REVIEW ARTICLE

Control of adipocyte differentiation

Cynthia M. SMAS and Hei Sook SUL*
Department of Nutritional Sciences, University of California, Berkeley, CA 94720, U.S.A.

INTRODUCTION

Adipose tissue allows for triacylglycerol storage in periods of energy excess and the subsequent use of triacylglycerol stores during energy deprivation with the lipogenic/lipolytic balance under tight hormonal control. Information is incomplete on the origins of adipose cells and adipose tissue. White adipose tissue is not detected during embryonic life or at birth in rats and mice, but is present at birth in humans and pigs. Adipocytes represent between one-third and two-thirds of cells in adipose tissue. The remaining cells comprise the stromal vascular fraction and include blood cells and endothelial cells, along with adipocyte precursor cells [1]. Studies to date suggest the adipocyte lineage derives from an embryonic stem cell precursor with the capacity to differentiate to mesodermal cell types such as adipocytes, chondrocytes and myocytes. Treatment of murine C3H10T1/2 mesodermal stem cells with a demethylating agent generates loci of muscle, cartilage and fat cells, and suggests that relatively few genes may regulate adipocyte differentiation [2,3]. Animal studies demonstrate that the potential to make new fat cells continues throughout the lifespan. Rats increase fat-cell number in response to high-carbohydrate or high-fat diets. Fat depots of very old mice contain cells that express early differentiation markers, suggesting an ongoing process of differentiation. The cells of the stromal vascular fraction of adipose tissue from aged rats and humans differentiate *in vitro* into adipose cells [4,5].

This review addresses the regulation of adipose development. Since this area has been the subject of intensive research, we focus here on conclusions attained through the use of *in vitro* model systems of adipocyte differentiation. The first section describes the various preadipocyte cell lines and reviews the changes in gene expression that occur during their differentiation. Next, the culture conditions requisite for, and the hormonal regulation of, adipocyte differentiation are reviewed and the involvement of cytoskeletal and extracellular matrix is examined. This is followed by an overview of *cis*-elements and DNA-binding proteins that are involved in the expression of adipocyte genes. Lastly, we present the approaches taken to attempt to identify genes that may control not just the expression of specific adipocyte genes, but the adipocyte differentiation programme.

IN VITRO MODELS OF ADIPOCYTE DIFFERENTIATION

Many adipocyte differentiation studies employ animals and adipose-derived stromal vascular precursor cells as model systems. However, the development of established preadipocyte cell lines greatly facilitated the study of the molecular details of adipocyte differentiation. Preadipocyte cell lines commonly used for the *in vitro* study of adipocyte differentiation are listed in Table 1. 3T3-F442A and 3T3-L1 cells derive from the Swiss 3T3 cell line prepared from disaggregated 17–19-day-old Swiss 3T3 mouse embryos [6,7]. 3T3-C2 cells derive from the same source but are not preadipocyte in nature as they do not differentiate to adipocytes. They can be utilized to compare the responses of differentiation-defective with differentiation-competent cell types. The TA1 cell line was established by treating 10T1/2 mouse embryo fibroblast cells with the demethylating agent 5′-azacytidine [8]. Adipose precursors present in the epididymal fat pads of genetically obese *ob/ob* adult mice [9] were the source of Ob17 cells and their derivatives. During growth, preadipocytes resemble fibroblasts. However, upon growth arrest in the presence of fetal-calf serum, spontaneous differentiation to adipocytes occurs. The overall process and the various classes of signals that influence adipocyte differentiation are schematically illustrated in Figure 1. Cells convert to a spherical shape and accumulate small lipid droplets. These droplets later fuse to give the rounded, signet-ring appearance of cells similar to those of white adipose tissue. The mature adipocyte produced by *in vitro* differentiation has many characteristics of adipose cells *in vivo*. They have sensitivity to hormones and metabolic effectors similar to that of adipose tissue [10]. Subcutaneous implantation of these cells in mice, at a site normally lacking adipose tissue, produces fat-cell lobules and tissue masses that are histologically indistinguishable from white adipose tissue [11,12].

Terminal differentiation to adipocytes involves alterations in the levels of over 100 proteins [13] with the time course of differentiation reflected by the appearance of early and late mRNA markers and triacylglycerol accumulation. These changes are primarily at the transcriptional level, although post-transcriptional regulation occurs for some adipocyte genes [14]. Growth arrest and not cell confluence *per se* appears to be a prerequisite for adipocyte differentiation. Confluent 3T3-F442A cells shifted to suspension culture undergo differentiation [15]. Following growth arrest the cells undergo at least one round of DNA replication and cell doubling, and this has been proposed to lead to the clonal amplification of committed cells [15]. Studies on 3T3-F442A and Ob17 cells show that an increase in DNA synthesis precedes expression of late mRNA markers and that inhibition of DNA synthesis prevents the formation of fat cells [16,17]. The requirement of post-confluent mitoses for subsequent differentiation has been shown in 3T3-F442A cells [15] and in the Ob17-derived cell line, Ob1754, which requires putrescine supplementation for DNA synthesis, cell division and terminal differentiation [17]. Growth-arrested cells that have undergone post-confluent mitoses are apparently at a crossroads in the differentiation programme. Mitogenic challenge or replating cells

Abbreviations used: bFGF, basic fibroblast growth factor; CUP, C/EBP undifferentiated protein; DAP, differentiation-associated protein; DSE, differentiation-specific enhancer; ECM, extracellular matrix; EDHB, ethyl-3,4-dihydroxybenzoate; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; LPL, lipoprotein lipase; MIX, methylisobutylxanthine; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; PRE, preadipocyte repressor element; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SV40, simian virus 40; T_3, thyroid hormone; TNFα, tumour necrosis factor α; TGF-α, transforming growth factor-α.

*To whom correspondence should be addressed.

Table 1 Selected murine preadipocyte cell lines

Abbreviation: GH, growth hormone.

Cell line	Derivation	Comments	Reference
3T3-L1	A clonal subpopulation derived from the Swiss 3T3-M line, originally prepared from 17–19-day disaggregated mouse embryo.	Since it is derived from whole mouse embryo, cellular origin largely unknown. Differentiation accelerated by dexamethasone and MIX. IGF-1 may be crucial.	[7]
3T3-F442A	Same as above.	As with the 3T3-L1 line, spontaneous differentiation begins at confluence; lipid accumulation usually accelerated by insulin treatment.	[6]
TA1	Treatment of mouse-embryo-derived 10T-1/2 mesenchymal stem cells with demethylating agent 5′-azacytidine.	Since parental line multipotential, may represent an earlier preadipocyte stage.	[8]
Ob17	Epididymal fat pads of genetically obese (*ob/ob*) adult mice; sublines include Ob1771.	Adult derivation may represent a later preadipocyte stage, and may explain lack of GH requirement for *in vitro* differentiation. Since derived from a genetically obese animal, could theoretically have different properties than other preadipocyte cell lines.	[9]

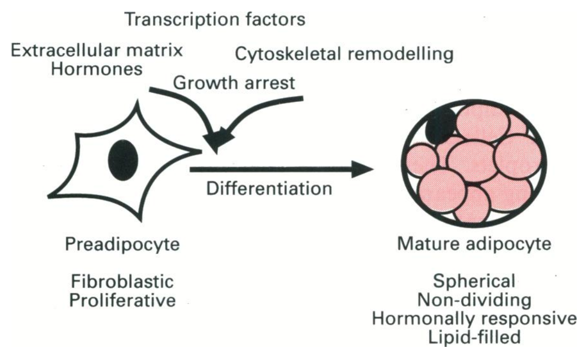

Figure 1 Multiple classes of signals influence the differentiation of preadipocytes to adipocytes

The process of adipocyte differentiation involves conversion of proliferative preadipocytes into terminally differentiated adipocytes containing multiple large lipid droplets. This is accompanied both by changes in cell shape and by dramatic increases in lipid synthesis and hormonal responsiveness integral to the specialized role of the adipocyte in lipid metabolism. Under the influence of various transcription factors, ECM components and hormones, as well as cytoskeletal remodelling and growth arrest, differentiation ensues.

at low density initiates cell division and interrupts differentiation, while with appropriate culture conditions terminal differentiation ensues [17].

c-Myc is implicated in the ability of preadipocytes to respond to mitogenic signals [18]. Enforced expression of c-Myc in 3T3-L1 cells blocks differentiation of these cells when tested in the presence of inducing agents. Expression of antisense c-Myc sequences in these cells leads to adipocyte differentiation upon treatment with inducing agents. c-Myc does not affect the ability of the cells to arrest growth or to replicate the genome, two steps that are requisite for initiation of the differentiation programme. However, challenge of the growth-arrested cells with 30% (v/v) serum reveals that while c-Myc-expressing cells re-entered the cell cycle, the non-transfected controls did not. This suggests that growth-arrested 3T3-L1 cells are in a particular state of the cell cycle, and that enforced c-Myc expression precludes entry to this state. This state is distinct from cells arrested by confluence since the c-Myc-expressing preadipocytes are unresponsive to mitogenic stimuli such as serum challenge [18]. Thus c-Myc may act as a molecular switch directing cells to a pathway of proliferation or terminal differentiation.

Cell growth arrest and/or high cell densities, under conditions favouring differentiation, induce the expression of early marker mRNAs. The expression of early markers is not dependent on additional agents required for subsequent terminal differentiation and appears to be restricted to those cells capable of adipose conversion. Confluence in TA1 cells increases clone 5 mRNA, an early marker of differentiation in these cells [19]. Ob17 and 3T3-F442A cells increase lipoprotein lipase (LPL) and the α-2 chain of type-VI collagen [20] mRNAs at confluence, while non-differentiating 3T3-C2 cells do not [21]. Ob17 cells in growth-factor-depleted serum express early mRNAs at confluence although terminal differentiation does not ensue, since required serum factors are lacking. Replating cells at low density reverses expression of early marker mRNAs. It therefore appears that growth-arrested cells expressing early markers can either differentiate to express terminal markers, or continue to divide with the loss of early marker mRNAs, with this decision dependent on culture conditions [17].

During the terminal phase of differentiation, adipocytes exhibit marked increases in *de novo* lipogenesis. The activity levels of proteins and/or mRNAs, for ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase, glycerol-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase and glyceraldehyde-3-phosphate dehydrogenase increase 10–100-fold [22–24]. Numbers of glucose transporters [25] and insulin receptors increase as does insulin sensitivity [26]. Before activation of these adipocyte genes, the mRNAs for C/EBPα [27] and mPPARγ appear [27a]. These transcription factors are involved in the regulation of some of the above genes and a full discussion of both is presented below. Besides increases in mRNAs for proteins directly related to lipid metabolism, alterations occur in the type and level of extracellular matrix (ECM) components secreted [28,29]. As cells convert from fibroblastic morphology to a spherical shape the levels of actin and tubulin decline [30]. Adipocytes also synthesize other adipose-tissue-specific products. These include an adipocyte-specific fatty-acid-binding protein aP2 [24], a lipid droplet-associated protein perilipin [31], monobutyrin, an angiogenic agent [32], and adipsin, a homologue of the serine protease complement factor D that is markedly decreased in some rodent models of obesity [33,34]. The expression of mRNAs during the various stages of adipocyte differentiation is illustrated in Figure

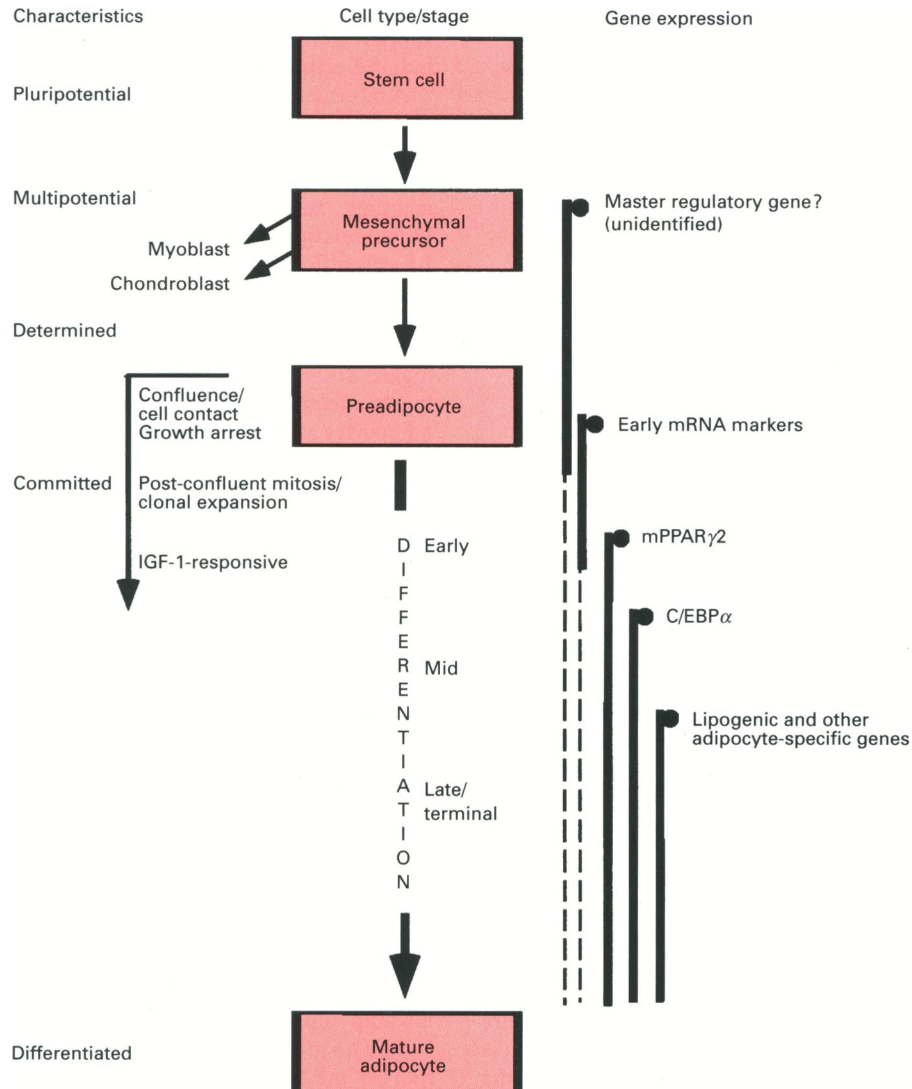

Figure 2 Stages in adipocyte differentiation

As the pluripotential precursor stem cell differentiates to the mature adipocyte (centre of diagram), its cell-fate becomes restricted first to that of the multipotential mesenchymal cell type with an ability to give rise to preadipocytes as well as myoblasts and chondroblasts. Following subsequent environmental and gene expression cues, the preadipocyte becomes committed to its terminal differentiation pathway. A presumed master regulatory gene expressed at the mesenchymal state commits cells to the preadipocyte lineage. Differentiation is divided into early, mid and late terminal phases. The corresponding pattern of gene expression is shown to the right with broken lines indicating possible continued expression.

2. When considering the time course of gene expression, where earlier-expressed genes are often assumed to have important roles, it should be remembered that these data are limited by the sensitivity of the methods employed, i.e. usually Northern blot. In such analyses, an inadvertent assumption may be made that mRNAs that are not detected are not expressed, or that those genes expressed below the detection limit are less important. There may exist a repertoire of yet to be identified low-abundance mRNAs that contribute to the differentiation process. An additional consideration is that although preadipocyte cell lines have many outward characteristics of adipose tissue *in vivo*, these cell lines are aneuploid, and therefore could express genes at levels that are different from those *in vivo*. Furthermore, their study is largely out of the *in vivo* context, where fat cells are intimately associated with ECM components and blood vessels. Use of transgenic mice models can bridge this gap and test the conclusions from *in vitro* studies in an *in vivo* setting.

HORMONAL SIGNALS REGULATING ADIPOCYTE DIFFERENTIATION

Preadipocyte cell lines are restricted in developmental potential to the adipose lineage. However, the use of inducing agents that accelerate differentiation, but are not required to maintain the differentiated phenotype, gives clues to the biochemical pathways that may function during adipocyte differentiation. For example, 3T3-L1' cells can spontaneously differentiate over a period of several weeks into fat-cell clusters [6]. Exposure of cells to a combination of the synthetic glucocorticoid dexamethasone and the phosphodiesterase inhibitor methylisobutylxanthine (MIX) for 48 h post-confluence results in more cells converting and shortens the conversion process to 5–7 days [26]. Addition of insulin, while not affecting the number of cells converting, accelerates lipid accumulation in these cells. Growth hormone, glucocorticoid, thyroid hormone (T_3), insulin-like growth factor-

Table 2 Selected agents influencing adipocyte differentiation

Agent	Comments	References
Growth hormone	Required for some cell lines.	[36–38]
IGF-1	Required for some cell lines.	[39]
EGF, TGFα	Inhibitory.	[46–48]
bFGF	Inhibitory.	[50,51]
TNFα	Inhibits differentiation and suppresses a subset of adipocyte marker mRNAs in mature fat cells.	[52]
Glucocorticoid	Stimulates differentiation of 3T3-L1 and TA1 cells.	[8,26]
Arachidonic acid	Variable depending on model system.	[60–65]
Retinoic acid	At supraphysiological doses, inhibits differentiation and suppresses a subset of adipocyte marker mRNAs in mature fat cells.	[69–74]
	At lower concentrations, accelerates differentiation of Ob1771 cells.	[75]

1 (IGF-1) and prostaglandins are adipogenic when tested in *in vitro* adipocyte differentiation. Although the pathways of protein kinase A, protein kinase C, prostaglandins and nuclear signalling are implicated when examined independently, the fact that one modulator can in some cases substitute for an agent from a separate signal transduction pathway probably indicates a redundancy of certain signalling systems in adipocyte differentiation. These results also neither indicate the relative dominance of one pathway over another, nor do they necessarily reflect the *in vivo* situation.

Another consideration is that use of different preadipocyte cell lines, of both embryonic and adult origin, may result in differential requirements for differentiation. Cells of the various lines may represent distinct stages along the adipocyte developmental pathway with perhaps inherently different responses to various drugs and hormones. Furthermore some culture conditions include fetal-calf serum. The composition of serum is not well defined and may vary considerably with regard to the relative levels of both stimulatory and inhibitory components that can enhance and/or replace the agents tested. However, the use of various preadipocyte cell lines and differentiation of *in vivo*-derived adipocyte precursors also offers an advantage. If multiple model systems generate the same results, then that response is more likely to be integral to the differentiation process rather than characteristic of only a particular cell line or culture condition. Table 2 summarizes some of the agents that affect adipocyte differentiation.

Growth hormone and IGF-1

All preadipocyte cell lines exhibit some degree of spontaneous conversion into adipocytes at confluence when grown in fetal-calf serum. The focus of initial studies was to detect the serum component(s) responsible for the full expression of the adipose phenotype. Studies addressing the role of growth hormone and IGF-1 in adipocyte differentiation illustrate the problems in comparing results obtained under various culture conditions and with different cell lines. 3T3-F442A preadipocytes grown in serum-supplemented conditions require growth hormone for differentiation. This was determined through use of anti-(growth hormone) antibody to deplete growth hormone in serum. IGF-1 could not substitute for growth hormone in this system [36,37]. Adipocytes newly differentiated by growth hormone treatment are much more sensitive to the mitogenic effects of IGF-1 than the untreated precursor cells. This suggests that IGF-1 acts to support the selective multiplication of young growth hormone-induced cells during clonal expansion to adipocytes [38].

A more definitive demonstration of the role of growth hormone and IGF-1 comes from experiments that have attempted to remove the variability due to serum by use of serum treated with charcoal to remove growth factors and hormones, or by supplementing serum-free medium with various components. Differentiation does not occur if the standard protocol for 3T3-L1 cell differentiation is modified by using charcoal-absorbed fetal-calf serum. Supplementation with growth hormone, epidermal growth factor (EGF) and platelet-derived growth factor does not result in adipocyte differentiation. However, physiological doses of IGF-1 and pharmacological doses of insulin are highly effective in restoring the ability of the cells to develop into adipocytes as judged by levels of late marker adipocyte mRNAs [39]. Since IGF-1 receptors are present in preadipocytes whereas insulin receptor expression is differentiation-dependent, it is likely that IGF-1 receptors mediate this effect. Transfection of either normal or transforming alleles of H-*ras* apparently bypasses the need for insulin or IGF-1, and substitutes for IGF-1 treatment in the above system [40]. Ras may either mediate IGF-1 function or activate a distinct pathway in adipocyte differentiation.

Other preadipocyte cell lines have a requirement for growth hormone and not IGF-1. Under conditions similar to those used to show an IGF-1 requirement by 3T3-L1 cells, Ob1771 cells require growth hormone for adipose conversion. Here, IGF-1 fails to substitute for the growth hormone requirement, indicating that growth hormone function is at least in part independent of its stimulation of IGF-1 production. However, protein kinase C activators including phorbol esters, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and diacylglycerol [41,42] mimic the growth hormone effect. Growth hormone may also act via c-Fos since growth hormone induces a transient increase in c-Fos mRNA [43]. A separate study in Ob17 cells tested growth hormone effects in serum-free medium supplemented with transferrin, fetuin and T_3. In this system no growth hormone was necessary for expression of early marker mRNAs such as LPL and the α-2 chain of type-VI collagen, but it was necessary for terminal differentiation of these cells [44]. A possible explanation for these differences is that perhaps the cell lines have inherently different requirements. The requirement for growth hormone by Ob17 cells, which are of adult origin, is not consistent with the general observation that adipose precursors isolated from animals do not require growth hormone for their differentiation. It has been proposed that the growth hormone requirement is obviated in primary cultures by their prior *in vivo* exposure to circulating growth hormone and that these cells are primed for subsequent sensitivity to other adipogenic agents [45]. Despite these variable observations, the general conclusion is that growth arrest is a requirement for terminal differentiation and that, depending on the cell line employed, either growth hormone or IGF-1 is obligatory.

Polypeptide hormones, growth factors and related agents

EGF, transforming growth factor-α (TGFα), basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNFα), and other cytokines inhibit adipocyte differentiation, although their mode of action is largely uncharacterized. bFGF and TNFα also decrease the expression of adipocyte-specific genes in mature fat cells. Inhibitors may, in theory, affect the round of DNA replication and cell division that initiates clonal expansion of preadipocytes [15] or affect the expression of all, or a subset of, adipocyte genes. Furthermore their effect may be dependent on

when during the adipocyte differentiation process they are employed. Several studies indicate a role for those growth factors that interact with the EGF receptor in adipose development. TGFα inhibits the differentiation of 3T3-F442A preadipocytes [46] and EGF inhibits the differentiation of primary cultures of preadipocytes [47]. Transgenic mice studies support these *in vitro* findings. Transgenic mice overexpressing TGFα have a 50% reduction in total body fat compared with non-transgenic controls that is not attributable to differences in metabolic rates [46]. Subcutaneous injection of EGF into newborn rats for 10 days decreased fat pad weight in a dose-dependent manner. This is apparently due to decreased differentiation of precursor cells to adipocytes [48]. Furthermore, it was found that adipose precursors from EGF-treated animals showed a lower *in vitro* differentiation capacity than those from untreated animals [48].

Inhibition of *in vitro* adipocyte differentiation, as judged by triacylglycerol accumulation and expression of various marker mRNAs, is also reported for a number of cytokines. Interleukin-11 has a dose-dependent inhibitory effect on both 3T3-L1 and the bone-marrow stroma-derived H-1/A cells. Inhibition is dominant over the effect of standard inducing agents [49]. Interferon-γ decreases the proportion of differentiating cells and reduces the extent of adipose conversion in those cells that do differentiate [50]. TGFβ is an active inhibitory agent described for freshly isolated rat adipose precursors but not for mature adipocytes. This differential effect may be attributable to the 10-fold decrease in cell-surface TGFβ-1-binding sites following differentiation [51].

bFGF inhibits differentiation when added with necessary inducing agents and at higher concentrations it decreases adipose gene expression in differentiated TA1 adipocytes [52]. As the bFGF effect occurs even in those cells made deficient in protein kinase C, bFGF apparently acts separately from protein kinase C. Although c-Fos mRNA is elevated by bFGF, bFGF action in adipocyte differentiation does not appear to involve c-Fos, since the elevation of c-Fos mRNA occurs at much lower concentrations of bFGF than those that inhibit differentiation. Not all agents that block differentiation cause a decrease in the levels of adipocyte-specific mRNAs in mature fat cells. Phorbol 12-myristate 13-acetate (PMA) is effective at inhibiting adipocyte conversion, but neither PMA or the calcium ionophore ionomycin alone decreases adipocyte gene expression in mature fat cells. However, the combination of PMA and ionomycin is very effective at reversing adipocyte gene expression [52].

One of the earliest identified inhibitors of adipocyte differentiation was TNFα. TNFα, secreted by activated macrophages, alters lipid and protein metabolism in adipose and skeletal muscle cells *in vivo* [53]. When added concurrently with standard differentiation agents, TNFα inhibits adipose conversion of TA1 cells as judged by triacylglycerol accumulation and levels of adipocyte-specific mRNAs [54]. A dose-dependent decrease in the number of developing fat cells has also been noted for human adipocyte precursor cells exposed to TNFα. The TNFα effect is dominant over the adipogenic effects of glucocorticoids and indomethacin in this system. In addition to its inhibition of adipocyte differentiation, TNFα also suppresses adipocyte-specific genes in mature adipocytes, leading to what has been termed 'dedifferentiation'. Treatment of mature adipose cells with TNFα, in addition to causing lipolysis, decreases the expression of some adipocyte-specific genes. Chronic exposure drastically suppresses glycerol-3-phosphate dehydrogenase activity in human adipocytes [55] and the GLUT4 glucose transporter, adipocyte fatty acid-binding protein aP2 [56], stearoyl-CoA desaturase and acyl-CoA synthetase [22] mRNAs in 3T3-L1 cells. Exposure of TA1 adipocytes to TNFα for 4–6 days causes decreases in adipose-specific mRNAs; this precedes the loss of lipid and occurs within 24 h of treatment [54]. Cells show marked delipidation, and morphological changes including long spindle-shaped cytoplasmic extensions [55]. The study of additional adipocyte-specific genes reveals that TNFα affects only a subset of adipocyte genes. In developing and mature 3T3-L1 adipocytes LPL and glyceraldehyde-3-phosphate dehydrogenase mRNAs decrease only transiently and malic enzyme mRNA levels do not change [22]. Whether the nearly complete suppression of some adipocyte mRNAs by TNFα is a permanent alteration of their phenotype is unclear. Adipocyte mRNAs do not increase upon TNFα removal following a 20-day treatment of 3T3-L1 cells [56]. Upon TNFα removal, adipocyte morphology and transcriptional activity of adipocyte genes return when examined in TA1 cells [54]. The shorter treatment time of 4–6 days for the TA1 study versus the 20-day exposure used for 3T3-L1 cells may explain the variability observed regarding the reversibility of the TNFα effects.

Suppression of only those genes related to long-chain fatty acid synthesis [22] or those transactivated by C/EBPα [57,58] are two proposed explanations for the selectivity of the TNFα effect in mature adipocytes. *Cis*-acting DNA elements for the down-regulation of adipocyte genes by TNFα have not yet been identified. TNFα treatment of fully differentiated TA1 adipocytes indicates that mRNA and protein levels for the transcription factor C/EBPα decrease before those for adipocyte-specific genes decline [57,58]. TNFα induces c-Myc expression in TA1 preadipocytes and adipocytes. This is partially blocked by antisense c-Myc oligonucleotides [59]. Additionally, the regulatory region of the C/EBPα gene contains possible c-Myc-binding sites. These observations suggest that in mature adipocytes, repression of gene expression by TNFα may occur via down-regulation of C/EBPα. That a similar mechanism is responsible for the inhibition of preadipocyte differentiation by TNFα is unclear. Any agent that blocks differentiation would, by definition, result in a lack of expression of all genes normally activated during adipocyte differentiation. The fact that one such gene, for example C/EBPα, is not expressed and the concurrent observation that adipocyte differentiation is inhibited does not in itself indicate a causal effect.

Arachidonic acid and related agents

Arachidonic acid and its metabolites have been shown to both accelerate and inhibit adipocyte differentiation, depending on the cell lines and culture conditions tested. Prostacyclin and prostaglandin E_2 inhibit differentiation of 3T3-L1 cells, and $PGF_{2\alpha}$ inhibits differentiation of adipocyte precursors in primary cultures [60]. The glucocorticoid dexamethasone, commonly used to accelerate 3T3-L1 differentiation, has been proposed to act by decreasing the production of these prostaglandins by induction of an inhibitor of phospholipase A_2, the enzyme that synthesizes arachidonic acid from phospholipids. Cyclo-oxygenase catalyses the initial step in the breakdown of arachidonic acid to its metabolites, and in 3T3-L1 [61] and TA1 [62] cells the cyclo-oxygenase inhibitor indomethacin promotes differentiation. The concentration of indomethacin required for a significant stimulation of differentiation is much greater than that needed for complete inhibition of cyclo-oxygenase. Additionally, other inhibitors of prostaglandin production in TA1 cells have no effect on their differentiation. Thus it appears that the inhibition of prostaglandin synthesis does not completely account for the effect of indomethacin on differentiation [62].

In contrast to the inhibition observed in 3T3-L1 and TA1 preadipocytes, arachidonic acid stimulates the differentiation of

Ob17 cells that already express early markers when tested in serum-free medium supplemented with growth hormone, T_3 and IGF-1 [63]. This effect is blocked by cyclo-oxygenase inhibitors and mimicked by prostacyclin and $PGF_{2\alpha}$. This indicates that metabolism of arachidonic acid to these products is probably required for the stimulatory effect on differentiation [63–65]. Prostacyclin is much more effective at promoting Ob1771 cell conversion than other cyclic AMP-potentiating agents and leads to increased intracellular Ca^{2+}, independent of cyclic AMP breakdown. This indicates that in Ob1771 cells prostacyclin acts by elevating cyclic AMP as well as by intracellular Ca^{2+} mobilization. The results obtained with Ob1771 cells may be attributable to the use of serum-free and chemically defined media, and perhaps the fact that these cells are of adult origin, rather than embryo-derived.

Role of the steroid hormone superfamily

Besides those growth factors that act through membrane receptors, the steroid hormone receptor superfamily also influences adipocyte differentiation. Their mechanism of action in adipocyte differentiation is not well characterized. However, in general, steroid hormones diffuse into cells, interact with cognate receptors and exert nuclear effects through *cis*-acting hormone-response elements. T_3 is required for optimal expression of the adipocyte phenotype in Ob17 cells cultured in serum-free, hormone-supplemented conditions, as judged by levels of adipsin, aP2 and glycerol-3-phosphate dehydrogenase mRNAs [66]. However, T_3 is not required when culture medium is supplemented with 8-bromo-cyclic AMP. This suggests that T_3 and cyclic AMP may have redundant function(s) in adipocyte differentiation [67]. Glucocorticoids stimulate adipocyte differentiation of 3T3-L1 [26], TA1 [8] and RCJ 3.1 rat calvarial cells [68].

Supraphysiological concentrations of retinoic acid (RA) inhibit adipocyte differentiation [69–71] and specifically decrease the expression of certain adipocyte mRNAs in differentiated cells [72]. Addition of 10 μM RA concurrently with inducing agents in 3T3-L1 cells does not affect the rate of cell growth or DNA replication. However, RA inhibits differentiation as assessed by triacylglycerol accumulation and levels of aP2 and stearoyl-CoA desaturase mRNAs [69]. No subsequent differentiation occurred during the 4 days following RA removal. RA addition, either prior to or after treatment with inducing agents, does not affect differentiation, indicating that RA acts at an early stage in differentiation. This finding is supported by the observation that RA treatment blocks the induction of C/EBPα mRNA that normally occurs early in differentiation [73]. RA also inhibits differentiation in 3T3-F442A cells [70,71,74]; however, in this system the RA effect is reversible. Differentiation ensues after removal of RA and growth under normal differentiation conditions, with a dose-dependent delay in the onset of differentiation. This last observation may explain the discrepancy in the 3T3-L1 and 3T3-F442A findings in regard to reversibility of RA inhibition. The effect of retinoids on adipocyte differentiation appears to be concentration-dependent. For example in Ob1771 cells, supraphysiological concentrations are inhibitory, while concentrations close to the K_d value for the RA receptor (RAR) (1 pM to 10 nM) accelerate adipose differentiation [75]. One proposed hypothesis is that at higher concentrations retinoids may bind other nuclear hormone receptors, thus indirectly influencing transcriptional events [75].

The inhibitory signals provided by high concentrations of RA are apparently balanced by stimulatory signals of fatty acids and other peroxisome proliferators. 3T3-L1 cells cultured in serum lacking lipids and retinoids continued to proliferate after control cells had reached confluence. Treatment with fatty acids or clofibrate prevents and reverses changes induced by the delipidated serum, although the inhibitory action of RA is dominant. Peroxisome proliferators apparently induce the growth arrest requisite for adipocyte differentiation. Peroxisome proliferators in serum-containing media initiate differentiation in the absence of additional inducing agents, while a 48 h exposure of preadipocytes cultured in delipidated serum with 10 μM RA induces apoptosis. Growth under serum-containing culture conditions apparently offsets the RA-induced apoptosis, perhaps through fatty acid activation of the peroxisome proliferator-activated receptor (PPAR). Since the RAR and the PPAR can heterodimerize with the retinoid X receptors (RXRs), the heterodimerization partner of the RXR may govern the differentiation process. mRNA levels for PPAR-α and the related receptors Nucl, RXR-α and RXR-γ are increased during adipocyte differentiation [76]. This suggests that preadipocytes are in a state intermediate between proliferative growth and terminal differentiation. At the molecular level, this may be co-ordinated by differential heterodimerization of the RAR and the PPAR to the RXR [77].

The connection between PPAR activation and adipocyte differentiation is further supported by two recent findings. (1) Fatty acids, activators of the PPAR, increase aP2 mRNA in preadipocytes. Here, fatty acid-mediated gene activation occurs outside of the full array of changes that occur during adipocyte differentiation. The mechanism appears to be post-transcriptional [78]. This response was specific for cells of the preadipose lineage, since NIH 3T3 and 3T3-C2 cells did not respond. Exposure of preadipose Ob1771 cells to palmitate activates the genes for aP2 and acyl-CoA synthetase, although in this system the response is primarily transcriptional [79]. Treatment during the initial 3 days post-confluence during differentiation results in a strong augmentation of cell differentiation, as reflected by subsequent increases in glycerol-3-phosphate dehydrogenase and aP2 mRNAs. The effect is apparently restricted to long-chain fatty acids and the fact that it occurs with 2-bromopalmitate, a non-metabolizable fatty acid, indicates that metabolism is not required [80]. (2) The 5′ flanking region of the aP2 gene (discussed in detail below) that is responsible for adipose-tissue-specific expression contains a binding site for the adipocyte-specific nuclear factor ARF6. The sequence of the ARF6-binding site contains a direct repeat of the hormone response element that is the preferred binding site for RXR and PPAR heterodimers. DNA binding and transactivation studies indicate that ARF6 consists of a heterodimer of RXR and a newly cloned murine adipocyte-expressed factor mPPARγ2 [27a]. This PPAR isoform may be involved in the mechanism whereby fatty acids and retinoids influence adipose differentiation and adipose-specific gene expression. It is discussed further in the section on aP2 gene expression, below.

The above observations indicate that a variety of agents influence adipocyte differentiation, with effects somewhat dependent on the cell lines and culture conditions employed. With this variability in mind, a general picture emerges that suggests that the multitude of diverse regulatory signals may converge at a few classic signal transduction pathways. The effects of T_3, glucocorticoid, RA and peroxisome proliferators make a decisive argument for mediation of adipocyte differentiation by steroid hormone superfamily receptors. The existence of cross-talk between various signalling mechanisms is demonstrated, for example, by the observation that RA inhibition of Ob1771 differentiation is accompanied by a marked decrease in T_3 receptors [82]. Studies addressing the role of arachidonic acid

and prostaglandins in adipocyte differentiation suggest the involvement of these agents, and Ca^{2+} mobilization, in adipocyte differentiation. The role of cyclic AMP is less clear. MIX accelerates the differentiation of 3T3-L1 and TA1 cells in culture. Whether MIX functions via its action as a phosphodiesterase inhibitor, thus potentiating the effects of cyclic AMP, is not resolved. In other systems MIX competes with adenosine for adenosine receptor binding [83], and may also function via calmodulin [84]. Contradictory results have been obtained in studies that address whether the cyclic AMP-elevating agents dibutyryl cyclic AMP or forskolin can replace the effect of MIX in stimulating adipocyte differentiation [61,85,86]. G-proteins mediate adipocyte differentiation in 3T3-L1 cells in a manner apparently independent of adenylate cyclase. Antisense oligodeoxynucleotides to $G_s\alpha$ accelerate adipocyte differentiation and agents that activate $G_s\alpha$ block differentiation [86]. Likewise, expression of the inhibitory subunit $G_i\alpha 2$ promotes lipid accumulation [87]. In this study, increasing intracellular cyclic AMP did not affect differentiation, leading to the conclusion that the effect of G-proteins in adipocyte differentiation may be distinct from their coupling to adenylate cyclase.

CYTOSKELETAL AND ECM COMPONENTS IN ADIPOCYTE DIFFERENTIATION

While the increases in levels of proteins related to lipid metabolism have been studied in detail, less attention has focused on the alterations in cytoskeletal and ECM components that accompany adipocyte differentiation. The effect of ECM components on cell differentiation in general is demonstrated by the decrease in tissue-specific markers that often occurs upon plating of primary cells on tissue culture dishes. These effects have been specifically demonstrated in a number of systems [88] including the induction of β-casein by basement membrane substratum during mammary epithelium development [89], apparent inhibition of differentiation by basement membrane in keratinocyte differentiation [90], and the regulation of N-CAM cell adhesion molecule gene expression by cell aggregation [91]. Cell–ECM interaction may lead to cytoskeletal network rearrangement and an intracellular cascade of signal transduction that influences differentiation. Additionally, a physical connection between the ECM and nuclear matrix, via the cytoskeleton, has been proposed [92]. A secondary effect of the ECM is its ability, through altering cell spreading, to expose the cell surface to various growth factors present in the environment [93,94]. A summary of cytoskeletal, ECM and related components with demonstrated roles in adipocyte differentiation is presented in Table 3.

Cytoskeletal and ECM alterations

The ECM of adipose tissue interconnects adipocytes and gives rise to fat-cell clusters *in vitro* and to fat lobules of adipose tissue *in vivo*. One of the first ultrastructural changes seen in *in vivo* adipocyte differentiation is the deposition of collagen at the cell–ECM border and biogenesis of an extracellular basement membrane [95]. Fibroblasts in developing fat tissue of newborn rats exhibit loose interactions with the ECM. This loose interaction may be necessary for the morphological changes that accompany differentiation and may serve to keep cells in close proximity during differentiation, thus increasing exposure to juxtacrine signals. Electron microscopy of 3T3-F442A cells reveals that preadipose cells have limited granular ECM deposits, mainly on top of the cells. In contrast, 3T3-F442A adipocytes appear interconnected by abundant ECM rods and fibres that resemble the fat-cell clusters similar to the lobules of adipose tissue [96]. Histochemical studies show that the gap junctional communication between cells is lost during differentiation. Down-regulation of a gap junction component, connexin 43, occurs prior to the expression of glycerol-3-phosphate dehydrogenase during the adipocyte differentiation of H-A/1 cells [97].

During adipocyte differentiation, drastic changes occur in cell morphology, cytoskeletal components and the level and type of ECM components secreted. The latter is evident by the increased viscosity of the medium. Studies in 3T3-L1 cells indicate that chondroitin sulphate proteoglycan-I in the medium and the cell-associated form increases [29]. Secretion of type-IV collagen and entactin/nidogen increases [28]. The relative concentrations of fibroblast-expressed type-I and type-III procollagen mRNAs decline by 80–90% during 3T3-L1 differentiation. This is via transcriptional mechanisms [98], although transcription of the

Table 3 Cytoskeletal, ECM and related molecules in adipocyte differentiation

Abbreviation: CSPG-1, chondroitin sulphate proteoglycan-1.

Molecule	Modulation during adipocyte differentiation	References
Actin	Decreases.	[30]
	Disorganization of stress-fibre patterns.	[100]
	May allow for cytoskeletal modelling.	
Tubulin	Decreases.	[30]
	May allow for cytoskeletal remodelling.	
Laminin	Production constant but differentiating cells produce unorthodox subunit in place of the A subunit.	[28]
Entactin/Nidogen	Increases.	[28]
Fibronectin	Decrease in cellular synthesis and pericellular fibronectin.	[99]
	Culture on fibronectin markedly decreases differentiation.	[103,105]
Collagen type I	Decreases.	[98]
Collagen type III	Decreases.	[98]
Collagen type IV	Increases.	[28]
Collagen type VI (α-2)	Increases at confluence and early in differentiation then gradually decreases.	[20]
CSPG-1	Increases in cell-associated and soluble forms.	[29]
Connexin 43	This gap junction protein decreases; may modulate communication between cells during differentiation.	[97]
Pref-1	Decreases. The EGF-like domains of this membrane protein are hypothesized to interact with ECM molecules to maintain a preadipose phenotype.	[107]

α-1 and α-2 chains of type-I collagen are reported not to change significantly during the adipose conversion of 3T3-F442A cells. While laminin production remains constant, cells produce an unorthodox laminin complex [28]. The amount of pericellular fibronectin, as well as cellular synthesis of fibronectin, decreases by 4–5-fold during the differentiation of 3T3-F442A cells [99]. Perhaps one of the earliest mRNA markers of differentiation is the α-2 type-VI collagen mRNA. It is first detectable upon confluence in Ob1771 cells and increases sharply following confluence in 3T3-F442A cells. It increases to maximal at 4 days post-confluence and gradually decreases to 50% of maximal levels during differentiation [20]. Many of these components, for example laminin and entactin/nidogen, are known to interact with each other, as well as with the cell surface. Modulation of ECM components could permit release of cell–cell adhesion and remodelling of cell components necessary for morphological changes that occur during adipocyte differentiation.

Cell shape is drastically altered as the fibroblast form of preadipocytes changes to the rounded cell type distinctive of mature fat cells. This is reflected at the cytoskeletal level. Immunofluorescence studies of differentiating 3T3-F442A cells indicate that F-actin fibres are highly structured in preadipocytes with a well-developed stress-fibre pattern. In adipocytes they are disassembled and disorganized with non-parallel fibres [100]. Expression of actin and tubulin decreases during differentiation [30]; this precedes overt changes in morphology and the expression of adipocyte-specific genes. These changes in cell shape reflect a distinct process in differentiation and are not the result of accumulated lipid stores. Cells induced to differentiate in the absence of biotin [101] or differentiated in the presence of lipolytic agents [102] do not accumulate triacylglycerol but still undergo biochemical and morphological differentiation.

Regulation of differentiation by ECM and other extracellular components

Subjecting preadipocytes to differentiation conditions in the presence of ECM components demonstrates that cell-shape changes, perhaps through contacts with the ECM, are requisite for differentiation. Culture of 3T3-F442A preadipocytes on fibronectin matrices decreases gene expression of lipogenic enzymes and leads to decreased triacylglycerol accumulation [103] although the differentiation-associated decrease in synthesis of actin and tubulin is still observed. The inhibitory effect of fibronectin is not observed on its addition to culture medium, and the inhibition is overcome by keeping cells in a rounded configuration. These observations indicate that the inhibitory effect of fibronectin requires cell spreading. Cytochalasin D, an agent that disrupts actin filaments, overcame the inhibitory effects of fibronectin [103], further indicating that cytoskeletal rearrangement is a prerequisite for terminal differentiation. In a separate study, differentiation of 3T3-F442A cells was accelerated by cytochalasin D treatment, perhaps by allowing earlier cytoskeletal remodelling. Furthermore, with long-term cytochalasin exposure some cells of the non-differentiating 3T3-C2 cell line undergo morphological and biochemical conversion [104]. Addition of soluble fibronectin and growth on fibronectin matrices markedly decreases differentiation of ST-13 preadipocytes. This is reversed by antibody against the $\alpha_5\beta_1$-integrin and by a short peptide corresponding to the cell attachment domain of fibronectin. Interestingly, differentiation of these cells is stimulated by addition of a thermolysin digest of fibronectin [105]. These experiments suggest that fibronectin has the ability to both inhibit and stimulate adipocyte differentiation, depending on the state of the molecule.

Use of ethyl-3,4-dihydroxybenzoate (EDHB), a specific inhibitor of collagen synthesis, demonstrates the role of collagens in modulating adipocyte differentiation. Exposure of TA1 cells to EDHB during differentiation results in a dose-dependent inhibition of differentiation as indicated by the aP2 mRNA level [106]. Whether ECM components are directly involved in initiating or transducing signals for adipose differentiation or merely allow cells differential access to the growth and/or inhibitory factors present in the surrounding environment is undetermined.

A recently described transmembrane protein, pref-1, is hypothesized to link adipocyte differentiation signals from ECM components to the cell interior [107,108]. The pref-1 mRNA is easily detected in preadipocytes, but decreases to non-detectable levels during their differentiation to mature adipocytes. A similar decrease in pref-1 protein content is observed during adipocyte differentiation. The mRNA is also absent from adult fat and most other adult tissues surveyed, with a wider, although still restricted, expression in embryos. Multiple pref-1 protein species ranging from 45 to 60 kDa are present in preadipocytes, with this heterogeneity arising from both post-translational modification and alternate splicing. Pref-1 mRNA is expressed 3–5-fold higher in the differentiation-defective 3T3-C2 cells than in 3T3-L1 cells and is down-regulated by fetal-calf serum, an essential component for adipocyte differentiation. Moreover, constitutive expression of pref-1 in 3T3-L1 preadipocytes inhibits differentiation, as judged by lipid accumulation and the levels of adipocyte mRNAs. The predicted structure of the pref-1 protein reveals six tandem EGF-like repeats in the putative extracellular domain. The EGF-like motif is characterized by an approximately 40-amino-acid stretch with conserved spacing of six cysteines and conservation at a number of other residues. Although the prototype molecule is EGF, this motif is found in a variety of proteins. These include all growth factors that bind the EGF receptor [109–111], proteins of the blood clotting cascade [112] and a variety of ECM molecules [113,114]. The unifying theme of this protein motif is mediation of protein–protein interaction. The involvement of the EGF-like motif in development is best illustrated in the Notch and Delta proteins of the fly, where specific EGF-like repeats of these proteins interact to affect a number of cell fate decisions [115]. The cysteine spacing and other amino acids within the EGF-like motif of pref-1 indicate that it would not bind the EGF receptor, and is most similar to the EGF-like motif of the Delta protein. Delta has a demonstrated role in cell fate decisions, best described in the developing nervous system. Analysis of the intron placement also suggests that the pref-1 gene is more related to invertebrate than vertebrate genes. The observation that a number of ECM components including versican, tenascin, laminin, and cell adhesion molecules contain EGF-like repeats [113,114] and the demonstrated ability of specific EGF-like motifs to mediate protein–protein and/or cell–cell interactions suggests several hypotheses for the mechanism of pref-1 action in adipocyte differentiation. Given the importance of cell shape modulation and of the ECM components collagen and fibronectin in adipocyte differentiation, pref-1 may function by interaction of its EGF-like repeats with EGF-like or other protein motifs present in ECM molecules. Such interaction may maintain the preadipocyte phenotype by blocking the requisite morphological changes or cytoskeletal remodelling.

TRANSCRIPTION FACTORS AND DNA ELEMENTS INVOLVED IN ADIPOCYTE GENE EXPRESSION

Studies of gene regulation during adipocyte differentiation address several aspects: (1) Numerous investigators utilize

differentiated adipocytes for identification of *cis*-acting DNA sequences that regulate basal activity and hormone/drug responsiveness. While outside the scope of this review, examples include insulin [116,117], cyclic AMP and glucocorticoid [85]. (2) The transcriptional activation of specific genes during differentiation. The myoblast master control gene myoD initiates myoblast differentiation and directly transactivates an array of muscle-specific genes. Identification of DNA-binding proteins that govern adipocyte differentiation-dependent gene transcription will allow investigators to work back along the regulatory hierarchy and may lead to the eventual identification of a master regulatory gene in the adipocyte programme. (3) To search for a master regulatory gene(s) that controls activation of the complete differentiation programme.

Differentiation-dependent increases in gene expression account for more than 40% of the total soluble protein of the adipocyte [118]. Considerable progress has been made in defining *cis*-acting elements that govern the expression of adipose-specific genes, with those required for the metabolic role of the adipocyte receiving much attention [22–24]. Post-transcriptional mechanisms are also involved in increasing levels of some mRNAs during adipocyte differentiation. Increased mRNA stability appears to be responsible for enhanced fatty acid synthetase mRNA expression in adipocytes [14]. A few genes that are down-regulated during the process have also been identified but are not characterized with regard to 5′ flanking sequences responsible for basal promoter activity, preadipose-specific expression or differentiation-dependent down-regulation. These include a Na^+/K^+ pump isoform [119], collagen type I and type III [98], the gap junctional protein connexin 43 [97] and the EGF-like protein pref-1 [107], which is completely absent in mature adipocytes. The mRNA for the calcium-dependent phospholipid-binding protein annexin I [120] declines by 15-fold during differentiation. Although no role is demonstrated for annexin I in the control of adipocyte differentiation, it is proposed that annexin I may inhibit phospholipase A_2 by sequestering its phospholipid substrate.

Preadipocyte cell lines have been used extensively to identify DNA sequences conferring adipocyte-specific expression on heterologous promoters via specific DNA–protein interactions. Some of the *cis*-acting elements identified in this manner resemble known transcription factor-binding sites. The majority of the studies focus on the 5′ flanking region in the control of gene expression, and regulatory elements that could be present in introns and/or in the 3′ flanking region have received little attention. With the exception of the aP2 gene, these elements have been tested only in cell culture and not in transgenic mice models. The following discussion focuses on the identification of *cis*-elements involved in adipocyte differentiation. It begins with a discussion of the transcription factor C/EBP. This is followed by recent work in less-well-characterized systems, and concludes with the identification of an adipocyte-specific enhancer in the aP2 gene.

The C/EBP family

A transcription factor extensively characterized in adipocyte differentiation is the CCAAT/enhancer **b**inding **p**rotein α (C/EBPα). C/EBPα is a 42 kDa transcription factor of the bZIP family. These proteins have a basic transcriptional activation domain and an adjoining leucine zipper motif that mediates homo- and hetero-dimerization. C/EBPα has a central role in the expression of some adipocyte genes *in vitro*. While not strictly adipocyte-specific [121], cell culture studies of adipocyte differentiation, primarily in 3T3-L1 cells, provide extensive evidence for a role for C/EBPα in adipocyte differentiation. C/EPBα expression precedes the expression of adipocyte-specific genes in *in vitro* adipocyte differentiation [27]. Co-transfection studies demonstrate that C/EBPα binds to and transactivates the promoters of several genes that are co-ordinately expressed during adipocyte differentiation. These include the aP2, stearoyl-CoA desaturase [122], GLUT-4 glucose transporter [123], phosphoenolpyruvate carboxykinase, and the insulin receptor [124] genes. Mutation of the C/EBPα site in these genes abolishes transactivation. However, no C/EBPα-binding site is present in that portion of the aP2 regulatory region that governs adipose-tissue-specific expression in transgenic mice [125,126].

Since C/EBPα possesses anti-mitotic activity, the effect of C/EBPα expression on adipocyte differentiation has been tested employing a C/EBPα–oestrogen receptor fusion protein [127]. Activation of the fusion protein alone did not trigger differentiation of preadipocytes; however, when done in concert with differentiation agents it led to the premature expression of adipose-specific genes and an acceleration of adipocyte differentiation. In separate studies, expression of C/EBPα was sufficient to convert a small fraction of preadipocytes into adipocytes in the absence of exogenous inducing agents [128]. High levels of C/EBPα expression in 3T3-L1 preadipocytes, using an inducible promoter system, activate adipocyte-specific genes and cytoplasmic lipid accumulation in the absence of exogenous inducing agents [128a]. Furthermore, expression of C/EBPα antisense RNA inhibits lipid accumulation and adipocyte conversion. Transfection of antisense-expressing cells with C/EBPα sense mRNA rescues the adipocyte phenotype [129]. The anti-mitotic activity of C/EBPα suggests it may function in growth arrest. That stably transformed preadipocytes can be established that express p30, a shorter alternative translation product of the C/EBPα gene, maps the anti-mitotic function of C/EBPα to within the first 12 kDa of the protein [130]. However, use of antisense C/EBPα to reduce C/EBPα expression blocks triacylglycerol accumulation but not the expression of the early marker LPL, evidence that C/EBPα functions in the terminal phase of adipocyte differentiation [131].

Fine-tuning of the control of adipocyte gene expression by C/EBPα involves homo- and hetero-dimerization with other C/EBP isoforms, i.e. C/EBPβ, C/EBPγ and C/EBPδ, that have similar binding specificities [27]. C/EBPβ, C/EBPγ and C/EBPδ are less well-characterized with regard to adipocyte differentiation. While expression of these three C/EBP isoforms is not strictly adipocyte-specific they are expressed to relatively high levels in cell types that possess high lipogenic activity. Each isoform has a distinct temporal and spatial expression pattern during adipocyte differentiation [27,132]. The relative ratios of the isoforms may modulate the effect of C/EBP proteins in adipocyte gene expression. A fourth protein, CHOP-10, has overall sequence similarity to C/EBP proteins in the DNA-binding and dimerization domain. However, the DNA-binding domain of CHOP-10 also has changes in two amino acid residues that are critical for DNA interaction and does not appear to bind the C/EBP consensus sequence or form homodimers [132]. Rather, CHOP-10 appears to function as a negative modulator through heterodimerization with C/EBPα. Furthermore, interaction of the C/EBP isoforms with members of other transcription factor families, including NF-κB [133] and CREB/ATF [134], adds to the complexity of gene regulation by C/EBP transcription factors.

Study of the regulation of the C/EBPα gene may link the extrinsic signals for adipocyte differentiation to the expression of adipocyte-specific genes. The C/EBPα gene is autoregulated and DNA elements that may function in the control of the C/EBPα

promoter have been identified [135]. One protein, C/EBP undifferentiated protein (CUP), binds in concert with the general transcription factor Sp1 to bipartite CUP–Sp1 sites [136]. CUP is expressed only in tissues and cell lines that do not express C/EBPα. During differentiation of 3T3-L1 cells, CUP activity or expression decreases as expression of C/EBPα increases, suggesting that CUP may repress transcription of C/EBPα. A second bipartite element consists of adjoining C/EBP and Sp1 sites and might interact with the nearby CUP–Sp1 site through a common complex to repress transcription of the C/EBPα gene. A third element contains a binding site for c-Myc, and studies indicate that c-Myc may function through C/EBPα. Expression of c-Myc blocks the normal induction of C/EBPα and prevents differentiation. Subsequent transfection of these cells with C/EBPα allows a small fraction of cells to undergo adipogenesis, indicating that C/EBPα action is dominant over c-Myc [128]. This is expected if c-Myc functions through repression of the C/EBPα gene. The identification of possible c-Myc-binding sites in the C/EBPα promoter suggests that c-Myc may act by repressing the C/EBPα promoter.

Differentiation-dependent regulatory elements of adipocyte-expressed genes

The C/EBP sites present in the 5′ flanking regions of many adipocyte genes clearly influence the expression of these genes *in vitro*. However, this element is not strictly adipocyte-gene-specific as it is also involved in expression of several liver-expressed genes. Sequences that appear to regulate gene expression during adipocyte differentiation have been identified in a number of genes. We concentrate here on those studies that identify short well-defined regulatory elements that interact with preadipocyte or adipocyte DNA-binding proteins. Whether any of these elements are restricted solely to a role in preadipocyte/adipocyte expression has not been determined. LPL is one of the first genes induced during differentiation. In most systems, an increase in LPL mRNA requires only cell confluence, and not other agents required for the expression of terminal markers. Two *cis*-elements appear important for the gradual activation of the LPL gene [137]. LP-α and LP-β, map to (-)702 to (-)666 and (-)468 to (-)430 respectively. These elements have similarity to a consensus sequence that binds the HNF-3 and forkhead transcription factors. Factors with properties similar to those of the HNF-3/forkhead family are present in the adipocyte and bind at the LP sites. However, the ability of these proteins to transactivate via these sequences is not known.

Transcriptional activation of the stearoyl-CoA desaturase-2 gene occurs during adipocyte differentiation. Reporter gene constructs and DNA-binding studies identified a preadipocyte repressor element (PRE) located between (-)435 and (-)410 [138]. The core DNA sequence of the PRE has some similarity to the β-interferon gene PRD1 elements, to which a repressor protein binds. The PRE interacts with a 58 kDa nuclear protein present at high levels in 3T3-L1 preadipocytes and HeLa cells but not detected in adipocytes. A single copy of the PRE placed upstream of the simian virus 40 (SV40) enhancer/promoter strongly represses its transcription in preadipocytes but not adipocytes. The PRE and its cognate binding protein may regulate transcription of the stearoyl-CoA desaturase-2 gene, and possibly other genes, by inhibiting their expression in preadipocytes. The angiotensinogen gene shows a developmentally regulated increase during adipocyte differentiation of 3T3-L1 cells. A 14 bp element, termed the differentiation-specific enhancer (DSE) [139], is present at (-)1000. The DSE is similar to homeotic and pou transcription factor binding sites. Binding of preadipocyte proteins to the DSE is competed for by known pou-binding sites. The DSE could serve as a developmental switch to repress expression of the angiotensinogen gene in preadipocytes.

Study of the adipsin gene indicates that the same DNA sequence can function as an enhancer or repressor element, presumably depending on the interacting DNA-binding protein(s). Transfection of a construct containing (-)950 bp of 5′ flanking region of the adipsin gene gives preferential expression in adipose versus preadipose cells. Deletional analyses identified a region from (-)114 to (-)38 that results in negative regulation of expression in preadipocytes and positive regulation in adipocytes. This area contains a large inverted repeat sequence that may form a stem-loop structure and contains multiple binding sites for nuclear factors, some that appear to be preadipose-specific. Additionally, a protein(s) induced upon adipocyte differentiation bound only single-stranded DNA [140]. The work with these systems indicates that a variety of elements may have the ability to confer differentiation-dependent gene expression; however, their cognate DNA-binding proteins have yet to be cloned. Once the cloned binding proteins are in hand, their tissue distribution, regulation during adipocyte differentiation and their ability to transactivate via these *cis*-elements can be tested.

From cell culture to mouse: the aP2 gene

Extensive characterization of the 5′ regulatory region of the aP2 gene reveals that cell culture models of gene regulation do not necessarily reflect the *in vivo* situation. The promoter proximal 168 bp of the 5′ flanking sequence confers preferential expression of reporter genes in 3T3-F442A adipocytes, but not in transgenic mice [125,126,141]. This region contains a C/EBP site and an FSE2 site. Homology of the FSE2 to the viral core enhancer AP-1 sequence [142–145], and the presence of an FSE2 site in the glycerol-3-phosphate dehydrogenase gene, suggested a potential role for this sequence element in adipocyte gene expression. The pattern of protein binding to the FSE2 site is altered upon adipocyte differentiation [125]. That c-Fos was in the protein complex from both preadipocytes and adipocytes was the first demonstration that c-Fos had a role in DNA binding [146]. As it became evident that the FSE2/AP-1 site enhances aP2 gene expression and does not confer fat-specific responses, attention shifted to other regions of the 5′ flanking sequence of the aP2 gene in the search for an adipocyte-specific/differentiation-dependent transcription factor.

A construct containing a 5.4 kb portion of the flanking region, and not those containing 168 bp, 247 bp or 1.7 kb, directs adipose-tissue-specific expression in transgenic mice. When linked to a minimal aP2 or SV40 promoter, a 540 bp fragment at (-)4.9 to (-)5.4 kb directs tissue-specific expression of a reporter gene in transgenic mice [125,126,141]. Cell culture studies map the adipose-specific enhancer to a 122 bp subfragment. This tissue-specific enhancer sequence does not contain AP-1 or C/EBP consensus sites, and co-transfections with AP1 or C/EBPα fail to transactivate reporter constructs. This newly identified fat-specific enhancer comprises several *cis*-elements needed for full activity. An NF-1-like site binds to an NF-1-like complex present in preadipocytes and adipocytes. A pair of elements, ARE6 and ARE7, binds a distinct factor ARF6. ARF6 is detected in nuclear extracts derived from adipocytes and not preadipocytes [147]. Multimers of ARE6 and ARE7 stimulate promoter activity in a strictly adipocyte-specific manner in cell culture, with mutations in ARE6 greatly reducing the activity of the enhancer. Interestingly, the ARE6 and ARE7 sites contain sequences very similar to the well-characterized hormone-

responsive element that binds to RXR-containing heterodimers [27a]. The ARF6 complex contains the RXR; and heterodimers of the RXR and a newly cloned adipocyte-expressed PPAR isoform, mPPARγ2, bind the ARE7 site. mPPARγ2 is expressed very early in differentiation of 3T3-L1 and 3T3-F442A cells, and its levels peak late in differentiation. While the mPPARγ2 mRNA is very abundant in adipose tissue, very low levels of this mRNA have been detected in other tissues, although this expression is attributed to contaminating adipocytes. Recent evidence suggests that mPPARγ2 may act as a molecular link between adipose-specific gene expression and regulation via RA and PPAR activators such as fatty acids. Under culture conditions permissive to differentiation including serum, dexamethasone and insulin, expression of a retrovirally transduced mPPARγ2 gene stimulates adipose differentiation of NIH 3T3 fibroblasts. This occurs in a dose-dependent response to PPAR activators [148].

IN SEARCH OF AN ADIPOCYTE MASTER REGULATORY GENE

With the identification of the genes of the myoD bHLH-leucine zipper transcription factor family that control myoblast differentiation *in vitro* [149], the search was on to find a master regulatory gene for adipocyte differentiation. Evidence for such a molecular switch is suggested by the observation that treatment of the mesodermal 10T1/2 cell line with demethylating agents leads to formation of adipocyte, chondrocyte, and myotube loci. The frequency of loci indicates that activation of relatively few genes is required for initiation of adipocyte differentiation [2]. Transfection of human fat cell or 3T3-F442A genomic DNA into normally differentiation-defective 3T3-C2 cells results in their differentiation when cultured in the presence of insulin. This indicates that a DNA sequence(s) present in 3T3-F442A cells and human fat is capable of initiating the adipose differentiation programme [150]. The adipose commitment activity is present in two non-identical sequences of 1.2 and 2.0 kb [151]. The mechanism by which these sequences confer adipose commitment is unknown. They may encode a gene product that directs differentiation, or allow rearrangement of the 3T3-C2 DNA to activate silent gene(s) that in turn initiate adipocyte differentiation.

The general criteria for the evaluation of candidate genes are expression in preadipocytes or very early in the differentiation programme, adipocyte-specificity, and regulation by various agents consistent with a role in adipocyte differentiation. Recent evidence suggests that two transcription factors, C/EBPα and mPPARγ2, in addition to their demonstrated effects in the differentiation of preadipocyte cells, may have a role in the initiation and/or commitment of mesenchymal precursors to the adipocyte differentiation programme. The temporal expression of mPPARγ2, prior to C/EBPα expression, suggests a sequential model of action, with mPPARγ2 hypothesized to control the expression of C/EBPα [148]. High levels of expression of C/EBPα, via retroviral transduction, promotes differentiation of NIH 3T3 and Balb/c 3T3 fibroblasts to adipocytes [151a]. Since C/EBPα is not adipocyte-specific, development to the adipocyte lineage cannot be attributable solely to C/EBPα expression. Expression of the adipocyte-specific gene mPPARγ2 stimulates adipocyte differentiation of NIH 3T3, Balb/c 3T3 and 3T3-C2 cells [148] when tested in the presence of dexamethasone and PPAR activators. However, co-expression of C/EBPα and mPPARγ2 in NIH-3T3 cells leads to adipocyte differentiation in the absence of inducing agents and/or PPAR activators. This indicates that these two transcription factors may co-operate in specifically activating the adipocyte differentiation programme [148]. Neither C/EBPα nor mPPARγ2 is detected in preconfluent preadipocytes. Therefore, initial activation of these genes may depend on environmental cues and/or a transcription factor expressed at the determination stage that subsequently activates the C/EBPα and/or mPPARγ2 genes. Several other gene products have also been hypothesized to regulate the adipocyte differentiation programme; however, as they are not extensively characterized, their possible function in the control of adipocyte differentiation remains speculative.

Exposure of 3T3-L1 cells to the inducing agents MIX, dexamethasone and insulin results in the alteration of the relative rate of synthesis of over 100 proteins [13]. The hypothesis that potential regulatory genes should be expressed very early in response to agents that initiate/promote differentiation has led to the recent identification of five differentiation-associated proteins (DAPs) whose function in adipocyte differentiation remains to be determined. The time course of their expression during differentiation and their response to various combinations of MIX, dexamethasone and insulin [152] is consistent with a possible regulatory role in adipocyte differentiation. Two early markers of adipocyte differentiation have been proposed to be involved in governing adipocyte differentiation. One of these is clone 5, a protein of unknown function. High cell density and treatment with adipogenic agents increase clone 5 mRNA levels and agents that inhibit clone 5 expression inhibit differentiation [19]. Reduction of clone 5 expression in TA1 cells by antisense RNA inhibits differentiation [153]. Clone 5 mRNA is present in differentiation-defective 3T3-C2 cells. This indicates that clone 5 expression alone is not sufficient to permit cell differentiation. Given the established role of morphogenic gradients in differentiation, it is proposed that as clone 5 rises above a threshold level, differentiation initiates. The evidence that a second early mRNA marker, α-2 collagen type VI, has a role in initiating adipocyte differentiation is somewhat circumstantial. The mRNA is expressed in white adipose tissue but not in a variety of other tissues. In Ob1771 and 3T3-F442A cells the mRNA increases as cell density increases early in adipocyte differentiation and then decreases. Two agents that inhibit adipocyte differentiation, TNFα and TGFβ, decrease α-2 collagen type VI expression [20]. It is not adipose-cell- or tissue-specific [154], and the response of adipocyte differentiation to the modulation of α-2 collagen type VI levels is not reported.

Master regulatory gene(s) identified for *in vitro* muscle cell differentiation are bHLH-leucine zipper transcription factors. Under the hypothesis that this protein motif may have a role in adipocyte differentiation, a novel adipocyte bHLH-leucine zipper factor, ADD1, was cloned using degenerate oligonucleotides [155]. While the native DNA-binding site for ADD1 is undetermined, the protein transactivates via the E-Box sequence-binding site for bHLH-leucine zipper transcription factors. ADD1 mRNA is highest in brown adipose tissue, and is detected at low levels in liver, white fat, and kidney. Its expression increases an additional 5-fold during differentiation of 3T3-F442A cells to adipocytes. The presence of ADD1 in 3T3-C2 cells, taken with the fact that its expression is not specific for adipose tissue, makes it difficult at this time to predict the role of ADD1 in adipocyte differentiation.

CONCLUSIONS

The study of adipocyte differentiation serves as a paradigm for addressing the general mechanisms involved in the expression of a terminally differentiated phenotype, as well as those specific mechanisms governing fat-cell differentiation. Current knowledge indicates that initiation and progression through the

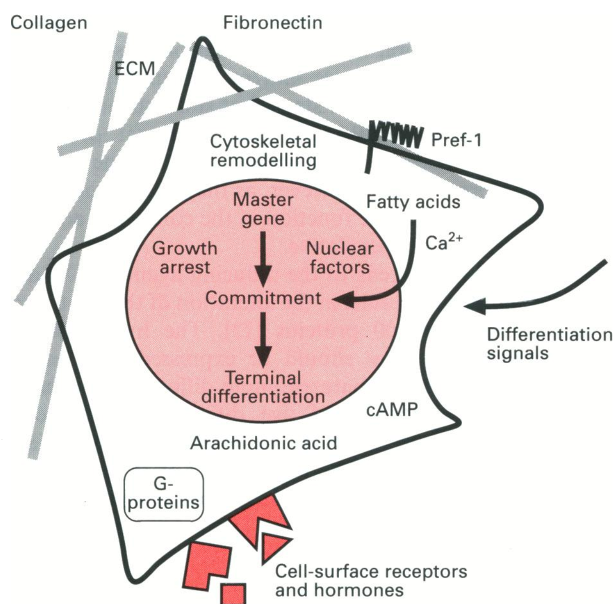

Figure 3 Summary of the multiple agents influencing adipocyte differentiation

Selected aspects of the regulation of adipocyte differentiation that have been discussed in the text are presented. Evidence to date suggests that the decision to differentiate involves the combinatorial and possibly synergistic effects of multiple differentiation signals. These include ECM components such as collagen and fibronectin, shown as rods in the upper left of the diagram. These molecules may interact with cell-surface components, for example the membrane-bound molecule pref-1, to influence requisite cytoskeletal remodelling. Other signals at the membrane include a large array of cell-surface receptors and hormones. These may be coupled to G-proteins, cyclic AMP (cAMP), or calcium mobilization. Nuclear signals include that of an as yet unidentified possible master regulatory gene. Additional nuclear influences include the novel effects of fatty acids on adipocyte gene expression, as well as multiple transcription factors discussed in the text, including C/EBPα and mPPARγ2.

adipocyte differentiation programme involves the integration of multiple signals as indicated in Figure 3. These include those that act via traditional signal transduction at the cell membrane and in the nucleus. In addition to these molecules, components of the ECM are pivotal in the differentiation process. On another level, the identification of genes that are regulated in this process, and more fundamentally genes that regulate the process, allows definition of the molecular details of adipocyte differentiation. In other systems, genes required for tissue-specific expression in the adult are crucial in the embryonic development of the corresponding organs and tissues. For example, the transcription factor Pit-1 not only controls the tissue-specific expression of some anterior-pituitary-specific genes in the adult, but also has a role in the organogenesis and development of the pituitary gland [156]. The study of those transcription factors involved in adipose tissue-specific expression may also identify proteins that function at earlier stages in adipocyte tissue development. However, in the study of adipocyte differentiation, as is often the case, the whole appears to be more that just the sum of the parts. The missing pieces to this puzzle include those studies that link the various exogenous signals for adipocyte differentiation to nuclear events, identification of a master regulatory gene(s) for adipocyte differentiation, and testing the conclusions reached in cell culture models in transgenic mice systems.

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