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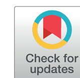
Therapeutic silencing of FSP27 reduces the progression of atherosclerosis in *Ldlr*$^{-/-}$ mice



Ananthi Rajamoorthi [a], Richard G. Lee [b], Ángel Baldán [a, c, d, *]

[a] Edward A. Doisy Department of Biochemistry & Molecular Biology, Saint Louis University, Saint Louis, MO, 63104, USA
[b] Cardiovascular Group, Antisense Drug Discovery, Ionis Pharmaceuticals, Carlsbad, CA, 92010, USA
[c] Center for Cardiovascular Research, Saint Louis, MO, 63104, USA
[d] Liver Center, Saint Louis University, Saint Louis, MO, 63104, USA

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ABSTRACT

Background and aims: Obesity, hepatosteatosis, and hypertriglyceridemia are components of the metabolic syndrome and independent risk factors for cardiovascular disease. The lipid droplet-associated protein CIDEC (cell death-inducing DFFA-like effector C), known in mice as FSP27 (fat-specific protein 27), plays a key role in maintaining triacylglyceride (TAG) homeostasis in adipose tissue and liver, and controls circulating TAG levels in mice. Importantly, mutations and SNPs in *CIDEC* are associated with dyslipidemia and altered metabolic function in humans. Here we tested whether systemic silencing of *Fsp27* using antisense oligonucleotides (ASOs) was atheroprotective in LDL receptor knock-out (*Ldlr*$^{-/-}$) mice.

Methods: Atheroprone *Ldlr*$^{-/-}$ mice were fed a high-fat, high-cholesterol diet for 12 weeks while simultaneously dosed with saline, ASO-ctrl, or ASO-Fsp27.

Results: Data show that, compared to control treatments, silencing *Fsp27* significantly reduced body weight gain and visceral adiposity, prevented diet-induced hypertriglyceridemia, and reduced atherosclerotic lesion size both in *en face* aortas and in the aortic root.

Conclusions: Our findings suggest that therapeutic silencing of *Fsp27* with ASOs may be beneficial in the prevention and management of atherogenic disease in patients with metabolic syndrome.

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1. Introduction

Central obesity, non-alcoholic fatty liver disease, insulin resistance, and hypertriglyceridemia are core manifestations of the metabolic syndrome (MetS) (reviewed in Ref. [1]). A global epidemic afflicting both adults and children, MetS is associated with increased risk for atherogenic cardiovascular disease, which ultimately leads to myocardial infarction and stroke. Although the mechanisms that link MetS and atherogenesis are yet to be fully elucidated, it was proposed that persistent systemic inflammation and dyslipidemia synergize to promote endothelial dysfunction, fatty streak formation, and other intimal perturbations in the arterial wall.

Lipid droplets (LDs) are critical organelles for intracellular metabolic regulation [2]. LD-associated proteins define the metabolic fate of the lipids stored within the LD. *CIDEC* (cell death-inducing DFF45-like effector C; referred to as *Fsp27* or fat specific protein 27 in mice) was originally identified as an abundant transcript in white and brown adipocytes [3], where it facilitates LD growth by both promoting LD fusion and inhibiting the action of lipases [4–7]. Two *CIDEC/Fsp27* isoforms (α and β) that differ in 10 aa at the N-terminus are expressed via alternative promoters [8]. *CIDEC/Fsp27* is barely detectable in the normal liver, but its expression is drastically elevated in the livers of obese patients [8–10] and mice [11–14], as well as in response to fasting in mice [11,15]. Recent studies showed that FSP27 activity modulates different physiological responses related to MetS. *Fsp27*$^{-/-}$ mice are lean, resistant to diet-induced obesity, and show enhanced insulin sensitivity [16,17]. Paradoxically, though, high-fat diet-fed *Fsp27*$^{-/-}$

Abbreviations: ASO, antisense oligonucleotide; CVD, cardiovascular disease; CIDEC, cell death-inducing DFFA-like effector C; FSP27, fat-specific protein 27; gWAT, gonadal white adipose tissue; HDL, high-density lipoprotein; HFD, high-fat diet; LD, lipid droplet; LDL, low-density lipoprotein; MetS, metabolic syndrome; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; TAG, triacylglyceride; VLDL, very low-density lipoprotein; WD, western diet.

* Corresponding author. Doisy Research Center, Saint Louis University, Room 615, Saint Louis, MO, 63104, USA.
E-mail address: angel.baldan@health.slu.edu (Á. Baldán).

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[18], *ob/ob* × *Fsp27*$^{-/-}$ [18], and adipocyte-specific *Fsp27*$^{-/-}$ [19] mice develop severe lipodystrophy, fatty liver, and insulin resistance. On the other hand, antisense oligonucleotide (ASO)-mediated silencing of *Fsp27* in genetic or dietary murine models of obesity and diabetes results in decreased visceral adiposity, reduced triacylglyceride (TAG) contents in fat pads, multilocular brown-like white adipocytes, reduced circulating VLDL-TAG, and improved whole-body glycemic control and multi-organ insulin sensitivity [12,20]. Acute shRNA-mediated knock-down of hepatic *Fsp27* reduces fasting and diet-induced hepatosteatosis [11,14,21]. In contrast, long-term systemic ASO-mediated *Fsp27* silencing abolishes diet-induced hepatic TAG accumulation only when used in combination with a fibrate [20], but not by itself [12,20]. Collectively, these reports suggest that silencing *Fsp27* may provide atheroprotection by ameliorating several independent cardiovascular risk factors. Herein we tested the effects of ASO-based anti-Fsp27 therapy on the progression of arterial disease in high fat, high cholesterol-fed atheroprone *Ldlr*$^{-/-}$ mice.

2. Materials and methods

2.1. Chemicals

Chimeric 2′-methoxyethyl control (5′-CCTTCCCTGAAGGTT CCTCC) and anti-Fsp27 (5′-CAGACTCTAATACCATTCAC) antisense oligonucleotides (ASOs) were synthesized and purified as described [22], suspended in saline, and stored at −20 °C until use.

2.2. Mouse studies

All animals were maintained in a 12 h/12 h light/dark cycle with *ad libitum* access to food and water. LDL-receptor knockout (*Ldlr*$^{-/-}$) mice (Jackson Laboratories stock 002207) were bred in our facility and kept on a normal diet (PicoLab Rodent Diet 20). At 12 weeks of age, male mice were switched to a western diet (WD) containing 21% fat and 1.25% cholesterol (Research Diets D12108) for 12 weeks. While on WD, 100 μL ASOs (25 mg/kg) or saline were injected *i. p.* twice weekly (Monday and Thursday). Mice were sacrificed at 9–10 a.m. without prior fasting, and plasma, liver, and gonadal white adipose tissue (gWAT), were harvested for analysis. In a subset of mice within each experimental group, resident macrophages were collected from peritoneal lavages. Studies were conducted in conformity with the Public Health Service policy on humane care and use of laboratory animals, and approved by the IACUC at Saint Louis University.

2.3. Plasma analysis

Blood samples were collected one week prior to start of WD and treatment regimen via superficial temporal vein bleeds, and upon sacrifice via inferior vena cava. Total cholesterol and triglycerides were assayed enzymatically using colorimetric kits (Wako Chemicals, Richmond, VA). Lipoprotein profiles were obtained by a modified Column Lipoprotein Profile (CLiP) method [23]. Briefly, 20 μL of pooled plasma were diluted in 60 μL of saline, and 40 μL of this mixture was auto-injected into a Superose-6 column (GE Healthcare, Wilmington, MA) using elution buffer (saline, 2 mmol/L EDTA, pH 7.4) at a flow rate of 0.6 mL/min at 40 °C. The eluate was immediately mixed with cholesterol or triglyceride reagent (Pointe Scientific, Ann Arbor, MI) at a flow rate of 0.2 mL/min, and incubated at 40 °C in a 5 m KOT coiled reactor. The final mixture entered a capillary spectrophotometric detector set at 500 nm, and the profiles were collected in real time using LC Solution software (Shimadzu, Kyoto, Japan).

2.4. Tissue lipid analysis

Lipids were extracted into chloroform by a modified Folch method [24], solubilized in water, and quantitated enzymatically using kits for triglycerides, total cholesterol, and FFAs (Wako Chemicals). Results were normalized to protein.

2.5. RNA analysis

RNA was isolated from tissues using Direct-zol RNA miniprep kit (ZYMO Research, Irvine, CA), and analyzed by real-time quantitative PCR using PowerSybrGreen (Life Technologies, Carlsbad, CA) and a LightCycler LC480 instrument (Roche, Indianapolis, IN). Values were normalized to *36b4*, and relative expression calculated using the $\Delta\Delta C_t$ method. Primer sequences are available upon request.

2.6. Histology

Samples of livers and gWAT were fixed in 10% formalin and embedded in paraffin blocks. Sections (5 μm) were processed for hematoxylin and eosin staining using standard techniques.

2.7. Analysis of atherosclerotic lesions

Whole aortas were dissected from the heart to the iliac bifurcation. Aortas and the upper half of the hearts were fixed in formalin-sucrose buffer (10% formalin, 20 μmol/L EDTA, 5% sucrose, pH 7.4), and stored at 4 °C. *En face* preparations of the aorta were pinned and stained with oil red O. Hearts were embedded in paraffin, and 20 serial sections (5 μm) of the aortic root (covering 1 mm around the aortic valve) were stained with hematoxylin and eosin. Atheromata were quantified using ImageJ software by a single operator blind to the identity of the samples, as described [25]. Select sections of the aortic root were stained with a monoclonal rat anti-mouse CD68 antibody (1:400 dilution; AbD Serotec) or Masson's trichrome to visualize macrophage and collagen content, respectively.

2.8. Macrophage studies

Macrophages from saline- and ASO-treated mice ($n = 5$ each) were isolated from the peritoneal cavity 4 days after injection of 2 mL of 3% thioglycollate broth. Red blood cells were removed with ACK lysis buffer (150 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA, pH 7.4), and pellets frozen for RNA extraction. Bone marrow cells were obtained from the tibias and femurs of chow-fed C57BL/6 mice, and differentiated to macrophages in media supplemented with 20 ng/mL M-CSF for 4 days, as described [26]. Cells were then incubated in media supplemented with PBS or 40 μg/mL oxLDL (AlfaAesar, Tewksbury, MA) overnight.

2.9. Statistical analysis

Data are shown as mean ± s. e.m. Differences between groups were analyzed by one-way ANOVA followed by post-hoc Bonferroni's test, using SPSS version 20.0 (IBM, Armonk, NY). Differences were considered significant at $p \leq 0.05$.

3. Results

To test the usefulness of therapeutic silencing of *Fsp27* on atherogenesis, male *Ldlr*$^{-/-}$ mice were fed a western diet for 12 weeks, while dosed with saline, ASO-control, or ASO-Fsp27. Fig. 1A and B show that treatment with ASO-Fsp27 reduced body weight

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