Biochemical and Biophysical Research Communications 644 (2023) 122–129

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

FOXO1 represses PPARα-Mediated induction of FGF21 gene expression

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ARTICLE INFO

Article history:
Received 26 December 2022
Accepted 5 January 2023
Available online 6 January 2023

Keywords:
FGF21
PPARα
FOXO1
Metabolic diseases

ABSTRACT

Fibroblast growth factor 21 (FGF21) has emerged as a metabolic regulator that exerts potent anti-diabetic and lipid-lowering effects in animal models of obesity and type 2 diabetes, showing a protective role in fatty liver disease and hepatocellular carcinoma progression. Hepatic expression of FGF21 is regulated by PPARα and is induced by fasting. Ablation of FoxO1 in liver has been shown to increase FGF21 expression in hyperglycemia. To better understand the role of FOXO1 in the regulation of FGF21 expression we have modified HepG2 human hepatoma cells to overexpress FoxO1 and PPARα. Here we show that FoxO1 represses PPARα-mediated FGF21 induction, and that the repression acts on the FGF21 gene promoter without affecting other PPARα target genes. Additionally, we demonstrate that FoxO1 physically interacts with PPARα and that FoxO1/3/4 depletion in skeletal muscle contributes to increased Fgf21 tissue levels. Taken together, these data indicate that FOXO1 is a PPARα-interacting protein that antagonizes PPARα activity on the FGF21 promoter. Because other PPARα target genes remained unaffected, these results suggest a highly specific mechanism implicated in FGF21 regulation. We conclude that FGF21 can be specifically modulated by FOXO1 in a PPARα-dependent manner.

1. Introduction

Insulin regulates glucose production in the liver through the phosphorylation-dependent inactivation of the transcription factor Forkhead box protein O1 (FOXO1), which acts as a metabolic sensor to coordinate changes in gene expression in response to nutritional conditions [1,2]. FOXO1 is a member of the evolutionarily conserved O-subfamily of forkhead (FOX) transcription factors. In mammals this protein family has four isoforms, FOXO1, FOXO3, FOXO4 and FOXO6. FOXO transcription factors regulate cellular metabolism and survival in response to nutrient and environmental stress [3,4]. The transcriptional activity of FOXO factors is regulated by insulin through the phosphoinositide-3 kinase (PI3K)/Akt signaling pathway. Both insulin and insulin-like growth factor 1 (IGF-1) induce Akt-dependent phosphorylation of FOXO1, which facilitates its interaction with 14-3-3 protein, leading to nuclear exclusion and ubiquitination-dependent proteasomal degradation [5,6]. FOXO1 stimulates gluconeogenesis by activating transcription of several genes including glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). Consistent with FOXO1-mediated regulation of gluconeogenic key enzymes, liver-

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https://doi.org/10.1016/j.bbrc.2023.01.012

specific FoxO1 knock-out (KO) mice (l-FoxO1) showed hypoglycemia following a prolonged fast [7]. Interestingly, hepatic FoxO1 ablation also led to mild increase in fibroblast growth factor 21 (FGF21) production and secretion from the liver in fasted mice, but a more dramatic increase after fasting in streptozotocin-treated diabetic mice [8]. Furthermore, FGF21 was repressed in response to the activation of FoxO1 in the liver, by combined deletion of IRS-1 and IRS-2, leading to systemic metabolic dysregulation, which was reversed with hepatic FoxO1 deletion [9].

FGF21 is a member of the atypical fibroblast growth factors (FGFs), that can diffuse from its tissues of origin and function in an endocrine manner [10]. FGF21 is a small peptide with different sites of production that acts as a hormone by binding to a FGF tyrosine kinase receptor and the coreceptor β-klotho. Tissue specificity of FGF21 is determined by the coexpression of these two receptors [11]. FGF21 has been shown to mediate the adaptive response to starvation inducing ketogenesis, gluconeogenesis, lipolysis, and lipid β-oxidation [12–14]. Hepatic expression of FGF21 is known to be regulated by peroxisome proliferator-activated receptor α (PPARα) and is highly induced by fasting [14]. PPARα is a fatty acid-activated nuclear receptor that regulates metabolic changes in liver in particular affecting lipid metabolism in response to fasting [15]. Many additional targets for PPARα transactivation have been described [16], including mitochondrial carnitine palmitoyltransferase 1 A (CPT1A) and hydroxymethylglutaryl CoA synthase 2 (HMGCS2), key enzymes of fatty acid oxidation and ketogenesis, respectively [17–19]. The convergence of the FOXO1 and PPARα signaling pathways may represent a crucial mechanism in the regulation of energy metabolism and insulin sensitivity. Due to great interest in FGF21 as a potential therapeutic for obesity and other metabolic disturbances, it is imperative to better evaluate the molecular mechanisms that regulate the expression of FGF21.

2. Materials and methods

2.1. In vivo studies

Mice studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at both the Joslin Diabetes Center and the University of Iowa (M-TKO mice). Muscle-specific FoxO1, FoxO3, and FoxO4 triple knockout (M-FoxO-TKO) male mice were generated as previously described [20,21], and maintained on a standard chow diet (Mouse Diet 9F, 5020; LabDiet) before sacrifice.

2.2. Cell culture and treatments

Cellular studies were performed in HepG2 cells, a well-characterized cell line derived from human hepatoma. Though of cancer origin, HepG2 are widely used as hepatic in vitro model since these cells may retain many liver-specific functions [22]. HepG2 cells display a detectable response to PPARα activation, which confirms that the needed auxiliary machinery exists in these cells, namely its heterodimer partner RXRα [23] and detectable FGF21 levels [24]. HepG2 cells (ATCC HB-8065) were maintained in MEM (Minimum Essential Medium, Invitrogen®), a low glucose media (1000 mg/L D-Glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics (100 U/ml penicillin G, 100 μg/mL streptomycin) and glutamine (4 mM), and seeded at a density of 1×10^5 cells/cm^2 for each individual experiment. All cells and subsequent experiments were maintained under 5% CO_2 at 37 °C. The following treatment was performed in cells overexpressing PPARα: WY14643 (C7081, Sigma®, St. Louis, SO) 10 μM or 30 μM for 24 h or 15 h, respectively, as indicated in figure legends.

2.3. Plasmids

hHMGCS2luc (−1149/+28) containing a 1177-bp fragment of human HMGCS2, including the described PPAR response element (PPRE) (−111/-84) [25], was previously described [26]. mFgf21luc (−1497/+5) containing a 1502-bp fragment of murine Fgf21, including the described PPRE (−1119/-1044) and E-box (−51,-45) sequences [27,28], was amplified by PCR and cloned in the SmaI/XhoI restriction sites of the pGL3basic vector. pSG5-PPARα, pJCXRα, and pcDNA3-FOXO1 contained the cDNAs for mouse PPARα, human RXRα and human FOXO1, respectively. Bmal1 and Clock expression vectors were from Addgene [29]. pRL-CMV (Promega®) contained the Renilla (RL) luciferase gene under the control of the cytomegalovirus (CMV) intermediate-early enhancer/promoter.

2.4. Transient transfection assays and luciferase assays

HepG2 cells were seeded in 12-well plates 24 h before transfection. Cells were transfected using Lipofectamine LTX reagent (Invitrogen®), following the manufacturer's instructions. pRL-CMV plasmid was included as an internal transfection control. After 24 h, cells were harvested using the passive lysis method (Promega®) and cell lysates were collected for luciferase assays, performed using the Dual Luciferase Reporter Assay System (Promega®). Firefly and Renilla (RL) luciferase activities were determined in a Berthold Sirius Luminometer.

2.5. Adenovirus infection

HepG2 cells were infected with the following adenoviruses: AdCMV-hPPARα; AdCMV-mFoxO1 (WT or ADA) (provided by MA. Valverde); and AdCMV-GFP (abbreviated AdGFP, from CBATEG, Barcelona, Spain) for 48 h in MEM 10% FBS. The multiplicity of infection (MOI) of each adenovirus is indicated in figure legends. FoxO1 adenoviruses have been described previously [30]. When indicated, AdGFP was used as mock infection to adjust the total MOIs used with the adenovirus of interest, namely in AdPPARα alone (Fig. S1) or AdFoxO1 alone (Fig. 1A), and considered as control.

2.6. RNA isolation and real-time PCR analyses

Total RNA was extracted from HepG2 cells with Tri-Reagent (Ambion®) and was subsequently treated with DNase I (Ambion®), following the manufacturer's instructions. For quantitative real-time analysis of mRNA expression, TaqMan RT-PCR was performed on ABI PRISM 7700 sequence detection system (Applied Biosystems®, Foster City, CA). RNA was transcribed into cDNA using a M-MLV reverse transcripase (Invitrogen®) and random-hexamer primers (Roche Diagnostics®). The reactions were performed using TaqMan universal PCR master mix, No-AmpErase UNG reagent, and the specific gene expression primer pair probes. The Assay-on-Demand probes used were Hs00157079 for human CPT1A, Hs00173927 for human FGF21, Hs00985427 for human HMGCS2, and Mm00490672 for murine FoxO1, and eukaryotic 18S (Ref: 4319413E) as an endogenous control. The relative amount of gene expression in each sample was normalized to a reference control using the comparative ($2^{-\Delta\Delta Ct}$) method and following the manufacturer's instructions [31].

In order to analyze gene expression in muscle tissue, RNA was extracted from mixed quadriceps and gastrocnemius muscles from WT and M-FoxO-TKO mice using Trizol reagent (Invitrogen®), and was subsequently reverse transcribed into cDNA (Applied Biosystems®) according to the manufacturer's protocol. RT-PCR was carried out using SYBR green (Bio-Rad®) with specific primers, and

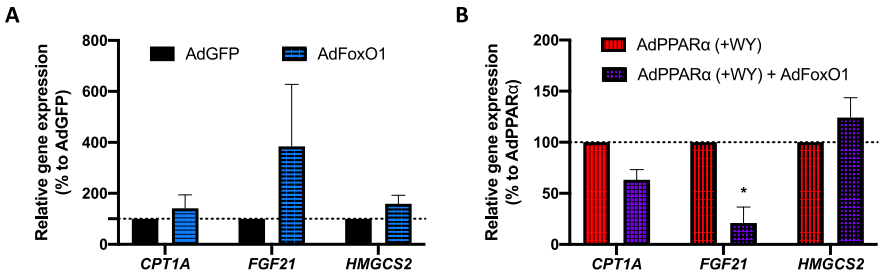

Fig. 1. - FoxO1 overexpression inhibits PPARα activation of *FGF21* mRNA levels. (**A, B**) *CPT1A*, *FGF21* and *HMGCS2* mRNA relative levels in response to FoxO1 overexpression (AdFoxO1-ADA, 10 MOIs), represented as fold change to respective controls (filled black or red bars). (**A**) HepG2 cells infected with AdGFP (100 MOIs) correspond to 100%. (**B**) HepG2 cells infected with AdPPARα (100 MOIs) correspond to 100%. Cells overexpressing PPARα were treated with 30 μM of WY14643 for 15 h before harvesting (48 h after infection). Results show mean ± SEM of three independent experiments. *$p < 0.05$ relative to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

normalized to TATA-binding protein (TBP). Primer sequences (5′-3′) were as following:

Fgf21 (Fwd) AGCTCTCTATGGATCGCCTCACTT; Fgf21 (Rev) ACACATT-GTAACCGTCCTCCAGCA; Cpt1b (Fwd) GCACACCAGGCAG TAGCTTT; Cpt1b (Rev) CAGGAGTTGATTCCAGACAGGTA; Hmgcs2 (Fwd) TTTTACCACAAGGTGAACTT; Hmgcs2 (Rev) AATTGTTGCATG-GATCTCCA; Ppara (Fwd) CAGTGGGGAGAGAGGACAGA; Ppara (Rev) AGTTCGGGAACAAGACGTTG; TBP (Fwd) ACCCTTCACCAATGACTCC-TATG; TBP (Rev) TGACTGCAG-CAAATCGCTTGG.

2.7. Protein extraction and western blotting

Whole cell extracts from HepG2 cells were prepared by homogenization in a NP40 lysis buffer (NaCl 150 mM, TrisHCl 50 mM, NP40 1%) supplemented with a cocktail of protease inhibitors (P8340, Sigma®, St. Louis, SO) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Subcelullar fractionation was performed as previously described [32]. Total protein concentrations were obtained by using Bradford reagent. Proteins were separated in an 8% SDS/PAGE gel, transferred to Immobilon-P membranes (Millipore®, Bedford, MA) and probed with an antibody against PPARα (1:1000) (sc-1985, Santa Cruz Biotechnology®, Santa Cruz, CA) or anti-HA (1:250) (hybridome 12CA5) to detect overexpressed heterologous FoxO1. Incubation with anti-β-actin antibody (Sigma®, A2066), anti-αTubulin (Calbiochem®, CP06) or anti-Sp1 (Santa Cruz Biotecnology®, 07–645) was performed to establish equal loading of protein samples. Detection was carried out using an enhanced chemiluminescence (ECL) detection kit for horseradish peroxidase (HRP) (Biological Industries®) with autoradiography (clear-blue X-ray) films.

2.8. GST-pull down assay

Glutathione S-transferase (GST) and GST-PPARα fusion proteins were produced in *Escherichia Coli* and purified on glutathione-sepharose beads (Amersham Pharmacia Biotech®), as described previously [33]. For the GST-pull down assay, equivalent amounts of protein extracts from HepG2 cells infected with AdCMV-mFoxO1 (WT), harvested in NP40 lysis buffer, were incubated in the presence of equivalent amounts of immobilized GST or GST-PPARα (15 μg) in 1 mL of binding buffer [NETN + 0.5% milk + protease inhibitor cocktail (Sigma®)] for 4 h, at 4 °C with agitation. The samples were then centrifuged for 1 min at 2000 rpm and the resin was washed twice in NETN at room temperature. After that, the samples were boiled, mixed with 2x Laemmli Buffer and resolved by SDS-PAGE in a 10% polyacrylamide gel. FoxO1 bands were visualized with anti-HA antibody by immunoblotting.

2.9. Statistical analysis

Numerical data are presented as mean ± SEM and were analyzed by two-tailed Student's t tests, followed by Welch correction where appropriate. Only p values < 0.05 were considered statistically significant and denoted on figures.

3. Results

3.1. FoxO1 overexpression inhibits PPARα activation of FGF21 mRNA levels

To better understand the mechanism underlying FGF21 regulation by FOXOs and PPARα, adenoviruses encoding a constitutively active form of FoxO1 (ADA; AdFoxO1) were transduced into HepG2 cells and real time PCR analysis was performed for selected PPARα target genes, such as CPT1A, HMGCS2 and FGF21. Fig. 1A shows that FoxO1 overexpression alone has only a mild effect on the expression of these genes. The transcript levels of CPT1A and HMGCS2 slightly increased compared to cells infected with AdGFP (Fig. 1A), while FGF21 mRNA expression tended to be more elevated but without reaching statistical significance (Fig. 1A), largely due to the high variability between independent experiments (Fig. S1).

Since CPT1A, HMGCS2 and FGF21 are known to be upregulated upon fasting, mainly due to ligand-activation of the PPARα transcription factor and subsequent PPARα binding to their promoters [13,14,17,18], we investigated the effect of FoxO1 overexpression on PPARα-mediated regulation of these genes. To this end, we transduced cells with adenoviruses encoding PPARα (AdPPARα) and treated them with WY14643, a specific synthetic PPARα ligand, in the presence or absence of FoxO1 overexpression. As expected, the overexpression and ligand-mediated activation of PPARα resulted in a dramatically increased expression of the selected genes, where the transcript levels of CPT1A, HMGCS2 and FGF21 were 15.7 ± 4.5 ($p = 0.08$), 322.7 ± 44.5 ($p = 0.019$) and 463,4 ± 198,5 ($p = 0.145$) times higher, respectively, when compared to cells without PPARα overexpression (cells transduced only with AdGFP). A preliminary experimental validation proved that ligand treatment further increased the PPARα-dependent induction of its target genes (Fig. S2). In the presence of FoxO1 co-overexpression, we observed a repression of PPARα-mediated induction of FGF21 (Fig. 1B). While FoxO1 had only a mild positive effect on the PPARα-induced

expression of HMGCS2 (24% increase, $p = 0.338$), it negatively affected CPT1A induction (37% decrease, $p = 0.067$). Notably, we observed a significant reduction of over 80% of PPARα-induced FGF21 expression ($p = 0.037$) (Fig. 1B). These data suggest that FOXO1 specifically interferes with the transcriptional regulation of FGF21 by PPARα.

3.2. FoxO1 repression is specific for PPARα activation in FGF21 promoter

In order to investigate if FOXO1 can repress PPARα-mediated regulation of FGF21 gene expression at the gene promoter level, luciferase reporter assays were performed using the mouse Fgf21 promoter. Specific regions for PPARα binding called peroxisome proliferator response elements (PPRE) are conserved among species and have been previously identified [14]. A scheme of the mouse Fgf21 promoter/luciferase reporter construct used is shown in Fig. 2A mFgf21 promoter activity, measured by calculating the luciferase/renilla ratio, was normalized to values obtained with cells transfected with pcDNA3 (Fig. 2C). Consistent with the gene expression data, FOXO1 overexpression alone did not significantly alter the promoter activity, while PPARα strongly induced the luciferase activity. However, when co-transfected together with PPARα, FOXO1 repressed Fgf21 promoter activation by 50% (Fig. 2C). In order to assess whether FOXO1-mediated suppression of PPARα was specific for FGF21 or would affect other PPARα target genes, we performed promoter activity assays using a reporter construct containing the promoter of HMGCS2 (Fig. 2B), another PPARα target gene [18,19]. Fig. 2D shows that, in line with the gene expression data, PPARα-induced HMGCS2 promoter activity was not altered by FOXO1 overexpression. In order to rule out an effect of FOXO1 on other regulatory regions such as the E-box sequence present in the mFgf21 reporter construct (see Fig. 2A), we measured the promoter activity co-transfecting constructs encoding the basic helix-loop-helix (bHLH) proteins brain and muscle Arnt-like protein-1 (Bmal1) and Clock, known to bind as heterodimers to E-box elements [27]. Fig. 2E shows that Bmal1/Clock increases the activity of the mFgf21 promoter, but the luciferase signal remained unchanged when FOXO1 was overexpressed. These data indicate that the effect of FOXO1 on FGF21 expression is specific for the FGF21 gene promoter and depends on PPARα.

3.3. FoxO1 physically interacts with PPARα

FOXO1 protein was shown to antagonize peroxisome proliferator-activated receptor γ (PPARγ) activity through disruption of its binding to DNA [34], and to transrepress PPARγ via physical interaction in adipocytes [35], where the γ2-isoform is preferentially expressed [36]. To test whether FOXO1 acts in a similar fashion on PPARα protein, we investigated if FoxO1 physically interacted with PPARα. To this end, an in vitro glutathione-S-transferase (GST)-pull down assay, using recombinant GST-PPARα and extracts from HepG2 cells overexpressing FoxO1, was performed as previously described [23]. Briefly, whole cell extracts from HepG2 cells transduced with HA-tagged AdFoxO1 (WT) were incubated with GST alone or GST-PPARα and pulled down. As shown in Fig. 3A, Western blot analysis with an anti-HA antibody shows that GST-PPARα pulled down FoxO1 proteins, suggesting a physical interaction between PPARα and FOXO1.

3.4. FoxO1 overexpression favors PPARα nuclear localization

Since transcriptional activation occurs when transcription factors bind nuclear target DNA sequences, we sought to evaluate PPARα cellular localization before and after FoxO1 overexpression. HepG2 cells were infected with AdPPARα and AdFoxO1 and Western blot analysis of nuclear (N) and cytoplasmic (C) extracts were performed (Fig. 3B). In the absence of FoxO1, PPARα almost exclusively localizes to the cytoplasm. Upon overexpression, FoxO1 was evenly distributed between both cellular compartments, whereas PPARα nuclear levels increased, while its amount in cytoplasmic extracts decreased (Fig. 3B). Taken together, these data suggest that

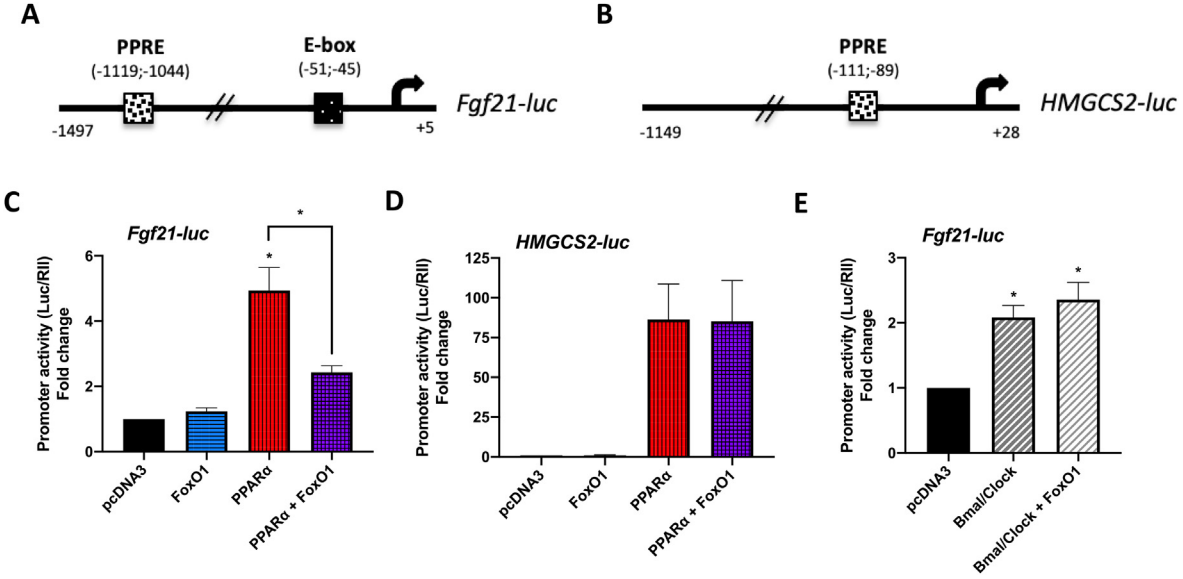

Fig. 2. - FOXO1 repression is specific for PPARα activation in Fgf21 promoter. (**A**) Scheme of the mouse Fgf21 and (**B**) human HMGCS2 promoter/luciferase reporter construct. (**C, D**) HepG2 cells transiently cotransfected with pFgf21-luc reporter (**C**) or pHMGCS2-luc reporter (**D**) in the presence or absence of PPARα/RXRα, and FOXO1 expression vectors, as indicated. (**E**) HepG2 cells transiently cotransfected with pFgf21-luc reporter in the presence or absence of Bmal1/Clock, and FOXO1 expression vectors, as indicated. (**C-E**) After 24 h of transfection, cells were harvested, and luciferase activity was measured. Cells overexpressing PPARα were concomitantly treated with 10 μM of WY14643. Results show Renilla-normalized luciferase activity, represented as mean ± SEM of three independent experiments performed in duplicate, represented as fold change to pcDNA3-empty vector (control). *$p < 0.05$ relative to control, or as indicated in figure.

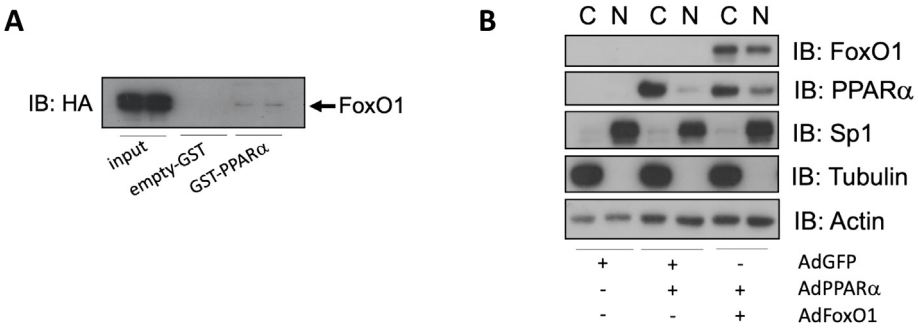

Fig. 3. FoxO1 physically interacts with PPARα and favors its nuclear localization. (A) Foxo1 interaction with recombinant PPARα. GST-pull down was performed in duplicate with HepG2 whole cell extracts (infected with AdFoxO1-HA, WT, 10 MOIs) with empty-GST or GST-PPARα. IB anti-HA. (B) FoxO1 overexpression effect on PPARα cellular localization. Cellular fractions enrichment from HepG2 cells infected with AdGFP, as mock control, or AdPPARα (100 MOIs), in the absence (AdPPARα alone) or presence of AdFoxO1 (10 MOIs). Sp1 and Tubulin immunoblots were used as nuclear (N) and cytoplasmic (C) loading control, respectively, as indicated.

FOXO1 either retains or drives PPARα into the nucleus but that the FOXO1-PPARα complex is unable to efficiently drive the activation of the FGF21 gene promoter.

3.5. *Fgf21* is upregulated in the muscle of mice with a muscle-specific deletion of FoxO1/3/4

Although circulatory FGF21 is predominantly from hepatic sources [13,14], skeletal muscle can increase FGF21 expression and secretion under certain conditions [37–40]. To uncover whether our observations were limited to liver tissue [8], or could be observed in other tissues, such as skeletal muscle, the expression levels of *Fgf21* were evaluated in the mixed quadriceps and gastrocnemius muscle of mice with a skeletal muscle-specific deletion of FoxO1/3/4 (M-TKO, muscle triple knock-out) [20]. We determined the transcript level of *Fgf21* through RT-qPCR in fed, control and tissue-specific triple KO, animals. Importantly, the absence of the three major FoxO isoforms in muscle significantly increased levels of *Fgf21* in this tissue (Fig. 4). In line with our results obtained in HepG2 cells (Fig. 1), carnitine palmitoyltransferase 1 b (*CPT1b*) and *Hmgcs2* expression remained unchanged in the skeletal muscle from control and M-FoxO-TKO (Fig. 4). *Ppara* expression revealed a non-statistically significant ($p = 0.149$) reduction of 35% in M-TKO (Fig. 4). Collectively, these data suggest a prominent role of FoxO1 in the regulation of the levels of FGF21 expression in various tissues.

4. Discussion

The beneficial metabolic effects of FGF21, which include weight loss, improved glycemia, and protection against non-alcoholic steatohepatitis (NASH), have raised the hope for developing FGF21-based therapeutics as agents for the treatment of metabolic conditions such as obesity, non-alcoholic fatty liver disease (NAFLD), insulin resistance and type 2 diabetes (T2D) [11,42,43]. Moreover, although FGF21 is increased upon obesogenic diet consumption or genetic induced hepatocellular carcinogenesis [44], deficiency of FGF21 promoted the development of hepatocellular carcinoma (HCC) [45]. Therefore, FGF21 might act as a stress response factor, playing a substantial role in limiting the progression from fatty liver to HCC by normalizing systemic metabolic parameters. Hence, understanding the mechanisms involved in the regulation of FGF21 expression has potential therapeutic value. Based on the observation that liver-specific FoxO1 knock out (L-FoxO1) mice showed elevated levels of hepatic and systemic FGF21 levels [8], the main goal of our work was to investigate the role of FoxO1 in the regulation of FGF21 expression.

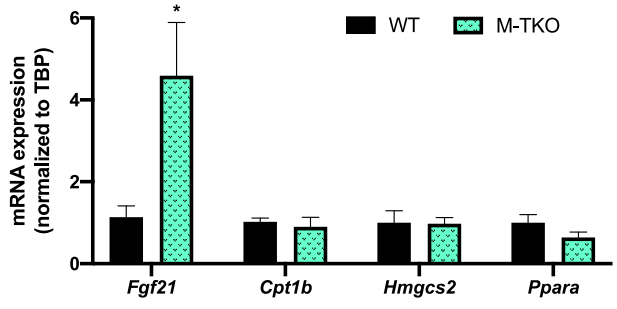

Fig. 4. - *Fgf21* is upregulated in the muscle of mice with a muscle-specific deletion of FoxO1/3/4. Quantitative RT-PCR of *Fgf21*, *Cpt1b*, *Hmgcs2* and *Ppara* in skeletal muscle from control (wild type, WT) and FoxO-TKO (M-TKO) mice (n = 4–6 each group; average fold change ± SEM) is represented. *$p < 0.05$ vs. control.

In the liver, FOXO1 activity is mainly regulated by nutritional status. Upon insulin stimulation FOXO1 is inactivated by Akt-mediated phosphorylation at three sites, including serine positioned at 256 (Ser256), leading to its exclusion from the nucleus [2]. HMGCS2 has been characterized as a transcriptional target of both PPARα and FOXO1 transcription factors [18,46], but we find only a modest induction of HMGCS2 with co-expression of PPARα and FoxO1. The cooperation of both transcription factors in the upregulation of FOXO1 target genes has been described for other genes such as LPL in C2C12 cultured myocytes [47]. However, the regulation of PPARα and FOXO targets is not always synergistic. Indeed, PPARα-induced expression of CPT1A [17], was found to be partially blocked by FoxO1 overexpression, which is in agreement with the upregulation of CPT1A previously observed in the L-FoxO1 mice liver [8]. A very recent study confirmed that hepatic FoxO1 suppressed Fgf21 in a mouse model of severe insulin resistance and in mouse primary hepatocytes [9]. In agreement with our findings, the authors showed that hepatic FoxO1 suppressed Fgf21 production from the liver in a cell-autonomous fashion. They further show that this repression of Fgf21, in a model of hepatic insulin resistance worsens the metabolic phenotype [9]. Our data indicate that overexpression of FoxO1 by itself fails to affect the expression of FGF21, significantly. In line with this finding, FGF21 has not been identified as a target gene of FOXO proteins. However, the strongest repression of FGF21 mRNA levels was only observed upon co-transfection of FoxO1 and PPARα, whereas FoxO1 overexpression alone did not decrease FGF21 expression. It was previously shown that glucose stimulation, via ChREBP transcription factor activation, induced FGF21 gene expression in rat hepatocytes in a time-dependent manner [48], while another study reported that PPARα was required for this induction [49]. Though we have not

challenged the cells with different glucose supplies, our findings agree with those asserting FGF21 as a nutritional adaptation factor. In agreement with the observation that hepatic Fgf21 mRNA levels increased upon fasting but not refeeding in liver-specific FoxO1-KO mice [8], FGF21 expression was highly attenuated by FoxO1 protein overexpression upon ligand-PPARα activation in HepG2 cells. It is still to be determined *in vivo*, if post-translational modifications of FOXO proteins, such as acetylation or ubiquitination, which can peak and fall at different times and change the biology of FOXO interactions [50], are involved in the mechanism herein proposed.

Since the identification of FGF21 as a new member of the FGF family back in 2000 [51], the FGF21 promoter has been exhaustively characterized. Besides the description of the PPARα binding site (PPRE) present in FGF21 regulatory sequences [14], several other transcription factors have been identified to bind to particular promoter sequences and to induce transcriptional activity, such as activating transcription factor 2 (ATF2) [52], ATF4 [53], Sp1 [54], among several others [55]. Contradictory results on Bmal1/Clock-mediated activation of the Fgf21 promoter have been previously reported. While some showed absence of activation [28], others, in line with our results, have reported transactivation [27]. Of interest, other transcription factors such as E4-binding protein 4 (E4BP4), were reported to repress PPARα activation of the Fgf21 promoter [27]. However, while our results showed that FoxO1 specifically represses PPARα transactivation without affecting Bmal1/Clock induction, E4BP4 indistinctly blocked both Bmal1/Clock and PPARα induction [27]. Similarly, FOXO1 has been described as a promoter-specific repressor of PPARγ target genes that regulate adipogenesis. FOXO1 was shown to be recruited to PPAR response elements (PPRE) in the promoters of PPARγ target genes and to interfere with promoter DNA occupancy of the receptor [34]. Since the observed repression was reported to be carried out by direct FOXO1—PPARγ interactions, we decided to explore whether a similar mechanism drives FoxO1-mediated repression of PPARα. Our results show analogous protein-protein interaction between PPARα and FoxO1. Intriguingly, PPARα has been shown to functionally antagonize FOXO1 in hepatic apolipoprotein C-III (apoC-III) expression [56], suggesting, together with our data, a mutual antagonism between FOXO1 and PPARα acting on specific target gene promoters. Although FGF21 is mainly expressed and secreted in liver [13,14], adipose tissues have been also described to contribute to the amount of secreted FGF21 found in circulation upon certain conditions [52,57]. Studies reported that FGF21 is induced by PPARγ in the white adipose tissue (WAT) [57], and that FGF21 regulates PPARγ activity [58]. Therefore, it might be expected that FOXO1 similarly represses PPARγ transactivation of FGF21 in fat tissue, although to the best of our knowledge, this has not been confirmed.

FOXO1 exhibits both positive and negative transcriptional properties [59], although it is controversial whether repression of gene expression mediated by FOXO proteins proceed through a direct mechanism or generally occurs through DNA-binding independent mechanisms [60]. In line with the latter view, we show that FoxO1 negatively affects transcriptional regulation by an indirect mechanism, through binding to PPARα. It remains to be determined whether the interaction of FoxO1 with PPARα interferes with the direct binding of PPARα to DNA and in which specific conditions these interactions can be detected at the endogenous level.

We provide evidence that FOXO1 affects the subcellular localization of PPARα. PPARα nuclear distribution increased upon FoxO1 overexpression suggesting that FoxO1 could be retaining PPARα in the nucleus. Intriguingly, this is coincident with a decreased capacity of PPARα to transactivate FGF21. As PPARα has been shown to be degraded by the ubiquitin-proteasome system [61], the interaction with FoxO1 may stabilize the PPARα protein leading to an inactive PPARα pool in the nucleus or the formation of a co-repressor complex.

FOXO1 is known to be a key regulator of muscle cellular metabolism [62]. Fasting activates FoxO1 expression in skeletal muscle [63], which in turn induces the key regulatory enzyme pyruvate dehydrogenase kinase (PDK) 4, a FoxO1 target gene, thereby suppressing muscle glucose oxidation [63]. Recent work performed in mice showed that skeletal muscle-specific deletion of FoxO1/3/4 prevented diabetes-induced muscle atrophy albeit not affecting glucose homeostasis [20]. FGF21 is expressed at very low levels in skeletal muscle in the basal state, but it seems to play a role in muscle homeostasis as its expression is induced in response to muscle stress [11]. Recently, a study showed that ablation of FGF21 protects mice from fasting-induced muscle loss [64]. Here, we provide evidence for the first time that, a mechanism highly specific for FGF21 regulation, similar to the one described for the liver, might operate in skeletal muscle. We showed that in the absence of FoxO1/3/4, *Fgf21* gene expression is upregulated in skeletal muscle, while other PPARα target genes such as *Cpt1b* and *Hmgcs2*, or fatty acid import genes (*Cd36*) and acyl-CoA modifying enzymes (*Slc27a1*), previously examined [20], remained unaffected. Interestingly, it was recently described that FoxO1/3/4 triple knock-out (M-TKO) mice, while displaying the expected muscle hypertrophy, gained less fat mass and showed improved glucose levels and glucose homeostasis on a high-fat diet (60% HFD) [65]. Nevertheless, only minimal changes were observed in the browning of white adipose tissue or circulating adipokines [65]. We hypothesize that while the observed 4-fold upregulation of *Fgf21* in skeletal muscle of M-TKO demonstrates the regulation of Fgf21 mRNA by FOXO proteins in more than one tissue, the secreted amount of protein is unlikely to be sufficient to affect circulating FGF21, which often require many thousand-fold increased expression [39,41], and those observed metabolic improvements are most likely to be FGF21-independent.

In summary, our data provide evidence for a FOXO1-mediated repressive effect on PPARα, as one of the potential mechanisms by which FoxO1 deletion increases [8], and excess of FoxO1 activity represses [9], hepatic FGF21 expression. Here we identified FOXO1 as a PPARα-interacting protein that antagonizes PPARα activity in the nucleus, specifically down-regulating PPARα-mediated induction of FGF21, both at the promoter and gene expression levels. We propose that the repression of PPARα-dependent induction of FGF21 by FOXO1 might play a protective role in several disease states such as diabetes, NAFLD or HCC, limiting excessive free fatty acid release from adipose tissue and the lipotoxic cell death that may result from an excessive fatty acid oxidation rate induced by PPARα. On the other hand, selective pharmacological inhibition of FoxO1 [66,67] might relieve its repression on PPARα, promoting the expression of FGF21 peptides and their beneficial effects on glucose homeostasis and lipid metabolism.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. WL is cofounders of Refoxy Pharmaceuticals GmbH, Berlin. W.L. is required by his institution to state so in his publications. The other authors declare no conflict of interest.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding

This work was supported by the Spanish Ministry of Science, Innovation and Universities through Grant RTI2018-094629-B-I00 to WL and by the "Ministerio de Educación y Ciencia" BFU2004-01925 to DH and BFU2007-67322/BMC to PFM; and the "Ajut de Suport als Grups de Recerca de Catalunya" 2005SGR857.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. WL is cofounders of Refoxy Pharmaceuticals GmbH, Berlin. W.L. is required by his institution to state so in his publications. The other authors declare no conflict of interest.

Acknowledgments

We thank C. Ronald Kahn for intellectual and technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.01.012.

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