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Prostaglandins, Leukotrienes and Essential Fatty Acids 73 (2005) 31–34
Prostaglandins Leukotrienes AND Essential Fatty Acids
www.elsevier.com/locate/plefa

The transcriptional basis of adipocyte development

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Abstract

Adipogenesis is the developmental process by which a multipotent mesenchymal stem cell differentiates into a mature adipocyte. This process involves a highly regulated and coordinated cascade of transcription factors that together lead to the establishment of the differentiated state. In the presence of the correct hormonal cues, committed pre-adipocytes express the bZIP factors C/EBPb and C/EBPd. These factors in turn induce the expression of C/EBPa and peroxisome proliferator-activated receptor g (PPARg). C/EBPa and PPARg together promote differentiation by activating adipose-specific gene expression and by maintaining each others expression at high levels. We have investigated the relative contributions of PPARg and C/EBPa to adipogenesis by selectively ablating these genes in mouse embryonic fibroblasts (MEFs). MEFs that lack C/EBPa are able to undergo adipogenesis, but only when PPARg is ectopically expressed. Interestingly, these cells are not sensitive to the metabolic actions of insulin. By way of contrast, cells that lack PPARg are utterly incapable of adipogenic conversion, even when supplemented with high levels of C/EBPa. Our current investigations are centered on the identification of novel adipogenic transcription factors, utilizing a variety of techniques, ranging from BAC transgenics to computational approaches. These approaches will be discussed, along with the roles of some new transcriptional players in adipogenesis, including the O/E family of proteins.

Adipose tissue has traditionally occupied a lowly position for biologists and clinicians, appreciated primarily for its insulatory attributes and as a depot for storing excess calories. This capacity to store energy has earned increasing notoriety for adipose tissue, as we have become ever fatter and more susceptible to the sequelae of obesity, including type 2 diabetes, cardiovascular disease, and certain cancers [1,2]. The ''obesity epidemic'', as it has been popularly dubbed, has pushed the adipocyte into the limelight, and has led to intensive investigations into all aspects of fat cell biology, including adipogenesis.

Most of what we know about adipogenesis comes from studies using a handful of cultured cell lines. Perhaps the best studied model is the 3T3-L1 system, developed by Howard Green and his colleagues in the 1970s [3,4]. These murine cells appear morphologically and proliferate like fibroblasts. If they are grown to confluence and then treated with an empirically derived cocktail of ''pro-adipogenic'' agents like insulin, dexamethasone, and a phosphodiesterase inhibitor, they undergo differentiation over the course of approximately a week. This in vitro model of differentiation appears to be quite faithful to the in vivo process, inasmuch as the cultured cells accumulate lipid, express virtually all markers of mature adipocytes, and become insulin sensitive. Their ready availability, lack of heterogeneity (as contrasted to the native fat pad, which contains a large number of non-adipocytes), and synchronous development all contribute to their utility. Nonetheless, these cells are only a model, and lessons learned from their use must be tested in vivo or in other cellular systems (e.g. mouse embryonic fibroblasts). Additionally, 3T3-L1 pre-adipocytes are already committed to the adipocytic lineage. This means that they can provide no real insight into the process of determination, which must be studied in vivo or in multipotent cells such as NIH-3T3 fibroblasts or C3H10T1/2 cells.

The differentiation of all specific cell types depends upon the coordinated regulation of cascades of transcription factors. Each of these proteins is responsible

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doi:10.1016/j.plefa.2005.04.004

for inducing downstream transcription factors or genes that typify the differentiated phenotype. In fact, most of the transcriptional regulators of adipogenesis (or of other differentiation programs such as myogenesis) seem to operate in a "feed-forward" fashion, whereby they induce both other pro-adipogenic factors and then cooperate with those factors to promote downstream gene expression. An example would be PPARγ (discussed in greater detail below), which induces the expression of the adipogenic transcription factor C/EBPα and then binds with C/EBPα to the promoter/enhancer of the gene encoding the adipocyte fatty acid binding protein aP2 (FABP4) [5,6].

The first transcription factors found to participate in adipogenesis were members of the bZIP family of CCAAT/enhancer binding proteins (C/EBPs), specifically C/EBPα, C/EBPβ, and C/EBPδ. Upon the addition of adipogenic cocktail to 3T3-L1 pre-adipocytes, there is a rapid and transient elevation in C/EBPβ and C/EBPδ [7,9] The appearance of these proteins is followed rapidly by the expression of C/EBPα, and by the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ). These two factors remain elevated for the rest of the differentiation process and indeed for the life of the mature adipocyte. C/EBPα and PPARγ are the most critical factors yet discovered for adipogenesis, as will be discussed below. For the moment, suffice it to say that virtually every single downstream target gene whose expression defines the adipocyte appears to be regulated by one or both of these proteins.

All of the aforementioned factors, C/EBPα, C/EBPβ, C/EBPδ, and PPARγ, can induce or accelerate differentiation in non-adipogenic NIH-3T3 cells [7]. These gain-of-function studies have demonstrated the sufficiency of these proteins for adipogenesis, and combined with the temporal sequence of the appearance of these factors already mentioned, suggests a transcriptional cascade whereby C/EBPβ and δ induce PPARγ and C/EBPα, which in turn promote the terminally differentiated state.

On the basis of these studies, important questions arose about the relative roles of PPARγ and C/EBPα in adipogenesis. To answer this, loss-of-function models needed to be generated. For C/EBPα, early studies with anti-sense oligonucleotides suggested that reducing the expression of this factor would inhibit fat cell formation in vitro [10]. We used mouse embryonic fibroblasts (MEFs) from C/EBPα null embryos (see below) to study this issue in greater detail [11]. These cells had little capacity for differentiation, and in particular did not express much PPARγ mRNA. When PPARγ expression was restored with retroviral gene transfer, however, they differentiated well, suggesting that C/EBPα plays an important role in adipogenesis primarily by inducing the expression of PPARγ. Ectopic expression of PPARγ circumvents the requirement for C/EBPα in adipogenesis, at least with respect to lipid accumulation and most gene expression. Interestingly, C/EBPα $-/-$ adipocytes created by ectopic expression of PPARγ are not insulin sensitive, a deficiency that could be restored by re-introducing C/EBPα. The reason for this is at least in part due to reduced expression of insulin receptor and IRS-1 in the absence of C/EBPα, as well as a poorly characterized post-receptor defect that prevents optimal tyrosine phosphorylation of proximal components of the insulin signaling cascade. There is also a downstream effect of the absence of C/EBPα centered on abnormal subcellular localization of Glut4 in the absence of insulin (Tim McGraw, personal communication). Farmer and colleagues have noted a similar phenomenon; NIH-3T3 cells that ectopically express PPARγ will differentiate into adipocytes but will not demonstrate normal insulin-stimulated glucose uptake unless C/EBPα is also added [12].

Attempts to extend these findings in vivo, however, were initially hampered by perinatal lethality of homozygote C/EBPα null mice, which perish due to hypoglycemia secondary to a failure of hepatic gluconeogenesis [13]. Two different groups subsequently developed ways to work around this problem. One group expressed C/EBPβ from the endogenous C/EBPα locus (so-called C/EBPβ/β mice) [14]. This rescued the hepatic phenotype, presumably because the requirement for a C/EBP in liver is not restricted to the C/EBPα isoform. In white fat, however, C/EBPα function could not be replaced by C/EBPβ, and these animals had small fat pads with reduced lipid content per cell. There was also dramatically reduced expression of some adipocyte genes, such as those encoding leptin and adipsin, while others like *Glut4* and *Fabp4* were not altered. Interestingly, brown fat deposits were not affected to nearly the same degree as white fat. Darlington and colleagues confirmed most of these findings by transgenically restoring C/EBPα expression to liver only in C/EBPα null mice, which rescued the pups from a hypoglycemic death [15]. These animals also showed significant reductions in white fat, although there were important depot-specific differences. In particular, there was no subcutaneous or visceral fat, but intramammary white adipose tissue was still present. As with the C/EBPβ/β mice, brown fat was largely normal.

Loss-of-function studies with PPARγ were also made more difficult by unexpected morbidity among null embryos. Unlike C/EBPβ null mice that survive until birth, PPARγ $-/-$ embryos die at approximately e10, due to a failure of normal placentation [16,17]. Because most adipogenesis occurs post-natally in rodents, this model was not a particularly useful tool. To circumvent this difficulty, we studied chimeric mice made by mixing PPARγ $+/+$ and $-/-$ ES cells [18]. The tissues of these mice develop normally, but the relative contribution of each ES cell genotype allows inferences to be made

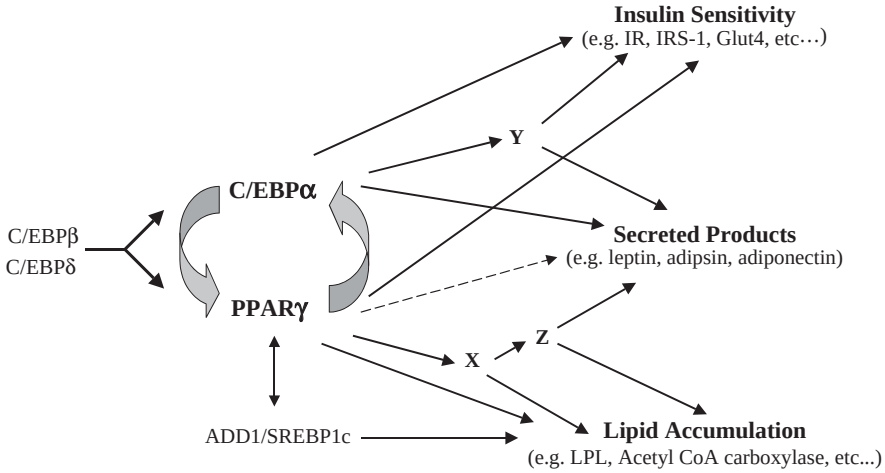

Fig. 1. A model of the transcriptional cascade leading to adipogenesis. C/EBPβ and C/EBPδ induce the expression of C/EBPα and PPARγ, which in turn promote the expression of the differentiated state. X, Y, and Z denote factors acting downstream of PPARγ and C/EBPα which are postulated to cooperate with those factors in adipocyte-selective gene expression. The dashed line refers to the inhibitory actions of PPARγ on some genes, including leptin and adipsin.

about the importance of PPARγ to the development of any specific cell type or organ. Using this approach, we were able to show that PPARγ is required for the differentiation of adipose tissue in vivo. We also performed in vitro differentiation of wild-type and PPARγ $-/-$ ES cells. PPARγ $+/+$ cells were able to undergo adipogenesis with high efficiency and reproducibility, while PPARγ $-/-$ cells never accumulated lipid, expressed markers of differentiation, or acquired insulin sensitivity.

These results were corroborated by other groups using different approaches. In one set of experiments, PPARγ $-/-$ primary MEFs were shown to be incompetent for differentiation [16] while another group used tetraploid rescue of placenta function to allow the birth of a single PPARγ $-/-$ pup [17]. This animal died shortly after birth, but necropsy showed no obvious brown or white adipose stores.

As mentioned earlier, lack of C/EBPα could be compensated for by the ectopic expression of PPARγ. This begged the question, then, of whether PPARγ was absolutely required for adipogenesis, or whether it's absence could be similarly compensated for by the ectopic expression of C/EBPα. We generated a MEF cell line from embryos carrying a PPARγ allele flanked by loxP sites [19]. These cells were immortalized using a standard 3T3 protocol, and then infected with an adenovirus expressing cre recombinase. This resulted in the generation of PPARγ $-/-$ fibroblasts that are much more tractable than the ES cell models previously discussed. These cells do not differentiate when treated with pro-adipogenic inducers unless PPARγ is added back with a retroviral vector. In contrast, the addition of C/EBPα does not allow differentiation in the absence of PPARγ. Our conclusion was that PPARγ is absolutely required for adipogenesis, but C/EBPα plays a more ancillary role that consists primarily of keeping PPARγ levels elevated in the cell. C/EBPα also plays an important role in maintaining insulin sensitivity in differentiated adipocytes.

One important challenge that we face now is understanding the genetic regulatory circuits that promote and define the differentiated state (see Fig. 1). Part of this entails the identification of other transcription factors that act upstream, downstream, or in parallel with the C/EBPs and PPARγ in adipocytes. Certainly, a large number of transcription factors are known to be expressed in adipocytes in vitro and in vivo, including members of virtually all families and classes of these proteins. Despite this broad array of factors, little is known about the roles played by any of them in promoting or maintaining the differentiated state. The helix-loop-helix (HLH) factor SREBP1c has been shown to be highly expressed in adipocytes, and to promote differentiation when over-expressed [20]. This may occur in part through direct actions of SREBP1c on target promoters (including that of PPARγ, and also indirectly by inducing the generation of a ligand for PPARγ [21,22]. The absence of SREBP1c does not, however, impair adipogenesis in vivo.

In the last few years, putative roles in adipogenesis have been proposed for a wide array of transcription factors, including LXRα [23], FBI-1 [24], KLF-15 [25] and the O/E family of bHLH proteins [26]. Most of these proteins appear to act upstream of PPARγ, as ascertained by their appearance prior to the induction of PPARγ, their ability to induce the expression of PPARγ, C/EBPα, or both, and their inability (when tested) to promote adipogenesis in cells lacking PPARγ.

We are also searching for factors that act downstream of PPARγ and C/EBPα, utilizing a wide variety of techniques ranging from differential display to screening adipocyte cDNA libraries in PPARγ $-/-$ adipocytes. We are also attempting to identify *cis*-regulatory elements that enable tissue-specific gene expression in adipocytes. These approaches include DNAse hypersensitivity mapping, BAC transgenics, and, in collaboration with Eric Lander and colleagues, computational strategies to identify over-represented motifs flanking adipose-selective genes.

We believe that these techniques and others will help us to piece together the developmental pathway by which cells become differentiated to mature adipocytes, and will also shed light on the physiological and pathological roles played by these cells in health and disease.

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