The Chicken Ovalbumin Upstream Promoter-Transcription Factors Modulate Genes and Pathways Involved in Skeletal Muscle Cell Metabolism*

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The chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are “orphan” members of the nuclear hormone receptor (NR) superfamily. COUP-TFs are involved in organogenesis and neurogenesis. However, their role in skeletal muscle (and other major mass tissues) and metabolism remains obscure. Skeletal muscle accounts for ~40% of total body mass and energy expenditure. Moreover, this peripheral tissue is a primary site of glucose and fatty acid utilization. We utilize small interfering RNA (siRNA)-mediated attenuation of COUP-TfI and II (mRNA and protein) in a skeletal muscle cell culture model to understand the regulatory role of COUP-Tfs in this energy demanding tissue. This targeted NR repression resulted in the significant attenuation of genes that regulate lipid mobilization and utilization (including Ppara, Fabp3, and Cpt-1). This was coupled to reduced fatty acid β-oxidation. Additionally we observed significant attenuation of Ucp1, a gene involved in energy expenditure. Concordantly, we observed a 5-fold increase in ATP levels in cells with siRNA-mediated repression of COUP-TfI and II. Furthermore, the expression of “classical” liver X receptor (LXR) target genes involved in reverse cholesterol transport (Abacl and Abcg1) were both significantly repressed. Moreover, we observed that repression of the COUP-Tfs ablated the activation of Abacl, and Abcg1 mRNA expression by the selective LXR agonist, T0901397. In concordance, COUP-Tf-siRNA-transfected cells were refractory to Lxr-mediated reduction of total intracellular cholesterol levels in contrast to the negative control cells. In agreement Lxr-mediated activation of the Abacl promoter in COUP-Tf-siRNA cells was attenuated. Collectively, these data suggest a pivotal role for COUP-Tfs in the regulation of lipid utilization/cholesterol homeostasis in skeletal muscle cells and the modulation of Lxr-dependent gene regulation.

Nuclear receptors (NRs)3 comprise a large family of DNA-binding transcription factors that control many cellular and physiological processes crucial for development and survival. Dysregulation of nuclear receptor signaling leads to proliferative, reproductive, and metabolic disorders such as cancer, infertility, dyslipidemia, and insulin resistance/type II diabetes (1). Consequently, NRs are a major focal point of intense clinical and pharmaceutical studies. The NR superfamily includes receptors for steroid hormones, vitamin D, edcysone, retinoic acids (all-trans and 9-cis isoforms), and thyroid hormones. In addition to these known receptors for known ligands, there are a number of NRs to which no ligand has been described, and thus, designated “orphan” receptors.

Of the orphan nuclear receptors, the chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) have received considerable attention for their role in neurogenesis, organogenesis, and embryogenesis (2–4). In mouse there are two highly related Coup-Tfs (Coup-TfI and Coup-TfII) encoded by distinct genes on chromosomes 13 and 7, respectively (5). Interestingly, Coup-Tfs can modulate the activity of genes that are fundamental in the orchestration of glucose and lipid metabolism. These include, bile acid synthesis (Cyp7A1, cholesterol 7a-hydroxylase) (6), ketogenesis (mitochondrial hydroxymethylglutaryl-CoA synthase) (7), cholesterol transport (cholesterol ester transfer protein and apolipoprotein AI) (8, 9), and fatty acid β-oxidation (medium-chain acyl-coenzyme A dehydrogenase) (10). Furthermore, organ-specific (pancreatic β cells) Coup-TfI null mutants in mice demonstrated a role for Coup-Tf in glucose homeostasis and insulin sensitivity (11). In addition, the Coup-Tf ortholog in Drosophila (seven up) is required for the expression of alcohol dehydrogenase and type IV collagen, two genes that are essential for fat cell differentiation (12). Although Coup-Tfs are implicated in metabolism in these systems, little is known of the role of these NRs with respect to metabolism in skeletal muscle, and other major mass peripheral tissues.

Skeletal muscle is one of the most metabolically active tissues in the body that accounts for >30% of total energy expenditure. By virtue of its large mass (~40% of body weight) and signifi-
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cant energy demands, skeletal muscle provides a vital milieu for fatty acid and glucose homeostasis (13). Furthermore, it is also involved in cholesterol efflux (i.e. reverse cholesterol transport) (14). Consequently, skeletal muscle plays a considerable role in insulin sensitivity and the blood lipid profile. These characteristics alone suggest that skeletal muscle has an integral role in metabolism and provides an attractive target tissue to study the role of orphan nuclear receptors with respect to metabolism in this system.

Previous studies from this laboratory have identified a role for the orphan nuclear receptors (including Rora, Rev-erbβ, and Nur77) in the regulation of the genetic programs controlling lipid homeostasis in the mouse C2C12 skeletal muscle cell line (15–17). Moreover, other studies have successfully utilized this model system to study Coup-Tfs. For example, constitutive expression of Coup-Tfl repressed the activity of the human muscle glyceron phosphorylase gene promoter in C2C12s (18). In fact, the C2C12 cell line has been used to study the functional role of many orphan nuclear receptors (14). The ability of this cell line to differentiate into multinucleated fibers in tissue culture (19), coupled to the acquisition of a contractile and metabolic phenotype, provides a robust system to study the role of COUP-TF in skeletal muscle metabolism.

In this study we tested the hypothesis that Coup-Tfs are implicated in the regulation of pathways controlling metabolism. Our studies suggest a role for Coup-Tfs in (i) the modulation of key genes involved in lipid utilization (Ucp1, Ucp3, Fabp3, and Cpt-1) and (ii) the regulation of total cholesterol levels, and liver X receptor (Lxr)-dependent expression of the ATP-binding cassette proteins that control cholesterol efflux (Abca1 and Abcg1). The role of Coup-Tfs in this system presents an essential platform to further study lipid utilization and cholesterol efflux in skeletal muscle. Importantly, these processes have implications for disease states such as dyslipidemia.

MATERIALS AND METHODS

Cell Culture—Proliferating mouse C2C12 myoblasts were cultured and maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated Serum Supreme (Cambrex Bio Science, Victoria, Australia). Differentiation of myoblasts into post-mitotic, multi-nucleated myotubes was induced by mitogen withdrawal, i.e. DMEM supplemented with 2% horse serum (differentiation medium, DM) (Trace, Scientfic, Victoria, Australia) for 4 days.

RNA Extraction and cDNA Synthesis—Total RNA was extracted from C2C12 cells using TRI-Reagent (Sigma) according to the manufacturer’s protocol. Total RNA was then treated with 2 units of Turbo DNase I (Ambion, Austin, TX) for 30 min at 37 °C followed by purification of the RNA through an RNeasy purification column system (Qiagen, Victoria, Australia). RNA was electrophoresed to determine the integrity of the preparation. SuperScript III was used to synthesize cDNA from 4 μg of total RNA using random hexamers according to the manufacturer’s instructions (Invitrogen). The cDNA was then diluted to 300 μl in nuclease-free water.

Protein Extraction—Total soluble protein was extracted from C2C12 cells by the addition of lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) containing protease “mixture” inhibitors (Sigma). Lysates were passed through a 26-gauge needle and centrifuged at 10,000 x g for 20 min. The supernatant was collected and total protein concentration was determined by the BCA as outlined by the manufacturer’s instructions (Pierce).

Generation of Coup-Tfl-siRNA Construct—A 21-mer siRNA duplex specific to the annotated mouse cDNA sequence of Coup-Tfl (NM_010151) was generated with the siRNA target finder from Ambion. The target sequence, Coup-Tfl-5’-AAAGCACTACGGCCAATTCCAC-3’, was chosen based on having no sequence similarity with other members of the nuclear receptor family and low sequence homology with non-target murine sequences. The sense and antisense siRNA oligomers were synthesized (Geneworks, SA, Australia) and cloned into the pSilencer 3.1 neomycin vector as described by the manufacturer (Ambion). Target sequences were confirmed by direct sequencing at the Australian Genome Research Facility, University of Queensland, Brisbane, Australia.

Transient Transfections of siRNA Constructs—C2C12 myoblasts were grown to ~80% confluence and three independent transfections of Coup-Tfl (4 μg/10-cm dish) and the pSilencer 3.1 negative control siRNA sequence (a sequence not found in the rat, human, or the mouse genome, Ambion) were performed in the presence of Lipofectamine 2000 as outlined by the manufacturer’s protocol (Invitrogen). Twenty-four h post-transfection, fresh DM was added for a further 24 h and RNA was collected as outlined above.

Generation of C12C2 Stable Coup-Tfl-siRNA Cell Lines—C2C12 cells were transfected at 50% confluence with 4 μg of pSilencer-3.1-Coup-Tfl and 4 μg of pSilencer-3.1-negative control in 2 × HEPEs, 10 μl of N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salt (DOTAP), and 10 μl of Metafectene (Scientific, Victoria, Australia) per 25-cm² flask. Cells were subsequently passed into 10-cm² round flasks followed by 8–12 days selection with 700 μg/ml G418 in DMEM. Following the selection process and the death of the non-transfected control population, viable siRNA-transfected stable cell populations were maintained in DMEM supplemented with 300 μg/ml G418. The large number of stable transfectants (>100 colonies) were passaged into three independent polyclonal pools (to avoid clonal bias) and independently, passaged, cultured, and propagated for various experiments. Each independent stable myoblasts were then differentiated into myotubes as outlined above. Myoblasts and differentiated myotubes were analyzed on a Nikon Eclipse TS100 microscope (Coherent Scientific, SA, Australia) and digital images were captured on a Nikon Coolpix 4500 digital camera.

T9091317 Treatment of Stable C2C12 Cell Lines—C2C12 cell lines stably expressing the pSilencer negative 3.1 control and the Coup-Tfl-siRNA were grown to confluence and then induced to differentiate over a 4-day period. At day 3 of differentiation, the cells were treated with either vehicle (Me₃SO) or LXR agonist, T9091317 (10 μM), until day 4 of differentiation. The cells were then collected for RNA processing as outlined above.

Quantitative Real-time PCR (Q-RT-PCR)—Q-RT-PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) in triplicate on three independent RNA
preparations. Target cDNA levels were analyzed in 25-μl reactions with either SYBR Green or TaqMan Technologies (Applied Biosystems). Primers (200 nM) used for the amplification of target gene sequences have been described in detail (15–17). PCR was performed with 5 μl of cDNA and 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative level of target gene expression was normalized to Gapdh or 18 S ribosomal RNA as described under “Results” and associated errors were calculated using the guidelines described by Bookout and Mangelsdorf, on the Nuclear Receptor Signaling Atlas website (NURSA). Statistical analysis was performed on the average of three independent assays and a Student’s t test was performed to calculate significance.

**Western Blot Analysis**—Total soluble protein from the pSilencer 3.1 negative control and pSilencer 3.1 Coup-Tf-siRNA stable C2C12 cell lines was resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked overnight or for 1 h in 5% skim milk in Tris-buffered saline/Tween 20 followed by an overnight incubation with either Coup-Tfi (1:3000, sc-6577), Coup-TfII (1:2000, sc-5578), Abca1 (1:1000, sc-20794) (Santa Cruz Biotechnology, Inc.), or Gapdh (1:10,000) (R&D Systems, Minneapolis, MN) antibodies. Following 4 × 15-min washes the membrane was incubated with either anti-goat horseradish peroxidase (Coup-Tfi and II) or anti-rabbit horseradish peroxidase (Gapdh and Abca1) (1:2000) for 1 h. Immunoreactive signals were detected using enhanced chemiluminescence SuperSignal West Pico Substrate (Pierce) and visualized by autoradiography on an X-Omat film developer (Kodak).

**Luciferase Assay**—C2C12 stable Coup-Tf-siRNA and the negative 3.1 control cells were grown in DMEM in 24-well plates to ~80% confluency. Transient transfection of the ABCA1-Luc promoter (20) (0.5 μg/well) was performed in the presence of Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, the cells were treated with either vehicle (Me3SO) or LXR agonist, T0901317 (10 μM). Luciferase assays were performed using the LucLite luminescence reporter gene assay system as outlined by the manufacturer (PerkinElmer Life Sciences). Luminescence was recorded on a MicroBeta luciferase system (PerkinElmer Life Sciences) (20).

**ATP Assay**—A total of 10,000 C2C12 cells (counted by microscopy via the trypan blue exclusion method) per well (96-well plate) expressing the pSilencer 3.1 negative control and Coup-Tf-siRNA were seeded and maintained in DMEM. At 100% confluence, DM was added and the cells were induced to differentiate for 4 days. The measurement of ATP was performed using the ATPlite luminescence ATP detection assay system as outlined by the manufacturer (PerkinElmer Life Sciences). The results obtained are from three independent polyclonal pools, assayed in triplicate. ATP concentration was normalized to total soluble protein. A Student’s t test was performed to calculate statistical significance.

**Amplex Red Cholesterol Assay**—The assay for free cholesterol and cholesterol esters was performed as described by the manufacturer’s instructions (Invitrogen). Briefly, cell samples containing the pSilencer 3.1 negative control and the Coup-Tf-siRNA C2C12 stable cells were seeded at ~10,000 cells per well (96-well plate). The cells were then maintained in DMEM until confluent and differentiated in 10% charcoal-stripped DMEM prior to the addition of 10 μmol/liter of the LXR agonist, T0901317 for 24 h. The cells were then resuspended in Amplex Red reaction buffer (Invitrogen) and incubated at 100 °C for 30 min to inactivate endogenous cellular cholesterol esterase. Following a 30-min incubation at 37 °C with the Amplex Red assay reagents, fluorescence was measured at an excitation of 530 nm and emission of 590 nm. The results obtained are from three independent polyclonal pools, assayed in triplicate. Cholesterol concentration was normalized to total soluble protein and a Student’s t test was performed to calculate statistical significance.

**RESULTS**

**The Coup-TfI and II mRNA and Protein Are Expressed during Skeletal Muscle Differentiation**—To elucidate the role of Coup-TfI and II in skeletal muscle with respect to metabolism, we initially investigated the expression profile of both Coup-TfI and II mRNA relative to Gapdh in the mouse C2C12 myoblast cell line. Proliferating myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by mitogen withdrawal over a 24–96-h period. This transition from a non-muscle to a contractile phenotype is associated with activation and repression of a structurally diverse group of genes responsible for contraction and the extreme metabolic demands placed on this tissue. During this period of differentiation, we observed by Q-RT-PCR that both Coup-TfI and II mRNA were expressed during skeletal muscle cell differentiation (Fig. 1, a and b). To assess the levels of Coup-TfI and Coup-TfII proteins during differentiation we performed Western blotting analysis. High levels of immunoreactive Coup-TfI and II were observed in the proliferating myoblasts and confluent myoblasts and down-regulated (relative to Gapdh) during myogenesis (Fig. 1, a and b).

To assess the differentiation status of the C2C12 cells and demonstrate that they had acquired a muscle-specific, contractile and metabolic phenotype, Q-RT-PCR was performed on several important marker genes encoding myogenin, a gene that encodes the hierarchical basic helix loop regulator and is specifically required for differentiation (21), the slow twitch (type I) and the fast twitch (type II) isoforms of the contractile
protein troponin I, and the metabolic genes Abca1 and Abcg1 (ATP-binding cassette proteins), Fabp3 (fatty acid-binding protein 3), Cpt-1 (carnitine palmitoyltransferase 1), and Ucp3 (uncoupling protein 3).

Both the marker of differentiation (myogenin) and the contractile protein genes (types I and II, Tnni1 and Tnni2, respectively) were dramatically induced during the myogenic process and thus confirmed the differentiated state of the C2C12 cell (Fig. 2, a–c). Furthermore, the expression of these key myogenic markers of differentiation are entirely consistent with previous studies (15, 17, 22). Additionally, genes involved in lipid metabolism (Abca1, Abcg1, Cpt-1, and Fabp3) and energy expenditure (Ucp3) were all induced during skeletal myogenesis, and confirmed that the muscle cells had acquired a metabolic phenotype (Fig. 2, d–h). Due to the availability of antibodies against Abca1, we assessed the levels of this protein across the developmental time course. From these data, the Abca1 protein correlated with the Abca1 mRNA profile (Fig. 2d).

**Coup-Tf-siRNA Expression Represses Endogenous Levels of Coup-Tfi and Coup-Tfii mRNA and Protein in Skeletal Muscle Cells**—To elucidate the biological role of Coup-Tf in skeletal muscle with respect to lipid utilization, we preceded to selectively ablate the expression of Coup-Tfi and Ii. RNA interference was utilized to create stable cell lines expressing Coup-Tf-siRNA (having no sequence similarity to other nuclear receptors) in an attempt to attenuate mRNA levels of both Coup-Tfi and Ii. As a control we produced stable C2C12 cells expressing the pSilencer 3.1 negative control (a sequence not found in the mouse, rat, or human genome). Accordingly, triplicate polyclonal C2C12 cell lines stably expressing the pSilencer 3.1 negative control siRNA (negative control) and the specific COUP-TF-siRNA (Fig. 3a) were subsequently differentiated for 4 days and total RNA and soluble protein was extracted. Representative images of the C2C12 myoblasts and myotubes are shown for the negative 3.1 control and the Coup-Tf-siRNA cells. Both negative 3.1 controls and the Coup-Tf-siRNA stable cell lines differentiated from a proliferating mononucleated phenotype to a post-mitotic multinucleated morphology indicative of a contractile phenotype (Fig. 3b).

Quantitative RT-PCR was then performed to measure the expression of both Coup-Tfi and Coup-Tfii mRNA normalized to both Gapdh and 18 S rRNA in the RNA isolated from the differentiated negative control and Coup-Tf-siRNA transfected stable cell lines. We observed a significant attenuation of both the Coup-Tfi (5.0-fold, \( p < 0.0001 \)) and Coup-Tfii (4.75-fold, \( p < 0.0001 \)) mRNA when compared with the negative 3.1 control and normalized to Gapdh (Fig. 3, c and d). Moreover, the relative expression of Coup-Tfi and Ii mRNA was similar when normalized to 18 S rRNA (Coup-Tfi, 5.65-fold, \( p = 0.0170 \), and Coup-Tfii, 4.38-fold, \( p = 0.0114 \)) (Fig. 3, c and d). To determine that the attenuation of both Coup-Tfi and Ii mRNA was not due to differential Gapdh mRNA expression, Q-RT-PCR was also performed on Gapdh mRNA normalized to 18 S rRNA. No change was observed in the levels of Gapdh mRNA expression in the negative control relative to the Coup-Tf-siRNA cell lines and thus, validated the attenuated expression of Coup-Tfi and Ii mRNA in the C2C12 Coup-Tf-siRNA stable lines (Fig. 3e).

To determine whether the repression of Coup-Tfi and Coup-Tfii mRNA expression also correlated with Coup-Tf protein levels, Western blot analysis was performed using specific Coup-Tfi and Ii antibodies. Western blot analysis also confirmed both Coup-Tfi and Ii proteins were dramatically reduced in the C2C12 Coup-Tf-siRNA stables relative to the negative 3.1 control (Fig. 3, f and g). The effects of Coup-Tf attenuation by the specific siRNA (relative to the negative 3.1
control) were further validated by analyzing the expression of both Coup-TfI and II mRNA after transient transfection of the Coup-Tf-siRNA. C2C12 cells were transiently transfected with the negative 3.1 control and Coup-Tf-siRNA and the endogenous levels of both Coup-TfI and II mRNA were measured in Q-RT-PCR experiments. Both Coup-TfI and II mRNA were clearly attenuated in the Coup-Tf-siRNA transfection (Fig. 3). Moreover, these data show that the attenuation of both Coup-TfI and II is not a result of the G418 selection process for the generation of stable cell lines, and is specific to the expression of the siRNA.

Expression of Coup-Tf-siRNA Modulates the Expression of Genes Involved in Lipid Utilization—Genes involved in lipid utilization (from intramyocellular lipid depots) and catabolism, respectively, for example, Fabp3 and Cpt-1 were dramatically attenuated (14-fold, \( p = 0.0211 \) and 10.5-fold, \( p = 0.0199 \), respectively) (Fig. 4, a and b). Consequently, we examined whether these changes in gene expression had functional consequences on lipid oxidation in these cells utilizing a \(^3\)H\(_2\)O-based palmitate assay. In concordance with the dramatic attenuation of Cpt-1, we observed reduced fatty acid \( \beta \) oxidation in the Coup-Tf-siRNA (relative to the negative control) transfected cells (Fig. 4c). A number of other genes important in fatty acid homeostasis were analyzed (Table 1), however, minimal nonspecific changes were observed between the negative control and the Coup-Tf-siRNA cells.

We next analyzed a number of important nuclear receptors that are modulators of lipid metabolism and homeostasis, for example, Ppars, Lxra, Lxrβ, Rora, Errα, and Rev-erba (14). From these studies, we observed a dramatic repression of Ppara (master regulator of lipid catabolism) mRNA of up to 30-fold \(( p < 0.0001 \)) in the Coup-Tf-siRNA cells (Fig. 4d). Moreover, the specificity of Ppara attenuation was supported by the minimal changes observed in the expression of the other nuclear receptors in the Coup-Tf-siRNA-transfected cells (Table 2).
The myogenic cytokine, Il-15 (Fig. 4e) was down-regulated 5.6-fold ($p < 0.0084$). Il-15 has been demonstrated to function in a muscle to fat endocrine axis to modulate fat: lean body composition and insulin sensitivity (23). We also observed a dramatic attenuation of phosphoenolpyruvate carboxykinase ($Pepck-1$) a gene involved in gluconeogenesis. $Pepck-1$ was undetectable in $Coup-Tf$-siRNA cells (Fig. 4f).

Other genes involved in inflammation and glucose homeostasis were analyzed; however, minimal changes were observed (Tables 1 and 2).

**The Attenuation of $Coup-Tf$ I and II Expression Modulates the Expression of the Uncoupling Genes and Steady State ATP Levels**—Subsequently, we analyzed the mRNAs encoding the uncoupling proteins ($Ucp1–3$) and other genes ($Ampk, \beta, \gamma$) involved in energy expenditure, and balance. For example, the expression of genes encoding $Ucp1$ and $Ucp3$ that are involved in energy uncoupling and preferential lipid utilization, respectively, were significantly modulated. Specifically, $Ucp1$ mRNA was dramatically repressed (and undetectable), whereas in contrast, $Ucp3$ mRNA expression was induced 6.24-fold ($p < 0.0024$) in the $C2C12$ $Coup-Tf$-siRNA cell line (Fig. 5, a and b). $Ucp2$ and other genes involved in energy expenditure and balance were not substantially modulated or significantly changed in this system (Table 1). To assess the effect of the dramatic attenuation of $Ucp1$ mRNA in the $Coup-Tf$-siRNA $C2C12$ stable cells, we measured and compared the levels of ATP in the negative control and $Coup-Tf$-siRNA-transfected $C2C12$ cells. In concordance with the striking reduction of $Ucp1$ mRNA expression in the $Coup-Tf$-siRNA stable cells, we observed an approximate 5-fold increase in ATP levels in the
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Involved in Reverse Cholesterol Transport (Abca1 and Abcg1)

We further analyzed a number of important genes implicated in lipid efflux. We observed the mRNAs encoding the reverse cholesterol transporters, Abca1 and Abcg1, were both significantly attenuated (4.5-fold, \( p = 0.0073 \), and 3.86-fold, \( p = 0.0063 \), respectively) (Fig. 5, d and e).

The Effects of COUP-TF Repression on Lipid Homeostasis Are Independent of Differentiation—To evaluate whether the changes observed in the COUP-TF-siRNA stable cell lines were not due to aberrant differentiation, the mRNAs levels of the differentiation (myogenin) and contractile (Tnni1 and Tnni2) markers were analyzed in the RNA samples derived from both the differentiated negative controls and the COUP-TF-siRNA stable cell lines. Minimal non-significant changes in the expression of the mRNA encoding myogenin and troponin I were observed when compared with the negative 3.1 control (Table 3) relative to the dramatic significant changes observed in the metabolic genes reported above. Moreover, and more importantly, we observed both induction (e.g., Ucp3, Fig. 5b) and repression (e.g., Cpt-1 and Abca1, Figs. 4b and 5d, respectively) of genes in the stable cells that are induced during the normal differentiation process (Fig. 2, d, f, and h). These data emphasize that the observed phenomenon are not differentiation-dependent and further validate that the modulating effects of COUP-TF attenuation on metabolism were not due to aberrant differentiation.

Furthermore, to establish that the attenuation of COUP-TF and the modulation of core metabolic genes was not an artifact of the G148 selection process, we performed Q-RT-PCR on these key genes from cells transiently transfected with the COUP-TF-siRNA. In these studies we observed identical modulation of these key genes (for example, Ppara, Abca1, Ucp3, and Cpt-1 (supplementary materials Fig. S1, a–d) suggesting the effects observed in the COUP-TF-siRNA cells was not inherent to the stable C2C12 cell lines.

Repression of COUP-TF and II Compromises LXR-mediated Activation of Abca1, Abcg1, and Sreb-1c mRNA Expression—We observed that suppression of COUP-TF and II mRNA expression attenuated the expression of several “classical” Lxr target genes including Abca1, Abcg1, and Sreb-1c. Therefore, we explored the potential cross-talk between COUP-TF and Lxr signaling in skeletal muscle cells and examined the effect of attenuated COUP-TF expression on Lxr signaling. Consequently, we treated the negative control and the COUP-TF-siRNA-transfected stable C2C12 cell lines with vehicle (Me2SO), and the potent/selective LXR agonist, T0901317, and examined the activation of several classical LXR target genes. T0901317 treatment of the negative control cells resulted in a robust activation of the LXR target genes Abca1, Abcg1, and Sreb-1c (approxim-
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**Table 2**

| Gene          | Relative expression C2C12 negative | Relative expression C2C12 siRNA | Fold change and p value |
|---------------|-----------------------------------|--------------------------------|-------------------------|
| **Nuclear receptors** |                                   |                                |                         |
| Egrγ          | 3.04 ± 0.52                      | 1.69 ± 0.34                    | 1.8 p = 0.1624          |
| Egrα          | 509.9 ± 103                     | 286.3 ± 32                    | 1.78 p = 0.1085         |
| Lrxb          | 109.4 ± 15.40                    | 44.13 ± 1.284                  | 2.5 p = 0.0134          |
| Lrxb          | 3072 ± 133.6                     | 2045 ± 176.6                   | 1.5 p = 0.0098          |
| Pparβ/β       | 1917 ± 94.20                 | 1004 ± 104.4                   | 1.9 p = 0.0012          |
| Pparγ         | 882.1 ± 81.33                   | 323.3 ± 23.54                  | 2.7 p = 0.0027          |
| Rev-erba       | 9319 ± 1610                   | 7456 ± 770                     | 1.2 p = 0.3178          |
| Rev-erβb       | 21645 ± 1508                   | 7790 ± 965.8                   | 2.8 p = 0.0015          |
| Rora          | 91.07 ± 3.638                    | 76.06 ± 95.80                  | 1.2 p = 0.2169          |
| Ropy          | 2199 ± 282.9                     | 1760 ± 421.5                   | 1.2 p = 0.4779          |
| Nur1          | 7983 ± 271.6                     | 8483 ± 1486                    | 1.06 p = 0.7572         |
| Nur77         | 1843 ± 361.9                     | 2237 ± 227.8                   | 1.2 p = 0.4090          |
| **Inflammatory mediators** |                                 |                                |                         |
| Il-6          | 2482 ± 177.5                        | 2383 ± 148                     | 1.0 p = 0.1990          |
| Ibesα         | 212500 ± 1508                     | 159700 ± 15210                | 1.5 p = 0.0695          |
| Nfκβ-p65      | 330500 ± 32320                    | 201100 ± 39470                | 1.6 p = 0.0642          |

COUP-TFs with respect to the control of genetic programs associated with metabolism in skeletal muscle (or any other major mass peripheral tissue) has not been addressed. Here, we have utilized siRNA-mediated targeting of COUP-TFII and II to investigate whether the COUP-TFs regulate critical pathways of metabolism in C2C12 skeletal muscle cells. We utilized the mouse C2C12 skeletal muscle cell culture model as previous data derived from this in vitro model with LXR and PPARβ agonists (20) were verified/validated in subsequent in vivo mouse studies (24, 25).

The COUP-TFII and II mRNAs are constitutively expressed during C2C12 skeletal muscle myogenesis. In contrast, COUP-TF protein levels are down-regulated as the C2C12s exit the cell cycle and undergo myogenic differentiation. This suggests that during differentiation, and/or cell cycle withdrawal that COUP-TFs is regulated by translational control mechanisms. Whether this involves mechanisms targeting the 5′ and 3′ untranslated regions and/or subcellular localization is not currently known. However, sea urchin homologs of COUP-TFs are stored as maternal RNA in the egg, a common site of translational regulation (26).

Stable expression of the COUP-TFII-siRNA (relative to the pSilencer 3.1 negative control) in the C2C12 skeletal muscle cells significantly attenuated both COUP-TFI and II mRNA (5- and 4.75-fold, respectively) relative to two controls, Gapdh and 18 S rRNA. Moreover, these effects were independent of the differentiation status of the C2C12 cells.

Our study demonstrates that a subgroup of genes (including, Fabp3, Cpt-1, and Ppara) involved in intramyocellular lipid mobilization/utilization and catabolism are dependent on the expression of COUP-TFs. This is further highlighted by the fact that related genes such as Fabp4, Ppar β/β, and Pparγ (and genes in similar pathways for example, Mcad, Lpl, and several other NRs) were refractory to the attenuation of COUP-Tf expression (see Tables 1–3). The observed reduction of these key metabolic target genes is noteworthy for a number of reasons. First, correlation between Fabp3 and Ppara expression has been described during transcriptional adaptation of lipid homeostasis in the skeletal muscle of endurance trained human males (27). Second, the attenuated expression of Cpt-1 correlates with reduced Ppara expression. Cpt-1 is a well-characterized Ppara (and δ) target gene (28). The roles of the above genes as important regulators of the lipolytic mobilization and catabolism of fatty acids (Ppara (29), Fabp3 (30), and Cpt-1 (30)), and their modulation in our C2C12 cell line with attenuated COUP-TFI and II expression, suggests that COUP-TFs plays a critical role in modulating lipolysis in this system. This is underscored by reduced palmitate/b-oxidation in the COUP-TFII-siRNA-transfected cells.

The role of the COUP-TFs in lipid utilization may appear contrary to the differentiation dependent regulation of COUP-Tf in the cell culture model. However, the COUP-TFs are abundantly expressed in skeletal muscle tissue (4). Second, it is pertinent to point out that Ppara was described as a regulator of lipid storage that mediated the actions of insulin sensitizers in adipose tissue. Later studies with muscle-specific Ppara null mice underscored the role of this NR in skeletal muscle in the...
context of insulin sensitivity, despite weak expression in this lean tissue (31).

The observed attenuation of Il-15 in the Coup-Tf-siRNA was interesting. Il-15 (and several other cytokines Il-6 and -8 (32, 33)) are expressed in skeletal muscle tissue and C2C12 cells and induced during myogenesis (34). Furthermore, it has been demonstrated that IL-15 has an anabolic effect of skeletal muscle in vitro and in vivo (33). In contrast, adipogenic cells do not express Il-15. Moreover, IL-15 administered to rodents decreased white adipose tissue mass and induced adiponectin secretion (23). In fact, recent studies have suggested that Il-15 functions in a muscle-to-fat endocrine axis to reciprocally regulate muscle/adipose tissue mass and to control insulin sensitivity (23). The repression of Il-15 in the Coup-Tf-siRNA cell line provides further support for a critical regulatory role of this orphan NR in metabolism. Moreover, the link between Coup-Tfs and Il-15 in muscle, and the role of Il-15 in the muscle/fat axis and insulin sensitivity correlates with the effects of the pancreatic Coup-TfII "knock-out" on glucose intolerance (11).

The attenuation of the Pepck-1 mRNA in the Coup-Tf-siRNA cells is consistent with previous studies demonstrating Coup-TfII-dependent induction of Pepck-1 transcription by glucocorticoids (2, 35).

We report that the attenuation of Coup-Tfs and Il in C2C12 cells modulates key genes involved in lipid utilization. Specifically, we observed that the mRNA encoding uncoupling proteins 1 and 3 (Ucp1 and Ucp3) were significantly repressed and activated, respectively, in the Coup-Tf-siRNA stable cells. In this context the C2C12 Coup-Tf-siRNA cells expressed significantly elevated steady state levels of total ATP (∼5-fold) relative to the negative 3.1 control cells. This may correlate with the reduction of Ucp1 mRNA expression in these cells. Along these lines ectopic overexpression of Ucp1 in HepG2 cells demonstrated a 23% reduction in ATP steady state levels (36). We should note that Ucp1 is almost exclusively expressed in brown adipose tissue. However, studies have reported Ucp1 mRNA expression is induced during myogenesis in C2C12 skeletal muscle cells (22, 37) and demonstrated that Ucp1 expression in muscle increases in a pathophysiological context. For example, Ucp1 is up-regulated in the soleus of the kyphoscoliosis (ky)

![Figure 5](image-url)

**FIGURE 5.** Attenuation of Coup-Tf expression affects expression of the uncoupling genes and the reverse cholesterol transporter genes (Abca1 and Abcg1). a, Ucp1; b, Ucp3; and c, total ATP production. The Coup-Tf-siRNA produced an approximate 5-fold increase in total ATP (36 μmol/liter, p = 0.0002) relative to the pSilencer 3.1 negative control (7 μmol/liter) (c). d, Abca1; e, Abcg1. -Fold changes of gene expression are relative to Gapdh. Mean ± S.D. was derived from three independent RNA preparations (each analyzed in triplicate). Significance was calculated using the Student’s t test; ***, p < 0.0001. Note: Ucp1 was undetectable in the Coup-Tf-siRNA stable C2C12 cell lines (a).
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Mouse mutant (38), a strain with muscular dystrophy localized to type I muscle fibers.

Ucp3 is highly expressed in skeletal muscle, in contrast to the role of Ucp1 in β-oxidation associated energy expenditure; the role of Ucp3 is less understood. Ectopic and overexpression of Ucp3 in transgenic mice and in cell culture (39–43) suggest that Ucp3 is involved in adiposity, and preferential lipid utilization. Our study indicates that repression of Coup-Tfs leads to a significant increase in Ucp3 mRNA expression. Although, a paradoxical observation in the context of reduced palmitate oxidation, Fabp3, Cpt-1, and Ppara expression; induction of Ucp3 has been reported as a compensatory response to the attenuation of Ucp1 expression in a non-obese Ucp1 null mice model (44). From this perspective, Ucp3 may be implicated in a more protective role in the mitochondria against lipid-induced oxidative damage. Second, the expression of the Ucp3 (a Ppara target gene) is surprisingly maintained in the skeletal muscle of Ppara null mice (45). These reports are in concordance with our observations.

The observed significant repression of classical LXR target genes involved in reverse cholesterol efflux and lipogenesis (Abca1, Abcg1, and Srebp-1c etc.) in the Coup-Tf-siRNA transfected cells is of interest for several reasons. First, Coup-TfIII has been demonstrated to activate the cholesterol 7α-hydroxylase (CYP7A1) (6, 46), and the cholesterol ester transfer protein (CETP) gene promoters (8), important genes in cholesterol homeostasis. Second, the Cyp7a1 and Cetp genes have also been demonstrated to be directly trans-activated by LXR agonists (47, 48). Indeed, studies of Lxra−/− null mice failed to induce the expression of the Cyp7a1 (49), which has also been demonstrated to be dependent on Coup-Tf expression (6, 46, 50, 51). This suggested to us the existence of cross-talk between and Lxr and Coup-Tf signaling in muscle. In concordance with this hypothesis we clearly observed that repression of the Coup-Tfs significantly diminished the classical and very reproducible activation of Abca1 (mRNA and promoter), Abcg1, and Srebp-1c mRNA (20, 52) expression by the selective LXR agonist, T0901317, independent of major changes in Lxr and Lxrβ expression. Moreover, this was consistent with no change in intracellular cholesterol levels in the Coup-Tf-siRNA cell line (in contrast to the negative control) after treatment with the LXR agonist (T0901317). In humans, mutations in the ABCA1 gene have been linked to Tangier disease, where these patients have a decreased ability to efflux cholesterol and consequently accumulate cellular cholesterol (53). Recently, it has been shown that a Ppara-Rxr-Lxr axis in mouse liver modulates an overlapping set of genes involved in both fatty acid catabolism and synthesis (54). This may also be true in our C2C12 cell model.

FIGURE 6. Coup-Tf-siRNA expression represses Lxr-mediated activation of Abca1, Abcg1, and Srebp-1c. C2C12 differentiated myotubes were treated with vehicle (Me2SO) or 10 μmol/liter of the LXR agonist, T0901317, for 24 h. Total RNA was extracted and Q-RT-PCR was performed. a–e, Abca1, Abcg1, Srebp-1c, Lxra, and Lxrb mRNA expression, respectively, in the pSilencer 3.1 negative control and the Coup-Tf-siRNA. Mean ± S.D. of three independent stable cell preparations are shown. Significance was calculated using the Student’s t test, where *, 0.01–0.05; **, 0.001–0.01; and ***, p < 0.0001. N.S. indicates not significant. Note: for each experiment in the negative control and the Coup-Tf-siRNA, the Me2SO vehicle was set at 1.
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A number of studies have demonstrated a role for COUP-TFs in cholesterol metabolism. For example, COUP-TfII was shown to abrogate the transactivation of reporter constructs containing the ApoA-II, ApoB, and ApoC-III gene promoters (55). These proteins are major components of the high density lipoproteins and are critical for their role in transporting cholesterol from extrahepatic tissues to the liver for processing and excretion in the form of bile acids. Moreover, they are intrinsically linked with the pathogenesis of atherosclerosis. The ability of COUP-Tf to modulate Lxr-inducible gene expression involved in cholesterol homeostasis may have utility in the future (if novel small molecule regulators are identified) in the context of cholesterol homeostasis.

In summary, these links between the COUP-Tfs, Lxr, and cholesterol efflux in skeletal muscle are consistent with other studies on COUP-Tf- and Lxr-mediated gene regulation and cholesterol homeostasis in several other major mass tissues. Moreover, these studies underscore the role of COUP-Tfs in skeletal muscle lipid utilization and mobilization, and provide a robust system to further study the molecular mechanisms involved in COUP-Tf/Lxr-dependent control of cholesterol homeostasis. Further studies are underway to examine the role of these orphan NRs in tissue-specific animal models.

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whereby the ablation of COUP-Tf and subsequent Ppara attenuation results in the modulation of LXR-dependent genes involved in metabolism.
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