SUPPLEMENTAL MATERIAL

Supplemental Methods

Materials

Antibodies against phosphorylated AMPK (Thr-172), AMPK, ACC and phosphorylated Smad1 (Ser463/465) / Smad5 (Ser463/465) / Smad8 (Ser426/428) were purchased from Cell Signaling Technology. BMP-4 (N-16) and Smad1/5/8 antibodies were purchased from Santa Cruz biotechnology. Phosphorylated ACC (Ser-79) antibody was purchased from Millipore. c-Myc antibody was purchased from Upstate Biotechnology. Human FSTL1 antibody was purchased from GeneTex. Mouse Fstl1 antibody was purchased from R&D systems. β-actin antibody was purchased from Abcam. Recombinant human BMP-4 protein (<1.0 EU per 1 μg of the protein by the LAL method) was purchased from R&D systems. LPS was purchased from Sigma. Compound C was purchased from Calbiochem. Small interfering RNAs (siRNAs) against BMP-4 and unrelated siRNAs were purchased from Thermo Scientific. Adenovirus vectors containing the gene for β-galactosidase (Ad-β-gal) and c-myc-tagged dominant-negative AMPK (Ad-dnAMPK) were prepared as previously described [1, 2]. ELISA kits for measurement of pig and mouse plasma Troponin-I concentration were purchased from Kamiya Biomedical and Life Diagnostics, respectively. ELISA kits for measurement of pig serum creatine phosphokinase-MB (CKMB) were purchased from Blue Gene. RAW264.7 cell line was purchased from the American Type Culture Collection (Manassas, VA).

Preparation of recombinant human FSTL1 protein

The pMIB/V5-His insect cell expression vector expressing full-length human FSTL1 cDNA lacking signal peptide (1-20 AA), tagged with FLAG at the C terminus, was

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transfected into insect Sf9 cells, and a stable cell line was generated by blasticidin selection as previously described [3] with some modifications. The culture supernatants were collected and incubated with anti-FLAG M2 affinity gel (Sigma) for 16 hours. FSTL1 protein was eluted by incubation with 3×FLAG peptide (Sigma) and dialyzed with PBS.

Mouse model of ischemia-reperfusion injury

Male C57BL/6 mice were purchased from Oriental BioService, Inc. We subjected mice at the age of 10-12 weeks to myocardial ischemia-reperfusion as previously described [4]. Briefly, after anesthetization (pentobarbital 50 mg/kg i.p.) and intubation, the LAD artery was ligated for 60 minutes with a suture using a snare occluder and then loosed. An initial experiment demonstrated that human FSTL1 was detected in plasma at the concentration of 232 ng/ml at 5 minutes after intravenous injection of recombinant human FSTL1 protein (100 ng/g mouse)(Supplemental Figure 1). Because this concentration of Fstl1 in the blood stream was similar to the level of Fstl1 that is effective at reducing hypertrophic responses in cultured cardiac myocytes[5], we injected recombinant human FSTL1 protein (100 ng/g mouse) or vehicle (PBS) through the right jugular vein before the induction of ischemia or 5 minutes after reperfusion. At 24 hours after reperfusion, the suture was re-tied, and Evans blue was systemically injected into mice to determine the non-ischemic tissue. The heart was excised, cut and incubated with 2,3,5-triphenyltetrazolium chloride (TTC) to determine the infarcted region. Left ventricular (LV) area, the area at risk (AAR) and infarct area (IA) were assessed by computerized planimetry using Image J. In some experiments, AMPK inhibitor compound C (20 mg/kg) dissolved in dimethyl sulfoxide (DMSO) or DMSO was intraperitoneally injected into mice before the

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operation and after reperfusion[6]. Study protocols were approved by the Institutional Animal Care and Use Committees at Nagoya University.

Pig model of ischemia-reperfusion injury

This study used domestic female Yorkshire-Duroc pigs (2 to 3 months old, Nihon Crea, Tokyo, Japan). The ischemia-reperfusion procedure was performed as previously described [7]. Briefly, animals were anesthetized with ketamine hydrochloride (20 mg/kg) and xylazine (3.5 mg/kg) and maintained with isoflurane (1% to 2.5%) by ventilator after intubation. Hemodynamic measurements were performed with a 6F catheter-tip manometer (CA-6100-PLB; CD Leycom Instrument, Zoetermeer, The Netherlands). Power Laboratory recording system and analysis software (AD Instruments, Oxfordshire, UK) were used for data analyses as previously described [7]. Coronary angiography was performed to determine the optimal location of the occlusion using a 6F guiding catheter. After an over-the-wire-type angioplasty balloon catheter (diameter, 3.0 ± 0.5 mm; length, 18 mm; Boston Scientific Japan) was placed in the LAD distal to the first major diagonal branch, the balloon was inflated to occlude the LAD at 6 to 8 atm for 45 minutes. After occlusion of the LAD, an intracoronary bolus of recombinant human FSTL1 protein (3 μg/kg pig) or vehicle (saline) as a control was given through the wire lumen of the inflated balloon catheter during the first 10 minutes of coronary ischemia. The dose of FSTL1 protein for intracoronary injection for pigs was calculated based upon the findings that coronary blood flow represents 5% of the total cardiac output, about half of which are estimated to enter into left coronary arteries (100 (ng/g) x 0.05 x 0.5 = 3.0 (μg/kg), which is rounded to the nearest number). After 24 hours of reperfusion, we anesthetized animals, measured hemodynamic parameters and euthanized them

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with an overdose of pentobarbital. All procedures were approved by the institutional animal care and use committee and were conducted according to the institutional guidelines of Nagoya University.

Echocardiographic analysis

We performed transthoracic echocardiography to evaluate cardiac function of mice at 24 hours after I/R surgery. Left ventricular (LV) end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) were measured by M-mode images using an Acuson Sequioa C-256 machine with a 15-MHz probe, and LV fractional shortening was calculated as (LVEDD-LVESD)/LVEDD X 100 (%).

Cell Culture

Primary cultures of neonatal rat ventricular myocytes were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) as previously described [8]. After 12 hours of serum starvation, cardiac myocytes were treated with FSTL1 protein (100 or 250 ng/ml) or vehicle for the indicated lengths of time. For hypoxia/reoxygenation studies, cells were exposed to 12 hours of hypoxia followed by 24 hours of reoxygenation. Hypoxic conditions were generated using an AnaeroPack (Mitsubishi GAS Chemical). RAW264.7 (mouse macrophage cell line) cells were maintained in RPMI1640 supplemented with 10% FBS. RAW264.7 cells were treated with FSTL1 protein (250 ng/ml) or vehicle for the indicated lengths of time. In some experiments, cardiac myocytes and RAW264.7 cells were administered with BMP-4 protein (100 ng/ml) and/or FSTL1 protein for 18 hours. In some experiments, cardiac myocytes and RAW264.7 cells were pretreated with FSTL1 protein or vehicle for 30 minutes followed by stimulation with LPS for 6

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hours. In some experiments, these cells were infected with Ad-dnAMPK or Ad-β-gal as a control at a multiplicity of infection (MOI) of 10 for 24 hours. In some experiments, NRVMs were transfected with siRNAs targeting BMP-4 or unrelated siRNAs at 40 nM using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Immunofluorescent analysis

To detect apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining for the frozen heart sections or cultured cardiac myocytes was performed using the In Situ Cell Death detection kit (Roche Diagnostics) as described previously [4]. Cryo-sections (5 μm thickness) were fixed with 4 % paraformaldehyde in PBS, permeabilized with 0.1 % Triton X-100. DAPI was used for nuclear staining. TUNEL-positive cells were counted in randomly selected three fields of the slide and the experiments were repeated at least three times in duplicates.

Determination of mRNA levels

Gene expression levels were quantified by real-time PCR method. Total RNA was extracted from cultured cardiac myocytes and macrophages using RNeasy Micro Kit (Qiagen) and from heart tissues using RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was prepared using a SuperScript RT-PCR system (Invitrogen). PCR procedure was performed with a Bio-Rad real-time PCR detection system using SYBR Green I as a double-standard DNA-specific dye. The primers were listed in Supplemental Table 1.

Western blot analysis

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Heart tissue and cell samples were prepared in lysis buffer containing 1mM PMSF (Sigma). The protein concentration was calculated using a BCA protein assay kit (Thermo Scientific). The equal amounts of proteins were separated by denaturing SDS-PAGE. Proteins were transferred onto PVDF membrane (GE Healthcare) and probed with the primary antibody followed by incubation with the HRP-conjugated secondary antibody. ECL or ECL plus system (GE Healthcare) were used for detection of the protein signal. 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 β-actin signal.

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Supplemental Table 1. Primers used in RT-PCR protocols.

Mouse Primers		Sequence
TNF-α	forward	5'-ACCACCATCAAGGACTC-3'
	reverse	5'-TGACCACTCTCCCTTTG-3'
IL6	forward	5'-TTCCAATGCTCTCCTAACAG-3'
	reverse	5'-CTAGGTTTGCCGAGTAGATC-3'
β-actin	forward	5'-TCCTTCTTGGGTATGGAATC-3'
	reverse	5'-TAGAGGTCTTTACGGATGTC-3'
Pig Primers		**Sequence**
TNF-α	forward	5'-ACTGCACTTCGAGGTTATCG-3'
	reverse	5'-GCTTTGACATTGGCTACAAC-3'
IL6	forward	5'-CTTCCAATCTGGGTTCAATC-3'
	reverse	5'-GACATTTCCCTTATTGCTCTC-3'
β-actin	forward	5'-TTTCTTCTCTGACCTGAGTC-3'
	reverse	5'-AGCCAGTGTTAGTACCTATAC-3'
Rat Primers		**Sequence**
TNF-α	forward	5'-CCAATCTGTGTCCTTCTAAC-3'
	reverse	5'-GTTTCTGAGCATCGTAGTTG-3'
IL6	forward	5'-AAGGTCACTATGAGGTCTAC-3'
	reverse	5'-CATATTGCCAGTTCTTCGTA-3'
BMP-4	forward	5'-GACTTCGAGGCGACACTTC -3'
	reverse	5'-CGGTAAAGATCCCTCATGTAATCC-3'
β-actin	forward	5'-GGTCATCACTATCGGCAATG-3'
	reverse	5'-GTTTCTGAGCATCGTAGTTG-3'

TNF-α: Tumor necrosis factor -α, IL6: Interleukin 6, BMP-4: Bone morphogenetic protein-4

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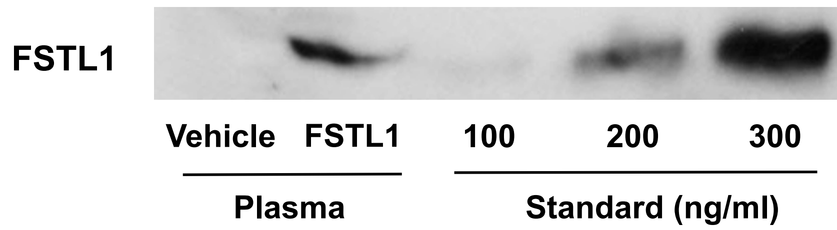

Plasma levels of human FSTL1 in mice after systemic injection of human recombinant FSTL1 protein. Blood was collected from mice at 5 minutes after intravenous injection of recombinant human FSTL1 protein (100 ng/g mouse). FSTL1 protein levels in plasma (10 μl) was determined by Western blot analysis. The signal intensities were standardized by recombinant human FSTL1 protein (100, 200, 300 ng/ml) and quantified by using Image J software.

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Supplemental Figure 1

Supplemental References

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