GDF15 is a heart-derived hormone that regulates body growth

Ting Wang,1,2,†, Jian Liu,1,2,†, Caitlin McDonald,1,2, Katherine Lupino,1,2, Xiandun Zhai,1,2,3, Benjamin J Wilkins,2,4, Hakon Hakonarson,5,6 & Liming Pei1,2,4,*

Abstract

The endocrine system is crucial for maintaining whole-body homeostasis. Little is known regarding endocrine hormones secreted by the heart other than atrial/brain natriuretic peptide discovered over 30 years ago. Here, we identify growth differentiation factor 15 (GDF15) as a heart-derived hormone that regulates body growth. We show that pediatric heart disease induces GDF15 synthesis and secretion by cardiomyocytes. Circulating GDF15 in turn acts on the liver to inhibit growth hormone (GH) signaling and body growth. We demonstrate that blocking cardiomyocyte production of GDF15 normalizes circulating GDF15 level and restores liver GH signaling, establishing GDF15 as a bona fide heart-derived hormone that regulates pediatric body growth. Importantly, plasma GDF15 is further increased in children with concomitant heart disease and failure to thrive (FTT). Together these studies reveal a new endocrine mechanism by which the heart coordinates cardiac function and body growth. Our results also provide a potential mechanism for the well-established clinical observation that children with heart diseases often develop FTT.

Keywords body growth; failure to thrive; GDF15; heart disease; heart-derived hormone

Subject Categories Cardiovascular System; Metabolism

Introduction

A central question in physiology is how different organs communicate with each other to maintain whole-organism homeostasis. The classical endocrine system is well documented for its essential role in inter-organ communication. In addition, research in the past 20 years has revealed that certain non-glandular organs including adipose tissue, liver, skeletal muscle, intestine, and bone have secondary endocrine functions and secrete various hormones that regulate whole-body metabolism (Zhang et al., 1994; Deng & Scherer, 2010; Potthoff et al., 2012; Stefan & Haring, 2013; Liu et al., 2014; Wang et al., 2015a; Gribble & Reimann, 2016; Karsenty & Olson, 2016). In contrast, little is known regarding heart-derived hormones besides atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) discovered more than 30 years ago (de Bold, 1985; Frohlich, 1985; Karsenty & Olson, 2016). Cardiac synthesis and secretion of ANP and BNP are increased in various heart diseases, and plasma level of BNP is used clinically to diagnose heart failure (Yancy et al., 2013). ANP and BNP in turn signal to target tissues such as kidneys and vascular smooth muscles and increase natriuresis (excretion of sodium in the urine) and decrease blood pressure, reducing the burden on the distressed heart. Identification of new heart-derived hormones and study of their biological functions will significantly advance our understanding of cardiac biology and whole-organism homeostasis.

Failure to thrive refers to poor physical growth of children, typically evaluated by height and body weight gain. FTT is seen in 5–10% of children in the U.S. primary care settings (Cole & Lanham, 2011). It is well established that pediatric heart diseases such as congenital heart disease often cause FTT, but the underlying mechanism is poorly understood (Menon & Poskitt, 1985; Poskitt, 1993; Forchielli et al., 1994; Nydegger & Bines, 2006). Intriguingly, FTT associated with pediatric heart diseases often features lower circulating insulin-like growth factor 1 (IGF1) and IGF binding protein 3 (IGFBP3) levels (Barton et al., 2007; Dinleyici et al., 2007; Surmel-Onay et al., 2011; Peng et al., 2013). GH-IGF1 signaling is a dominant mechanism regulating postnatal mammalian growth (Pilecka et al., 2007; Baik et al., 2011; Savage et al., 2011; Rotwein, 2012; Milman et al., 2016). GH secreted from the pituitary signals to the liver to stimulate the production of
IGF1, IGFBP3, and IGFBP acid-labile subunit (IGFALS) via the JAK2-STAT5 pathway. Circulating GDF15 forms a ternary complex with IGFBP3 and IGFALS and is a major mediator of GH's effect on mammalian postnatal body growth (Czech, 1989; Pilecka et al., 2007; Baik et al., 2011; Savage et al., 2011; Rotwein, 2012). Mutations of almost every gene in this pathway cause severe to mild growth inhibition in humans and animals.

GDF15 (also known as MIC-1, NAG-1, PLAB, or PTGFB) is a distant member of the transforming growth factor beta (TGFβ) family of secreted proteins with pleiotropic functions (Bootcov et al., 1997; Yokoyama-Kobayashi et al., 1997; Paralkar et al., 1998; Bottner et al., 1999; Fairlie et al., 1999; Baek et al., 2001; Johnen et al., 2007; Unsicker et al., 2013). GDF15 has been shown to have a local cardioprotective role (Kempf et al., 2006; Xu et al., 2006), presumably due to its autocrine/paracrine function. Plasma GDF15 was found elevated in various heart diseases in many independent biomarker studies and in animal models (Wollert & Kempf, 2012; Baggen et al., 2017; Wollert et al., 2017). However, the organ source and biological mechanism of increased circulating GDF15 in heart disease are little known.

Intriguingly, although loss of both ERRα and ERRγ is essential for maintaining normal cardiac metabolism and function, overall health and survival (Wang et al., 2015b). Using cardiomyocyte-specific Myh6-Cre, we recently generated mice lacking ERRα and cardiac ERRγ (zKOyKO mice; genotype is ERRα−/−/ERRγfloX/floXMyh6-Cre−/−). Control mice and those lacking ERRα or cardiac ERRγ alone exhibited normal cardiac metabolism and function, overall health and survival (Wang et al., 2015b). In contrast, zKOyKO mice developed lethal dilated cardiomyopathy and heart failure soon after birth (median life span of 14–15 days), featuring metabolic, contractile, and conduction dysfunctions. These results demonstrated that ERRα and ERRγ are essential for maintaining normal cardiac metabolism and function. Intriguingly, although loss of both ERRα and ERRγ occurred exclusively in cardiomyocytes (Wang et al., 2015b), zKOyKO mice exhibited secondary FTT as often observed in children with pediatric heart disease. Using these zKOyKO mice as a model of primary pediatric cardiomyopathy with secondary FTT, here we identify GDF15 as a heart-derived hormone that inhibits pediatric body growth. We therefore used zKOyKO mice as a model to investigate the mechanism of FTT associated with pediatric heart disease.

The reduced body growth (about 30% less body weight) in zKOyKO mice is similar to that seen in animal and humans with defective GH-IGF1 signaling (Cui et al., 2007; Baik et al., 2011; Savage et al., 2011; Rotwein, 2012). We therefore measured their plasma GH and IGF1 concentrations. We found that zKOyKO mice had normal plasma GH (Fig 1C and D). In addition, expression of Gh in the pituitary and GH-releasing hormone (Ghrh) in the hypothalamus remained unchanged, indicating normal GH production and secretion (Appendix Fig S1B). Hypothalamic expression of appetite-regulating neuropeptide Y (Npy) and pro-opiomelanocortin (Pomc) was not changed either (Appendix Fig S1B). In contrast, plasma IGF1 level in zKOyKO mice was significantly decreased across multiple ages and was about 70% lower than controls by 16 days of age (Fig 1C and D). Normal plasma GH but significantly decreased IGF1 suggests that impaired liver GH signaling underlies FTT in zKOyKO mice. We therefore next examined the key components of liver GH signaling in zKOyKO mice. STAT5 phosphorylation was significantly reduced in zKOyKO mouse livers (Fig 1E and F, and Appendix Fig S1C) while upstream JAK2 phosphorylation remained unchanged (Fig 1F). Moreover, expression of STAT5 target genes Igf1, Igfbp3, and Igfals was all significantly decreased in zKOyKO mouse livers (Fig 1G). These result in reduced production and secretion of IGFBP3 (Fig 1H) in addition to IGF1 (Fig 1C and D). These results demonstrate that liver GH resistance underlies FTT in zKOyKO mice.

Circulating factors mediate impaired liver GH signaling in cardiac zKOyKO mice

In our zKOyKO mouse model, loss of both ERRα and ERRγ is restricted to cardiomyocytes, which was confirmed by unchanged ERRγ expression in every other tissue examined (Wang et al., 2015b). In addition, only zKOyKO mice exhibit primary pediatric cardiomyopathy and secondary FTT, while control littermates including ERRα KO mice retain normal cardiac function and liver GH signaling (Fig 1C–E and G). We asked how primary cardiac genetic defects affected liver GH signaling and caused secondary FTT. We considered the possibility that the heart was communicating its functional status to the liver via nervous or endocrine mechanisms. Unfortunately, the severe cardiomyopathy and early postnatal lethality (median life span of 14–15 days) in zKOyKO mice prevented us from using surgical procedures such as vagectomy or parabiosis to investigate these possibilities. As an alternative approach to test a potential endocrine mechanism, we treated wild-type (WT) mouse primary hepatocytes with plasma from zKOyKO or control littermate zHet/WT mice. Although zKOyKO mouse plasma contained the same amount of GH (Fig 1C and D, and Appendix Fig S2), it induced significantly less STAT5 phosphorylation in WT hepatocytes (Fig 2A), recapitulating in vivo observations (Fig 1E and F, and Appendix Fig S1C). This suggests that the

Results

Cardiac zKOyKO mice exhibit FTT with impaired liver GH signaling

Both male and female zKOyKO mice were significantly slower at gaining weight (slope of the weight curve) and were visibly smaller and shorter (height) from 5 to 7 days of age (Fig 1A and B, and data not shown), despite that weighing similar to controls around birth suggests little preterm growth defects. We hereafter chose body weight over bone or body length to determine body growth because body weight can be more accurately measured (0.01 gram precision with digital scale). Most internal organs except the heart of zKOyKO mice showed decreased absolute weight but maintained same relative weight (Appendix Fig S1A), indicating FTT-like whole-body growth inhibition rather than organ-specific developmental defects. We therefore used zKOyKO mice as a model to investigate the mechanism of FTT associated with pediatric heart disease.

In our zKOyKO mouse model, loss of both ERRα and ERRγ is restricted to cardiomyocytes, which was confirmed by unchanged ERRγ expression in every other tissue examined (Wang et al., 2015b). In addition, only zKOyKO mice exhibit primary pediatric cardiomyopathy and secondary FTT, while control littermates including ERRα KO mice retain normal cardiac function and liver GH signaling (Fig 1C–E and G). We asked how primary cardiac genetic defects affected liver GH signaling and caused secondary FTT. We considered the possibility that the heart was communicating its functional status to the liver via nervous or endocrine mechanisms. Unfortunately, the severe cardiomyopathy and early postnatal lethality (median life span of 14–15 days) in zKOyKO mice prevented us from using surgical procedures such as vagectomy or parabiosis to investigate these possibilities. As an alternative approach to test a potential endocrine mechanism, we treated wild-type (WT) mouse primary hepatocytes with plasma from zKOyKO or control littermate zHet/WT mice. Although zKOyKO mouse plasma contained the same amount of GH (Fig 1C and D, and Appendix Fig S2), it induced significantly less STAT5 phosphorylation in WT hepatocytes (Fig 2A), recapitulating in vivo observations (Fig 1E and F, and Appendix Fig S1C). This suggests that

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Figure 1. GDF15 is a heart-derived hormone

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αKOγKO mouse plasma contains altered amount of specific factors that regulate endogenous hepatocyte GH signaling.

To determine the chemical nature of such circulating factors, we used size fractionation to separate plasma into high molecular weight (HMW, >3 KD) and low molecular weight (LMW, <3 KD) fractions. We then recombined different HMW and LMW fractions of the littermate control αHet/WT and αKOγKO mouse plasma to treat WT mouse hepatocytes (Fig 2B). Reconstituted αKOγKO HMW and LMW fractions recapitulated its inhibitory effect on GH signaling. Intriguingly, αKOγKO HMW and αHet/WT LMW combination also inhibited STAT5 phosphorylation (behaving as the αKOγKO plasma). In contrast, αHet/WT HMW and αKOγKO LMW combination had no effect on STAT5 phosphorylation (behaving as the αHet/WT plasma). This result suggests that the putative GH signaling regulating factors exist in the HMW fraction of αKOγKO mouse plasma and are therefore most likely proteins rather than small chemicals or cellular metabolites.

CDF15 is a candidate heart-derived hormone that inhibits liver GH signaling and body growth

We employed two independent, unbiased strategies aiming to identify such heart-derived circulating factors that impact pediatric body growth. We used SOMAscan, an aptamer-based multiplexed proteomic platform which measures relative levels of more than 1,000 plasma proteins at one time (Gold et al., 2010), to identify proteins with altered plasma concentrations in the αKOγKO mice (Appendix Table S1). IGF1 appeared as the top decreased plasma protein in the SOMAscan assay, validating the power of this approach. To take account of proteins not covered by SOMAscan, we also performed RNA-Seq in αKOγKO and littermate control mouse hearts and identified genes with altered cardiac expression that encode secreted proteins (Chen et al., 2005; Appendix Table S2). We combined the candidates obtained from both approaches and focused on genes that showed significantly higher expression in the
heart than in other tissues such as the liver (by qRT–PCR). In case of proteins with similar sequence/structure/functions such as BNP and ANP, we chose to prioritize testing one of them (e.g., BNP) first. With these criteria, we came up with eight prioritized candidates (Fig 3A). We then tested whether any of them impacted liver GH signaling in young WT mice in vivo, using a 96-well ELISA-based screen with plasma IGF1 as the readout. Only GDF15 significantly altered plasma IGF1 level, resulting in about 30% decrease 2 days after injection (Fig 3A). To determine whether GDF15 impacts postnatal body growth by interfering with liver GH signaling, we next performed daily GDF15 injections to WT mice from 3 days of age and monitored their daily body growth (Appendix Fig S3A). GDF15 was found to inhibit liver STAT5 phosphorylation without altering JAK2 phosphorylation (Fig 3B), decrease liver expression of STAT5 target genes Igf1, Igfbp3, and Igfals (Fig 3C), and reduce plasma IGF1 and IGFBP3 concentrations without affecting GH level (Fig 3D–F). Importantly, GDF15 consistently reduced body weight gain in multiple independent cohorts of WT mice as the result of this constant inhibition of liver GH signaling (Fig 3G). Individual organs such as kidneys were proportionally lighter with relative weight remaining constant (Appendix Fig S3B). GDF15 did not change hypothalamic Npy and Pomc expression (Appendix Fig S3C), suggesting that this growth-inhibiting effect is distinct from its appetite-suppressing function seen in adult mice (Johnen et al, 2007). This almost complete molecular and phenotypic recapitulation of the zKOyKO mice (Fig 1 and Appendix Fig S1) strongly suggests that GDF15 is a major mediator of the FTT phenotype in zKOyKO mice. In contrast, injection of BNP did not alter liver GH signaling, plasma IGF1 level, or body weight in WT mice (Appendix Fig S3D–G).

To determine whether GDF15 directly acts on the liver to alter GH signaling, we next tested whether GDF15 can inhibit GH signaling in primary hepatocytes from young WT mice. We found consistently that GDF15 inhibits hepatocyte GH signaling in a dose-dependent manner (Fig 3H). In particular, pathological concentration of GDF15 seen in the zKOyKO mouse plasma (2 ng/ml, see Fig 4C) was found to inhibit signaling of physiological level of GH based on STAT5 phosphorylation. The total cellular protein tyrosine phosphatase (PTP) activity remained unchanged (Appendix Fig S3H), suggesting that GDF15 does not affect the activity of potential PTPs known to deactivate STAT5 under these conditions (Chen et al, 2003; Rigacci et al, 2003). Together these results suggest that GDF15 directly acts on hepatocytes to inhibit GH signaling.

**GDF15 is increased in pediatric heart disease and is synthesized in cardiomyocytes**

GDF15 is believed to be synthesized in the cell as a pro-protein with the C-terminal mature peptide secreted (Appendix Fig S4A; Bauskin et al, 2000). We found that cardiac Gdf15 expression in zKOyKO mice was similar to control at 3 days of age, but continued to quickly rise with the development of cardiomyopathy and reached over 30-fold by 13 days of age over control mice (Fig 4A). This resulted in a significant increase of GDF15 protein in both the heart and the plasma in a similar kinetic pattern (Fig 4B–D and Appendix Fig S4A). Immunohistochemistry further showed that while completely absent in the control mouse hearts, GDF15 protein is abundant in the zKOyKO mouse hearts (Fig 4D).

Coimmunostaining with cardiomyocyte marker troponin I allowing muscle fiber visualization revealed that GDF15 was located in the cytoplasm of cardiomyocytes and did not appear to be present in any other cell types of the heart (Fig 4E).

**GDF15 is a bona fide heart-derived hormone that regulates liver GH signaling**

These findings extend beyond the zKOyKO mouse model and have broad implications. Plasma GDF15 level was reported to be elevated in many forms of adult heart disease in both patients and animal models and was therefore recently proposed as an independent biomarker for heart diseases (Wollert & Kempf, 2012; Baggen et al, 2017; Wollert et al, 2017). However, the exact organ source and biological function of increased circulating GDF15 in heart disease remain little understood. To determine whether GDF15 is essential in mediating body growth inhibition in zKOyKO mice, we first tested this in vitro using GDF15 antibody to specifically deplete GDF15 in control and zKOyKO mouse plasma (Appendix Fig S4B). GDF15-depleted zKOyKO plasma largely lost its ability to inhibit GH signaling in primary hepatocytes (Appendix Fig S4C). This result suggests that GDF15 is the major GH-inhibiting factor in zKOyKO plasma. We next aimed to determine whether cardiac-derived GDF15 is critical in inhibiting liver GH signaling in vivo. We designed an AAV9-Gdf15 shRNA vector to specifically knockdown Gdf15 expression in the mouse heart (Fig 5A). Pericardial injection of AAV9 has been shown to achieve stable and relatively cardioc-specific expression of transgenes compared to other AAV serotypes (Bish et al, 2008). Since Gdf15 is exclusively expressed in zKOyKO mouse cardiomyocytes (Fig 4E), we designed the AAV9 vector to ensure that Gdf15 shRNA is solely expressed in zKOyKO mouse cardiomyocytes (Myh6-Cre+) but not elsewhere (Myh6-Cre−) (Fig 5A). The specificity of our strategy was experimentally confirmed as non-cardiac expression of Gdf15 was not affected (Appendix Fig S4D). One week after pericardial injection of AAV9-shRNA, we found that cardiomyocyte-specific knockdown of Gdf15 almost completely normalized cardiac Gdf15 expression and plasma GDF15 concentration (Fig 5B and C). Importantly, the development of lethal cardiomyopathy in zKOyKO mice was little affected by cardiac Gdf15 knockdown based on cardiac histology and cardiomyopathy marker Bnp expression (Fig 5D). These results provide definitive evidence that the elevated plasma GDF15 is exclusively produced by cardiomyocytes. In addition, normalization of circulating GDF15 in zKOyKO mice restored liver GH signaling (significantly increased STAT5 phosphorylation, Fig 5E) and doubled circulating IGF1 (Fig 5F) without affecting circulating GH levels (Fig 5G). Although the early lethality of zKOyKO mice (regardless of receiving control or Gdf15 shRNA due to heart failure; Wang et al, 2015b) prevented us from keeping monitoring their body weight following this molecular reversal of liver GH inhibition, these results demonstrate that GDF15 is a bona fide heart-derived hormone that regulates liver GH signaling.

**Plasma GDF15 is elevated in children with heart disease and FTT**

Last, to substantiate the clinical significance of our findings beyond animal models, we asked whether GDF15 potentially underlies clinical FTT associated with pediatric heart disease, which often features...
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**GDF15 is a heart-derived hormone**

**Figure 3.**

**A**

Plasma IGF1 (ng/ml)

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**B**

Liver relative gene expression

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Liver relative gene expression

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Plasma IGF1 (ng/ml)

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Plasma IGFBP3 (ng/ml)

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Plasma GH (ng/ml)

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**Figure 3.**
lower circulating IGF1 and IGFBP3 levels (Barton et al., 1996; Dinleyici et al., 2007; Surmeli-Onay et al., 2011; Peng et al., 2013). We measured plasma GDF15 concentrations in children diagnosed with heart disease and with either normal body weight or FTT. Both groups of children with heart disease had significantly higher plasma GDF15 levels than gender- and age-matched healthy control children (Fig 6A), consistent with previous findings that plasma GDF15 was increased in adult heart disease (Wollert & Kempf, 2012; Baggen et al., 2017; Wollert et al., 2017). Importantly, plasma GDF15 level is further significantly increased (another 80% higher) in children with concomitantly heart disease and FTT than those with heart disease but normal body weight (Fig 6A), supporting elevated GDF15 as an underlying mechanism linking pediatric heart disease and FTT.

Discussion

The rapid body growth and increased nutrients/energy demand during the pediatric period require accompanying increase of cardiac function. Here, we identified a novel endocrine mechanism by which the heart communicates with the rest of the body to coordinate body growth and cardiac function. The heart synthesizes and secretes GDF15 to inhibit body growth, thereby relieving potential extra cardiac burden as well as helping the body adapt to decreased cardiac output, a theme also seen in ANP and BNP (Fig 6B). Both GDF15 and ANP/BNP are synthesized as prohormones and processed to become active hormones. Cardiac and circulating levels of both GDF15 and ANP/BNP are highly elevated in many forms of heart disease. BNP is well established and widely used clinically for diagnosis of heart disease especially heart failure; GDF15 activity, and determine the detailed molecular mechanism of non-cardiac sources. Our cardiac-specific Gdf15 knockout studies suggest that cardiac-derived GDF15 in the heart disease condition contributed to all the elevated circulating GDF15 above basal level (Fig 5). This additional circulating GDF15 from the heart clearly has a significant biological impact on liver GH signaling (Fig 5). One possible explanation is that such a fourfold to fivefold increase in GDF15 concentration is enough to make a significant difference in its signaling. Another possible explanation is that heart-derived GDF15 is biochemically distinct from the basal circulating GDF15 from non-cardiac sources and potentially possesses higher biological activities. Future studies will investigate the factors that modulate GDF15 activity, and determine the detailed molecular mechanism of GDF15 signaling.

We designed AAV9-shGdf15 vectors to specifically knockdown cardiomyocyte Gdf15 in zKOyKO mice (Fig 5). In this experiment, we injected AAV-shRNA to 2-day-old mice, the age right when initial molecular changes of cardiac dysfunction were observed in cardiac zKOyKO mice (Wang et al., 2015b). It was expected that injected AAV9-Gdf15 shRNA would take some time (probably in days) to be well expressed and take effect to completely block cardiac GDF15 synthesis. By this time, it is highly likely that liver GH signaling and circulating IGF1 would have already significantly decreased. We therefore anticipated that reduced liver GH signaling in zKOyKO mice would be partially reversed in the days following AAV9-shGdf15 injection. We did observe that once cardiac and circulating GDF15 levels were completely normalized 1 week after AAV9-shGdf15 injection, downstream liver STAT5 phosphorylation was largely restored and circulating IGF1 level was partially normalized.
Figure 4. GDF15 is a heart-derived hormone.
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**Figure 4. GDF15 is increased in pediatric heart disease and produced in cardiomyocytes.**
A. Expression of Gdf15 in 3- (n = 5–7 mice per group), 7- (n = 6 mice per group), 10- (n = 8–10 mice per group), and 13-day-old (n = 12–13 mice per group) littermate mouse hearts quantified by qPCR.
B. GDF15 protein level in 3-, 7-, 10-, and 13-day-old littermate mouse hearts determined by Western blot. β-Actin serves as a loading control.
C. Plasma GDF15 concentrations in 3- (n = 5–7 mice per group), 7- (n = 6 mice per group), and 10-day-old littermate mice (n = 8–10 mice per group) measured by ELISA.
D. Representative pictures of 3-, 7-, 10-, and 13-day-old littermate αHet/WT and αKO/KO mouse heart sections stained with GDF15 antibody (brown) and counterstained with hematoxylin (purple). Scale bar: 100 μm.
E. Representative pictures of 16-day-old αKO/KO mouse hearts stained with GDF15 (red) and cardiac troponin I (green) antibodies. Arrows point to the nucleus of cardiomyocytes, and asterisks mark the nucleus of non-cardiomyocytes. Scale bar: 20 μm.

Data information: *P < 0.05, **P < 0.01, and ***P < 0.001 between αKO/KO and all other littermate control genotypes by t-test. All values are mean ± s.e.m.

reversed. Although it appears that GDF15 is the major circulating factor in αKO/KO plasma that inhibits hepatocyte GH signaling in vitro (Appendix Fig S4B and C), it is possible that additional GDF15-independent mechanisms are involved. These include mechanisms through the nervous system that sense cardiac health and in turn regulate body growth, other heart-derived endocrine signals (including those unexamined candidates from our plasma proteomics and cardiac RNA-Seq studies), or a combination of nervous and endocrine mechanisms. Future studies in these areas will further broaden our understandings of the communication between the heart and rest of the body. Nevertheless, our current results clearly demonstrate that heart-derived GDF15 is essential for altered liver GH signaling in FTT associated with pediatric heart disease.

**Figure 5. GDF15 is a bona fide heart-derived hormone that regulates liver GH signaling.**
A. Design of AAV9-mGDF15 shRNA construct to specifically knockdown GDF15 in Cre+ cardiomyocytes.
B-G. Cardiac Gdf15 expression quantified by qPCR (B), plasma GDF15 concentrations measured by ELISA (C), heart phosphorylated STAT5 level measured by ELISA (D), and plasma IGF1 (F) and GH concentrations (G) measured by ELISA in 9- to 10-day-old littermate control and αKO/KO mice (n = 8–12 mice per group) that received pericardial injection of AAV9-control or Gdf15 shRNA at 2 days of age. *P < 0.05, **P < 0.01, and ***P < 0.001 by t-test. Values are mean ± s.e.m.
The vital function of the heart has been known for thousands of years. Besides ANP and BNP discovered over 30 years ago (de Bold, 1985; Frohlich, 1985; McGrath et al., 2005; Clerico et al., 2011; Ogawa & de Bold, 2014), only small numbers of heart-secreted factors are known (Shimano et al., 2012; Karsenty & Olson, 2016). However, they are not established as endocrine hormones because their known functions to date are largely limited to mere biomarkers of cardiac health or autocrine/paracrine factors that affect cardiomyocyte or cardiac fibroblast biology locally (Shimano et al., 2012; Karsenty & Olson, 2016). Potential heart-derived endocrine factors were suggested that impact lipid and adipose tissue lipid metabolism in few recent studies (Grueter et al., 2012; Baskin et al., 2014; Magida & Leinwand, 2014), but the exact identities of such factors remain to be determined (Karsenty & Olson, 2016). By identifying GDF15 as a new heart-derived hormone and revealing its biological function, our findings support the importance of the endocrine function of the heart and advance our understanding of the heart and its role in whole-organism homeostasis. Our studies also uncover an underlying mechanism of FTT associated with pediatric heart disease. Intriguingly, plasma GDF15 was recently reported to be increased in mitochondrial disease patients, which often features slowed body growth as well (Yatsuga et al., 2015; Fujita et al., 2016; Montero et al., 2016). Whether GDF15 is critical in this context remains to be determined.

Materials and Methods

Animal studies

All animal studies were approved by and performed under the guidelines of the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia (CHOP). All mice were backcrossed at least six generations to and maintained in the C57BL6/J background. Mice were maintained in a temperature- and light-controlled environment with ad libitum access to water. Mice in holding cages (after weaning at around 28 days of age) received a standard chow diet (Lab Diet 5L0D, 58% calories from carbohydrate, 13.5% calories from fat, and 28.5% calories from proteins); nurturing moms and their pups before weaning received a breeder diet (Lab Diet 5058, 55% calories from carbohydrate, 22% calories from fat, and 23% calories from proteins). The breeding strategy to generate experimental cohorts (♂Het°WT, ♀Het°KO, ♂KO°WT, and ♀KO°KO littermates at 1:1:1:1 ratio) was previously described (Wang et al., 2015b). For postnatal body growth analysis, the breeding pairs were monitored daily for birth of pups. The first day we observed new pups born was deemed as P0, and the pups were toe-clipped for identification and genotyping. We chose body weight over bone or body length to determine body growth because body weight can be more accurately measured using a digital scale with 0.01 gram precision. Mice were weighed daily between 10 am and 2 pm. Both male and female pups exhibited lethal cardiomyopathy and FTT phenotypes, and therefore, both genders were included in the study. We determined the minimum number of animals needed using power calculations based on the literature, our preliminary results and sample variation aiming to ensure 90% power. Only littermate mice were used and they were randomized to ensure that the same age, gender, and weight (mean and variation, where applicable) are represented in all groups. Investigators were blinded to the sample group allocation wherever possible. The number of mice used in each experiment and number of times experiments are replicated are described in figure legends or related Materials and Methods sections. Only mice living for at least 13 days were included in the body weight curve (Fig 1; because some ♀KO°KO die before this age). All tissues were collected and weighed between 12 and 4 pm to avoid the impact of circadian rhythm.

Figure 6. Plasma GDF15 is elevated in children with concomitant heart disease and FTT.

A. Plasma GDF15 concentrations in 2- to 3-year-old children diagnosed with heart disease with either normal body weight (n = 35) or FTT (n = 45) and in age- and gender-matched healthy controls (n = 45) were measured by ELISA. *P < 0.05 and ***P < 0.001 by t-test. Values are mean ± s.e.m.

B. Cartoon illustrating how GDF15 and ANP/BNP relieve cardiac burden and coordinate cardiac function with the rest of the body.
ELISA

Mouse blood was collected in lithium-heparin-coated microvette CB300LH (Sarstedt), and plasma was collected after spinning down at 3,000 g for 5 min at 4°C. Plasma protein levels were measured using ELISA kits: mouse GH (Millipore EZRMGH-45K), mouse IGF1 (Abcam ab108874), mouse IGFBP3 (R&D MGB300), mouse GDF15 (R&D MGD150), and human GDF15 (R&D DY957). Separately, mouse livers were homogenized with kit-provided lysis buffer containing protease and phosphatase inhibitors (Roche). Phosphorylated and total STAT5 and JAK2 levels were measured using the following ELISA kits and normalized to total protein amount: p-STAT5 (Tyr694, Cell Signaling #7113C), total STAT5 (Abcam ab205714), p-JAK2 (Tyr1007/1008, ThermoFisher Scientific KH05621), and total JAK2 (Invitrogen KH05521).

Plasma proteomics

EDTA plasma was collected from 16-day-old littermate zHet/WT and zKOy/KO mice (n = 3 mice per group) and stored at −80°C until analysis. Measurement of relative plasma protein levels using the SOMAscan platform was performed at SomaLogic Inc. (Gold et al., 2010).

Gene expression analysis

We isolated total RNA from mouse tissues or cells using RNAzol RT (Molecular Research Center) following the manufacturer’s instructions. We synthesized cDNA from 1 μg total RNA using iScript cDNA synthesis kit (Bio-Rad) and quantified mRNA levels by real-time qPCR using SYBR Green (Bio-Rad). We ran samples in technical triplicates and calculated relative mRNA levels using a standard curve and normalized to 36b4 mRNA level in the same sample. The qPCR primer sequences are listed in Appendix Table S3.

RNA-Seq

Total RNA from 16-day-old littermate mouse (zHet/WT, zHet/KO, zKOy/WT, and zKOy/KO) hearts was extracted using RNAzol RT. We prepared two independent samples per genotype for RNA-Seq, with each sample comprised of equal amount of pooled RNA from three biological replicates (total of six littermate hearts per genotype used). Library was constructed using TrueSeq Library Prep kit (Illumina), and sequencing and bioinformatic analysis were performed by BGI@CHOP (PE100, 20 million reads). The raw and processed data are deposited in the GEO database (accession number GSE88761, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=n=cpqnciqrzcxhnlg&acc=GSE88761).

Protein analysis

For whole-cell extract preparation, cells or 20 mg of tissues was homogenized and kept in 400 μl cold RIPA buffer containing protease/phosphatase inhibitors on ice for 10 min. The homogenates were spun down at 13,000 g for 10 min, and the supernatant was collected as cytosolic extract. The pellets were resuspended in 300 μl cold buffer containing 10 mM HEPES (pH 7.4), 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, and protease/phosphatase inhibitors and shaken overnight at 4°C. They were then spun down at 13,000 g for 20 min, and the supernatant was collected as nuclear extract. Protein concentration was quantified by a BCA assay kit (ThermoFisher Scientific). Western blot was performed as previously described following standard procedures (Wang et al., 2015b). Primary antibodies used were p-STAT5 (Cell Signaling #9359, 1:1,000), STAT5 (Cell Signaling #9358, 1:1,000), p-JAK2 (Cell Signaling #3776, 1:1,000). JAK2 (Millipore 06-1310, 1 μg/ml), GDF15 (Abcam ab189358, 1 μg/ml), and β-actin (Cell Signaling #4970, 1:1,000), and TFIIF (Santa Cruz sc-293, 1:2,000).

Plasma fractionation and GDF15 depletion by immunoprecipitation

Pooled plasma from 3 to 4 16-day-old zHet/WT or zKOy/KO mice was used for each fractionation or GDF15 depletion experiment. Plasma was separated into high (> 3 KD) and low (< 3 KD) molecular weight fractions using Amicon Ultra 0.5-ml centrifugal 3 KD Filters (Millipore) following the manufacturer’s instructions. For GDF15 depletion, plasma was incubated with 1 μg mouse GDF15 capture antibody (from ELISA kit R&D DY6385) and shaken at 4°C overnight. 250 μl dynabeads protein G (ThermoFisher Scientific 100.04D) was then added to the plasma and shaken at room temperature for 2 h. The mixture was separated using magnet, and the supernatant was collected as GDF15-depleted plasma. GDF15 concentration in plasma before and after GDF15 depletion was determined using ELISA (R&D DY6385).

Mouse primary hepatocyte isolation and GH signaling studies

Primary hepatocytes were isolated from young WT mice as previously described (Pei et al., 2006). Briefly, 3- to 4-week-old mouse was anesthetized and perfused at 37°C with 10 mM HEPES-buffered HBSS (1 ml/min for 5 min) and then with 0.1% type I collagenase (Worthington LS004194) in Williams E media (1 ml/min for 5 min), entering the hepatic portal vein and exiting the inferior vena cava. The perfused liver was removed and shaken to disperse cells into hepatocyte attachment media (DMEM/F12 supplemented with 0.2 mg/ml BSA, 1 mg/ml D-galactose, 0.03 mg/ml proline, 5 mM sodium pyruvate, 1× insulin-transferrin-selenium-A (ThermoFisher Scientific 51300044), 1× antibiotic-antimycotic (ThermoFisher Scientific 15240062), and 10% FBS). After mixing cells with Percoll (final concentration of Percoll is 45%) and centrifugation to collect all the viable cells, we resuspended cells in hepatocyte attachment media and plated them on collagen I-coated 24-well plates with a density of 0.2–0.3 million cells per well. 3 h later, cells were washed with DMEM to remove non-attached cells and fasted overnight in DMEM. The next morning, cells were treated with DMEM, DMEM plus 50% plasma, or 50% plasma fractions for 1 h. Alternatively, cells were first treated with different concentration of GDF15 (R&D 8146-GD/CF) for 30 min and then treated with 20 ng/ml GH (R&D 1067-GH-025/CF) for 15 min. For Western blot, cells were washed with...
ice-cold PBS for three times and lysed with 60 μl lysis buffer (30 μl RIPA buffer and 30 μl 2× Laemmli sample buffer from Bio-Rad, plus protease and phosphatase inhibitors and 2.5% β-mercaptoethanol). For determining protein tyrosine phosphatase (PTP) activity, cells were washed three times with ice-cold PBS and lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, and protease inhibitors (Sorensen & Sheibani, 2002). 1 μl cell lysates (around 2 μg proteins) from each group were incubated at 37°C for 30 min with 0.2 mM PTP substrate RRLIEDAEpYAARG (Upstate) in 12-217) in a final volume of 50 μl of assay buffer (25 mM HEPES pH 7.2, 50 mM NaCl, 5 mM DTT, and 2.5 mM EDTA) (Kakazu et al., 2008). Amount of phosphate released was measured by malachite green phosphate assay kit (Cayman Chemical 10009325) following manufacturer’s instructions. This was normalized to total protein, which was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific 23225).

**Protein injection**

Recombinant proteins were purchased from commercial sources: GDF15 (R&D 8146-C/D-CF), IGFBP1 (R&D 1588-B1), Sema3B (R&D 5440-S3-CF), GPC3 (R&D 2119-GP-CF), TWEAKR/TNFRSF12 (R&D 1610-TW), TSP4 (R&D 7860-TH), IGFlsR (IGFlR 1-936, R&D 6630-GR-CF), and BNP 1-32 (Tocris Bioscience 3522). They were reconstituted per vendor’s recommendations and further diluted in PBS for in vivo i.p. injection. For in vivo experiments, we used gender- and body weight (BW)-matched WT mice all from the same litter to exclude the impact of mom’s nurturing ability on BW among litters. We repeated all short- and long-term GDF15 injections at least four times and other protein injections at least two times and obtained the same results. For short-term injection, gender- and BW-matched 5-day-old WT mice all from the same litter (n = 3–5 per group) were i.p. injected between 11 am and 1 pm with control (same solvent diluted in PBS) or indicated protein every 24 h, three times total. Mouse plasma was collected 1.5 h after the last injection (7 days old). The injection doses were based on literature and product information from the vendors: 500 μg/kg BW for BNP and GPC3, 100 μg/kg BW for GDF15, TWEAKR, Sema3B, TSP4, IGFBP1, and IGFlsR. For long-term injection (illustrated in Appendix Fig S3A), gender- and BW-matched (BW of all littermate pups within 10% variation) 3-day-old WT mice all from the same litter (n = 4–5 per group) received daily i.p. injection between 11 am and 1 pm of control, 400 μg/kg BW GDF15 or 500 μg/kg BW BNP. Plasma and tissues were collected at the end of the experiment, 1.5 h after the final injection.

**Histology**

Histological studies were performed as previously described (Pei et al., 2011, 2015). Mice were euthanized and perfused with 3 ml PBS. Tissues were then dissected and fixed in 4% paraformaldehyde in PBS overnight. Tissues were embedded in paraffin or frozen blocks. For heart immunohistochemistry, 5–8 μm paraffin sections were used. After deparaffinization, sections were incubated in AR buffer (Vector Lab H-3300) for antigen retrieval (microwave for 5 min). Sections were then rinsed in PBST and blocked with 2% horse serum for 1 h and incubated with GDF15 (Abcam ab189358, 10 μg/ml) or troponin I (Abcam ab47003, 10 μg/ml) antibodies overnight at 4°C. The sections were then incubated with biotin-labeled secondary antibodies (5 μg/ml) and ABC reagent (Vectastain Elite ABC Reagent RTU, Vector Lab PK-7100), and stained using peroxidase substrate solution (Vector Lab). After counterstain with hematoxylin (Vector Lab H-3401) and dehydration, the sections were mounted and imaged using a Zeiss Axio microscope. For immunofluorescence, the sections were incubated with fluorescence-labeled secondary antibodies and mounted and imaged using a Zeiss LSM710 confocal microscope.

**AAV injection**

AAV9-Sico-mouse Gdf15 shRNA (based on shAAV-260008) or scramble control shRNA was custom-built and manufactured by Vector Biolabs. Pericardial injection of 3 × 1011 genome copies AA9 was performed in 2-day-old mice using a Hamilton syringe by adapting procedures previously described without using ultrasound guidance (Laakmann et al., 2013). αK0T/WT mice were randomized to receive AAV9-Gdf15 or control shRNA, and littermate control αHet/WT mice received AA9-control shRNA. As quality control and based upon pre-established criteria, mice dead before 9 days of age or with unsuccessful cardiac Gdf15 knockdown (presumably due to unsuccessful injection or ineffective AAV infection) were excluded from the study (2 of 10 αK0T/αKO mice that received AA9-sh Gdf15 and survived to 9–10 days of age were excluded from the study shown in Fig 5; similar success rate was observed in control groups).

**Human plasma samples**

Plasma samples were from the CHOP Center for Applied Genomics biobank. Plasma samples from three groups of children were used (Appendix Table S4). Group 1: age- and gender-matched healthy controls. Group 2: 2- to 3-year-old children diagnosed with congenital heart disease and/or pediatric cardiomyopathy, with normal body weight (within 40–70% range of the standard body weight chart of the individual’s age; 50% is the median body weight) at the time of sample collection. Group 3: 2- to 3-year-old children diagnosed with congenital heart disease and/or pediatric cardiomyopathy, with FTT (within 0–8.5% range of the standard body weight chart of the individual’s age; most were also diagnosed with FTT) at the time of sample collection. Informed consent was obtained from all subjects and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

**Statistical analysis**

Two-tailed distribution, two-sample unequal variance t-test was used to determine the statistical significance between results of two independent groups, using either Microsoft Excel (animal studies) or GraphPad Prism (human studies, allowing automatic identification and exclusion of no more than 1 outlier out of > 35 biological samples per group based on the built-in ROUT method), with P < 0.05 deemed as statistically significant. The P-values of all figures are provided in Appendix Table S5.

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The paper explained

Problem
Endocrine organs and the hormones they secrete regulate many essential functions in our body. The vital role of the heart has been known for thousands of years, but its function as an important endocrine organ remains little understood.

Results
Here, we identify a new heart-derived hormone called growth differentiation factor 15 (GDF15) that regulates body growth. GDF15 synthesis and secretion by cardiomyocytes are increased in pediatric heart disease. Circulating GDF15 in turn acts on the liver to inhibit growth hormone (GH) signaling and body growth. Blocking cardiomyocyte production of GDF15 normalizes circulating GDF15 level and restores liver GH signaling, establishing GDF15 as a bona fide heart-derived hormone. Plasma GDF15 is increased in children with concomitant heart disease and failure to thrive (FTT).

Impact
Our study advances the field of cardiac endocrinology by revealing GDF15 as a heart-derived hormone that coordinates cardiac function and body growth. These results also provide a potential mechanism for the well-established clinical observation that children with heart disease and failure to thrive (FTT).

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Author contributions
LP conceived and directed the project. TW, JL, and LP performed most of the experiments including independently repeated experiments. CM, KL, and XZ provided technical assistance. BJW is a pathologist who evaluated, read, and scored the histology studies. HH provided human plasma samples and contributed to the analysis and interpretation of the results. TW and LP wrote, and all authors reviewed and/or edited the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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GDF15 is a heart-derived hormone  Ting Wang et al

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