HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2022 August 01.

Published in final edited form as:

Hypertension. 2021 August ; 78(2): 342–352. doi:10.1161/HYPERTENSIONAHA.120.16888.

Mir379 Regulates Klotho Deficiency-induced cardiomyocyte Apoptosis *via* Repression of Smurf1

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Abstract

Klotho is an aging-suppressor gene. *Klotho* gene deficiency impairs heart function leading to heart failure, but the underlying mechanism remains poorly understood. MicroRNAs are increasingly recognized to play important roles in the pathogenesis of cardiomyopathy. The objective of this study is to investigate whether microRNA 379 (Mir379) regulates Klotho deficiency-associated cardiomyocyte apoptosis. Using inducible Cre-Loxp recombination technology, we first found that kidney-specific deletion of the *Klotho* gene caused heart failure. Using microRNA sequencing analysis, we found that Mir379 may be a target of Klotho. In cultured H9c2 heart cells, we found that treatment with Klotho-free medium increased Mir379 levels and induced apoptosis. To test whether Mir379 mediates Klotho deficiency-induced apoptosis, H9c2 cells were transfected with Mir379 inhibitor. Interestingly, Mir379 inhibitor (anti-Mir379) prevented Klotho deficiency-induced H9c2 cell apoptosis. On the other hand, Mir379 mimic itself caused apoptosis in H9c2 cells. These findings suggest that Mir379 may mediate Klotho deficiency-induced apoptosis in H9C2 cells. Using the mRNA-miRNA target interaction assay, we found that Smurf1 mRNA contained the 3-UTR binding site for Mir379. Importantly, Mir379 mimic suppressed Smurf1 expression, and the Mir379 mimic-induced apoptosis can be rescued by treatment with exogenous Smurf1 protein. Therefore, Smurf1 repression may be involved in Mir379-induced H9c2 cells apoptosis. In conclusion, Mir379 may mediate Klotho deficiency-associated cardiomyocyte apoptosis through repression of Smurf1 which is required for Mir379-induced apoptotic cell death. Mir379 may be a potential therapeutic target for cardiomyocyte apoptosis-associated heart failure due to Klotho deficiency.

Keywords

Mir379; Klotho; Smurf1; cardiomyocyte; apoptosis; microRNA sequencing

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Disclosures

Kai Chen: None; Bo, Zhang: None; Zhongjie Sun: None

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Apoptosis, a highly regulated cell death process, contributes to cardiac pathology, including heart failure, myocardial infarction, and reperfusion injury.[1, 2] Since cardiomyocyte loss is the most important determinant of patient morbidity and mortality, fully understanding the regulatory mechanisms of apoptotic signaling is crucial. In fact, the inhibition of cardiac apoptosis holds promise as an effective therapeutic strategy for heart diseases.

Klotho is an anti-ageing gene which was discovered in 1997.[3] The *Klotho* gene encodes a single-pass transmembrane protein predominantly expressed in kidney distal tubule cells.[4, 5] The transmembrane Klotho serves as a coreceptor of FGF1 and regulates the Na-Pi co-transporter activity and phosphate reabsorption in kidneys.[5] *Klotho* mutation leads to hyperphosphatemia.[5] The extracellular domain of Klotho can be cleaved and released into the circulation known as soluble Klotho.[5, 6] Two forms of soluble Klotho were found in the serum, long-form soluble Klotho (130 kDa) and short-form soluble Klotho (65 kDa). In contrast, secreted Klotho (65 kDa) is generated due to alternative mRNA splicing and released to the blood.[5, 7] Recently, Klotho deficiency in cardiovascular disease has attracted much attention and a number of observations underscore its clinical relevance.[8–11] Serum level of soluble Klotho protein declines with age after age 40[12–14], so does heart function.[15–18] Thus, it is important to determine whether Klotho deficiency impairs heart function. Because *Klotho* is primarily expressed in kidneys, we investigated whether deletion of *Klotho* gene in kidneys causes heart failure and further explored the underlying mechanisms of Klotho deficiency-induced cardiomyopathy.

MicroRNAs (miRNAs) are small, non-coding RNA molecules approximately 22 nucleotides in length which act as post-transcriptional regulators of gene expression. Individual miRNAs have been shown to regulate the expression of multiple genes. Conversely, the expression of individual genes can be regulated by multiple miRNAs. Some miRNAs may regulate cardiac function. The *miRNA-379* gene (Mir379) is located on chromosome 14q32.31 and has been reported to act as a tumor suppressor in different kinds of cancer.[19–23] It is not known, however, whether MiR379 is involved in cardiomyocyte apoptosis. Smurf1 is an E3 ubiquitin-protein ligase that acts as a negative regulator of BMP signaling pathways.[24, 25] It plays a key role in the regulation of cell motility, cell signaling, and cell polarity.[26].

In this study, we first found that kidney specific deletion of *Klotho* gene caused cardiac dysfunction. By miRNA sequencing analysis, we identified that Mir379 may be an important target for Klotho deficiency-induced heart failure. Using H9C2 cells, we found that Mir379 may mediate cell apoptosis induced by Klotho-free medium. Using the miRDB database, we found that Smurf1 is a top predicted target for mmu-miR-379-3p. Mir379 negatively regulated Smurf1 expression, and the Mir379 mimic-induced apoptosis was inhibited by treatment with exogenous Smurf1 protein. Our findings suggest that Mir379 may be a potential therapeutic target for Klotho deficiency-induced cardiomyocyte apoptosis.

Materials and Methods

Data available on request from the authors

Animal protocol

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Oklahoma Health Science Center. Conditional kidney-specific deletion of *Klotho* gene was generated using Cre-Loxp recombination (Fig. 1A) as described previously[27, 28]. For detailed information, refer to Online Supplemental Methods and Data. Briefly, the Cre-Loxp mice and the Cre mice were given five-dose tamoxifen injections (20 mg/kg, IP) at the age of 6 months for achieving Klotho deletion. All mice were fed a purified low phosphate diet containing with 0.1% inorganic phosphate (wt/wt, normal diets contain 0.35% inorganic phosphate) (TD.10080, Harlan Teklad, Madison, WI) for one week before the first tamoxifen injection. The mice were harvested at week 5 after *Klotho* gene deletion.

H9C2 Cell culture and treatment

H9c2 cells, a cardiomyocyte cell line derived from rat ventricles, were cultured as we described previously.[29–31] For detailed information, refer to Online Supplemental Methods and Data.

In Vivo Measurement of Cardiac Function

Echocardiography was used for assessing cardiac function[32]. For detailed information, refer to Online Supplemental Methods and Data.

Cardiomyocytes isolation

Cardiomyocytes were isolated by adult M/R cardiomyocytes isolation kit (Cellutron Life Technology, Baltimore, MD) as per the manufacturer's protocol. For detailed information, refer to Online Supplemental Methods and Data.

MicroRNA sequencing analysis

For detailed information, refer to Online Supplemental Methods and Data.

Western Blot Analysis

Western blot analysis was carried out as we described recently[33–38]. For detailed information, refer to Online Supplemental Methods and Data.

Quantitative Real-time PCR

Quantitative Real-time PCR was performed as we described previously.[38] For detailed information, refer to Online Supplemental Methods and Data.

TUNEL assay and cardiac fibrosis measurement

TUNEL staining was performed as we described previously[39, 40]. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) using the Click-iT® Plus TUNEL Assay Kit (Thermo Fisher, Foster city, CA) as per the

manufacturer's protocol. Percentage of TUNEL positive cells were measured using Image J (NIH) from 3 fields per sample, 4 samples per group. Cardiac collagen was quantified by Picro-Sirius red staining as described previously.[11]

mRNA-miRNA target interaction assay

Smurf1 is the predicated target gene for Mir379. H9c2cells were transfected with Smurf1 3' UTR target sequence expression plasmid (GeneCopoeia, Rockville, MD) alone or co-transfected with Mir379 mimic expression plasmid (Thermo Fisher, Foster city, CA). Smurf1 3' UTR sequences were inserted downstream of a secreted Gaussia luciferase (GLuc) reporter gene, which is expressed under the control of the SV40 promoter in mammalian cells. Thus, the study of mRNA-miRNA target interaction can be easily performed with a live cell assay for GLuc using only 10 μl of the cell culture medium. Besides using GLuc as the miRNA 3' UTR target reporter, a secreted Alkaline Phosphatase (SEAP) reporter driven by a CMV promoter is also cloned into the same vector and serves as the internal control. The dual-reporter vector system enables transfection normalization for accurate across-sample comparison. The cell culture medium was collected 48 hours post-transfection, both the GLuc activity and an internal control SEAP activity were determined using secrete-pair dual luminescence Assay Kit (GeneCopoeia, Rockville, MD).

Statistical Analysis

Quantitative data were presented as the Means ± SE. Differences between experimental groups were examined by t-test or two-way ANOVA followed by the Bonferroni post-test using Prism software (GraphPad, La Jolla, CA). For all analysis, $p<0.05$ was considered statistically significant.

Results

Klotho deficiency-induced cardiac dysfunction and remodeling is associated with the increase of Mir379 level in the heart

Western blot analysis showed that full-length and short-form Klotho protein expressions were barely detectable in kidneys of the kidney-specific Klotho knockout (KSP-KL$^{-/-}$) mice, indicating effective deletion of the *Klotho* gene (Fig. 1B). Kidney-specific knockout of the *Klotho* gene largely decreased serum level of soluble-Klotho (130 kDa) (Fig. 1C) which is responsible for heart failure in KSP-KL$^{-/-}$ mice. Surprisingly, serum level of short-form Klotho did not decrease significantly (KSP-KL$^{-/-}$) mice (Fig. 1C), indicating that short-form Klotho in the serum may primarily originate from other tissues or organs rather than kidneys. This hypothesis is supported by the recent finding that global knockout of Klotho diminishes serum short-form Klotho.[31] Thus, our data suggest that the kidney is the major source of soluble Klotho but not short-form Klotho in the blood. Although Klotho is primarily expressed in the kidney[5], it is also moderately expressed in other tissues or organs[41]. Additional studies are required to determine what tissue is the major source of circulating secreted Klotho.

Echocardiography showed that stroke volume began to decline at 2 weeks after kidney specific deletion of Klotho, then continued to decrease over time (Fig. 1D). This decline led

to a decreased cardiac output (Fig. 1E). There was no difference in heart rate between the kidney-specific Cre control (KSP-CON) mice and kidney-specific Klotho knockout (KSP-KL$^{-/-}$) mice (Fig. 1F). Fractional shortening and ejection fraction decreased in KSP-KL$^{-/-}$ mice (Fig. S1A, B), suggesting that Klotho deficiency impairs systolic function. The E/A ratio was also decreased in KSP-KL$^{-/-}$ mice (Fig. S1C), indicating that Klotho deficiency impairs cardiac diastolic function. These results suggest that downregulation of soluble Klotho levels due to kidney-specific deletion of Klotho causes cardiac dysfunction. Furthermore, echocardiography showed that left ventricular end-systolic anterior wall thickness (LVAW, d) increased significantly in KSP-KL$^{-/-}$ mice (Fig. S1D), suggesting that Klotho deficiency may cause LV hypertrophy. Left ventricular end-systolic posterior wall thickness (LVPW, d) also increased although it did not reach a significant level in KSP-KL $^{-/-}$ mice (Fig. S1E). Heart weight increased significantly in KSP-KL$^{-/-}$ mice (Fig. S1F), confirming cardiac hypertrophy.

At the dose of 20 mg/kg, tamoxifen did not alter heart function in KSP-CON (control) mice (Figures 1D, E; Figure S1), indicating that a low dose of tamoxifen does not affect the cardiac phenotypes.

The WGA staining analysis showed a significant increase in cardiomyocyte sizes as measured by cross-section areas in KSP-KL$^{-/-}$ mice (Fig. S1G), suggesting that Klotho deficiency causes cardiac hypertrophy. The picrosirius red staining (PRS) demonstrated fibrosis formation in KSP-KL$^{-/-}$ mice (Fig. S1H), indicating that Klotho deficiency causes fibrotic remodeling.

By miRNA sequencing analysis, we identified a set of 16 miRNAs that were differentially expressed in the cardiomyocytes isolated from Klotho knockout mice: 15 miRNAs were upregulated and one microRNA was downregulated (Fig. 2A). Among these miRNAs, Mir379 was on the very top of the list. The upregulation of Mir379 in the cardiomyocytes isolated from Klotho knockout mice was confirmed by RT-PCR (Fig. 2C). The function of Mir379 has not been recognized, especially in the cardiovascular system. So, we chose Mir379 for further study. There are 240 predicted targets for mmu-miR-379-3p in miRDB database. Smurf1 was on the very top of the list (Fig. 2B). Meanwhile, western blot analysis showed that Smurf1 expression was significantly decreased in cardiomyocytes isolated from Klotho knockout mice (Fig. 2D).

Klotho deficiency caused apoptosis and increased Mir379 level in H9c2 cells

TUNEL-positive cardiomyocytes increased in KSP-KL$^{-/-}$ mice (Fig. S2A, B), indicating that downregulation of soluble Klotho levels caused cardiomyocyte apoptosis. This finding is supported by increased cleaved-caspase 3 expression in the heart in KSP-KL$^{-/-}$ mice (Fig. S1C).

In H9c2 cells, TUNEL-positive cells were increased by Klotho-free medium treatment for 24 hours (Fig. 3A). Cleaved-caspase 3 expression was also significantly increased by Klotho-free medium treatment (Fig. 3B). These results suggest that Klotho deficiency causes H9c2 cells apoptosis.

Real-time PCR results showed that Mir379 levels were dramatically increased by Klotho-free medium treatment (Fig. 4A). The protein expression of Smurf1, a predicted target of Mir379, was decreased by Klotho-free medium treatment (Fig. 4B, C). Concomitantly, BMP4, negatively regulated by Smurf1, was increased by Klotho-free medium treatment (Fig. 4B, D). These results suggest that Klotho deficiency increases Mir379 expression and activity in H9c2 cells. Mir379 may play a role in Klotho deficiency-induced apoptosis in H9c2 cells.

Mir379 is essential for Klotho deficiency-induced apoptosis in H9c2 cells

To test whether Mir379 plays a role in Klotho deficiency-induced apoptosis, H9c2 cells were transfected with Mir379 inhibitor (anti-Mir379) for 48 hours, which was followed by Klotho-free medium treatment for another 24 hours. Expectedly, Klotho-free medium treatment decreased Smurf1 expression and increased BMP4 expression (Fig. 5A). However, Mir379 inhibitor abolished these effects (Fig. 5A). These results testified inhibition of the activity of Mir379. We then tested the necessity of Mir379 in Klotho deficiency-induced apoptosis. Cleaved-caspase 3 expression (Fig. 5B) and TUNEL positive cells (Fig. 5C) were markedly increased by Klotho-free medium treatment. However, Mir379 inhibitor prevented the increase of cleaved-caspase 3 expression (Fig. 5B) and TUNEL positive cells (Fig. 5C). These results indicate that Mir379 is essential for Klotho deficiency-induced apoptosis in H9c2 cells.

Mir379 is sufficient to cause H9c2 cells apoptosis

If Mir379 mediates the pro-apoptotic effects of Klotho deficiency, Mir379 itself should be able to induce apoptosis in H9c2 cells. Therefore, we test the ability of Mir379 to induce apoptosis. H9c2 cells were transfected with Mir379 mimic for 48 hours. Mir379 mimic down-regulated Smurf1 expression and up-regulated its target BMP4 expression (Fig. 6A). Consequently, Mir379 mimic dramatically upregulated cleaved-caspase 3 expression (Fig. 6B) and increased TUNEL positive cells (Fig. 6C). Collectively, our data suggest that Mir379 is not only sufficient to cause apoptosis on its own but also necessary for Klotho deficiency to induce apoptosis in H9c2 cells.

Smurf1 repression is necessary for Mir379 induced H9c2 cells apoptosis

Next, we tested whether Smurf1 is the target gene of Mir379. Using mRNA-miRNA target interaction assay, we found that Mir379 mimic suppressed the luciferase activity from the GLuc-Smurf1-3'-UTR clone by 66.0% (Fig. S4A). This study fulfilled the Mir379 target identification and functional validation of predicted targets. Thus, Smurf1 is the target of Mir379.

To test whether Smurf1 repression is necessary for Mir379-induced apoptosis, H9c2 cells were transfected with Mir379 mimic for 48 hours, which was followed by treatment with exogenous Smurf1 protein (100 ng/ml) for another 24 hours. As expected, Mir379 mimic upregulated cleaved-caspase 3 expression (Fig. S4B) and increased TUNEL positive cells (Fig. S4C). Interestingly, exogenous Smurf1 protein prevented the Mir379 mimic-induced increases in cleaved-caspase 3 expression (Fig. S4B) and TUNEL positive cells (Fig. S4C). These results suggest that Smurf1 repression is essential for Mir379-induced apoptosis in

H9c2 cells. Although the data support that Smurf1 regulates apoptosis as measured by cleaved caspase 3, we cannot exclude that other factors may also induce caspase cleavage.

Discussion

Herein, we found that kidney-specific deletion of *Klotho* gene causes cardiac dysfunction and remodeling, a syndrome resembling heart aging. Klotho is expressed in renal tubular epithelial cells and brain choroid plexus.[3, 39, 40, 42–44] Two types of Klotho protein with potentially different functions have been identified: a full-length transmembrane Klotho and a circulating Klotho.[5] The full-length Klotho is mainly expressed in kidney distal tubule cells and serves as a coreceptor of FGF1 and enhances FGF23 signaling to maintain phosphate homeostasis.[5] *Klotho* gene mutation causes severe hyperphosphatemia.[5] The circulating Klotho, including short-form Klotho (65 kDa) and long-form Klotho (130 kDa), may act as a hormone and regulate the functions of tissues or cells that do not express Klotho.[5, 45] In this study, all mice were fed a low-phosphate diet containing 0.1% (wt/wt) inorganic phosphate which kept serum phosphate in a normal range in Klotho knockout mice (Fig. 5S). Low phosphate diet also prevented kidney dysfunction in KL-deficient mice (Fig. S5). Thus, heart failure due to kidney-specific deletion of the *Klotho* gene is caused by the downregulation of circulating soluble Klotho protein rather than hyperphosphatemia. Therefore, it is important to investigate how downregulation of circulating Klotho causes heart failure in mice. This finding establishes that the circulating Klotho plays an important role in the regulation of heart function in mice. Klotho is not expressed in mouse cardiomyocytes[46] although it was reported that Klotho is expressed in human cardiomyocytes.[47] These findings suggest species differences in Klotho expression in the heart.

Heart is rich in microRNAs (miRNAs).[48] miRNAs, a class of ~22-nt non-coding RNAs, are often conceptualized as ‘fine-tuners’ of gene expression, whose functional influences are due to the cumulative effects of coordinated modulation of multiple downstream mRNA transcripts. miRNAs are increasingly recognized to play important roles in cardiovascular diseases.[49, 50] As a result, miRNAs have become interesting novel drug targets, leading to the development of miRNA mimics and anti-miRNAs. Thus, we performed miRNA sequencing analysis in the cardiomyocytes isolated from the hearts of *Klotho* gene knockout mice to identify putative miRNAs that may mediate the action of Klotho in cardiomyocytes. The microRNA microarray data revealed that 15 miRNAs were upregulated while one miRNA was downregulated in cardiomyocytes due to Klotho deficiency (Fig. 2). Among these miRNAs, Mir379 emerges as a top candidate, but its function in cardiovascular function and has never been investigated.

In H9c2 myoblasts, a cell model used as an alternative for cardiomyocytes, Klotho-free medium increased Mir379 levels and induced apoptosis (Fig. 2 and 3). Treatment with soluble Klotho decreased Mir379 expression (Fig. S3B). These findings suggest that Klotho may negatively regulate Mir379 expression. Mir379 is located at a highly conserved imprinted DLK1-DIO3 genomic region on 14q32.31, which shows great developmental importance and signatures in schizophrenia and metabolic disease.[51] Disruption of Mir379 has been reported in cancers,[20, 51, 52] but little is known about its roles in cardiovascular

diseases. In this study, we found that Klotho-free medium induced cell apoptosis through Mir379 up-regulation. Upregulation of Mir379 is sufficient and necessary for Klotho deficiency to induce apoptosis in H9c2 cells. Mir379 mimic caused H9c2 cells apoptosis while the Mir379 inhibitor prevented the Klotho-free medium induced apoptosis. Although some miRNAs (miR-423-5, miR-103) have been shown to be involved in heart failure,[48] this study provides the first evidence that Mir379 mediates Klotho deficiency-induced cardiomyocyte apoptosis, an important contributor to heart failure. In contrast, other reported miRs in heart failure[48] did not appear on the list of miRs altered due to Klotho deficiency (Fig. 2). These data suggest that different models of heart failure may share different miR profiles and that the roles of miRs in heart failure may be model-specific. It should be mentioned that H9c2 cells may not fully recapitulate the cardiomyocyte phenotypes, the *in vitro* findings in H9c2 cells need to be further validated in animal models.

Interestingly, Mir379 expression was also upregulated in cardiomyocytes in aging mice (Fig. S3) which may be attributed to downregulation of Klotho because serum level of Klotho decreases in aging mice.[36] In humans, serum level of Klotho declines with age after age 40[12–14], so does heart function.[15–18] A decrease in serum Klotho is also found in patients with chronic kidney disease (CKD)[53] which often leads to heart failure. Therefore, upregulation of MiR379 could contribute to aging- and CKD-associated cardiomyocyte apoptosis and heart failure. Supplement with Klotho or inhibition of MiR379 may be a new therapeutic strategy for aging- and CKD-associated cardiomyopathy and heart failure. The circulating Klotho is derived from kidneys. Thus, a decrease in circulating Klotho may be a new and important mediator of cardiorenal syndrome.

As ‘fine-tuners’ of gene expression, each miRNA has its own target genes. In this study, we further found that Smurf1 is the target gene of Mir379. Smurf1 repression is essential for Mir379 induced apoptosis. Smurf1 is a ubiquitin ligase that is specific for receptor-regulated SMAD proteins in the bone morphogenetic protein (BMP) pathway.[24, 25] Diseases associated with Smurf1 include colonic benign neoplasm and Wolfram syndrome.[54, 55] Herein, we showed that Smurf1 takes part in cardiomyocytes apoptosis that plays an important role in various cardiovascular diseases. Smurf1 may be a potential target for cardiomyopathy therapy.

It should be mentioned that that H9c2 cells may not fully recapitulate the cardiomyocyte phenotypes, the *in vitro* findings in H9c2 cells need to be further validated in animal models and cardiomyocytes sourced from human left ventricular tissues.

Perspective

Mir379 level is increased in cardiomyocytes while heart function is impaired in Klotho-deficient mice. Klotho deficiency upregulates Mir379 levels which cause apoptosis in H9c2 cells. Furthermore, Mir379 is sufficient and necessary for Klotho deficiency-induced apoptosis. Smurf1 is the target gene of Mir379. Smurf1 repression is essential for Mir379 induced apoptosis. The results from this study suggests that anti-Mir379 (inhibitor) could be a potential therapeutic target for cardiomyopathy. Overall, this study demonstrates a new pathway (i.e., Klotho-Mir379-Smurf1) that mediates Klotho deficiency-induced cardiomyocyte apoptosis and heart failure (Fig. S5). We fully realized that Klotho

deficiency-induced cardiomyocyte apoptosis is a complicated process and may involve multiple factors although this study focuses on the Mir379-Smurf1 pathway. Further mechanistic investigation is needed to gain a deeper understanding on how Smurf1 mediates Klotho deficiency-induced apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Source of Funding

This work was supported by NIH R01 AG049780, AG062375, HL154147 and HL118558.

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Novelty and Significance

What is new?

- This study reported for the first time that Mir379 may mediate Klotho deficiency-associated cardiomyocyte apoptosis.
- This study reveals a new finding that Smurf1 is a direct target of Mir379.

What is relevant?

- Deficiency of the aging-suppressor gene Klotho impairs heart function leading to heart failure. Unfortunately, there currently is no effective intervention for Klotho deficiency-associated heart failure.
- This study reveals that Mir370 may be a potential therapeutic target for cardiomyocyte apoptosis associated with heart failure due to Klotho deficiency.

Summary

This study provides the first evidence that Mir379 may mediate Klotho deficiency-associated cardiomyocyte apoptosis through repression of Smurf1, which is required for Mir379-induced apoptotic cell death. Further mechanistic investigation is needed to gain a deeper understanding on how Smurf1 mediates Klotho deficiency-induced apoptosis. The results from this study suggests that anti-Mir379 (inhibitor) could be a potential therapeutic strategy for cardiomyopathy.

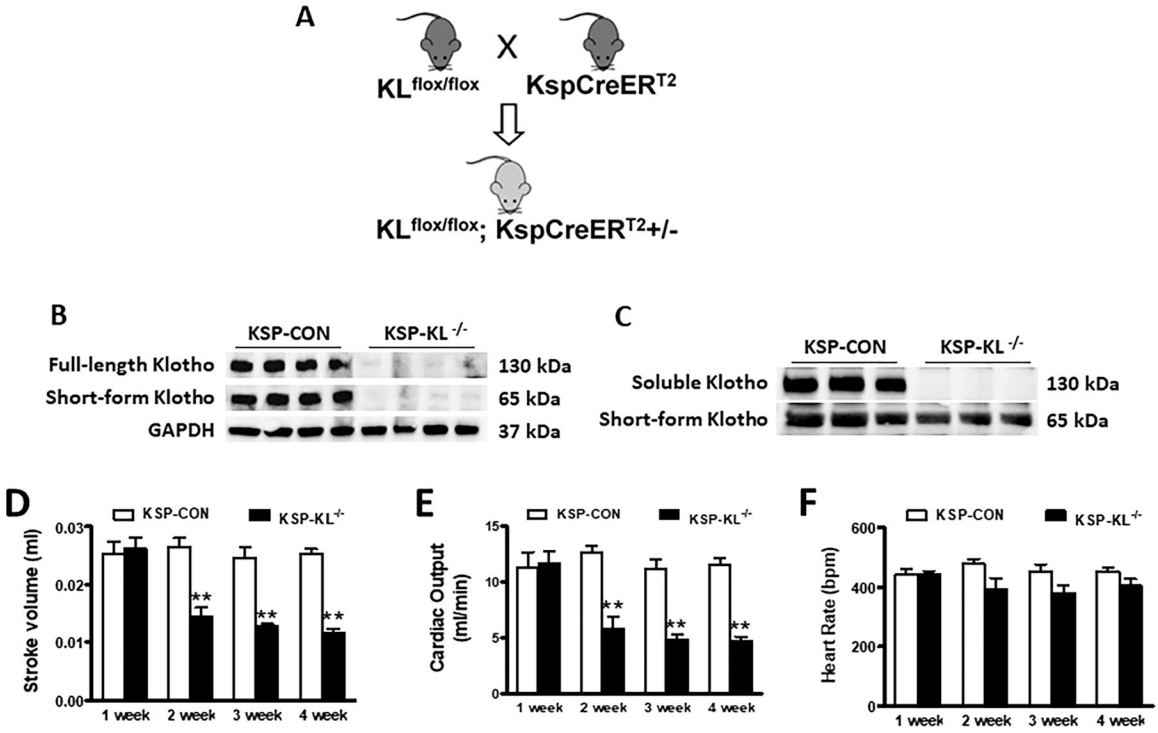
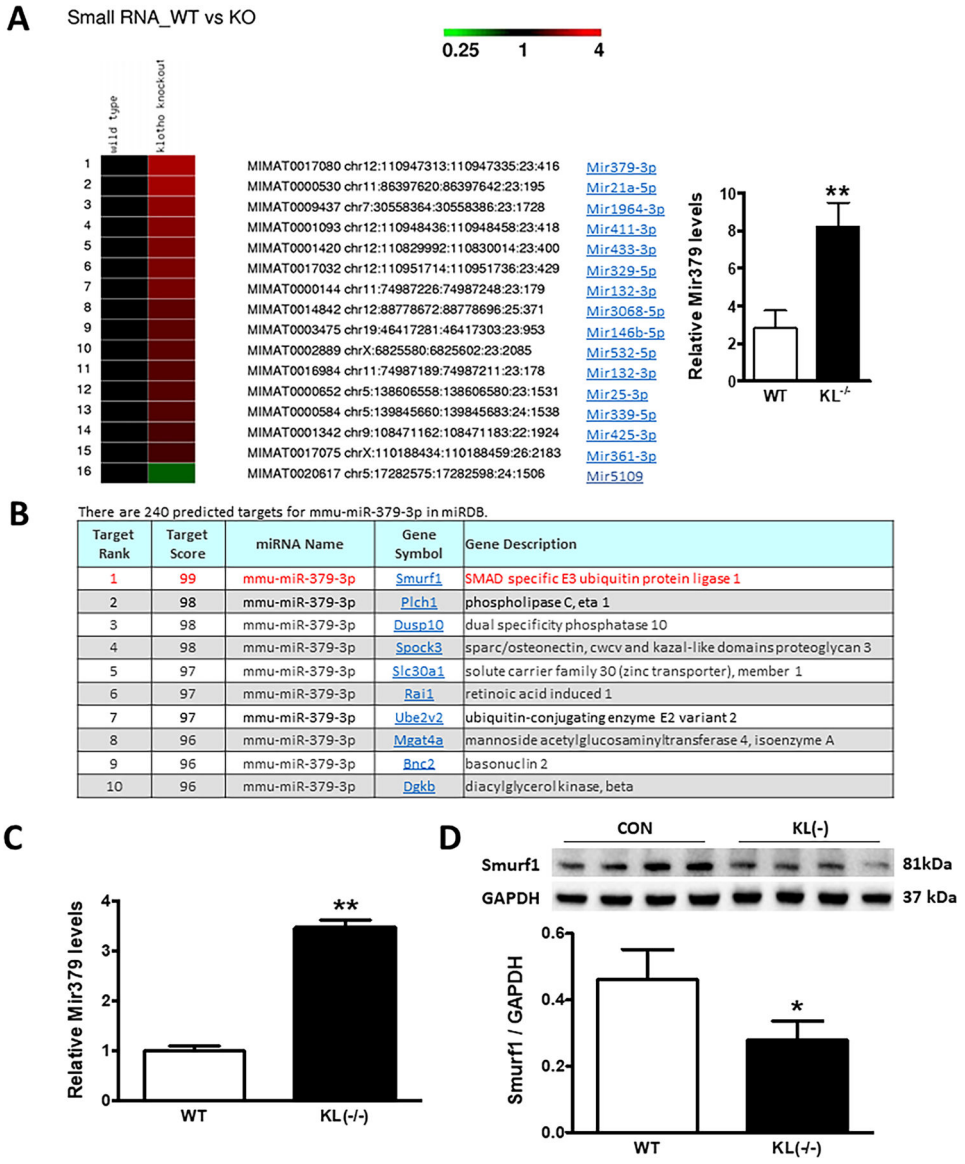


Fig 1. Klotho deficiency impaired cardiac function.

(A) The schematic diagram for the generation of kidney specific Klotho knockout mice. (B) WB of Klotho in the kidney. (C) WB of Klotho in the serum. Serum Klotho was pulled down using anti-Klotho antibody (R&D, AF1819) and magnetic bead (Cell Signaling 73778) according to the immunoprecipitation protocol. IP products were further subjected to western blotting analysis. Time course of changes in stroke volume (D), cardiac output (E), and heart rate (F) in kidney-specific Cre control (KSP-CON) mice and kidney-specific Klotho knockout (KSP-KL$^{-/-}$) mice. Data are expressed as mean ± SE and analyzed by t-test, **p<0.01 vs Control mice. N=5.



Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
1	99	mmu-miR-379-3p	Smurf1	SMAD specific E3 ubiquitin protein ligase 1
2	98	mmu-miR-379-3p	Plch1	phospholipase C, eta 1
3	98	mmu-miR-379-3p	Dusp10	dual specificity phosphatase 10
4	98	mmu-miR-379-3p	Spock3	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 3
5	97	mmu-miR-379-3p	Slc30a1	solute carrier family 30 (zinc transporter), member 1
6	97	mmu-miR-379-3p	Rai1	retinoic acid induced 1
7	97	mmu-miR-379-3p	Ube2v2	ubiquitin-conjugating enzyme E2 variant 2
8	96	mmu-miR-379-3p	Mgat4a	mannoside acetylglucosaminyltransferase 4, isoenzyme A
9	96	mmu-miR-379-3p	Bnc2	basonuclin 2
10	96	mmu-miR-379-3p	Dgkb	diacylglycerol kinase, beta

Fig 2. Klotho deficiency increased cardiac Mir379 level.

(A) MicroRNA sequencing analysis of WT and Klotho $^{-/-}$ mice. N=3. (B) MicroRNA target prediction for mmu-miR-379-3p in miRDB database. (C) RT-PCR results of Mir379. N=4. (D) WB of Smurf1. N=4. Data are expressed as mean ± SE and analyzed by t-test, *p<0.05; **p<0.01 vs control mice.

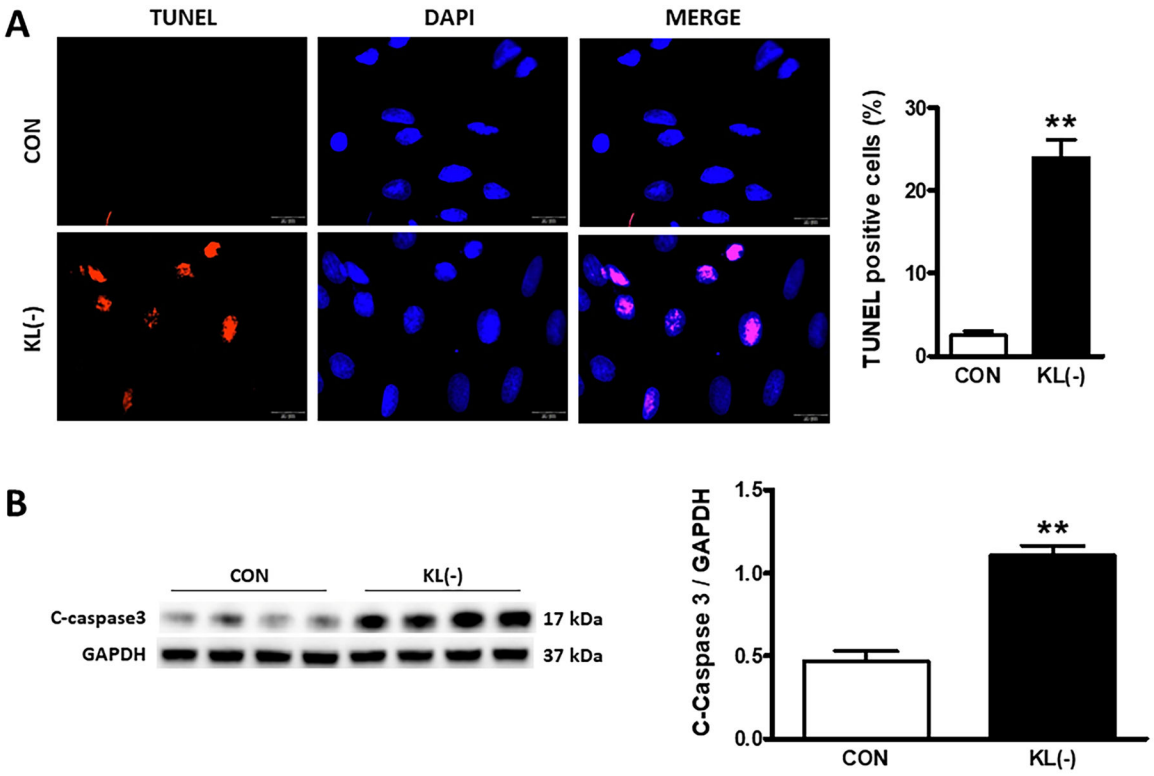


Fig 3. Klotho deficiency caused H9c2 cells apoptosis.

(A) TUNEL labeling of H9c2 cells. (B) WB of cleaved caspase 3. Data are expressed as mean ± SE and analyzed by t-test, **p<0.01 vs Mir control group. N=4.

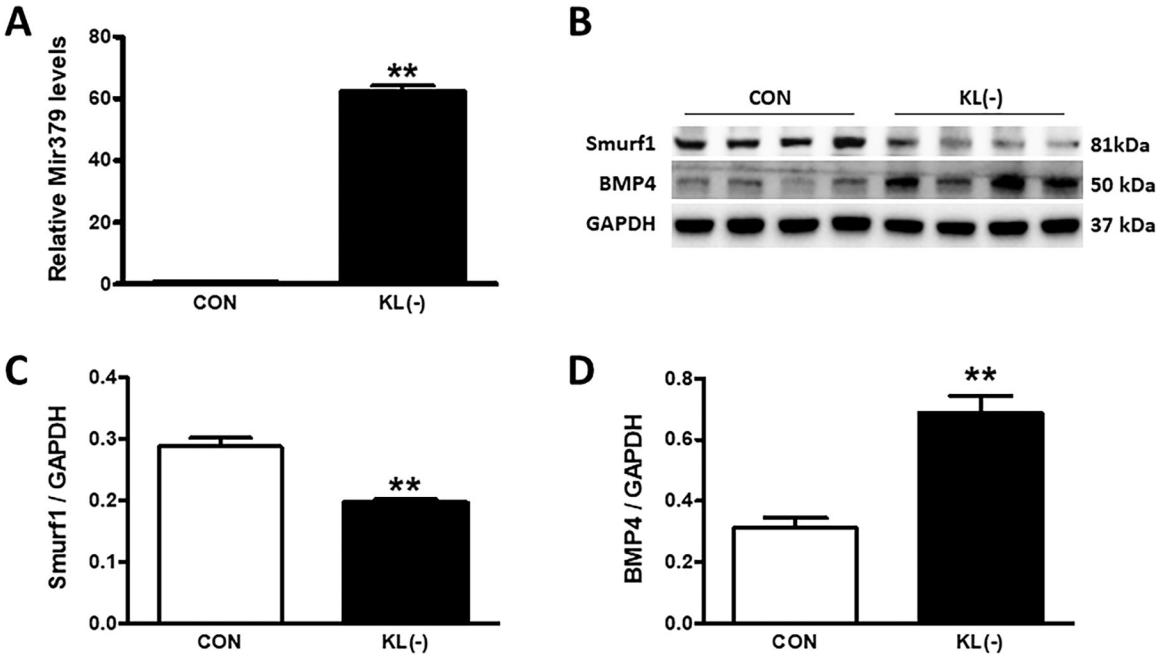


Fig 4. Klotho deficiency increased Mir379 level and Smurf1 expression and activity in H9c2 cells.

(A) RT-PCR results of Mir379. (B) WB of Smurf1 and its target BMP-4. (C) Quantification of Smurf1 protein expression. (D) Quantification of BMP-4 protein expression. Data are expressed as mean ± SE and analyzed by t-test, **p<0.01 vs control group. N=4.

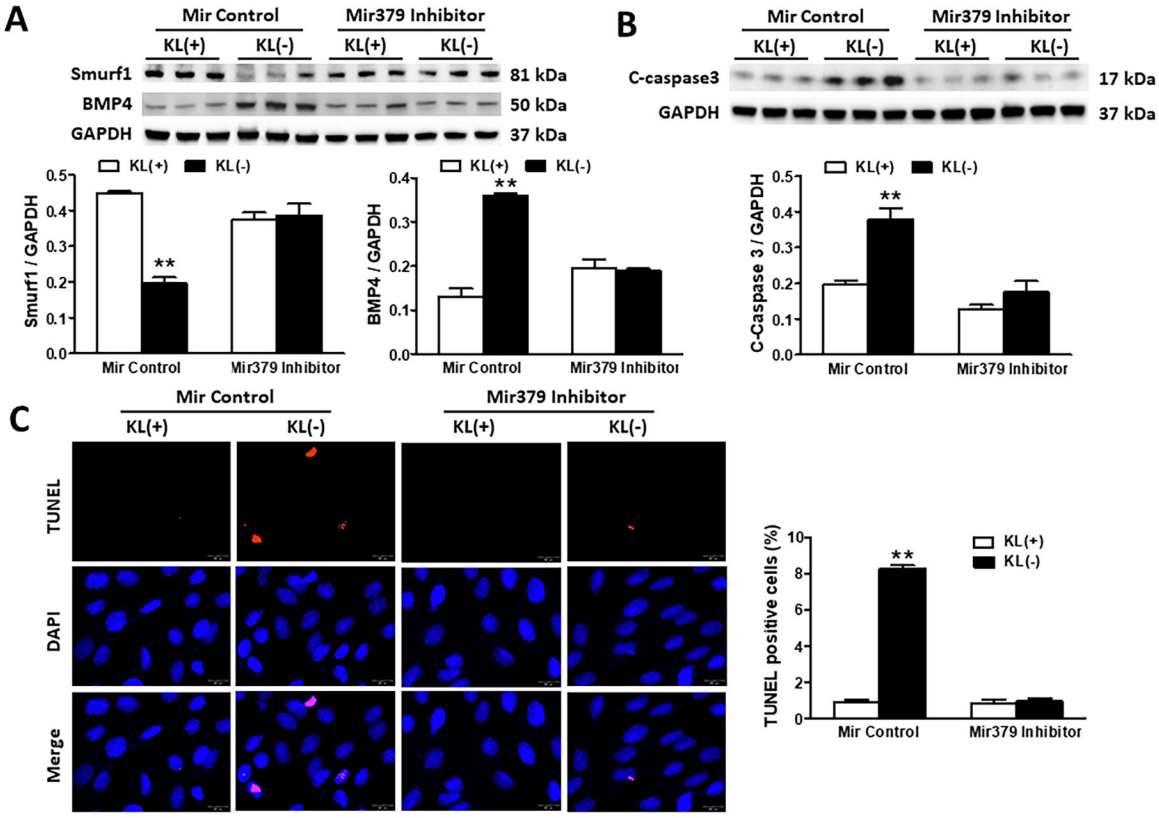


Fig 5. Mir379 inhibitor abolished the effects of Klotho deficiency in H9c2 cells.

(A) WB of Smurf1 and its target BMP-4. N=3. (B) WB of cleaved caspase 3. N=3. (C) TUNEL labeling of H9c2 cells. N=4. Data are expressed as mean ± SE and analyzed by a two-way ANOVA, **p<0.01 vs Mir control only group.

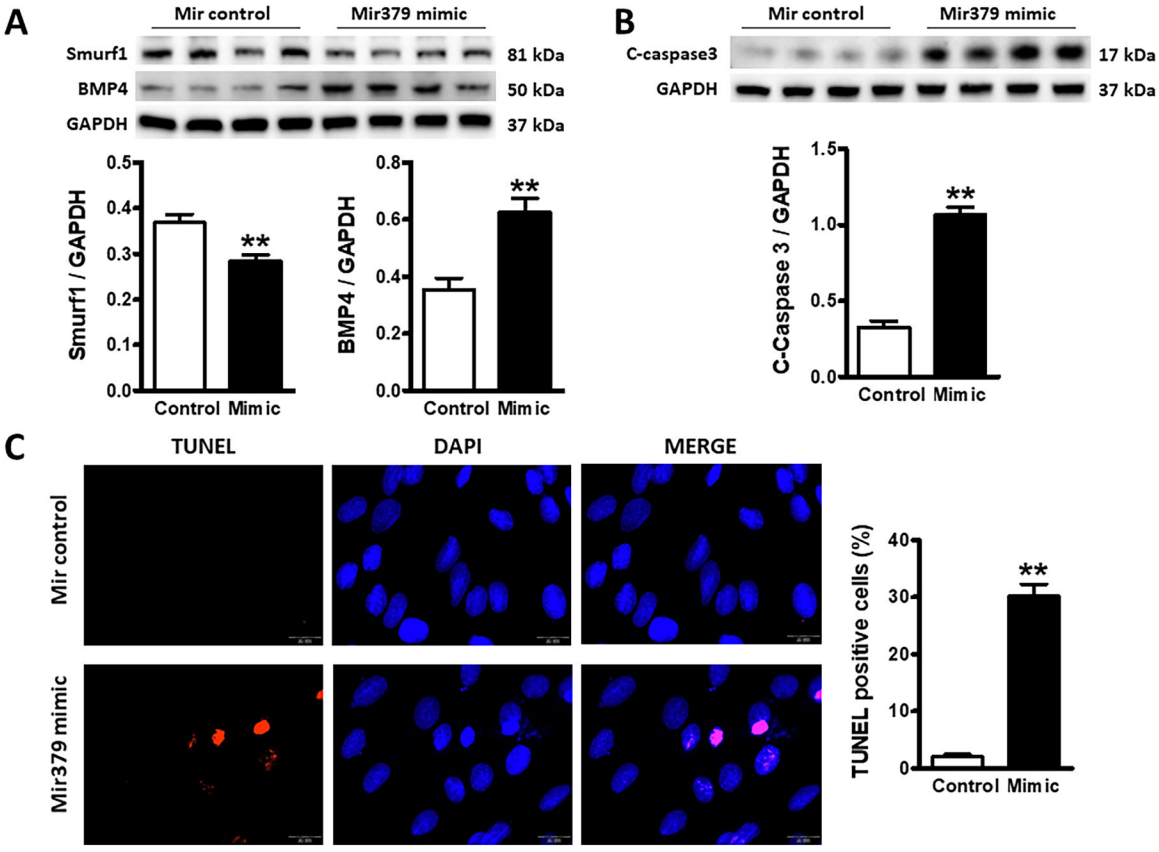


Fig 6. Mir379 mimic inhibited Smurf1 expression and activity and caused H9c2 cell apoptosis.

(A) WB of Smurf1 and its target BMP-4. (B) WB of cleaved caspase 3. (C) TUNEL labeling of H9c2 cells. Data are expressed as mean ± SE and analyzed by t-test, **p<0.01 vs Mir control group. N=4.