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Retinoic Acid Inhibits Adipogenesis Modulating C/EBPβ Phosphorylation and Down Regulating *Srebf1a* Expression

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ABSTRACT

Adipogenesis comprises a complex network of signaling pathways and transcriptional cascades; the GSK3β-C/EBPβ-*srebf1a* axis is a critical signaling pathway at early stages leading to the expression of PPARγ2, the master regulator of adipose differentiation. Previous work has demonstrated that retinoic acid inhibits adipogenesis affecting different signaling pathways. Here, we evaluated the anti-adipogenic effect of retinoic acid on the adipogenic transcriptional cascade, and the expression of adipogenic genes *cebpb*, *srebf1a*, *srebf1c*, *pparg2*, and *cebpa*. Our results demonstrate that retinoic acid blocks adipose differentiation during commitment, returning cells to an apparent non-committed state, since they have to be newly induced to adipose conversion after the retinoid is removed from the culture medium. Retinoic acid down regulates the expression of the adipogenic genes, *srebf1a*, *srebf1c*, *pparg2*, and *cebpa*; however, it did not down regulate the expression of *cebpb*, but it inhibited C/EBPβ phosphorylation at Thr188, a critical step for the progression of the adipogenic program. We also found that RA inhibition of adipogenesis did not increase the expression of *dlk1*, the gene encoding for Pref1, a well-known anti-adipogenic factor. J. Cell. Biochem. 117: 629–637, 2016.

KEY WORDS: ADIPOGENESIS; TRANSCRIPTIONAL CASCADE; RETINOIC ACID; CELL DIFFERENTIATION; C/EBPβ; SREBP1a; 3T3-F442A

Obesity results from fat cell hypertrophy and/or increased number of adipocytes through the differentiation of preadipose cells in the stromal fraction of adipose tissue. Adipogenesis occurs when progenitor cells are recruited to differentiate into fat cells. It is driven by a transcriptional cascade that has been amply studied in cell culture models such as the 3T3-L1 and 3T3-F442A sister cell lines. The 3T3-L1 cells undergo adipogenesis when cultured with adipogenic serum in the presence of insulin, methylisobutyl xanthine (Mix) and dexamethasone (Dex) [Russell and Ho, 1976]. The 3T3-F442A cells differentiate into adipocytes under physiological signals of the body when implanted in nude mice [Green and Kehinde, 1979]; they can be triggered to adipogenesis with adipogenic serum, or solely with growth hormone or staurosporine (St) in the absence of any other adipogenic factor [Kuri-Harcuch and Green, 1978; Morikawa et al., 1984; Diaz-Velasquez et al., 2008]. In any case, these signals trigger the adipogenic transcriptional cascade and cells undergo adipose differentiation. Induction of adipogenesis with St and Dex has the advantage of using a short time treatment with the inducer, without additional signals from serum that could represent a bias in the characterization and modulation of adipogenesis [Ayala-Sumuano et al., 2008; Diaz-Velasquez et al., 2008]. In this model, St is the inducer of adipose conversion whereas Dex acts as an enhancer of the expression of adipose phenotype [Ayala-Sumuano et al., 2013]. This model of 3T3-F442A cells, allows an early response to adipogenesis, since only 4-hours induction with St is sufficient to trigger the adipose program, whereas conditions involving adipogenic serum or Mix/Dex require at least 48 h for induction in 3T3-F442A cells or 3T3-L1 cells, respectively. Also, the St model allows the study of

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commitment without the presence of Mix/Dex, or of any other adipogenic factor, therefore facilitating the analysis of the intracellular events without those triggered by adipogenic serum, growth hormone or Mix/Dex and their signaling pathways. St induces two well-defined stages of commitment to adipogenesis that take place before clonal expansion, thus allowing for the identification and analysis of early gene regulation [Ayala-Sumuano et al., 2008; Diaz-Velasquez et al., 2008; Ayala-Sumuano et al., 2011]. The first stage consists of 4 h of induction, where St induces progenitor cells to differentiate, and the second stage consists of a subsequent 44 h of stabilization, where commitment continues in the absence of the inducer but can still be reversed by anti-adipogenic substances or cytokines [Diaz-Velasquez et al., 2008]. Retinoic acid (RA) or TNFα inhibit adipogenesis and cells are blocked in an uncommitted stage showing fibroblastic gene expression and morphology [Castro-Munozledo et al., 2003; Diaz-Velasquez et al., 2008]. These four adipogenic pathways described above, seem to involve mechanisms that differ in the early steps of signaling but converge in the expression and phosphorylation of C/EBPβ.

C/EBPβ expression and phosphorylation at Thr188 are critical events during early adipogenesis [Piwien-Pilipuk et al., 2002; Park et al., 2004; Ayala-Sumuano et al., 2011] since their inhibition blocks the expression of the adipogenic Sterol Responsive Element Binding Protein 1a (SREBP1a; gene: *srebf1a*) and Peroxisome Proliferator-Activated Receptor γ2 (PPARγ2; gene: *pparg2*) [Ayala-Sumuano et al., 2011]. It is postulated that PPARγ2, together with the CCAAT Enhancer Binding Protein α (C/EBPα; gene: *cebpa*) and the Sterol Responsive Element Binding Protein 1c (SREBP1c; gene: *srebf1c*), orchestrate the expression of the adipose phenotype [Farmer, 2006]. *Srebf1* gene encodes two isoforms, *-1a* and *-1c*. Both isoforms are involved in the transactivation of lipogenic enzymes [Amemiya-Kudo et al., 2002] and their overexpression in mice lead to different biological effects: SREBP1a favors hypertrophy of adipose tissue; whereas, SREBP1c favors lipodystrophy and diabetes [Horton et al., 2003]. In a previous report, we demonstrated that the early expression of *srebf1a* isoform, which occurs earlier than that of *pparg2*, is critical for the subsequent steps of adipogenic transcriptional cascade to take place; expression of *srebf1a* depends on GSK3β activity via phosphorylation of C/EBPβ and knockdown of *srebf1a* prevents the expression of the genes encoding for PPARγ2, C/EBPα and SREBP1c [Ayala-Sumuano et al., 2011], the three transcription factors that drive the expression of adipose phenotype.

RA, a metabolite of vitamin A, has been described as a molecule that can regulate adipose metabolism in mice, resulting in weight loss and increased insulin sensitivity [Berry and Noy, 2009; Bonet et al., 2012]. This molecule prevents adipose differentiation in a variety of adipogenesis models, including the St-induced model [Castro-Munozledo et al., 1987; Bost et al., 2002; Berry et al., 2012; Diaz-Velasquez et al., 2008; Lee et al., 2011; Kim et al., 2013; Kuri-Harcuch, 1982]. Its mechanism of action is not well understood but there is some evidence that suggests that RA up regulates expression of anti-adipogenic factor Pref1, affects MAPK signaling, and C/EBPβ transcriptional activity [Schwarz et al., 1997; Lee et al., 2011; Berry et al., 2012]. Neither the RA mechanism to regulate lipid metabolism and energy homeostasis in fat cells, nor its mechanism of action during inhibition of adipogenesis is well known. In this work, we describe the modulating effect of RA on the expression of adipogenic genes at different stages of adipogenesis.

MATERIALS AND METHODS

CELL CULTURE

We seeded 3T3-F442A cells in culture dishes at 1.25×10^3 cell/cm^2 density in non-adipogenic growing medium consisting of DMEM (Invitrogen, Carlsbad, CA), supplemented with 4% adult cat serum, 5 μg/ml insulin, and 1 μM d-biotin (Sigma Chemical Co., Saint Louis, MO). We fed 1-day post-confluent cultures with a basal medium consisting of DMEM supplemented with 2% adult cat serum, 0.2% adult bovine serum, 5 μg/ml insulin, 5 μg/ml transferrin, 1 μM d-biotin, 2 nM triiodothyronine, 40 μM β-mercaptoethanol (all from Sigma Chemical Co.) and 0.01 ng/ml epidermal growth factor (IMCERA; Terre Haute, IN). Adipogenesis was induced in post-confluent cultures with 10 nM St and 0.25 μM Dex [Ayala-Sumuano et al., 2013], both from Sigma Chemical Co., for 4 h in the basal medium. Afterwards, we incubated cultures for up to 144 h at 37°C and 10% CO_2. To evaluate adipose conversion, we fixed cultures with 4% formalin then stained them with Oil Red O. We manually counted adipose clusters under a stereoscope. Since preadipocytes undergo low levels of spontaneous adipose conversion, parallel control cultures were followed for a maximum of 144 h in the basal media. These cultures represent the background level of adipose conversion. We obtained adult cat serum by bleeding domestic adult cats in observance of the NIH guidelines on the welfare of research animals and we used protocols approved by the Internal Committee for the Care and Use of Laboratory Animals of CINVESTAV-IPN.

RELATIVE-QUANTITATIVE RT-PCR

We prepared total RNA with Trizol® from induced and non-induced cultures. Complementary DNA was obtained using oligo-dT primer, and SuperScript II™ reverse transcriptase reaction mix (Invitrogen), according to manufacturer's instructions. Relative-quantitative PCR was carried out using FastStart Universal SYBR Green I Master (Rox) kit (Roche Applied Science, Manheim, Germany) to monitor reaction amplification on a 7,500 real time thermal cycler (Applied Biosystems, Foster City, CA). Specific primers are listed in Supplementary Table S1. Gene expression was normalized to the expression of ribosomal phosphoprotein large P0 gene (*rplp0*) amplified from the same sample. Expression values were determined by using the $2^{-\Delta\Delta CT}$ formula and expressed as fold change.

IMMUNOBLOT

We washed cultures with ice-cold PBS. Then, we extracted proteins with ProteoJet Mammalian Cell Lysis Reagent (Fermentas Inc. Glen Burnie, MA) supplemented with protein and phosphatase inhibitors. Proteins were separated by SDS-PAGE and blotted onto nitro-cellulose membranes. The antibodies used were: anti-human Thr235 phospho-C/EBPβ (the equivalent for murine Thr188) from Cell Signaling Technology (Berkeley, CA), anti-mouse C/EBPβ (Santa Cruz Biotechnology. Santa Cruz, CA), and mouse anti-Rab Guanine Nucleotide Dissociation Inhibitor 1 (GDI) from Invitrogen.

IN SILICO GENE PROMOTER ANALYSIS

For the analysis of gene promoter sequences we used the Champion ChIP Transcription Factor Search Portal (URL: http://www.sabiosciences.com) and the DECODE database (SABiosciences; Valencia, CA). This algorithm uses sequence data from UCSC Genome Browser (University of California; Santa Cruz, CA) and a proprietary text mining application to compile a list of predicted binding sites.

DATA MANAGEMENT AND STATISTICAL ANALYSIS

Data are presented as the mean ± standard deviation of the mean from two independent experiments by triplicate (n = 6). Qualitative data corresponds to one representative experiment carried out by duplicate. Data were analyzed by Mann–Whitney test for median analysis when comparing two groups, or Kruskal–Wallis test when comparing three groups. Statistical differences were set when *P*-value was lower than 0.05.

RESULTS

RA INHIBITS ADIPOGENESIS IN 3T3-F442A CELLS INDUCED WITH ST AND DEX

When 3T3-F442A cells are induced with St/Dex for 4 h, after 6 days adipose differentiation appears as fat cell clusters easily identifiable by staining intracytoplasmic neutral lipids with Oil Red O (Fig. 1A). Since each cluster arises from clonal amplification of one committed cell, the number of adipose clusters is related to the number of committed cells [Pairault and Green, 1979]. When St/Dex-induced cells were incubated in presence of RA, adipose conversion decreased to levels lower than control cells cultured in non-adipogenic conditions (Fig. 1A and B). Since the inhibitory effect of RA decreases as cells traverse the adipogenesis program, we studied the anti-adipogenic effect of RA at different stages during adipose conversion. Postconfluent cultures were induced with St/Dex and treated with RA as indicated in Fig. 1C and D. We found that when cells were treated with RA alongside, or immediately after, adipogenic induction (4 h), still during commitment stage, the number of clusters considerably decreased (Fig. 1C). When Dex was added to the culture medium immediately after removal of St and RA at 4 h of treatment, the number of adipose clusters was about 50%, compared to control cultures non-treated with RA (Fig. 1C). This suggests a rescuing effect by the glucocorticoid, or the presence of a sub-population of progenitor cells stimulated to differentiation by Dex after St induction, as we previously showed [Ayala-Sumuano et al., 2013]. RA did not only inhibit adipose commitment, but it reverted the cells to a possibly non-committed previous state. In order to undergo differentiation again, RA had to be removed from

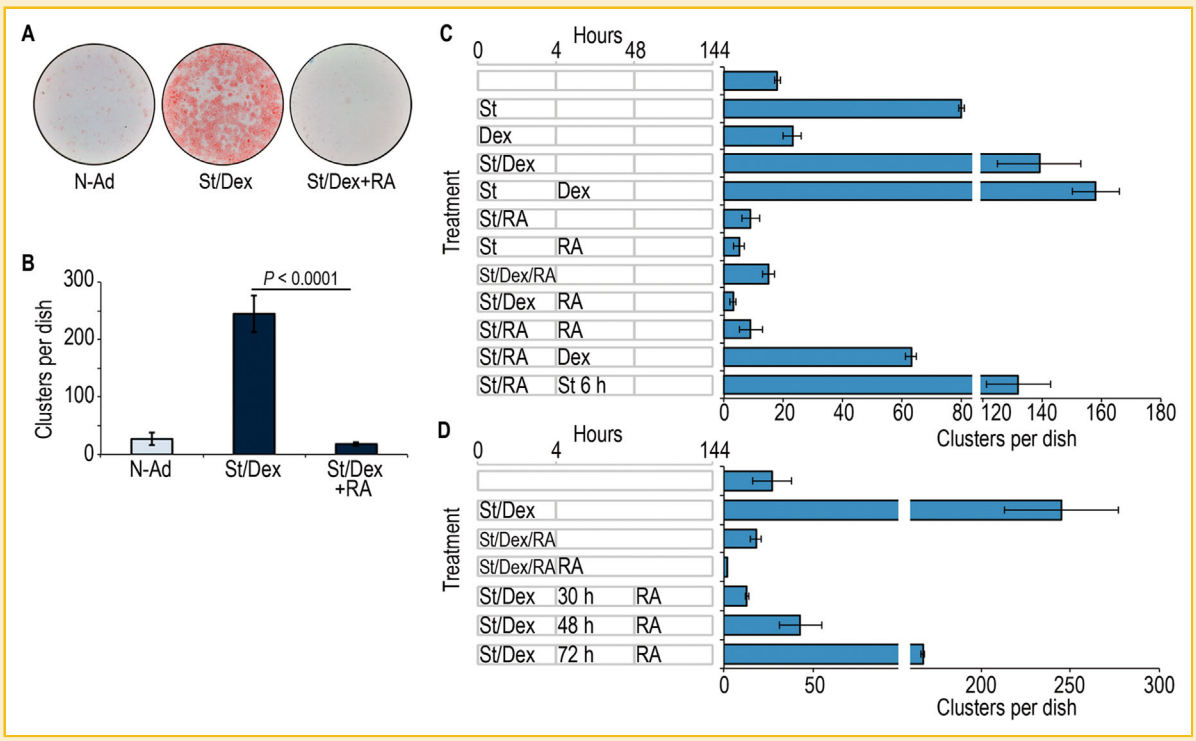

Fig. 1. Effect of retinoic acid on adipogenesis of 3T3-F442A cells induced with St/Dex. A: Representative photographs of adipose conversion of cultured 3T3-F442A cells. Post-confluent cultures were treated with non-adipogenic medium (N-Ad), or St/Dex for 4 h (St/Dex), or St/Dex plus RA for 4 h (St/Dex+RA). Cultures were followed up to 144 h and stained with Oil Red O. B: Quantification of adipose conversion: differentiated cultures treated as in A were stained with Oil Red O and the number of adipose clusters was determined by counting under a stereoscope. C and D: Effect of RA at different stages of adipogenesis: post-confluent cultures were treated as indicated, and after 144 h, the number of adipose clusters per dish was determined for each condition. Data are presented as mean plus/minus standard deviation of two independent experiments by triplicate (n = 6).

the culture medium, and St had to be added back to reach a similar number of adipose clusters as control cultures (Fig. 1C).

To elucidate the time frame at which RA exerts its anti-adipogenic effect, we induced postconfluent cultures with St/Dex; then, RA was added to the cultures at distinct time frames. When RA was applied during the first 48 h of incubation, it decreased the number of adipose clusters to the non-adipogenic control levels (Fig. 1D). However, when RA was added after the commitment stage (72 h), and a high proportion of committed cells began to express the adipose phenotype, the number of adipose clusters was larger than in non-adipogenic cultures, reaching about 68% in comparison to the non-inhibited control cultures (Fig. 1D). These data suggest that RA mainly interferes with the early events that take place during adipose commitment, after which the cells do not respond to inhibitory action of the retinoid.

RA INHIBITS ADIPOGENIC GENE EXPRESSION DURING ADIPOGENESIS OF 3T3-F442A CELLS

To evaluate the transcriptional effect of RA during adipogenesis, we determined, at the end of experiment (144 h), the expression levels of the adipogenic genes *srebf1a, pparg2*, *srebf1c*, and *cebpa*, as indicated in Fig. 2. When RA was added to the cultures simultaneously with St/Dex (0–4 h or up to 48 h) during commitment, the expression of *srebf1a*, *pparg2*, *cebpa*, and *srebf1c* decreased to low levels as those found in cells cultured with non-adipogenic medium (Fig. 2B). In another experiment, we incubated the cultures with RA at various times after induction with St/Dex: at 30 h (during commitment stage), at 48 h (end of commitment) and at 72 h (phenotype expression stage). Then, we evaluated gene expression at the end of experiment (144 h). The expression of *srebf1a*, *pparg2*, *cebpa*, and *srebf1c* remained down regulated when RA was added during commitment (30 h) or at the end of it (48 h). However, when RA was added at 72 h (during terminal differentiation), *pparg2* expression levels reached about half of its expression in comparison with the adipogenic cultures; whereas, that of *cebpa*, *srebf1a* and *srebf1c* reached levels comparable to those found in adipogenic cultures (Fig. 2B). These results suggested that *pparg2*, a gene regarded as the master transcriptional regulator of adipogenesis seems to be more sensitive to a direct RA action, or to some other factor that regulates its transcription, in comparison to the other adipogenic genes. Therefore, the expression of these adipogenic genes can be modulated by retinoic acid mainly during the early stages of adipogenesis but at lower extent during terminal differentiation.

RA INHIBITS ADIPOGENESIS IN A PREF1-INDEPENDENT MANNER IN 3T3-F442A CELLS

A possible mechanism of adipogenesis inhibition by RA could be through activating the expression of Pref1, Sox9 and other related genes [Berry et al., 2012]. Pref1 (gene: *dlk1*) is a transmembrane EGF-repeat containing protein whose soluble form is a ligand of an unknown receptor that activates the MAPK kinase/ERK signaling pathway [Kim et al., 2007]. Pref1 controls mesenchymal cell fate, since ablation of its gene leads to a high perinatal lethality and growth retardation, skeletal malformation, and increased adiposity in the surviving animals [Moon et al., 2002]. In adipose tissue, Pref1 gene, *dlk1*, is expressed by preadipocytes and its over-expression blocks adipogenesis in 3T3-L1 cells [Smas and Sul, 1993; Mitterberger et al., 2012]; for the adipogenic program, its expression should be down regulated early during adipogenesis in 3T3-L1 cells. Recently, it was reported that Pref1 decreases its expression during adipogenesis of human adipose-derived stromal/progenitor cells, which highlights its role in modulating adipogenesis [Mitterberger et al., 2012]. On this basis, we studied the effect of RA on *dlk1* expression and attempted to study if RA inhibition is mediated by preventing down-regulation of this gene expression. First, we determined the expression of *dlk1* along adipogenic process. We

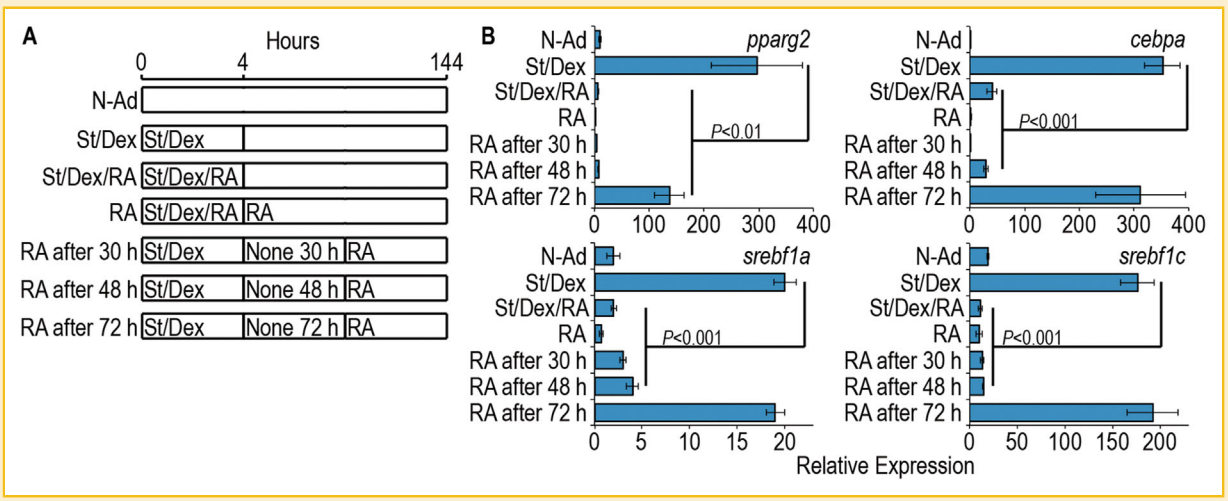

Fig. 2. Effect of retinoic acid on the expression of genes involved in the adipogenic transcriptional cascade. A: Description of each treatment used to evaluate the effect of RA. B: Expression levels of the adipogenic genes under distinct adipogenic conditions plus RA. Post-confluent cultures of 3T3-F442A cells were induced as indicated in panel A, then cultures were allowed to differentiate for up to 144 h and gene expression was assessed by qRT-PCR. Data are presented as mean plus/minus standard deviation of two experiments by triplicate (n = 6).

induced post-confluent cultures of 3T3-F442A cells with St/Dex for 4 h as described above. After induction, cells were followed throughout the experiment (144 h), and cultures evaluated for adipogenesis with Oil Red O staining. We collected samples at several time points and evaluated the expression of *dlk1* by quantitative RT-PCR. The kinetic analysis of the expression of this gene demonstrated that, after an early slight increase during the first 4 h of induction, its expression was down regulated at stabilization stage of commitment and remained down during phenotype expression (Fig. 3A). Kinetics of *dlk1* down-regulation resembled the mirror image of *pparg2* expression during commitment, as we described previously [Ayala-Sumuano et al., 2008]. Therefore, we induced post-confluent cultures of 3T3-F442A cells with St/Dex for 4 h plus 10 μM RA for up to 48 h. In the RA treated cultures, despite that adipogenesis was completely inhibited; the expression of *dlk1* was also down-regulated to similar levels and comparable to cultures without RA. As expected, RA also significantly decreased the expression of adipogenic markers *pparg2*, *cebpa*, and *srebf1c* at 144 h, a time when most cells are fully differentiated and appear as mature adipocytes (Fig. 3B). These data suggest that the anti-adipogenic effect of RA does not involve the Pref1 pathway. However, an up-regulation of *dlk1* has been reported in 3T3-L1 cells when differentiation is induced with Mix/Dex in presence of RA [Berry et al., 2012]. These contradictory results for *dlk1* expression might be related to an alternative pathway of adipogenesis independent of Pref1 in 3T3-F442A cells, or to the alternative pathway of induction of adipose differentiation with Mix/Dex.

TNFα is another well-known anti-adipogenic substance. This molecule has great importance in adipose tissue metabolism because hypertrophic adipocytes produce and release this cytokine [de Ferranti and Mozaffarian, 2008] and halts adipogenesis mainly at commitment stage [Castro-Munozledo et al., 2003]. To evaluate the expression of *dlk1* under the anti-adipogenic effect of TNFα, we induced post-confluent cultures of 3T3-F442A cells with St/Dex for 4 h and treated them with 10 ng/ml TNFα for up to 48 h; then we evaluated adipose differentiation as above. As expected, TNFα strongly blocked adipogenesis as revealed by adipose cluster staining (Fig. 3C). This cytokine also significantly decreased the expression levels of *pparg2*, *cebpa*, and *srebf1c*, compared to cultures induced with St/Dex (Fig. 3D). The expression of *dlk1* was not up-regulated by the effect of TNFα showing similar levels of expression as those found in the cytokine non-treated cultures (Fig. 3D). These results demonstrate that similarly to RA, TNFα blocks adipogenesis without increasing the expression of the gene encoding for anti-adipogenic factor Pref1, as previously reported [Xing et al., 1997]. It is interesting to note that these physiologically important anti-adipogenic substances, RA and

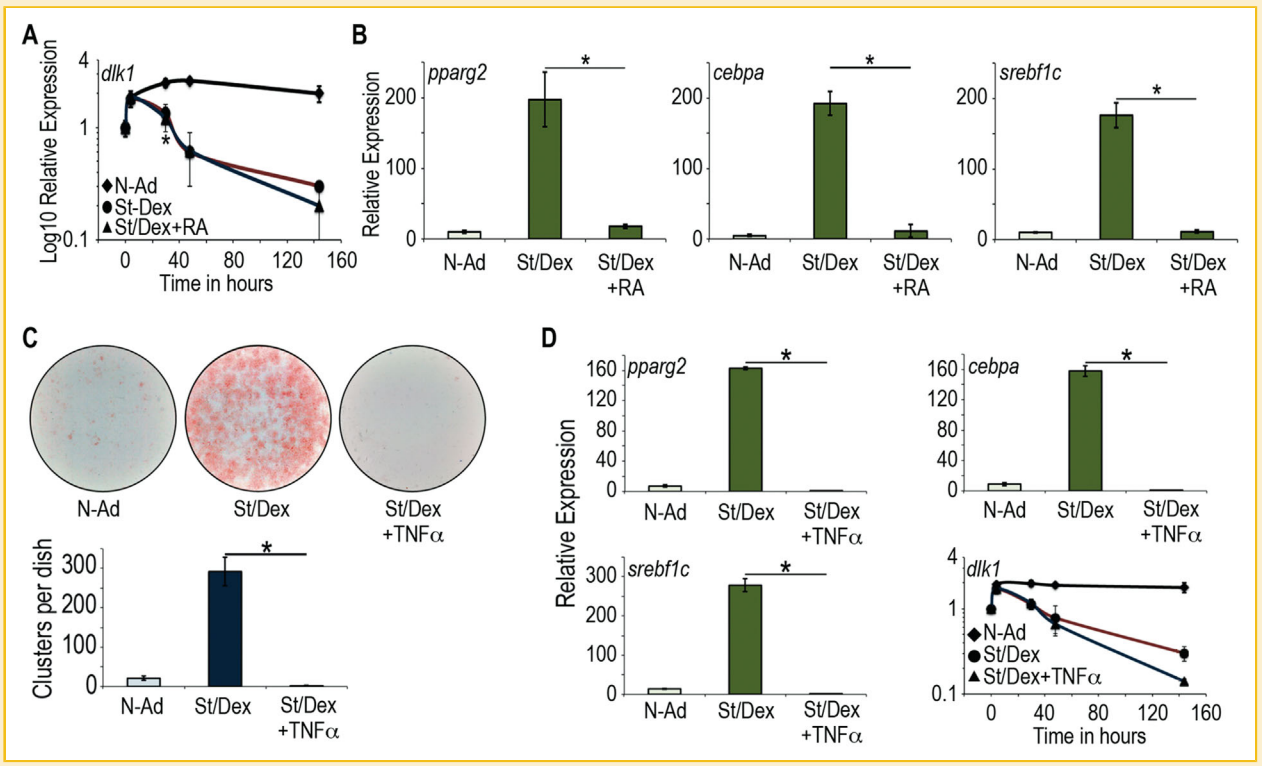

Fig. 3. RA and TNFα inhibit commitment to adipogenesis in a Pref1-independent way. Post-confluent cultures were induced to adipose conversion with St/Dex with or without 10 μM RA. A: Kinetic analysis of *dlk1* expression under the effect of RA. B: Expression levels of *pparg2*, *cebpa* and *srebf1c* were evaluated by quantitative RT-PCR. Gene expression was determined by quantitative RT-PCR. C: Effect of TNFα on adipogenesis of 3T3-F442A cells adipogenesis. Post-confluent cultures were induced to adipose conversion with St/Dex with or without 10 ng/ml TNFα. Cultures were followed for 144 h and stained with Oil Red O for manually counting. D: Expression levels of *pparg2*, *cebpa* and *srebf1c* and *dlk1* under TNFα effect. For all panels data are presented as the mean plus/minus standard deviation of two experiments done by triplicate (n = 6). N-Ad; cultures under non-adipogenic conditions. Asterisks indicate statistical differences between data groups relative to St/Dex control with a *P* value lower than 0.05.

TNFα, with different mechanisms of action, do not up-regulate *dlk1* expression to inhibit adipogenesis, which indicates that their mechanism of action is not related to the Pref1 signaling pathway.

RETINOIC ACID INHIBITS C/EBPβ PHOSPHORYLATION DURING ADIPOGENESIS

Since RA did not prevent down-regulation of *dlk1*, we looked into other mechanisms that could explain inhibition of adipogenesis by this retinoid. In 3T3-F442A cells induced with St/Dex, *cebpb* is transiently expressed 2 h after induction reaching a peak at 4 h [Ayala-Sumuano et al., 2011]. In parallel, there is a transient phosphorylation of C/EBPβ at Thr188 that arises around 30 min and disappears about 24 h after adipogenic induction [Ayala-Sumuano et al., 2011]. This first phosphorylation primes subsequent phosphorylation at Ser184 and Thr179, increasing DNA-binding activity with a peak about 24 h from induction [Tang et al., 2005]. It was reported that RA blocks the transcription of C/EBPβ-dependent genes [Schwarz et al., 1997] and GSK3β activity is indispensable for transcriptional activity of C/EBPβ [Park et al., 2004; Ayala-Sumuano et al., 2011] but the effect of RA on C/EBPβ gene expression and/or phosphorylation during adipogenesis has not been elucidated. We treated 3T3-F442A cells with St/Dex with RA, and at the end of induction (4 h), we determined the expression of *cebpb* by quantitative RT-PCR, when it reached its peak. At this time-point, in cells induced with St/Dex, *cebpb* expression increased about 6-fold, whereas, in cells treated simultaneously with RA, a similar increase in the expression of *cebpb* was evident in comparison to the St/Dex condition (Fig. 4A). This rules out that the inhibitory effect of RA is mediated by blocking the expression of this adipogenic gene.

Since phosphorylation of C/EBPβ is also a critical step during early stages of adipogenesis [Park et al., 2004; Tang et al., 2005; Ayala-Sumuano et al., 2011], we evaluated whether RA might exert its inhibitory action during commitment through changes in the phosphorylation pattern of C/EBPβ. Therefore, we induced the 3T3-F442A cells with St/Dex for 4 h and simultaneously treated them with RA. After treatment, we extracted total proteins, separated them by SDS-PAGE and blotted. Membranes were challenged with anti-human phospho-Thr215C/EBPβ (the equivalent for murine

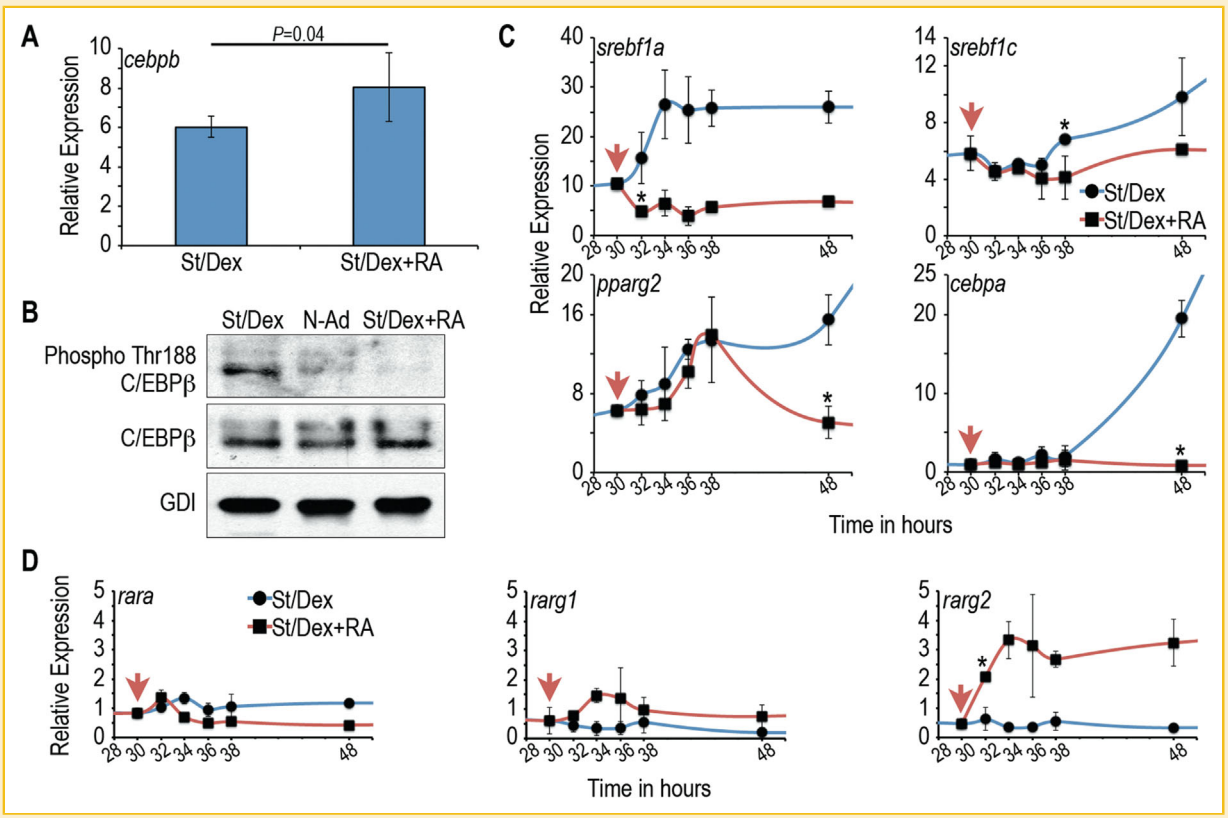

Fig. 4. Retinoic acid down regulates gene expression and phosphorylation of C/EBPβ during adipogenesis. A: Effect of RA on *cebpb* expression. Cells were induced with St/Dex plus RA during 4 h then the expression of *cebpb* was evaluated by qRT-PCR. B: Phosphorylation of C/EBPβ in non-adipogenic conditions (N-Ad) or after induction with St/Dex or St/Dex+RA. Cells were induced as in A and, at 4 h total protein was extracted and separated by SDS-PAGE for Western blot assay. Samples were challenged with anti-phospho Thr215C/EBPβ (the human equivalent for murine Thr188), total C/EBPβ, and GDI was used to show that the same amount of protein was loaded in all lanes. C: Effect of RA on kinetics of gene expression during adipogenesis. Post-confluent cells were induced with St/Dex for 4 h, and at 30 h cultures were incubated with RA. From this point, samples were obtained every two hours and at the end of commitment (48 h from induction), as indicated. Gene expression was evaluated by qRT-PCR. D: Expression of RA receptor genes (*rara*, *rarg1*, and *rarg2*) during adipogenesis of 3T3-F442A cells. Cultures were treated as indicated in A and gene expression was determined by qRT-PCR. Data are presented as mean plus/minus standard deviation of two experiments done by triplicate (n = 6). Red arrows indicate the time at which the RA was added.

Thr188), anti-C/EBPβ and anti-GDI antibodies. When St/Dex-induced cultures were treated with RA, a significant decrease in the phosphorylation of C/EBPβ at Thr188 was evident in comparison to cells not treated with RA (Fig. 4B). This result demonstrates that RA acid inhibits adipose conversion by affecting phosphorylation of C/EBPβ, one of the critical steps during early stages of adipogenesis [Park et al., 2004; Tang et al., 2005; Ayala-Sumuano et al., 2011].

The anti-adipogenic action of RA occurs for up to 48 h, which suggests that phosphorylation of C/EBPβ is not the only factor inhibited by the retinoid, and that other factors during adipogenic commitment might be affected. Thus, to evaluate the effect of RA on the expression of the other adipogenic genes, we induced post-confluent 3T3-F442A cells with St/Dex for 4 h. Thirty hours later we switched cultures to fresh medium supplemented with RA. From this point, we evaluated gene expression at intervals of 2 h during the first 8 h (at 30, 32, 34, 36 and 38 h from induction), and at 18 h later at the end of commitment (at 48 h from induction). Kinetic gene expression assays demonstrated that RA inhibited primarily *srebf1a*, an indispensable adipogenic gene expressed during commitment, which precedes the expression of *pparg2* [Ayala-Sumuano et al., 2011]. Its expression decreased rapidly to low levels just 2 h after RA addition (at 32 h from induction), whereas the expression of *srebf1c*, the other isoform of *srebf1* gene [Shimomura et al., 1997], continued for up to 6 h from RA addition, after which its expression remained at about 60% of those found in the non-treated cultures with RA (Fig. 4C). The addition of RA did not strongly affect the expression of *pparg2* during the first 8 h after RA exposure. Its expression increased similarly to non-inhibited cultures, but at 48 h from induction and 18 h from RA, it decreased suddenly to about 30% (Fig. 4C); whereas, expression of *cebpa*, which it was expected at the end of commitment [Ayala-Sumuano et al., 2011], did not arise at any time when cultures where exposed to RA (Fig. 4C). These results showed that *srebf1a* is the first gene in this cascade that is inhibited by RA. They also support our conclusion that *srebf1a* and its product, the transcription factor SREBP1a, are part of the early molecular events that follow GSK3β activation, orchestrating the adipogenic transcriptional cascade during commitment prior to *pparg2* expression. The early expression of *srebf1a* is necessary for the occurrence of adipogenic transcriptional cascade and it depends on GSK3β activity and C/EBPβ phosphorylation [Ayala-Sumuano et al., 2011]; when this phosphorylation is inhibited (i.e., when RA is present), expression of *srebf1a* does not arise.

RA regulates gene expression through several pathways, but the classical pathway occurs via all-*trans* retinoic acid bound to the Retinoic Acid Receptor (RAR): Retinoic X Receptor (RXR) dimer [Mark et al., 2006; Theodosiou et al., 2010]. The 3T3-L1 cells express mainly RARα and RARγ2, both nuclear receptors that mediate inhibition of adipose conversion and are functionally redundant in their inhibitory effect [Xue et al., 1996; Schwarz et al., 1997]. Since RARs act as transcriptional co-repressors, we decided to evaluate the expression of these nuclear receptors. In a similar experiment as above, we analyzed the expression of *rara*, *rarg1* and *rarg2*. In cultures treated only with St/Dex, expression of *rar* genes during commitment remained constant at low levels (Fig. 4D). When cultures where treated with RA at 30 h, expression of *rarg1* and *rarg2* increased within 2 h from RA addition reaching a peak of 1.5-fold and 3.5-fold higher, respectively, at 34 h (Fig. 4D). This expression of *rarg1* and *rarg2* correlates with the decrease seen in expression of adipogenic genes. The increase in *rarg2* followed a mirror image kinetic as that of *srebf1a*, suggesting that RA induced the expression of its RA receptor genes to mediate its inhibitory effect. It might be thought that *rarg2* is a regulator of *srebf1a*; but, analysis of the *srebf1a* promoter with DECODE database, for the search of probable transcription factor binding sites, did not reveal any probable site for RAR binding, which suggests that these receptors are not direct repressors of the expression of *srebf1a*. However, a similar analysis on *rarg2* promoter revealed several binding sites for SREBP1a and -1c near the translation start site. It suggests that SREBP1a or -1c could be acting as transcriptional repressors for *rarg2* and when *srebf1a* expression is down-regulated, its repressing activity disappears. Further research is needed to assess this issue.

DISCUSSION

Retinoic acid is a biologically active molecule that has become important for the study of molecular events related to differentiation of mesenchymal cells [Kuri-Harcuch, 1982; Ide and Aono, 1988; Skillington et al., 2002]. In this work, we demonstrate that RA blocks adipogenesis during commitment by inhibiting the phosphorylation of C/EBPβ at Thr188, a critical step during early adipogenesis [Park et al., 2004; Tang et al., 2005; Ayala-Sumuano et al., 2011].

The inhibitory effect of RA on adipogenesis has been previously studied and several reports describe different pathways. Recent work showed that RA inhibits adipogenesis by increasing the expression of genes encoding Pref1 and Sox9 [Berry et al., 2012]. Pref1 is an anti-adipogenic factor that its gene expression is down-regulated as adipogenesis progresses [Smas and Sul, 1993; Ayala-Sumuano et al., 2008]; whereas, Sox9 is a mesenchymal fate transcription factor that depends on Pref1 signaling and down regulates the expression of C/EBPβ and C/EBPδ [Wang and Sul, 2009], two important transcription factors during early stages of adipogenesis [Tanaka et al., 1997; Gregoire et al., 1998]. We did not measure the expression of Sox9 during inhibition of adipogenesis by RA. However, RA did not up regulate the expression of *dlk1* nor down regulated that of *cebpb*, ruling out the possibility that RA might mediate its effect through the expression of Sox9 affecting C/EBPβ at transcriptional level, but its effect is downstream of its transcription.

GSK3β activity is indispensable for adipogenesis [Diaz-Velasquez et al., 2008; Ayala-Sumuano et al., 2011]. This enzyme phosphorylates C/EBPβ at several sites and phosphorylation at Thr188 favors its transcriptional activity and correlates with the expression of *srebf1a* [Park et al., 2004; Tang et al., 2005; Ayala-Sumuano et al., 2011]. Activation of Wnt signaling pathway, which involves inactivation of GSK3β, decreases C/EBPβ phosphorylation and inhibits adipogenesis [Kim et al., 2013; Ayala-Sumuano et al., 2011]. It is noteworthy that RA activates the Wnt signaling pathway [Kim et al., 2013]. Our results in Fig. 1C showed that Dex somewhat rescued adipose differentiation after RA inhibition. Dex stimulates adipogenesis by inhibiting Wnt/β-catenin pathway [Naito et al., 2012] and increases the phosphorylation of C/EBPβ [Ayala-Sumuano et al., 2013]. All these data

highlight the importance of C/EBPβ as a central hub during adipogenesis, linking several adipogenic and anti-adipogenic pathways that modulate the expression of adipose phenotype (Fig. 5).

Phosphorylation of C/EBPβ occurs during the first 24 h of adipogenesis [Ayala-Sumuano et al., 2011]; however, the larger anti-adipogenic effect of RA occurs during the first 48 h from induction. At 30 h from induction, well into the stabilization stage of commitment before the expression of adipose phenotype had begun, RA is still able to somewhat inhibit adipose differentiation. This result suggests that there are other pathways through which RA can inhibit the expression of adipose phenotype. The canonical pathway of all-*trans* retinoic acid inhibition involves binding of the retinoid to RAR, then forming dimers with RXR [Mark et al., 2006; Theodosiou et al., 2010], a nuclear receptor indispensable for the transcriptional activity of PPARγ [Imai et al., 2001].

Recently, it was shown that RA treated mice did not gain weight despite a higher food intake, and their adipocytes looked smaller in comparison with control animals. In addition, RA treatment blunted diet-induced elevation in cholesterol or triglycerides plasma levels, without affecting free fatty acids plasma levels [Berry et al., 2012]. It is important to note, that in the same study, RA decreased the amount of terminally differentiated adipocytes in white adipose tissue, suggesting that adipogenesis in vivo was also inhibited by the retinoid. This result is in complete agreement with the inhibitory effect of RA in cell culture models of adipogenesis. Since we report that RA inhibits the adipogenic transcriptional cascade at a very early stage by impeding the phosphorylation of C/EBPβ at Thr188, and therefore adipogenesis, it is surprising that animals with a high fat diet whose mechanism of recruitment of new adipocytes is impaired by the retinoid, showed normal lipid and energy homeostasis [Berry et al., 2012]. It is interesting that other actions of RA should compensate for this inconsistency. For example, we can hypothesize that RA might be able to change the adipocyte metabolism into a more dissipating energy cell, such as a brown or brite fat cell. In fact, there are some data that seem to suggest a similar instance, since in mice treated with RA there was an increased lipid oxidation and inhibition of lipid biosynthesis in liver [Amengual et al., 2010]. However, it is important to keep in mind that in human cancer patients RA led to hypertriglyceridemia [Conley et al., 1997]. The different response to RA might be due to species specificity or to a toxic effect of high doses of RA in the treated patients. It is likely that lower doses of RA might lead to a metabolic response in humans similar to that observed in mice. If this is the case, it would be interesting to test analogs of RA that might lack its toxic side effects in hopes of treating adipose cells to change their lipid and energy metabolism. However, a word of caution is necessary regarding the treatment of obesity with RA. Since, as we and others have described, RA inhibits commitment into adipogenesis, it is possible that impairment of this differentiation process in mammals that are fed a high fat diet and treated with the retinoid, might lead to significant adverse effects on other organs or systemic lipid metabolism not yet elucidated.

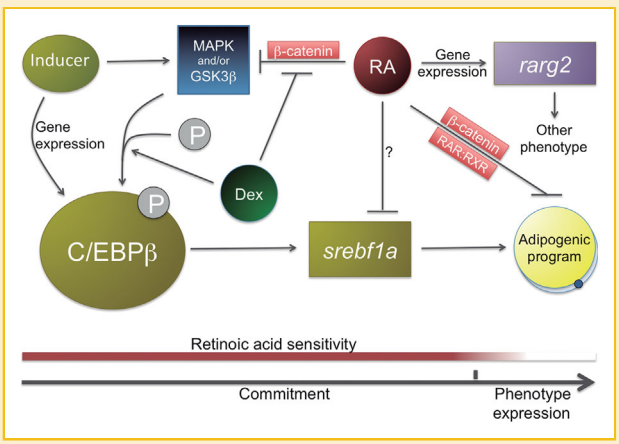

Fig. 5. Schematic representation of retinoic acid effect on commitment of 3T3-F442A cells during adipogenesis. C/EBPβ might act as a hub for the adipogenic program and as a central target of RA inhibitory effect. By decreasing phosphorylation of C/EBPβ, expression of the main genes of the adipogenic transcriptional cascade is down regulated. Based on data from this paper and on data reported in Park et al. [2004], Ayala-Sumuano et al. [2011, 2013], Lee et al. [2011], Naito et al. [2012], and Kim et al. [2013].

REFERENCES

Amemiya-Kudo M, Shimano H, Hasty AH, Yahagi N, Yoshikawa T, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Sato R, Kimura S, Ishibashi S, Yamada N. 2002. Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. J Lipid Res 43:1220–1235.

Amengual J, Ribot J, Bonet ML, Palou A. 2010. Retinoic acid treatment enhances lipid oxidation and inhibits lipid biosynthesis capacities in the liver of mice. Cell Physiol Biochem 25:657–666.

Ayala-Sumuano JT, Velez-Del Valle C, Beltran-Langarica A, Hernandez JM, Kuri-Harcuch W. 2008. Adipogenic genes on induction and stabilization of commitment to adipose conversion. Biochem Biophys Res Commun 374:720–724.

Ayala-Sumuano JT, Velez-Delvalle C, Beltran-Langarica A, Marsch-Moreno M, Cerbon-Solorzano J, Kuri-Harcuch W. 2011. Srebf1a is a key regulator of transcriptional control for adipogenesis. Sci Rep 1:178.

Ayala-Sumuano JT, Velez-Delvalle C, Beltran-Langarica A, Marsch-Moreno M, Hernandez-Mosqueira C, Kuri-Harcuch W. 2013. Glucocorticoid paradoxically recruits adipose progenitors and impairs lipid homeostasis and glucose transport in mature adipocytes. Sci Rep 3:2573.

Berry DC, DeSantis D, Soltanian H, Croniger CM, Noy N. 2012. Retinoic acid upregulates preadipocyte genes to block adipogenesis and suppress diet-induced obesity. Diabetes 61:1112–1121.

Berry DC, Noy N. 2009. All-trans-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor beta/delta and retinoic acid receptor. Mol Cell Biol 29:3286–3296.

Bonet ML, Ribot J, Palou A. 2012. Lipid metabolism in mammalian tissues and its control by retinoic acid. Biochim Biophys Acta 1821:177–189.

Bost F, Caron L, Marchetti I, Dani C, Le Marchand-Brustel Y, Binetruy B. 2002. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem J 361:621–627.

Castro-Munozledo F, Beltran-Langarica A, Kuri-Harcuch W. 2003. Commitment of 3T3-F442A cells to adipocyte differentiation takes place during the first 24-36h after adipogenic stimulation: TNF-alpha inhibits commitment. Exp Cell Res 284:163–172.

Castro-Munozledo F, Marsch-Moreno M, Beltran-Langarica A, Kuri-Harcuch W. 1987. Commitment of adipocyte differentiation in 3T3 cells is inhibited by retinoic acid, and the expression of lipogenic enzymes is modulated through cytoskeleton stabilization. Differentiation 36:211–219.

Conley BA, Egorin MJ, Sridhara R, Finley R, Hemady R, Wu S, Tait NS, Van Echo DA. 1997. Phase I clinical trial of all-trans-retinoic acid with correlation of its pharmacokinetics and pharmacodynamics. Cancer Chemother Pharmacol 39:291–299.

de Ferranti S, Mozaffarian D. 2008. The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. Clin Chem 54:945–955.

Diaz-Velasquez CE, Castro-Munozledo F, Kuri-Harcuch W. 2008. Staurosporine rapidly commits 3T3-F442A cells to the formation of adipocytes by activation of GSK-3beta and mobilization of calcium. J Cell Biochem 105:147–157.

Farmer SR. 2006. Transcriptional control of adipocyte formation. Cell Metab 4:263–273.

Green H, Kehinde O. 1979. Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. J Cell Physiol 101:169–171.

Gregoire FM, Smas CM, Sul HS. 1998. Understanding adipocyte differentiation. Physiol Rev 78:783–809.

Horton JD, Shimomura I, Ikemoto S, Bashmakov Y, Hammer RE. 2003. Overexpression of sterol regulatory element-binding protein-1a in mouse adipose tissue produces adipocyte hypertrophy, increased fatty acid secretion, and fatty liver. J Biol Chem 278:36652–36660.

Ide H, Aono H. 1988. Retinoic acid promotes proliferation and chondrogenesis in the distal mesodermal cells of chick limb bud. Dev Biol 130:767–773.

Imai T, Jiang M, Chambon P, Metzger D. 2001. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinol X receptor a mediated by tamoxifen-inducible chimeric Cre recombinase (Cre-ER^{T2}) in adipocytes. Proc Natl Acad Sci USA 98:224–228.

Kim DM, Choi HR, Park A, Shin SM, Bae KH, Lee SC, Kim IC, Kim WK. 2013. Retinoic acid inhibits adipogenesis via activation of Wnt signaling pathway in 3T3-L1 preadipocytes. Biochem Biophys Res Commun 434:455–459.

Kim KA, Kim JH, Wang Y, Sul HS. 2007. Pref-1 (preadipocyte factor 1) activates the MEK/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation. Mol Cell Biol 27:2294–2308.

Kuri-Harcuch W. 1982. Differentiation of 3T3-F442A cells into adipocytes is inhibited by retinoic acid. Differentiation 23:164–169.

Kuri-Harcuch W, Green H. 1978. Adipose conversion of 3T3 cells depends on a serum factor. Proc Natl Acad Sci USA 75:6107–6109.

Lee JS, Park JH, Kwon IK, Lim JY. 2011. Retinoic acid inhibits BMP4-induced C3H10T1/2 stem cell commitment to adipocyte via downregulating Smad/p38MAPK signaling. Biochem Biophys Res Commun 409:550–555.

Mark M, Ghyselinck NB, Chambon P. 2006. Function of retinoid nuclear receptors: Lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol 46:451–480.

Mitterberger MC, Lechner S, Mattesich M, Kaiser A, Probst D, Wenger N, Pierer G, Zwerschke W. 2012. DLK1(PREF1) is a negative regulator of adipogenesis in CD105(+)/CD90(+)/CD34(+)/CD31(−)/FABP4(−) adipose-derived stromal cells from subcutaneous abdominal fat pats of adult women. Stem Cell Res 9:35–48.

Moon YS, Smas CM, Lee K, Villena JA, Kim KH, Yun EJ, Sul HS. 2002. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol Cell Biol 22:5585–5592.

Morikawa M, Green H, Lewis UJ. 1984. Activity of human growth hormone and related polypeptides on the adipose conversion of 3T3 cells. Mol Cell Biol 4:228–231.

Naito M, Omoteyama K, Mikami Y, Takahashi T, Takagi M. 2012. Inhibition of Wnt/β-catenin signaling by dexamethasone promotes adipocyte differentiation in mesenchymal progenitor cells, ROB-C26. Histochem Cell Biol 138:833–845.

Pairault J, Green H. 1979. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. Proc Natl Acad Sci USA 76:5138–5142.

Park BH, Qiang L, Farmer SR. 2004. Phosphorylation of C/EBPbeta at a consensus extracellular signal-regulated kinase/glycogen synthase kinase 3 site is required for the induction of adiponectin gene expression during the differentiation of mouse fibroblasts into adipocytes. Mol Cell Biol 24:8671–8680.

Piwien-Pilipuk G, MacDougald O, Schwartz J. 2002. Dual regulation of phosphorylation and dephosphorylation of C/EBPb modulate its transcriptional activation and DNA binding in response to growth hormone. J Biol Chem 277:44557–44565.

Russell TR, Ho R. 1976. Conversion of 3T3 fibroblasts into adipose cells: Triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutyl xanthine. Proc Natl Acad Sci USA 73:4516–4520.

Schwarz EJ, Reginato MJ, Shao D, Krakow SL, Lazar MA. 1997. Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription. Mol Cell Biol 17:1552–1561.

Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. J Clin Invest 99:838–845.

Skillington J, Choy L, Derynck R. 2002. Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol 159:135–146.

Smas CM, Sul HS. 1993. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 73:725–734.

Tanaka T, Yoshida N, Kishimoto T, Akira S. 1997. Defective adipocyte differentiation in mice lacking the C/EBPb and/or C/EBPδ gene. EMBO J 16:7432–7443.

Tang QQ, Gronborg M, Huang H, Kim JW, Otto TC, Pandey A, Lane MD. 2005. Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. Proc Natl Acad Sci USA 102:9766–9771.

Theodosiou M, Laudet V, Schubert M. 2010. From carrot to clinic: An overview of the retinoic acid signaling pathway. Cell Mol Life Sci 67:1423–1445.

Wang Y, Sul HS. 2009. Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. Cell Metab 9:287–302.

Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL, Ringold GM. 1997. TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARgamma without effects on Pref-1 expression. Endocrinology 138:2776–2783.

Xue JC, Schwarz EJ, Chawla A, Lazar MA. 1996. Distinct stages in adipogenesis revealed by retinoid inhibition of differentiation after induction of PPARgamma. Mol Cell Biol 16:1567–1575.

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