H3K27 (lysine 27 of histone 3) (Berga-Bolanos, 2010), while the estrogen receptor can downregulate CD24 in breast cancer cells by chromatin remodelling via histone deacetylase (Kaipparettu, 2008). In addition, the transcription factor Twist can repress CD24 expression in breast cancer cells by binding to the CD24 promoter, and thereby downregulating its expression (Vesuna, 2009). Twist is a transcription factor involved with cell lineage determination and differentiation, and when overexpressed in mesenchymal stem cells (MSC), the MSC display a decreased capacity for osteo/chondrogenic differentiation and an enhanced capacity to undergo adipogenesis (Cakouros et al., 2010). Furthermore, both NFAT5 (also known as TonEBP) (Ferraris, J., 2004) and Twist (Firulli, A., 2008) can be activated by PKA, a mediator in 3T3-L1 adipocyte differentiation.

CD24 mRNA levels can also be regulated by mRNA stability. CD24 mRNA is composed of a short 0.23 kb open reading frame and a long 1.3 kb 3'untranslated region (3' UTR). Zhou et al. (Zhou et al., 1998) demonstrated that CD24 mRNA stability could be regulated by both positive and negative cis elements in the 3'UTR, which modulate CD24 mRNA degradation.

The factor(s) that regulate CD24 expression in adipocytes are not known. Identification of CD24 regulators could make it possible to regulate CD24 expression in a tissue-specific manner and therefore, regulate cell survival and/or differentiation.

1.6 Hypothesis and Objectives of Thesis

Deciphering the early molecular events in adipogenesis is essential for fully understanding the process of adipogenesis and for the possible development of therapeutics. The purpose of this thesis was to examine the role of CD24 in adipogenesis using both *in vitro* and *in vivo* approaches. My major hypothesis is that **CD24 directly regulates adipogenesis.** To address this hypothesis, my objectives were:

1. Determine the CD24 mRNA and protein expression levels over the course of adipocyte development *in vitro* using the 3T3-L1 pre-adipocyte cell line.

2. Identify the intracellular mediators regulating CD24 expression *in vitro*.

3. Determine the CD24 mRNA and protein expression levels in C57BL/6 mice fed a high fat diet compared to those fed a low fat diet.

4. Determine if activation of CD24 signalling, by antibody mediated clustering, is sufficient to affect adipogenesis *in vitro* and *in vivo*.

2.0 Materials and Methods

2.1 Antibodies

The antibodies (Abs) specific for CD24 (HIS50) and secondary Ab were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The Ab specific for actin was purchased from Sigma (St. Louis, MO).

2.2 3T3-L1 Adipogenesis assay

All media and supplements were obtained from Life Technologies (Burlington, ON) unless otherwise indicated. 3T3-L1 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% newborn calf serum and 5% antibiotic/antimycotic (DMEM/NCS). 3T3-L1 cells were grown to confluency in 6-well (for protein analysis) or 12-well plates (for RNA analysis). To initiate adipogenesis, 48 hours post-confluency, 0.5mM IBMX (Millipore, Billerica, MA) and 0.1μM DEX (Millipore) in DMEM supplemented with 10% fetal bovine serum and 5% antibiotic/antimycotic (DMEM/FBS) was added to the cells. 48 hours later, media was replaced with 10 μg/ml insulin (Millipore) in DMEM/FBS. 48 hours later, media was replaced with progression media composed of only DMEM/FBS. Cells were maintained in this media for 5 days, replacing media every 2 days, until mature adipocytes were observed. Control cells were maintained in DMEM/NCS. Samples were taken 6 hours after addition of IBMX and DEX, insulin and progression media in Trizol (Invitrogen, Carlsbad, CA), for mRNA analysis or radioimmunoprecipitation assay (RIPA) lysis buffer (RIPA: 50mM Tris-HCL, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150mM NaCl; plus inhibitors: 1mM phenylmethanesulfonylfluoride,

1mM sodium orthovanadate, 1ug/ml aprotinin, 1x HALT Phosphatase inhibitor (Thermoscientific) for protein analysis.

2.2.1 Cycloheximide, actinomycin and forskolin treatment

3T3-L1 cells were treated with 20μg/ml of cycloheximide (CHX) (Calbiochem) or 1μg/ml of actinomycin-D (ACT) (Calbiochem) for 1 hour before addition of IBMX and DEX. Cells were treated with forksolin (Calbiochem) or inactive forskolin (iForskolin; Calbiochem) at various concentrations (1μm, 2.5μm, 10μm, 20μm). Control samples were treated with equivalent volumes of dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Six hours after treatment, samples were taken for mRNA analysis.

2.2.2 CD24 antibody treatment

CD24 antibody (HIS50) or normal mouse serum IgM was added to 3T3-L1 cells at various concentrations (400ng/ml, 800ng/ml, 1200ng/ml) along with IBMX/DEX and refreshed at every media change throughout the assay. Cells were maintained in this media for 5 days, until mature adipocytes were observed in the positive control well and then samples were taken for mRNA analysis.

2.3 Animal diets and procedures

2.3.1 HFD experiment performed by Dr. Hirasawa in 2009

The first HFD experiment was completed by Dr. Michriu Hirasawa, who donated a portion of the WAT from the epididymal fat depot. Male C57BL/6 mice (3 weeks old) (Charles River, Wilmington, MA) were fed a high fat diet (HFD) containing 45% energy from fat (Research Diets, Inc., New Brunswick, NJ D12451) or a low fat control diet

(LFD) containing 10% energy from fat (Research Diets, Inc. D12450B) for a period of 0-7 days. Mice were anethezised using halothane, sacrificed through cervical dislocation and the epididymal WAT was immediately frozen in liquid nitrogen for RNA extraction.

2.3.2 HFD experiment performed by Christian lab in 2012

Three-week-old C57BL/6 mice were fed a HFD (45% energy from fat) or LFD (10% energy from fat) for 1-7 days. Mice were anethezised using isoflurane, sacrificed through cervical dislocation and the epididymal WAT was immediately weighed and frozen in liquid nitrogen for RNA extraction or solubilized in RIPA buffer for protein analysis.

2.3.3 Clustering of CD24 by anti-CD24 antibody injection in vivo

To induce clustering of CD24 *in vivo,* 3-week-old C57BL/6 male mice were injected intraperitoneally with 200μg of CD24 (M1/69) functional grade antibody in 200μl saline (10mM NaH2PO4+150mM NaCl), or 200μg of Rat IgG2b K isotype control (eB149/10H5) in 200μl saline, or 200μl of saline every 2 days, starting on day 0, over a period of 6 days. Mice were fed HFD (45% energy from fat) or LFD (10% energy from fat), starting on day 0. Mice were anesthetised using isoflurane, sacrificed by cervical dislocation and the epididymal WAT was immediately weighed and either frozen in liquid nitrogen for RNA extraction or fixed in 4% phosphate buffered paraformaldehyde for histology.

All animal procedures were approved by Memorial University's Institutional Animal Care Committee.

2.6 RNA Isolation, Dnase Treatment and cDNA synthesis.

All procedures were carried out according to the manufacturer's protocol. RNA was isolated using Trizol (Invitrogen), contaminating DNA removed with TURBO Dnase (Life Technologies) and cDNA synthesized from 500ng of RNA using MMLV-RT (Invitrogen).

2.7 Quantitative Reverse Transcriptase PCR (qRT-PCR)

Fermentas Maxima SYBR Green qPCR Master Mix (2X) (ThermoScientific, Waltham, MA) was used to carry out Reverse transcriptase polymerase chain reaction (RT qPCR) using 10ng of cDNA in an Eppendorf Mastercycler instrument. Sequence, efficiency and amplicon size of all primer sets can be found in Table 1. Ct values were analyzed after 40 cycles according to the Pfaffl equation, which is a modified version of Δ/Δ cycle threshold (Ct) that takes into account the empirically determined efficiency of each primer set (Pfaffl, 2001). All primer sets have been validated and primer efficiencies determined according to the MIQE guidelines (Bustin et al., 2009).

2.8 Western Blot

Protein content was determined using the Bicinchoninic Acid (BCA) Protein Assay Reagent (ThermoScientific) according to the manufacturer's protocol and 20 μg of protein was run on a 10% SDS-PAGE gel. Antibodies were diluted in TBST+ 5% skim milk, CD24 (HIS50) 1:500, actin 1:2000, goat-anti-rabbit IgG 1:2000, and goat-anti-

Table 1. qRT PCR primer sequences

Gene	Sequence	Efficiency	Amplicon Size
CD24	F: 5'-ACT CAG GCC AGG AAA CGT CTC T-3' F: 5'-AAC AGC CAA TTC GAG GTG GAC-3'	1.07	109
RPLP0	F: 5'-TCA CTG TGC CAG CTC AGA AC-3' R: 5'-AAT TTC AAT GGT GCC TCT GG-3'	1.03	101
GAPDH	F: 5'-ATG TGT CCG TCG TGG ATC TGA-3' R: 5'-TGC CTG CTT CAC CAC CTT CTT-3'	1.00	164
Pref-1	F: 5'- CCC CCT TCG TGG TCC GCA AC-3' R: 5'-GGT GGG TCG CAT TCA GCC CC-3'	1.01	182
CEBPα	F: 5'-TGG ACA AGA ACA GCA ACG AG-3' R: 5'-TCA CTG GTC AAC TCC AGC AC-3'	1.00	127
PPARγ	F: 5'-TGT TAT GGG TGA AAC TCT GGG-3' R: 5'-AGA GCT GAT TCC GAA GTT GG-3'	1.00	145
Leptin	F: 5'-ACC AGG CAC CCT TGG AGG GG-3' R: 5'-TGT GGG GCC CTC ACT CCC TG-3'	1.05	78
Adiponectin	F: 5'-GTT GCA AGC TCT CCT GTT CC-3' R: 5'-TCT CCA GGA GTG CCA TCT CT-3'	1.00	192
Perilipin	F: 5'-CTC GGT GTT ACA GGC AAG GA -3' R: 5'-TGT GCC CCT GGT CTC TCA T -3'	0.98	121

mouse IgM 1:2000. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used for signal detection. Images were acquired using an AlphaImager Gel Documentation system with FluorChem HD2 software, version 3.4.0. Image manipulation was limited to adjustments to brightness and contrast of the entire image.

2.9 Histology

WAT and liver samples were processed by the Histology Unit, Faculty of Medicine, Memorial University. Fixed WAT was paraffin embedded, sliced at 5μm and stained with hematoxylin and eosin (H&E). Liver was frozen and stained with Oil Red O. Images were analyzed using ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MA) and Cell Profiler (Carpenter et al., 2006) and cell number determined as previously described (Jo et al). Image manipulation was limited to adjustments to brightness and contrast of the entire image.

2.10 Statistical Analysis

Statistical analysis was performed using a one-way ANOVA if data were normally distributed when more than 2 samples were being compared, followed by a Tukey post hoc test, if significant, and using the Student's t-test when only 2 samples were being compared. The data on cell surface area was not normally distributed; therefore a Kruskal-Wallis test, followed by a Kolmogorov-Smirnov *post hoc* test if significant, was performed. Differences were considered significant at $p<0.05$. Statistical analysis was determined using Graphpad version 6.0 for Mac (GraphPad Software, La Jolla California USA) and VassarStats (Lowry R., 2010).

3.0 Results and Discussion

3.1 CD24 mRNA and protein increase *in vitro* at initiation of adipogenesis

To determine if CD24 plays a role in adipogenesis, changes in CD24 gene expression, along with known adipogenic markers, were examined in the 3T3-L1 pre-adipocyte cell line undergoing adipogenesis, by qRT-PCR and western blot. If CD24 were involved in adipocyte differentiation, then changes in CD24 gene expression would be observed over the course of the adipogenesis assay. The adipogenic markers were chosen based on their known pattern of expression during 3T3-L1 adipocyte development (Burton et al., 2004; Cao et al., 2012; Ntambi and Young-Cheul, 2000; Smas et al., 1998). Pref-1 is expressed on pre-adipocytes and is down-regulated on mature adipocytes (Smas, 1993). PPARγ and CEBPα are major regulators of adipogenesis (Gregoire et al., 1998) and are upregulated early in adipogenesis. Adiponectin is a marker of mature adipocytes (Lihn et al, 2005).

As expected, Pref-1 mRNA expression decreased with adipocyte differentiation, while PPARγ, CEBPα and adiponectin expression increased at the later stages (Fig. 2). CD24 expression significantly increased 6 h after addition of initiation media (IBMX and DEX) and then returned to initial levels as the adipocytes matured (Fig. 2).

To determine if changes to CD24 mRNA expression correlate with changes to protein expression, CD24 protein expression was examined by western blot. Similar to the changes in mRNA expression, CD24 protein levels increased 6 hours after initiation of adipogenesis with IBMX and DEX, and remained high 24 hours later, followed by a return to baseline as the adipocyte matured (Fig.3).

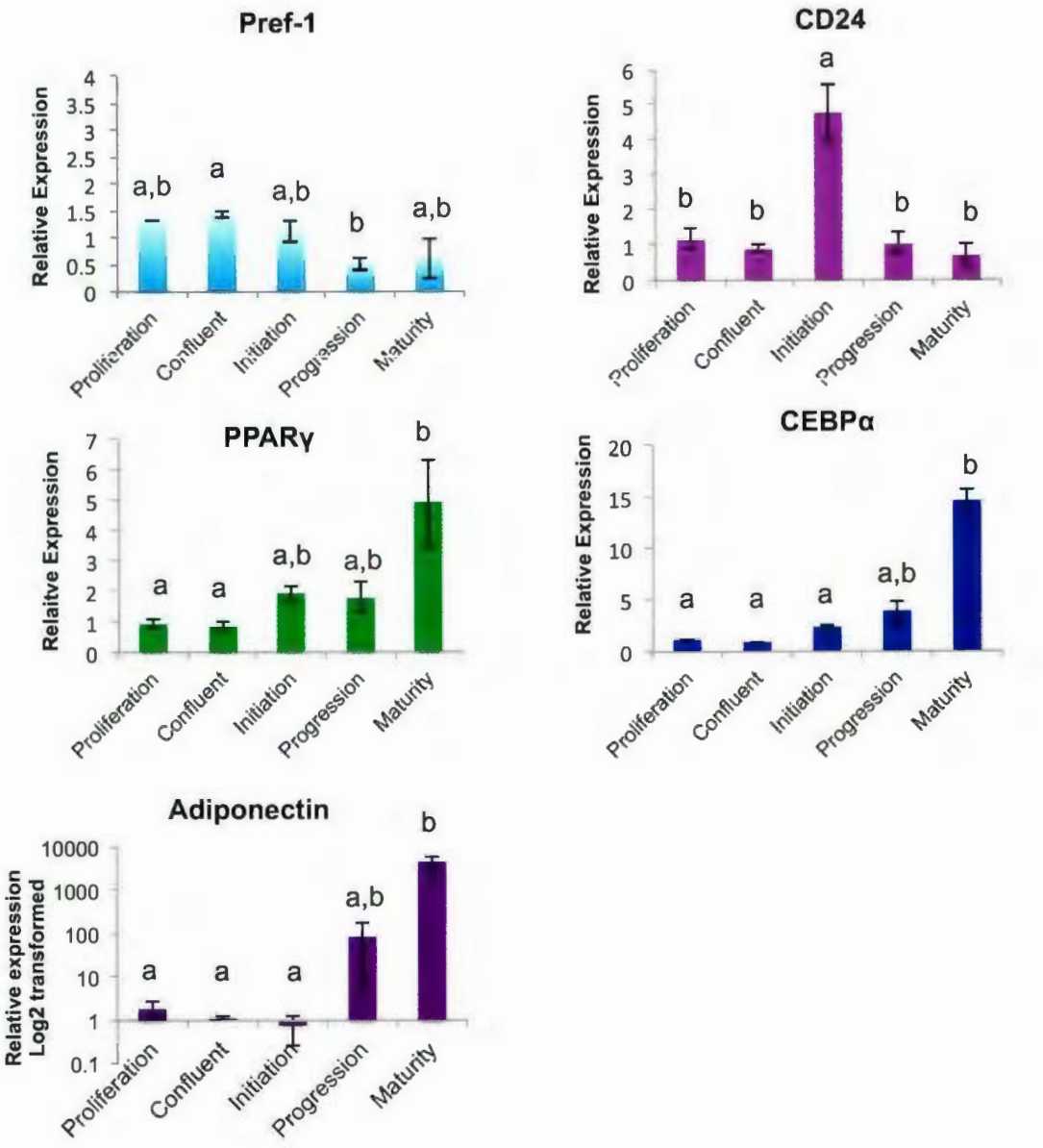

Fig. 2. **Initiation of adipogenesis with addition of IBMX and DEX increased CD24 mRNA expression in 3T3-L1 pre-adipocytes.** mRNA expression of CD24 and known adipogenic markers in the 3T3-L1 cell line was determined throughout the various stages of the adipogenesis assay by qRT PCR. Proliferation: proliferating pre-adipocytes; Confluent: cells are 100% confluent; Initiation: 6 hours after addition of dexamethasone and IBMX; Progression: 6 hours after addition of insulin; Maturity: 5 days after addition of insulin, lipid filled adipocyte. Expression levels were normalized to the internal control RPLP0 using the Pffafl equation (Pfaffl, 2001) and calculated relative to levels in proliferating cells. Data shown as mean ± SE. Expression changes in adiponectin were very large, therefore data for adiponectin is shown as log2 transformed. Statistical significance was determined using one-way ANOVA, Tukey *post hoc*, n=3-4, different lower case letters indicate a significant difference of $p<0.05$ between groups.

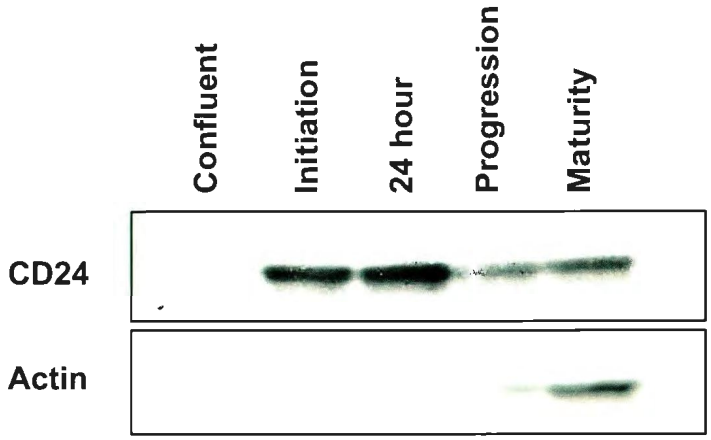

Fig. 3. **Initiation of adipogenesis with addition of IBMX and DEX increased CD24 protein expression in 3T3-L1 pre-adipocytes.** Protein expression of CD24 in the 3T3-L1 cell line during the various stages of the adipogenesis assay was determined by western blot analysis. Confluent: cells are 100% confluent; Initiation: 6 hours after addition of IBMX and DEX; 24 hour: 24 hours after DEX and IBMX addition; Progression: 6 hours after addition of insulin; Maturity: 5 days after addition of insulin, lipid filled adipocyte. Actin is used a loading control. A representative of 3 experiments is shown.

The expression pattern of the adipogenic genes Pref-1, CEBPα and adiponectin that was observed throughout 3T3-L1 adipocyte development was expected and similar to that of previous studies (Burton et al., 2004; Cao et al., 2012; Ntambi and Young- Cheul, 2000; Smas et al., 1998). There was significant decrease in Pref-1 mRNA expression and a significant increase in PPARγ, CEBPα and adiponectin mRNA expression as the adipocytes matured. The increase in CD24 expression was observed earlier than the increase in PPARγ, CEBPα and adiponectin suggesting that CD24 functions during the initial stages of adipogenesis. Furthermore, the return of CD24 to baseline level expression suggests that down-regulation of CD24 may be necessary for full differentiation.

3.2 cAMP is sufficient to regulate CD24 mRNA expression in response to adipogenic media

To begin to elucidate the signalling pathway that regulates CD24 mRNA expression levels in response to IBMX and DEX, the contribution of each individual component of the adipogenic media in regulating the increase in CD24 expression was examined. Confluent 3T3-L1 cells were treated with IBMX, DEX or both IBMX and DEX for 6 hours, which is when the increase in CD24 mRNA was observed, and CD24 mRNA expression was determined by qRT-PCR. While treatment with IBMX or DEX significantly increased CD24 expression compared to confluent cells, it was the combination of both that caused the highest increase in expression, suggesting that IBMX and DEX function additively to regulate CD24 mRNA levels (Fig.4a).

IBMX is a cyclic nucleotide phosphodiesterase inhibitor that can also stimulate adenylyl cyclase activity, resulting in increased cAMP levels (Parsons et al., 1988). DEX

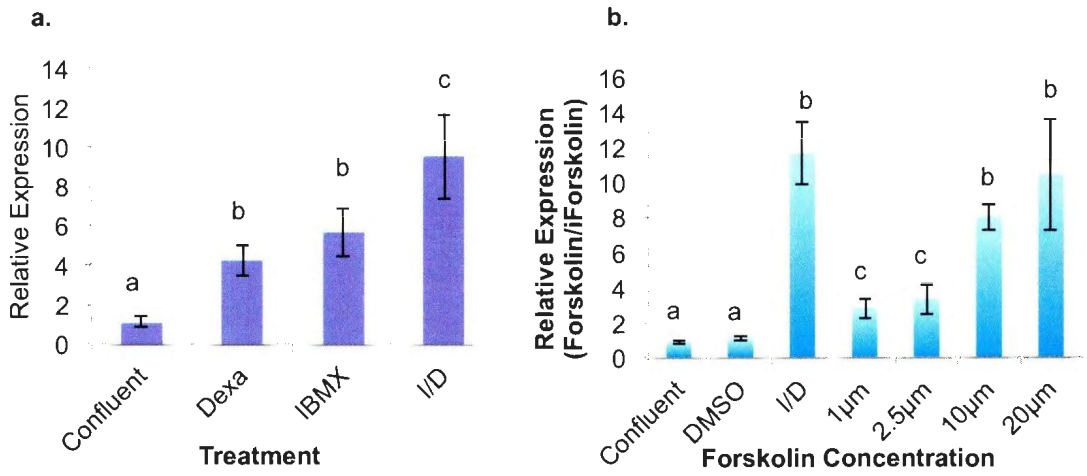

Fig. 4. **A. IBMX and DEX act additively to increase CD24 expression while B. Forksolin increased CD24 mRNA expression in 3T3-L1 pre-adipocytes. a.** 3T3-L1 cells were treated with DEX, IBMX or IBMX and DEX for 6 hours and mRNA expression of CD24 was determined by qRT PCR. **b.** 3T3-L1 cells treated with Forskolin at the concentrations indicated and CD24 mRNA expression was determined by qRT PCR. Ratio of CD24 expression in Forskolin treatment vs inactive Foskolin (iForskolin) was calculated. CD24 expression levels were normalized to the internal control RPLP0 using the Pffafl equation and calculated relative to levels in **a.** untreated confluent cells **b.** DMSO-treated cells. Data shown as mean ± SE. Statistical significance determined using one-way ANOVA, Tukey *post hoc*, n=4, different lower case letters indicate a significant difference of $p<0.05$

is a glucocorticoid that activates the glucocorticoid receptor, and can also act to increase cAMP levels by decreasing expression of cAMP-phosphodiesterase (Ahlstrom et al., 2005).transcription factors involved in adipogenesis (Elks and Manganiello, 1985). DEX has also been shown to inhibit cAMP phosphodiesterase expression, further increasing cAMP levels (Ahlström M, 2005). To determine if an increase in cAMP is sufficient to regulate CD24 mRNA expression, 3T3-L1 cells were treated with forskolin, its inactive form (1,9-dideoxy-forskolin) or DMSO alone, as both forskolin and its inactive form were solubilized in DMSO. Forskolin is an activator of adenylate cyclase (de Souza et al., 1983), which increases cAMP levels. Treatment with forskolin increased CD24 mRNA in a dose-dependent manner, while inactiave forskolin had no affect on CD24 mRNA expression. Therefore this indicates that an increase in cAMP is sufficient to regulate CD24 expression in pre-adipocytes (Fig. 4b).

The regulation of CD24 by cAMP has never before been demonstrated and provides a clear explanation for the previously observed discrepancy in the ability of CD24- cells to undergo adipogenesis *in vivo* compared to *in vitro* (Rodeheffer et al., 2008). As shown here, addition of supraphysiological levels of IBMX and DEX, increases the expression level of CD24 *in vitro* (Figs. 1 and 2). Therefore, it is reasonable to propose that CD24 expression was induced in cells that were originally identified as CD24- in response to initiation of adipogenesis *in vitro,* which would have resulted in sufficient CD24 expression to allow normal adipogenesis to occurs. Future experiments determining the ability of cells expression low levels of CD24 expression to increase expression in response to supraphysiological levels of IBMX and DEX are necessary to definitively answer this outstanding question.

3.3 The increase in CD24 mRNA in response to IBMX and DEX is dependent on transcription and not mRNA stability.

CD24 mRNA levels can be regulated at the level of transcription (Vesuna et al., 2009; Wenger et al., 1993) or by mRNA stability (Zhou et al., 1998). To determine if CD24 mRNA is regulated by transcription, 3T3-L1 cells were treated with actinomycin-D (ACT), a transcription inhibitor (Sobell, 1985), for 1 h followed by IBMX/DEX for 6 h. If the increase in CD24 expression was inhibited with ACT pre-treatment then that would suggest that the increase in CD24 was dependent on transcription. Results showed that treatment with ACT inhibited the increase in CD24 expression stimulated with addition of IBMX/DEX, demonstrating that active transcription is necessary for the increase in CD24 mRNA expression in 3T3-L1 cells (Fig. 5a).

To determine if CD24 mRNA expression was regulated through *de novo* protein synthesis of a regulatory protein, 3T3-L1 cells were pre-treated with cycloheximide for 1h followed by IBMX/DEX for 6h. Cycloheximide inhibits translational elongation, therefore blocking all *de novo* protein synthesis (Schneider-Poetsch, 2010). If blocking protein synthesis by treatment with CHX prevented the increase in CD24 mRNA expression observed after IBMX and DEX treatment, then CD24 mRNA levels are likely regulated by *de novo* expression of a protein regulator, most likely a transcription factor. Conversely, if the increase in CD24 mRNA expression is not blocked by CHX pre-treatment, then the protein regulator must be pre-existing. A significant increase in CD24 mRNA was observed with the pre-treatment of CHX followed by IBMX and Dex treatment. This increase was to the same level as IBMX and Dex only treatment, demonstrating that the increase in CD24 mRNA expression does not require *de novo* protein synthesis (Fig. 5b).

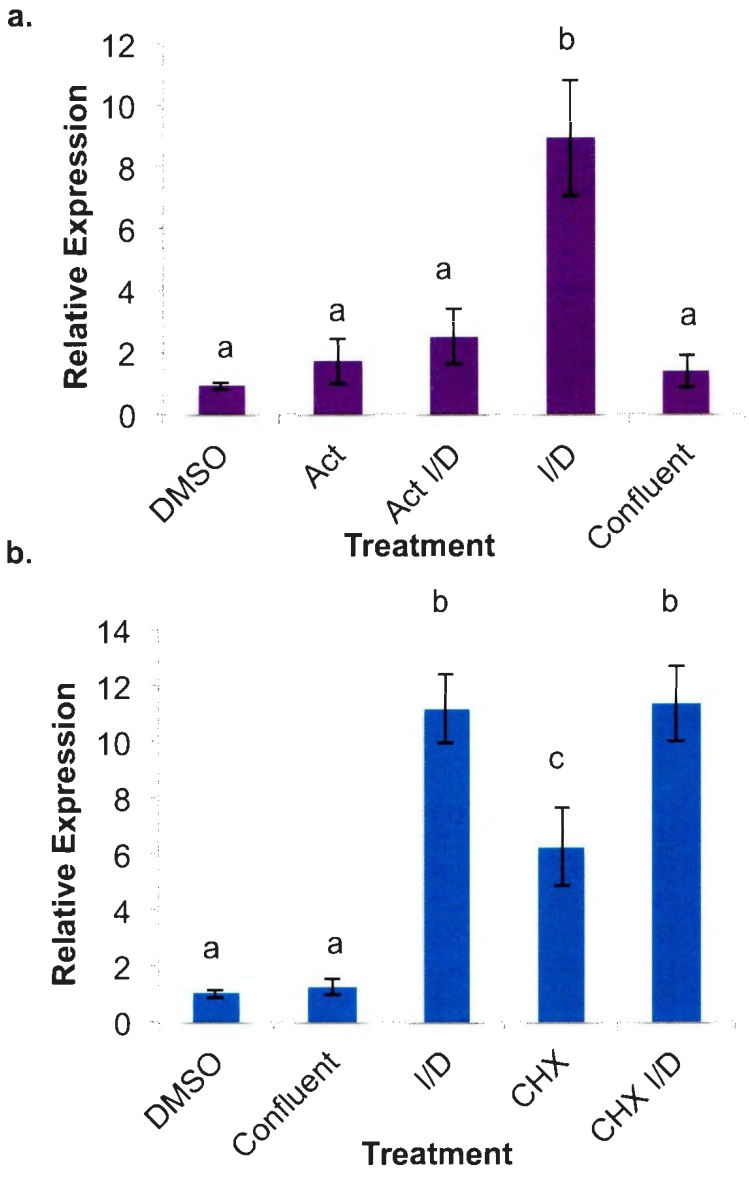

Fig 5. **A. Blocking transcription with actinomycin-D inhibited the increase in CD24 expression seen with IBMX and DEX addition in 3T3-L1 pre-adipocytes, while B. blocking protein synthesis with cyclohexamide had no significant effect on CD24 expression. a.** 3T3-L1 cells were treated with actinomycin-D (act) for 1 h to inhibit transcription, followed by IBMX and DEX. Six hours after addition of IBMX and DEX, mRNA expression of CD24 was determined by qRT PCR. **b.** 3T3-L1 cells treated with cyclohexamide (CHX) for 1 h to inhibit *de novo* protein synthesis, followed by IBMX and DEX. Six hours after addition of IBMX and DEX, mRNA expression of CD24 was determined by qRT PCR. CD24 expression levels were normalized to the internal control RPLP0 using the Pffafl equation and calculated relative to levels in cells treated with DMSO. Data are shown as mean ± SE. Statistical significance was determined using one-way ANOVA, Tukey *post hoc*, n=4, different lower case letters indicate a significant difference of $p<0.05$ between groups.

CHX treatment alone also increased CD24 mRNA expression. Increased mRNA expression of early and primary response genes has been reported with CHX treatment, as well as with other protein biosynthesis inhibitors, through a process called superinduction (Hershko et al., 2004; Okazaki R et al., 1992; Newton et al., 1996). Superinduction is poorly understood, but it is thought to be regulated by decreased mRNA degradation (Hershko et al..,2004). Although there is an increase in CD24 mRNA with CHX treatment, it is not to the same level as treatment with CHX followed by IBMX and DEX. Moreover, there was no reduction in CD24 mRNA in the presence of CHX compared to IBMX and Dex alone. Therefore, the mediator regulating CD24 expression in response to IBMX and DEX is not affected by inhibiting protein synthesis, and hence must be pre-existing.

3.4 Antibody mediated clustering of CD24 decreases adipogenesis in 3T3-L1 cells

Previous work has shown that both CD24+ and CD24- primary pre-adipocytes can undergo adipogenesis *in vitro*, while only CD24+ cells can develop into functional WAT depots *in vivo* (Rodeheffer et al., 2008). This suggests that the expression level of CD24 may not directly regulate adipocyte differentiation, but that activation of downstream signalling in response to ligand binding may be more important. Addition of anti-CD24 Ab to crosslink and cluster CD24 has been shown in cancer cell lines (Kim et al., 2008) and B cell lines to induce activation (Suzuki et al., 2001) of signal transduction pathways downstream of CD24. Therefore, in an attempt to mimic ligand binding of CD24 at the cell surface, 3T3-L1 cells were treated with anti-CD24 Ab (HIS50) or the isotype control (normal mouse IgM) starting at the initiation stage, which is the stage when the increase in CD24 expression levels was observed (Figs. 2 and 3). The isotype control has no

specificity for CD24, but retains all the non-specific characteristics of the CD24 Ab. It was used to confirm the specificity of the CD24 Ab binding, as well as to rule out non-specific Fc receptor binding or other non-specific interactions. This experiment would determine if clustering of CD24 by addition of anti-CD24 Ab was sufficient to affect adipogenesis. Samples were taken at the end of the assay, when it was visually conformed that lipid filled adipocytes had formed in the positive control wells not treated with Ab, and the mRNA levels of the pro-adipogenic genes, C/EBPα, PPAR-γ and adiponectin, were examined using qRT-PCR to determine the effect on adipogenesis.

With addition of increasing concentrations of anti-CD24 Ab, C/EBPα and adiponectin expression decreased in a dose-dependent manner (Fig. 6). While there was no significant change in PPAR-γ, there was a decreasing trend in expression with increasing Ab concentration. In addition, the accumulation of lipid droplets was visually assessed and was found to decrease with increasing concentration of anti-CD24 antibody treatment, compared to the isotype control (data not shown). Interestingly, with increasing concentrations of anti-CD24 Ab, CD24 mRNA expression also increased suggesting that Ab-mediated clustering of CD24 can positively regulate CD24 mRNA expression. Increasing CD24 mRNA expression correlated with decreased levels of C/EBPα, adiponectin and PPAR-γ, suggesting that CD24 expression is inversely correlated with known markers of adipogenesis. There were no significant changes in gene expression in the isotype control samples (Fig. 6).

It is important to note that there is no assay for CD24 activation and it is unknown what happens to the CD24 protein when it is bound by Ab. It is possible that binding of the Ab may actually block CD24 expression by down regulating the receptor expression

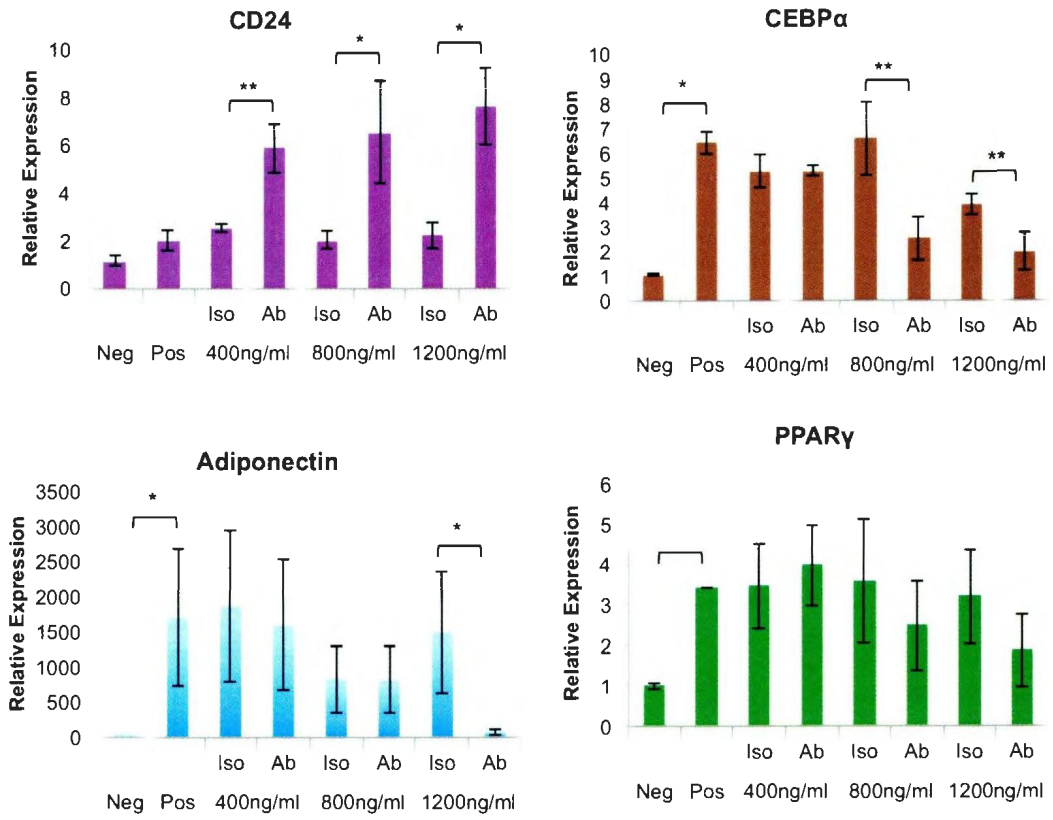

Fig. 6. **Increased CD24 expression resulted in decreased mRNA expression of late adipogenic genes.** CD24 Ab (HIS50) or isotype control (normal mouse serum IgM) was added to 3T3-L1 cells, along with IBMX/Dex and refreshed at every media change throughout the assay. Cells were sampled when mature adipocytes developed in the positive sample and mRNA expression was determined by qRT-PCR. Neg: confluent, non-treated cells; Pos: cells treated with IBMX/Dex; Iso: cells treated with isotype control; Ab: cells treated with CD24 antibody. The mRNA expression levels are shown for CD24, adiponectin, CEBPα and PPARγ. Expression levels were normalized to RPLP0 using the Pffafl equation and calculated relative to levels in confluent cells. Data is shown as mean ± SE. Statistical significance was determined using a Student t-test between cells treated with isotype and antibody of the same concentration, or neg and pos, n=3, *$p<0.01$, **$p<0.05$

through internalization, as in the case of other GPI-linked proteins (Deckert et al., 1996). To determine if addition of anti-CD24 Ab is causing internalization of CD24, CD24 could be tagged with a fluorescently labelled probe and internalization followed by confocal microscopy.

To determine if increased expression of CD24 throughout adipogenesis inhibits adipogenesis, CD24 should be constitutively over-expressed in the 3T3-L1 cell line and adipogenic outcome measured by changes in mRNA expression of adipogenic markers, as well as Oil Red O staining of neutral lipid accumulation. However, the 3T3-L1 cell line is a very difficult cell line to transfect, and after many unsuccessful attempts, Ab-mediated clustering of the CD24 Ab was used as a way to cluster endogenously expressed CD24 and activate intracellular signalling. To determine if Ab-mediated clustering of CD24 is activating intracellular signalling in 3T3-L1 cells as demonstrated in several other cells lines, activation levels of the mitogen-activated protein kinases ERK 1/2, Lyn, a member of the Src family of proteins, and p38, all downstream of CD24, should be determined (Sammar et al., 1997; Su et al., 2012; Suzuki et al., 2001)

Genes associated with later stages of adipogenesis (PPAR-γ and C/EBPα) and mature adipocyte function (adiponectin) were measured as a quantitative indication of adipogenesis. A difference in expression of these genes between anti-CD24 Ab treated cells and isotype control cells suggested an effect on adipogenesis. While there was decreased expression of PPARy, it was not significant. Therefore, a better indication of overall adipogenesis is needed. Analyzing more adipogenic markers such as the glucose transporter (GLUT4) which markedly increases in mature adipocytes, as well as markers involved in triglyceride synthesis and lipogenesis such as acetyl-CoA carboxylase (ACC),

stearoyl-CoA desaturase (SCD1), and fatty acid synthase (Spiegelman et al., 1983; Weiner et al., 1991; Garcia de Herreros and Birnbaum,1989), would allow for a more global gene analysis and thus a better indication of adipogenesis. Alternatively, global gene expression analysis by DNA microarray would give a complete view of the response of the entire genome.

While lipid accumulation was visibly decreased in response to Ab-mediated clustering, this was not imaged by Oil Red O staining, which is a qualitative measure of neutral lipid accumulation. Future experiments will include imaging of the cells after Oil Red O staining to verify overall changes to adipogenesis.

Even though the mechanism for regulation of adipogenesis by CD24 remains unclear, there is a clear inverse correlation of CD24 gene expression and mature adipogenic gene expression. As CD24 expression increased in the adipocyte, the late stage markers of adipogenesis C/EBPα and adiponectin decreased. This suggests that CD24 expression must decrease to allow adipogenesis to occur, supporting the hypothesis that an increase in CD24 expression at an early stage in adipogenesis, followed by its downregulation, is necessary for full differentiation and/or mature adipocyte function.

3.5 High fat diet increases CD24 mRNA expression in WAT at an early time point during adipogenesis

Addition of the initiation media (IBMX and DEX), promoting adipogenesis, was found to increase CD24 expression in 3T3-L1 pre-adipocytes, suggesting that regulation of CD24 expression contributes to adipogenesis *in vitro*. To determine if CD24 expression is regulated during adipogenesis *in vivo,* in a similar manner to what was found *in vitro,* expression of CD24, along with the other adipogenic genes, was examined in 3 week-old

mice fed a high fat diet (HFD, 45% of energy from fat) or a control low fat diet (LFD, 10% of energy from fat) for 0 to 7 days.

After 1 week of feeding, there was no significant difference in caloric intake or body weight between diets (Fig. 7). As only a portion of the fat depot was sampled in this donated tissue, WAT mass was not recorded. After 2 days of feeding, mice fed a HFD had significantly higher Pref-1 and CD24 mRNA expression levels compared to mice fed a LFD (Fig. 8). The increase in CD24 expression decreased by day 7, however it remained significantly higher than the LFD group. No other changes in adipogenic gene expression were observed suggesting that adipogenesis is at its initial stages. The increase in CD24 expression is observed concurrently with an increase in the pre-adipocyte marker Pref-1 and before an increase in any later markers of adipogenesis, suggesting that a HFD can increase CD24 expression at a very early time point during adipogenesis *in vivo*.

There are several studies examining changes in adipocyte gene expression during diet induced obesity in rodents, however these studies examine much longer feeding periods (2-18 weeks) and with older mice (6-8 weeks old). There are very few studies that have looked at gene expression during a short term HFD feeding in young (3 week old) mice. It is known though that within 1 week of a HFD, mice have increased epididymal fat mass and increased adipocyte size compared to mice fed a LFD (Lee et al., 2011; Winzell and Ahrén, 2004). From birth until 3-4 week of age, adipose tissue growth in rodents is hyperplastic and overfeeding during this period can result in permanent weight gain and increased fat cell number. From 4-14 weeks, adipose tissue growth occurs through both hypertrophy and hyperplasia, and after 14 weeks, adipose tissue grows primarily by hypertrophy (Jo et al., 2009; Li et al., 2002). Further, mice fed a HFD for 1

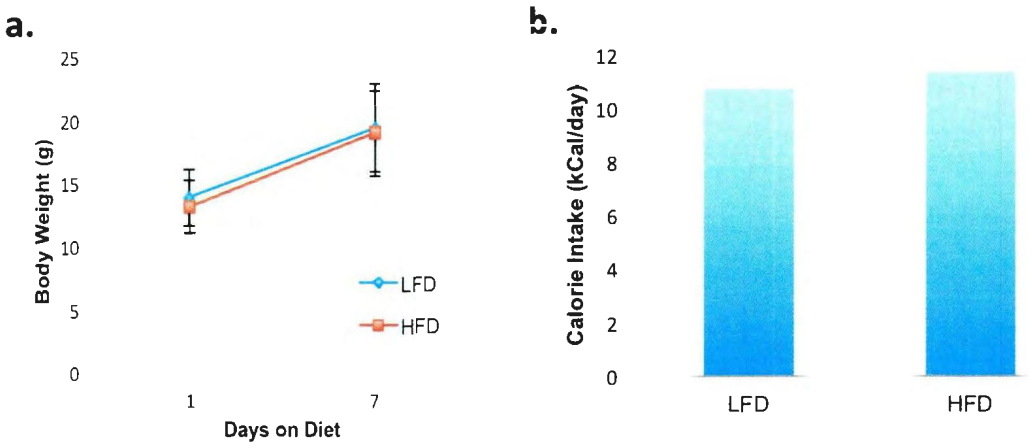

Fig. 7. **No difference in body weight or caloric intake was observed in mice fed a LFD or HFD for one week. Three-week-old** C57BL/6 mice were fed a 10% energy from fat low-fat diet (LFD) or 45% energy from fat high-fat diet (HFD) for 1 week. This experiment was part of a larger experiment completed by Dr. Michiru Hirasawa. **a.** Body weight in grams **b.** Calorie intake in kCal/day. Data is shown as mean ± SE. Calorie intake was measured per group, not per mouse, therefore no standard deviation was calculated. Statistical significance was determined using a Student t-test, n=5-7

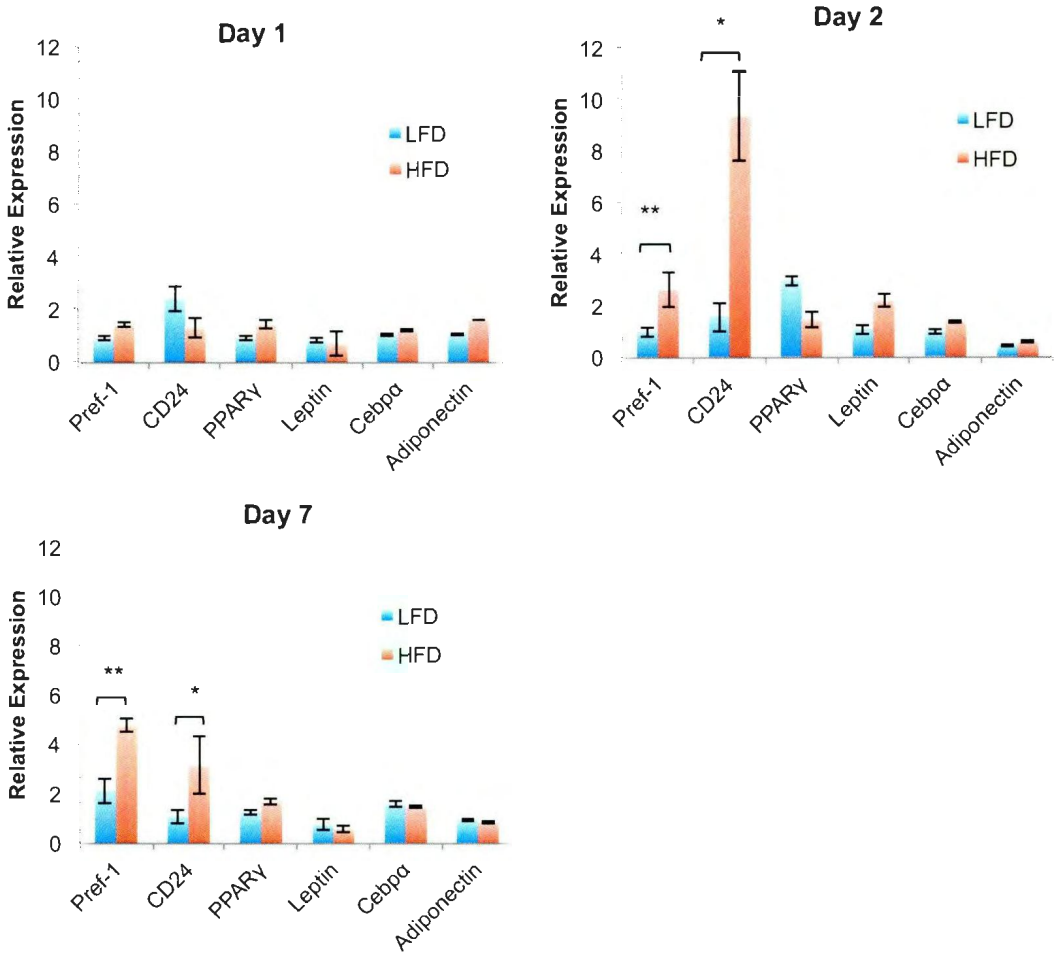

Fig. 8. **CD24 and Pref-1 mRNA expression increased in WAT of mice after 2 days of HFD. Three-week-old** C57BL/6 mice were fed a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) for up to 1 week. mRNA was isolated from the epididymal white adipose tissue at days 1, 2 and 7, and gene expression analysis conducted by RT-qPCR. Expression levels were normalized to RPLyoutube.com P0 using the Pffafl equation and calculated relative to one LFD control mouse. Data shown as mean ± SE. Statistical significance was determined using a Student t-test, n=5-7, *$p<0.01$, **$p<0.05$

week exhibit increased plasma glucose and insulin levels (Marques et al., 2000; Winzell and Ahrén,2004). This data suggests that within 1 week of a HFD, mice are on the path to obesity and adipogenesis is likely initiated. Because the mice in this study are less than 4 weeks of age, the changes to adipose tissue growth is most likely due to hyperplasia, although there may be hypertrophic effects as well (Jo et al., 2009; Li et al., 2002).

3.6 High Fat Diet increases mRNA expression of late adipogenic markers but no effect on CD24 mRNA expression

To examine both mRNA and protein expression levels of CD24 in WAT, the HFD vs LFD feeding study was repeated. Expression levels of CD24 and the known adipogenic genes were determined by qRT-PCR, while CD24 protein expression levels was determined by western blot analysis. There was no significant difference in food intake or overall body weight between mice fed LFD or HFD, however mice on a HFD had significantly greater WAT mass after only 1 day of feeding (Fig. 9).

Unexpectedly, the increase in neither Pref-1 nor CD24 mRNA expression in response to a HFD was not observed. However, there was a significant increase in mRNA levels of PPARγ, Leptin and Adiponectin after 1 day of a HFD (Fig. 10) indicating a very rapid induction of adipogenesis by the HFD. Analysis of protein expression by western blot found no significant difference in CD24 protein levels (Fig. 11). The densitometry analysis does however suggest a possible trend for increased CD24 protein expression after 2 days of HFD (Fig. 11e). The increase in PPARγ, Leptin and adiponectin mRNA expression after 1 day of HFD suggests that the HFD is inducing adipogenesis very rapidly and in a different time frame than the first HFD experiment and therefore the

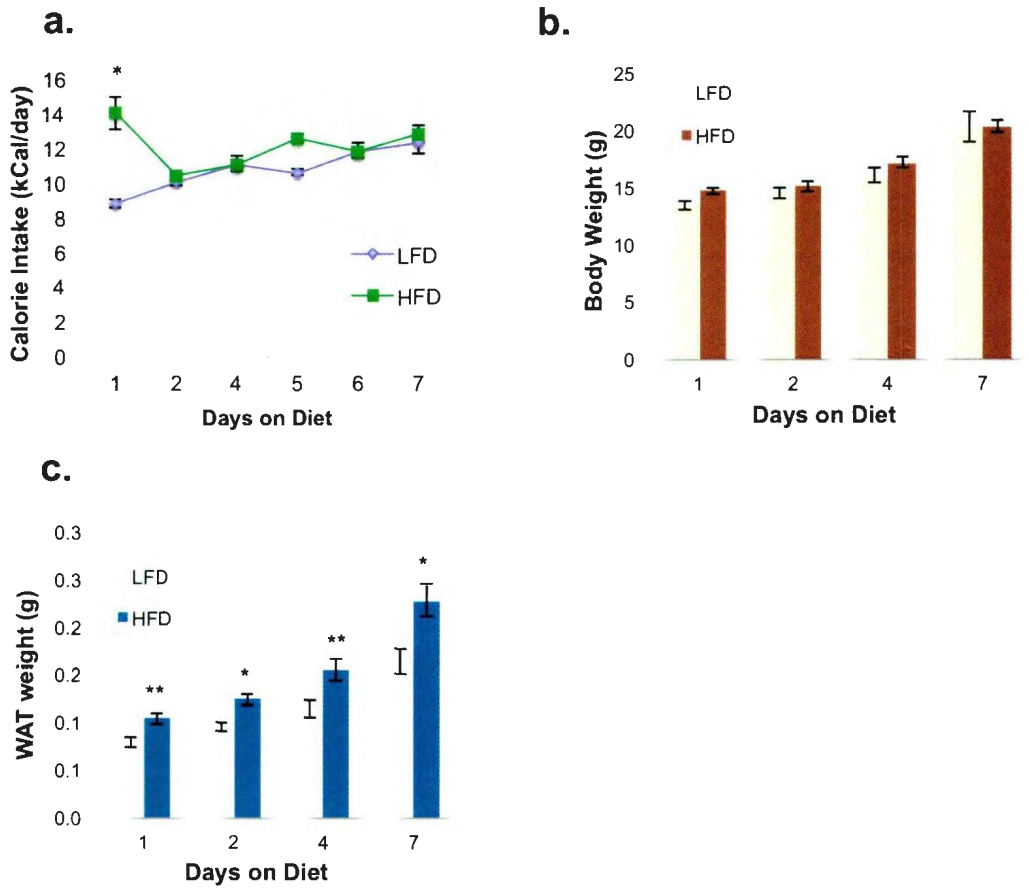

Fig. 9. **Mice fed a HFD had significantly more WAT but not body weight after one day of HFD.** Three-week-old C57BL/6 mice were fed a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) starting at 3 weeks of age for up to one week. **a.** Calorie intake in kCal/day **b.** Body weight in grams and **c.** WAT weight in grams. Data in **b.** and **c.** shown as mean ± SE. Statistical significance was determined using a Student t-test, n=8, *$p<0.01$, **$p<0.05$

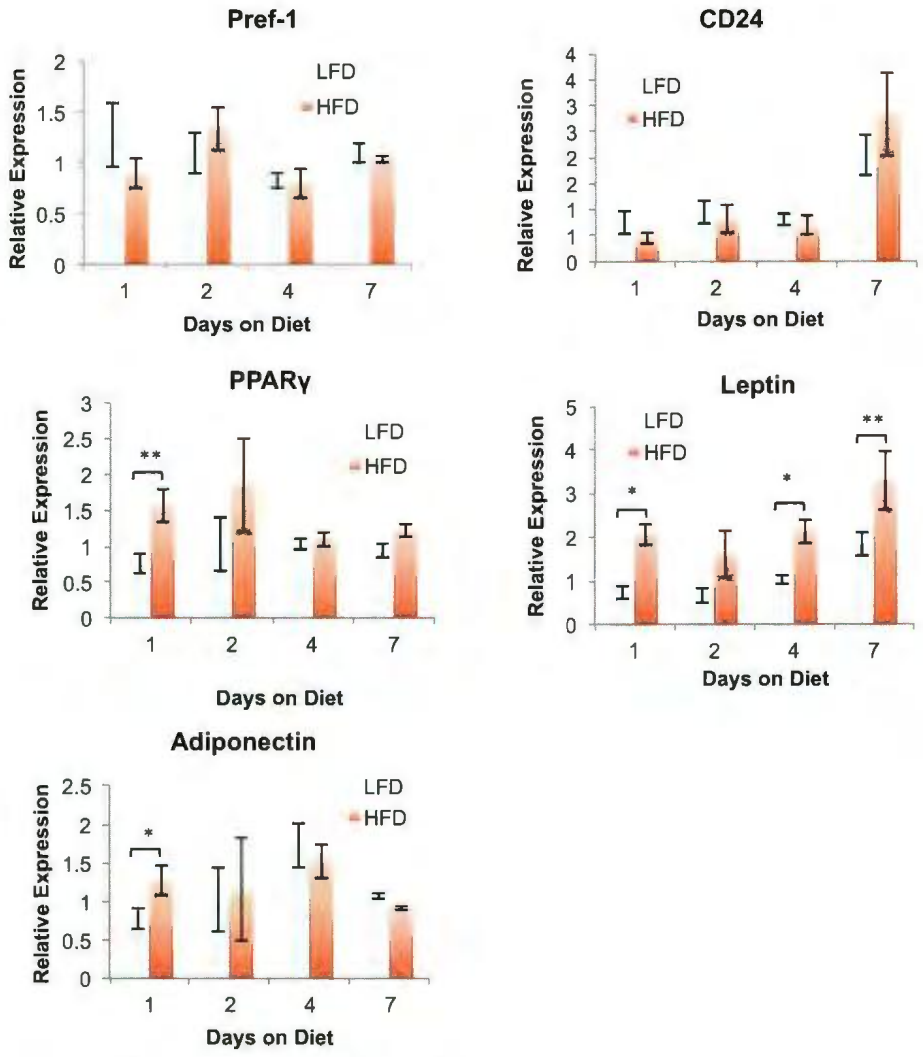

Fig. 10. **No change in CD24 or Pref-1 mRNA expression with HFD but a significant increase in expression of late adipogenic markers with HFD was observed.** mRNA expression in WAT of 3-week-old C57BL/6 mice fed a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) for up to one week, as determined by qRT PCR. Expression levels were normalized to RPLP0 using the Pffafl equation and calculated relative to one LFD control mouse. Data shown as mean ± SE. Statistical significance was determined using a Student t-test, n=8, *$p<0.01$, **$p<0.05$

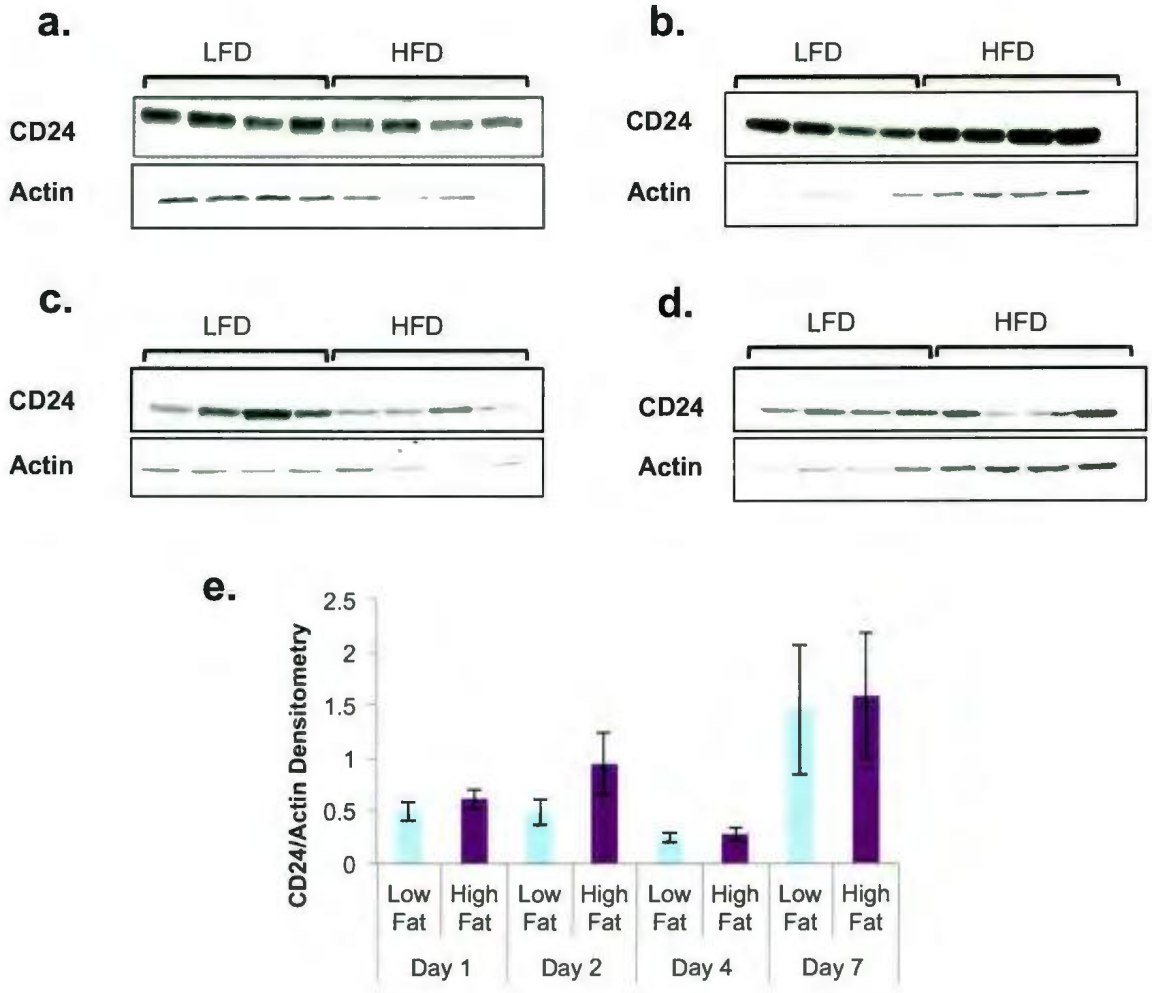

Fig. 11. **No significant difference in CD24 protein expression was observed in mice fed a HFD or LFD.** Representation of protein expression of CD24 in C57BL/6 mice fed a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) starting at 3 weeks old after **a.** 1 day, **b.** 2 days, **c.** 4 days or **d.** 7 days of feeding. Densitometry analysis of CD24/Actin is displayed in **e.** Data shown as mean ± SE. Actin was used as a loading control, showing western blot for half of each diet group, n=8

increase in CD24 expression may have been missed. Again, there are no other studies examining gene expression of these common adipogenic markers during a short term HFD. This is the first time such a rapid increase in PPARγ, Leptin and adiponectin mRNA expression in response to a HFD has been shown.

Although diet induced obesity in mice is commonly used to study adipogenesis, there are often differences in response to the HFD between strains, age and sex of the mice (see section 1.2.1.1). Different responses to a HFD have even been found between C57BL/6 mice within the same litter, which may account for some variability between experiments (Koza et al., 2006).

Differences in the procedures used to obtain the WAT may also account for the different results. The first HFD experiment was completed by Dr. Michiru Hirasawa, who donated the WAT for our analysis. Halothane was used to anaesthetize the mice, while in the second HFD experiment isoflurane was used. While all volatile anaesthetic agents will produce a drop in blood pressure, the effects are mediated in different ways. While halothane produces a direct depression on myocardial function causing hypotension, isoflurane contributes to hypotension through peripheral vasodilation (Gelman et al., 1984). Pre-adipocytes have been found to be located in the vasculature (Cawthorn et al., 2011). By using isoflurane, the vasculature in the WAT may have been altered, which could be hypothesized to change the localization of pre-adipocytes in the WAT, however there are no studies demonstrating the effects of isoflurane on pre-adipocyte localization. Furthermore, when removing the adipose tissue, we were careful not to include any of the gonadal tissue located next to the WAT and to avoid vasculature within the WAT. Therefore by removing the vasculature, we may also have lost a population of pre-adipocytes which could explain why there was no change in Pref-1 mRNA expression.

The 3T3-L1 experiments suggest that CD24 expression is regulated specifically on pre-adipocytes, therefore, a loss of pre-adipocytes, as suggested by the lack of change in Pref-1 expression would also result in no observable changes to CD24 expression.

3.7 Serial injections of anti-CD24 antibody reduced WAT and overall body weight of mice fed HFD

Antibody mediated clustering of CD24 in 3T3-L1 cells resulted in decreased expression of adipogenic markers. To determine if clustering of CD24 by anti-CD24 Ab injection would have an effect on adipogenesis *in vivo*, 3-week-old mice were injected with a monoclonal anti-CD24 Ab or saline every 2 days over the course of 1 week. Like the 3T3-L1 Ab experiment (Section 3.4), this was a pilot experiment to determine if clustering of CD24 by anti-CD24 Ab injection would have any effect on adipogenesis in mice fed a HFD. Again, it is important to note that it is unknown if CD24 is being activated or possibly blocked with anti-CD24 injection, and although anti-CD24 antibody was injected directly into the inter-peritoneal region, CD24 is expressed on many different cell types and therefore the response could be non-specific to adipocytes.

Saline injected mice were fed either a LFD or HFD while the group of mice injected with anti-CD24 were fed a HFD. A second set of mice were injected with a control Ab of the same isotype as the anti-CD24 Ab (rat IgG2a) or saline and fed a HFD. All mice were introduced to the experimental diets on the same day as the first injection.

Mice injected with anti-CD24 antibody weighed significantly less, had significantly less caloric intake per day and had significantly less WAT mass compared to mice injected with saline (Fig 12). Mice injected with saline and fed a HFD had

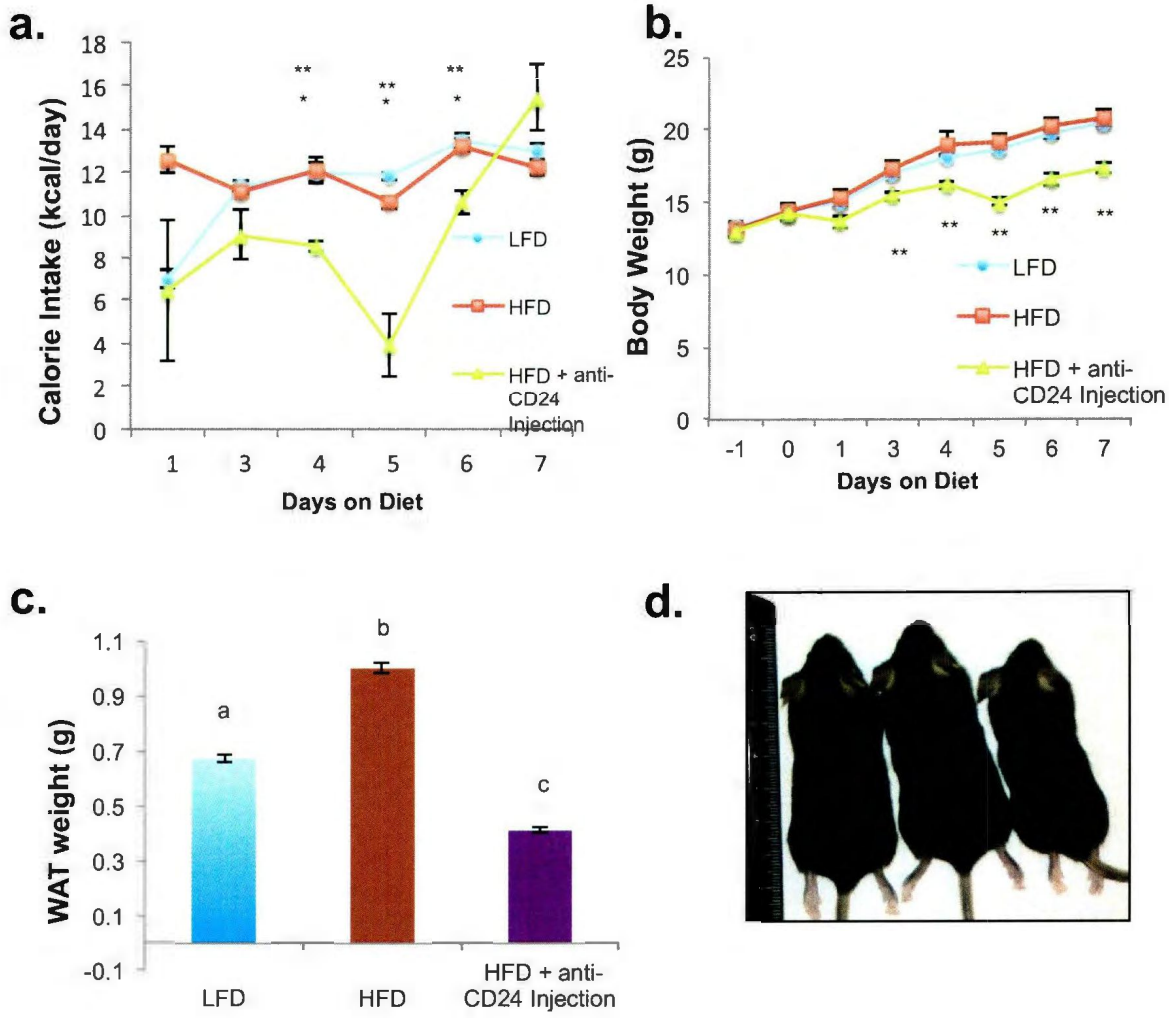

Fig. 12. **Mice injected with anti-CD24 antibody consumed significantly less calories, weighed less and had less WAT then mice injected with saline.** Three-week-old C57BL/6 mice were fed a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) for one week. Every 2 days, starting on day 0, mice were injected intra-peritoneally with 200µg, in 200µl, of anti-CD24 antibody (M1/69) or 200µl saline, for a total for 4 injections. **a.** Calorie intake in kCal/day **b.** Body weight in grams **c.** WAT mass on day 7 **d.** Dorsal view of mice, from left to right, LFD, HFD, HFD + CD24-Injected. Data for **b.** and **c.** shown as mean ± SE. Statistical significance was determined using a One-way ANOVA followed by Tukey *post-hoc*, n=6. For **a.*** HFD vs HFD+anti-CD24 injection, $p<0.01$ on day 4 and 5, $p<0.05$ on day 6. **LFD vs HFD+anti-CD24 injection, $p<0.01$. For **b.*** HFD vs HFD+anti-CD24 injection, $p<0.05$ on day 1, $p<0.01$ for all other significant days. ** LFD vs HFD+anti-CD24 injection, $p<0.05$ on day 3 and 4, $p<0.01$ on day 5 and 7. For **c.** $p<0.01$. n=6

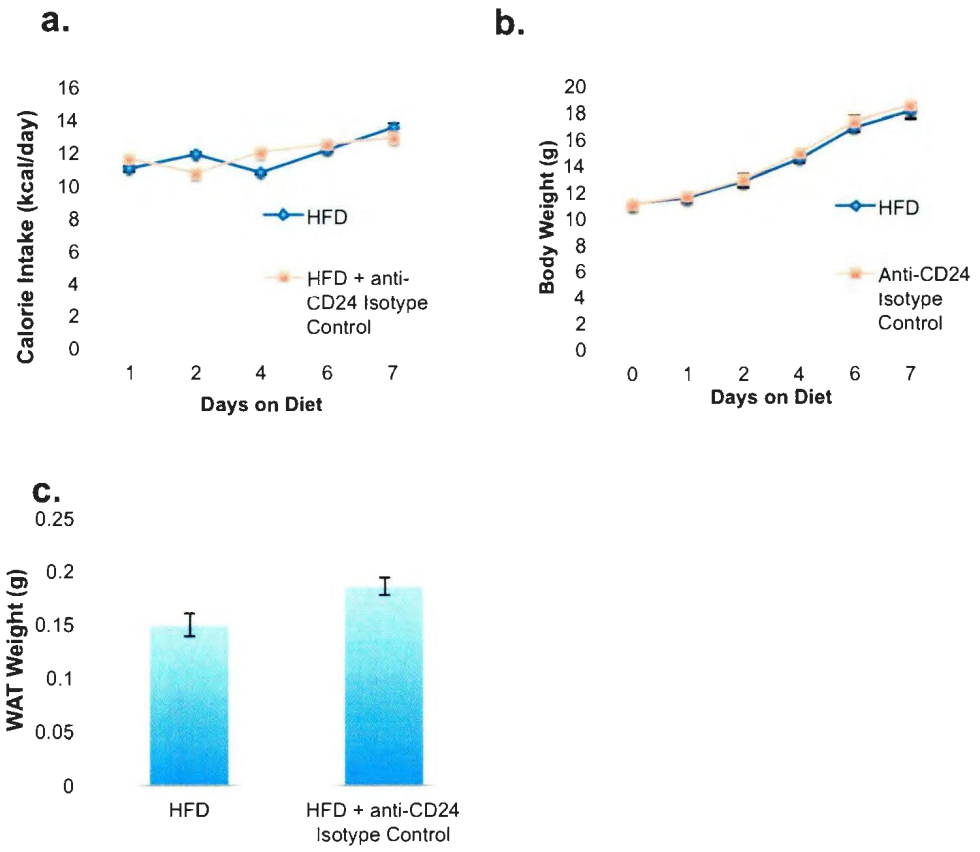

Fig. 13. **No significant difference was observed in calorie intake, body weight and WAT in mice injected with anti-CD24 isotype or saline.** Three-week-old C57BL/6 mice were fed a high fat diet (HFD, 45% energy from fat) and given an intra-peritoneal injection of 200 μg anti-CD24 isotype control or saline following the injection schedule in Fig 15. **a.** Calorie intake in kCal/day **b.** Body weight in grams **c.** WAT weight in grams. Data shown as mean ± SE. Statistical significance was determined using a Students t-test, n=3

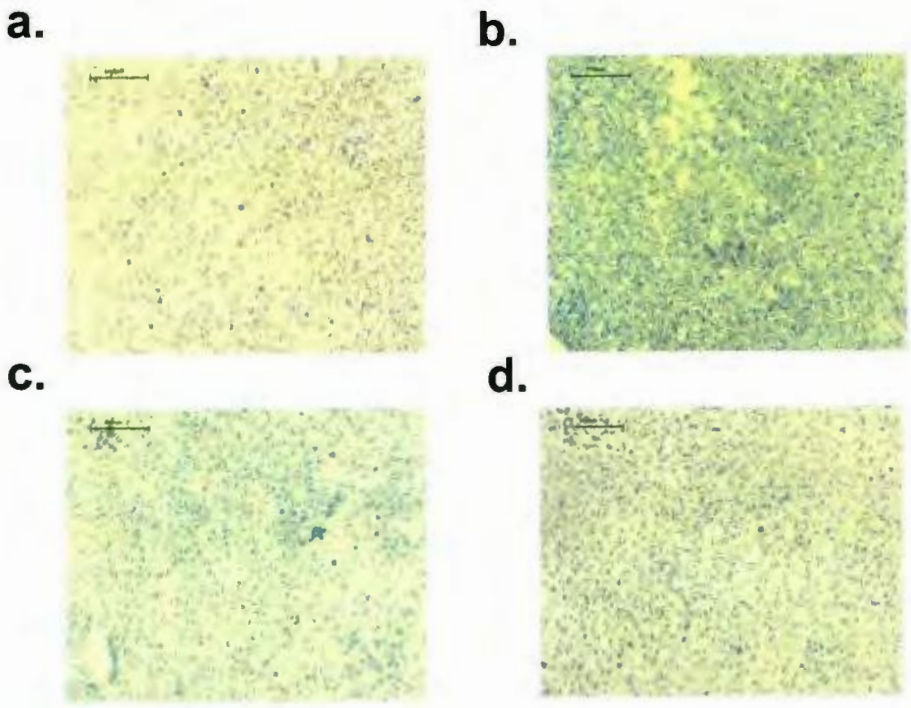

Fig. 14. **No difference in lipid accumulation was observed in the liver of mice injected with anti-CD24 antibody, anti-CD24 isotype control or saline.** Liver histology samples were stained with Oil Red O to determine lipid accumulation. **a.** Low fat diet, saline injected, **b.** High fat diet, saline injected, **c.** High fat diet, anti-CD24 antibody injected, **d.** High fat diet, isotype control antibody injected

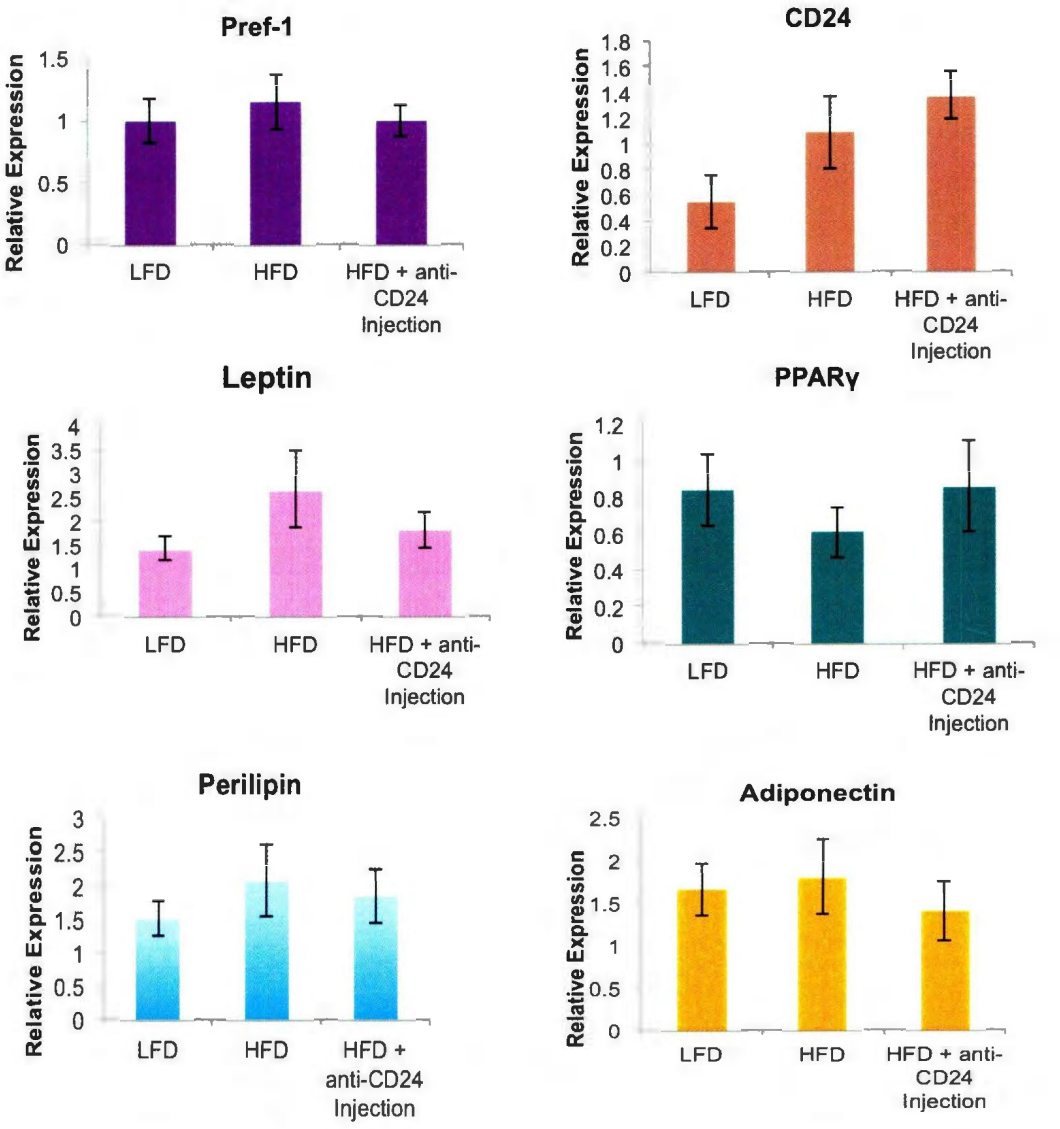

Fig. 15. **No significant difference was observed in CD24 mRNA expression or other adipogenic gene expression in mice injected with anti-CD24 antibody or saline.** mRNA expression of 3 week old mice fed either a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) and injected with saline or anti-CD24 antibody for 1 week, as determined by qRT PCR. Expression levels were normalized to RPLP0 using the Pffafl equation and calculaied relative to one LFD control mouse. Data shown as mean ± SE. Statistical significance was determined using a One-Way ANOVA, n=6

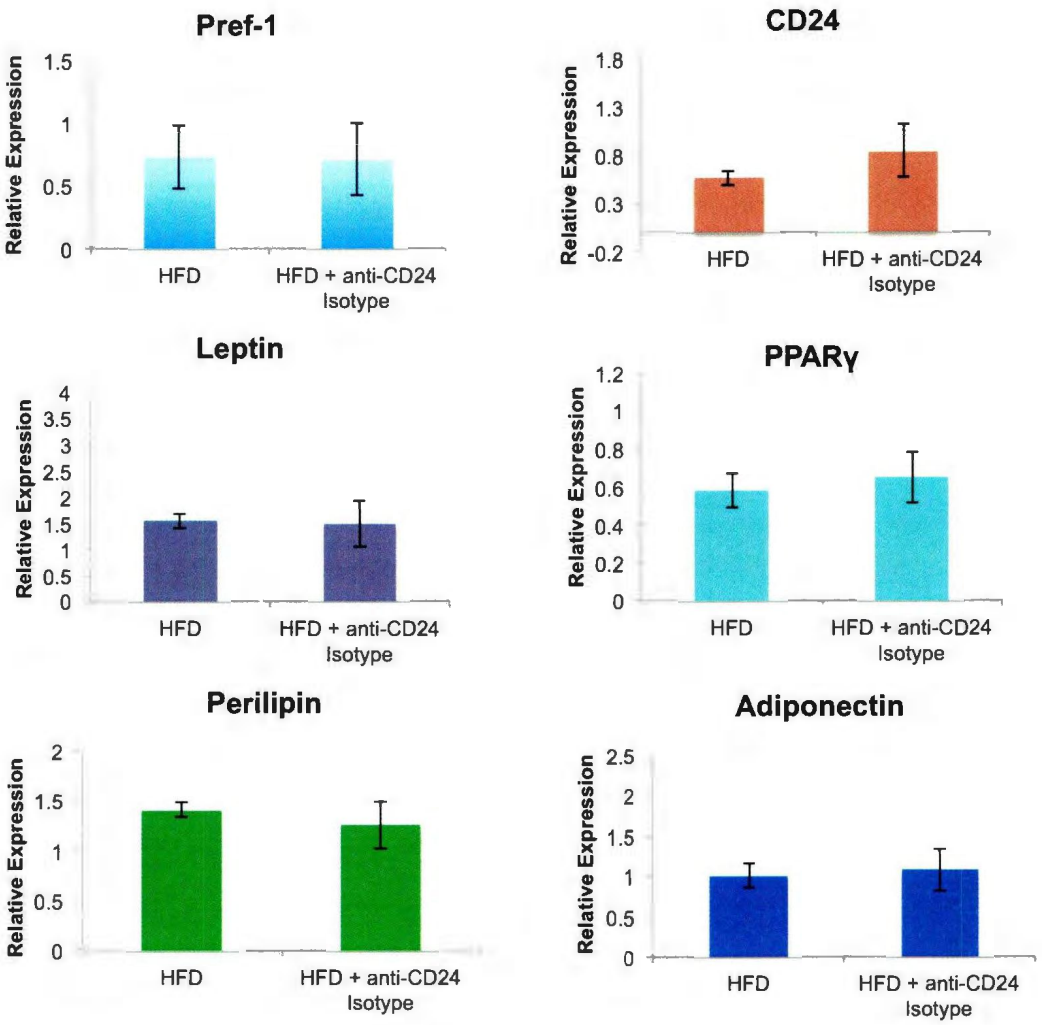

Fig. 16. **No significant difference was observed in CD24 mRNA expression or other adipogenic gene expression in mice injected with anti-CD24 isotype or saline.** mRNA expression of 3 week old mice fed either a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) and injected with saline or anti-CD24 isotype control for 1 week, as determined by qRT PCR. Expression levels were normalized to RPLP0 using the Pffafl equation and calculated relative to one LFD control mouse. Data shown as mean ± SE. Statistical significance was determined using a student T-test, n=3

significantly more WAT than mice injected with saline and fed a LFD (Fig. 12). There was no significant difference in body weight, calorie intake or WAT mass in mice fed a HFD injected with saline and mice fed a HFD injected with anti-CD24 isotype control (Fig. 13).

Because there was such a dramatic difference in WAT in mice injected with anti-CD24 Ab compared to mice fed a HFD, the lipid accumulation in the liver was examined to determine if the reduced WAT size was leading to fat deposition in other tissues. There was no difference found in Oil Red O staining of lipid in liver histology sections from saline injected animals or anti-CD24 injected animals on HFD (Fig. 14).

No significant difference in mRNA expression of CD24 or any of the adipogenic genes examined by qRT-PCR was found (Fig. 15, 16). In addition to the known adipogenic genes studied previously, the expression of perilipin, a protein that acts as a protective coating on lipid droplets in adipocytes (Brasaemle, 2007), was examined as an indicator of lipid droplet size. Similar to the other adipogenic genes, no changes in mRNA expression were observed (Fig. 15, 16).

There was however a trend for an increase in CD24 mRNA expression ($p=0.086$) in mice injected with anti-CD24 Ab suggesting a possible positive feedback whereby clustering of anti-CD24 Abmay regulate CD24 expression itself, which is similar to the findings in the 3T3-L1 cells (Fig. 6).

To determine the cause of the large difference in the WAT mass of the anti-CD24 injected mice, the cell surface area of the adipocytes was examined to determine if anti-CD24 injection affected cell size (hypertrophy) or cell number (hyperplasia). Cell surface area of histology samples from the LFD, HFD and HFD + anti-CD24 injection, as well as the HFD and HFD + anti-CD24 isotype control groups, was determined using CellProfiler

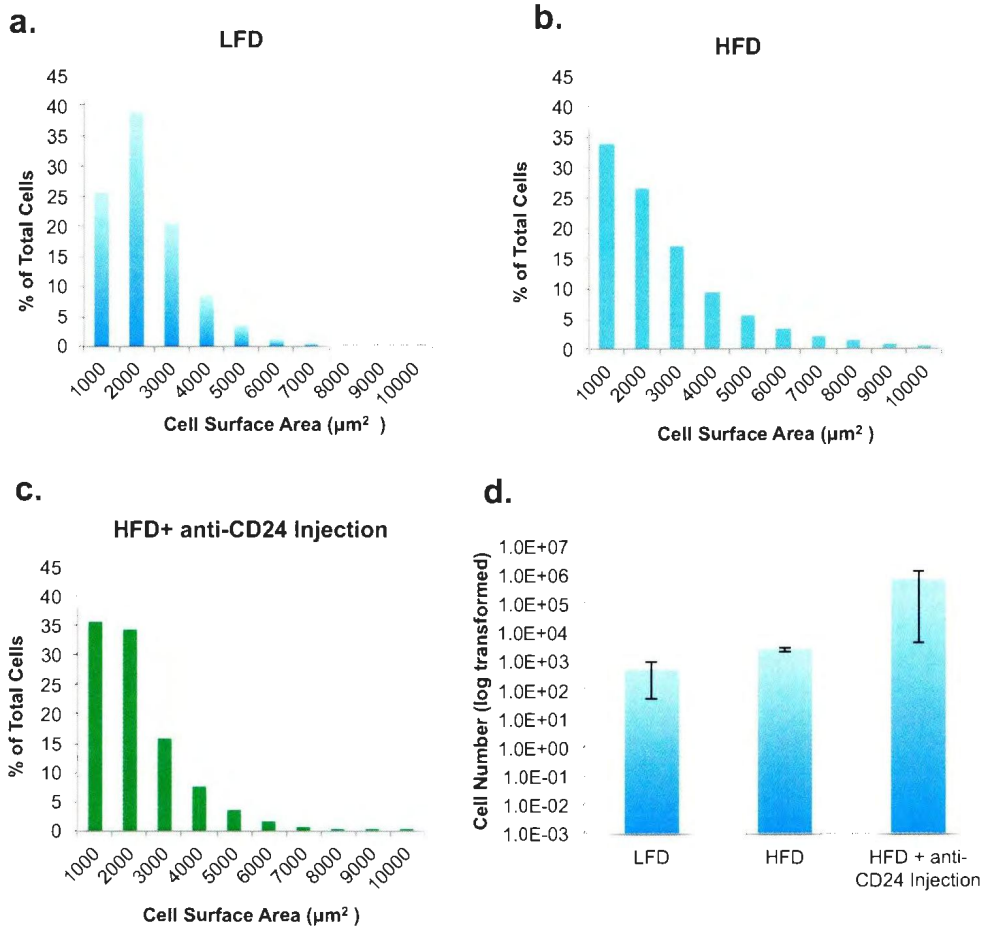

Fig. 17. **A-C. Significant differences were observed in the distribution of cell surface area in WAT of mice fed a LFD, HFD and HFD + anti-CD24 antibody. D. No difference in adipocyte cell number between diets.** Cell surface area of 3 week old mice fed either a low fat diet (LFD, 10% energy from fat) or a high fat diet (HFD, 45% energy from fat) and injected with saline or anti-CD24 antibody for 1 week. Frequency distribution for **a.** low fat diet, **b.** high fat diet or **c.** high fat + anti-CD24 injection is shown. Data shown as percentage of total cells analyzed. **d.** The number of cells in the WAT was calculated base on the adipocyte size distribution and fat depot mass using the previously described formula (Jo et al., 2009). No significant difference was found. Data shown as mean ± SE and y-axis is log transformed. Statistical significance was determined using a Kruskal-Wallis test, followed by a Kruskal--Smirnov post hoc, $p<0.0001$ in **a-c.** Statistical significance was determined using a One-way ANOVA in **d.**

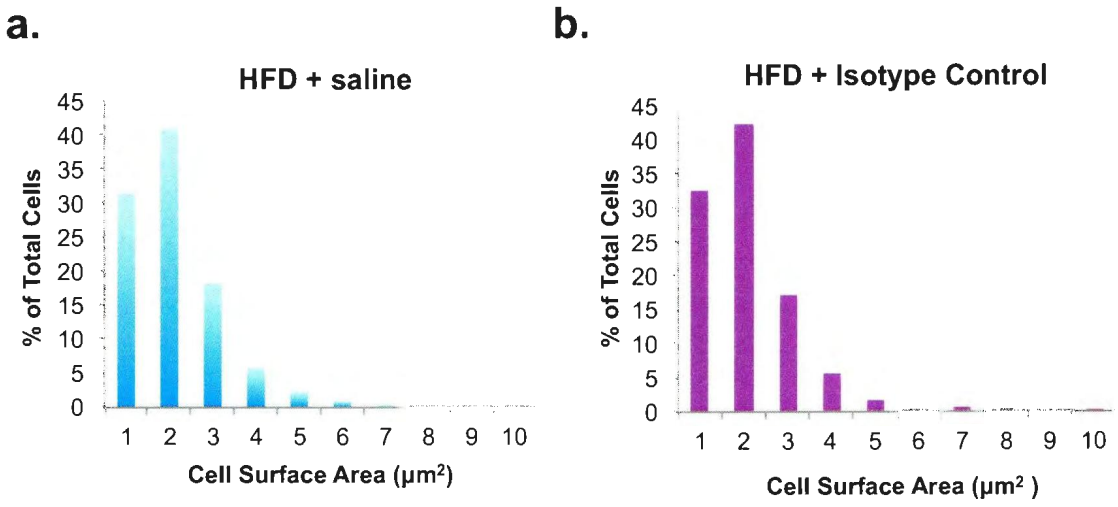

Fig. 18. **No significant difference was observed in the distribution of cell surface area in WAT of mice fed a HFD + saline and HFD + anti-CD24 isotype control.** Cell surface area of 3 week old mice fed a high fat diet (HFD, 45% energy from fat) and injected with either saline or anti-CD24 isotype control for 1 week. Frequency distribution for **a.** HFD + saline, **b.** HFD +anti-CD24 isotype control. Data shown as percentage of total cells analyzed. Statistical significance was determined using a Kruskal-Wallis test.

(Carpenter et al., 2006). To determine if anti-CD24 injection had an effect on hypertrophy, the resulting histograms of cell surface area were analyzed (Fig. 17). There was a significant difference in cell surface area distribution between the LFD, HFD and HFD + anti-CD24 injection groups. The greatest percentage of cells within the HFD groups had a surface area of less than 1000μm with a declining distribution through the larger sizes while the LFD group had the greatest percentage of cells between 1000μm and 2000μm and was much less distributed through the larger sizes. The greatest percentage of cells within the HFD + anti-CD24 injected mice had a surface area of less than 1000μm (34%) and between 1000-2000μm (34%) and was much less distributed through the larger sizes suggesting that anti-CD24 injection affected cell size. There was no significant difference found in the distribution of the HFD or HFD + anti-CD24 isotype control groups (Fig. 18).

To determine if there was a hyperplasic effect, the total cell number, was determined using the mean cell size distribution and fat depot mass (Jo et al., 2009). Although there was an increase in number of cells in the anti-CD24 injection group, there was no significant difference in cell number between the LFD, HFD and HFD + anti-CD24 injection groups, suggesting there was no change in hyperplasia (Fig. 17d). Therefore the differences in fat depot mass in response to a short term HFD are most likely due to changes in cell size, where anti-CD24 injection resulted in smaller adipocytes.

Adipose tissue can grow by both hyperplasia and hypertrophy. The results showed a significant increase in WAT weight after 1 week of a HFD compared to the LFD, while there was a significant decrease in WAT after 1 week of a HFD + anti-CD24 injection compared to the LFD and HFD. There was a larger increase in cell size distribution in

mice fed a HFD compared to LFD, where mice fed a HFD had more large cells, while mice fed a HFD + anti-CD24 injection had significantly more smaller cells (<2000μm) compared to both LFD and HFD. This suggests that a HFD is inducing hypertrophy, increasing cell size, while anti-CD24 injection is preventing hypertrophy. However, there were no changes in mRNA expression of any of the adipogenic genes tested. It is expected that with increasing cell size, leptin, adiponectin and perilipin expression would increase. As stated in section 3.4, future experiments should examine a larger variety of adipogenic genes such as GLUT 4, ACC, SCD1 and fatty acid synthase, to obtain a more global gene analysis, allowing for a better indication of changes in adipogenesis.

Like the previous *in vivo* experiment, isoflurane was used as the anaesthetic, which may have affected the results in a similar manner. Mice that were injected with anti-CD24 antibody were hypophagic, having significantly less calorie intake on days 4-6. Serum sickness is a common syndrome that occurs with antibody injection, due to the formation of Ab-Ab immune complexes in response to the foreign Ab (D'Arcy, 2001). Hypophagia is a symptom of serum sickness, however because the anti-CD24 isotype control injected mice did not exhibit hypophagia, it is unlikely that the mice were experiencing serum sickness. Furthermore, CD24 is expressed on a variety of cell types and therefore any effects on adipogenesis may be due to secondary/indirect effects on other cell types. Mice injected with anti-CD24 antibody also had a severely enlarged spleen compared to the other groups (data not shown). The spleen consists of many cells that express CD24 such as T cells, B cells and dendritic cells. CD24 can act as a co-stimulator, acting synergistically with B7, to cause the clonal expansion of T cells (Liu et al., 1992; Wang et al., 1995), therefore the injected antibody may have been activating T cells in the spleen, rather then adipocytes, causing proliferation and growth of the T cell

population and enlarging the spleen. Such a large immune reaction may have resulted in sick mice, causing reduced appetite and therefore resulting in decreased adipose tissue development as a secondary effect.

This experiment was completed with two different cohorts of mice whose physical response to the HFD was different. Mice fed a HFD in the second cohort did not gain as much weight as mice fed a HFD in the first cohort. They also had much less WAT on day 7, having less WAT then both mice fed a LFD or HFD in the first cohort. Again, it has been previously demonstrated that there can be differing responses to a HFD between and within strains of mice. Therefore, because there was such a drastic difference in response to a HFD between the mice cohorts, caution must be used when interpreting the results from this experiment.

4.0 Conclusion and Future Directions

This research is the first to show that CD24 expression can be regulated during adipogenesis. Upon initiation of adipogenesis, CD24 mRNA and protein levels increased in pre-adipocytes *in vitro.* This increase in CD24 expression is regulated by transcription, likely through activation of a pre-existing protein regulator such as a transcription factor. Increasing cAMP levels were found to be sufficient to increase CD24 mRNA levels, demonstrating a role for cAMP as a primary intracellular mediator regulating CD24 expression in pre-adipocytes *in vitro* (Fig. 19).

To continue to elucidate the signalling pathway regulating CD24 mRNA expression levels in response to IBMX and DEX, the downstream target of cAMP should be determined. The two major downstream targets of cAMP that are essential for adipogenesis are Protein Kinase A (PKA) (Taylor et al., 1990) and exchange proteins activated by cAMP (EPAC-1, EPAC-2) (de Rooij et al., 1998). Future work will

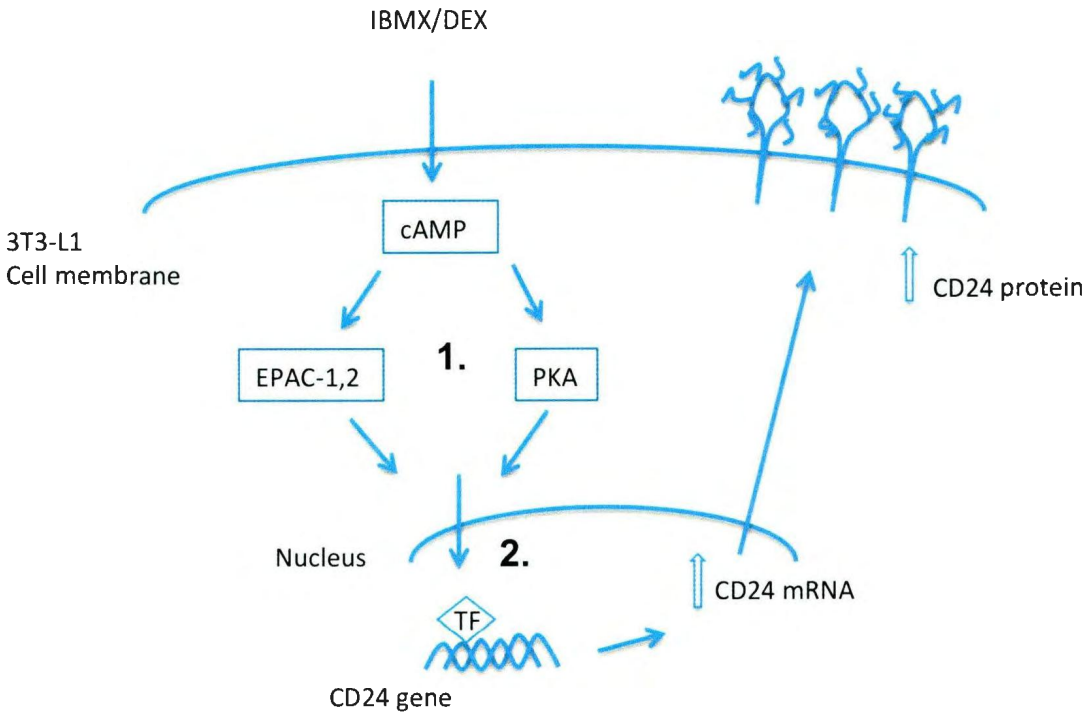

Fig 19. **IBMX and DEX regulate CD24 mRNA expression by increasing cAMP resulting in transcription of the CD24 gene.** Schematic diagram of the regulation of CD24 expression in 3T3-L1 pre-adipocytes. IBMX and DEX increase cAMP, which can activate downstream mediators PKA and/or EPAC-1-2. Further downstream, IBMX and DEX result in transcription of the CD24 gene, likely by a pre-existing transcription factor (TF), resulting in increased CD24 mRNA and protein expression. Numbers represent future directions in elucidating this pathway; 1. to determine mediator directly downstream of cAMP 2. to determine the TF regulating CD24 expression

determine the downstream target of cAMP by treating 3T3-L1 cells with inhibitors specific for PKA or EPAC, followed by IBMX and DEX and measuring CD24 expression. The increase in CD24 mRNA expression in 3T3-L1 cells in response to IBMX and DEX is dependent on transcription. There are several transcription factors that can regulate CD24 expression, including NFAT5, which upregulates CD24 expression in T-lymphocytes and Twist, which downregulates CD24 expression in breast cancer cells. Furthermore, overexpression of Twist in mesenchymal stem cells induces adipogenesis. Additionally, both NFAT5 (also known as TonEBP) (Ferraris, J., 2004) and Twist (Firulli, A., 2008) can be activated by PKA, a possible mediator of CD24 expression in 3T3-L1 cells. Future work will include identification of the transcription factor regulating CD24 mRNA expression in pre-adipocytes by promoter analysis and/or chromatin immunoprecipitation (ChIP) analysis.

Increased CD24 expression was found to associate with decreased adipogenic gene expression. To determine if a loss of CD24 function affects adipogenesis, future work would include knocking out CD24 expression in 3T3-L1s using siRNA and measuring adipogenesis by both mRNA expression of the adipogenic markers, as well as Oil Red O staining, to determine if loss of CD24 abrogates the effect of antibody mediated CD24 expression. Conversely, to determine if increased CD24 function affects adipogenesis, CD24 should be overexpressed in 3T3-L1 cells and adipogenesis measured by both mRNA expression of the adipogenic markers, as well as Oil Red O staining.

An increase in CD24 and Pref-1 mRNA was observed after 2 days of a HFD suggesting an increase in the CD24 cell population at the initial stages of adipogenesis. However, when this study was replicated a second time with the same diet, an increase in

mRNA of the late adipogenic markers was observed after 1 day of a HFD, while no change in Pref-1 or CD24 expression was observed. Injection with the anti-CD24 Ab had no effect of gene expression, but there was a dramatic reduction in WAT in the anti-CD24 antibody injected mice.

To discern the role of CD24 *in vivo,* future studies would focus on culturing primary cells from the SVF of both mouse and human WAT and determining expression of CD24 mRNA and protein during adipogenesis of primary cells. It is expected that CD24 would follow a similar pattern of expression as in the 3T3-L1 cells, where it increases expression at initiation of adipogenesis, then decreases to baseline as the adipocyte matured.

It is unknown if CD24 is actually being activated with anti-CD24 Ab injection, or if the anti-CD24 Ab is interfering with ligand binding or perhaps inducing internalization. Several ligands have been identified for CD24 including P-selectin in cancer and immune cells (Aigner et al, 1997; Xia et al., 2002), L1 in neuronal cells (Kleene et al., 2001) and Siglec-10 in dendritic cells (Chen et al., 2009). In future studies to investigate the role of CD24 in adipogenesis *in vivo*, CD24 could be activated by adding a ligand of CD24 to cultured stromal vascular fraction (SVF) cells or 3T3-L1 cells and determining the change in adipogenesis.

If the diet-induced obesity experiments were to be repeated, there should be several changes to the experimental design. The mice should be sacrificed by decapitation, without volatile anaesthetics, as to not affect the vasculature and possibly losing the pre-adipocyte population. In addition, analysis should focus on the SVF, rather then the WAT, to ensure the enrichment of mRNA from pre-adipocytes. A baseline of CD24 expression should be established by sampling the mice at time 0, before the

addition of a HFD or LFD. Some adipogenic genes are differentially expressed even before introduction to a HFD, which may partly explain why C57BL/6 mice can have varying responses to a HFD (Koza et al., 2006). Determining baseline expression levels would help account for this variability and allow for a better analysis of changes in gene expression due to a HFD.

Finally, a larger number of mice should be used, especially in the anti-CD24 Ab injection experiment, where only 6 animals were tested. A power test revealed that a sample of 8 is required for a power of 0.8 and significance of 0.5. There was a trend for increased CD24 mRNA in the anti-CD24 antibody injected mice, therefore if there was a larger sample, this may have been significant. In future experiments, more genes involved in adipogenesis, such as those involved in glucose transport and lipogenesis, should be also be examined to get a better overall indication of adipogenesis.

To conclude, the data presented in this thesis demonstrate that CD24 is regulated during pre-adipocyte differentiation and that CD24 expression is inversely correlated with fully developed adipocytes. Furthermore, this is the first study to report the positive feedback regulation of antibody-mediated CD24 clustering leading to increased CD24 expression. Future work is required to determine the signalling pathway regulating CD24 expression in 3T3-L1 pre-adipocytes, as well as to identify how CD24 is activated *in vivo.* Deciphering the precise role of CD24 in pre-adipocytes is important for determining the early molecular events regulating adipogenesis and contributing to the development of therapeutics to combat excess or paucity white adipose tissue.

5.0 References

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Appendix

6.0 Effects of western diet on CD24 expression

The increase in CD24 expression in the first HFD experiment was observed concurrently with an increase in the pre-adipocyte marker Pref-1 and before an increase in any later markers of adipogenesis, suggesting that a HFD can increase CD24 expression at a very early time point during adipogenesis *in vivo*. Because WAT contains a heterogeneous population of cells, experiments were performed to try to identify the cell type in WAT with increased CD24 expression in response to a western diet (WD), a modified version of the HFD. The WD includes a variety of saturated fats, minerals and vitamins, making it isocaloric compared to the HFD and more similar to the diet consumed in western society as compared to the HFD. Five week old mice were used in an attempt to decrease the effect of animal development including hormonal effects that may affect adipogenesis at younger ages and because adiposity during the first 3 week of a HFD may not correlate with difference in food intake or feeding efficiency (Koza et al., 2006).

6.1 Materials and Methods

6.1.1 Antibodies

The antibodies (Abs) specific for CD24 (91), and all secondary ABS were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The Abs specific for CD11b (M1/70), PerCP-Cy 5.5 (Mac-1 marker) and isotype control were purchased from eBioScience (San Diego, CA).

6.1.2 Animal Diets and Procedures

5 week-old male C57BL/6 mice were fed a western diet (WD) containing 40% energy from fat (TestDiet®, Richmond, IN, 5TJN) or a control diet (LFC) containing 12% energy from fat (TestDiet®, Richmond, IN, 5TJS). Mice were sacrificed by cervical dislocation and the epididymal white adipose tissue (WAT) was immediately weighed and either frozen in liquid nitrogen for RNA extraction or minced in Krebs-Ringer Hepes Buffer (KRH) (Joost and Schürmann, 2001) for stromal vascular fractionation. All animal procedures were approved by Memorial University's Institutional Animal Care Committee.

6.1.3 Stromal Vascular Fraction Isolation

WAT in KRH buffer was minced using razor blades followed by incubation in KRH buffer containing 15mg type 1 collagenase (Life Technologies, Burlington, ON), at 37°C for 1 hour with periodic shaking. WAT was then passed through a 100 μM filter and centrifuged for 10 min at 200xg to pellet the stromal vascular fraction (SVF) while causing the mature adipocytes to float to the top. The mature adipocytes and interface were removed and the SVF was washed 3 times in KRH buffer, centrifuging for 8 min at 400xg between each wash. After the final wash, the SVF pellet was re-suspended in 1ml of KRH buffer and cells were stained with Tryphan Blue (Invitrogen), staining the dead cells, and non-stained cells were counted using a haemocytometer.

6.1.4 Flow Cytometry

Cells ($5x10^6$ cells/ml) were used for each analysis and all antibodies were diluted in FACS buffer (1% PBS +5% FBS) to a total volume of 100 µl. Cells were incubated in 1µg of CD24 (91) antibody for 30 min followed by AlexFluor 488 donkey-anti-rat antibody, 1:100, for 30 min. Cells were washed 3 times in FACS buffer between all incubations. Cells were then incubated in 20µl of normal rat serum (Santa Cruz Biotechnologies, sc-45135) for 20 min followed by 10µg of Fab fragment Donkey-anti-rat IgG antibody (Jackson ImmunoResearch, 712-007-003) for 20 min. Finally, cells were incubated in 0.26µg of Mac-1 antibody for one hour. Isotype controls were incubated in AlexFluor 488 donkey-anti-rat antibody(1:100) for 30 min, followed by normal rat serum for 20 min, Fab fragment for 20 min and Rat IgG2b K Isotype Control PerCP-Cy5.5 (0.26µg) for one hour. All cells were re-suspended in 1% paraformaldehyde and analyzed with a BD FACSCalibur. Analysis was completed using Cyflogic (www.cyflogic.com).

6.1.5 RNA Isolation, Dnase Treatment and cDNA synthesis.

As completed in section 2.5.

6.1.6 Quantitative PCR (qRT-PCR)

As completed in section 6.5.

6.1.7 Statistical Analysis

Statistical analysis was performed using the Student's t-test. Differences were considered significant at $p<0.05$. Statistical analysis was determined using VassarStats (Lowry R., 2010).

6.2 Results and Discussion

6.2.1 Western diet increases expression of CD24 at a single cell level after 2 days of feeding

Five week old C57BL/6 mice were fed a western diet (WD, 40% of energy from fat) or low fat control diet (LFC, 12% of energy from fat) for up to 42 days and mRNA and protein expression in the SVF of WAT was examined by qRT-PCR and flow cytometry, respectively. The use of flow cytometry allowed the examination of surface protein expression in cells from the SVF at a single cell level. As WAT is a heterogeneous population of cells, it is unknown if the increase in CD24 expression is on pre-adipocytes or another cell population within the WAT. Macrophages are located in adipose tissue and are known to express CD24; therefore the SVF was co-stained with antibodies for CD24 and Mac-1 (CD11b), a marker of macrophages, and analyzed by flow cytometry, to determine if the increase in CD24 expression was on macrophages.

Mice fed the WD ate significantly more kCal/day, were significantly heavier then LFC mice after 8 days of feeding, and had significantly more WAT after 42 days (Fig 20). There was no significant difference in mRNA levels of CD24 or any adipogenic gene examined between diets until day 42 when there was an increase in PPARγ suggesting that the WD may not be inducing adipogenesis compared to LFC (Fig. 21). The WD caused an increase in body weight after 8 days of feeing, but no change in WAT, while the HFD used in previous experiments, caused an increase in WAT after 1 week but no change in body weight. This may be due to the different diet compositions. The WD is composed of a variety of different fats, vitamins and minerals compared to the HFD,

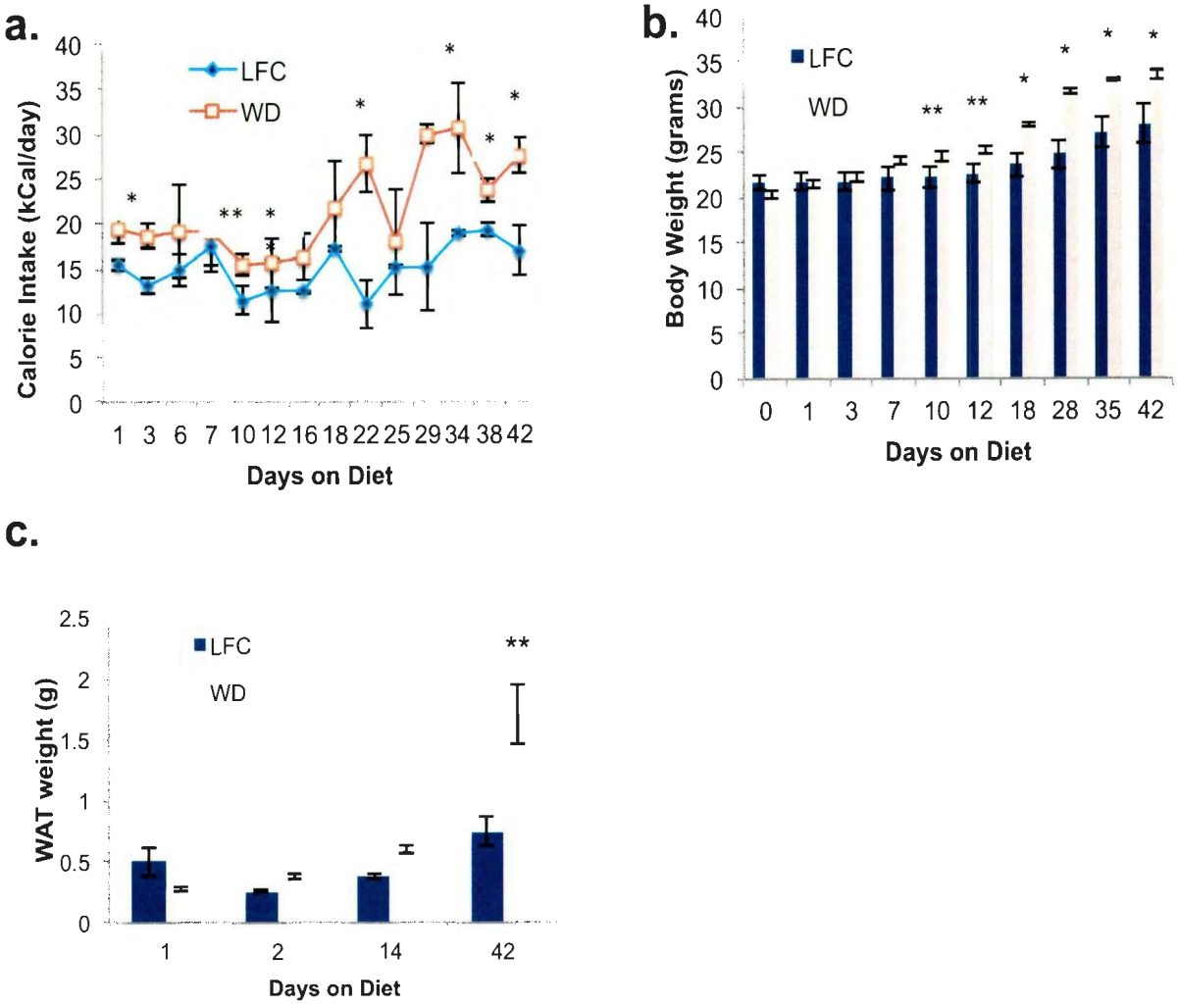

Fig 20. **Mice fed a western diet had increased caloric intake, body weight and WAT compared to LFC.** Five week old C57BL/6 mice were fed a 12% energy from fat low fat control (LFC) or 40% energy from fat western diet (WD) for 1-42 days. **a.** Calorie intake in kCal/day **b.** Body weight of day 42 mice, **c.** WAT weight. Data in **a.** and **c.** shown as mean ± SE. Statistical significance was determined using a Student t-test, n=4, *p<0.01, **p<0.05

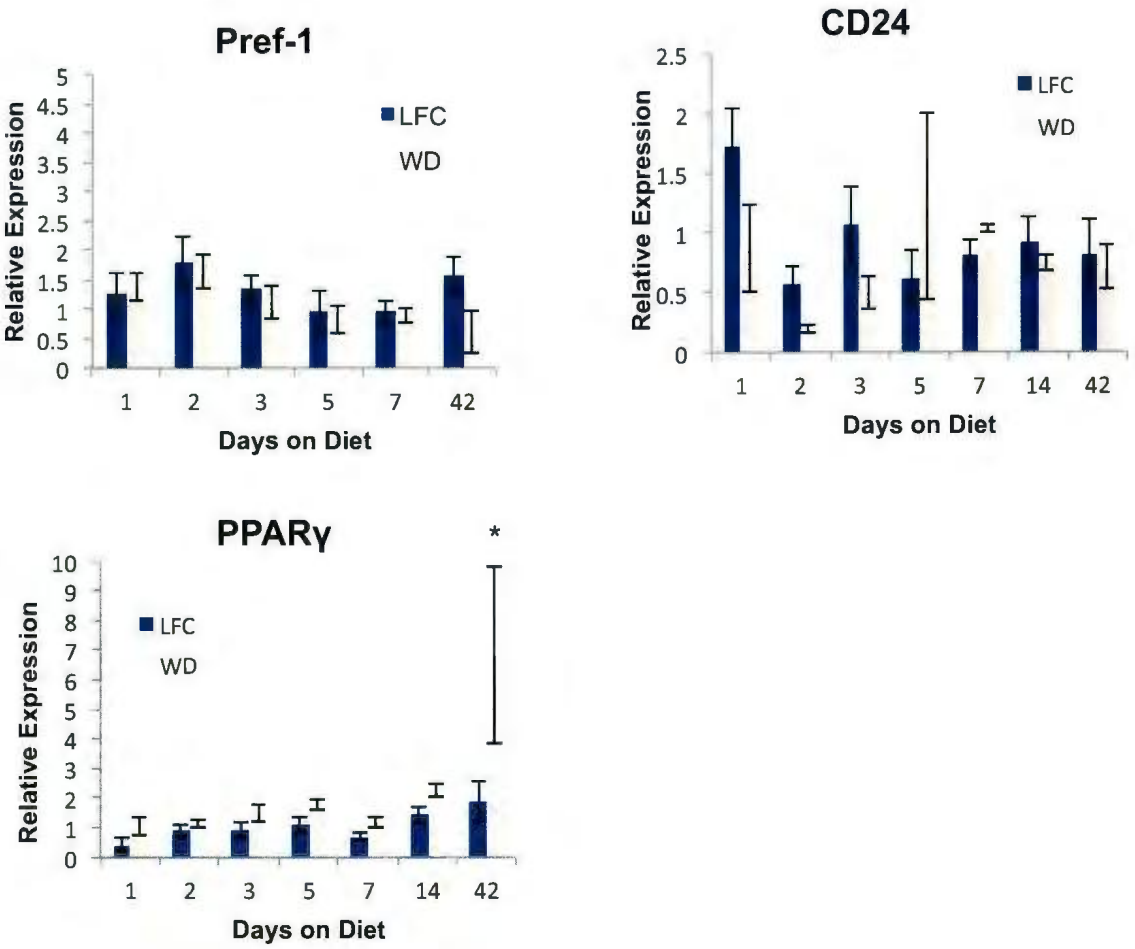

Fig 21. **Western diet had no effect on CD24 mRNA expression or other adipogenic genes until day 42 when there was an increase in PPARγ.** mRNA expression in WAT of 5 week old C57BL/6 mice fed a 12% energy from fat low fat diet (LFC) or a 40% energy from fat western diet (WD) for up to 42 days as determined by qRT PCR. Expression levels were normalized to RPLP0 using the Pffafl equation and calculated relative to one LFC mouse. Data shown as mean ± SE. Statistical significance was determined using a Student t-test, n=4, *p<0.05

which consists mostly of lard, and therefore one hypothesis is that the WD may have affected overall bone, muscle and adipose tissue growth differently then the HFD. Further, there was a high degree of variability in mRNA levels within groups making any biological effects on adipogenesis difficult to discern. As stated in section 1.2.1.1, different responses to a HFD have been found within strains of mice, which may account for the variability within groups, as well as the different response observed between the HFD and WD.

There was no significant difference in the number of cells expressing CD24 (CD24+) until day 42, when there was an increase in cells that expressed both CD24 and Mac-1 (CD24+Mac+) in mice fed a WD (Fig. 22). Obesity is associated with low-grade chronic inflammation, therefore the increase in the CD24+Mac+ cell population after 42 days of the WD was likely due to a recruitment of macrophages or neutrophils, contributing to inflammation during obesity (Subramanian, V., 2009). To determine if macrophage infiltration was the cause of increased CD24 expression after 42 days, mice could be injected intraperitoneally with clodronate liposome to deplete macrophages in the adipose tissue (Pang et al., 2008), and expression of CD24 measured after 42 days of the WD. If no increase in CD24 expression was observed after 42 days of the WD, then the increase in CD24 expression was likely due to infiltration of macrophages expressing CD24.

Analysis of protein expression at a single cell level, measured by mean fluorescence intensity (MFI), revealed a significant increase in high levels of CD24 protein expression after 2 days of the WD (Fig. 23), followed by a decrease in expression levels by day 7. Examination of MFI of CD24 on the Mac+ cell population on day 2

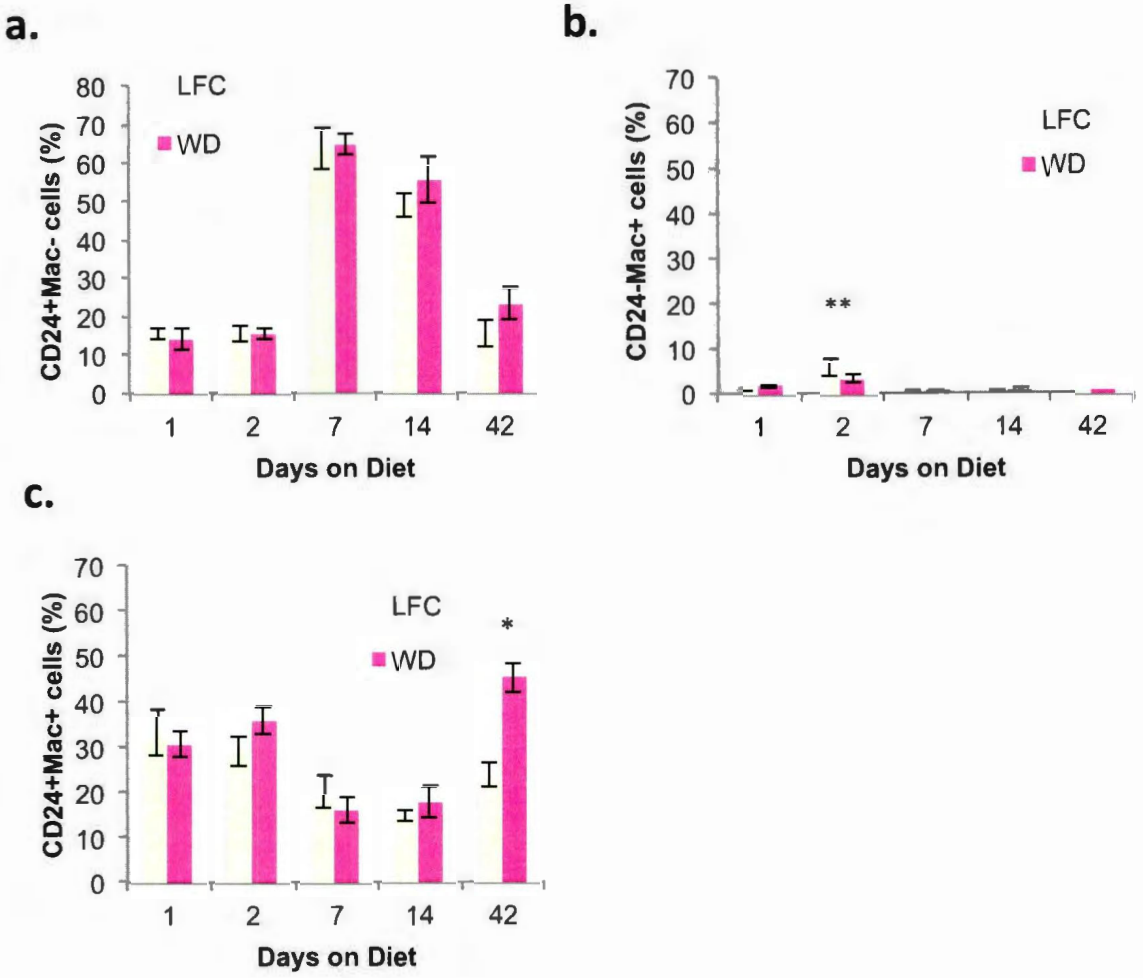

Fig 22. **No change in CD24 protein expressing cells was observed in mice fed a WD compared to the LFC until day 42.** Protein levels of CD24 and Mac-1, a macrophage marker, were determined in the stromal vascular fraction (SVF) of WAT from 5 week old C57BL/6 mice fed a 12% energy from fat low fat control (LFC) or a 40% energy from fat western diet (WD), by flow cytometry and expressed as percent populations. **a.** CD24+Mac- cell population **b.** CD24-Mac+ cell population **c.** CD24+Mac+ cell population. Data shown as mean ± SE. Statistical significance was determined using a Student t-test, n=4, *$p<0.01$, **$p<0.05$

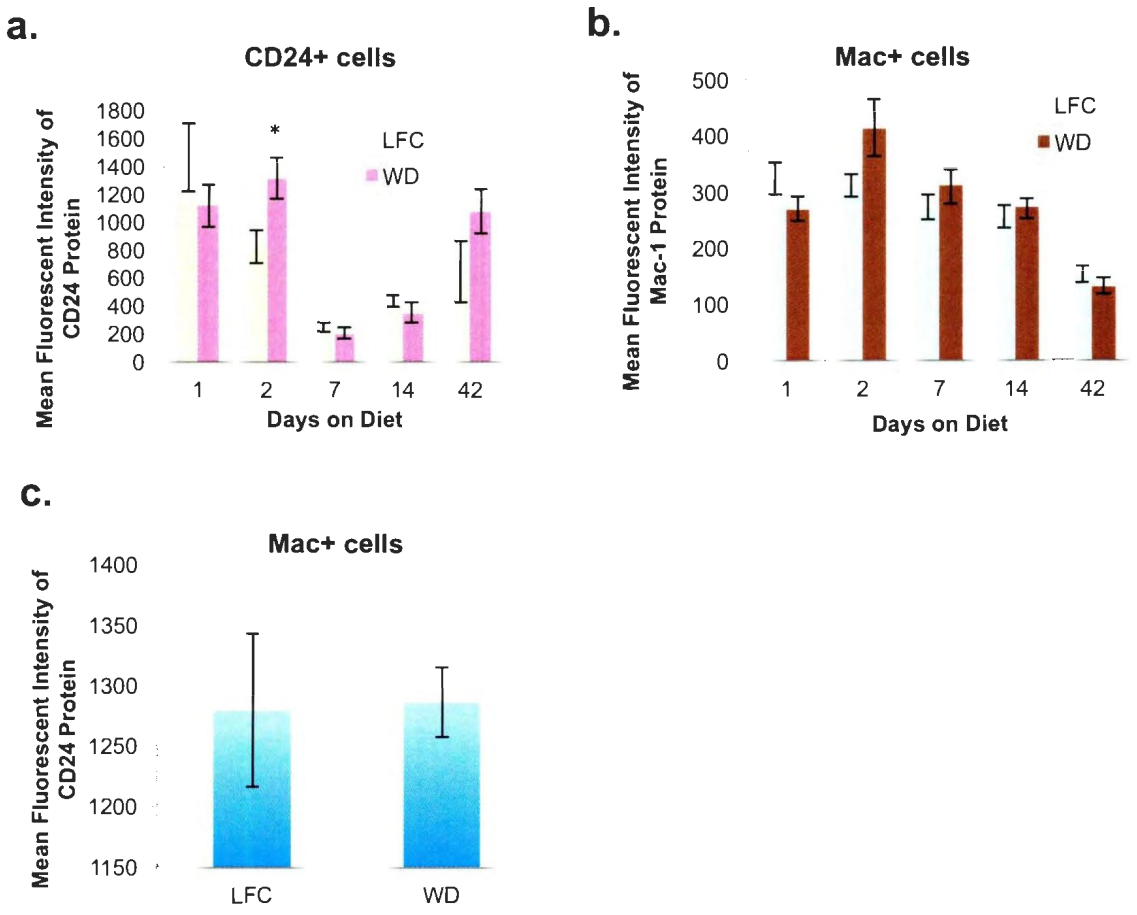

Fig 23. **The amount of CD24 protein per cell increased after 2 days of WD but not on macrophages.** Single cell analysis of protein expression in the stromal vascular fraction (SVF) of WAT from 5 week old C57/BL mice fed a 12% energy from fat low fat control (LFC) or a 40% energy from fat western diet (WD) was determined using flow cytometry and expressed as mean relative intensity of fluorescence of the specified protein. **a.** Cells expressing CD24 protein **b.** Cell expressing Mac-1 protein, **c.** Expression of CD24 protein on Mac-1 protein expressing cells on day 2. Data shown as mean ± SE. Statistical significance was determined using a Student t-test, n=4, *$p<0.05$

determined that the increase in high levels of CD24 protein was not on macrophages but another cell type in the SVF (Fig. 23c).

Significant changes in mRNA levels were found in the HFD vs LFD experiment but not the WD vs LFC diet. There could be several reasons that may contribute to these differences. In the WD experiment, there was high variability between mice in each group, suggesting that a larger sample size may have been required for analysis. Again, variability in responses to a HFD has been observed within strains of mice (Koza et al., 2006). The difference in diet compositions may have also had an effect on the rate of adipogenesis where the WD may be inducing adipogenesis, as well as muscle and bone growth, at a different rate compared to the HFD, although this has never been examined. Finally, the difference in the age of mice (3 week in the HFD vs 5 week in the WD) may have contributed to the different results.

It is hard to discern any conclusions from this experiment as there was a large variability in the effects on adipogenic gene expression with this diet. In future experiments to address the cell type in WAT that expresses CD24, the stromal vascular fraction (SVF) should isolated from the WAT and magnetic cell sorting used to separate specific cells types, such as macrophages or pre-adipocytes. CD24 expression could then be analyzed to determine which cell type is expressing CD24.