Vitamin D and adipogenesis: new molecular insights

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The focus of the current review is to highlight some new insights into the molecular mechanism by which vitamin D, a potentially nutritionally modulated factor, influences adipogenesis. Recent studies, predominantly using the mouse 3T3-L1 pre-adipocyte cell culture model, have shown that the role of vitamin D in inhibiting adipogenesis is mediated at the molecular level through a vitamin D receptor (VDR)-dependent inhibition of CCAAT enhancer binding protein-alpha (C/EBPα) and peroxisome proliferator-activated receptor-gamma (PPARγ) expression and a decrease in PPARγ transactivating activity in the pre-adipocyte. The latter action may reflect a vitamin D-induced decrease in endogenous PPARγ-ligand availability and a competition between VDR and PPARγ for a limiting amount of retinoid X receptor (RXR), a common heterodimeric binding partner of both nuclear receptors.

INTRODUCTION

The process of cell type-specific differentiation from multipotent mesenchymal stem cells is complex. The relative balance of different molecular factors determines whether a mesenchymal stem cell will undergo adipogenesis, osteogenesis, or myogenesis. This initial determination step, of which we know few details, seals the fate of the cell along a given cell lineage. In the case of adipogenesis, a mesenchymal stem cell develops into a pre-adipocyte, which can undergo terminal differentiation into a mature adipocyte, but has lost the ability to become any of the other cell types. During the process of terminal differentiation, the pre-adipocyte undergoes changes in morphology, biochemical expression, and cellular function as it is transformed into a mature adipocyte, which is able to transport and synthesize lipids, respond metabolically to insulin, and secrete adipocyte-specific proteins (Figure 1).

The process of adipogenesis requires a sequential series of gene expression events. However, specific molecular factors that may be critical during one phase of differentiation may not be as important at another phase. For example, cyclic AMP-dependent ligand activation of PPARγ is necessary early in the initiation of differentiation, but it is not required for maintaining PPARγ-dependent gene expression in the mature adipocyte.

In addition to a series of complex intracellular factors that regulate adipogenesis, a large number of extracellular factors have also been identified that can influence adipogenesis. In light of the global obesity epidemic, there is increasing interest in the role of potentially modifiable factors in adipogenesis. The focus of the current review is to describe some recent insights into the molecular mechanism by which vitamin D, a potentially nutritionally modulated factor, influences adipogenesis.

OBESITY AND VITAMIN D STATUS

Accumulating evidence suggests there is a potential link between obesity and vitamin D insufficiency among many populations worldwide. Although there is a substantial body of circumstantial evidence that excess body fat is associated with an increased risk of suboptimal vitamin D status, it is unclear to what extent poor vitamin D status is a consequence of obesity or is in some way involved in its development.
Figure 1 Vitamin D and adipogenesis. Current evidence suggests that as pre-adipocytes differentiate from spindle-like fibroblast-like cells into mature lipid storing functional adipocytes (left boxed panel) 1,25-dihydroxyvitamin D and the vitamin D receptor (VDR) may play an important role in adipogenesis. An early response to the adipogenic stimulus in pre-adipocytes is a rapid increase, then decrease, in the availability of VDR, which creates a short window of opportunity for 1,25-dihydroxyvitamin D to influence differentiation of pre-adipocytes into adipocytes. These early changes in VDR expression are accompanied by an increase in the transcription factors C/EBPα and C/EBPβ. 1,25-dihydroxyvitamin D may influence C/EBP-dependent gene expression via effects on C/EBPβ or ETO, an inhibitor of C/EBPβ activity, but these effects are currently controversial and require further study. Nevertheless, 1,25-dihydroxyvitamin D and the VDR can clearly inhibit the expression of C/EBPα and PPARγ, which is the master regulator of adipogenesis, disrupt PPARγ-dependent gene expression, and inhibit the subsequent maturation of adipocytes. The molecular mechanism by which 1,25-dihydroxyvitamin D/VDR inhibit PPARγ and PPARγ-dependent gene expression remains to be fully resolved, but may work on several fronts including inhibition of endogenous PPARγ ligand production and sequestration of limiting amounts of RXR.

The metabolism and cellular action of vitamin D is quite complex. Vitamin D is produced in the skin in response to ultraviolet B radiation and then converted in the liver to 25-hydroxyvitamin D, which acts as a prohormone for the renal production of the 1,25-dihydroxyvitamin D hormone. This bioactive vitamin D metabolite acts as a steroid hormone and a high-affinity ligand for the cellular vitamin D receptor (VDR). The ligand-activated vitamin D receptor forms a heterodimer with the retinoid X receptor (RXR), which can bind to vitamin D response elements in various genes and cause the transactivation or repression of vitamin D-responsive genes in a variety of tissues. Intracellular 1,25-dihydroxyvitamin D activates the VDR in a dose-dependent manner. The concentration of 1,25-dihydroxyvitamin D in the cell is controlled by a number of factors, including the following: circulating plasma 1,25-dihydroxyvitamin D concentrations, which depends primarily on renal 1,25-dihydroxyvitamin D production; and, in some cell types, the intracellular availability of the prohormone 25-hydroxyvitamin D, which is a reflection of overall vitamin D status. Locally, the prohormone can be converted to the bioactive hormone by the relative rates of cellular 1,25-dihydroxyvitamin D synthesis via CYP27B1 (1-hydroxylase) and hormone breakdown via CYP24 (24-hydroxylase).

Despite the ability to synthesize vitamin D in the skin and the availability of vitamin D in some foods, vitamin D deficiency is increasingly recognized as a widespread global nutritional problem. Vitamin D insufficiency has ramifications not only for bone health, but also in other non-skeletal areas of vitamin D function, such as immune cells, muscle cells and, perhaps, adipocytes. Peroxisome proliferator-activated receptor-gamma (PPARγ) is both sufficient and necessary for conversion of pre-adipocytes into adipocytes, and is the likely "master
regulator" of adipogenesis. Peroxisome proliferator-activated receptors are members of the nuclear-receptor superfamily of proteins and act as nuclear transcription factors where they form a heterodimer with the retinoid X receptor (RXR). The PPAR-RXR heterodimer binds to PPAR response elements (PPRE) in the promoter of PPAR-responsive genes. There are three different types of PPARs (PPARα, PPARγ, and PPARδ), which are expressed from three different genes. In addition, PPARγ has three different isoforms (PPARγ1, PPARγ2, and PPARγ3) that result from alternative processing of PPARγ mRNA. The protein product for PPARγ1 and PPARγ3 are identical, but PPARγ2 is 30 amino acids longer. PPARγ2 is predominantly expressed in adipose tissue, while the other PPARγ isoforms are expressed in adipocytes and other tissues.17

GENETIC PROGRAMMING IN ADIPOGENESIS

To better understand the signaling pathways that promote terminal differentiation, and factors that can modulate adipocyte function, many studies have utilized cell culture models that have already been committed to an adipocyte lineage, such as mouse 3T3-L1, 3T3-F442A, and C3H10T1/2 cells and human pre-adipocytes. For example, the mouse 3T3-L1 pre-adipocyte experimental system has proven to be a useful and popular cell culture model to investigate the molecular steps involved in regulating the progression of pre-adipocytes to mature lipid-accumulating adipocytes. The 3T3-L1 pre-adipocytes can be induced to differentiate into adipocytes by treatment with 'DIM', a hormonal cocktail of dexamethasone, insulin, and 3-isobutyl-1-methylxanthine, which is a phosphodiesterase inhibitor that causes an increase in intracellular cyclic AMP. The differentiation program in the 3T3-L1 pre-adipocyte cell line proceeds in a predictable temporal pattern consisting of one or two rounds of clonal expansion within the first 24 h or so followed by cell cycle arrest. Continued stimulation with the DIM cocktail for 48 h promotes the development of evident morphologic changes in most of the cells, from spindle-shaped fibroblast-like pre-adipocytes to a more rounded adipocyte phenotype. Continued insulin exposure is needed to complete the morphologic, biochemical and functional terminal differentiation of all the cells into mature lipid-synthesizing adipocytes over the next four to five days in culture (Figure 1).

Our current understanding of genetic programming in adipocytes reveals a great deal of underlying complexity.17 For example, in addition to the master regulator of adipogenesis PPARγ, there are more than 100 other transcriptional factors in adipocytes, including all the nuclear receptor families. Other important adipogenic transcriptional factors include several members of the C/EBP (CCAAT-enhancer binding protein) family (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ and CHOP) and Krüppel-like factor family (KLF2, KLF5, KLF15), which bind to PPAR promoters, as do GATA transcription factors. Depending on the balance of these factors bound to gene promoters, the effect on the pre-adipocyte may be either pro- or anti-adipogenic.17 In addition, certain transcription factors, such as sterol response element-binding protein-1c (SREBP1c)18 and C/EBPγ,19 can influence the production of an endogenous PPARγ ligand early in adipogenesis, which can influence PPARγ transactivation activity. This panoply of adipogenic regulators indicates there are likely many layers of transcriptional control woven into a complex temporal network of gene expression control points that regulate adipogenesis. Relatively little is known about the role of the VDR and its activating ligand 1,25-dihydroxyvitamin D in adipogenesis.

VITAMIN D AND ADIPOGENESIS

Studies conducted in Japan almost 20 years ago indicated that treatment of 3T3-L1 pre-adipocytes in culture with 1,25-dihydroxyvitamin D, the bioactive form of vitamin D, affected differentiation and adipocyte metabolism.20 These early studies indicated that nanomolar concentrations of 1,25-dihydroxyvitamin D could inhibit adipogenesis and reduce the accumulation of triacylglycerol by 50% compared to fully differentiated control cells. In addition, treatment of pre-adipocytes with other vitamin D metabolites, such as 24,25-dihydroxyvitamin D, could also inhibit pre-adipocyte differentiation, but at higher concentrations than 1,25-dihydroxyvitamin D that paralleled their reduced affinity for the vitamin D receptor. These early studies also noted that specific 1,25-dihydroxyvitamin D binding was evident in pre-adipocyte 3T3-L1 cells, but there was no evidence of specific binding for 1,25-dihydroxyvitamin D in mature adipocytes.21 This finding is consistent with the idea that any influence of vitamin D on adipogenesis would likely be exerted early in the pre-adipocyte to adipocyte transition when more VDR was available. Theoretically, the sensitivity of the pre-adipocyte to 1,25-dihydroxyvitamin D would be increased by a greater amount of vitamin D receptor or other limiting VDR-associated proteins. More than a decade ago, it was shown that one of the early effects of 1,25-dihydroxyvitamin D treatment of 3T3-L1 pre-adipocytes was an increase in vitamin D receptor mRNA expression in pre-adipocytes, which occurs within 4 h of treatment in cell culture.22 Little was appreciated, however, about any direct importance of the unliganded VDR in adipogenesis, or the molecular details whereby 1,25-dihydroxyvitamin D treatment inhibited differentiation of pre-adipocytes.
The anti-diabetic drug thiazolidinedione is a specific ligand for PPARγ, the master regulator of adipogenesis, and acts as a strong inducer of terminal differentiation in pre-adipocytes. An important molecular insight was gained several years ago by Hida et al. who found that treatment of 3T3-L1 cells with 1,25-dihydroxyvitamin D inhibited thiazolidinedione-induced pre-adipocyte differentiation. Moreover, this effect of the bioactive vitamin D metabolite was associated with inhibition of the upregulation of PPARγ protein expression, which normally occurs during the first 48 h after initiation of pre-adipocyte differentiation.

NEW MOLECULAR INSIGHTS INTO THE MECHANISM OF 1,25-DIHYDROXYVITAMIN D ACTION IN PRE-ADIPOCYTES

Although it has been known for many years that 1,25-dihydroxyvitamin D can affect PPARγ expression and block pre-adipocyte differentiation, the molecular mechanism responsible for this action had not been well clarified. Recently, independent work from the research laboratories of Kong and Li in Chicago and Blumberg et al. in Boston have elucidated some of the layers of complexity of the vitamin D effect on adipogenesis.

A short window of opportunity for vitamin D action in adipogenesis

In Chicago, Kong and Li confirmed in 3T3-L1 mouse pre-adipocytes that 1,25-dihydroxyvitamin D treatment inhibits adipocyte differentiation, and they observed that the normal induction of a number of genes involved with the early and later stages of adipocyte development, such as CCAAT/enhancer binding protein alpha (C/EBPα), PPARγ, lipoprotein lipase (LPL) and adipocyte protein 2/adipocyte-specific fatty acid-binding protein (aP2), as well as sterol-regulatory element-binding protein (SREBP)-1 and fatty acid synthase (FAS), which are genes characteristic of the mature adipocyte, were blunted in a dose-dependent manner by 1,25-dihydroxyvitamin D. Interestingly, however, they also observed that 1,25-dihydroxyvitamin D treatment was only efficacious in blocking fat cell differentiation and gene expression when administered within the first 48 h of initiating differentiation with the hormonal cocktail. In addition, they observed that removal of 1,25-dihydroxyvitamin D after three days of treatment allowed the differentiation process to be reinitiated. This important observation suggests that the main locus of the vitamin D effect on adipogenesis must reside in the suppression of a key reversible molecular event very early in the pre-adipocyte differentiation process.

Clonal expansion in 3T3-L1 cells does not mediate the effects of vitamin D on adipogenesis

In 3T3-L1 cells, there is an obligatory clonal cell population expansion step, involving one to two cell divisions, that occurs immediately following exposure of the cells to the differentiating cocktail. Given that 1,25-dihydroxyvitamin D is well known to have anti-proliferative properties, the Chicago investigators expected that the early vitamin D effect would be evident at the clonal expansion step. However, they observed that 1,25-dihydroxyvitamin D had no effect on mitotic clonal expansion. A similar finding was also made by the Boston group in 3T3-L1 cells.

In addition, Kong and Li provided further supporting evidence that a clonal expansion step was not critical to the vitamin D effect on adipogenesis by studying the effects of 1,25-dihydroxyvitamin D in primary cultures of mouse epididymal fat pads, an experimental system that does not undergo an initial clonal expansion step. Ex vivo treatment of primary cultures of these fat cells with 1,25-dihydroxyvitamin D also caused a decrease in the expression of C/EBPα and PPARγ mRNA seen in 3T3-L1 cells, confirming that a clonal expansion step is not necessary to observe an effect of vitamin D on adipogenesis.

1,25-dihydroxyvitamin D inhibits endogenous PPARγ ligand activity

PPARγ is a ligand-activated nuclear receptor. There is evidence that 3T3-L1 cells produce increased amounts of a novel endogenous PPARγ ligand on day 1 and day 2 following exposure to the adipogenic cocktail. The Boston group focused their attention on determining to what extent 1,25-dihydroxyvitamin D influenced the formation of this unidentified endogenous PPARγ ligand. Using a unique stable 3T3-L1 5B2 cell line expressing an ingenius reporter gene that is sensitive to changes in the availability of endogenous ligand for PPARγ, Blumberg et al. demonstrated that 1,25-dihydroxyvitamin D treatment partially inhibited endogenous PPARγ ligand formation, suggesting that 1,25-dihydroxyvitamin D-mediated inhibition of PPARγ ligand production could account, at least in part, for the reduced PPARγ transactivation activity in 1,25-dihydroxyvitamin D-treated cells. Although the identity of the endogenous PPARγ ligand has not yet been determined, the observations of the Boston group using the functional PPARγ-ligand assay indicate that at least one of the 1,25-dihydroxyvitamin D-mediated effects on pre-adipocyte differentiation in 3T3-L1 cells may be upstream of the endogenous PPARγ ligand production step, which is evident within the first 24 h following DIM administration.
Vitamin D receptor is necessary for 1,25-dihydroxyvitamin D-mediated effects in pre-adipocytes

The importance of VDR on adipogenesis was investigated by the Chicago group in VDR knockout mice by using mouse embryonic fibroblasts (MEFs). The MEFs, which could be stimulated to differentiate into adipocytes, were isolated from wild-type and vitamin D receptor knockout mice. Differentiation was monitored by measuring PPARγ expression. 1,25-dihydroxyvitamin D was able to block the expected increase in PPARγ expression in the wild-type MEFs, but not in the MEFs from the VDR knockout, supporting the absolute importance of the VDR in mediating the effects of 1,25-dihydroxyvitamin D on PPARγ expression and pre-adipocyte differentiation.

Kong and Li also found that VDR protein expression was quite low in mouse 3T3-L1 pre-adipocyte. However, VDR increased dramatically by 4 h following treatment with the differentiation cocktail, and then declined back to baseline levels by day two. Treatment with 1,25-dihydroxyvitamin D was able to stabilize the expression of VDR for at least one additional day. The importance of VDR stabilization by 1,25-dihydroxyvitamin D treatment in mediating the effects of 1,25-dihydroxyvitamin D on adipogenesis is currently unknown, but overall, these observations are consistent with the idea of an early VDR-dependent window of opportunity for 1,25-dihydroxyvitamin D to inhibit adipogenesis, at least in the 3T3-L1 cell culture model.

The role of the VDR in pre-adipocyte differentiation in 3T3-L1 cells was also addressed in the studies conducted in Boston by Blumberg et al. In searching for the early induction of cAMP-dependent genes using cDNA microarray analyses in 3T3-L1 cells, the researchers identified the VDR gene as a potential early cAMP target in the adipogenesis pathway. Similar to the Chicago group, they found that VDR mRNA increased to a maximum by 6 h following initiation of differentiation, and VDR protein accumulated in the nucleus as early as 4 h post initiation reaching a maximum by 12 h and then declining over the next 2 days. Consistent with their observation that the early induction of VDR gene expression in 3T3-L1 cells was cAMP-dependent, they identified 3-isobutyl-1-methylyxanthine, the phosphodiesterase inhibitor in the DIM hormonal cocktail, as the critical cocktail component responsible for the induction of VDR.

Overall, then, the observations of both the Chicago and Boston groups are consistent with earlier findings that the availability of the VDR is not equal at all points during differentiation of pre-adipocytes into adipocytes. This early temporal window of opportunity for vitamin D-mediated effects on the differentiation program in pre-adipocytes is likely governed by the temporary increased availability of VDR, or some VDR transactivation factor, within the first day or two following the triggering of the differentiation program.

Controversial role of C/EBPβ in 1,25-dihydroxyvitamin D-induced inhibition of C/EBPα and PPARγ expression

Kong and Li and Blumberg et al. both observed that the vitamin D-induced block of pre-adipocyte differentiation in 3T3-L1 cells was associated with an inhibition of PPARγ and C/EBPα expression. C/EBPα and PPARγ are critical molecular components of adipogenesis and can be regulated by C/EBPβ. Blumberg et al. found that C/EBPβ mRNA and protein rose equally in control and 1,25-dihydroxyvitamin D-treated cells, but declined more precipitously in the vitamin D-treated group. A sharper decline in C/EBPβ in the vitamin D-treated 3T3-L1 cells could indicate that reduced C/EBPβ is a possible proximate cause for the inhibition of C/EBPα and PPARγ expression, or the production of endogenous PPARγ ligand and subsequent derailment of the terminal differentiation program. In support of this idea, Blumberg et al. investigated the effects of 1,25-dihydroxyvitamin D on the expression of ETO/MTG8, which has been recently identified as a potent inhibitor of adipogenesis because it binds to C/EBPβ and prevents its interaction with the C/EBPα gene promoter. Normally, ETO/MTG8 mRNA expression declines quite quickly over the first 12 h following administration of differentiating medium to 3T3-L1 cells. However, in the presence of 1,25-dihydroxyvitamin D, the Boston group found there was a clear rebound of ETO/MTG8 mRNA expression back to baseline levels by 24 h and these levels persisted throughout differentiation. The continued presence of ETO/MTG8 in 1,25-dihydroxyvitamin D-treated pre-adipocytes would presumably bind to any C/EBPβ still present and inhibit C/EBPα expression, which could in turn inhibit adipogenesis. A key unanswered question, however, was whether C/EBP activity was reduced in pre-adipocytes triggered to differentiate and treated with 1,25-dihydroxyvitamin D.
construct allowing them to directly monitor C/EBP-mediated transactivation activity in the presence or absence of 1,25-dihydroxyvitamin D treatment in pre-adipocytes, they found there was no effect of 1,25-dihydroxyvitamin D treatment on reporter gene activity after 16 h of treatment. This latter observation would appear to argue strongly against an important role of increased ETO and apparent decreased C/EBPβ, as found by the Boston group, as an apparent cause for the vitamin D-mediated inhibition of C/EBPα and PPARγ expression and adipogenesis. However, it should be noted that C/EBP transactivation measurements were made at a time point (16 h) that may precede the rebound in ETO expression (24 h). If C/EBP response element binding activity in 1,25-dihydroxyvitamin D-treated pre-adipocyte cells is normal, then it suggests that the vitamin D-associated decrease in C/EBPα and PPARγ is likely to be a proximate cause, rather than a consequence, of the vitamin D-dependent interruption of the terminal differentiation program.

**Does the unliganded vitamin D receptor control adipogenesis by monopolizing RXR and blocking PPARγ activity?**

In cell culture studies, when VDR was overexpressed in mouse 3T3-L1 cells, even in the absence of 1,25-dihydroxyvitamin D treatment, pre-adipocyte differentiation was inhibited completely. This finding is noteworthy because it points to a possible role of the unliganded VDR in controlling adipogenesis. This observation is interesting because it could have ramifications for adipogenesis, irrespective of vitamin D status, in people with different VDR polymorphisms, or suggest a possible interaction between vitamin D status and VDR polymorphisms on adipogenesis; thus, it warrants further study in the future. Moreover, the availability of increased amounts of the unliganded VDR early in the differentiation process, identified by both the Chicago and Boston research groups, is of biological and nutritional interest because these changes in unliganded VDR availability may have variable effects on adipogenesis and the risk of developing obesity, depending upon the intracellular 1,25-dihydroxyvitamin D concentration. Intracellular 1,25-dihydroxyvitamin D concentration can be controlled by a number of factors, including overall vitamin D nutritional status and the relative activity of intracellular enzymes involved in the cellular synthesis of 1,25-dihydroxyvitamin D from the 25-hydroxyvitamin D prohormone by CYP27B1 (1-hydroxylase) and the intracellular catabolism of vitamin D metabolites by CYP24 (24-hydroxylase). Future investigations of polymorphisms in these vitamin D metabolism genes may offer additional insights into how gene-diet interactions can serve as risk factors for the development of obesity.

The Chicago group pursued the question of the unliganded VDR even further by investigating whether overexpression of VDR alone, in the absence of added 1,25-dihydroxyvitamin D ligand, could affect PPARγ transactivation activity in either the basal or induced state. They found that increased expression of VDR reduced both the basal activity of a PPARγ-responsive reporter gene and the ability to induce reporter gene activity by increasing PPARγ protein expression. This intriguing observation directed their attention to the specific details by which the VDR might block PPARγ activity and adipogenesis (Figure 1).

3T3-L1 pre-adipocytes express relatively low levels of RXR, the heterodimeric receptor partner of VDR. Several years ago, Hida et al. speculated that 1,25-dihydroxyvitamin D might inhibit pre-adipocyte differentiation by a mechanism wherein VDR competes with PPARγ for their common heterodimeric partner RXR. The Chicago group tested the hypothesis that additional VDR may influence PPARγ-mediated gene expression activity by sequestering low amounts of endogenous RXR and thereby squelching PPARγ transactivation activity in 3T3-L1 cells. To test this squelching hypothesis, 3T3-L1 cells were co-transfected with the PPARγ-responsive PPRE-luciferase reporter gene cDNA and VDR cDNA, either with or without RXR cDNA. Consistent with the squelching hypothesis, they observed that an increase in VDR expression reduced basal PPRE-driven luciferase reporter activity, which was also inhibited by 1,25-dihydroxyvitamin D treatment alone. However, increased expression of RXR protein was able to increase PPRE-luciferase activity in the presence of increased VDR and prevent inhibition by 1,25-dihydroxyvitamin D. Thus, these observations are consistent with the hypothesis that the vitamin D receptor inhibits adipogenesis by blocking PPARγ activity, at least in 3T3-L1 cells, by a mechanism apparently due to VDR monopolizing the limiting amounts of shared endogenous RXR. The importance of this potential mechanism of 1,25-dihydroxyvitamin D-induced inhibition of adipogenesis will need additional testing under in vivo conditions.

**CONCLUSION**

Overall, the recent observations made by the Chicago and Boston groups make a strong case for the role of vitamin D in adipogenesis being mediated at the molecular level through VDR-dependent inhibition of C/EBPα and PPARγ expression and a decrease in PPARγ transactivation activity. However, additional research is needed to fully clarify the role of C/EBPβ, to determine why 1,25-dihydroxyvitamin D causes a decrease in PPARγ
expression, and to investigate to what extent the interaction of VDR and PPARγ is influenced in vivo by limiting levels of RXR in pre-adipocytes.

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