Midkine Deteriorates Cardiac Remodeling via Epidermal Growth Factor Receptor Signaling in Chronic Kidney Disease

Yuki Honda, Tetsuro Shishido, Tetsuya Takahashi, Tetsu Watanabe, Shunsuke Netsu, Daisuke Kinoshita, Taro Narumi, Shinpei Kadowaki, Satoshi Nishiyama, Hiroki Takahashi, Takanori Arimoto, Takuya Miyamoto, Satoshi Kishida, Kenji Kadomatsu, Yasuchika Takeishi, Isao Kubota

Abstract—In chronic kidney disease, activation of the epidermal growth factor receptor (EGFR) leads to cardiac hypertrophy, which affects morbidity and mortality. In patients with renal insufficiency and heart failure, the expression of midkine, a heparin-binding growth factor, is increased. Therefore, we investigated the association between midkine and EGFR in the induction of cardiac hypertrophy and dysfunction in chronic kidney disease. We performed subtotal nephrectomies in midkine-knockout mice and wild-type mice. We found that subtotal nephrectomy-induced cardiac hypertrophy and phosphorylation of extracellular signal–regulated kinase 1/2 and AKT were attenuated in midkine-knockout mice compared with wild-type mice. An antiphosphotyrosine receptor antibody array was used to demonstrate that EGFR phosphorylation in the heart was also lower in midkine-knockout mice than in wild-type mice. Midkine induced EGFR, extracellular signal–regulated kinase 1/2, and AKT phosphorylation and led to hypertrophy in neonatal rat cardiomyocytes. Pretreatment with EGFR inhibitors or EGFR silencing suppressed midkine-stimulated phosphorylation of extracellular signal–regulated kinase 1/2 and AKT, induction of fetal cardiac gene expression, and hypertrophy in cardiomyocytes. To confirm the association between midkine and EGFR in vivo, mice subjected to subtotal nephrectomy were treated with the EGFR inhibitor gefitinib. Gefitinib treatment attenuated subtotal nephrectomy-induced cardiac hypertrophy. These results indicate that midkine might be a key mediator of cardiorenal interactions through EGFR activation, which plays a crucial role in cardiac hypertrophy in chronic kidney disease. (Hypertension. 2016;67:857-865. DOI: 10.1161/HYPERTENSIONAHA.115.06922.) • Online Data Supplement

Key Words: cardiomegaly • hypertrophy, left ventricular • midkine • nephrectomy • receptor, epidermal growth factor • renal insufficiency, chronic

Chronic kidney disease (CKD) is a major global health problem, the prevalence of which is estimated to be 8% to 16% worldwide.1 CKD leads to volume overload, anemia, hypertension, and activation of the renin–angiotensin–aldosterone system. All of these conditions eventually contribute to the development of cardiac remodeling, which affects cardiovascular morbidity and mortality. In patients with CKD, the most frequently observed type of cardiac remodeling is left ventricular (LV) hypertrophy (LVH).2,3 LVH is a powerful risk factor of morbidity and mortality4 because chronic LVH eventually results in LV dysfunction.5 LV dysfunction reduces blood supply to the kidney and deteriorates renal functions. Therefore, the coexistence of CKD and LVH results in the formation of a vicious cycle6-8 and worsens the prognosis.9 In patients with CKD, the initial manifestation of cardiac remodeling was shown to be LVH.10 Therefore, preventing the development of LVH is a key strategy for reducing morbidity and mortality in patients with CKD.

The epidermal growth factor receptor (EGFR), a member of the ErbB receptor tyrosine kinase family, plays a critical role in cell survival and proliferation.11 EGFR signaling has recently been suggested to be associated with the development of chronic kidney injury and cardiac dysfunction through activation of the extracellular signal–regulated kinase (ERK) and phosphatidylinositol-3′-phosphate kinase signaling pathways.12-14

Midkine is a 13-kDa heparin-binding growth factor that plays diverse physiological roles such as promoting the survival of embryonic neurons and the migration of inflammatory cells.15-17 Midkine is expressed during various pathological
conditions such as cancer, renal failure,\textsuperscript{18,19} and cardiovascular diseases,\textsuperscript{20–22} after which it is released into the systemic circulation.\textsuperscript{19,23} Midkine secreted from the kidney and lungs might be associated with cardiorenal interactions, but the precise mechanism by which midkine induces cardiac remodeling remains unclear. Here, we performed receptor tyrosine kinase-array analysis and identified an association between midkine and EGFR phosphorylation in the heart. Therefore, we hypothesized that midkine-mediated cardiorenal interactions are associated with EGFR signaling. In this study, we also demonstrated that circulating midkine induced by subtotal nephrectomy evoked the activation of EGFR and downstream signaling, eventually contributing to the development of cardiac dysfunction.

Methods

The Methods/Protocols used in this study are detailed in the online-only Data Supplement.

Subtotal Nephrectomy Models

Midkine systemic knockout mice (MK-KO; C57BL/6 background) were established as previously reported.\textsuperscript{14} A major branch of the right renal artery was ligated and then the entire left kidney was removed 1 week later. Sham operations were performed similarly, except that the right renal artery was not ligated and the left kidney was not removed. Successful subtotal nephrectomy was confirmed based on the elevated levels of serum urea nitrogen and serum creatinine, and cardiac function after subtotal nephrectomy or sham operation was evaluated by means of transthoracic echocardiography performed using a Vevo2100 (VisualSonics, Toronto, ON, Canada).

Primary Culture of Neonatal Rat Cardiomyocytes

Hearts were excised from 1- to 2-day-old Sprague–Dawley rat pups, promptly after euthanasia by decapitation, and primary cultures of neonatal cardiomyocytes were prepared as described previously.\textsuperscript{25}

Ethics Statement

All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine, and the study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Statistical Analysis

Data are presented as mean±SEM. Differences between groups were evaluated using 1-way ANOVA with post hoc Bonferroni test. Survival curves after subtotal nephrectomy were generated using Kaplan–Meier analysis and compared using the log-rank test. P<0.05 was considered significant. All statistical analyses were performed using a standard statistical program package (JMP version 9; SAS institute Inc., Cary, NC).

Results

Effects of Subtotal Nephrectomy on Cardiac Hypertrophy in MK-KO Mice

To investigate the effects of midkine deletion on cardiac hypertrophy in vivo, we performed subtotal nephrectomies or sham operations with MK-KO and wild-type (WT) mice. Midkine expression in the kidney (Figure 1A; Figure S1 in the online-only Data Supplement) and serum midkine concentrations (Figure 1B; Figures S2 and S3) were higher in WT mice subjected to a subtotal nephrectomy than in sham-operated WT mice. The expression levels of midkine were faint in the heart, and the mRNA but not protein expression levels of midkine in the heart were elevated after subtotal nephrectomy (Figure S4). In Western blots, equal sample loading for evaluating serum midkine was confirmed by staining the membranes with Ponceau S (Figure S5). Moreover, serum urea nitrogen and creatinine levels were statistically increased after subtotal nephrectomy, and MK-KO mice showed lower concentrations of urea nitrogen and creatinine compared with WT mice (Table S1). Both the systolic and mean blood pressure significantly increased after subtotal nephrectomy, but we detected no statistically significant difference in blood pressure between WT and KO mice after subtotal nephrectomy (Figure 1C). Echocardiography revealed a statistically significant increase in the LV end-diastolic and end-systolic diameter and LV wall thickness, as well as a decrease in fractional shortening in WT mice subjected to subtotal nephrectomy, as compared with the corresponding measurements for sham-operated mice. The heart weight/tibia length ratio and cell-surface area were significantly increased in WT mice that received subtotal nephrectomy (Table S1; Figure 1D and 1E). Importantly, serum levels of midkine increased (before the heart weight significantly increased) after subtotal nephrectomy (Figure S3 and Table S2). However, these geometric changes were attenuated in MK-KO mice when compared with those observed in WT mice (Table S1). We also performed terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining and Masson trichrome staining to evaluate the degree of apoptosis\textsuperscript{26} and fibrosis\textsuperscript{27} occurring in the heart after nephrectomy. However, no significant differences in the degrees of apoptosis and fibrosis between MK-KO and WT mice were observed (Figure S6).

Effect of Midkine on Cardiomyocyte Hypertrophy

Because midkine performs various biological functions that are mediated through ERK and AKT activation,\textsuperscript{15} we evaluated the phosphorylation statuses of ERK1/2 and AKT in the heart. Both ERK1/2 and AKT were phosphorylated after subtotal nephrectomy, but the phosphorylation levels were lower in MK-KO mice than those in WT mice (Figure 2A and 2B). To confirm the role of circulating midkine in the development of cardiac hypertrophy, we stimulated neonatal rat cardiomyocytes with midkine, and the results showed that ERK1/2 and AKT were phosphorylated after the stimulation (Figure 2C; Figure S7). These results suggested that midkine secreted from the kidney contributes to ERK1/2 and AKT phosphorylation in the heart after subtotal nephrectomy.

Effect of Midkine on EGFR Signaling

To clarify the mechanisms by which midkine phosphorylates ERK1/2 and AKT, we evaluated the activities of receptor tyrosine kinase in the hearts using the Proteome Profiler Mouse Phospho-RTK Array Kit. We found that several receptor tyrosine kinases including EGFR were activated in the nephrectomized WT mice compared with in the nephrectomized KO mice (Figure S8). It has been reported that midkine contributed to renal failure and cardiac hypertrophy.\textsuperscript{10,21,24} Similarly, EGFR activity is associated with various
pathological states, including renal insufficiency and cardiac remodeling.\textsuperscript{13,14,29–31} Therefore, among the activated receptor tyrosine kinases, we focused on the EGFR and hypothesized that circulating midkine might modify EGFR signaling in the heart and lead to cardiac hypertrophy after subtotal nephrectomy.

Figure 1. Systemic midkine (MK) knockdown suppresses left ventricular (LV) hypertrophy after subtotal nephrectomy. A, Kidney tissues were labeled with antibodies against MK, which demonstrated increased renal MK expression after subtotal nephrectomy. Scale bar, 50 μm. B, At 12 wk after subtotal nephrectomy, serum MK concentrations were evaluated by Western blotting analysis. Serum MK concentrations increased after subtotal nephrectomy. C, Changes in blood pressure were examined at the indicated times after subtotal nephrectomy. D, Representative M-mode echocardiography of wild-type (WT) or MK with systemic knockout mice (MK-KO) at 12 wk after subtotal nephrectomy or sham-operation. E, Representative images of hematoxylin–eosin staining of LV transverse sections at 12 wk after subtotal nephrectomy or sham operation. Scale bar, 50 μm. Data are expressed as the mean±SEM in experiments performed with 6 to 10 mice per group. *P<0.05 and **P<0.01 vs control mice of the same strain. MBP indicates mean blood pressure; Nx, subtotal nephrectomy; and SBP, systolic blood pressure.
Next, we stimulated neonatal rat cardiomyocytes with midkine and evaluated EGFR phosphorylation. Midkine induced EGFR autophosphorylation in dose- and time-dependent manners (Figure 3A; Figure S9). We also examined the impact of EGFR phosphorylation on ERK1/2 and AKT phosphorylation using EGFR-family inhibitors and an EGFR-specific small interfering RNA. Neonatal rat cardiomyocytes were treated with lapatinib (10 nmol/L), which inhibits both EGFR and ErbB2; AG1478 (3 nmol/L), which inhibits EGFR; and mubritinib (4 nmol/L), which inhibits ErbB2. Pretreatment with lapatinib (Figure 3B) or AG1478 (Figure 3C), but not mubritinib (Figure 3D), strongly inhibited midkine-induced phosphorylation of ERK1/2 and AKT. A specific small interfering RNA against EGFR mRNA blocked its expression by >70%, and small interfering RNA–mediated EGFR silencing also inhibited midkine-induced phosphorylation of ERK1/2 and AKT (Figure 3E; Figure S10). These results indicated that midkine stimulated EGFR autophosphorylation and downstream signaling in cardiomyocytes.

Impact of Midkine on Cardiac Fetal Gene Expression and Cardiomyocyte Hypertrophy
To determine the role of midkine in cardiac hypertrophy, we quantified brain natriuretic peptide promoter activity by performing luciferase reporter assays. Midkine stimulation increased brain natriuretic peptide promoter activity in neonatal rat cardiomyocytes (Figure 4A), which was abolished after pretreatment with lapatinib or AG1478. However, pretreatment with mubritinib did not affect brain natriuretic peptide promoter activity. Next, we measured cell-surface areas after midkine stimulation in cardiomyocytes transfected with pCMV6-AC-GFP. We found that after a 48-hour stimulation, the surface areas of cardiomyocytes increased. However, pretreatment with the EGFR-selective blocker gefitinib significantly inhibited midkine-induced hypertrophic changes in cardiomyocytes (Figure 4B), as did small interfering RNA–mediating EGFR silencing (Figure 4C). These results suggested that midkine induced cardiomyocyte hypertrophy in an EGFR-dependent manner.

Effect of EGFR Inhibition on Cardiac Remodeling After Subtotal Nephrectomy
Our results showed that subtotal nephrectomy increased midkine expression, which activated EGFR and downstream signaling; thus, we expected gefitinib to inhibit cardiac remodeling after subtotal nephrectomy in WT mice, but not MK-KO mice. Examination revealed that LVH after subtotal nephrectomy was also attenuated in WT mice treated with gefitinib (Figure 5A and 5B; Table S3). As expected, gefitinib administration inhibited ERK1/2 and AKT phosphorylation after subtotal nephrectomy (Figure 5C; Figure S11). Kaplan–Meier analysis revealed...
that after subtotal nephrectomy, WT mice treated with gefitinib exhibited a higher survival rate than did their untreated littermates (Figure 5C). However, gefitinib did not affect the degree of cardiac remodeling and survival rate after subtotal nephrectomy in MK-KO mice.

**Discussion**

In this study, we found that (1) subtotal nephrectomy increased the expression of midkine in the kidney and the levels of circulating midkine; (2) midkine induced EGFR autophosphorylation and caused subsequent ERK1/2 and AKT phosphorylation in cardiomyocytes; (3) midkine-induced hypertrophic changes and fetal gene expression levels were suppressed after the inhibition of EGFR signaling in cardiomyocytes; (4) cardiac dysfunction after subtotal nephrectomy were attenuated in MK-KO mice as compared with those in WT mice; and (5) gefitinib-dependent EGFR inhibition suppressed downstream kinase activation and the development of cardiac dysfunction after subtotal nephrectomy in WT mice, but not in MK-KO mice.

**Renal Injury Increased Midkine Expression and Circulating Midkine Levels, Which Exacerbated Renal Injury and Cardiac Hypertrophy**

Here, we determined that midkine expression was induced in the kidney and that midkine was secreted into the circulation after subtotal nephrectomy, in agreement with previous
Hobo et al. showed that midkine regulates the renin–angiotensin system by inducing expression of the lung angiotensin-converting enzyme. We also found that renal function was relatively preserved in MK-KO mice after subtotal nephrectomy, which suggests that midkine might indirectly lead to LVH by modulating the renin–angiotensin system. However, our results revealed that cardiac function was preserved to a greater extent in MK-KO mice than in WT mice, although the blood pressure after subtotal nephrectomy was similar in WT and MK-KO mice. Previously, circulating midkine was reported to affect various organs through ERK and AKT activation. Moreover, we recently

Figure 5. Gefitinib exerts inhibitory effects on cardiac remodeling after subtotal nephrectomy. A, Representative images of hematoxylin–eosin staining of left ventricular transverse sections at 12 wk after subtotal nephrectomy, with or without epidermal growth factor receptor (EGFR) inhibitor administration, in wild-type (WT) and MK with systemic knockout mice (MK-KO) mice. Scale bar, 50 μm. B, Cardiomyocyte surface areas were decreased after subtotal nephrectomy in WT mice after gefitinib administration. The data are expressed as the means±SEM. C, Phosphorylation of ERK1/2 and AKT after subtotal nephrectomy (Nx) was suppressed by gefitinib administration. D, Survival curves after subtotal nephrectomy, obtained for WT and MK-KO, mice with and without gefitinib treatment.
reported that cardiac-specific overexpression of midkine exacerbates cardiac remodeling after pressure overload. In this study, we confirmed that midkine induced ERK1/2 and AKT phosphorylation and hypertrophy in cardiomyocytes. These data suggest that circulating midkine might directly induce strong and broad effects in the heart through ERK and AKT activation.

Increase in Midkine Levels and EGFR Activation Contribute to Cardiorenal Interactions

Although various candidate receptors for midkine have been recognized in several cell types, the mechanisms by which midkine contributes to cardiac remodeling have not yet been identified. We demonstrated that EGFR inhibition attenuated midkine-induced hypertrophic changes in cardiomyocytes. We also showed that gefitinib restored cardiac remodeling after subtotal nephrectomy in WT mice. In the case of CKD, EGFR phosphorylation was increased in the heart, and EGFR inhibition was capable of preserving renal function and improving the clinical course. Moreover, excessive EGFR activity is associated with various pathological states, including renal fibrosis and cardiac hypertrophy. Although noncardiomyocytes are suspected to potentially account for the cardiac side effects related to EGFR inhibition, several recent studies have revealed unanticipated effects of EGFR inhibitors, including cardiomyopathy and heart failure. We found that circulating midkine levels were increased after subtotal nephrectomy and that the activities of kinases downstream of EGFR were attenuated after subtotal nephrectomy in MK-KO mice. These findings suggest that circulating midkine might function as a key molecule that mediates CKD and LVH by modulating EGFR signaling. Therefore, inhibition of circulating midkine might serve as a novel therapeutic approach against CKD-induced LV dysfunction.

Limitations

In this study, we did not identify the mechanism(s) though which midkine induces EGFR phosphorylation. EGFR can be activated directly by ligand binding or indirectly via crosstalk with nonreceptor tyrosine-kinase pathways (referred to as transactivation). Recently, pleiotrophin, which is 50% homologous to midkine, was reported to contribute to EGFR transactivation in cultured osteoblasts. Therefore, it should be determined how midkine activates EGFR in cardiomyocytes in the future. Moreover, the dose of midkine used in this study was higher than midkine levels observed in patients with malignancy or heart failure. Because it is difficult to stimulate neonatal cardiomyocytes with midkine for a long duration, we used doses of 50 to 200 ng MK/mL, as done previously.

Another study limitation is that we used systemic MO-KO mice in our in vivo study. Therefore, other organs besides the heart (such as the kidneys) might have influenced our results, and additional studies must be conducted to clarify this point. The cardiac phenotypes of the nonsurviving mice varied depending on the length of the time after nephrectomy. In the early phase after nephrectomy, cardiac hypertrophy was not frequently observed in the nonsurviving mice. In contrast, at 1 to 2 months after nephrectomy, almost all deceased mice showed cardiac hypertrophy with dilated cardiac cavities. In addition, accumulation of effusion in the pleural cavity was observed in most of the deceased animals irrespective of the phase after nephrectomy. Therefore, we speculate that the causes of deaths were because of either acute renal failure or decompensated heart failure. However, we did show that midkine induced EGFR autophosphorylation and hypertrophic changes in cardiomyocytes. Therefore, circulating midkine might directly participate in the modulation of cardiac remodeling. In contrast, we could not exclude the possibility that midkine synthesized in the heart, rather than circulating from the kidney, contributed to the pathogenesis of the cardiac hypertrophy. In the future study, we need to generate cardiomyocyte-specific MK-KO mice to elucidate this point.

Perspectives

Midkine released from the kidney induces LVH and subsequently causes cardiac dysfunction through EGFR-dependent signaling. Thus, midkine might play a crucial role in mediating cardiorenal interactions. Our results imply that midkine inhibition might serve as a novel therapeutic strategy for preventing cardiac remodeling in patients with CKD.

Acknowledgments

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- We have shown for the first time that midkine induces epidermal growth factor receptor autophosphorylation and activates its downstream signaling, and that kidney-secreted midkine is associated with cardiac hypertrophy in chronic kidney disease.

**What Is Relevant?**

- The findings of this study suggest a new approach for preventing cardiac morbidity and mortality in patients with chronic kidney disease.

**Summary**

Subtotal nephrectomy in mice upregulates the expression of midkine and its release from the kidney, and midkine induces left ventricular hypertrophy and causes subsequent cardiac dysfunction through epidermal growth factor receptor–dependent signaling. These findings suggest a critical role for midkine in mediating cardiorenal interactions and identify midkine inhibition as a potential treatment strategy for preventing cardiac remodeling in patients with chronic kidney disease.
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Short title: Midkine Mediated Cardio-renal Association

Key words: midkine, cardio-renal interaction, cardiac hypertrophy, growth factor, epidermal growth factor receptor, chronic kidney disease

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Supplemental Methods

Ethics Statement

All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine, and the study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Materials and Reagents

The following reagents were from commercial sources: recombinant human MK (Peptide Institute, Osaka, Japan); antibodies against ERK1/2, phospho-ERK1/2 (p-ERK1/2), AKT, phospho-AKT (p-AKT), phospho-EGFR (p-EGFR Tyr 1068), STAT3, phospho-STAT3, and β-tubulin (Cell Signaling Technology, Danvers, MA, USA); antibodies against MK and EGFR (Sigma, St. Louis, MO, USA); EGFR-family inhibitors lapatinib, gefitinib, and mubritinib (Selleck Chemicals, Houston, TX, USA); EGFR inhibitor AG1478 (Cell Signaling Technology); and EGFR-specific small interfering RNA (siRNA) and negative-control siRNA (Cell Signaling Technology). The luciferase reporter construct BNP/luc was generated and used to examine the effects of MK on fetal gene expression.1, 2

Primary Culture of Neonatal Rat Cardiomyocytes

Hearts were excised from 1–2-day-old Sprague-Dawley rat pups, promptly after euthanasia by decapitation, and primary cultures of neonatal cardiomyocytes were prepared as described previously. Cells were seeded in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and cultured for 4 days, after which the cardiomyocytes were maintained in serum-free medium for 24 h and then stimulated with MK. After MK stimulation, samples were collected to examine the expression levels of EGFR, p-EGFR, ERK1/2, p-ERK1/2, AKT, and p-AKT by means of western blotting. EGFR siRNA, control siRNA, and expression vectors were transfected into cardiomyocytes using the Lipofectamine LTX & Plus Reagent (Life Technologies Japan) according to the manufacturer’s instructions. MK-induced BNP promoter activities were evaluated by performing luciferase reporter gene assays with BNP/luc, pRL-TK vectors, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Transcriptional activities were calculated from 3 separate assays performed in triplicate. Cardiomyocytes transfected with pCMV6-AC-GFP (OriGene, Rockville, MD, USA) were incubated with or without MK for 48 h, and then cell-surface areas were measured using Image J software (US National Institutes of Health, Bethesda, MD, USA). Cardiomyocytes cotransfected with pCMV6-AC-GFP and EGFR siRNA or control siRNA were incubated with or without MK, and then cell-surface areas were measured as described previously.
Protein Extraction and Western Blotting

Total proteins were extracted from the left ventricle, lung, and kidney using ice-cold lysis buffer, as described previously\(^3\). The protein concentrations of each sample were determined using the BCA protein assay (BioRad Laboratories, Inc., Hercules, CA). Equal amounts of protein were electrophoresed on 8%–14% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes. Membranes were blocked with 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.1% Tween (TBS-T) and 5% milk and then probed with primary antibodies diluted in TBS-T. After incubation with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T containing 5% milk, immunoreactive bands were detected using an ECL kit (Amersham Biosciences, Piscataway, NJ, USA).

EGFR-Inhibitor Experiments

In the in vitro study, the EGFR-family inhibitors lapatinib (which inhibits both EGFR and ErbB2 receptor), gefitinib (an EGFR-specific inhibitor), AG1478 (an EGFR-specific inhibitor), and mubritinib (an ErbB2-specific inhibitor) were dissolved in dimethyl sulfoxide and added to the culture medium 60 min before MK stimulation. The concentrations of lapatinib, gefitinib, AG1478, and mubritinib were 10 nM, 1 μM, 3 nM, and 4 nM, respectively. These concentrations were determined based on the manufacturer’s recommendations. Cardiac toxicity has been reported to be induced by tyrosine kinase inhibitors such as imatinib mesylate, dasatinib, nilotinib, sunitinib, sorafenib, and lapatinib, but gefitinib and erlotinib have not been related to toxic effects on the heart\(^4\). Therefore, in our in vivo study, gefitinib was used as a representative EGFR inhibitor; 100 mg/kg gefitinib was orally administered once daily\(^5\) during the period from 2 to 6 weeks after subtotal nephrectomy.

Subtotal Nephrectomy Models

MK systemic knockout mice (MK-KO; C57BL/6 background) were established as previously reported\(^6\). We anesthetized 8–12-week-old male MK-KO mice and wild-type (WT) littermates by intraperitoneal injection with a mixture of ketamine (80 mg/kg). The mice were maintained in the prone position and low-back incisions were used during the surgeries. A major branch of the right renal artery was ligated and then the entire left kidney was removed 1 week later. Sham operations were performed similarly, except that the right renal artery was not ligated and the left kidney was not removed. This method differed from that of Hobo and colleagues, who used renal ablation rather than renal artery ligation\(^7\). Mice were housed in a facility under a 12/12-h light-dark cycle and were allowed free access to water and standard rodent chow. The room was maintained under specific pathogen-free conditions. Blood pressures were measured in conscious mice using the standard tail-cuff method and a MK-2000ST NP-NIBP MONITOR (Muromachi Kikai CO., LTD, Tokyo, Japan). Cardiac function at 12 weeks after subtotal nephrectomy or sham operation was evaluated by means of transthoracic echocardiography performed using a Vevo2100 (VisualSonics, Toronto,
Canada) under anesthesia induced through intraperitoneal injection of pentobarbital (30 mg/kg). Mice subjected to subtotal nephrectomy or sham operation were euthanized 12 weeks after operation by intraperitoneal injection with pentobarbital (30 mg/kg). Blood was sampled from the inferior vena cava, and then the hearts, lungs, and kidneys were rapidly excised. Serum levels of MK were evaluated using an ELISA kit for MK (Wuhan USCN Business Co., Ltd., Hubei, China). Successful subtotal nephrectomy was confirmed based on the elevated levels of serum urea nitrogen (UN) and serum creatinine, which were measured using the DRICHEM and BUN-PIII, CRE-PIII Kit (FUJIFILM, Tokyo, Japan). To normalize heart weights, the tibia length of the left posterior limb was measured for each mouse.

**Receptor Tyrosine Kinase Array**

The activities of receptor tyrosine kinases in the hearts of MK-KO mice and WT littermates were measured using the Proteome Profiler Mouse Phospho-RTK Array Kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer’s instructions. Briefly, extracted heart proteins were applied onto the anti-RTK antibody-coated membrane and incubated overnight. Membranes were washed and then exposed to a chemiluminescent reagent.

**Assessment of the Histology of Heart and Kidney**

Tissues were excised and immediately fixed with 4% paraformaldehyde in phosphate-buffered saline for 24 h, embedded in paraffin, and cut serially. The heart sections were stained with hematoxylin and eosin. For immunofluorescence staining, kidney tissue sections were treated with a blocking agent before incubation with a primary antibody against MK. Sections were then treated with fluorescent dye-conjugated secondary antibody and counterstained with 4’,6-diamidino-2-phenylindole (Cell Signaling Technology).
Supplemental References


Table S1
Comparison of gravimetric data, echocardiography, blood pressure, and renal function of MK-KO mice and their WT littermates after subtotal nephrectomy.

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<tr>
<td>LVEDD (mm)</td>
<td>3.03 ± 0.13</td>
<td>3.09 ± 0.14</td>
<td>3.30 ± 0.25</td>
<td>3.07 ± 0.11</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>54.5 ± 3.0</td>
<td>55.4 ± 1.5</td>
<td>43.0 ± 6.4</td>
<td>57.1 ± 5.6</td>
</tr>
<tr>
<td>Renal Function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>29.3 ± 4.8</td>
<td>29.7 ± 6.6</td>
<td>55.1 ± 10.3</td>
<td>45.5 ± 7.9</td>
</tr>
<tr>
<td>sCr (mg/dl)</td>
<td>0.18 ± 0.07</td>
<td>0.15 ± 0.05</td>
<td>0.61 ± 0.28</td>
<td>0.32 ± 0.19</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

WT, wild-type mice; KO, midkine knockout mice; Nx, subtotal nephrectomy; BW, body weight; HW, heart weight; TL, tibia length; IVS, inter ventricular septal diameter; PW, left ventricular posterior wall diameter; LVEDD, left ventricular end diastolic diameter; LVFS, left ventricular fractioning shortening; BUN, blood urea nitrogen; sCr, serum creatinine. *P < 0.05 and **P < 0.01 vs. sham-operated mice, #P < 0.05 vs. WT-Nx mice.
Table S2
Changes in gravimetric and echocardiographic data after subtotal nephrectomy in WT mice.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Nx</th>
<th>4w</th>
<th>8w</th>
<th>12w</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW / TL (mg / mm)</td>
<td>6.22 ± 0.24</td>
<td>6.43 ± 0.46</td>
<td>6.99 ± 0.23*</td>
<td>7.19 ± 0.50**</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.66 ± 0.02</td>
<td>0.69 ± 0.07</td>
<td>0.75 ± 0.03</td>
<td>0.81 ± 0.01*</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>0.67 ± 0.04</td>
<td>0.66 ± 0.08</td>
<td>0.70 ± 0.08</td>
<td>0.77 ± 0.08*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.13 ± 0.14</td>
<td>3.08 ± 0.11</td>
<td>3.23 ± 0.15</td>
<td>3.30 ± 0.25*</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>55.8 ± 4.0</td>
<td>55.3 ± 1.9</td>
<td>50.9 ± 5.4</td>
<td>43.0 ± 6.4**</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

Nx, subtotal nephrectomy; HW, heart weight; TL, tibia length; IVS, inter ventricular septal diameter; PW, left ventricular posterior wall diameter; LVEDD, left ventricular end diastolic diameter; LVFE, left ventricular fractioning shortening. *P < 0.05 and **P < 0.01 vs. sham-operated mice, #P < 0.05 vs. WT-Nx mice.
Table S3
Comparison of heart weight and renal function after subtotal nephrectomy, with or without EGFR-inhibitor administration.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Vehicle</th>
<th>Gefitinib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT-Nx</td>
<td>KO-Nx</td>
</tr>
<tr>
<td></td>
<td>N = 15</td>
<td>N = 15</td>
</tr>
<tr>
<td>Pre-BW (g)</td>
<td>25.5 ± 2.0</td>
<td>24.2 ± 1.8</td>
</tr>
<tr>
<td>HW / TL (mg / mm)</td>
<td>7.53 ± 0.79</td>
<td>6.26 ± 0.68*</td>
</tr>
</tbody>
</table>

Renal Function

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Gefitinib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT-Nx</td>
<td>KO-Nx</td>
</tr>
<tr>
<td></td>
<td>N = 15</td>
<td>N = 15</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>55.0 ± 11.2</td>
<td>46.4 ± 9.5</td>
</tr>
<tr>
<td>sCr (mg/dl)</td>
<td>0.51 ± 0.28</td>
<td>0.31 ± 0.17*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.
WT, wild-type mice; KO, midkine knockout mice; Nx, subtotal nephrectomy; BW, body weight; HW, heart weight; TL, tibia length; BUN, blood urea nitrogen; sCr, serum creatinine.
*P < 0.01 vs. vehicle treated WT mice receiving subtotal nephrectomy.
Figure S1. MK expression in the kidney after subtotal nephrectomy.
After subtotal nephrectomy, kidney tissues were harvested and western blotting was performed. MK expression in the kidney increased after subtotal nephrectomy.
Figure S2. Serum MK expression increased after subtotal nephrectomy. Serum MK expression levels were higher in WT mice subjected to subtotal nephrectomy. Data are expressed as the mean ± SEM (n = 6).
Figure S3. Changes in the MK concentration after subtotal nephrectomy. Serum MK concentrations were evaluated by ELISAs. Serum MK concentrations significantly increased at 4 weeks after subtotal nephrectomy. Data are expressed as the mean ± SEM (n = 4–8). *P < 0.05 vs. control.
Figure S4. Expression of mRNA MK in the heart after subtotal nephrectomy.
Expression of mRNA MK expression in the heart increased after subtotal nephrectomy. Bars represent the mean ± SEM (n = 6 each).
Figure S5. Ponceau S staining of membrane blots. Equal sample loading was confirmed by means of Ponceau S staining of western blot membranes.
We evaluated cardiac fibrosis after subtotal nephrectomy; however, no significant difference was observed between WT and MK-KO mice. Bars represent the mean ±SEM (n = 8 each).

Figure S6. cardiac fibrosis after subtotal nephrectomy.
Figure S7. Phosphorylation of ERK1/2 and AKT after MK stimulation.
Neonatal rat cardiomyocytes were stimulated with MK, which induced ERK1/2 and AKT phosphorylation. Bars represent the mean ± SEM (n = 6 each).
*P < 0.05 vs. control.
Figure S8. Phosphorylation of receptor tyrosine kinases in the heart after subtotal nephrectomy. The level of EGFR phosphorylation was higher in WT mice subjected to subtotal nephrectomy than in MK-KO mice subjected to subtotal nephrectomy or sham operation.
Figure S9. Phosphorylation of EGFR after MK stimulation.
Neonatal rat cardiomyocytes were stimulated with MK, which induced EGFR phosphorylation in dose- and time-dependent manners. Bars represent the mean ± SEM (n = 6 each). *P < 0.05, **P < 0.01 vs. control.
Figure S10. A. A specific siRNA against EGFR blocked its expression by >70%. B, C. Silencing of EGFR attenuated MK-induced phosphorylation of ERK1/2 and AKT. Bars represent the mean ± SEM (n = 4-6 each).
Figure S11. Gefitinib attenuated phosphorylation of ERK1/2 and AKT after subtotal nephrectomy.
Bars represent the mean ± SEM (n = 4–6 each).