Fibroblast Growth Factor 21 Prevents Atherosclerosis by Suppression of Hepatic Sterol Regulatory Element-Binding Protein-2 and Induction of Adiponectin in Mice

Running title: Lin et al.; The anti-atherosclerotic effect of FGF21

Zhuofeng Lin, PhD1; Xuebo Pan, PhD1; Fan Wu, MS2; Dewei Ye, PhD3,4; Yi Zhang, PhD4; Yu Wang, PhD3,4; Leigang Jin, MS1; Qizhou Lian, PhD, MD3,4; Yu Huang, PhD5; Hong Ding, MD, PhD6; Chris Triggle, PhD6; Kai Wang, PhD, MD7; Xiaokun Li, MD, PhD1,2; Aimin Xu, PhD1,3,4,5

1School of Pharmaceutical Science, Wenzhou Medical University, Wenzhou China; 2Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun China; 3State Key Laboratory of Pharmaceutical Biotechnology, the University of Hong Kong, Hong Kong, China; 4Dept of Medicine & Dept of Pharmacology and Pharmacy, the University of Hong Kong, China; 5School of Biomedical Sciences, Chinese University of Hong Kong, China; 6Dept of Pharmacology, Weill Cornell Medical College in Qatar, Doha, Qatar; 7Dept of Neurology, the 1st Affiliated Hospital of Anhui Medical University, Hefei, China

Address for Correspondence:
Aimin Xu, PhD Xiamen University, Lab Block
Department of Medicine University of Hong Kong, Lab Block
Wenzhou Medical University 21 Sassoon Rd
Hong Kong, China Wenzhou 325035
Tel: 852-28199754 Tel: 86-577-86699350
Fax: 852-28162095 Fax: 86-577-86699238
E-mail: amxu@hku.hk E-mail: xiaokunli@wzmc.edu.cn.

Abstract

Background—Fibroblast growth factor 21 (FGF21) is a metabolic hormone with pleiotropic effects on glucose and lipid metabolism and insulin sensitivity. It acts as a key downstream target of both peroxisome proliferator-activated receptor (PPAR) α and PPARγ, the agonists of which have been used for lipid-lowering and insulin-sensitization respectively. However, the role of FGF21 in cardiovascular system remains elusive.

Methods and Results—The roles of FGF21 in atherosclerosis were investigated by evaluating the impact of FGF21 deficiency and replenishment with recombinant FGF21 in apoE−/− mice. FGF21 deficiency causes a marked exacerbation of atherosclerotic plaque formation and premature death in apoE−/− mice, which was accompanied by hypoadiponectinemia and severe hypercholesterolemia. Replenishment of FGF21 protects against atherosclerosis in apoE−/− mice via two independent mechanisms: inducing the adipocyte production of adiponectin, which in turn acts on the blood vessels to inhibit neointima formation and macrophage inflammation, and suppressing the hepatic expression of the transcription factor Sterol regulatory Element-Binding Protein-2 (Srebp-2), thereby leading to reduced cholesterol synthesis and attenuation of hypercholeseterolemia. Chronic treatment with adiponectin partially reverses atherosclerosis without obvious effects on hypercholesterolemia in FGF21-deficient apoE−/− mice. By contrast, the cholesterol-lowering effects of FGF21 are abrogated by hepatic expression of Srebp-2.

Conclusions—FGF21 protects against atherosclerosis via fine-tuning the multi-organ crosstalk between liver, adipose tissue and blood vessels.

Key words: atherosclerosis, fibroblast growth factor, adipokine, FGF21, metabolic hormones
Fibroblast growth factor (FGF) 21 is a member of the endocrine FGF subfamily that is produced predominantly in the liver\(^1\). Physiologically, FGF21 plays a key role in mediating the metabolic responses to fasting/starvation by enhancing fatty acid oxidation, ketogenesis and inducing growth hormone resistance\(^2,^3\). Pharmacologically, therapeutic intervention with recombinant FGF21 has been shown to counteract obesity and its related metabolic disorders in both rodents and nonhuman primates, including reduction of adiposity and amelioration of hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia and fatty liver disease\(^4,^5\). Furthermore, FGF21 is the downstream target of both peroxisome proliferator-activated receptor (PPAR)\(\alpha\) and PPAR\(\gamma\), and a growing body of evidence suggest that the glucose-lowering and insulin-sensitizing effects of the PPAR\(\gamma\) agonists (thiazolidinediones, TZDs) and the therapeutic benefits of the PPAR\(\alpha\) agonists (fenofibrates) on lipid profiles are mediated in part by induction of FGF21\(^6\).

FGF21 exerts its metabolic actions by binding to the complex receptor between FGF receptor (FGFR) and \(\beta\)-klotho, a single transmembrane protein which is highly expressed in adipose tissue, liver, pancreas and hypothalamus\(^4,^7,^8\). Adipocytes are the primary target of FGF21, where it increases glucose uptake, modulates lipolysis\(^9\), enhances mitochondrial oxidative capacity, enhances PPAR\(\gamma\) activity\(^10\), and also promotes browning of white adipose tissue\(^11\). Furthermore, therapeutic administration of FGF21 has been shown to increase the production of adiponectin\(^12,^13\), an adipocyte-secreted hormone with insulin-sensitizing, anti-inflammatory and vascular protective activity. Adiponectin knockout mice are resistant to the effects of FGF21 on alleviation of insulin resistance, hyperglycemia, dyslipidemia and fatty liver disease associated with dietary or genetic obesity\(^12\), suggesting that adiponectin acts as an obligatory downstream mediator of FGF21 on energy metabolism and insulin sensitivity. In
addition, FGF21 has also been shown to exert its direct actions on pancreas, hypothalamus, heart and liver\textsuperscript{14-18}, acting as a mediator to coordinate the multi-organic crosstalk under various pathophysiologica1 conditions.

Although the metabolic functions of FGF21 are well characterized, little is known about its pathophysiological roles in atherosclerosis, a chronic inflammatory disease intimately associated with metabolic syndrome. A number of clinical studies have observed an increased circulating level of FGF21 in atherosclerotic patients or those individuals who are at the high risk of developing this disease\textsuperscript{19,20}. In both rhesus monkey and humans with obesity and diabetes, chronic administration of FGF21 decreases LDL-cholesterol and increases HDL-cholesterol\textsuperscript{5,21,22}. However, whether such a beneficial effect of FGF21 on lipid profiles is sufficient to render a protection against atherosclerotic diseases has not been explored. To address this issue, we investigated the impact of both FGF21 deficiency and replenishment on the pathogenesis of atherosclerosis in apoE\textsuperscript{-/-} mice. Our results showed a markedly aggravated atherosclerotic phenotype of FGF21 knockout mice, which can be reversed by replenishment of FGF21. Therefore, we further investigated the mechanisms whereby FGF21 protects atherosclerosis via its multiple actions in both adipose tissue and liver.

**Methods**

Additional details of mice and experimental procedures are included in the online-only Data Supplement. All the animal studies were approved by the animal research ethics committee of Wenzhou Medical University and the University of Hong Kong.

**Statistical Analysis**

Statistical analysis was performed using either the Mann-Whitney U test or the Kruskal-Wallis
test when >2 experimental conditions were compared. When the global Kruskal-Wallis test was significant, pairwise comparisons were performed with the Dunn-Sidak procedure for multiple corrections. Repeated measure ANOVA was used to compare circulating FGF21 levels between WT and apoE<sup>−/−</sup> mice at different time points, as well as serum levels of FGF21 and adiponectin in FGF21 and apoE<sup>−/−</sup> double deficiency (DKO) mice at different time points after administration with FGF21 or adiponectin. The survival of mice was compared using Kaplan-Meier survival analysis with a log-rank test. All statistical analyses were performed with IBM SPSS version 20.0. A value of p < 0.05 was considered statistically significant.

**Results**

**FGF21 Deficiency Accelerates Atherosclerotic Plaque Formation in apoE<sup>−/−</sup>Mice**

Several clinical studies have observed a significantly elevated serum level of FGF21 in patients with atherosclerosis. Consistently, both circulating levels of FGF21 and its hepatic mRNA expression were progressively elevated in apoE<sup>−/−</sup> mice with spontaneous development of hypercholesterolemia and atherosclerosis (Figure 1A-1B in the online-only Data Supplement). To explore the pathophysiological roles of FGF21 in atherosclerosis, we generated DKO mice by backcrossing FGF21 knockout mice into apoE<sup>−/−</sup> mice in C57BL/6J background for over 10 generations. DKO mice were confirmed by both PCR analysis and Western blot analysis of the liver tissue (Figure 2 in the online-only Data Supplement). There were no obvious differences in food intake and body weight between apoE<sup>−/−</sup> mice and DKO mice on standard chow (Figure 3A in the online-only Data Supplement). However, the atherosclerotic lesion area in DKO, as determined by oil red O staining of entire aorta, was 1.6-fold and 1.8-fold greater at 24 weeks and 52 weeks than age-d and sex-matched apoE<sup>−/−</sup> mice (p<0.01, Figure 1A). Further histological
evaluation showed that the plaque areas in aortic sinus and brachiocephalic artery of 24-week-old DKO mice were a 2.1-fold and 2.9-fold greater than in apoE<sup>−/−</sup> mice (Figure 1B-1C). Likewise, both macrophage infiltration and smooth muscle proliferation in the atherosclerotic lesion area of the aortic sinus in DKO mice were significantly higher than in apoE<sup>−/−</sup> mice (Figure 1D-1E). Cholesterol ester contents extracted from the brachiocephalic artery of DKO mice were also much higher than those in apoE<sup>−/−</sup> mice (Figure 1F), suggesting that FGF21 deficiency renders apoE<sup>−/−</sup> mice more susceptible to atherosclerosis.

To investigate whether accelerated atherosclerosis in DKO mice decreases longevity, we monitored DKO (n=20) and apoE<sup>−/−</sup> mice (n=20) on standard chow for 18 months. The surviving rate of DKO was decreased to approximately 45%, which was significantly lower than that in apoE<sup>−/−</sup> mice (80%, Figure 1G).

**DKO Mice Display Exacerbated Hyperlipidemia and Augmented Inflammation**

Since FGF21 is an important metabolic regulator, we next investigated whether atherosclerosis-prone phenotype of DKO mice is attributed to impaired glucose and/or lipid metabolism. Glucose and insulin levels were comparable between DKO and apoE<sup>−/−</sup> mice (Figure 3B in the online-only Data Supplement). Glucose tolerance test showed a similar glucose excursion in response to intraperitoneal glucose challenge (Figure 3C-3D in the online-only Data Supplement). On the other hand, DKO mice exhibited a 1.5-fold and 2.1-fold increase in plasma levels of total triglyceride and cholesterol respectively (Figure 2A-2B). Further analysis of lipoprotein compositions demonstrated a significantly increased LDL and VLDL, but decreased HDL levels in DKO mice as compared to apoE<sup>−/−</sup> controls (Figure 2C-2E).

Quantitative real-time PCR analysis demonstrated a significantly increased expression of the adhesion molecules ICAM-1 and VCAM-1, and the pro-inflammatory cytokines MCP-1 and
TNF-α in aortic tissues of DKO mice as compared to apoE^{-/-} mice (Figure 2F). Likewise, the circulating levels of these pro-inflammatory chemokines and cytokines in DKO mice were much higher than those in apoE^{-/-} mice (Figure 2G-2J), suggesting that FGF21 deficiency exacerbates both local inflammation in atherosclerotic lesions and systemic inflammation.

Similar to the above findings in chow-fed mice, high fat high cholesterol (HFHC)-induced atherosclerotic plaque formation, hypertriglyceridemia, hypercholesterolemia and production of the pro-inflammatory cytokines were significantly exacerbated in DKO mice as compared to apoE^{-/-} mice (Figure 4 in the online-only Data Supplement), suggesting that FGF21 is also an important protector against Western diet-induced dyslipidemia and atherosclerosis in mice.

**FGF21 Exerts Its Anti-atherosclerotic Effects via both Adiponectin-Dependent and Independent Mechanisms**

Adipocytes are the primary target of FGF21, where it induces the expression and secretion of adiponectin, an adipokine with insulin-sensitizing, anti-inflammatory and anti-atherosclerotic activities. Since the insulin-sensitizing actions of FGF21 are mediated by adiponectin, we next investigated whether FGF21 exerts its anti-atherosclerotic activities via induction of adiponectin. As expected, both circulating levels of adiponectin and its mRNA expression in different adipose depots, including epididymal, subcutaneous, perivascular and perirenal adipose tissues, were significantly reduced in DKO mice as compared to apoE^{-/-} mice (Figure 3A-3B). Daily administration of recombinant mouse FGF21 (rmFGF21) for a period of 16 weeks led to higher circulating levels of adiponectin in DKO mice (Figure 5A-5B in the online-only Data Supplement), which was accompanied by a significant reduction of atherosclerotic lesion area, as determined by both oil red O staining of entire aorta and histological quantification of plaque area.
areas between sinus aorta and brachiocephalic arteries (Figure 3C-3E). Chronic administration of recombinant mouse adiponectin (Figure 5C in the online-only Data Supplement) also alleviated atherosclerotic plaque formation in DKO mice, whereas the magnitude of reduction in atherosclerosis by adiponectin was significantly smaller than that by rmFGF21.

Further histological analysis demonstrated that rmFGF21 and adiponectin caused a similar degree of decrease in collagen composition, smooth muscle proliferation and macrophage infiltration (Figure 4A). The magnitude of reduction in expression of pro-inflammatory chemokines intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion protein-1 (VCAM-1) and cytokines tumor necrosis factor alpha (TNFα) and monocyte chemotactic protein-1 (MCP-1) was also comparable between rmFGF21- and adiponectin-treated DKO mice (Figure 4B-4F). However, in adiponectin-treated DKO mice, cholesterol ester contents in brachiocephalic arteries were reduced only by 22%, which was significantly lower than rmFGF21-mediated reduction (56%) (Figure 4G). Notably, while rmFGF21 decreased total cholesterol in DKO mice to a level comparable to apoE−/− mice, adiponectin had no effect on hypercholesterolemia caused by FGF21 deficiency (Figure 4H), despite that both rmFGF21 and adiponectin had a similar potency in decreasing hypertriglyceridemia in DKO mice (Figure 4I).

We next compared the direct effect of adiponectin and rmFGF21 in several types of blood vessel cells. Consistent with previous reports26,27, recombinant adiponectin directly inhibited PDGF-induced proliferation and migration of human smooth muscle cells (Figure 6A-6B in the online-only Data Supplement), and also reduced the uptake of acetylated LDL in peritoneal macrophages (Figure 6C in the online-only Data Supplement). However, rmFGF21 had no direct effect on these cells.
FGF21 Suppresses Cholesterol Biosynthesis and Enhances Cholesterol Efflux in Mice

Since our data suggest that the cholesterol-lowering effects of rmFGF21 are independent of adiponectin, we further explored the mechanisms by which FGF21 modulates cholesterol metabolism in mice. The intestinal absorption of cholesterol, as measured by the fecal dual isotope ratio of $^{14}$C to $^3$H in feces, was comparable between apoE$^{-/-}$ mice and DKO mice, and was not affected by treatment with either rmFGF21 or adiponectin (Figure 5A). There was a modest, but significant decrease in cholesterol contents in the feces of DKO mice, and this change was reversed by treatment with rmFGF21, but not adiponectin (Figure 5B). On the other hand, the excretion of bile acids into the feces was not altered by either FGF21 deficiency or replenishment with rmFGF21 (Figure 5C). The de novo biosynthesis of cholesterol in the liver, as measured with the amount of [1-$^{14}$C]-acetate incorporated into sterols in liver, was markedly increased by 1.49 folds in DKO mice as compared to apoE$^{-/-}$ mice, and this augmented cholesterol synthesis was completely rectified by replenishment with rmFGF21, but not adiponectin (Figure 5D). Likewise, hepatic cholesterol accumulation was elevated by FGF21 deficiency, but was suppressed by treatment with rmFGF21 (Figure 5E).

We next evaluated the impact of FGF21 on expression of key genes involved in cholesterol metabolism in the liver. In DKO mice, hepatic expression of HMG-CoA reductase (a rate-limiting enzyme involved in cholesterol synthesis) and several other cholesterologenic genes was significantly elevated when compared to apoE$^{-/-}$ mice, whereas this elevation in DKO mice was inhibited by administration of rmFGF21, but not adiponectin (Figure 6A-6B). On the other hand, the expression levels of key genes involved in bile acid metabolism and secretion, including cholesterol 7-alpha-monooxygenase (CYP7A1), sterol 27-Hydroxylase (CYP27A1), sterol 12-alpha-hydroxylase (CYP8B1) and small heterodimer partner (SHP), were not altered by
either FGF21 deficiency or administration (Figure 6C). DKO mice exhibited a modest elevation in expression of ABC5 and ABCG8 (Figure 6D), the two ATP-binding cassette transporters that promote cholesterol secretion. The reduced expression of ABCG5 and ABCG8 was reversed by replenishment with rmFGF21, but not adiponectin.

**FGF21 Inhibits Cholesterol Biosynthesis via Suppression of Srebp-2**

Cholesterol homeostasis is orchestrated by a number of transcriptional factors, including sterol responsive element protein (Srebp)-1a, 1c and 2, liver X receptors (LXRs) and farnesoid X receptor (FXR). We next investigated whether FGF21 modulates cholesterol metabolism via these transcription factors. There was no obvious difference in either mRNA or protein expression of LXRα, FXR and Srebp-1 between DKO mice and apoE−/− mice (Figure 7A-7B). In contrast, DKO mice exhibited a marked elevation in both mRNA and protein expression of Srebp-2, and this change was reversed by administration of rmFGF21, but not adiponectin (Figure 7C-7D). Consistently, the transcriptional activity of nuclear Srebp-2 in the liver of DKO mice was approximately 2.9-fold higher than in apoE−/− mice, as determined by the binding of Srebp-2 in the nuclear extracts to the specific DNA sequences (Figure 7E). Elevated transcriptional activity of Srebp-2 in DKO mice was largely reversed by treatment with rmFGF21, but not adiponectin. On the other hand, the transcriptional activity of nuclear Srebp-1 in the liver was not altered by either FGF21 deficiency or supplementation (Figure 7F).

To explore whether FGF21 lowers cholesterol via inhibition of Srebp-2, adenovirus delivery system was employed to knockdown or overexpress Srebp-2 in the liver. Following tail-vein injection of recombinant adenovirus encoding Srebp-2-specific siRNA, an obvious reduction in Srebp-2 expression was observed at 2 days post infection (data not shown), and its expression continued to decline to a level comparable to apoE−/− mice at day 6 and 12 after Ad-
Srebp-2 siRNA infection (Figure 8A-8B). Notably, suppression of Srebp-2 expression reversed hypercholesterolemia in DKO mice caused by FGF21 deficiency, and concurrently reduced the expression of several cholesterologenic genes, including HMG-CoA reductase (HMGCR), farnesyl diphosphate synthetase (Fdps), squalene synthase (Sqle) and HMG-CoA synthetase (HMGCS), which are all well-known downstream targets of Srebp-2 (Figure 8C). Conversely, the effects of rmFGF21 administration on alleviation of hypercholesterolemia and suppression of cholesterologenic gene expression were abrogated by adenovirus-mediated expression of Srebp-2 (Figure 8D-8F).

**The suppressive effects of FGF21 on cholesterol biosynthesis are mediated by β-klotho and FGFR2 in the liver.**

FGF21 exerts its actions by binding to FGF receptors (FGFR) and its co-receptor β-klotho, the latter of which is highly expressed in the liver. To determine whether the regulatory effects of FGF21 on cholesterol homeostasis are attributed to its direct hepatic actions, we generated the β-klotho liver-specific knockout (β-klotho-LKO) mice by intravenous injection of adenovirus-associated virus encoding Cre recombinase into β-klotho-floxed mice (Figure 7A-7B in the online-only Data Supplement). Daily administration of FGF21 significantly decreased HFHC diet-induced hypercholesterolemia, which was accompanied by decreased expression of Srebp-2 and several cholesterologenic genes in β-klotho-floxed mice injected with AAV encoding GFP as wild-type control, whereas these effects of FGF21 were largely abrogated in β-klotho-LKO mice. By contrast, the stimulatory effects of FGF21 on adiponectin production were comparable between β-klotho-LKO mice and β-klotho-floxed mice, suggesting that hepatic β-klotho mediates the effects of FGF21 on lowering cholesterol, but not on elevating adiponectin levels (Figure 7C-7H in the online-only Data Supplement).
Among four major subtypes of FGFRs, FGFR1 plays a key role in mediating the FGF21 actions in adipose tissues\textsuperscript{31}. However, hepatic expression levels of FGFR1 were hardly detectable (Figure 8A in the online-only Data Supplement). Instead, FGFR4 and FGFR2 were abundantly present in the liver, followed by FGFR3\textsuperscript{31}. We next explored the role of these FGFRs in mediating the hepatic actions of FGF21 on cholesterol metabolism using adenovirus-mediated knockdown of their expression. Notably, the inhibitory effects of FGF21 on expression of Srebp-2 and cholesterologenic genes, and hypercholesterolemia were significantly compromised in mice with reduced hepatic expression of FGFR2 (Figure 8B-8G in the online-only Data Supplement). By contrast, these FGF21 actions on cholesterol metabolism were little affected by knocking down the expression of other three FGFRs despite over 70\% knocking down efficiency (data not shown). Taken together, these findings suggest that the regulatory effects of FGF21 on cholesterol homeostasis are mediated at least in part by the FGFR2-\(\beta\)-klotho complex.

### Discussion

Despite intensive research on metabolic functions of FGF21, its role in cardiovascular system has scarcely been explored. This study provides novel evidence that FGF21 deficiency causes a marked exacerbation of atherosclerosis and increased mortality of apoE\textsuperscript{-/-} mice, suggesting that FGF21 is a physiological protector against vascular diseases. In this connection, elevated circulating FGF21 levels in patients and rodents with atherosclerosis may represent the body’s defense mechanism to prevent vascular damage. In support of this notion, upregulated FGF21 has been shown to act as a compensatory mechanism to protect against cerulein-induced pancreatitis\textsuperscript{15}, endotoxin-induced sepsis\textsuperscript{32}, and acetaminophen-induced acute liver injury\textsuperscript{33}.

Atherosclerosis is a chronic inflammatory disease involving multiple cell types at various
stages of the plaque formation, including endothelial cells, lymphocytes, monocytes/macrophages and smooth muscle cells. Our histological and immunological analysis demonstrated that depletion of FGF21 in apoE−/− mice causes a markedly increased endothelial activation (as determined by expression of endothelial adhesion molecules), augmented macrophage infiltration and foam cell formation, exacerbated smooth muscle cell proliferation and collagen deposition, all of which can be reversed by replenishment of exogenous rmFGF21, suggesting that FGF21 is able to inhibit almost every key pathogenic event of atherosclerosis. However, these anti-atherosclerotic effects of FGF21 are not attributed to its direct actions on the vascular walls, but due to the ability of FGF21 in induction of adiponectin in adipocytes and reduction of cholesterol biosynthesis in the liver. In support of this notion, the expression of β-klotho, an obligatory co-receptor of FGF21, is hardly detectable in any type of blood vessel cells (Lin Z and Xu A, unpublished observation), despite its high abundance in adipose tissue and liver.

Recent studies have demonstrated the effects of FGF21 on elevation of circulating adiponectin in both rodents and humans. In adipocytes, FGF21 can stimulate the gene expression as well as the protein secretion of adiponectin in a PPARγ-dependent manner. Adiponectin possesses potent anti-inflammatory and anti-atherosclerotic activities via its multiple actions on blood vessels. In humans, hypoadiponectinemia is an independent risk factor for vascular inflammation and atherosclerosis. In contrast, elevation of circulating adiponectin by either pharmacological or genetic intervention can decrease neointima formation and atherosclerosis in both rodents and rabbits. Adiponectin accumulates in the atherosclerotic lesion area, where it protects vascular endothelium by promoting nitric oxide and alleviating oxidative stress, suppresses smooth muscle cells proliferation and migration, inhibits
macrophage infiltration and foam cell formation, and ameliorates the collagen deposition\textsuperscript{35}. In line with these reports, our results demonstrated that adiponectin, but not FGF21, suppresses PDGF-induced proliferation and migration of smooth muscle cells and blocks LDL uptake and cholesterol accumulation in macrophages. On the other hand, the exacerbated smooth muscle proliferation and macrophage infiltration in the atherosclerotic plaques of DKO mice can be largely reversed by replenishment with adiponectin. Taken together, these findings suggest that the effects of FGF21 on smooth muscle cells and macrophages in the vessel walls are indirect, mediated in part by induction of adiponectin.

Dyslipidemia, especially elevated LDL-cholesterol, is a major contributor to atherosclerotic plaque formation. The cholesterol-lowering drugs, such as statins, have been used clinically to reduce the risk of coronary heart disease. Therapeutic administration of FGF21 has been shown to alleviate dyslipidemia in rodents\textsuperscript{4}, obese monkeys\textsuperscript{22} and patients with type 2 diabetes\textsuperscript{38}, including reductions in total and LDL-cholesterol and triglycerides, elevations in HDL-cholesterol and a shift to a less atherogenic apolipoprotein profile. Consistent with these pharmacological studies, our present study showed that FGF21 deficiency in apoE\textsuperscript{-/-} mice causes a further aggravation of hypercholesterolemia and a shift of apolipoprotein profiles from HDL to LDL. Notably, the severe hypercholesterolemia in DKO mice is accompanied by augmented de novo cholesterol biosynthesis and increased expression of several cholesterologenic genes in the liver, suggesting that endogenous FGF21 is a physiological suppressor of hepatic cholesterol production. However, while adiponectin replenishment reverses hypertriglycerideremia, it has little effect on hypercholesterolemia and augmented hepatic cholesterologenesis in DKO mice, suggesting that the cholesterol-lowering activity of FGF21 is independent of adiponectin. Given that hepatic FGF21 expression is progressively elevated with the development of hypercholesterolemia in
apoE−/− mice, it is possible that FGF21 acts as a sensor of cholesterol overload, which in turn prevents further worsening of hypercholesterolemia via its autocrine inhibition of hepatic cholesterologenesis.

Srebps, which structurally belong to the basic helix-loop-helix-leucine zipper transcription factor family, are the principal regulator of lipid synthesis29. Unlike other members of this class of transcription factor, Srebps are synthesized as membrane-bound precursors that require cleavage by a two-step proteolytic process in order to release their amino-terminal transactivation domain into the nucleus to bind to a specific DNA sequence (sterol regulatory element), and activate their target genes29. Hepatic expression and activity of Srebps are tightly regulated at both transcriptional and posttranslational levels by metabolic hormones and nutritional factors29. Srebp-1a and 1c preferably activate transcription of genes involved in fatty acid synthesis, whereas Srebp-2 displays strong specificity for genes involved in cholesterol biosynthesis39,40. Our present study demonstrated that the expression and transcriptional activity of Srebp-2, but not Srebp-1, is significantly enhanced by FGF21 deficiency, but is markedly suppressed by FGF21 treatment.

Furthermore, adenovirus-mediated silencing of hepatic Srebp-2 expression is sufficient to counteract exacerbation of hypercholesterolemia and augmentation of hepatic cholesterol biosynthesis caused by FGF21 deficiency, whereas the therapeutic benefits of systemic FGF21 administration on inhibition of hepatic cholesterologenesis and reduction of hypercholesterolemia are abrogated by overexpression of Srebp-2. Thus, our study identifies hepatic Srebp-2 as a key intracellular mediator conferring the regulatory effects of FGF21 on cholesterol homeostasis.

Although the precise signaling pathways whereby FGF21 selectively suppresses hepatic Srebp-2 remain unclear, differential regulation of Srebp-1 and Srebp-2 has been reported in several previous studies41,42. High carbohydrate diet induces the mRNA and protein expression of Srebp-1
but not Srebp-2\textsuperscript{41}, whereas dietary cholesterol enhances the expression of Srebp-2 and Srebp-1c but not Srebp-1a\textsuperscript{42}. The NAD\textsuperscript{+}-dependent deacetylase sirtuin (Sirt) 6 and FOXO3 suppress the transcriptional activation of the Srebp-2 gene without any obvious effect on Srebp-1\textsuperscript{43}. Notably, FGF21 has been shown to form a regulatory loop with Sirt1 to reduce diet-induced fatty liver disease\textsuperscript{44}. Further investigation is warranted to interrogate the role of the sirtuin family members in mediating FGF21-induced suppression of hepatic Srebp-2.

In summary, our present study uncovers the protective effects of FGF21 against atherosclerosis via induction of adiponectin in adipose tissue, reduction of hypercholesterolemia by suppression of hepatic Srebp-2, and augmentation of cholesterol efflux possibly by increasing ABCG5/8 expression (\textbf{Figure 9} in the online-only Data Supplement). Consistent with our animal data, a recent clinical trial in obese patients with type 2 diabetes showed that chronic administration of a long-acting form of FGF21 causes a marked elevation of adiponectin and an obvious reduction in total and LDL cholesterol, but has little effect on hyperglycemia\textsuperscript{38}. Therefore, our present study, together with this clinical data, raises the possibility that FGF21 or its agonists might be more effective for treatment of atherosclerosis, instead of diabetes.

There are several limitations in our study. First, our observations are solely based on rodent models. In light of the fact that there is a difference in lipid metabolism and cardiovascular structure between rodents and humans, the pathophysiological relevance of our findings remains to be confirmed in humanoid large animals (such as pigs) and in clinical studies. Second, although our data demonstrated the obligatory role of β-klotho and FGFR2 in mediating the cholesterol-lowering effects of FGF21 via suppression of Srebp-2 in the liver, the signaling pathways that link FGF21 receptor to its regulation of cholesterol metabolism need further investigation.
**Acknowledgments:** We thank Dr. Ruby C.L. Hoo from faculty of Medicine, the University of Hong Kong for technical assistance.

**Funding Sources:** This work was supported by the National Natural Science Foundation of China (Major Program 91439123; General Program 81471075); General Research Fund (784111M), Collaborative Research Fund (CUHK2/CRF/12G and HKU4/CRF/10) and Theme-Based Research Scheme Grant T12-705/11 from the Research grant Council of Hong Kong, and Qatar National Research Fund (NPRP 6-428-3-113).

**Conflict of Interest Disclosures:** None.

**References:**


19. Lin Z, Wu Z, Yin X, Liu Y, Yan X, Lin S, Xiao J, Wang X, Feng W, Li X. Serum levels of fgf-21 are increased in coronary heart disease patients and are independently associated with...


**Figure Legends:**

**Figure 1.** ApoE−/− mice with FGF21 deficiency exhibit exacerbated atherosclerosis and premature death. Aortas were dissected from 24-week-old and 52-week-old apoE−/− mice and apoE−/−FGF21−/− (DKO) mice. n=8 in each group. (A) *En face* staining of entire aortas of 24-week-old mice with oil red O. (B, C) Cross-sections of aortic sinuses and brachiocephalic arteries of 24-week-old mice respectively. (D, E) Macrophage infiltration and smooth muscle proliferation in aortic sinus as determined by immunostaining for F4/80 and α-actin respectively. (F) Cholesterol ester levels in brachiocephalic arteries (BCA) of 24 week-old mice. (G) The surviving rate of apoE−/− mice (n=20) and DKO mice (n=20) on standard chow was monitored for 18 months. Data are presented as dot plots with the line indicating the median. The Mann-Whitney U test was used for two-group comparisons(A-F); The survivals of mice were compared using Kaplan-Meier survival analysis with the log-rank test (G).

**Figure 2.** FGF21 deficiency worsens lipid profiles and exacerbates inflammation in apoE−/− mice. DKO and apoE−/− mice fed with standard chow were sacrificed at 24 weeks after birth. Plasma samples were collected for measurement of triglycerides (TG, A), total cholesterol (TC, B), HDL (C), LDL (D) and VLDL (E). (F) The mRNA expression of ICAM-1, VCAM-1, MCP-1
and TNF-α in aortic tissue as well as (H-J) plasma levels of these pro-inflammatory chemokines and cytokines were measured with real-time PCR and ELISA respectively. n=6-7. Data are presented as dot plots with the line indicating the median. The Mann-Whitney U test was used for comparison of two groups.

**Figure 3.** Recombinant mouse FGF21 and adiponectin ADN attenuate the atherosclerotic plaque formation in DKO mice. 8-week-old DKO mice were treated with recombinant mouse FGF21 (0.1mg/kg.day), adiponectin (10mg/kg.day) or vehicle by daily intraperitoneal injection for a period of 16 weeks. (A, B) Plasma levels of adiponectin and its mRNA expression in epididymal adipose tissues (EPAT), subcutaneous (SAT), perivascular (PVAT) and perirenal (PRAT) adipose tissues. (C) En face staining of entire aortas with oil red O. (D, E) Cross-section analysis of aortic sinuses and brachiocephalic arteries with oil red O respectively. n=6-7. Data are presented as dot plots with the line indicating the median. The Mann-Whitney U test was used to compare two groups (A,B). The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure (C-E).

**Figure 4.** Differential effects of FGF21 and adiponectin on lipid profiles and atherosclerotic plaque composition in DKO mice. DKO mice were treated with recombinant mouse FGF21, adiponectin (ADN) or vehicle for 16 weeks as in Figure 3. (A) Immunohistological analysis of atherosclerotic lesion areas in aortic sinuses with antibodies against the smooth muscle marker α-actin, the macrophage marker F4/80, or with Masson trichrome staining for the collagen composition as indicated. (B-F) The mRNA expression of several pro-inflammatory chemokines and cytokines in the aortic sinus and their plasma levels as determined by real-time PCR and ELISA respectively. (G) Cholesterol ester content in the brachiocephalic arteries. (H, I) Plasma
cholesterol and triglyceride levels in DKO mice treated with rmFGF21, ADN or vehicle respectively. n=5-7. Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.

**Figure 5.** Effects of FGF21 and ADN on cholesterol metabolism in mice. DKO mice were treated with recombinant mouse FGF21, ADN or vehicle for 4 weeks as in Figure 4. apoE<sup>−/−</sup> mice were used as a control. (A) The absorption rate of dietary cholesterol was determined by oral gavage with [14C] cholesterol and [3H] sitostanol, followed by measurement of the ratio of the two isotopes in feces. (B) Fecal cholesterol and (C) bile acids were measured with the corresponding commercial kits respectively. (D) The rate of de novo cholesterol synthesis as measured by determining the amount of [1-14C]-acetate incorporated into sterols per minute per gram liver tissue. (E) Hepatic cholesterol contents determined by a Cholesterol Assay Kit. n=6-7. Data are presented as dot plots with the line indicating the median. The global significance among four groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.

**Figure 6.** FGF21, but not ADN, alters hepatic expression of the key genes involved in cholesterol biosynthesis and transport. Total RNA extracted from the liver of apoE<sup>−/−</sup> mice or DKO mice treated with recombinant mouse FGF21, ADN or vehicle as in Figure 4 was subjected to real-time PCR analysis. The relative mRNA expression levels of genes involved in cholesterol synthesis including HMGCR, HMGCS, Sqle and Fdps (A) genes involved in bile acids metabolism including CYP7A1, CYP8B1, CYP27A1 and SHP (B) as well as genes involved in cholesterol transports including ABCG5 and ABCG8 (C). n=5-7. Data are presented as dot plots
with the line indicating the median. The global significance among four groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.

**Figure 7.** Effects of FGF21 and ADN on several key transcription factors involved in cholesterol metabolism. The liver samples from apoE<sup>−/−</sup> mice or DKO mice treated with recombinant mouse FGF21, ADN or vehicle as in Figure 4 were subjected to real-time PCR or Western blot analysis. (A, B) The relative mRNA and protein expression levels of LXRα, FXR, and Srebp-1. (C, D) The relative mRNA and protein expression of Srebp-2. (E, F) The DNA binding activities of Srebp-1 and Srebp-2 in the nuclear extracts of liver tissues. n=5-7. Data are presented as dot plots with the line indicating the median. The global significance among four groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.

**Figure 8.** FGF21 decreases hypercholesterolemia via inhibition of hepatic Srebp-2. (A-C) DKO mice were infected with adenovirus encoding siRNA specific to Srebp-2 or scrambled control (5x10<sup>8</sup> p.f.u/mouse) for various periods. Age-matched apoE<sup>−/−</sup> mice were used as a control. (A) Protein expression levels of hepatic Srebp-2 at day-6 and day-12 after adenoviral infection. (B) Circulating levels of total cholesterol (TC), (C) the expression levels of cholesterologenic genes determined by real-time PCR analysis at day-12 (n=6). (D-E) DKO mice were infected with adenovirus encoding Srebp-2 (Ad-Srebp-2) or luciferase (Ad-Luc) for 6 days (as control), followed by treatment with daily intraperitoneal injection of rmFGF21 (0.1mg/kg.day) for another six days. (D) The protein expression levels of Srebp-2 in the liver, (E) serum levels of total cholesterol, (F) The mRNA expression of cholesterologenic genes at 12 days after
adenoviral infection (n=6). Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A
Srebp-2
β-actin

6 days 12 days

Global p=0.0006 Global p=0.001

Srebp-2/β-actin (fold change)

B

6 days 12 days

Global p=0.0001 Global p=0.0001

Srebp-2/β-actin (fold change)

C

HMGCR Fdps Sqle HMGCS

Relative mRNA levels (Fold change)

D

Ad-Srebp-2 Ad-Luc

6 days 12 days

Global p=0.0002

E

Ad-Srebp-2 Ad-Luc

6 days 12 days

Global p=0.0002

F

Ad-Srebp-2 Ad-Luc

Relative mRNA levels (Fold change)
Fibroblast Growth Factor 21 Prevents Atherosclerosis by Suppression of Hepatic Sterol Regulatory Element-Binding Protein-2 and Induction of Adiponectin in Mice
Zhuofeng Lin, Xuebo Pan, Fan Wu, Dewei Ye, Yi Zhang, Yu Wang, Leigang Jin, Qizhou Lian, Yu Huang, Hong Ding, Chris Triggle, Kai Wang, Xiaokun Li and Aimin Xu

Circulation. published online March 20, 2015;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2015/03/20/CIRCULATIONAHA.115.015308
Free via Open Access

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2015/03/20/CIRCULATIONAHA.115.015308.DC1
SUPPLEMENTAL MATERIAL

Supplementary methods

Mice

FGF21 knockout mice in C57BL/6J background were generated as previously described\(^1\). C57BL/6J apoE\(^{-/-}\) mice were obtained from Jackson Laboratory. ApoE\(^{-/-}\) mice were backcrossed with FGF21\(^{+/+}\) mice for at least ten generations to obtain apoE\(^{-/-}\) / FGF21\(^{+/+}\) mice (namely DKO mice). β-klotho-floxed mice were generated by Shanghai Nanfang Center for Model Organisms, and were used to produce β-klotho liver specific knockout mice (β-klotho LKO) by injecting with adeno-associated virus encoding Cre recombinase (AAV-Cre) under the control of the mouse apoE gene promoter. All the mice were housed in a room at controlled temperature [23±1°C] with a 12 hour light-dark cycle, and had free access to water and standard rodent diet. For the intervention studies, 8-week-old DKO and β-klotho LKO mice were treated with recombinant full-length adiponectin produced from mammalian cells\(^2\) and recombinant mouse FGF21\(^3\) by daily intraperitoneal injection respectively. Glucose and insulin tolerance tests were performed as described previously\(^4\). All the animal studies were approved by the animal research ethics committee of Wenzhou Medical University and the University of Hong Kong.

Analysis of Atherosclerotic Lesions

Oil red O staining was used to assess the size of the atherosclerotic lesion as previously described\(^5, 6\) with slight modifications. For en face analyses of lesions in the entire aorta, after perfusion, whole aorta was dissected out, opened longitudinally from heart to the iliac arteries, pinned on a black wax pan, and stained with Oil red O. The images of the aorta were captured using a SONY DXC-970MD color video camera, analyzed with the Image-Pro plus program.
(Media Cybernetics, Silver Spring, MD), and presented as the percentage of lesion area in whole aorta.

For analysis of plaque lesion in aortic sinus, the heart and proximal aorta were removed and embedded in optimum cutting temperature compound. Serial 10μm-thick cryosections from the middle portion of the ventricle to the aortic arch were collected. For analysis of lesion in brachiocephalic arteries, serial 10μm-thick cryosections from a 150μm-distance position to aortic arch were collected. Then, cryosections of aortic sinus and brachiocephalic arteries were stained with Oil Red O and hematoxylin. The lipid-containing area on each section was determined in a blinded fashion, using an ocular piece with a 20×20 μm² grid on a light microscope. The average lesion area per aorta, calculated from 5 to 10 sections of each aorta, was determined.

**Immunocytochemistry**

The cryosections of mouse aortic arches were fixed in acetone and blocked with non-immune rabbit serum, followed by incubation with a rabbit polyclonal antibody against the macrophage marker F4/80 (Abcam, Cambridge, MA) or smooth muscle α-actin (Sigma, St Louis, MO) for 90 minutes. After washing, the sections were incubated with a FITC- or Cy3-labeled secondary goat anti-rabbit antibody for 30min. All the slides were examined under the Olympus biological microscope BX41, and the images were recaptured with an Olympus DP72 color digital camera. Planimetry on the photographed cross-sections was performed using Image-Pro Plus version 5.0.1 (Media Cybernetics, Inc, Bethesda, MD). The lesion areas were defined as an area between the lumen and internal elastic lamina, and the cell contents were presented as the percentage of positive surface areas in the lesion area.

**Measurement of Cholesterol/cholesteryl Ester Levels in the Brachiocephalic arteries**

The analysis of cholesterol/cholesteryl ester abundance was measured with a commercial kit according to the manufacturer instruction (abcam 65359, Abcam, Cambridge, UK). Briefly, the brachiocephalic artery was dissected free of adventitial tissue, weighed, extracted with 200μl of chloroform: Isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer after weighting, and centrifuged at 15,000 x g for 10 minutes. The organic phase was transferred to a new
tube and air-dried at 50°C followed by vacuum drying for 30 min to remove trace organic solvent. The lipids were then dissolved with 200μl of Cholesterol Assay Buffer. 50μl of the samples or standard cholesterol were incubated with the reaction mixture including cholesterol esterase, cholesterol enzyme mix and cholesterol probe for 60 minutes at 37°C, followed by measurement of absorbance at 570nm with a microplate reader. Data were expressed as μmole cholesterol/cholesteryl ester per g tissue.

**Biochemical and Immunological Assays**

Plasma lipid profiles including total triglycerides, total-, HDL- and LDL-cholesterol levels were measured with commercial kits from Sigma, (St. Louis, MO). Liver total lipids were extracted with acetone and chloroform/methanol (2/1, v/v) as described7, and total cholesterol levels in the liver were quantified as above and expressed as mg cholesterol per gram liver tissue (wet). Plasma levels of insulin and total adiponectin were measured using immunoassays from Antibody and Immunoassay Services at the University of Hong Kong. MCP-1 and TNF-α concentrations were analyzed with immunoassays from R&D System Inc (Minneapolis, MN).

**Measurement of Cholesterol Absorption**

Intestinal cholesterol absorption was determined by a fecal dual-isotope ratio method8. Briefly, mice were injected with 2.5 μCi 3H-cholesterol in Intralipid (Sigma, St Louis, MO, USA) via tail vein, followed by oral gavage of 1 μCi 14C-cholesterol in median-chain triglycerides (MCT oil, Mead Johnson, Evansville, IN). Mice were returned to cage with free access to food and water. After 72 h, blood samples were collected and the radioactivity of 14C and 3H were determined by scintillation counting. Intestine cholesterol absorption was determined as the ratio of 14C/3H in 1 ml of plasma.

**Hepatic de novo Cholesterol synthesis**

Hepatic cholesterol synthesis was determined as previous described9. Mice fasted for 4 hours were intraperitoneally injected with 10 μCi of [1-14C]-sodium acetate (Perkin Elmer, Waltham, MA), and were then sacrificed at 30min after injection to harvest liver tissue. Approximately 250mg of liver tissue was rinsed in ice-cold PBS, and then saponified in 2.2
ml mixture of 50% KOH: 95% ETOH (1:10, v: v) at 70°C overnight. $^3$H-cholesterol (1 μCi) was added to the same tube as a recovery control. Sterols were extracted with 3 ml hexane, dried and redissolved in 300μl mixture of acetone : ETOH (1:1, v: v), followed by precipitation with 1 ml of digitonin (0.5% in 95% ETOH) overnight at room temperature. The radioactivity of $^3$H and $^{14}$C in the precipitates was determined in a scintillation counter. Cholesterol synthesis rate was expressed as the amount of [1-$^{14}$C]-acetate incorporated into sterols per minute per gram liver tissue.

**Measurement of Fecal Cholesterol and Bile Acids**

Feces were collected from individually housed mice over a 3-day period and were dried, weighed, and ground to a powder. Lipids were extracted from feces with chloroform /methanol (2:1), dried, and dissolved with 5% Triton X-100 in isopropanol. Cholesterol contents were quantified using a biochemical assay. For analysis of bile acids, 50 mg of dried feces were added to 2.2 ml ethanolic NaOH (0.08M) and heated (95°C) for 2 h. After cooling, neutral sterols were extracted three times with 5 ml hexane, followed by acidification with 2.5 ml of 0.16M HCl and extraction with 5 ml of ethyl acetate. The dried extract was solubilized in 1.25% Triton X-100 in 20% methanol. Bile acids in each sample were determined with a Bile Acid Assay Kit (Genzyme Diagnostic, Framingham, MA).

**RNA Extraction and Real-Time PCR**

Total RNA was extracted from liver or adipose tissues with TRIzol reagent (Invitrogen), and complementary DNA was synthesized from 0.5μg total RNA by reverse transcription with an ImProm-II reverse transcription kit (Promega) with random hexamer primers. Quantitative real-time PCR was performed on the Applied Biosystems Prism 7000 sequence detection system, with specific primers described in [Supplementary Table 1](#). The amplification efficiency, as calculated from the slope of each standard curve using the formula $E=10^{(-1/Slope)} \times 100$, is between 92 and 105%. The relative expression level of each gene was calculated with the Pfaffl methods as previously described$^{10}$, using the β-actin gene as the reference control for normalization.
Srebp-1 and Srebp-2 DNA Binding Activity Assay

Srebp-1 and -2 DNA binding activities were measured by a commercial kit according to the manufacturer instruction (ab133125 and ab133111, Abcam Cambridge, UK). In brief, approximately 10mg nuclear extracts isolated from liver tissue were added to 96-well microplates coated with a specific double strand DNA (dsDNA) sequence containing with Srebp-1 or -2. The unbound reagents were removed by extensive washing, followed by sequential incubation with a primary antibody against Srebp-1 or -2 for 2 h, and a HRP-conjugated secondary antibody was added and incubated for 30 minutes. After washing, the substrate reagents were added, and the absorbance values at 450nm were measured with a microplate reader.

Construction of Adenoviral Vectors for Knockdown Srebp-2, FGFR2, FGFR3 and FGFR4 or Overexpression of Srebp-2

An adenovirus delivery system was used for knocking down Srebp-2, FGFR2, FGFR3 and FGFR4 expression with small hairpin RNA as well as for overexpression of Srebp-2. The oligonucleotides for generation of siRNA against these genes as well as the corresponding scrambled controls are listed in Supplementary Table 2. The forward and reverse oligonucleotides were annealed, ligated into pENTR/U6 entry vector, and then subcloned into pAd/BLOCK-iT DEST vector through recombination. To construct adenoviral vectors for overexpression of Srebp-2, cDNA encoding amino acids 1–460 of mouse Srebp-2 was inserted into pshuttle-CMV vector, and then subcloned into pAdeasy-1 adenoviral backbone vector (Stratagene) through recombination in Escherichia coli.

To package adenovirus, the adenoviral vectors were linearized with the restriction enzyme PacI and transfected into HEK293 cells using Lipofectamine 2000. After several rounds of propagation, recombinant adenovirus was purified by an AdEasy virus purification kit (Stratagene), and the titer was determined with an endpoint assay as described11.

Smooth Muscle Proliferation and Migration Assays

Human aortic smooth muscle cells (HASMCs) were maintained in a humidified 37 °C and 5% CO₂ environment in OPTI-MEM® supplemented with 10% fetal bovine serum, 0.01 mg/ml
Supplemental materials_FGF21 and atherosclerosis by Lin Z.

insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, and 0.05 mg/ml ascorbic acid. Cells were routinely split at a 1:4 ratio, and cultures between the third to eighth passages were used. For proliferation analysis, cells were plated in 24-well plates (8000 and 10000 cells/well for HASMCs) and grown for 24 h. Medium was then replaced with DMEM with 0.1% BSA for 24 h. The medium was subsequently changed to fresh DMEM with 1% fetal bovine serum plus PDGF-BB (10ng/ml), recombinant mouse FGF21 (50ng/ml) and/or recombinant mouse adiponectin (2.0μg/ml). 18 h after incubation, 1 μCi of [methyl-³H] thymidine was added into each well for another 6 h, and the amount of [³H] thymidine incorporated into DNA in each well was determined as we described previously12.

To assess cell migration, a Boyden chamber assay was performed using Transwell chambers (6.5mm, model 3422; Costar, Cambridge, MA, USA) with an 8-mm pore polycarbonate membrane. Growth-arrested HASMC were harvested, suspended in serum-free DMEM. Cells were added to the upper chamber of the Transwell at 5 × 10⁵ cells in 100 μl/well. A total of 600 μl of serum-free DMEM was added to the lower chamber, followed by treatment with PDGF-BB(10ng/ml), recombinant full-length adiponectin (2.0μg/ml) or recombinant mouse FGF21(50ng/ml) for 24 h respectively. Afterwards, the nonmigratory cells were removed from the upper surface of the membrane by scraping with cotton swabs. Membrane was then fixed with methanol, stained with Diff-Quik solution (Baxter, McGaw Park, IL, USA) and mounted on a glass slide. Migrated cells were counted at x400 magnification in 10 microscope fields per filter.

Analysis of Macrophage Uptake of Acetylated LDL (AcLDL)

AcLDL labeled with the fluorescent probe 3,3,39,39-tetramethylindocarbocyanine perchlorate (Dil-AcLDL) and unlabeled AcLDL were purchased from Biomedical Technologies (Ward Hill, MA 01835 USA). Primary mouse peritonealmacrophages grown in a serum-free RPMI-1640 medium were treated with mouse adiponectin (2.0μg/ml) or rmFGF21(50ng/ml) for 24 h, and were then incubated with 10μg/ml Dil-AcLDL dissolved in RPMI-1640 containing 2% lipoprotein-deficient serum (Sigma, St. Louis, Missouri, USA) for 3 hours. For competition assays, unlabeled AcLDL in excess amounts (50-fold) were added
together with Dil-AcLDL. The cells were resuspended in PBS and analyzed using a FACScan flow cytometer (Becton Dickinson). Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from the mean fluorescent intensity of Dil-labeled cells. The uptake of Dil-AcLDL was calculated by subtracting binding in presence of excess amount of unlabeled AcLDL from total binding in absence of unlabeled acLDL, and expressed as fold changes over untreated cells.

**Generation of β-klotho Liver specific knockout mice**

C57BL/6J mice with the β-klotho gene floxed were generated by Shanghai Nanfang Center for Model Organisms. The targeting construct containing the two loxP sites flanking the exon 2 of the β-klotho gene and the FRT-flanked neomycin selection cassette (supplementary Fig.7A) was electroporated into embryonic stem cells, followed by selection of positive ES clones, microinjection and chimera identification as described previously14. Chimeric males were mated with C57BL/6J females for at least 8 generations to produce homologous β-klotho-floxed mice (β-klotho^fl^) in C57BL/6 background. Genotypes of mice were determined by PCR using the following primers: P1 (5’- TGTTGGGGCCATCTAAAATGG-3’), P2 (5’- GCCAAGACAAACATATTCGGG-3’), which produces a 216-bp fragment for the β-klotho-floxed locus, and a 132-bp fragment for wild-type (WT) locus respectively.

To generate β-klotho liver-specific knockout (Klb-LKO) mice, 1 × 10^{12} genomic particles of adeno-associated virus encoding Cre recombinase (AAV-Cre) or green fluorescent protein (AAV-GFP, as wild-type control) under the control of the mouse apoE gene promoter (which drives hepatocyte-specific gene expression) were intravenously injected into β-klotho^fl^ mice as previously reported15. After 4 weeks of AAV injection, mice were sacrificed to obtain various tissues for Western blot analysis to obtain liver-specific deletion of β-klotho.

**References**

2. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin...


**Supplementary Table 1:** A list of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse primer 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse adiponectin</td>
<td>AGACCTG GCCCATTTCTCCTCAT T</td>
<td>AGAGGAACAGGAGAGGCTTGCAACA</td>
</tr>
<tr>
<td>Mouse CYP7A1</td>
<td>CTGT CATACCAAAAGTCTTATGTCA</td>
<td>ATGCTTCTG TGTCCAAATGCC</td>
</tr>
<tr>
<td>Mouse CYP8B1</td>
<td>GCAAAGAAGATCCACACTAC</td>
<td>ATCTCGTGAGGGCAAAG</td>
</tr>
<tr>
<td>Mouse HMGCR</td>
<td>TCAAGTGGG CACTTGTCCAGCA</td>
<td>TGGAGTACCGCTTACAAACAC</td>
</tr>
<tr>
<td>Mouse HMGCS</td>
<td>GATGA AAGACACAGAAGAAC</td>
<td>CCTACACAGATATCTTAATG</td>
</tr>
<tr>
<td>Mouse SHP</td>
<td>CTATTCTGTATGCACTTCTGTAGCACC</td>
<td>GGCAGTGGCTGTAGATGC</td>
</tr>
<tr>
<td>Mouse ICAM-1</td>
<td>CCACTCAGGTGTATCGTTTC</td>
<td>AGGTCTTGTGCTACTTGT</td>
</tr>
<tr>
<td>Mouse VCAM-1</td>
<td>TGGGGAGGCTCTAACCAGTACT</td>
<td>GCAATGTTTGTGATTAGGCAA</td>
</tr>
<tr>
<td>Mouse MCP-1</td>
<td>GCAGCAGGG TGTCCCAAAAGAA</td>
<td>ATTTACGGCTAACCACATC</td>
</tr>
<tr>
<td>Mouse TNF-α</td>
<td>TCTCTCAGACACACCTCAACC</td>
<td>GAGGCCCGATTTTGAAATCC</td>
</tr>
<tr>
<td>Mouse Faqs</td>
<td>GGAGTTCCCTAGA TAACATGCC</td>
<td>AAGCCTGAGGAGTGTACAC</td>
</tr>
<tr>
<td>Mouse Scle</td>
<td>AGGAAGTTTTTGCCCTCAGGA</td>
<td>AAACACACTTGTTGCAGG</td>
</tr>
<tr>
<td>Mouse Srebpb-1</td>
<td>ACA CGGCTTTTTGAACGATC</td>
<td>CAGAGAGGAGGGCACAGAGAA</td>
</tr>
<tr>
<td>Mouse Srebpb-2</td>
<td>AAGCTGGGCGATGGTAGG</td>
<td>ATCTCGTGATG TCCCG</td>
</tr>
<tr>
<td>Mouse LXRa</td>
<td>GAGTGTGGAAGAAGACAGACCTCAA</td>
<td>GGCCATCCGCTCCCTC</td>
</tr>
<tr>
<td>Mouse FXR</td>
<td>CTGAGCCGCA GCAATCTCA</td>
<td>CACTTTTGGTAGACATTCA</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>GGCTGTATTCCCCTCACG</td>
<td>CCAGTTGGAACAAATGCCATG</td>
</tr>
</tbody>
</table>

**Supplementary Table 2:** The oligonucleotides used for construction of adenoviral siRNA for Srebpb-2, FGFR2, FGFR3, FGFR4 and scrambled control.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sh-Srebpb-2 (forward)</td>
<td>5’-CAC CGA AGA GTG GAT CTG TAC AAT AACGAA GAG GAA GCC CAT TGA TTA C -3’</td>
</tr>
<tr>
<td>Sh-Srebpb-2 (reverse)</td>
<td>5’-AAA AAA GAG TGG ATC TGT ACA ATA ATTCGGAG GAA GCC CAT TGA TTA C -3’</td>
</tr>
<tr>
<td>Sh-scramble (forward)</td>
<td>5’-CACCGAATATTATTAAGGACAGACAGCAGAATCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-scramble (reverse)</td>
<td>5’-AAAAAAATATATTATTAAGGACAGACAGCAGAATCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-FGFR4(forward)</td>
<td>5’-CAC CGAGGCGGACGAACAAACAAGAAGAUGAACAGGAGAAG GAA GCC CAT TGA TTA C -3’</td>
</tr>
<tr>
<td>Sh-FGFR4(reverse)</td>
<td>5’-AAA AAAAUGAUGUUCUUGUGUUGUGUGCTTGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Srebpb-2 (forward)</td>
<td>5’-CACCGAUUCUCCGACAGACGACAGACGACACTCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Srebpb-2 (reverse)</td>
<td>5’-AAA AAAAAGCGAAGGACGAGGAGAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-FGFR2(forward)</td>
<td>5’-CAC CGAGGAGCAAGCAGACAGACGACAGACACTCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-FGFR2(reverse)</td>
<td>5’-AAAAAAAGCGAAGGACGAGGAGAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Sclare (forward)</td>
<td>5’-CACCGAUUCUCCGACAGACGACAGACGACACTCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Sclare (reverse)</td>
<td>5’-AAAAAAAGCGAAGGACGAGGAGAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-FGFR3(forward)</td>
<td>5’-CAC CGA CGACAAGGAGGACTCTAGAGGTT CGAAGCTCTGTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-FGFR3(reverse)</td>
<td>5’-AAAAAAAGCGAAGGACGAGGAGAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Sclare (forward)</td>
<td>5’-CACCGATCTTAATCGGTATAAAGGCGCAAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
<tr>
<td>Sh-Sclare (reverse)</td>
<td>5’-AAAAAAATCTTATCGGTATAAAGGCGCAAATGCTGTCGCTTAAATATATT -3’</td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Dynamic changes in circulating levels and hepatic mRNA expression of FGF21 in apoE<sup>−/−</sup> mice and C57BL/6J mice. The tissue samples were collected at 4-, 12- and 24-week-old male mice. (A) Serum levels of FGF21 measured with an immunoassay. (B) The mRNA expression level of FGF21 in the liver as determined by real-time PCR. n=6. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed using repeated measure ANOVA.
Supplemental Figure 2. Genotyping of FGF21−/−apoE−/− (DKO) mice. FGF21 KO mice were generated by replacing most of exon 1 and all of exons 2 and 3 of the FGF21 gene with the IRES-LacZ-polyA/PGK-neo cassette. Genotypes of mice were determined by PCR using the following primers: P1-Fgf21 (5′-GACTGTTCAGTCAGGGATTG-3′), P2-Fgf21 (5′-CCCGTGATATTGCTGAAGAG-3′), and P3-Fgf21 (5′-ACAGGGTCTCAGGTTCAGA-3′). P1 and P3 produced a 541-bp fragment of the wild-type (WT) Fgf21 locus. P2 and P3 produced a 243-bp fragment of a mutant Fgf21 locus. Genotypes of apoE−/− mice were determined by PCR using the following primers: P1-apoE (5′-TAT CTA AAC AGACTCACAGCCTCCAGACC-3′), P2-apoE (5′-GACTGGGACCA ACA GAC AAT CGG CTG CTCT-3′), P3-apoE (5′-CGAAGGCTGAGGTTACAGAATGGATC-3′). P1 and P3 produced a 400-bp fragment of the wild-type apoE locus. P2 and P3 produced a 600-bp fragment of a mutant apoE locus. (A) Representative PCR genotyping results. (B) Confirmation of FGF21 protein deficiency in the liver tissue of DKO mice by immunoblotting analysis.
Supplemental Figure 3. FGF21 deficiency does not influence body weight, glucose tolerance and insulin sensitivity in apoE\(^{-/-}\) mice. DKO and apoE\(^{-/-}\) mice on standard chow were monitored for (A) body weight, (B) glucose and insulin levels, (C,D) Glucose and insulin tolerance tests were performed at 19 and 20 weeks after birth respectively. n=6. Data are presented as mean ± SEM (A, C and D) or dot plots with the line indicating the median (B).
**Supplemental Figure 4.** FGF21 deficiency exacerbates HFHC diet-induced atherosclerosis, hyperlipidemia and inflammation. 8-week-old apoE−/− mice or DKO mice were fed with HFHC diet for a period of 12 weeks. STC-fed apoE−/− mice were included as a control. (A) *En face* staining of entire aortas with Oil red O. (B) Quantification of aortic plaque areas. (C-G) Serum lipid profiles, including triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL). (H,I) Serum levels of MCP-1 and TNF-α. n = 6-8. ***, p<0.001 vs. vehicle.** Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with a Dunn-Sidak procedure.
Supplemental Figure 5. Circulating levels of FGF21 and adiponectin in DKO mice after intraperitoneal administration of recombinant mouse FGF21 or adiponectin. Serum samples were collected every hour until 6 hours after DKO mice were intraperitoneally injected with FGF21 (0.1 mg/Kg.day), adiponectin (10 mg/Kg.day) or PBS as vehicle control. (A-B) Serum levels of FGF21 and adiponectin at different time points after injection with FGF21. (C) Changes in serum levels of adiponectin after administration with recombinant mouse adiponectin. Note that serum FGF21 is not detectable in DKO mice. n = 5. ***, p<0.001 vs. vehicle. Data are presented as mean ± SEM. Repeated measure ANOVA was used for comparison between two groups at each time points.
Supplemental Figure 6. Adiponectin, but not FGF21, directly suppresses smooth muscle cell proliferation and migration, and LDL uptake of macrophages. (A,B) Human aortic smooth muscle cells were treated with PDGF-AA (0.1μg/ml), adiponectin (ADN, 2.0μg/ml) or recombinant mouse FGF21 (50ng/ml) for 24 hours. The cell proliferation (A) and migration (B) was determined by ³H-thymidine incorporation assay and a modified Boyden chamber assay described in respectively. (C) Primary mouse peritoneal macrophages were treated with ADN (2.0μg/ml), recombinant mouse FGF21 (50ng/ml) or vehicle (PBS) for 24 hours, loaded with Dil-AcLDL (10μg/ml). The uptake of Dil-AcLDL was determined by flow cytometry as described in the method, and expressed as fold changes over untreated cells. n = 5. Data are presented as dot plots with the line indicating the median. The global significance among three groups was determined by Kruskal-Wallis test, followed by pairwise comparisons with the Dunn-Sidak procedure.
**A**

- Diagram showing gene expression and exon regions of WT, Klb-LKO, and Klb-floxed mice.

**B**

- Western blots for liver and WAT showing expression levels of β-klotho, β-actin, Klb-LKO, and Klb-floxed.

**C**

- Timeline of experiments:
  - Week 8: Klb-floxed C57BL/6J mice treated with AAV-Cre or AAV-GFP.
  - Week 14: rmFGF21 (daily i.p. administration).
  - Week 16: Sacrificing.

**D**

- Graph showing relative mRNA levels of Steap-2 in WT and Klb-LKO mice with and without FGF21 treatment.

**E**

- Graph showing DNA binding activity in WT and Klb-LKO mice with and without FGF21 treatment.

**F**

- Graph showing relative mRNA levels of HMGCR, Fdps, and HMGCS in WT, Klb-LKO, and their respective treatments with and without FGF21.

**G**

- Graph showing cholesterol levels in WT and Klb-LKO mice with and without FGF21 treatment.

**H**

- Graph showing adiponectin levels in WT and Klb-LKO mice with and without FGF21 treatment.

**Legend:**
- WT: Wild Type
- Klb-LKO: Klb-Low-Knockout
- β-klotho
- β-actin
- Klb-LKO
- Klb-floxed
- AAV-Cre
- AAV-GFP
- HFHC diet
- rmFGF21
- Sacrificing
- Gene expression
- DNA binding
- Cholesterol
- Adiponectin

**Statistical significance:**
- p=0.002
- p=0.001
- p=0.009
- p=0.012
- p=0.013
- NS

**Additional notes:**
- AA V- Cre
- AA V-GFP
- HMGCR
- Fdps
- HMGCS
- Relative mRNA levels (fold change)
- DNA binding activity (fold change)
- Cholesterol (mg/dl)
- Adiponectin (μg/ml)
**Supplemental Figure 7.** The inhibitory effects of FGF21 on cholesterol biosynthesis are abrogated by liver-selective depletion of β-klotho. (A) Strategies for generating β-klotho liver-specific knockout mice (Klb-LKO) in C57BL/J background. 8-week-old β-klotho-floxed mice (Klb-floxed) were intravenously injected with $1 \times 10^{12}$ genomic particles of adeno-associated virus encoding Cre recombinase (AAV-Cre) to delete the exon-2 region floxed with the two loxp sites for generation of β-klotho-LKO, or AAV encoding GFP as a wild-type (WT) controls. (B) Western blot analysis to confirm the specific deletion of β-klotho in the liver tissue in β-klotho-LKO mice, after 4 weeks of injection with AAV encoding Cre recombinase. Note that β-klotho remains unchanged in epididymal white adipose tissue (WAT) of Klb-LKO mice. The deletion of β-klotho can sustain for over 30 weeks after injection with AAV-Cre recombinase (data not shown). (C) A schematic diagram showing the study design for panel D-H. (D) The relative expression levels of Srebp-2 in the liver. (E) The DNA binding activities of Srebp-2 in the nuclear extracts of liver tissues. (F) The relative mRNA expression levels of genes involved in cholesterol synthesis including HMGCR, HMGCS and Fdps. (G) Serum total cholesterol levels. (H) Circulating levels of adiponectin. NS, not significant. n = 7. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed with two-way ANOVA (D-H).
**Supplemental Figure 8.** Hepatic FGFR2 is involved in FGF21-mediated suppression of Srebp-2, expression of cholesterologenic genes and hypercholesterolemia. (A) The relative mRNA expression levels of the four FGFRs in the liver tissues of 12-week-old male C57BL/6J mice. (B-G) 10-week-old C57BL/6J mice on HFHC diet were intravenously injected with 1x10⁹ p.f.u of adenovirus encoding siRNA specific to FGFR2 (Ad-siR2) or scramble control (Ad-scramble) for 7 days, followed by daily intraperitoneal administration of rmFGF21 (0.1 mg/Kg) for another 7 days. (B, C) The relative mRNA and protein expression of FGFR2 in the liver at 14 days after the adenoviral administration. (D,E) The relative mRNA levels and DNA binding activities of Srebp-2 in the nuclear extracts of liver tissues. (F) The relative mRNA expression levels of genes involved in cholesterol synthesis including HMGCR, HMGCS and Fdps. (G) Serum total cholesterol (TC) levels. n = 6. Data are presented as dot plots with the line indicating the median. Statistical analysis was performed with Mann-Whitney U test (B) or two-way ANOVA (D-G) to compare two groups or multiple groups respectively.
**Supplemental Figure 9.** The proposed mechanism whereby FGF21 suppresses atherosclerosis via induction of adiponectin (ADN) and suppression of Srebp-2.