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Mapping the response of human fibroblast growth factor 21 (*FGF21*) promoter to serum availability and lipoic acid in HepG2 hepatoma cells

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ABSTRACT

The hormone-like polypeptide, fibroblast growth factor 21 (FGF21), is a major modulator of lipid and glucose metabolism and an exploratory treatment strategy for obesity related metabolic disorders. The costs of recombinant FGF21 and mode of delivery by injection are important constraints to its wide therapeutic use. The stimulation of endogenous FGF21 production through diet is being explored as an alternative approach. To that end, we examined the mechanism(s) by which serum manipulation and lipoic acid (a dietary activator of *FGF21*) induce FGF21 in human hepatocellular carcinoma HepG2 cells. Serum withdrawal markedly induced FGF21 mRNA levels (88 fold) and FGF21 secreted in the media (19 fold). Lipoic acid induced FGF21 mRNA 7 fold above DMSO-treated control cells and FGF21 secretion 3 fold. These effects were several-fold greater than those of PPARα agonist, Wy14643, which failed to induce *FGF21* above and beyond the induction seen with serum withdrawal. The use of transcription inhibitor, actinomycin D, revealed that de novo mRNA synthesis drives FGF21 secretion in response to serum starvation. Four previously unrecognized loci in *FGF21* promoter were nucleosome depleted and enriched in acetylated histone H3 revealing their role as transcriptional enhancers and putative transcription factor binding sites. FGF21 did not accumulate to a significant degree in induced HepG2 cells, which secreted FGF21 time dependently in media. We conclude that lipoic acid cell signaling connects with the transcriptional upregulation of *FGF21* and it may prove to be a safe and affordable means to stimulate FGF21 production.

1. Introduction

Fibroblast growth factor 21 (FGF21) is a pleiotropic hormone-like protein expressed in liver, pancreas, adipose and muscle tissue [1–5]. FGF21 was classified as a fibroblast growth factor based on its apparent structure similarity with other FGFs [1]; however, FGF21 has no demonstrated activity in fibroblasts and does not promote growth in vivo [6]. FGF21 functions as an endocrine hormone through binding to cell-surface FGF receptor (FGFR)/β-Klotho complex found in the liver, white and brown adipose tissues, and brain in mice [1,4,7–12]. FGF21 is a potent modulator of lipid and glucose metabolism and an exploratory treatment strategy for obesity related metabolic disorders [13,14]. The administration of FGF21 to obese rats and mice increases fat utilization and energy expenditure, and lowers plasma and liver triglycerides, blood glucose and insulin [6]. These positive outcomes were also observed in diabetic rhesus monkeys [15,16]. Recombinant wild-type and optimized FGF21 variant were investigated clinically as therapeutic against type-2 diabetes with meaningful effects, notably in the control of serum lipids and body weight [17], while the effect on blood glucose was not as robust as first anticipated [18]. Potentially adverse effects related to bone density have been described in mice administered recombinant FGF21 or mice overexpressing FGF21 [19]. In particular, high serum and synovial fluid FGF21 were associated with radiographic bone loss of knee osteoarthritis [20]. However, an association between high plasma FGF21 and bone loss (bone mineral density was measured by dual-energy X-ray absorptiometry) was not observed in healthy women [21].

Despite a plethora of positive outcomes, the high costs of producing FGF21 and mode of delivery by daily injection are important constraints to the wide therapeutic use of engineered FGF21. A cost-effective and practical alternative may be to stimulate endogenous FGF21 production through diet. We previously showed that naturally occurring dietary compound *R*-α-lipoic acid (LA, a cofactor found in green leafy

Abbreviations: ACTB, β-actin; *CPT1A*, carnitine palmitoyltransferase 1α; CBP, CREB binding factor; CREB, cAMP response element-binding protein; DHLA, DL-α-dihydrolipoic acid; Elk-1, ETS domain-containing Elk-1; Elk-4, ETS domain-containing Elk-4 (aka SAP-1); ENCODE, encyclopedia of DNA elements; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; ERK5, extracellular signal-regulated kinase 5; ETS, E26 transformation specific sequence; FAIRE, formaldehyde-assisted isolation of regulatory elements; FGF21, fibroblast growth factor 21; FGFR, FGF receptor; FUT1, fucosyltransferase 1; *HMOX 1*, heme oxygenase 1; IR, insulin receptor; LA, *R*-α-lipoic acid; MAPK, mitogen-activated protein kinase; PPARα, peroxisome proliferator-activator receptor α; PPIA, cyclophilin A; p38MAPK, p38 mitogen-activated protein kinase; PPRE, peroxisome proliferator response elements; SPZ1, spermatogenic leucine zipper protein 1; SRF, serum response factor; TCF, ternary complex factor; TRED, transcriptional regulatory element database; TSS, transcription start site.

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vegetables, red meats, and in supplement form) stimulates liver *Fgf21* expression and blood FGF21 levels in rats [5,22,23]. The objective of the present study was to examine the mechanism of induction of the human *FGF21* gene in HepG2 liver cells under conditions known to stimulate *FGF21* gene expression, namely the fasting state (reproduced by withdrawing serum) and the intake of LA (reproduced by treating the cells with LA or its reduced form dihydrolipoic acid, DHLA). Our results show that serum withdrawal, LA and DHLA induced the gene expression and secretion of FGF21 without hepatocellular accumulation of FGF21. We used the FAIRE (formaldehyde-assisted isolation of regulatory elements) assay coupled to qPCR to isolate, within the *FGF21* 5′-flanking region, nucleosome-depleted chromatin regions that are predictive of regulatory elements and transcription factor binding under the aforementioned experimental conditions. Results show that serum withdrawal led to structural changes in chromatin at two distinct non-overlapping distal regions of the *FGF21* promoter (−3578/−3348 and −2628/−2421 bp from TSS); while LA and DHLA altered two overlapping regions located −1524/−1286 and −1317/−1124 bp from TSS. These loci were enriched with acetylated histone H3, which indicated that they were transcriptional competent and hot spots for the binding of transcriptional factors. Overall, these results provide novel mechanistic insight into the transcriptional induction of *FGF21* by serum starvation and by an organosulfur dietary molecule, *R*-α-lipoic acid.

2. Materials and methods

2.1. Cell culture

Human hepatocellular carcinoma HepG2 cells (ATCC, HB-8065) were cultured in DMEM (pH 7.4, 5.5 mM D-glucose, Life Technologies/Gibco, 11885084) supplemented with 10% FBS (ATCC, 30–2020) and antibiotics (Sigma, A5955). Cultures were maintained at 37 °C in a CO_2 incubator supplied with humidified air and 5% CO_2. Cells were cultured in 10% FBS until 50% confluence (time = 0 h), then in the absence of FBS for up to 58 h. In specific experiments, FBS (10%) or PBS control was reintroduced at time = 24 h. Media was changed at least 12 h before sample collection to eliminate artifactual response arising from fresh media. To investigate the effects of LA (*R*-α-lipoic acid, MAK Wood, Grafton, WI), DHLA (DL-α-dihydrolipoic acid, EMD Millipore/Calbiochem, 437694), PPARα agonist Wy14643 (Sigma, C7081), and actinomycin D (Cayman, 11421), 1000× stocks were freshly prepared in sterile DMSO and cells were treated with 50 μM LA, 50 μM DHLA, 50 μM Wy14643, 10 μg/ml actinomycin D or 0.1% DMSO control for the indicated times.

2.2. Trypan blue exclusion assay

Cells were trypsinized and collected by centrifugation. An aliquot of the cell suspension was combined with HBSS and trypan blue solution to a final concentration of 0.2% (*w*/*v*), allowed to stand for 5 min, and analyzed microscopically on a hemocytometer. Cells excluding the dye were counted as viable and blue cells as nonviable. Cell viability (%) = total viable cells/total cells (stained and unstained) × 100. The cell viability data obtained from this assay is not affected by the change of cell proliferation due to serum withdrawal.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using BioRad Aurum Total RNA kit and treated with DNase I. RNA integrity of representative isolates was confirmed on an Agilent Bioanalyzer 2100. First strand cDNA was synthesized with oligo(dT) and random primers using BioRad iScript. qRT-PCR was performed on a BioRad CFX96 using SYBR Green supermix. Amplicon authenticity was confirmed by melt curve analysis and agarose gel electrophoresis. PCR efficiencies were assessed with serial dilutions of the template (0.001–100 ng cDNA/reaction) and 0.3 μM of each primer, and plotting quantification cycle (Cq) vs. log amount of template. PCR efficiencies between target genes and housekeeping genes were comparable, thus unknown amounts of target in the sample were determined relative to cyclophilin A (*PPIA*). Primer sequences are shown in Supplementary data.

2.4. Western blotting and ELISA

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer supplemented with 1 mM DTT, Halt protease and phosphatase inhibitor cocktails (Thermo Scientific/Pierce). After overnight agitation at 4 °C and centrifugation (14,000 ×*g*, 10 min, 4 °C), protein content in the clear supernatant was quantified by the bicinchoninic acid assay (Thermo Scientific/Pierce). Cellular proteins contained in an aliquot of the supernatant were precipitated with cold acetone, centrifuged (14,000 ×*g*, 10 min, 4 °C), resuspended in PBS, and heat denatured in Laemmli sample buffer (5 min, 95 °C). FGF21 secreted in culture media was determined following centrifugation of debris, ammonium sulfate precipitation, desalting (Thermo Scientific/Pierce, Zeba Spin Desalting Columns, 7K MWCO), and heat denaturation in Laemmli sample buffer. Proteins were subjected to reducing SDS-PAGE, transferred to nitrocellulose membrane (Odyssey, 926-31092), and immunoblotted with anti-FGF21 antibodies (LifeSpan BioSciences, LS-B5864 IHC-plus). Antibodies to β-actin (Sigma, A5441), α-tubulin (Sigma, T6793) and albumin (Midland Bioproducts, 71907) were also used as controls. Antibody binding was visualized using LiCOR IR Dye secondary antibodies and Odyssey scanner. Specificity of the primary antibody was evaluated by pre-incubating 3 μg IgG with 8 μg blocking peptide (LifeSpan BioSciences, LS-E20001) prior to Western blotting. Recombinant human FGF21 (Toronto Bioscience, 41189) was used as a positive control. FGF21 secreted in media was also determined by ELISA (AIS, Hong Kong).

2.5. Polypeptide glycosylation

To determine whether FGF21 (~30 kDa) secreted by HepG2 cells was modified with *O*-linked or *N*-linked glycosylation, proteins resulting from the ammonium sulfate precipitation of conditioned media were subjected to enzymatic deglycosylation using an enzyme cocktail (New England Biolabs, P6039S), which contains *O*-glycosidase, peptide:*N*-glycosidase F, neuraminidase, β(1–4) galactosidase and β-*N*-acetylglucosaminidase. Bovine fetuin, which bears sialylated *N*-linked and *O*-linked glycans, was used as a positive control. To assess whether inhibitors of enzymatic deglycosylation were present in the ammonium sulfate-processed conditioned media, the deglycosylation of fetuin was also carried out in the presence of processed conditioned media. Band shift was assessed by Western blotting for FGF21 or Coomassie blue R-250 staining for fetuin.

2.6. Formaldehyde-assisted isolation of regulatory elements (FAIRE)-coupled qPCR

FAIRE exploits the difference in formaldehyde cross-linking efficiency between nucleosomes and sequence-specific DNA-binding factors [24]. During phenol–chloroform extraction, DNA fragments not cross-linked to proteins (euchromatin) segregate to the aqueous phase, while DNA cross-linked to proteins (nucleosomes) remains in the organic lower phase. DNA fragments isolated from the aqueous phase are then identified by quantitative PCR (FAIRE-qPCR). Two promoters (P1 and P2) within the *FGF21* 5′-flanking region were retrieved from TRED (Cold Spring Harbor Lab) and included in the 4451-bp region surveyed (Supplementary Figures 1S and 2S). This region is located within a region dense in acetylated histone (H3K27Ac) marks, which are predictive of regulatory elements. The enrichment among euchromatin of fragments of the 4451-bp 5′-flanking region was determined by qPCR analysis of 25 genomic overlapping fragments spanning the 4451-bp