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FGF21 is an Akt-regulated myokine

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Abstract **Fibroblast growth factor-21 (FGF21) functions as a metabolic regulator. The FGF21 transcript is reported to be abundantly expressed in liver, but little is known about the regulation of FGF21 expression in other tissues. In this study, we show that levels of FGF21 protein expression were similar in skeletal muscle and liver from fasted mice. FGF21 transcript and protein expression were upregulated in gastrocnemius muscle of skeletal muscle-specific Akt1 transgenic mice. Serum concentration of FGF21 was also increased by Akt1 transgene activation. In cultured skeletal muscle cells, FGF21 expression and secretion was regulated by insulin, Akt transduction and LY294002. These data indicate that skeletal muscle is a source of FGF21 and that its expression is regulated by a phosphatidylinositol 3-kinase (PI3-kinase)/Akt1 signaling pathway-dependent mechanism.**

Keywords: FGF21; Akt; Metabolism; Transcript; Transgenic mice

1. Introduction

Fibroblast growth factor-21 (FGF21) is a member of FGF super family that is reported to be primarily expressed in liver and expressed to a lower extent in thymus [1]. Recent evidence indicates that FGF21 is an important endogenous regulator for systemic glucose and lipid metabolism. It has been demonstrated that FGF21 enhances glucose uptake in an insulin-independent manner and attenuates lipolysis in mouse and human adipocytes in vitro [2,3]. It has also been shown that transgenic mice that overexpress FGF21 in liver are resistant to diet-induced obesity. Therapeutic administration of FGF21 to diabetic rodents (*ob*/*ob*, *db*/*db* and DIO mice) or diabetic rhesus monkeys improved plasma glucose and lipid profile to near normal levels, and corrects obesity [2,4,5]. Knockdown of hepatic FGF21 transcript leads to hyperlipidemia and fatty liver in mice fed a ketogenic diet [6], whereas overexpression of FGF21 in liver leads to ketone body production [7], suggesting that FGF21 functions as a regulator of the organism's adaptation to starvation. In this regard, hepatic FGF21 gene expression is regulated by PPARα [6–8]. Furthermore, the FGF21 gene is a direct target of PPARγ [9,10], and the synergy between FGF21 and PPARγ pathways was demonstrated in 3T3-L1 adipocytes [11].

Akt1 is serine–threonine protein kinase that is activated by various extracellular stimuli through a phosphatidylinositol 3-kinase (PI3-kinase) pathway. Numerous studies have implicated Akt in insulin signaling as well as the control of cellular growth and organ size and cellular hypertrophy [12,13]. Overexpression of Akt in skeletal muscle results in fiber hypertrophy in vitro and in vivo [14–16]. We have recently demonstrated that Akt1-mediated skeletal muscle growth in obese mice leads to a reduction in accumulated white adipose tissue and improvements in metabolic parameters [17]. These findings led us to the hypothesize that activation of Akt1 signaling in skeletal muscle could lead to the secretion of hormonal factors or "myokines" that act on adipose, hepatic or central nervous system tissues to normalize metabolic parameters. Myokines are defined as proteins that are produced and secreted from muscle that have paracrine or endocrine functions [18]. While it has been well-established that muscle secretes factors required for blood vessel recruitment [16,19], the secretion of metabolic-regulatory proteins by this tissue has only received recent attention. Interleukin-6 is an example of a myokine that is released into the circulation in response to exercise and modulates the function of remote metabolically important tissues [20]. Recently, it was also shown that visfatin, a putative regulator of inflammation and glucose metabolism, is produced at higher levels in skeletal muscle than visceral adipose tissue in chickens [21]. Here, we show that skeletal muscle is a source of secreted FGF21 and that its expression is regulated by an Akt1 signaling pathway-dependent mechanism.

2. Materials and methods

2.1. Cell culture and adenoviral infection

C2C12 mouse myoblasts (American Type Culture Collection) were maintained in growth medium (DMEM supplemented with 20% FBS) as described elsewhere [16]. To induce differentiation, cells were shifted to differentiation medium (DMEM supplemented with 2% heat-inactivated horse serum) for 4 days. Cells were then infected with adenoviral constructs encoding constitutively-active Akt1

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Abbreviations: DOX, doxycycline; FGF21, fibroblast growth factor-21; KD, ketogenic diet; PI3-kinase, phosphatidylinositol 3-kinase; QRT-PCR, quantitative real-time PCR

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(Adeno-myrAkt1) or β-galactosidase (Adeno-βgal) at a multiplicity of infection (MOI) of 250 for 16 h. The transfection efficiency was greater than 90% under these conditions [16]. In some experiments, C2C12 myocytes were pretreated with LY294002 (20 μmol/L) or vehicle for 1 h before stimulation with insulin (10 nmol/L).

2.2. Animals

Generation and phenotypic characterization of skeletal muscle-specific inducible Akt1 transgenic (TG) mice have been previously described elsewhere in detail [17]. In brief, 1256 [3Emut] MCK-rtTA TG mice [22] were crossed with TRE-myrAkt1 TG mice [23] to generate double TG (DTG) mice. For Akt1 transgene expression, DTG mice were induced with doxycycline (DOX, 0.5 mg/ml) in their drinking water. 1256 [3Emut] MCK-rtTA single TG littermates were treated with DOX in the same manner as DTG mice and used as controls. Three weeks after DOX treatment, sera and gastrocnemius muscle from control or transgenic mice were harvested for mRNA and protein analysis. For fasting experiments, food pellets were removed from the cages of ad libitum-fed male C57BL/6 mice for 48 h. Mice were then anesthetized, gastrocnemius muscles and liver were immediately removed for protein analysis.

2.3. Determination of FGF21 mRNA

Total RNA was prepared by Qiagen using protocols provided by the manufacturer and cDNA was produced using ThermoScript RT-PCR Systems (Invitrogen). Real-time PCR was performed on iCycler iQ Real-Time PCR Detection System (BIO-RAD). SYBR Green I was used as a double-stranded DNA-specific dye as described previously [24]. Transcript levels were determined relative to the signal from GAPDH, and normalized to the mean value of samples from control. Primers were as follows: 5′-GCTGCTGGAGGACGGTTACA-3′ and 5′- CACAGGTCCCCAGGATGTTG-3′ for mouse FGF21 and 5′-GCTAAGCAGTTGGTGGTGCA-3′ for mouse GAPDH. Microarray analysis was carried out as described previously [25].

2.4. Western blots and immunoprecipitations

C2C12 cell lysates, culture media, and skeletal muscle tissue samples were subjected to SDS–PAGE and Western blot analysis. Antibodies used in Western blots were against: phospho-Akt from Cell Signaling Technology (Beverly, MA); FGF21 (monoclonal; clone#84-E12; raised against full length human FGF21 protein); GAPDH [11]. Immunoprecipitation from lysates was performed using the same anti-FGF21 antibodies. Immune complexes were probed with anti-FGF21 (polyclonal; clone PR050928A; raised

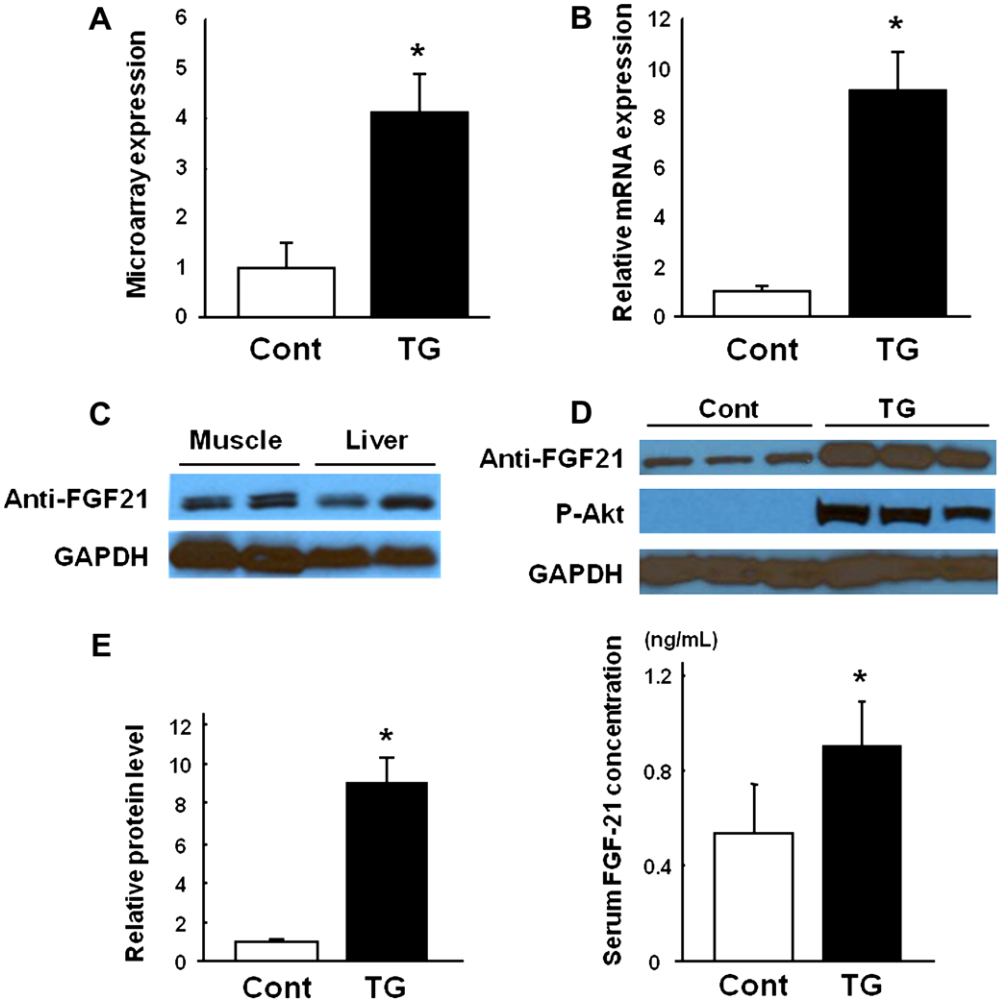

Fig. 1. Skeletal muscle FGF21 transcript and protein levels are upregulated in skeletal muscle-specific inducible Akt1 transgenic mice. (A) Microarray analysis of FGF21 transcript regulation in gastrocnemius muscle harvested from skeletal muscle-specific inducible Akt1 transgenic mice 3 weeks after Akt1 transgene activation. (B) Relative transcript expression levels of FGF21 as measured by QRT-PCR in control and transgenic gastrocnemius muscle. (C) Comparable levels of FGF21 protein expression in gastrocnemius muscle and liver of wild-type mice that have been fasted for 48 h. (D) Representative western blot analysis and quantified relative protein expression levels of FGF21 in gastrocnemius muscle from control or skeletal muscle-specific Akt1 transgenic mice 3 weeks after transgene induction. (E) Serum FGF21 levels in control or skeletal muscle-specific inducible Akt1 transgenic mice. Results are presented as mean ± S.E.M. ($n = 4$). $^{*}P < 0.05$ vs. control. Cont; control mice, TG; skeletal muscle-specific inducible Akt1 transgenic mice.

against full length human FGF21 protein). For immunodetection, goat anti-mouse and anti-rabbit HRP conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the ECL detection system from Amersham Biosciences (Piscataway, NJ) was used.

2.5. FGF21 ELISA

Serum samples were measured for concentrations of FGF21 using anti-FGF21 antibodies by sandwich ELISA method [26]. The wells of a 96-well microtiter plate were coated overnight at 4 °C with monoclonal anti-FGF21 antibody at a concentration of 5 μg/mL (0.1 mL/

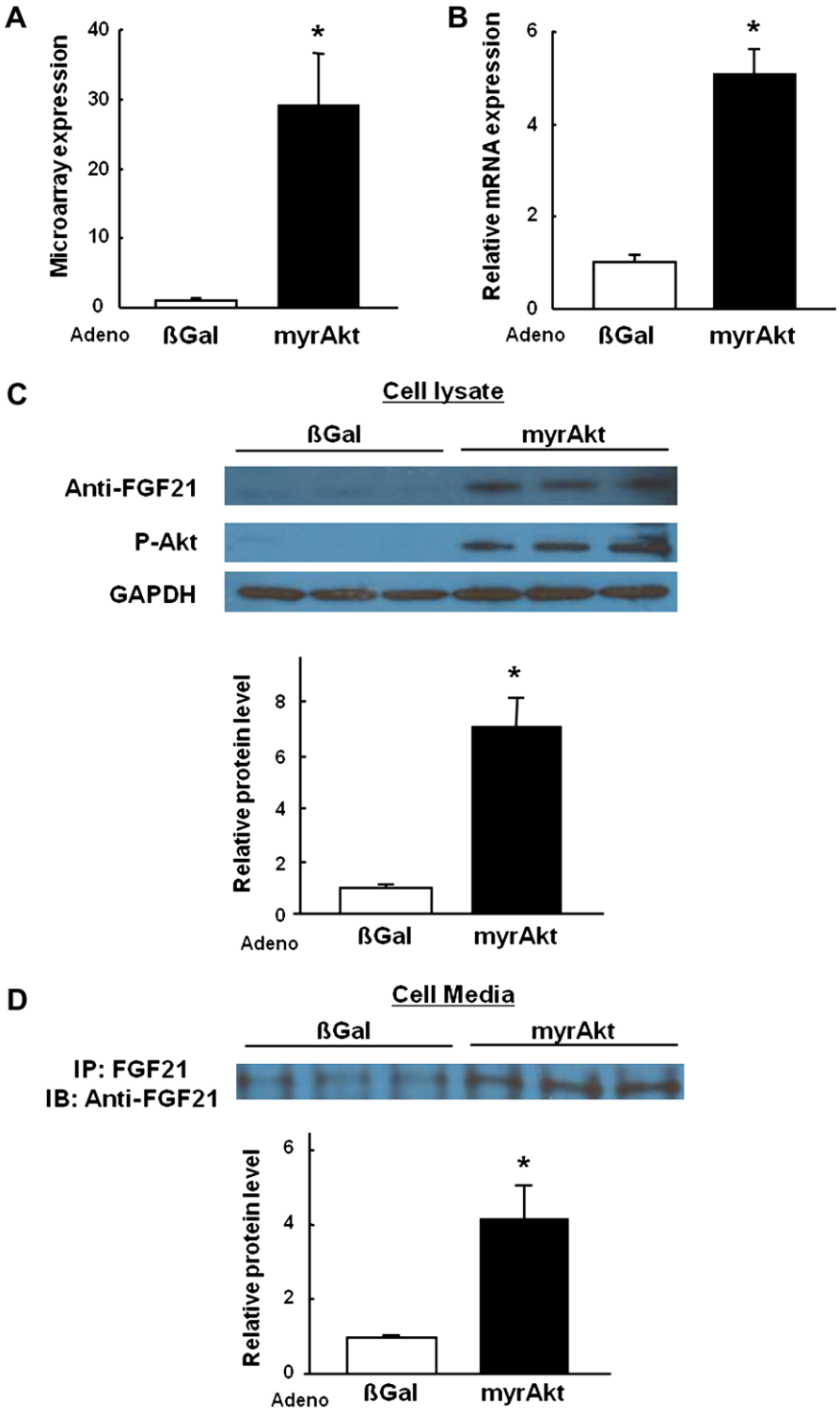

Fig. 2. FGF21 transcript and protein levels are upregulated in cultured C2C12 myocytes by Akt1 overexpression. (A) Microarray analysis of FGF21 transcript expression in Adeno-myrAkt1-treated C2C12 myocytes cell lysates compared with control. (B) Relative transcript expression levels of FGF21 as measured by QRT-PCR. (C) Representative western blot analysis and quantified relative protein expression levels of FGF21 in adeno-βGal or adeno-myrAkt1-treated C2C12 myocyte cell lysates. (D) Representative Western blot analysis and quantified relative protein expression levels of FGF21 in C2C12 cell culture media. Results are presented as mean ± S.E.M. ($n = 4$). $^{*}P < 0.05$ vs. control.

well). All assay steps were carried out in 0.1 mL/well additions with 1 h incubations at room temperature. After washing and blocking, standards and samples were added to the wells. Polyclonal anti-FGF21 (1:10000 dilution) was added and detected with a 1:5000 dilution of donkey-anti-rabbit-HRP from Jackson ImmunoResearch (West Grove, PA). Samples were analyzed in duplicate. The standard curve range for the assay was 0.39–50 ng/mL.

2.6. Statistical analyses

All data are presented as mean ± S.E.M. Statistical comparison of data from two experimental groups were made by using Student's *t*-test. Comparison of data from multiple groups was made by ANOVA with Fisher's PLSD test. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. FGF21 induction by Akt signaling in skeletal muscle

Microarray analysis was performed on inducible, skeletal muscle-specific Akt1 transgenic mice [17]. The constitutively-active Akt1 transgene is under the control of a mutated, muscle creatine kinase promoter [22]. This promoter construct has little or no activity in type I muscle fibers or heart [27], and we have shown that Akt expression from this promoter leads to the selective growth of type IIb fibers [17]. This analysis revealed that FGF21 was upregulated approximately 4-fold in gastrocnemius muscle at the 3 week time point following transgene induction (Fig. 1A). Akt-mediated regulation of FGF21 transcript in the transgenic mice was confirmed by quantitative real-time PCR (QRT-PCR) (Fig. 1B).

FGF21 protein expression in skeletal muscle tissue samples were examined by western blot analysis using a monoclonal antibody, clone#84-E12, directed to FGF21. Because the hepatic FGF21 transcript is dramatically induced by fasting [6,7], we used 48-h fasted liver tissues as a positive control. Comparison of gastrocnemius muscle and liver from fasted wild-type mice revealed similar levels of FGF21 protein in these tissues (Fig. 1C).

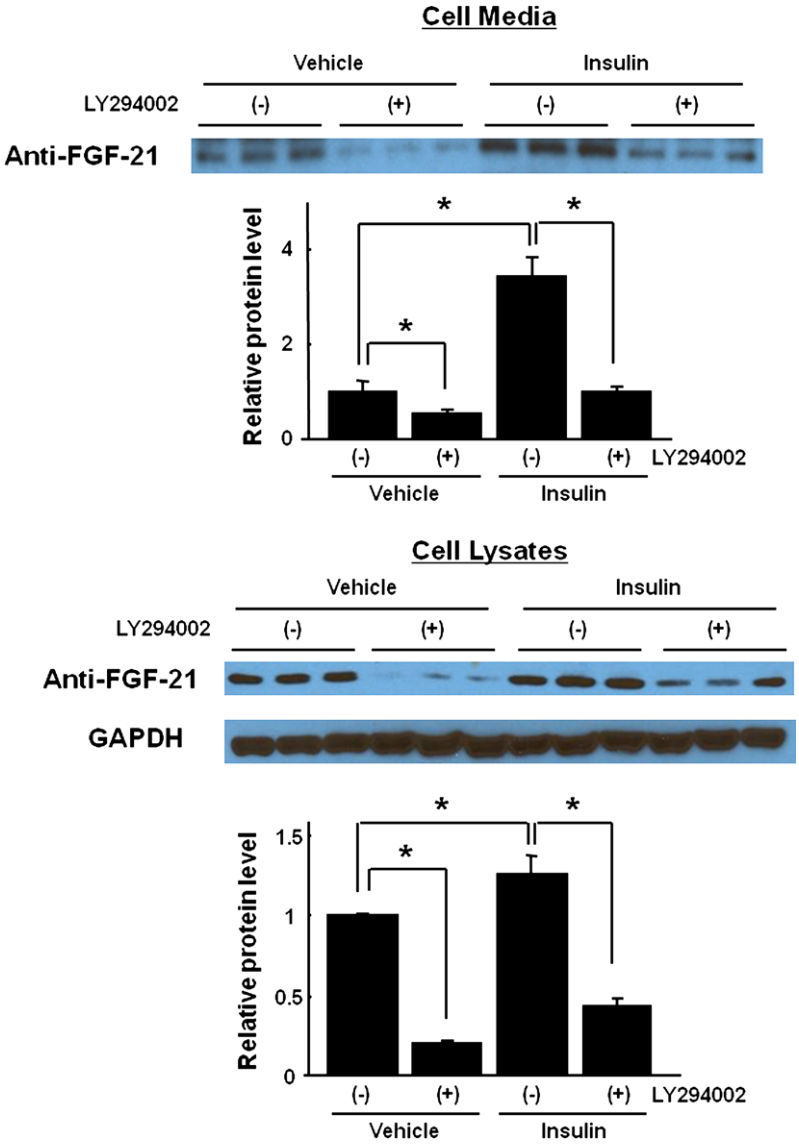

Fig. 3. FGF21 protein expression is upregulated by insulin, and inhibited by PI3-kinase inhibitor in cultured C2C12 myocytes. Representative western blot analysis and quantified relative protein expression levels of FGF21 in C2C12 myocytes culture media and cell lysates pretreated with LY294002 (20 μmol/L) or vehicle for 1 h before stimulation with insulin (10 nmol/L) or vehicle.

We next examined the effects of Akt1 activation on FGF21 protein expression using skeletal muscle-specific Akt1 transgenic mice. FGF21 protein expression was robustly upregulated in gastrocnemius muscle following 3 weeks of Akt1 transgene induction (Fig. 1D). Serum FGF21 concentration was determined by FGF21-specific ELISA. Serum levels of FGF21 were also increased by the induction of the Akt1 transgene in skeletal muscle compared with control mice (Fig. 1E). These results indicate that activation of Akt1 signaling in skeletal muscle lead to increase in circulating and tissue-resident FGF21 levels.

3.2. FGF21 is upregulated by Akt1 in C2C12 myocytes

To examine changes in gene expression in cultured myocyte in response to Akt1 overexpression, differentiated C2C12 myocytes were transfected with Adeno-βgal or Adeno-myrAkt1 for 16 h and microarray analysis was performed. FGF21 transcript was markedly upregulated in C2C12 cells treated with Adeno-myrAkt1 (Fig. 2A). The upregulation of FGF21 transcript was confirmed by QRT-PCR (Fig. 2B).

FGF21 protein expression levels in culture media and cell lysates were examined by Western blot analysis (Fig. 2C). FGF21 protein expression was induced 7-fold in Adeno-myr-Akt1-treated C2C12 myocytes cell lysates compared with control. FGF21 protein in culture medium was increased 4-fold by overexpression of constitutively active Akt1 (Fig. 2D).

3.3. FGF21 expression is upregulated by insulin in a PI3-kinase-dependent manner in cultured C2C12 myocytes

To examine the regulation of FGF21 expression by insulin, a hormone known to naturally induce Akt activation. C2C12 myocytes were treated with physiological levels of insulin (10 nmol/L) or vehicle in the presence or absence of pre-treatment with the PI3-kinase inhibitor LY294002. FGF21 expression in the cell lysate and the culture media was significantly increased in response to insulin stimulation (Fig. 3). Pretreatment with LY294002 inhibited both basal and insulin-stimulated FGF21 expression. These results indicate that FGF21 protein is produced in cultured myocytes and is a downstream target of PI3-kinase/Akt1 activation.

4. Discussion

Since its identification by Nishimura et al. as a member of FGF super family, it has generally been assumed that FGF21 is predominantly expressed in liver, and a number of recent reports describing the exceptionally dynamic regulation of FGF21 mRNA expression in liver [6–8] have confirmed these initial observations. Nevertheless, FGF21 has also been detected in pancreas and in cells of various origins, suggesting that the FGF21 expression profile may indeed be broader than initially thought [28].

In the present study, we examined for the first time skeletal muscle expression of FGF21 transcript and protein using monoclonal antibodies raised against the full length FGF21. We found that in skeletal muscle FGF21 protein expression is essentially comparable to that in fasted liver. Because hepatic FGF21 transcript is upregulated by fasting [6,7], these results indicate that skeletal muscle can be an important source of FGF21 production.

In this study, we also demonstrated that FGF21 expression in skeletal muscle is upregulated upon activation of the PI3-kinase/Akt1 signaling pathway. We found that both FGF21 mRNA and protein were induced by Akt1 overexpression or insulin stimulation in skeletal muscle cells. Conversely, insulin-stimulated FGF21 expression was inhibited by incubation with a PI3-kinase inhibitor. Under these conditions we also detected FGF21 protein in the media. Furthermore FGF21 protein levels in the media were increased following transduction with an activated Akt transgene or treatment with insulin, and reduced by treatment with a PI3K inhibitor, indicating that FGF21 can be secreted by skeletal muscle cells in PI3K/AKT-dependent fashion. Overexpression of constitutively active Akt1 in the type IIb fibers of transgenic mice also led to an increase in circulating FGF21, suggesting that the secretion of this factor from muscle may be physiologically significant. Thus, in addition of being direct target of PPARα in liver in response to fasting [7,8] or ketogenic diet [6] and PPARγ in adipose [9,10], FGF21 expression and secretion in skeletal muscle can be under control of PI3K/AKT pathway.

While in vitro FGF21 does not appear to be an insulin mimetic or potentiator [2], the ability of physiological levels of insulin to induce the expression of FGF21 in muscle cells as well as in 3T3-L1 adipocytes (data not shown), and of FGF21 to stimulate insulin production/secretion in pancreatic islets and β-cells [29], raises the intriguing possibility of cross-talk between these metabolic hormones. Indeed, FGF21 levels are significantly elevated in overtly insulinemic *ob/ob* mice [8]. In healthy animals, however, insulin levels are low during fasting and peak after feeding, whereas FGF21 expression is regulated in the opposite manner [7,8]. Furthermore, to exert its anti-hyperglycemic in vivo effects, FGF21 is likely to function as an insulin "sensitizer" since it effectively lowers insulin levels in diabetic animals, and improves total insulin sensitivity in glucose tolerance tests [28]. Thus, additional studies on the interplay between insulin and FGF21 under normal physiological conditions and in diseased states are warranted.

Myogenic Akt signaling is preferentially activated by resistance training, which leads to the growth of type IIb muscle fibers that are referred to as fast/glycolytic [30–32]. We recently reported, that this system of Akt1 transgene activation in skeletal muscle of mice leads to the hypertrophy of type IIb, but not type I or IIa, muscle and the upregulation of glycolytic pathways, whereas oxidative pathways are decreased [17]. We also reported that Akt1-induced type IIb fiber growth in obese mice leads to appreciable reductions in fat mass and a normalization of metabolic parameters. These metabolic improvements result, at least in part, from muscle-induced changes in the phenotypic and transcriptional profile of the liver that promote hepatic fatty acid oxidation and ketone body production. Based upon the findings of the current study, we speculate that FGF21 released from skeletal muscle may be one of the critical factors involved in this inter-tissue communication. Consistent with this hypothesis, the phenotype of myogenic Akt1 transgene activation in obese mice is similar to the effects of FGF21 administration to diabetic rodents and non-human primates [2,4,6,7]. Furthermore, FGF21 induces transcription of enzymes and transporters required for hepatic fatty acid oxidation, lipogenesis and ketogenesis [5–8], and the same

transcript changes are also observed in the liver of skeletal muscle-specific Akt1 transgenic mice [17].

Here, we provide evidence that FGF21 is an Akt1-mediated skeletal muscle-derived secreted protein or myokine. These findings support the hypothesis that stimulators of Akt1 signaling in skeletal muscle, such as resistance training exercise, could attenuate obesity-related metabolic disorders through production and secretion of metabolic-regulatory myokines.

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