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Fibroblast growth factor 21 is elevated in metabolically unhealthy obesity and affects lipid deposition, adipogenesis, and adipokine secretion of human abdominal subcutaneous adipocytes

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ABSTRACT

Objective: Serum concentrations of the hepatokine fibroblast growth factor (FGF) 21 are elevated in obesity, type-2 diabetes, and the metabolic syndrome. We asked whether FGF21 levels differ between subjects with metabolically healthy vs. unhealthy obesity (MHO vs. MUHO), opening the possibility that FGF21 is a cross-talker between liver and adipose tissue in MUHO. Furthermore, we studied the effects of chronic FGF21 treatment on adipocyte differentiation, lipid storage, and adipokine secretion.

Methods: In 20 morbidly obese donors of abdominal subcutaneous fat biopsies discordant for their whole-body insulin sensitivity (hereby classified as MHO or MUHO subjects), serum FGF21 was quantified. The impact of chronic FGF21 treatment on differentiation, lipid accumulation, and adipokine release was assessed in isolated preadipocytes differentiated *in vitro*.

Results: Serum FGF21 concentrations were more than two-fold higher in MUHO as compared to MHO subjects (457 ± 378 vs. 211 ± 123 pg/mL; $p < 0.05$). FGF21 treatment of human preadipocytes for the entire differentiation period was modestly lipogenic ($+15\%$; $p < 0.05$), reduced the expression of key adipogenic transcription factors (*PPARG* and *CEBPA*, -15% and -40%, respectively; $p < 0.01$ both), reduced adiponectin expression (-20%; $p < 0.05$), markedly reduced adiponectin release (-60%; $p < 0.01$), and substantially increased leptin ($+60\%$; $p < 0.01$) and interleukin-6 ($+50\%$; $p < 0.001$) release.

Conclusions: The hepatokine FGF21 exerts weak lipogenic and anti-adipogenic actions and marked adiponectin-suppressive and leptin and interleukin-6 release-promoting effects in human differentiating preadipocytes. Together with the higher serum concentrations in MUHO subjects, our findings reveal FGF21 as a circulating factor promoting the development of metabolically unhealthy adipocytes.

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Keywords FGF21; Hepatokine; Adiponectin; Adipokine; Secretome; Type-2 diabetes

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1. INTRODUCTION

Fibroblast growth factor (FGF) 21, FGF19 (murine homologue: FGF15), and FGF23 constitute a subfamily of FGFs with hormone-like rather than growth factor functions [1].

In mice, FGF21 is produced by liver, pancreas, skeletal muscle, brown and white adipose tissue, and major stimuli for FGF21 release are fasting and ketogenic diets, cold exposure, and exercise [2]. In murine models of obesity and diabetes, and less evident or even absent in normal mice, pharmacological administration or transgenic over-expression of FGF21 exerts beneficial metabolic effects, i.e., reduction of body weight and liver fat, improvement of insulin sensitivity, hyperlipidaemia, and hyperglycaemia, and this is most probably due to the induction of thermogenesis and stimulation of energy expenditure [3–5].

In humans, FGF21's role in physiology and metabolic disease is more complicated and far from being understood: circulating FGF21 is mainly produced by the liver and is independent of nutritional status [1]; furthermore, its plasma concentrations are elevated in abdominal obesity, fatty liver disease, hyperlipidaemia, insulin resistance, type-2 diabetes, metabolic syndrome, and coronary artery disease [6–10]. Since this is in sharp contrast to data obtained in mice and the existence of a postulated FGF21-resistant state in humans is still insufficiently proven, there is a clear need for further studies to better understand FGF21's role in humans.

We previously succeeded in describing metabolically healthy and unhealthy obesity (MHO and MUHO) with hepatic fat content and inflammation representing major determinants of these states [11,12]. In a very recent metabolomics study, we have shown that human abdominal subcutaneous (hasc) preadipocytes differentiated in vitro to adipocytes reveal intra- and extracellular metabolite signatures that also allow discriminating MUHO from MHO [13]. Thus, these cells reveal stable, mitotically inherited properties that may reflect and/or contribute to these states.

In this study, we asked whether serum FGF21 concentrations differ between MHO and MUHO subjects. This would open the possibility that FGF21 acts as a cross-talker between the liver and adipose tissue affecting (pre)adipocyte properties in a way that could explain metabolic features of MUHO versus MHO. Therefore, we studied the effects of chronic FGF21 treatment on typical adipocyte functions, such as adipose conversion, lipid storage, and adipokine secretion, in hasc preadipocytes differentiated in vitro.

2. MATERIAL AND METHODS

2.1. Study participants

The 20 hasc adipose tissue donors reported earlier [13] were morbidly obese (body mass index (BMI) > 40 kg/m^2 all), well matched for gender, age, and body fat content (as measured by bioelectrical impedance), but discordant for their whole-body insulin sensitivity (as estimated by a five-point oral glucose tolerance test-derived insulin sensitivity index calculated as 10,000/[fasting glucose {mmol/L} x fasting insulin {pmol/L} x mean glucose {mmol/L} x mean insulin {pmol/L}]$^{1/2}$). According to their insulin sensitivity, they were stratified into ten MHO (insulin-sensitive; insulin sensitivity index $\geq 5.0 \times 10^{19}$ L^2 x mol^{-2}) and ten MUHO (insulin-resistant; insulin sensitivity index $\leq 3.5 \times 10^{19}$ L^2 x mol^{-2}) subjects, and the clinical characteristics of the two groups were reported recently [13]. The study adhered to The Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants gave informed written consent to the study, and the study protocol was approved by the local ethics board.

2.2. Preadipocyte culture, differentiation, and treatment

Preadipocytes were isolated from the 20 hasc adipose tissue explants as described earlier [14] and expanded in α-MEM/Ham's nutrient mixture F12 (1:1) containing 20% fetal calf serum, 1% chicken embryo extract (Sera Laboratories, Haywards Heath, UK), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 0.5 μg/mL fungizone, and 2 mmol/L glutamine. Second-pass cells were used for experiments. At confluence, adipose conversion was induced by shifting the cells into DMEM/Ham's nutrient mixture F12 (1:1), 5% fetal calf serum, 17 μmol/L pantothenate, 1 μmol/L biotin, 2 μg/mL apo-transferrin, 1 μmol/L human insulin, 1 μmol/L dexamethasone, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 0.5 μg/mL fungizone, and 2 mmol/L glutamine (differentiation medium) supplemented with 0.5 mmol/L 3-isobutyl-1-methyl-xanthine, 2 nmol/L triiodo-thyronine, and 50 μmol/L indomethacin for seven days. Thereafter, the cells were allowed to terminally differentiate for another 11 days in differentiation medium alone. Differentiating preadipocytes were left untreated or were chronically treated for the whole 18-day period with 50 ng/mL recombinant human (rh)FGF21 (PeproTech, Rocky Hill, NJ, USA). Culture media and supplements were obtained from Lonza (Basel, Switzerland) and Biochrom (Berlin, Germany).

2.3. Staining of intracellular neutral lipids

Differentiation of preadipocytes to adipocytes was qualitatively monitored by microscopy after Oil Red O staining according to Greenberger et al. [15]. For quantification of intracellular neutral lipids, Oil Red O was extracted with isopropanol and photometrically measured at 500 nm as described earlier [16].

2.4. RNA isolation and real-time quantitative polymerase chain reaction (qPCR)

(Pre)adipocytes were harvested with Qiazol lysis reagent (Qiagen, Hilden, Germany). Total RNA was isolated with miRNeasy columns (Qiagen), treated with RNase-free DNase I, and reverse transcribed into cDNA using Qiagen's QuantiTect reverse transcription kit. Real-time qPCR was performed in technical duplicates with QuantiTect primer assays on a LightCycler™ 480 II (Roche Diagnostics, Mannheim, Germany). All mRNA data were normalized to the housekeeping gene RPS13 using the ΔCt method.

2.5. Enzyme-linked immunosorbent assays

FGF21 in serum and adiponectin, leptin, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in the conditioned cell culture media were measured with immunoassays from R&D Systems (Wiesbaden-Nordenstadt, Germany) according to the manufacturers' instructions.

2.6. Protein extraction and immunoblotting

Cellular protein was extracted using RIPA buffer (50 mmol/L Tris—HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/L phenyl-methyl-sulfonyl-fluoride, 1 mmol/L dithiothreitol) containing a protease and phosphatase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were sonicated and cleared by centrifugation. Protein concentration was determined with the RC DC kit from Bio-Rad (Hercules, CA, USA). Equal amounts of protein (30 μg/lane) were loaded onto 10-% sodium dodecyl sulfate polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes and incubated with antibodies against phospho-threonine 172 of the catalytic α-subunit of AMP-activated protein kinase (AMPK), AMPKα protein, phosphorylated extracellular signal-regulated kinases (ERK) 1 and 2, ERK1 and 2 protein, or glyceraldehyde 3-phosphate dehydrogenase

(GAPDH), respectively. All primary antibodies were from Cell Signaling Technologies (Danvers, MA, USA), the secondary anti-rabbit antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Proteins were visualized by electrochemiluminescence (PerkinElmer, Waltham, MA, USA).

2.7. Genome-wide gene expression analysis

Total RNA was isolated as described. The Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) was used to assess RNA quality, and only high-quality RNA (RNA integrity number >9) was used for microarray analysis. Total RNA (30 ng) was amplified using the Ovation PicoSL WTA System V2 in combination with the Encore Biotin Module (NuGEN, Leek, The Netherlands). Amplified cDNA was hybridized on Affymetrix Human Gene ST 1.0 arrays (Affymetrix, Santa Clara, CA, USA). Staining (Fluidics script FS450_0007) and scanning was done according to the Affymetrix expression protocol including minor modifications as suggested in the Encore Biotin protocol (NuGEN). Expression console (Affymetrix) was used for quality control and to obtain annotated normalized robust multi-array analysis gene level data (standard settings including sketch-quantile normalization). Statistical analyses were performed by utilizing the statistical programming environment R implemented in CARMAweb [17]. Genewise testing for differential expression was done employing the limma t-test (paired) and Benjamini-Hochberg correction for multiple testing (false discovery rate <10%). Gene Ontology and Kyoto Encyclopedia of Genes and Genomes term and pathway enrichment analyses were performed with GePS (Genomatix, Germany) and significant terms ($p < 0.01$) were determined. In addition, Ingenuity Pathway Software (http://www.ingenuity.com) was used to identify enriched pathways. Array data have been submitted to GEO (GSE67279).

2.8. Further statistical analyses

To approximate normal distribution, serum FGF21 concentrations were log_e-transformed prior to statistical analysis. Differences in serum FGF21 between the MHO and MUHO groups were tested by multiple linear regression analysis with FGF21 concentration as outcome variable, the nominal MHO/MUHO classification as independent variable, and gender, age, and BMI as confounding variables. All *in vitro* data, except microarray data (see above), were analyzed by two-group comparisons (FGF21-treated vs. untreated) using two-tailed paired Student's t-test. A p-value <0.05 was considered statistically significant. For these analyses, the statistical software package JMP 10.0 (SAS Institute, Cary, NC, USA) was used.
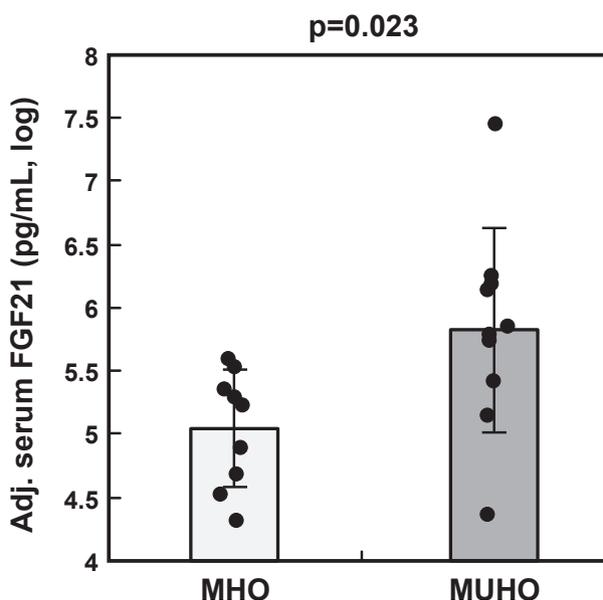
3. RESULTS

3.1. FGF21 concentrations in MHO and MUHO subjects

After adjustment for the putative confounders gender, age, and BMI by multiple linear regression modeling, serum FGF21 concentrations were more than twice as high in MUHO subjects as compared to MHO subjects (unadjusted 457 ± 378 vs. 211 ± 123 pg/mL, means $\pm$ SD; adjusted data presented in Figure 1; $p = 0.023$).

3.2. FGF21 responsiveness of differentiating hasc preadipocytes

The FGF21 receptor complex, composed of FGF receptor-1 and β-klotho, was readily detectable on the gene expression level as determined by qPCR (mean Cp-values in undifferentiated preadipocytes: *FGFR1* 22.8, *KLB* 33.9). To further provide evidence that hasc preadipocytes are responsive to rhFGF21, differentiating preadipocytes (from day 8 of differentiation) were incubated for 10 min with 50 ng/mL (2.6 nmol/L) rhFGF21, a concentration that is above the physiological

Figure 1: Serum FGF21 concentrations in MHO and MUHO subjects Data adjusted for gender, age, and BMI are shown as log-transformed individual data and means $\pm$ SD ($N = 10$ per group). Adjustment was performed by multiple linear regression modeling. MHO — metabolically healthy obesity; MUHO — metabolically unhealthy obesity.

range (reported to reach up to 3 ng/mL [1]), but well within the pharmacological range reported for novel FGF21 analogues/mimetics (17.5—150 ng/mL [18]), and below the high concentrations usually used in *in vitro* settings (≥ 1 μg/mL [19—21]). This acute treatment induced robust phosphorylation of AMPKα, ERK1, and ERK2 (Figure 2), well-known FGF21 downstream signaling events [19,22]. Therefore, we used 50 ng/mL rhFGF21 in all subsequent experiments.

3.3. Effect of chronic FGF21 treatment on lipid accumulation

As shown by qualitative Oil Red O staining (Figure 3A) and photometric quantification of the intracellular Oil Red O content over time (Figure 3B), triglyceride accumulation was significantly increased by rhFGF21 during the early stages of differentiation. This effect, however, was modest (+15%, days 4 and 8) and diminished to a statistical trend at later time-points ($p = 0.09$, days 12 and 18; Figure 3B).

3.4. Effects of chronic FGF21 treatment on white and brown adipocyte marker gene expression

Chronic rhFGF21 treatment significantly impaired the expression of the key adipogenic transcription factors peroxisome proliferator-activated receptor-γ (PPAR-γ; -15%, day 18; Figure 4A) and CCAAT/enhancer-binding protein-α (C/EBP-α; up to −40%, days 8—18; Figure 4B). In addition, rhFGF21 reduced the expression of the adipokine adiponectin (up to −20%; days 4 and 18; Figure 4C), whereas leptin expression remained unaffected ($p > 0.08$). Unexpectedly, the brown adipocyte marker genes encoding uncoupling protein-1 (UCP-1), PPAR-γ coactivator-1α (PGC-1α), and cell death-inducing DNA fragmentation factor-like effector a (CIDEA) were differently regulated with UCP-1 being 2-fold induced by rhFGF21 (days 12 and 18; Figure 4D) and PGC-1α and CIDEA being repressed by up to −40% and −50%, respectively (all time-points; Figure 4E,F). Similar regulations of the brown adipocyte marker genes were seen when rhFGF21 was sub-chronically added for three days to adipocytes (from day 18) that were differentiated in the absence of rhFGF21 (data not shown). It

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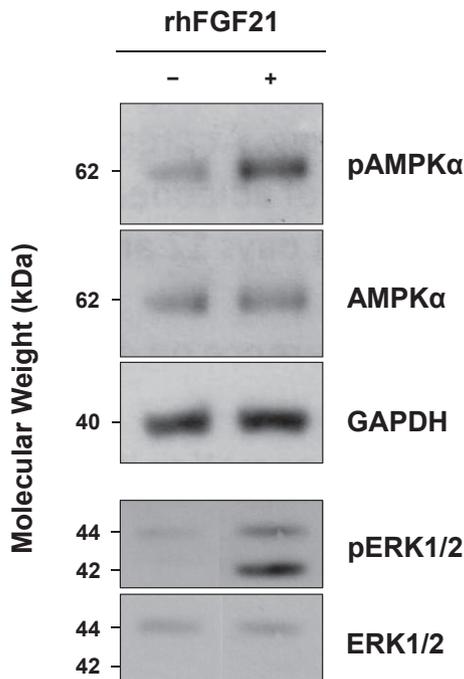
rhFGF21

Figure 2: FGF21 signaling in differentiating hasc preadipocytes Cells from day 8 of the differentiation protocol were left untreated (−) or were treated (+) for 10 min with rhFGF21 (50 ng/mL). Immunoblotting was performed with primary antibodies against phospho-threonine 172 of AMPKα, AMPKα protein, phosphorylated ERK1 and ERK2, and ERK1/2 protein. A primary antibody against GAPDH was used as loading control. One immunoblot representative of three biological replicates is shown. AMPK — AMP-activated protein kinase; ERK — extracellular signal-regulated kinase; hasc — human abdominal subcutaneous; GAPDH — glyceraldehyde 3-phosphate dehydrogenase; rh — recombinant human.

should be noted that the UCP-1 expression levels were very low (mean Cp-value on day 0: 35.9; mean Cp-value on day 18: 31.4). Moreover, we did not obtain higher expression levels using isoproterenol, a well-known and potent inducer of the gene. Therefore, we concluded that hasc preadipocytes represent a cell type with very limited capacity to brown. In accordance with this, we did not detect any convincing signal for UCP-1 protein by immunocytochemistry.

### 3.5. Effects of chronic FGF21 treatment on adipokine secretion

Secretion of the major adipokines adiponectin, leptin, IL-6, and TNF-α upon chronic treatment with rhFGF21 was assessed in 48-hour conditioned culture media at different time-points after start of differentiation. Adiponectin release into the medium was markedly reduced (up to −60%; days 8 and 18; Figure 5A), whereas that of leptin (up to +60%; days 12 and 18; Figure 5B) and interleukin-6 (up to +50%; all time-points; Figure 5C) was substantially increased by rhFGF21 treatment. TNF-α secreted by preadipocytes of day 0 ranged at the detection limit (<5 pg/mL) and became undetectable at later time-points both in the absence and presence of rhFGF21 (data not shown). To see whether the rhFGF21-induced increase in IL-6 release was due to enhanced IL-6 gene expression, we performed qPCR. In accordance with the release data, IL-6 gene expression was induced by rhFGF21 (up to +45%; days 4, 8, and 18; Figure 5D).

Upon a closer look at the temporal course of adiponectin release, as depicted in Figure 5A, it appeared as if FGF21 not only reduces overall adiponectin release but also alters the shape of the release curve: in the absence of chronic FGF21, adiponectin release appeared biphasic

with a prominent peak on day 8 (first phase) and half-maximum levels on day 12 that modestly further declined from day 12 on (second phase); chronic FGF21 treatment changed the curve into a bell-shaped one with a markedly diminished, broader, and delayed peak between days 8 and 12; as a result of this, there is a crossing point of the curves on day 12.

3.6. Role of candidate genes/pathways underlying FGF21-induced adiponectin suppression

Adiponectin is a major secretory product of adipocytes and an important insulin-sensitizing adipokine [23]. Therefore, we tried to further decipher the adiponectin-suppressive effect of chronic FGF21 treatment. Even though the repressive effect of FGF21 on adiponectin expression may be explained by impaired PGC-1α expression (and
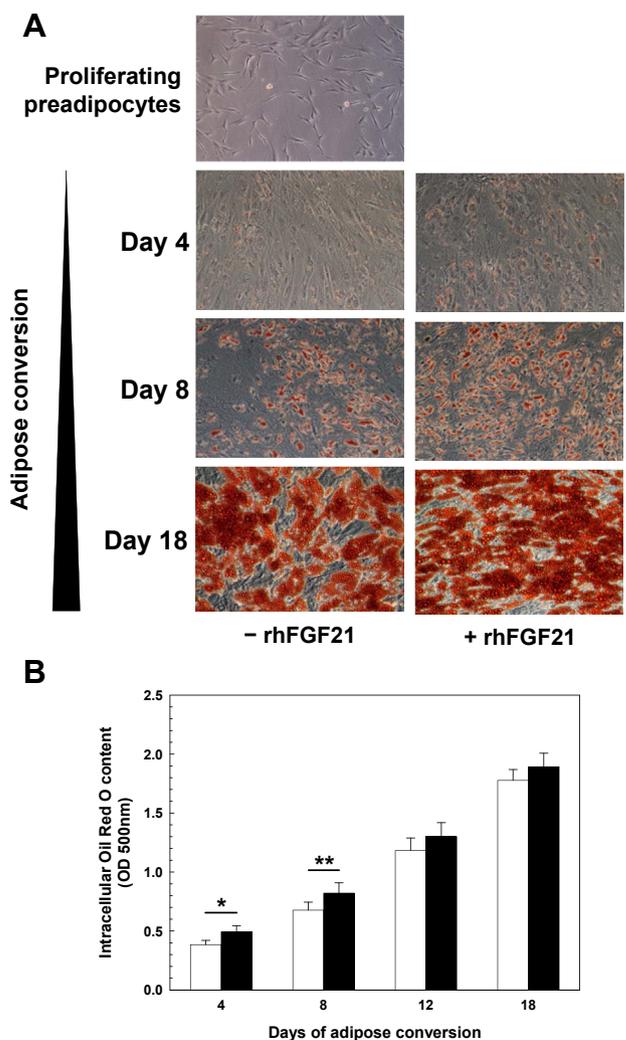
Figure 3: FGF21 effect on lipid accumulation in differentiating hasc preadipocytes (A) Triglyceride accumulation was visualized by Oil Red O staining of proliferating preadipocytes (topmost panel) and of differentiating adipocytes (from days 4, 8, and 18) left untreated (left column of images) or chronically treated with rhFGF21 (right column of images). Microscopical pictures of Oil Red O-stained cultures representative of 20 biological replicates are shown. (B) The Oil Red O content of cultures was photometrically quantified at 500 nm after stain extraction with isopropanol (means ± SEM; N = 20). Black bars — rhFGF21-treated cultures; white bars — untreated controls. hasc — human abdominal subcutaneous; OD — optical density; rh — recombinant human. *p < 0.05, **p < 0.01.
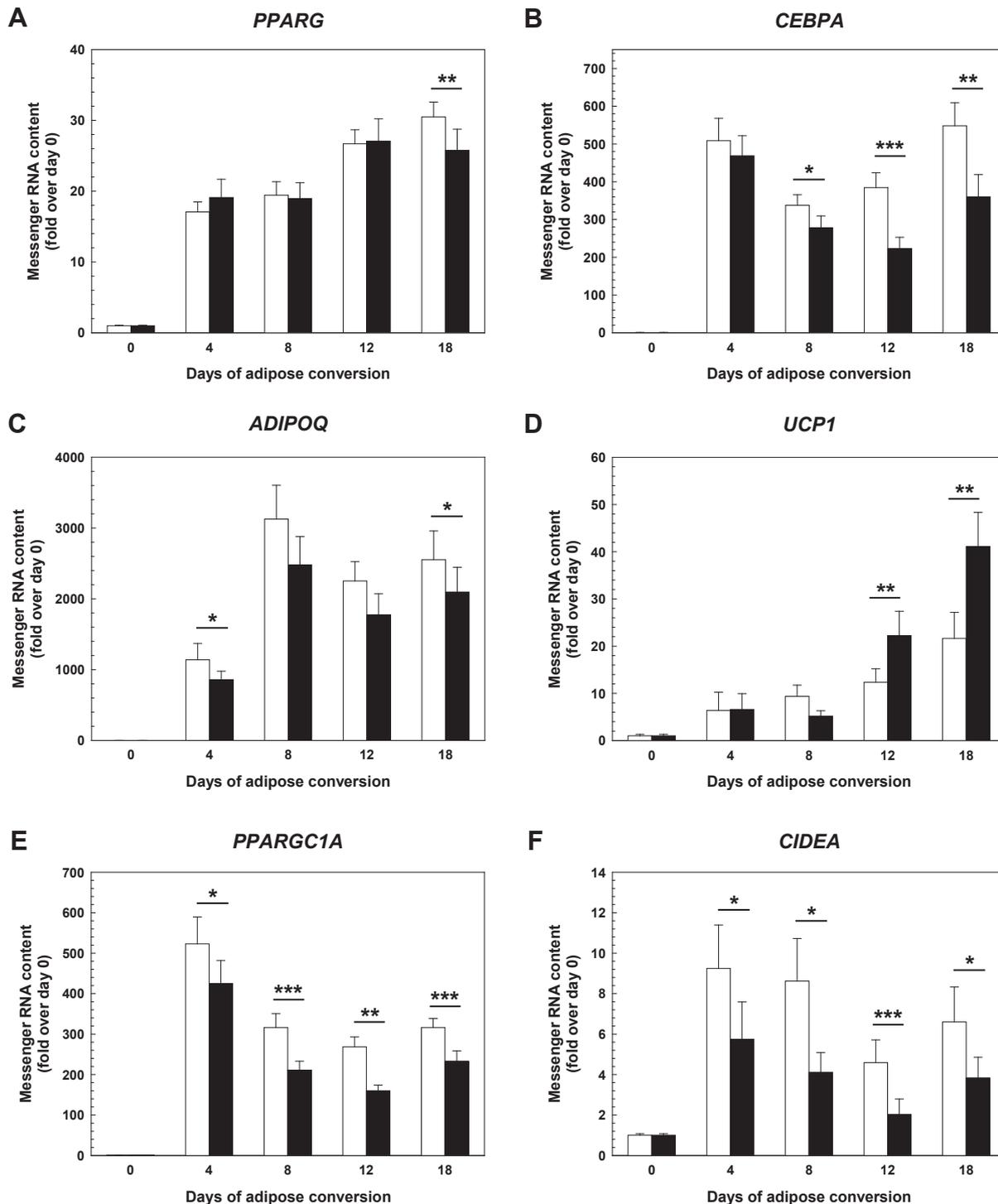
Figure 4: FGF21 effects on white and brown adipocyte marker gene expression in differentiating hasc preadipocytes The mRNA expression of *PPARG* encoding PPAR-γ (A), *CEBPA* encoding C/EBP-α (B), *ADIPOQ* encoding adiponectin (C), *UCP1* encoding UCP-1 (D), *PPARGC1A* encoding PGC-1α (E), and *CIDEA* encoding CIDEA (F) was monitored by real-time qPCR (means ± SEM; N = 20). Black bars — rhFGF21-treated cultures; white bars — untreated controls. C/EBP-α — CCAAT/enhancer-binding protein α; CIDEA — cell death-inducing DNA fragmentation factor-like effector a; hasc — human abdominal subcutaneous; PGC-1α — PPAR-γ coactivator 1α; PPAR-γ — peroxisome proliferator-activated receptor γ; qPCR — quantitative polymerase chain reaction; rh — recombinant human; UCP-1 — uncoupling protein 1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

consequently reduced activity of the adiponectin-inducing transcription factor PPAR-γ), the effect size of adiponectin gene repression was modest (−20%) and did not correspond to the marked adiponectin release-suppressive effect of FGF21 (−60%). Hence, we asked whether chronic rhFGF21 treatment affects the expression of genes involved in cellular protein synthesis, protein degradation, or exocytosis. Using whole-genome microarray gene expression analysis and subsequent single-gene as well as pathway analyses based on Gene

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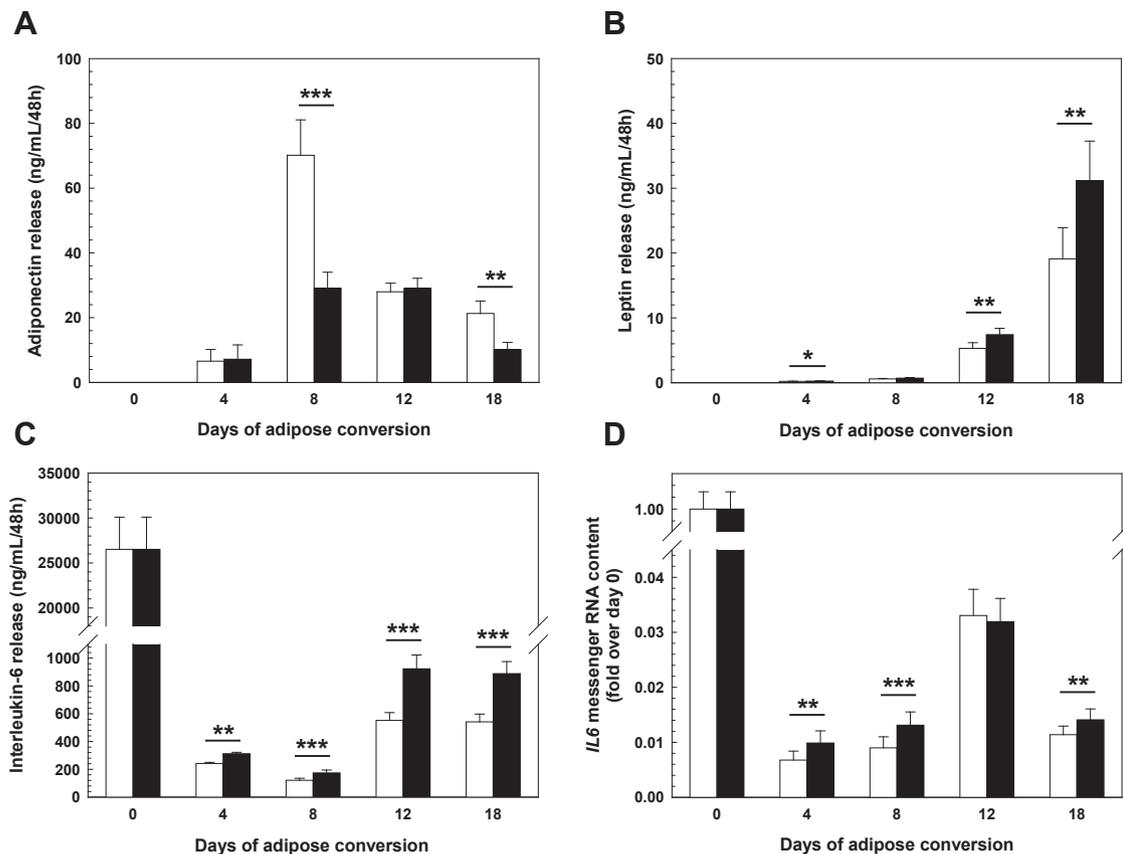
Figure 5: FGF21 effects on adipokine release and interleukin-6 gene expression in differentiating hasc preadipocytes The concentrations of adiponectin (A), leptin (B), and interleukin-6 (C) in media conditioned for 48 hours by differentiating preadipocytes was measured by enzyme-linked immunosorbent assays (means ± SEM; N = 20). The mRNA expression of *IL6* encoding interleukin-6 (D) was monitored by real-time qPCR (means ± SEM; N = 20). Black bars — rhFGF21-treated cultures; white bars — untreated controls. hasc — human abdominal subcutaneous; rh — recombinant human. *p < 0.05, **p < 0.01, ***p < 0.001.

Ontology and Kyoto Encyclopedia of Genes and Genomes terms did not reveal any significant rhFGF21-induced changes in these pathways (Supplementary Figure S1, Supplementary Tables S1 and S2). This whole-genome gene expression analysis, however, confirmed the IL-6-inducing (+50%; day 8) and PGC-1α-repressing (−30%; day 8) effects of rhFGF21 (+49% and −29%; Supplementary Table S1). As a second hypothesis, we tested whether the enhanced IL-6 expression of FGF21-treated cells might affect, in an auto-/paracrine mode, adiponectin release. Neither the chronic use of a neutralizing anti-IL-6 antibody nor that of a pharmacological inhibitor of IL-6 signaling (small molecule STAT3 inhibitor stattic) significantly influenced rhFGF21-dependent suppression of adiponectin release (data not shown).

4. DISCUSSION

In the present study of morbidly obese human subjects, we asked whether FGF21 is a potential cross-talker between the liver, where it originates in humans, and adipose tissue, affecting human (pre) adipocyte properties in a way that may discriminate MUHO from MHO. In humans, FGF21 concentrations in blood directly correlate with BMI, overall body fat mass, visceral fat mass, pericardial fat mass, and ectopic lipid deposition [10,24–31]. On the other hand, anorexia nervosa is associated with lower plasma FGF21 levels [32], and acute fat loss due to bariatric surgery was also reported to reduce plasma

FGF21 [33]. Here we show that circulating FGF21 levels are more than two-fold higher in MUHO as compared to body fat-matched MHO subjects, pointing to an adiposity-independent role of FGF21 in insulin resistance in humans. This is in keeping with previous studies reporting BMI- and body fat mass-independent associations of plasma FGF21 levels with hepatic and whole-body insulin resistance [6,26,27]. This finding opened the possibility that pathophysiologically high plasma concentrations of the human hepatokine FGF21, as measured in the morbidly obese subjects of the MUHO group, can exert metabolically detrimental effects and thus may contribute to the MUHO phenotype.

To explore whether chronically elevated concentrations of FGF21 alter human (pre)adipocyte properties, we applied a moderately supraphysiological concentration (50 ng/mL) for 18 days and studied its impact on adipose conversion, lipid storage, and adipokine secretion of differentiating hasc preadipocytes. We detected modest lipogenic and anti-adipogenic effects of this treatment. Most impressively however, chronic FGF21 treatment markedly altered the release of the classical adipokines adiponectin, leptin, and IL-6: FGF21 potently suppressed adiponectin release and enhanced leptin and IL-6 release with the effects on adiponectin and IL-6 being, at least in part, reflected by FGF21's impact on the expression of the respective genes. Since the FGF21 concentration used in these experiments is well within the range of pharmacological blood concentrations reached with FGF21 analogues/mimetics [18], the described unfavorable FGF21 effects on

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(pre)adipocyte properties could be of particular relevance for the clinical use of these novel compounds currently in development for the treatment of diabetes. Even though the adiponectin-suppressive effect of FGF21 observed in this study is in agreement with results from several human cross-sectional and intervention studies showing an inverse relationship between plasma FGF21 and plasma adiponectin concentrations [29,31−35], a recent 28-day clinical trial with LY2405319, an FGF21 variant engineered to improve biopharmaceutical properties, revealed treatment-induced elevations of plasma adiponectin [18]. The origin of this discrepancy is unclear but may be due to the structural peculiarities of LY2405319 resulting from the introduction of an additional disulfide bond, deletion of four N-terminal amino acids, and elimination of an O-linked glycosylation site [36]. With respect to physiology and to adiponectin's well-known insulin-sensitizing properties, adiponectin suppression by the natural form of human FGF21, as observed in our study in differentiating hasc pre-adipocytes, could be a possible reason for FGF21's association with insulin resistance in humans.

As to the molecular mechanism(s) underlying adiponectin suppression, the observed FGF21-induced repression of adiponectin gene expression may, of course, be relevant. Adiponectin gene repression could, in turn, result from impaired PGC-1α gene expression as PGC-1α is an essential coactivator of the transcription factor PPAR-γ, a well-described transcriptional inducer of the adiponectin gene [37,38]. However, the repressive effect of FGF21 on adiponectin gene expression was modest compared to the FGF21-induced reduction in adiponectin release. Thus, additional mechanisms might contribute to FGF21-dependent adiponectin suppression. In this context, we assessed whether IL-6, which is induced and released upon FGF21 treatment, acts as an auto-/paracrine negative feedback regulator of adiponectin release, as was hypothesized in earlier studies [39,40]. However, our attempts to interfere with IL-6 signaling were without any effect on adiponectin release. Our results obtained with a neutralizing anti-IL-6 antibody and an inhibitor of IL-6 signaling are certainly insufficient to ultimately exclude a role of adipocyte-derived IL-6 in FGF21-induced adiponectin suppression, and further studies are needed to clarify this issue. Finally, we tested whether adiponectin suppression is due to FGF21-induced alterations in the expression of genes involved in more general pathways, such as protein synthesis, protein degradation, and/or exocytosis. Since our whole-genome microarray analysis was well-powered (N = 20) and did not reveal any significant FGF21 effects on these pathways, we suggest that other, still unknown, mechanisms may contribute to FGF21-dependent adiponectin suppression.

Even though mechanistically unresolved, FGF21-induced adiponectin suppression may establish a novel hypothesis as depicted in Figure 6: fatty liver, a hallmark of MUHO [11,41], produces elevated levels of circulating FGF21 [10,42]. FGF21, in turn, suppresses adiponectin production in adipose tissue as suggested by our data herein, and this is in line with the reduced serum adiponectin levels of our MUHO subjects [11,13] as well as with the inverse relationship between FGF21 and adiponectin levels observed in several cross-sectional and longitudinal studies [29,31−35]. As FGF21 is repressed by low- and high-molecular-weight forms of adiponectin in primary human hepatocytes [43] and adiponectin knockdown *vice versa* results in increased hepatic FGF21 expression [44], FGF21-induced hypoadiponectinaemia may further enhance hepatic FGF21 production and establish a vicious cycle aggravating whole-body insulin resistance.

One might argue that a bias of our work is that we do not discuss our data with respect to the wealth of findings about FGF21 in mice. However, we are convinced that such a discussion, against the background of obvious species-specific differences with respect to the
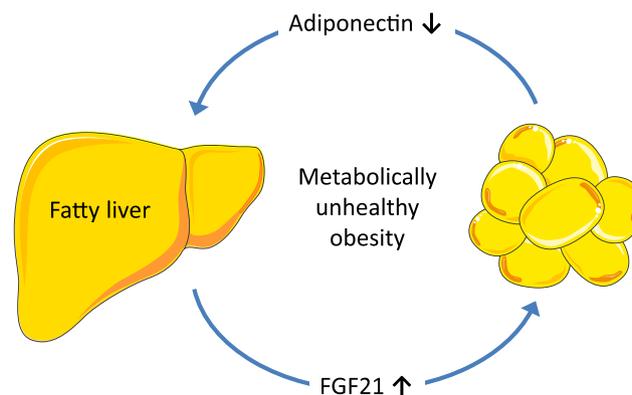
Figure 6: Hypothetical model describing the role of FGF21 and adiponectin in metabolically unhealthy obesity Fatty liver, a hallmark of metabolically unhealthy obesity, produces elevated levels of circulating FGF21 which, in turn, suppress adiponectin production in adipose tissue. As adiponectin acts as a physiological inhibitor of hepatic FGF21 expression, hypoadiponectinaemia results in unblocked FGF21 production in the liver. This establishes a vicious cycle that aggravates whole-body insulin resistance.

sites of FGF21 production, the regulation of FGF21 expression, the relationship of circulating FGF21 levels with pathophysiological states, and certain FGF21 effects (as was recently reviewed in [2]), is not helpful as long as we do not know the exact molecular basis of these discrepancies. Therefore, the primary focus of our work was to get closer to an understanding of FGF21's role in humans by discussing our human *in vivo* and *in vitro* findings in the light of what is known about FGF21 in humans.

5. CONCLUSIONS

The hepatokine FGF21 exerts weak lipogenic and anti-adipogenic effects and marked adiponectin-suppressive and leptin and interleukin-6 release-promoting effects in cultured hasc adipocytes. Together with the markedly higher serum concentrations in MUHO subjects, our findings point to FGF21 as a circulating factor promoting the development of metabolically unhealthy adipocytes and hypoadiponectinaemia. These metabolically unfavorable FGF21 effects could be of particular relevance for the clinical use of FGF21 analogues/mimetics currently in pharmaceutical development.

CONFLICT OF INTEREST

The authors have no conflicts of interest related to this study.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.04.002.

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