Nonalcoholic fatty liver disease (NAFLD) has emerged as one of the most common chronic liver diseases over the past decade, with a prevalence of 20% to 30% in the general population. It encompasses a spectrum of liver damage ranging from steatosis to steatohepatitis, cirrhosis, and even hepatocellular carcinoma. Although the “two-hit” hypothesis has become a widely accepted framework to guide current

**Abbreviations:** C/EBP, CCAAT/enhancer binding protein; DIGE, difference gel electrophoresis; ECHS1, enoyl-CoA hydratase; FABPL, liver fatty acid binding protein; FFA, free fatty acid; HFD, high-fat diet; IPA, ingenuity pathway analysis; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; PPAR, peroxisome proliferator activated receptor; siRNA, small interfering RNA; TG, triglyceride.
studies in this area, the pathogenesis of NAFLD remains largely unknown. There is an urgent need to better understand how NAFLD occurs and to develop treatment strategies for afflicted patients.

It is increasingly recognized that both genetic and acquired factors contribute to the pathogenesis of NAFLD. Among the acquired determinants, diet and nutrition (in particular the amount and type of fat intake) can directly affect hepatic fatty infiltration and oxidative damage in different types of liver disease. Higher intake of saturated fat and a lower intake of the antioxidant vitamins C and E were characteristics of patients with nonalcoholic steatohepatitis (NASH) compared with obese people presenting no evidence of liver disease. Feeding Sprague-Dawley rats a high-fat diet (HFD) resulted in the reproduction of many key features of human NAFLD, including obesity, liver steatosis, inflammation, insulin resistance, abnormal mitochondria, oxidative stress, and increased collagen content. However, the exact role of saturated fat in the development of NAFLD remains poorly understood.

Proteomics may constitute an essential approach for elucidating the complex pathogenesis of NAFLD. Proteomic tools, such as two-dimensional difference gel electrophoresis (DIGE), provide a global approach to the discovery and quantification of the functional dynamics of the cell. As a modified form of two-dimensional electrophoresis, two-dimensional DIGE is based on labeling two to three samples with different fluorescent cyanine dyes that are then co-separated on the same two-dimensional gel, which minimizes spot pattern variability. Moreover, by incorporation of the same internal standard on every two-dimensional gel, DIGE has reduced the problem of inter-gel variation and improved quantification accuracy. With high reproducibility and reliability, two-dimensional DIGE technology has been applied widely to comparative proteomics.

In the current study, we attempted to define the liver proteins that contribute to the pathogenesis and progression of NAFLD in animal models. Male Sprague-Dawley rats fed with an HFD for 4, 12, and 24 weeks replicated the natural history of human NAFLD: simple steatosis, nonspecific inflammation, and then NASH. High-throughput proteomic tools, two-dimensional DIGE combined with tandem mass spectrometry, revealed a cluster of proteins exhibiting phase-specific changes at each stage of the disease. Among them, enoyl–coenzyme A hydratase (ECHS1), an enzyme that catalyzes the second step of mitochondrial fatty acid beta-oxidation, underwent further investigation. The reduced level of ECHS1 was validated both in rat models and in patients with simple steatosis. We further demonstrated that the down-regulation of ECHS1 by small interfering RNA (siRNA) significantly exacerbated lipid accumulation in hepatocytes induced by free fatty acid (FFA) overload both in vitro and in vivo.

Materials and Methods

Animals Models of NAFLD. Male 12-week-old Sprague-Dawley rats weighting 160 to 170 g were purchased from the Medical Science Institution of Zhejiang Province (Hangzhou, China). All rats were maintained in a 12-hour day/night cycle at controlled room temperature and provided free access to food and water. The rats were acclimatized to laboratory conditions for 1 week before the study and then randomly divided into two groups: (1) control group (n = 30): animals treated with the standard chow diet (8% rice bran, 51% maize, 30% soybean powder, 3% bone powder, 1.3% multivitamin, and 6.7% mineral); (2) HFD group (n = 30): animals given an HFD (80.5% SCD, 2% cholesterol, 7% lard, 10% yolk powder, and 0.5% bile salt). Ten rats of each group were sacrificed at weeks 4, 12, and 24 after being placed on the respective diet. Blood was collected just before sacrifice for serum biochemical analysis. Livers were quickly excised, cleaned completely with ice-cold phosphate-buffered saline (PBS), and preserved in liquid nitrogen until use. Pieces of the remaining liver tissues were processed for histology. All animal studies were approved by the Animal Care and Use Committee of Zhejiang University in accordance with the Chinese guidelines for the care and use of laboratory animals.

Patients and Samples. Access to human tissues complied with the guidelines of the Ethics Committee of Zhejiang Province. Twenty patients with biopsy-proven NAFLD were enrolled from Medical Treatment Center of Lihuili Hospital for further validation. All of the patients were defined as simple steatosis according to the histological manifestation. Patients were excluded if alcohol consumption was more than 40 g/day for men and more than 20 g/day for women and if other liver diseases were detected by serological testing and imaging studies. For comparison purpose, 14 healthy controls undergoing hemangioma surgery at Lihuili Hospital without clinical signs or symptoms of other cause of liver disease, and no history of chronic illnesses, were analyzed (Supporting Table 1).

Histological Examination. See Supporting Material. Two-Dimensional DIGE and Matrix-Assisted Laser Desorption Ionization Time of Flight/Time of Flight Analysis. The prepared liver tissue lysates were labeled with Cy2, Cy3, and Cy5 following the protocols described in the Ettan DIGE User Manual Supporting Table 2). Gel electrophoresis in the first dimension was
performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 3-10 linear, 18 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12%, 20 × 24 cm² sodium dodecyl sulfate polyacrylamide gels. Cy2-labeled, Cy3-labeled, and Cy5-labeled images were acquired on a Typhoon 9410 scanner (GE Healthcare) at the excitation/emission values (nm) of 488/520, 532/580 and 633/670, respectively. The resulting DIGE images were analyzed using DeCyder version 5.02 software (GE Healthcare). Statistically significant spots (P < 0.05) as compared by Student test and with an average volume ratio over 1.5-fold between control and HFD groups were defined as “differentially-expressed proteins” and excised from preparative gels for in-gel digestion and matrix-assisted laser desorption ionization time of flight/time of flight identification (see Supporting Material).

**Functional Annotation and Pathway Analysis.** Differentially expressed proteins during the development of NAFLD were characterized in more detail using various annotation tools, including the “Panther Classification System” (www.pantherdb.org) for functional annotation and the “Ingenuity Pathway Analysis” (IPA) (www.ingenuity.com) for pathway and network-oriented analysis.

**Immunoblotting and Immunohistochemistry.** See Supporting Material.

**Small Interfering RNA.** For RNA silencing, four different sequences of siRNA targeting mouse ECHS1 were designed and synthesized by GenePharma. The siRNA 1 was raised against the sequence beginning at nt 344 (5'-GAACATTTCAGGACTGTTA-3'), siRNA 2 at 413 (5'-cgctggcagaa-3'), siRNA 3 at 480 (5'-catctatgcttgagtgaag-3'), and siRNA 4 at 766 (5'-gaccttgagatggttaaa-3'). Nonsilencing siRNA was a commercially available duplex (GenePharma) that was used as a negative control siRNA. The siRNA 4 with 2'-O-Me modification was used for in vivo studies.

**Cell Transfection and In Vitro Model of Cellular Steatosis.** For gene silencing, the mouse hepatocyte cell lines, AML12 hepatocytes were transfected with 200 pmol siRNA using the Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. Seventy-two hours after transfection, protein extracts were analyzed by immunoblotting to confirm protein knockdown.

Fat overloading induction of cells was done mainly according to previously established methods in which AML12 cells were exposed to a mixture of FFAs (oleate and palmitate) at a final ratio of 2:1 and final concentration of 1 mM. To assess the influence of ECHS1 knockdown on cellular steatosis, cells were treated with FFAs after 72 hours of siRNA transfection and harvested after 24 hours incubation with FFAs.

**Hydrodynamic Tail Vein Injection and In Vivo Model of Hepatic Steatosis.** Male Balb/c mice, 8 weeks of age, were purchased from Vital River and acclimated for 1 week after arrival before they were used for experiments. Synthetic ECHS1 siRNA 4 with 2’-OMe modification were delivered in vivo using a modified “hydrodynamic transfection method” by which 60 μg siRNA dissolved in 1.8 mL PBS was rapidly injected into the tail vein. The control mice were injected with an equal volume of negative control siRNA. The animals were then treated with control or HFD diet after 24 hours of siRNA injection and sacrificed at the fourth week after being placed on the diet. The siRNA injection was repeated once per week during the diet.

**Oil Red O Staining.** Cells were washed twice with PBS and fixed with 7.5% formaldehyde in PBS for 30 minutes. After two washes in PBS, cells were stained for 15 minutes in freshly diluted Oil Red O solution. The dishes were then rinsed in water and counterstained with hematoxylin for 10 seconds. To determine hepatic lipid accumulation, frozen sections of liver (10 μm) were stained with Oil Red O for 10 minutes, washed, and counterstained with hematoxylin for 20 seconds. Representative photomicrographs were captured at 200× magnification using a system incorporated in the microscope.

**Triglyceride and FFA Assay.** Intracellular and liver triglycerides were assayed using a triglyceride assay kit (GPO-POD; Applygen Technologies Inc., Beijing, China). Intracellular and hepatic free fatty acids were estimated using an ultrasensitive assay kit for free fatty acids (Applygen Technologies Inc.) according to the manufacturer’s recommended protocol.

**Statistical Analysis.** Results are expressed as means ± standard error. The significance of the difference in means was determined by two-tailed Student t test.

**Results**

**Effects of High-Fat Diet on Sprague-Dawley Rats.** The HFD successfully induced hepatic steatosis and steatohepatitis in rats (Fig. 1). After 4 weeks of HFD treatment, approximately 30%-60% of all hepatic parenchymal cells were filled with multivesicular fat (mainly microvesicular), but no necroinflammatory lesion was observed (Fig. 1B). At 12 weeks, besides a marked increase in fat droplets, focal hepatocyte necrosis was notable in the centrilobular region with mild to moderate infiltration of inflammatory cells (Fig. 1C). By 24 weeks, most of the hepatic lobules lost their normal architecture, which was associated with ballooned hepatocytes in centrilobular parenchyma and plenty of macrovesicular fat (Fig. 1D). No histological abnormalities were ob-
erved in the livers of the control group (Fig. 1A). The HFD resulted in a significant \( (P < 0.01) \) increase in the circulating levels of alanine aminotransferase, aspartate aminotransferase, triglycerides (TG), and cholesterol. There was also an increase in the levels of insulin \( (P < 0.05) \) and a decrease in leptin \( (P < 0.05) \). However, there were no significant effects of HFD on body weight, serum TG, and glucose levels (Table 1).

**Quantitative Proteomic Analysis.** The liver protein profiles of Sprague-Dawley rats fed with HFD and control diet (Chow) for 4, 12, or 24 weeks were determined by a two-dimensional DIGE approach. Based on the results of the image analysis with DeCyder software (GE Healthcare), an average of 1600–1800 protein spots were detected per gel. Differentially expressed spots were produced by the two-dimensional pattern comparison between control and HFD groups at each time point. According to the Student \( t \) tests, 111, 63, and 68 protein spots showed more than 1.5-fold difference with statistical significance \( (P < 0.05) \) by 4, 12, and 24 weeks, respectively. These protein spots were picked from the preparative gels and subject to in-gel digestion and mass spectrometry/mass spectrometry analysis. For each time point, 53, 42, and 49 differentially expressed proteins were identified (Fig. 2), with 95 unique proteins observed altogether (Supporting Table 3).

Immunoblot analyses of several proteins with commercially available antibodies were performed to confirm the DIGE result. Among them are enzymes involved in lipid metabolism (liver fatty acid binding protein [FABPL], ECHS1) and amino acid metabolism (carbamoyl-phosphate synthase, argininosuccinate synthase), endoplasmic reticulum chaperones (protein disulfide isomerase [PDI], protein disulfide isomerase A3), and proteins in other processes (14-3-3 epsilon, coflin 1, and macrophage migration inhibitory factor). As shown in Fig. 3, the results of western blot were in accordance with that of proteomic analysis.

**Functional Annotation of Differentially Expressed Proteins.** Differentially expressed proteins at each time point were classified into several functional categories using the tools at www.pantherdb.org, which showed phase-specific biological characteristics during the development of NAFLD (Supporting Fig. 1). At the simple steatosis stage, almost half of the identified proteins were metabolic enzymes and transporters, involved in lipid, amino acid, carbohydrate, and other metabolic processes. By the second stage (nonspecific inflammation), mitochondrial proteins were mostly affected (approximately 25%), including four enzymes involved in mitochondrial electron transduction and oxidative phosphorylation. At the last stage (NASH), nearly 20% were cytoskeleton proteins, which showed significantly increased expression levels during the development of NASH.

Biological functions and signaling networks were further identified by IPA, a robust tool to develop biological

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**Table 1. Serum Biochemical Parameters of Animal Models**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow (4w n = 10)</th>
<th>HFD</th>
<th>Chow (12w n = 10)</th>
<th>HFD</th>
<th>Chow (24w n = 10)</th>
<th>HFD</th>
</tr>
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<tbody>
<tr>
<td>Weight (g)</td>
<td>376.5 ± 23.3</td>
<td>385.7 ± 25.8</td>
<td>453.7 ± 34.9</td>
<td>483.4 ± 36.0</td>
<td>553.0 ± 45.3</td>
<td>554.1 ± 65.0</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.33 ± 0.14</td>
<td>0.40 ± 0.21</td>
<td>0.47 ± 0.09</td>
<td>0.51 ± 0.18</td>
<td>0.50 ± 0.14</td>
<td>0.54 ± 0.26</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.42 ± 0.35</td>
<td>2.05 ± 0.46**</td>
<td>1.71 ± 0.18</td>
<td>2.57 ± 0.49**</td>
<td>1.95 ± 0.32</td>
<td>2.79 ± 0.48**</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>37.9 ± 7.8</td>
<td>64.2 ± 27.6**</td>
<td>44.9 ± 4.5</td>
<td>217.5 ± 31.2**</td>
<td>46.6 ± 6.79</td>
<td>140.1 ± 58.0**</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>148.4 ± 54.3</td>
<td>179.6 ± 42.6</td>
<td>127.7 ± 16.9</td>
<td>328.6 ± 45.2**</td>
<td>136.6 ± 21.7</td>
<td>215.9 ± 56.5**</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>5.59 ± 1.87</td>
<td>4.45 ± 1.06</td>
<td>5.33 ± 0.46</td>
<td>4.83 ± 0.54</td>
<td>5.67 ± 0.75</td>
<td>5.34 ± 0.62</td>
</tr>
<tr>
<td>Insulin (ng/dL)</td>
<td>24.79 ± 1.47</td>
<td>27.25 ± 3.05*</td>
<td>27.05 ± 8.92</td>
<td>29.84 ± 5.04</td>
<td>27.82 ± 3.46</td>
<td>28.45 ± 4.78</td>
</tr>
<tr>
<td>Leptin (ng/dL)</td>
<td>0.59 ± 0.25</td>
<td>0.39 ± 0.07*</td>
<td>0.74 ± 0.26</td>
<td>0.55 ± 0.17</td>
<td>0.91 ± 0.42</td>
<td>0.56 ± 0.34</td>
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Data are the mean ± SEM. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

* \( P < 0.05 \), Chow versus HFD.

** \( P < 0.01 \), Chow versus HFD.
networks based on the Ingenuity Pathways Knowledge Base. Based on the 95 unique proteins identified by DIGE, three highly ranked networks were identified, and the top ranked network incorporated 16 focus proteins identified by DIGE (Fig. 4A). This network identified peroxisome proliferator activated receptor α (PPARα), CCAAT/enhancer binding protein α (C/EBPα), and sterol regulatory element binding proteins as the key signaling “nodes,” which interacted with many differentially expressed proteins involved in urea cycle (carbamoylphosphate synthase, arginase-1, and argininosuccinate synthase), fatty acid β-oxidation (peroxisomal multifunctional enzyme type 2 [HSD17B4] and fatty acid-binding protein, liver [FABpL]), ketogenesis (hydroxymethylglutaryl-CoA synthase [HMGCS2]), and lipid transport (adipose differentiation-related protein [ADRP]), and other processes. In view of their central position in the network, the expression of PPARα and C/EBPα were confirmed by semiquantitative western analysis. PPARα belongs to the peroxisome proliferators activated receptor superfamily and regulates the transcription of many genes encoding enzymes involved in fatty acid β-oxidation. Its expression level was decreased at all the three stages of NAFLD (Fig. 4B, 4C), in accordance with the decreased expression of enzymes involved in fatty acid β-oxidation in our results. The abundance of C/EBPα was increased at the early two stages of NAFLD and decreased at the latest stage (Fig. 4B, 4D).

ECHS1 Level in Patients with Simple Steatosis. One of the differentially expressed proteins, ECHS1, was
Further validated in patients with simple steatosis. ECHS1 functions in the second step of the mitochondrial fatty acid beta-oxidation pathway. The relationship between ECHS1 and NAFLD has never been reported. Immunohistochemistry staining of ECHS1 was performed on paraffin sections that contained both steatotic and normal liver tissue from 20 patients with simple steatosis and 14 controls. Results showed that the positive staining rate of ECHS1 was 75% to 100% in control tissues. In steatotic liver tissues, only a minor portion of hepatocytes (approximately 25%-50%) were positively stained (Table 2 and Supporting Fig. 2). The ECHS1 was stained in the cytoplasm of hepatocyte (Fig. 5).

Silencing ECHS1 Expression Exacerbates Hepatocellular Steatosis In Vitro Models. To determine the role of ECHS1 on hepatic steatosis, four pairs of siRNA duplexes were constructed for down-regulation of ECHS1 in the mouse hepatocyte cell lines, the AML12 hepatocytes. Immunoblot analysis showed that the inhibitory efficiency of the fourth pair of siRNAs was more significant than that of the other three pairs of siRNAs and they were therefore used in the following experiment (Fig. 6A, B).

To determine whether knockdown of ECHS1 has a crucial role on lipid accumulation in hepatocytes, AML12 cells transfected with ECHS1 siRNA or negative siRNA were cultured in full media with or without FFA mixtures. As depicted in Fig. 6C, Oil Red O staining demonstrated that increased lipid stores were present in hepatocytes when cells were treated with FFAs for 24 hours. The increases were markedly augmented in cells transfected with ECHS1 siRNA. As expected, the siRNA knockdown of ECHS1 resulted in a significant 60% to 70% increase in cellular triglyceride (P < 0.05) (Fig. 6D). Because of the role of ECHS1 in fatty acid oxidation, the cellular FFAs levels were assessed. Figure 6E showed a 30% to 40% increase in FFA levels with ECHS1 siRNAs as compared with negative siRNA after FFAs exposure (P < 0.05).

Silencing ECHS1 Expression Exacerbates Hepatic Steatosis In Vivo Models. On the basis of in vitro results, we next examined whether ECHS1 siRNA treatment also exacerbated HFD-induced hepatic steatosis in mice. We adopted a modified “hydrodynamic tail vein injection” to delivered synthetic siRNAs into mouse hepatocytes in vivo. ECHS1 protein expression was measured by immunoblotting after 72 hours of injection. The result showed that ECHS1 siRNA 4 reduced ECHS1 protein expression by approximately 80% as compared with negative siRNA injection (Fig. 7A, B).

To evaluate further the effect of ECHS1 on HFD-induced hepatic steatosis, mice treated with ECHS1 siRNA 4 or negative siRNA were fed with either control or high-fat diet for 4 weeks. Because siRNA-mediated
suppression of genes in vivo in mouse liver was maintained for approximately 10 days,\textsuperscript{15,16} the siRNA injection was repeated once per week for 4 consecutive weeks during the diet. As shown in Fig. 7C, Oil Red O staining demonstrated that an HFD resulted in increased hepatic lipid stores in mice. Silencing ECHS1 expression by siRNA further exacerbated steatotic changes in the liver (Fig. 7C). In accordance with morphological findings, hepatic triglyceride content increased by 35% in mice injected with ECHS1 siRNAs than with negative siRNAs after 4 weeks of HFD diet ($P < 0.05$) (Fig. 7D). There was also a significant increase in hepatic FFAs with ECHS1 siRNAs ($P < 0.05$) (Fig. 7E).

Discussion

Despite the high prevalence and the potential to progress to end-stage liver disease, the molecular mechanisms underlying NAFLD initiation and progression remains poorly understood. The current study systematically analyzed the liver proteomes during different stages of NAFLD in an HFD-induced rat models, which may help elucidate the mechanism involved in the progression of the disease.

**Table 2. Immunohistochemistry Results for ECHS1 in Patients with Simple Steatosis and Controls**

<table>
<thead>
<tr>
<th></th>
<th>−</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>+++++</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>NAFLD</td>
<td>0</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

Specimens with 0, −25, −50, −75, and −100% positively stained hepatocytes were scored as −, +, ++, ++++, and +++++, respectively.

**Fig. 5.** Immunohistochemistry results of ECHS1. Liver sections from simple steatosis patients and controls were used for immunohistochemistry staining. The positive staining rate of ECHS1 was 75% to 100% in control tissues (A). In steatotic liver tissues, only a minor portion of hepatocytes (approximately 25%-50%) were positively stained (B). The ECHS1 was stained in the cytoplasm of the hepatocyte.
Ninety-five unique proteins associated with the development of NAFLD were identified through a comparative proteomics approach. Several of the proteins, including ATP5B, FABPL, and prohibitin, have already been indicated in published studies addressing protein expressions in hepatic steatosis or NASH. Nonetheless, functional annotation of the identified proteins indicated phase-specific biological characteristics during different stages of NAFLD, which were not reported in previous proteomic studies. We found that enzymes involved in fatty acid oxidation (ECHS1, short chain 3-hydroxyacyl-CoA dehydrogenase [HADH], HSD17B4, and FABPL) and mitochondrial respiration chain (ubiquinol-cytochrome c reductase core protein [IUQCRC1], cytochrome c oxidase polypeptide Va [COX5A], electron-transfer-flavoprotein, beta polypeptide [ETFB], and ATP synthase beta chain [ATP5B]) were down-regulated during simple steatosis and nonspecific inflammation stage, respectively, whereas cytoskeleton proteins were up-regulated in NASH. These findings indicated that impairment of fatty acid oxidation may contribute to the initiation of hepatic steatosis, whereas mitochondrial dysfunction and cytoskeleton disturbance were associated with disease progression. The findings also provide a molecular explanation for the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of NASH. Those cytoskeleton proteins with increased expression level in the last stage would be potential markers of NASH. One of these proteins, cytokeratin 18, has already been considered as a novel biomarker of NASH. The level of caspase 3–generated cytokeratin 18 fragment was reported to be correlated with disease severity in NAFLD patients.

IPA showed that several identified proteins involved in lipid metabolism were regulated by PPARα, a transcriptional activator of fatty acid oxidation genes. In our study, a reduced protein level of PPARα was found in rat models of NAFLD, in accordance with the down-regulation of its downstream genes involved in fatty acid oxidation (HSD17B4 and FABPL). These findings demonstrated that impairment of fatty acid β-oxidation occurred in our model, which may contribute to the pathogenesis of NAFLD. Another transcription factor identified by IPA was C/EBPα, which is generally considered a master regulator of adipose tissue development. More recently, the C/EBP family has emerged as an important group of regulators of hepatic lipogenesis. The up-regulation of C/EBPα and its target gene ADRP were detected in the current study, indicating an enhance-
ment of lipogenesis in response to HFD treatment. Taken together, the underlying cause of fat accumulation in NAFLD is mostly attributable to the inhibition of fatty acid oxidation and enhancement of lipid synthesis.

These results emphasized the power of proteomic approaches in providing high-throughput information to elucidate the complex pathogenesis of NAFLD. To give a more profound insight into the mechanism of NAFLD, one of the identified proteins, ECHS1, underwent further investigation. ECHS1 catalyzes the second step in the physiologically important β-oxidation pathway of fatty acid metabolism. Oxidation of fatty acids occurs in three subcellular organelles, with β-oxidation confined to mitochondria and peroxisomes and ω-oxidation occurring in the endoplasmic reticulum.23,24 Although impairment of mitochondrial β-oxidation of fatty acid was considered to account for excess lipid storage in several animal models of hepatic steatosis,28-30 the expression level and the potential role of ECHS1 in the pathogenesis of NAFLD have not been fully elucidated. In the current study, we found that the expression of ECHS1 protein was significantly down-regulated in rats fed with HFD. The results were further confirmed in patients with simple steatosis. Moreover, siRNA-mediated knockdown of ECHS1 significantly exacerbates lipid accumulation in hepatocytes in in vitro and in vivo models of NAFLD. The increases of FFA levels by ECHS1 silencing were in parallel with that of TG levels. These findings indicated that ECHS1 down-regulation contributed to the defect of mitochondrial fatty acid β-oxidation, which subsequently leads to triglyceride storage in the liver. However, whether decreased mitochondrial fatty acid oxidation induced by ECHS1 down-regulation is regulated by PPARα or whether it plays a primary role in causing the insulin resistance,28,31 remains to be determined.

One limitation of the current study is that the HFD dietary animal model used here did not manifest obesity, high serum TG level, or insulin resistance, although this model developed hepatic steatosis and steatohepatitis that were morphologically similar to human NAFLD. Many published studies used HFD-induced NAFLD models that differed in phenotypes. Some manifested obesity,9,32 and others did not.33,34 The reasons are implicated. The species, strain, or sex of the animals, the amount and type of dietary fat, and the diet duration are all important in influencing vulnerability to dietary effects. Because few animal models can entirely reflect the natural course and the causative background of human NAFLD, it would be better to validate differentially expressed proteins obtained from animal models in human samples. The 2D-

Fig. 7. Silencing ECHS1 expression in vivo exacerbated hepatic steatosis induced by the HFD. (A) Immunoblot of liver lysate obtained from mice treated with modified ECHS1-s4 or negative siRNA 72 hours after injection. (B) Relative protein level of ECHS1. ECHS1-s4 significantly reduced ECHS1 protein level in mice livers. (C) Oil Red O staining of liver sections obtained from mice treated with modified ECHS1-s4 or negative siRNA after chow or HFD for 4 weeks. Original magnification, 200×. (D) Influence of ECHS1 knockdown on hepatic TG and FFA (E) content in mice treated with HFD. Data are mean ± standard deviation in B, D, and E; n = 5, *P < 0.05 versus negative siRNA-treated mice. **P < 0.01 versus negative siRNA-treated mice.
DIGE method we used in the current study also has its own shortcomings. As a two-dimensional technique, it does not generally permit the resolution of proteins with high (>150 kDa) or low (<10 kDa) molecular masses, or very basic or hydrophobic proteins, thus limiting the proteomic coverage of most biological sample types.

In conclusion, we comprehensively studied the nonalcoholic fatty liver proteome in rat models by quantitative global protein profiling using DIGE technology. We identified proteins that were differentially expressed during various stages of NAFLD. Analysis of biological processes and regulatory networks provided both global and specific information regarding the molecular events that cause this complex disease. The impairment of liver fatty acid oxidation, as well as mitochondrial dysfunction, and the changes of cytoskeleton organization provide a molecular feature for the pathogenesis and progression of NAFLD. More importantly, the down-regulation of ECHS1 was found to be associated with mitochondrial proteins as targets of S-adenosylmethionine. Proc Natl Acad Sci U S A 2003;100:3065-3070.

References