Adipogenesis: From Stem Cell to Adipocyte

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Abstract
Excessive caloric intake without a rise in energy expenditure promotes adipocyte hyperplasia and adiposity. The rise in adipocyte number is triggered by signaling factors that induce conversion of mesenchymal stem cells (MSCs) to preadipocytes that differentiate into adipocytes. MSCs, which are recruited from the vascular stroma of adipose tissue, provide an unlimited supply of adipocyte precursors. Members of the BMP and Wnt families are key mediators of stem cell commitment to produce preadipocytes. Following commitment, exposure of growth-arrested preadipocytes to differentiation inducers [insulin-like growth factor 1 (IGF1), glucocorticoid, and cyclic AMP (cAMP)] triggers DNA replication and reentry into the cell cycle (mitotic clonal expansion). Mitotic clonal expansion involves a transcription factor cascade, followed by the expression of adipocyte genes. Critical to these events are phosphorylations of the transcription factor CCATT enhancer-binding protein β (C/EBPβ) by MAP kinase and GSK3β to produce a conformational change that gives rise to DNA-binding activity. “Activated” C/EBPβ then triggers transcription of peroxisome proliferator–activated receptor-γ (PPARγ) and C/EBPx, which in turn coordinately activate genes whose expression produces the adipocyte phenotype.
INTRODUCTION

Animals possess highly integrated systems to regulate energy storage and expenditure. These systems have evolved to promote energy storage during periods of food surplus and mobilization of these stores when food is scarce (1–4). Such systems are regulated both at the cellular level and in the whole organism by the coordinated actions of circulating hormones and efferent neural signals from the central nervous system both to higher brain centers and to peripheral tissues, including the liver, muscle, and adipose tissue. When food is abundant, carbohydrates are stored as small reserves of glycogen in the liver and as a much larger reserve as fat—primarily in adipocytes. When food is limited, these stores are mobilized to meet energy needs (1, 2). Despite these control mechanisms to survive feast-or-famine situations, lifestyles in developed societies have led to excessive consumption of energy-rich foods and sedentary behavior. As a consequence obesity and its accompanying pathological consequences, most notably insulin resistance, type 2 diabetes, and heart disease, have become serious medical problems (5–8).

Excessive caloric intake relative to expenditure produces a metabolic state that promotes hyperplasia and hypertrophy of adipocytes (5). Hyperplasia involves stem cell recruitment to the adipocyte lineage and a consequent increase in adipocyte number. Here, we review both the mechanisms by which pluripotent mesenchymal stem cells (MSCs) undergo commitment to become preadipocytes and the program by which preadipocytes differentiate into adipocytes. Together, these processes are responsible for the increase in adiposity produced by excessive energy intake, which is accompanied by low energy expenditure. The reader is referred to several recent reviews that deal with related aspects of this topic (2, 6–9).

THE ADIPOCYTE

The mature adipocyte contains a single large fat droplet surrounded by a thin rim of cytoplasm that lies between the droplet and the plasma membrane. When triggered by lipolytic hormones, cytoplasmic hormone-sensitive lipase (10) and adipocyte-triglyceride lipase (11, 12) translocate to the surface of the fat droplet, where lipolysis of triglyceride occurs (13). Also bound to the surface of the fat droplet are accessory proteins, such as perilipin (14), that facilitate this process. Thus, fatty acids derived from triglyceride are released into the bloodstream to supply peripheral tissues, especially those of the skeletal and heart muscle and the liver, with an energy-rich fuel. During periods of excess caloric intake, lipogenic enzymes, localized in the cytoplasm and endoplasmic reticulum (ER), synthesize triglyceride, which is incorporated into the fat droplet. As discussed in the section Differentiation: Preadipocyte to Adipocyte, see below, the genes encoding these
and other adipocyte proteins are coordinately expressed during preadipocyte differentiation to produce the adipocyte phenotype.

Two technical approaches have been of paramount importance in advancing progress in studies on the metabolism and differentiation with adipocytes and their progenitors. The use of collagenase dissociation to isolate functional adipocytes from adipose tissue (15–17) made possible the biochemical analysis of adipocyte function. Using isolated adipocytes, it was shown that these cells are exquisitely responsive to hormones, such as insulin, that promote glucose uptake and lipogenesis as well as to adrenergic agents, such as epinephrine and cyclic AMP (cAMP), that promote triglyceride mobilization (5, 15, 17). Studies on gene expression/regulation and differentiation, however, had to await the development of clonal preadipocyte cell lines that could be differentiated into adipocytes in cell culture (18–20). Established cell lines, e.g., the 3T3-L1 preadipocyte line, that can be cultured indefinitely have served as faithful model systems for characterizing the differentiation program.

Recent studies have revealed that adipocytes also have endocrine functions and secrete hormones and cytokines that play key roles in global energy metabolism. Certain of these hormones act locally as paracrine factors, and others, such as leptin (21–25) and adiponectin (26–28), have long-range effects and act on the feeding centers of the central nervous system/brain, notably the hypothalamus. Leptin, which is expressed and secreted by adipocytes in proportion to adipose tissue mass, is anorectic and acts to limit energy storage when adipose tissue reserves have been filled (29). Leptin interacts with specific receptors in the hypothalamus (30) to reduce food intake. In the obese state, however, resistance to leptin occurs, limiting its effectiveness. Adipose tissue is also under the control of the central nervous system because it is innervated by neurons of the sympathetic nervous system that secrete adrenergic hormones (epinephrine/norepinephrine) that promote fat mobilization (17).

Origin of Adipocytes
Adipocytes are derived from pluripotent MSCs that have the capacity to develop into several cell types, i.e., adipocytes, myocytes, chondrocytes, and osteocytes (7, 31, 32). These stem cells reside in the vascular stroma of adipose tissue as well as in the bone marrow, and when appropriately stimulated undergo a multistep process of commitment in which the progenitor cells become restricted to the adipocyte lineage. Recruitment to this lineage gives rise to preadipocytes, which, when induced, undergo multiple rounds of mitosis (mitotic clonal expansion) and then differentiate into adipocytes. Model cell culture systems (discussed below) have been indispensable in identifying/characterizing the steps in the commitment and differentiation programs.

Pluripotent Stem Cell Lines
Several cell lines faithfully mimic the functional characteristics of the mesenchymal pluripotent cell type in vivo and can be induced to undergo commitment to the adipose, muscle, cartilage, and bone lineages. The best characterized of these pluripotent lines is the CH310T1/2 line established in 1973 in the Heidelberger laboratory at the University of Wisconsin (33). In addition, embryonic stem cell knockdowns and mouse knockouts have proven useful (32, 34). The adipocyte commitment process can be initiated by factors such as BMP4 or BMP2 (bone morphogenetic proteins) (35) or factors in the downstream signaling pathway (36).

Preadipocyte Cell Lines
Preadipocyte lines that represent the next step (differentiation) in adipocyte development were first established in Green’s laboratory (18, 19, 37). Notable among these is the 3T3-L1 preadipocyte line, which has become the “gold standard” for investigating preadipocyte differentiation. This line (along with the 3T3-F442A line) faithfully recapitulates the steps in adipocyte differentiation. Induction of
Adipogenesis: the processes by which mesenchymal stem cells commit to the adipose lineage and differentiate into adipocytes

differentiation is triggered with a “cocktail” of agents to initiate the cascade of synchronous steps in the differentiation process.

Mesenchymal Stem Cells

Pluripotent MSCs derived from the embryonic mesoderm (7, 31) have the capacity to differentiate into adipocytes and have been used to verify many findings made with the established 3T3-L1 preadipocyte line (38). It was definitively shown that a subpopulation of MSCs is pluripotent and capable of committing to the adipocyte lineage (36, 38).

COMMITMENT: PLURIPOTENT STEM CELL TO PREADIPOCYTE

The vascular stroma of adipose tissue possesses a resident population of pluripotent MSCs that has the potential to undergo commitment and differentiation into adipocytes, chondrocytes, myocytes, and osteocytes (7, 31, 35, 39). Use of these cells for genetic studies is limited, however, because of their short lifetime in culture. Pluripotent C3H10T1/2 cells in culture have served as a faithful MSC model for long-term genetic studies of the adipocyte developmental program. When appropriately induced, C3H10T1/2 stem cells can be prompted to commit and differentiate into cells of the adipocyte, myocyte, chondrocyte, or osteocyte lineages.

Recruitment to the adipocyte lineage in vivo is prompted by excessive energy intake and elevated glucose uptake (5) over an extended time period. This metabolic state appears to generate a signal (or signals), yet to be identified, that induces MSCs to enter the commitment pathway leading to hyperplasia and the preadipocyte phenotype. Several factors have been identified that commit or inhibit the conversion of pluripotent stem cells to the adipocyte lineage. These include BMP family members BMP4 and BMP2 (6, 35), Wnt (40, 41), and Hh (hedgehog) (42–44). BMP4 and BMP2 have an activating role, whereas Hh signaling has an inhibitory role, and Wnt appears to have both an activating role in commitment (41) and an inhibitory role in adipocyte differentiation, see below (45).

Mounting evidence indicates that lineage determination is regulated by a network of extracellular signaling factors that ultimately impinge on the promoters of lineage-specific transcription factors. It is the balance of these signaling molecules that determines the developmental pathway, often simultaneously promoting one pathway while inhibiting another. For example, Wnt10b promotes osteogenesis and possibly myogenesis, and inhibits adipogenesis (46); BMP4 promotes adipogenesis while inhibiting myogenesis (45). Conversely, peroxisome proliferator–activated receptor-γ (PPARγ) inhibits chondrogenesis and stimulates adipogenesis, whereas Msx2 stimulates osteogenesis while inhibiting adipogenesis (47, 48) by inhibiting the transcriptional activity of PPARγ.

Bone Morphogenetic Protein Signaling

BMP4 and BMP2 have been implicated in the commitment of pluripotent stem cells to the adipocyte lineage (32, 35, 36, 41, 49–52). Thus, exposure of dividing C3H10T1/2 stem cells to either BMP4 or BMP2 gives rise to preadipocyte-like cells which, when treated at growth arrest with differentiation inducers, enter the adipose development pathway, express adipocyte markers, and acquire the adipocyte phenotype (6, 32, 35).

The role for BMP4 in the commitment process has been validated using another approach, clonal selection after blocking DNA methylation (6). Thus, exposure of proliferating C3H10T1/2 stem cells to 5-azacytidine, an inhibitor of DNA methylation, generated clonal subpopulations of cells, e.g., the A33 line, that converted into adipocytes when exposed to differentiation inducers in the absence of exogenous BMP4 (6). Remarkably, A33 cells express and secrete BMP4 in the same proliferation time window at which exogenous BMP4 must be added to induce adipocyte commitment of “naive” C3H10T1/2 cells.
Moreover, exposure of A33 cells to the naturally occurring BMP4-binding antagonist, noggin, during this critical time window blocked conversion/differentiation into adipocytes (Figure 1). The role of BMP4 in adipocyte lineage commitment is further supported by the expression in proliferating C3H10T1/2 stem cells and A33 preadipocytes of genes and proteins that are known to be involved in the BMP signaling pathway (6, 35, 36). These include BMP4; the BMP receptors, BMPR2 and BMPR1α; and Smad-1, -5, -8. BMPs are known to signal through two receptor types, BMPR1 and BMPR2, which form cell-surface complexes with serine/threonine kinase activity (53, 54). Binding of BMP to the BMPR1:BMPR2 complex induces phosphorylation and, thus, activation of the BMPR1 kinase. The BMP receptor phosphorylates Smad-1,-5,-8, which forms a complex with Smad4 that translocates into the nucleus and regulates gene expression (32). Furthermore, overexpression of a constitutively active (CA) BMP receptor, CA-BMPR1A or CA-BMPR1B, induces commitment in the absence of BMP2 or BMP4, whereas overexpression of a dominant-negative receptor, dominant-negative-BMPR1A, suppresses commitment induced by BMP. Also, knockdown of the expression of Smad4 (a coregulator in the BMP/Smad signaling pathway) with RNAi disrupts commitment by the BMPs (see Figure 2).

**BMP promoter switch during commitment.**

The mouse BMP4 gene is known to have two promoters that are differentially regulated during development (55, 56). Analysis of RNA early in the commitment program revealed that promoter 1 is used to drive expression of BMP4 in A33 cells but not in C3H10T1/2 cells (6). On day seven when BMP4 synthesis decreases, use of promoter 1 and expression of BMP4 ceases. In contrast, mRNA from uncommitted C3H10T1/2 cells showed that promoter 2 was used exclusively to drive expression of the BMP4 gene (6). These findings suggest that commitment to the adipocyte lineage induced by 5-azacytidine altered the methylation status of the BMP4 gene, rendering promoter 1 more accessible for transcriptional activation, and indicate that a region of the BMP4 locus, rich in CpG islands, has fewer methylated cytosines in genomic DNA from A33 cells than...
Figure 2

Proposed schema of events for the commitment of mesenchymal stem cells (MSCs) to the adipocyte lineage. BMP refers to bone morphogenetic protein. Frizzled refers to the Wnt cell-surface receptor. The Wnt and BMP pathways are shown as both linear and parallel events since both pathways may be involved. Wnt appears to function both in an activating and an inhibitory capacity in MSC commitment (10, 42, 50) and differentiation (41). Dashed lines indicate some uncertainty.

from 10T1/2 cells. The BMP4 gene locus in naive C3H10T1/2 stem cells possesses a highly methylated region of CpG islands, whereas this region in committed 10T1/2 cells possesses fewer methylated cytosines (6). This difference correlates with the switch in BMP promoter usage (promoter 2 to promoter 1) during commitment that is induced by the methylation inhibitor, suggesting that a change in methylation status renders promoter 1 more accessible for transcriptional activation.

**Downstream targets of BMP signaling.** Proteomic analysis revealed that three cytoskeleton-associated proteins [i.e., lysyl oxidase (Lox), translationally controlled tumor protein 1 (Tpt1), and αB-crystallin] are downstream target genes in the BMP signaling pathway and play important roles during adipocyte lineage commitment (52). Eight proteins were found to be upregulated by BMP2, and 27 proteins were upregulated by BMP4. Five unique proteins were upregulated ≥10-fold by both BMPs, including three cytoskeleton-associated proteins (i.e., Lox, Tpt1, and αB-crystallin). Commitment was completely blocked by knockdown of Lox, whereas it was partially inhibited by knockdown of Tpt1 and αB-crystallin expression. Dramatic changes in cell shape normally occur during commitment. Knockdown of these cytoskeleton-associated proteins prevented these cell shape changes and restored F-actin organization into stress fibers and inhibited commitment to the adipocyte
lineage. These differentially expressed proteins may determine the ability of MSCs to commit to the adipocyte lineage via cell shape regulation.

**Wnt Signaling**

The Wnts comprise a family of secreted signaling glycoproteins whose effects are mediated through the frizzled receptor and low-density lipoprotein-related protein 5/6 coreceptor (57). The Wnt proteins can act via the more prominent “canonical” Wnt signaling pathway (58–60) or a “noncanonical” Wnt signaling pathway (61). A linkage between Wnt signaling and adipogenesis was first recognized through the finding that expression of Wnt10b decreased dramatically during adipocyte differentiation (40). Consistent with this finding, forced expression of Wnt10b prevented adipocyte differentiation by blocking the expression of the key adipogenic transcription factors, PPARγ and C/EBPα. Surprisingly, the Wnts act at two points in the adipose development program (Figure 2), both early in the program as an activator of lineage commitment (41) and late in the program as an inhibitor of adipocyte differentiation (40, 45), perhaps through the actions of different Wnt proteins.

**Wnt activates lineage commitment of MSCs.** The canonical Wnt pathway functions early in the lineage commitment process of the canonical pathway. In this pathway cytosolic β-catenin is embedded in a “destruction complex” containing adenomatous polyposis coli, axin, and glycogen synthase kinase-3β (GSK-3β) (60). In the absence of Wnt stimulation, GSK-3β phosphorylates β-catenin, priming it for ubiquitination and proteasomal degradation (60). Conversely, activation of Wnt signaling through binding of Wnts to their cell-surface receptor, frizzled, and the associated low-density lipoprotein receptor-related coactivator 5/6 promotes dissociation of the destruction complex, thereby allowing β-catenin to accumulate and translocate to the nucleus (59, 62). Thus, during Wnt signaling, β-catenin accumulates in the nucleus, where it activates the transcription factors lymphoid enhancer factor and/or T-cell factor (Figure 2), triggering the transcription of downstream genes (including c-myc and Cyclin D1) (39).

Microarray studies combined with quantitative RT-PCR analyses identified several genes of the Wnt signaling pathway that are differentially expressed by A33 cells (preadipocytes derived from C3H10T1/2 stem cells, see above) (41). Of particular interest are the newly described R-spondins-2 and -3, which activate the canonical Wnt signaling pathway, and are dramatically upregulated in proliferating A33 preadipocytes compared with their progenitor C3H10T1/2 stem cells. Likewise, Lef1 and Tcf, downstream transcription factors of the Wnt signaling pathway, are expressed differently in A3 preadipocytes relative to their expression in C3H10T1/2 stem cells (41). These events occur concurrently with the accumulation of β-catenin in the nuclei of proliferating A33 cells. Taken together, these findings implicate Wnt signaling as an early event in stem cell commitment to the adipose lineage.

**Wnt inhibits terminal differentiation.** Late in the adipogenic program, the canonical Wnt signaling pathway regulates the balance among myogenic, osteoblastogenic, and adipogenetic fates (40, 46, 57) and, by doing so, decreases adipogenesis. Myoblasts in cell culture retain their plasticity in developmental potential because they can be induced to undergo myogenic, adipogenic, or osteoblastogenic differentiation (57). β-catenin-dependent signaling has been reported to promote both myogenesis (40) and osteogenesis (46) while inhibiting the differentiation of preadipocytes into adipocytes (41, 63). Consistent with the inhibitory effect of Wnt on adipogenesis, myoblasts isolated from Wnt10b-null mice exhibit increased adipogenic potential (57). In addition, activation of the Wnt signaling pathway enhances myogenesis and inhibits adipogenesis in cultured MSCs (40, 64).

The Wnt signaling pathway is more active in proliferating A33 cells (a clonal line with preadipocyte characteristics) than in its C3H10T1/2 progenitors, perhaps because
Wnt signaling serves to increase the number of preadipocytes during commitment. It has been speculated that Wnt signaling serves to increase the number of preadipocytes during commitment and mitotic clonal expansion (41) but that this signal must ultimately be terminated as preadipocytes/A33 cells enter growth arrest, before the newly recruited cells can undergo terminal differentiation into adipocytes (see the Mitotic Clonal Expansion section below).

**Hedgehog Signaling**

Three vertebrate Hh ligands have been identified, Sonic (Shh), Indian (Ihh), and Desert (Dhh), which initiate a signaling cascade mediated by Patched (Ptc-1 and Ptc-2) receptors (65, 66). In the presence of an Hh ligand, the membrane-spanning protein Smoothened (Smo), a homolog of G protein–coupled receptors, is activated, and the signal is transmitted via phosphorylation and nuclear localization of GliA (65, 66).

Hh signaling has an inhibitory effect on adipogenesis in murine cells, e.g., C3H10T1/2, KS483, calvaria MSC lines, and mouse adipose-derived stromal cells (67) as visualized by decreased cytoplasmic fat accumulation and the expression of adipocyte marker genes (42–44). In genetically obese (ob/ob) mice, a negative effect on adipogenesis is accompanied by a reduction in the expression of Smo, Gli1, Gli2, and Gli3. Moreover, reduced total white fat mass and epididymal adipocyte cell size were observed in naturally occurring spontaneous mesenchymal dysplasia (mes) adult mice (Ptc1 mes/mes), which carry a deletion of Ptc1, a negative regulator of Hh signaling, at the C-terminal cytoplasmic region (68). Although it is generally agreed that expression of Ihh has an inhibitory effect on preadipocyte differentiation, the mechanisms linking Hh signaling and adipogenesis remain poorly defined.

**Retinoblastoma Protein Signaling**

The retinoblastoma protein (Rb) inhibits the cell cycle by binding to and repressing the transcriptional activity of E2F (69). Upon hyperphosphorylation of Rb (⇒ pRb) by cyclin-dependent kinases, E2F is released and promotes transcriptional activation of genes that encode cell-cycle regulators required for S-phase entry and progression of the cell cycle (70). These events are critical for mitotic clonal expansion (see the Mitotic Clonal Expansion section below), an obligate step in the adipocyte differentiation program (38).

Additionally, pRb has been shown to regulate several transcription factors that are key differentiation inducers (38, 71). Depending on the differentiation factor and its cellular context, pRb can either suppress or promote transcriptional activity of such factors. Thus, pRb binds to Runx2 and potentiates its ability to promote osteogenic differentiation (72). In contrast, Rb acts with E2F to suppress peroxisome proliferator-activated receptor c (PPARγ2-c) subunit, the master activator of adipogenesis (73, 74). Because osteoblasts and adipocytes can both arise from MSCs, these observations suggest that pRb may play a role in the choice between these two lineage fates. And these findings indicate that Rb status plays a key role in establishing fate choice between bone and brown adipose tissue in vivo (75).

**DIFFERENTIATION: PREADIPOCYTE TO ADIPOCYTE**

The synchronous events undergone by preadipocytes during differentiation into adipocytes are described below. Cell culture studies with established preadipocyte lines have been indispensable for the identification and characterization of the key steps in the differentiation program. A large body of evidence shows that these preadipocyte models faithfully recapitulate differentiation of mouse embryonic fibroblasts (MEFs) in cell culture (34, 38, 76). Likewise, the authenticity of model systems has been validated in vivo by demonstrating that these preadipocyte lines give rise to normal adipose tissue when implanted subcutaneously into athymic mice without exogenous inducers (40, 77, 78).
**Figure 3**

Synchrony of DNA replication during mitotic clonal expansion of the adipocyte differentiation program. After staining with propidium iodide, DNA content was determined by FACS analysis and expressed (y axis) in arbitrary units. Time (h) refers to the elapsed time (in hours) after induction of differentiation of 3T3-L1 preadipocytes with the inducer cocktail. G1 refers to the growth-arrested phase of the cell cycle. S, M, and G2 refer to the subsequent phases of the cell cycle. The plots were constructed using data from Reference 38.

Abbreviations: 1n and 2n, one- to twofold; FLZ, fluorescence intensity.

**Induction of Differentiation**

Protocols have been developed to induce differentiation and track the synchronous progression of cells through the program. Preadipocytes, e.g., 3T3-L1 preadipocytes, or MEFs are grown to growth arrest, i.e., at the G1 phase of the cell cycle. At this point, differentiation is initiated with a cocktail of inducers, including a high level of insulin¹ (or low level of IGF1) (79) and dexamethasone, as well as an agent to elevate cellular cAMP in fetal calf serum-containing medium (80). These inducers activate the IGF1-, glucocorticoid-, and cAMP-signaling pathways, respectively. Induction initiates a series of events that regulate staging of the differentiation program. Following a delay of 16–20 h after induction, preadipocytes synchronously reenter the cell cycle (Figure 3) (39, 80) and undergo several rounds of mitosis, referred to as mitotic clonal expansion (see below). The cells then exit the cell cycle, lose their fibroblastic morphology, accumulate cytoplasmic triglyceride, and acquire the appearance and metabolic features of adipocytes (18, 80). Triglyceride accumulation is closely correlated with an increased rate of de novo lipogenesis and a coordinate rise in expression of the enzymes of fatty acid and triacylglycerol biosynthesis (79–81). Likewise, numerous regulatory proteins, characteristic of adipocytes in situ, are coordinately expressed, including insulin receptors (82, 83), the insulin-responsive glucose transporter GLUT4 (84), leptin (85, 86), and others (79, 86, 87).

¹It has been established that the IGF1, rather than insulin, is the true inducer ligand. IGF1 is active as an inducer at much lower concentrations than insulin binding; IGF1 binds ~100-fold more tightly than insulin to the IGF receptor (79).

**Mitotic Clonal Expansion**

Following induction of G1-phase growth-arrested cells, preadipocytes reenter the cell
Figure 4
Schema for the dual phosphorylation-induced conformational changes in C/EBPβ, which activates DNA binding. Dual phosphorylation (indicated by P) of C/EBPβ occurs on Thr188 by MAPK and on Ser184 or Thr179 by GSK3β; this induces a conformational change (or changes) that promotes dimerization and gives rise to binding activity. G1 → S refers to the mitotic clonal expansion phase of the cell cycle 16–24 h after the induction of differentiation. The data is from References 93 and 114. Abbreviation: C/EBPβ, CCAAT/enhancer-binding protein.

Active DNA binding

Cyclic AMP response element-binding protein. Because cAMP itself or forskolin or other agents, which elevate the cAMP I level, can substitute for methylisobutylxanthine in the differentiation inducer cocktail, CREB was considered a likely intermediate in the signaling pathway. Many lines of evidence support this view; these include the following:

1. The cellular target of cAMP, protein kinase A, catalyzes the phosphorylation and activation of CREB (95).
2. Forced expression of CREB in 3T3-L1 preadipocytes promotes differentiation (34).

3. The proximal promoter of the C/EBPβ gene possesses dual cis-regulatory elements that contain core CREB-binding sites (34, 96). C/EBPβ promoter-reporter genes with 5′-truncations or site-directed mutations in the TGA regulatory elements revealed that both are required for maximal promoter function.

4. Electromobility shift assay and chromatin immunoprecipitation analyses of wild-type MEFs and 3T3-L1 preadipocytes show that CREB associates with the proximal promoter and that interaction of phospho-CREB, the active form of CREB, with the C/EBPβ gene promoter occurs only after induction of differentiation of 3T3-L1 preadipocytes or MEFs (34, 90).

5. Constitutively active CREB activates C/EBPβ promoter-reporter genes (34, 90), induces expression of endogenous C/EBPβ, and promotes adipogenesis in the absence of hormonal inducers.

6. Conversely, dominant-negative CREB blocks promoter-reporter activity, as well as the expression of C/EBPβ and adipogenesis (90).

7. Subjecting wild-type MEFs to the standard differentiation protocol induces differentiation into adipocytes, whereas
CREB−/− MEFs treated similarly exhibit reduced expression of C/EBPβ and differentiation (90).

Consistent with the role of CREB in the differentiation process, C/EBPβ expression and accumulation of cytoplasmic triacylglycerol are markedly reduced in CREB−/− MEFs. Together these findings show that CREB functions early in the adipocyte differentiation program by transcriptionally activating the C/EBPβ gene.

The CCAAT/enhancer-binding family. Although C/EBPα was the first C/EBP family member to be cloned and to have its function shown in adipogenesis, three other isoforms have also been implicated, notably C/EBPβ (97–99), C/EBPδ (104, 105), and CHOP10 (106). Three of the isoforms contain a DNA-binding domain (C/EBPα, C/EBPβ, and C/EBPδ) and an adjacent C-terminal leucine zipper dimerization domain that allows formation of homo- or heterodimers with other C/EBP family members; dimerization is required for DNA binding (107). CHOP10 has a short nonfunctional DNA-binding domain and b-ZIP type C-terminal leucine zipper dimerization domain and can heterodimerize with the other isoforms to produce dominant-negative C/EBP dimers (106). Both C/EBPα and C/EBPβ also occur as N-terminally truncated polypeptides whose functions remain obscure (49, 108, 109).

CCAAT/enhancer-binding protein-β and -δ. C/EBPβ and C/EBPδ are rapidly (in <4 h) expressed following induction of differentiation (38). The importance of C/EBPβ is indicated by its ability to promote adipogenesis when overexpressed in 3T3-L1 preadipocytes or in NIH-3T3 cells in the absence of hormone inducers—an activity not shared by C/EBPδ (110). Although rapidly expressed upon induction of differentiation, C/EBPβ is inactive and unable to bind DNA until later in the differentiation program. Acquisition of DNA-binding activity is achieved at ~16 h after induction, approximately concomitant with entry into S phase and mitotic clonal expansion (see below). Another indicator of multiple C/EBP consensus-binding sites is the centromeric satellite DNA (38, 111). The role of this binding to centromeres has not been established.

Although C/EBPβ is required for adipocyte differentiation in cell culture (38, 93, 112, 113), a knockout of the C/EBPβ gene in mice has little effect on adipose tissue accumulation (113). Nevertheless, adipose tissue mass in the double knockout [C/EBPβ−/−/C/EBPδ−/−] is markedly reduced (113). This reduction of adipose tissue mass is because of the decreased cell number, supporting the view that C/EBPβ functions in mitotic clonal expansion. That the double C/EBPβ−/−/C/EBPδ−/− knockout produces a significant effect, whereas single knockouts of C/EBPβ−/− or C/EBPδ−/− do not, suggests redundancy of function by members of the C/EBP family (113).

Supporting the dependence of adipogenesis upon C/EBPβ, disruption of the gene prevents mitotic clonal expansion, which is itself required for differentiation. Thus, C/EBPβ−/− MEFs fail to undergo mitotic clonal expansion and do not differentiate into adipocytes, while C/EBPβ−/− MEFs undergo mitotic clonal expansion and differentiate normally (89, 92). Further support for the role of C/EBPβ in clonal expansion is derived from the use of A-C/EBP, which contains a leucine zipper but lacks the functional DNA-binding and transactivation domains and thus acts as a dominant-negative of C/EBP proteins. Forced expression of A-C/EBP disrupts both mitotic clonal expansion and differentiation in 3T3-L1 cells (90). Moreover, the turnover of p27—a requirement for progression from G1-growth arrest to S phase—is blocked by the forced expression of A-C/EBP (88, 114). The mechanism for this effect is that formation of a tight C/EBPβ:A-C/EBP heterodimer prevents the translocation of C/EBPβ into the nucleus, a process that is necessary for mitotic clonal expansion (90). Thus, the primary site of A-C/EBP—the first C/EBP family member to be expressed in the
transcription factor cascade—is most likely to block C/EBPβ function. Moreover, when A-C/EBP, under the control of the aP2 adipocyte-specific promoter, is expressed in transgenic mice, the animals become fatless, i.e., devoid of white adipose tissue (115). This finding provides further compelling proof that C/EBPβ is required for adipogenesis in vivo.

Phosphorylation is an important posttranslational modification of C/EBPβ and leads to the acquisition of DNA-binding function as preadipocytes traverse to the G1-S checkpoint at the onset of mitotic clonal expansion (93). C/EBPβ is phosphorylated sequentially, first by MAP kinase and then much later by GSK-3β (93). Phosphorylation on Thr188 by MAP kinase occurs ~4 h after induction of differentiation and is required for mitotic clonal expansion, C/EBPβ DNA-binding activity, and terminal differentiation; however, Thr188 phosphorylation is insufficient on its own to produce DNA-binding activity by C/EBPβ (93). Phosphorylation on Thr179 or Ser184 by GSK3β occurs between 12 and 16 h after induction. Dual phosphorylation of C/EBPβ at two of these sites (Thr188 and Thr179 or Thr188 and Ser184) leads to acquisition of DNA-binding activity. Hence, it appears that a phosphorylation-induced conformational change is involved. On the basis of these and other findings (116), it is thought that, following induction of differentiation, C/EBPβ is first rapidly (in ~2 h) phosphorylated by MAPK, and then phosphorylated at a second site ~14 h later by GSK3β (Figure 4). A conformational change is induced that renders the leucine zipper of monomeric C/EBPβ accessible for dimerization (117). It appears that dimerization brings the basic regions of the two monomers of C/EBPβ together to create a “scissors-like” DNA-binding pocket just above the coiled-coil leucine zipper as suggested by Vinson et al. (118).

CCAAT/enhancer-binding protein-α. C/EBPα and PPARγ function together as pleiotropic transcriptional activators of the large group of genes that produce the adipocyte phenotype (79, 86, 119). Within their proximal promoters, both the C/EBPα and PPARγ genes possess C/EBP regulatory elements at which C/EBPβ binds to coordinately activate transcription (111, 120–122). Once expressed, C/EBPα is thought to maintain expression of both the C/EBPα and PPARγ genes via transactivation mediated by their respective C/EBP regulatory elements (111, 120–122).

The promoters of many adipocyte genes contain C/EBP and PPAR regulatory elements and are trans-activated by C/EBPα and PPARγ (79). Forced expression of C/EBPα or PPARγ in 3T3-L1 preadipocytes induces adipogenesis in the absence of hormonal induction (123–125). Furthermore, blocking expression of C/EBPα with antisense RNA suppressed adipogenesis (126), and knocking out the C/EBPα gene in mice led to decreased lipid accumulation (127, 128). These and other findings indicate the requirement for C/EBPα in the adipocyte differentiation program. Beginning 18–24 h after the induction of differentiation, the C/EBPα and PPARγ genes are transcriptionally activated by C/EBPβ through C/EBP regulatory elements in their proximal promoters.

Once C/EBPα is expressed, its expression is maintained through autoactivation (120). The following question is raised: Why is there such redundancy in the expression of the C/EBP genes during adipogenesis? One possible explanation is that C/EBPα is antimitotic, and thus, its premature expression would prevent preadipocytes from entering mitotic clonal expansion, a required step for subsequent differentiation. Therefore, it appears that C/EBPα must remain repressed until the opportune time window. Several mechanisms cause a delay in the expression of C/EBPα. Binding of AP-2α (129) and Sp1 (130) to the C/EBPα promoter repress promoter activity, and delay acquisition of DNA binding by C/EBPβ and C/EBPδ (see the section titled CCAAT/enhancer-binding protein-β and -δ, above). Both AP-2α and Sp1 are downregulated concurrent with the upregulation of C/EBPβ.
**CHOP10.** CHOP10 was cloned from a 3T3-L1 adipocyte cDNA library on the basis of its ability to interact with the C/EBPβ C-terminal leucine zipper domain (106). CHOP10 contains proline and glycine residues in the DNA-binding region, which abolishes its ability to bind DNA but not its ability to form heterdimers. Thus, CHOP10 acts as a dominant-negative isoform (106) and when expressed ectopically in 3T3-L1 cells blocks adipogenesis (131). CHOP10 is normally expressed by G1-phase, growth-arrested preadipocytes, but is downregulated by C/EBPβ (132), CHOP10 provides a “fail-safe” mechanism to prevent the acquisition of DNA-binding activity by C/EBPβ. Because heterodimerization of CHOP10 with PPARγ prevents it from acquiring DNA-binding activity (132), CHOP10 provides a “fail-safe” mechanism to prevent the acquisition of DNA-binding activity by C/EBPβ until preadipocytes have entered mitotic clonal expansion.

**Peroxisome proliferator-activated receptor-γ.** PPARγ exists as three isoforms (PPARγ1, PPARγ2, and PPARγ3) that are transcribed from the same gene through alternative splicing and promoter usage (133, 134); PPARγ2 is the primary adipocyte-specific isoform (9). All isoforms possess transactivation, DNA-binding, and dimerization domains (9). To bind at peroxisome proliferator response elements in target genes, PPARs must first form heterodimers with the retinoid X receptor (9). Although naturally occurring ligands for PPARγ have not yet been identified, several potent synthetic thiazolidinediones and prostanoids, e.g., 15-deoxy-D12,14 prostaglandin J2 (37, 38), bind with high affinity. It is unlikely, however, that the 15-deoxy-D12,14 prostaglandin J2 concentration in vivo is of biological importance (9).

Although PPARγ2 appears to act as a “master” regulator of the adipogenesis program, like C/EBPα, it also participates in other diverse systems, including hepatic lipogenesis (9). The ectopic expression of PPARγ2 in naive preadipocytes and nonadipogenic fibroblasts activates expression of adipocyte genes and differentiation (9, 10, 135). Moreover, dominant-negative mutants (49, 136) and knockouts of the mouse gene block these functions.

As indicated above, C/EBPβ (and C/EBPδ) are rapidly (in <4 h) expressed after induction of differentiation. Following a delay of 16–20 h, C/EBPβ coordinates the expression of PPARγ2 and C/EBPα through C/EBP regulatory elements in the proximal promoters of their respective genes (38, 89, 137). Following their expression, PPARγ2 and C/EBPα coordinately transactivate a large group of genes that produce the adipocyte phenotype. Once expressed, PPARγ and C/EBPα positively cross activate each other through their respective C/EBP regulatory elements (136, 138, 139). Presumably, this action perpetuates the adipocyte phenotype in the mature adipocyte.

**Sterol regulatory element-binding protein.** The sterol regulatory element-binding proteins (SREBPs) are basic helix-loop-helix-leucine zipper proteins. SREBP2 regulates transcription of the genes of cholesterol metabolism, while SREBP1c and its mouse homolog, ADD1, regulate lipogenesis (140, 141). In the adipocyte differentiation program, the expression of SREBP1c/ADD1 mRNA is activated after the expression of C/EBPα and PPARγ, i.e., at ∼20 h after induction of differentiation (142). Current evidence implicates SREBP1c/ADD1 in the following terminal events of adipocyte differentiation. (a) SREBP1c/ADD1 is expressed as a membrane-bound precursor bound to SCAP (SREBP-cleavage-activating protein) and tethered in the ER by Insig-2a. (b) Upon its release from the ER (stimulated by insulin), SCAP-SREBP1c/ADD1 moves to the Golgi apparatus, where proteolytic cleavage frees its basic helix-loop-helix component for translocation to the nucleus. (c) Once in the nucleus, transcription of genes encoding lipogenic enzymes occurs—events that produce adipocyte characteristics.

Recent evidence (143, 144) indicates that insulin regulates the release of SCAP-SREBP1c/ADD1 from the ER by downregulating the level of Insig-2a through increased turnover of its mRNA. Reducing
the level of Insig-2α facilitates export of SCAP-SREBP1c/ADD1 from the ER to the Golgi for proteolytic cleavage and subsequent translocation of the basic helix-loop-helix component to the nucleus. As a result, the transcription of lipogenic genes is activated, including those that support fatty acid synthesis, desaturation, and uptake, as well as triacylglycerol synthesis. In a metabolic sense, this is consistent with the recent finding that SREBP1c/ADD1 is phosphorylated by AMP kinase (145), which promotes energy mobilization. Insulin, on the other hand, promotes energy storage by activating lipogenic enzyme expression.

**Histones and Chromatin Remodeling**

Genome-wide mapping studies have revealed changes in chromatin structure that occur during the differentiation of 3T3-L1 preadipocytes into adipocytes (145, 146). DNase I-hypersensitive site analysis revealed that alteration of chromatin structure occurs early in the differentiation program, coinciding with the cooperative binding of early transcription factors, such as C/EBPβ and C/EBPδ, to regulatory element hot spots. Of particular interest, C/EBPβ hot spots were observed prior to induction of differentiation and chromatin remodeling. Furthermore, a subset of the early remodeled C/EBPβ hot spots persisted throughout differentiation and was later occupied by PPARγ, suggesting that early C/EBP family members may act as initiating factors for subsequent binding of PPARγ. These findings are, however, inconsistent with those obtained in cell culture studies (93). These studies showed that, in the G1-S growth-arrested state prior to induction, C/EBPβ is in its dephosphorylated inactive state and unable to bind DNA (i.e., to consensus C/EBP promoter-binding sites); synchronous entry into S phase occurs. Synchronous entry into S phase occurs only after completion of dual phosphorylation of C/EBPβ and acquisition of DNA-binding activity (Figure 4) (93). A possible explanation for this discrepancy is that the preadipocytes in this study were not in the G1-growth-arrested state (147). Dividing preadipocytes are known to express high levels of active phosphorylated C/EBPβ. Thus, C/EBPβ hot spots, attributed to “uninduced” preadipocytes, may well have been due to cells that had traversed the G1-S cell-cycle checkpoint. See the sections above (i.e., Mitotic Clonal Expansion and CCAAT/enhancer-binding protein-β above). C/EBPβ must be phosphorylated multiple times to bind DNA. Thus, it will be necessary to definitively prove by cell-cycle analysis that all preadipocytes prior to induction were in the G1-growth-arrested state as illustrated in Figure 3.

**Role of microRNAs in Adipogenesis**

A number of microRNAs have been identified that appear to play a role in adipogenesis. Some of these microRNAs seem to accelerate adipogenesis (148, 149), while others negatively regulate adipogenesis (150, 151). Mammalian homologs of miR-8 promote adipogenesis by inhibiting Wnt signaling (148). Ectopic introduction of let-7 into 3T3-L1 cells inhibited clonal expansion as well as terminal differentiation (151). However, the mechanisms by which these microRNAs act have not been definitively linked to specific aspects of the differentiation program. This should be a fertile area for future research.

**SUMMARY POINTS**

1. Excessive caloric intake leads to adipocyte hyperplasia and adiposity.
2. Adipocyte hyperplasia is caused by recruitment of pluripotent mesenchymal stem cells (MSCs) from the vascular stroma of adipose tissue.
3. The BMP and Wnt families are key mediators of MSC commitment to produce preadipocytes.

4. Exposure of growth-arrested preadipocytes to differentiation inducers (IGF1, glucocorticoid, and cAMP) triggers DNA replication, reentry of the cell cycle (a process known as mitotic clonal expansion), and a transcription factor cascade, which leads to expression of adipocyte genes.

5. The transcription factor cascade includes the following: Induction $\rightarrow$ $\uparrow$[CREB $\rightarrow$ P-CREB] $\rightarrow$ $\downarrow$CHOP10 $\rightarrow$ $\uparrow$[C/EBP$\beta$ $\rightarrow$ P$_2$-C/EBP$\beta$] $\rightarrow$ $\uparrow$[C/EBP$\alpha$ / PPAR$\gamma$] $\rightarrow$ $\uparrow$SREBP1c $\rightarrow$ $\uparrow$adipocyte genes.

FUTURE ISSUES

1. A more detailed understanding is needed of the mechanisms by which lineage commitment and differentiation occur.
2. Are the Wnt and BMP pathways, involved in MSC commitment, linear/sequential or parallel?
3. Characterization is lacking of the conformational changes, induced by phosphorylation of C/EBP$\beta$, that promote dimerization and acquisition of DNA-binding activity. The three-dimensional structures of phospho- and dephospho-C/EBP$\beta$ by X-ray crystallography/magnetic resonance imaging are needed.
4. The role of microRNAs in lineage commitment and differentiation needs to be determined.

DISCLOSURE STATEMENT

The authors have nothing to disclose regarding potential bias and are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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