Cardiac Myocyte-Derived Follistatin-Like 1 Prevents Renal Injury in a Subtotal Nephrectomy Model

Satoko Hayakawa,* Koji Ohashi,† Rei Shibata,* Yoshiyuki Kataoka,* Megumi Miyabe,* Takashi Enomoto,* Yusuke Joki,* Yuuki Shimizu,* Takahiro Kambara,* Yusuke Uemura,* Daisuke Yuasa,* Hayato Ogawa,* Kazuhiro Matsuo,* Mizuho Hiramatsu-Ito,* Maurice J.B. van den Hoff,‡ Kenneth Walsh,§ Toyoaki Murohara,* and Noriyuki Ouchi†

Departments of *Cardiology and †Molecular Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan; ‡Department of Anatomy, Embryology & Physiology, Heart Failure Research Center, Academic Medical Center, Amsterdam, The Netherlands; and §Molecular Cardiology, Boston University School of Medicine, Boston, Massachusetts

ABSTRACT

Heart disease contributes to the progression of CKD. Heart tissue produces a number of secreted proteins, also known as cardiokines, which participate in intercellular and intertissue communication. We recently reported that follistatin-like 1 (Fstl1) functions as a cardiokine with cardioprotective properties. Here, we investigated the role of cardiac Fstl1 in renal injury after subtotal nephrectomy. Cardiac-specific Fstl1-deficient (cFstl1-KO) mice and wild-type mice were subjected to subtotal (5/6) nephrectomy. cFstl1-KO mice showed exacerbation of urinary albumin excretion, glomerular hypertrophy, and tubulointerstitial fibrosis after subtotal renal ablation compared with wild-type mice. cFstl1-KO mice also exhibited increased mRNA levels of proinflammatory cytokines, including TNF-α and IL-6, NADPH oxidase components, and fibrotic mediators, in the remnant kidney. Conversely, systemic administration of adenoviral vectors expressing Fstl1 (Ad-Fstl1) to wild-type mice with subtotal nephrectomy led to amelioration of albuminuria, glomerular hypertrophy, and tubulointerstitial fibrosis, accompanied by reduced expression of proinflammatory mediators, NADPH oxidase components, and fibrotic markers in the remnant kidney. In cultured human mesangial cells, treatment with recombinant FSTL1 attenuated TNF-α–stimulated expression of proinflammatory mediators. Treatment of mesangial cells with FSTL1 augmented the phosphorylation of AMP-activated protein kinase (AMPK), and inhibition of AMPK activation abrogated the anti-inflammatory effects of FSTL1. These data suggest that Fstl1 functions in cardiorenal communication and that the lack of Fstl1 production by myocytes promotes glomerular and tubulointerstitial damage in the kidney.


Accumulated evidence documents a bidirectional, pathologic interaction between heart and kidney that can contribute to the progressive dysfunction of both tissues. CKD is an independent risk factor for heart diseases,1–4 and microalbuminuria is a recognized risk factor as well,5–7 even when GFR is within the normal range. Conversely, heart disease, including chronic heart failure, is also involved in the development of CKD. Alterations in renal function are observed in patients with idiopathic dilated cardiomyopathy without renal insufficiency.8 Furthermore, abnormal glomerular changes are found at high frequencies in patients with chronic heart failure whose estimated GFR is within normal range.9

Several studies demonstrate that heart tissue produces a variety of secreted proteins, termed “cardiokines.”10,11 These factors help maintain normal...
cardiac function and control pathologic cardiac remodeling. Classic cardiokines, such as atrial natriuretic peptide and brain natriuretic peptide (BNP), exert favorable actions on myocardial remodeling through autocrine/paracrine actions on cardiac cells.12 Furthermore, both atrial natriuretic peptide and BNP exhibit endocrine actions and regulate vascular tone and affect water and electrolyte excretion in the kidney.12 Thus, the identification and characterization of novel cardiokines could improve understanding of the complex regulatory and counter-regulatory mechanisms that contribute to cardiorenal disease.

Follistatin-like 1 (Fstl1), also called TSC-36, is a member of the follistatin family of proteins.13,14 Previously, we reported that Fstl1 is a cardiokine upregulated by various heart stresses, including cardiac ischemia/reperfusion injury, pressure overload, and myocardial infarction.15,16 Cardiac myocyte–derived Fstl1 reduces pathologic cardiac hypertrophy induced by pressure overload.16 Systemic administration of Fstl1 improves myocardial ischemia/reperfusion damage through reduction of myocyte apoptosis and inflammatory responses.15,17 Thus, Fstl1 can modulate cardiac phenotypes in an autocrine or paracrine manner. However, the functional role of cardiac Fstl1 in regulating remote organ phenotypes has not been examined previously. Here, we investigated whether cardiac myocyte–derived Fstl1 affects the development of CKD in an endocrine manner using a mouse subtotal nephrectomy model.

RESULTS

Fstl1-KO Mice Exhibit Exacerbation of Renal Injury after Subtotal Nephrectomy

To assess the role of cardiac myocyte–derived Fstl1 in renal injury, cardiac-specific Fstl1-deficient (cFstl1-KO) mice were produced by crossing Fstl1fl/flxox mice with α-myosin heavy chain–Cre transgenic mice. No differences were observed in body weight, food intake, kidney weight, systolic BP, or heart rate between control and cFstl1-KO mice at age 8 weeks (Table 1). cFstl1-KO mice and control mice were subjected to 5/6 nephrectomy operation. At 1 week after subtotal nephrectomy, control mice showed 2.0±0.3-fold increase in plasma Fstl1 levels, which was accompanied by an increase in cardiac Fstl1 protein levels (Figure 1A). These increases were diminished in cFstl1-KO mice, indicating that cardiomyocytes are an important source of circulating Fstl1 in mice after renal injury. Echocardiographic analysis revealed no significant differences in left ventricular diastolic diameter, interventricular septum thickness, posterior wall thickness, and fractional shortening after nephrectomy between control and cFstl1-KO mice (Table 2).

Eight weeks after subtotal nephrectomy or sham operation, cFstl1-KO and control mice were euthanized for analyses after collection of urine and blood samples. Renal injury significantly increased urinary albumin excretion and plasma levels of urea nitrogen and creatinine levels in control mice. Urinary excretion of albumin was further increased in cFstl1-KO mice after subtotal renal ablation compared with control mice (Figure 1B). However, plasma urea nitrogen and creatinine levels after renal injury did not significantly differ between control and cFstl1-KO mice (Figure 1B).

To histologically evaluate glomerular damage, remnant kidneys of control and cFstl1-KO mice were stained with periodic acid-Schiff (PAS). The cFstl1-KO mice showed increased glomerular area and intraglomerular cell number after renal injury compared with control mice (Figure 1C). To analyze interstitial fibrosis in injured kidneys, remnant kidneys of control and cFstl1-KO mice were stained with Masson’s Trichrome (Figure 1D). The cFstl1-KO mice exhibited more kidney fibrosis compared with control mice after subtotal nephrectomy.

Inflammation is closely associated with the progression of CKD, and inflammation promotes oxidative stress, contributing to renal failure.18,19 Thus, to investigate the contribution of inflammation and oxidative stress to enhanced renal injury in cFstl1-KO mice, mRNA levels of proinflammatory cytokines and NADPH oxidase components were measured by real-time PCR. At 8 weeks after partial nephrectomy, cFstl1-KO mice showed higher expression levels of proinflammatory cytokines, including TNF-α, IL-6, IL-1β, monocyte chemotactic protein (MCP)-1, and NADPH oxidase components, including P40phox, P67phox, P47phox, and P22phox, compared with control mice (Figure 2, A and B). Consistent with the Masson’s Trichrome–staining data, mRNA levels of fibrosis markers, including collagen I, collagen III, TGF-β1, and connective tissue growth factor, were significantly higher in the remnant kidneys of Fstl1-KO mice than in those of control mice (Figure 2C). Collectively, these data show that the loss of cardiac-derived Fstl1 exacerbates renal injury after subtotal nephrectomy, which is accompanied by increased proinflammatory state and oxidative stress within the kidney.

Systemic Delivery of Fstl1 Ameliorates Renal Injury after Subtotal Nephrectomy

To assess the therapeutic effects of Fstl1 overexpression on renal injury, Ad-Fstl1 or an adenoviral vector producing β-galactosidase (Ad-β-gal) as a control was intravenously injected into wild-type mice at 4 weeks after subtotal nephrectomy. Ad-Fstl1–treated mice showed 2.6±0.2-fold increase in plasma Fstl1 levels at 4 weeks after Ad-Fstl1 injection (Figure 3A).

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Table 1. Characteristics of control and cFstl1-KO mice

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<tr>
<th>Characteristic</th>
<th>Control (n=6)</th>
<th>cFstl1-KO (n=6)</th>
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<tr>
<td>Body weight (g)</td>
<td>29.4±1.3</td>
<td>28.7±1.1</td>
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<td>Food intake (g/d)</td>
<td>3.7±0.1</td>
<td>3.8±0.5</td>
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<td>Kidney weight (mg)</td>
<td>169.1±4.8</td>
<td>166.4±6.9</td>
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<td>Systolic BP (mmHg)</td>
<td>105.5±3.0</td>
<td>104.0±2.4</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>627.2±18.3</td>
<td>624.6±6.3</td>
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Values are the mean±SEM.
Treatment with Ad-Fstl1 significantly reduced urinary excretion of albumin at 4 weeks after Ad-Fstl1 injection (i.e., at 8 weeks after subtotal nephrectomy), whereas Ad-Fstl1 did not affect urinary albumin excretion levels in sham-operated mice (Figure 3B). In histologic analyses, Ad-Fstl1–treated mice exhibited significantly smaller glomerular area, fewer intraglomerular cells, and smaller fibrosis area compared with control, Ad-β-gal–treated mice at 8 weeks after subtotal nephrectomy (Figure 3, C and D). In addition, Ad-Fstl1–treated mice showed significantly lower mRNA levels of TNF-α, IL-6, MCP-1, P40phox, P47phox, P22phox, collagen I, collagen III, TGF-β1, and connective tissue growth factor in the remnant kidney than did the Ad-β-gal–treated mice (Figure 3E).

Figure 1. cFstl1-KO mice show exacerbation of albuminuria, glomerular hypertrophy, and fibrosis after renal ablation. (A) Plasma and cardiac Fstl1 levels of control and cFstl1-KO mice after nephrectomy or sham operation. Left panels show representative bands of Fstl1 by Western blot analysis. Right panel shows quantitative analysis of plasma Fstl1 protein levels evaluated by ImageJ program. n=4 in each group. (B) Urine and plasma parameters of renal function in control and cFstl1-KO mice after nephrectomy or sham operation. Left panel shows urinary albumin excretion normalized to urinary creatinine. Center and right panels show plasma concentration of urea nitrogen and creatinine. n=5–8 in each group. (C) Histologic evaluation of glomerular hypertrophy. Upper panels show representative photos of remnant kidneys from control and cFstl1-KO mice as determined by PAS staining. Lower panels show quantitative analyses of glomerular cross-sectional area (left) and intraglomerular cell number (right). n=4 in each group. Scale bars represent 50 μm. (D) Evaluation of interstitial fibrosis in injured kidneys. Upper panels show representative photos of remnant kidney from control and cFstl1-KO mice as stained with Masson’s Trichrome. Lower panel shows quantitative analysis of fibrosis area as measured by WinROOF program. UN, urea nitrogen. All data are presented as mean±SEM. n=4 in each group. Scale bars represent 50 μm.
FSTL1 Attenuates Inflammatory Responses to TNF-α in Mesangial Cells

To examine the actions of Fstl1 on inflammatory response in the kidney at a cellular level, human mesangial cells were pretreated with recombinant human FSTL1 protein or vehicle for 1 hour, followed by 4-hour stimulation with TNF-α. Pretreatment of mesangial cells with human FSTL1 protein at 100 and 250 ng/ml partially suppressed the mRNA induction of IL-6 in response to TNF-α stimulation (Figure 4A). Treatment with FSTL1 protein also attenuated the induction of TNF-α mRNA expression following stimulation with TNF-α peptide.

FSTL1 Stimulates AMP-Activated Protein Kinase Signaling in Mesangial Cells

Because AMP-activated protein kinase (AMPK) has been reported to have anti-inflammatory and antioxidative stress properties in a variety of cell types, we investigated the effects of Fstl1 on the AMPK signaling pathway in mesangial cells. Treatment with FSTL1 protein, at 250 ng/ml, increased the phosphorylation levels of AMPK and its downstream target acetyl-CoA carboxylase (ACC) in human mesangial cells in a time-dependent manner (Figure 5A). In the in vivo model, strong signal intensity for the phosphorylated AMPK was detected by histologic analysis in the remnant kidney of control mice after renal injury (Figure 5B). In contrast, the signal intensity for the phosphorylated AMPK was markedly lower in the remnant kidney of cFstl1-KO mice compared with that of control mice. Conversely, the signal intensity for the phosphorylated AMPK was much higher in the remnant kidney of Ad-Fstl1–treated mice than in that of control Ad-β-gal–treated mice after renal ablation (Figure 5C). Consistent with these histologic findings, the nephrectomy-induced phosphorylation of AMPK was reduced in the remnant kidney in cFstl1-KO mice compared with control mice as assessed by Western blot analysis (Figure 5D).

FSTL1 Exerts Anti-Inflammatory Effects through an AMPK-Dependent Manner

To investigate the potential causal relationship between AMPK activation and the anti-inflammatory function of Fstl1, human mesangial cells were transduced with an adenoviral vector expressing the dominant negative mutant form of AMPK tagged with c-myc (Ad-dn-AMPK) or control Ad-β-gal. Treatment of human mesangial cells with Ad-dn-AMPK abrogated FSTL1-induced phosphorylation of ACC (Figure 6A). Treatment of mesangial cells with Ad-dn-AMPK reversed the suppressive effects of FSTL1 on TNF-α–induced upregulation of IL-6 and TNF-α transcript (Figure 6B). These findings indicate that FSTL1 exerts anti-inflammatory function on mesangial cells via an AMPK-dependent mechanism.

Table 2. Echocardiographic analysis of cFstl1-KO mice

<table>
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<th>Variable</th>
<th>Control (n=6)</th>
<th>cFstl1-KO (n=6)</th>
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<tr>
<td>Left ventricular diastolic</td>
<td>2.49±0.05</td>
<td>2.45±0.06</td>
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<tr>
<td>diameter (mm)</td>
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<tr>
<td>Interventricular septum</td>
<td>0.79±0.03</td>
<td>0.75±0.02</td>
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<tr>
<td>thickness (mm)</td>
<td></td>
<td></td>
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<tr>
<td>Posterior wall thickness (mm)</td>
<td>0.83±0.03</td>
<td>0.80±0.04</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>66.5±0.5</td>
<td>67.4±0.3</td>
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Values are the mean±SEM.
Our major findings are the following: (1) renal injury increased plasma Fstl1 levels, which were suppressed by cardiomyocyte-specific ablation of Fstl1; (2) ablation of Fstl1 in a cardiomyocyte-specific manner led to exacerbation of albuminuria, glomerular hypertrophy and tubulointerstitial fibrosis after subtotal nephrectomy, which were accompanied by increased expression of inflammatory cytokines and oxidative stress markers; (3) systemic administration of Fstl1 to mice improved albuminuria, glomerular hypertrophy, and tubulointerstitial fibrosis after renal ablation, which was accompanied by attenuation of inflammatory response and oxidative stress; (4) treatment of cultured human mesangial cells with recombinant FSTL1 protein attenuated the inflammatory responses to TNF-α through an AMPK-dependent manner.

Several factors known to be secreted by the heart with beneficial actions on the kidney have been identified. For example, BNP expression undergoes compensatory upregulation under stressed conditions of the heart, including heart failure and cardiac hypertrophy. BNP transgenic mice exhibit attenuated albuminuria and renal damage after subtotal nephrectomy, and the overexpression of BNP in mice also improves immune-mediated renal injury. Adrenomedullin is abundantly expressed in heart tissue and upregulated by cardiac stress, including pressure overload and myocardial infarction. Heterozygous adrenomedullin-deficient mice exhibit more severe cardiac and renal damage in mouse models of angiotensin II infusion and transverse aortic constriction. However, neither BNP nor adrenomedullin has been evaluated for their effects on kidney function using genetic models that specifically ablate their expression in heart. Here, we show that cardiac myocyte-specific ablation of Fstl1 exacerbates kidney damage after subtotal nephrectomy, indicating the importance of Fstl1 as a cardiokine with renal protective properties. Thus, Fstl1 could represent a novel mediator involved in communication between the heart and kidney.

Patients with CKD exhibit higher circulating levels of proinflammatory cytokines, including TNF-α and IL-6. Proinflammatory cytokines, such as TNF-α, promote renal injury and dysfunction, which are accompanied by increased generation of reactive oxygen species. In this regard, anti-inflammatory agents have been reported to attenuate the progression of kidney damage. Our study with loss-of-function genetic manipulations indicates that reduced levels of circulating Fstl1 exacerbate renal injury after subtotal nephrectomy, with an accompanying upregulation of proinflammatory cytokines and NADPH oxidase components that are the major source of reactive oxygen species. Conversely, systemic delivery of Fstl1
using adenovirus-mediated gene transfer led to downregulation of proinflammatory mediators in the remnant kidney. Consistent with our findings, it has been reported that a germline reduction of Fstl1 expression, by a gene trap method to generate a hypomorphic allele, leads to enhancement of acute cisplatin nephrotoxicity and increased IL-1β expression in the kidney.41 In addition, the present study showed that Fstl1 negatively regulates the inflammatory response to TNF-α in mesangial cells in vitro.

Recently, we reported that Fstl1 attenuates the expression of TNF-α and IL-6 in response to proinflammatory stimuli in cultured cardiac myocytes and macrophages, thereby protecting against acute cardiac injury.17 Administration of Fstl1 protein ameliorates joint inflammation induced by antibody-induced arthritis,42 and overexpression of Fstl1 by adenovirus gene transfer prolongs survival of heart allograft accompanied by reduced expression of proinflammatory cytokines.43 Thus, Fstl1 may act as an anti-inflammatory molecule that minimizes tissue injury. On the other hand, overexpression of Fstl1 increases the expression of proinflammatory cytokines, thereby exacerbating arthritis.44 A study with Fstl1-hypomorphic mice also demonstrated that reduced levels of Fstl1 contribute to an improvement in a model of collagen-induced arthritis.45 These contradicting results may be explained by differences in the experimental models and paradigms as well as the target cells or organs of Fstl1 under pathologic conditions, and will require further studies to delineate.

AMPK acts as a master energy sensor that regulates glucose and lipid metabolisms.46–48 AMPK activation also helps attenuate inflammatory responses in a variety of cell types.17,48–50 It has been reported that an AMPK activator, AICAR, attenuates renal hypertrophy, urine H2O2, and urine and renal MCP-1 in high-fat-diet–induced obese mice.51 AICAR also abrogates

Figure 3. Systemic delivery of Fstl1 ameliorates renal injury in wild-type mice after subtotal nephrectomy. (A) Plasma Fstl1 levels in wild-type mice at 4 weeks after intravenous administration of Ad-β-gal (1 × 10⁹ plaque-forming units) or Ad-Fstl1 (1 × 10⁹ plaque-forming units). Lower panel shows quantitative analysis of plasma Fstl1 levels evaluated by ImageJ program. n=6 in each group. (B) Quantitative analysis of urinary albumin excretion normalized to urinary creatinine in sham-operated and subtotal nephrectomy–operated wild-type mice at 4 weeks after intravenous administration of Ad-β-gal or Ad-Fstl1. n=4 in each sham-operated group. n=8 in each nephrectomy–operated group. (C) Histologic assessment of renal hypertrophy by PAS staining. Upper panels show representative photos of remnant kidneys from Ad-β-gal–treated or Ad-Fstl1–treated wild-type mice. Lower panels show quantitative analyses of glomerular cross-sectional area (left) and intraglomerular cell number (right). n=6 in each group. Scale bars represent 50 μm. (D) Evaluation of interstitial fibrosis by Masson’s Trichrome staining. Upper panels show representative photos of remnant kidney from Ad-β-gal–treated or Ad-Fstl1–treated wild-type mice. Lower panel shows quantitative analysis of fibrosis area evaluated by a WinROOF program. n=6 in each group. Scale bars represent 50 μm. (E) Relative mRNA levels of proinflammatory mediators (TNF-α, IL-6, and MCP-1), NADPH oxidase components (P40phox, P47phox, and P22phox), and fibrosis parameters (collagen I, collagen III, TGF-β1, and CTGF) in remnant kidneys in wild-type mice treated with Ad-β-gal (black bars) or Ad-Fstl1 (white bars). mRNA levels were measured by quantitative RT-PCR method. All samples are normalized to 36B4. n=8 in each group. CTGF, connective tissue growth factor. All data are presented as mean±SEM. *P<0.05 for Ad-β-gal group.
the MCP-1 induction by palmitic acid in cultured mesangial cells.51 Another AMPK activator, metformin, is reported to ameliorate kidney function and fibrosis after subtotal nephrectomy.52 Our data demonstrated that Fstl1 promotes AMPK signaling in injured kidney and cultured mesangial cells, and that the inhibition of AMPK activation abolishes the anti-inflammatory effects of Fstl1 in vitro. Collectively, these data suggest that Fstl1 exerts beneficial actions against renal injury through its ability to activate AMPK, thereby reducing inflammatory responses and oxidative stress in the kidney. Circulating levels of Fstl1 are increased in association with acute coronary syndrome and systolic heart failure.53,54 Fstl1 expression is increased in cardiac myocytes and endothelium in human heart failure and returns to normal after myocardial recovery.55 In mouse models, plasma Fstl1 levels are increased in response to cardiac ischemia-reperfusion, myocardial infarction, or pressure overload.15–17 Hind-limb ischemia also upregulates Fstl1 expression in ischemic skeletal muscle of mice.15 Treatment of cultured adipocytes with TNF-α leads to increased expression of Fstl1.56 Thus, it is plausible that Fstl1 is a counter-regulatory, anti-inflammatory cardiokine that is induced by stress, in particular ischemia and inflammation, and mitigates this damage in the heart and remote tissues. In the present study, plasma Fstl1 levels were increased after subtotal nephrectomy in control mice, whereas this increase was largely abolished in cardiac-specific Fstl1-KO mice. These findings suggest that cardiac myocytes are a major source of Fstl1 in the murine blood stream during the development of renal failure and that this factor may play a role in the communication associated with cardiorenal disease.

Our results from cardiac specific loss-of-function experiments indicate that endogenous Fstl1, produced by the heart, protects against renal damage in response to subtotal nephrectomy. Furthermore, our findings with gain-of-function genetic manipulations, through the systemic delivery of an adenoaviral vector, show that exogenous Fstl1 can have therapeutic utility in the treatment of kidney disease. Intravenous administration of an adenoaviral vector will largely target the liver, and the increase in circulating Fstl1 under these conditions will be hepatic—rather than cardiac—derived.57 In this regard, it has been noted that Fstl1 can be subjected to cell type–specific differences in post-translational modifications.58 Although the functional roles of the differential post-translational modification of Fstl1 are unknown, it is conceivable that hepatic- and cardiac-derived Fstl1 differ in their nephroprotective actions. Thus, further research is necessary to elucidate how differences in the source and structure of Fstl1 impact its function on the kidney.

In conclusion, we demonstrated for the first time that cardiac myocyte-derived Fstl1 protects against renal injury following subtotal nephrectomy. Additional studies demonstrate that systemic delivery of Fstl1 improves glomerular damage through reduction of proinflammatory response and oxidative stress. Thus, Fstl1 could represent a novel therapeutic target for the treatment of CKD.

CONCISE METHODS

Animal and Surgical Procedure

To generate cardiac-specific Fstl1-deficient (cFstl1-KO) mice, mice with loxP sites flanking the exon 1 of the Fstl1 gene (Fstl1lox/lox)16,59 were crossed with mice overexpressing Cre recombinase under the control of the α-myosin heavy chain promoter (α–myosin heavy chain–Cre) (The Jackson Laboratory). Both cFstl1-KO and control mice (8–11 weeks old) were assigned to two groups with or without subtotal renal ablation. Subtotal (5/6) nephrectomy was performed by the surgical excision method.18 Briefly, the upper and lower poles of the left kidney (two thirds of the left kidney) were resected. After 1 week, the remaining right kidney was removed through a right paramedian incision after ligation of the right renal artery, vein, and ureter. Eight weeks after ablation, cFstl1-KO and control mice were euthanized for analysis. The Institutional Animal Care and Use Committee approved all animal procedures.

Figure 4. FSTL1 protein attenuates inflammatory response to TNF-α in cultured human mesangial cells. These cells were pretreated with FSTL1 protein, at 100 or 250 ng/ml, or vehicle for 1 hour, followed by stimulation with or without TNF-α (10 ng/ml). mRNA levels of IL-6 (left panel) and TNF-α (right panel) were determined by quantitative RT-PCR method. Baseline in the absence of FSTL1 and TNF-α means vehicle-treated control. All data are presented as mean±SEM. n=4 in each group. All samples are normalized to 36B4.
Committee at Nagoya University approved the study protocols. For additional details on the study methods, see the Supplemental Material.

**Histology and Immunohistochemistry**
Paraffin or optimal cutting temperature compound (Sakura, Tokyo, Japan)–embedded sections were immunohistochemically analyzed. To evaluate renal injury and fibrosis, paraffin-embedded sections were stained with the PAS method and Masson’s Trichrome method (Sigma-Aldrich). Intraglomerular cell number, glomerular size, and fibrosis area were measured by using an image analysis system.60

**Laboratory Methods**
Eight weeks after surgery, mice were euthanized for analysis. Collected blood and urine samples were used for analysis. Urinary albumin excretion was evaluated as albumin/g urinary creatinine.

**Cell Culture**
Mesangial cells were maintained in mesangial cell basal medium containing 5% FBS and antibiotics (50 μg/ml gentamicin, 50 ng/ml amphotericin B). After 24 hours of serum starvation, mesangial cells were treated with FSTL1 protein (100 or 250 ng/ml) or vehicle for the indicated lengths of time.

**Figure 5.** Fstl1 activates AMPK signaling in cultured mesangial cells and remnant kidney. (A) Time course analysis of phospho-ACC (pACC), ACC, phospho-AMPK (pAMPK), AMPK, and α-tubulin (tubulin) by Western blot analysis. Mesangial cells were treated with FSTL1 protein (250 ng/ml) for the indicated length of time (0, 5, 15, 30, and 60 minutes). (B) Immunostaining of phosphorylated AMPK and DAPI in remnant kidney from control and cFstl1-KO mice after renal ablation. Original magnification, ×200. (C) Immunostaining of phosphorylated AMPK and DAPI in remnant kidney from Ad-β-gal–treated and Ad-Fstl1–treated wild-type mice after renal ablation. Original magnification, ×200. (D) Protein levels of pAMPK, AMPK, and α-tubulin in the remnant kidneys from control and cFstl1-KO mice after sham or subtotal nephrectomy surgery. Right panel shows quantitative analysis of pAMPK/α-tubulin as evaluated by ImageJ program. All data are presented as mean±SEM. n=4 in each group. DAPI, 4,6-diamidino-2-phenylindole.
Determination of mRNA Levels
Gene expression levels were quantified by real-time PCR method. We used the primers listed in Supplemental Table 1. All results were normalized to 36B4.

Western Blot Analysis
Tissue and cell samples were prepared in lysis buffer (Cell Signaling Technology) containing 1 mM PMSF (Sigma-Aldrich). The expression level was determined by measurement of the corresponding band intensities by using ImageJ software, and the relative values were expressed relative to a-tubulin signal.

Statistical Analyses
Data are presented as mean±SEM. Differences between groups were evaluated by the t test or ANOVA with the Fisher protected least significant difference test. A P value <0.05 denoted the presence of a statistically significant difference. All calculations were performed by using SPSS for Windows.

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DISCLOSURES
None.

REFERENCES


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