EFFECTS OF MACROPHAGE-CONDITIONED MEDIUM ON PREADIPOCYTE CYCLIN-DEPENDENT KINASE REGULATION DURING ADIPOGENESIS

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ABSTRACT

Macrophage-conditioned medium (MacCM) inhibits the differentiation of rodent and human preadipocytes. Previous studies report that murine J774A.1-MacCM inhibits clonal expansion (early required phase of adipogenesis), including Rb phosphorylation. I hypothesized that MacCM induced alterations in cyclins and/or cyclin-dependent kinases (CDKs) were responsible for impairing Rb phosphorylation. My first objective was to assess the effect of J774A.1-MacCM on CDK4, CDK2, and their regulatory cyclins. Murine 3T3-L1 preadipocytes were differentiated with control medium or J774A.1-MacCM. Expression of cyclin D and A was inhibited by J774A.1-MacCM. Inhibition of cyclin A expression was associated with reduced differentiation-induced CDK2 activity. My second objective was to assess the expression patterns of cell cycle proteins in differentiating human abdominal subcutaneous preadipocytes, which do not undergo clonal expansion in culture. Cyclin E expression increased with differentiation. THP-1-MacCM (a human macrophage cell line) further enhanced this increase. My studies suggest MacCM leads to alterations in cyclin/CDK regulation during adipogenesis in murine and human preadipocyte models.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ATM – adipose tissue macrophages
BAT – brown adipose tissue
BMI – body mass index
BSA – bovine serum albumin
CCL – C-C motif chemokine ligand
CDK – cyclin-dependent kinase
C/EBP – CCAAT/enhancer binding protein
CKI – cyclin-dependent kinase inhibitor
CREB – cAMP response element binding protein
CS – calf serum
DEX – dexamethasone
DMEM – Dulbecco's modified Eagle's medium
ERK1/2 – extracellular signal-regulated kinase 1/2
FAS – fatty acid synthase
FBS – fetal bovine serum
FFA – free fatty acid
HDAC – histone deacetylase
HSL – hormone sensitive lipase
IBMX – isobutylmethylxanthine
IFN – interferon
IGF-1 – insulin-like growth factor-1
IR – insulin receptor
IRS – insulin receptor substrate
IOD – integrated optical density
MacCM – macrophage-conditioned medium
MCE – mitotic clonal expansion
MCP-1 – monocyte chemoattractant protein 1
MSC – mesenchymal stem cell
NF-κB – nuclear factor-κB
PBS – phosphate-buffered saline
PDGF – platelet-derived growth factor
PI3K – phosphatidylinositol 3-kinase
PKA – protein kinase A
PP1– protein phosphatase 1
PPAR – peroxisome proliferator-activated receptor
pTyr – phosphotyrosine
Rb – retinoblastoma protein
RPMI – Roswell Park Memorial Institute
T2D – type 2 diabetes
TGFβ – transforming growth factor β
TL4 – Toll-like receptor 4
TNFα – tumor necrosis factor α
TPA – phorbol ester 12-O-tetradecanoylphorbol-13-acetate

WAT – white adipose tissue
WHO – World Health Organization

LIST OF FIGURES

INTRODUCTION

Obesity

Obesity as a global epidemic

The alarming increase in the prevalence of obesity threatens millions of lives around the world. Currently, the World Health Organization (WHO) reports that obesity has reached global epidemic proportions, with over 1 billion adults overweight and approximately 300 million of them obese. By 2030, it is estimated that 1.12 billion individuals will be obese worldwide (Kelly et al., 2008). In Canada, the most recent statistics show that approximately 60% of the adult population is overweight and over 20% are obese (Lau et al., 2007). These alarming increases in obesity are a cause for concern since obesity has been shown to cause or exacerbate a wide array of health problems (Kopelman, 2000).

Definition of obesity

Obesity is defined as an excess amount of white adipose tissue that could potentially impair health (Arner and Spalding, 2010). Body mass index (BMI) is commonly used as a measurement of obesity and is calculated as weight in kilograms divided by height in meters squared (kg/m^2). Individuals with a BMI equal to or above 25 kg/m^2 are classified as overweight and those with a BMI equal to or over 30 kg/m^2 are considered obese (WHO, 2010). BMI provides a convenient population-level measurement of overweight and obesity, but should be used with some caution since it fails to distinguish body fat from lean mass and ignores the anatomical distribution of fat. Consequently, other measurements, such as the waist circumference and waist-to-hip ratio, have been implemented to quantify body fat mass distribution more accurately (Chan et al., 2003; Lau et al., 2007).

Health complications associated with obesity

Hippocrates once stated "Corpulence is not only a disease itself, but the harbinger of others," reflecting the fact that obesity leads to a considerable amount of detrimental consequences. Obesity has been shown to be a risk factor for type 2 diabetes (T2D), cardiovascular disease, hypertension, dyslipidaemia and some cancers (Kopelman, 2000; Haslam and James, 2005).

One of the factors that links obesity to the majority of the previously mentioned health complications is insulin resistance, which is defined as the decreased ability for the muscle, liver and adipose tissue to respond to insulin (Kahn and Flier, 2000; Kopelman, 2000). Unresponsiveness to insulin results in impaired suppression of gluconeogenesis within the liver and reduced glucose uptake into the muscle and adipose tissue, leading to increased blood glucose levels (i.e. hyperglycemia). Insulin also regulates lipid metabolism by suppressing lipolysis in adipocytes. In the state of insulin resistance, ineffective suppression of lipolysis results in the increased release of non-esterified free fatty acids (FFAs) into the bloodstream. These FFAs become ectopically stored as triglycerides in the muscle and liver, further contributing to the diminished insulin sensitivity within these tissues (Reaven, 1988).

Excess accumulation of adipose tissue does not always result in insulin resistance and other adverse metabolic effects. Other factors, such as anatomical fat deposition, play a large role in determining the susceptibility to many metabolic disorders. Central obesity, characterized by increased intra-abdominal or visceral fat, is more closely associated with the increased risk of developing insulin resistance and other metabolic complications, compared to peripheral obesity, characterized by increased subcutaneous fat around the thighs and hips (Despres and Lemieux, 2006). This difference in susceptibility to metabolic disorders could

be due to the fact that the adipocytes found within the subcutaneous depot have different metabolic characteristics than those within the visceral depot (Ibrahim, 2010). For instance, compared to subcutaneous adipocytes, it has been observed that visceral adipocytes are more sensitive to lipolytic stimuli (Arner, 1995) and less sensitive to the antilipolytic effects of insulin (Bolinder et al., 1983; Zierath et al., 1998) . From these observations, it was initially postulated that the visceral depot accounted for the majority of FFAs that promote systemic insulin resistance. Furthermore, the anatomical location of the visceral fat depot is in close proximity to the liver, making it possible for the products from the visceral adipocytes to drain into the portal vein (O'Connell et al., 2010). As a result, these portal FFAs are delivered directly to the liver and could contribute to insulin resistance within the liver. However, more recent studies show that only approximately 20% of portal FFAs originate from visceral fat depots, with the majority being derived from subcutaneous fat in obese subjects (Nielsen et al., 2004; Miles and Jensen, 2005).

In addition to FFA release, visceral and subcutaneous fat depots differ from one another by altered secretion of adipose tissue derived bioactive molecules, also referred to as adipokines (Ibrahim, 2010). Studies have shown that the accumulation of visceral adipose tissue in obese subjects is associated with enhanced levels of pro-inflammatory cytokines (i.e. interleukin (IL)-6 and tumor necrosis factor α (TNFα)) and reduced levels of adiponectin (Fontana et al., 2007; Cartier et al., 2008). Therefore, it is possible that visceral adipose tissue could be causally involved in the development of insulin resistance through the expression and secretion of an altered adipokine profile.

White adipose tissue

Adipose tissue structure and function

The adipose tissue is highly complex, both structurally and functionally and is composed of two types of fat: white and brown (Ahima, 2006). White adipose tissue (WAT) is the predominant type found within mammals and is distributed throughout the human body, with the majority residing in the subcutaneous regions and around the viscera (Gesta et al., 2007). The primary function of the WAT is to store energy and secrete adipokines that modulate whole-body metabolism. On the other hand, brown adipose tissue (BAT), which most commonly found in the cervical–supraclavicular depot, plays an important role in thermogenesis and energy expenditure (Cypess et al., 2009).

BAT is predominantly found in infants and was thought to be almost non-existent and physiologically insignificant in adults. However, recent studies have shown that the existence of BAT in adults is not as rare as once believed. Using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron-emission tomographic (PET) and computed tomographic (CT) scanning, the presence of metabolically functional BAT in adults has been detected (Cypess et al., 2009; Cypess and Kahn, 2010). Furthermore, an inverse correlation was found between the prevalence of BAT and BMI, suggesting a possible role for BAT in adult human metabolism and that BAT may be a potential therapeutic target for obesity and T2D. Since obesity is defined as excess accumulation of WAT, the focus of my thesis is on WAT rather than on BAT.

The cellular composition of WAT is heterogeneous. Besides mature adipocytes, which account for 50-70% of the cell population, the adipose tissue contains various cell types within the stromal fraction (Hauner, 2005). Specifically, the majority of the stromal fraction contains preadipocytes that have the potential to differentiate into mature fat cells (Rodeheffer et al., 2008). Additionally, the stromal fraction includes macrophages, which constitute 1-30% of the cells within the adipose tissue (Hauner, 2005). The exact

physiological role of these adipose tissue macrophages (ATMs) is not well defined; however, there is strong evidence that implicate these macrophages in promoting a pro-inflammatory state that can cause adipose tissue dysfunction in obesity (Lumeng et al., 2007b; Shah et al., 2008). Studies have focused on other possible physiological roles of ATMs, and these will be discussed later.

In addition to maintaining energy homeostasis by storing triglycerides during caloric excess and releasing FFAs when energy is needed, the adipose tissue is a dynamic endocrine organ due to its ability to secrete adipokines. These factors act locally (autocrine/paracrine) or systemically (endocrine) to control appetite, thermogenesis, immunity and neuroendocrine function (Ahima, 2006). The adipose tissue became recognized as a true endocrine organ after the identification and characterization of the adipokine, leptin. Leptin is a polypeptide hormone that is released almost exclusively from adipocytes and acts on the hypothalamus to suppress appetite (Hauner, 2005). As well, adiponectin, alternatively referred to as apM1, Acrp30, adipoQ, and GBP28, is secreted exclusively from adipocytes and plays a critical role in promoting insulin sensitivity (Kershaw and Flier, 2004). Adipocytes and cells within the stromal fraction, such as macrophages, secrete inflammatory molecules that play a role in inflammation, growth, differentiation and apoptosis. It has been documented that the adipose tissue secretes over 100 products, indicative of its dynamic endocrine function (Hauner, 2005).

Adipose tissue expansion

In a state of positive energy balance, there is a demand for additional storage space to accommodate excess calories. In response to this, the adipose tissue expands by two coordinated processes. The first process is called hypertrophy, which is the increase in

adipocyte size that occurs by accumulating triglycerides within the lipid droplet. The second process is hyperplasia, characterized by an increase in cell number that is due to the recruitment of new preadipocytes that undergo proliferation and differentiation (adipogenesis) into mature adipocytes (Jo et al., 2009). It is thought that an initial phase of limited hypertrophy precedes hyperplasia, and once a critical size is reached, these enlarged cells signal neighbouring preadipocytes to undergo adipogenesis. This is supported by the observation that medium conditioned by larger adipocytes induces higher proliferative activity of preadipocytes, in comparison to medium conditioned with smaller adipocytes (Marques et al., 1998). Additionally, an orderly morphological sequence during adipose expansion in diet-induced obesity has been observed in rats, where an increase in adipocyte size occurred, followed by an increase in adipocyte number (Faust et al., 1978).

In lean individuals, it is believed that the total adipocyte number increases in childhood and early adolescence, but levels off and remains constant in adulthood (Spalding et al., 2008). Recent research has shown that adipocyte number is maintained in adulthood by a dynamic and highly regulated process. Using carbon-14 dating, is has been demonstrated that adipose tissue undergoes continuous remodelling, during which new adipocytes are recruited to replace dead adipocytes, such that 10% of adipocytes are renewed annually (adipocyte turnover) (Spalding et al., 2008; Arner and Spalding, 2010).

In obese individuals, adipose tissue expansion appears to be more complex and less clear. In comparison to lean individuals, overweight individuals have larger adipocytes with no marked difference in adipocyte number, whereas severely obese individuals have both larger and more adipocytes (Arner and Spalding, 2010). These differences in adipose cellularity between overweight and obese individuals could be a result of hypertrophy preceding hyperplasia. In obese adults, it is not known whether the increase in adipocyte

number is due to the recruitment of new cells that differentiate into adipocytes or rather a consequence of having more adipocytes at childhood or adolescence. Short-term studies do support the latter, where significant weight gain in adult humans does not result in an increase in adipocyte number (Salans et al., 1971). However, long-term longitudinal studies have yet to be conducted to determine whether individuals are capable of acquiring new fat cells in adulthood.

In addition to regulating fat mass, adipocyte turnover is important for adipocyte function. A deficit in adipogenesis, accompanied with adipocyte death, results in fewer adipocytes that can accommodate excess calories. Consequently, excess calories are stored only in existing adipocytes, leading to the formation of overly hypertrophied cells that become metabolically dysfunctional (Danforth, 2000). Dysfunctional adipocytes are characterized by an altered inflammatory cytokine profile, increased insulin resistance and enhanced release of FFAs (Skurk et al., 2007). As a consequence of acquiring dysfunctional adipocytes, excess calories are redirected to the muscle and liver, diminishing insulin sensitivity in these tissues (Ravussin and Smith, 2002). These consequences that arise from a deficit in adipogenesis are supported by studies that have shown that increased cell size correlates with insulin resistance and an increased risk of developing T2D (Weyer et al., 2000; Lonn et al., 2010). Moreover, it has been shown that obese subjects with fewer but larger adipocytes display higher glucose intolerance and hyperinsulinemia compared to obese subjects with the same degree of adiposity with many small fat cells (hyperplasia) (Arner et al., 2010). Therefore, understanding the molecular mechanisms that regulate adipogenesis will provide insight on the development and function of adipose tissue.

Preadipocyte models

Adipocytes are thought to originate from a multipotent mesenchymal stem cell (MSC) that resides within the stromal fraction of the adipose tissue and that has the potential to also differentiate into bone, muscle or cartilage (Gesta et al., 2007). The stages that the MSC progresses through before committing to the adipocyte lineage are not exactly known. However, it is believed that the MSC gives rise to an early precursor cell, called an adipoblast, which becomes a committed preadipocyte that can undergo differentiation into a mature adipocyte (Gesta et al., 2007). To study the different developmental stages of the adipocyte lineage, various cell models have been established.

Murine 3T3-L1 preadipocyte model

The generation of preadipocyte cell lines has been instrumental in elucidating the molecular and cellular events that regulate adipogenesis. The 3T3 cell line was derived from disaggregated 17 to 19 day-old mouse embryos (Todaro and Green, 1963). Isolation of specific clones that exhibited high susceptibility to adipose conversion led to the generation of the immortalized 3T3-L1 and 3T3-F442A preadipocyte cell lines (Green and Meuth, 1974; Green and Kehinde, 1976). Both cell lines are considered to have undergone determination and commitment to the adipocyte lineage, allowing them to accumulate triglycerides and acquire the morphological and biochemical characteristics of mature adipocytes with the appropriate stimulation (Ntambi and Young-Cheul, 2000).

Although both the 3T3-L1 and 3T3-F442A cell lines have been extensively characterized and continue to serve as reliable *in vitro* models for studying the adipogenic program, there is one difference between them. Injection of only 3T3-F442A preadipocytes into nude mice results in the formation of fat pads that are identical to endogenous ones. This is thought to be due to the lower expression of the anti-adipogenic Wnt-10b protein in 3T3-

F442A in comparison to 3T3-L1 preadipocytes (Ross et al., 2000). It has been suggested that 3T3-F442A preadipocytes are at a further point along the preadipocyte lineage pathway and represent a later stage of development, making this cell line less ideal for studying the early events that initiate adipogenesis (Ross et al., 2000). For this reason, I have chosen the 3T3-L1 preadipocyte cell line to study the early phases of adipogenesis.

Prior to adipogenic stimulation, 3T3-L1 preadipocytes are growth arrested and are morphologically similar to the fibroblastic preadipocytes found within the stromal fraction of the adipose tissue (Gregoire et al., 1998). Once the preadipocytes are exposed to adipogenic inducers, they undergo an early proliferative phase, referred to as mitotic clonal expansion (MCE). This phase is followed by growth arrest and subsequent terminal differentiation. As the preadipocytes progress through the differentiation process, they begin to acquire the characteristics of mature adipocytes (i.e. a round and lipid-filled morphology) (Gregoire et al., 1998).

Several features of 3T3-L1 preadipocytes provide many advantages when studying the cellular events that occur during differentiation. Firstly, 3T3-L1 preadipocytes can be maintained in culture for long periods of time and passaged repeatedly without resulting in any alterations that would affect the differentiation process (Green and Meuth, 1974). Secondly, the 3T3-L1 cell line is a convenient model to study differentiation since the differentiation process (6-8 days) occurs over a shorter period of time in comparison to the process for cultured human primary preadipocytes (14-15 days). Lastly, the 3T3-L1 cell line provides an unlimited supply of cells. This is in contrast to the study of primary human preadipocytes that is constrained by the limited availability of human adipose tissue and the variable yield and behaviour of preadipocytes due to inter-donor variation.

The use of an immortalized murine cell line does have some limitations. Some of the disadvantages include species difference and aneuploidy (Cornelius et al., 1994). In addition, metabolic differences between subcutaneous adipocytes and visceral adipocytes cannot be studied using this model. To circumvent these limitations, primary human preadipocytes can be studied to confirm 3T3-L1 preadipocyte results when indicated.

Human primary preadipocyte model

The development of primary preadipocyte culture models has made the study of human adipocyte differentiation possible. Protocols have been established to isolate preadipocytes from the stromal fraction within human adipose tissue. Although these primary human preadipocytes are similar to immortalized clonal cell lines, in that they can be placed in culture and hormonally induced to differentiate into adipocytes, there are some significant differences between the two model types.

In comparison to the 3T3-L1 system, human primary preadipocyte cultures provide the advantage of a more physiologically relevant system for studying adipogenesis (Gregoire et al., 1998). Isolation of preadipocytes from different anatomical locations allows for the study of depot-specific characteristics.

However, there are some drawbacks to using human primary preadipocytes. In culture, human primary preadipocytes differentiate into adipocytes without undergoing MCE (Ross et al., 2008). It is thought that these primary cells represent a stage that is further along the adipogenic pathway (Gregoire et al., 1998). As a result, primary preadipocyte cultures cannot be used for the study of early differentiation events. Additionally, in comparison to clonal cell lines, primary cell models have a limited life span in culture, grow slowly and vary in adipogenic potential according to the donor (Newell et al., 2006). Despite these

drawbacks, they provide a means to verify and confirm the observations made in clonal cell lines regarding the adipogenic program (Cornelius et al., 1994).

Adipocyte differentiation

Adipogenic program

Adipogenesis involves a complex network of transcription factors and cell-cycle regulators. With the use of immortalized preadipocyte cell lines, much progress has been made in defining the transcriptional events that control the adipogenic program. The cascade of events that lead to the formation of mature lipid-laden adipocytes is described below and outlined in Figure 1.

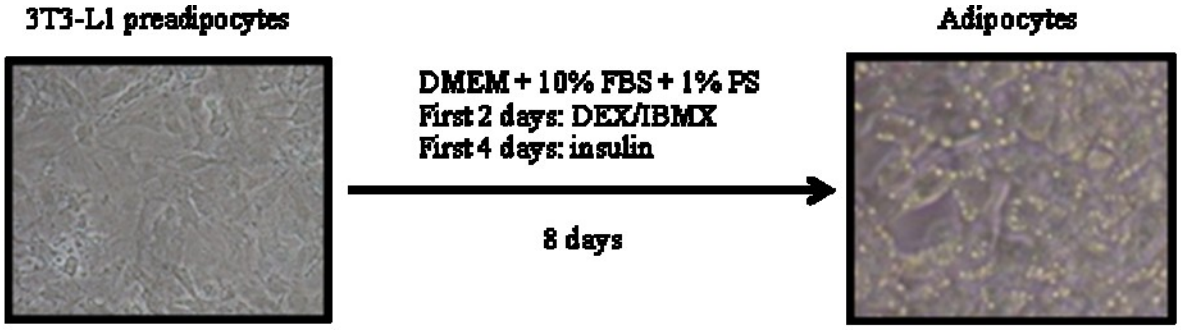
Adipogenic transcriptional cascade

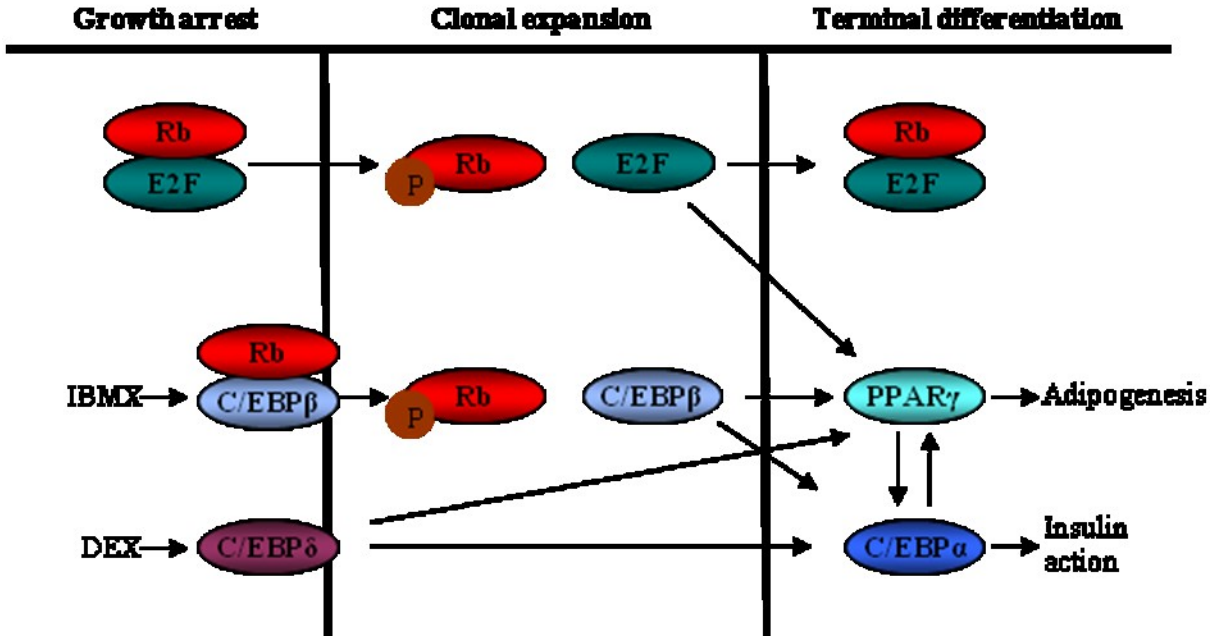
Growth arrest and hormone induction

In culture, preadipocytes grow exponentially until confluence is reached and they become growth-arrested in G_0 by contact inhibition (cell-cell contact). It is believed that growth arrest is required for adipogenesis (Pairault and Green, 1979). Growth arrested preadipocytes can then be induced to differentiate upon exposure to a cocktail of inducers and serum. The differentiation process for both 3T3-L1 and human primary preadipocytes will be discussed below.

For the differentiation of 3T3-L1 preadipocytes, the adipogenic inducers within the cocktail include a synthetic glucocorticoid (dexamethasone, DEX), a phosphodiesterase inhibitor that acts to elevate cyclic AMP (isobutylmethylxanthine, IBMX) and supraphysiological concentrations of insulin (Smith et al., 1988). Since preadipocytes express many more insulin-like growth factor-1 (IGF-1) receptors relative to insulin

Figure 1. Overview of 3T3-L1 adipocyte differentiation. Two-day post-confluent, growth-arrested preadipocytes are induced to differentiate upon exposure to insulin, dexamethasone (DEX), isobutylmethylxanthine (IBMX) and FBS. Following adipogenic induction, preadipocytes re-enter the cell cycle to undergo 1-2 rounds of cell division (i.e. MCE). MCE requires the phosphorylation of Rb and this allows for the release of E2F and C/EBPβ. The activation of these transcription factors, along with C/EBPδ, lead to the upregulation of C/EBPα and PPARγ. Together, C/EBPα and PPARγ direct the adipogenic program following MCE, allowing the cells to acquire the phenotype of mature adipocytes. See text for further details and abbreviations.


3T3-L1 preadipocytes
Adipocytes
DMEM + 10% FBS + 1% PS
First 2 days: DEX/IBMX
First 4 days: insulin
8 days


Growth arrest
Clonal expansion
Terminal differentiation
Rb
E2F
P
Rb
E2F
E2F
Rb
E2F
Rb
IBMX
C/EBPβ
P
Rb
C/EBPβ
PPARγ
Adipogenesis
DEX
C/EBPδ
C/EBPα
Insulin action

receptors (IR), insulin is thought to act primarily through IGF-1 receptor to induce differentiation (Smith et al., 1988). However, studies have shown that insulin signaling through the IR can also mediate differentiation (Accili and Taylor, 1991; Gagnon and Sorisky, 1998).

For the differentiation of primary human preadipocytes in culture, the differentiation cocktail consists of the adipogenic inducers mentioned above in addition to indomethacin, which serves as a ligand that binds and activates peroxisome proliferator-activated receptor (PPAR) γ, a transcription factor that is necessary and sufficient for differentiation (Rosen et al., 2000).

Mitotic clonal expansion

Following stimulation of 3T3-L1 preadipocytes with the adipogenic inducers, the cells synchronously re-enter the cell cycle to undergo 1-2 rounds of mitotic division (i.e. MCE). During the DNA replication phase of MCE, changes in chromatin structure enable access to adipocyte-specific promoter sites, therefore facilitating the upregulation of adipogenic genes (MacDougald and Lane, 1995). MCE has shown to be required for adipogenesis in 3T3-L1 preadipocytes, but does not occur in primary human preadipocytes that are induced to differentiate *in vitro* (Ross et al., 2008; Tang et al., 2003b). This may be due to the possibility that human preadipocytes have already undergone a proliferative phase that is similar to MCE *in vivo* prior to isolation.

MCE involves the expression of specific transcription factors and cell-cycle regulators that work in concert to promote the upregulation of adipogenic genes. Similar to subconfluent proliferating cells, cell cycle progression through the G_1/S boundary is regulated by the phosphorylation status of retinoblastoma protein (Rb) during MCE. In the

G_1 phase of the cell cycle, the hypophosphorylated form of Rb inhibits proliferation and differentiation by binding to essential regulatory proteins, including members of the E2F and CCAAT/enhancer binding protein (C/EBP) family of transcription factors. E2F transcription factors are responsible for upregulating genes that participate in progression through the cell cycle and differentiation (i.e. PPARγ) (Mudryj et al., 1990; Fajas et al., 2002b; Satyanarayana and Kaldis, 2009). On the other hand, C/EBP transcription factors are involved in the initiation of the adipogenic signaling cascade that results in the transcriptional activation of genes that lead to the adipocyte phenotype. The inhibitory action of Rb is relieved once it becomes phosphorylated at the G_1/S boundary by serine/threonine cyclin-dependent kinases (CDKs) that form active complexes with their cyclin partners (Farmer, 2006). The two primary cyclin/CDK complexes that are involved in Rb phosphorylation include CDK4/6 and its cyclin partner, cyclin D, as well as CDK2 that binds to either cyclin E or cyclin A. Rb phosphorylation occurs sequentially, with cyclin D/CDK4/6 initially phosphorylating Rb during the early G_1 phase, followed by cyclin E/A/CDK2 during the late G_1 phase and entrance into the S phase (Sherr, 1994). The activity of cyclin/CDK complexes is negatively regulated by cyclin-dependent kinase inhibitors (CKIs). The two widely studied CKIs include $p27^{Kip1}$ and $p21^{Waf1/Cip1}$, which have been shown to be downregulated during MCE to allow for cyclin/CDK activation and subsequent Rb phosphorylation (Morrison and Farmer, 1999).

When Rb becomes phosphorylated, E2F transcription factors are released, gain access for DNA binding and promote the expression of genes involved in cell cycle progression through S phase (i.e. cyclin E, cyclin A and c-Myc). Once MCE is completed, $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ are induced to inactivate cyclin/CDK complexes and the hypophosphorylated form of Rb re-associates with E2F, allowing cells to exit the cell cycle.

In addition to Rb, two other Rb-related pocket proteins (p107 and p130) negatively regulate E2F transcription factors, a characteristic that does not occur in preconfluent proliferation and is unique to MCE (Richon et al., 1997). At confluence, p130 associates with E2F. As preadipocytes progress through MCE, p130 is downregulated and p107 expression increases. At this point, E2F/p130 complexes subside, while E2F/p107 complexes appear. After the completion of MCE, the expression pattern observed in confluent cells is re-established. Interestingly, the switch in p130 and p107 expression is also observed during differentiation in human adipose-derived stem cells, despite the absence of MCE (Ross et al., 2008). This suggests that cell-cycle regulators may be important for the differentiation process in human primary preadipocytes even though the cells do not undergo MCE.

Similar to the regulation of E2Fs, it has been previously reported that the phosphorylation of Rb removes the inhibitory constraint on C/EBPs, which can initiate the transcriptional cascade that is necessary for adipogenesis (Cole et al., 2004). In addition to E2Fs and C/EBPs, Rb is also a negative regulator of PPARγ (Fajas et al., 2002a). Rb has the ability to recruit histone deacetylase 3 (HDAC3) to PPARγ target promoter sites, leading to the formation of PPARγ-Rb-HDAC3 repressor complexes that block PPARγ transcriptional activity. In order for adipogenesis to occur, the disruption of the repressor complex via the phosphorylation of Rb or inhibition of HDAC3 activity is essential.

Transcriptional regulation of adipogenic genes

The two C/EBP transcription factors that are induced almost immediately after adipogenic induction include C/EBPβ and C/EBPδ. The upregulation of C/EBPβ and C/EBPδ are directly induced by IBMX and DEX, respectively (Cao et al., 1991; Yeh et al., 1995). The expression of these early adipogenic transcription factors has been shown to be

required for adipogenesis *in vivo* (Tanaka et al., 1997). More specifically, studies have revealed that C/EBPβ and C/EBPδ play important roles in the progression through MCE (Tang et al., 2003a; Zhang et al., 2004; Hishida et al., 2009).

Although both C/EBPβ and C/EBPδ are expressed very early on during the differentiation process, they do not gain DNA binding until the cells transition into the S phase of the cell cycle, which occurs approximately18-20 hours after induction (Tang and Lane, 1999). This delay in DNA binding is thought to allow the cells to progress through MCE and to prevent the early expression of C/EBPα and PPARγ, which are both anti-mitotic. It is possible that this delay is regulated by Rb, MAPK/ERK or cyclin A/CDK2 (Chen et al., 1996; Li et al., 2007). Specifically, the phosphorylation status of Rb regulates the transcriptional activity of C/EBPβ, as described above. In addition, studies have shown that C/EBPβ undergoes successive phosphorylations before binding to its target promoters. C/EBPβ is first phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2) or cyclin A/CDK2 (on Thr188) in G_1 and later by glycogen synthase kinase (GSK3β; on Ser184 or Thr179) at the onset of S phase (Li et al., 2007). It is believed that the phosphorylation of C/EBPβ induces a conformational change that is necessary to facilitate DNA binding activity and transcription (Kim et al., 2007).

Cell cycle exit and terminal differentiation

Once C/EBPβ and C/EBPδ gain DNA binding activity, together, they direct the adipogenic program by promoting the expression of two master regulators of adipogenesis, C/EBPα and PPARγ (Koutnikova and Auwerx, 2001; Farmer, 2006). Due to the anti-mitotic activity of C/EBPα and PPARγ, the upregulation of these factors lead to permanent withdrawal from the cell cycle and entrance into terminal differentiation (Timchenko et al.,

1996; Altiok et al., 1997). PPARγ has been shown to be both necessary and sufficient for adipogenesis (Rosen et al., 2000). In contrast, C/EBPα is unable to induce differentiation in the absence of PPARγ. However, C/EBPα plays an important role in mature adipocytes by maintaining PPARγ expression and glucose transport, an event that cannot be accomplished by PPARγ alone (Hamm et al., 1999).

In the terminal stage of differentiation, C/EBPα and PPARγ induce the expression of several genes that produce the adipocyte phenotype. Some of these genes are involved in glucose and lipid metabolism. In particular, increased expression of insulin-dependent glucose transporters GLUT-4 and insulin receptors allows the adipocytes to acquire insulin sensitivity (Rubin et al., 1978; Hamm et al., 1999). Additionally, the expression of several genes that participate in triglyceride synthesis and lipolysis, including fatty acid synthase (FAS) and hormone sensitive lipase (HSL), is enhanced (Rosen and Spiegelman, 2000). Other proteins specific to the mature phenotype are also synthesized, including fatty acid binding protein (aP2), perilipin and adiponectin (Gregoire et al., 1998).

Insulin/IGF-I Signaling

Insulin and/or IGF-I is known to be a required component of the differentiation cocktail in triggering the adipogenic program (Smith et al., 1988). Unlike insulin/IGF-I, DEX or IBMX alone are unable to initiate adipogenesis. Preadipocytes express twice the amount of IGF-I receptor (IGFR) in comparison to the IR and therefore, it is believed that supraphysiological concentrations of insulin induce differentiation by signaling primarily through the IGFR (Rubin et al., 1978; Smith et al., 1988). However, inactivation of the IR using gene targeting approaches resulted in a markedly reduced ability for preadipocytes to differentiate, suggesting that signaling through the IR plays a direct role in differentiation

(Accili and Taylor, 1991; Gagnon and Sorisky, 1998). Given that IR and IGF-I signaling transduction events are similar, only IR signaling will be described below.

The IR is a heterotetramer, composed of two αβ dimers that are linked by disulfide bonds (White, 2003). Insulin binds to two extracellular α subunits, inducing a conformational change that results in autophosphorylation of the intracellular portions of the transmembrane β subunits (Saltiel and Kahn, 2001). Activation of the IR allows for the recruitment and tyrosine phosphorylation of insulin receptor substrate (IRS) proteins that contain phosphotyrosine-binding domains that recognize a specific juxtamembrane phosphotyrosine (pTyr) residue on the IR. The phosphorylated tyrosine residues on the IRS proteins create specific docking sites that are recognized by the Src homology 2 (SH2) domains of various proteins. In particular, the recruitment of phosphatidylinositol 3-kinase (PI3K) and Grb2 initiate the activation of the Akt and ERK1/2 signalling cascades, respectively (White, 2003).

The activation of the Akt pathway is a critical signaling component for differentiation. *In vivo* studies have shown that deficiency in Akt results in impaired adipogenesis (Peng et al., 2003). On the other hand, studies have demonstrated that constitutively active Akt can lead to the spontaneous differentiation of preadipocytes (Kohn et al., 1996; Magun et al., 1996). Although the exact downstream targets of Akt that participate in differentiation are not well defined, it has been suggested that this pathway regulates cell cycle proteins that are involved in MCE. In particular, insulin-induced activation of the PI3K pathway is associated with the upregulation of cyclin D during the early phase of differentiation (Muise-Helmericks et al., 1998; Usui et al., 2000). In addition, Akt was shown to phosphorylate and inhibit GSK3β from inducing the proteasomal degradation of cyclin D1 (Cross et al., 1995; Diehl et al., 2003). As a result, cyclin D1 expression is stabilized, promoting the assembly of cyclin D/CDK4 complexes that partially

phosphorylate Rb. The initial phosphorylation of Rb releases E2F, leading to the transcription of cyclin E and cyclin A, which bind to CDK2 to further phosphorylate Rb (Ohtani et al., 1995). This results in the release of more E2F and allows for the transcription of necessary genes that drive cells into the S phase for DNA replication.

The regulation of adipogenesis by the activation of the ERK1/2 pathway is complex. There is evidence to support that early transient ERK1/2 activation is required for MCE by promoting the progression through G_1 (Prusty et al., 2002; Tang et al., 2003b). Cell cycle progression could be due to the fact that ERK1/2 positively regulates the expression of cyclin D1, leading to the increase in associated CDK activity (Lavoie et al., 1996). In addition, it has been reported that ERK1/2 participates in the stabilization of c-Myc protein, which regulates the transcription of genes that are necessary for cell proliferation (Sears et al., 2000).

Adipose tissue dysfunction and macrophages

Adipose tissue dysfunction is characterized by limited ability to store excess energy due to impaired adipogenesis, reduced insulin sensitivity, ectopic storage of triglycerides, abnormal inflammatory expression and increased macrophage infiltration into the adipose tissue. Although the exact factors that are responsible for causing adipose dysfunction are not known, there is emerging evidence to suggest that macrophages may play a role.

Firstly, obesity is commonly described as a state of chronic low grade inflammation, characterized by enhanced levels of circulating pro-inflammatory cytokines. It is well accepted that the adipose tissue, more specifically the macrophage population that resides within the tissue, serves as the primary source of these pro-inflammatory mediators (Fain et al., 2004). This is supported by the observation that macrophages can account for up to 50%

of the cell population within adipose tissue in obese mice and humans, compared to 5-10% in adipose tissue from lean ones (Weisberg et al., 2003; Xu et al., 2003). Pro-inflammatory cytokines are implicated in promoting insulin resistance in obesity by activating the c-Jun NH(2)-terminal kinase (JNK) pathway that induces the phosphorylation of serine residues on IRS-1, preventing the normal tyrosine kinase cascade from occurring (Aguirre et al., 2002). Thus, the chronic state of inflammation in obesity can lead to diminished insulin sensitivity within the adipocytes, leading to the formation of dysfunctional adipose tissue.

Secondly, macrophages have been implicated in adipose tissue dysfunction by limiting adipogenesis. This is evidenced by the observation that medium conditioned by different macrophage models (MacCM) inhibits adipogenesis of various preadipocyte models (Constant et al., 2006; Lacasa et al., 2007; Stienstra et al., 2008; Keophiphath et al., 2009; Lu et al., 2010). The inhibitory effect is characterized by impaired cell rounding, reduced lipid accumulation and decreased expression of adipogenic markers. The exact mechanism by which MacCM inhibits adipogenesis is unknown. However, using the established 3T3-L1 preadipocyte model, our laboratory showed that MacCM prevents MCE and that exposure to MacCM at the beginning of differentiation is required for the complete inhibitory effect. We recently reported that MacCM disrupts the essential phosphorylation of Rb, providing a possible explanation for the block in MCE and therefore adipogenesis (Yarmo et al., 2009).

In general, the MacCM studies reveal an important interaction between macrophages and adipose cells that could have profound physiological relevance in adipose tissue remodelling and function *in vivo*. The consequence of restricted adipogenesis includes the formation of hypertrophied adipocytes, which are highly insulin resistant, inflamed and hyperlipolytic (Le Lay et al., 2001; Jernas et al., 2006; Franck et al., 2007; Skurk et al.,

2007). Studies have shown that adipocyte hypertrophy is linked to altered cellular metabolism through a mechanism that involves the redistribution of cholesterol from the plasma membrane to the lipid droplet. Reduction in membrane cholesterol was shown to be related to adipocyte insulin resistance and enhanced secretion of adipocyte derived factors, including TNFα, angiotensinogen and IL-6 (Le Lay et al., 2001). In addition, the increase in FFA release correlates with cell size and is associated with enhanced inflammation (Suganami et al., 2005). Thus, the formation of hypertrophied adipocytes as a result of impaired adipogenesis can give rise to an inflammatory and insulin resistant state.

Adipose tissue macrophages

Adipose tissue macrophage origin and activation

The discovery that macrophages are present in obese adipose tissue and are capable of inducing local and systemic insulin resistance by promoting a chronic state of inflammation has prompted studies on the origin, phenotypes and functions of ATMs. Macrophages are phagocytic cells that can be found in virtually all tissues and are thought to originate from bone marrow derived peripheral-blood mononuclear cells that differentiate into monocytes, circulate in the blood and enter into the tissue (Mosser and Edwards, 2008). The monocyte population is highly heterogeneous, and whether specific monocyte populations give rise to specific tissue macrophages is not known (Mosser and Edwards, 2008). It was previously suggested that ATMs could be derived from adipocyte progenitor cells, since preadipocytes exhibit phagocytic activity and have a similar gene expression profile as macrophages (Charriere et al., 2003). However, bone marrow transplant studies have shown that 85% of the ATM population is composed of bone marrow-derived cells (Weisberg et al., 2003).

The phenotypes of ATMs are dependent on the distinct programs of macrophage activation: classical (or M1) or alternative (or M2) (Lumeng et al., 2007a). Macrophages that are stimulated by type1 T-helper (T_H1) cytokines, such as interferon γ (IFNγ), or by bacterial by-products, induce the M1phenotype. These macrophages have high bactericidal potential and produce high amounts of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-12. In contrast, the promotion of M2 macrophages is through the stimulation of type 2 T-helper (T_H2) cytokines, including IL-4 and IL-13. These macrophages participate in tissue remodelling and produce anti-inflammatory mediators, such as IL-1 receptor antagonist and IL-10 (Mantovani et al., 2004).

The activation state of macrophages plays a role in the modulation of insulin sensitivity in obesity. Previous studies have demonstrated that obesity induces a switch in ATM phenotype from an anti-inflammatory M2 state to a pro-inflammatory M1 state, resulting in inflammation and insulin resistance in mice (Lumeng et al., 2007a; Fujisaka et al., 2009). However, some studies have identified ATMs with a mixed M1/M2 expression pattern after high-fat feeding in mice and humans (Zeyda et al., 2007; Shaul et al., 2010). These ATMs are thought to have a "remodelling phenotype" that act in the process of adipose tissue development (Bourlier et al., 2008; Shaul et al., 2010).

Macrophage models

Murine J774A.1 macrophage model

Macrophage cell lines have been established to study immune-related functions, such as cytokine production, chemotaxis and phagocytosis. In particular, the establishment of the J774A.1 macrophage cell line, a murine reticulum cell sarcoma line, has provided a means for studying eukaryotic cell motility and immunomechanics (Ralph and Nakoinz, 1975; Lam

et al., 2009). This macrophage cell line was derived from a tumor originating in a female BALB/c/NIH mouse (Ralph et al., 1975).

J774A.1 macrophages exhibit macrophage-defining characteristics, including adherence, expression of receptors for immunoglobulin and the ability to carry out antibody-dependent phagocytosis (Ralph and Nakoinz, 1975). Therefore, this macrophage cell line serves as an appropriate model system to study macrophage functional properties. In addition, given that macrophage cell lines can be easily maintained and genetically manipulated, they are highly recognized as the preferred model systems for studying macrophage-related functions (Lam et al., 2009).

However, given that J774A.1 macrophages are of cancer origin, they may vary in biological and biochemical properties compared to normal macrophages. In fact, a study has shown that J774A.1 macrophages respond poorly to certain chemotactic stimuli, in comparison to normal macrophages (Snyderman et al., 1977). Despite this, with respect to their anti-adipogenic action, J774A.1 macrophages behave similarly to human monocyte-derived macrophages in suppressing adipogenesis.

Human THP-1 macrophage model

The THP-1 monocyte cell line was the first human leukemic cell line with monocytic properties and immunological functions to be established (Tsuchiya et al., 1980). The cells were cultured from the blood of a 1 year old boy suffering from acute monocytic leukemia. In culture, the THP-1 cells are in suspension and maintain a human monocytic nature, characterized by morphology, secretory products, and expression of membrane antigens and other genes that participate in lipid metabolism (Tsuchiya et al., 1980; Auwerx, 1991). When the cells are treated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA),

proliferation is halted and they differentiate into mature macrophages (Tsuchiya et al., 1982). Morphological and functional changes occur, including adherence to the culture surface, expression of IgG Fc receptors, and phagocytic activity (Tsuchiya et al., 1982).

In comparison to other human myeloid cell lines, TPA-treated THP-1 cells have been shown to mimic native monocyte-derived macrophages in several ways, including changes in morphology, expression of membrane molecules and secretion of a wide range of bioactive factors (i.e. polypeptide hormones, enzymes and reactive oxygen species) (Auwerx, 1991). Therefore, the THP-1 cell line serves as an appropriate model for the study of macrophage-specific gene expression and functions. Also, in comparison to native human blood monocytes, the THP-1 cell line offers the advantage of a more homogenous cell population (Auwerx, 1991).

However, there are some limitations with the use of the THP-1 cell line. As a cancer cell line, there are still concerns that THP-1 cells may differ from native human macrophages in gene expression, phenotype and function. In addition, TPA is an artificial inducer of macrophage differentiation and it is possible that activation of particular signal transduction pathways may differ according to the choice of inducer, resulting in different macrophage phenotypes (Auwerx, 1991).

Adipose tissue macrophage recruitment

The exact mechanisms underlying increased macrophage recruitment into obese adipose tissue is unclear. However, there is growing evidence to show that factors derived from the adipose tissue, including chemokines and inflammatory mediators, may be involved in macrophage recruitment. In particular, C-C motif chemokine ligand (CCL) 2, also known as monocyte chemoattractant protein 1 (MCP-1), is one of the most studied chemokines

implicated in macrophage recruitment (Kanda et al., 2006). Studies have shown that elevated plasma concentrations of CCL2 are associated with increased macrophage accumulation within the adipose tissue in obesity. Conversely, these effects are reduced with deficiency in CCL2 (Kanda et al., 2006). A recent report has shown that the chemokine, CCL5 (also known as RANTES), participates in WAT macrophage recruitment in obese humans (Keophiphath et al., 2010). Although deficiency or antagonism of chemokines has shown to lessen obesity-induced macrophage infiltration, accumulation of macrophages is not completely abolished, suggesting that non-chemokine factors may play a role in recruitment during obesity. Other mediators that have been reported include complement factors and toll-like receptor 4 (TL4). In addition, previous studies have indicated the possibility that macrophages are recruited into the adipose tissue by dying adipocytes (Cinti et al., 2005; Strissel et al., 2007). Obesity in mice and humans has been shown to be associated with an increase in adipocyte necrosis. More than 90% of adipose tissue macrophages are localized around what appear to be dead adipocytes, forming crown-like structures. Thus, it is possible that ATMs are involved in clearing cellular debris from the adipose tissue.

Interactions between macrophages and preadipocytes/adipocytes

The molecular basis by which macrophages and adipocytes may communicate has been studied using *in vitro* co-culture systems. It has been postulated that adipocyte-derived FFAs and macrophage-derived TNFα create a paracrine loop that exacerbates inflammatory changes within obese adipose tissue. Suganami *et al.* demonstrated that co-culturing 3T3-L1 adipocytes with murine RAW264 macrophages resulted in an upregulation of TNFα and IL-6 in both adipocytes and macrophages and a downregulation of adiponectin in adipocytes (Suganami et al., 2005). These results were observed without direct contact between the

adipocytes and macrophages, indicating that communication between these cell types involves a paracrine mechanism. It has been suggested that macrophage-derived TNFα induces lipolysis within hypertrophied adipocytes, resulting in increased FFA secretion. In turn, these FFAs serve as naturally occurring ligands for TLR4, activating the nuclear factor-κB (NF-κB) pathway within macrophages, therefore leading to increased TNFα production (Suganami et al., 2007). *In vivo* studies have further supported these findings by showing that TLR4 deficient mice displayed reduced macrophage infiltration into the adipose tissue, decreased obesity-associated adipose tissue inflammation and improved insulin sensitivity (Davis et al., 2008). Additionally, other co-culture studies have demonstrated that factors derived from macrophages block insulin action in mature adiopcytes by downregulating IRS-1, leading to decreased PI3K/Akt signaling and subsequent impairment of GLUT4 translocation to the plasma membrane (Lumeng et al., 2007b). Together, these studies have provided valuable insight into the interactions between macrophages and adipocytes.

Our laboratory and others have recently investigated the effects of macrophage-secreted factors on adipocyte differentiation. These studies implicate macrophages in the restriction of adipose tissue expansion and adipose tissue dysfunction. As previously stated, we have focused on the effect of MacCM on 3T3-L1 preadipocyte differentiation in order to determine the molecular mechanism underlying the anti-adipogenic effect. The recent finding that MacCM disrupts the early adipogenic events including MCE and Rb phosphorylation has prompted us to determine the effects of MacCM on cell cycle factors that regulate Rb phosphorylation. We believe that this investigation will further our understanding of the mechanism by which MacCM inhibits adipogenesis, offering potential relevance to the interaction between macrophages and adipose cells *in vivo*.

HYPOTHESIS AND OBJECTIVES

Hypothesis

The overall hypothesis is that the anti-adipogenic effect of MacCM is associated with alterations in cyclins and/or cyclin-dependent kinases (CDKs) that impair Rb phosphorylation.

Objective 1

My first objective was to assess the effects of J774A.1-MacCM on CDK4, CDK2, and their regulatory cyclins during MCE, the early phase of 3T3-L1 preadipocyte differentiation.

Objective 2

My second objective was to assess the expression patterns of cell cycle proteins in differentiating human abdominal subcutaneous preadipocytes, which do not undergo clonal expansion in culture, and to assess the effect of THP-1-MacCM on these cell cycle proteins.

MATERIALS AND METHODS

Part I: Studies with 3T3-L1 preadipocytes and J774A.1 macrophages

Culture of murine J774A.1 macrophages and collection of conditioned medium

Murine J774A.1 macrophages (American Tissue Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml penicillin and 0.1 mg/ml streptomycin). The growth medium was changed every 2 days. The macrophages were passaged by detaching them from the cell culture plate with a cell scraper; a split ratio of 1:6 was never exceeded.

At 90% confluence, the growth medium was removed and replaced with fresh medium. After 24 hours, the resulting macrophage conditioned medium (J774A.1-MacCM) was collected and centrifuged (Megafuge 1.0R; Heraeus Instruments) at 150×g for 5 min. The supernatants were then stored at −20°C until needed for preadipocyte studies. Growth medium that was not exposed to macrophages (control medium) underwent a similar preparation to that of the J774A.1-MacCM to ensure that any observed effects on preadipocyte responses were not due to medium processing procedures (i.e. centrifugation and freezing). For the cyclin A2 mRNA experiments, J774A.1-MacCM was generated over 24 h in the absence of serum and subsequently supplemented with 10% FBS and adipogenic inducers prior to being added to preadipocytes. All media were thawed prior to experiments in a 37°C water bath.

Cell culture and differentiation of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes (American Tissue Culture Collection, Manassas, VA) were grown in DMEM supplemented with 10% calf serum (CS) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). The growth medium was changed every 2 to 3 days.

Subconfluent cultures were passaged using trypsin-EDTA to detach the preadipocytes from the culture plates.

Two day post-confluent preadipocytes were induced to differentiate for the indicated times in either control medium or in J774A.1-MacCM supplemented with 0.25 μM DEX, 0.5 mM IBMX, for the first 2 days (Rubin et al., 1978), and 1 μM insulin (Roche, Indianapolis, IN) for the first 4 days. Non-differentiating preadipocytes were maintained in the corresponding medium in the absence of adipogenic inducers. On day 8 of differentiation, cultures were photographed with a digital camera (Coolpix 995; Nikon, Mississauga, ON, Canada) that was mounted on a microscope (Eclipse TS-100; Nikon).

Preparation of 3T3-L1 whole cell lysates and protein quantification

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Laemmli buffer (Laemmli, 1970) containing 5% β-mercaptoethanol, 1 mM sodium orthovanadate (Na_3VO_4), 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM sodium pyrophosphate (NaPPi) and 50 mM sodium fluoride (NaF). Culture plates were scraped with a cell scraper and lysates were passed though a 26 ½ gauge syringe, boiled for 5 minutes and stored at -20ºC. Quantification of protein concentration was performed using the modified Lowry assay with bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA). Colourimetric assays were quantified using the FLUOstar Galaxy spectrophotometer (BMG, Offenburg, Germany).

Immunoblotting

Following the quantification of protein concentration, equal amounts of solubilized protein (5-20 μg, depending on the experiment) were resolved by 7.5%, 10% or 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Apparatus). Non-specific

binding sites were blocked for 1 hour in PBS containing 0.1% Tween20 and 5% skim milk powder (or with 3% BSA for pTyr immunoblots). The membranes were incubated at 4ºC overnight in PBS containing 3% BSA, 0.02% sodium azide, and the indicated primary antibodies to detect the following: cyclin D1 (mouse monoclonal; 1µg/ml), cyclin A (mouse monoclonal; 1µg/ml), CDK2 (rabbit polyclonal; 0.2µg/ml), CDK4 (rabbit polyclonal; 0.4µg/ml), histone H1 (mouse monoclonal; 1µg/ml), C/EBPα (rabbit polyclonal; 1µg/ml), C/EBPβ (rabbit polyclonal; 1µg/ml) and PI 3-kinase p85α (rabbit polyclonal; 1µg/ml) all from Santa Cruz Biotechnology (Santa Cruz, CA); c-Myc (rabbit polyclonal; 1:1000), cyclin E (mouse monoclonal; 1:1000), $p27^{Kip1}$ (rabbit polyclonal; 1:250), phospho-Rb (rabbit polyclonal; 1:250), PPARγ (rabbit monoclonal; 1:1000), pTyr (mouse monoclonal; 1:1000) and pC/EBPβ (rabbit polyclonal; 1:1000) all from Cell Signaling (Danvers, MA); Rb (mouse polyclonal; 1:250; BD Biosciences, Mississauga, ON, Canada); cyclin D2 (mouse monoclonal; 1:500; Abcam, Cambridge, MA); aP2 (goat polyclonal; 0.1µg/ml; R&D Systems, Minneapolis, MN) or ERK1/2 (rabbit polyclonal; 0.25µg/ml; Upstate Biotechnology, Charlottesville, VA).

The membranes were then incubated in PBS, containing 5% skim milk powder (or with 3% BSA for pTyr immunoblots) and the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences or Jackson Laboratories, West Grove, PA). Immunoreactivity was detected using chemiluminescence horseradish peroxidase substrate solution (Millipore, Billerica, MA) and subsequent exposure to Bioflex scientific imaging film (Clonex). A Kodak M35A X-OMAT Processor was used. Relative band intensity was determined with AlphaEaseFC$_{TM}$ Software (version 4.0.0) and expressed as integrated optical density (IOD) units.

CDK2 immunoprecipitation and kinase assay

CDK2 immunoprecipitation was performed as described in (Phelps and Xiong, 1998). Briefly, two day post-confluent 3T3-L1 preadipocytes were maintained under non-adipogenic conditions or induced to differentiate in the presence of control medium or J774A.1-MacCM for 20 hours. Cells were washed three times with ice-cold PBS, placed in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1 mM phenylmethanesulfonylfluoride (PMSF), 25 μg/ml aprotinin, 25 μg/ml leupeptin, 4 μg/ml benzamidine) and sonicated for 10 seconds with Vibra-Cell sonicator (Sonics and Materials, Newton, CT). Soluble proteins were quantified using the modified Lowry assay with BSA as a standard (Bio-Rad, Hercules, CA). Whole cell lysates were exposed to protein A-sepharose beads for 1 hour at 4°C on a rotator for preclearing. Subsequently, lysates containing equal amounts of protein (700-800μg of protein, depending on experiment) were incubated with 1μg of CDK2 antibody bound to protein A-sepharose beads for 90 minutes at 4°C on a rotator. Immunoprecipitates were washed 5 times with lysis buffer, followed by two washes with kinase buffer (50 mM HEPES pH 7.0, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 5 mM ATP).

Kinase reactions were performed in 25μl of kinase buffer containing 5μCi [γ^{32}P] ATP and 4μg histone H1 (Calbiochem, Gibbstwon, NJ) at 30°C for 30 minutes. Reactions were terminated by the addition of 50μl of Laemmli buffer containing 5% β-mercaptoethanol, 1 mM Na_3VO_4, 5 mM EGTA, 5 mM NaPPi and 50 mM NaF. Samples were boiled for 5 minutes to ensure complete denaturation of immunoprecipitated proteins. Immunoprecipitates were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose. Phosphorylation of histone H1 was visualized by autoradiography with a Kodak M35A X-

OMAT Processor. Immunoprecipitated proteins (CDK2 and cyclin A) and total histone H1 substrate were detected by immunoblotting, as described above.

IRS-1 immunoprecipitation

Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in the presence of control medium or J774A.1-MacCM for the indicated times. Cells were washed once with ice-cold PBS and lysed with ice-cold lysis buffer (1% NP40, 200 uM Na_3VO_4, 0.1 mg/ml PMSF, 10 ug/ml aprotinin, 10 ug/ml leupeptin, 4 ug/ml benzamidine, 50 mM NaF, 1 uM β-glycerophosphate, 5 mM NaPPi). Soluble proteins were quantified using the modified Lowry assay with BSA as a standard (Bio-Rad, Hercules, CA). Whole cell lysates were exposed to protein A-sepharose beads for 1 hour at 4°C on a rotator for preclearing. Subsequently, lysates containing equal amounts of protein (750-1200μg of protein, depending on experiment) were incubated with 1μg of IRS-1 antibody bound to protein A-sepharose beads for 90 minutes at 4°C on a rotator. Immunoprecipitates were washed 5 times with lysis buffer and then resuspended in 75μl of Laemmli buffer containing 5% β-mercaptoethanol, 1 mM Na_3VO_4, 5 mM EGTA, 5 mM NaPPi and 50 mM NaF. Samples were boiled for 5 minutes to ensure complete denaturation of imunnoprecipitated protein. Immunoprecipitates were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose. Immunoprecipitated proteins (IRS-1, p85) and their tyrosine phosphorylation status were detected by immunoblotting, as described above.

Cyclin A2 RNA preparation and Real Time PCR

Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in the presence of control medium or J774A.1-MacCM for the indicated times. RNA was extracted with TRI reagent and subsequently treated with DNase I, according to the manufacturer's

instructions (Ambion, Austin, TX). 1 µg of total RNA (heat denatured) was reversed transcribed in a reaction volume of 20 µl, containing 50 mM Tris-HCl, pH 8.3, 75 mM potassium chloride (KCl), 3 mM magnesium chloride ($MgCl_2$), 5 mM DTT, 6 µg of random primers, 0.5 mM of each dNTP, 10 U of RNase inhibitor and 100 U of reverse transcriptase (all reagents from Invitrogen). Control assays, without reverse transcriptase, were performed for all reactions. Real time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with 900 nM target primers, or QuantiTect Probe RT-PCR kit with 18S rRNA primers (Qiagen), according to the manufacturer's protocol for assays using the Roche Light Cycler Real-Time PCR System. Data were analyzed using Light Cycler Software 3.0, and are expressed as relative quantification (RQ). Cyclin A2-specific primer pairs were:
forward, 5'-GGCACGGCTGCTATGCTGCT-3', and
reverse, 5'-TGTGCCAATGACTCAGGCCAGC-3', with 18S as an internal control.

Part II: Studies with human subcutaneous abdominal preadipocytes and THP-1 macrophages

Culture of human THP-1 macrophages and collection of conditioned medium

Human THP-1 monocytes (American Tissue Culture Collection, Manassas, VA) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate ($NaHCO_3$), 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 10% FBS, 0.05 mM β-mercaptoethanol and antibiotics. The growth medium was changed every 2 days. Cell culture suspensions were maintained at a concentration below 1 x 10^6 cells per ml. Cell counts were performed using a Neubauer hemocytometer.

At a concentration of 1 x 10^6 cells per ml, monocytes were induced to differentiate with 100 nM TPA for 24 hours. Subsequently, this medium was removed and replaced with

fresh medium, which did not contain TPA, for 24 hours. This conditioned medium (THP-1-MacCM) was collected, centrifuged (Megafuge 1.0R; Heraeus Instruments) at 150×g for 5 min and stored at −20°C until used in preadipocyte studies. Growth medium that was not exposed to macrophages (THP-1 control medium) underwent similar preparation as the THP-1-MacCM to ensure that any observed effects on preadipocyte responses were not due to medium processing procedures (i.e. centrifugation and freezing). The medium was thawed prior to experiments in a 37°C water bath.

Isolation and culture of human abdominal subcutaneous stromal preadipocytes

Subcutaneous adipose tissue samples were obtained from 7 consenting female patients undergoing elective abdominal surgery. All protocols and procedures were approved by The Ottawa Hospital Research Ethics Board. Mean age was 49 ± 5 years and mean BMI was 27.9 ± 3.7 kg/m^2 (± SD; range from 21.8 to 48.4 kg/m^2).

Isolation of the stromal preadipocytes from tissue biopsies was performed as previously described (Hauner et al., 2001; Artemenko et al., 2005). Briefly, connective tissue and capillaries were removed by careful dissection and the adipose tissue was then subjected to collagenase CLS type 1 (600 U/g of tissue) digestion for 1 hour at 37°C on a rotating mixer. The digestion was followed by size filtration (200 micron sterile nylon filter) and centrifugation at 200 x g for 20 minutes. The fraction containing floating mature adipocytes was removed. 10% FBS was added to the infranatant and was subjected to progressive size filtration (100, 50 and 25 micron sterile nylon filters) and further centrifuged at 200 x g for 20 minutes to remove any remaining mature adipocytes. For the removal of red blood cells, pellets were incubated in erythrocyte lysis buffer, containing 155 mM ammonium chloride, 5.7 mM potassium phosphate and 0.1 mM EDTA (pH 7.3) for 5 minutes. The stromal cells

were centrifuged at 200 x g for 5 minutes and resuspended in DMEM supplemented with 10% FBS, antibiotics and 50 U/ml nystatin. Stromal preadipocytes were expanded by seeding them at a density of 6,000 cells/cm^2 in DMEM supplemented with 10% FBS and antibiotics and grown to 80-90% confluence. Cells were subsequently cryopreserved in 10% dimethyl sulfoxide (DMSO) growth media and stored in liquid nitrogen until required. Cryopreservation did not alter their adipogenic capacity (Ort et al., 2005).

When needed, cryopreserved stromal preadipocytes were thawed in a 37°C water bath and grown in DMEM supplemented with 10% FBS, antibiotics and 50 U/ml nystatin. At 80-90% confluence, the number of preadipocytes was expanded for a maximum of three passages (Hutley et al., 2003).

Differentiation of human abdominal subcutaneous stromal preadipocytes

Stromal preadipocytes were seeded at a density of 3 x 10^4 cells per cm^2 and grown to confluence in DMEM supplemented with 10% FBS, antibiotics and 50 U/ml nystatin. For the human abdominal subcutaneous preadipocyte time course experiments, the preadipocytes were induced to differentiate for the indicated times in growth medium that was supplemented with 5 μg/ml insulin, 100 μM indomethacin, 0.5 μM DEX and 0.25 mM IBMX (Lehmann et al., 1997; Artemenko et al., 2005). Non-differentiating preadipocytes were maintained in growth medium in the absence of adipogenic inducers. On day 14 of differentiation, cultures were photographed with a digital camera (Coolpix 995; Nikon, Mississauga, ON, Canada) mounted on a microscope (Eclipse TS-100; Nikon).

For the experiments that assessed the effects of THP-1-MacCM on cell cycle protein expression in human abdominal subcutaneous preadipocytes, the preadipocytes were induced to differentiate in the presence of control medium or THP-1-MacCM, supplemented with

adipogenic inducers for the indicated times. Non-differentiating preadipocytes were maintained in the corresponding medium in the absence of adipogenic inducers.

Preparation of human abdominal subcutaneous stromal preadipocyte whole cell lysates and protein quantification

Processing of cell lysates and protein quantification was performed as described above for 3T3-L1 preadipocyte studies.

Immunoblotting

Following the quantification of protein concentration, equal amounts of solubilized protein (7-35μg, depending on the experiment) were resolved by 7.5%, 10% or 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Apparatus). Non-specific binding sites were blocked for 1 hour in PBS containing 0.1% Tween20 and 5% skim milk powder. The membranes were incubated at 4ºC overnight PBS containing 3% BSA, 0.02% sodium azide, and the indicated primary antibodies to detect the following: cyclin A (mouse monoclonal; 1μg/ml), CDK2 (rabbit polyclonal; 0.2μg/ml), CDK4 (rabbit polyclonal; 0.4μg/ml), histone H1 (mouse monoclonal; 1μg/ml) and actin (rabbit polyclonal; 1μg/ml) all from Santa Cruz Biotechnology (Santa Cruz, CA); cyclin E (mouse monoclonal; 1:1000), $p27^{Kip1}$ (rabbit polyclonal; 1:250), phospho-Rb (rabbit polyclonal; 1:250), and PPARγ (rabbit monoclonal; 1:1000) all from Cell Signaling (Danvers, MA); Rb (mouse polyclonal; 1:250; BD Biosciences, Mississauga, ON, Canada), or ERK1/2 (rabbit polyclonal; 0.25μg/ml; Upstate Biotechnology, Charlottesville, VA).

The membranes were then incubated in PBS, containing 5% skim milk powder and the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences or Jackson Laboratories, West Grove, PA). Immunoreactivity was detected

using chemiluminescence HRP substrate solution (Millipore, Billerica, MA) and subsequent exposure to Bioflex scientific imaging film (Clonex). A Kodak M35A X-OMAT Processor was used. Relative band intensity was determined with AlphaEaseFC$_{TM}$ Software (version 4.0.0) and expressed as IOD units.

CDK2 immunoprecipitation and kinase assay

Human preadipocytes were maintained under non-adipogenic conditions or induced to differentiate for 4 days. CDK2 immunoprecipitation and kinase reactions were performed as described above for 3T3-L1 studies. Immunoprecipitates were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose. Phosphorylation of histone H1 was visualized by autoradiography with a Kodak M35A X-OMAT Processor. Immunoprecipitated proteins (CDK2 and cyclin E) and total histone H1 substrate were detected by immunoblotting, as described above.

Statistical analysis for all studies

Statistical significance was determined either by one-way ANOVA (Instat, version 3.05; GraphPad, San Diego, CA) or two-way ANOVA (Minitab Release 15.1.0.0 software) followed by a Newman–Keuls post-hoc test or paired t-tests (Instat, version 3.05; GraphPad, San Diego, CA) respectively, to assess the differences between means. $p<0.05$ was considered significant.

RESULTS

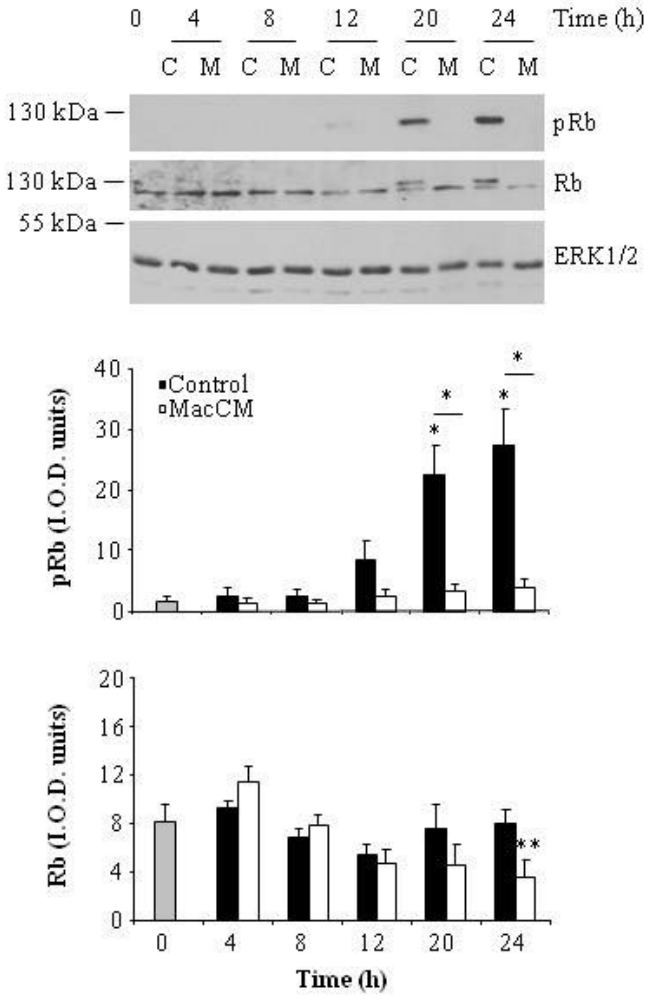
Part I: Studies with 3T3-L1 preadipocytes and J774A.1 macrophages

Our laboratory has previously reported that J774A.1-MacCM completely inhibits 3T3-L1 adipogenesis and disrupts early differentiation events, including MCE and Rb phosphorylation (Constant et al., 2008; Yarmo et al., 2009). We demonstrated that Rb phosphorylation was inhibited by J774A.1-MacCM 24 hours after adipogenic induction. To further evaluate the anti-adipogenic effect of J774A.1-MacCM on 3T3-L1 adipogenesis, I examined key cell cycle regulatory events during MCE by performing more extensive time courses.

J774A.1-MacCM inhibits differentiation-induced Rb phosphorylation in 3T3-L1 preadipocytes

3T3-L1 preadipocytes were induced to differentiate for up to 24 hours in the absence or presence of J774A.1-MacCM. Rb phosphorylation levels were assessed by using a phospho-Rb antibody in addition to observing a mobility shift of Rb. Phospho-Rb levels were initially undetectable and then increased considerably by the 20 and 24 hour time points under control adipogenic conditions (Fig. 2). The mobility shift observed on the Rb immunoblot at the 20 and 24 hour time points is consistent with the increase in Rb phosphorylation. Overall, Rb levels did not change over time under these conditions. The differentiation-induced Rb phosphorylation was inhibited by J774A.1-MacCM by 92% (versus time-matched control, $P<0.05$, $n=4$) and 91% (versus time-matched control, $P<0.05$, $n=4$) at 20 and 24 hours, respectively (Fig. 2). The corresponding mobility shift in Rb was not evident at either time point. Rb mass was reduced by 56% (versus time-matched control,

Figure 2. J774A.1-MacCM inhibits differentiation-induced Rb phosphorylation during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against pRb, Rb, and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


0
4
8
12
20
24
Time (h)
C M C M C M C M C M
130 kDa
pRb
130 kDa
Rb
55 kDa
ERK1/2
Control
MacCM
pRb (I.O.D. units)
Rb (I.O.D. units)
Time (h)

n=4) at 24 hours with J774A.1-MacCM, but did not reach significance. For all studies, ERK1/2 served as a loading control, since its expression does not change with differentiation (Prusty et al., 2002).

J774A.1-MacCM inhibits differentiation-induced cyclin D expression in 3T3-L1 preadipocytes

Since Rb is sequentially phosphorylated by cyclin D/CDK4, followed by cyclin E/A/CDK2, I first assessed the effect of J774A.1-MacCM on the expression of CDK4 and its cyclin D partners during MCE. My results reveal a non-significant downward trend over time in CDK4 expression under both control and J774A.1-MacCM conditions, with no differences observed between the two media conditions (Fig. 3). One of the most widely studied CKIs that inhibits CDK4 activity is $p27^{Kip1}$. Following adipogenic induction, $p27^{Kip1}$ is downregulated, permitting the activation of cyclin/CDK complexes that phosphorylate Rb (Patel and Lane, 2000; Tang et al., 2003b). Our laboratory has previously published results showing that J774A.1-MacCM does not affect $p27^{Kip1}$ expression at 6 and 24 hours post induction (Yarmo et al., 2009). The results that I obtained over the 24 hour time course were consistent with those previous findings (Fig. 3).

The D-type family of cyclins includes cyclin D1, D2, and D3. In general, increases in cyclin D1 and D2 have been shown to occur during MCE, while cyclin D3 expression is not induced during MCE, but rather during later stages of differentiation (Reichert and Eick, 1999; Sarruf et al., 2005). Therefore, I assessed cyclin D1 and D2 expression in preadipocytes that were exposed to adipogenic inducers in control medium or J774A.1-MacCM for 24 hours. Under control adipogenic conditions, cyclin D1 transiently increased, reaching peak levels at 8-12 hours (Fig. 4A). J774A.1-MacCM reduced this differentiation-

Figure 3. J774A.1-MacCM does not affect CDK4 or $p27^{Kip1}$ expression during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against CDK4, $p27^{Kip1}$ or ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01 compared to 0 time point. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.

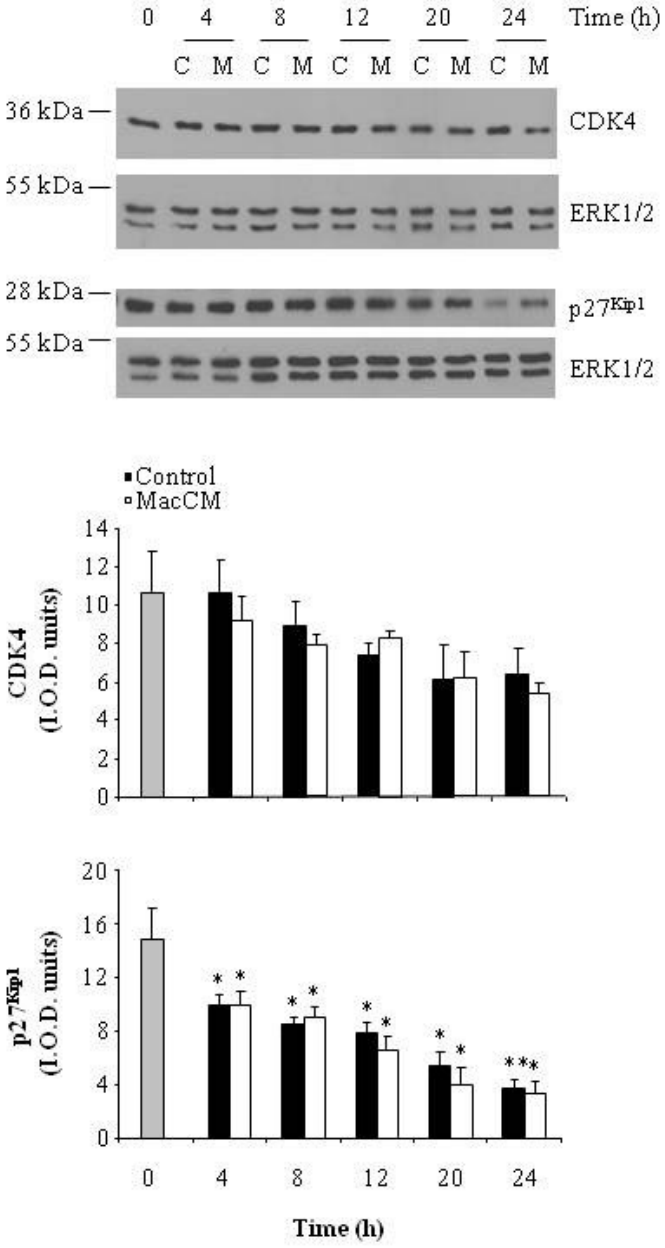
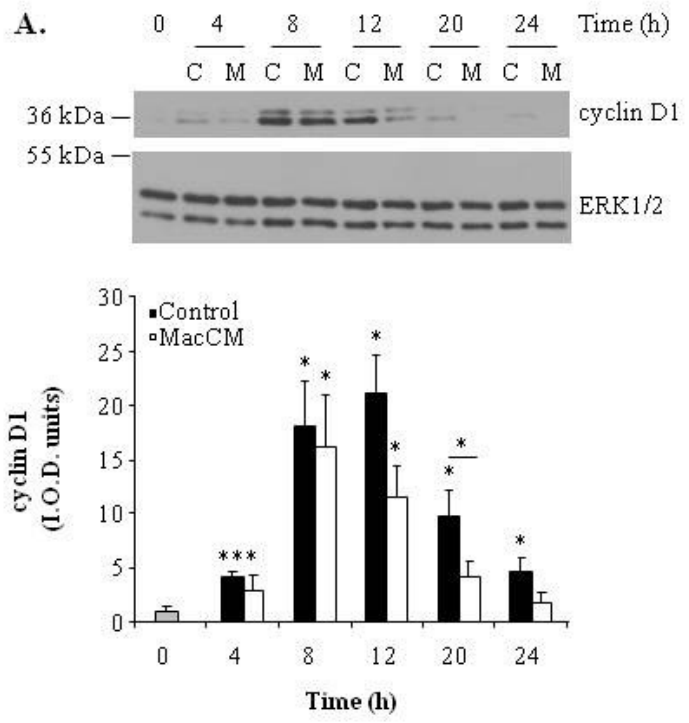
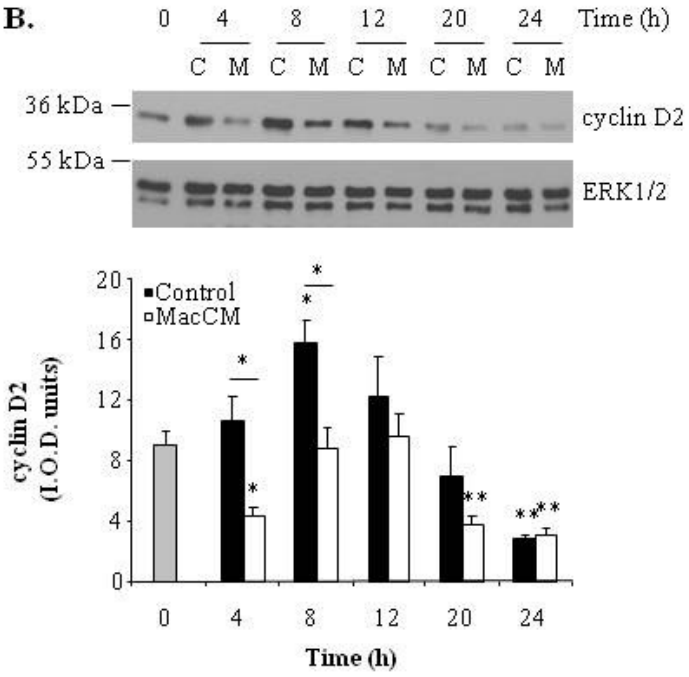

0 4 8 12 20 24 Time (h)
C M C M C M C M C M
36 kDa
CDK4
55 kDa
ERK1/2
28 kDa
p27Kip1
55 kDa
ERK1/2
Control
MacCM
CDK4 (I.O.D. units)
p27Kip1 (I.O.D. units)
Time (h)

Figure 4. J774-A.1 MacCM reduces differentiation-induced cyclin D1 and D2 expression during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against **(A)** cyclin D1, **(B)** cyclin D2 and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01, ***P<0.001 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


A.
0
4
8
12
20
24
Time (h)
C M C M C M C M C M
36 kDa
55 kDa
cyclin D1
ERK1/2
Control
MacCM
cyclin D1 (I.O.D. units)
Time (h)


B.
0
4
8
12
20
24
Time (h)
C M C M C M C M C M
36 kDa
55 kDa
cyclin D2
ERK1/2
Control
MacCM
cyclin D2 (I.O.D. units)
Time (h)

induced increase of cyclin D1, leading to a significant inhibition of 64% at 20 hours (versus time-matched control, $P<0.05$, n=4). Cyclin D2 levels transiently increased by 2-fold (versus basal, $P<0.05$, n=4) at 8 hours under control adipogenic conditions. This differentiation-dependent increase was completely inhibited by J774A.1-MacCM (Fig. 4B).

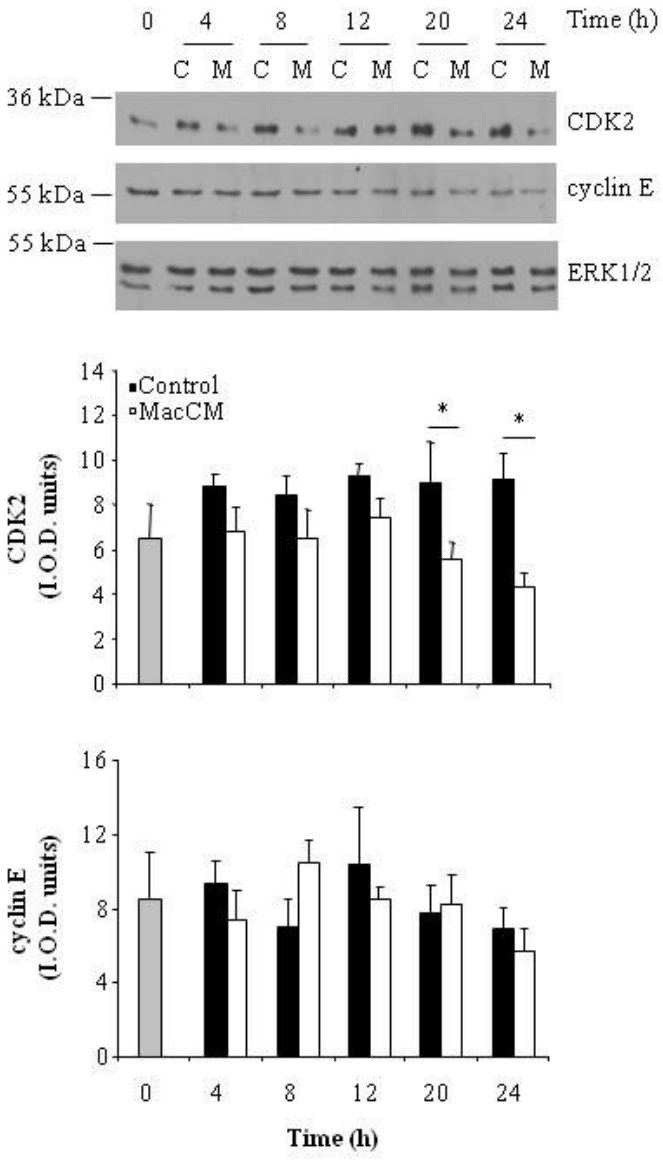
Based on the findings that J774A.1-MacCM reduced cyclin D1 and D2, my intention was to determine if these reductions were associated with decreased CDK4 activity. 3T3-L1 preadipocytes were maintained under non-adipogenic conditions or induced to differentiate in the absence or presence of J774A.1-MacCM for 20 hours. This time point was chosen because Rb phosphorylation was observed at 20-24 hours following induction of differentiation. I attempted to immunoprecipitate CDK4 and assay its kinase activity, using two different commercial CDK4 antibodies and immunoprecipitation protocols. However, I was unable to reliably measure CDK4 activity, since I could not efficiently immunoprecipitate CDK4 with commercially available antibodies.

J774A.1-MacCM inhibits differentiation-induced CDK2 and cyclin A expression in 3T3-L1 preadipocytes

To determine the effect of J774A.1-MacCM on other upstream cell cycle regulators of Rb, I next examined whether J774A.1-MacCM altered the expression of CDK2 and its cyclin partners in response to adipogenic inducers. When the preadipocytes were induced to differentiate in control medium, no changes in CDK2 expression were observed over time. In contrast, the exposure to adipogenic inducers in J774A.1-MacCM resulted in a 38% and 53% decrease (versus time-matched control, $P<0.05$, n=4) in CDK2 expression at 20 and 24 hours, respectively (Fig. 5).

Cyclin E expression did not change over time in cells induced to differentiate in control medium or J774A.1-MacCM (Fig. 5). In contrast, the expression of cyclin A was

Figure 5. J774A.1-MacCM reduces CDK2 expression during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against CDK2, cyclin E and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *$P<0.05$ between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


0
4
8
12
20
24
Time (h)
C M C M C M C M C M
36 kDa
CDK2
55 kDa
cyclin E
55 kDa
ERK1/2
Control
MacCM
CDK2 (I.O.D. units)
cyclin E (I.O.D. units)
*
*
Time (h)

initially undetectable and then dramatically rose by 20 and 24 hours following adipogenic induction in control medium (Fig. 6A). J774A.1-MacCM inhibited these increases in cyclin A by 73% (versus time-matched control, $P<0.05$, n=4) and 74% (versus time-matched control, $P<0.05$, n=4), respectively.

To assess whether this inhibition by J774A.1-MacCM occurred at the transcriptional level, RNA was extracted from preadipocytes that were induced to differentiate in the absence or presence of J774A.1-MacCM for 16 or 20 hours. Cyclin A2 mRNA expression dramatically increased by 22-fold (versus basal, $P<0.05$, n=4) at 16 hours in the presence of adipogenic inducers in control medium (Fig. 6B). This response was inhibited by 82% (versus time-matched control, $P<0.05$, n=4) in J774A.1-MacCM.

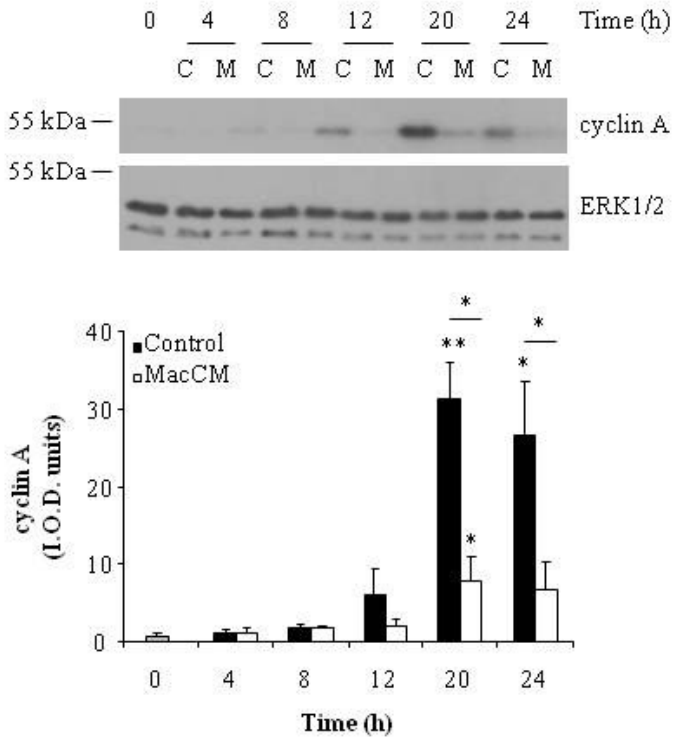
Since c-Myc has been reported as a regulator of cyclin A and is upregulated during the early phases of differentiation (Rudolph et al., 1996; Reichert and Eick, 1999), I determined if J774A.1-MacCM altered c-Myc expression, which could account for the reduced cyclin A expression. When preadipocytes were induced to differentiate in control medium, c-Myc levels, initially undetectable, rose to maximal levels at 4-12 hours. These increases were blunted by J774A.1-MacCM by 41% at 4 hours, 26% at 8 hours, and 40% at 12 hours (versus time-matched controls, $P<0.05$, n=4) (Fig. 7).

J774A.1-MacCM inhibits differentiation-induced CDK2 activity in 3T3-L1 preadipocytes

Based on the earlier findings that J774A.1-MacCM inhibited CDK2 and cyclin A expression, I determined if these inhibitions would alter CDK2 activity. 3T3-L1 preadipocytes were either maintained under non-adipogenic conditions or induced to differentiate in control medium or J774A.1-MacCM for 20 hours. This time point was chosen based on Rb phosphorylation levels being maximal at 20-24 hours after adipogenic

Figure 6. Differentiation-induced cyclin A protein and mRNA expression are inhibited by J774A.1-MacCM during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. **A.** Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against cyclin A and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. **B.** RNA was extracted from 3T3-L1 preadipocytes that were induced to differentiate in the presence of control medium or J774-MacCM for the indicated times, and quantified by real-time PCR. Data are expressed relative to 18S rRNA levels and represented as mean ± SE of 4 separate experiments. *$P<0.05$, **$P<0.01$ compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.

A.


0 4 8 12 20 24 Time (h)
C M C M C M C M C M
55 kDa
cyclin A
55 kDa
ERK1/2
Control
MacCM
cyclin A (I.O.D. units)
Time (h)

B.

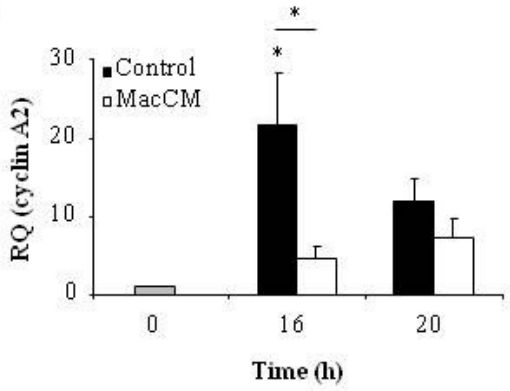
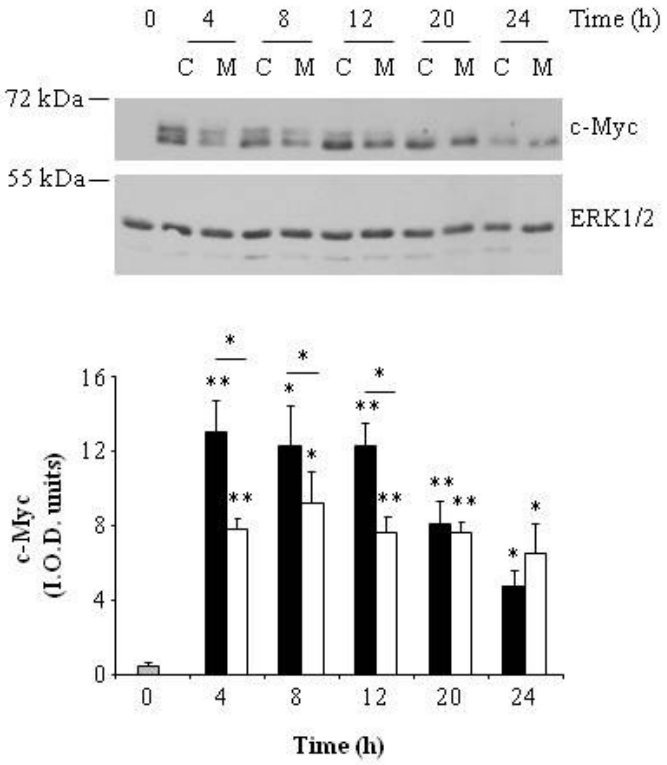

Control
MacCM
RQ (cyclin A2)
Time (h)

Figure 7. Differentiation-induced c-Myc expression is inhibited by J774A.1-MacCM during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against c-Myc and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


0
4
8
12
20
24
Time (h)
C M C M C M C M C M
72 kDa
c-Myc
55 kDa
ERK1/2
c-Myc
(I.O.D. units)
16
12
8
4
0
Time (h)

induction in control medium. CDK2 was immunoprecipitated and assayed for kinase activity, using histone H1 as a substrate in the presence of [γ^{32}P]-ATP. A 5-fold increase (versus non-differentiated control medium, $P<0.01$, n=4) in CDK2 activity was observed in preadipocytes that were induced to differentiate in control medium (Fig. 8). J774A.1-MacCM reduced the differentiation-induced increase in CDK2 activity by 75% (versus control adipogenic condition, $P<0.01$, n=4). Equal amounts of histone H1 substrate were present in each reaction sample, indicated by the histone H1 immunoblot (Fig. 8).

To determine whether the reduction in CDK2 activity was associated with a decrease in the amount of cyclin A present, the proteins immunoprecipitated with anti-CDK2 antibody were immunoblotted with cyclin A antibody. There was a 3-fold increase (versus non-differentiated control medium, $P<0.05$, n=4) in the amount of cyclin A that co-immunoprecipitated with CDK2 under control adipogenic conditions and J774A.1-MacCM reduced this association by 79% (versus control adipogenic condition, $P<0.05$, n=4, Fig. 9). Equal amounts of immunoprecipitated CDK2 were detected for each condition (Fig. 9).

In addition to Rb, C/EBPβ has been reported to be a downstream target of cyclin A/CDK2 in MCE (Li et al., 2007). Therefore, I determined whether the reduction in CDK2 activity that was induced by J774A.1-MacCM decreased C/EBPβ phosphorylation. Through the process of alternative translation, various N-terminally truncated C/EBPβ isoforms can be produced. The two C/EBPβ isoforms, liver-enriched transcriptional activating protein (LAP) and liver-enriched transcriptional inhibitory protein (LIP) have been shown to have a functional significance in adipogenesis, where LAP acts to positively regulate differentiation and LIP acts to inhibit the transcriptional activity of LAP (Welm et al., 1999). Under control adipogenic conditions, phosphorylated levels of C/EBPβ (LAP) were initially very low and

Figure 8. Differentiation-induced CDK2 activity is inhibited by J774A.1-MacCM during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were either induced to differentiate (+DMI) or not (-DMI) in control medium or J774A.1-MacCM for 20 hrs. Immunoprecipitated CDK2 was assayed for kinase activity, using histone H1 as a substrate, in the presence of [γ^{32}P]-ATP. Immunoprecipitates were resolved by SDS-PAGE and incorporation of ^{32}P into histone H1 was visualized by autoradiography. A representative autoradiograph and histone H1 immunoblot are shown. Densitometric results are from 4 (autoradiographs) or 3 (immunoblots) independent experiments, expressed as mean ± SE. IOD, integrated optical density. **P<0.01 between indicated pairs. Statistical significance was determined by a one-way ANOVA followed by Newman–Keuls post-hoc test to assess differences between means.

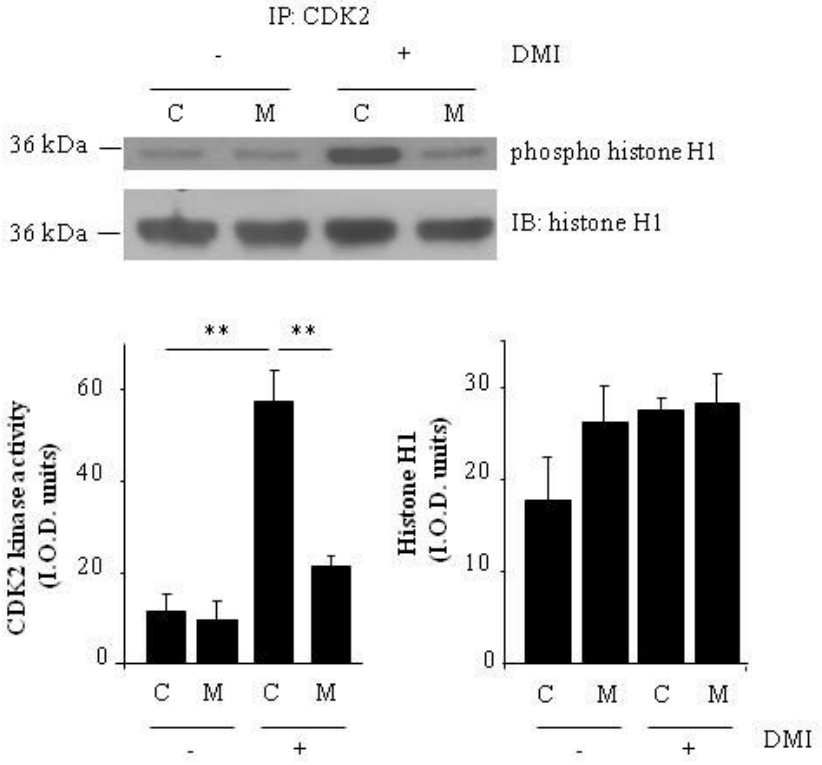
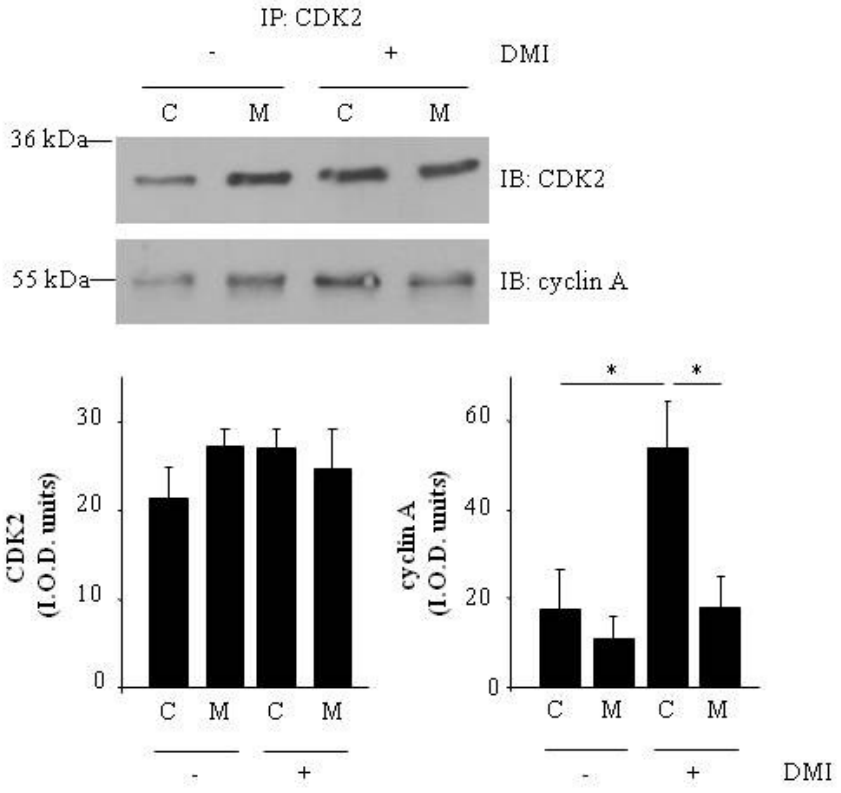

IP: CDK2
-
+
DMI
C
M
C
M
36 kDa
phospho histone H1
36 kDa
IB: histone H1
**
**
CDK2 kinase activity
(I.O.D. units)
60
40
20
0
C
M
C
M
-
+
Histone H1
(I.O.D. units)
30
20
10
0
C
M
C
M
-
+
DMI

Figure 9. Inhibition of differentiation-induced CDK2 activity by J774A.1-MacCM is associated with a reduction in the amount of cyclin A co-immunoprecipitation. Two day post-confluent 3T3-L1 preadipocytes were either induced to differentiate (+DMI) or not (-DMI) in control medium or J774A.1-MacCM for 20 hrs. Cell lysates were incubated with anti-CDK2 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with antibodies against CDK2 and cyclin A. Representative immunoblots are shown. Densitometric results from 4 independent experiments, expressed as mean ± SE, are shown. IOD, integrated optical density. *$P<0.05$ between indicated pairs. Statistical significance was determined by a one-way ANOVA followed by Newman–Keuls post-hoc test to assess differences between means.


IP: CDK2
-
+
DMI
C
M
C
M
36 kDa
IB: CDK2
55 kDa
IB: cyclin A
CDK2 (I.O.D. units)
30
20
10
0
C
M
C
M
-
+
cyclin A (I.O.D. units)
60
40
20
0
*
*
C
M
C
M
-
+
DMI

then increased considerably by 4 hours (Fig. 10). J774A.1-MacCM reduced the phosphorylation of C/EBPβ (LAP) by 31% (versus time-matched control, $P<0.01$, n=4) and 62% (versus time-matched control, $P<0.05$, n=4) at 20 and 24 hours, respectively. The phosphorylation of C/EBPβ (LIP) was too faint to detect. Additionally, C/EBPβ (LAP) expression was initially very low at basal levels and then increased dramatically when the cells were exposed to adipogenic inducers in control medium (Fig. 10). This increase was blunted by J774A.1-MacCM by 21% (versus time-matched control, $P<0.05$, n=4) and 35% (versus time-matched control, $P<0.05$, n=4) at 20 and 24 hours, respectively. Since the reduction in C/EBPβ (LAP) phosphorylation with J774A.1-MacCM could be due to the decreased C/EBPβ (LAP) expression, phospho-C/EBPβ (LAP) levels were normalized to C/EBPβ (LAP). After normalization, there were no longer any significant differences in C/EBPβ (LAP) phosphorylation between the two medium conditions at either 20 or 24 hours. C/EBPβ (LIP) expression increased significantly from basal levels under adipogenic control conditions and there were no differences observed with J774A.1-MacCM (Fig. 10).

Effect of J774A.1-MacCM on proximal insulin signaling events in 3T3-L1 preadipocytes

Since the insulin signaling pathway can regulate cyclin expression, I evaluated the effect of J774A.1-MacCM on IRS1-tyrosine phosphorylation and IRS1-PI3K (p85) association. IRS1 was immunoprecipitated from 3T3-L1 preadipocytes that were exposed to adipogenic inducers in control medium or J774A.1-MacCM for up to 1 hour. IRS1 tyrosine phosphorylation increased 3-fold (versus basal, $P<0.05$; n=3) at 5 minutes of adipogenic induction under control conditions and was sustained at 60 minutes. This increase was not affected by J774A.1-MacCM (Fig. 11). IRS1-p85 association increased 2-fold (versus basal,

Figure 10. Differentiation-induced CEBPβ (LAP) expression is reduced by J774A.1-MacCM during mitotic clonal expansion in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against pCEBPβ, CEBPβ and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01, ***<P<0.001 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.

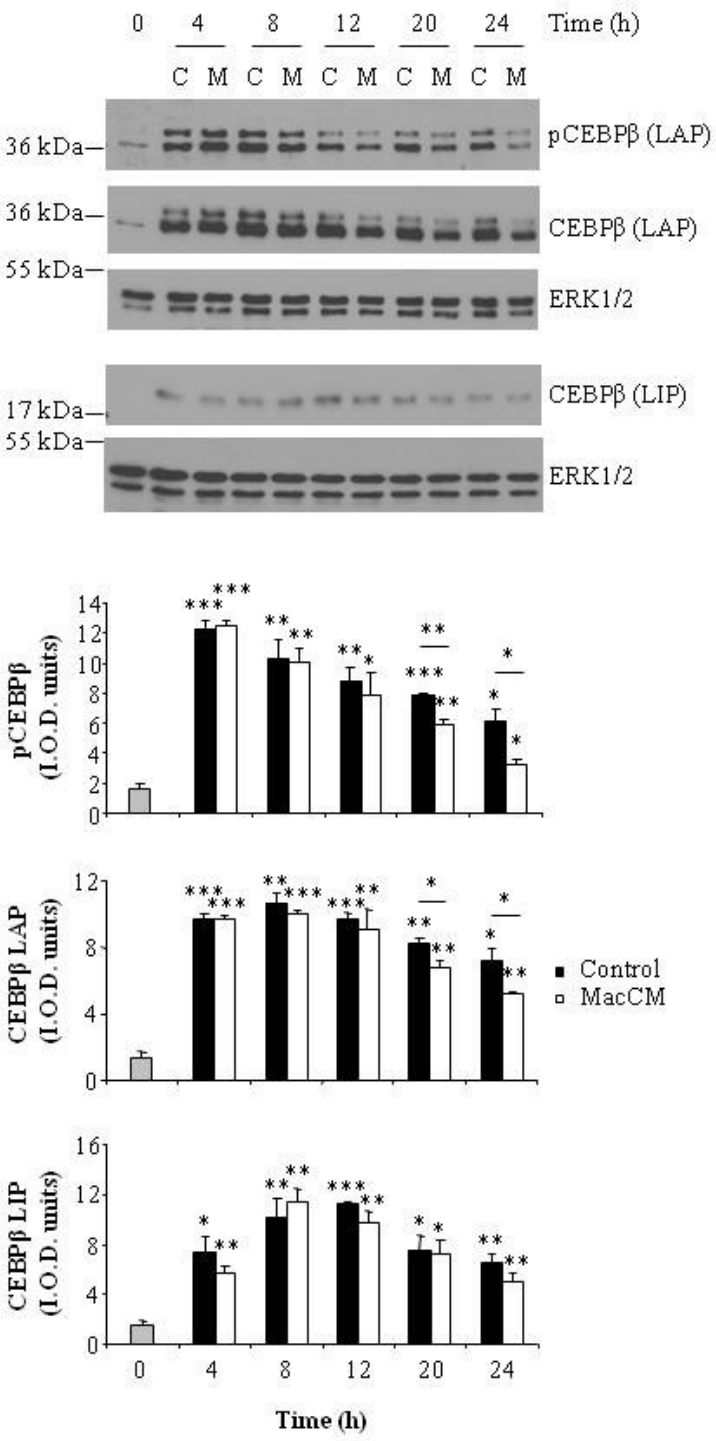
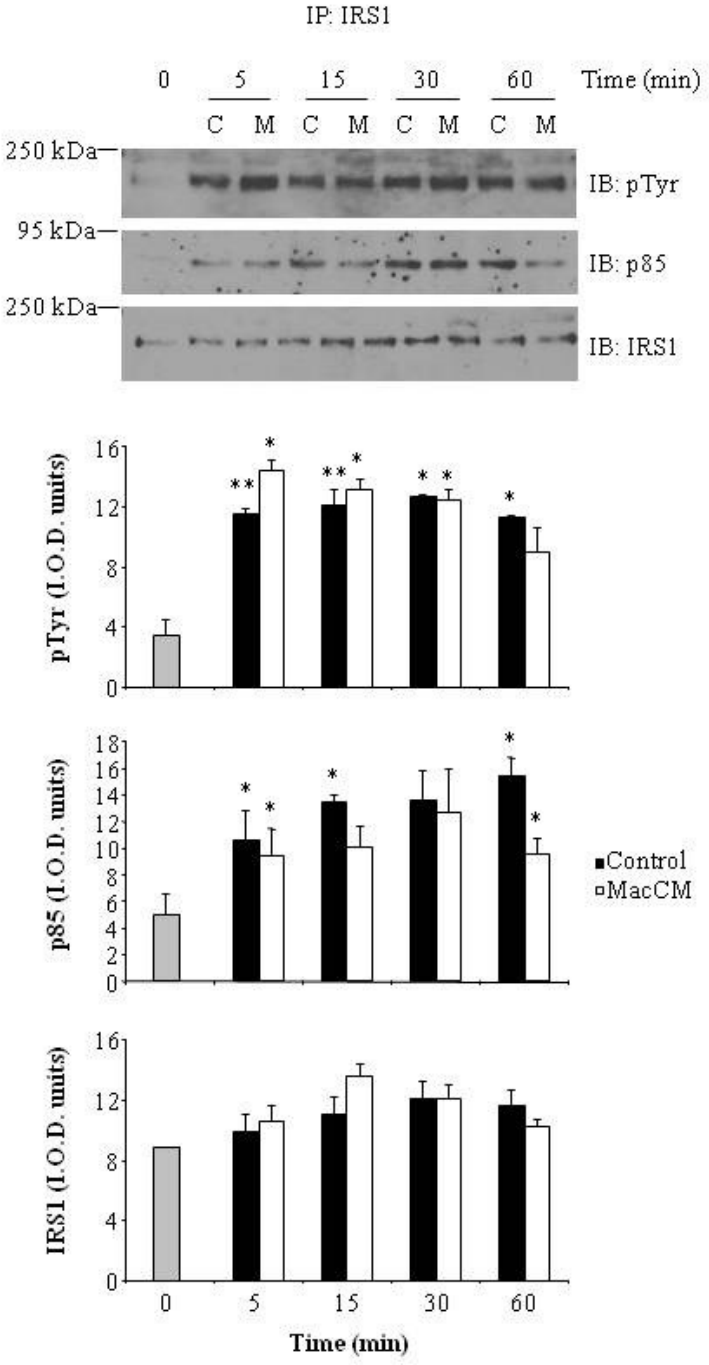

0 4 8 12 20 24 Time (h)
C M C M C M C M C M
36 kDa
pCEBPβ (LAP)
36 kDa
CEBPβ (LAP)
55 kDa
ERK1/2
17 kDa
CEBPβ (LIP)
55 kDa
ERK1/2
pCEBPβ (I.O.D. units)
CEBPβ LAP (I.O.D. units)
CEBPβ LIP (I.O.D. units)
Control
MacCM
Time (h)

Figure 11. Effect of J774A.1-MacCM on IRS1 Tyr phosphorylation and IRS1-p85 association in 3T3-L1 preadipocytes. Two day post-confluent 3T3-L1 preadipocytes were induced to differentiate in control medium (C) or J774A.1-MacCM (M) for the indicated times. Cell lysates were incubated with anti-IRS1 antibody. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with antibodies against pTyr, p85, and IRS1. Representative immunoblots are shown. Densitometric data from 3 independent experiments are expressed as mean ± SE. IOD, integrated optical density. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


IP: IRS1
0
5
15
30
60
Time (min)
C M C M C M C M
250 kDa
IB: pTyr
95 kDa
IB: p85
250 kDa
IB: IRS1
pTyr (I.O.D. units)
p85 (I.O.D. units)
IRS1 (I.O.D. units)
Control
MacCM
Time (min)

P<0.05, n=3) at the 5 minutes of induction and then further increased (3-fold versus basal, P<0.05, n=3) by 60 minutes under control adipogenic conditions. The increase at 60 minutes was reduced by 56% (P=0.06) with J774A.1-MacCM. Equal amounts of immunoprecipitated IRS1 were detected for each condition (Fig. 11).

Part II: Studies with human subcutaneous abdominal preadipocytes and THP-1 macrophages

Cyclin E expression increases with differentiation in human abdominal subcutaneous preadipocytes

THP-1-MacCM is known to inhibit human adipogenesis, but the underlying mechanism of this anti-adipogenic effect is unknown. Although human preadipocytes do not undergo MCE, some studies have suggested an alternate role for cell cycle regulators in human adipogenesis (Abella et al., 2005; Ross et al., 2008). Therefore, I examined the expression patterns of various cell cycle regulators in differentiating human preadipocytes.

Confluent human abdominal subcutaneous preadipocytes were either left undifferentiated or induced to differentiate for up to 14 days. The preadipocytes that were maintained under non-adipogenic conditions did not accumulate lipid (Fig. 12A) or express the adipogenic marker, PPARγ (Fig. 12B). In contrast, preadipocytes treated with adipogenic inducers accumulated lipid and displayed an upregulation of PPARγ.

Rb phosphorylation levels were initially detectable and then decreased over time in non-differentiating conditions (Fig. 13). This downward pattern was also observed in differentiating cells; however, the decrease occurred more rapidly, such that there was a 52% reduction in levels of Rb phosphorylation at day 1 (compared to time matched-control, P<0.05, n=4). The levels of Rb in the non-differentiated preadipocytes remained unchanged

Figure 12. The expression of PPARγ increased in differentiating human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either maintained under non-differentiating conditions (U) or induced to differentiate (D). **(A)** Cultures were photographed at 200x magnification. **(B)** Cells were lysed at the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against PPARγ and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.

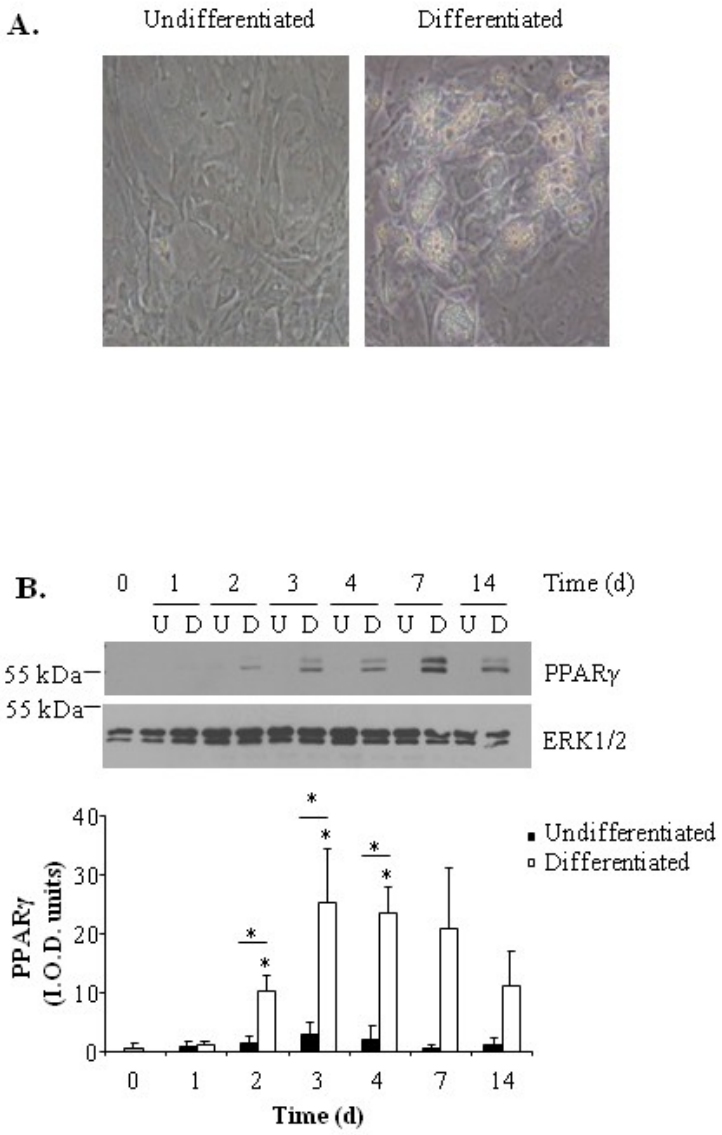
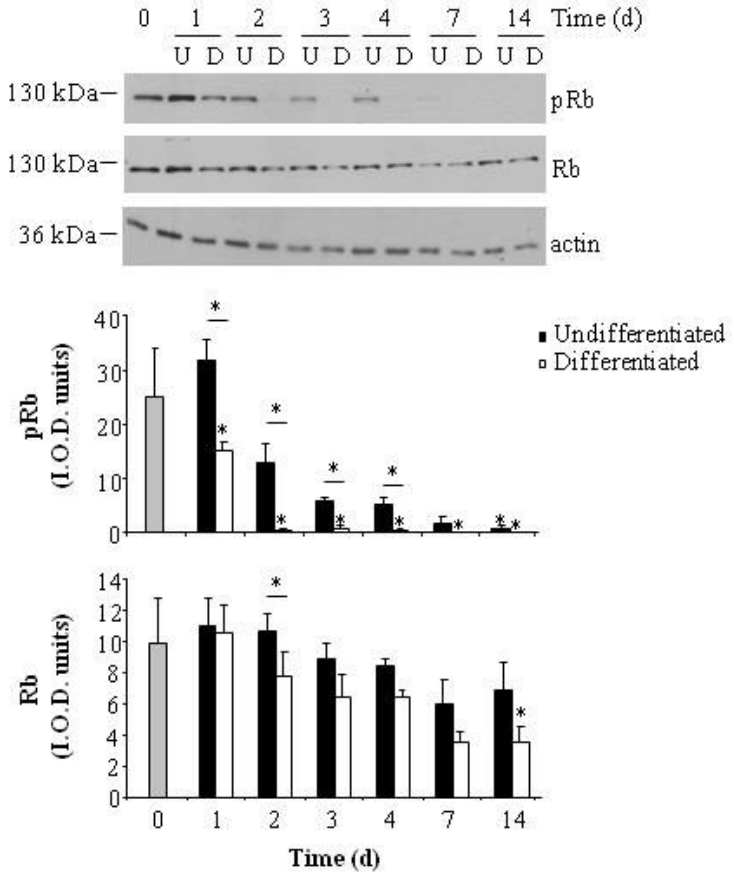

A.
Undifferentiated
Differentiated
B.
0 1 2 3 4 7 14 Time (d)
U D U D U D U D U D U D U D
55 kDa
PPARγ
55 kDa
ERK1/2
PPARγ (I.O.D. units)
40
30
20
10
0
0 1 2 3 4 7 14
Time (d)
Undifferentiated
Differentiated

Figure 13. The decrease in Rb phosphorylation is enhanced in differentiating human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either maintained under non-differentiating conditions (U) or induced to differentiate (D) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against pRb, Rb and actin (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05 compared to 0 time point or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.

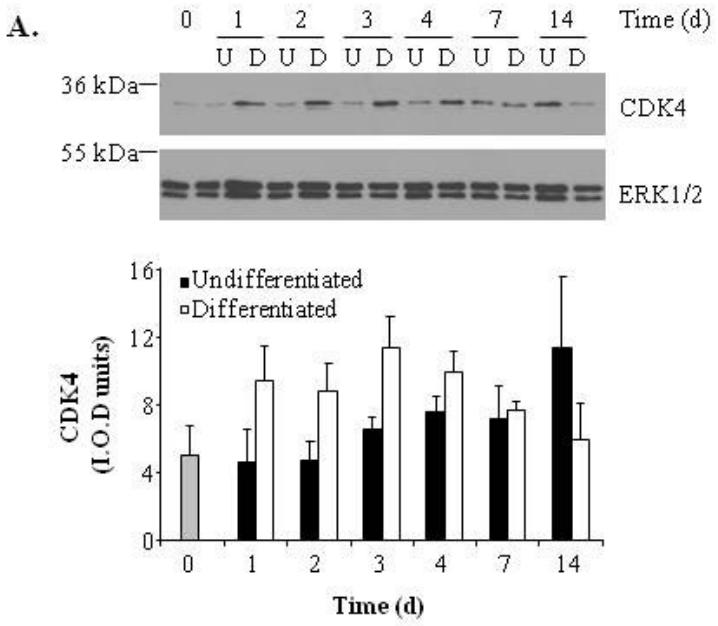

0 1 2 3 4 7 14 Time (d)
U D U D U D U D U D U D
130 kDa
pRb
130 kDa
Rb
36 kDa
actin
Undifferentiated
Differentiated
pRb (I.O.D. units)
Rb (I.O.D. units)
Time (d)

from basal levels. In contrast, Rb mass was reduced by 28% (versus time-matched control, $P<0.05$, $n=4$) at day 2 under adipogenic conditions. Differences in phospho-Rb levels between the conditions observed on day 2 were still significant after phospho-Rb levels were normalized to Rb.

I also examined the expression of CDKs and their regulatory binding partners. CDK4 expression in non-differentiated preadipocytes remained stable at basal levels over time. An upward trend in CDK4 expression was observed in differentiating preadipocytes; however, these differences did not reach significance (Fig. 14A). The expression of $p27^{Kip1}$ rose significantly over time in undifferentiated cells, reaching a 3-fold increase (versus basal, $P<0.05$, $n=4$) on day 14 (Fig. 14B). Similarly, a 3-fold increase (versus basal, $P<0.05$, $n=4$) in $p27^{Kip1}$ expression was observed in differentiating cells on day 4. No significant differences were observed in $p27^{Kip1}$ expression between non-differentiated and differentiating conditions. Under non-differentiating conditions, levels of cyclin E remained stable at basal levels. In contrast, cyclin E expression increased for the first 4 days, reaching a 3-fold increase (versus basal, $P<0.05$, $n=4$) at day 4 under adipogenic conditions and then returned towards basal levels (Fig. 15). The expression levels of CDK2 and cyclin A were also examined; however, the variability between independent experiments was too large to determine any distinct expression patterns.

To assess whether the differentiation-induced increase in cyclin E expression was associated with an increase in CDK2 activity, CDK2 was immunoprecipitated from human abdominal subcutaneous preadipocytes that were either maintained under non-adipogenic conditions or induced to differentiate for 4 days. This particular time point was chosen since the maximal increase in cyclin E levels were observed on day 4 of differentiation. CDK2 activity was unaffected by the addition of adipogenic inducers (Fig. 16). Additionally, the

Figure 14. CDK4 and $p27^{Kip1}$ expression is not altered by differentiation in human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either maintained under non-differentiating conditions (U) or induced to differentiate (D) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against $p27^{Kip1}$, CDK4 and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05 compared to 0 time point. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


A.
0 1 2 3 4 7 14 Time (d)
U D U D U D U D U D U D U D
36 kDa
CDK4
55 kDa
ERK1/2
Undifferentiated
Differentiated
CDK4 (I.O.D units)
16
12
8
4
0
0 1 2 3 4 7 14
Time (d)

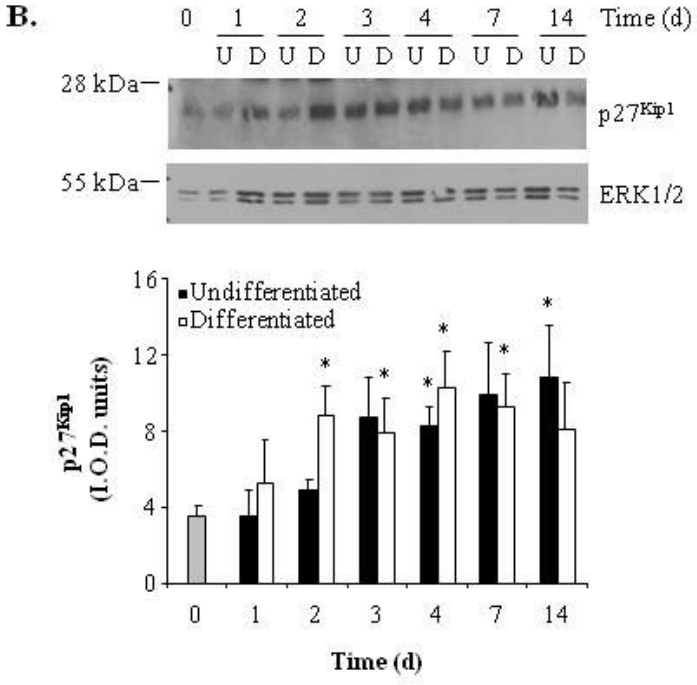
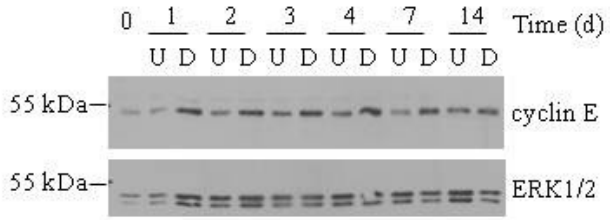

B.
0 1 2 3 4 7 14 Time (d)
U D U D U D U D U D U D U D
28 kDa
p27Kip1
55 kDa
ERK1/2
Undifferentiated
Differentiated
p27Kip1 (I.O.D. units)
16
12
8
4
0
0 1 2 3 4 7 14
Time (d)

Figure 15. Cyclin E expression increases during differentiation in human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either maintained under non-differentiating conditions (U) or induced to differentiate (D) for the indicated times. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against cyclin E and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 4 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *$P<0.05$ compared to time 0 or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


0 1 2 3 4 7 14 Time (d)
U D U D U D U D U D U D U D
55 kDa
cyclin E
55 kDa
ERK1/2

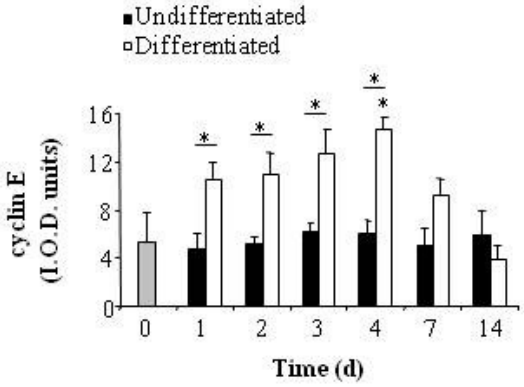
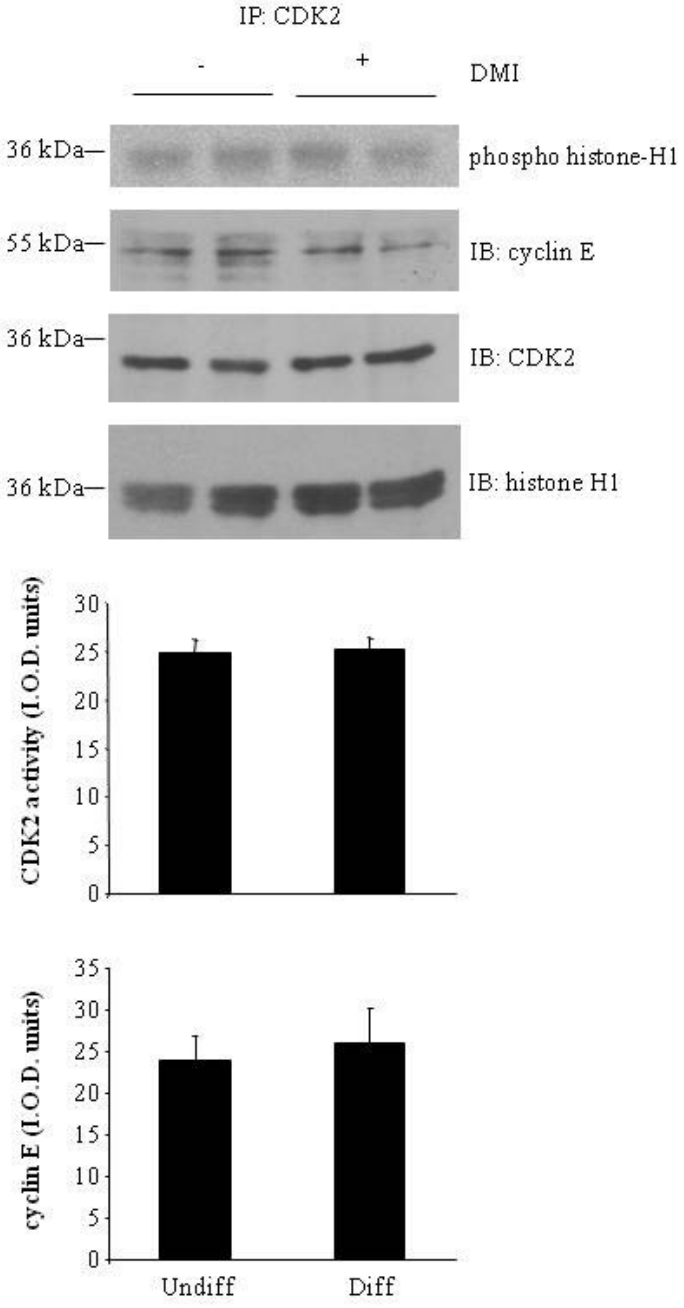

Undifferentiated
Differentiated
cyclin E (I.O.D. units)
16
12
8
4
0
0 1 2 3 4 7 14
Time (d)

Figure 16. Differentiation-induced increase in cyclin E expression is not associated with CDK2 activation in differentiating human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were induced to differentiate (+DMI) or not (-DMI) for 4 days. Immunoprecipitated CDK2 was assayed for kinase activity, using histone H1 as a substrate in the presence of radiolabelled ATP. Immunoprecipitates were resolved by SDS-PAGE and incorporation of ^{32}P into histone H1 was visualized by autoradiography. Representative autoradiograph and immunoblots are shown. Densitometric results from 3 independent experiments are expressed as mean ± SE. IOD, integrated optical density. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


IP: CDK2
-
+
DMI
36 kDa—
phospho histone-H1
55 kDa—
IB: cyclin E
36 kDa—
IB: CDK2
36 kDa—
IB: histone H1
CDK2 activity (I.O.D. units)
30
25
20
15
10
5
0
cyclin E (I.O.D. units)
35
30
25
20
15
10
5
0
Undiff
Diff

amount of cyclin E that co-immunoprecipitated with CDK2 was unchanged with differentiation. Equal amounts of CDK2 and histone H1 were detected for each condition.

THP-1-MacCM prevents the differentiation-dependent decrease in Rb phosphorylation in human abdominal subcutaneous preadipocytes.

Based on the previous results showing that Rb phosphorylation rapidly decreases with differentiation (Fig. 13), it is possible that the reduction in phospho-Rb levels is important for adipogenesis. Since MacCM has been shown to inhibit human adipogenesis, I wanted to determine if the anti-adipogenic effect of THP-1-MacCM was associated with alterations in the Rb phosphorylation pattern. To test this, human primary preadipocytes were left undifferentiated or induced to differentiate in the presence or absence of THP-1-MacCM for 1 day. The usual decrease in Rb phosphorylation under control adipogenic conditions was observed (Fig. 17). In contrast, phospho-Rb levels trended upward (although not reaching significance) when the preadipocytes were induced to differentiate in the presence THP-1-MacCM. No differences in Rb mass were observed between the various conditions.

THP-1-MacCM enhances the differentiation-induced cyclin E in differentiating human abdominal subcutaneous preadipocytes.

The increase in cyclin E expression during differentiation suggests the possibility of a role for cyclin E in human adipogenesis. I determined if the inhibition of human preadipocyte differentiation by MacCM was associated with alterations in cyclin E expression. To test this, cyclin E expression was assessed in human preadipocytes that were induced to differentiate or not in control medium or THP-1-MacCM for 4 days (Fig. 18). The usual differentiation-induced increase in cyclin E was observed under control adipogenic conditions. Interestingly, rather than reducing cyclin E expression, THP-1-MacCM further

Figure 17. THP-1-MacCM prevents the differentiation-dependent decrease in Rb phosphorylation in human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either induced to differentiate (+DMI) or not (-DMI) in the presence of control medium or THP-1-MacCM for 1 day. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against pRb, Rb and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 3 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *$P<0.05$ compared to time 0 or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by by paired t-tests for post-hoc comparisons of means.

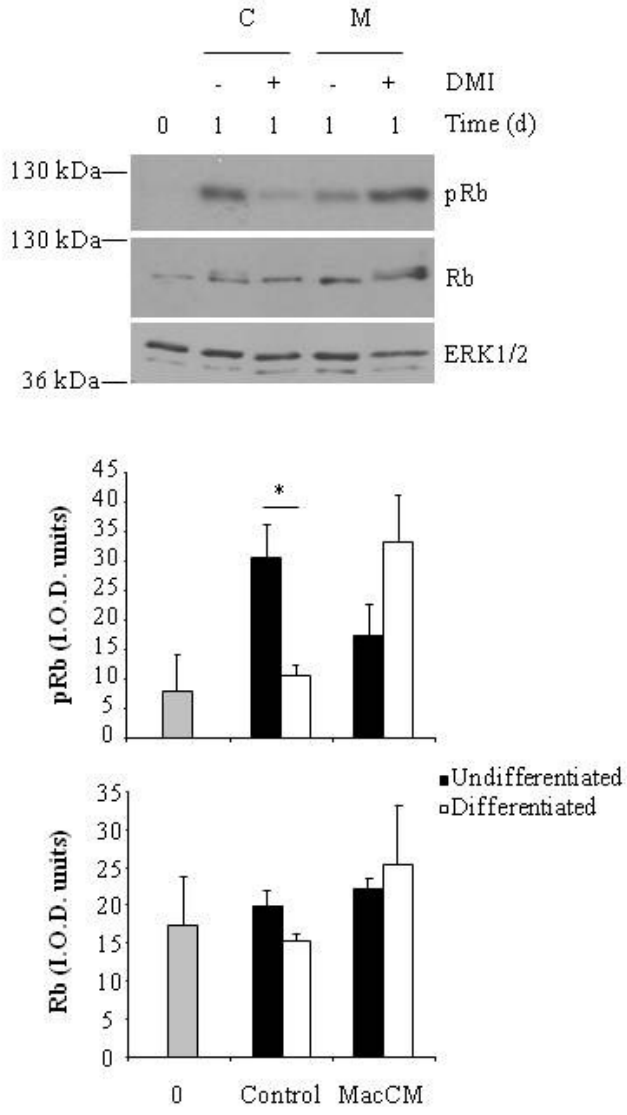
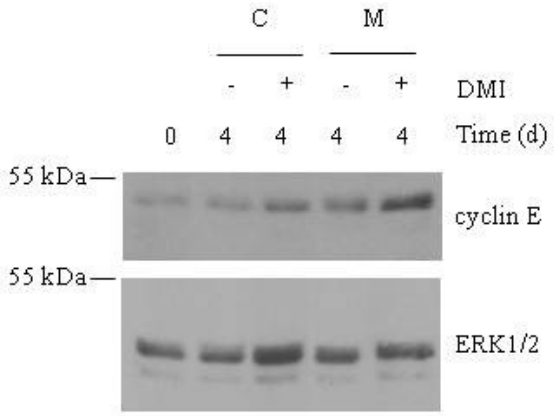
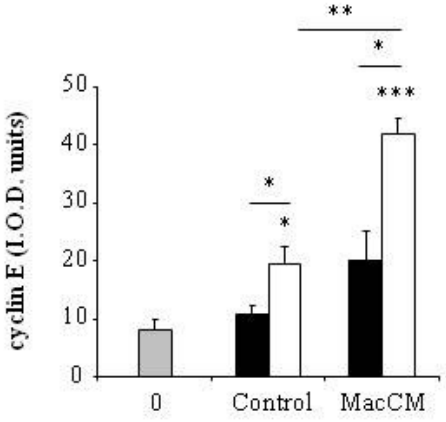

C
M
-
+
-
+
DMI
0
1
1
1
1
Time (d)
130 kDa
pRb
130 kDa
Rb
ERK1/2
36 kDa
pRb (I.O.D. units)
45
40
35
30
25
20
15
10
5
0
*
Undifferentiated
Differentiated
Rb (I.O.D. units)
35
30
25
20
15
10
5
0
0
Control
MacCM

Figure 18. THP-1-MacCM enhances the differentiation-induced cyclin E expression in human subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were either induced to differentiate (+DMI) or not (-DMI) in the presence of control medium or THP-1-MacCM for 4 days. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibodies against cyclin E and ERK1/2 (loading control). Representative immunoblots are shown. Densitometric data from 3 independent experiments are expressed as mean ± SE. IOD, integrated optical density. *P<0.05, **P<0.01, ***P<0.001 compared to time 0 or between indicated pairs. Statistical significance was determined by a two-way ANOVA, followed by paired t-tests for post-hoc comparisons of means.


C
M
-
+
-
+
DMI
0
4
4
4
4
Time (d)
55 kDa
cyclin E
55 kDa
ERK1/2


**
*
***
*
*
50
40
30
20
10
0
cyclin E (I.O.D. units)
0
Control
MacCM

enhanced the differentiation-induced levels of cyclin E by 2-fold (versus control adipogenic condition, $P<0.01$, $n=3$, Fig. 18).

DISCUSSION

Recent studies have reported that MacCM inhibits adipogenesis in 3T3-L1 and human primary preadipocytes. This finding suggests a potential role for macrophages in restricting adipose tissue expansion, consequently leading to the development of dysfunctional adipocytes and diminished insulin sensitivity in the muscle and liver due to ectopic deposition of fatty acids within these tissues. In 3T3-L1 preadipocytes, it has been recently reported that J774A.1-MacCM acts on early events of adipogenesis, including MCE and Rb phosphorylation (Yarmo et al., 2009). To further characterize the anti-adipogenic effect, I investigated the J774A.1-MacCM induced alterations in cell cycle events that lie upstream of Rb. My research reveals that J774A.1-MacCM has profound inhibitory effects on adipogenesis-related CDK2 activity in 3T3-L1 preadipocytes. This would be expected to contribute to the dramatic reduction in Rb phosphorylation seen when 3T3-L1 adipogenesis is inhibited by J774A.1-MacCM.

CDK2 is a key participant in MCE and governs the transition from G_1 to S phase by phosphorylating Rb. Pharmacological inhibition of CDK2 has been shown to impair adipogenesis by preventing MCE (Tang et al., 2003b; Li et al., 2007). Therefore, it was important to identify the possible mechanisms that explain the observed J774A.1-MacCM–dependent reduction in CDK2 activity. One possible mechanism could be decreased CDK2 protein expression. Under control adipogenic conditions, CDK2 expression remained unchanged throughout the 24 hour time course. This observation is consistent with a previous study conducted by Reichart and Eick (Reichert and Eick, 1999). However, some studies have indicated a gradual increase in CDK2 expression over a 24 hour period (Tang et al., 2003b; Li et al., 2007; Saito et al., 2009). The exact reasons for these inconsistencies are

not clear. In contrast to the control adipogenic conditions, when differentiation was induced in the presence of J774A.1-MacCM, the level of CDK2 was reduced. This reduction would be expected to contribute to the decrease in CDK2 activity and subsequent Rb phosphorylation during MCE.

CDK2 activity is dependent on its interaction with cyclin A, therefore the differentiation-induced upregulation of cyclin A is critical for MCE (Morrison and Farmer, 1999; Tang et al., 2003b). Under standard adipogenic conditions, my results indicate an upregulation of cyclin A mRNA and protein. This trend has been demonstrated by others and appears to be one of the few cell cycle protein expression patterns that is consistent between different studies (Morrison and Farmer, 1999; Reichert and Eick, 1999; Tang et al., 2003b). J774A.1-MaCM strongly inhibited the expression of cyclin A mRNA and protein, suggesting an additional mechanism, besides the lower levels of CDK2 itself, to explain the reduction in CDK2 activity that I observed.

To determine how J774A.1-MacCM acts to inhibit cyclin A expression, I evaluated the effects of J774A.1-MacCM on c-Myc, a transcriptional regulator of cyclin A. c-Myc was an attractive candidate since it has been shown to regulate cyclin A in other cell types (Rudolph et al., 1996), to promote 3T3-L1 preadipocyte proliferation, and to be upregulated during MCE (Rudolph et al., 1996; Reichert and Eick, 1999). Although J774A.1-MacCM reduced the upregulation of c-Myc normally observed under control adipogenic conditions, there was still a strong induction of c-Myc from basal levels. The mechanisms that mediate c-Myc expression are not completely known, given the complex regulation of its promoter. Previous studies provide evidence that the induction of c-Myc by growth factors is mediated by E2F (Mudryj et al., 1990). Although this may be the case, the upregulation of c-Myc precedes the observed differentiation-induced phosphorylation of Rb and the subsequent

release of E2F, suggesting a role for other transcriptional regulators of c-Myc. A recent report has identified a factor, referred to as EMYCS, that is distinct from E2F and that is implicated in the activation of the c-Myc promoter during the transition from quiescence into the cell cycle (Alvaro-Blanco et al., 2009). Furthermore, the activation of ERK1/2 has been shown to stabilize and enhance the accumulation of c-Myc protein (Sears et al., 2000). Therefore, numerous factors and pathways could be contributing to the induction of c-Myc, with some but not all negatively affected by J774A.1-MacCM.

Given that the degree of inhibition of c-Myc was less (26-41%, $P<0.05$, $n=4$) than the observed inhibition of cyclin A (73-74%, $P<0.05$, $n=4$), it is plausible that regulators of cyclin A expression, besides c-Myc, may be altered by J774A.1-MacCM. In particular, cAMP response element binding protein (CREB) has been shown to initiate 3T3-L1 preadipocyte differentiation and to regulate cyclin A transcription in human fibroblasts (Desdouets et al., 1995; Reusch et al., 2000). Signaling pathways that are responsible for the phosphorylation and activation of CREB include the ERK1/2 and protein kinase A (PKA) pathways (Desdouets et al., 1995; Klemm et al., 1998), which are activated by the stimulation with insulin and cAMP-elevating reagents (i.e. IBMX), respectively (Prusty et al., 2002; Martini et al., 2009). Future studies investigating the effects of J774A.1-MacCM on CREB phosphorylation, as well as on the signaling pathways that lie upstream of CREB, should be conducted.

In addition to CREB, E2F is a known regulator of cyclin A (Soucek et al., 1997). Since the observed time points that Rb phosphorylation is induced under control conditions coincide with those showing upregulation of cyclin A, it may be that E2F is an additional factor responsible for regulating cyclin A expression. In the presence of J774A.1-MacCM, the inhibition of the differentiation-induced Rb phosphorylation also corresponded with the

reduction in cyclin A expression at the same time points, suggesting that the restraint on E2F activity due to the inhibition of Rb phosphorylation may contribute to the reduction in cyclin A expression. Efforts in our laboratory have been made to look at the effects of J774A.1-MacCM on the ability of E2F to dissociate from Rb and gain DNA binding activity using an electrophoretic mobility shift assay (EMSA). However, alterations in the E2F DNA binding activity in response to J774A.1-MacCM could not be determined due to technical difficulties and limitations of the E2F antibody being used. Therefore, further research will be required in order to demonstrate the effect of impaired Rb phosphorylation on E2F DNA-binding activity in response to J774A.1-MacCM.

Given that full phosphorylation of Rb by cyclin E/A/CDK2 complexes cannot occur without the initial phosphorylation by cyclin D/CDK4, it is possible that the inhibition of Rb phosphorylation by J774A.1-MacCM may arise from a reduction in CDK4 activity (Planas-Silva and Weinberg, 1997; Patel and Lane, 2000). Disruption of CDK4 activity would impede the partial release of E2F, resulting in the inhibition of cyclin A expression and subsequent CDK2 activity. A previous study conducted by Abella *et al.* demonstrated that the inhibition of CDK4 results in impaired MCE and subsequent differentiation, highlighting the critical role of CDK4 for the differentiation process (Abella et al., 2005). My attempts to measure CDK4 activity were not successful, since sufficient amounts of immunoprecipitated CDK4 could not be obtained with commercially available antibodies. One group that has successfully immunoprecipitated CDK4 using an in-house-generated antibody observed an increase in CDK4 activity within 8 hours of adipogenic induction (Phelps and Xiong, 1998).

Although I was unable to directly measure CDK4 activity, I was able to observe changes in the expression of cyclin D1 and D2, which are known regulators of CDK4. Consistent with some previous studies, an upregulation of cyclin D1 was observed in 3T3-L1

preadipocytes in response to adipogenic inducers (Hishida et al., 2008; Saito et al., 2009). Other studies have reported no change in cyclin D1 expression during the early phase of differentiation (Reichert and Eick, 1999; Tang et al., 2003b). The reason for this discrepancy is unknown. The observed transient increase in cyclin D2 expression after adipogenic induction is consistent with another study (Hishida et al., 2008). The differentiation-induced increases in cyclin D1 and D2 were blunted with J774A.1-MacCM and this would be expected to inhibit differentiation-induced CDK4 activity.

Activation of the insulin signaling pathway has been shown to lead to Rb phosphorylation as well as the upregulation of cyclin expression during the early phase of differentiation (Muise-Helmericks et al., 1998; Usui et al., 2000). Therefore, I was interested in determining if the effects of J774A.1-MacCM on Rb phosphorylation and cyclin expression were associated with the disruption of early insulin signaling events, including IRS1 tyrosine phosphorylation and IRS1 association with PI3K (p85). Consistent with a previous study that involved the stimulation of 3T3-L1 preadipocytes with insulin alone, acute stimulation with adipogenic inducers under control conditions resulted in an increase in IRS1 tyrosine phosphorylation and IRS1-p85 association (Gagnon et al., 1998). J774A.1-MacCM did not significantly alter these early insulin-dependent responses. However, due to the reduction in IRS1-p85 association with J774A.1-MacCM (56% versus time matched control, P=0.06, n=3) that was observed at the 60 minute time point, there is the possibility that there is a difference in early insulin signaling events between the media types, but the time course was too short to make it apparent. It is possible that longer stimulation of the insulin signaling pathway is necessary for sustained activation of downstream pathways that lead to the upregulation of cyclin proteins. For example, some studies have shown in other cell types that sustained activation of the ERK1/2 pathway during G_1 progression is

necessary for the induction of cyclin D expression and entrance into S phase (Pages et al., 1993; Lavoie et al., 1996). During the early stages of 3T3-L1 adipogenesis, ERK1/2 is rapidly activated by becoming phosphorylated within 5 minutes from induction and persists for approximately 6 hours, which is then followed by the progression through G_1 of the cell cycle (Prusty et al., 2002). We have previously shown that J774A.1-MacCM does not alter ERK1/2 phosphorylation for the first 60 minutes of differentiation, but have yet to determine the ERK1/2 phosphorylation status at later time points in response to J774A.1-MacCM (Yarmo et al., 2009). In addition to ERK1/2, insulin can activate the PI3K/Akt pathway, which has been shown to be associated with cyclin D regulation (Muise-Helmericks et al., 1998; Usui et al., 2000). Similar to ERK1/2, activation of Akt can persist for hours following adipogenic induction (Xu and Liao, 2004), but whether or not prolonged activation of these pathways is required for cyclin upregulation, is not known. Perhaps J774A.1-MacCM may cause the insulin signaling pathway to shut down too quickly, leading to the disruption in downstream pathways that participate in the regulation of cyclin expression. Therefore, a longer time course should be performed to confirm whether there are alterations in proximal insulin signaling events and downstream signaling pathways in response to J774A.1-MacCM.

In addition to examining the effects of J774A.1-MacCM on events that lie upstream of CDK2 activation, a portion of my project also involved identifying possible downstream events that would be affected by impaired CDK2 activity. One important adipogenic target of CDK2 is C/EBPβ (Li et al., 2007). C/EBPβ (Thr188) is initially phosphorylated by ERK1/2 immediately following adipogenic induction, but does not gain DNA binding activity until several hours later when it becomes phosphorylated by GSK3β (on Ser184 and Thr179) during S phase. ERK1/2 activity is downregulated before S phase is completed, so

CDK2 was identified as the kinase that sustains C/EBPβ (Thr188) in the primed phosphorylation state during S phase (Li et al., 2007). The observed differentiation-induced phosphorylation of C/EBPβ (Thr188) was consistent with previous studies (Tang et al., 2005; Li et al., 2007) and was not affected by J774A.1-MacCM. This result agrees with previously published data from our laboratory showing that MacCM does not negatively alter the differentiation-induced C/EBPβ DNA binding (Yarmo et al., 2009). It is possible that J774A.1-MacCM is capable of activating ERK1/2 long enough to sustain the primed phosphorylation state of C/EBPβ, compensating for the reduced CDK2 activity.

Although exposure to J774A.1-MacCM did not reduce C/EBPβ phosphorylation, it significantly reduced C/EBPβ (LAP) expression. Previous data from our laboratory that investigated the effect of J774A.1-MacCM on C/EBPβ (LAP) expression showed a reduced trend in C/EBPβ (LAP) expression under J774A.1-MacCM differentiating conditions; however, it did not reach significance (Yarmo et al., 2009). The exact reason for this variation is not clear, but may be attributed to the fact that MacCM was generated from different stocks of J774 macrophages, possibly resulting in varying levels of potency. Since C/EBPβ (LAP) is an essential regulator for adipogenesis (Tanaka et al., 1997), a decrease in its expression would be expected to provide another mechanism by which J774A.1-MacCM inhibits preadipocyte differentiation. It has been reported that the transcription of C/EBPβ (LAP) is regulated by CREB (Niehof et al., 1997; Zhang et al., 2004). Therefore, future experiments should be conducted in order to determine the effects of J774A.1-MacCM on CREB activation.

In the second aim of my studies, I investigated the expression of cell-cycle regulators during human adipogenesis. Although human primary preadipocytes do not undergo MCE (i.e. an early proliferative phase) during differentiation *in vitro*, their ability to differentiate is

susceptible to MacCM. Conditioned medium generated from different macrophage models have exerted anti-adipogenic effects on human preadipocytes. The exact mechanism underlying this anti-adipogenic effect is unknown. This study considered the possibility that the inhibition of human adipocyte differentiation in response to THP-1-MacCM may be associated with alterations in cell cycle protein expression.

The adipogenic program in human preadipocytes has not been as extensively characterized as the 3T3-L1 preadipocyte model. The expression and function of many cell-cycle regulators during human adipogenesis have not been explored. However, their role in human preadipocyte differentiation has been suggested by several studies. For example, Rb-related pocket proteins, p130 and p107, have been shown to exhibit distinct expression patterns over the course of human adipocyte differentiation. For the first four days of differentiation, p130 is predominantly expressed. As preadipocytes progress though differentiation, a switch from p130 to p107 expression occurs (Ross et al., 2008). The functional relevance of this distinct expression pattern in human adipogenesis is not known. Interestingly, a very similar expression pattern occurs in 3T3-L1 preadipocytes, despite the fact that MCE is not observed during human adipogenesis *in vitro* (Richon et al., 1997). Furthermore, Abella *et al.* have suggested a cell cycle-independent role for CDK4 in terminal differentiation during human adipogenesis through its modulation of PPARγ activity (Abella et al., 2005). Together, these studies suggest the possibility of a role for cell cycle regulators in human preadipocyte differentiation. Therefore, I sought to determine if the reported inhibition of human adipogenesis by MacCM is associated with changes in cell cycle-related proteins.

A 14 day time course was performed to assess the kinetics of Rb phosphorylation in differentiating human preadipocytes. Under non-adipogenic conditions, phospho-Rb levels

were evident at the beginning of the time course and then significantly decreased over time. Similarly, under differentiating conditions, phospho-Rb levels were detectable at the outset, and significantly decreased during the course of adipogenesis; however, this decrease occurred more rapidly compared to non-adipogenic conditions. Whether or not this accelerated reduction in Rb phosphorylation is important for human adipogenesis is not known. When the effect of THP-1-MacCM on Rb phosphosphorylation was assessed during differentiation, the usual decrease in phospho-Rb levels was prevented. This observation suggests that the anti-adipogenic effect of THP-1-MacCM on human preadipocyte differentiation could be associated with a delay in the usual phospho-Rb decline. Potential approaches to test this could involve manipulating the phosphorylation state of Rb. For instance, the phosphorylation state of Rb could be maintained by stimulating the preadipocytes to differentiate in the presence of a phosphatase inhibitor of protein phosphatase 1 (PP1), whose action has been shown to be associated with direct dephosphorylation of phospho-Rb (Yan and Mumby, 1999). This would mimic the effect of THP-1-MacCM on the phosphorylation state of Rb and allow us to investigate whether the prevention in Rb dephosphorylation would be associated with a block in differentiation.

The most striking observation obtained from the 14 day time course of standard differentiation was the transient increase in cyclin E expression. A study conducted by Sarruf *et al.* observed a similar pattern during differentiation in 3T3-L1 preadipocytes (Sarruf et al., 2005). However, other 3T3-L1 preadipocyte studies reveal different cyclin E expression patterns during differentiation; some show that cyclin E expression remains unchanged from basal levels, while others report a decrease (Morrison and Farmer, 1999; Reichert and Eick, 1999; Tang et al., 2003b; Saito et al., 2009). Despite the fact that cyclin E is classically known for mediating events during the G1 phase of the cell cycle, others have also observed

cyclin E upregulation in various cell types during differentiation, suggesting the possibility that cyclin E may have functions independent of its role in cell cycle regulation (Gao and Zelenka, 1997).

Despite the induced levels of cyclin E that were observed in human preadipocytes undergoing differentiation, CDK2 activity was not enhanced within these cells. Several studies have reported similar findings (i.e. elevated levels of cyclin E are not associated with increased levels of CDK2 activity). For example, during the differentiation of PC12 cells, CDK2 activity is suppressed, while cyclin E expression is significantly enhanced (Dobashi et al., 1995). *In vitro* studies looking at rat calvarial osteoblast differentiation showed that nuclear cyclin E is upregulated, while CDK2 expression declined (Smith et al., 1995). In addition, it has been reported that exposure to cAMP-inducing reagents causes an antiproliferative effect in human adipose stem cells and that this response was associated with reduced Rb phosphorylation and CDK2 activity. Along with these responses, levels of cyclin E were markedly increased upon cAMP stimulation (Ugland et al., 2008). Together, these studies indicate that the induction of cyclin E is uncoupled from CDK2 activity during a postproliferative state, suggesting a role for cyclin E in functions other than being a CDK2 activator. Cyclin E has been reported to play cell cycle independent roles in apoptosis regulation (Mazumder et al., 2007; Ugland et al., 2008) and neural cell fate determination (Berger et al., 2010). However, the exact mechanisms by which cyclin E mediates these functions are not fully elucidated. To investigate the role of cycle E in human adipogenesis, future studies could begin by identifying the proteins that interact with cyclin E.

Based on the observation that cyclin E expression increases during differentiation and the possibility that it is important for human adipogenesis, I was interested in assessing the effect of THP-1-MacCM on cyclin E expression. My results indicate that the differentiation-

induced increase in cyclin E expression was enhanced in response to THP-1-MacCM. It has been previously reported that the activation of the ERK1/2 pathway is implicated in the upregulation of cyclin E (Bokemeyer et al., 2007). In addition, unpublished data from our laboratory have provided preliminary evidence to suggest that THP-1-MacCM activates the ERK1/2 pathway (Yarmo and Sorisky, unpublished observations). This was based on the observation that ERK1/2 phosphorylation was markedly enhanced in human preadipocytes undergoing differentiation in the presence of THP1-MacCM. Therefore, it can be postulated that THP1-MacCM activates ERK1/2, leading to an increase in cyclin E levels that surpass optimal levels during differentiation and somehow excessive cyclin E expression disrupts other cellular responses involved in the differentiation program. To determine if the ERK1/2 pathway participates in the regulation of cyclin E expression, studies using pharmacological inhibitors of the ERK1/2 pathway (i.e. PD98059), could be performed to see if this could reduce levels of cyclin E back to usual levels which might permit restoration of differentiation. In addition, to discern whether extreme levels of cyclin E are detrimental for the adipogenic response, cyclin E could be overexpressed to levels observed under THP-1-MacCM adipogenic conditions in order to determine if this would be sufficient to disrupt adipogenesis.

Currently, the macrophage-secreted factor(s) in MacCM that is (are) responsible for inhibiting adipogenesis is (are) unknown. Their abundance may be affected by BMI, which should be considered in future studies. Pro-inflammatory cytokines that are secreted by macrophages have been shown to inhibit adipogenesis. For example, a recent study has implicated IL-1β as an anti-adipogenic factor in J774A.1-MacCM. This was based on the observation that treatment with growth hormone down-regulated IL-1β in J774A.1 macrophages and consequently decreased the ability of J774A.1-MacCM to inhibit 3T3-L1

preadipocyte differentiation (Lu et al., 2010). In addition, TNFα has been shown to inhibit the differentiation of both 3T3-L1 and human preadipocytes. In 3T3-L1 adipogenesis, the inhibitory effect of TNFα is thought to be associated with a disruption in the normal regulation and expression pattern of pocket proteins (i.e. p130 and p107), resulting in a complete block in MCE (Lyle et al., 1998). Although TNFα has been shown to inhibit human adipogenesis, immunoneutralization of TNFα only mildly reversed the inhibitory effect of MacCM from human monocyte-derived macrophages, suggesting that other factors in MacCM may be working in concert with one another to inhibit adipogenesis (Lacasa et al., 2007). Furthermore, other macrophage-secreted factors, including platelet-derived growth factor (PDGF), IL-6, and transforming growth factor β (TGFβ) have been shown to inhibit preadipocyte differentiation. Of these factors, PDGF and TGFβ have been shown to be secreted by both J774A.1 and THP-1 macrophages. However, unpublished data from our laboratory suggests that PDGF (Molgat and Sorisky, unpublished observations) and TGFβ are not responsible for the anti-adipogenic effect in 3T3-L1 adipogenesis.

Further research will be needed in order to identify the factors that contribute to the inhibition of adipogenesis. Different approaches could be taken to determine the species of the factor. Heat denatuaration methods could be used to determine if the factor is a protein. Charcoal filters could be used to deplete the MacCM of lipids (Chen, 1967) and then this lipid-depleted media could be used in the adipogenesis studies to see if there is a reversal of the inhibitory effect. Additionally, another approach to identify the factor could involve the use of size-fractionation filters to obtain various fractions of MacCM. The different fractions could then be placed individually or in combination on the preadipocytes in the presence of adipogenic inducers to determine their ability to inhibit differentiation. The fraction(s) that display anti-adipogenic activity could then be subjected to mass spectrometry to identify

potential peptide/protein factors. This could be followed by immunodepletion or immunoneutralization studies to evaluate the requirement of the identified factors for the anti-adipogenic effect.

CONCLUSION

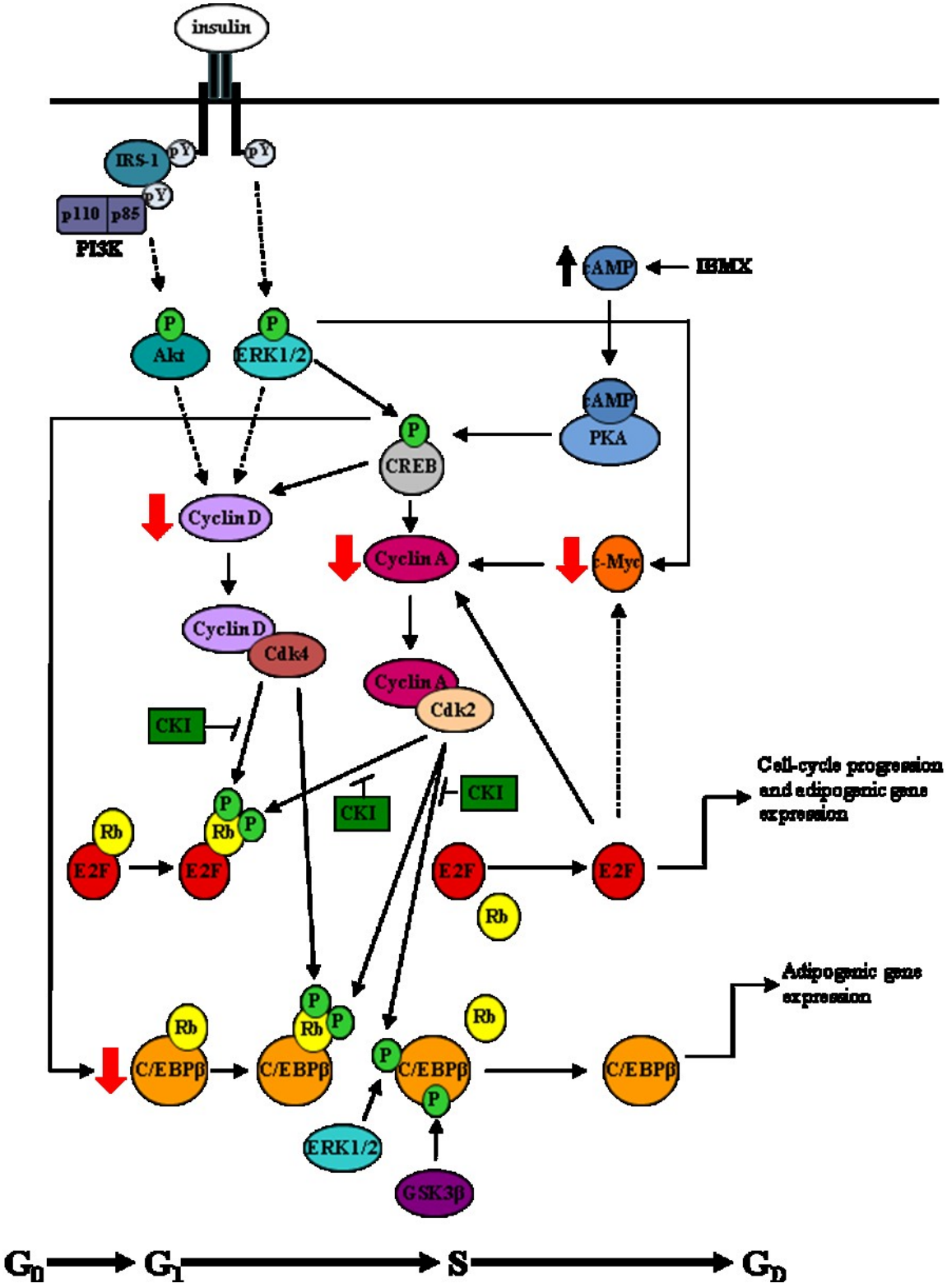
The findings presented in this thesis offer new insights into the complex interactions between macrophages and adipose cells. My results build on the previous discovery that MacCM inhibits the early events that are pivotal for adipogensis to occur, including MCE and Rb phosphorylation. Firstly, I have identified novel negative effects of MacCM on key cell cycle proteins that regulate Rb phosphorylation. More specifically, my results show that cyclin A and CDK2 expression, as well as CDK2 activity, are inhibited when preadipocytes were induced to differentiate in the presence of MacCM. In addition, MacCM reduced the expression of cyclin D1 and D2, which may have profound effects on CDK4 activity. These studies suggest a potential mechanism to explain the inhibition of Rb phosphorylation and therefore, the disruption in MCE and subsequent adipogenesis. Secondly, my results reveal a negative effect of MacCM on C/EBPβ (LAP) expression, providing another mechanism by which MacCM may inhibit adipogenesis. Lastly, I have shown that human adipogenesis is associated with a decline in phospho-Rb levels and an increase in cyclin E expression, suggesting the possibility of roles for cell cycle proteins in human preadipocyte differentiation. THP-1-MacCM prevented the usual decline in Rb phosphorylation and enhanced the upregulation of cyclin E. Further studies will have to be conducted to determine the specific relevance of these findings to adipogenesis. Together, my studies provide more information pertaining to the underlying molecular mechanisms for abnormal adipose tissue remodelling that lead to adipose tissue dysfunction associated with obesity.

PROPOSED MODEL

I propose the following model based on the findings from the *in vitro* 3T3-L1 studies (Fig. 19). At growth arrest (G_0), Rb is hypophosphorylated and bound to E2F. Following adipogenic induction, the activation of the PKA pathway, which is stimulated by elevated levels of cAMP, leads to the activation of CREB. CREB induces the upregulation of C/EBPβ, which has been suggested to be sequestered by Rb. Upon entry into the G_1 phase of the cell cycle, cyclin D is upregulated by the activation of CREB and the insulin-dependent activation of the ERK1/2 and PI3K/Akt pathways. Cyclin D forms a complex with CDK4, which then phosphorylates Rb, resulting in the partial release of E2F and allowing E2F to induce the expression of cyclin A and c-Myc. CREB also is a transcriptional regulator of cyclin A. The upregulation of cyclin A leads to the formation of cyclin A/CDK2 complexes that complete the phosphorylation of Rb and allows for the release of more E2F. The enhancement in E2F results in the transcription of cell cycle genes that are essential for S phase entry and DNA replication. The phosphorylation of Rb also allows the release of C/EBPβ. In order for C/EBPβ to gain DNA binding activity, it undergoes sequential phosphorylation. C/EBPβ is initially phosphorylated by ERK1/2 and CDK2, priming C/EBPβ for further phosphorylation by GSK3β. Once C/EBPβ gains DNA-binding activity, it is responsible for initiating the adipogenic transcriptional cascade by transcribing the master regulators of adipogenesis.

Upon adipogenic stimulation in the presence of J774A.1-MacCM, an impairment in MCE and Rb phosphorylation occurs. The block in the differentiation-induced Rb phosphorylation can be attributed to the inhibitory effects of J774A.1-MacCM on cyclin expression (i.e. cyclin A, D1 and D2) and CDK2 activity. With the disruption in CDK

Figure 19. Proposed model for the anti-adipogenic effect of J774A.1-MacCM on 3T3-L1 preadipocyte differentiation. Stimulation with an adipogenic cocktail induces growth-arrested 3T3-L1 preadipocytes to re-enter the cell cycle and undergo 2 rounds of mitoses (MCE). The phases of the cell cycle (G_0, G_1 and S) are indicated as well as the growth-arrested state in which the cells become terminally differentiated (G_D). At growth-arrest (G_0), Rb is hypophosphorylated and bound to E2F. During the early G_1 phase of the cell cycle, cyclin D expression is induced. The induction of cyclin D results in the formation of cyclin D/CDK4 complexes, which are responsible for early phosphorylation of Rb, partially relieving the constraint on E2F. The E2F-mediated upregulation of cyclin A, as well as the increase in c-Myc expression, leads to the formation of cyclin A/CDK2 complexes, which complete the phosphorylation of Rb. Once E2F is released, it can transcribe genes necessary for cell cycle progression and adipogenesis. The phosphorylation of Rb also alleviates the constraint on C/EBPβ, which is responsible for initiating the adipogenic transcriptional cascade by transcribing the master regulators of adipogenesis. J774A.1-MacCM inhibits 3T3-L1 preadipocyte differentiation by impairing proximal events in the adipogenic program. J774-MacCM interferes with the necessary expression of cyclin proteins and the activation of CDK2, leading to the inhibition of Rb phosphorylation. As a result, E2F remains bound to Rb, and so progression through the cell cycle and MCE does not occur. The effects of J774A.1-MacCM are indicated by the red arrows. See text for further details and abbreviations.


insulin
IRS-1
pY
pY
pY
p110
p85
PI3K
cAMP
IBMX
P
Akt
P
ERK1/2
cAMP
PKA
P
CREB
Cyclin D
Cyclin A
c-Myc
Cyclin D
Cdk4
Cyclin A
Cdk2
CKI
CKI
CKI
Rb
E2F
P
Rb
P
E2F
E2F
E2F
Rb
Cell-cycle progression and adipogenic gene expression
Adipogenic gene expression
Rb
C/EBPβ
P
Rb
P
C/EBPβ
P
C/EBPβ
Rb
P
C/EBPβ
C/EBPβ
ERK1/2
GSK3β
G0
G1
S
G0

activity, Rb phosphorylation cannot occur and the constraint on E2F is not alleviated. Therefore, cell cycle progression cannot resume. As a result, the preadipocytes are restricted from entering into S phase and gaining access to adipogenic-specific promoter sites during DNA replication and subsequent upregulation of adipogenic genes cannot occur. Furthermore, J774A.1-MacCM reduces the expression of C/EBPβ, which is expected to contribute to the inhibition of differentiation. Further research will need to be conducted in order to determine the mechanisms that explain how J774A.1-MacCM acts to reduce the expression of cell-cycle regulators and C/EBPβ.

Overall, this study demonstrates a relationship between macrophages and adipose cells, whereby macrophage-secreted factors impair adipogensis. One can speculate that, if this occurs *in vivo*, it would prevent the number of differentiated adipocytes from increasing to accommodate excess calories, leading to compensatory adipocyte hypertrophy and subsequent cellular dysfunction.

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CURRICULUM VITAE

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EDUCATION

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- NSERC Alexander Graham Bell Canada Graduate Scholarship (CGS) — Sept 2009
- Ontario Graduate Scholarship (OGS) (declined) — March 2009
- Admission Scholarship — Sept 2008 – April 2010

Carleton University — Ottawa, Ontario
- Senate Medal for Outstanding Academic Achievement — June 2008
- NSERC Undergraduate Student Research Award — May 2008 – Aug 2008
- General In-Course Scholarship — Sept 2006 – April 2007
- Gerhard Herzberg Scholarship — Sept 2007
- Deans' Honour List — Sept 2005 – April 2007

ABSTRACTS AND PUBLICATIONS

Abstract
- **Ide, J.**, Gagnon, A., Sorisky, A. (2009). Macrophage secreted factors inhibit CDK2 activity in preadipocytes. International Diabetes Federation's 20th World Diabetes Congress Conference (October 18-22, 2009; Montreal, Quebec). *(poster)*

Publication
- **Ide, J.**, Gagnon, A., Molgat, A., Sorisky, A. Macrophage-Conditioned Medium Inhibits the Activation of Cyclin-Dependent Kinase 2 by Adipogenic Inducers in 3T3-L1Preadipocytes. *Journal of Cellular Physiology.*

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