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FGF21 reloaded: challenges of a rapidly growing field

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Fibroblast growth factor 21 (FGF21) is a pleiotropic hormone-like protein and a major metabolic regulator. However, several key aspects of FGF21 biology remain poorly understood. Indeed, the list of controversies in the FGF21 field spans a variety of topics, from basic matters such as the anatomic distribution of FGF21 expression and the molecular composition of the FGF21 receptor, to the ultimate question of therapeutic relevance of FGF21-dependent pathways in humans. In this paper, we focus on current challenges in the field in an attempt to provide a balanced overview of FGF21 biology and guide future research into this exciting metabolic target.

Fibroblast growth factor 21 is not a growth factor

Fibroblast growth factor 21 (FGF21) was classified as a fibroblast growth factor based on its structure, as it contains a canonical 'FGF-like' domain and shares 10–30% sequence identity with other FGFs [1]. Unfortunately, this name is misleading as FGF21 has no demonstrable activity in fibroblasts and despite numerous attempts, has never been shown to promote growth *in vivo*. For instance, FGF21 transgenic mice are devoid of excess tissue proliferation including malignancies, and chronic systemic delivery of FGF21 to rodents and primates has no effect on tissue growth [2,3]. If anything, forced expression of FGF21 in mice reveals a delayed process of chemically induced liver tumors, although this effect is rather subtle and noticeable only at early stages of cancer initiation [4]. Mechanistically, the antiproliferative actions of FGF21 might be a result of the reduced activity of this protein on fibroblast growth factor receptor 4 (FGFR4) and its deficiency in heparin binding [5]. Thus, FGF21 is a unique member of a fairly distinct so-called 'hormone-like' subgroup within the FGF superfamily that also contains FGF19 and FGF23. These three FGFs were put together in one subfamily based on the highest level of structural resemblance, molecular similarities in receptor activation mechanism, a putative 'endocrine' mode of action and the involvement of FGF21, FGF19 and FGF23 in the regulation of metabolic processes such as glucose/lipid homeostasis, cholesterol/bile acid synthesis, and phosphate/vitamin D handling, respectively [6,7].

Where in the body is FGF21 expressed?

The initial cloning report pointed mouse liver as a predominant tissue of FGF21 mRNA expression [1]. Subsequently, FGF21 was detected in fat [8] and muscle [9], and the concepts of FGF21 being a novel hepatokine, adipokine and even a myokine, were suggested. However, FGF21 is also present in the exocrine pancreas and β-cells, at drastically higher levels than in liver, adipose and muscle [10–12]. Although the latter points to the pancreas as a major source for *in vivo* production of FGF21, the mechanisms of its expression/regulation in this tissue are rather understudied. Moreover, the pattern of tissue-specific FGF21 localization requires further and more refined examination because of the exceptionally dynamic alterations in FGF21 expression after various challenges [3], the necessity to confirm mRNA-driven data at the protein level that can deliver new and reasonably unexpected findings (as in [9]), and the need to extend FGF21 expression analysis to a human background.

What is the target tissue of FGF21?

Although the FGF21 target tissue profile remains limited, it goes far beyond fat cells and adipose tissue as initially postulated [13]. For example, FGF21 is active on isolated pancreatic islets and insulinoma-derived INS-1E cells, where it suppresses glucose-mediated glucagon release, stimulates insulin production, and provides protection from cell apoptosis [10]. These *in vitro* effects appear to be present *in vivo* as well, because FGF21 delivery to mice and non-human primates leads to decreased glucagon release and preservation of β-cell mass/function [2,10,14]. However, both of these effects could also be indirect and rather secondary to FGF21-induced improvement of peripheral insulin action and consequential diminution of islet glucotoxicity. FGF21 also signals in pancreatic acinar cells [11], and protects them from overt damage during acute pancreatitis by limiting *in vivo* tissue inflammation and fibrosis. Current studies report FGF21-induced signaling in hepatocytes and direct functional effects of FGF21 in liver [15–19]. Of interest, a handful of initial studies failed to demonstrate hepatic actions of FGF21 [13,20], which was probably a result of the use of a truncated or tagged FGF21 protein preparation that could have affected FGF21 bioactivity [21,22]. Thus, it appears that liver, pancreas and adipose tissue are the main FGF21 targets, which is consistent with the restricted expression profile of transmembrane βKlotho (KLB) [23]. Therefore, it is up to KLB to provide the necessary mechanistic basis for the tissue-specific selectivity of FGF21 actions, rather than to the FGF receptors that are widely expressed in the body.

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1043-2760/$ – see front matter doi:10.1016/j.tem.2010.11.003 Trends in Endocrinology and Metabolism, March 2011, Vol. 22, No. 3

What does the FGF21 receptor look like?

FGF21 activates typical FGF receptor-mediated signaling [2]. However, in contrast to the classic FGFs, FGF21 does not bind FGFRs directly. This led to the hypothesis of existence of a non-FGFR cofactor that is crucially required for FGF21 mechanism of action [2]; transmembrane KLB appears to play such a role. This conclusion is based on the following evidence: 1) KLB reconstitutes FGF21 activity when expressed in non-FGF21-responsive cells [24–26]; 2) induction of KLB expression increases cell sensitivity to FGF21 [27]; and 3) small interfering RNA (siRNA)-mediated attenuation of KLB levels in turn impairs FGF21 activity [24]. Thus, the FGF21 receptor consists structurally of two precomplexed components: FGFR and KLB [25]. Both KLB and FGFR are necessary but individually insufficient to support FGF21 signals, as each protein has its own unique role, KLB being an adaptor-like molecule able to bind FGF21 directly thus allowing this factor to activate FGFR, and FGFR serving as an activity-competent subunit (Figure 1).

Because four individual FGF receptors (FGFR1 to FGFR4) exist, it is still unclear whether there is a preferred FGFR for constitution of a fully functional FGF21 receptor complex. Although FGFR1 was suggested to play this role and the other FGFRs were ruled out [13], the hypothesis of FGFR1 being the only binding partner of FGF21 is contradictory to several observations. For example, FGF21 also activates FGFR2 and FGFR3 [2,25–27], soluble KLB strongly binds several FGFRs, and FGF21 associates with KLB in a complex with various FGFR isoforms including FGFR4 [24,25]. Finally, in liver, where FGFR1 is expressed at low levels compared with FGFR2, FGFR3 and FGFR4 [18], FGF21 induces a multitude of direct effects [15–19], suggesting its ability to partner with FGFRs beyond FGFR1. Together, these data indicate that KLB constitutes a necessary interaction partner with many, if not all, FGFR isoforms required for FGF21-induced activation of target cells.

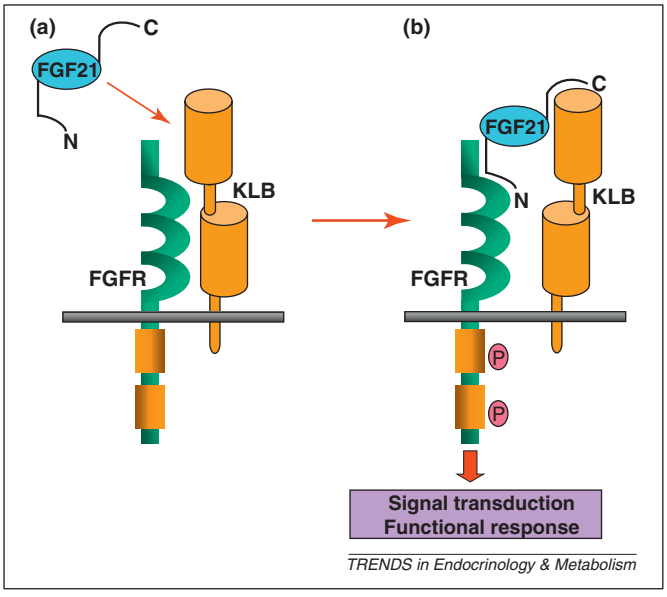

Figure 1. The proposed mechanism of FGF21 receptor activation. **(a)** FGFRs and KLB that are constitutively associated on the plasma membrane comprise the FGF21 receptor, but the receptor complex is silent without FGF21 [24–26]. **(b)** Once FGF21 comes into the vicinity of the receptor, it associates with its receptor in a two-step motion. At first, FGF21, via its C-terminus, binds to KLB. This interaction leads to a conformational change in FGFR or FGF21, or even both, allowing FGF21 through its N-terminal part to contact FGFR [21,22]. Binding of FGF21 to FGFR/KLB complex triggers the intrinsic tyrosine kinase activity of FGFR, followed by receptor crossphosphorylation, downstream signal transduction and a cellular functional response.

Interestingly, the concept of an obligatory KLB/FGFR interaction as a prerequisite for FGF21 bioactivity has been recently challenged by a study conducted in mice carrying a global deletion of the KLB gene [28]. This report reconfirmed the requirement of KLB for the propagation of FGF21 signal *in vitro* [24–26], and also provided some evidence of generic transcriptional activity in FGF21-dosed KLB knockout (KO) mice. Although the latter observation led the authors to state that FGF21, via an unidentified mechanism(s), acts *in vivo* in a KLB-independent mode, this hypothesis needs to be substantiated with more evidence than that provided to date [28]. At the very least, FGF21 has to be tested in KLB-deficient mice for its functional actions, such as lowering of glucose, plasma insulin, lipid and/or body weight. Moreover, because the *in vivo* data in this study [28] were obtained using concentrated supernatants from FGF21-expressing cells, and the molecular integrity and purity of FGF21 in these preparations was not revealed, the overall significance of this provocative finding remains to be independently verified.

Is FGF21 an endocrine factor?

Given the presence of FGF21 in blood [29–31], its ability to target adipose tissue *in vivo* [2], and the assumption that the main organ of FGF21 production is the liver, FGF21 was postulated to be an endocrine factor comprising a pivotal component of a liver to adipose tissue axis of communication [32]. However, to qualify as a genuine hormone, FGF21 must be released into and be present in the bloodstream in its active form, which has yet to be proven. To date, the presence of FGF21 protein in plasma of animals and humans has only been demonstrated using immunochemical methods, and the currently available radioimmunoassay/ELISA kits are unable to discriminate between bioactivity-competent, inactive or even antagonistic forms of FGF21 that might in fact be generated by removal of a handful of amino acids from either the N- or C-termini [21,22]. Furthermore, recent data indicate direct FGF21 function not only in fat cells, but also in the pancreas and liver [10,11,15–19]. Thus, the fact that FGF21 is mainly expressed in liver, pancreas and adipose, the same tissues upon which this factor acts, makes unclear the biological need for FGF21 to support cross-tissue communication, and rather suggests that this protein functions in a paracrine/autocrine manner as proposed recently [11,18].

Determining whether FGF21 is a true hormone or a molecule acting locally at the site of its expression is crucial in elucidating FGF21 biology; however, testing this is a technically challenging task likely to require the generation of animals with FGF21 ablation in a tissue-specific manner. It is also currently unclear why circulating FGF21 shows an exceptionally broad variability in humans, ranging from single digit pg/ml to low ng/ml levels [31]. This could be a reflection of distinctive interindividual patterns of FGF21 secretion, modification and clearance, and is

suggestive of associations between FGF21 levels with the development and pathophysiology of the metabolic disease.

What is the physiologic role of FGF21?

Available evidence points to a role for FGF21 in the adaptation of the body to starvation. As hepatic lipid metabolism accelerates in response to fasting, increased amounts of immunoreactive FGF21 are released, which might subsequently affect whole-body substrate utilization. It was recently demonstrated that dual-action glucagon-like peptide-1 (GLP-1)/glucagon co-agonists led to increased FGF21 expression in mouse liver [33]. Because GLP-1 agonists have no effect on hepatic FGF21 expression, it seems most likely that glucagon mediates this effect, thereby suggesting that FGF21 is released to modulate the classic counter-regulatory responses of glucagon. However, it is worth mentioning that most starvation studies that found induced effects on FGF21 physiology have been conducted in small rodents with limited intrahepatic glycogen depots, capable of sustaining only a few hours of glycogenolyis before fatty acid oxidation takes over as the most prominent source of metabolic fuel source [34,35].

When free fatty acids (FFAs) released during prolonged fasting reach the liver, they activate peroxisome proliferator-activated receptor alpha (PPARα) signaling in this tissue, leading to their conversion into ketone bodies, a major energy source during starvation. Of note, plasma levels of FGF21 are dramatically elevated in fasted rodents [32,36,37], and the induction of FGF21 expression correlates with a starvation-induced rise in serum ketone bodies [36]. Further, ketogenic diets (low carbohydrate, high protein) also induce elevated plasma FGF21 levels, and knockdown of FGF21 leads to impairments in ketogenesis, and to hepatosteatosis and overt dyslipidemia [36]. Thus, consistent with ketogenesis being part of the physiologic response to fasting, FGF21 has been suggested to be a starvation, ketogenic or 'Atkins' hormone [32,36,37]. Nevertheless, recently obtained data in FGF21 KO animals sheds new light on the physiology of FGF21. During fasting, when FGF21 is destined to increase and therefore to function, FGF21 null mice still respond normally, with increased lipolysis in adipose tissue and elevated plasma levels of FFAs [38]. This observation confirms the previously demonstrated anti-lipolytic role of FGF21 in fat cells and tissue [36,39,40], and is certainly not indicative of the increased mobilization of adipocyte fat stores, as suggested previously [32]. Furthermore, FGF21 expression in liver is under tight control by PPARα [32,36,37]. It is therefore counterintuitive to expect FGF21 to act before fasting-induced elevation of plasma levels of FFAs, a prerequisite for full PPARα activation. Thus, FGF21 is unlikely to be directly involved in FFA release from adipose upon initiation of starvation.

Although the phenotype of FGF21 transgenic mice largely appears to be consistent across different investigations [2,4,32], the functional consequences of FGF21 ablation seem to vary significantly, based on the findings reported in three independently generated strains of animals [38,41,42]. For example, FGF21 null mice on a ketogenic diet for 28–30 weeks developed fatty liver, hypertriglyceridemia, increased serum FFAs and cholesterol, attenuated glucose handling and insulin sensitivity, and impaired ketogenesis, [41], whereas another cohort of FGF21 KO mice fed normal chow for 8 weeks only had reduced fasting plasma FFA levels, with otherwise unchanged metabolic parameters [38]. Finally, consistent with the first [41] but in contrast to the second [38], the third line of FGF21 KO animals of undisclosed age and on an unknown diet regimen [42] had elevated fasting FFAs and mild hypoglycemia. The latter is rather counterintuitive, given the well-documented glucose-lowering function of FGF21 [3,32]. Although these data controversies are puzzling and require reconciliation through future research, they might be related in part to the differences in the animal work protocols used by each of these individual research groups. Indeed, the most striking phenotype of FGF21 ablation was observed in aged animals fed a high-fat diet [41], as opposed to young and healthy mice [38,42]. Thus, the discrepancies between these studies [38,41,42] could simply be a reflection of a metabolic challenge/disease state required to reveal the true physiologic function of this factor, which would then be consistent with the basic aspects of FGF21 pharmacology [3].

What are the mechanisms of FGF21 actions *in vivo*?

As shown in a variety of independent studies, systemically administered FGF21 induces a sustained lowering of blood glucose and triglycerides, improved insulin sensitivity, enrichment in brown adipocytes [2], preservation of β-cell function and mass [10], amelioration of obesity and hepatosteatosis [15,16,36], improvements in leptin resistance [2,15], lowering of low-density lipoprotein cholesterol, and elevation of high-density lipoprotein cholesterol and adiponectin, and beneficial changes in several cardiovascular risk factors/markers [14]. Although these effects can be observed during chronic studies run over weeks, [3] and in the acute setting just a few hours after protein delivery [19], they require animals to be in a state of metabolic imbalance, as FGF21 does not appear to otherwise alter metabolic parameters at the 'healthy' levels. This may be the reason why preclinical assessment of FGF21-based therapy appears to be devoid of any apparent side effects, such as hypoglycemia, edema, pancreatitis, liver toxicity or increased mitogenicity, suggesting the 'sensitizing' nature and safety advantages of the mechanism of action of FGF21 [3].

The multitude of the metabolic effects coupled with a clean side effect profile of FGF21 action in animals raises the ultimate question of what could be the mechanism(s) by which a single molecule induces all these beneficial outcomes. Although the complete understanding of an *in vivo* mechanism for FGF21 is not yet in place, several educated guesses can be made based on the currently available literature.

The KLB requirement for the propagation of FGF21 signal *in vivo* provides a mechanistic rationale for many. if not all, the unique features of FGF21 biology. Indeed, KLB expression in metabolically competent liver, pancreas and adipose permits FGF21 to selectively target these tissues, thus allowing this factor to influence glucose, lipid and bodyweight homeostasis. Furthermore, as the mechanisms

by which each of these tissues control metabolism are unique and diverse, FGF21 becomes predetermined to be a truly pleiotropic factor using distinct molecular pathways that are shaped by its receptor composition/distinctive signaling and functional milieu in each organ FGF21 acts upon. Finally, tissue-restricted character of KLB localization in the body as opposed to the broad character of FGFR expression limits FGF21 activity to a select few targets, rendering FGF21-based therapies to be more compelling as a result of a lesser chance of inducing body wide effects.

Another important aspect of FGF21 mechanism relates to the fact that its various functional effects *in vivo* seem in general to be achieved independently of one another rather than in a causative or sequential manner. This is based on distinct FGF21-dependant dose response relationships for different metabolic readouts, for example, glucose, triglycerides and bodyweight lowering and FGF21 ability to affect in the same animal the metabolic parameters only at the abnormal state as opposed to those at the 'normal' level [2,15–19].

Although FGF21 was discovered as an inducer of GLUT1 in adipocytes [2], it is unlikely that this effect underlies the mechanism of its glycemic action. Rather, FGF21 exerts its anti-hyperglycemic function through insulin sensitization. Indeed, FGF21 directly regulates insulin accumulation and secretion *in vitro*, effectively lowers insulin levels *in vivo* and improves total insulin sensitivity [2,10,15–17]. Importantly, FGF21 reverses hepatic insulin resistance [17] and FGF21-dependent induction of insulin receptor in liver might be a mechanistic trigger for this effect [15]. Alternatively, or even in concert, FGF21 antilipolitic activity on adipocytes with concomitant lowering of plasma FFAs could be a contributing factor as well [39,40].

Of notice, insulin is also able to influence the FGF21 pathway as it stimulates FGF21 expression via an AKT1-dependent mechanism [9]. Furthermore, significant elevation of FGF21 in plasma during insulin infusion [43] and in skeletal muscle in hyperinsulinemic condition [44], and tight associations between plasma FGF21 and insulin/insulin sensitivity indexes [29,30] are all indicative of a functional interplay between insulin and FGF21 pathways in human. Insulin is released in a fed state although FGF21 is induced at fast and both hormones might physiologically control expression of each other; therefore, they might function in a consequent manner to assure a concerted metabolic regulation on periphery at any time. As for clinical implications, the efficient insulin/FGF21 crosstalk in the body in a disease state might have a positive effect on the therapeutic benefits of each therapy alone or when both molecules are used in a combination.

The actions of FGF21 in liver and adipose are likely to underlie its lipid and body weight-lowering effects. In the liver, FGF21 increases FFA oxidation and suppresses *de novo* lipogenesis [15,16,19]. In adipose tissue, FGF21 simultaneously induces lipid accumulation, uncoupling, biogenesis and inhibition of lipolysis, indicative of a state of futile cycling [15,19]. Consistent with this hypothesis, FGF21-infused mice have higher energy expenditure and elevated core body temperature, suggesting that FGF21 has catabolic functions [15,16]. Of note, FGF21 was initially shown to promote the opposite effects, that is, lowering body temperature and even directly inducing torpor, but only in a fasted state [32]; the basis for this conspicuous difference is currently unclear. Nevertheless, the report [32] has been recently further challenged by data showing that FGF21 promotes thermogenic activation of brown fat [45] and is dispensable during starvation-induced torpor [46]. Given the potent effect FGF21 has on energy expenditure *in vivo* [15,16], FGF21-overexpressing animals under severe nutritional stress are likely to experience an extreme energy-deficient state. Thus, the conclusions of the previous study [32] need to be re-evaluated in regard to the relative energy abundance, a shortage of which could have been an underlying reason for the observed hibernation-like phenotype, rather than due to FGF21 being a direct inducer of torpor [32]. If so, the findings [32] will be a lesser safety concern for FGF21 therapy given the social prevalence of excessive caloric intake in the western hemisphere.

Beyond the pancreas, adipose tissue and liver, FGF21 might also target the hypothalamus, where KLB and FGFRs are both present, and Agouti-related peptide and neuropeptide Y are induced upon systemic delivery of FGF21 [15]. Furthermore, centrally administered FGF21 induces energy expenditure and insulin sensitivity [47], raising the intriguing possibility of FGF21-mediated crosstalk between brain and periphery. Although it is already known that systemically delivered FGF21 can access the brain [48], it is likely to happen via an active transport mechanism given the FGF21 protein size and charge.

FGF21 resistance

Given a consistent elevation of FGF21 plasma levels in rodents and humans in a state of a metabolic disease, which is reminiscent of hyperinsulinemia and hyperleptinemia, the idea of FGF21 resistance has been suggested [17,18,37]. Indeed, ob/ob and diet-induced obesity (DIO) mice carry elevated FGF21 blood levels and are less sensitive to acute FGF21 administration [17,18]. Mechanistically, reduced response to FGF21 treatment in a metabolically compromised state can be explained at the FGF21 receptor level, as FGFRs and KLB transcripts are reduced in FGF21-responsive tissues in ob/ob and DIO mice compared with 'normal' animals [18]. Nevertheless, even though the acute response to FGF21 appears to be attenuated in diabetic or obese animals, it does not prohibit a robust FGF21-driven pharmacodynamic effect in the same disease models when the protein is delivered chronically [17,18], implying the need for long-term administration studies to fully explore the metabolic benefits of FGF21 therapy. Furthermore, while FGF21 can be elevated in metabolically-compromised rodents by a couple of orders of magnitude, reaching single digits (ng/ml levels) [18,37], in people with metabolic disease FGF21 is increased to only a modest 2–3 times the average [29–31], and the evidence of attenuation of acute FGF21 pharmacology in humans is yet to be reported.

Never say never

The understanding of FGF21 as a major metabolic regulator is rapidly evolving. Noticeably, the number of FGF21-related manuscripts has been exponentially growing and

Box 1. Outstanding research questions

- Is there a preferred FGFR to constitute FGF21 receptor complex?
- In what form is FGF21 present in blood?
- Is FGF21 a true hormone, or does it function in an autocrine/paracrine manner?
- Why are FGF21 levels in plasma so interindividually variable?
- What are the specifics of interplay between the FGF21 pathway and physiology of insulin, glucagon and leptin?
- What is the precise role of KLB in propagation of FGF21 signals *in vivo*?
- Is the FGF21 pathway therapeutically relevant in humans?
- Are there any pharmacologic deficiencies of the native FGF21?

in fact, doubling each year since 2005. In spite of this accelerated pace of investigation, the scientific appreciation of FGF21 biology is still fragmented and controversial, and these knowledge gaps are yet to be filled through prioritized research focused on the most fundamental questions. Some of those unanswered questions, reflecting our personal views, are listed (Box 1), with the therapeutic relevance of FGF21 pathway in humans being the primary inquiry.

Several FGF21 variants are now in early stages of clinical development. What makes FGF21 attractive as a drug candidate is the fact that its animal pharmacology is consistent and reproducible, while the *in vitro* structure-activity relationship assays can be established in a variety of ways. It is very intriguing that thiazolidinediones affect expression of FGF21 and its co-receptor KLB [8,27,49], leading to a concomitant increase in FGF21 sensitivity [27], suggesting that these anti-diabetic drugs in part function via the FGF21 pathway. In people, plasma levels of FGF21 typically average several hundred pg/ml, comparable with that of insulin, and are clearly lower than those of leptin, which failed initial therapeutic promise. Even though FGF21 resistance has been demonstrated in rodents, this does not preclude FGF21 inducing a robust pharmacologic response in these species [17,18]. Thus, it seems plausible that chronically delivered pharmacologic doses of more potent FGF21 agonists will be capable of overcoming FGF21 resistance in humans, if this is indeed the case. Ongoing clinical investigations should soon bring definitive answers to fully assess the therapeutic potential of FGF21 agonists in the field of metabolic disease.

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