Retinoid-related orphan receptor γ regulates several genes that control metabolism in skeletal muscle cells: links to modulation of reactive oxygen species production

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Abstract

Retinoid-related orphan receptor γ (RORγ) is an orphan nuclear hormone receptor (NR) that is preferentially expressed in skeletal muscle and several other tissues, including pancreas, thymus, prostate, liver and testis. Surprisingly, the specific role of RORγ in skeletal muscle, a peripheral tissue, has not been examined. Muscle is one of the most energy demanding tissues which accounts for ~40% of the total body mass and energy expenditure, >75% of glucose disposal and relies heavily on β-oxidation of fatty acids. We hypothesize that RORγ regulates metabolism in this major mass lean tissue. This hypothesis was examined by gain and loss of function studies in an in vitro mouse skeletal muscle cell culture model. We show that RORγ mRNA and protein are dramatically induced during skeletal muscle cell differentiation. We utilize stable ectopic over-expression of VP16-RORγ (gain of function), native RORγ and RORγΔH12 (loss of function) vectors to modulate RORγ mRNA expression and function. Ectopic VP16 (herpes simplex virus transcriptional activator)-RORγ and native RORγ expression increases RORγ mRNA expression. Candidate-driven expression profiling of lines that ectopically express the native and variant forms of RORγ suggested that this orphan NR has a function in regulating the expression of genes that control lipid homeostasis (fatty acid-binding protein 4, CD36 (fatty acid translocase), lipoprotein lipase and uncoupling protein 3), carbohydrate metabolism (GLUT5 (fructose transporter), adiponectin receptor 2 and interleukin 15 (IL-15)) and muscle mass (including myostatin and IL-15). Surprisingly, the investigation revealed a function for RORγ in the pathway that regulates production of reactive oxygen species.

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Introduction

Nuclear hormone receptors (NRs) have been demonstrated to regulate metabolism in an organ-specific manner. The importance of NRs in the context of promoting and maintaining human health is underscored by the therapeutic utility of drugs that target dysfunctional hormone signalling in the context of human disease. NRs function as ligand-dependent DNA-binding proteins which translate physiological/nutritional/metabolic signals into gene regulation. The NR superfamily includes the ‘orphan NRs’, which have no recognized ligands in the ‘orthodox sense’.

The orphan nuclear receptor NR1F subgroup, retinoic acid receptor-related orphan receptor (ROR/RZR) includes three ROR/RZR genes; RORz encodes four RORz isoforms and is predominantly expressed in blood, brain, skeletal muscle and fat cells (Becker-Andre et al. 1993, Giguere et al. 1994). RORβ/RZRβ is expressed specifically in the brain (Carlberg et al. 1994), and RORγ encodes two isoforms RORγ1 and RORγt. RORγ1 is preferentially expressed in skeletal muscle and several other tissues, including pancreas, thymus, prostate, liver and testis of human (Hirose et al. 1994, Ortiz et al. 1995). RORγt isoform lacks 20 amino acids at amino terminus and is restricted to thymocytes (He et al. 1998).

The NR1F subgroup is closely related to the NR1D subgroup, including Rev-erbα, Rev-erbβ and the Drosophila orphan receptor, E75A, particularly in the DNA-binding domain and the putative ligand-binding domain. ROR, Rev-erbα and RVR bind as monomers to an asymmetric (A/T) 6 RGGTCA motif. ROR functions as a constitutive transactivator of gene expression, whereas Rev-erbα and RVR do not activate transcription, rather they mediate transcriptional repression, and can repress RORz-mediated transactivation from this motif (Harding & Lazar 1993, Bonnelye et al. 1994, Dumas et al. 1994, Forman et al. 1994, Giguere et al. 1994, Retnakaran et al. 1994, Adelmant et al. 1996).

In vivo and in vitro (cell culture) genetic studies have implicated RORz in the regulation of lipid homeostasis. First, NR1F1 (RORz)-deficient mice have a dyslipidaemic phenotype, hypo-α-lipoproteinaemia, muscular atrophy and heightened inflammatory responses which lead to
atherosclerosis (Vu-Dac et al. 1997, Mamontova et al. 1998, Raspe et al. 1999, Jetten & Ueda 2002, Jetten 2004). Secondly, ectopic over-expression of RORαΔDE in a muscle cell culture model (Lau et al. 2004) leads to the modulation of genes involved in lipid absorption and β-oxidation. RORα (NR1F1) and Rev-erbα (NR1D1) opposingly regulate the expression of apolipoprotein CIII, a component of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) that regulates triglyceride (TG) levels and lipoprotein lipase (LPL) activity (Vu-Dac et al. 1997, He et al. 1998, Mamontova et al. 1998, Raspe et al. 1999).

Characterization of RORγ−/− mice, identified several important immunological functions for RORγ, in the control of lymph node and Peyer’s patch development, thymopoiesis and T cell homeostasis. RORγ−/− mice display increased apoptosis in the cortex of the thymus, and RORγ−/+ thymocytes placed in culture rapidly undergo apoptosis (Kurebayashi et al. 2000).

RORγ is highly expressed in skeletal muscle (Hirose et al. 1994). Muscle accounts for ~40% of the total body mass and energy expenditure, and > 75% of glucose disposal (Baron et al. 1988). This lean tissue relies heavily on β-oxidation of fatty acids to supply the extreme energy demands of this organ. However, the fundamental role of RORγ in skeletal muscle lipid and energy homeostasis has not been addressed. We hypothesize that RORγ regulates metabolism in this major lean tissue. This hypothesis was tested by ectopic stable over-expression of RORγ ‘gain and loss’ of function vectors in an in vitro mouse skeletal muscle cell culture model. These studies demonstrated that RORγ has a role in controlling the expression of genes that regulate muscle and fat mass (including myostatin and interleukin 15 (IL-15)), and lipid homeostasis (fatty acid-binding protein 4 (FABP4), CD36 and LPL). Unexpectedly, the study demonstrated a role for RORγ in the regulation of reactive oxygen species (ROS) production.

Materials and methods

Cell culture

Mouse myogenic C2C12 cells were cultured in growth medium called Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated Serum Supreme (Cambrex Bio Science, Mt. Waverly, Victoria, Australia) in 6% CO₂. Confluent myoblasts were differentiated into post-mitotic multinucleated myotubes by 5 days (MT5) of serum withdrawal (i.e. cultured in DMEM supplemented with 2% horse serum). Cells were harvested at the indicated time points, usually 24–120 h (1–5 days) after mitogen withdrawal, unless indicated differently. African green monkey kidney COS-1 cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum.

RNA extraction and cDNA synthesis

Total RNA was extracted from C2C12 cells using TRI-Reagent (Sigma–Aldrich) according to manufacturer’s protocol. Total RNA was then treated with 2 U Turbo DNase 1 (Ambion, Austin, TX, USA) at 37 °C for 30 min followed by purification of the RNA through an RNeasy purification column system (Qiagen). RNA was electrophoresed to determine the integrity of the preparation. SuperScript III was used to synthesize cDNA from 3 μg total RNA using random hexamers according to 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 and 5 mM EDTA) containing protease ‘cocktail’ inhibitors (Rosch). Lysates were passed through a 26-gauge needle and centrifuged at 10 000 g for 20 min. The supernatant was collected and total protein concentration was determined by the bicinchoninic acid (BCA) as outlined by manufacturer’s instructions (Pierce Biotechnology Inc., Rockford, IL, USA).

Transient transfections

Each well of a 24-well plate of COS-1 cells (~60% confluence) was transfected with a total of ~0.6–1.0 μg of DNA per well using the liposome-mediated transfection procedure as described previously (Lau et al. 1999). Briefly, cells were transfected using an N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate 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 Na₂HPO₄ and 11 mM dextrose (pH 7.1)). The DNA/N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulphate/ metafectene mixture was added to the cells in 0.5–0.6 ml DMEM supplemented with 10% fetal calf serum. The culture medium was changed 16–24 h later and the cells were subsequently harvested for the assay of luciferase activity 48 h after the transfection period. Fold activation is expressed relative to activity obtained after co-transfection of the promoter–reporter and pSG5/pNL-VP16 vectors only, arbitrarily set at 1. The mean fold activation values and S.D. were derived from a minimum of three independent triplicate experiments.
C2C12 stable transfection

Myogenic C2C12 cells, cultured in growth medium, were co-transfected with pSG5-RORγΔH12, pSG5-RORγ and pNL-VP16-RORγ (and pCMVNEO at a 20:1 ratio of expression vector/NEO vector) by the liposome-mediated procedure in triplicates. The cells were then grown for another 24 h to allow cell recovery and neomycin resistance expression before G418 selection. After 10- to 14-day selection with 700 μg/ml G418 (Promega) in culture medium, the three independent polyclonal pools of stable transfectants were cultured and maintained on 300 μg/ml G418 medium.

Primers

Primers used for qPCR analysis of the mRNA populations have been described in detail (Lau et al. 2004), with the exception of primers designed for the detection of endogenous RORγ using SYBR green (endo RORγ forward, CCGGCCACTCTATAAGGAACTCT and endo RORγ reverse, AGGGCTGAAGGA AATAGAAAGTTGT).

Plasmids

pSG5-RORγ1, mPCP-2x4-tk-LUC(RORγRE) containing four copies of mouse PCP-2 (GGTATAGTAC-TGGTGAGGGGACT) and pSG5 have been described previously (Lau et al. 2004). Mouse RORγ1 cDNAs were amplified from C2C12 total RNA using Pfu Turbo (Stratagene) as per manufacturer’s instructions and subsequently cloned into pBluescript. After DNA sequencing, this insert was cloned into eukaryotic expression vectors pSG5 and pNL-VP16. Subsequently, a truncated version of RORγ was amplified (coding 1–494 aa) and cloned into pSG5 vectors and used as dominant negative version of RORγ vectors pSG5 and pNL-VP16. Subsequently, a truncated insert was cloned into eukaryotic expression vectors pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript. After DNA sequencing, this insert was cloned into pBluescript.

Quantitative real-time PCR (qPCR)

qPCR was performed on an ABI Prism 7500 sequence (detection system (Applied Biosystems, Foster city, CA, USA) in triplicate on three independent RNA preparations. Target cDNA levels were analysed in 25 μl reactions with either Syber Green or Taqman Technologies (Applied Biosystems). Primers (200 nM) used for the amplification of target gene sequences have been described in detail (Lau et al. 2004, Ramakrishnan et al. 2005). PCR was performed with 5 μl cDNA and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative level of expression or fold change and associated errors were calculated using the guidelines described by Bookout & Mangelsdorf (2003) on the Nuclear Receptor Signaling Atlas website (NURSA; www.nursa.org/index.cfm) in accord with the accepted qPCR standards for the National Institutes of Health supported NURSA research.

Western blot analysis

Total soluble protein from the wild-type C2C12 myotubes stable pSG5-RORγΔH12, pSG5-RORγ and pNL-VP16-RORγ C2C12 myotubes were resolved on a 10% SDS–PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked overnight or for 1 h in 5% skimmed milk in TBS–Tween 20 followed by an overnight incubation with either RORγ (1:5000, Santa Cruz-28559; Santa Cruz Biotechnology Inc., Santa Cruz, CA, 95060, USA) or RORα (1:2000, Santa Cruz 28612), and GAPDH (1:10 000; R&D Systems, Minneapolis, MN, USA) antibodies. Following 4×15-min washes, the membrane was incubated with anti-rabbit horseradish peroxidase (HRP) (1:2000) for 1 h. Immunoreactive signals were detected using enhanced chemiluminescence Super Signal West Pico Substrate (Pierce) and visualized by autoradiography on an XOMAT film developer (Kodak).

Measurement of ROS

Ten-thousand stable C2C12 cells/well were seeded in a 96-well view plate (Perkin–Elmer) and grown in normal cultured medium (DMEM) with 10% serum. Confluent myoblasts were differentiated into post-mitotic multinucleated myotubes by 5 days of serum withdrawal. Myotubes were loaded with 15 μM H2DCFDA (ROS probe, Invitrogen) in phenol red-free media for 30 min at 37 °C and fluorescence was measured. To induce ROS production, after loading ROS probe, we treated the cells with 50 μM H2O2 for about 30 min. The results obtained are from three independent polyclonal pools, assayed in triplicate.

Statistical analysis

Statistical analysis was performed on the average of three independent assays using Student’s t-test or one-way ANOVA, followed by either Tukey’s or Dunnett’s multiple comparison test.
Results

The RORγ mRNA and protein are expressed in a differentiation-dependent manner during skeletal muscle differentiation

We initially investigated the expression of RORγ mRNA (and protein) relative to GAPDH in the mouse C2C12 in vitro skeletal muscle cell culture model. Proliferating myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by mitogen withdrawal over several days. This transition from a non-muscle to a contractile phenotype is associated with the activation and repression of a structurally diverse group of genes that are responsible for the contractile and energy demands on this tissue. We utilized qPCR and western blot analysis to show that RORγ mRNA and protein expression (relative to GAPDH) increased during skeletal muscle cell differentiation (Fig. 1A).

The differentiation-dependent RORγ (mRNA and protein) expression was concomitant with the expression of several (muscle specific) contractile and metabolic markers. For example, qPCR demonstrated concomitant induction of myogenin mRNA (a gene that encodes the hierarchical basic helix loop regulator), slow (type I) and fast (type II) isoforms of the contractile protein troponin I (Fig. 1B–D). Similarly, differentiation-dependent expression of the genes involved in metabolism (and regulation) of muscle mass was observed. For instance, fatty acid translocase (CD36), FABP4, myostatin and uncoupling protein 3 (UCP3; Fig. 1E–H).

VP16-RORγ and RORγΔH12 operate as gain and loss of function vectors

To investigate the metabolic role of RORγ in skeletal muscle cells by gain and loss of function strategies, we proceeded to design vectors (for ectopic expression) that enhanced and/or attenuated RORγ expression function. In order to construct the constitutively activated RORγ (gain of function) vector, we cloned the VP16 transactivation domain (411–456 aa) to the N-terminus of RORγ (Fig. 2A). We utilized VP16-activated receptor because it still remains unknown whether a natural agonist/ligand exists for the RORγ nuclear orphan receptor. Hypothetically, this fusion would produce a hyperactive receptor. This approach has been used to mimic ligand/agonist-activated nuclear receptor (Schreiber et al. 2004, Wang et al. 2004). In addition, to perturb RORγ function and to disrupt RORγ-mediated gene expression, we constructed the RORγΔH12 (dominant negative) expression vector. This construct encodes amino acids 1–494 and consequently lacks helix 12 (H12) of the ligand binding domain (LBD) that encodes the AF2 transactivation domain (Fig. 2A). The AF2 domain contains the LYKELF aromatic amino acid residues and is conserved in all members of the ROR family. These aromatic amino acid residues are a crucial component for recruiting co-factors containing LXXLL motifs (Xie et al. 2005). To measure the RORγ-mediated transactivation on reporter gene, we constructed the heterologous reporter vector containing seven copies of RORγE (GGTAAGTAGGTTCAT) in pTKLuc (Medvedev et al. 1996).

Initially, we demonstrated that the constitutively active RORγ vector (VP16-RORγ) significantly increased transactivation of the synthetic reporter in COS-1 cells relative to the native RORγ-mediated transactivation (Fig. 2B). Subsequently, we demonstrated that the loss of function vector suppressed native RORγ-mediated transactivation of a heterologous reporter in COS-1 cells (Fig. 2C). Further, the loss of function vector operated in a dominant negative manner and repressed the RORγ-mediated transactivation of the synthetic reporter in a dose-dependent manner (Fig. 2D).

Stable ectopic native RORγ, VP16-RORγ and RORγΔH12 expression modulates endogenous RORγ mRNA expression

Further, native RORγ, VP16-RORγ and pSG5-RORγΔH12 expression vectors were transfected into skeletal muscle cells, and polyclonal pools of cells (to avoid clonal bias) were isolated after 10–14 days of G418 selection. Subsequently, wild-type C2C12 cells, C2:RORγΔH12, native C2:RORγ and C2:VP16-RORγ cell lines were harvested after 5 days of serum withdrawal (n = 3). Initially, we measured total RORγ mRNA expression in these stably transfected cell lines relative to RORγ mRNA expression in wild-type C2C12 cells. The primers utilized measured total (i.e. both endogenous and ectopic) RORγ mRNA expression. This demonstrated that the stably transfected cell line expressed ~20-fold more RORγ mRNA. In contrast, western blot analysis indicated that RORγ protein levels did not correlate with the relative total mRNA levels (Fig. 3A). In this context, studies have demonstrated that differential expression of mRNAs encoding contractile proteins in cardiac muscle is not reflected in protein levels (dos Remedios et al. 1996, Coumans et al. 1997). It should be noted that the antibody utilized cannot discriminate between endogenous (native) and exogenous hybrid RORγ proteins, and therefore reflects total protein levels.

Furthermore, we noticed that although the levels of total RORγ mRNA expression were similar (and not significantly different) in the three stable cell lines, endogenous levels of RORγ transcripts were significantly reduced in the cell line expressing the dominant negative form of RORγ. Correspondingly, the lines that express native RORγ and constitutively active RORγ, displayed increased levels of endogenous RORγ transcripts (Fig. 3B, albeit less than twofold).
Figure 1 Expression of RORγ mRNA and protein during skeletal muscle differentiation in C2C12 myoblasts and differentiated myotubes. Total RNA and soluble protein from proliferating myoblasts (PMBs), confluent myoblasts (CMBs) and MT1–MT5 (days 1–5) of differentiation were analysed by qPCR and western blot analysis. (A) qPCR analysis of RORγ mRNA and western blot analysis of RORγ protein, GAPDH was used as a loading control in western blot. (B–D) Expression of markers indicative of the acquisition of the muscle specific (myogenin), contractile (TNNI1 and TNNI2) and (E–H) some metabolic genes CD36, FABP4, myostatin and UCP3 during differentiation. Data are expressed as the mean of triplicate samples ± S.D.
In conclusion, we have produced stable cell lines that ectopically over-express dominant negative RORγ native RORγ and constitutively active RORγ.

**Stable ectopic VP16-RORγ expression modulates myogenin mRNA expression**

The cell lines that ectopically express the dominant negative, native and activated forms of RORγ cell lines were differentiated and retained the potential to morphologically differentiate. In order to confirm that the changes observed in these cell lines were not due to aberrant differentiation, we measured the levels of myogenic markers in RNA from differentiated cells. Comparable expression of the mRNAs encoding the sarcomeric slow troponin I (TNNI1) and fast troponin I (TNNI2) contractile proteins was observed in stable and wild-type myotubes (Fig. 4A and B). Similar levels of myogenin transcripts were noticed in the lines that over-express the dominant negative and native forms of RORγ. Curiously, the line that over-expressed constitutively active RORγ showed significantly increased levels of myogenin mRNA expression (Fig. 4C). However, as observed above the increased levels of myogenin mRNA did not impact on contractile protein mRNA expression.

Further, we tested the potential of ROR (α and γ) to transactivate the myogenin promoter. We transiently transfected COS-1 cells with the reporter plasmid containing −1565 bp of the myogenin promoter and examined the effect of co-transfected (exogenous)
RORγ and RORα expression. Interestingly, the myogenin promoter was significantly transactivated by RORγ (not RORα) (Fig. 4D).

Ectopic VP16-RORγ expression induces RORα and Rev-erbα mRNA expression

We subsequently profiled the expression of several NRs involved in metabolism (Table 1). This was performed to investigate whether ectopic over-expression of dominant negative, native and activated RORγ vectors altered the expression of other NRs.

Interestingly, the cell lines that ectopically expressed native and constitutively active RORγ were characterized by significantly increased expression of RORα transcripts (Fig. 5A). Furthermore, ectopic VP16-RORγ induced significantly greater levels of RORα, relative to native RORγ expression (Fig. 5A). Previous studies have demonstrated that RORα regulates Rev-erbα expression (Delerive et al. 2002). Moreover, VP16-RORγ expression resulted in a corresponding increase in Rev-erbα mRNA expression. This suggested a threshold of RORα mRNA expression may be required for the induction of Rev-erbα mRNA expression. Surprisingly, the dominant negative form of RORγ did not alter RORα or Rev-erbα expression.

The profiling of the other NRs involved in metabolism indicated that the effects of ectopic RORγ were relatively specific. For example, profiling of many NRs involved in the regulation of lipid homeostasis demonstrated the majority of relevant NRs were refractory to ectopic RORγ expression (Table 1). For example, the expression of liver x receptor (LXR)α and LXRβ, peroxisome proliferator activated receptor (PPAR)α, PPARδ and PPARγ was refractory to the effects of increased RORγ (and the corresponding changes in RORα and Rev-erbα).

Previous studies have demonstrated that RORα transactivates the Rev-erbα promoter (Delerive et al. 2002). Hence, we co-transfected the Rev-erbα promoter with RORα and RORγ. This demonstrated that unlike RORα, RORγ does not significantly transactivate the Rev-erbα promoter (Fig. 5C). Moreover, it suggests that the increased Rev-erbα mRNA expression in these cells (i.e. C2:VP16-RORγ) is a reflection of increased RORα (not RORγ) mRNA and protein expression.

Stable over-expression of dominant negative, native and activated RORγ vectors perturbs the expression of several genes in lipid and carbohydrate metabolism

We then examined each of the stable cell lines for changes in expression of critical genes involved in metabolism relative to wild-type C2C12 cells. Surprisingly, expression profiling analysis of the cell line that over-expressed the dominant negative form of RORγ (and resulted in suppression of endogenous RORγ mRNA expression)
did not significantly affect programmes of gene expression that control metabolism of lipids and carbohydrates (Table 2). Interestingly, expression profiling of the cell lines that exogenously expressed native RORγ and activated RORγ did reveal a functional (and distinct) role of RORγ in the regulation of metabolism.

Exogenous expression of the native RORγ and the activated RORγ leads to concordant effects on specific genes. For example, we observed significant activation of GLUT5 mRNA expression in the lines that exogenously expressed native RORγ and activated RORγ (Fig. 6A). GLUT5 mRNA encodes the ‘fructose-specific’ glucose transporter, and its expression is elevated in the muscle from diabetic patients (Stuart et al. 2007) and normalized after pioglitazone treatment.

Interestingly, exogenous expression of activated RORγ (but not the native RORγ) resulted in increased expression of the adiponectin receptor 2 (AdipoR2) mRNA (Fig. 6B). AdipoR2 is the receptor for adiponectin, an ‘adipokine’ that regulates glucose uptake and fatty acid oxidation in skeletal muscle (Yamauchi et al. 2003).

Subsequently, we observed significant activation of IL-15 mRNA expression in the lines that ectopically over-express native RORγ and activated RORγ cell lines (Fig. 6C). IL-15 is highly expressed in skeletal muscle and previous studies have demonstrated that IL-15

Figure 4 (A–C) qPCR analysis of mRNA expression of markers indicative of the acquisition of the muscle contractile (TNNI1 and TNNI2) and muscle specific (myogenin), during differentiation respectively in wild-type C2C12 myotubes and stably transfected dominant negative, native and activated RORγ C2C12 myotubes. Normalized mRNA expression is expressed as the mean ± S.E.M. of three (n=3) independent samples, each assayed in triplicate. (D) Promoter analysis of myogenin was performed by co-transfecting with ROR (α and γ) in non-skeletal muscle cell lines. COS-1 cells were co-transfected with 1.8 μg reporter (myogenin promoter), and 0.7 μg of each of pSG5, pSG5-RORγ and pSG5-RORα. pSG5 was added to normalize the final amount of DNA transfected to 3 μg in three wells of 24-well plate. Fold activation is expressed (mean ± S.E.M, and derived from three independent transfections (n=3); each transfection was composed of three replicates) relative to luciferase activity obtained after co-transfection of the reporter, and pSG5 vector only was arbitrarily set at 1. Significance was calculated using the one-way ANOVA and Tukey’s multiple comparison test where **P<0.001.
Table 1 Relative mRNA expression of selected nuclear receptors from wild-type C2C12, stably over expressed dominant negative (retinoid-related orphan receptor) RORγ; C2C12, native RORγ; C2C12 and activated RORγ; C2C12 cell lines

<table>
<thead>
<tr>
<th>NRs</th>
<th>Wild-type C2C12s</th>
<th>C2C12-RORγΔH12</th>
<th>C2C12-native RORγ</th>
<th>C2C12-VP16-RORγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LXRβ</td>
<td>3.67±0.06</td>
<td>2.12±0.08</td>
<td>1.7±0.3</td>
<td>1.5±0.07</td>
</tr>
<tr>
<td>LXRβ</td>
<td>6.7±0.5</td>
<td>1.7±0.3</td>
<td>1.5±0.05</td>
<td>1.19±0.56</td>
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<tr>
<td>PPARγ</td>
<td>0.44±0.47</td>
<td>0.49±0.07</td>
<td>1.0±0.05</td>
<td>0.23±0.25</td>
</tr>
<tr>
<td>PPARγ</td>
<td>3.3±0.08</td>
<td>4.5±2.8</td>
<td>1.3±0.02</td>
<td>5.9±2.7</td>
</tr>
<tr>
<td>NRs</td>
<td>8.0±0.03</td>
<td>18.7±1</td>
<td>2.3±0.00</td>
<td>17.8±0.7</td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>19.0±2.9</td>
<td>26.0±2.3</td>
<td>1.3±0.01</td>
<td>12.4±4.9</td>
</tr>
<tr>
<td>Rev-erbβ</td>
<td>30.6±1.4</td>
<td>34.7±2.1</td>
<td>1.1±0.00</td>
<td>33.6±0.99</td>
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<tr>
<td>RORγ</td>
<td>41.2±2.4</td>
<td>96.13±4.9</td>
<td>2.3±0.00</td>
<td>181.0±5.3</td>
</tr>
<tr>
<td>RORγ</td>
<td>1.81±0.19</td>
<td>2.8±0.16</td>
<td>1.5±0.05</td>
<td>2.7±0.19</td>
</tr>
<tr>
<td>Nur77</td>
<td>35.6±2.3</td>
<td>35.5±1.1</td>
<td>1.0±0.00</td>
<td>45.5±1.5</td>
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<tr>
<td>Nurr1</td>
<td>10.4±1.3</td>
<td>7.05±0.3</td>
<td>1.4±0.00</td>
<td>12.6±1.06</td>
</tr>
<tr>
<td>NOR1</td>
<td>0.469±0.053</td>
<td>0.514±0.042</td>
<td>1.1±0.00</td>
<td>0.55±0.071</td>
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<tr>
<td>COUP-TFI</td>
<td>9.4±1.3</td>
<td>15.5±2.8</td>
<td>1.6±0.05</td>
<td>23.3±2.8</td>
</tr>
<tr>
<td>COUP-TFI</td>
<td>68.2±8.7</td>
<td>74.6±3.5</td>
<td>1.0±0.00</td>
<td>98.5±7.2</td>
</tr>
</tbody>
</table>

*a: Relative expression expressed as a number of transcripts per GAPDH transcript (×10⁻⁶) ± s.d.

*b: Fold change and associated P value.

*c: Denotes significant up- or downregulation.

Increases glucose utilization (Busquets et al. 2006) and administration of IL-15 to rats and mice inhibits white adipose tissue deposition (Quinn et al. 2005). In summary, several genes involved in carbohydrate metabolism were modulated by ectopic expression of native and activated RORγ.

In addition, we observed significant increases in myostatin (and IL-15) mRNA expression in the lines that ectopically over-express native RORγ and activated RORγ cell lines (Fig. 6C and D). Myostatin is a negative regulator of muscle mass and positive regulator of adiposity (McPherron et al. 1997, Thomas et al. 2000). Moreover, IL-15 has anabolic effects on skeletal muscle protein synthesis both in vitro and in vivo (Quinn et al. 2005).

Furthermore, we observed that the expression of the mRNA encoding LPL (Fig. 6E) was significantly enhanced in the lines transfected with the native and constitutively active RORγ. LPL is involved in preferential lipid utilization and TG hydrolysis. Subsequently, to understand the molecular mechanisms involved in the significant increase in LPL transcripts in the RORγ over-expressing lines, we analyzed the LPL promoter. We transiently transfected COS-1 cells with reporter plasmid containing −1800 bp of LPL promoter and examined the reporter activity after co-transfection of exogenous RORγ and RORβ plasmids. Interestingly, LPL was significantly transactivated by RORγ (not RORβ; Fig. 6F). Thus, we speculate that LPL expression is modulated by RORγ expression in skeletal muscle cells.

The expression of the mRNAs encoding CD36 and FABP4 (involved in fatty acid and oxysterol absorption) was significantly attenuated in the stably over-expressing activated RORγ cell line relative to the wild type. Surprisingly, the over-expression of the dominant negative and native RORγ vectors resulted in increased CD36 and FABP4 mRNA expression (Fig. 7A and B).

In summary, analysis of the lines that over-express ectopic native RORγ and activated RORγ suggested that this orphan NR is involved in the regulation of lipid and carbohydrate homeostasis, and the control of muscle mass. However, the latter observations (Fig. 7A and B) present us with two apparent paradoxes. First, over-expression of the dominant negative and the native NR produced similar effects (for example, see 6A vs 7A) on specific genes. There are a number of possible explanations. As discussed previously, it still remains unknown whether a natural agonist/ligand exists for RORγ and this coupled to the observation that agonist-dependent NRs can function as repressors in the absence of ligand complicates the interpretation of native NR over-expression. The issue is further convoluted by several observations that underscore the permutations associated with NR-mediated regulation: i) loss of LXR in mice leads to derepression (increased expression) of ABCA1, a classical target gene, whereas another target gene, SREBP-1c remains silenced (Wagner et al. 2003); ii) agonist activation of PPARγ and PPARβ can lead to transcriptional repression (Lee et al. 2003, Ghisletti et al. 2007); and iii) orphan NRs (e.g. chicken ovalbumin upstream promoter transcriptional factor (COUP-TF)) have been reported to function as either activators and repressors of transcription, or accessory factors. These observations can be partially explained by differential co-repressor/coactivator recruitment and displacement in gene- and cell-specific manner.
RORγ gain and loss of function studies identify a role for RORγ in the regulation of UCP3 mRNA expression and ROS production

We further investigated the physiological role of RORγ in skeletal muscle cells by measuring steady-state ATP levels, the rate of fatty acid oxidation and ROS production. Surprisingly, we did not observe any significant change in fatty acid oxidation and total ATP content (data not shown) in the stably over-expressing dominant negative, native and activated RORγ cell lines relative to wild-type C2C12 cells.

Additional expression profiling revealed that the expression of the mRNA encoding UCP3 (Fig. 8A) was significantly increased in the cell line over-expressing VP16-RORγ. UCPs are inner mitochondrial membrane transporters that uncouple substrate oxidation from ATP synthesis, converting fuel to heat. However, many studies have suggested that UCP3 is involved in preferential lipid utilization and modulation of ROS production in skeletal muscle cells. UCP3 knockout mice and adenoviral over-expression in skeletal muscle cells have demonstrated that aberrant UCP3 expression effects ROS production (Vidal-Puig et al. 2000, MacLellan et al. 2005). We investigated whether VP16-RORγ mediated increases in UCP3 mRNA expression in skeletal muscle cells, altered ROS levels relative to the wild-type (and dominant negative) RORγ expression in C2C12 cells. Interestingly, the line that over-expressed ectopic-activated RORγ showed a significant decrease in endogenous and (hydrogen peroxide (H2O2)) induced ROS production relative to the dominant negative and wild-type cells (Fig. 8B).

Discussion

Genetic, molecular and biochemical studies have demonstrated that RORα has a distinct role in lipid metabolism. For example, RORα-deficient mice develop severe atherosclerosis, hypo-α-lipoproteinaemia, C2C12 myotubes. (B) qPCR analysis of Rev-erbα mRNA in wild-type C2C12 myotubes and stably transfected dominant negative, native and activated RORγ C2C12 myotubes. Normalized mRNA expression is expressed as the mean ± S.E.M. of three (n=3) independent samples, each assayed in triplicate. (C) Promoter analysis of Rev-erbα was performed by co-transfecting with ROR (α and γ) in non-skeletal muscle cell lines. COS-1 cells were co-transfected with 1.8 μg reporter (Rev-erbα promoter), 0.7 μg of each of pSG5, pSG5-RORγ and pSG5-RORα. pSG5 was added to normalize the final amount of DNA transfected to 3 μg in three wells of 24-well plate. Fold activation is expressed (mean ± S.E.M. and derived from three independent transfections (n=3); each transfection was composed of three replicates) relative to luciferase activity obtained after co-transfection of the reporter, and pSG5 vector only was arbitrarily set at 1. Significance was calculated using one-way ANOVA Tukey’s multiple comparison test where **P<0.001.

Figure 5 (A) qPCR analysis of total RORα mRNA and western blot analysis of RORα protein in wild-type C2C12 myotubes and stably transfected dominant negative, native and activated RORγ.

Although, RORγ is highly expressed in skeletal muscle (a major mass peripheral metabolic tissue), the functional role of this orphan NR in metabolism has not been explored. We utilized the C2C12 skeletal muscle cell culture model to investigate whether RORγ regulates genetic programmes implicated in metabolism. This in vitro model system is robust and data derived from

Table 2 Relative mRNA expression of genes involved in metabolism from wild-type C2C12, stably over expressed dominant negative (retinoid-related orphan receptor) RORγ; C2C12, native RORγ C2C12 and activated RORγ C2C12 cell lines

<table>
<thead>
<tr>
<th>Genes</th>
<th>C2C12-RORγΔH12</th>
<th>C2C12-native RORγ</th>
<th>C2C12-VP16-RORγ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transcrip levels</td>
<td>Fold change and</td>
<td>Transcrip levels</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>P value b</td>
<td></td>
</tr>
<tr>
<td>Energy expenditure and balance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCP2</td>
<td>458±42-20</td>
<td>1-0; P=0.418</td>
<td>643±4-38-0</td>
</tr>
<tr>
<td>UCP3</td>
<td>2-89±0-31</td>
<td>2; P=0.003</td>
<td>8-48±0-72</td>
</tr>
<tr>
<td>AMPKβ1</td>
<td>23-9±1-6</td>
<td>1-1; P=0.049</td>
<td>18-8±0-8</td>
</tr>
<tr>
<td>AMPKγ3</td>
<td>61-2±5-3</td>
<td>1-0; P=0.008</td>
<td>100-2±6-9</td>
</tr>
<tr>
<td>Fatty acid and lipid absorption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td>716±6-550</td>
<td>2-3; P=0.001</td>
<td>1740±3-90-0</td>
</tr>
<tr>
<td>FABP4</td>
<td>0-18±0-01</td>
<td>0-3±0-4</td>
<td>0-3±0-4</td>
</tr>
<tr>
<td>FABP3</td>
<td>12-06±0-6</td>
<td>1-3; P=0.040</td>
<td>12-9±0-6</td>
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<tr>
<td>Lipid catabolism</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACS4</td>
<td>69-5±3-3</td>
<td>1-95; P=0.002</td>
<td>140-0±12-0</td>
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<td>LPL</td>
<td>43-9±1-7</td>
<td>1-34; P=0.008</td>
<td>411-2±31-0</td>
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<tr>
<td>AdipoR1</td>
<td>529-6±19-5</td>
<td>1-3; P=0.050</td>
<td>688-2±22-8</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>2-1±0-9</td>
<td>1-4; P=0.159</td>
<td>3-7±0-37</td>
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<tr>
<td>CPT-1</td>
<td>2-4±0-14</td>
<td>1-08; P=0.035</td>
<td>2-5±0-25</td>
</tr>
<tr>
<td>PDK-2</td>
<td>48-4±1-5</td>
<td>1-73; P=0.002</td>
<td>83-45±5-1</td>
</tr>
<tr>
<td>PDK-4</td>
<td>10-4±1-1</td>
<td>1-4; P=0.023</td>
<td>17-3±2-1</td>
</tr>
<tr>
<td>PGC-1</td>
<td>4-3±0-26</td>
<td>1-16; P=0.026</td>
<td>3-8±0-23</td>
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<tr>
<td>MCAD</td>
<td>47-4±2-7</td>
<td>1-1; P=0.041</td>
<td>59-6±4-1</td>
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<td>Lipid efflux and homeostasis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ABCA1</td>
<td>15-9±2-4</td>
<td>5; P=0.068</td>
<td>16-7±1-5</td>
</tr>
<tr>
<td>ABCG1</td>
<td>2-98±0-24</td>
<td>5-1±0-5</td>
<td>3-5±0-21</td>
</tr>
<tr>
<td>ApoE</td>
<td>27-9±0-4</td>
<td>1-1; P=0.580</td>
<td>88-5±5-5</td>
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<tr>
<td>CAV-3</td>
<td>199-8±4-9</td>
<td>1-08; P=0.063</td>
<td>166-2±8-4</td>
</tr>
<tr>
<td>Glucose and lipid storage</td>
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<td></td>
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<tr>
<td>GYG1</td>
<td>29-02±0-9</td>
<td>2-24; P=0.001</td>
<td>58-8±4-9</td>
</tr>
<tr>
<td>ADRP</td>
<td>189-1±11-2</td>
<td>2-01; P=0.001</td>
<td>340-9±20-9</td>
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<td>Lipogenesis</td>
<td></td>
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<tr>
<td>FAS</td>
<td>133-0±9-9</td>
<td>228-4±20-4</td>
<td>153-0±10-4</td>
</tr>
<tr>
<td>SCD-1</td>
<td>3899-6±234-0</td>
<td>6194-3±293-0</td>
<td>5694-3±262-0</td>
</tr>
<tr>
<td>SCD-2</td>
<td>892-0±24-0</td>
<td>1723-0±65-0</td>
<td>1619±14-60</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>18-3±1-7</td>
<td>22-7±1-4</td>
<td>25-4±1-8</td>
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<tr>
<td>Myokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>11-41±1-2</td>
<td>15-99±1-2</td>
<td>1-4; P=0.145</td>
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<tr>
<td>Myostatin</td>
<td>0-38±0-2</td>
<td>1-1±0-2</td>
<td>2-8; P=0.026</td>
</tr>
<tr>
<td>Sugar uptake</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GLUT4</td>
<td>0-12±0-003</td>
<td>0-218±0-02</td>
<td>0-204±0-01</td>
</tr>
<tr>
<td>GLUT5</td>
<td>0-03±0-008</td>
<td>0-04±0-01</td>
<td>0-11±0-02</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>86-5±3-3</td>
<td>93-4±2-7</td>
<td>1-07; P=0.487</td>
</tr>
</tbody>
</table>

aRelative expression ascribed of transcription per GAPDH transcript (×10^-4) ± s.d.
bFold change and associated P value.
cDenotes significant up- or downregulation.

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the analysis of differentiated post-mitotic multinucleated myotubes with LXR and PPARδ agonists have been validated/reproduced in mice (Muscat et al. 2002, Dressel et al. 2003, Holst et al. 2003, Wang et al. 2004).

We probed RORγ function in skeletal muscle cells by stable ectopic expression of vectors that encode dominant negative, native and activated RORγ. The experiments indicated that the RORγ dominant negative vector in skeletal muscle cells significantly repressed the endogenous levels of the RORγ transcripts, and RORγ-dependent gene expression. However, we did not observe any significant change in gene expression involved in metabolism. This is consistent with the phenotype of RORγ-deficient mice that appear normal in the context of lipid and carbohydrate homeostasis.

Interestingly, we observed that ectopic overexpression of activated RORγ significantly increased the expression of RORα and Rev-erbα mRNA. These observations are consistent with the previous reports demonstrating that RORα directly transactivates the Rev-erbα promoter (Delerive et al. 2002).

In the context of carbohydrate metabolism, we observed the lines expressing native and activated GLUT5, AdipoR, IL-15, myostatin and LPL mRNA from wild-type C2C12 myotubes and stably transfected dominant negative, native and activated RORγ C2C12 myotubes. Normalized mRNA expression is expressed as the mean ± S.E.M. of three (n=3) independent samples, each assayed in triplicate. (F) Analysis of the LPL promoter was performed by co-transfecting with ROR (α and γ) in non-skeletal muscle cell lines. COS-1 cells were co-transfected with 1.8 μg reporter (LPL promoter), 0.7 μg of each of pSG5, pSG5-RORγ and pSG5-RORα. pSG5 was added to normalize the final amount of DNA transfected to 3 μg in three wells of 24-well plate. Fold activation is expressed (mean ± S.E.M. and derived from three independent transfections (n=3); each transfection was composed of three replicates) relative to luciferase activity obtained after co-transfection of the reporter, and pSG5 vector only was arbitrarily set at 1. Significance was calculated using one-way ANOVA and Tukey’s multiple comparison test where **P<0.001.
RORγ expressed significantly elevated levels of GLUT5 mRNA. This transcript is increased in skeletal muscle and repressed in adipose in the insulin-resistant diabetic state (Litherland et al. 2004) and normalized by pioglitazone therapy. Concordantly, we noted a significant increase in IL-15 mRNA expression that encodes a cytokine expressed in skeletal muscle which induces glucose uptake and oxidation (Busquets et al. 2006).

Exogenous expression of the VP16-RORγ in skeletal muscle cells resulted in the significant elevation of transcripts encoding UCP3, which correlated with attenuated production of ROS. UCPs are inner mitochondrial membrane transporters that uncouple substrate oxidation from ATP synthesis, converting fuel to heat. However, UCP3 has been implicated in the transport of fatty acid anions (Himms-Hagen & Harper 2001) and regulating the accumulation of ROS (Vidal-Puig et al. 2000). For example, reduced UCP3 expression has been linked to increased ROS production (Vidal-Puig et al. 2000). Moreover, increased expression minimizes ROS production (MacLellan et al. 2005). In this context, decreased UCP3 expression in skeletal muscle is associated with insulin resistance in diabetic individuals (Schrauwen et al. 2001, Patti et al. 2003, Houstis et al. 2006). Increased ROS production may critically damage cell membranes and reduce mitochondrial number. In the course of mitochondrial respiration, increased ROS production may lead to deleterious changes in transmembrane potential. Therefore, UCP3 may influence energy expenditure because of a secondary effect on the integrity of the mitochondria (Schrauwen & Hesselink 2002).

We also demonstrated that the expression of mRNA which encodes myostatin, a negative regulator of skeletal muscle mass and positive regulator of adiposity, is significantly increased in the stable lines which ectopically express native and activated RORγ (McPherron et al. 1997). In this context, we observe elevated levels of IL-15 that have been demonstrated to have anabolic effects in muscle, and decrease white adipose tissue mass in rodents (Quinn et al. 2005). In summary, it appears that RORγ modulates genes involved in the regulation of muscle and adipose mass.

Notably, over-expression of native RORγ and chimeric VP16-RORγ implicated the orphan NR in the regulation of lipid metabolism. For example, in the RORγ gain of function cell lines, we observed elevated LPL mRNA expression. LPL is a rate-limiting enzyme, which hydrolysates the TG-rich lipoproteins into fatty acids in skeletal muscle. Furthermore, we demonstrated that RORγ (but not RORα) transactivates the LPL promoter. In the context of lipid homeostasis, we observed significant repression of CD36 and FABP4 mRNAs (that encode proteins involved in fatty acid absorption) in the VP16-RORγ cell line. Whether RORγ regulates the LPL promoter by primary (direct) and/or secondary mechanisms remain unclear at present. Future studies utilizing ChIP, deletion and EMSA analysis will be used to address these questions.

These latter observations raise questions about the complex mechanisms that mediate the sometimes

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Figure 7 qPCR analysis of (A) CD36 mRNA and (B) FABP4 mRNA from wild-type C2C12 myotubes and stably transfected dominant negative, native and activated RORγ C2C12 myotubes. Normalized mRNA expression is expressed as the mean ± S.E.M. of three (n=3) independent samples, each assayed in triplicate. Significance was calculated using one-way ANOVA Tukey’s and Dunnett’s multiple comparison test where **P<0.001.
apparently disparate effect of ectopic native RORγ versus activated RORγ expression in skeletal muscle cells on a subset of target genes. One clear explanation is that VP16-RORγ (but not native RORγ) expression activates both RORα and Rev-erbα. These are opposingly acting NRs that respectively function as activators and repressors of transcription. This expression pattern of NRs may account for the contrasting effects of native and VP16-RORγ over-expression in skeletal muscle cells. In addition, differential effects of either NR gain or loss of function on specific targets are not unforeseen paradoxes. As discussed previously, it is not clear whether RORγ is modulated by natural compounds (Stehlin-Gaon et al. 2003), and this impacts on whether the NR can be additionally modulated. For example, oestrogen-related receptors can function independently of ligands, but retain the capacity to modulate by agonists and antagonists (Kamei et al. 2003, Rodriguez-Calvo et al. 2006). Furthermore, gain and loss of NR function can differentially effect target genes (LXR, PPARδ and COUP-TF) due to differential cofactor recruitment and displacement in diverse cells and tissues (Lee et al. 2003, Wagner et al. 2003).

In conclusion, we suggest that RORγ in the skeletal muscle cells controls several programmes of gene expression that have important roles in the control of muscle growth, lipid and carbohydrate metabolism. Moreover, we present evidence that this orphan NR may modulate mitochondrial function via the UCP3-mediated regulation of ROS production.

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