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Rev-erb beta regulates the *Srebp-1c* promoter and mRNA expression in skeletal muscle cells

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ABSTRACT

The nuclear hormone receptor, Rev-erb beta operates as a transcriptional silencer. We previously demonstrated that exogenous expression of Rev-erbβΔE in skeletal muscle cells increased *Srebp-1c* mRNA expression. We validated these *in vitro* observations by injection of an expression vector driving Rev-erbβΔE expression into mouse tibialis muscle that resulted in increased *Srebp-1c* mRNA expression. Paradoxically, Rev-erbβ siRNA expression in skeletal muscle cells repressed *Srebp-1c* expression, and indicated that Rev-erbβ expression was necessary for *Srebp-1c* expression. ChIP analysis demonstrated that Rev-erbβ was recruited to the *Srebp-1c* promoter. Moreover, Rev-erbβ trans-activated the *Srebp-1c* promoter, in contrast, Rev-erbβ efficiently repressed the Rev-erbα promoter, a previously characterized target gene. Finally, treatment with the Rev-erb agonist (hemin) (i) increased the trans-activation of the *Srebp-1c* promoter by Rev-erbβ; and (ii) increased Rev-erbβ and *Srebp-1c* mRNA expression. These data suggest that Rev-erbβ has the potential to activate gene expression, and is a positive regulator of *Srebp-1c*, a regulator of lipogenesis.

Introduction

Rev-erb beta (Rev-erbβ/RVR, NR1D2), is a member of the nuclear receptor superfamily [1–3]. Recent studies show that the Rev-erb subfamily modulates genes involved in lipid metabolism, circadian rhythm and inflammation [4]. Rev-erbβ is a transcriptional repressor and known target genes for this receptor include, *Rev-erbα*, *apoCIII*, *Afp*, and *N-Myc* [5–9]. Until recently, this receptor has been coined as an "orphan". However, recent reports show that "heme" binds to this receptor and can function as a physiological ligand [10,11].

Skeletal muscle, a major mass peripheral tissue, initially identified to be a locomotive organ, has also been shown to have a prominent role in various aspects of lipid metabolism [12]. Many nuclear receptors are expressed in skeletal muscle and play important roles in maintaining lipid homeostasis. Abnormal nuclear receptor signaling in skeletal muscle leads to many diseases [14]. *In vitro* studies performed in our laboratory show that Rev-erbβΔE modulates genes involved in lipid homeostasis and inflammation such as *Srebp-1c*, *Fabp4*, *Il-6*, and *Myostatin* [13,15].

Srebp-1c, a member of *s*terol-*r*egulatory *e*lement (SRE)-binding proteins (SREBPs), belongs to the basic helix-loop-helix leucine zipper family of transcription factors [16–21]. Recently, members of the nuclear receptor family have been identified to regulate *Srebp-1c* gene expression in muscle and non-muscle tissues [22–28].

We hypothesized that Rev-erbβ regulated *Srebp-1c* expression in muscle. We verified this hypothesis through several *in vitro* and *in vivo* approaches. We noted that Rev-erbβ is a direct regulator of *Srebp-1c* mRNA expression. We also identified that Rev-erbβ operated as a transcriptional activator. In summary, our studies suggest that Rev-erbβ is a novel regulator of *Srebp-1c* gene expression in skeletal muscle and propose a role for this receptor in lipogenic pathways.

Materials and methods

Cell culture, RNA extraction and cDNA synthesis, quantitative real-time PCR and luciferase assays have been described previously [15,26].

RNAi. Two 21-mer siRNA duplexes (targeting N-terminus and hinge regions of Rev-erbβ) specific to the annotated mouse cDNA

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sequence of Rev-erbβ (NM_011584) was generated with the siRNA target finder from Ambion. The target sequences, Rev-erbβ1–5′-GAACGCTGATATCTCTAGC-3′, and Rev-erbβ2–5′-AGGAGGA AGTGATTGGTAT-3′ were cloned into the pSilencer 2.1 neomycin vector.

Plasmid constructs. Mouse *Srebp-1c* promoter construct has been described previously [29]. Tk-Luc–*Srebp-1c* (−525/−401) construct spanning RERE2 site was PCR amplified from mouse tail DNA with Pfu Turbo polymerase (Stratagene) using the following primers—Forward: CCCCCTCCTTGAAACAAGTGT and Reverse: GCAGCAAG ATTTGCCTACAGTCT.

Chromatin immunoprecipitation (ChIP) assay: as described previously [30]. Samples were analyzed using quantitative RT-PCR using the following primers: RERE1—Forward: CTCAGATGTC AGAAGGAGCAGAGTAG and Reverse: GTTCTGTCCAGCCTGCAAGTG; RERE2—Forward: CCCCCTCCTTGAAACAAGTGT and Rev: GCAGCAA GATTTGCCTACAGTCT; GAPDH—Forward: GCTCACTGGCATGGCC TTCCG and Reverse: GTAGGCCATGAGGTCCACCAC; +2 kb region—Forward: GCAGGAATTCAGCCCAGAAA and Reverse: AGTGAGCCC CATTGCACCTA. All of the amplification data were first normalized to input (non-immunoprecipitated chromatin) and expressed as fold enrichment over those obtained with immunoprecipitations using a non-immune serum.

In vivo electrotransfer into mice. DNA was extracted, purified using endotoxin-free maxi-prep kits and re-suspended in sterile 0.9% saline. Anaesthesia was induced in mice using 5% halothane in oxygen and maintained with 1–2% halothane in oxygen with administration of 5 mg/kg ketoprofen to provide post-operative analgesia. The hind limbs of mice were prepared with a chlorhexidine–ethanol solution, and one tibialis cranialis muscle was injected in oblique fashion transcutaneously along their length with 30 μl of saline containing 0.5 mg/ml of each vector (pEGFP and pSG5-Rev-erbβΔE using a 29-gauge needle. The injection was immediately followed by the application of a pair of caliper electrodes across the injected leg connected to an ECM-830 electroporator device (BTX, Holliston, MA). Eight 20-ms pulses of 200 V/cm at a frequency of 1 Hz were administered as described previously [31]. The other tibialis cranialis muscle was injected with the appropriate control vectors (pEGFP and pSG5) and electroporated. One week after electrotransfer mice were killed, and the tibialis cranialis muscles were collected. All experimental procedures were approved by the Garvan Institute Animal Experimentation Ethics Committee and were in accordance with the NHMRC of Australia Guidelines on Animal Experimentation.

Results

Electroporation of Rev-erbβΔE into mouse hind limbs activates Srebp-1c mRNA expression

Studies from our laboratory in the C2C12 *in vitro* skeletal muscle cell culture system demonstrated that constitutive ectopic expression of Rev-erbβΔE induces expression of the mRNA encoding *Srebp-1c* [15]. We confirmed whether Rev-erbβ expression modulated *Srebp-1c* mRNA expression *in vivo*. We injected and

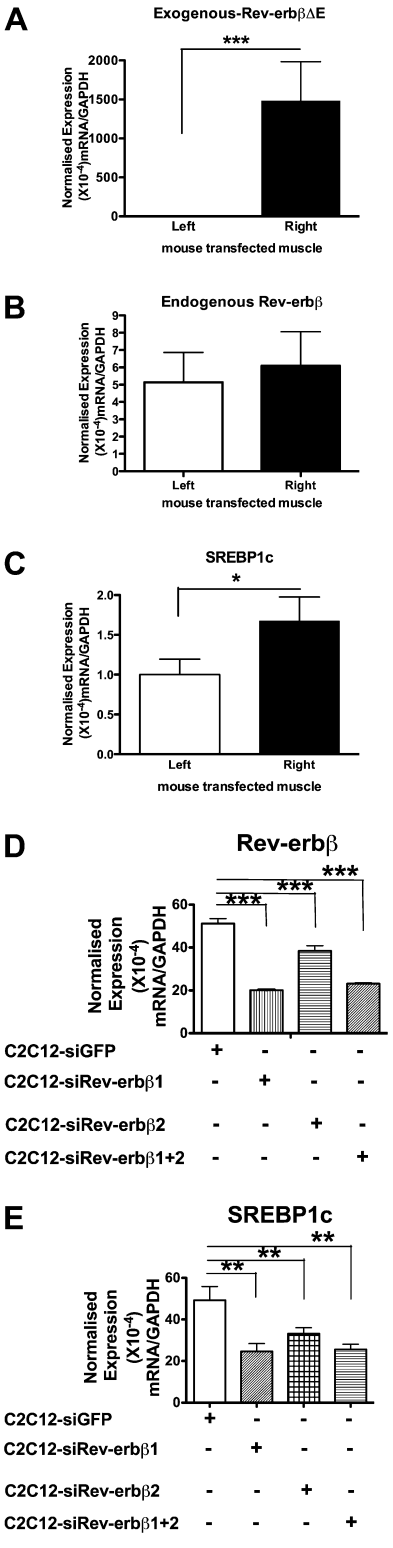

Fig. 1. *Srebp-1c* mRNA expression is regulated in mouse tibialis muscles transiently expressing Rev-erbβΔE transcript; and skeletal muscle cells expressing Rev-erbβ siRNAs. Mouse tibialis cranialis muscles were injected with a pSG5-Rev-erbβΔE expression vector or pSG5 control vector. Muscles were collected 1 week later and processed for RNA, and the level of (A) exogenous Rev-erbβΔE, (B) endogenous Rev-erbβ, and (C) *Srebp-1c*, mRNA expression was determined by quantitative RT-PCR. Values obtained from five animals were pooled, normalized, and expressed as the number of target transcripts per GAPDH transcript. Data represent mean ± SEM ($n = 5$). Statistical significance was measured using paired two-tailed Student's *t*-test where $P < 0.05$ (∗); $P < 0.0001$ (∗∗∗). (D) C2C12 cells were transfected with C2C12-siGFP, C2C12-siRev-erbβ1, C2C12-siRev-erbβ2, and C2C12-siRev-erbβMix (with both siRev-erbβ1 and siRev-erbβ2). After 48–72 h post-transfection, RNA was isolated for quantitative RT-PCR analysis of Rev-erbβ expression (relative to GAPDH control). Data represent mean ± SEM ($n = 3$). Statistical significance was measured using unpaired two-tailed Student's *t*-test where $P < 0.0001$ (∗∗∗). (E) C2C12 cells were individually transfected with C2C12-siGFP, C2C12-siRev-erbβ1, C2C12-siRev-erbβ2, and C2C12-siRev-erbβMix (with both siRev-erbβ1 and siRev-erbβ2). After 48–72 h post-transfection, RNA was isolated for quantitative RT-PCR analysis of *Srebp-1c* expression (relative to GAPDH control). Data represent mean ± SEM ($n = 3$). Statistical significance was measured using unpaired two-tailed Student's *t*-test where $P < 0.01$ (∗∗).

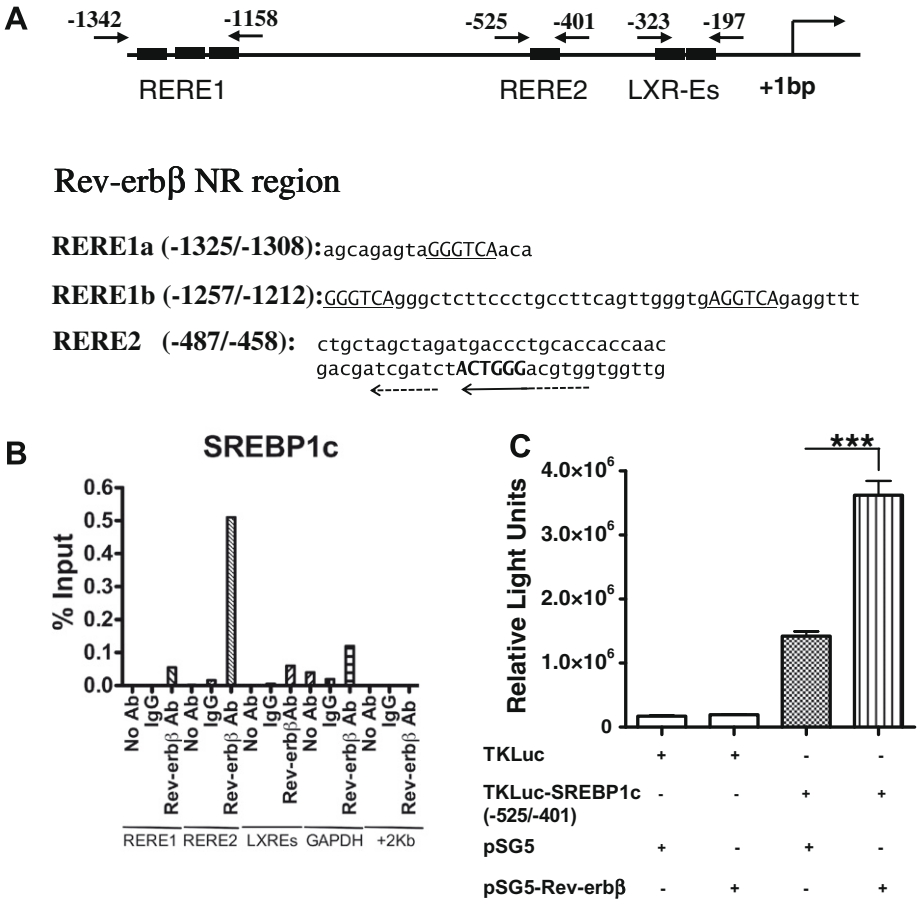

Fig. 2. Rev-erbβ is recruited to the *Srebp-1c* promoter. (A) Diagrammatic representation of two predicted Rev-erbβ sites on SREBP-1c promoter. RERE2 site is on the reverse strand of *Srebp-1c* promoter. (B) The recruitment of Rev-erbβ onto the *Srebp-1c* promoter in C2C12 skeletal muscle cells by ChIP assay (data represent mean of one representative assay, from three independent experiments). (C) Transfection of pTK-LUC–SREBP-1c (−525/−401) reporter with pSG5-Rev-erbβ or pSG5 vector in COS-1 cells. Data represent mean ± SEM ($n = 3$). Statistical significance was measured using unpaired two-tailed Student's t-test where $P < 0.0001$ (***).

electroporated the right hind limb of mouse tibialis muscle with the pSG5-Rev-erbβΔE expression vector. The left hind limb was injected with pSG5 control vector and electroporated using same procedure. Mice were sacrificed a week after electroporation and muscles were collected for RNA preparation and subsequently processed for RT-PCR analysis. Over-expression of pSG5-Rev-erbβΔE in these muscles produced abundant ectopic Rev-erbβΔE transcript compared to control vector (Fig. 1A). However, the endogenous Rev-erbβ expression remained unchanged (Fig. 1B). Interestingly, in agreement with our previous results from the *in vitro* cell culture model, we observed that Rev-erbβΔE expression induced *Srebp-1c* mRNA expression *in vivo* (Fig. 1C).

Rev-erbβ siRNA expression suppresses Srebp-1c mRNA expression

We hypothesized that Rev-erbβ regulated *Srebp-1c* gene expression. Secondly, that Rev-erbβ siRNAs would induce *Srebp-1c* expression, based on the observation that Rev-erbβ represses transcription, and that the E-region/LBD is required for repression. To test this hypothesis, we designed two siRNAs targeting N-terminus and hinge regions of Rev-erbβ, and cloned these into the pSilencer 2.1 plasmid vector. Expression of siRev-erbβ1, siRev-erbβ2 and a mixture of both siRNAs (siRev-erbβMix) significantly repressed endogenous Rev-erbβ mRNA expression 30–50%, relative to the GFP siRNA transfected control cells (Fig. 1D). Paradoxically, the expression of the mRNA encoding *Srebp-1c* was significantly decreased (Fig. 1E). These experiments suggested that Rev-erbβ expression was necessary for *Srebp-1c* expression.

Rev-erbβ is recruited to the Srebp-1c promoter

We subsequently examined whether Rev-erbβ is recruited to *Srebp-1c* promoter in skeletal muscle cells. Putative Rev-erbβ binding sites were identified using programs MatInspector and Consite. We identified two potential Rev-erbβ binding sites in the promoter [hereafter denoted as Rev-erbβ response elements (RERE1 and 2) located between nt positions −1257/−1212 and −487/−458, respectively]. Through ChIP analysis, we identified selective recruitment of Rev-erbβ to the RERE2 site (Fig. 2A and B) compared to IgG, GAPDH, and the no antibody controls. Interestingly, Rev-erbβ is not recruited to the RERE1 site which has been reported to interact with PPARα and RORα [24,27]. We cloned a single copy of the −525/−401 region spanning RERE2 into the basal TK-Luc promoter to study whether this fragment responds to Rev-erbβ. Interestingly, this region is activated by Rev-erbβ (Fig. 2C). These results identified a novel RERE in *Srebp-1c* promoter and demonstrated that Rev-erbβ is specifically recruited to this element.

Rev-erbβ activates the Srebp-1c and represses Rev-erbα promoter

The *in vivo* and *in vitro* expression experiments coupled to the ChIP experiments strongly suggested that Rev-erbβ expression regulates *Srebp-1c* expression, and the mechanism involves recruitment of Rev-erbβ to the *Srebp-1c* promoter. We subsequently examined whether Rev-erbβ modulated the *Srebp-1c* promoter. Initial experiments performed with *Srebp-1c* promoter showed that Rev-erbβ trans-activated this promoter (data not shown). This

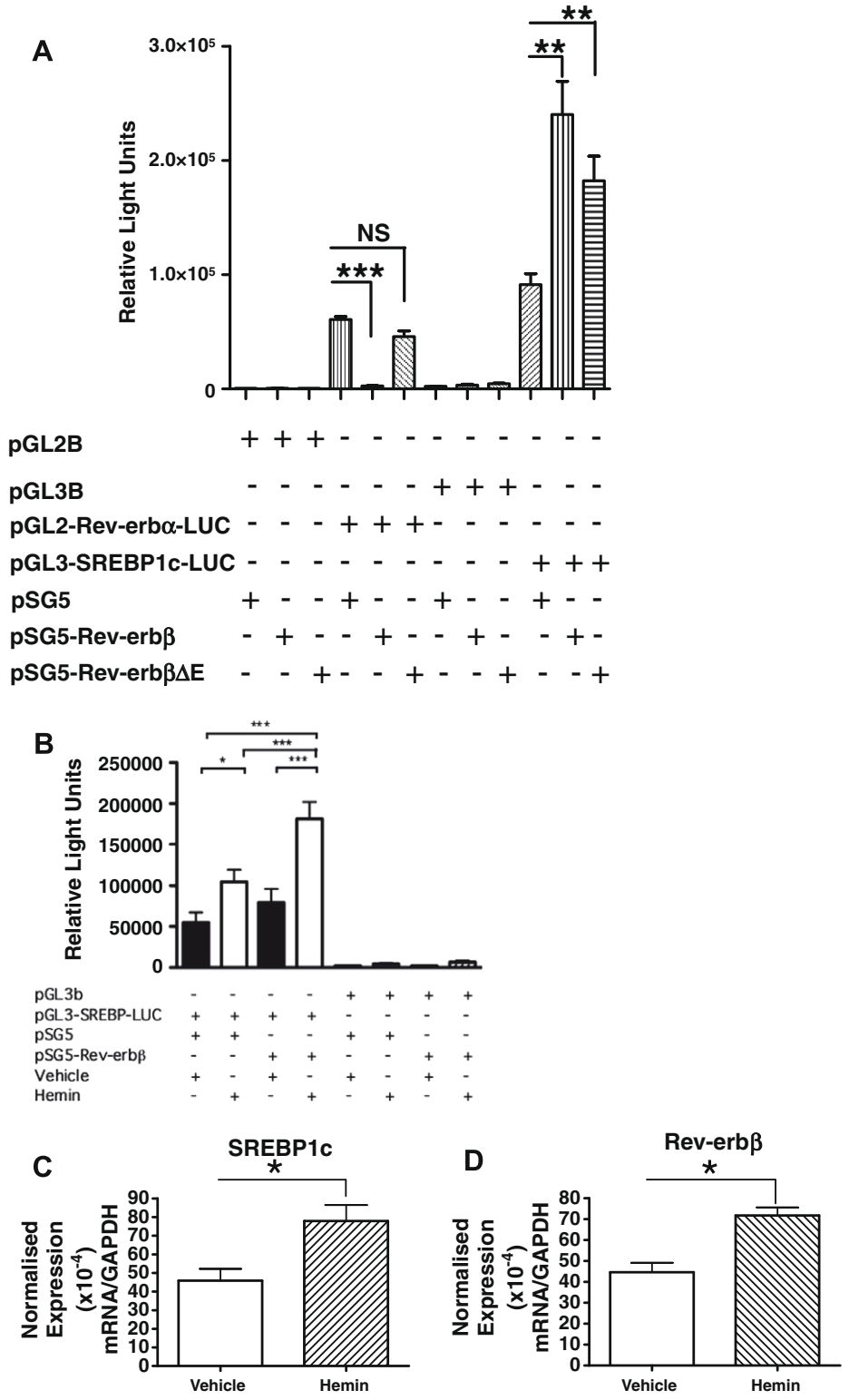

Fig. 3. Differential regulation of the Rev-erbα and *Srebp-1c* promoter by Rev-erbβ in skeletal muscle cells. (A) Each well of a 24-well plate of C2C12 cells (~50% confluence) was co-transfected with either 0.33 μg of pGL2/pGL3-basic, human Rev-erbα gene reporter and the mouse *Srebp-1c* gene reporter with either pSG5-Rev-erbβ, pSG5-Rev-erbβΔE or pSG5 (i.e. a total of ~0.66 μg of DNA per well) using the liposome-mediated transfection procedure. Data represent mean ± SEM ($n = 3$). Statistical significance was measured using unpaired two-tailed Student's *t*-test where $P < 0.01$ (**); $P < 0.0001$ (***). (B) C2C12 cells were co-transfected with 5.6 μg of the SREBP-pGL3 promoter, and 0.4 μg of pSG5-Rev-erbβ or pSG5 over 6 wells. Growth media was changed to 10% charcoal stripped media 24 h after transfection to contain 50 μM hemin or vehicle, and luciferase activities were determined in cell lysates 48 h after transfection. The data represent the mean fold change in luciferase activity ±SEM from three independent transfections each performed in triplicate. (C, D) C2C12 skeletal muscle cells were grown to confluence and differentiated for 3 days. The cells were then treated with vehicle (NaOH) or hemin (30 μM). After 24 h post-treatment, the cells were harvested and processed for RNA extraction and quantitative RT-PCR analysis of (C) SREBP-1c and (D) Rev-erb beta. Data represent mean ± SEM ($n = 3$). Statistical significance was measured using paired two-tailed Student's *t*-test where $P < 0.05$ (*).

observation appeared paradoxical, since studies performed over the past-decade showed that Rev-erbβ is a transcriptional silencer [2,5,9]. However, it is consistent with ectopic expression of the Rev-erb siRNA and expression vectors, *in vitro* and *in vivo*. Moreover, the literature has suggested that some nuclear receptors can perform dual functions as transcriptional activators and repressors [32]. Therefore, we simultaneously examined the ability of Rev-erbβ to modulate the activity of the hRev-erbα promoter (previously characterized to be repressed by Rev-erbβ [5]) and the *Srebp-1c* promoter.

Transfection experiments performed in skeletal muscle cells showed that both Rev-erbβ and Rev-erbβΔE trans-activate *Srebp-1c* promoter (Fig. 3A), and not the vector controls. In contrast, (as expected) Rev-erbβ (but not Rev-erbβΔE) significantly repressed the hRev-erbα promoter. These observations highlight that Rev-erbβ can specifically and selectively modulate gene expression in a gene specific manner.

Hemin treatment induces Srebp-1c promoter activity and mRNA expression in skeletal muscle cells

Hemin, a derivative of heme binds to the E-region/ligand binding domain of Rev-erbβ [10,11]. However, these studies did not report whether heme binding to Rev-erbβ modulates Rev-erbβ target gene expression. We tested the hypothesis of whether hemin treatment modulates *Srebp-1c* promoter activity and mRNA expression in skeletal muscle cells.

Transfection experiments performed in skeletal muscle cells showed that hemin treatment increases the activity of the *Srebp-1c* promoter in the presence and absence of Rev-erb (Fig. 3B), compared to the vector controls. Moreover, skeletal muscle cells were differentiated for three days and treated with hemin for 24 h. Quantitative RT-PCR analysis showed that hemin treatment increased endogenous *Srebp-1c* (Fig. 3C) and Rev-erbβ mRNA (Fig. 3D) expression in skeletal myotubes. These results show that heme, a Rev-erbβ agonist, regulates *Srebp-1c* mRNA expression, in a subtle but significant manner.

Discussion

Previously, we identified that the nuclear receptor Rev-erbβ modulated *Srebp-1c* mRNA expression in skeletal muscle cells [15]. We have followed up this observation, and are reporting data that suggests Rev-erbβ is a direct regulator of *Srebp-1c* gene expression in murine skeletal muscle cells. *In vivo* analysis showed that ectopic Rev-erbβΔE expression induces *Srebp-1c* mRNA expression in mice, in concordance with the previous and current *in vitro* studies. Our study suggests that Rev-erbβ is a key modulator of *Srebp-1c*.

Srebp-1c has been shown to be a regulator of genes involved in fatty acid biosynthesis [18,19]. *Srebp-1c* was initially shown to play major role in adipocyte metabolism [33–36], however in recent years this transcription factor has been reported to play a role in skeletal muscle metabolism. In this context, nuclear receptors have been identified as important modulators *Srebp-1c* expression in skeletal muscle. For example, agonists for the nuclear receptor Liver X Receptor (LXR) increase *Srebp-1c* mRNA expression in skeletal muscle cells/tissue [29]. Studies from our laboratory show that expression of the COUP-TFs in skeletal muscle cells is necessary for LXR-mediated *Srebp-1c* activation [26]. Moreover, *staggerer* mice, which carry a natural mutation for orphan receptor RORα displayed decreased *Srebp-1c* gene expression and serum triglycerides [27].

Several orphan NRs interact with DNA efficiently as monomers to an extended hexameric AGGTCA half site flanked by a 5′ A/T rich sequence (R/T_5AGGTCA, R = AorG) [37]. We observed that the region between nt positions −525/−401 in the SREBP-1c promoter mediated trans-activation by Rev-erbβ, and selectively recruited Rev-erbβ in the ChIP assay. This region has the hexameric half site within the extended region between nt positions −487/−458. Whether, Rev-erbβ is binding to the monomeric well conserved AGGTCA half site, or whether there is homodimeric binding to weak Rev-DR2 motif in this Rev-erbβ responsive region is not clear at present (Fig. 2A and B). Mutation of the two proximal LXR response elements between nt positions −323 and −197 do not compromise Rev-erbβ mediated trans-activation of the SREBP-1c promoter (data not shown). Finally, the basal SREBP-1c promoter supported weak Rev-erbβ mediated trans-activation, however, ChIP analysis did not identify any selective recruitment of Rev-erbβ to this region (data not shown).

In summary, we have identified a novel function of Rev-erbβ, being a transcriptional activator in the presence and absence of hemin. Studies have shown that apart from the C-terminal Ligand Binding Domain (LBD), nuclear receptors can also modulate gene expression using their N-terminus Activation Function-1 (AF-1) domain [38,39]. Interestingly, the Rev-erbβ AF-1 domain has been shown to be functional [40]. Raghuram and colleagues have recently shown that heme associates with the LBD of Rev-erbα and β [10,11] and demonstrated that the LBD of these NRs form several different confirmations structures in response to heme, NO or CO and redox state. Interestingly, our data shows that Rev-erbβ activates the *Srebp-1c* promoter in skeletal muscle cells. These observations raise the interesting question whether Rev-erb ligands function in tissue specific manner, and strongly indicate that Rev-erbβ can directly induce and silence gene expression.

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