

Michael E. Symonds *Editor*

Adipose Tissue Biology

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Chapter 1

Adipocyte Precursors: Developmental Origins, Self-Renewal, and Plasticity

Christian Dani and Nathalie Billon

Abstract The current epidemic of obesity and overweight has caused a surge of interest in the study of adipose tissue formation. Much progress has been made in defining the transcriptional networks controlling the terminal differentiation of preadipocytes into mature adipocytes. However, the early steps that direct mesenchymal stem cells down the adipocyte lineage remain largely unknown. Similarly, the study of the developmental origin of adipocytes during embryogenesis has been largely disregarded until now. This review summarizes the surprising findings that have recently emerged from in vivo lineage tracing studies, unraveling unsuspected developmental origins for white adipocytes. We will propose that the differential origin of adipocytes could also reflect functional differences and site-specific regulations of adipose tissue. This chapter also reports recent work that has led to the identification of discrete immature cell populations from which white adipocytes are derived in mice.

A pool of adipocyte progenitors remains present in adipose tissue during adult life. This pool is responsible for the renewal of adipocytes and the potential of this tissue to expand in response to chronic energy overload. However, factors controlling proliferation and differentiation of human adipocyte progenitors are largely unknown. We will present stem cells derived from human adipose tissue (human Multipotent Adipose tissue Derived Stem (hMADS) cells) for studying proliferation and differentiation of adipocyte progenitors and will show that fibroblast growth factor 2 and activin A are key regulators of human adipocyte precursor self-renewal. Finally, we will discuss about the plasticity of hMADS cells.

Keywords Adipocyte precursors • Stem cells • Adipose tissue • Adipocyte development

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1.1 Introduction

Two adipose tissues with different functions coexist in humans, i.e. white and brown adipose tissues. White adipose tissue (WAT) is mainly involved in energy storage and mobilization. WAT is localized in various sites of the body, has an enormous capacity for expansion, and excess of fat accumulation is associated with metabolic disorders. Brown adipose tissue is specialized in energy expenditure. It is a key thermogenic organ, and brown adipocytes burn fat. We will concentrate in this chapter on adipocyte precursors (APs) giving rise to white adipocytes.

The main cellular components of WAT are mature adipocytes and stromal-vascular cells, which include immune cells, endothelial cells, and APs. Expansion of WAT during normal development and in obesity is the result of an increase in size and number of adipocytes. As mature adipocytes do not divide *in vivo*, regeneration of adipocytes and the increase in adipocyte number depend on self-renewal of a pool of APs that remains present during adult life and that can be recruited to form new fat cells (Hauner et al. 1989; Spalding et al. 2008). Therefore, characterization of the cellular and molecular events involved in the generation of APs and the identification of factors regulating their self-renewal could provide a means for better understanding the mechanisms that lead to hyperplasia and excessive development of adipose tissue.

Adipogenesis is described as a two-step process. The first step consists in the generation of APs, also named preadipocytes, or adipose-derived stem cells depending on their potential to differentiate in adipocyte only or in additional cell types. The second step involves the terminal differentiation of these precursors. Key events controlling terminal differentiation of preadipocytes into adipocytes have been identified. Transcription factors such as CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) are known to play a critical role in this process, whereas Wnt and Hedgehog signaling pathways are critical regulators of terminal differentiation (Rosen and Spiegelman 2000; Longo et al. 2004; Fontaine et al. 2008). Terminal differentiation has been extensively studied (Rosen and MacDougald 2006) and will not be reviewed in this chapter. We will focus on the earliest steps of adipogenesis, i.e. the generation of APs and regulators of their self-renewal and plasticity (Fig. 1.1).

1.2 Developmental Origins of Adipocyte Precursors

Strikingly, the study of the developmental origin of APs has received very little attention until now. APs are generally described to derive from mesenchymal stem cells (MSCs), which themselves are thought to arise from mesoderm. It is worth noting that during development of higher vertebrates, the mesoderm is not the only germ layer source of mesenchymal cells. In the head, for instance, the facial bones have been shown to derive from the neural crest (NC). The NC is a vertebrate cell

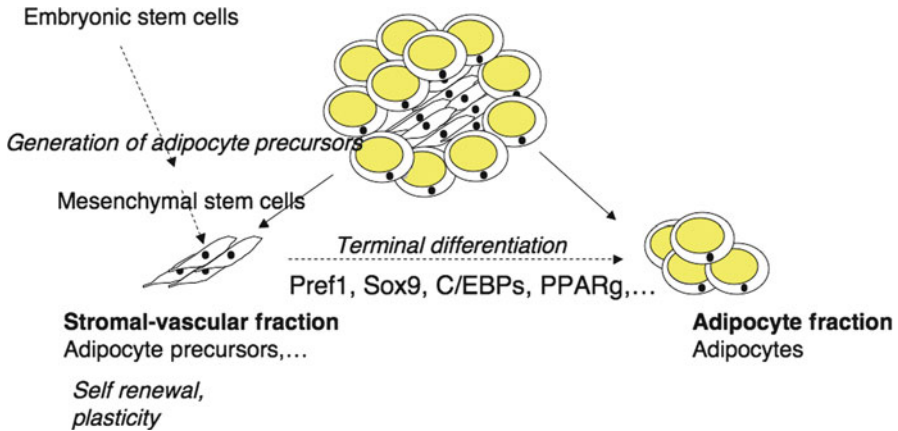


Fig. 1.1 Different steps of the adipocyte development. Adipose tissue is composed of mature adipocytes and stromal-vascular cells including adipocyte precursors (APs). Key events controlling terminal differentiation of APs have been identified. The developmental origins of APs, factors regulating AP self-renewal, as well as the plasticity of APs are discussed in this chapter

population that arises from the neuroectoderm. After neural tube closure, NC cells (NCCs) undergo an epithelio–mesenchyme transition and migrate to diverse regions in the developing embryo, where they differentiate into various cell types. In the head and neck, the NC also yields mesenchymal precursors differentiating into connective tissue cells (reviewed in Dupin et al. 2006). Adipogenesis of mouse embryonic stem (mES) cells in vitro provided a powerful model to investigate the earliest steps of adipocyte development and revealed the surprising conclusions regarding the ontogeny of such cells in the NC.

1.2.1 Adipocyte Development in Mouse Embryonic Stem Cells

Mouse embryonic stem cells (mESCs) are proliferating, pluripotent stem cells that have been isolated from the epiblast of blastocyst-stage mouse embryos. They can be propagated indefinitely at the undifferentiated state in vitro. Furthermore, when transplanted into a mouse blastocyst, mESCs integrate into the embryo and contribute to all cell lineages, including germ cells (Smith 1992). When aggregated to form embryoid bodies (EBs) in vitro, they undergo differentiation in ectodermal, mesodermal, and endodermal derivatives (Keller 1995). In addition, ESCs are easily genetically modifiable and can be produced in large numbers, thus offering a unique cell culture model to study the earliest steps of mammalian development. Directed differentiation of mESCs towards the adipocyte lineage was first accomplished in 1997 by Dani et al. (1997), who showed that functional adipocytes could be obtained when mESCs were exposed to appropriate extracellular cues. In this system, the generation of adipocytes is dependent on an early and transient exposure of EBs to

retinoic acid (RA) and a subsequent treatment with conventional adipogenic factors (e.g., insulin, triiodothyronine, and rosiglitazone). Both, lipogenic and lipolytic activities, as well as high levels of expression of adipocyte-specific genes, could be detected in mESC-derived adipocytes. Remarkably, the sequence of expression of key transcription factors known to govern preadipocyte differentiation, such as members of the C/EBP and the PPAR families, was closely conserved during mESC adipogenesis. Thus, this model has provided a powerful system to address the different steps of adipocyte development (Wdziekonski et al. 2003, 2006, 2007; Billon et al. 2010; Schulz et al. 2009; Carnevalli et al. 2010; Tong et al. 2000; Takashima et al. 2007). More recently, adipocytes have been obtained from human ESCs and from human induced pluripotent stem (iPS) cells using a protocol based on mESC studies (e.g., Xiong et al. 2005; Taura et al. 2009; T. Mohsen-kanson and C. Dani, unpublished data).

1.2.1.1 Mesenchymal Stem Cells and Adipocytes Developing from RA-Treated mESCs Derive from the Neuroectoderm, Rather Than from the Mesoderm

In a first attempt to unravel the events underlying the formation of mesenchymal derivatives in RA-treated mES cells, Kawaguchi et al. (2005) examined the expression of various mesodermal and mesenchymal markers in early EBs. Surprisingly, they noticed that treatment with RA resulted in a sharp reduction in several mesodermal markers, as well as in the suppression of cardiomyocyte formation, suggesting that RA reduces overall mesoderm formation in mESCs (Kawaguchi et al. 2005). Since at high concentrations, RA was shown to promote neural differentiation of mESCs and since some mesenchymal tissues are known to be generated by the NC, which itself derives from neuroectoderm, these authors then analyzed the expression of various NC markers in mES cells. They showed that *sox9*, *sox10*, *foxD3*, and *runx2*, which all play an important role in NC formation and/or mesenchymal condensation, were upregulated upon RA treatment. Together, these data suggest that neuroectoderm/NC is the major source of mesenchymal cells in RA-treated mESCs. To test this hypothesis with respect to adipocytes, we have developed a genetic lineage selection approach in mESCs, which is outlined in Fig. 1.2. We used genetically engineered, selectable *Sox2-βgeo/oct4-tk* mESCs that allow selection for neuroepithelial precursors (*Sox2*⁺) and eliminate residual undifferentiated mESCs (*Oct4*⁺). After induction of neural differentiation via RA treatment, highly enriched populations of neuroepithelial cells were selected in the presence of G418 and Gancyclovir. We then exposed them to adipogenic signals and showed that indeed, they could give rise to mature adipocytes within 14 days. Interestingly, a significant increase in *sox9*, *sox10*, and *FoxD3* mRNAs was observed prior to adipocyte formation, suggesting that NC-like cells present in the selected population could undergo adipocyte differentiation (Billon et al. 2007, 2008). Together, these data suggest that neuroectoderm/NC is the major source of adipocytes, at least in mESCs exposed to RA (Fig. 1.2a).

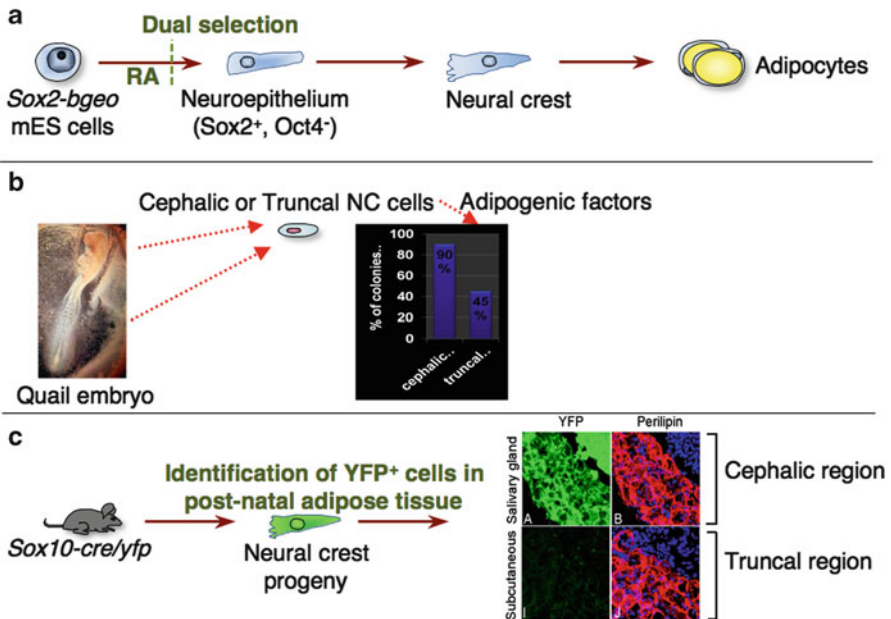


Fig. 1.2 Subset of adipocytes that originated from the neural crest. (a) Genetic selection strategy used for the generation of adipocyte of neuroepithelium origin in mESCs. (b) Adipogenic potential of cephalic and truncal NCCs isolated from quail embryo. (c) Permanent genetic lineage-labeling approaches used in mouse to reveal NC-derived adipocytes in adult cephalic adipose tissues

These findings were later corroborated by Takashima et al. who used an elegant approach to unravel the NCC origin of MSCs in both mESC culture and during mouse development (Takashima et al. 2007). All together, these studies suggest that MSCs, as well as adipocytes generated from RA-exposed mES cells, arise from the neuroectoderm/NC, rather than from the mesoderm.

1.2.2 Study of Adipocyte Precursor Developmental Origins in Quail and Mouse Embryos

To better understand adipocyte lineage specification from the NC, we checked whether adipocytes could be obtained from NCCs isolated from a normal developing embryo. We used primary cultures of quail NCCs, since they have been instrumental in establishing the developmental potentialities of the NC. NCCs were isolated from both the cephalic and thoracic level and maintained in culture media permissive for adipocyte differentiation (Rodriguez et al. 2004). This analysis revealed that typical mature adipocytes could readily be produced from cephalic

NCCs and, to a lesser extent, from truncal NCCs (Billon et al. 2007). Therefore, quail NCCs from both the cephalic and the thoracic level exhibit an adipogenic potential *in vitro* (Fig. 1.2b). Finally, we have used a lineage tracing approach in mouse to address the origin of the adipocyte lineage *in vivo* and to provide direct evidence for the contribution of the NC. We have investigated whether subsets of adipocytes originate from the NC using *Sox10-cre/yfp* transgenic mice to map NC derivatives *in vivo* because to date, Sox10 is considered as the best bona fide NC marker. Indeed, Sox10 is strongly and specifically expressed in the NC from early embryonic development and is not expressed in mesoderm. This study revealed adipocytes derived from NC in cephalic adipose depots, between the salivary gland and the ear area. In contrast, no NC-derived adipocytes could be detected in truncal adipose depots, including subcutaneous, perirenal, periepididymal, and interscapular tissues (Fig. 1.2c). These data therefore provide new information about the ontogeny of the adipocyte lineage and demonstrate that during normal development, a subset of adipocytes in the face originates from NC, and not from mesoderm (Billon et al. 2007). The role of RA in the early steps of adipocyte development remains to be demonstrated *in vivo* in mouse. Interestingly, RA has recently been shown to be required for differentiation of cephalic NCCs into adipocytes in developing zebrafish embryos (Li et al. 2010), which is reminiscent of the role of RA in mESC adipogenesis. Due to the lack of specific markers of undifferentiated APs in the studies described above, these cells were functionally traced by the appearance of adipocytes, or identified *a posteriori*, by their potential to differentiate into adipocytes. The AP phenotype allowing their identification in a prospective manner, as well as their tissue localization in adult mice, has recently been addressed (see below).

1.3 Cellular Origins and Tissue Localization of Adipocyte Precursors

Recently, Graff and Friedman laboratories performed critical experiments to identify and localize APs in mouse adipose tissue. Rodeheffer et al. used Fluorescence Activated Cell Sorting (FACS) analysis to isolate various cellular subpopulations from stromal-vascular fraction (SVF) and tested their adipogenic potential both *in vitro* and *in vivo* after transplantation in lipoatrophic A-Zip mice. By this approach, the authors identified mouse APs in the SVF of adipose tissue as $lin^{-}/CD34^{+}/CD29^{+}/sca-1^{+}/CD24^{+}$ cells (Rodeheffer et al. 2008). Whether APs originated from NC or from mesoderm display the same immunophenotype remains to be determined. By a different approach, based on the expression of PPAR γ in SVF of adipose tissue, (Tang et al. 2008) isolated undifferentiated cells able to undergo adipogenesis *in vitro* and *in vivo* in *nude* mice. These cells express markers of preadipocytes but not those of mature adipocytes, indicating that PPAR γ can also be used to trace APs. Interestingly, these cells are $CD45^{-}/Ter119^{-}/CD34^{+}/sca1^{+}$, indicating that they are similar, if not identical, to cells isolated by Friedman laboratory. Thanks to the expression of a reporter gene under the control of PPAR γ promoter, APs have

been localized in the mural cell compartment of adipose tissue vasculature in mice (Tang et al. 2008). The immunophenotype of human APs has not yet been fully characterized, although they have been shown to reside in the CD34⁺/CD31⁻ subpopulation of stromal-vascular cells of adipose tissue (Sengenès et al. 2005).

APs are resident in adipose tissue, but other sources have been recently reported. Bone marrow appeared to be a source of APs in the adipose tissue as it has been reported that a small subpopulation of adipocytes in WAT might arise from bone marrow progenitors (Crossno et al. 2006). More recently, a hematopoietic origin of APs has also been proposed. Indeed, clonal analysis and cell sorting-based studies of hematopoietic progenitors suggested that adipocytes could be derived from hematopoietic stem cells via progenitors for monocytes/macrophages or via myeloid intermediates in mice. These conclusions are supported by previous studies showing that the phenotypes of adipocyte and macrophages are closed. Interestingly, hematopoietic-derived adipocytes seem to accumulate with age in visceral fat depot, where they display higher expression of inflammatory genes than “conventional” adipocytes (Sera et al. 2009; Majka et al. 2010; Cousin et al. 1999). The contribution of these nonresident APs on metabolic diseases remains to be determined.

1.4 Do Adipocyte Precursors Produced from Different Sources Differ in Their Biological Properties?

It is well established that APs isolated from different depots display different features in terms of proliferation, differentiation, and gene expression profiles (Tchkonina et al. 2007; Gesta et al. 2006). In addition, adipocytes derived from these APs have different functional properties and have different contributions to metabolic diseases (Montague et al. 1998). The cellular and molecular mechanisms underlying these fat depot-dependent differences are currently unknown. However, several observations suggest that developmental mechanisms contribute to regional variation in function. Therefore, studies on the origins of APs open at least two questions: are adipocytes derived from different developmental origins or cellular sources functionally different? And what are the developmental origins of APs in humans? As adipocytes can be now generated from human ESCs and from human iPS cells (Xiong et al. 2005; Taura et al. 2009; T. Mohsen-kanson and C. Dani, unpublished data), studies of APs properties related to their cellular and developmental origins can be now addressed in human cellular models.

1.5 Self-Renewal of Human Adipocyte Precursors

A pool of APs remains present in adipose tissue during adult life. This pool is responsible for the renewal of adipocytes and the potential of this tissue to expand in response to chronic energy overload. Therefore, the identification of factors

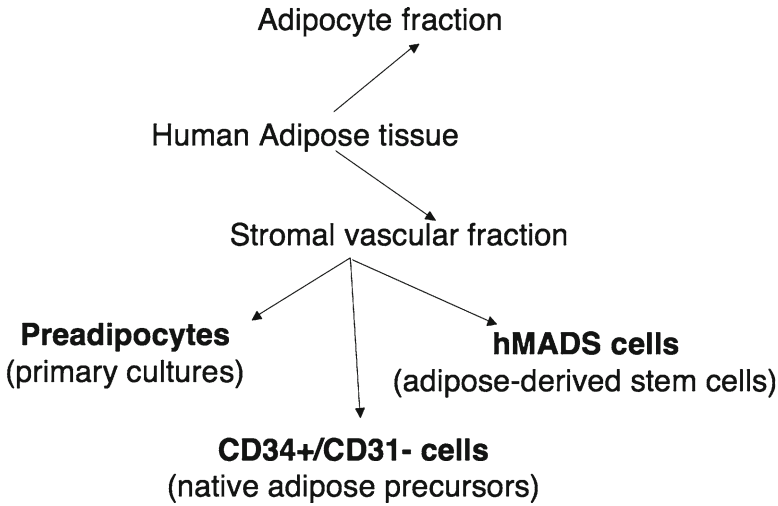


Fig. 1.3 Three types of adipocyte precursors isolated from the stromal-vascular fraction of human adipose progenitors

regulating self-renewal of APs cells could provide a means for better understanding the mechanisms that lead to hyperplasia and excessive development of adipose tissue and could ultimately be translated into clinical interventions. Ex vivo and in vitro cellular models are used to gain insight into cellular and molecular mechanisms of early steps of adipogenesis.

1.5.1 Cellular Models to Investigate Self-Renewal of Human Adipocyte Precursors

Three types of AP populations can be isolated from the SVF of human adipose tissue (Fig. 1.3) to study the regulation of human adipogenesis. Primary cultures of preadipocytes derived from SVF of adipose tissue, although being able to differentiate into adipocytes in vitro, undergo a dramatic decrease in their ability to differentiate, and replicative senescence occurs with serial subculturing, making it difficult to investigate molecular mechanisms in a fully reproducible manner. It has been clearly demonstrated that native APs are contained in the CD34⁺/CD31⁻ cell population. This population can be easily isolated from the SVF using the immunoselection/depletion protocol as previously described (Bourlier et al. 2008). Finally, human Multipotent Adipose tissue Derived Stem (hMADS) cells are adipocyte progenitors isolated from the SVF of infant adipose tissues (Rodriguez et al. 2005a). As these cells display the characteristics of MSCs, they have been termed hMADS cells.

1.5.1.1 Human Adipose-Derived Stem (hMADS) Cells

hMADS cells exhibit the capacity to self-renew, as cells can be expanded in vitro for more than 160 population doublings (i.e., around 30 passages) while maintaining a normal diploid karyotype. They also differentiate under serum-free adipogenic condition into cells able to exhibit characteristics of human fat cells (Rodriguez et al. 2004). Within 14 days after induction of adipocyte differentiation, more than 90% of cells accumulate intracellular lipids present as multiple droplets. These cells express the major molecular markers, key transcription factors, and nuclear receptors of human white adipocytes. Then, after differentiation, they exhibit the panoply of lipolytic responses, which are characteristic of human adipocytes. Interestingly, hMADS cells respond to the atrial natriuretic peptide, a unique characteristic both in vitro and in vivo of adipocytes from primates (Lafontan et al. 2000). An important feature of differentiated hMADS cells is their ability to secrete leptin and adiponectin within values reported for isolated human adipocytes. More recently, hMADS cells have been described as a faithful model to study human fat cell metabolism (Poitou et al. 2009; Bezaire et al. 2009). Altogether, these data indicate that hMADS cells commit to the adipose lineage at a high rate and differentiate into cells that display a unique combination of properties similar, if not identical, to those of native human adipocytes making them a powerful cellular model to investigate human adipogenesis.

1.5.2 *Fibroblast Growth Factor 2 (FGF2) and Activin A, Both Secreted by hMADS Cells, Are Key Regulators of Self-Renewal*

Regarding factors regulating proliferation and differentiation of hMADS cells, it has been shown that FGF2 plays a crucial autocrine role (Zaragosi et al. 2006). Analysis of FGF2 secretion revealed that FGF2 is exported to hMADS cell surface without being released into the culture medium, suggesting a strictly autocrine loop. Indeed, treatment of FGF2-expressing hMADS cells with PD173074, a specific FGF receptor inhibitor, decreased dramatically their clonogenicity and differentiation potential. Thus, hMADS cells express a functional autocrine FGF loop that allows maintenance of their self-renewal ability in vitro. Inhibition of Mitogen-Activated Protein Kinase (MEK1) reduced the clonogenic potential of hMADS cells but did not affect their differentiation potential, indicating that the Extracellular Signal Regulated Kinase (ERK)1/2 signaling pathway is partly involved in FGF2-mediated self-renewal. FGF1 is also expressed in human adipose tissue (Widberg et al. 2009). However, the involvement of FGFs in human WAT growth remains to be investigated. Activin A is expressed in the SVF of human adipose tissue and is secreted by undifferentiated hMADS cells and by preadipocytes isolated from different human fat depots. However, its expression is down regulated as soon as cells undergo adipocyte differentiation and is not only a marker of undifferentiated cells

but plays also a functional role in proliferation as observed by activin A supplementation and activin A knockdown expression (Zaragosi et al. 2010). Altogether, data support the hypothesis that activin A represents a novel crucial player controlling self-renewal of human adipose progenitors. We have proposed a model in which activin A is involved in the maintenance of the pool of adipose progenitors in adipose tissue of lean subjects by promoting proliferation and inhibiting differentiation. The molecular mechanisms involved in activin A effects have been identified. Sustained activation or inhibition of the activin A pathway impairs or promotes adipocyte differentiation via C/EBP β -LAP and Smad2 pathway, respectively, in an autocrine/paracrine manner (Zaragosi et al. 2010). It has been proposed recently that the bone morphogenetic protein pathway, which shares signaling components with the activin pathway, regulates both adipose cell fate determination, differentiation of committed preadipocytes, as well as function of mature adipocytes in mouse models (Schulz and Tseng 2009). Altogether, these data support the hypothesis that the Smad pathway regulates different steps of adipogenesis. Therefore, we propose a model in which FGF2 and activin A, both secreted by undifferentiated cells, are involved in the maintenance of the pool of APs in adipose tissue by promoting proliferation and inhibiting differentiation.

1.5.2.1 Regulation of AP Self-Renewal by Obese Adipose Tissue Microenvironment

Obesity is associated with new macrophages that are recruited into adipose tissue and is accompanied by chronic low-grade inflammation in this tissue (Weisberg et al. 2003; Xu et al. 2003). Interestingly, an increase in the proportion of CD34⁺/CD31⁻ cells exhibiting proliferative potential is observed in obese adipose tissue (Maumus et al. 2008). In addition, it has been recently reported that the differentiation potential of human preadipocytes is inversely correlated with obesity, whereas the pool of precursors cells was positively correlated to BMI (Permana et al. 2004; Isakson et al. 2009), suggesting that the obese microenvironment is capable of inducing proliferation of human preadipocytes while inhibiting their differentiation. Concordantly, human macrophages conditioned medium stimulates proliferation of human preadipocytes in vitro (Lacasa et al. 2007; Keophiphath et al. 2009). Therefore, a model of cross talk between APs and macrophages, in which immunoinflammatory cells that accumulate within adipose tissue with obesity might contribute to fat mass enlargement through paracrine effects on APs, can be proposed. We observed that levels of secreted activin A and of FGF2 are dramatically increased in hMADS cells maintained in the presence of factors secreted by macrophages isolated from obese adipose tissues. Adipose-tissue macrophage secreted factors involved in stimulation of activin A expression remain to be identified. IL-1 β and/or TNF α are potent candidates as previous studies have shown in other cell models that activin A secretion is increased upon treatment with these two cytokines (Mohan et al. 2001). Therefore, we propose a model in which FGF2 and activin A, both secreted by undifferentiated cells and induced by signals secreted from

adipose tissue-derived immune cells, are involved in the maintenance of the pool of APs in adipose tissue by promoting proliferation and inhibiting differentiation. The disappearance of macrophages (Cancello et al. 2005), and by consequences, the reduction of activin A levels in adipose tissue, for instance as a consequence of dieting, might be favorable to the formation of additional adipocytes from adipose progenitors upon ending dietary restriction, a situation reminiscent of the “yoyo” phenomenon. Related with the concept of adipose tissue expandability (Sethi and Vidal-Puig 2007), blocking activin A signals that prevent differentiation of APs in adipose tissue of obese patients could represent a new therapeutic avenue to increase the number of new adipocytes and therefore to decrease the accumulation of fat in ectopic tissues not specialized to store large amount of triglycerides. Further studies are required to validate activin A as a candidate biomarker for obesity and associated metabolic complications.

Finally, we would like to point out that APs are also present in skeletal muscles. In 2010, two papers have been published showing a critical role of undifferentiated APs in muscle of mouse models (Uezumi et al. 2010; Joe et al. 2010). Proliferation and differentiation of APs seem to be controlled in healthy skeletal muscle. However, in several pathological situations including obesity, type II diabetes, aging, and muscular dystrophies (Wren et al. 2008; Goodpaster and Wolf 2004), APs undergo adipocyte differentiation, and adipocytes accumulate and replace a large proportion of muscle fibers. As previously described in human adipose tissue, APs are contained in the CD34⁺ cell population of human skeletal muscle (Pisani et al. 2010). However, it is not known whether skeletal muscle and adipose tissue APs are identical. Nevertheless, clinical knowledge of muscular dystrophy disease may lead to the identification of new regulators of AP biology.

1.6 Plasticity of Human Adipocyte Precursors

Zuk et al. first reported that human adipose tissue contains a population of uncharacterized cells, harvested by liposuction, able in vitro to undergo adipogenic, osteogenic, chondrogenic, and myogenic differentiation (Zuk et al. 2001, 2002), suggesting that APs could be multipotent stem cells. A few years later, isolation and characterization of hMADS cells demonstrated that human adipose tissue is a rich source of multipotent stem cells (Rodriguez et al. 2005a, b). hMADS cells display the potential to undergo differentiation into adipocytes, osteoblasts, and chondrocytes at the single cell level (Rodriguez et al. 2005a; Zaragosi et al. 2006) (Fig. 1.4). The plasticity of hMADS cells led us to investigate their therapeutic potential. Actually, transplantation of hMADS cells into *mdx* mouse, an animal model for Duchenne muscular dystrophy, results in substantial expression of human dystrophin on a long-term basis, and engraftment takes place in non-immunocompromised animals (Rodriguez et al. 2005a). hMADS cells have a weak intrinsic myogenic potential. However, ectopic expression of MyoD1 dramatically increases the ability of hMADS cells to form myotubes in vitro and in vivo (Goudenege et al. 2009).

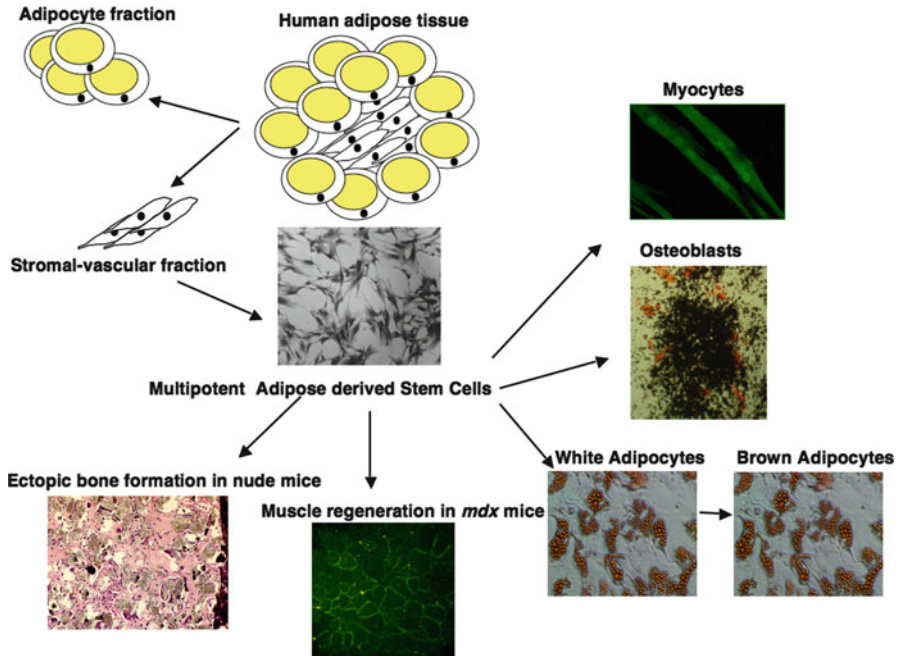


Fig. 1.4 Plasticity of human adipose derived Stem (hMADS) cells. hMADS cells are isolated from the stromal-vascular fraction of young donor adipose tissues. In vitro, they can undergo differentiation into osteoblasts, skeletal myocytes (after ectopic expression of MyoD gene), and white adipocytes, which can turn into brown adipocytes. In vivo, they are able to contribute to muscle regeneration after transplantation into mdx mice or to form ectopic bone after subcutaneous transplantation

When transplanted with a scaffold, hMADS cells are able to form ectopic bone in mouse, suggesting that cells can be used for bone repair (Elabd et al. 2007). More recently, culture conditions to turn hMADSc-white adipocytes into brown adipocytes have been reported (Elabd et al. 2009). Upon chronic exposure to a specific PPAR γ agonist, but not to a PPAR β/δ or PPAR α agonists, white adipocytes derived from hMADS cells are able to switch to a functional brown phenotype by expressing uncoupling protein 1 (UCP1) protein. This switch is accompanied by an increase in oxygen consumption and uncoupling. The existence of a common precursor for white and brown adipocytes has been a debate for several years. Recently, elegant experiments in mouse have reported the surprising findings of a common precursor between brown adipocytes and skeletal myocytes while white adipocytes derived from a different lineage (Timmons et al. 2007; Seale et al. 2009). These studies also report the existence of a second type of brown adipocytes, localized in WAT, that do not derive from an adipocyte/myocyte precursor. The ability of hMADS cells to differentiate into both white and brown adipocytes strongly suggests that a common precursor for these two types of adipocytes may exist in humans.

Altogether, the plasticity of APs suggests that these cells could be an important tool for cell-mediated therapy. They also represent an invaluable cell model to screen for drugs stimulating the formation and/or the uncoupling capacity of human brown adipocytes that could help to dissipate excess caloric intake of individuals.

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Chapter 2

Adipocyte Differentiation

José María Moreno-Navarrete and José Manuel Fernández-Real

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, hyperplastic (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

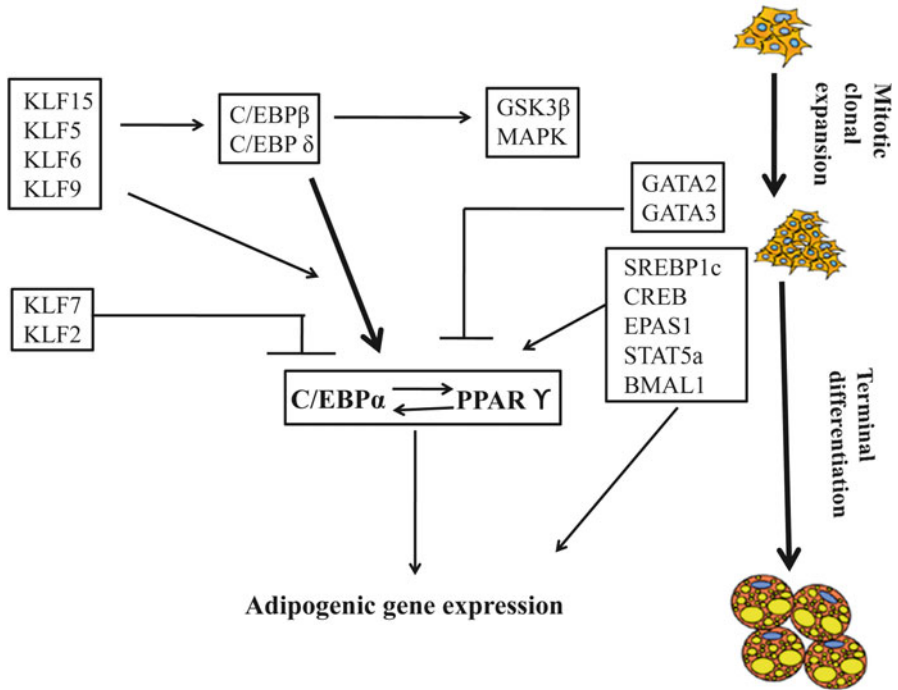


Fig. 2.1 Transcriptional regulation of adipocyte differentiation during 3T3-L1 mitotic clonal expansion and terminal differentiation

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).