The Forkhead Transcription Factor FoxC2 Inhibits White Adipocyte Differentiation*

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In this study, we show that expression of FoxC2 blocks the capacity of 3T3-L1 preadipocytes to undergo adipogenesis in the presence of dexamethasone, isobutylmethylxanthine, and insulin. This block is characterized by an extensive decrease in the expression of proteins associated with the function of the mature fat cell, most notably C/EBPα, adiponectin, perilipin, and the adipose-specific fatty acid-binding protein, FABP4/aP2. Since the expression of these proteins lies downstream of PPARγ, we overexpressed PPARγ in Swiss mouse fibroblasts to promote adipocyte differentiation. We show that FoxC2 blocks the ability of PPARγ to induce adipogenic gene expression in response to exposure of the cells to dexamethasone, isobutylmethylxanthine, insulin, and a PPARγ ligand. Interestingly, the expression of aP2 escapes the inhibitory action of FoxC2 under conditions that promote maximum PPARγ activity. In contrast, FoxC2 inhibits the expression of C/EBPα, perilipin, and adiponectin even in the presence of potent PPARγ ligands. Finally, we show that FoxC2 does not affect the ability of PPARγ to bind to or transactivate from a PPARγ response element. These data suggest that FoxC2 blocks adipogenesis by inhibiting the capacity of PPARγ to promote the expression of a subset of adipogenic genes.

Adipose tissue, once thought to be just a storage depot for excess energy supply, is now realized to be a fully functioning endocrine organ, secreting numerous factors that help to maintain normal whole body metabolism (1, 2). Significant changes in adipose mass or perturbation in adipocyte signaling can result in the disruption of overall metabolic homeostasis (3, 4). Changes in adipose mass can result from increased lipid storage in preexisting adipocytes as well as an increase in adipocyte number through the proliferation and differentiation of preadipocytes (5). The differentiation of preadipocytes into mature insulin-responsive adipocytes results from coordinated signaling cascades descending on many transcription factors that promote the expression of terminal adipogenic genes (4, 6). Much of what is known regarding the transcriptional events of adipogenesis has been gained through the use of cell lines predisposed to the adipocyte lineage. The most frequently used are the 3T3-L1 and 3T3-F442A cell lines (7–9). Signaling events that induce adipogenesis promote both the increased expression of some factors as well as the down-regulation of others. Early events in adipogenesis include up-regulation of C/EBPβ and C/EBPδ, which in turn activate transcription of PPARγ and C/EBPα (4, 10–13). These two factors work in a cooperative fashion to promote the expression of terminally adipogenic genes. In fact, the expression of both PPARs and C/EBPα is necessary and sufficient for complete adipogenesis to occur (14–17). In addition, the inducers of adipocyte differentiation promote the down-regulation of factors that maintain the undifferentiated state of preadipocytes. Members of both the GATA family as well as factors in the Wnt signaling pathway act as negative regulators of adipogenesis, and their maintained expression during differentiation blocks terminal adipogenesis (18–21). More recently, members of the forkhead (FOX) family of transcription factors have been identified as playing both positive and negative roles in adipocyte differentiation.

Forkhead transcription factors have been shown previously to play varying roles during early development by regulating growth, differentiation, and apoptosis of different cell types (22–24). They bind as monomers to the consensus sequence 5’-TGACCTTGACCT-3’, and selective binding is dictated by flanking sequences as well as the relative abundance of individual members. The affinity of FOX proteins to bind to sequences overlaps to a great extent between members, and in addition, some FOX proteins primarily act as transcriptional activators while others act as transcriptional repressors (24). Recently, it has been shown that specific FOX family members, FoxO1, FoxA2, and FoxC2, play a role in maintaining normal metabolic homeostasis in the adult organism. FoxO1 expression increases during adipogenesis, and its transcriptional activity is negatively regulated by insulin signaling through Akt phosphorylation during the early phase of adipogenesis. Overexpression of a constitutively active FoxO1 blocks adipocyte differentiation as a result of induced expression of the tumor suppressor, p21CIP1, and the concurrent block in clonal expansion (25, 26). Expression of FoxA2 also inhibits adipocyte differentiation, and it is induced in an obese diabetic mouse, proposing a role for negative feedback against adipocyte differentiation. FoxA2 induces the expression of pref-1, a known inhibitor of adipogenesis (27), as well as genes that function in glucose and lipid metabolism (28). FoxC2 has been shown to have an effect on the formation of white and brown adipose tissue in rodents. Its expression in mice is restricted to adipose tissue, and overexpression of FoxC2 targeted to adipose tissue results in attenuation of white adipose tissue formation while brown adipose development is grossly enhanced (29).

1 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor γ; MIX, isobutylmethylxanthine; DMEM, Dulbecco’s modified Eagle’s medium; DEX, dexamethasone; CREB, cAMP-response element-binding protein; LUC, luciferase; TET, tetracycline; RT, reverse transcription; PPRE, PPAR response element.
from those mice indicate an increase in insulin sensitivity and more efficient signaling through β-adrenergic receptors. Furthermore, the expression of FoxC2 is up-regulated in mature 3T3-F442A adipocytes upon exposure to insulin and tumor necrosis factor α (30). The dramatic decrease in the white adipose depots in FoxC2 transgenic mice suggested that FoxC2 might act to suppress white adipogenesis. Consequently, our goal in this study is to determine whether FoxC2 blocks the differentiation of these white preadipocytes and, if so, to begin to identify the molecular mechanisms underlying an inhibitory action. We show that inducible overexpression of FoxC2 in 3T3-L1 preadipocytes inhibits adipogenesis and that, more specifically, it blocks the expression of several known PPAR downstream targets that function in the mature adipocyte. These observations led us to create a cell line whose capacity to undergo adipogenesis is directly dependent on the expression and activity of PPARγ. To this end, we employed Swiss mouse fibroblasts, which constitutively express PPARγ. In these cells, we inducibly overproduced FoxC2 and found that its expression results in a significant attenuation in the capacity of these fibroblasts to undergo adipogenesis. Furthermore, the degree of attenuation appears to be selective for individual PPARγ downstream targets. Finally, we show that FoxC2 does not alter the ability of PPARγ to bind or transactivate a PPARγ response element, indicating that FoxC2 functions at a location downstream of PPARγ.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—Stocks of 3T3-L1 cells expressing the Tet-Off regulator protein (3T3-TET) were a kind gift of Jacques Pairault (UMR7079 CNRS UPMC, Paris, France). The FoxC2-pLNCX plasmids (mhf1) was a kind gift of Dr. Brigid Hogan (Vanderbilt University, Nashville, TN). FoxC2 cDNA was amplified out of FoxC2-pLNCX by PCR and then cloned into His-Topo-pCDNA3-V5 (Invitrogen). The FoxC2 cDNA now tagged with the V5 epitope sequence was provided by Dr. David Bernlohr (University of Minnesota). Antibodies of specific proteins.

Antibodies—The periplasm antibody was a gift of Dr. Andrew Greenberg (Tufts University, Boston, MA). Anti-aP2 serum was kindly provided by Dr. David Bernalohr (University of Minnesota). Antibodies against PPARγ, CEBPα, and C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-V5 antibody was purchased from Invitrogen, and the adiponectin antibody was purchased from Affinity BioReagents (Golden, CO).

Electromobility Shift Assays—Electromobility shift assays were performed as described previously (32). Sequences for PPAR response element (PREF) (DR-1) and FOXRE were 5'-50% confluency at which time they were changed to media containing 2.5 μΜ troglitazone. Several colonies were selected and analyzed. A single colony, termed Swiss-PFoxC2, was chosen on the basis that it was representative of the other populations and was subjected to further manipulation. The FoxC2-V5-pBI-G plasmid, along with a puromycin selection plasmid (pBabe-puro), was transfected into 3T3-L1-TET cells using FuGENE 6 (Roche Applied Science). Colonies of cells resistant to 2 μg/ml puromycin were selected and analyzed for expression of the pBGI-vector on the basis of tetracycline-responsive β-galactosidase production. The initial selection gave rise to several non-homogeneous colonies with only 10–20% of the cells expressing β-galactosidase activity. One of these colonies was subjected to serial dilution and cloning. Colonies were selected on the basis that nearly the entire population of cells expressed β-galactosidase activity in a tetracycline-responsive manner. The pBabe-PAR-PPARγ plasmid was a kind gift of Dr. Bruce Spiegelman (Dana Farber Institute, Harvard Medical School, Boston, MA). The pBabe-PAR-PPARγ plasmid was transfected into Swiss mouse 3T3 fibroblasts constitutively expressing the Tet-Off activator protein (Clontech). Primary colonies were selected using 100 μg/ml hygromycin. This initial selection gave rise to several non-homologous colonies, one of which was subjected to serial dilution. Colonies were selected based on their ability to undergo adipogenesis when exposed to the standard induction mixture along with 5 μM troglitazone. Several colonies were selected and analyzed. A single colony, termed Swiss-Pγ, was chosen on the basis that it was representative of the other populations and was subjected to further manipulation. The FoxC2-V5-pBGI plasmid, along with pBabe-puro, was transfected into Swiss-Pγ cells using FuGENE 6. Colonies of cells resistant to 2 μg/ml puromycin were selected and analyzed for expression of the pBGI-vector on the basis of tetracycline-responsive β-galactosidase production. The initial selection gave rise to several non-homogeneous colonies with only 10–20% of the cells expressing β-galactosidase activity. One of these colonies was subjected to serial dilution and cloning. Colonies were selected on the basis that nearly the entire population of cells expressed β-galactosidase activity in a tetracycline-responsive manner. The Swiss-Pγ-FoxC2 colonies were transfected using the previously mentioned mixture of inducers along with 5 μM troglitazone (Roche-Davis/Warner Lambert, Ann Arbor, MI). Additional ligands were also used at 1 μM of a specific PPARγ agonist, GW7845 (GlaxoSmithKline), and 1 μM rosiglitazone (Cayman Chemicals, Ann Arbor, MI). The PPARγ agonist T0070907 was obtained from CalBiochem, Inc. (Boston, MA) and used at 10 μM as described previously (31). Cyclohexamide (Sigma) and MG132 (Calbiochem) were used at 5 μg/ml and 12.5 μM, respectively.

Oil Red O Staining—Staining was performed following the procedure described previously (32). The cells then were photographed using phase-contrast microscopy.

Preparation of Whole Cell and Nuclear Extracts—Cultured cells were rinsed with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.4). For total extracts, cells were harvested in ice-cold buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxicholate, 4% Nonidet P-40, 0.4% SDS, 5 μM aprotinin, and 50 μM leupeptin and lysates were incubated on ice for 15 min and then centrifuged at 13,000 rpm. For nuclear protein extracts, cells were lysed in buffer (10 mM Tris, pH 7.6, 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40). After a low speed centrifugation, nuclei were resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.0, 25% glycerol) and then incubated on ice for 20 min and centrifuged at full speed (13,000 rpm) at 4 °C. The resulting supernatants were stored at −80 °C, and protein concentrations were determined using the BCA kit (Pierce, Rockford, IL).

Gel Electrophoresis and Immunoblotting—Proteins were analyzed by SDS-12% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences) in 25 mM Tris, 192 mM glycine, and 10% methanol. Following transfer, membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline-0.1% Tween 20 and probed with the antibodies specified below. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and ECL substrate kit (PerkinElmer Life Sciences) were used for detection of specific proteins.

Analysis of RNA—Total RNA was extracted with TRIzol (Invitrogen) according to manufacturer’s instructions. Following quantification, messenger RNA was assayed in equivalent amounts of total RNA by reverse transcriptase-PCR (RT-PCR) as described previously (33). Primer sequences used for amplification were synthesized by Integrated DNA Technologies, Inc., based on the published sequences in the GenBank database (sequences are available upon request). All of the PCR reactions were performed in the linear range of cycle number for each set of primers, and the corresponding products were analyzed by 1.5% agarose gel electrophoresis.

Reporter Assays—Swiss-Pγ-FoxC2 cells were plated and grown to ~50% confluency at which time they were changed to media containing the appropriate tetracycline condition. Two days later, at ~85% confluency, the cells were transfected with 4 μg of Dr-1-LUC (a gift from Dr. Jackie Stephens, Louisiana State University) and 0.04 μg of pRL-CMV (Promega, Madison, WI) using FuGENE 6. 24 h post-transfection, the cells were split into the standard induction mixture of DMI and their appropriate ligand and tetracycline condition. 48 h later, the medium was changed to the established tetracycline and ligand condition. The cells were harvested the following day and analyzed for luciferase activity and protein expression. Luciferase activity was measured using the DLRII kit (Promega, Madison, WI).
data indicate that the inducible expression system is producing physiological levels of FoxC2 in 3T3-L1 preadipocytes. Fig. 1b shows that the expression of the ectopic mRNA results in the production of a V5-tagged FoxC2 protein only in cells cultured in the absence of tetracycline.

To determine how FoxC2 expression affects adipogenesis, 3T3-L1-FoxC2 cells were induced to differentiate in the presence or absence of tetracycline for 4 days using the standard differentiation mixture of dexamethasone (DEX), isobutyryl-ethylxanthine (MIX), and insulin. Samples were analyzed by Western blot analysis for various transcription factors and markers of terminal adipogenesis (Fig. 2). In the absence of FoxC2, both PPARγ and C/EBPα are induced by day 2 and abundantly expressed by day 3, leading to increased expression of perilipin, adiponectin, and aP2 by day 4. Culture in the absence of tetracycline results in abundant expression of the V5-tagged FoxC2 protein, which leads to the inhibition of the adipogenic proteins including PPARγ and C/EBPα.

**FoxC2 Inhibits Adipogenesis at a Step Downstream of PPARγ—**It is generally accepted that terminal differentiation of 3T3-L1 preadipocytes depends to a great extent on the activity of PPARγ. In fact, the expression of PPARγ itself requires its continual activation through a positive feedback loop involving C/EBPα (34). Consequently, it is possible that the effects of FoxC2 on adipogenic gene expression observed in Fig. 2 may result from an inhibition of PPARγ activity. To better understand the mechanisms by which FoxC2 inhibits differentiation of 3T3-L1 preadipocytes, we employed a system in which adipogenesis in Swiss mouse fibroblasts is driven by ectopic PPARγ expression and activity. In this cell line, we conditionally overexpressed FoxC2 using the Tet-Off regulator protein, giving rise to a cell line termed Swiss-P-FoxC2. RT-PCR analysis of RNA isolated from these cells cultured in the presence or absence of tetracycline demonstrates that the expression of FoxC2 is tightly regulated by tetracycline (Fig. 3a). Furthermore, the ectopic FoxC2 tagged with a V5 epitope can bind to a FOX response element and this complex can be supershifted with the addition of a V5 antibody (Fig. 3b). Swiss-Py-FoxC2 cells were induced to differentiate by exposure to DEX, MIX, and
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**Fig. 3.** FoxC2 blocks the expression of adipogenic genes in mouse fibroblasts expressing an ectopic PPARγ. a, Swiss-Pγ-FoxC2 cells were cultured in the presence or absence of tetracycline for 2 days and harvested for total RNA. Samples were subjected to RT-PCR analysis using oligonucleotide primers against ectopic FoxC2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. b, Swiss-Pγ-FoxC2 cells were induced to differentiate using the standard induction mixture in the presence or absence of tetracycline. Extracts were subjected to gel shift analysis using a FOXRE consensus oligonucleotide. Prior to incubation with the radiolabeled oligonucleotide, the -TET sample was incubated with an anti-V5 antibody shown by the supershift in the far right lane. An aliquot of the -TET sample was also incubated with a 100-fold excess of the unlabeled oligonucleotide prior to the addition of the radiolabeled probe (cold oligo). c, cells were manipulated as in b and were harvested for total protein. Extracts were subjected to Western blot analysis.

![Image](214x464 to 562x737)

**Fig. 4.** Activation of PPARγ with troglitazone does not overcome the inhibitory action of FoxC2. a, Swiss-Pγ-FoxC2 cells were differentiated with the standard induction mixture in the presence or absence of tetracycline with (+TROG) or without (-TROG) 5 µM troglitazone (for 4 days at which time the cells were stained with Oil Red O (see “Experimental Procedures”) and photographed. b, Swiss-Pγ-FoxC2 cells were differentiated using the standard induction mixture in the presence of increasing concentrations of troglitazone with or without tetracycline. Cells were harvested at day 5 for total protein and samples were subjected to Western blot analysis.

Insulin for 4 days in the presence or absence of tetracycline, and total cell proteins were harvested for Western blot analysis. In the absence of FoxC2, PPARγ promotes the expression of several markers of adipogenesis including perilipin, adiponectin, and aP2 (Fig. 3c). Under these culture conditions (i.e. exposure to DEX, MIX, and insulin), ~20% of the cells become round and accumulate lipid droplets (Fig. 4a, -TROG, +TET). Ectopic expression of FoxC2 blocked this low level of morphological differentiation (Fig. 4a, -TROG, -TET) and also blocked the expression of adipogenic mRNAs (Fig. 3c). The small percentage of lipid-laden cells following exposure to DEX, MIX, and insulin suggests that the activity of PPARγ is below optimal levels when compared with the level of differentiation in 3T3-L1 cells. To assess whether FoxC2 is capable of inhibiting adipogenesis when PPARγ is fully active, we exposed the cells to a potent PPARγ ligand in addition to the standard adipogenic mixture. In fact, in the absence of FoxC2, differentiation of the cells in the presence of 5 µM troglitazone (PPARγ ligand) results in greater than 90% of the population accumulating lipid droplets (Fig. 4a, +TROG, +TET) compared with only 20% of the population in the absence of the exogenous PPARγ ligand (Fig. 4a, -TROG, +TET). More importantly, FoxC2 is capable of inhibiting lipid accumulation under conditions (+ troglitazone) promoting optimal PPARγ activity (Fig. 4a, +TROG, -TET). To analyze the relationship between PPARγ activity and the inhibitory effect of FoxC2 on adipogenic gene expression, cells were stimulated to differentiate in the presence or absence of tetracycline with increasing concentrations of troglitazone for 6 days and proteins were analyzed by Western blot (Fig. 4b). Fig. 4b demonstrates that, in the absence of FoxC2 (+ tetracycline), there is a troglitazone dose-dependent increase in the expression of perilipin, adiponectin, C/EBPα, and aP2 and a corresponding decrease in the abundance of the PPARγ2 protein. In contrast, the ectopic expression of FoxC2 significantly attenuates the expression of the adipogenic genes at all concentrations of troglitazone tested. Interestingly, higher doses of troglitazone appear to partially overcome the inhibitory effect of FoxC2 on aP2 expression (Fig. 4b, lanes 9 and 11). Previous studies have shown that the proteasomal-mediated degradation of PPARγ2 correlates with PPARγ activity (35, 36). However, even in the presence of FoxC2, troglitazone is still capable of facilitating the down-regulation of PPARγ2 protein, suggesting that FoxC2 is not blocking this process. However, it is important to point out...
that, in the absence or at low doses of troglitazone, FoxC2 appears to increase the abundance of PPARγ (Figs. 3b and 4b, lanes 5 and 6), which is consistent with the notion that at suboptimal PPARγ activity (low doses of ligand) FoxC2 can directly inhibit PPARγ activity resulting in its extended lifespan. Furthermore, at these same low doses of troglitazone, FoxC2 significantly attenuates aP2 expression. To determine whether PPARγ ligands other than troglitazone are capable of counteracting the inhibitory effect of FoxC2, Swiss-Pγ-FoxC2 cells were induced to differentiate in the presence of GW347845, rosiglitazone, or troglitazone (Fig. 5a). The inhibitory effect of FoxC2 is evident in the presence of all three ligands as shown by a FoxC2-dependent inhibition of perilipin, C/EBPα, and adiponectin. Again, aP2 seems to escape this inhibitory action of FoxC2. A plausible explanation for this apparent absence of effect on aP2 expression is that the aP2 protein may be significantly more stable than the other markers of terminal adipogenesis. To determine whether FoxC2 is
inhibiting transcription of the aP2 gene as well as the other genes, we measured the abundance of the corresponding mRNAs by RT-PCR analysis. Fig. 5b demonstrates that, whereas perilipin, GLUT4, C/EBPα, and adiponectin gene expression are all blocked by the FoxC2, aP2 gene expression is completely unaffected. The analyses presented in Fig. 5 were performed in differentiated cells; therefore, to gain a better understanding of the sequence of events that lead to the inhibitory action of FoxC2, we analyzed protein expression in Swiss-Py-FoxC2 cells during the course of the differentiation process. As expected from previous data, FoxC2 blocks expression of the adipogenic markers, C/EBPα, perilipin, and adiponectin; however, it has little effect on aP2. Driven by the overexpression and activity of PPARγ, these adipogenic genes are all expressed and attenuated by FoxC2 by 2–3 days post-induction (Fig. 6a). However, it appears that the PPARγ-dependent induction of C/EBPα expression during the initial 1–2 days of differentiation is not as sensitive to the inhibitory action of FoxC2 as the other proteins. Consequently, we performed a more detailed time course to assess whether FoxC2 is acting downstream of C/EBPα rather than acting directly on PPARγ. The data presented in Fig. 6b demonstrate that, in the absence of FoxC2, perilipin expression is induced as early as 8–16 h and that this event is blocked by overexpression of FoxC2. In contrast, the induction of C/EBPα during this same 8–16-h time period is completely resistant to the action of FoxC2 (Fig. 6b). In fact, the down-regulation of C/EBPα expression by FoxC2 does not become apparent until 44–48 h post-induction. Adiponectin expression is induced several hours later than perilipin and C/EBPα (40–44 h) and only under conditions where FoxC2 expression is repressed. The expression of aP2 occurs to a limited extent prior to treatment with troglitazone (0 h), but it is enhanced many fold in response to the PPARγ ligand and is unaffected by the presence of FoxC2. We conclude from these data that FoxC2 inhibits the capacity of PPARγ to promote the expression of select downstream target genes, which might result from a direct effect on PPARγ as well as inhibition of other factors. In this regard, we have recently demonstrated that C/EBPα is required for the optimum expression of adiponectin (37); thus, the FoxC2-associated down-regulation of adiponectin might be due to the block in C/EBPα expression.

Of additional interest is the observation that FoxC2 appears to enhance the abundance of the ectopically expressed PPARγ. Other studies have shown that ligand activation of PPARγ results in a destabilization of the protein and its subsequent proteasomal degradation (35, 36). Therefore, it is conceivably that FoxC2 might be enhancing PPARγ expression by blocking its activity and consequently preventing its degradation by the proteasome. To determine whether the expression of FoxC2 was causing a stabilization of PPARγ, a short 24-h time course was performed where Swiss-Py-FoxC2 cells were differentiated using the standard protocol in the presence or absence of tetracycline (Fig. 7). 8 h post-stimulation, the cells were treated with cyclohexamide to inhibit protein synthesis and control samples were also treated with the proteasomal inhibitor MG132. Western blot analysis shows that, when cells are cultured in the absence of FoxC2, the turnover of PPARγ over a 16-h time period is significantly faster than when cells express FoxC2. Additionally, inhibiting the proteasome with MG132 arrests the decay of PPARγ in the presence or absence of FoxC2. These data are consistent with the notion that FoxC2 may be inhibiting the capacity of PPARγ to promote transcription of adipogenic genes by decreasing its transcriptional activity.
FoxC2 Does Not Interfere with the Ability of PPARγ to Bind to or Transactivate from a PPRE Under Conditions Where It Blocks Adipogenesis

**DISCUSSION**

Targeted overexpression of FoxC2 in adipose tissue has previously been shown to result in a significant decrease in the mass of the major white adipose depots in the mouse. An explanation for this observation is that FoxC2 blocks the differentiation of preadipocytes into mature white fat cells in vivo. The goal of the present study was to uncover potential mechanisms through which FoxC2 may block adipogenesis. To this end, we employed both the 3T3-L1 preadipocyte cell model as well as a fibroblast cell line in which adipogenesis is driven by PPARγ. Initially, we showed that adipogenesis in 3T3-L1 preadipocytes is inhibited by FoxC2. This is most clearly demonstrated in Fig. 2, which shows that conditional ectopic expression of FoxC2 inhibits the expression of genes associated with...
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terminal differentiation. Studies during the last several years have positioned PPARγ as a principal regulator of terminal adipogenesis. In fact, it is likely that many signaling pathways responsible for initiating the adipogenic process converge on PPARγ. Additionally, many transcriptional events regulating the expression of proteins responsible for establishing the mature fat cell phenotype are themselves regulated by PPARγ. With this in mind, we questioned whether FoxC2 is inhibiting the differentiation of preadipocytes by blocking the activity of PPARγ. Using fibroblasts that constitutively express PPARγ2, we demonstrate that FoxC2 blocks the capacity of these cells to differentiate in the presence of an exogenous PPARγ ligand regardless of its potency (Figs. 5b and 4a). It is interesting, however, that the expression of α2 escapes the inhibitory action of FoxC2 particularly under conditions that promote maximum PPARγ activity. In addition, the capacity of FoxC2 to inhibit the expression of other adipogenic proteins is somewhat selective. Specifically, Fig. 6b shows a rapid induction of both perilipin and C/EBPα during the initial 8–16 h following activation of PPARγ but FoxC2 only blocks perilipin expression during this time period. The eventual inhibition of C/EBPα by FoxC2 occurs at 44–48 h post-induction, suggesting that the PPARγ-associated expression of C/EBPα versus perilipin is regulated by different mechanisms. It is interesting that the induction of adiponectin expression lags behind that of perilipin and C/EBPα, since it is not detected by Western blot analysis until 40–44 h post-induction. These data suggest that PPARγ alone is not a direct activator of adiponectin but requires the cooperation of another factor that itself is induced by PPARγ. The most likely factor is C/EBPα, because recent studies have demonstrated that the induction of adiponectin expression during terminal adipogenesis requires both PPARγ and C/EBPα (37). Consequently, the FoxC2-associated block in adiponectin expression may result from a corresponding inhibition of C/EBPα that occurs at approximately the same time as the induction of adiponectin expression (40–44 h). It is also noteworthy that FoxC2 appears to restrict the stabilization of PPARγ, which could be attributed to decreased transcriptional activity (Fig. 7). These results suggest that FoxC2 may affect the transcriptional activity of PPARγ but only on specific promoters. Interestingly, FoxC2 has no effect on the capacity of PPARγ to bind to or transactivate from a PPRE (Fig. 8), supporting the idea that the capacity of FoxC2 to block PPARγ-driven transcription is promoter-selective.

Mechanisms by Which FoxC2 Inhibits Adipogenesis in 3T3-L1 Preadipocytes—The data presented in Fig. 2 demonstrate that the expression of FoxC2 at the commencement of the differentiation process in 3T3-L1 preadipocytes blocks the production of both PPARγ2 and C/EBPα. The expression of these two transcription factors is regulated by several upstream factors, which potentially could be targets of FoxC2. The induction of adipogenesis in 3T3-L1 preadipocytes involves the exposure of a confluent, quiescent population of cells to a mixture of adipogenic hormones including DEX, MIX, and insulin. This treatment activates a burst of proliferation (referred to as clonal expansion), which occurs during the initial 2–3 days of the differentiation process. Various cell cycle-associated proteins expressed during this time appear to play a direct role in regulating the expression of PPARγ and C/EBPα as the preadipocytes exit clonal expansion to undergo terminal differentiation (4, 38–40). It is possible that FoxC2 somehow interferes with this well-coordinated transition from clonal expansion to terminal adipogenesis. In fact, Nakae et al. (28) have shown that the overexpression of a constitutively active form of another member of the forkhead family, FoxO1, inhibits the differentiation of 3T3-F442A preadipocytes by preventing the cells from undergoing clonal expansion. This event appears to result from the FoxO1-associated induction of the cyclin-dependent kinase inhibitor, p21CIP. Other studies have shown that the expression of FoxA2 also blocks adipogenesis, but the mechanism appears to be somewhat different from that of FoxO1. In this case, FoxA2 activates the expression of pref1, a known inhibitor of differentiation. Expression of pref-1 is normally down-regulated during differentiation, but in the presence of FoxA2, its expression is sustained resulting in a block in the adipogenic process (28). Therefore, it is conceivable that FoxC2 may alter the expression of cell cycle factors, which in turn regulate expression specific terminal markers.

The data presented in Figs. 5 and 6 demonstrate that FoxC2 is capable of inhibiting the expression of proteins normally regulated by PPARγ. It is possible that the principal target of FoxC2 is PPARγ, even in 3T3-L1 cells in which FoxC2 inhibits PPARγ2 expression. The activation of PPARγ2 in 3T3-L1 cells occurs at 2 days post-induction, coinciding with the cessation of clonal expansion. This event involves an earlier expression of C/EBPβ, C/EBPδ, and PPARγ1. Our previous studies have identified C/EBPβ as a potent inducer of PPARγ2, but we have also shown that the activation of PPARγ1 can also initiate adipogenesis by mechanisms that involve establishing a positive feedback loop comprising C/EBPα (41). It is conceivable that FoxC2 may prevent the induction of PPARγ2 by blocking the activity of PPARγ1. Clearly, in mouse fibroblasts whose adipogenic potential depends exclusively on the activation of an ectopic PPARγ2, the forced expression of FoxC2 blocks adipogenesis, indicating that PPARγ is a potential target of FoxC2.

Mechanisms by Which FoxC2 Inhibits Expression of Select PPARγ2 Target Genes—The data presented in Fig. 6 show that FoxC2 inhibits the expression of proteins that are induced in response to activation of an ectopic PPARγ2. FoxC2 may be acting directly at the level of transcriptional complexes responsible for mediating the adipogenic activity of PPARγ2. This may involve a direct interaction with PPARγ as has been suggested for FoxO1 (42) or an association of FoxC2 with coactivators such as CREB-binding protein/p300. Data presented in this paper indicates that FoxC2 blocks adipogenesis by inhibiting a subset of genes that ultimately prevents the acquisition of the mature fat cell phenotype. Although the details are poorly understood, PPARγ promotes transcription through the assembly of a complex of coactivators and chromatin-remodeling factors. The nature of these complexes is probably unique for a given promoter, and the factors involved most probably change over the course of differentiation. The data in this study suggest that FoxC2 is disrupting one or more components of these transcriptional complexes, altering PPARγ transcriptional activity on select promoters that results in decreased adipogenic potential. It is also possible that FoxC2 acts directly to repress the transcription of select PPARγ2 target genes that are required for the expression of the other genes associated with terminal adipogenesis. For instance, C/EBPα is induced in direct response to the activation of PPARγ2 and its presence appears to be critical for the differentiation of preadipocytes into mature fat cells. Specifically, the activation of PPARγ2 in fibroblasts lacking C/EBPα gives rise to immature adipocytes that do not express GLUT4 or β3-adrenergic receptors and that express low levels of other proteins including adiponectin (34, 43–45). Furthermore, sustained expression of PPARγ2 during terminal adipogenesis depends on the availability of C/EBPα (34). Consequently, decreased C/EBPα expression through inhibition of PPARγ by FoxC2 would have a significant impact on adipogenic gene expression. It is possible that FoxC2 may act directly on the promoter/enhancer of
the C/EBPα gene, thereby antagonizing the action of PPARγ, which promotes C/EBPα expression.

The forkhead family of transcription factors is rapidly coming into light as important regulators of metabolic homeostasis. FoxC2 expression is restricted to adipose tissue in mouse and human (29) and is increased in adipose tissue of ob/ob mice (28). Previous studies have shown that targeted overexpression of FoxC2 in adipose tissue results in a significant ablation of white fat depots and hypertrophy of brown adipose depots (29). Furthermore, the adipocytes that do form in the white adipose depots have a brown-like phenotype. Although humans lack distinct brown depots, it is evident that white depots have brown adipocytes dispersed throughout the tissue (46–48). In addition, it has also been shown that white and brown adipocytes can transdifferentiate in response to external conditions (49–52). More recently, FoxC2 has been shown to be down-regulated in insulin-resistant human subjects that coincided with the down-regulation of brown adipogenic genes (53). Although we could not find any evidence for FoxC2-induced transdifferentiation in our system, FoxC2 may act to suppress white adipogenesis in favor of enhancing brown fat cell formation. In this regard, the engineered fibroblasts employed in this study may lack the positive effectors required to induce the expression of brown adipogenic genes. Further study of FoxC2 and its role in regulating a possible switch from white to brown adipogenesis may provide important information leading to therapies for obesity-associated disorders and metabolic syndrome.

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