

Chapter 2

Adipocyte Differentiation

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Abstract Adipocyte differentiation is a highly controlled process that has been extensively studied for the last 25 years. Two different kinds of in vitro experimental models, essential in determining the mechanisms involved in adipocyte proliferation, differentiation and adipokine secretion, are currently available: preadipocyte cell lines, already committed to the adipocyte lineage, and multipotent stem cell lines, able to commit to different lineages including adipose, bone and muscle lineage. Many different events contribute to the commitment of a mesenchymal stem cell into the adipocyte lineage, including the coordination of a complex network of transcription factors, cofactors and signalling intermediates from numerous pathways. New fat cells constantly arise from a preexisting population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes. Analysis of adipocyte turnover has shown that adipocytes are a dynamic and highly regulated population of cells. Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development. Several positive and negative regulators of this network have been elucidated in recent years. This review is focused in the main molecular and cellular processes associated with adipocyte differentiation, including transcriptional factors and cofactors and extranuclear modulators. The role of epigenetic factors, microRNAs and chronobiology in adipogenesis is also summarized.

Keywords Adipocyte • Adipogenesis regulatory factors • PPAR- γ • C/EBP- α • Preadipocyte cell lines • Adipose-derived stem cells • Mesenchymal stem cells

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2.1 Introduction

Adipose tissue is characterized by a marked cellular heterogeneity: among its cellular components, we can find adipocytes, preadipocytes, fibroblasts, endothelial cells and multipotent stem cells able to differentiate into several cell types. Overall, fat tissue consists of approximately one-third of mature adipocytes. The remaining two-thirds are a combination of small mesenchymal stem cells (MSCs), T regulatory cells, endothelial precursor cells, macrophages and preadipocytes in various stages of development. Preadipocytes have the ability to proliferate and differentiate into mature adipocytes, conferring adipose tissue a constant functional plasticity, which determines its ability to expand throughout the entire lifespan.

Adipocytes, also known as fat cells and lipocytes, are found in stereotypical depots throughout the body and mixed with other cell types in some other positions, such as loose connective tissue. There are two kinds of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), both of which differ in a few significant properties. Most of our understanding about adipocyte differentiation and adipogenesis comes from *in vitro* studies of fibroblasts and preadipocytes (Rosen and MacDougald 2006). White adipocytes contain single, large lipid droplets that appear to comprise the majority of cell volume, while the cytoplasm and nucleus are found at the cell periphery. Preadipocytes that resemble fibroblasts are cultured and after differentiation is induced, the cell cultures may be used for metabolic studies. Brown adipocytes, which are characterized by multilocular lipid droplets and high mitochondrial content, are derived from distinct adipose tissue depots that are highly vascular and innervated.

Obesity can be characterized into two main types, hyperplasic (increase in adipocyte number) and hypertrophic (increase in adipocyte volume). Hypertrophy, to a certain degree, is characteristic of all overweight and obese individuals. Hyperplasia, however, is correlated more strongly with obesity severity and is most marked in severely obese individuals (Hirsch and Batchelor 1976). Prolonged periods of weight gain in adulthood may result in an increase in adipocyte number. Indeed, animal studies suggest that increases in adipocyte size precede increases in adipocyte number. Adipose hypertrophy might be diabetogenic, with two independent prospective studies showing that adipose hypertrophy is an independent risk factor for developing type 2 diabetes (Weyer et al. 2000; Lonn et al. 2010).

At the cellular level, obesity was originally considered an hypertrophic disease resulting from an increase in the fat cell number or the size of individual adipocytes. New fat cells constantly arise from a preexisting population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes. In both cases, the generation of new fat cells plays a key role in the development of obesity. Given that in adulthood, adipocyte number stays constant, and weight changes are predominantly accompanied by changes in adipocyte volume, one may conclude that at some critical point in development, the final fat cell number is attained, and after this point, no fat cell turnover occurs. Analysis of adipocyte turnover using carbon-14 dating, however, has recently shown that this is not the case, but rather that adipocytes are

a dynamic and highly regulated population of cells. New adipocytes form constantly to replace lost adipocytes, such that approximately 50% of adipocytes in the human subcutaneous fat are replaced every 8 years (Spalding et al. 2008).

Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development. Several positive and negative regulators of this network have been elucidated in recent years (Lefterova and Lazar 2009). The first hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cells convert from fibroblastic to spherical shape. These morphological modifications are paralleled by changes in the level and type of extracellular matrix (ECM) components and the level of cytoskeletal components (Gregoire et al. 1998). Mediation of the proteolytic degradation of the stromal ECM of preadipocytes by the plasminogen cascade is required for cell-shape change, adipocyte-specific gene expression and lipid accumulation (Selvarajan et al. 2001). Ectoderm-Neural Cortex-1 (ENC-1), a *Drosophila* kelch-related actin-binding protein, may also play a regulatory role early in adipocyte differentiation by affecting cytoskeletal reorganization and cell-shape change. In preadipocytes, ENC-1 colocalizes with actin filaments, and its mRNA levels are transiently increased 8–12-fold early in adipocyte differentiation, preceding peroxisome proliferator-activated receptor- γ (PPAR- γ) and CCAAT/enhancer binding protein- α (C/EBP- α) gene expression (Zhao et al. 2000).

During the terminal phase of differentiation, activation of the transcriptional cascade leads to increased activity, protein and mRNA levels for enzymes involved in triacylglycerol synthesis and degradation. Glucose transporters, insulin receptor number and insulin sensitivity also increase. Synthesis of adipocyte-secreted products including leptin, adiponectin, resistin and adipocyte-complement-related protein (Acrp30) begins, producing a highly specialized endocrine cell that will play key roles in various physiological processes.

We here review the main molecular and cellular processes associated with adipocyte differentiation. First, we summarize the main cellular models to study and characterize these fascinating cellular changes.

2.2 In Vitro Experimental Systems to Study Adipocyte Differentiation

Two different kinds of cell lines are currently available: preadipocyte cell lines, already committed to the adipocyte lineage, and multipotent stem cell lines, able to commit to different lineages including adipose, bone and muscle lineage.

2.2.1 Preadipocyte Cell Lines

3T3-F442A and 3T3-L1 cells, isolated from the Swiss 3T3 cell line, derived from disaggregated 17–19-day-old Swiss 3T3 mouse embryos, are the most frequently

used preadipocyte lines (Green and Meuth 1974; Green and Kehinde 1976). Importantly, clonal cell lines are homogenous in terms of cellular population, and their cell types are all at the same differentiation stage. This allows a homogeneous response to treatments. In addition, these cells can be passaged indefinitely, which provides a consistent source of preadipocytes for study. For all these reasons, clonal cell models are an interesting and complementary tool to animal models for the study of relevant biological questions. 3T3-F442A are generally regarded as a model with a more advanced commitment in the adipose differentiation process than 3T3-L1 (Gregoire et al. 1998). During proliferation, all preadipose cell models show a similar morphology to fibroblasts. Induction of differentiation triggers deep phenotypical changes of preadipocytes that become spherical and filled with lipid droplets, displaying many morphological and biochemical characteristics of adipocytes differentiated *in vivo*.

Ob17 cells, derived from adipose precursors present in epididymal fat pads of genetically obese (ob/ob) adult mice, are employed less frequently. In comparison to 3T3-F442A and 3T3-L1 cells, adult derivation of Ob17 cells represents a later preadipocyte stage. The derivation from an obese animal could also confer properties different from those of embryonic origin (Negrel et al. 1978).

Most available models of murine preadipocyte (3T3-L1, 3T3-F442A and Ob17), once they reach confluence and growth arrest, upon opportune hormonal induction, re-enter cell cycle and undergo several rounds of postconfluent mitosis, known as mitotic clonal expansion (MCE). This is a fundamental requirement for terminal adipocyte differentiation. In fact, blocking the entry of 3T3-L1 cells into S phase at the time of MCE completely inhibits the adipose conversion program (Tang et al. 2003). Also, inhibition of DNA synthesis in 3T3-F442A cells prevents formation of fat cells (Kuri-Harcuch and Marsch-Moreno 1983). However, confluent 3T3-F442A cells shifted to suspension culture maintain their ability to differentiate, suggesting that growth arrest but not confluency is required for adipocyte formation (Pairault and Green 1979). Similarly, C3H10T1/2 cells treated with bone morphogenetic protein-4 (BMP-4) that triggers commitment to adipose lineage undergo MCE in the presence of differentiation inducers (Tang et al. 2004).

The availability of adipose clonal cell lines and primary preadipocytes has allowed us to investigate the adipogenic or antiadipogenic potential of hormones, growth factors and various pharmacological compounds. Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail. Maximal differentiation is achieved upon early hormonal induction for 48 h with a combination of insulin, GCs and methylisobutylxanthine (MIX), which elevates intracellular cAMP levels, in the presence of fetal bovine serum. Dexamethasone (DXM), a synthetic GC agonist, is traditionally used to stimulate the GC receptor. After the first 48 h, insulin alone is required to continue the differentiation program. Interestingly, DXM is a powerful inductor of adipogenesis at early stages of differentiation, but displays antiadipogenic effects when added at later stages of adipose maturation, indicating that the effects of hormones are strictly time dependent (Caprio et al. 2007).

Differentiation of 3T3-F442A preadipocytes does not require early induction with GCs, since their commitment in adipogenesis is more advanced compared to

3T3-L1 cells. It is worthy to note that treatment of 3T3-442A cells with DXM represses adipogenesis, confirming that observed in 3T3-L1 cells exposed to GC at a later stage of adipose conversion.

2.2.2 Mature Adipocyte-Derived Dedifferentiated Fat Cells

Recently, several authors showed that mature adipocytes derived from fat tissue retain the ability to dedifferentiate in vitro into fibroblast-like cells. The culture technique developed to dedifferentiate adipocytes is known as ceiling culture (Sugihara et al. 1986; Yagi et al. 2004; Matsumoto et al. 2008; Nobusue et al. 2008). In this protocol, floating unilocular mature adipocytes adhere to the top inner surface of a culture flask filled completely with medium. After about 7 days of culture, the adipocytes change morphology, spread and show fibroblast-like shape with no lipid droplets. These cells, known as dedifferentiated fat (DFAT) cells, retain remarkable proliferative ability and are able to differentiate again into mature adipocytes both in vitro and in vivo. Human DFAT cells from human subcutaneous adipocytes do not express adipocyte markers such as LPL, leptin, glucose transporter-4 (GLUT-4) and C/EBP- α , showing low levels of PPAR- γ , C/EBP- β and C/EBP- δ transcripts. Interestingly, these cells express RUNX2 and SOX9, critical factors for osteogenesis and chondrogenesis respectively, and are able to undergo osteogenic and chondrogenic differentiation in vitro in the presence of appropriate culture conditions. Moreover, they are able to form osteoid matrix when implanted in nude mice, after osteogenic induction in vitro (Matsumoto et al. 2008). The ability of DFAT cells to proliferate and differentiate into multiple mesenchymal lineages confers to these cells the characteristics of adult stem cells.

2.2.3 Mesenchymal Stem Cells

C3H10T1/2 cells, established in 1973 from 14- to 17-day-old C3H mouse embryos, are MSCs which, following treatment with 5-azacytidine, can be differentiated into cells showing morphology and biochemical features of muscle, bone, cartilage and adipose tissue. Unlike 3T3-L1 cells, pluripotent C3H10T1/2 stem cells do not differentiate into adipocytes in the presence of adipose differentiation inducers (Konieczny and Emerson 1984). Treatment of proliferating C3H10T1/2 cells with BMP-4 is required to induce commitment to adipocyte lineage cells, which can differentiate into adipocytes when exposed to adipocyte differentiation inducers.

2.2.4 Adipose-Derived Stem Cells (ADSCs)

Adipose-derived stem cells (ADSCs) show a cell surface antigen profile similar to that observed on MSCs in adult bone marrow, but are more simple to purify, given

that their source is easily available. MSCs and ADSCs are characterized by a heterogeneous population that contains also differentiated cells, contaminating the stem cell preparation. Removal of the contaminating differentiated cells requires several passages. In fact, flow cytometer analysis shows that DFAT cells are more homogeneous than ADSCs, representing an interesting cell source for cell engineering and regenerative medicine applications (Matsumoto et al. 2008). Thanks to the adipose differentiation potential of DFAT cells, they represent a valuable cell system to study adipocyte development and metabolism, which could potentially replace conventional primary preadipocyte cultures.

ADSCs can be isolated and differentiated *in vitro* into mature adipocytes. Primary preadipocyte cultures may better reflect the context of adipose function *in vivo*, representing a suitable cellular system to confirm data deriving from preadipocyte lines. In addition, primary preadipocytes do not undergo continuous passages, hence they keep a diploid status, better reflecting the context *in vivo*. Interestingly, proliferation and differentiation of primary preadipocytes is clearly influenced by the anatomic site of the depots as well the age of the donor. In particular, aging reduces replicative ability of primary preadipocytes in cell culture. Subcutaneous ADSCs replicate and differentiate better than visceral ADSCs (Djian et al. 1983).

Cells corresponding to the adipose-derived stromal cells are defined by the following phenotype: CD31⁻, CD34⁺, CD45⁻, CD90⁺, CD105⁻, CD146⁻, and represent 70–90% of the total CD45⁻ adipose cells. Stromal Vascular Fraction (SVF) also includes endothelial cells, defined as CD34⁺/CD31⁺ cells, and macrophages, which express CD14 and CD31. Cells capable of differentiating into adipocytes are included in the CD34⁺/CD31⁻ cell fraction and do not express the MSC marker CD105 (Sengenès et al. 2005). For this reason, adipose committed preadipocytes express a specific pattern of cell surface markers, allowing selective purification by immune-magnetic beads or by flow cytometric cell sorting.

2.3 Stages of Adipocyte Differentiation

Two phases of adipogenesis have been extensively characterized:

Determination phase: This stage results in the conversion of the stem cell to a preadipocyte, which cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types.

Terminal differentiation phase: In this stage, the preadipocyte takes on the characteristics of the mature adipocyte. It acquires the machinery that is necessary for lipid transport and synthesis, insulin action and the secretion of adipocyte-specific proteins. The molecular regulation of terminal differentiation is more extensively characterized than determination because most studies have used cell lines that have a restricted potential to differentiate into other cell types. Some preadipocyte models (such as the mouse cell lines 3T3-L1, 3T3-F442A) need one or two rounds of cell division prior to differentiation, whereas others (such as mouse C3H10T1/2 and

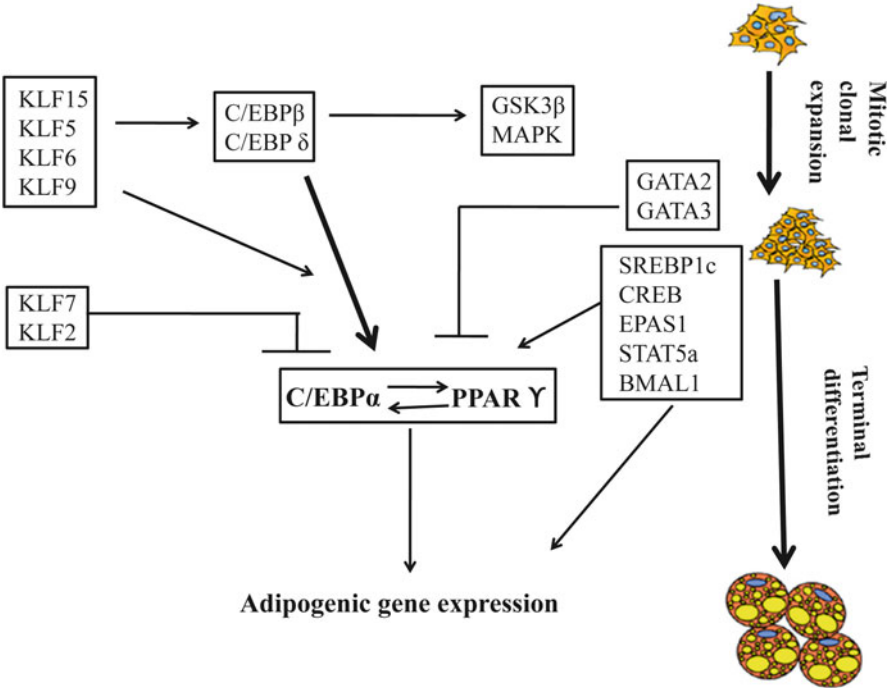


Fig. 2.1 Transcriptional regulation of adipocyte differentiation during 3T3-L1 mitotic clonal expansion and terminal differentiation

human preadipocytes) differentiate without postconfluence mitosis. In MCE of preadipocytes, cells re-enter the cell cycle and undergo several rounds of supplementary cell divisions (Ntambi and Young-Cheul 2000). These events depend on a complex coordinated cascade of cell-cycle proteins, such as members of E2F and retinoblastoma protein, that are necessary for terminal adipocyte differentiation of murine preadipocytes (Fajas et al. 2002a, b). The mitosis is believed necessary to unwind DNA, allowing transcription factors access to regulatory response elements present in genes involved in adipocyte differentiation (Cornelius et al. 1994). Growth arrest is followed by expression of final adipogenic genes. It is clear that some of the checkpoint proteins for mitosis also regulate aspects of adipogenesis.

The course of adipocyte differentiation has been well studied using cell lines and primary preadipocyte cell cultures (reviewed above). In the presence of a hormonal cocktail consisting of insulin, DXM, and 3-isobutyl-1-methylxanthine, 3T3-L1 and 3T3-F422A preadipocytes can differentiate into mature adipocyte cells, expressing specific adipocyte genes and accumulating triacylglycerol lipid droplets (Cornelius et al. 1994). Differentiation requires the activation of numerous transcription factors which are responsible for the coordinated induction and silencing of more than 2,000 genes related to the regulation of adipocyte in both morphology and physiology (Farmer 2006) (Fig. 2.1).

2.4 Nuclear Regulation of Adipocyte Differentiation

2.4.1 *Transcriptional Regulation of Adipocyte Differentiation*

Terminal adipocytes differentiation involves a series of transcriptional processes. The first stage of adipogenesis consists of the transient dramatic induction of C/EBP- β and C/EBP- δ , stimulated *in vitro* by hormonal differentiation cocktail (Ramji and Foka 2002). C/EBP- β and C/EBP- δ begin to accumulate within 24 h of adipogenesis induction and the cells re-take the cell cycle and execute MCE synchronously (Tang et al. 2003). In the conversion from G1 to S stage, C/EBP- β is hyperphosphorylated and sequentially activated by glycogen synthase kinase-3 β and mitogen-activated protein kinase (MAPK). Then, both C/EBP- β and C/EBP- δ directly induce expression of PPAR- γ and C/EBP- α , the key transcriptional regulators of adipocyte differentiation (Tang et al. 2005). PPAR- γ and C/EBP- α initiate positive feedback to induce their own expression and also activate a large number of downstream target genes whose expression determines the adipocyte. By day 2 of the differentiation course, C/EBP- α protein initiates to accumulate, and then is phosphorylated by the cyclin D3, inducing a proliferation inhibition effect on the cells, which allow to begin final differentiation phase of adipogenesis (Wang et al. 2006). By day 8 after differentiation induction, more than 90% of the adipocytes are already mature (Huang and Donald 2007) (Fig. 2.2).

C/EBP- α induces many adipocyte genes directly, and *in vivo* studies indicate an important role for this factor in the development of adipose tissue. PPAR- γ is a member of the nuclear receptor superfamily of ligand-activated transcription factors and is a prerequisite for the differentiation of both brown and white adipocytes (Kajimura et al. 2008). All the studies performed on PPAR- γ gain and loss of function models confirmed that PPAR- γ is both necessary and sufficient for fat formation (Farmer 2006). Ectopic expression of C/EBP- α in fibroblasts can induce adipogenesis only in the presence of PPAR- γ (Freytag et al. 1994). Accordingly, PPAR- γ ectopic expression can induce adipogenesis in mouse embryonic fibroblasts lacking C/EBP- α , but C/EBP- α cannot rescue adipogenesis when PPAR- γ is not expressed, showing that PPAR- γ is a master regulator of adipogenesis (Rosen et al. 2002). No factor has been discovered that promotes adipogenesis in the absence of PPAR- γ , and most pro-adipogenic factors seem to function at least in part by activating PPAR- γ expression or activity. The action of PPAR- γ is mediated through two protein isoforms: PPAR- γ 1 and PPAR- γ 2. PPAR- γ 1 is constitutively expressed, and PPAR- γ 2 expression is restricted to adipose tissue. Expression of each isoform is driven by a specific promoter that confers distinct tissue-specific expression and regulation (Zhu et al. 1995). Both isoforms are strongly induced during preadipocyte differentiation *in vitro*, and both are highly expressed in adipose tissues in animals. PPAR- γ 1 is induced earlier than PPAR- γ 2 and is maintained at high levels during adipocyte differentiation (Morrison and Farmer 1999). PPAR- γ is also required for maintenance of the differentiated state. Adenoviral introduction of a dominant-negative PPAR- γ into mature 3T3-L1 adipocytes causes dedifferentiation

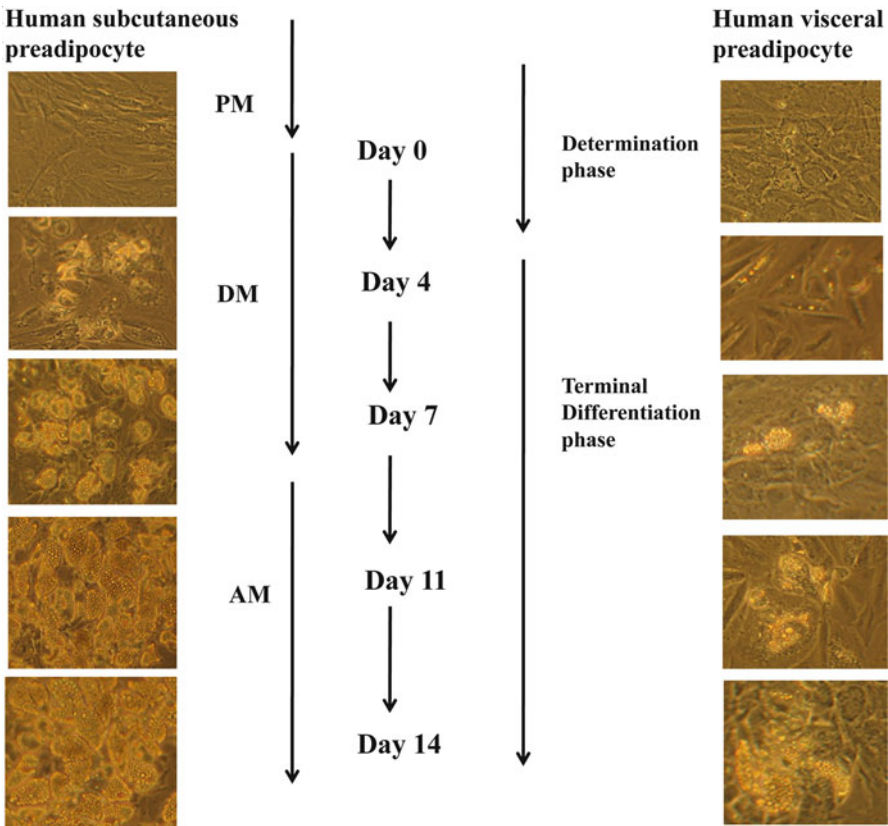


Fig. 2.2 Adipogenesis phases of human subcutaneous and visceral preadipocytes. PM is proliferation medium and composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin and streptomycin. DM is differentiation medium and composed of PM, human insulin, DXM, isobutylmethylxanthine and peroxisome proliferator-activated receptor- γ agonists (rosiglitazone). AM is adipocyte maintenance medium and composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, biotin, panthothenate, human insulin, DXM, penicillin, streptomycin and amphotericin

with loss of lipid accumulation and decreased expression of adipocyte markers (Tamori et al. 2002).

In addition to PPAR- γ and C/EBPs, several other transcription factors are likely to play an important role in the molecular control of adipogenesis. These proteins include pro- and anti-adipogenic transcription factors, and the adipocyte differentiation process is thus the result of an equilibrium between these intervening factors.

The Kruppel-like factors (KLFs) are a large family of C2H2 zinc-finger proteins that regulate apoptosis, proliferation and differentiation. The range of KLF genes that are expressed in adipose tissue, the variability in their expression patterns

during adipocyte differentiation and their effects on adipocyte development and gene expression indicate that a cascade of KLFs function during adipogenesis. For example, KLF15 promotes adipocyte differentiation (Mori et al. 2005) and induces expression of the insulin-sensitive GLUT-4 (Gray et al. 2002). KLF5 is induced early during adipocyte differentiation by C/EBP- β and C/EBP- δ and activates the *Pparg2* promoter, functioning in concert with the C/EBPs. KLF6 inhibits the expression of preadipocyte factor-1 (Pref-1) in 3T3-L1 cells and fibroblasts. Although overexpression of KLF6 is not sufficient to promote adipocyte differentiation, cells with reduced amounts of KLF6 show decreased adipogenesis (Li et al. 2005). Recently, KLF9 has been reported as a key pro-adipogenic transcription factor through regulation of PPAR- γ 2 expression with C/EBP- α at the middle stage of adipogenesis. The expression of KLF9 was markedly upregulated during the middle stage of 3T3-L1 adipocyte differentiation and inhibition of KLF9 by RNAi impaired adipogenesis (Pei et al. 2011). However, not all KLFs promote adipocyte differentiation. KLF2 and KLF7 are both anti-adipogenic factors, and KLF2 represses the *Pparg2* promoter (Wu et al. 2005; Kanazawa et al. 2005a, b). KLF factors would presumably be functioning through the differential recruitment of co-repressors and co-activators to the *Pparg2* promoter.

Sterol regulatory element binding transcription factor 1 (SREBP1c) was identified as a pro-adipogenic basic helix-loop-helix transcription factor that induces PPAR- γ expression and possibly generation of an as-yet-unknown PPAR- γ ligand (Kim et al. 1998a; Kim and Spiegelman 1996). SREBP1c also mediates the induction of lipid biosynthesis by insulin in adipocytes increasing the gene expression of the main lipogenic genes, as fatty acid synthase and acetyl-CoA carboxylase (Kim et al. 1998b).

Cyclic AMP response element-binding protein (CREB) also seems to have a possible role in the control of adipogenesis. CREB expression in 3T3-L1 preadipocytes is necessary and sufficient to induce adipogenesis, whereas silencing of CREB expression blocks adipogenesis (Reusch et al. 2000; Zhang et al. 2004). Other transcription factors that promote adipogenesis include Endothelial PAS domain Protein 1 (EPAS1) (Shimba et al. 2004), the signal transducer and activator of transcription-5a (Nanbu-Wakao et al. 2002; Floyd and Stephens 2003) and the circadian regulator Brain and Muscle ARNT-like Protein 1 (BMAL1) (Shimba et al. 2005).

Many transcription factors repress adipogenesis, including several members of the GATA-binding and forkhead families (Forkhead Box O1 (FOXO1) and Forkhead Box A2 (FOXO2)). GATA2 and GATA3, two members of the GATA family of transcription factors which are zinc-finger DNA-binding proteins involved in developmental processes, are expressed in preadipocytes and downregulated during terminal maturation (Tong et al. 2000). Forced expression of GATA2 reduces adipogenesis, and GATA2-deficient embryonic stem cells displayed enhanced adipogenic potential. Constitutive expression of GATA2 and GATA3 blunts adipocyte differentiation and traps cells at the preadipocyte stage. This inhibitory effect on adipogenesis could be mediated through reduced PPAR- γ promoter activity. Although GATA factors can bind to and inhibit the *Pparg2* promoter, a mutant GATA2 protein that does not bind to DNA retains anti-adipogenic activity by binding to C/EBPs and inhibiting their ability to transactivate *Pparg* (Tong et al. 2005).

2.4.2 *Transcriptional Cofactors in Adipogenesis*

Nuclear cofactors do not bind to DNA directly but participate in the formation of large transcriptionally active (co-activator) or inactive (co-repressor) complexes that link transcription factors to the basal transcription machinery.

Some cofactors modify chromatin directly, such as the histone acetyltransferases (HATs) and the ATP-dependent chromatin remodeling proteins of the SWI/SNF family, whereas other cofactors that do not have enzymatic activity function as platforms for the recruitment of chromatin modifiers. Many co-activators, including members of the p160 family, function as scaffolds and also have some HAT activity.

TRAP220 (or PPAR-binding protein) is a known binding partner of PPAR- γ , and the absence of this protein prevents adipogenesis (Ge et al. 2002), as well as the absence of a related co-activator called PPAR-interacting protein (Qi et al. 2003).

Another interesting example involves TATA binding protein-associated factor-8 (TAF8), which is a member of the TFIID complex of basal-promoter binding factors. TAF8 expression is upregulated during adipogenesis, and its expression is necessary for adipocyte differentiation (Guermah et al. 2003).

Several checkpoint-control proteins might also function as cofactors in adipogenesis. The cyclin D3–cyclin-dependent kinase-6 (CDK6) complex binds to and phosphorylates PPAR- γ and leads to increased transcriptional activity of PPAR- γ , which promotes adipogenesis (Sarruf et al. 2005). CDK4 also interacts with and activates PPAR- γ through the kinase domain of CDK4 (Abella et al. 2005). Conversely, cyclin D1 represses PPAR- γ activity and inhibits adipocyte differentiation (Fu et al. 2005). TAZ (transcriptional co-activator with PDZ-binding motif), represses PPAR- γ activity in adipocytes but activates RUNX2 activity in osteoblasts (Hong et al. 2005).

Some co-repressors recruit histone deacetylases (HDACs) to target promoters, thereby blocking transcription. HDACs repress adipogenesis and show coordinated reduction of expression during adipocyte differentiation. Mammalian sirtuins (SIRT1) with HDAC activity represses 3T3-L1 adipogenesis through its interaction with PPAR- γ . Other co-repressors, such as the nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptors, are anti-adipogenic, and their reduction promotes differentiation (Yu et al. 2005).

2.5 *Extranuclear Regulation of Adipocyte Differentiation*

Adipogenesis can be influenced in a positive or negative way by many hormones, cytokines, growth factors and some pharmacological compounds.

2.5.1 *Adipogenic Factors*

It is well known that insulin, insulin-like growth factor-1 (IGF-1), thyroid hormones, GCs, mineralocorticoids and PPAR- γ agonists promote differentiation.

Insulin has marked effects on adipogenesis. Downstream components of the insulin/IGF-1 signalling cascade are also crucially important for adipogenesis. The loss of individual insulin-receptor substrate (IRS) proteins inhibits adipogenesis (Smith et al. 1988; Bluher et al. 2002). Downstream effectors of insulin action cascade, such as phosphatidylinositol-3 kinase, AKT1/2 and mammalian target of rapamycin, have been shown to be involved in adipogenesis (Garofalo et al. 2003; Kim and Chen 2004). IRS signalling also promotes CREB phosphorylation, which is important for adipogenesis of cultured cells (Klemm et al. 2001).

Thyroid hormone (T3) plays a central role in normal development, differentiation and metabolic homeostasis. It is well known that thyroid hormone stimulates basal metabolic rate and adaptive thermogenesis. In mammals, there are two major thyroid receptors isoforms, thyroid receptor $\alpha 1$ (TR $\alpha 1$) and thyroid receptor $\alpha 2$ (TR $\alpha 2$), which are functionally antagonistic. T3 induced adipogenesis through TR $\alpha 1$ -induced lipogenic gene expression, whereas TR $\alpha 2$ antagonizes T3 action. In obese subjects, subcutaneous fat, with higher expression of TR $\alpha 1$, is more T3 responsive than visceral fat (Ortega et al. 2009).

GCs are potent inducers of adipogenesis *in vitro*, and hypercortisolism is associated with obesity and disturbances in fat tissue distribution (Joyner et al. 2000). GC receptors are present in human preadipocytes, and GCs activate the expression of C/EBP- δ and PPAR- γ (Wu et al. 1996). The enzyme 11- β -hydroxysteroid-dehydrogenase 1 (11BHSD1), which ensures the conversion of inactive cortisone to active cortisol (or corticosterone in rodent), is expressed in preadipocytes and adipocytes, and is thus able to sensitize adipose tissue to GCs. Interestingly, mice overexpressing 11BHSD1 in adipose tissue exhibit metabolic disturbances, including visceral adiposity, insulin resistance, dyslipidaemia and hypertension (Masuzaki et al. 2001). In contrast, mice lacking 11BHSD1 have reduced adiposity (Stewart and Tomlinson 2002). Moreover, obesity is associated with increased 11BHSD1 expression in adipose tissue in both rodents and humans (Rask et al. 2001). Locally produced cortisol may thus act in a paracrine manner to promote adipogenesis in visceral fat tissue.

Several studies have reported the effects of MAPK family members on adipogenesis with conflicting results. ERK1 is required in the proliferative phase of differentiation, and blockade of ERK activity in 3T3-L1 cells or in mice inhibits adipogenesis. Conversely, in the terminal differentiation phase ERK1 activity leads to phosphorylation of PPAR- γ , which inhibits differentiation (Bost et al. 2005). p38 MAPK is required for adipogenesis in 3T3-L1 but not in primary preadipocytes (Aouadi et al. 2006).

Some fibroblast growth factors (FGFs), as FGF1, FGF2 and FGF10, show pro-adipogenic activity on human preadipocytes, and their neutralization inhibits adipogenesis (Hutley et al. 2004).

In recent years, the influence of environmental factors on adipogenesis is being increasingly recognized. For instance, infection with human adenovirus type 36 (Ad-36) has been demonstrated to promote adipogenesis, increasing adipose tissue-induced glucose uptake in the context of increased insulin action, similar to the effects of thiazolidinodiones. Ad-36 modulated regulatory points that covered the entire adipogenic cascade ranging from the upregulation of cAMP, phosphatidylinositol

3-kinase and p38 signaling pathways, downregulation of Wnt10b expression, and increased expression of CEBP β and PPAR- γ 2 and consequential lipid accumulation via its E4 orf-1 gene (Rogers et al. 2008a, b).

2.5.2 Antiadipogenic Factors

The Wnt family of secreted glycoproteins act through autocrine or paracrine mechanisms to influence the development of many cell types. Wnt completely blocks induction of the key adipogenic transcription factors C/EBP- α and PPAR- γ . In contrast, inhibition of Wnt signalling in preadipocytes results in spontaneous differentiation, indicating that preadipose cells produce endogenous Wnt that is a potent inhibitor of differentiation. Ectopic expression of the Wnt gene potently represses adipogenesis (Ross et al. 2000). In particular, the constitutive expression of *WNT10b*, a gene which is highly expressed in preadipocytes and downregulated during the course of differentiation, inhibits adipogenesis (Longo et al. 2004). Ectopic expression of *WNT10b* stabilizes free cytosolic β -catenin and is a potent inhibitor of adipogenesis. In vivo, transgenic expression of *WNT10b* in adipocytes results in a 50% reduction in WAT mass and the development of BAT is absent. In this sense, WNT10a and WNT6 have also been identified as determinants of brown-adipocyte development.

β -catenin functions as a Wnt effector, binds to the androgen receptor and is translocated to the nucleus in response to testosterone where it interacts with the TCF/LEF transcription factors to inhibit adipogenesis. Loss of β -catenin in myometrial tissue causes its conversion to adipose tissue, which shows that the Wnt- β -catenin pathway is an important regulator of adipogenesis and mesenchymal-cell fate in vivo (Kanazawa et al. 2005a, b; Singh et al. 2006).

The transforming growth factor β (TGF β) superfamily members, TGF β , BMPs and myostatin regulate the differentiation of many cell types, including adipocytes. TGF- β is a cytokine that stimulates preadipocyte proliferation and inhibits adipogenesis in vitro. TGF β and its signalling components are expressed in cultured adipocytes and adipose tissue. Transgenic overexpression of TGF β impairs the development of adipose tissue (Clouthier et al. 1997). Blockade of endogenous TGF β signalling by inhibition of SMAD3 increases adipogenesis. SMAD3 binds to C/EBPs and inhibits their transcriptional activity (Choy and Derynck 2003). Exposure of multipotent mesenchymal cells to BMP4 commits these cells to the adipocyte lineage, allowing them to undergo adipose conversion. The effects of BMP2 are more complex and are dependent on the presence of other signalling molecules. BMP2 stimulates adipogenesis of multipotent C3H10T1/2 cells at low concentrations, but favors chondrocyte and osteoblast development at higher concentrations. Myostatin, positively or negatively regulates adipogenesis in vitro, depending on the type of cell and culture conditions (Rebbapragada et al. 2003).

Pref-1 is a transmembrane protein that belongs to a family of epidermal-growth-factor-like repeats containing proteins and is activated by proteolytic cleavage (Villena et al. 2002). Pref-1 cleavage releases an extracellular moiety that inhibits

adipogenesis, possibly through interaction with Notch. Expression of Pref-1 is high in preadipocytes and normally declines during differentiation, and forced Pref-1 expression in 3T3-L1 cells blocks adipogenesis. A soluble form of Pref-1 is sufficient to decrease adipose tissue mass and insulin sensitivity (Lee et al. 2003). Pref-1 is implicated in the regulation of adipogenesis by FOXA2 (Wolfrum et al. 2003), KLF2 (Li et al. 2005) and KLF6 (Wu et al. 2005).

Exposure of preadipocytes to pro-inflammatory cytokines inhibits adipogenesis by reducing PPAR- γ and C/EBP- α expression and by blocking insulin action. TNF- α and IL-1 suppress adipose conversion by activation of the TAK1/TAB1/NIK cascade, which in turn inhibits PPAR- γ activity (Suzawa et al. 2003). In fact, cytokines have the potential to decrease adipocyte numbers through multiple points in the adipogenic program and by activation of several distinct intracellular signalling pathways (Constant et al. 2006; Lumeng et al. 2007; Yarmo et al. 2009).

Some drugs show a strong influence on adipogenesis. Highly active antiretroviral therapy on human immunodeficiency virus (HIV) infection, has been associated with metabolic syndrome including insulin resistance, dyslipidemia, peripheral lipoatrophy and visceral adiposity (Leow et al. 2003). Studies in cell culture have shown that several protease inhibitors, for example nelfinavir and indinavir, decrease preadipocyte differentiation and lipogenesis, while increasing apoptosis and lipolysis (Dowell et al. 2000; Lenhard et al. 2000; Zhang et al. 1999). In addition, studies in patients with HIV-associated lipoatrophy display an increase in pro-inflammatory cytokines in adipose tissue, suggesting that the reducing effects of protease inhibitors on adipogenesis could be the consequence of the local overproduction of these cytokines (Bastard et al. 2002; Kannisto et al. 2003).

Metformin, a widely prescribed drug in the treatment of patients with type 2 diabetes, inhibited the differentiation of mouse 3T3-L1 cell line and primary human preadipocytes, decreasing lipogenic gene expression and increasing AMPK activity and glucose intake (Lenhard et al. 1997; Huypens et al. 2005; Alexandre et al. 2008; Fischer et al. 2010). Metformin effects on human adipocytes are likely to mediate through organic cationic transporter 1, which is induced during adipocyte differentiation (Moreno-Navarrete et al. 2011).

2.5.3 Other Players in the Regulation of Adipogenesis

2.5.3.1 Epigenetic Factors in Adipogenesis

Epigenetic regulation plays a critical role in several differentiation processes and possibly in adipocyte differentiation (D'Alessio et al. 2007). Recently, differentiation of 3T3-L1 cells was demonstrated to be associated with genome-wide epigenetic changes, as evidenced by the ratio of demethylation/methylation and furthermore maintenance of a static demethylated/methylated state, both of which depend on differentiation phase (Sakamoto et al. 2008). DNA methylation might be associated with the course of determination phase.

In addition, the study of 3T3-L1 cells using microarray-based integrated method clarified that adipogenesis is regulated by a ras homologue guanine nucleotide exchange factor (RhoGEF, WGEF) expression through DNA methylation change (Horii et al. 2009). Furthermore, like DNA demethylation, the methylation of histone H3 lysine 4 was related to transcriptional activation. In order to detect the change of histone methylation, 3T3-L1 fibroblast cells were treated with low dose of the methyltransferase inhibitor methylthioadenosine, which eliminates this epigenetic sign from the promoters, and generates a significant decreased adipogenesis, therefore, suggesting the crucial role of this histone modification in the regulation of adipocyte differentiation (Musri et al. 2006). The transcription factors and co-regulators involved in preserving appropriate levels of histone methylation and modification at the late adipogenic genes remain unknown. Above all, the role of DNA and histone modification in adipogenesis is very important, and some functions remain unknown.

2.5.3.2 The Role of miRNAs in Adipogenesis

MicroRNAs (miRNAs) are small non-coding RNAs that bind to regulatory sites of target mRNA and modify their expression, either by translational repression or target mRNA degradation, resulting in decreased protein production. MiR-143 was the first miRNA associated with regulation of adipocyte differentiation. Its expression increases in differentiating adipocytes, and antisense oligonucleotides against miR-143 inhibit human-cultured adipocyte differentiation and lead to a decrease in triglyceride accumulation and the downregulation of PPAR- γ 2, adipocyte fatty acid binding protein and GLUT-4. Several miRNAs (including miR-103, miR-107 and miR-143) are induced during adipogenesis, which may play a role in accelerating adipocyte differentiation, and then be downregulated in the obese state. Conversely, miR-222 and miR-221 are decreased during adipogenesis but upregulated in obese adipocytes. Forced miR-103 and miR-143 expression accelerate the rate of 3T3-L1 differentiation, increasing triglyceride accumulation and the expression of many adipocyte important genes at early stages of adipogenesis (Xie et al. 2009).

miRNA378/378 is highly expressed during adipocyte differentiation. Overexpression of miRNA378/378 during adipogenesis also increased triglyceride triacylglycerol accumulation, and lipogenic genes, PPAR- γ 2 and GLUT-4 expression. In addition, in the presence of microRNA378/378, C/EBP- α and C/EBP- β activity on the GLUT-4 promoter was increased (Gerin et al. 2010).

The miRNA expression profile has been recently demonstrated to change during adipocyte differentiation (Ortega et al. 2010). These authors found a differential expression of 70 miRNAs during adipocyte differentiation. In addition. The miRNA expression profile of visceral and subcutaneous adipose tissue is different in obese and non-obese subjects (Ortega et al. 2010; Klöting et al. 2009). A genome-wide miRNA profiling study of 723 human miRNAs have disclosed the expression of 40 (in preadipocytes) and 31 (in adipocytes) mature miRNAs that significantly differed according to obese status. The expression pattern of 22 miRNAs in human subcutaneous adipose

tissue was also associated with parameters of adipose tissue physiology, glucose metabolism and obesity status. This study revealed that miRNAs may constitute biomarkers for obesity and obesity-related complications. For example, some miRNAs (miR-221, miR-125b, miR-34a and miR-100) were upregulated in fat depots from obese subjects and downregulated during adipocyte differentiation. On the contrary, miR-185 was upregulated in mature adipocytes while downregulated in obese men. Others, as 130b and miR-210, were both downregulated during adipocyte differentiation and in fat depots from obese subjects. Only miR-34a was found to be positively upregulated during adipogenesis and associated positively with BMI (Ortega et al. 2010).

2.5.3.3 Chronobiology in Adipogenesis

Some clock genes, especially *Bmal1* and *Rev-Erba*, may play a part in adipocyte differentiation and lipogenesis. It has also been shown that clock genes can oscillate accurately and independently of the central nervous system in human AT explants and that this intrinsic oscillatory mechanism may participate in regulating the timing of other clock-controlled gene such as *PPAR-γ* and GC metabolism genes. Moreover, these circadian patterns differ between visceral and subcutaneous AT depots (Gómez-Santos et al. 2009; Hernández-Morante et al. 2009).

A number of adipocyte-specific factors show rhythmic expression. Some examples are leptin, adiponectin, resistin, adiponectin and visfatin, all of them showing circadian rhythmicity. For example, adiponectin shows both ultradian pulsatility and a diurnal variation (Gómez-Abellan et al. 2010). Recently, nocturnin, a circadian-regulated gene, has been demonstrated to promote adipogenesis by stimulating *PPAR-γ* nuclear translocation and enhancing its transcriptional activity (Kawai et al. 2010).

2.6 Future Perspectives

This review provides a brief overview on various adipocyte cell lines that could be used in appropriate experiments to gain insight in the molecular mechanisms that underlie adipocyte differentiation. The selection and use of an *in vitro* system must consider all known levels of regulation of proliferation, differentiation and function to ensure relevant results.

The information summarized here concerning intracellular pathways and nuclear and extranuclear modulators of adipocyte differentiation is continuously expanding. Further research is necessary to gain insight in the molecular processes that are involved in adipocyte differentiation, connecting extranuclear and nuclear mediators. New areas, as epigenetic, microRNAs and circadian clock, also need to be more investigated. An in-depth knowledge of adipocyte differentiation is absolutely essential to gain insight in the treatment of important metabolic diseases associated

with obesity and adipose tissue expandability, such as type 2 diabetes, atherosclerosis, cardiovascular disease and cancer.

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