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Rev-erbβ Regulates the Expression of Genes Involved in Lipid Absorption in Skeletal Muscle Cells

EVIDENCE FOR CROSS-TALK BETWEEN ORPHAN NUCLEAR RECEPTORS AND MYOKINES*

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Rev-erbβ is an orphan nuclear receptor that selectively blocks trans-activation mediated by the retinoic acid-related orphan receptor-α (RORα). RORα has been implicated in the regulation of high density lipoprotein cholesterol, lipid homeostasis, and inflammation. Rev-erbβ and RORα are expressed in similar tissues, including skeletal muscle; however, the pathophysiological function of Rev-erbβ has remained obscure. We hypothesize from the similar expression patterns, target genes, and overlapping cognate sequences of these nuclear receptors that Rev-erbβ regulates lipid metabolism in skeletal muscle. This lean tissue accounts for >30% of total body weight and 50% of energy expenditure. Moreover, this metabolically demanding tissue is a primary site of glucose disposal, fatty acid oxidation, and cholesterol efflux. Consequently, muscle has a significant role in insulin sensitivity, obesity, and the blood-lipid profile. We utilize ectopic expression in skeletal muscle cells to understand the regulatory role of Rev-erbβ in this major mass peripheral tissue. Exogenous expression of a dominant negative version of mouse Rev-erbβ decreases the expression of many genes involved in fatty acid/lipid absorption (including *Cd36*, and *Fabp-3* and *-4*). Interestingly, we observed a robust induction (>15-fold) in mRNA expression of interleukin-6, an "exercise-induced myokine" that regulates energy expenditure and inflammation. Furthermore, we observed the dramatic repression (>20-fold) of *myostatin* mRNA, another myokine that is a negative regulator of muscle hypertrophy and hyperplasia that impacts on body fat accumulation. This study implicates Rev-erbβ in the control of lipid and energy homoeostasis in skeletal muscle. In conclusion, we speculate that selective modulators of Rev-erbβ may have therapeutic utility in the treatment of dyslipidemia and regulation of muscle growth.

Members of the nuclear receptor (NR)[1] superfamily bind to specific DNA elements and function as transcriptional regulators (1, 2). In addition to the ligand-activated NRs, many members within this superfamily have no known ligand, and are referred to as "orphan NRs" (3). The orphan receptor *Rev-erbβ* (NR1D2, also known as *Rev-erbα*-related receptor, RVR) belongs to the family of "*Reverbs*" that also contain *Rev-erbα* (4, 5). The primary structure of these two receptors together with retinoic acid-related orphan receptor-α (RORα) and the *Drosophila* orphan receptor, E75A, is very similar especially in the DNA-binding domain and the putative ligand-binding domain (6).

Two Rev-erbβ genes have been identified; *Rev-erbβ*1 and *Rev-erbβ*2, which are alternatively spliced products of the *Rev-erbβ* gene (7). The mRNA expression data shows that *Rev-erbβ* is abundantly expressed in most tissues, although higher levels of expression are observed in skeletal muscle, brain, kidney, and liver (Refs. 4 and 8 and references therein).

Rev-erbα, Rev-erbβ, and ROR bind as monomers to the nuclear receptor half-site motif, PuGGTCA flanked 5′ by an AT-rich sequence ($(A/T)_6$PuGGTCA). Although these receptors are closely related, and bind to the same motif, they function in an opposing manner. Whereas ROR activates gene transcription, Rev-erbα and Rev-erbβ mediate transcriptional repression, and can repress trans-activation mediated by ROR (6, 9–12). The inter-relationship between these nuclear receptors is underscored by the evidence that demonstrates RORα trans-activates the *Rev-erbα* promoter (13).

Loss of function studies in cell culture and in animal models has demonstrated a role of RORα in lipid metabolism. RORα-deficient *staggerer* mice are predisposed to the development of atherosclerosis. The *staggerer* mice possess an aberrant blood-lipid profile with low circulating levels of major lipoproteins such as HDL cholesterol, apolipoprotein (apo) C-III, and plasma triglycerides. Furthermore, decreases in specific apolipoprotein compartments, namely *Apoa-I*, the major constituent of HDL, and *Apoa-II* that lead to hypo-α-lipoproteinemia have been reported in *staggerer* mice (15). Moreover, our recent studies demonstrate that RORα regulates the expression of genes involved in lipid homeostasis and energy balance in skeletal muscle cells (16).

Several reports have demonstrated the role of Rev-erbα in lipid metabolism and inflammation. In the context of lipid metabolism, Rev-erbα, together with peroxisome proliferator activated receptor-α (PPAR-α) has been shown to regulate the expression of *ApoA1* in a species-specific manner (17). *Apoa1* has an important role in the formation of nascent HDL particles and apolipoprotein-mediated cholesterol efflux in mice (18, 19). Furthermore, Rev-erbα has also been shown to regulate the expression of the *ApoC-III* gene that plays a key role in maintaining serum triglyceride levels (20, 21). The inter-relationship between Rev-erbβ and lipid metabolism has not been investigated. Within inflammatory pathways, Rev-erbα and ROR act opposingly on the NFκB pathway. RORα has been

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[1] The abbreviations used are: NR, nuclear receptor; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate; ROR, retinoic acid-related orphan receptor; HDL, high density lipoprotein; PPAR, peroxisome proliferator-activated receptor; Q-RT, quantitative real time; IL, interleukin; SREBP, sterol regulatory element-binding protein.

shown to directly up-regulate the expression of IκBα, the major inhibitor of the NFκB signaling pathway by binding to the IκBα promoter (22). The functional role of Rev-erbβ in NFκB-mediated inflammation still remains obscure.

Rev-erbβ is abundantly expressed in skeletal muscle and brown fat. Skeletal muscle is one of the most metabolically demanding major mass peripheral tissues that accounts for 50% of energy expenditure. Moreover, this lean tissue relies heavily on fatty acids as an energy source, accounts for 75% of glucose disposal, and is involved in cholesterol efflux. Consequently, muscle has a significant role in insulin sensitivity and the blood-lipid profile. However, the role of Rev-erbβ in skeletal muscle lipid and energy homeostasis has not been well studied.

The C2C12 *in vitro* cell culture model system has been used to investigate the regulation of lipid metabolism and cholesterol homeostasis by nuclear receptors such as LXR (23), PPARα, -β/δ, -γ (24–28), and RORα (16). Selective and synthetic agonists to these nuclear receptors induce similar effects on mRNAs encoding *Abca1/g1*, *ApoE*, sterol regulatory element-binding protein (*Srebp*)-1c, *Scd-1*, *Fabp3*, fatty acid synthase, lipoprotein lipase, *Glut5*, *Ucp-2*, and *Ucp-3* in differentiated C2C12-myotubes and *Mus musculus* skeletal muscle tissue (23–25). The physiological validation of the cell culture model with respect to lipid homeostasis in the mouse corroborates the utility of this model system. This cell culture model provides an ideal platform to identify the Rev-erbβ-dependent regulation of genes involved in metabolism.

We have examined the regulation of gene expression involved in lipid homeostasis by Rev-erbβ "loss of function" in skeletal muscle cell culture model. Our investigation demonstrates that Rev-erbβ controls the expression of genes involved in lipid absorption. Moreover, we observed the regulation of genes encoding critical myokines that influence energy expenditure and inflammation.

MATERIALS AND METHODS

Cell Culture—Mouse myogenic C2C12 cells were cultured in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated Serum Supreme (BioWhittaker, Edward Keller Pty. Ltd., Hallam, Victoria, Australia)) in 6% CO_2. Myoblasts were differentiated into post-mitotic multinucleated myotubes by 4 days of serum withdrawal (*i.e.* cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum + 1% serum supreme). Cells were harvested 96 h (4 days) after mitogen withdrawal.

C2C12 Transfections—Each well of a 24-well plate of C2C12 cells (~50% confluence) was transfected with DNA using the liposome-mediated transfection procedure as described previously (16). Cells were transfected using a DOTAP and Metafectene (Biontex Laboratories GmbH, Munich, Germany) liposome mixture in 1× HBS (HEPES-buffered saline (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na_2HPO_4, 11 mM dextrose (pH 7.1)). The DNA/DOTAP/Metafectene mixture was added to the cells in 0.6 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum for experiments with RORα or 10% serum supreme. After 16–24 h, the culture medium was changed and after a further 24 h the cells were harvested for the assay of luciferase activity. Luciferase activity was measured as described previously (16).

C2:Rev-erbβΔE Stably Transfected Cell Line—The transfection of pSG5-Rev-erbβΔE into mouse myogenic C2C12 cells and the selection of stably transfected polyclonal cells using G418 has been described previously (29). The cells were cultured in growth medium with G418, and differentiated into post-mitotic multinucleated myotubes by 4 days of serum withdrawal.

RNA Extraction and cDNA Synthesis—Total RNA was extracted from C2C12 cells using Tri-Reagent (Sigma) according to the manufacturer's protocol. After treatment with 2 units of Turbo DNase (Ambion, Austin, TX) for 60 min, DNase was inactivated by heating to 75 °C for 10 min. RNA for quantitative real time (Q-RT)-PCR was further purified using the RNeasy RNA extraction kit (Qiagen, Clifton Hill, Victoria, Australia) according to the manufacturer's instructions.

Q-RT-PCR—RNA was normalized using UV spectrometry and agarose gel electrophoresis. Complementary DNA was synthesized from 3

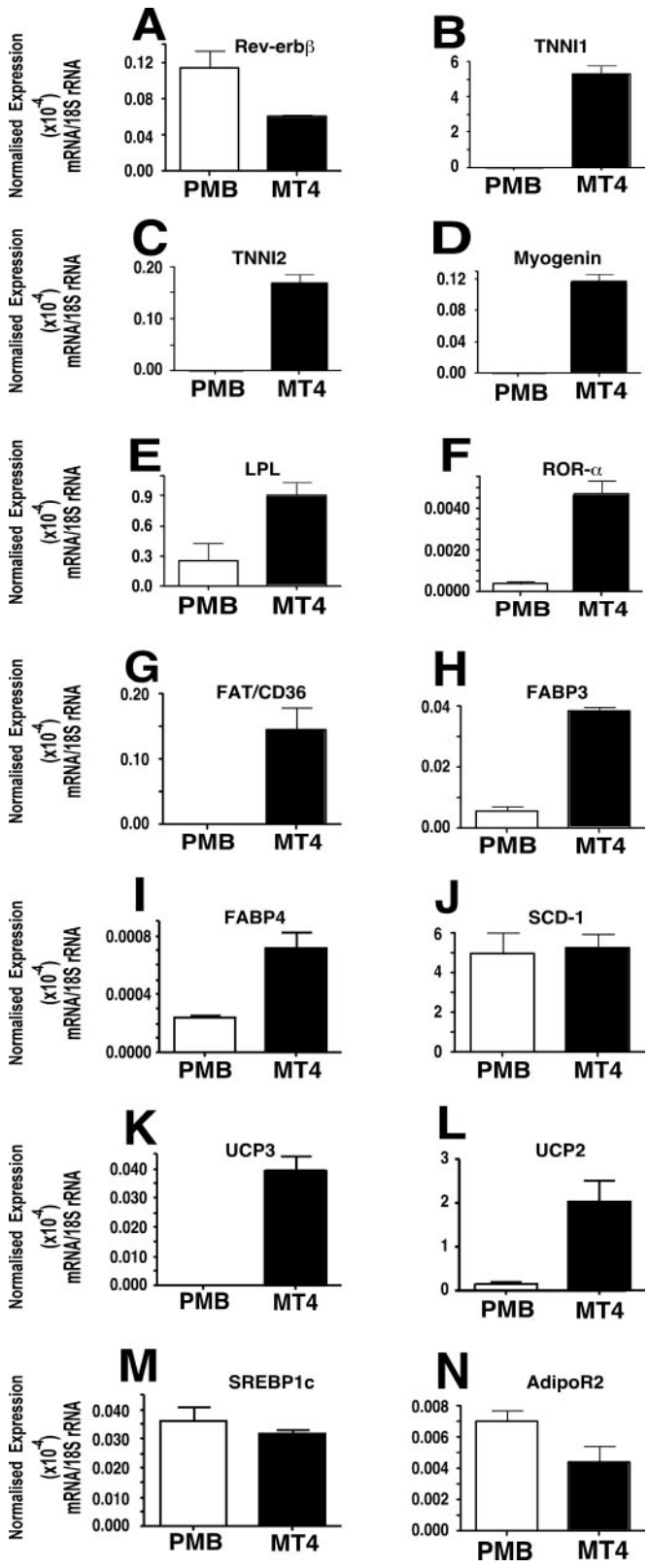

FIG. 1. **Q-RT-PCR analysis of mRNA expression during skeletal muscle myogenesis.** Total RNA from wild type C2C12 proliferating myoblasts (*PMB*), and myotubes after 4 days of serum withdrawal (*MT4*) was reverse transcribed to cDNA and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of: *A*, *Rev-erbβ*; *B*, *Tnni1*; *C*, *Tnni2*; *D*, *myogenin*; *E*, lipoprotein lipase; *F*, *RORα*; *G*, *Fat/Cd36*; *H*, *Fabp3*; *I*, *Fabp4*; *J*, *Scd-1*; *K*, *Ucp-3*; *L*, *Ucp-2*; *M*, *Srebp1c*; and *N*, *AdipoR2*. Normalized expression is relative to the expression of 18S rRNA determined with the same cDNA then multiplied by 10,000. All reactions were performed in triplicate and results are shown as average ± S.D.

TABLE I
Key target genes in this study

	Metabolic genes
Abca1 and *Abcg8*	ATP binding cassette proteins: transporters that transfer cholesterol to the HDL acceptors, *i.e.* reverse cholesterol efflux
Acs4	Acyl-CoA synthetase-4. Enhances the uptake of fatty acids by catalyzing their activation to acyl-CoA esters for subsequent use in catabolic fatty acid oxidation pathways.
Adrp/adipophilin	Adipocyte differentiation-related protein. Involved in lipid storage.
AdipoR1 and *R2*	Adiponectin receptors 1 and 2. Cell surface membrane receptors for adiponectin that regulate glucose uptake and fatty acid oxidation.
Ampk	5′AMP-activated protein kinase. A fuel-sensing enzyme that responds to cellular stress by regulating carbohydrate and fat metabolism.
ApoE	Apolipoprotein-E: facilitates cholesterol and lipid efflux.
Cav3	Caveolin 3. Muscle-specific caveolin involves in membrane protein anchoring, caveolae, t-tubules formation and lipid trafficking.
Cpt-1 and *-2*	Carnitine palmitoyltransferases. Transfers the long-chain fatty acyl group from coenzyme A to carnitine, the initial reaction of mitochondrial import of long-chain fatty acids and their subsequent oxidation.
Fas	Fatty acid synthase. Involved in *de novo* fatty acid production.
Fat and *Fabp3*/*Fabp4*	Fatty acid translocase, and fatty acid-binding proteins. Facilitate uptake of long chain fatty acids (LFCAs) and low density lipoproteins (LDLs).
Lpl	Lipoprotein lipase. Hydrolysis of lipoprotein triglycerides into free fatty acids and responsible for the uptake of free fatty acids.
Mcad	Medium chain acyl coenzyme A dehydrogenase. A key enzyme involved in fatty acid oxidation pathway.
Pdk-2 and *-4*	Pyruvate dehydrogenase kinases: inhibiting the pyruvate dehydrogenase complex, thereby controlling glucose oxidation and maintaining pyruvate for gluconeogenesis.
Scd-1 and *-2*	Stearoyl CoA desaturase-1, and -2. Enzymes associated with adiposity, *i.e.* storage and esterification of cholesterol, and responsible for the *cis* saturation of stearoyl and palmitoyl-CoA converting them to oleate and palmitoleate, which are the monounsaturated fatty acids of triglycerides.
Srebp-1c	Sterol regulatory element-binding protein-1c, the hierarchical transcriptional activator of lipogenesis.
Ucp-2 and *-3*	Uncoupling proteins. Mitochondrial proteins that uncouple metabolic fuel oxidation from ATP-synthesis, regulating energy expenditure.
	Myokines and inflammatory mediators
IL-6	Interleukin-6. An inflammatory cytokine and exercise induced myokine. A key gene that acts as functional link between inflammation and metabolism.
IL-15	Interleukin-15. An inflammatory cytokine. Stimulates skeletal muscle fiber protein synthesis. Inhibits fat deposition *in vivo* in muscle.
IκBα	Inhibitor of NFκappaB α. A cytoplasmic protein. Regulates the expression of inflammatory genes through interaction with NFκB.
Cox-2	Cyclooxygenase-2. An inflammatory protein. Involved in the synthesis of prostaglandin-H upon tumor necrosis factor stimulation. Also plays role in regulating blood pressure.
Myostatin	Member of the transforming growth factor β family. Operates as a negative regulator of skeletal muscle mass.

μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen Australia Pty. Ltd., Mulgrave, Victoria, Australia) and random hexamers according to the manufacturer's instructions. Target cDNA levels were analyzed by Q-RT-PCR in 25-μl reactions containing either SYBR green (ABI, Warrington, UK) or Taqman PCR master mix (ABI, Branchburg, NJ), 200 nM each of forward and reverse primers or Assays-on-Demand Taqman primers (ABI, Foster City, CA), and cDNA (derived from 50 nanograms of RNA) by using an ABI Prism 7000 Sequence Detector system. PCR was conducted over 45 cycles at 95 °C for 15 s and 60 °C for a 1-min two-step thermal cycling preceded by an initial 95 °C for 10 min for activation of Amplitaq Gold DNA polymerase.

Primers—Primers used for the Q-RT-PCR analysis of the mRNA populations have been described in detail (16), with the exception of primers designed for the detection of *Rev-erbβΔE* (pSG5-RVR-F, TGGGCAACGTGCTGGTTA; pSG5-RVR-R, CCATGGTGGGATCCGAATT), *Mcad* (MCAD-F, CAGCCAATGATGTGTGCTTACTG; MCAD-R, ATAACATACTCGTCACCCTTCTTCTCT), *Errα* (ERRα-F, CTCTGGCTACCACTACGGTGTG; ERRα-R, AGCTGTACTCGATGCTCCCCT), IL-6 (IL-6-F, AGCCAGAGTCCTTCAGA; IL-6-R, GGTCCTTAGCCACTCCT) using SYBR green. *Rev-erbβ*, *Fabp4*, *NFκB* (*relA*), *IκBα*, *Cox-2*, *IL-15*, and *myostatin* were detected using Assay-on-demand primer/probe sets.

Plasmids—pSG5, pSG5-Rev-erbβ, and pSG5-Rev-erbβΔE have been described previously (29). The reporters containing the human Rev-erbAα promoter (pRev-erbAαWT) or four copies of the mouse Purkinje cell protein-2 RORE (mPCP-2tk LUC) linked to the luciferase gene have been described previously (9, 16).

RESULTS

Rev-erbβ mRNA Is Expressed in Skeletal Muscle Cells and Repressed during Myogenic Differentiation—Many studies indicate that the C2C12 cell line is an excellent cell culture model to investigate myogenesis, and the NR-mediated regulation of lipid homeostasis in skeletal muscle (23–26). In this culture system, proliferating C2C12 myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by serum withdrawal in culture over a 48–96-h period. This transition from a non-muscle phenotype to a contractile phenotype is associated with activation/expression of a structurally diverse group of genes responsible for contraction and the extreme metabolic demands on this tissue.

Initially, total RNA was isolated from proliferating myoblasts and post-mitotic myotubes after 4 days of serum withdrawal, converted to cDNA for the analysis of mRNA expression by Q-RT-PCR. We utilized the GenBank™ sequences of mouse *Rev-erbβ* to design specific primers for detection of mouse *Rev-erbβ* by Q-RT-PCR.

We observed that *Rev-erbβ* is expressed in proliferating myoblasts and the transcript was decreased 2–3-fold as the cells exit the cell cycle and form differentiated multinucleated myotubes (Fig. 1*A*). Concomitant with this decrease in *Rev-erbβ* mRNA was the striking induction of expression of mRNA that encodes the slow (type I, *Tnni1*) and fast (type II, *Tnni2*) isoforms of the contractile protein, *troponin I*, and *myogenin* that encodes the hierarchical basic helix loop helix regulator (Fig. 1, *B–D*). These data confirmed that these cells had terminally differentiated and had acquired a contractile phenotype.

Furthermore, we analyzed the expression of several genes that encode major metabolic enzymes and regulators that are involved in the regulation of energy expenditure and lipid homeostasis (as described in Table I). Acquisition of the muscle-specific phenotype saw an increase in expression of mRNAs encoding lipoprotein lipase (Fig. 1*E*), RORα (Fig. 1*F*), fatty acid translocase/CD36 (*Fat/Cd36*, Fig. 1*G*), fatty acid binding proteins 3 and 4 (Fig. 1, *H* and *I*), and the uncoupling proteins (*Ucp2* and *-3*, Fig. 1, *K* and *L*). Finally, there was no change in the expression of the mRNAs encoding *Srebp-1c* (Fig. 1*M*), stearoyl-CoA desaturase 1 (*Scd-1*, Fig. 1*J*), and adiponectin receptor 2 (*adipoR2*, Fig. 1*N*).

In summary, these data demonstrate that the mRNA transcript encoding *Rev-erbβ* was expressed during myogenesis. Second, during the acquisition of a contractile and metabolic phenotype (consistent with the increased utilization of lipids in skeletal muscle) we observed an ~2.5-fold repression of this orphan NR. However, we note that *Rev-erbβ* is still expressed at a significant level in differentiated cells, in concordance with the expression of this orphan NR in adult muscle tissue.

Deletion of the Rev-erbβ Ligand Binding Domain Compromises Its Ability to Repress RORα-mediated Trans-activation—To understand the metabolic role of Rev-erbβ in skeletal muscle lipid and energy homeostasis, and to identify target genes of this orphan receptor in muscle cells, we examined the effect of perturbing Rev-erbβ function. To disrupt Rev-erbβ-mediated trans-repression of gene expression, we utilized Rev-erbβΔE, that encodes amino acids 1–394 but lacks the entire E region that encodes the putative ligand binding domain of Rev-erbβ. Deletion of this region has been reported to have a dominant negative effect on Rev-erbβ-mediated trans-repression of gene expression (29). Moreover, this region has been implicated in associating with the nuclear receptor co-repressors (30). In transient transfection assays in C2C12 skeletal muscle cells, deletion of the E region ablated the ability of Rev-erbβ to trans-repress the human Rev-erbAα promoter (Fig. 2*A*). Moreover, the capacity of *Rev-erbβ* to repress RORα-mediated transcription is diminished when the E region is absent (Fig. 2, *B* and *C*). This demonstrates that deletion of the E-region that encodes the ligand binding domain of Rev-erbβ compromises the capacity of this orphan NR to trans-repress the *Rev-erbα* promoter, and to attenuate RORα-mediated trans-activation.

Ectopic Dominant Negative (Rev-erbβΔE) Expression Induces Precocious Differentiation and RORα mRNA Expression—The stable C2C12 cell line expressing Rev-erbβΔE (C2:Rev-erbβΔE) has been described previously (29). Prior to more extensive analysis of this cell line, we decided to ascertain the Rev-erbβ status of this cell line. We designed a SYBR green-mediated Q-RT-PCR assay to selectively detect the ectopically expressed *Rev-erbβΔE* transcript. We observed that the C2:Rev-erbβΔE cell line specifically expresses the ectopically introduced mRNA in myoblasts and myotubes (Fig. 3, *A* and *B*). The expression of the exogenous transcript is constitutive (Fig. 3, *A* and *B*) in contrast to the endogenous transcript that is down-regulated during differentiation of proliferating myoblasts to postmitotic myotubes (Fig. 1*A*). Moreover, the steady state levels of *myogenin* mRNA were elevated in myotubes from the C2:Rev-erbβΔE cell line relative to wild-type C2C12 cells (Fig. 3*C*). This is consistent with the previously reported precocious biochemical and morphological differentiation of the C2:Rev-erbβΔE cell line relative to wild-type C2C12 cells (29). For example, Burke *et al.* (29) reported induction of differentiation upon serum withdrawal leads to accelerated activation and expression of the *myogenin*, and $p21^{Cip\text{-}1/Waf\text{-}1}$ mRNAs. Moreover, reduced expression of *cyclin D1* was observed, which

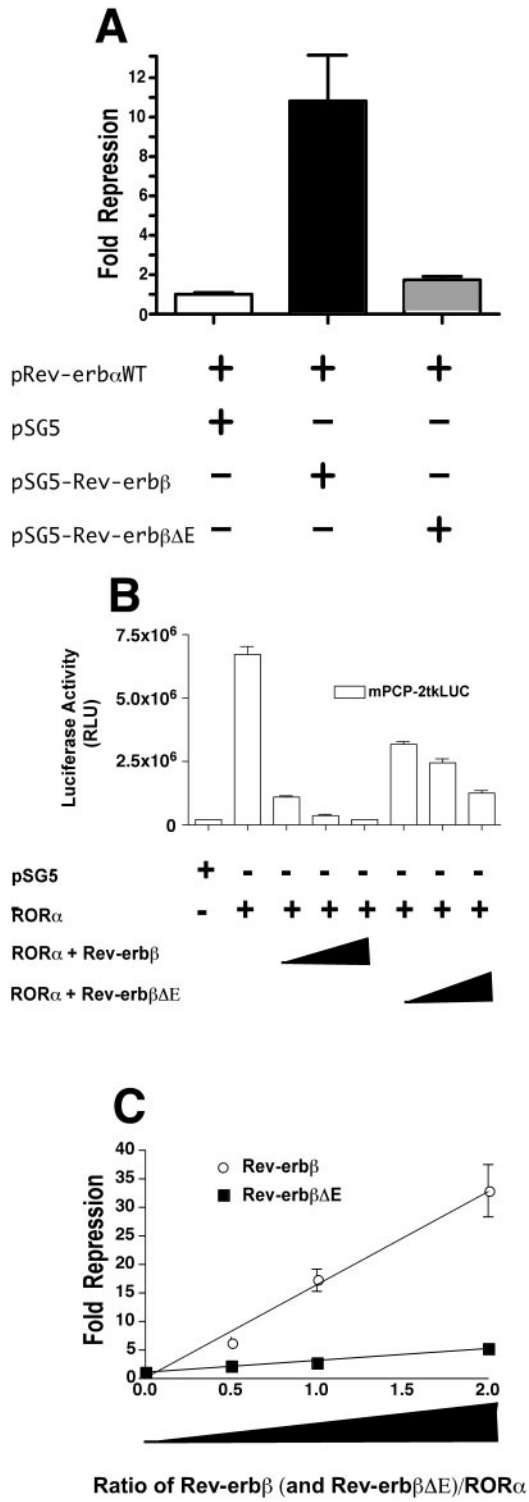

FIG. 2. **Deletion of the E region of Rev-erbβ compromises its trans-repression activity.** *A*, C2C12 cells were co-transfected with 0.5 μg of the reporter, pRev-erbAαWT, and 0.16 μg each of pSG5, pSG5-Rev-erbβ, and pSG5-Rev-erbβΔE. -Fold repression is expressed relative to luciferase activity obtained after co-transfection of the reporter and pSG5 vector only, arbitrarily set at 1. All transfections comprised six replicates and results are shown as average ± S.D. *B*, C2C12 cells were co-transfected with 0.33 μg of the RORα responsive reporter, mPCP-2tk-LUC, and 0.48 μg of pSG5 or 0.16 μg of pSG5-RORα and increasing amounts (0.05, 0.1, and 0.2 μg) of pSG5-Rev-erbβ or pSG5-Rev-erbβΔE. pSG5 was added to normalize the final amount of DNA transfected to 0.63 μg in each well. All transfections comprised six replicates and results are shown as average ± S.D. *C*, data from *B* are represented in terms of -fold repression. -Fold repression is expressed relative to luciferase activity obtained after co-transfection of the reporter and pSG5-RORα, arbitrarily set at 1.

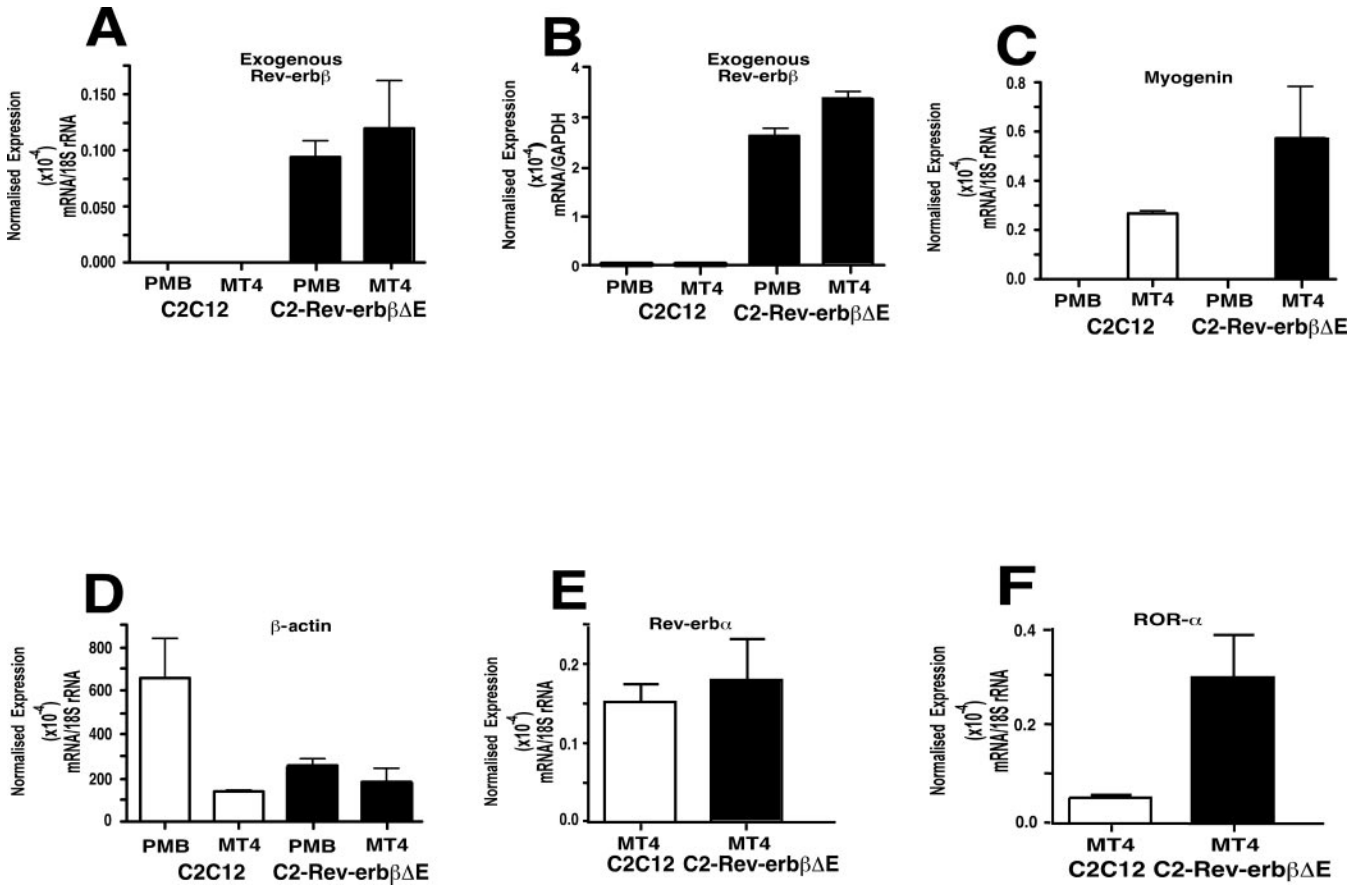

FIG. 3. **Ectopic expression of the dominant negative Rev-erbβΔE accelerates myogenesis and induces expression of RORα.** Total RNA was extracted from the wild type (C2C12) and the dominant negative (C2:Rev-erbβΔE) cell lines from proliferating myoblasts (*PMB*), and myotubes after 4 days of serum withdrawal (*MT4*), reverse transcribed to cDNA, and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of: *A* and *B*, *Rev-erbβΔE*; *C*, *myogenin*; *D*, *β-actin E*, *Rev-erbα*; *F*, *RORα*. Normalized expression is calculated relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) RNA (*B*) or 18 S rRNA (*A*, *C–F*) determined with the same cDNA. All reactions were performed in triplicate and results are shown as average ± S.D.

correlated with the increased expression of the cdk inhibitor, p21 (29). In addition, the levels of the non-muscle cytoskeletal *β-actin* are repressed in the C2:Rev-erbβΔE cell line, relative to wild-type C2C12 cells (Fig. 3*D*) in concordance with the elevated *myogenin* mRNA and precocious differentiative capacity of this cell line (29).

We used Q-RT-PCR with an ABI Taqman primer set that does not discriminate between ectopic *Rev-erbβΔE* and endogenous *Rev-erbβ* mRNA expression to determine the total quantity of *Rev-erbβ* mRNA in the stable cell line. We observed that the total pool of *Rev-erbβ* mRNA is increased by ~20–30% in myotubes from the C2:Rev-erbβΔE cell line relative to wild type C2C12s (data not shown). Considering the efficient expression of the exogenous *Rev-erbβΔE* mRNA transcript (Fig. 3, *A* and *B*), this data suggests that the endogenous transcript expression is reduced, in concordance with the previous analysis of this cell line (29). The down-regulation of the endogenous transcript is not surprising. During myogenesis, mRNA pool sizes in muscle tissue are under strict control (31) and mechanisms in skeletal muscle exist that sense total output from exogenous and endogenous genes (32). Furthermore, exogenous expression of a number of different contractile protein transgenes in the mouse (*e.g.* myosin light chain 2, troponin I fast, skeletal and cardiac actin) results in the decline of the expression of the corresponding endogenous gene (32–36).

Ectopic *Rev-erbβΔE* mRNA expression does not affect the mRNA expression level of the other member of the Rev-erb family, *Rev-erbAα* (Fig. 3*E*). Interestingly, the closely related but opposingly acting orphan receptor, RORα, was dramatically increased ~6-fold (Fig. 3*F*) in the C2:Rev-erbβΔE cell line. However, the levels of ROR-γ remain unchanged (see Table IV). These data indicate that there is cross-talk between these two related, but opposingly acting, orphan receptors in skeletal muscle cells.

Dominant Negative Rev-erbβ Regulates the Expression of Genes Involved in Lipid Absorption and Energy Expenditure— The expression of many metabolic markers is altered during skeletal muscle differentiation and acquisition of the muscle-specific and contractile phenotype (Fig. 1). We therefore analyzed the expression of the genes involved in differentiation and skeletal muscle metabolism in the C2:Rev-erbβΔE cell line, relative to wild-type C2C12 cells (see Table I and II). Before analysis of the mRNAs encoding the metabolic enzymes we first characterized the key markers of differentiation in the C2:Rev-erbβΔE cell line to determine a threshold of significance with which to assess putative changes in a relevant context.

We observed that after mitogen withdrawal, the C2:Rev-erbβΔE cells formed post-mitotic multinucleated myotubes and thus had morphologically differentiated. The induction and activation of myogenin (Fig. 3*C*) demonstrated that the cells had also acquired a muscle-specific phenotype. Transcripts for the contractile proteins troponin I slow ((*Tnni1*) Fig. 4*A*), and troponin I fast ((*Tnni2*) (Fig. 4*B*)) were also induced after serum withdrawal demonstrating that the C2:Rev-erbβΔE cell line had acquired a contractile phenotype and was biochemically differentiated. Analysis of the relative expression of these biochemical markers in wild type C2C12 *versus* C2:Rev-erbβΔE myotubes after 4 days of serum withdrawal revealed that *myo-*

TABLE II
Relative changes in the expression of genes involved in lipid metabolism in skeletal muscle cells constitutively expressing Rev-erbβΔE

Gene	-Fold change
Fatty acid and lipid absorption	
Fat/Cd36	**↓5.4**
Fabp-3	**↓4.3**
Fabp-4	**↓24.4**
Lipid catabolism	
Lpl	↓2.1
Cpt-1	↓1.9
Cpt-2	↓1.8
Acs4	↑1.6
AdipoR1	↓1.2
AdipoR2	↓1.4
Pdk-2	↓1.8
Pdk-4	↓1.4
Mcad	↓1.1
Energy expenditure	
Ucp3	**↓7.5**
Ucp2	↓1.8
Energy balance	
Ampkβ1	↑1.7
Ampkγ1	↓1.0
Ampkγ3	↓1.4
Lipid efflux and homeostasis	
Abca1	↑1.0
Abcg8	↑1.7
ApoE	↓2.0
Cav3	↓2.1
Lipid storage	
Adrp	↓1.3
Lipogenesis	
Srebp-1c	**↑2.8**
Scd-1	**↓3.9**
Scd-2	↑1.3
Fas	↓1.0
Contractility/myogenic markers	
Tnni1	↓1.2
Tnni2	↑1.6
Myogenin	↑2.1
β-Actin	↑1.4

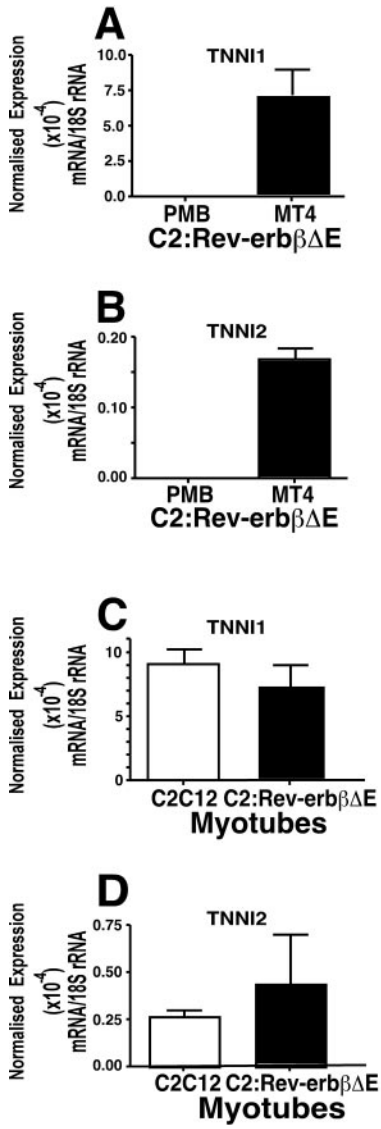

FIG. 4. **The C2:Rev-erbβΔE cell line acquires contractile markers of skeletal muscle.** *A* and *B*, expression of troponin I type I (slow; *Tnni1*) and II (fast; *Tnni2*) mRNA levels, respectively, in the C2:Rev-erbβΔE cell line. Total RNA was extracted from proliferating myoblasts (*PMB*) or myotubes were harvested after 4 days of serum withdrawal (*MT4*), reverse transcribed to cDNA, and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of *Tnni1* (*A*) and *Tnni2* (*B*). *C* and *D*, total RNA was extracted from wild type (C2C12) and dominant negative (C2:Rev-erbβΔE) cell lines from myotubes after 4 days of serum withdrawal, reverse transcribed to cDNA, and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of *Tnni1* (*C*) and *Tnni2* (*D*). Normalized expression was calculated relative to the expression of 18S rRNA determined with the same cDNA. All reactions were performed in triplicate and results are shown as average ± S.D.

genin (Fig. 3*C*) and fast type II (*Tnni2*) mRNA expression (Fig. 4, *C* and *D*) were increased ~1.5–2-fold. The TNNI1 mRNA encoding the slow type I isoform was actually slightly reduced.

This indicated that any major changes in the expression of metabolic markers were not because of compromised morphological and/or biochemical differentiation. Moreover, these data provide a threshold of significance whereby altered expression of genes involved in skeletal muscle metabolism would only be considered to be of importance if it varied more than 2-fold.

Analysis of the expression of the genes involved in skeletal muscle metabolism in these cell lines revealed that attenuation of Rev-erbβ had a number of significant effects in C2C12 cells (Table II). In the context of lipid and fatty acid absorption we observed repression of the mRNAs encoding *Fabp-3* and *-4* (~5 and ~24-fold) and *Fat/Cd36* (~5-fold) (Fig. 5, *A–C*). Analysis of genes involved in the regulation of energy expenditure and lipid utilization showed that the expression of *Ucp3* mRNA (Fig. 5*D*) was significantly repressed (~7.5-fold). In contrast, *Ucp2* mRNA showed minimum changes in the cell line overexpressing *Rev-erbβΔE*, relative to the ~2-fold changes observed in the markers of differentiation (Table II). Moreover, the mRNA encoding *Ampkγ3* (preferentially expressed in glycolytic fast twitch muscle fibers), a regulator of glucose uptake, and energy balance was unchanged by Rev-erbβΔE expression. The expression of *Scd-1*, a key enzyme involved in adiposity, is repressed ~4-fold in the cell line overexpressing *Rev-erbβΔE* (Fig. 5*E*). In contrast, the expression of mRNA encoding the *Scd-2* was relatively unaffected. Surprisingly, *Srebp-1c* mRNA expression increased 2.8-fold (Fig. 5*F*, Table II).

The expression of the transcripts encoding *Cpt1*, *Mcad*, and *Acs-4* that are involved in lipid catabolism and preferential fuel utilization were not affected in the Rev-erbβΔE expressing cell line (Table II). Additionally, we did not observe any changes in the expression of mRNAs encoding ABCA1, ABCA8/G1, apoE, Cav-3, and ADRP, which are involved in cholesterol homeostasis and lipid storage (Table II). Analysis of the expression of many other nuclear hormone receptors that have been demonstrated to regulate lipid and carbohydrate metabolism, including *ERRα*, *PPAR-α*, *-β/δ*, and *-γ*, *LXRα* and *-β*, *Rev-erbα*, and *Nur77* demonstrated that *Rev-erbβΔE* expression did not affect

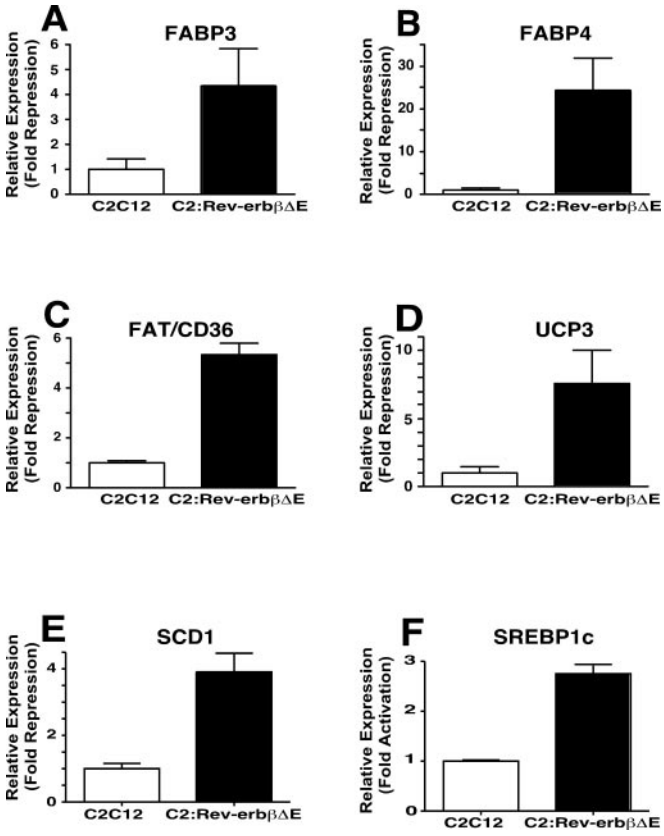

FIG. 5. **Rev-erbβ controls genes that regulate lipid metabolism and energy expenditure.** Total RNA was extracted from wild type (C2C12) and dominant negative (C2:Rev-erbβΔE) cell lines from myotubes after 4 days of serum withdrawal, reverse transcribed to cDNA, and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of: *A*, *Fabp-3*; *B*, *Fabp-4*; *C*, *Cd36*; *D*, *Ucp-3*; *E*, *Scd-1*; and *F*, *Srebp1c*. Relative expression is shown as -fold repression and -fold activation calculated relative to the expression in the wild type C2C12 cell line, which was arbitrarily set at 1. All reactions were performed in triplicate and results are shown as average ± S.D.

the expression of the mRNAs encoding these NRs (see Table IV).

Rev-erbβ Modulates Myokine Expression—Muscle cytokines (myokines) include IL-6, IL-15, and *myostatin* control inflammation, energy expenditure, muscle growth, and fat deposition (see Table I). The Rev-erbα nuclear receptor has been implicated in the control of inflammation, and the modulation of the NFκB-dependent pathway. Therefore, we decided to investigate the expression of several myokines and inflammatory markers/regulators in the wild-type and C2C12 cells expressing Rev-erbβΔE.

Initially, we examined the expression of IL-6, IL-15, *myostatin*, and IκBα (the regulator of NFκB) during differentiation of native C2C12 cells. We isolated total RNA from wild type C2C12 proliferating myoblasts, and post-mitotic multinucleated myotubes after 4 days of serum withdrawal, and analyzed the expression levels of mRNAs by Q-RT-PCR. First, we observed that these myokines, and modulators of the inflammatory process, were expressed in wild type C2C12 cells (Fig. 6). Second, we observed that IL-15 and *myostatin* were induced (~31- and 25-fold, respectively), whereas IκBα and IL-6 were repressed (~2.5- and 5.5-fold, respectively) during myogenic differentiation (Fig. 6, *A–D*).

Subsequently, we examined the effect of Rev-erbβΔE on the expression of the myokines, and inflammatory regulators in C2:Rev-erbβΔE cells after 4 days of serum withdrawal, relative to similarly differentiated native C2C12 cells by Q-RT-PCR (see Table III). Interestingly, we observed that ectopic expression of Rev-erbβΔE leads to an induction in the inhibitor of NFκB-mediated gene expression, IκBα (Fig. 6*E*). Consistent with increased expression of IκBα, mRNAs encoding the NFκB and tumor necrosis factor target genes *IL-15* (Fig. 6*G*) and *Cox-2* (Table III) were not induced in cells expressing Rev-erbβΔE.

TABLE III
Relative changes in the expression of genes encoding myokines and inflammatory markers in skeletal muscle cells constitutively expressing Rev-erbβΔE

Gene	-Fold change
IL-6	↑ **15.1**
IL-15	↑ 1.0
IκBα	↑ 2.8
NFκB (*relA*)	↑ 1.0
Cox-2	↓ 1.3
Myostatin	↓ **23.7**

Curiously, we found that IL-6 mRNA is robustly increased (~15-fold; Fig. 6*F*), and *myostatin* mRNA is dramatically reduced (~24-fold; Fig. 6*H*) in the cells expressing *Rev-erbβΔE*, concordant with the precocious differentiation of the C2:Rev-erbβΔE cell line. In summary, this demonstrates that Rev-erbβ regulates myokine expression, and as such is a critical modulator of muscle growth and inflammation in this tissue.

DISCUSSION

We have utilized the C2C12 *in vitro* cell culture model system and ectopic overexpression of dominant negative Rev-erbβ to investigate the role of this orphan receptor in skeletal muscle lipid, and energy homeostasis. Here we report that perturbation of Rev-erbβ function decreases the expression of genes involved in lipid absorption. Additionally, we observed dramatic induction and repression of two myokines, IL-6, and *myostatin*, respectively, which are involved in energy expenditure, inflammation, muscle hypertrophy and hyperplasia, and the accumulation of body fat.

These observations are consistent with genetic and molecular studies that demonstrate that the NR1D subfamily of nuclear receptors (Reverbs) has a direct role in lipid homeostasis. For example, the NR1D subfamily of orphan receptors regulate the expression of *ApoC-III* (20, 21), a major component of triglyceride-rich remnant lipoprotein and associated with hypertriglyceridemia (37). In addition, Rev-erbα −/− mice have elevated levels of *ApoC-III* and very low density lipoprotein triglycerides. Moreover, Rev-erbα regulates ApoA1 gene expression in rodents treated with the hypolipidemic fibrate drugs. Finally, Rev-erbα is preferentially expressed in fast twitch/type IIB glycolytic, and intermediate type IIA oxidative fibers and null mutations lead to a transition to type I fibers (38). This suggests that this orphan NR subgroup regulates muscle fiber type, which can influence insulin sensitivity and the utilization of aerobic/anaerobic metabolism for the generation of ATP.

Specifically, we demonstrated that attenuation of Rev-erbβ-mediated gene expression suppresses the expression of a subgroup of genes that include *Fabp-3* and -4, *Cd36*, *Ucp-3*, and *Scd-1* that are involved in lipid absorption and utilization. In this context, it is interesting to note that mice with a targeted disruption of SCD-1, a key target for Rev-erbβ, had lower levels of very low density lipoprotein, impaired triglyceride and cholesterol ester biosynthesis, and a lean phenotype (39). The *Scd-1* −/− phenotype is very similar to the natural mutation called *staggerer* ($ROR\alpha^{sg/sg}$) in an obese mouse strain, which leads to a functional knockout of RORα. The *staggerer* mice exhibit an aberrant blood-lipid profile with lower circulating plasma levels of HDL-C, apoC-III, and plasma triglycerides. It

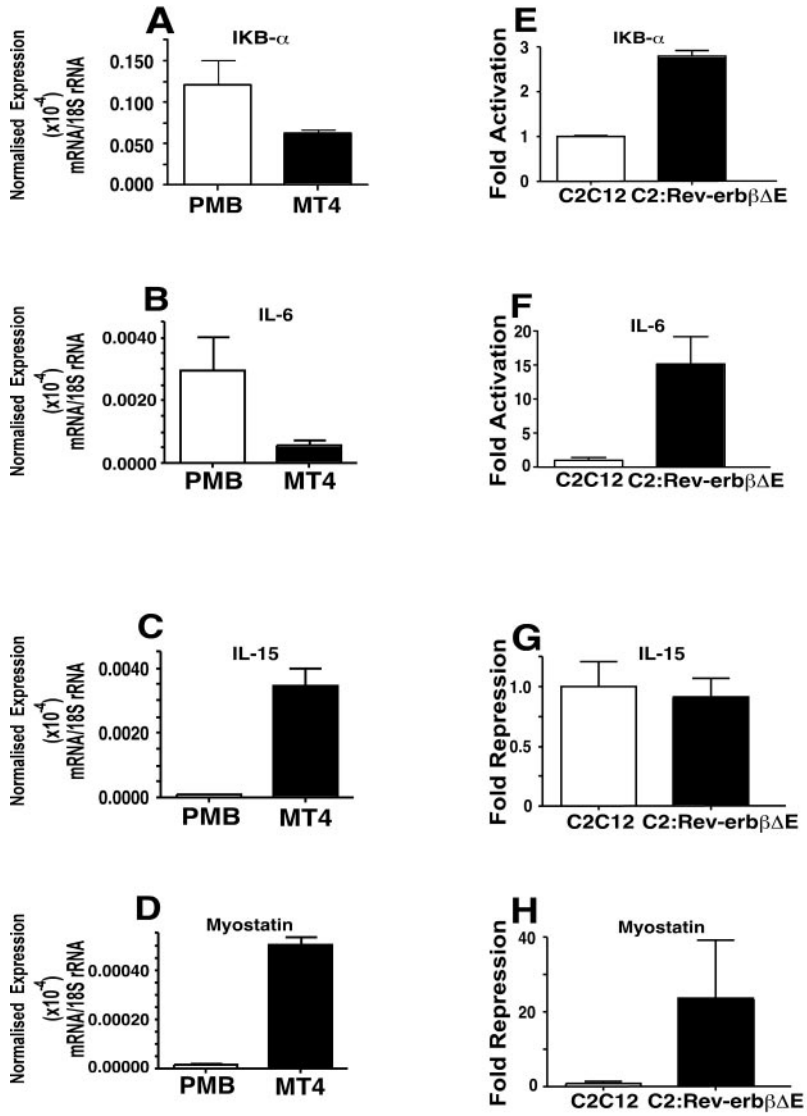

FIG. 6. **Rev-erbβ regulates myokine expression.** Total RNA was isolated from wild type C2C12 proliferating myoblasts (*PMB*) and post-mitotic multinucleated myotubes after 4 days of serum withdrawal (*MT4*), *A–D*, or wild type (C2C12) and dominant negative (C2:Rev-erbβΔE) cell lines from myotubes after 4 days of serum withdrawal (*E–H*). RNA was reverse transcribed to cDNA and analyzed by Q-RT-PCR. Gene-specific primers were used to examine the expression of: *A* and *E*, IκBα; *B* and *F*, IL-6; *C* and *G*, IL-15; and *D* and *H*, *myostatin*. Relative expression is shown as -fold repression and -fold activation calculated relative to the expression in the wild type C2C12 cell line that was arbitrarily set at 1. All reactions were performed with at least triplicates and results are shown as average ± S.D.

TABLE IV
Relative changes in the expression of the nuclear hormone receptors in skeletal muscle cells constitutively expressing Rev-erbβΔE

Gene	-Fold change
Rev-erbβ	↑ 1.5
Rev-erbα	↑ 1.2
RORα	↑ **5.8**
RORγ	↑ 1.0
ERRα	↑ 1.0
PPAR-α	↑ 1.1
PPAR-β/δ	↑ 1.0
PPAR-γ	↓ 1.4
LXRα	↑ 1.2
LXRβ	↓ 1.0
Nur77	↑ 1.8
NOR-1	↑ 1.0

should be noted that a dominant negative RORα represses both *Rev-erbα* and -β mRNA expression in skeletal muscle (16). Moreover, lack of *Fabp4*, a key target for Rev-erbβ in our model, protected the mice deficient in ApoE against atherosclerosis (40).

Interestingly, we observed a suppression in the expression of genes involved in lipid absorption, and in *Scd-1* that is involved in the formation of cholesterol esters. Moreover the activity of *Scd-1* has also been linked to adiposity. Curiously, we observed an increase in *Srebp-1c* expression, the master regulator of fatty acid metabolism. This apparent contradiction is in concordance with several reports in the literature. For example, Raspe *et al.* (15), showed that *staggerer* mice have reduced plasma triglycerides, and Lau *et al.* (16) demonstrated that a dominant negative RORα expression in muscle reduced *Srebp-1c* mRNA expression. The increase in SREBP-1c expression correlates with the >5-fold increase in RORα mRNA expression in the C2:Rev-erbβΔE cell line. Second, in most tissues SREBP-1c and SCD-1 mRNA are coordinately expressed and regulated. However, it has been previously reported in skeletal muscle cells, and tissue treated with LXR agonists, that *Srebp-1c* and *Scd-1* mRNA expression are uncoupled, and not co-ordinately regulated (23).

Our studies also demonstrate that Rev-erbβ has a crucial role in regulating UCP3. Abundant expression of this gene correlates with preferential lipid utilization, and increased energy expenditure. The role of uncoupling proteins in regulating energy balance have been extensively investigated through transgenic mice and cell culture studies (41–43). Repression of UCP3 mRNA also correlates with the decreased expression of genes involved in lipid absorption and utilization, in concordance with the observations derived from this study.

Recent studies (22, 44, 45) have demonstrated a role of RORα in the inflammation process. For example, RORα has an anti-

inflammatory role by inhibiting tumor necrosis factor-α-induced gene expression (22). The molecular basis of this modulation involves the direct induction of IκBα transcription, thereby inhibiting the NFκB signaling cascade. Conversely, Rev-erbα induces the NFκB-mediated activation of the inflammatory cascade, for example, *Cox-2* (44). Our studies show that attenuation of Rev-erbβ-mediated gene regulation leads to elevated RORα and IκBα expression in skeletal muscle cells. This correlates with no change in expression of the NFκB/tumor necrosis factor target genes, *Cox-2* and *IL-15*.

Surprisingly, these studies have revealed a dramatic induction (~15-fold) of interleukin-6, in the presence of increased IκBα expression. However, IL-6 is an exercised induced myokine that induces lipolysis in adipose tissue, and suppresses tumor necrosis factor production. The increased expression of IL-6 is consistent with decreased *myostatin* expression. For example, it has been reported that IL-6 deficiency leads to late-onset obesity (46), whereas myostatin deficiency leads to the reduced accumulation of body fat (47). Therefore, the inverse correlation between IL-6 and *myostatin* expression in our Rev-erbβ cell line is in concordance with the phenotypic effects of these myokines on metabolism (48–50). The study has implicated Rev-erbβ in the regulatory cascade controlling genes involved in lipid homeostasis. Furthermore, the significant impact on myokine expression that regulates inflammation, lipolysis, muscle growth, and the accumulation of body fat underscores the potential therapeutic utility of Rev-erb modulation by inverse agonists/antagonists. However, identification of small molecule regulators will be hindered by the lack of a significant pocket in the ligand binding domain in the Rev-erb/NR1D subgroup of orphan nuclear receptors (51).

It is becoming increasingly apparent that skeletal muscle is a critical target tissue in the fight against metabolic disorders associated with diet, lifestyle, and metabolism. For example, LXR, PPARα, -β/δ, and -γ in skeletal muscle have been shown to be involved in enhancing the insulin-stimulated glucose disposal rate, decreasing triglycerides, increasing energy expenditure, and increasing lipid catabolism, cholesterol efflux, and plasma HDL-C levels (14, 23–28).

Activation of these NRs in muscle has lead to increased insulin sensitivity, resistance to diet-induced obesity, and atherosclerosis. Hence, orphan nuclear receptors (for example, Rev-erbβ) that regulate lipid homeostasis and inflammation in skeletal muscle have enormous homeostatic ramifications. As discussed, skeletal muscle is a major mass peripheral tissue, and this lean tissue accounts for ~40% of the total body mass (36% for females, and 42% for males) and 30–50% of the energy expenditure. These results indicate significant homeostatic interrelationships between the muscular and other body systems including the cardiovascular, endocrine, and lymphatic networks for the treatment of dyslipidemia, syndrome X, and inflammation. In conclusion, we suggest that in skeletal muscle cells, Rev-erbβ programs a cascade of gene expression that controls the regulatory cross-talk between lipid metabolism and inflammation.

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Rev-erbβ Regulates the Expression of Genes Involved in Lipid Absorption in Skeletal Muscle Cells: EVIDENCE FOR CROSS-TALK BETWEEN ORPHAN NUCLEAR RECEPTORS AND MYOKINES

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