Supplemental data

Supplemental methods

Materials

Antibodies for phosphorylated AMPK (Thr-172), AMPK, ACC and α-tubulin were purchased from Cell Signaling Technology. Phosphorylated ACC (Ser-79) antibody was purchased from Millipore. C-Myc antibody was purchased from Upstate Biotechnology. Mouse Fstl1 antibody was purchased from R&D systems. Recombinant human TNF-α protein was purchased from R&D systems. Recombinant human FSTL1 protein, tagged with FLAG at the C terminus, was produced by using insect sf9 cell system as previously described [1]. The adenoviral vectors expressing β-galactosidase (Ad-β-gal) or Fstl1 (Ad-Fstl1) were prepared as previously described [2, 3].

Animal and surgical procedure

To generate cardiac specific Fstl1 deficient (Fstl1-KO) mice, mice with loxP sites flanking the exon 1 of the Fstl1 gene ($Fstl1^{flox/flox}$) [4, 5] were crossed with mice over-expressing Cre recombinase under the control of the α-MHC promoter

(αMHC -Cre) (Jackson Laboratory). αMHC –$Cre^{+/-}$; $Fstl1^{flox/flox}$ mice were used as Fstl1-KO mice, whereas αMHC –$Cre^{-/-}$; $Fstl1^{flox/flox}$ mice were used as control mice. Both Fstl1-KO and control mice (8 to 11-week-old) were assigned to two groups with or without subtotal renal ablation. In some experiments, Ad-Fstl1 (1×10^9 pfu/mouse) or Ad-β-gal (1×10^9 pfu/mouse) was injected into wild type mice via right jugular vein at 4 weeks after subtotal nephrectomy operation. Subtotal (5/6) nephrectomy was performed by the surgical excision method[18]. Pentobarbital (30 mg/kg) was used as anesthesia in all surgical procedure. Briefly, the upper and lower poles of 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, Fstl1-KO and control mice were sacrificed for analysis. Tissues were fixed by 4% paraform aldehyde at 4°C for 16 hours. Study protocols were approved by the Institutional Animal Care and Use Committee at Nagoya University. Our study conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Echocardiographic analysis

Transthoracic echocardiography was performed to evaluate cardiac function of mice at 7 days after subtotal nephrectomy operation [1]. Left ventricular diastolic diameter (LVDd), left ventricular systolic diameter (LVSd), interventricular septum (IVS) thickness and posterior wall (PW) thickness were measured by M-mode images by using an Acuson Sequioa C-256 machine with a 15-MHz probe. Fractional shortening was calculated as (LVDd-LVSd)/LVDd X 100 (%).

Histology and Immunohistochemistry

Five-μm paraffin or optimal cutting temperature (OCT) compound (Sakura, Tokyo, JAPAN) -embedded sections were immunohistochemically analyzed. As to phosphorylated AMPK, the fluorescent-labeled secondary antibody, Alexa Fluor 594-conjugated goat anti-rabbit antibody (Invitrogen), was used. To evaluate renal injury and fibrosis, paraffin-embedded sections were stained with periodic acid-Schiff (PAS) method and Masson's trichrome method (Sigma). More than 10 consecutive sections in each mouse were examined. Intraglomerular cell number, glomerular size and fibrosis area were measured by using an image analysis system [6].

Laboratory methods

At 8 weeks from operation, mice were sacrificed for analysis. Collected blood and urine samples were used for analysis. Plasma concentrations of urea nitrogen (UN) and creatinine (Cr) and urine concentrations of Cr were measured in commercial laboratory (SRL). Urinary albumin concentration was measured by a murine albumin enzyme-linked immunosorbent assay kit (Exocell). Urinary albumin excretion was evaluated as albumin / gram of urinary Cr.

Cell culture

Human mesangial cells were purchased from Lonza. Mesangial cells were maintained in MsBM containing 5% fetal bovine serum (FBS), antibiotics (50μg/ml gentamicin, 50ng/ml amphotericin B). After 24 hrs. of serum starvation, mesangial cells were treated with FSTL1 protein (100 or 250 ng/ml) or vehicle for the indicated lengths of time. In some experiments, mesangial cells were transduced with Ad-dn-AMPK or Ad-β-gal at a multiplicity of infection (MOI) of 100 for 24 hrs.

Determination of mRNA levels

Gene expression levels were quantified by real-time PCR method. Total RNA was extracted with RNeasy-Mini Kit (Qiagen) according to the manufacture's protocol [1]. Extracted RNA was reverse-transcribed using the Revatra Ace (Toyobo) or SuperScript RT-PCR system (Invitrogen). PCR procedure was performed with a Bio-Rad real-time PCR detection system using SYBR Green I or THUNDERBIRD SYBR qPCR Mix as a double-standard DNA-specific dye. 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 1mM PMSF (Sigma). Immunoblot analysis was performed with antibodies at a 1:1000 dilution, followed by incubation with a secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. An ECL system Western blotting detection kit (GE healthcare) was used. The expression level was determined by measurement of the corresponding band

intensities by using Image J software, and the relative values were expressed relative to α-tubulin signal.

Statistical Analysis

Data are presented as mean ± S.E. Differences between groups were evaluated by the Student's *t* test or analysis of variance with Fisher's 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.

References

1. Ogura, Y, Ouchi, N, Ohashi, K, Shibata, R, Kataoka, Y, Kambara, T, Kito, T, Maruyama, S, Yuasa, D, Matsuo, K, Enomoto, T, Uemura, Y, Miyabe, M, Ishii, M, Yamamoto, T, Shimizu, Y, Walsh, K, Murohara, T: Therapeutic Impact of Follistatin-like 1 on Myocardial Ischemic Injury in Preclinical Animal Models. *Circulation,* 126**:** 1728-1738, 2012.

2. Ouchi, N, Oshima, Y, Ohashi, K, Higuchi, A, Ikegami, C, Izumiya, Y, Walsh, K: Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. *The Journal of biological chemistry,* 283**:** 32802-32811, 2008.

3. Enomoto, T, Ohashi, K, Shibata, R, Higuchi, A, Maruyama, S, Izumiya, Y, Walsh, K, Murohara, T, Ouchi, N: Adipolin/C1qdc2/CTRP12 protein functions as an adipokine that improves glucose metabolism. *The Journal of biological chemistry,* 286**:** 34552-34558, 2011.

4. Shimano, M, Ouchi, N, Nakamura, K, van Wijk, B, Ohashi, K, Asaumi, Y, Higuchi, A, Pimentel, DR, Sam, F, Murohara, T, van den Hoff, MJ, Walsh, K: Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy

following pressure overload. *Proceedings of the National Academy of Sciences of the United States of America,* 108**:** E899-906, 2011.

5. Sylva, M, Li, VS, Buffing, AA, van Es, JH, van den Born, M, van der Velden, S, Gunst, Q, Koolstra, JH, Moorman, AF, Clevers, H, van den Hoff, MJ: The BMP antagonist follistatin-like 1 is required for skeletal and lung organogenesis. *PloS one,* 6**:** e22616, 2011.

6. Ohashi, K, Iwatani, H, Kihara, S, Nakagawa, Y, Komura, N, Fujita, K, Maeda, N, Nishida, M, Katsube, F, Shimomura, I, Ito, T, Funahashi, T: Exacerbation of albuminuria and renal fibrosis in subtotal renal ablation model of adiponectin-knockout mice. *Arteriosclerosis, thrombosis, and vascular biology,* 27**:** 1910-1917, 2007.

Supplemental Table 1

Used primers for quantitative RT-PCR

Mouse Primers

36B4:	forward	5'-GCTCCAAGCAGATGCAGCA-3'
	reverse	5'-CCGGATGTGAGGCAGCAG-3'
TNF-α:	forward	5'-CGGAGTCCGGGCAGGT-3'
	reverse	5'-GCTGGGTAGAGAATGGATGAACA-3'
MCP-1:	forward	5'-CAGCCAGATGCAGTTAACGC-3'
	reverse	5'-GCCTACTCATTGGGATCATCTTG-3'
IL1β:	forward	5'- AGTTGACGGACCCCAAAAG -3'
	reverse	5'-AGCTGGATGCTCTCATCAGG -3'
IL6:	forward	5'-CTTCCATCCAGTTGCCTTCTTG-3'
	reverse	5'-AATTAAGCCTCCGACTTGTGAAG-3'
collagen I:	forward	5'-GTCCCAACCCCCAAAGAC-3'
	reverse	5'-CAGCTTCTGAGTTTGGTGATA-3'
collagen III:	forward	5'-TGGTTTCTTCTCACCCTTCTT-3'
	reverse	5'-TGCATCCCAATTCATCTACGT-3'
TGF-β1 :	forward	5'-CACCGGAGAGCCCTGGATA-3'
	reverse	5'-TTCCAACCCAGGTCCTTCCT-3'
CTGF:	forward	5'-GAGTGGGTGTGTGACGAGCCCAAGG-3'
	reverse	5'-ATGTCTCCGTACATCTTCCTGTAG-3'
$P47^{phox}$:	forward	5'-GATGTTCCCCATTGAGGCCG-3'
	reverse	5'-GTTTCAGGTCATCAGGCCGC -3'
$P22^{phox}$:	forward	5'-GTCCACCATGGAGCGATGTG-3'
	reverse	5'-CAATGGCCAAGCAGACGGTC-3'
$P67^{phox}$:	forward	5'-CTGGCTGAGGCCATCAGACT-3'
	reverse	5'-AGGCCACTGCAGAGTGCTTG-3'
$P40^{phox}$:	forward	5'-GCCGCTATCGCCAGTTCTAC-3'
	reverse	5'-GCAGGCTCAGGAGGTTCTTC-3'

Human Primers

36B4:	forward	5'-GAGTGATGTGCAGCTGATCAAGAC-3'
	reverse	5’- GGATGACCAGCCCAAAGGA-3'
TNF-α:	forward	5'-CGAGTGACAAGCCTGTAGC-3'
	reverse	5'-GGTGTGGGTGAGGAGCACAT-3'
IL6:	forward	5'-AGTGCCTCTTTGCTGCTTTCAC-3'
	reverse	5'-TGACAAACAAATTCGGTACATCCT-3'