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Lipasin, a novel nutritionally-regulated liver-enriched factor that regulates serum triglyceride levels

Ren Zhang *

Center for Molecular Medicine and Genetics, School of Medicine, Wayne State University, Detroit, MI 48201, USA

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ABSTRACT

The metabolic syndrome, a common disorder including glucose intolerance and dyslipidemia, poses a major public health issue. Patients with high blood lipids, such as triglycerides, are at high risk in developing atherosclerotic cardiovascular diseases. To identify genes involved in metabolism, we performed RNA-seq experiments on the liver and fat in mice treated with a high-fat diet or fasting, and identified Gm6484 (named Lipasin) as a novel nutritionally regulated gene. Human *LIPASIN* is liver specific, while the mouse one is enriched in the liver and fat, including both brown and white adipose tissues. Obesity increases liver *Lipasin*, whereas fasting reduces its expression in fat. ANGPTL3 (Angiopoietin-like 3) and ANGPTL4 are critical regulators of blood lipids. LIPASIN shares homology with ANGPTL3's N-terminal domain that is needed for lipid regulation, and with ANGPTL4's N-terminal segment that mediates lipoprotein lipase (LPL) binding. Lipasin overexpression by adenoviruses in mice increases serum triglyceride levels, and a recombinant Lipasin inhibits LPL activity. Therefore, a potential mechanism for Lipasin-mediated triglyceride elevation is through reduced triglyceride clearance by LPL inhibition. Lipasin is thus a novel nutritionally-regulated liver-enriched factor that plays a role in lipid metabolism.

1. Introduction

The metabolic syndrome, a common metabolic disorder including glucose intolerance and dyslipidemia, has received significant attention, because the number of patients with this syndrome has increased dramatically in the past two decades [1], largely due to the global obesity epidemic [2–4]. Patients with metabolic syndrome are at high risk in developing atherosclerotic cardiovascular disease and diabetes, posing a major public health issue [5]. Of note, levels of blood lipids, such as triglycerides, are major contributors to cardiovascular diseases [6,7]. There is an urgent need to develop novel therapeutic approaches to alleviate the burden from these diseases.

To identify genes involved in metabolism, the microarray technique has been routinely used to examine differential expression of genes from metabolic active organs, such as the liver, white adipose tissue (WAT) and brown adipose tissue (BAT), at different nutritional states, such as diet induced obesity and fasting [8,9]. However, microarray technology has low sensitivity, and is limited to only known genes. In contrast, RNA-seq, a new sequencing based technology, is sensitive and identifies both known and unknown transcripts [10]. We therefore performed RNA-seq experiments on the liver and WAT in mice treated with a high-fat diet (HFD) or fasting.

Among the novel nutritionally regulated genes identified by the RNA-seq experiment (to publish elsewhere), we here focus on Gm6484 (Table 1), because of following reasons. (1) In humans, variations of C19ORF80, the human homologue of Gm6484, are linked to blood lipid levels by genome wide association studies [11]. (2) In mice, being among the mouse knockout library for secreted and transmembrane proteins, Gm6484 deletion largely reduced serum triglyceride levels [12]. We name the gene Lipasin (lipase inhibition). We here show that human *LIPASIN* is liver specific, while the mouse one is enriched in the liver and fat. Obesity increased *Lipasin* expression in the liver, while fasting reduced its expression in fat. Being evolutionarily conserved, LIPASIN is homologous to ANGPTL3's N-terminal domain that regulates blood lipids, and to ANGPTL4's N-terminal segment that mediates lipoprotein lipase (LPL) binding. Indeed, adenovirus-mediated overexpression of Lipasin increased serum triglycerides and a recombinant Lipasin inhibited LPL activity. Therefore, Lipasin is a novel nutritionally regulated liver-enriched factor that plays a role lipid metabolism.

2. Materials and methods

2.1. Mice

Mice were housed at 22–24 °C with a 14-h light, 10-h dark cycle and provided with *ad libitum* water and a chow diet (6% calories from fat, 8664; Harlan Teklad, Indianapolis, IN) unless otherwise

* Fax: +1 313 577 5218.
E-mail address: rzhang@med.wayne.edu

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Table 1
IDs of Lipasin in databases.

	Mouse	Human
Name	Lipasin	LIPASIN
Symbol	Gm6484	C19ORF80
Synonyms	EG624219	TD26; PRO1185; PVPA599
Chromosome	Chr9	Chr19
Location	21639954–21641790 bp	11350295–11352619 bp
RefSeq	NP_001074409	NP_061157
Ensembl	ENSMUSP00000058951	ENSP00000252453
Uniprot	Q8R1L8	Q6UXH0

indicated. To examine nutritional stimulation induced gene expression, 10 4-week-old male C57B6 mice (Jackson Laboratory, Bar Habor, ME) were placed on either a chow diet or a high-fat, high-sucrose diet (58% kcal from fat, 26% kcal from sucrose, D-12331; Research Diets, New Brunswick, NJ) for 3 months. Five 8-week-old mice were treated with 24-h fasting with four fed mice as controls. To examine the expression pattern of *Lipasin* in various mouse tissues, 3 8-week-old mice were used. All animal protocols were approved by the Animal Care and Use Committee of Wayne State University.

2.2. RNA extraction and quantitative real-time PCR

Dissected tissues were immediately placed into RNAlater solution (Ambion, Austin, TX) for subsequent RNA extraction. Total RNA was isolated from tissues with RNeasy tissue minikit with deoxyribonuclease treatment (QIAGEN, Valencia, CA). One microgram of RNA was reverse transcribed to cDNA using random hexamers (Superscript; Ambion). Relative expression levels were calculated and β-actin was used as an internal control. Primer sequences for *Lipasin* were: forward, 5′-CACTGTACGGAGACTACAA GTGC-3′; reverse, 5′-GTGGCTCTGCTTATCAGCTCG-3′. Primer sequences for β-actin were: forward, 5′-GTGACGTTGACATCCGTAA-AGA-3′; reverse, 5′-GCCGGACTCATCGTACTCC-3′. Two human normal cDNA arrays (Origene, Rockville, MD) were used to examine *LIPASIN* expression pattern in human tissues. Primer sequences for LIPASIN were: 5′-GCAAGCCTGTTGGAGACTCAG-3′; 5′-CTGTC CCGTAGCACCTTCT-3′.

2.3. Adenovirus injection, recombinant protein production and LPL activity assay

Mice were divided into three groups with five mice in each group. Each mouse was injected into the tail vein with 200 μl of saline (sham), or 5×10^8 pfu (diluted in 200 μl of saline) of adenoviruses expressing GFP (Vector Biolabs, Philadelphia, PA) or Lipasin (Applied Biological Materials, Richmond, BC). Blood was collected through tail nicks 48 h after virus injection, and serum triglyceride levels were determined by triglyceride quantification kit (Biovision, Milpitas, CA).

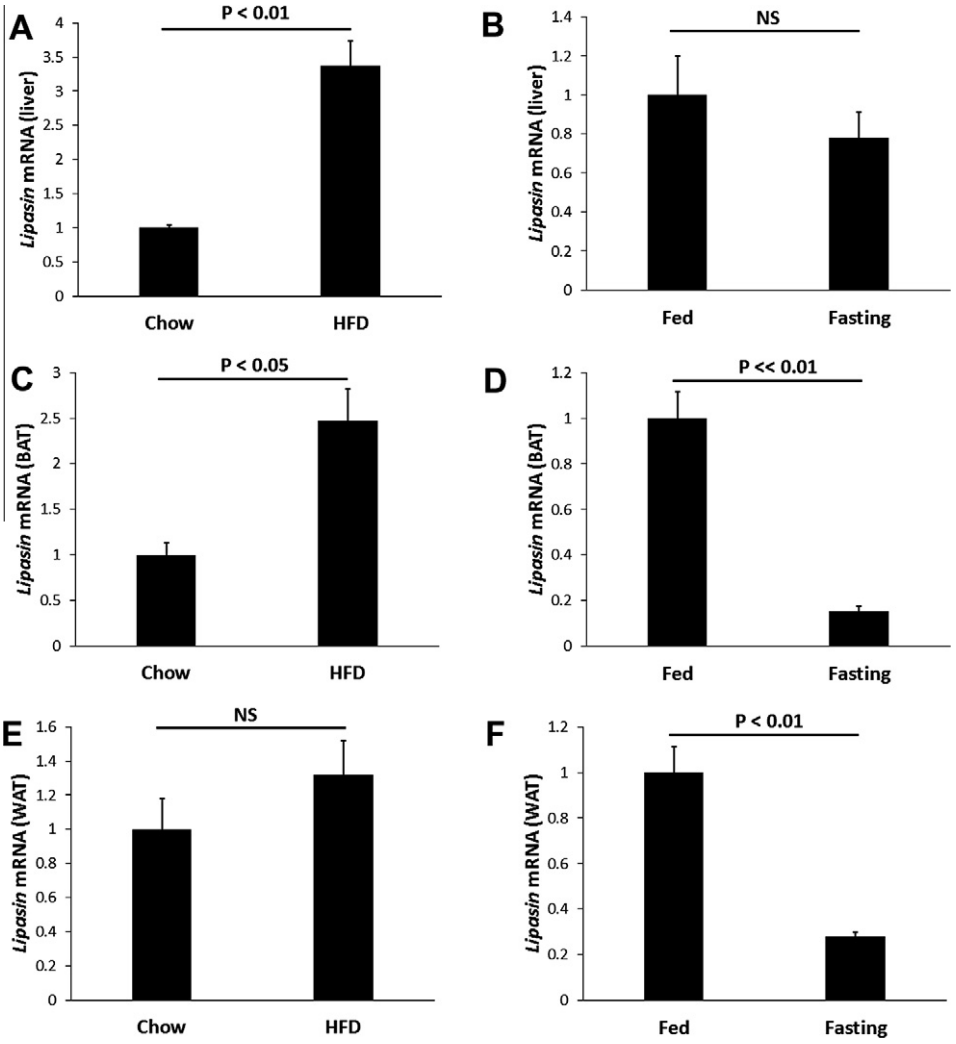

Fig. 1. Nutritional regulation of Lipasin. *Lipasin* mRNA levels in the liver (A and B), brown adipose tissue (C and D) and white adipose tissue (E and F) in mice treated with a high-fat diet for 3 months or 24-h fasting, respectively. Data are represented as mean ± SEM.

The DNA sequence of Lipasin was synthesized (Genscript, Piscataway, NJ) and cloned into pET 19 (Novagen, Billerica, MA) with an N-terminal 6-His tag. The recombinant protein was expressed in *Escherichia coli*, and the purified protein was resolved by 4–20% gradient gel, and then visualized by Coomassie Blue staining, and confirmed by Western blotting analysis using an antibody against the 6-His tag. The recombinant Lipasin in concentrations up to 100 nM was incubated with a purified LPL (Sigma, St. Louis, MO) for 30 min before assessing its activity by LPL Activity Assay Kit (Roar Biomedical, Calverton, NY) according to the manufactory instruction.

2.4. Multiple alignments

Lipasin protein IDs for *Homo sapiens*, *Pan troglodytes*, *Mus musculus* and *Rattus norvegicus* were NP_061157.3, XP_003316163.1, NP_001074409.1 and XP_002729923.1, respectively. The software ClustalW [13] was used to perform multiple alignments, and Blast [14] was used to perform pairwise comparison between two sequences.

2.5. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was tested with unpaired two-tailed Student's *t* tests. The differences were considered statistically significant if $P < 0.05$.

3. Results and discussion

To comprehensively identify nutritionally regulated genes, we performed RNA-seq experiments on the liver and white adipose tissue, in mice treated with 3-month HFD or 24-h fasting, together with controls. Here, we focus on the analysis of the novel gene Lipasin, which was one of the identified genes that were sensitive to nutritional stimulation. We performed qPCR analysis to confirm the expression levels of *Lipasin*. Indeed, in obese mice induced by HFD, liver *Lipasin* was increased for more than threefold ($P < 0.01$), while no significant change was observed in fasted mice (Fig. 1A and B). In BAT, HFD induced a more than twofold increase ($P < 0.05$); conversely, fasting induced an about 85% decrease in *Lipasin* expression ($P \ll 0.01$) (Fig. 1C and D). In WAT, although no significant change in obese mice, fasting induced more than 70% decrease ($P < 0.01$) in *Lipasin* expression (Fig. 1E and F). Therefore, *Lipasin* is nutritionally regulated.

Expression pattern of a novel gene can be an informative indicator of its potential functions. From qPCR experiments, we observed that the expression of *Lipasin* was relative high in the liver, because it was about 1/16 of the expression level of β-actin (the difference in CT values was about 4). To examine the expression pattern of *Lipasin* in other mouse tissues, we dissected 20 tissues for each of the three mice, and then performed qPCR analysis to examine *Lipasin* mRNA. The liver was the tissue with the highest expression. In addition, *Lipasin* was also highly expressed in fat, including brown fat, and subcutaneous fat and perigonadal fat

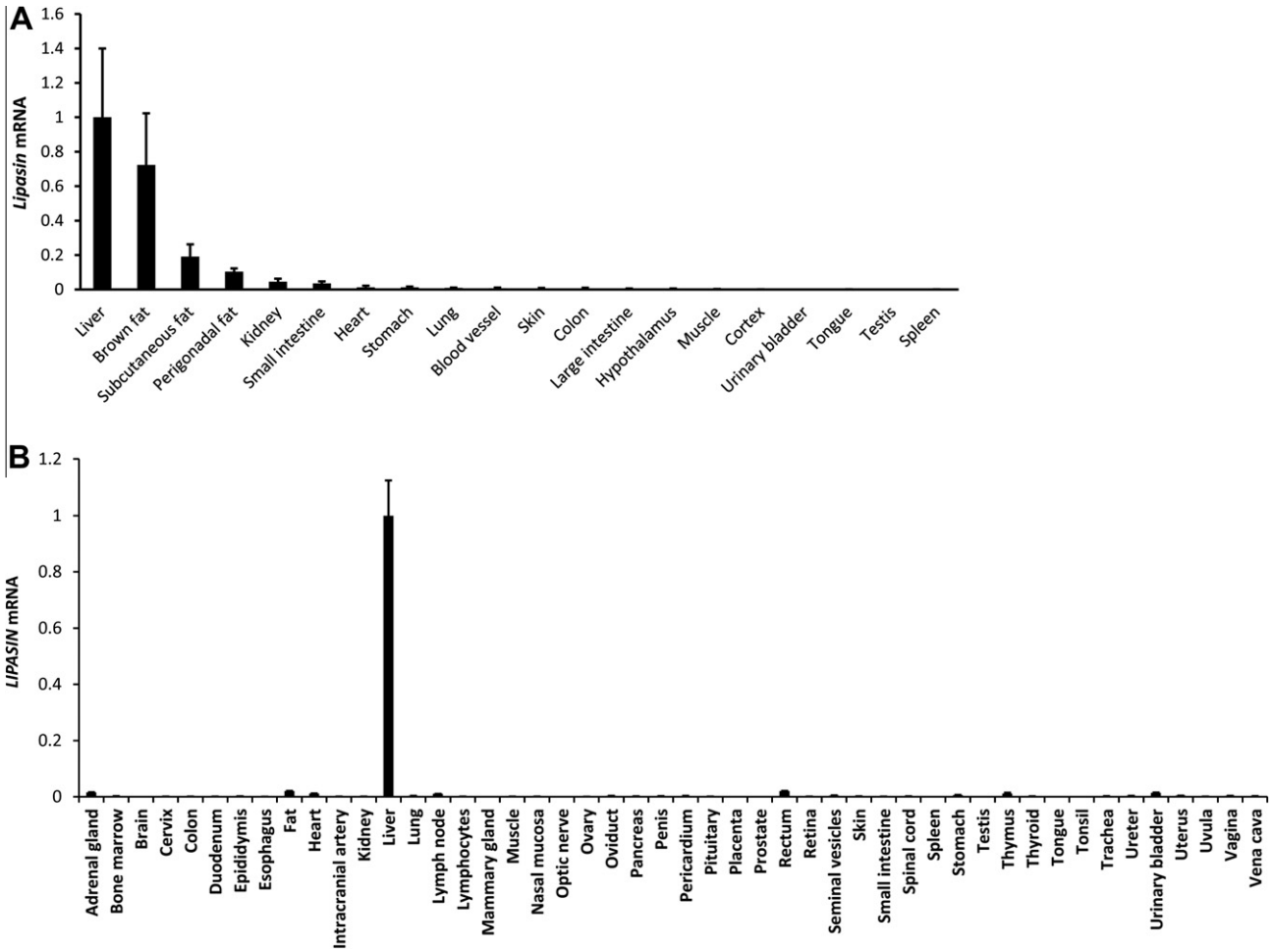

Fig. 2. Expression pattern of *Lipasin* in mice and humans. (A) *Lipasin* is enriched in mouse liver and fat, including brown fat, subcutaneous fat and perigonadal fat. (B) Human *LIPASIN* is liver specific.

(Fig. 2A). To examine *LIPASIN* expression patterns in human tissues, we used the human tissue cDNA array that contains cDNAs from 48 human tissues. Strikingly, *LIPASIN* was specific to human liver (Fig. 2B). Therefore, mouse *Lipasin* is enriched the liver and fat, while human *LIPASIN* is liver specific, suggesting its roles in metabolism.

Alignment of human and mouse Lipasin protein sequences showed that 144 of the 198 residues (73%) were identical, suggesting that the protein is evolutionarily conserved (Fig. 3A). A Blast search against NCBI protein database showed that LIPASIN was homologous to the N-terminal domain of ANGPTL3 from multiple species.

ANGPTL3 is a secreted protein that has been demonstrated to be critical in lipid metabolism [15]. ANGPTL3 has an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, and it is cleaved between the two domains (Fig. 3B). It has been demonstrated that the N-terminal domain is necessary and sufficient in increasing serum triglycerides [16]. ANGPTL4 has similar domain structure and is also critical in lipid regulation [17,18].

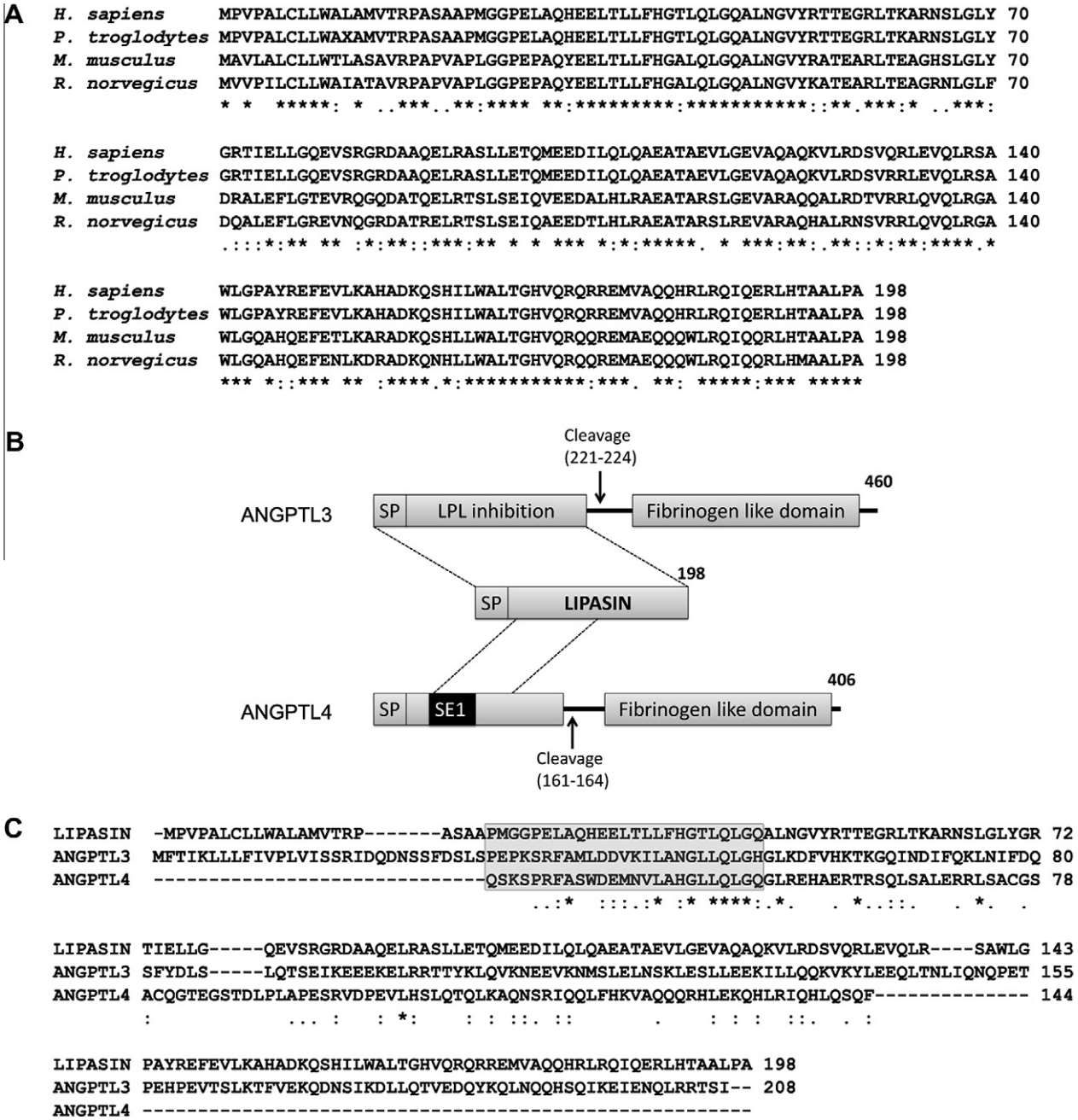

Fig. 3. LIPASIN is evolutionarily conserved, and homologous to the N-terminal domains of ANGPTL3 and ANGPTL4. (A) Alignments of Lipasin protein sequences from human, chimpanzee, mouse and rat. Seventy-three percent of human and mouse sequences (144 of 198) were identical. (B) LIPASIN is homologous to ANGPTL3's N-terminal domain that is critical for serum triglyceride regulation, and homologous to ANGPTL4's N-terminal domain that mediates LPL binding. Dotted lines denote homologous regions. SP, signal peptide; LPL, lipoprotein lipase; SE1, a segment that mediates LPL binding. The figure is not drawn to scale. (C) Alignments of protein sequences of LIPASIN and N-terminal domains of human ANGPTL3 and ANGPTL4. Human LIPASIN and ANGPTL3's N-terminal domain shared 22% identical residues with a P value being 6×10^{-12}, and shares homology with ANGPTL4's N-terminal domain with a P value being 2×10^{-5}.

Interestingly, LIPASIN is homologous to ANGPTL3's N-terminal domain that is released following cleavage in the linker region and mediates lipid regulation. Alignments of LIPASIN with human ANGPTL3 N-terminal domains showed 50% residues with similarities (Fig. 3C). This result suggests LIPASIN has similar activity as the N-terminal domain of ANGPTL3. In addition, LIPASIN also shared homology with ANGPTL4, albeit with shorter segment (about 100 residues at the N-terminal) (refer to Supplementary Fig. 1 for alignments of LIPASIN with ANGPTL3 and 4, separately). Of note, the homologous region contains the SE1 segment, which mediates binding of ANGPTL4 and LPL [17].

Overexpression of the N-terminal domain of ANGPTL3 increases serum triglyceride levels [16]. We therefore hypothesized that Lipasin overexpression increases serum triglyceride levels. To test this hypothesis, we injected adenoviruses that express recombinant Lipasin into five male mice via the tail vein (each mouse was infected with 5×10^8 pfu of adenoviruses diluted in 200 μl of saline). The Controls mice received injection of either 200 μl saline or the same amount of adenovirus overexpressing green fluorescent protein (GFP). Blood was collected 48 h after virus injection, followed by serum triglyceride determination. In sham treated mice, serum triglyceride level was about 80 mg/dL, similar to that in mice infected with GFP expressing adenoviruses (Fig. 4A). Injection of Lipasin expressing adenoviruses, however, increased serum triglyceride levels for more than fivefold, with an average of 452 mg/dL ($P < 0.01$) (Fig. 4A). Because ANGPTL3 inhibits LPL, we also tested if Lipasin has similar effects. We then expressed and purified the recombinant Lipasin with a 6-His tag at the N-terminal (Fig. 4B), which was verified by Western blotting analysis using an antibody against the 6-His tag (Fig. 4C). The activity of a purified bovine LPL was examined in the presence of the purified recombinant Lipasin in different concentrations, with a co-incubation for 30 min before the assay. Indeed, the recombinant Lipasin dose-dependently inhibited the LPL activity, and at the concentration of 100 nM, the LPL activity was reduced for more than 40% ($P < 0.05$) (Fig. 4D).

In humans *LIPASIN* is specific to the liver, clearly suggesting its role in metabolism. In addition to the liver, in mice *Lipasin* is also abundant in fat; nevertheless, expression in BAT appears to be much higher than in WAT. It is increasingly being recognized that adult humans have BAT as well, and its amount is inversely correlated with body mass index [19,20]. The 48 human tissues that we examined do not include BAT, therefore it cannot be ruled out that LIPASIN is also abundant in human BAT and plays a role in human BAT functions.

ANGPTLs are a family of secreted proteins that have seven members, and among them only ANGPTL3 and 4 have been demonstrated to be critical in controlling serum triglyceride levels [15,18,21,22]. In humans both ANGPTL3 and ANGPTL4 have been linked to circulating lipids in a number of genome wide association studies [11,23–26], and loss of function mutations in human ANGPTL3 were found to explain low plasma triglyceride [27,28]. In mice, consistently, serum triglyceride levels were decreased or increased by either overexpression or deletion, respectively, for both

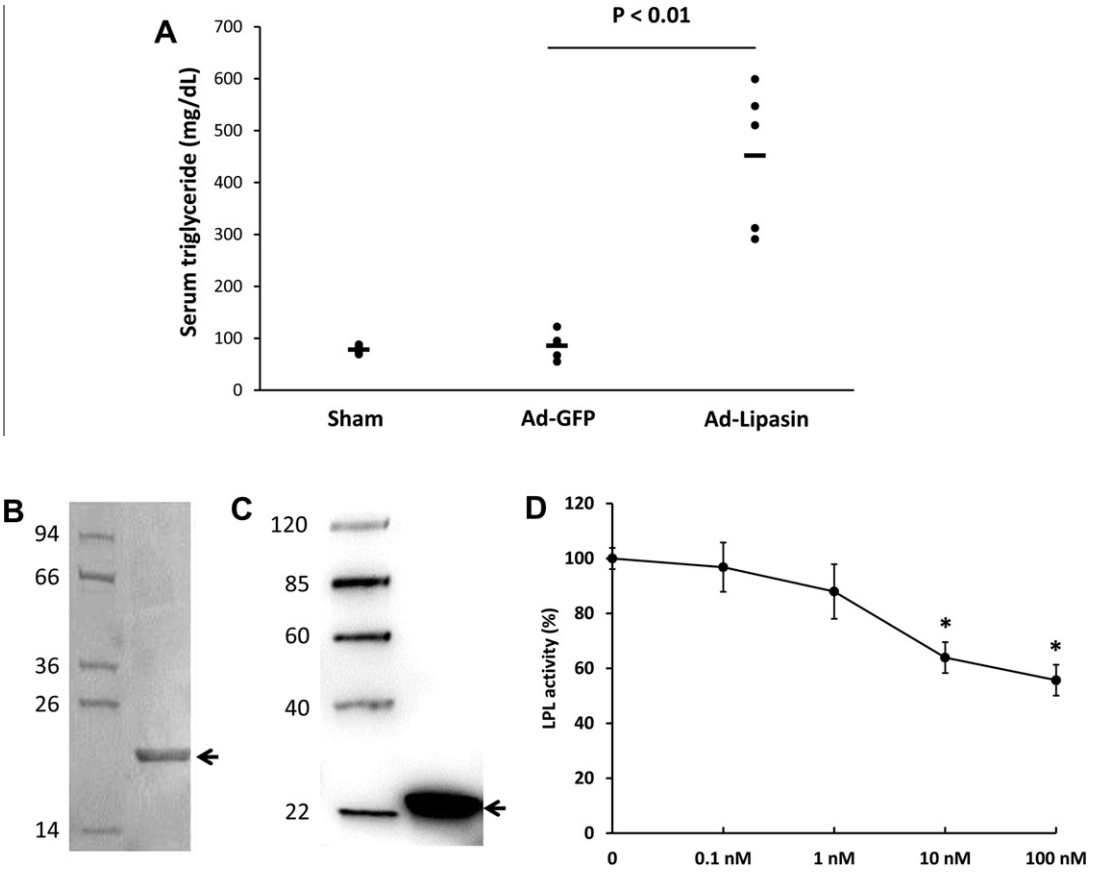

Fig. 4. Lipasin increases serum triglycerides, and inhibits lipoprotein lipase. (A) Adenovirus-mediated expression of Lipasin increased mouse serum triglycerides. Each group contains five mice, and each mouse was injected with 200 μl of saline (sham), or 5×10^8 pfu (diluted in 200 μl of saline) of adenoviruses expressing either green fluorescent protein (GFP) or Lipasin. Serum triglyceride levels were determined in blood samples collected 48 h after adenovirus infection. Bars denote average values. (B) A recombinant Lipasin with the 6-His tag at the N-terminal was expressed in *E. coli*, purified and then visualized by Coomassie Blue staining and (C) confirmed by Western blotting analysis using an antibody against the 6-His tag. Arrows indicates the position of the recombinant protein. (D) The recombinant Lipasin inhibited LPL activity. The activity of a purified LPL was examined in the presence of the purified recombinant Lipasin at indicated concentrations, with a co-incubation for 30 min before the assay. $^*P < 0.05$.

Angptl3 and Angptl4 [15,17,18,29,30]. LIPASIN shares homology with ANGPTL3's N-terminal domain that is necessary and sufficient to mediate its function in triglyceride regulation, and it also shares homology with ANGPTL4's N-terminal domain that mediates LPL binding. Nevertheless, LIPASIN is distinct from the other two ANGPTLs from some aspects. First, LIPASIN does not have a linker domain, and does not need to be cleaved. Second, nutritional regulation is different for the three genes. ANGPTL3 seems not sensitive to nutritional status, while fasting induces ANGPTL4 [29]. In contrast, Lipasin is nutritionally regulated; fasting dramatically reduced *Lipasin* in BAT and WAT, while obesity increased its expression in the liver and BAT. In addition, Lipasin may be inhibitory to other lipases, such as the endothelial lipase, which is inhibited by ANGPTL3 [31]. It is likely that Lipasin has both similar and distinct functions to the other two ANGPTLs, and there are complex regulations in coordinating the effects of the three genes.

In summary, Lipasin is a novel liver-enriched factor that is nutritionally regulated. Human *LIPASIN* is liver specific, while the mouse one is enriched in the liver and fat. Obesity induces *Lipasin* in the liver and BAT, while fasting reduces its levels in BAT and WAT. Being evolutionarily conserved, LIPASIN shares homology with ANGPTL3's N-terminal domain that is necessary and sufficient for lipid regulation, and with ANGPTL4's N-terminal segment that mediates LPL binding. Adenovirus-mediated overexpression of Lipasin increases serum triglycerides, and a recombinant Lipasin inhibits LPL activity. Therefore, a potential mechanism for Lipasin-mediated triglyceride increase is, in part, through reduced triglyceride clearance by LPL inhibition. Lipasin is thus a novel nutritionally regulated liver-enriched factor that plays a role in lipid metabolism, and is a potential drug target for treating dyslipidemia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.038.

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