The process of adipocyte differentiation is driven by a highly coordinated cascade of transcriptional events that results in the development of the mature adipocyte and lipid accumulation. One of the early events of differentiation is the upregulation of C/EBPβ expression. C/EBPβ then acts to upregulate the expression of adipogenic factors such as C/EBPα, which control the late stage of adipogenesis. Retinoic acid (RA) is a potent inhibitor of adipogenesis, and its action appears to block C/EBPβ transcriptional potential early during differentiation. Using preadipocytes and mesenchymal stem cell models, we show that RA specifically blocks the occupancy of C/EBPβ of the C/EBPα promoter, thereby abrogating the differentiation process. RA does not act directly on C/EBPβ but rather stimulates the expression of the Transforming Growth Factor β-effector protein Smad3, which can interact with C/EBPβ via its Mad Homology 1 (MH1) domain and can interfere with C/EBPβ DNA binding. The RA-induced increase in Smad3 expression results in increased cytoplasmic and nuclear Smad3, an important event as ectopic expression of Smad3 in preadipocytes in the absence of RA treatment only modestly inhibits adipogenesis and C/EBPβ DNA binding, suggesting that Smad3 alone is not sufficient to completely recapitulate the effects of retinoic acid treatment during differentiation. However, in the absence of Smad3, RA is not able to inhibit adipocyte differentiation or to elicit a decrease in C/EBPβ DNA occupancy suggesting that Smad3 is necessary to convey the inhibitory effects of retinoic acid during adipogenesis.

**INTRODUCTION**

In times of caloric restriction, an organism relies on stores of fat to provide the energy necessary for survival. Adipocytes are capable of storing large amounts of lipid, and when caloric intake far exceeds the energy requirement of the organism, can increase in size and in number to accommodate the excess.

The process of adipocyte differentiation is driven by a highly coordinated cascade of transcriptional events that results in the development of the mature adipocyte and in lipid accumulation. When confluent preadipocytes are treated with a hormonal induction cocktail containing insulin, a cyclic AMP phosphodiesterase inhibitor (isobutylmethyl-xanthine, MIX), and glucocorticoids they differentiate efficiently into mature adipocytes within 7-10 days (1,2). Treatment with this induction cocktail also causes the rapid and transient induction of the early transcriptional regulators C/EBPβ and δ (3). Transcription by these two factors leads to the induction of other factors involved in the development of the mature adipocyte phenotype, notably C/EBPα and PPARγ.

C/EBPβ and δ are members of the CCAAT/Enhancer Binding Protein family of bzip transcription factors (4). While ablation of C/EBPβ in vivo results in reduced white adipose tissue in mice and increased insulin sensitivity, the loss of C/EBPδ is without effect (5,6). Although C/EBPδ can compensate partially for the loss of C/EBPβ in vivo, the regulation of C/EBPβ transcriptional activity is an important control point during adipogenesis (7,8). In this regard, fibroblastic cell lines such as NIH 3T3, which express only low levels of endogenous
C/EBPβ, are unable to differentiate into adipocytes if ectopic C/EBPβ is not provided (7,9).

In addition to a role in adipogenesis, a role for C/EBPβ activity has been identified in numerous other biological processes including osteoblast differentiation, mammary gland development, female reproduction and liver regeneration (10-16). Due to its important role in differentiation processes, C/EBPβ activity is tightly regulated (17). In particular, the transcriptional activity of C/EBPβ is modulated by members of the nuclear hormone receptor superfamily such that steroid hormone receptors such as the glucocorticoid and progesterone receptor enhance its activity (8), while retinoic acid receptor α/γ attenuate C/EBPβ-mediated transcription (13,18).

Unlike glucocorticoids, co-treatment of preadipocytes with induction cocktail and retinoic acid (RA) leads to the inhibition of differentiation (18,19). In fact, RA is a potent repressor of adipocyte differentiation, both in vivo and in vitro (18-22). Pharmacological use of oral retinoids for the treatment of skin conditions (acne, psoriasis) or cancer causes weight loss in humans (23,24). Obese rats fed diets supplemented with vitamin A exhibit a decrease in adiposity without change in food intake (20), while a vitamin A-deficient diet elicits an increase in both adiposity and body weight (22). Dietary supplementation with all-trans RA reduces adipose marker expression, including C/EBPα (22) and, in mice, RA treatment triggers a remodelling of white adipose tissue depots such that they contain less lipid and express markers of brown adipose tissue (21).

Treatment of preadipocytes with RA during the early stages of differentiation triggers a profound inhibition of both adipocyte gene expression and lipid accumulation (19). Interestingly, the period of sensitivity to RA appears to be confined to early differentiation, which overlaps with the period of C/EBPβ activity, suggesting that retinoid signalling directly interferes with early transcriptional events (19). Indeed, while both C/EBPβ and C/EBPδ are normally induced in RA treated cells, expression of C/EBPα (a C/EBPβ target gene) as well as other adipocyte markers is abrogated (18,19).

In mesenchymal stem cell (MSC) lines, such as C3H10T1/2, ectopic expression of C/EBPβ stimulates adipogenesis and potently represses osteoblast differentiation (13) during which C/EBPβ prevents the expression of the master osteoblast regulator runx2 via direct promoter interaction (13). Osteoblastic differentiation of these cells can be evoked by treating confluent cultures continuously with RA for a period of 3-4 weeks. Within forty-eight hours of RA treatment there is a loss of C/EBPβ occupancy at its negative response element in the runx2 promoter (13) and results in runX2 expression. Interestingly, the effect of RA, while mediated by RARα/γ occurs in the absence of interaction with the runX2 promoter or C/EBPβ itself and requires forty-eight hours of treatment to be induced. These results suggest that the effect of RA on C/EBPβ DNA occupancy is dependent on an intermediary.

Herein, we demonstrate that retinoic acid can interfere with C/EBPβ occupancy of the C/EBPα promoter during adipogenesis of MSCs and 3T3-L1 preadipocytes. RA acts to specifically stimulate the expression of Smad3, its nuclear accumulation, and its transcriptional activity. We provide evidence that Smad3 is necessary for the inhibition of adipogenesis by RA and does so by specifically interfering with C/EBPβ DNA occupancy of the C/EBPα promoter.

EXPERIMENTAL PROCEDURES

Plasmids. The retroviral vector pLPCX-Smad3 (Addgene plasmid 12638), pGEX-Smad3 (Addgene plasmid 12630) and pEXL-FLAG-Smad3 (Addgene plasmid 10920) were purchased through Addgene and were kindly deposited by Dr. Rik Derynck and Dr. Bob Weinberg respectively (25-27). pLXSN, pLXSN-C/EBPβ, RSV-βgal and the wild type and mutant C/EBPα-reporter construct have been previously described (8). Smad transcriptional activity was monitored using the Cignal Smad reporter (luc) kit (SABiosciences) according to manufacturer’s instructions. A specific small hairpin RNA directed against the Smad3 sequence (5'
TGGAAAGGACGAAACACCGGTGCGAGA
AGGCGGTCAAGAGA
ctcgagTCTCTTGACC
CCTTCTCGCA
CCTTTTTGAATTCGCCAGC
ACAGTGGT-3′ was cloned into pMKO.1 (Addgene plasmid 8452) which was kindly deposited by Dr. Bob Weinberg (28). This Smad3 target sequence has been used previously (29) and is known to not affect the expression of other Smad factors.

**Antibodies.** Smad expression was evaluated with the following antibodies: Smad2/3 FL-425, Smad3 38-Q, Smad1/5/8 N-18, and Smad4 H-552 all from Santa Cruz Biotechnology. To evaluate adipocyte differentiation the following antibodies were used: C/EBPβ C-19, C/EBPα 14AA, adipin M-120, PPARγ H-100 (all from Santa Cruz), anti-actin and anti-tubulin (Sigma).

**Cell culture and Differentiation.** 3T3-L1 preadipocytes and NIH 3T3 fibroblasts (ATCC) were cultured in DMEM supplemented with 10% calf serum in a humidified incubator at 10% CO₂. C3H10T1/2 mesenchymal stem cells were cultured in DMEM supplemented with 10% fetal bovine serum in a humidified incubator at 5% CO₂. Replication incompetent retroviruses were generated in Phoenix Amphi packaging cells (ATCC). 10 cm dishes of 50% confluent target cells were infected using 1 ml of viral supernatant in the presence of 4 µg/ml polybrene (Sigma). Cells were selected in media containing 400 µg/mL G418 (Sigma) or 1 µg/mL puromycin (Sigma) for 10 days before differentiation to ensure retroviral expression in all cells.

To induce the differentiation of 3T3-L1 preadipocytes, two-days post-confluent cultures were treated with regular culture media supplemented with 100nM insulin, 1 X 10⁻⁶ M dexamethasone (DEX) and 500µM isobutylmethylxanthine (MIX) (induction cocktail) with the addition of vehicle or 1 X 10⁻⁶ M all trans retinoic acid (RA) as indicated in figure legends. Cells were refed every two days with media containing 100nM insulin, and vehicle or RA as required for a total of 8-10 days.

Adipogenic induction of C3H10T1/2 cells was achieved induction with insulin, DEX and MIX as described above or by the treatment of sub-confluent cultures with 3µM 5-azaacytidine (Sigma) for 48 hours after which cells were refed with growth media containing vehicle or 10⁻⁶ M RA as indicated in figure legends for a total of 14 days.

**Transient Transcription Assays.** NIH 3T3 or C3H10T1/2 cells were transiently transfected with the indicated plasmids using Lipofectamine 2000™ (Invitrogen) according to manufacturer’s instructions. Twenty-four hours after transfection, media was changed to include vehicle, 1 X 10⁻⁶ M RA or 1 ng/ml TGFβ (Sigma) as indicated for 24 hours after which cells were harvested and processed for luciferase activity and β-galactosidase activity according to standard procedures. Relative Light Units were corrected for transfection efficiency using the cotransfected RSV-βgal construct. Results are representative of three independent experiments. Error bars are the standard error of the mean.

**GST pulldown.** GST and GST-C/EBPβ were expressed in E.coli strain BL21. Bacteria were lysed and GST constructs were captured on glutathione-sepharose beads (Sigma) and washed extensively in lysis buffer before use. Full length Smad3 protein and the N-terminally truncated Smad3 lacking the MH1 domain (∆MH1) were produced by in vitro translation (IVT) following the manufacturers protocol (Promega). Each binding assay was achieved by mixing 0.5 µg GST protein with 10 µl of Smad3 translation product followed by incubation with rotation at 4°C for 90 min. After extensive washing in binding buffer, precipitates were separated by SDS-PAGE and detection of bound Smad3 protein was achieved by Western analysis (anti-Smad2/3 antibody, FL-425, Santa Cruz Biotechnology). Results are representative of three independent experiments.

**Nuclear localization studies.** Differentiating 3T3-L1 preadipocytes were harvested in PBS and immediately fractionated into cytoplasmic and nuclear extracts according to standard procedures. Equal amounts of protein were analyzed by Western blotting as determined by Bradford assay. Integrity of the nucleus was
verified using tubulin expression as a cytoplasmic marker and C/EBPβ as a nuclear marker. Results are representative of three independent experiments.

**ABCD Assays.** Five hundred nanograms of whole cell lysates from C3H10T1/2 cells was incubated with 2 μg of 5’ biotin-tagged double-stranded oligo in binding buffer [20 mM HEPES (pH 7.7), 50 mM KCl, 20% glycerol, 0.1% Nonidet P-40, 2 μg of sheared salmon sperm DNA] in the presence of recombinant GST or GST-Smad3. Oligos were then immunoprecipitated with streptavidin-conjugated magnetic beads (Dynal, Invitrogen) and precipitates were washed extensively in binding buffer and resolved by SDS-PAGE. Binding was evaluated by Western blot analysis using anti-C/EBPβ antibody (C-19; Santa Cruz Biotechnology). Results are representative of three independent experiments.

**Semi-quantitative RT-PCR.** For RT-PCR, RNA was extracted using the RNaseasy™ kit (QIAGEN) and was reverse transcribed using oligo-deoxythymidine and Superscript III™ (Invitrogen) according to manufacturer’s instructions. PCRs were optimized to determine the linear phase of amplification and results were compared with glyceraldehyde-3-phosphate message. Primer sequences used for amplification are available upon request. Results are representative of three independent experiments.

**Chromatin Immunoprecipitation.** C3H10T1/2 and 3T3-L1 cells were treated as indicated in the figure legends for 48 hrs and cells were washed 2x in serum-free media and treated with 1% formaldehyde at room temperature for 10 min. ChIP was performed essentially as described (12) using C/EBPβ C-19 (Santa Cruz Biotechnology) for precipitation at 4°C overnight. A type-matched nonspecific antibody was used as a negative control. DNA fragments were purified using the Qiaquick PCR purification™ kit (QIAGEN) and amplified by PCR. Results shown are representative of a minimum of three independent experiments.

**RESULTS**

**Retinoic acid is a potent inhibitor of adipogenesis.** Treatment of preadipocytes with RA, especially during the first 48 hours of differentiation, results in a profound inhibition of adipogenesis (18). This blockade in differentiation has been attributed to abrogated C/EBPβ-mediated transcription that is normally responsible for initiating the differentiation process (6,8,30-32). However, the mechanism of inhibition has remained elusive. Given that retinoic acid has been shown to enhance osteoblastic differentiation of MSC cultures, and that this activity has been attributed to a decrease in C/EBPβ occupancy of the runx2 promoter, we asked whether RA treatment could inhibit the differentiation of these cells into adipocytes (13). To address this question, confluent cultures of C3H10T1/2 mesenchymal stem cells, which are known to differentiate into adipocytes, osteoblasts, chondrocytes and skeletal muscle (33), were induced to differentiate into adipocytes with a cocktail containing glucocorticoids, insulin and a CAMP phosphodiesterase inhibitor (MIX) in the presence or absence of RA. Two days following induction, the cells were refed every 48 hours with medium containing insulin and vehicle or RA and allowed to differentiate for a total of 8 days. It was noted that while C3H10T1/2 cells spontaneously differentiated into adipocytes when kept in culture, treatment with adipogenic cocktail had little additional stimulatory effect, despite identical treatment evoking robust differentiation of preadipocytes such as 3T3-L1. Indeed, while both mesenchymal stem cells and preadipocytes share many similarities in their adipogenic programs, including the transcriptional cascade which drives adipogenesis and an early phase of clonal expansion. However, in preadipocytes, clonal expansion is a necessary step that is regulated by C/EBPβ and is required for the normal expression of the adipocyte master regulator PPARγ. While MSCs undergo clonal expansion, this process can be blocked without impacting PPARγ expression (34).

Despite the low levels of observed differentiation in C3H10T1/2 cells, pockets of lipid-laden cells could be observed in
differentiating cultures in the absence of RA treatment (Fig. 1A) and adipocyte markers C/EBPα, PPARγ and adipsin could be detected by Western Blotting (Fig. 1B). The addition of RA to the induction cocktail profoundly inhibited adipogenesis, resulting in a marked reduction in Oil Red O staining and adipocyte marker expression following 8 days of differentiation (Fig. 1A, B). It should be noted that while RA treatment reduced the expression of PPARγ2, the PPARγ isoform associated with adipogenesis, it did not impact on PPARγ1 expression (Fig. 1B). As demonstrated previously, RA treatment did not impact C/EBPβ expression in these cells (Fig. 1C) (13).

The work of Schwarz et al. suggested that the retinoic acid-induced block of adipogenesis in preadipocytes occurred downstream of C/EBPβ induction, and, since RA has no effect on C/EBPβ induction or protein expression, it likely involves a crippling of C/EBPβ transactivation activity (18). Consistent with this report, overexpression of C/EBPβ in C3H10T1/2 mesenchymal stem cells by retroviral transduction resulted in a robust stimulation of adipogenesis following induction to differentiate with induction cocktail (data not shown). This strong induction of differentiation by C/EBPβ expression prevented the direct comparison of adipocyte marker expression in control and C/EBPβ cells. To overcome this difficulty, we induced differentiation of empty virus control (pLX) and C/EBPβ-expressing cells with the DNA demethyltransferase inhibitor 5-azacytidine. 5-azacytidine treatment promotes the differentiation of C3H10T1/2 cells into all possible fates, though adipocytes are the first to appear in culture. Induction of differentiation with 5-azacytidine stimulated modest adipocyte differentiation in empty virus control cells (pLX) as evidenced by increased lipid accumulation (Fig. 2B) and adipocyte marker expression (Fig. 2C). Addition of RA to the induction and growth media of control cultures inhibited lipid accumulation completely (Fig. 2B), and markedly reduced adipsin and PPARγ2 expression (Fig. 2C).

Differentiation of C/EBPβ-expressing C3H10T1/2 cultures resulted in a large increase in the number of lipid-laden cells (Fig. 2B) and robust expression of PPARγ2 and C/EBPα (Fig. 2C) over control cells. Similar to control cells, co-treatment with RA resulted in the inhibition of both lipid accumulation and adipocyte marker expression. However, while treatment with RA potently inhibited differentiation of both control and C/EBPβ-expressing cells, overexpression of C/EBPβ offered partial protection from the effects of RA, as evidenced by the presence of neutral fat (Fig. 2B) and increased levels of both C/EBPα and PPARγ2 expression as compared to control cells treated with RA (Fig. 2C). When C/EBPβ was overexpressed in 3T3-L1 cells, these cells began to differentiate in the absence of hormonal stimulation. As expected, the ectopic expression of C/EBPβ in these cells potentiated both the development of the adipocyte phenotype and adipocyte marker expression following hormonal induction (Fig. 2D,E). Addition of RA to the media blocked the differentiation of the control cells and reduced their adipocyte marker expression (Fig. 2D,E). In contrast to the C3H10T1/2 model, in C/EBPβ-expressing 3T3-L1 cells, RA was no longer able to block differentiation, as evidenced by the robust adipocyte marker expression comparable to that of cells not treated with RA (Fig. 2D,E). These results suggest that overexpression of C/EBPβ can overcome the inhibitory effects of RA during adipocyte differentiation of preadipocytes. Indeed, we have observed that as little as a two-fold overexpression of C/EBPβ is sufficient to block RA-induced osteoblastogenesis in C3H10T1/2 cells (12).

**Retinoic acid decreases C/EBPβ occupancy of the C/EBPα promoter.** We have observed previously that treatment of mesenchymal stem cells with RA promoted osteoblastogenesis by reducing C/EBPβ occupancy of the runx2 promoter, where it acts as an inhibitor (13). Since C/EBPβ is an important positive acting transcription factor during adipogenesis, and the inhibitory effects of RA can be blocked by C/EBPβ overexpression in preadipocytes, we asked whether RA treatment resulted in decreased occupancy of C/EBPβ at adipogenic promoters, notably the C/EBPα promoter. Indeed, in both C3H10T1/2 mesenchymal stem
cells and 3T3-L1 preadipocytes, RA treatment resulted in a decrease in C/EBPβ occupancy of the C/EBPα promoter, a principal C/EBPβ target gene (Fig. 3). In C3H10T1/2 cells induced to differentiate for 48 hours with adipogenic induction cocktail, C/EBPβ could be observed occupying the C/EBPα promoter in the absence of RA treatment as measured by chromatin immunoprecipitation (Fig. 3A, lane 3). Co-treatment with RA resulted in a dramatic decrease in C/EBPβ occupancy of the C/EBPα promoter without affecting C/EBPβ protein expression (Fig. 3A lane 4, 1C). This evidence in addition to the RA-evoked loss of C/EBPβ occupancy of the runx2 promoter during osteoblastogenesis suggests that RA acts to decrease C/EBPβ occupancy in multiple promoter contexts (12).

Unlike C3H10T1/2 cells, unstimulated 3T3-L1 preadipocytes express only very low levels of C/EBPβ. In these cells C/EBPβ cannot be detected interacting with the C/EBPα promoter in the absence of treatment with induction cocktail following immunoprecipitation with anti-C/EBPβ antibody (8) (Fig. 3B, lane 1). This condition therefore serves as a negative control for the ChIP. Following a 48 hour treatment with adipogenic induction cocktail (MID), C/EBPβ could be seen occupying the C/EBPα promoter (Fig. 3B, lane 2). As was observed in MSCs, addition of RA to the medium of stimulated cells decreased C/EBPβ occupancy (Fig. 3B, lane 4) at this promoter.

The long RA treatments (48 hours) required to elicit loss of C/EBPβ occupancy at target promoters suggests that the actions of RA are not direct. Rather, the time frame permits the retinoic acid receptor to act on other promoter targets and stimulate the upregulation of a second factor which in turn could act on C/EBPβ DNA binding. To determine if de novo protein synthesis is required for the RA-induced loss of C/EBPβ occupancy of the C/EBPα promoter, we treated C3H10T1/2 cells with RA and cycloheximide, a protein synthesis inhibitor. This experiment was not possible in 3T3-L1 preadipocytes, as the treatment with cycloheximide inhibited the upregulation of C/EBPβ. In untreated cells and those treated with cycloheximide, C/EBPβ can be seen occupying the C/EBPα promoter (Fig 3C, lane 2). As demonstrated in Fig. 3A, treatment with RA resulted in loss of C/EBPβ occupancy at this promoter. This effect of RA was abrogated by co-treatment with RA and cycloheximide, suggesting that the RA-induced reduction in C/EBPβ occupancy of the C/EBPα promoter requires de novo protein synthesis (Fig. 3C, lane 8).

Smad3 is a novel retinoic acid-target gene in preadipocytes. Based on the kinetics of C/EBPβ displacement, the lack of protein-protein interaction between C/EBPβ and retinoic acid receptors and the cycloheximide sensitivity (13), we hypothesized that RA induces the expression of a second protein which acts to modify C/EBPβ DNA affinity. We thus undertook the task of identifying a protein target that was both upregulated by RA treatment and able to modulate C/EBPβ DNA-binding affinity. Of interest, CHOP, a C/EBP family member and known negative regulator of C/EBPβ during adipogenesis has been shown to be upregulated by RA in the blood (35). However, in both 3T3-L1 preadipocytes and C3H10T1/2 mesenchymal stem cells, RA failed to stimulate CHOP expression (data not shown). Given that TGFβ signalling can also inhibit adipogenesis, that Smad3/4 have been shown to interfere with C/EBPβ-mediated transcriptional responses, and that Smad4 can disrupt C/EBPβ binding to the haptoglobin promoter in vitro (36), we hypothesized that a member of the Smad family of transcription factors may be responsible for transducing the effects of retinoic acid in our system. Smad3 has been shown to inhibit adipogenesis of 3T3 F442A preadipocytes, but it remains unclear whether Smad3 can itself inhibit C/EBPβ-dependent adipogenesis of 3T3-L1 preadipocytes or mesenchymal stem cells. Furthermore, Smad3 has not been described as a RA-target gene in either of these systems, though recently Smad3 expression has been shown to be induced by RA treatment in CD4+ T cells (37).

Following a 48 hour treatment with RA, we observed a robust induction of Smad3 mRNA expression as measured by semi-quantitative
RT-PCR in C3H10T1/2 cells (Fig. 4A). Further time course analysis revealed that while Smad3 mRNA is upregulated by a shorter (24 hr) RA treatment, mRNA continues to accumulate and reaches greater levels after 4 days of continuous RA treatment (Fig. 4B).

Treatment with retinoic acid also resulted in a robust but transient increase in Smad3 protein expression in these same cells (Fig. 4C). We observed an increase in Smad3 expression after 24 hours of RA treatment (Fig. 4C). Despite continued incubation with RA, Smad3 levels returned to baseline after 3 days of treatment (Fig. 4C). Interestingly, the time frame of Smad3 upregulation correlates tightly with the window of C/EBPβ activity during adipogenesis (7,8). In 3T3-L1 preadipocytes, a 48 hour RA treatment induced Smad3 protein expression in 3T3-L1 cells irrespective of co-treatment with adipogenic cocktail to induce C/EBPβ expression and differentiation (Fig. 4D). RA treatment did not stimulate the expression of Smad2 which has a greater molecular weight and is recognized by a Smad2/3 antibody. Rather, Smad2 levels are downregulated by treatment with MIX and insulin, but not further affected by the addition of the synthetic glucocorticoid dexamethasone. Furthermore, the levels of other receptor associated Smads (Smad1/5/8) were also downregulated by adipogenic induction media and unchanged by RA treatment (Fig. 4D). Taken together, these results suggest that Smad3 is positively regulated by RA treatment in both mesenchymal stem cells and preadipocytes.

While both protein and mRNA levels of Smad3 are rapidly induced by RA treatment, mRNA levels continue to rise while protein levels are downregulated, suggesting a more complex mechanism for regulation by RA. To establish whether de novo protein synthesis is required for the induction of Smad3 mRNA expression by, we treated C3H10T1/2 cells with the protein synthesis inhibitor cycloheximide (CHX) and evaluated Smad3 mRNA levels following RA treatment by semi-quantitative RT-PCR. While Smad3 mRNA is induced following a 48 hour RA treatment, co-treatment with CHX blocked this induction (fig. 4E), indicating that de novo protein synthesis is required for the induction of Smad3 mRNA by RA and suggesting that the regulation of Smad3 expression by RA occurs via an indirect mechanism.

C/EBPβ DNA occupancy is abrogated by Smad3. In HepG2 cells the association of C/EBPβ with Smad4 is thought to provoke a decrease in occupancy of the haptoglobin promoter in vitro (36). During both osteoblastogenesis and adipogenesis we have observed that RA treatment results in decreased DNA occupancy by C/EBPβ (Fig. 3) which may be attributed to an association of C/EBPβ with Smad factors (13). Using a GST-pulldown experiment, we evaluated the interaction of GST-C/EBPβ with in vitro translated Smad3 (Fig. 5A). Whereas GST alone did not interact with Smad3, we found a weak interaction between GST-C/EBPβ and Smad3 consistent with a previous report (38). Further analysis using an N-terminally truncated Smad3, which lacks the Smad MH1 domain (ΔMH1), revealed that this domain was necessary for the interaction of Smad3 with GST-C/EBPβ as the truncated Smad3 was unable to interact with GST-C/EBPβ (Fig. 5A). Previous studies concentrating on C/EBPδ have demonstrated that Smad3 interacts with this factor via its bzip domain, a highly conserved region responsible for heterodimerization and DNA binding (38). Interaction of Smad3 with the C/EBPβ bzip domain could conceivably inhibit DNA binding through steric interference.

To measure the impact of Smad3 expression on C/EBPβ DNA-binding ability, we used an in vitro Avidin-Biotin Conjugated DNA assay (ABCD Assay), which measures the occupancy of endogenous C/EBPβ on a short double stranded oligonucleotide encoding four consensus C/EBP elements. While C/EBPβ interacts efficiently with this element in the absence of Smad3, addition of GST-Smad3 reduced interaction with the promoter element by greater than 60% as compared to addition of GST alone (Fig. 5B, C). This is the first report implicating Smad3 in the modulation of C/EBPβ DNA binding activity, though Smad4-mediated inhibition of DNA binding has been described in vitro (36).
To test the effects of ectopic Smad3 expression on C/EBPβ DNA binding in vivo, we produced pooled stable 3T3-L1 cells lines expressing FLAG-tagged Smad3 by retroviral transduction (Fig. 5D). The expression of Smad3 in our stable cell line was approximately 5 times greater than the levels of Smad3 in control cell following induction with RA (Fig. 5D) and this level of expression did not impact on C/EBPβ expression levels as demonstrated by Western blotting (Fig. 5D).

To evaluate the effect of ectopic Smad3 expression on C/EBPβ promoter occupancy in vivo, we used chromatin immunoprecipitation to assess C/EBPβ occupancy of the C/EBPα and the resistin promoter (Fig. 5F). In cells overexpressing Smad3, the occupancy of C/EBPβ at both the C/EBPα promoter and the resistin promoter were reduced as compared to the occupancy observed in empty vector controls (Fig. 5E). However, occupancy of the C/EBPα promoter, though reduced, was still observed in Smad3 expressing cells, suggesting that ectopic of Smad3 alone cannot completely recapitulate the effects of RA in this system. Thus, while Smad3 can act to interfere with C/EBPβ DNA-binding in vitro, ectopic expression of Smad3 in itself is not sufficient for this effect in vivo. Despite the modest reduction of C/EBPβ occupancy in vivo following Smad3 overexpression, we observe a reduction of approximately 50% in the C/EBPβ-mediated transactivation from the C/EBPα promoter in a transient transcription assay in C3H10T1/2 cells (Fig. 5F). This inhibition of transcription by ectopic Smad3 is lost upon overexpression of C/EBPβ in these cells. Furthermore, Smad3 was unable to inhibit transcription from a mutant C/EBPα promoter, which has the C/EBP element abolished by site directed mutagenesis, suggesting that the repressive actions of Smad3 at this promoter are mediated through the C/EBP element (Fig. 5F).

Retinoic acid treatment induces nuclear accumulation and activation of Smad3.

Smad3 is a TGFβ receptor-associated transcription factor that following TGFβ binding to its receptor, is phosphorylated leading to its heterodimerization with Smad4 and its nuclear accumulation (39). While this mechanism appears to be the primary mode of nuclear accumulation for Smad3, Smad3 nuclear localisation has been described in the absence of Smad4 (40). Since C/EBPβ is a nuclear protein and RA treatment does not affect its subcellular localization (data not shown), Smad3 must gain access to the nucleus in the absence of TGFβ signalling to interfere with C/EBPβ DNA-binding activity. To assess the subcellular localisation of Smad3 following retinoic acid treatment, 3T3-L1 cells were treated with adipogenic induction cocktail including vehicle, TFGβ or RA for 48 hours and nuclear Smad3 was evaluated by subcellular fractionation and Western blotting (Fig. 6). Western analysis of differentiating 3T3-L1 cell nuclear extracts revealed that while adipogenic induction cocktail had little effect on Smad3 nuclear accumulation, TGFβ treatment resulted in nuclear accumulation of both Smad3 and Smad2 (Fig 6A). Treatment with RA, which we have demonstrated to increase cellular Smad3 levels, resulted in an increase in nuclear Smad3, but not Smad2. Peak levels of nuclear Smad3 were observed with 48 hours of RA treatment, corresponding to the peak of Smad3 protein expression (Fig. 6A,B and 4C,D). For TGFβ treated cells, maximal nuclear accumulation of Smad3 was observed between 12-24 hours of treatment (Fig . 6D), while levels of nuclear Smad3 were highest following 48 hours of RA treatment. The kinetics of Smad3 nuclear accumulation is in concordance with our previous observations which noted that the displacement of C/EBPβ by RA required at least 48 hours to be produced, with shorter hormone treatments being ineffective (13).

Given that cellular Smad3 levels increase following RA treatment, the increase in nuclear Smad3 may be a result of higher cellular levels of this factor. However, Smad3’s molecular weight precludes the passive accumulation into the cell nucleus. Despite this, many different modes of Smad3 transport into the nucleus have been described. The classic mode of Smad3 nuclear transport involves heterodimerization with Smad4. In RA treated cells we were unable to correlate Smad4 nuclear accumulation with the presence of nuclear Smad3 (Fig. 6C).
Nuclear and cytoplasmic extracts prepared from differentiating 3T3-L1 preadipocytes co-treated with RA or vehicle for 48 hours indicate that while RA treatment stimulates the upregulation of Smad3 expression in both the cytoplasm and the nucleus, Smad4 is not redistributed to the nucleus accordingly, suggesting that the increased nuclear Smad3 occurs independently of Smad4 (Fig. 6C). Tubulin and C/EBPβ are shown as markers of the cytoplasmic and nuclear fractions respectively. Furthermore, despite the reported ability of RA to stimulate Smad3 phosphorylation via MAPK activation, we were unable to correlate changes in phosphorylation with RA-induced nuclear accumulation of Smad3 (data not shown and (41)). Taken together these results suggest that RA may act at least in part to induce an increase in nuclear Smad3 in a Smad4-independent fashion.

Despite the novel mechanics of Smad3 nuclear accumulation in retinoic acid-treated cells, RA-induced Smad3 was able to drive Smad-mediated transcription, suggesting that this population of Smad3 is functional (Fig. 6D). Transient transcription experiments in NIH 3T3 cells revealed that treatment with RA in the absence of ectopically expressed Smad3 was sufficient to induce a 2-fold activation of a synthetic Smad-responsive promoter (Fig 6D). NIH 3T3 cells were chosen for this experiment because they have the same origin as 3T3-L1 cells but are easier to transfect. In contrast to the RA-treated cells, ectopic expression of Smad3 and treatment with TGFβ resulted in a 5-fold activation of this same promoter. Taken together, these results strongly suggest that treatment of cells with RA results in the upregulation of Smad3 expression such that transcription from Smad-responsive genes can be induced.

**Smad3 overexpression is not sufficient to inhibit adipogenesis.** To directly investigate the role of Smad3 in the inhibition of adipogenesis by RA, we used the pooled stable 3T3-L1 cell lines created by retroviral transduction described in Fig. 5. These cells express approximately 5 times the Smad3 as control cells (Fig. 5D). When these cells were induced to differentiate for 8 days, we observed a significant reduction in Oil red O positive cells (Fig. 7A) in cells overexpressing Smad3 as compared to empty vector controls. However, adipogenesis was more potently inhibited by the addition of RA to the adipogenic cocktail in both Smad3-expressing and empty vector control cell lines (Fig. 7A). Despite a significant reduction in the number of lipid-containing cells in cultures overexpressing Smad3, Western analysis of adipogenic marker expression indicated that while RA potently inhibited the expression of both C/EBPα and PPARγ2, ectopic expression of Smad3 alone was unable to entirely recapitulate these effects. While C/EBPα levels were unaffected by Smad3 expression, PPARγ2 levels were reduced two-fold in Smad3-expressing cells stimulated to differentiate for 8 days (Fig. 7B). Taken together with the ChIP data that indicates that Smad3 overexpression is insufficient to prevent C/EBPβ DNA binding in vivo, these results suggest that in 3T3-L1 preadipocytes, expression of Smad3 alone was not sufficient to inhibit adipocyte differentiation in the absence of RA treatment (7,8). Indeed, if RA acts to actively promote Smad3 nuclear accumulation in addition to stimulating its expression, ectopic expression of Smad3 alone would not be expected to exert its effects on a nuclear target such as C/EBPβ in the absence of RA.

**The upregulation of Smad3 by retinoic acid is necessary for inhibition of adipogenesis by RA.** To determine if the stimulation of Smad3 expression by RA is required for the inhibition of adipogenesis, we retrovirally transduced 3T3-L1 cells with virus to express a small hairpin RNA directed against Smad3 (shSmad3) or with empty virus. The shRNA was designed to a region specific to Smad3 and is not predicted to bind any other known Smad protein. This targeting sequence has been used with success by other groups without cross-reactivity (29). Expression of the shRNA reduced both the basal and RA-induced levels of Smad3 such that following RA treatment, cell expressing the shRNA did not upregulate Smad3 expression (Fig. 8A). In addition, as predicted the shRNA did not affect Smad2 levels, the Smad1/5/8 levels or C/EBPβ expression (Fig. 8A).
When these cells were induced to differentiate with standard adipogenic cocktail and cultured for 8 days, no apparent differences in morphology or lipid accumulation were observed between shSmad3-expressing and empty vector controls (Fig 8B). However, when cells were co-treated with RA, lipid accumulation was profoundly inhibited in empty vector control cells but largely unaffected in shSmad3-expressing cells (Fig. 8B). These results are supported by analysis of adipocyte marker expression (Fig. 8C). In the absence of RA, both empty vector control cells and shSmad3-expressing cells express similar levels of C/EBPα and PPARγ2. Following treatment with RA, while empty vector control cells display reduced levels of both C/EBPα and PPARγ2, shSmad3-expressing cells maintain high levels of these proteins (Fig. 8C).

Analysis of C/EBPβ occupancy of the C/EBPα promoter by ChIP in shSmad3-expressing cells revealed that Smad3 was required for the modulation of C/EBPβ DNA binding ability in 3T3-L1 cells following RA treatment. While RA treatment reduced C/EBPβ occupancy of the C/EBPα promoter in empty vector control cells induced to differentiate for 48 hours, C/EBPβ occupancy was unaffected by retinoic acid treatment in shSmad3-expressing cells. Taken together, these data suggest that the stimulation of Smad3 expression by retinoic acid is necessary for the inhibition of adipogenesis by this hormone and the reduction of C/EBPβ occupancy.

**DISCUSSION**

Despite a role for retinoids in the inhibition of adipocyte differentiation both in vivo and in vitro, the use of retinoids for weight loss is precluded by the varied toxic effects of these compounds and their teratogenicity. However, understanding how RA impacts on adipocyte differentiation reveals critical regulatory networks and may assist in the development of new pharmacological strategies to inhibit adipocyte hyperplasia.

Herein we provide compelling evidence that RA inhibits adipogenesis of both mesenchymal stem cells and preadipocytes through the upregulation of Smad3 and the stimulation of its nuclear accumulation. Interestingly, while overexpression of C/EBPβ in MSCs only partially rescues inhibition of adipogenesis by RA, it completely blocks the inhibition in preadipocytes. This observation highlights the differences between the two models studied. Indeed, while both models express C/EBPβ, the actions of C/EBPβ including the regulation of clonal expansion appear to be more important in preadipocytes than MSCs. Notwithstanding these differences, RA is a potent inhibitor of adipocyte differentiation in both models. In MSCs the effects of RA may extend to the promotion of alternative cell fates, making the study of the biological effects of C/EBPβ activity more complex in this model.

The activity of Smad3 in preadipocytes converges on the transcription factor C/EBPβ whose DNA-binding activity on target promoters is decreased in vitro by Smad3 and in vivo by Smad3 and RA. While the simple overexpression of Smad3 does not completely recapitulate the effects of RA in our models, this difference may be due to several factors. First, Smad3 expression is sustained in our system, whereas RA triggers only a transient rise in Smad3 levels, coinciding with the window of C/EBPβ activity. Since our stable cell lines maintain high levels of Smad3 expression throughout differentiation, this may influence downstream signalling pathways. Indeed, given that Smad3 has been shown to bind the C/EBPβ bzip domain, it is possible that Smad3 expression could, in our overexpression system impinge on C/EBPα transcriptional responses as well.

Second, ectopic expression of Smad3 alone may not permit the nuclear accumulation required to affect C/EBPβ-mediated transcriptional responses. RA may then act, in addition to upregulating Smad3 expression, to stimulate Smad3 nuclear accumulation in a Smad4-independent fashion. Indeed, in our system, overexpression of Smad3 was unable to completely recapitulate the actions of RA, suggesting that Smad3 alone is not sufficient to inhibit adipogenesis. However, we demonstrate that the upregulation of Smad3 by retinoic acid is a necessary step for the inhibition of adipogenesis by RA, and in the absence of...
Smad3, both inhibition of adipogenesis and the effect of C/EBPβ DNA binding are lost. Interestingly, while Smad3 mRNA is rapidly upregulated following RA treatment, its level continues to rise over a period of 4 days, a time frame which corresponds to the downregulation of Smad3 protein expression. This apparent discrepancy may be attributed to multiple actions of RA. RA may act to stimulate the transcription from the Smad3 gene through a mechanism that requires de novo protein synthesis and results in the accumulation of Smad3 protein. With continued RA treatment, while the mRNA levels continue to rise, the protein synthesis of this message may be inhibited or alternatively the Smad3 protein stability may be altered resulting in a decrease in Smad3 protein expression despite high mRNA levels.

The observed upregulation and nuclear accumulation of Smad3 occurs independently of TGFβ signalling. However, there have been numerous reports describing cross-talk, both positive and negative, between RA signalling and the TGFβ pathway. Our results may provide the link to explain the pleiotropic observed effects of retinoid treatment. Interestingly, during adipogenesis, TGFβ is also a potent inhibitor of differentiation, and this effect has been attributed to the actions of Smad3/4 (38). While TGFβ does not affect C/EBPβ protein levels, it does impinge on its ability to drive transcription from a synthetic C/EBP-responsive promoter and the leptin promoter in transient transcription assays (38). These results may be explained at least in part by the effects of Smad3 on C/EBPβ DNA-binding ability but also on reported reduction of C/EBPβ transactivation activity following TGFβ treatment (38).

C/EBPβ is a ubiquitously expressed transcription factor whose activity is tightly controlled by multiple translational starts, post-translational modification and homo- and heterodimerization with other bZIP transcription factors (7,32,42,43). Due to the numerous levels of control, C/EBPβ's transcriptional activity ranges the entire spectrum from repression to potent activation. Here we show that retinoic acid treatment can prevent the interaction of C/EBPβ with its target promoters and thereby can interfere with C/EBPβ-mediated transcriptional responses. We expect that the importance of this mechanism extends beyond adipogenesis. Indeed, our own results indicate that C/EBPβ, a repressor of osteoblastogenesis, can be prevented from interacting with the runX2 promoter by retinoic acid, thereby stimulating the osteoblast differentiation process (13). Interestingly, Smad3 is also a regulator of osteoblast differentiation; its expression stimulates early differentiation but represses late differentiation in a runx2-dependent fashion (44,45). These results are consistent with our own observations which demonstrate that C/EBPβ expression potently represses early osteoblast differentiation but can act as an activator at later stages (13).

The RA/Smad3 mediated interference of C/EBPβ activity may also be important outside of mesenchymal lineage decisions. Indeed, whereas C/EBPβ is required for both liver regeneration and ductal morphogenesis of the mammary gland, retinoic acid has the opposite effect in both systems (15,43,46-50). It is interesting to speculate that these effects may be mediated through the Smad3 pathway described herein.

FOOTNOTES
*This work is supported by a grant from the Heart and Stroke Foundation of Ontario Grant #NA 6375.
a The abbreviations used are:
   C/EBP, CCAAT/Enhancer Binding Protein
   MIX, isomethylxanthine
   DEX, dexamethasone
   RA, retinoic acid
   RAR, retinoic acid receptor
   MH1, Mad Homology Domain 1
The authors wish to thank Drs. Rik Derynck and Bob Weinberg for plasmids provided.
REFERENCES

FIGURE LEGENDS

Figure 1. Retinoic acid treatment inhibits adipocyte differentiation of mesenchymal stem cells. (A) Oil red O micrographs of C3H10T1/2 cells retrovirally transduced to express C/EBPβ or with empty virus and induced to differentiate with insulin, MIX and dexamethasone for two days in the presence or absence of RA. Following induction cells were refed every two days with growth media containing insulin and vehicle or RA as indicated for a total of 8 days. (B) Western analysis of adipocyte marker expression 8 days after induction to differentiate in the presence or absence of RA. Actin is shown as a loading control. (C) Western analysis of C/EBPβ expression following a 48 hour retinoic acid treatment. Actin serves as a loading control.

Figure 2. C/EBPβ overexpression prevents the inhibition of adipogenesis by RA. (A) Western analysis of C/EBPβ expression in C3H10T1/2 cells retrovirally transduced to express C/EBPβ or with empty virus (pLX) and induced to differentiate with a 2 day 5-azacytidine treatment in the presence or absence of RA. Actin serves as a loading control. (B) Oil red O micrographs of cells transduced and induced to differentiate as in (A) in the presence or absence of RA for 14 days. (C) Western analysis of adipocyte marker expression in cells transduced and induced as in (B). Actin serves as a loading control. (D) Oil red O micrographs of 3T3-L1 cells retrovirally transduced to express C/EBPβ or with empty virus (pLX) and induced to differentiate with insulin, MIX and dexamethasone for two days in the presence or absence of RA. Following induction cells were refed every two days with growth media containing insulin and vehicle or RA as indicated for a total of 10 days. (E) Western analysis of C/EBPβ and adipocyte marker expression in cells transduced and induced as in (D). Actin serves as a loading control.

Figure 3. Retinoic acid treatment reduces C/EBPβ occupancy of the C/EBPα promoter in vivo. (A) Chromatin immunoprecipitation (ChIP) analysis of C/EBPβ occupancy of the C/EBPα promoter in C3H10T1/2 cells following treatment with vehicle or RA for 48 hrs as indicated. Isolated chromatin was immunoprecipitated using anti-C/EBPβ antibody (β) or with a non-specific type matched antibody (NS). (B) ChIP analysis of C/EBPβ occupancy of the C/EBPα promoter in 3T3-L1 cells following induction to differentiate with adipogenic cocktail (MIX, insulin, dexamethasone =MID) and treated with vehicle or RA for 48 hours as indicated. Unlike C3H10T1/2 cells, in the absence of adipogenic cocktail, C/EBPβ protein levels are low and resulting in no occupancy of the C/EBPα promoter. Pulldown from unstimulated cells with anti-C/EBPβ antibody (lane 1) serves as a negative control for the ChIP. (C) ChIP analysis of C/EBPβ occupancy (β) of the C/EBPα promoter in C3H10T1/2 cells treated with RA and cycloheximide (CHX) as indicated for 48 hours. Input represents 25% of the material used for immunoprecipitation. NS = non-specific type matched antibody.

Figure 4. Retinoic acid treatment triggers upregulation of Smad3 protein expression. (A) Semi-quantitative RT-PCR analysis of Smad3 mRNA expression in C3H10T1/2 cells treated with retinoic acid (RA) or vehicle for 48 hours. Amplification of GAPDH is used as a loading control. (B) Semi-quantitative RT-PCR analysis of Smad3 mRNA expression in C3H10T1/2 cells treated with RA for the indicated times. Amplification of GAPDH is used as a loading control. (C) Western analysis of Smad3 expression in C3H10T1/2 cells treated with vehicle or RA for the indicated time period in days. Actin serves as a loading control. (D) Western analysis of Smad expression in 2-day post-confluent 3T3L1 cells induced to differentiate with the cAMP phosphodiesterase inhibitor MIX and insulin (+MI) in the presence or absence of the synthetic glucocorticoid dexamethasone (DEX) and RA as indicated for 48 hours. Actin serves as a loading control. (E) C3H10T1/2 cells treated with RA and cycloheximide (CHX) as indicated for 48 hours were harvested for RNA isolation and semi-quantitative RT-PCR for Smad3 and GAPDH.
Figure 5. Smad3 interferes with C/EBPβ occupancy of target promoters. (A) Interaction of in vitro translated full length Smad3 and a truncated Smad3 lacking the MH1 domain (ΔMH1) with GST and GST-C/EBPβ. Following binding, precipitated Smad3 was revealed by Western blotting. (B) ABCD assay evaluating the interaction of endogenous C/EBPβ from 3T3-L1 cells induced to differentiate for 24 hrs, with a double-stranded oligonucleotide coding for 4 repeats of a C/EBP consensus motif in the presence or absence of recombinant Smad3. Input represents 10% of the material used in the binding reaction. (C) Quantification of the interaction of C/EBPβ with a consensus DNA motif as in (A) by phosphorimager analysis. Data results from three experiments. Error bars represent the standard error of the mean. (D) Western analysis of Smad and C/EBPβ expression in 3T3-L1 cells retrovirally transduced to express Smad3 or with empty virus and induced to differentiate into adipocytes with standard cocktail for 48 hrs in the presence or absence of RA as indicated. Note that retrovirally expressed Smad3 is FLAG-tagged and thus migrates higher than endogenous Smad3. Endogenous Smad3 is indicated as the lower band observed in RA-treated empty virus control cells. The larger Smad2 is indicated by an arrowhead. Actin is used as a loading control. (E) ChIP analysis of C/EBPβ occupancy of the C/EBPα promoter and the resistin promoter in 3T3-L1 cells retrovirally transduced to express Smad3 or with empty virus and induced to differentiate for 48 hrs. Chromatin was immunoprecipitated using anti-C/EBPβ antibody (β) or a type matched non-specific antibody (NS). Inputs represent 25% of the material used for precipitation. (F) Transient transcription assay in C3H10T1/2 cells measuring activation of the mouse C/EBPα promoter by C/EBPβ and Smad3. Both the wild type promoter (WT) and a mutant reporter (MT) with the C/EBP response element abolished were used. Data is reported as fold induction over the activity of the respective promoters in the absence of C/EBPβ and Smad3. Luciferase activity was corrected with β-galactosidase activity from a cotransfected reporter to correct for transfection efficiency. Data represents the means of two independent experiments performed in duplicate. Error bars represent the standard error of the mean.

Figure 6. RA treatment increases nuclear Smad3. (A) Time course Western analysis of nuclear Smad3 in 3T3-L1 cells induced to differentiate with MIX, insulin and DEX (+MID) and treated with vehicle, RA or TGFβ treatment as in (A). (B) Quantification of nuclear Smad3 following RA or TGFβ treatment as in (A). (C) Nuclear and cytoplasmic localization of Smad3 and Smad4 in 3T3-L1 cells induced to differentiate for 48 hours in the presence or absence of RA. Tubulin is a cytoplasmic marker, whereas C/EBPβ is used to evaluate the integrity of the nuclear compartment. (D) Transient transcription assay measuring the activation of a synthetic Smad-responsive reporter construct by a 48 hr RA treatment in NIH 3T3 cells. Relative light units were corrected for β-galactosidase activity from a cotransfected constitutively active reporter construct to correct for transfection efficiency. Data represents three independent experiments and error bars are the standard error of the mean (* p<0.05).

Figure 7. Smad3 overexpression is not sufficient to inhibit adipocyte differentiation. (A) Oil Red O micrographs of 3T3-L1 cells retrovirally infected to express Smad3 or with empty vector (pLP) and induced to differentiate into adipocytes with standard cocktail for 8 days in the presence or absence of RA. (B) Western analysis of adipocyte marker expression in 3T3-L1 cells transduced and induced to differentiate as in (A). Actin expression is shown as a loading control.

Figure 8. Inhibition of adipogenesis by RA is abrogated by loss of Smad3 expression. (A) Western analysis of Smad and C/EBPβ expression in 3T3-L1 cells retrovirally transduced to express a small hairpin RNA directed against Smad3 (shSmad3) or with empty vector (pMKO) and induced to differentiate into adipocytes with standard cocktail for 48 hrs in the presence or absence of RA. (B) Oil Red O micrographs of 3T3-L1 cells retrovirally transduced as in (A) and induced to differentiate into adipocytes with induction cocktail for 8 days. (C) Western analysis of adipocyte marker expression in 3T3-L1 cells transduced and induced to differentiate as in (B). (D) ChIP analysis of C/EBPβ occupancy of the C/EBPα promoter in cells retrovirally transduced and induced to differentiate as in (A). Chromatin
was immunoprecipitated with anti-C/EBPβ antibody (β) or a type-matched non-specific antibody (NS) as indicated. Input represents 25% of the material used for immunoprecipitation.
Figure 1

A C3H10T1/2

-Oil Red O

-B +RA

-C/EBPβ (38 kDa)

-actin (42 kDa)

-actin (43 kDa)

48 hrs

B +RA

-RA

-C/EBPα (42 kDa)

-PPARγ2 (58 kDa)

-PPARγ1

-adipsin (28 kDa)

8 days

C +RA

-RA

-C/EBPβ (38 kDa)

-actin (42 kDa)

48 hrs
**Figure 2**

A. RA and C/EBPβ expression in C3H10T1/2 cells on day 2.

B. Oil Red O staining of C/EBPβ in pLX and RA conditions.

C. Western blot analysis of PPARγ2, C/EBPα, adipin, and actin in 3T3 L1 cells.

D. Immunohistochemistry of C/EBPβ with Oil Red O staining.

E. Western blot analysis of PPARγ1, PPARγ2, C/EBPβ, adipin, and actin in 3T3 L1 cells.
Figure 3

(A) C3H10T1/2

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C/EBPα
Figure 4
Figure 5
Figure 6

A. Time course of Fold Induction of Veh, RA, and TGF-β on various proteins.

B. Graph showing Relative Smad3 nuclear localisation over time post-induction.

C. Western blot analysis of Smad3 (55 kDa), Smad4 (55 kDa), tubulin (55 kDa), and C/EBPβ (38 kDa).

D. Bar graph showing Fold Induction of Veh, RA, and TGF-β.
Figure 7
Figure 8

A

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Smad3 (55 kDa)
Smad2 (60 kDa)
Smad1,5,8 (55 kDa)
C/EBPβ (38 kDa)
actin (42 kDa)

day 2

B

pMKO
shSmad3
+MID
+MID +RA

C

RA
shSmad3
- & + & - & +
- & - & + & +
C/EBPα (42 kDa)
PPARγ2 (58 kDa)
actin (42 kDa)

day 8

D

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Figure 8
Transcription factor SMAD3 is required for the inhibition of adipogenesis by retinoic acid
François Marchildon, Catherine St-Louis, Rahima Akter, Victoria Roodman and Nadine L. Wiper-Bergeron

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