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Adipose Development: From Stem Cell to Adipocyte

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ABSTRACT Cell culture models have been developed to study commitment and subsequent differentiation of preadipocytes into adipocytes. Bone morphogenetic protein 4 commits mesenchymal stem cells to the adipose lineage. Other factors, including Wnt signaling, cell density, and cell shape, play a role in lineage commitment. Following commitment to the adipose lineage, growth-arrested preadipocytes can differentiate to adipocytes by treatment with insulin-like growth factor 1, glucocorticoid and an agent that increases cAMP level. This process is characterized by a rapid and transient increase in CCAAT/enhancer binding protein (C/EBP) β and synchronous re-entry into the cell cycle. Acquisition of DNA-binding by C/EBPβ occurs after the transcription factor becomes phosphorylated. The cells enter a growth-arrested state and begin terminal differentiation. C/EBPα, peroxisome proliferator-activated receptor γ, and adipocyte determination, and differentiation-dependent factor 1 coordinate the expression of genes that create and maintain the adipocyte phenotype.

KEYWORDS commitment, differentiation, preadipocyte, C/EBP, PPAR, ADD1/SREBP-1c

INTRODUCTION

The primary role of adipocytes is to store energy in the form of triacylglycerol during times when input exceeds expenditure and to break down this stored lipid into free fatty acids when energy is required. Obesity results when energy input persistently exceeds energy expenditure, causing both hypertrophy and hyperplasia of adipocytes (Figure 1). The sedentary lifestyle and high-fat diet have contributed to a high frequency of obesity and its attendant health problems in the United States, with approximately 65% of the population being classified as overweight and about 30% as obese (Mokdad *et al.*, 2001). A large body of evidence now indicates that adipocytes also play a major role in the control of metabolism through secretion of paracrine and endocrine hormones. Adipocytes secrete a wide range of regulatory factors in proportion to adiposity that have effects on feeding behavior, metabolism, insulin sensitivity and secretion, and reproductive and immune functions (for review, see Kershaw & Flier, 2004). Furthermore, as the largest energy reserve in the body, adipose tissue has a major impact on energy flux, plasma lipid levels, and rates of glucose uptake. In order to manage the health implications of obesity and the role of adipose tissue in energy homeostasis, it is necessary to understand the development and regulation of adipogenesis.

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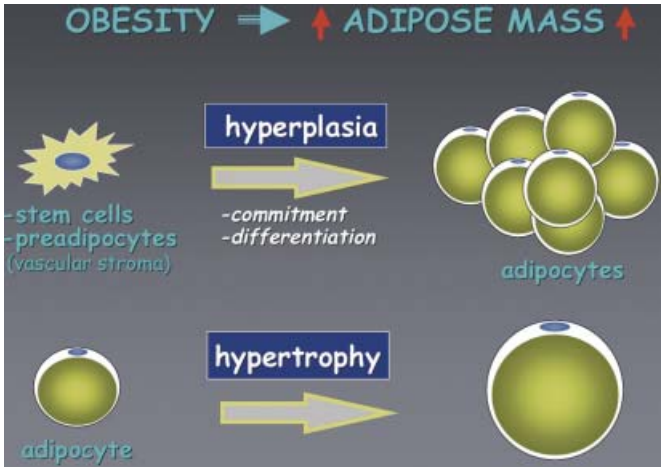


FIGURE 1 **Adipocyte hyperplasia and hypertrophy are responsible for the increase of adipose tissue mass associated with obesity. Recruitment of multipotent stem cells to the adipocyte lineage to produce new preadipocytes and mitotic clonal expansion of existing preadipocytes during adipogenesis contribute to hyperplasia. Increased lipogenesis and storage of the triacylglycerol in the unilocular fat droplet lead to the hypertrophy of mature adipocytes. Both multipotent stem cells and preadipocytes are present in the vascular stromal fraction of adipose tissue.**

The adipose lineage arises from a multipotent stem cell population of mesodermal origin. These precursor cells reside in the vascular stroma of adipose tissue and undergo a multi-step process comparising an initial commitment step, in which cells become restricted to the adipocyte lineage but do not yet express markers of terminal differentiation, and a subsequent program of activation of a network of transcription factors resulting in the adipocyte phenotype (Figure 2). It is likely

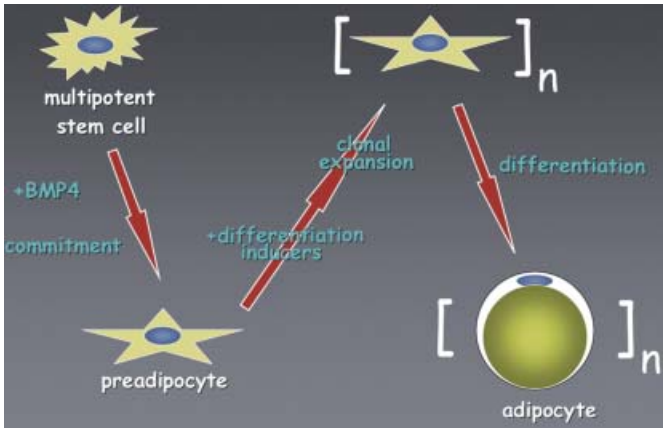


FIGURE 2 **Major phases in the development of adipocytes from multipotent stem cells. Commitment of multipotent stem cells to the adipocyte lineage to produce preadipocytes is initiated by BMP4. Exposure of preadipocytes to the appropriate differentiation inducers triggers ~2 rounds of mitotic clonal expansion, thereby increasing cell number by ~4-fold. Following mitotic clonal expansion, terminal differentiation occurs, in which the large family of adipocyte genes is transcriptionally activated.**

that the factors the initiating this process are secreted by cells within the stromal vascular population and/or adipocytes undergoing hypertrophy. Factors have been identified that lead to the commitment of mesenchymal stem cells to the adipose lineage *ex vivo*, but the molecular mechanisms by which these pathways are regulated have not been determined.

This review details our current understanding of stem cell commitment to the adipose lineage and the subsequent differentiation of preadipocytes into adipocytes. Emphasis is placed on the key transcriptional activators of adipogenesis.

CELL CULTURE MODELS OF ADIPOGENESIS

Much of what is known about adipocyte development has been discovered by studying adipocyte cell culture models. There are two types of model systems: (*a*) multipotent stem cell lines that have not undergone commitment to the adipocyte lineage, and (*b*) preadipocyte cell lines that have already been committed to the adipocyte lineage and can be induced to terminally differentiate into adipocytes. Multipotent stem cells undergo commitment to several distinct lineages, including the adipose, muscle, bone, and cartilage lineages. Once committed, preadipocytes can be induced to differentiate into adipocytes but not into other cell types (Figure 3). This discussion will be limited to the frequently used cell lines.

Commitment

The most commonly used multipotent stem cell line is the C3H10T1/2 (hereafter referred to as 10T1/2) line. This cell line was isolated from 14 to 17-day C3H mouse

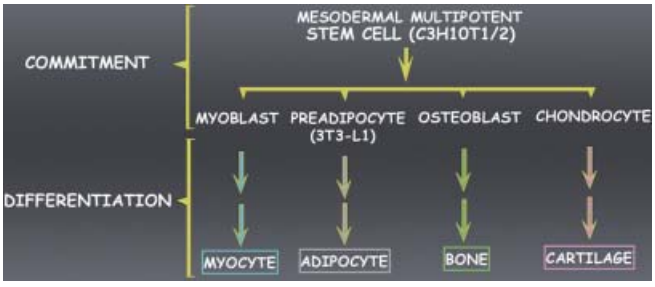


FIGURE 3 **Commitment of multipotent stem cells of mesodermal origin give rise to multiple cell types which have differing fates. When appropriately stimulated, mesodermal multipotent stem cells give rise to four major lineages: myoblast, preadipocyte, osteoblast, and chondroblast, which undergo differentiation into cells that produce muscle, adipose, bone and cartilage tissue, respectively.**

embryos and displays a fibroblastic morphology in culture (Reznikoff *et al.*, 1973). 10T1/2 cells are functionally similar to mesenchymal stem cells, in that treatment with the demethylating agent, 5-azacytidine, leads to the generation of multiple cell types that display the morphological and biochemical characteristics of muscle, adipose, or cartilage (Figure 3) (Taylor & Jones, 1979). The conversion to each of these cell types is a heritable trait, since clones can be isolated that retain these specific phenotypes without further treatment with 5-azacytidine (Taylor & Jones, 1979). It is believed that this phenotypic alteration results from hypomethylation of regulatory genes (Konieczny & Emerson, 1984). Transfection of genomic DNA isolated from 5-azacytidine-treated 10T1/2 cells results in the conversion of 10T1/2 cells into myoblasts at a frequency suggesting the modification of only one or a few regulatory genes (Lassar *et al.*, 1986). Using subtractive cDNA hybridization, Davis and colleagues (1987) identified MyoD, a transcription factor which converts the 10T1/2 cells to myoblasts. This finding led to the discovery of other myogenic regulatory factors (reviewed in, see Sabourin & Rudnicki, 2000). Attempts to identify an adipocyte commitment gene have thus far been unsuccessful.

10T1/2 cells will respond to bone morphogenetic proteins (BMPs), members of the transforming growth factor β superfamily. BMPs are well known for their roles as morphogens and, a controlling patterning in the embryo, particularly of mesoderm from which mesenchymal stem cells are derived (De Robertis & Kuroda, 2004). BMP2 and BMP4 have been shown to play a role in the control of mesenchymal stem cell commitment, promoting adipose, cartilage, or bone lineages, depending on culture conditions (Ahrens *et al.*, 1993; Wang *et al.*, 1993; Butterwith *et al.*, 1996; Denker *et al.*, 1999; Tang *et al.*, 2004a). Indeed, treatment of 10T1/2 cells with BMP4 is capable of inducing nearly complete commitment and subsequent differentiation to the adipocyte lineage (Butterwith *et al.*, 1996; Tang *et al.*, 2004a). This phenomenon should be valuable in deciphering the processes involved in adipose lineage commitment.

Differentiation

Primary and established preadipocyte cell lines have been useful in the study of adipogenesis. Primary preadipocytes can be isolated from the vascular stroma of adipose tissue and induced to differentiate when treated with a combination of adipogenic factors. However, the cells cannot readily be propagated and do not maintain a differentiated state for extended periods in culture. Conversely, established preadipocyte cell lines offer homogeneous cell populations and a stably differentiated state. The mouse 3T3-L1 and 3T3-F442A lines are the most widely characterized preadipocyte cell lines. These lines were selected from disaggregated mouse embryos for their ability to accumulate cytoplasmic triacylglycerol (Green & Kehinde, 1974; Green & Kehinde, 1975; Green & Kehinde, 1976) and are believed to be faithful models of preadipocyte differentiation as demonstrated by *in vivo* implantation studies. The subcutaneous implantation of 3T3-F442A preadipocytes into BALB/c athymic mice resulted in the development of an adipose depot that was virtually indistinguishable from endogenous adipose tissue (Green & Kehinde, 1979). Beta-galactosidase was utilized as a marker to confirm the origin of the tissue (Mandrup *et al.*, 1997). The leptin message level in the F442A-derived fat pad was similar to *in vivo* levels, and its expression was responsive to hormone injected into the host animal (Mandrup *et al.*, 1997). Unlike the 3T3-F442A cell line, 3T3-L1 preadipocytes do not differentiate into adipocytes after implantation into athymic mice. However, inhibiting the Wnt pathway through ectopic expression of the dominant-negative T-cell factor 4 (dnTCF4) allows these cells to develop into a fat pad upon subcutaneous injection (Ross *et al.*, 2000).

Agents which are efficient inducers of adipocyte differentiation in many cell systems include: insulin, insulin-like growth factor-1 (IGF-1), glucocorticoid, triiodothyronine, and cAMP (Spiegelman & Green, 1980; Student *et al.*, 1980; Amri *et al.*, 1986). Often these agents are most effective when used in combination. For example, methylisobutylxanthine (a cAMP phosphodiesterase inhibitor), dexamethasone (a synthetic glucocorticoid agonist), and a high concentration of insulin (MDI) in the presence of fetal bovine serum (FBS) have proven to be most effective for inducing differentiation of 3T3-L1 preadipocytes (Student *et al.*, 1980). In contrast, insulin and FBS are routinely used to differentiate 3T3-F442A preadipocytes (Spiegelman & Green, 1980). Insulin, which induces differentiation only at supraphysiological concentrations, exerts its effect through the IGF-1 receptor (Smith *et al.*, 1988). Insulin binds to IGF-1 receptors with low affinity and can mimic most of the biological effects of IGF-1. IGF-1, which is an effective differentiation inducer at a more physiological

concentration, is an authentic inducer of differentiation (Smith *et al.*, 1988).

Prior to differentiation, the preadipocyte cell lines are morphologically similar to fibroblastic preadipose cells in the stroma of adipose tissue. However, upon induction of differentiation, the cells lose their fibroblastic character, assume a rounded-up appearance and acquire the morphological and biochemical characteristics of adipocytes.

ADIPOCYTE DEVELOPMENT PROGRAM

Stem cell commitment to the adipocyte lineage and subsequent differentiation of preadipocytes into adipocytes occur throughout embryonic development and in adult animals. Extensive research of both multipotent and preadipocyte cell lines has identified several key events essential in adipocyte development. These events are summarized below.

Commitment

Commitment is the process by which a stem cell from the vascular stroma responds to signal(s) to undergo determination to the adipocyte lineage. Limited information is available about the commitment process of the adipocyte development program. Studies with 5-azacytidine in 10T1/2 cells enabled the Weintraub laboratory and others to discover and characterize a number of muscle-specific transcription factors that, when expressed in a multipotent cell line, commit those cells to the muscle lineage. An adipocyte-specific commitment factor has yet to be identified.

The factor or gene responsible for signaling the conversion of multipotent stem cells to the adipocyte lineage is unknown. The signal may be secreted by a mature adipocyte to recruit new cells to the adipocyte lineage. Evidence to support this theory comes from studies demonstrating that mature adipocytes secrete factors that promote preadipocyte proliferation and differentiation (Lau *et al.*, 1990; Considine *et al.*, 1996; Marques *et al.*, 1998). However, none of these factors has been identified. BMP4 has been shown to play a role in commitment *ex vivo*. 10T1/2 cells treated with BMP4 causes these cells to acquire the characteristics of preadipocytes at high frequency. Thus, with MDI treatment, the cells differentiate into adipocytes (Tang *et al.*, 2004a). This observation suggests that BMP4 commits the cells to preadipocytes that, once given the appropriate signals, can terminally differentiate. The BMP signaling pathway has been greatly studied in bone formation during development. It is known that the BMPs bind to a cell membrane surface receptor and activate the Smad signaling pathway (Shi & Massague, 2003). Smad proteins travel to the nucleus and activate the expression of genes that are responsible for the cartilage and bone phenotypes. Treatment of 10T1/2 cells rapidly activates the phosphorylation of Smad1 (Tang *et al.*, 2004a). Determining the transcription factor(s) targeted by the Smad pathway during 10T1/2 commitment could lead to the identification of the elusive adipocyte-lineage commitment gene.

Cell density and cell shape also appear to play a role in lineage commitment. In an elegant study by McBeath and coworkers (2004), human mesenchymal stem cells (hMSCs) plated at low density had higher potential to become osteoblasts, whereas cells plated at high density had a propensity to become adipocytes. Using micropatterned substrates as a means of controlling cell spreading, the researchers examined the significance of cell shape on commitment. Varying sizes of fibronectin "islands" were microcontact-printed onto polydimethylsiloxane substrates such that hMSCs attached as single cells on each island. Cells would only become adipocytes if they were plated on a small island of fibronectin, forcing them into an unspread, contracted morphology. On the other hand, cells would only become osteoblasts if they were plated on a large island of fibronectin, permitting them to flatten and spread. Further studies demonstrated a role for RhoA for mediating cell shape and commitment.

The Wnt signaling pathway is important in development and in the maintenance of adult tissues. Under normal conditions glycogen synthase kinase-3 (GSK-3) phosphorylates β-catenin, thereby promoting its proteosome-mediated degradation. Upon activation of the Wnt pathway, GSK-3 becomes inhibited, and β-catenin accumulates in the cytoplasm and nucleus. Once in the nucleus, β-catenin binds to TCF/lymphoid enhancer factor family of transcription factors and stimulates transcription of many genes (Logan & Nusse, 2004). A report by Ross and coworkers (2000) demonstrated that activation of the Wnt signaling pathway by expression of either Wnt-1 or a β-catenin mutant that increases β-catenin stability prevented adipogenesis by inhibiting expression of the adipogenic transcription factors CCAAT/enhancer binding protein (C/EBP)α and peroxisome proliferator-activated

receptor (PPAR) γ. Treatment with lithium, an inhibitor of GSK-3 which targets β-catenin for degradation, also blocked differentiation. Conversely, inhibition of the pathway in 3T3-L1 preadipocytes by ectopic expression of dnTCF4 resulted in spontaneous adipogenesis. 3T3-L1 preadipocytes are unable to differentiate into fat pads upon subcutaneous injection into athymic mice. These cells apparently cannot differentiate due to endogenous Wnt signaling, since dnTCF4 expression promoted formation of a fat pad. Wnt-10b is a likely candidate for signaling in preadipocytes as its expression is high in growing and confluent cells and decreases upon differentiation. Recently, Wnt10b has been implicated as a regulator of bone mass, promoting osteoblastogenesis and inhibiting adipogenesis (Bennett *et al.*, 2005).

Growth Arrest and Mitotic Clonal Expansion

In cell culture, preadipocytes become cell density-inhibited and arrest at the G_0/G_1 cell cycle boundary. Growth-arrest, and not cell-cell contact at confluence per se, is necessary for preadipocyte differentiation (Pairault & Green, 1979; Gregoire *et al.*, 1998). Growth-arrested cells receiving the appropriate combination of mitogenic and adipogenic signals will synchronously re-enter the cell cycle and undergo several rounds of cell division, referred to as mitotic clonal expansion, before exiting the cell cycle and terminally differentiating. It is believed that DNA replication and the changes in chromatin structure that occur during the clonal expansion process increase the accessibility of *cis*-elements to *trans*-acting factors, which activate or de-repress transcription of the gene(s) that gives rise to the adipocyte phenotype (MacDougald & Lane, 1995). Although the requirements of mitotic clonal expansion for adipogenesis have been controversial, preponderant evidence described below now shows that mitotic clonal expansion is a prerequisite for terminal differentiation. Numerous studies have demonstrated that inhibiting clonal expansion prevents adipogenesis. Blocking cell-cycle progression with rapamycin (Yeh *et al.*, 1995a), aphidicolin (Reichert & Eick, 1999), or roscovitine (Tang *et al.*, 2003b) disrupts differentiation. Similar results are achieved with the inhibition of calpain by the protease inhibitor *N*-acetyl-Leu-Leu-norleucinal, thereby preventing degradation of p27, a process required for entry of S-phase of the cell cycle (Patel & Lane, 2000). Conversely, blocking mitotic clonal expansion with the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD98059 (Qiu *et al.*, 2001; Liu *et al.*, 2002) or U0126 (Liu *et al.*, 2002) had no effect on adipogenesis. The latter results are in direct conflict to those obtained by Tang and colleagues (2003b), who found that only a delay of mitotic clonal expansion, and hence adipogenesis, was observed with PD98059. On the other hand, treatment with U0126 almost completely blocked the expression of cell-cycle markers and as a consequence prevented mitotic clonal expansion, the expression of adipocyte gene markers and the accumulation of cytoplasmic triacylglycerol (Tang *et al.*, 2003b). The discrepancy cited above could be due to drifting of the cell lines such that some cells have already traversed the stage of differentiation beyond mitotic clonal expansion (Fajas, 2003). However, the most compelling evidence that mitotic clonal expansion is required for subsequent differentiation derives from experiments in which disruption of signaling from C/EBPβ prevented mitotic clonal expansion and thereby adipogenesis (see below, *C/EBPβ and C/EBPδ* and Tang *et al.*, 2003a; Zhang *et al.*, 2004b).

Terminal Differentiation

As the cells complete clonal expansion, they enter a unique growth-arrested stage of the cell cycle, G_D, which is permissive for subsequent differentiation (Scott *et al.*, 1982). In 3T3-L1 preadipocytes, this process is characterized by changes in expression of cyclin-dependent kinase inhibitors p18, p21 and p27 (Morrison & Farmer, 1999). Even after differentiation has been initiated, the preadipocytes retain the ability to dedifferentiate and re-enter mitosis. However, once the cells have exited G_D they reach a poorlydefined point and become committed to terminal differentiation.

Terminal differentiation is maintained, at least in part, through the expression of C/EBPα and PPARγ. C/EBPα and PPARγ act synergistically to activate transcription of genes that produce the adipocyte phenotype (Figure 4) (Tontonoz *et al.*, 1994c). The promoter of the C/EBPα gene possesses a C/EBP regulatory element to which C/EBPα can bind and thereby autoactivate its expression and maintain the differentiated state (Christy *et al.*, 1991; Tang *et al.*, 2004b). C/EBPα also has anti-mitotic activity; its over-expression leads to cell cycle arrest (Umek *et al.*, 1991), which terminate mitotic clonal expansion. This anti-mitotic activity is through direct interaction with the cyclin-dependent

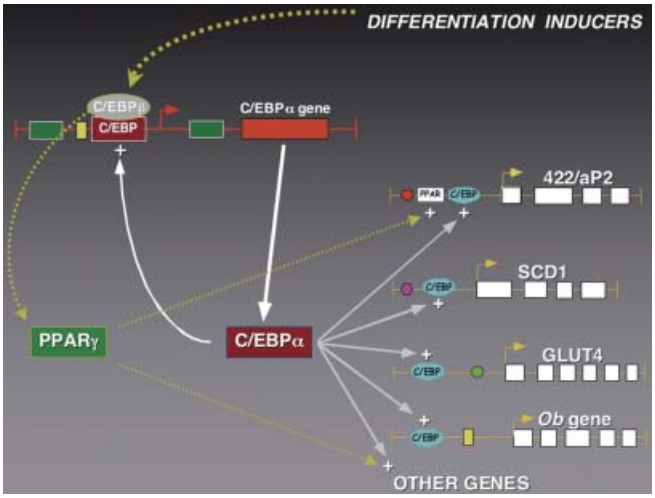


FIGURE 4 **Cascade of transcription factors leading to the pleiotropic activation of adipocyte genes. C/EBP = CCAAT/enhancer binding protein; PPAR = peroxisome proliferator-activated receptor; 422/aP2 = adipocyte protein 2, a fatty acid binding protein; SCD = stearoyl-CoA desaturase; GLUT4 = glucose transporter-4, the insulin-responsive glucose transporter; and Ob = Obese gene, the gene that encodes leptin.**

kinases cdk2 and cdk4 (Wang *et al.*, 2001). Like C/EBPα, PPARγ has been observed to induce growth arrest in certain cells. PPARγ expression inhibits E2F/DP-1 DNA-binding activity through decreased PP2A phosphatase levels, thereby increasing phosphorylation of E2F and leading to a withdrawal from the cell cycle (Altiok *et al.*, 1997).

TRANSCRIPTIONAL REGULATION OF ADIPOGENESIS

After treatment of preadipocytes with a cocktail of differentiation inducers (*i.e.*, MDI), the cells synchronously re-enter the cell cycle and begin to express a cascade of transcription factors that ultimately produce the adipocyte phenotype. The 3T3-L1 and -F442A cell lines have been extensively characterized so that a detailed picture of adipocyte differentiation is now well understood. This review will be limited to a discussion of the key trans-acting factors that regulate adipogenesis.

CCAAT/Enhancer Binding Proteins (C/EBP)

C/EBPα, the first protein to be identified in this family, was purified from rat liver nuclei and characterized for its ability to bind to the CCAAT motif found in several gene promoters as well as "core homology" sequences present in certain viral enhancers (Graves *et al.*, 1986; Johnson *et al.*, 1987; Landschulz *et al.*, 1988). To date, five other C/EBP isoforms have been cloned and sequenced: C/EBPβ (Akira *et al.*, 1990; Chang *et al.*, 1990; Descombes *et al.*, 1990; Poli *et al.*, 1990; Cao *et al.*, 1991; Williams *et al.*, 1991); C/EBPγ (Roman *et al.*, 1990); C/EBPδ (Cao *et al.*, 1991; Kageyama *et al.*, 1991; Williams *et al.*, 1991); C/EBPε (Williams *et al.*, 1991); and C/EBPζ (CHOP-10) (Luethy *et al.*, 1990; Ron & Habener, 1992). These proteins control a number of activities in a variety of tissues, including cell growth and differentiation and immune processes. Thus far, only C/EBPα, -β, -δ and CHOP-10 have been shown to be involved in adipogenesis.

All C/EBP family members contain a transactivation domain, DNA-binding basic region and a leucine-rich dimerization domain. The highly conserved C-terminal basic region/leucine zipper (bZIP) domain confers the ability to bind DNA and to form homo- or heterodimers with other C/EBP family members. Dimerization is a prerequisite to DNA-binding (Landschulz *et al.*, 1989). CHOP-10 has a non-functional DNA-binding domain so heterodimerization with this isoform acts as a dominant-negative of C/EBP function (Ron & Habener, 1992).

C/EBPα and C/EBPβ can produce N-terminally truncated polypeptides either by alternative translation through leaky ribosome scanning (Descombes & Schibler, 1991; Lin *et al.*, 1993; Ossipow *et al.*, 1993) or by regulated proteolysis (Welm *et al.*, 1999). C/EBPα mRNA yields a 42 kDa and 30 kDa protein. The 30 kDa protein is a less potent activator than the 42 kDa protein and lacks anti-mitotic activity (Lin *et al.*, 1993; Ossipow *et al.*, 1993). C/EBPβ mRNA is translated into at least three isoforms, a full length 38 kDa, a 35 kDa (LAP) and a 20 kDa (LIP) protein. LIP lacks the N-terminal transactivation domain and thus has been proposed to function as a dominant-negative regulator of C/EBP-induced transcription (Descombes & Schibler, 1991).

C/EBPβ and C/EBPδ

C/EBPβ and C/EBPδ are early regulators of preadipocyte differentiation. These isoforms are rapidly induced and maximal protein levels are detected within 4 hours of induction of differentiation (Tang & Lane, 1999). Their importance in adipogenesis is evidenced by the fact that over-expression of either protein in 3T3-L1 preadipocytes accelerates adipogenesis. Ectopic expression of C/EBPβ, but not C/EBPδ, can stimulate

differentiation in the absence of hormone inducers. C/EBPβ can also promote adipogenesis to a limited extent in the multipotent NIH-3T3 cell line (Yeh *et al.*, 1995b). Although they are expressed immediately after induction of differentiation, C/EBPβ and C/EBPδ are unable to bind DNA until much later in the program. Acquisition of DNA-binding occurs between 12 and 16 hours, coincident with entry into S-phase in mitotic clonal expansion. Concomitant with acquisition of DNA-binding function, C/EBPβ and C/EBPδ become localized to centromeres through C/EBP consensus binding sites in centromeric satellite DNA (Tang & Lane, 1999).

While C/EBPβ expression in cell culture is important in preadipocyte differentiation, C/EBPβ(−/−) mice have normal epidydimal adipose tissue (Tanaka *et al.*, 1997). Apparently C/EBPβ function is not required for differentiation *in vivo*. A similar result was observed in C/EBPδ (−/−) mice (Tanaka *et al.*, 1997). However, epidydimal tissue mass in C/EBPβ(−/−)-C/EBPδ(−/−) mice was markedly reduced compared to wild type animals. Despite this, adipocytes in the epidydimal tissue were normal in both size and morphology (Tanaka *et al.*, 1997). Therefore, the decrease in adipose tissue mass was due to a decrease in cell number, suggesting that C/EBPβ and C/EBPδ function in the increase in the number of adipocytes (Tanaka *et al.*, 1997).

Transcriptional activation of C/EBPβ is controlled by cAMP response element binding protein (CREB). C/EBPβ contains two cAMP responsive element-like *cis* regulatory sequences in the proximal promoter, and these sites are necessary in maintaining transcriptional activation (Niehof *et al.*, 1997; Zhang *et al.*, 2004a). Characterization of CREB in the early phase of differentiation revealed that CREB phosphorylation and hence its activation correlated with C/EBPβ expression. Chromatin immunoprecipitation experiments demonstrated that phospho-CREB binds to the proximal promoter of C/EBPβ as early as one hour after induction of differentiation in 3T3-L1 preadipocytes. C/EBPβ expression and accumulation of cytoplasmic triacylglyceride were markedly reduced in CREB(−/−) mouse embryonic fibroblasts (MEFs). It is likely that other CREB family members may partially compensate for the loss of CREB protein (Zhang *et al.*, 2004a). Phosphorylation of CREB is known to be activated by cAMP (Gonzalez & Montminy, 1989) and insulin (Klemm *et al.*, 1998), two inducers of preadipocyte differentiation.

Mitotic clonal expansion is dependent on C/EBPβ as this process was blocked in C/EBPβ(−/−) MEFs (Tang *et al.*, 2003a). Other evidence to support a role for C/EBPβ in clonal expansion comes from the use of A-C/EBP. A-C/EBP contains a leucine zipper but lacks the functional DNA-binding and transactivation domains and thus acts as a dominant-negative of C/EBP proteins. Expression of A-C/EBP disrupted both mitotic clonal expansion and differentiation in 3T3-L1 cells (Zhang *et al.*, 2004b). Examination of cell cycle proteins during the clonal expansion process showed that the degradation of p27 was blocked with expression of A-C/EBP. Degradation of p27 is necessary for cells to enter mitotic clonal expansion (Patel & Lane, 2000). A-C/EBP prevented entry of C/EBPβ into the nucleus as observed by immunofluorescence. Presumably, the acidic region of A-C/EBP heterodimerized with the bZIP region of C/EBPβ and formed a stable coiled-coil extension of the leucine zipper, thereby sequestering the nuclear localization signal.

A number of kinases can phosphorylate C/EBPβ *ex vivo* including protein kinase A (Trautwein *et al.*, 1994), protein kinase C (Trautwein *et al.*, 1994), MAP kinase (Nakajima *et al.*, 1993), Ca^{2+}-calmodulin-dependent kinase II (Wegner *et al.*, 1992), cdk2, (Shuman *et al.*, 2004), and GSK-3 (Piwien-Pilipuk *et al.*, 2001; Tang *et al.*, 2005). Phosphorylation is an important post-translational modification of C/EBPβ that leads to the acquisition of DNA-binding function as preadipocytes traverse to the G_1-S check-point at the onset of mitotic clonal expansion (Tang *et al.*, 2005). C/EBPβ is phosphorylated sequentially, first by MAP kinase and then much later by GSK-3β. Phosphorylation on Thr188 by MAP kinase occurs within 4 hours of induction of differentiation and is required for mitotic clonal expansion, C/EBPβ DNA-binding activity and terminal differentiation. Phosphorylation on Thr179 and Ser184 by GSK-3β occurs between 12 to 16 hours after induction. Hyperphosphorylation of C/EBPβ at these three sites activates its DNA-binding activity, and although the mechanism by which this occurs is not understood, it is likely that a phosphorylation-induced conformational change is involved.

C/EBPα

C/EBPα is a pleiotropic transcriptional activator of adipocyte-specific genes. Promoters from numerous adipocyte genes contain C/EBP regulatory consensus

sequences and are trans-activated by C/EBPα (Figure 4) (MacDougald and Lane, 1995). Forced expression of this isoform in 3T3-L1 preadipocytes stimulates adipogenesis in the absence of any hormonal induction (Freytag & Geddes, 1992; Freytag *et al.*, 1994; Lin & Lane, 1994). In addition, blocking its expression with antisense C/EBPα RNA inhibits adipogenesis (Lin & Lane, 1992). Furthermore, C/EBPα(−/−) mice have defects in lipid accumulation (Wang *et al.*, 1995; Flodby *et al.*, 1996). These observations establish the requirement for C/EBPα in preadipocyte differentiation.

Initially, C/EBPα is transcriptionally activated by C/EBPβ and C/EBPδ via a C/EBP regulatory element in the proximal promoter. Once C/EBPα is expressed, it appears the expression is maintained through auto-activation (Figure 4) (Christy *et al.*, 1991). Alternatively, C/EBPα stimulates USF binding at a consensus sequence within the C/EBPα promoter to auto-activate its expression (Timchenko *et al.*, 1995). Why does there exist such a redundancy in the expression of the C/EBP gene family in adipogenesis? This may be due to the fact that C/EBPα has anti-mitotic activity. Expression of C/EBPα early during adipogenesis would prevent preadipocytes from entering mitotic clonal expansion, a requirement for subsequent differentiation. Therefore, C/EBPα must be repressed until the opportune time window. Several mechanisms exist to delay the expression of C/EBPα, including binding of an AP-2α isoform (Jiang *et al.*, 1998) and Sp1 (Tang *et al.*, 1999), both of which have repressive effects on the C/EBPα gene promoter, as well as the delay in acquisition of DNA-binding of C/EBPβ and C/EBPδ (see *C/EBPβ and C/EBPδ*).

Phosphorylation of C/EBPα occurs at three sites in 3T3-L1 adipocytes. Two of these sites, Thr222 and Thr226, are insulin-sensitive sites phosphorylated by GSK-3. The third site, Ser230, is phosphorylated by an unidentified kinase. Thr222 and Thr226 become de-phosphorylated in response to insulin treatment through inactivation of GSK-3 (Ross *et al.*, 1999). Insulin has also been reported to decrease C/EBPα mRNA and protein levels (MacDougald *et al.*, 1995). Although the phosphorylation status of these sites does not affect transactivation by C/EBPα, they do appear to alter the conformation of the protein. Lithium treatment of 3T3-L1 preadipocytes blocks differentiation, implying that phosphorylation of C/EBPα and other proteins by GSK-3 is required for adipogenesis (Ross *et al.*, 1999).

CHOP-10

CHOP-10 was originally identified in a Chinese hamster ovary cell library while investigators were screening for genes activated upon growth arrest or DNA damage (Fornace *et al.*, 1989) and later was cloned from a 3T3-L1 adipocyte library based on its ability to interact with the C/EBPβ bZIP domain (Ron & Habener, 1992). Although a C/EBP family member, CHOP-10 is unique in that proline and glycine residues in the DNA-binding region interfere with its ability to bind DNA but do not alter its capacity to form heterodimers. Thus, CHOP-10 acts as a dominant-negative isoform (Ron & Habener, 1992). Indeed, ectopic expression of CHOP-10 blocks adipogenesis in 3T3-L1 cells (Batchvarova *et al.*, 1995). CHOP-10 is expressed by growth-arrested preadipocytes and is down-regulated with kinetics consistent with the centromeric localization of C/EBPβ. During preadipocyte differentiation, CHOP-10 heterodimerizes with C/EBPβ and prevents acquisition of DNA-binding to C/EBP regulatory elements (Tang & Lane, 2000). It appears that the interaction of CHOP-10 with C/EBPβ provides an additional "fail-safe" mechanism that prevents the acquisition of DNA-binding activity by C/EBPβ until preadipocytes have entered mitotic clonal expansion (see *C/EBPβ and C/EBPδ*). Even though CHOP-10/C/EBP heterodimers cannot bind traditional C/EBP sites, the complex can interact with a novel DNA sequence suggesting that CHOP-10 may redirect the binding of C/EBPs to other promoter elements (Ubeda *et al.*, 1996).

Peroxisome Proliferator-Activated Receptors (PPAR)

PPARs are members of the nuclear hormone receptor family which includes retinoic acid receptors, thyroid hormone receptors and vitamin D_3 receptors (Aranda & Pascual, 2001). As with most nuclear hormone receptors, PPARs are activated by ligands to control gene expression. The first PPAR to be discovered, PPARα, was cloned as a receptor activated by the lipid lowering drug clofibrate, which induced proliferation of peroxisomes in rodents (Issemann & Green, 1990). Since its discovery, two other members have been identified: PPARγ and PPARδ. Unlike PPARα, however, these receptors do not induce peroxisome proliferation (Francis *et al.*, 2003).

PPAR family members consist of a ligand-independent transactivation domain, DNA binding domain, and a ligand binding and dimerization domain with a ligand-dependent transactivation domain. PPAR must heterodimerize with the retinoid X receptor prior to binding DNA at the peroxisome proliferator response element in the promoter of target genes (Aranda & Pascual, 2001). The heterodimer binds to a direct repeat of the hormone response element half-site separated by one nucleotide (DR-1) (Mangelsdorf & Evans, 1995). Transcriptional activation by PPAR can be controlled through interactions with co-activators and co-repressors (Francis *et al.*, 2003). PPARγ exists as three isoforms, designated PPARγ_1, PPARγ_2 and PPARγ_3, which are transcribed from the same gene through alternative splicing and promoter use (Zhu *et al.*, 1995; Fajas *et al.*, 1998). PPARγ_1 and PPARγ_3 transcription products give rise to the same protein, while PPARγ_2 has a 30-amino-acid extension at its N-terminus. PPARγ_1 is widely expressed, including in adipose tissue, whereas PPARγ_3 has been found in adipose tissue, large intestine and macrophages. PPARγ_2 is the adipocyte-specific isoform (Tontonoz *et al.*, 1994b; Braissant *et al.*, 1996; Fajas *et al.*, 1998; Ricote *et al.*, 1998).

PPARγ

PPARγ is a central regulator during adipogenesis. The transcription factor is induced during differentiation and is responsible for activating a number of genes involved in fatty acid binding, uptake and storage, including 422/aP2 (Tontonoz *et al.*, 1994a; Tontonoz *et al.*, 1994b), lipoprotein lipase (Schoonjans *et al.*, 1996), acyl coenzyme A synthase (Schoonjans *et al.*, 1995) and phosphoenolpyruvate carboxykinase (Tontonoz *et al.*, 1994b). The importance of PPARγ in adipogenesis has been demonstrated in several ways. Over-expression of PPARγ in non-adipogenic fibroblasts stimulates adipogenesis (Tontonoz *et al.*, 1994c). PPARγ (−/−) mice die in utero secondary to a placental defect, but those that survive to term through tetraploid rescue are deficient in brown fat (Barak *et al.*, 1999). PPARγ (+/−) mice are resistant to diet-induced obesity and are more insulin-sensitive (Kubota *et al.*, 1999). To demonstrate that PPARγ expression is essential for white adipose tissue development, Rosen and colleagues (1999) designed a chimeric mouse composed of wild-type and PPARγ(−/−) embryonic stem cells. The development of white adipose tissue in these chimeric mice had a strong preference toward PPARγ expression.

Since both PPARγ_1 and PPARγ_2 are expressed at similar levels in adipocytes, it has been difficult to assign a specific role for each isoform during differentiation. To address this issue, Ren and coworkers (2002) generated 3T3-L1 preadipocytes that expressed neither PPARγ_1 nor PPARγ_2 by targeting zinc finger repressor proteins to specific regions in the PPARγ gene. Ectopic expression of PPARγ_2, and not PPARγ_1, was able to restore adipogenesis. Hence, PPARγ_2 has a unique role in the adipogenic program.

Natural ligands for PPARγ include fatty acids and fatty acid derivatives. The prostaglandin, 15-deoxy-delta12,14-prostaglandin J_2, is a potent activator of PPARγ (Forman *et al.*, 1995; Kliewer *et al.*, 1995). Thiazolidinediones are high-affinity synthetic ligands for PPARγ and are currently used to manage type 2 diabetes (Lehmann *et al.*, 1995). However, the endogenous ligand is currently unknown. Adipocyte determination and differentiation-dependent factor 1/sterol regulatory element binding protein-1c (ADD1/SREBP-1c) is implicated in stimulating endogenous PPARγ ligand production (Kim *et al.*, 1998b).

PPARγ is transcriptionally activated by C/EBPβ and C/EBPδ (Wu *et al.*, 1995), and once expressed, PPARγ and C/EBPα positively regulate each other's expression (Schwarz *et al.*, 1997; Shao & Lazar, 1997). There has been some debate in the past as to the specific roles of PPARγ and C/EBPα during adipogenesis. Established MEFs from C/EBPα(−/−) mice express low levels of PPARγ and have reduced ability to undergo adipogenesis. Ectopic expression of PPARγ in these cells can induce adipogenesis, but these cells are not insulin-sensitive (Wu *et al.*, 1999). However, over-expression of C/EBPα in PPARγ(−/−) fibroblasts is unable to stimulate adipogenesis (Rosen *et al.*, 2002). Although C/EBPα is required for differentiation, it appears that PPARγ is the central regulator of adipogenesis. Clearly there are genes that only C/EBPα can transcriptionally activate, but it is more likely to be in involved in insulin sensitivity and induction/maintenance of PPARγ. Indeed, C/EBPα can transcriptionally activate the PPARγ promoter in 3T3-L1 preadipocytes (Elberg *et al.*, 2000).

Sterol Regulatory Element Binding Proteins (SREBP)

SREBPs are basic helix-loop-helix-leucine zipper transcription factors which regulate the transcription of many genes that are important in cholesterol and

fatty acid metabolism (Horton *et al.*, 2002). Three family members have been identified: SREBP-1a, SREBP-1c and SREBP-2. Adipocyte determination and differentiation-dependent factor 1 (ADD1) was cloned from a rat adipocyte cDNA library and is homologous to human SREBP-1c (Tontonoz *et al.*, 1993). SREBP-1a and ADD1/SREBP-1c are derived from the same gene through alternative promoter usage, whereas SREBP-2 is produced from a separate gene (Hua *et al.*, 1995). SREBPs are expressed as precursor proteins that remain membrane-bound in the endoplasmic reticulum (ER). Lowered cholesterol content in the ER facilitates release of SREBP-2 for translocation to the Golgi. Proteolytic cleavage in the Golgi releases SREBP into the cytosol where it can enter the nucleus to bind target genes through the sterol response elements (for review, see Horton *et al.*, 2002). ADD1/SREBP-1c can also recognize an E-box motif (Yokoyama *et al.*, 1993; Kim *et al.*, 1995). SREBP-1a is a strong activator of all SREBP-responsive genes. SREBP-1c activates transcription of genes required for fatty acid synthesis, whereas SREBP-2 enhances cholesterol synthesis.

ADD1/SREBP-1c

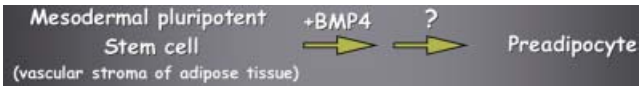
ADD1/SREBP-1c is responsible for the transactivation of lipogenic genes that encode enzymes of fatty acid synthesis, desaturation and uptake, as well as triacylglycerol synthesis. The mRNA levels of ADD1/SREBP-1c increase within the first 24 hours after induction of differentiation (Kim & Spiegelman, 1996), indicating its importance early during adipogenesis. Indeed, over-expression of ADD1/SREBP-1c in NIH-3T3 cells stimulates adipogenesis but only in the presence of PPARγ activators (Kim & Spiegelman, 1996). It is likely that SREBP-2 can compensate for the loss of ADD1/SREBP-1c since ADD1/SREBP-1c(−/−) mice have normal adipose depots (Shimano *et al.*, 1997).

As mentioned earlier, ADD1/SREBP-1c has been implicated in the transcriptional activation of PPARγ (Kim *et al.*, 1998b). This activation is specific for the PPARγ isoform and appears to be limited to the ligand-binding domain. Interestingly, conditioned media from the ADD1/SREBP-1c expressing cells can increase PPARγ transcriptional activity. This observation suggests that ADD1/SREBP-1c expression promotes synthesis/secretion of a natural PPARγ ligand. ADD1/SREBP-1c may activate transcription of PPARγ since over-expression of ADD1/SREBP-1c in 3T3-L1 cells induces PPARγ mRNA levels (Fajas *et al.*, 1999). Whether this activation is direct or indirect is unknown.

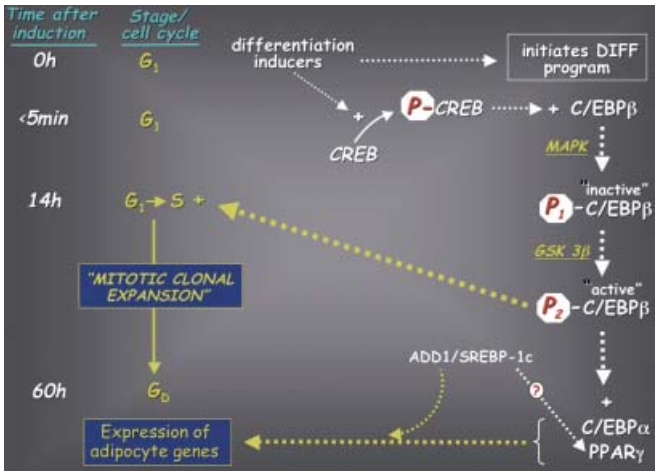
The insulin/glucagon ratio plays a role in ADD1/SREBP-1c mRNA levels. Insulin stimulates ADD1/SREBP-1c mRNA production in isolated rat hepatocytes, as well as the mRNAs of its target genes (Foretz *et al.*, 1999a; Shimomura *et al.*, 1999). Expression of a dominant-negative ADD1/SREBP-1c blocks the induction of target genes (Foretz *et al.*, 1999b). In contrast, primary hepatocytes incubated with glucagon decreases mRNA for ADD1/SREBP-1c and its target genes. Similar results have been observed *in vivo*. For instance, fasting mice lowers insulin and raises glucagon levels. These animals have reduced ADD1/SREBP-1c mRNA levels and target genes (Horton *et al.*, 1998; Kim *et al.*, 1998a). Given the precedent for the control of processing/activation of SREBP-2 by cholesterol, it is likely that a similar activation mechanism exists for ADD1/SREBP-1c related to energy status.

OVERVIEW OF ADIPOSE DEVELOPMENT

The commitment of stem cells to the adipose lineage and subsequent differentiation of preadipocytes to adipocytes occurs throughout life. Most of our current knowledge of adipose development has been discovered using both commitment and differentiation cell culture models. In culture, BMP4 commits mesenchymal stem cells to the adipocyte lineage (Figure 5A). Quiescent preadipocytes given the appropriate hormone inducers begin the differentiation program (Figure 5B). This process is characterized by a rapid increase in phosphorylation of CREB. CREB, in turn, transcriptionally activates C/EBPβ through cAMP responsive element-like *cis* regulatory elements in the proximal promoter. C/EBPβ does not have DNA-binding activity at this time. Sometime thereafter, the preadipocytes re-enter the cell cycle to complete two rounds of division, referred to as mitotic clonal expansion. Coincident with entry into S phase, C/EBPβ is phosphorylated by MAP kinase and later by GSK-3β. Phosphorylation leads to the acquisition of DNA-binding activity by C/EBPβ. C/EBPβ can now transcriptionally activate the two key adipogenic transcription factors, C/EBPα and PPARγ. These factors, along with ADD1/SREBP-1c, coordinately activate the genes responsible for maintaining the adipocyte phenotype. ADD1/SREBP-1c can activate



(A)



(B)

FIGURE 5 Key events in the pathway of adipocyte development. A. Stem cell commitment to the adipocyte lineage. BMP = bone morphogenetic protein. B. The adipocyte differentiation activation cascade. P = phosphoryl group; C/EBP = CCAAT/enhancer binding protein; CREB = cAMP regulatory element binding protein; GSK-3β = glycogen synthase kinase-3β; MAPK = mitogen activated protein kinase; PPAR = peroxisome proliferator-activated receptor; SREBP = sterol regulatory element binding protein-1; G_1 and S = G_1- and S-phases of the cell cycle, respectively.

expression of PPARγ, but its significance during differentiation is unclear.

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