Therapeutic Impact of Follistatin-like 1 on Myocardial Ischemic Injury in Preclinical Animal Models

Running title: Ogura et al; Follistatin-like 1 and myocardial ischemia reperfusion

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Abstract:

**Background** - Acute coronary syndrome (ACS) is a leading cause of death in developed countries. Follistatin-like 1 (FSTL1) is a myocyte-derived secreted protein, which is upregulated in the heart in response to ischemic insult. Here, we investigated the therapeutic impact of FSTL1 on acute cardiac injury in small and large preclinical animal models of ischemia/reperfusion (I/R) and dissected its molecular mechanism.

**Methods and Results** - Administration of human FSTL1 protein significantly attenuated myocardial infarct size in a mouse or pig model of I/R, which was associated with reduction of apoptosis and inflammatory responses in the ischemic heart. Administration of FSTL1 enhanced the phosphorylation of AMP-activated protein kinase (AMPK) in the I/R-injured heart. In cultured cardiac myocytes, FSTL1 suppressed apoptosis in response to hypoxia/reoxygenation and LPS-stimulated expression of pro-inflammatory genes through its ability to activate AMPK. I/R led to enhancement of bone morphogenetic protein-4 (BMP-4) expression and Smad1/5/8 phosphorylation in the heart, and FSTL1 suppressed the increased phosphorylation of Smad1/5/8 in ischemic myocardium. Treatment of cardiac myocytes with FSTL1 abolished the BMP-4-stimulated increase in apoptosis, Smad1/5/8 phosphorylation and pro-inflammatory gene expression. In cultured macrophages, FSTL1 diminished LPS-stimulated expression of pro-inflammatory genes via activation of AMPK, and also abolished BMP-4-dependent induction of pro-inflammatory mediators.

**Conclusions** - Our data indicate that FSTL1 can prevent myocardial I/R injury by inhibiting apoptosis and inflammatory response through modulation of AMPK- and BMP-4-dependent mechanisms, suggesting that FSTL1 could represent a novel therapeutic target for ACS.

**Key words:** apoptosis; inflammation; ischemia; myocytes; reperfusion
Introduction

Coronary heart disease including acute coronary syndrome (ACS) is one of the major causes of morbidity and mortality worldwide. Successful reperfusion therapy for ACS with percutaneous coronary intervention can minimize myocardial infarction and prevent heart failure, thus leading to reduced mortality. However, tissue damage and pathological remodeling can be a side-effect of myocardial reperfusion. Thus, a strategy to protect against cardiac injury and dysfunction in response to tissue ischemia and reperfusion could be useful as an adjunct therapy for the treatment of ACS patients.

Accumulating evidence indicates that heart tissue secretes a variety of bioactive molecules, also known as cardiokines, which modulate the cellular processes in the heart including cardiac remodeling in an autocrine, paracrine or endocrine manner. Cardiokines include atrial natriuretic peptide, brain natriuretic peptide, adrenomedullin and protease inhibitor. Several cardiokines are upregulated in response to cardiac stress and play important roles in cardiac pathology, implying that cardiokines may serve both as biomarkers and targets for the management of the disease process. Follistatin-like 1 (FSTL1), also known as TSC-36, is a secreted glycoprotein and belongs to follistatin family of proteins. Follistatin family members bind to TGF-β superfamily proteins and inhibit their functions. We have previously shown that FSTL1 is a cardiokine that is up-regulated in ischemic-injured and hypertrophic hearts of mice. It has been reported that FSTL1 transcript is increased in the myocardium in patients with end-stage heart failure. We have demonstrated that elevated levels of circulating FSTL1 are associated with chronic systolic heart failure and that FSTL1 protein expression is increased in the failing heart in humans. Furthermore, circulating FSTL1 levels are elevated in patients with acute coronary syndrome. Thus, FSTL1 can serve as a useful biomarker of...
cardiac disease.

The functional role of FSTL1 in regulation of cardiovascular disease has also been investigated. We have shown that systemic delivery of adenoviral vectors encoding murine Fstl1 can prevent myocardial ischemia/reperfusion (I/R) injury in mice, which is associated with reduced myocyte apoptosis 16. We have also shown that overexpression of Fstl1 promotes revascularization in response to hind limb ischemia in mice 21. Recently we have demonstrated that Fstl1 attenuates cardiac hypertrophy in response to pressure overload in mice 22. Based upon these genetic gain- and loss-of-function experiments, we conclude that Fstl1 has broad cardiovascular-protective activities. However, therapeutic impact of acute FSTL1 administration on cardiac injury in preclinical models has not been previously investigated. Furthermore, the molecular mechanism by which FSTL1 exerts the protective actions on the heart is incompletely understood. Here we investigated the therapeutic effects of human FSTL1 protein on cardiac injury and remodeling in preclinical models of myocardial I/R and assessed its molecular mechanism.

**Methods**

An expanded Method section can be found in online Data supplement.

**Statistical analysis**

Data are presented as mean ± S.E. Student's t test was performed for comparison between two independent groups. One-way analysis of variance test was performed for comparison of three or more independent groups. Fisher’s protected LSD test was used only if the overall comparison by one-way analysis of variance was statistically significant. All continuous variables were assumed to be normally distributed. A p value <0.05 denoted the presence of a statistically
significant difference. Given the large number of statistical comparisons performed, all at the 0.05 level of significance may result in the possibility of a type I error.

Results

Administration of FSTL1 protein reduces myocardial infarct size following I/R in murine and porcine models

To examine the role of Fstl1 in the ischemic heart, Fstl1 protein expression was measured in the myocardium and plasma in male wild-type C57BL/6J mice after I/R injury or sham surgery. Myocardial I/R injury led to an increase in Fstl1 protein expression in the heart compared with sham operation (Figure 1A). Plasma Fstl1 levels were also increased following myocardial I/R. These data indicate that Fstl1 may be released from the heart during I/R.

To test whether systemic delivery of FSTL1 protein affects acute cardiac ischemic injury in mice, male C57BL/6J mice were intravenously treated with recombinant human FSTL1 protein or vehicle, and subsequently subjected to 60 minutes of myocardial ischemia and 24 hours of reperfusion. Figure 1B shows the representative photographs of the heart sections stained with Evans blue dye to delineate the area at risk (AAR) and 2,3,5-triphenyl tetrazolium chloride (TTC) to delineate the infarct area (IA) at 24 hours after reperfusion. Administration of FSTL1 protein significantly reduced the IA/AAR and IA/LV ratios by 54 ± 3 % and 51 ± 3 %, respectively (Figure 1C). There were no significant differences in the AAR/LV ratios between two groups. Treatment of mice with FSTL1 protein also reduced the plasma levels of troponin I, a marker of heart damage, at 3 hours after I/R (Figure 1D). Furthermore, treatment with FSTL1 significantly increased left ventricular fractional shortening in mice at 24 hours after myocardial I/R as measured by echocardiography (Figure 1E).
To further assess whether FSTL1 administration during reperfusion can affect myocardial injury, we intravenously administered recombinant human FSTL1 protein or vehicle to mice at 5 min after reperfusion. The administration of FSTL1 protein reduced the IA/AAR and IA/LV ratios by 40 ± 4 % and 41 ± 4 %, respectively as compared with that of vehicle (Figure 1F). Thus, FSTL1 administration, either before ischemia or after reperfusion, is effective at minimizing cardiac ischemic damage in mice.

To examine the effects of FSTL1 on cardiac injury in a large animal model of I/R, female Yorkshire-Duroc pigs were subjected to 45 minutes of ischemia and 24 hours of reperfusion. Intracoronary injection of recombinant human FSTL1 protein or vehicle was performed via the wire lumen of the catheter during the first 10 minutes of cardiac ischemia. Figure 1G shows the representative photographs of the pig heart sections stained with Evans blue dye and TTC. Quantitative analysis of infarct size demonstrated that the intracoronary administration of FSTL1 protein attenuated the IA/AAR and IA/LV ratios by 55 ± 5 % and 54 ± 5 %, respectively (Figure 1H). Treatment of pigs with FSTL1 protein also reduced the circulating levels of troponin I and CKMB, another marker of heart damage, at 24 hours after I/R (Figure 1H).

To examine the actions of FSTL1 on cardiac function in the porcine model, hemodynamic parameters were determined before the induction of ischemia and at 24 hours after I/R by using a manometer-tipped catheter. All parameters before I/R operation were not significantly different between FSTL1- and vehicle-treated pigs. FSTL1 administration to pigs led to a decrease in LV end-diastolic pressure and an increase in dP/dt_{max} at 24 hours after I/R (Figure 1J).

Delivery of FSTL1 protein suppresses apoptosis and inflammatory responses in ischemic heart in vivo
Apoptosis is the key feature of various pathological heart conditions. To investigate whether administration of FSTL1 protein modulates the apoptosis in the ischemic hearts, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining in the AAR regions was performed. Figure 2A shows representative photographs of TUNEL-positive nuclei in the heart in mice. Systemic delivery of FSTL1 protein significantly reduced the frequency of TUNEL-positive cells in the mouse hearts following I/R compared with vehicle treatment (Figure 2A). Similarly, the intracoronary administration of FSTL1 to pigs led to a marked reduction of the percentage of TUNEL-positive cells in the myocardium after I/R (Figure 2B).

Because inflammation contributes to myocardial injury after I/R, the expression of proinflammatory cytokines including TNF-α and IL6 were evaluated in the ischemic hearts on the different experimental groups by quantitative RT-PCR. Systemic administration of FSTL1 protein to mice attenuated the mRNA levels of TNF-α and IL6 in the I/R-injured hearts (Figure 2C). Similarly, intracoronary injection of FSTL1 protein significantly reduced the transcript levels of TNF-α and IL6 in the pig heart after I/R (Figure 2D).

FSTL1 attenuates apoptosis of cardiac myocytes via activation of AMPK and inhibition of BMP-4 signaling

To investigate whether FSTL1 directly influences cardiomyocyte apoptosis, neonatal rat ventricular myocytes (NRVMs) were cultured under conditions of normoxia or hypoxia/reoxygenation (H/R) in the presence of recombinant human FSTL1 protein or vehicle. H/R significantly increased the percentage of TUNEL-positive cells, whereas treatment with FSTL1 protein at the concentrations of 100 and 250 ng/ml attenuated the frequency of TUNEL-positive cells (Figure 3A).

Because activation of AMP-activated protein kinase (AMPK) protects cardiomyocytes
from apoptosis, we investigated the effects of FSTL1 on AMPK signaling pathways. In a cell culture model, treatment of NRVMs with FSTL1 protein augmented the activating phosphorylation levels of AMPK at Thr-172, which was accompanied by increased phosphorylation of its downstream substrate acetyl-coenzyme A carboxylase (ACC) at Ser-79 (Figure 3B). Systemic injection of human FSTL1 protein also promoted the phosphorylation of AMPK and ACC in the ischemic heart of mice (Figure 3C), and the intracoronary delivery of FSTL1 protein enhanced the phosphorylation of AMPK and ACC in I/R-injured hearts of pigs (Figure 3D).

To investigate the involvement of AMPK signaling in anti-apoptotic effects of FSTL1 in cardiac myocytes, NRVMs were transduced with adenoviral vectors expressing a dominant-negative form of AMPK tagged with c-myc (Ad-dn-AMPK) or β-galactosidase (Ad-β-gal) as a control, followed by treatment with human FSTL1 protein or vehicle. FSTL1-induced phosphorylation of ACC in NRVMs was abolished by transduction with Ad-dn-AMPK (Figure 3E). Transduction with Ad-dn-AMPK also inhibited the suppressive effects of FSTL1 on H/R-induced apoptosis of NRVMs (Figure 3F), indicating that the actions of FSTL1 on myocyte survival are partly dependent on its ability to activate AMPK.

FSTL1 has been reported to bind to bone morphogenetic protein-4 (BMP-4) and to antagonize BMP-4, thereby regulating lung development. It has also been shown that BMP-4 promotes the apoptosis of cardiac myocytes. These findings led us to speculate that BMP-4 signaling contributes to the cardioprotective actions of FSTL1. Myocardial I/R in pigs led to an increase in BMP-4 protein expression in AAR regions, which was accompanied by an increase in phosphorylation levels of Smad1/5/8, a downstream target of BMP-4 (Figure 4A). Likewise, H/R stimulation significantly increased BMP-4 protein expression in cultured NRVMs (Figure 4B).
H/R also stimulated the phosphorylation of Smad1/5/8 in NRVMs, which was abolished by treatment with FSTL1 protein at the concentrations of 100 and 250 ng/ml (Figure 4C). Treatment of NRVMs with recombinant BMP-4 protein significantly increased the percentage of TUNEL-positive cells under normoxic conditions, and the simultaneous addition of FSTL1 protein to BMP-4 abolished BMP-4-induced increase in TUNEL-positive cells (Figure 4D). Transduction with Ad-dn-AMPK did not affect the inhibitory actions of FSTL1 on BMP-4-stimulated myocyte apoptosis under conditions of normoxia (Figure 4E). BMP-4 treatment also increased the phosphorylation levels of Smad1/5/8, which were diminished by the addition of FSTL1 protein (Figure 4F). Moreover, intracoronary infusion of FSTL1 protein significantly reduced the phosphorylation levels of Smad1/5/8 in the ischemic myocardium of pigs (Figure 4G). These data indicate that FSTL1 promotes the survival of cardiomyocytes, in part, via inhibition of BMP-4 signaling that is independent of AMPK.

To assess the relative contribution of BMP-4 antagonization and AMPK activation to the cardioprotection by FSTL1, NRVMs were transfected with siRNAs targeting BMP-4 or unrelated siRNAs and transduced with Ad-dn-AMPK or Ad-β-gal followed by treatment with FSTL1 protein or vehicle under H/R conditions. Transfection of NRVMs with siRNAs against BMP-4 led to reduction of BMP-4 mRNA expression by 69 ± 3 %. Knockdown of BMP-4 suppressed the apoptosis of NRVMs in the absence of FSTL1 under conditions of H/R (Figure 4H). Treatment of NRVMs with FSTL1 significantly attenuated the H/R-stimulated apoptosis under conditions of BMP-4 ablation, which was reversed by transduction with Ad-dn-AMPK. Furthermore, knockdown of BMP-4 did not affect the H/R-stimulated apoptosis of NRVMs in the presence of FSTL1. Collectively these data indicate that FSTL1 reduces H/R-induced apoptosis through two independent pathways involving activation of AMPK and inhibition of
BMP-4 signaling.

**FSTL1 reduces inflammatory responses in cultured cardiac myocytes**

To analyze the anti-inflammatory actions of FSTL1 at a cellular level, NRVMs were pretreated with human FSTL1 protein or vehicle followed by stimulation with LPS. LPS exposure increased the mRNA levels of TNF-α and IL6 in NRVMs, and FSTL1 treatment significantly suppressed the LPS induced-increases in TNF-α and IL6 expression (**Figure 5A**). Stimulation with BMP-4 protein also increased the mRNA levels of TNF-α and IL6 in NRVMs, and BMP-4-induced increases in TNF-α and IL6 expression were completely blocked by the addition of FSTL1 (**Figure 5B**).

To determine the potential contribution of AMPK signaling to the anti-inflammatory actions of FSTL1 in cardiomyocytes, NRVMs were transduced with Ad-dn-AMPK or Ad-β-gal in the presence or absence of FSTL1 protein followed by exposure to LPS. Transduction with Ad-dn-AMPK reversed the suppressive actions of FSTL1 on LPS-stimulated expression of TNF-α and IL6 in NRVMs (**Figure 5C**).

**FSTL1 inhibits expression of inflammatory mediators in cultured macrophages**

Macrophage is one of the major types of cells that produce pro-inflammatory cytokines during myocardial I/R \(^{33,34}\). To test the actions of FSTL1 on inflammatory responses in macrophages, RAW264.7 macrophage cells were pretreated with human FSTL1 or vehicle followed by stimulation with LPS. LPS treatment dramatically stimulated the mRNA levels of TNF-α and IL6 in cultured macrophages, which were significantly attenuated by FSTL1 protein (**Figure 6A**). BMP-4 stimulation also increased the expression of TNF-α and IL6 in macrophages, and treatment with FSTL1 completely blocked the up-regulation of TNF-α and IL6 induced by BMP-4 (**Figure 6B**).
To assess the participation of AMPK in the inhibitory actions of FSTL1 on inflammatory response in macrophages, AMPK signaling pathways were assessed in cultured macrophages by Western blot analysis. Treatment of macrophages with FSTL1 protein resulted in an increase in phosphorylation of AMPK and ACC (Figure 6C). Transduction with Ad-dn-AMPK blocked the FSTL1-induced increase in ACC phosphorylation in cultured macrophages (Figure 6D). Furthermore, transduction with Ad-dn-AMPK cancelled the inhibitory effects of FSTL1 on LPS-stimulated expression of TNF-α and IL6 in macrophages (Figure 6E). Thus, the anti-inflammatory actions of FSTL1 may be partly dependent on activation of AMPK in macrophages.

**AMPK contributes to the myocardial infarct-sparing effect of FSTL1**

To further analyze the involvement of AMPK in FSTL1-mediated protection from acute cardiac injury in vivo, we intraperitoneally injected AMPK inhibitor compound C into mice. Treatment with compound C significantly diminished FSTL1-induced increase in ACC phosphorylation in ischemic heart (Figure 7A). Compound C had little effects on ACC phosphorylation of ischemic heart in vehicle-treated mice. Although compound C did not influence myocardial infarct size in vehicle-treated mice, it significantly reversed the suppressive actions of FSTL1 on myocardial infarct area of mice after I/R (Figure 7B). These data suggest that AMPK is involved in the beneficial actions of FSTL1 on acute ischemic injury in the heart.

**Discussion**

Our study provides the first evidence that administration of FSTL1 protein improves acute myocardial injury and dysfunction following I/R in preclinical animal models. Systemic delivery of recombinant human FSTL1 protein to mice led to reductions of myocardial infarct size,
systolic dysfunction, apoptosis, and inflammatory responses following I/R. Intracoronary
injection of FSTL1 protein attenuated myocardial infarction and improved cardiac function in
pigs after I/R, which was associated with suppression of apoptosis and inflammation in the
ischemic heart. These data are consistent with our previous findings that adenovirus-mediated
delivery of mouse Fstl1 is effective at attenuating myocardial I/R injury in mice\textsuperscript{16}. Our in vitro
experiments showed that FSTL1 promotes the survival of cardiac myocytes in response to H/R
and that FSTL1 attenuated agonist-stimulated expression of pro-inflammatory mediators in
cardiac cells and macrophages. Thus, FSTL1 administration can protect the heart from ischemic
damage through at least two mechanisms involving reduction of cardiomyocyte death and
suppression of inflammatory responses in myocardial cells. Because therapeutic approaches to
minimize cell death and inflammation in the heart are believed to be logical strategies to treat
acute cardiac injury, administration of FSTL1 may be a useful adjunctive therapy for acute
coronary syndrome.

Increased apoptosis is a key feature of the pathological cardiac remodeling that occurs in
response to ischemia\textsuperscript{23,24}. In the present study, FSTL1 attenuated apoptosis in the ischemic heart
in animal models of I/R, which is associated with enhanced activation of AMPK. Our in vitro
data showed that FSTL1 promoted AMPK phosphorylation in cardiac myocytes and that FSTL1
suppressed cardiomyocyte apoptosis in response to H/R partly via activation of AMPK that is
independent of inhibition of BMP-4 signaling. Our data also showed that I/R injury led to an
increase in cardiac BMP-4 expression, which was associated with enhanced phosphorylation of
Smad1/5/8. Administration of FSTL1 diminished the phosphorylation of Smad1/5/8 in the
ischemic heart. In cultured myocytes, H/R stimulation increased BMP-4 expression and
enhanced the phosphorylation of Smad1/5/8, which was abolished by FSTL1. Furthermore,
FSTL1 abrogated BMP-4-induced apoptosis of myocytes via an AMPK-independent mechanism. It has been shown that BMP-4 heterozygous knockout mice exhibit reductions in myocardial infarct size and apoptosis after I/R \textsuperscript{32}. Therefore, the salutary effects of FSTL1 on cardiac injury under conditions of ischemia appear to be mediated, at least in part, through its abilities to both activate AMPK and inhibit BMP-4 signaling, leading to a suppression of cell death (Figure 7C).

Previous reports have demonstrated that BMP-4 acts as a pro-inflammatory mediator in endothelial cells \textsuperscript{35,36}. In line with these findings, treatment of cultured myocytes and macrophages with BMP-4 stimulated pro-inflammatory gene expression. The pro-inflammatory action of BMP-4 on myocytes and macrophages could be abolished by treatment with FSTL1. Similarly, FSTL1 abrogated the pro-apoptotic effects of BMP-4 on myocytes. Consistent with these observations, FSTL1 has been shown to directly bind to BMP-4 and inhibit its signaling in cultured hepatoma cells \textsuperscript{31}. Taken together, the favorable effects of FSTL1 on acute ischemic damage in the myocardium appear to be mediated through its abilities to antagonize both the pro-inflammatory and pro-apoptotic functions of BMP-4 (Figure 7C).

Our data indicate that the suppressive actions of FSTL1 on inflammatory responses in ischemic heart are partly dependent on its ability to activate AMPK signaling in its target cells (Figure 7C). Administration of FSTL1 protein markedly reduced the expression of pro-inflammatory mediators in ischemic areas of myocardium in vivo. Treatment of cultured cardiomyocytes or macrophages with FSTL1 protein led to a reduction of pro-inflammatory gene expression in response to LPS. Moreover, FSTL1 enhanced the phosphorylation of AMPK in ischemic regions of the heart as well as in cultured cardiac myocytes and macrophages, and the anti-inflammatory actions of FSTL1 in LPS-treated cells are reversed by inhibition of AMPK activation. These results are consistent with previous observations that activation of AMPK
signaling negatively regulates the inflammatory responses in various types of cells including macrophages.\textsuperscript{37}

Myocardial AMPK is activated during various stresses including ischemia and hypertrophy, and activation of AMPK signaling under these conditions is thought to confer a protective role.\textsuperscript{38} Thus, hormones or reagents that activate AMPK may protect the heart from ischemic injury and hypertrophy. In this regard, the cardioprotective effects of adiponectin and macrophage migration inhibitory factor are mediated in part through their ability to activate AMPK signaling.\textsuperscript{30, 39, 40} Recently we have shown that FSTL1 functions to reduce myocardial hypertrophy in response to pressure overload by promoting AMPK activation.\textsuperscript{22} Here we have extended these findings by showing that FSTL1-mediated activation of myocardial AMPK has salutary effects on ischemic heart disease in vivo and in vitro.

It has been shown that administration of FSTL1 protein improves joint inflammation in a model of antibody-induced arthritis.\textsuperscript{41} Overexpression of FSTL1 promotes heart allograft survival, which is associated with reduced expression of pro-inflammatory genes including IL6\textsuperscript{42}. In agreement with these findings, our data show that administration of FSTL1 attenuates the expression of TNF-\(\alpha\) and IL6 in ischemic heart in vivo and that FSTL1 treatment downregulates LPS- or BMP-4-stimulated expression of TNF-\(\alpha\) and IL-6 in cultured cardiac myocytes and macrophages. In contrast, Fstl1 overexpression is reported to exacerbate collagen-induced arthritis associated with enhanced expression of pro-inflammatory cytokines.\textsuperscript{43, 44} The discrepancies among these studies may result from differences in experimental models or the context of other regulatory molecules that influence FSTL1 function. Thus, future research will be required to clarify the role of FSTL1 in regulation of inflammatory responses under various pathological conditions.
In conclusion, we show that FSTL1 can prevent apoptosis and inflammatory responses in the heart during I/R through its ability to promote AMPK signaling and antagonize BMP-4 function, thereby contributing to protection against acute cardiac injury (Figure 7C). In particular, we demonstrated the effectiveness of FSTL1 treatment for myocardial damage and dysfunction in response to ischemia in a preclinical porcine I/R model that is applicable to human ACS \(^45\), indicating the potential clinical utility of FSTL1. Collectively, these data suggest that FSTL1 represents a novel target molecule for the treatment of the pathological cardiac remodeling.

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**Conflict of Interest Disclosures:** None.

**References:**


**Figure Legends:**

**Figure 1.** Administration of FSTL1 protein decreases myocardial infarct size in animal models of ischemia/reperfusion. A, Fst1 protein levels in the heart and plasma in mice at 24 hours after myocardial ischemia/reperfusion (I/R) injury or sham operation. Fst1 protein expression was analyzed by Western blotting, and expressed relative to β-actin levels (n=4). B and C, Systemic delivery of human FSTL1 protein reduces myocardial infarct size in mouse ischemia/reperfusion (I/R) models. After intravenous injection of human FSTL1 protein (100 ng/g mouse) or vehicle, wild-type mice were subjected to 60 minutes of ischemia followed by 24 hours of reperfusion. Representative photographs of the mouse heart sections stained with Evans blue and subsequent TTC are shown (B). Left ventricular (LV) area, the area at risk (AAR) and infarct area (IA) were measured, and quantitative analysis of infarct size is shown (C) (n=7-8). D, FSTL1 reduces plasma Troponin I levels of mice at 3 hours after I/R (n=4). E, Administration of FSTL1 protein improves left ventricular (LV) function as assessed by echocardiography. Representative M-mode echocardiograms for vehicle or FSTL1-treated mice at 24 hours after I/R are shown in upper panels. Quantitative analysis of LV fractional shortening (%FS) in mice treated with vehicle or FSTL1 protein is shown in lower panel (n=5). F, FSTL1 administration during reperfusion reduces infarct size in mice. Recombinant human FSTL1 protein (100 ng/g mouse) or vehicle was intravenously administered to mice at 5 minutes after reperfusion. Quantitative analysis of infarct size is shown (n=7). G and H, Intracoronary administration of human FSTL1 protein suppresses myocardial infarct size following I/R in pigs. Pigs were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion, and recombinant human FSTL1 protein (3 µg/kg pig) or vehicle was injected through the wire lumen of the catheter during the
first 10 minutes of ischemia. Representative photographs of the heart sections stained with Evans blue and TTC are shown (G). Quantitative analysis of the AAR/LV, the IA/AAR and the IA/LV ratios is shown (H) (n=5). I, FSTL1 treatment inhibits circulating levels of Troponin I and creatine phosphokinase-MB (CKMB) in pigs at 24 hours after I/R (n=5). J, FSTL1 decreases LV end-diastolic pressure (LVEDP) and increases LV dP/dt\text{max} in pigs at 24 hours after I/R (n=3-5).

**Figure 2.** Administration of FSTL1 protein attenuates apoptosis and inflammatory responses in the ischemic myocardium of mice and pigs. A, Systemic delivery of FSTL1 attenuates apoptosis in the ischemic heart of mice. Upper panels show representative photographs of mouse heart sections stained with TUNEL (green) and DAPI (blue). Lower graph shows quantitative analysis of TUNEL-positive nuclei (n=5). B, Intracoronary administration of FSTL1 to pigs inhibits apoptosis in the ischemic myocardium. Upper panels show representative pictures of heart sections stained with TUNEL (green) and DAPI (blue). Lower graph shows quantitative analysis of TUNEL-positive nuclei (n=4). C and D, Delivery of human FSTL1 suppresses expression of pro-inflammatory cytokines in the ischemic hearts of mice (C) and pigs (D). The mRNA expression of TNF-\alpha and IL6 was measured by RT-PCR method and expressed relative to \beta-actin levels (n=5).

**Figure 3.** Treatment of cardiac myocytes with FSTL1 inhibits apoptosis via activation of AMPK. A, FSTL1 suppresses hypoxia/reoxygenation (H/R)-induced apoptosis of neonatal rat ventricular myocytes (NRVMs). NRVMs were treated with FSTL1 protein (100 or 250 ng/ml) or vehicle under conditions of normoxia or H/R. NRVMs were stained with TUNEL (green) and DAPI (blue), and quantitative analysis of TUNEL-positive myocytes was performed (n=4). B,
FSTL1 promotes AMPK phosphorylation in NRVMs. NRVMs were treated with FSTL1 protein (250 ng/ml) or vehicle for 30 minutes. The phosphorylation status of ACC (p-ACC) and AMPK (p-AMPK) was analyzed by Western blotting. C and D, FSTL1 promotes AMPK signaling pathways in I/R-injured hearts of mice (C) and pigs (D). The phosphorylation of ACC (p-ACC) and AMPK (p-AMPK) was assessed by Western blot analysis. Representative blots are shown from 4 independent experiments. E, AMPK inhibition abolishes FSTL1-induced ACC phosphorylation as assessed by Western blot analysis. After transduction with Ad-dn-AMPK or Ad-β-gal at a MOI of 10 for 24 hours, NRVMs were treated with FSTL1 (250 ng/ml) or vehicle for 30 minutes. Representative blots are shown from 3 independent experiments. F, AMPK is involved in the suppressive action of FSTL1 on H/R-induced apoptosis of NRVMs. NRVMs were transduced with Ad-dn-AMPK or Ad-β-gal and cultured in the presence or absence of FSTL1 (250 ng/ml) under conditions of H/R. Apoptotic nuclei were identified by TUNEL staining (n=4).

**Figure 4.** FSTL1 antagonizes BMP-4 signaling in the ischemic heart and cardiac myocytes. A, BMP-4 is upregulated in the heart in response to I/R. Representative blots of BMP-4 and phosphorylated Smad1/5/8 (p-Smad1/5/8) at remote non-ischemic area (remote) and ischemic area at risk (AAR) of pigs are shown from 4 independent experiments. B, H/R increases the expression of BMP-4 in NRVMs. Representative blots of BMP-4 are shown from 4 independent experiments. C, FSTL1 attenuates the phosphorylation of Smad1/5/8 in NRVMs under conditions of H/R. NRVMs were treated with FSTL1 (100 or 250 ng/ml) or vehicle under conditions of normoxia or H/R. The phosphorylation levels of Smad1/5/8 (p-Smad1/5/8) were analyzed by Western blotting, and expressed relative to β-actin levels (n=3). D, FSTL1 abolishes
BMP-4-induced apoptosis of NRVMs. NRVMs were treated with BMP-4 protein (100 ng/ml) or vehicle along with FSTL1 protein (100 or 250 ng/ml) or vehicle for 18 hours under normoxic conditions. Upper panels show representative pictures of NRVMs stained with TUNEL (green) and DAPI (blue). Lower graph shows quantitative analysis of TUNEL-positive NRVMs (n=4).

E, Effect of AMPK inactivation on FSTL1-mediated inhibition of BMP4-stimulated apoptosis of NRVMs. NRVMs were transduced with Ad-dn-AMPK or Ad-β-gal at a MOI of 10 for 24 hours and treated with BMP-4 protein (100 ng/ml) or vehicle along with FSTL1 protein (250 ng/ml) or vehicle for 18 hours under normoxic conditions (n=4). F, FSTL1 suppresses BMP-4-stimulated phosphorylation of Smad1/5/8 in NRVMs. NRVMs were treated with BMP-4 protein (100 ng/ml) or vehicle along with FSTL1 protein (100 or 250 ng/ml) or vehicle for 18 hours under normoxic conditions. The phosphorylation levels of Smad1/5/8 (p-Smad1/5/8) were determined by Western blot analysis, and expressed relative to β-actin levels (n=4).

G, Intracoronary administration of FSTL1 attenuates the phosphorylation of Smad1/5/8 in the ischemic myocardium in pigs. Upper panels show representative blots of p-Smad1/5/8, Smad1/5/8 and β-actin. Lower chart shows quantitative analysis of relative phosphorylation levels of Smad1/5/8 (n=4).

H, Involvement of BMP-4 antagonization and AMPK activation in the inhibitory action of FSTL1 on H/R-induced myocyte apoptosis. NRVMs are transfected with siRNAs targeting BMP-4 or unrelated siRNAs (40 nM) and transduced with an Ad-dn-AMPK or Ad-β-gal followed by treatment with FSTL1 (250 ng/ml) or vehicle (n=4).

Figure 5. FSTL1 suppresses inflammatory responses in cultured cardiac myocytes. A, FSTL1 diminishes LPS-stimulated expression of pro-inflammatory genes in NRVMs. NRVMs were pretreated with FSTL1 (250 ng/ml) or vehicle for 30 minutes followed by stimulation with LPS...
(100 ng/ml) or vehicle for 6 hours. The mRNA expression of TNF-α and IL6 was measured by RT-PCR method and expressed relative to β-actin levels (n=4). B, Effect of FSTL1 on BMP-4-stimulated expression of pro-inflammatory cytokines. NRVMs were treated with BMP-4 protein (100 ng/ml) or vehicle along with FSTL1 protein (100 or 250 ng/ml) or vehicle for 18 hours. The transcript levels of TNF-α and IL6 were determined by RT-PCR method and expressed relative to β-actin levels (n=4). C, AMPK participates in the effect of FSTL1 on LPS-induced increase in pro-inflammatory gene expression. After transduction with an Ad-dn-AMPK or Ad-β-gal at a MOI of 10 for 24 hours, NRVMs were pretreated with FSTL1 (250 ng/ml) or vehicle for 30 minutes followed by treatment with LPS (100 ng/ml) or vehicle for 6 hours. The mRNA levels were analyzed by RT-PCR method and expressed relative to β-actin levels (n=4).

**Figure 6.** FSTL1 protein diminishes inflammatory responses in cultured macrophages. A, FSTL1 inhibits LPS-stimulated expression of pro-inflammatory genes in macrophages. Macrophage RAW264.7 cells were cultured in the presence of FSTL1 (250 ng/ml) or vehicle for 30 minutes followed by treatment with LPS (100 ng/ml) or vehicle for 6 hours. The mRNA expression of TNF-α and IL6 was quantified by RT-PCR analysis and expressed relative to β-actin levels (n=4). B, FSTL1 abolishes BMP-4-stimulated expression of pro-inflammatory mediators in macrophages. Macrophages were treated with BMP-4 protein (100 ng/ml) or vehicle along with FSTL1 protein (250 ng/ml) or vehicle for 18 hours. The transcript levels of TNF-α and IL6 were analyzed by RT-PCR method and expressed relative to β-actin levels (n=4). C, FSTL1 promotes the AMPK signaling pathway in macrophages. Macrophages were treated with FSTL1 (250 ng/ml) or vehicle for 15 minutes. The phosphorylation levels of ACC (p-ACC) and AMPK (p-AMPK) were determined by Western blot analysis. D, AMPK inactivation
cancels FSTL1-stimulated ACC phosphorylation in macrophages as determined by Western blot analysis. Macrophages were transduced with Ad-dn-AMPK or Ad-β-gal, and treated with FSTL1 (250 ng/ml) or vehicle for 15 minutes. E, AMPK signaling is involved in FSTL1-mediated inhibition of LPS-stimulated expression of pro-inflammatory cytokines. After transduction with Ad-dn-AMPK or Ad-β-gal, macrophages were treated with FSTL1 (250 ng/ml) or vehicle for 30 minutes followed by stimulation with LPS (100 ng/ml) or vehicle for 6 hours. Transcript levels were determined by RT-PCR analysis and expressed relative to β-actin levels (n=4).

Figure 7. AMPK is involved in the myocardial infarct-sparing effect of FSTL1. A, Effect of AMPK inactivation on ACC phosphorylation in the ischemic heart. AMPK inhibitor compound C (CC) (20 mg/kg) dissolved in dimethyl sulfoxide (DMSO) or DMSO was intraperitoneally injected into mice. After intravenous injection of human FSTL1 protein (100 ng/g mouse) or vehicle, wild-type mice were subjected to I/R. The phosphorylation of ACC (p-ACC) in the ischemic heart was assessed by Western blot analysis. Representative blots are shown from 4 independent experiments. B, Role of AMPK in FSTL1-mediated inhibition of infarct size. Quantitative analysis of the AAR/LV, the IA/AAR and the IA/LV ratios is shown (n=6). C, Proposed scheme for the mechanism by which FSTL1 protects the heart from ischemic injury. FSTL1 is upregulated in the heart and plasma in response to ischemic insult. FSTL1 promotes AMPK signaling pathways in the ischemic heart, thereby leading to reduction of inflammation and apoptosis. FSTL1 also antagonizes BMP-4-dependent pro-inflammatory and pro-apoptotic signals in the myocardium. Therefore, FSTL1 confers beneficial actions on the ischemic hearts by reducing inflammatory response and apoptosis through modulation of AMPK- and BMP-4-dependent mechanisms.
G

Vehicle  FSTL1

H

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<tr>
<th></th>
<th>Vehicle</th>
<th>FSTL1</th>
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<tbody>
<tr>
<td>AAR/LV</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>IA/AAR</td>
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<tr>
<td>IA/LV</td>
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p=0.753

p=0.009

p=0.009

I

Troponin I (ng/ml)

Vehicle  FSTL1

p=0.009

CKMB (ng/ml)

Vehicle  FSTL1

p=0.016

J

LVEDP (mmHg)

Before I/R  After I/R

p=0.003

LV dP/dt max (mmHg/s)

Before I/R  After I/R

p=0.036
(A) Mouse I/R

FSTL1

TUNEL positive cells (%)

+  +

-  -

p<0.001

(B) Pig I/R

FSTL1

TUNEL positive cells (%)

+  +

-  -

p=0.001

(C) Mouse I/R

FSTL1

TNF-α mRNA levels

+  +

-  -

p=0.021

(D) Pig I/R

FSTL1

TNF-α mRNA levels

+  +

-  -

p=0.034

(E) Mouse I/R

FSTL1

IL6 mRNA levels

+  +

-  -

p=0.043

(F) Pig I/R

FSTL1

IL6 mRNA levels

+  +

-  -

p=0.034
A.

TUNEL

DAPI

\[
p < 0.001 \quad p < 0.001 \quad p < 0.001
\]

TUNEL positive cells (%)

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<tr>
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<td>+</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>250</td>
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B.

p-ACC

ACC

p-AMPK

AMPK

β-actin

FSTL1

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C.

mouse I/R

p-ACC

ACC

p-AMPK

AMPK

β-actin

FSTL1

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D.

pig I/R

p-ACC

ACC

p-AMPK

AMPK

β-actin

FSTL1

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E.

p-ACC

ACC

c-myc

β-actin

FSTL1

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F.

TUNEL

DAPI

\[
p < 0.001 \quad p = 0.002 \quad p = 0.014
\]

TUNEL positive cells (%)

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Therapeutic Impact of Follistatin-like 1 on Myocardial Ischemic Injury in Preclinical Animal Models

Yasuhiro Ogura, Noriyuki Ouchi, Koji Ohashi, Rei Shibata, Yoshiyuki Kataoka, Takahiro Kambara, Tetsutaro Kito, Sonomi Maruyama, Daisuke Yuasa, Kazuhiro Matsuo, Takashi Enomoto, Yusuke Uemura, Megumi Miyabe, Masakazu Ishii, Takashi Yamamoto, Yuuki Shimizu, Kenneth Walsh and Toyoaki Murohara

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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. 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
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
operation and after reperfusion. 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. 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. 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.
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. 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
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**
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.
**Supplemental Table 1.** Primers used in RT-PCR protocols.

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<td><strong>TNF-α</strong></td>
<td>forward 5'-ACCACCATCAAGGACTC-3'</td>
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<td>reverse 5'-TGACCACTCCCTTTG-3'</td>
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<tr>
<td><strong>IL6</strong></td>
<td>forward 5'-TTCCAATGCTCTCTAACAAG-3'</td>
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<tr>
<td></td>
<td>reverse 5'-CTAGGTTTCGAGTAGATC-3'</td>
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<td><strong>β-actin</strong></td>
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<td><strong>IL6</strong></td>
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TNF-α: Tumor necrosis factor -α, IL6: Interleukin 6, BMP-4: Bone morphogenetic protein-4
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.
Supplemental References


