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Review

Going Back to the Biology of FGF21: New Insights

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Fibroblast growth factor 21 (FGF21) is a protein highly synthesized in the liver that exerts paracrine and endocrine control of many aspects of energy homeostasis in multiple tissues. In preclinical models of obesity and type 2 diabetes, treatment with FGF21 improves glucose homeostasis and promotes weight loss, and, as a result, FGF21 has attracted considerable attention as a therapeutic agent for the treatment of metabolic syndrome in humans. An improved understanding of the biological role of FGF21 may help to explain why its therapeutic potential in humans has not been fully realized. This review will cover the complexities in FGF21 biology in rodents and humans, with emphasis on its role in protection from central and peripheral facets of obesity.

Highlights

FGF21 is an endocrine factor shown to impact whole-body metabolism in preclinical species and adult humans.

FGF21 is under investigation as a novel therapeutic agent for the treatment of diabetes and its associated comorbidities. Expression of the FGF21 receptor/β-klotho complex (FGFR1-KLB) determines target tissue specificity; adipose tissue, liver, and the central nervous system have emerged as key target organs.

Engineered FGF21 analogs, at pharmacological levels, show considerable improvement in the metabolic syndrome phenotype in animal models. However, only some of these effects (reduced dyslipidemia and body weight) are evident in humans. This represents a significant challenge for the translational potential of engineered FGF21-based therapeutics.

FGF21: A Novel Metabolic Regulator

Fibroblast growth factor 21 (FGF21) is a hormone shown to play a key role in the regulation of energy homeostasis in both preclinical studies and man. Consequently, leveraging the broad metabolic effects of FGF21 for the treatment of metabolic syndrome has received significant interest from the pharmaceutical industry. The therapeutic potential of FGF21 was first recognized when it was reported that FGF21 reduces plasma glucose and triglycerides in mouse models of obesity, and that overexpression of FGF21 provided resistance to body weight gain in mice on a high-fat diet (HFD) [1,2]. However, contemporary data challenge existing concepts, and many facets of FGF21 biology remain divisive and/or poorly understood. Furthermore, only some of the metabolic effects characteristic of FGF21 action in preclinical studies using laboratory rodents are evident in humans, representing a significant challenge for the translational potential of engineered FGF21-based therapeutics. Indeed, several human clinical trials using FGF21 analogs have failed to observe meaningful blood glucose lowering and insulin sensitizing effects, although significant improvements in dyslipidemia and markers of fatty liver disease have been reported (Box 1). Consequently, FGF21 biology is rapidly moving into new arenas: control of macronutrient preference, providing protection from nonalcoholic steatohepatitis and nonalcoholic fatty liver disease (NASH/NAFLD, respectively), or more broadly as a biomarker for disease. Hence, there is a need to go back to the natural biology of FGF21 with the objective of better understanding its role in these diseases to maximize its therapeutic potential (see Boxes 2 and 3).

Since its initial isolation from liver in 2000 [9], FGF21 has been shown to be widely expressed in metabolic organs, including the gastrointestinal tract, adipose tissue, and pancreas [10]. FGF21 has been demonstrated to cross the blood–brain barrier in rats, is detectable in human cerebrospinal fluid (CSF), and there is a linear relationship between serum and CSF levels [11,12]. Furthermore, FGF21 biology demonstrates circadian rhythmicity (serum concentrations exhibit a clear diurnal rhythm) and dysregulation correlates with obesity-induced lipid disorders [13]. Despite this and its widespread distribution, various strains of FGF21 knockout (KO) mice show variable effects on body weight and composition and circulating glucose. This in itself is controversial, with some groups reporting fasting hypoglycemia, a possible consequence of the age of the animals and/or the fasting schedules [14–16]. Although these animals showed nor-

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Box 1. Clinical Trials with FGF21 Analogs

Long-acting FGF21 analogs have been utilized in several human clinical trials, showing improvement in some, but not all aspects of the metabolic syndrome. In overweight/obese patients with type 2 diabetes (T2D), treatment with LY2405319, a variant of FGF21, produced improvements in fasting insulin levels but only a modest, nonsignificant fasting glucose lowering effect [3]. Similarly, treatment with PF-05231023, a long-acting FGF21 analog, and Pegbelfermin (BMS-986036), a PEGylated human FGF21 analog, had no effects on glycemic control and HbA1c in overweight/obese patients with T2D [4–6]. The effects of those analogs on body weight was variable, with no change observed after 12 weeks of treatment with BMS-986036 [6], a small reduction of 1.8% in response to 4 weeks of LY2405319 [3], and a modest loss of 5%–6% after 4 weeks on PF-05231023 [4]. However, all trials reported improvements in dyslipidemia, with significant reductions in plasma triglycerides (up to 50%) and improved plasma lipoprotein profile (reductions of total cholesterol by up to 20% and low-density lipoprotein by ~30%, and increases in high-density lipoprotein by up to 25%). Overall, the effects of FGF21 analogs on aspects of glucose control and body weight loss are modest at best and cannot match the improvements observed with current pharmacological and lifestyle changes-based treatments targeting weight loss and/or improvement of β-cell function and insulin sensitivity in patients with T2D [7]. However, the therapeutic potential of FGF21 analogs may be realized when combined with other current therapies targeting glycemic control or as a monotherapy to improve plasma lipid profiles and for the treatment of NASH/NAFLD [8].

mal growth, development, and fertility, subsequent studies have suggested that FGF21 may have adverse effects on these processes but is not essential [1,16–20]. Thus, the physiological role of FGF21 is in the adaption to altered metabolic demand, for example, in times of stress; levels of FGF21 are increased under a variety of physiological interventions and pathogenic events, including starvation (acutely in mice, chronically in humans), obesity (in mice and humans), and exercise (in mice and humans) [17,21–23]. Furthermore, the effects of treatment with exogenous FGF21 are pleiotropic. Here we will explore the complexities in FGF21 biology and physiology in animal models and adult humans, its pharmacology, and metabolic actions from a preclinical and clinical perspective.

Adipose Tissue: A Key Determinant of FGF21 Action

Adipose tissue is a highly dynamic organ, which, in response to the internal environment, undergoes extensive remodeling. Caloric restriction, for example, promotes mitochondrial biogenesis and fatty acid oxidation, whereas white adipose tissue (WAT) rapidly expands in response to an obesogenic diet by increasing lipid storage and the number of adipocytes [58,59]. These are evolutionary protective mechanisms to ensure adequate glucose supply for the brain and delivery of free fatty acids (FFA) and amino acids to peripheral organs. Adipose tissue is known to play a crucial role in the buffering of diet-derived FFA in the postprandial period [60]. If this role, however, is impaired due to a disruption of insulin signaling in adipose tissue, excessive lipids accumulate in other tissues such as liver and skeletal muscle, and may result in the development of insulin resistance [61]. Therefore, insulin-sensitizing agents targeting adipose tissue are in high demand.

Initially, FGF21 was shown to stimulate glucose uptake in 3T3-L1 adipocytes via glucose transporter 1 (GLUT1) [2], an effect dependent on ERK1/2 and ELK-1 signaling [27]. Indeed, acute

Box 2. The Biology of FGF21

FGF21 (gene ID 56636) is located on chromosome 7, contains three coding exons, and produces a single transcript, a pre-protein of 210 amino acids (aa) with a N terminal signal peptide of 22aa. In humans, FGF21 (gene ID 26291) also contains three exons, however, this produces two transcripts via alternative promoters, both of which encode a pre-protein of 208aa, including a 28aa signal peptide. The sequence identity is circa 80%. Functionally, the FGF superfamily is grouped into three subfamilies: intracellular FGFs, which lack a signal peptide; FGFs, which lack heparin sulfate-binding capacity, indicating the potential for their escape into the circulation, exerting systemic action; and FGFs with a high heparin sulfate-binding capacity, thus potentially acting in an autocrine/paracrine manner [24]. At target tissues, FGF21 binds to and activates members of the FGFR superfamily of receptor tyrosine kinases [25]. FGFR activation by FGF21 *in vitro* and *in vivo* is dependent on obligate co-receptor βKlotho (KLB), an FGFR-binding, single-pass transmembrane protein [2,26]. Preference for both FGFR1c- and FGFR3c-KLB complexes has been demonstrated, so given the widespread expression of the FGFRs, tissue specificity and sensitivity is mainly governed by the presence and relative expression of KLB [27–30]. Activation of these receptor complexes by FGF21 leads to a plethora of rapid signaling events [31,32].

Box 3. Tissue-Specific Physiology

The highest level of FGF21 expression in the adult mouse is the liver. The liver is regarded as the major source of circulating FGF21; liver-specific deletion of FGF21 created by crossing FGF21-floxed mice ($FGF21^{fl/fl}$) with an albumin-Cre line results in dramatic reductions in circulating FGF21, and the increase typically observed in response to fasting is lost [33]. Peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor activated by fatty acids, is required for the normal adaptive response to starvation [34]. Mice lacking PPARα accumulate hepatic triglyceride and become hypoketonemic during fasting and starvation [35–37]. A downstream target of PPARα, hepatic FGF21, increases in response to dietary manipulation (fasting, ketogenic diet) and is rapidly suppressed by refeeding in mice [17,18,38]. Indeed, FGF21 was offered as the 'missing link' in the biology of fasting, inducing adipose tissue lipolysis, liver ketogenesis, and metabolic adaptation to the fasting state [39]. Furthermore, $FGF21^{-/-}$ mice showed impaired adaptation to ketosis, whilst adenoviral knockdown of FGF21 in the liver caused mice consuming a ketogenic diet to display fatty livers, hypertriglyceridemia, and reduced fat oxidation [15]. Having established that FGF21 was critical to the adaptive metabolic response to starvation in mice, data emerged demonstrating the induction of FGF21 in response to amino acid deprivation and protein restriction. This is mediated by the eukaryotic translation initiation factor (EIF) 2α-activating transcription factor (ATF)4-CHOP axis of the endoplasmic reticulum stress response in mice [40]. Mice consuming diets deficient in methionine, leucine, and alanine demonstrated increased serum FGF21 concentrations [40–42]. Furthermore, consumption of methionine/choline-deficient diets in mice results in NASH and is associated with increased hepatic expression and serum levels of FGF21. FGF21 knockout mice have an exacerbated phenotype on this deficient diet, in part due to attenuated activation of fatty acid oxidation, as well as increased expression of genes involved in lipid uptake and synthesis [43,44]. Pharmacological treatment of these mice with FGF21 resolved the phenotype.

administration of FGF21 to ob/ob and db/db mice or to mice with diet-induced obesity rapidly improved insulin sensitivity and decreased plasma glucose levels [51,62]. However, the effect on glucose uptake and GLUT1 in adipose tissue was not apparent in chronically treated animals, despite lower blood glucose, which was achieved via the suppression of hepatic glucose production [63]. Subsequently, FGF21 was shown to increase glucose uptake in brown adipose tissue (BAT) and WAT in lean mice, suggesting a differential response to FGF21 dependent on metabolic status and/or adiposity [64].

Chronic treatment of mice with FGF21 did, however, increase energy expenditure and result in weight loss [65,66] and, as a consequence, improve insulin sensitivity. However, the role of uncoupling respiration via activation of UCP1 to the pharmacological effects of FGF21 is not completely resolved. Studies using UCP1-KO mice have shown that several but not all metabolic endpoints are UCP1 independent [67,68]. Whereas UCP1-mediated thermogenesis was not required for FGF21 to lower body weight, improve glycemic control, or reduce circulating insulin and lipids [67,68], the ability of FGF21 to promote postprandial glucose tolerance was impaired in the absence of UCP1 in obese, glucose intolerant mice [69]. However, the ability of FGF21 to augment energy expenditure was significantly attenuated in one [67] but not another study [68]. Further research is required to elucidate these seemingly contradictory findings.

FGF21 signaling in adipose tissue was essential for the acute insulin-sensitizing effects of FGF21, specifically in brown adipocytes, but not for its effects on body weight; this effect was via non-adipose tissues [70]. Interestingly, BAT is present in humans and correlates negatively with body mass index and fasting blood glucose levels [71]. However, humans possess relatively less BAT when compared with rodents, which may account for the attenuated glucose lowering responses to FGF21 treatment observed in human clinical trials (Box 1). Given the variable distribution of BAT in different populations (e.g., more commonly associated with women and lean individuals) [71], further targeted clinical work is required to determine the contribution of BAT in the metabolic responses to FGF21 in human subjects.

The increase in circulating FGF21 in response to fasting resulted in the hypothesis that FGF21 induces lipolysis in adipose tissue. Indeed, this was demonstrated *in vivo* [15,18]. However, *in vitro*,

FGF21 was shown to be a potent repressor of lipolysis in mouse (and human) adipocytes [72,73]. Acute doses and chronic treatment with FGF21 reduce FFA [66,72]. Further work is required to dissect this duality and determine the precise role of FGF21 in lipolysis. At target tissues, FGF21 binds to and activates members of the FGFR superfamily of receptor tyrosine kinases [25]. FGFR activation by FGF21 is dependent on obligate co-receptor βKlotho (KLB), an FGFR-binding, single-pass transmembrane protein [2,26]. Mice lacking the FGFR1 receptor in adipose tissue show attenuated responses to the metabolic effects of FGF21 [74,75]. Similarly, mice with complete or adipose-specific ablation of KLB are resistant to acute insulin-sensitizing and glucose-lowering effects of FGF21 [74,76]. In mice, KLB expression is seen in adipose tissue, the liver, gall bladder, colon, and pancreas, as well as the suprachiasmatic and paraventricular nuclei of the hypothalamus and discrete regions of the brain stem [10,19]. Indeed, overexpression of KLB in adipose tissue sensitizes mice to endogenous FGF21 and offers protection from diet-induced obesity [29]. It was subsequently shown that KLB levels are markedly decreased in WAT during diet-induced obesity. Interestingly, TNF-α represses KLB expression and impairs FGF21 action in adipocytes *in vitro*, a mechanism mediated by TNF-α induced inflammation as the insulin-sensitizing anti-inflammatory drug rosiglitazone restored KLB levels [28]. Furthermore, high glucose represses KLB expression and impairs FGF21 action in mouse pancreatic islets [77]. Therefore, the concept of endogenous 'FGF21 resistance' has been raised, the biology of which requires further investigating as impairment of the pharmacological actions of FGF21 has not been shown. Furthermore, a hugely interesting aspect of FGF21 biology is the recent evidence from our animal studies (see Seasonal Lessons below), that the actions of FGF21 are enhanced in states of increased adiposity. Therefore, increasing KLB expression would be an interesting therapeutic option. Indeed, GLP-1 analogs and glitazones increase KLB expression *in vivo* [78,79] and may therefore increase FGF21 sensitivity, contributing to further metabolic improvements. However, maintenance of KLB protein expression in WAT does not alleviate impaired FGF21 signaling in this tissue or increase FGF21 sensitivity *in vivo* [80]. Despite these contradictory results on the role of KLB, adipose tissue appears to be important to the biology of FGF21 and its insulin sensitizing effects.

The Central Nervous System (CNS): The Final Frontier for FGF21

The CNS integrates a vast array of signals from the periphery to regulate feeding behavior and energy homeostasis; dysregulation results in obesity and T2D. These signals (hormones and nutrients, plus their by-products) act directly on the hypothalamus, the hindbrain, or peripheral neurons, which terminate in the nucleus of the solitary tract [81]. As noted above, FGF21 can cross the rodent blood–brain barrier [11] and is detectable in human CSF with a linear relationship between serum and CSF levels, suggesting that circulating FGF21 is the source of central FGF21 [12]; this is key for therapeutics whose primary targets are the CNS. The FGFR subtypes are widely expressed in the CNS, whilst KLB is highly expressed in the suprachiasmatic nucleus in the hypothalamus and in a number of hindbrain regions [19]. The film autoradiography/*in situ* hybridization strategy used by Bookout *et al.* [19] does not exclude the possibility that KLB might be expressed at lower levels or in isolated cells in other regions of the brain. Indeed Liang *et al*. [14] reported Western blot and immunostaining of KLB in the paraventricular nucleus of the hypothalamus of mice [14]. Overexpression of FGF21 lowers circulating levels of insulin and activation of the HPA axis and suppresses the reproductive axis [14,19,20]. Furthermore, intracerebroventricular injection of FGF21 increases sympathetic nerve activity in BAT; this effect is attenuated in mice lacking central KLB. Interestingly, treatment with a generic beta-blocker attenuated the effects of centrally administered FGF21 but not of FGF21 delivered peripherally. To exert its effects on energy expenditure, weight loss, and cholesterol in mouse models of obesity, central action of FGF21 may be required [82,83].

More recently, FGF21 has been shown to suppress the consumption of simple sugars and non-caloric sweeteners via actions on hypothalamic neurons [50]. In addition, FGF21 reduces alcohol preference in mice, whilst stimulating water drinking behavior [84,85]. The latter effect is inhibited by β-adrenergic receptor antagonists, suggesting a critical role of the sympathetic nervous system in mediating the effects of FGF21 in those responses. The emergence of FGF21 as a regulator of food preference both in rodents and humans, along with its role in metabolic regulation, raises the interesting prospect of its therapeutic potential in the dual regulation of appetite and hedonic behavior(s) and energy expenditure in obesity.

Seasonal Lessons

Whilst the ready availability of genetic tools have resulted in mice being the dominant animal model in recent years for studying mechanisms underlying energy metabolism, their high metabolic rate, rapid growth, and dependence on brown fat for thermogenesis when maintained under standard housing limits their translational value [86,87]. Most mammals are seasonal, in that they display profound annual cycles in appetite, fat metabolism, reproduction, and body weight [88]. Investigating the function of FGF21 in this context provides different insights into its biology, because deposition of body fat does not result in metabolic dysfunction. Thus, it becomes possible to study the effects of FGF21 in healthy obesity without the complications of the development of insulin resistance or NAFLD. Siberian hamsters (*Phodopus sungorus*) have been widely used as a seasonal rodent model because they can be readily maintained in animal facilities, and seasonal cycles in energy metabolism are generated simply by changing the environmental day length [89]. Under long-day conditions, typically with the light phase being 14 hours of light per day or more, these hamsters become hyperphagic and rapidly gain body weight, whereas under short days with the light phase being 8 hours per day or shorter they reduce their food consumption, and lose up to 30% of body weight over the course of a few months. This primarily reflects loss of visceral adipose depots, though significant loss of lean mass also occurs [90]. Thus, studies in this kind of seasonal mammal can address the role of FGF21 in natural situations of weight loss and gain, and provide the opportunity to investigate mechanisms of action of FGF21 in naturally occurring states of adiposity and leanness (Figure 1).

When exposed to short photoperiods, male hamsters show a significant increase in circulating FGF21 by 8 weeks that correlates with significant weight loss and decrease in epididymal fat pad mass at this time point [91]. Western blot analysis reveals that whilst FGF21 is detectable in liver, white fat, brown fat, and in muscle (gastrocnemius), only in the liver and brown fat are there significant increases in FGF21 concentrations as hamsters progress into short photoperiods [91]. The close correlation between liver and plasma FGF21 concentrations supports the argument that the liver is the predominant source of circulating FGF21. Similar observations have been made in another seasonal species, the 13-lined ground squirrel (*Ictidomys tridecemlineatus*). Serum FGF21 concentrations increase in squirrels exposed to short photoperiods in the fall, the increase being greater in animals in torpor than during bouts of arousal in winter, and concentrations then fall again as the photoperiod is increased in the following spring [92]. Again, these circulating FGF21 concentrations closely correlate with expression of FGF21 mRNA in the liver, as assessed by quantitative PCR [92]. A key question is whether the increases in circulating FGF21 contribute to the behavioral and physiological adaptations induced by the short photoperiod. Chronic infusion of hamsters maintained in summer long-day photoperiods with FGF21 results in some aspects of the short-day response, for example, decreased appetite and decreased body weight, reduced weight of epididymal fat depots, and a decrease in respiratory exchange ratio that would be consistent with increased whole-body fat oxidation [93,94] (Figure 1). These effects of FGF21 infusion were lost in hamsters tested after 12-week exposure to short-day winter photoperiods, at which point they had reached the nadir of the seasonal body

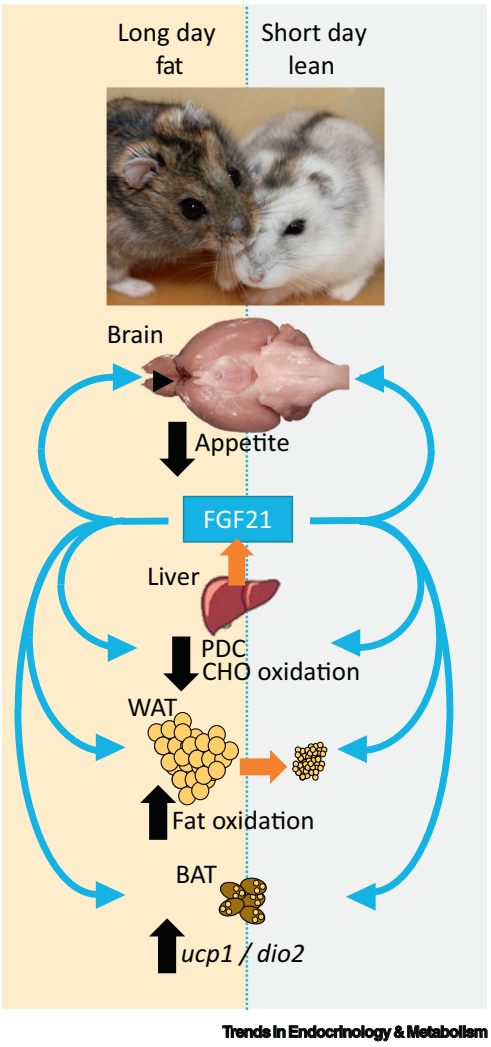

Figure 1. Actions of FGF21 in Fat and Lean Seasonal States. Exposure of Siberian hamsters to short days initiates molt from a summer agouti coat to a white winter coat (top) and programs a decrease in appetite and increased catabolism of visceral white fat (WAT) and hence body weight loss as a winter survival strategy. This is associated with increased hepatic fibroblast growth factor 21 (FGF21) production. Treatment of hamsters in the fat summer state with FGF21 induces behavioral and physiological changes that resemble the natural short-day response, including decreased appetite, promotion of fat oxidation rather than carbohydrate (CHO) oxidation (associated with decreased pyruvate dehydrogenase activity in liver; PDC), and increased thermogenic capacity of brown adipose tissue (BAT) via upregulation of uncoupling protein 1 (*ucp1*) and deiodinase II (*dio2*) gene expression.

weight cycle, and effects were also attenuated in ageing male Siberian hamsters that had reduced body weight [93]. These observations in naturally altered states of adiposity support the view that a major target of this hormone is WAT. This conclusion is supported by analysis of perirenal and subcutaneous WAT in hamsters treated with FGF21. For example, expression of *fgfr1c* and *βklotho* are upregulated in FGF21-treated hamsters, triglyceride lipase is increased and triglyceride content are reduced, and signal transduction events downstream of FGFR1c-βKlotho receptors including ERK1/2 phosphorylation are increased [93].

Although these studies in hamsters indicate that WAT is the main determinant of whole-body responsiveness to FGF21, they also identify additional targets (Figure 1). The thermogenic genes *ucp1* and *dio2* (deiodinase 2) were significantly upregulated in interscapular brown fat [93], which likely underlies increases in whole-body energy expenditure detected in hamsters infused with FGF21 [94]. Hepatic energy metabolism is also affected such that fatty acid oxidation is favored over carbohydrate oxidation. Indeed, FGF21 infusion reduced pyruvate dehydrogenase activity (PDC, the enzyme that catalyzes the rate-limiting step in glucose oxidation) and

acetylcarnitine accumulation (an index of reduced flux through PDC), but increased long-chain acylcarnitine content (suggesting an increased flux through β-oxidation) in the liver [54]. These dual effects of FGF21 on hepatic energy metabolism (Box 4) were mediated by increased protein abundance of PDK4 (a negative regulator of PDC), the phosphorylated (inhibited) form of acetyl-CoA carboxylase (a negative regulator of delivery of fatty acids into the mitochondria), and the transcriptional co-regulators of energy metabolism PGC1α and SIRT-1 [54].

Intriguingly, it appears likely that systemic FGF21 may also target the brain. There are consistent suppressive effects of FGF21 on appetite in hamsters [93,94]. This is in contrast to studies in mice, where FGF21 treatment has been associated with increased food intake, presumably a compensatory response to enhanced whole-body energy expenditure [65]. Whilst these effects do not necessarily indicate a direct action centrally, perhaps reflecting altered signaling from other peripheral signals to the brain, in hamsters the appetite-suppressing actions of FGF21 are associated with decreased levels of peripheral anorectic signals such as leptin and insulin [93]. Importantly, peripheral FGF21 induced upregulation of *dio2* in the hamster hypothalamus [95,96]. This enzyme, that is highly expressed in hypothalamic tanycytes, converts the thyroid hormone precursor thyroxine into the biologically active form tri-iodothyronine (T3). Altered local concentrations of T3 in the hypothalamus are considered to be the main driver of season cycles of appetite and energy expenditure [97,98], so this is a likely pathway of action for FGF21 regulating food intake in hamsters. Further evidence from studies in mice for central actions of FGF21 is considered in a previous section. The distribution of βKlotho has not yet been demonstrated in the hamster, but FGFR1c is expressed in the tanycyte cell layer of hamsters [99]. Intracerebroventricular treatment of hamsters with an anti-FGFR1c monoclonal antibody that may mimic the actions of FGF21 decreases appetite, increases whole-body energy expenditure and fat oxidation, and induces weight loss [99]. Given the recent evidence that tanycytes are in effect part of the blood–brain barrier in the hypothalamus and are critical in sensing nutrients and peripheral hormones [100], this is a likely target for peripheral FGF21 that requires further validation.

Box 4. Lessons from Rodent Models Provide a Liver-Centric View

One of the many paradoxes in the FGF21 field is that hepatic FGF21 expression is also increased in response to nutrient excess, a process described in rodents and humans that is likely mediated via the carbohydrate response element-binding protein (ChREBP), which may participate in the pathogenesis of NAFLD [9,21,45–47]. FGF21 levels in liver and adipose tissue are also elevated in db/db obese diabetic mice as compared with their lean db/+ littermates, in adult male rhesus macaque monkeys chronically maintained on a HFD, and in serum in overweight/obese humans [48,49]. Serum FGF21 is positively correlated with adiposity, fasting circulating insulin and triglycerides; logistic regression analysis demonstrated an independent association between serum FGF21 and metabolic syndrome [48]. It was subsequently shown that in response to carbohydrate intake, the liver produces FGF21 to selectively suppress sugar intake by acting on the paraventricular nucleus of the hypothalamus [50] (see the CNS section). Therefore, FGF21 is a key regulator of the nutritional state of the organism and coordinates the adaptive metabolic response; whilst the induction of FGF21 in response to changes in macronutrient ingestion is important for metabolic adaptation. Whether the liver is an important site of FGF21 action, however, is debatable. ERK1/2 phosphorylation and mRNA expression of immediate early gene markers downstream of the FGFR-KLB complex that are readily observed in adipose tissue in response to treatment with FGF21 are not so apparent in liver. However, Akt phosphorylation in the liver is reported in lean and ob/ob animals, in which FGF21 suppresses glucose production and increases insulin sensitivity [51]. Furthermore, other groups have shown that FGF21 does induce FRS2 and ERK1/2 phosphorylation in the liver of both lean and obese animals, with a time course and dose response similar to adipose tissue [28,52]. Furthermore, in HepG2 cells, FGF21 induces ERK1/2 phosphorylation, providing evidence that FGF21 may act directly on the hepatocyte [53]. Indeed, FGF21 exerts dual effects on hepatic energy metabolism, modulating net flux through both carbohydrate and fat metabolism [54] (see Seasonal Lessons section). Therefore, by stimulating hepatic fat oxidation, FGF21 may be an attractive target for protection from increased hepatic lipid content and insulin resistance that frequently accompanies obesity and T2D. Indeed, targeting hepatic fat accumulation and insulin resistance is a major therapeutic approach to the management of NAFLD and NASH, two of the most prevalent forms of liver disease that are closely associated with obesity and T2D [55]. An engineered variant of FGF21 has been shown to attenuate NASH progression in an animal model of NASH [56]; whereas a clinical Phase II trial (NCT02413372) with a recombinant FGF21 in NASH patients significantly reduced hepatic fat content and biomarkers of fibrosis [57]. Therefore, FGF21 may be emerging as a new class of drug for the treatment of fatty liver disease.

The catabolic effects of FGF21 are consistent with a role in facilitating the hypophagic and lipolytic state that occurs in hamsters in short days, but it should be borne in mind that the circulating concentrations of FGF21 delivered in these studies is in the supraphysiological range [94]. Nelson *et al*. tested the role of FGF21 in generating seasonal metabolic responses by overexpressing FGF21 in ground squirrels using an adenoviral approach [92]. This increased circulating FGF21 was associated with decreased circulating insulin and FFA concentrations, but was not sufficient to induce winter torpor bouts [92]. It is clear from analysis of hypothalamic gene expression that hamsters do not respond to the decrease in circulating leptin that reflects reduced caloric intake and increased lipolysis in short days with compensatory hyperphagia [101]. Perhaps the role of FGF21 in short days then is to promote metabolic adaptation to negative energy balance, rather than to be an underlying driver of winter physiology. Given the efficacy of FGF21 in hamsters in an obese but not metabolically compromised state, perhaps the optimal strategy for clinical use of FGF21 in humans would be in obese populations, particularly those with risk factors for cardiovascular and fatty liver diseases, prior to the development of T2D.

Advances in the Biology of FGF21: A Human Perspective

FGF21 has been implicated in a number of clinical and non-clinical conditions in humans, including the metabolic syndrome and T2D, hepatic liver disease, and metabolic regulation during exercise [48,102–105]. However, there are still unanswered questions remaining around its biological role in humans as a starvation hormone, exercise-induced myokine, and postprandial regulator of metabolism, in particular its link to insulin action and the metabolic syndrome.

FGF21 is often described as a key hormone in the biology of fasting in a variety of animal models. However, in humans, neither ketogenic diets (up to 3 months) nor short-term fasting (up to 48 hours) increase serum FGF21 levels [106]. Interestingly, elevations in FGF21 in humans are seen following prolonged fasting (7–10 days); a possible consequence of the divergent metabolic rates and adaptations to fasting between the species [22,107]. Since the ketogenic response in humans precedes the induction of FGF21 and ketogenic diets are associated with a reduction in circulating levels of FGF21, it appears that induction of FGF21 is not required to stimulate ketosis in humans but may serve to facilitate metabolic adaptations to prolonged periods of food deprivation [106].

Importantly, recent evidence suggests that FGF21 is an insulin-dependent postprandial hormone in humans (Figure 2). Indeed, short-term dietary carbohydrate, but not fat interventions, markedly increase the fasting circulating levels of FGF21 [47,108]. Furthermore, FGF21 secretion has been shown to respond to oral carbohydrate consumption, with fructose reported to increase plasma levels of FGF21 in humans to a greater extent than glucose [109]. Fructose ingestion was associated with elevated levels of circulating insulin, albeit to a lesser extent than glucose ingestion, which makes it difficult to dissociate its insulin-dependent and -independent effects. Using oral glucose tolerance tests and a combination of hyperglycemic and hyperinsulinemic clamps, Samms *et al*. [108] subsequently demonstrated that insulin rather than glucose *per se* increases both the total and the bioactive form of FGF21 in the postprandial period in humans. This supports observations from earlier cross-sectional studies showing a positive correlation between plasma insulin and total FGF21 levels in human subjects [48,110]. The fibroblast activation protein (FAP) is a serine protease that cleaves and inactivates FGF21 [111]. Interestingly, patients with T2D have elevated circulating levels of FAP when compared with nondiabetic controls, and this is associated with an attenuated ratio of bioactive to total FGF21 in response to an oral glucose tolerance test, suggesting that chronic states of insulin resistance may lead to inactivation of FGF21 and limit its metabolic effects [108]. Indeed, in response to a high-fat dietary intervention that is known to alter insulin-stimulated substrate utilization in healthy humans, the normal induction of circulating FGF21 in response to insulin is attenuated [108]. Confirming the mechanism by

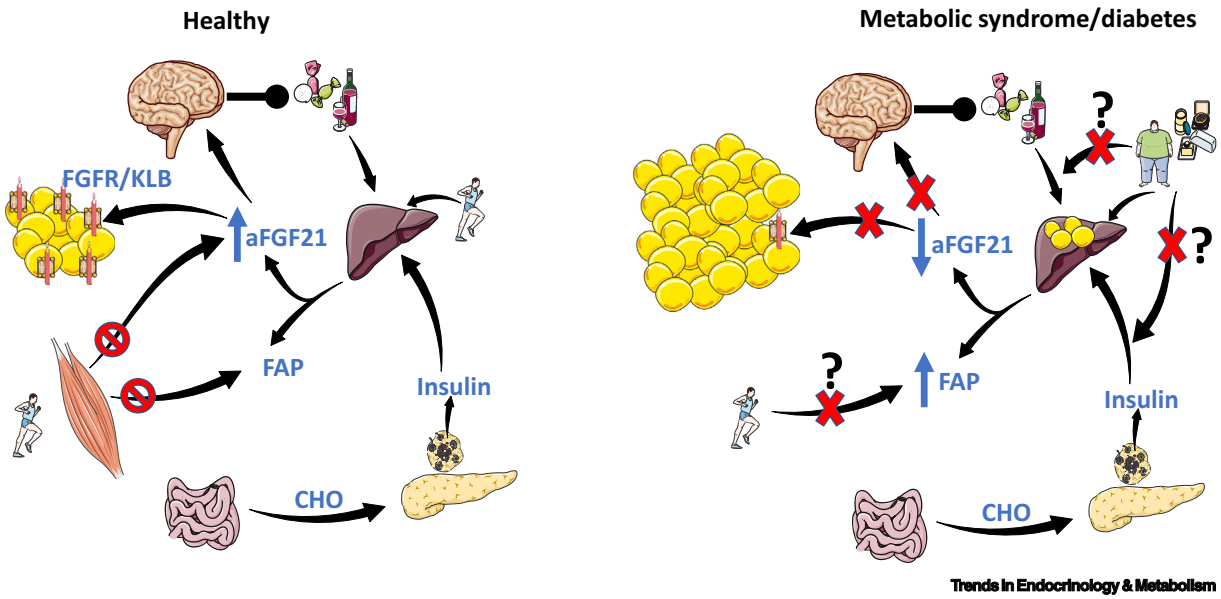

Figure 2. Role of FGF21 in Health and Disease. Blue arrows depict decreasing (downward) or increasing (upward) circulating concentrations of fibroblast activated protein (FAP) and the bioactive form of fibroblast growth factor 21 (FGF21) (aFGF21). Red 'stop' signs denote the absence of secretion in response to exercise. Red crosses and black block arrows indicate impairment in relevant biological processes, which when accompanied with questions marks, indicate outstanding biological questions that require further experimentation. In healthy humans (left panel), dietary carbohydrates (CHO) increase both the total and bioactive form of FGF21 in an insulin-dependent manner, whereas levels of FAP are unaffected. Exercise is a key stimulus in inducing hepatic, but not skeletal muscle, release of FGF21. Exercise also increases circulating levels of FAP with no apparent contribution from skeletal muscle. It remains to be established whether the liver releases FAP in response to exercise. Adipose tissue is the key target for the metabolic effects of FGF21. At target tissues, FGF21 binds to and activates members of the FGFR superfamily of receptor tyrosine kinases. This activation is dependent on obligate co-receptor βKlotho (KLB). FGF21 also markedly reduces sweet and alcohol preference via signaling through the paraventricular nucleus in the hypothalamus. The fact that consumption of dietary CHO increases the levels of FGF21, which in turn may suppress consumption of simple sugars and noncaloric sweeteners, likely indicates the presence of a negative feedback hormonal loop between the liver and the brain. Obese individuals with metabolic syndrome and patients with type 2 diabetes (right panel) have elevated circulating levels of FAP and this is associated with an attenuated ratio of bioactive to total FGF21 in response to an oral CHO (glucose) tolerance test, suggesting that chronic states of insulin resistance may lead to inactivation of FGF21 and limit its beneficial metabolic effects. KLB, the key co-receptor mediating the tissue sensitivity to FGF21, is associated with higher body mass index, and its protein levels are reduced in the adipose tissue of obese humans, which may further decrease its responsiveness to FGF21. Strategies aiming to minimize the release of FAP (either metabolic syndrome- or exercise-induced) may be of benefit in maximizing the metabolic effects of FGF21. Finally, it remains to be seen whether the negative feedback hormonal loop between the liver and the brain, as related to CHO consumption and sweet and alcohol preference, is in any way disturbed/impaired in obesity and diabetes, and whether it plays a role in appetite regulation under those conditions.

which insulin and/or insulin resistance regulates the hepatic secretion of FGF21 and FAP in humans and the subsequent tissue-specific actions of FGF21 require further investigation. Given that patients with T2D have elevated circulating levels of FAP, specific inhibitors for FAP in those patients (in combination with FGF21 mimetics) could be an appealing therapeutic strategy, particularly as FGF21 was shown to improve some but not all aspects of the metabolic syndrome in humans (Box 1).

Acute physiological elevations in plasma FFA levels through intravenous infusion of heparin and lipid emulsions was shown to produce modest elevation of circulating FGF21 [112], although in heathy human volunteers undergoing a lipid tolerance test, acute elevation of plasma FFAs was shown to decrease FGF21 [113,114]. In contrast, overfeeding with a HFD for 5 days resulted in a robust increase in circulating levels of FGF21 [115], possibly as a compensatory/adaptive mechanism to increased fat oxidation under conditions of energy oversupply. This is supported by the observations that short-term (6 days) isoenergetic HFDs do not alter fasting levels of either total or bioactive FGF21 [108]. Longer-term studies of the impact of HFDs have not been carried out, but obesity is generally associated with high serum FGF21 in humans, suggesting that chronic adiposity

may lead to an FGF21 resistant state [48,110]. This notion is particularly intriguing, given that a common single-nucleotide polymorphism (SNP) in KLB gene (rs2608819), the key co-receptor mediating the tissue sensitivity to FGF21, is associated with higher body mass index, and the protein levels of KLB are reduced in the adipose tissue of obese humans, which may decrease its responsiveness to FGF21 *in vivo* [110,116]. However, the hepatic expression of KLB and FGFR1 and FGFR3 are increased in obese individuals, which, in contrast to adipose tissue, may confer an improved hepatic responsiveness to increased FGF21 levels under those conditions. Large-scale RNA-sequencing studies reveal that mouse KLB gene is predominantly expressed in the liver, pancreas, and adipose tissue, with no KLB expression detected in skeletal muscle [117]. In humans, beyond the liver and adipose tissue, KLB is detected in breast, bone marrow, and pancreas [117]. It is conceivable that tissue differences at the KLB level between mice and humans may contribute to the divergent findings of the metabolic actions of FGF21 described for the two species.

In addition to its role as an insulin-dependent postprandial hormone, studies in mice have shown that FGF21 suppresses consumption of simple sugars and noncaloric sweeteners, but not complex carbohydrates, proteins, or lipids, via signaling through the paraventricular nucleus in the hypothalamus [50]. Furthermore, FGF21 administration markedly reduces sweet and alcohol preference in mice and sweet preference in cynomolgus monkeys. In mice, these effects require the FGF21 co-receptor KLB in the CNS and correlate with reductions in dopamine concentrations in the nucleus accumbens [84]. These actions of FGF21 may represent a conserved mechanism among species as genetic studies in humans have shown that SNPs in and around the FGF21 gene are associated with macronutrient preference, including carbohydrate, fat, and protein intake and alcohol consumption [44,118,119]. The fact that consumption of dietary carbohydrates (such as fructose and sucrose) may increase the fasting levels of FGF21, which in turn may suppress consumption of simple sugars and noncaloric sweeteners, likely indicates the presence of a negative feedback hormonal loop between the liver and the brain (Figure 2). Interestingly, recent studies have also linked variants in the KLB locus with levels of alcohol intake in humans [120]. FGF21 has also been shown to stimulate water drinking behavior in mice in response to alcohol; this response is dependent upon β-adrenergic signaling [85]. Furthermore, injection of recombinant FGF21 reduces *ad libitum* intake of alcohol in mice [119], whereas alcohol ingestion acutely and in the form of binge drinking increases plasma FGF21 in fasting humans [119,121]. Therefore, it is likely that alcohol-induced hepatic secretion of FGF21 serves as an adaptive mechanism aiming to inhibit alcohol appetite and stimulate water consumption in humans and therefore protect against long-term alcohol induced hepatic injury [121].

FGF21 has also been suggested to act as an exercise-induced myokine, as its serum levels are elevated in response to whole-body submaximal exercise [23,122,123]. However, recent elegant studies using tissue arteriovenous differences have shown that exercise is a key stimulus in inducing hepatic release of FGF21 in humans (Figure 2). Indeed, during submaximal exercise, most of the release of FGF21 into the systemic circulation occurs through the liver rather than skeletal muscle [104]. This was confirmed by a recent study showing that maximal eccentric contractions do not stimulate the release of either total or bioactive FGF21 from human skeletal muscle, whereas its FGF21 protein content is unchanged in response to exercise [105]. Interestingly, eccentric exercise resulted in significant elevations in arterialized and venous concentrations of its regulatory enzyme FAP, with no apparent contribution to its release from the exercised leg (Figure 2). These findings raise the possibility that increased levels of FAP may play a role in the inactivation of FGF21 during this type of exercise. Collectively, the results from the literature suggest that FGF21 may be an exercise-induced hepatokine but not myokine and that strategies aiming to minimize the release of FAP may be of benefit in maximizing the metabolic effects of FGF21, whether administered exogenously or produced in response to exercise.

FGF21 was first described as a key starvation hormone in a variety of animal models. However, recent studies in adult humans provide strong evidence that FGFF21 is an insulin-dependent postprandial hormone that plays a key role in the consumption of alcohol, water, simple sugars, and noncaloric sweeteners. Further understanding of the role of FGF21 in macronutrient preference and regulation of appetite in humans could be instrumental to uncovering its therapeutic potential in obesity, alcoholism, eating disorders, addictive behaviors, and other relevant disorders.

Concluding Remarks and Future Perspectives

Nearly two decades of research on FGF21 have identified it as a potent metabolic regulator when manipulated acutely by pharmacological means or by genetic approaches (in mice), and have characterized in detail its target tissues, receptor complexes, and degradation pathways. However, clinical trials with FGF21 analogs in overweight/obese patients with T2D have not progressed to Phase III because the improvements in circulating lipid profiles have not been matched by improvements in glycemic control and body weight loss. Perhaps the therapeutic potential of FGF21 analogs in T2D may be realized when combined with other current therapies targeting glycemic control. However, the emergence of FGF21 as a regulator of macronutrient preference both in rodents and humans and its role in the protection from fatty liver disease and dyslipidemia raises the interesting prospect of its therapeutic potential in obesity and some of its comorbidities. This is supported by the efficacy of FGF21 as a metabolic regulator in seasonal rodents that undergo natural cycles of adiposity and weight loss without the presence of metabolic dysfunction. The way forward with FGF21 may be to investigate its efficacy in obesity before T2D develops, targeting populations with specific polymorphisms that may confer an improved (tissue-specific) responsiveness to FGF21 treatment. This strategy may reveal how responsiveness to FGF21 can be improved and so insulin sensitivity maintained in the long term, reshaping our view of FGF21 as prevention rather than cure.

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R.J.S. is an employee of Eli Lilly and Company. J.E.L., F.J.P.E., and K.T. have had prior collaborative studies with Eli Lilly and Company.

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Outstanding Questions

Could the role of FGF21 in macronutrient preference be exploited in the regulation of appetite in obesity and diabetes?

Could FGF21 mimetics be combined in a dual therapy? For example, development of a dual antibody that could target both the FGF21 receptor and receptor (s) for other hormones/metabolites that can either maximize the positive impact of FGF21 or attenuate some of its side effects.

The duality between physiology and pharmacology: why would FGF21, a signal that protects against nutrient stress, drives energy expenditure when administered at pharmacological levels in obese animal models? And why does the increase in energy expenditure only occur during energy surfeit?

Do chronic states of insulin resistance (e.g., diabetes) lead to inactivation of FGF21 and limit its metabolic effects? What is the role of FAP in this process? Could specific inhibitors for FAP in diabetes patients (in combination with FGF21 mimetics) be an appealing therapeutic strategy?

What effect does FGF21 have on the vasculature given that FGF21-KO animals have reduced blood pressure when fed a ketogenic diet, whilst pharmacological administration increases blood pressure in rodents and humans?

Which are the quintessential neurons for the central effects of FGF21?

Can we modulate proteolytic degradation of FGF21 in a controlled/safe manner?

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