Elevated LDL-cholesterol is a risk factor for the development of cardiovascular disease. Thus, proper control of LDL-cholesterol homeostasis is critical for organismal health. Genetic analysis has identified PCSK9 (proprotein convertase subtilisin/kexin type 9) as a crucial gene in the regulation of LDL-cholesterol via control of LDL receptor degradation. Although biochemical characteristics and clinical implications of PCSK9 have been extensively investigated, epigenetic regulation of this gene is largely unknown. In this work we have discovered that Sirt6, an NAD+-dependent histone deacetylase, plays a critical role in the regulation of the Pcsk9 gene expression in mice. Hepatic Sirt6 deficiency leads to elevated Pcsk9 gene expression and LDL-cholesterol as well. Mechanistically, we have demonstrated that Sirt6 can be recruited by forkhead transcription factor FoxO3 to the proximal promoter region of the Pcsk9 gene and deacetylates histone H3 at lysines 9 and 56, thereby suppressing the gene expression. Also remarkably, overexpression of Sirt6 in high fat diet-fed mice lowers LDL-cholesterol. Overall, our data suggest that FoxO3 and Sirt6, two longevity genes, can reduce LDL-cholesterol levels through regulation of the Pcsk9 gene.

Elevated LDL-cholesterol is a risk factor for cardiovascular disease (1). High LDL-cholesterol can be caused by a number of dysregulated processes, including increased cholesterol biosynthesis, increased VLDL secretion, and decreased LDL clearance (2). Genetic studies have identified mutations in at least three genes that significantly contribute to autosomal dominant hypercholesterolemia, and they are LDL receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin kexin type 9 (PCSK9) (3). LDLR plays a major role in the LDL clearance. Apolipoprotein B, a protein component of LDL, also interacts with LDLR. PCSK9 can modulate the LDL metabolism through control of the LDLR degradation in the lysosome (3).

Since the discovery of PCSK9 mutations in the autosomal dominant hypercholesterolemia patients a decade ago (4), significant progress has been made in the understanding of PCSK9 biochemistry and pathophysiology (5). Now we know that PCSK9 is expressed mainly in the liver as a ~72-kDa precursor and can be auto-cleaved in the endoplasmic reticulum to an ~62-kDa mature form that is secreted to plasma. Circulating PCSK9 binds to the extracellular EGF-A domain of the LDLR and targets it for degradation in the lysosome (5). The physiological function of PCSK9 in the control of LDL-cholesterol has also been confirmed by mouse genetics. Overexpression of PCSK9 in mice leads to hypercholesterolemia, and the Pcsk9 gene knock-out in mice dramatically reduces LDL-cholesterol (6–13). Because of this biological function, PCSK9 has become a useful target for lowering LDL-cholesterol, and several clinical trials are in progress to validate the efficacy of targeting PCSK9 for cardiovascular disease (14–21).

PCSK9 gene expression can be induced by insulin and pioglitazone and also can be suppressed by glucagon, bile acids, berberine, fibrate, and oncostatin M (22–32). PCSK9 protein levels decrease in the course of fasting and increase after feeding (22, 27, 29, 32–34). A number of transcription factors or cofactors have been shown to regulate the PCSK9 gene expression, including sterol-response element binding proteins (SREBP-1/2), hepatocyte nuclear factor 1A (HNF1A), farnesoid X receptor, peroxisome proliferator-activated receptor γ, liver X receptor, and histone nuclear factor P (24, 28, 29, 33–37). However, how the PCSK9 gene expression is controlled by epigenetic

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2 The abbreviations used are: LDLR, LDL receptor; HFD, high fat diet; HNF1A, hepatocyte nuclear factor 1A; Mttp, microsomal triglyceride transfer protein; PCSK9, proprotein convertase subtilisin kexin type 9; SIRT6, sirtuin 6; SRE, sterol response element; SREBP, sterol-response element binding protein; IP, immunoprecipitation.
chromatin remodeling is not clear. In this work we have identified sirtn6 (Sirt6) as a critical histone deacetylase for the Pcsk9 gene regulation and LDL-cholesterol homeostasis.

MATERIALS AND METHODS

Animal Studies—FoxO1 (forkhead box O1), FoxO3, FoxO1/3/4, Sirt1, and Sirt6 liver-specific knock-out mice were produced by crossing floxed mice with an albumin-Cre line from The Jackson Laboratory. Animals were maintained on the following genetic background: FoxOs floxed mice on C57BL/6j: 129/Sv/FVB, Sirt1 floxed mice on C57BL/6j: 129/Sv, and Sirt6 floxed mice on NIH Black Swiss: 129/Sv:FVB. Genotyping was carried out as previously described (38–40). High fat diet (60% calories from fat) was purchased from Harlan Laboratories (Madison, WI). For the VLDL secretion analysis, mice were fasted for 4 h before a dose of 500 mg/kg body weight Triton WR1339 was injected via tail vein. Blood samples were collected and analyzed as previously described (41). All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine.

Plasmid Constructs and Adenoviruses—For mouse Pcsk9 gene promoter analysis, we cloned the short promoter (−128 to +330 bp relative to the transcriptional start site) together with the 5′-untranslated region (UTR) into pGL4.10 vector (Promega) using the primers mPcsk9-pro-forward (5′-GGCCCGAG-GAGGTTAGTTAATA-3′) and mPcsk9-pro-reverse (5′-ATGTCTCTGGGAGGCCAA-3′). Human FOXO3 and Sirt6 and mouse HNF1A, coding sequences were cloned into pcDNA3 (Invitrogen) with a FLAG or HA tag. Adenoviruses for Sirt6 and FOXO3 overexpression were generated as previously described (39, 42). Mouse Pcsk9 shRNAs were designed using the BLOCK-iT RNAi Designer (Invitrogen), and DNA oligos were cloned into a pENTR/U6 vector for further adenovirus generation. The sequence of the Pcsk9 shRNA used in this work is: 5′-CAGGACGAGGATGGAGATTAT-3′. For Sirt6 gene overexpression in vivo, adenoviruses were injected into mice via tail vein at a dose of 5 × 10⁹ pfu.

Serum and Liver Cholesterol Analysis—Blood samples were collected from overnight-fasted mice. Hepatic lipids were extracted as previously described (39). Total cholesterol and HDL- and LDL-VLDL-cholesterol were analyzed using assay kits from Wako Chemicals USA.

Luciferase Reporter Assays—Mouse Pcsk9 gene promoter (also including 5′-UTR) was analyzed in HEK293 cells using the pGL4.10 luciferase reporter system together with an internal control Renilla luciferase reporter as previously described (39).

mRNA Analysis—Total RNAs were isolated from cells and tissues using TRI Reagent (Sigma). Reverse transcription was performed using a cDNA synthesis kit (Applied Biosystems). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega). The primers used in PCR reactions were as follows: mMttp forward, 5′-ATGTCTCTGGGAGGCCAA-3′; mMttp reverse, 5′-TGAGAGGCCAGTGTGAC-3′; mPcsk9 forward, 5′-GAAAACCTTGAGGCCAA-3′; mPcsk9 reverse, 5′-CACCTGAGTGTGATATC-3′; mSirt6 forward, 5′-AGTCAGAGACACGGTGTC-3′; mSirt6 reverse, 5′-CCTC-TACAGGGCGGACAGTC-3′. Real-time PCR data were normalized to an internal control; Ppia and relative -fold changes (experimental group/control) were also calculated.

Protein Analysis—Cell and tissue extract preparation, immunoprecipitation, and immunoblotting were performed as described previously (39). The following antibodies were used: anti-actinin, anti-HA, anti-FoxO1, anti-FoxO3, anti-acetylated lysine (Cell Signaling Technology), anti-LDLR and anti-Pcsk9 (Cayman Chemical), anti-HNF1A (Santa Cruz Biotechnology), and anti-FLAG and anti-SIRT6 (Sigma).

Chromatin Immunoprecipitation (ChIP)—Chromatin association analysis was performed in mouse primary hepatocytes and mouse livers followed by chromatin preparation, immunoprecipitation with FLAG (Sigma), HA (Cell Signaling Technology), H3K9Ac, H3K56Ac, and histone H3 (Millipore) antibodies and the endogenous protein antibodies described above and real-time PCR analysis, as described previously (39). ChIP DNA amount for gene promoters of interest was normalized to that of a housekeeping gene, Ppia ChIP or total histone H3 ChIP. Primers used in the ChIP PCR reactions were: mPcsk9-ChIP forward, 5′-CGAACGCTTGGTGGTATTGAC-3′; mPcsk9-ChIP reverse, 5′-ATGTTCTGGGAGGCCAA-3′; mPpia-ChIP forward, 5′-CAGACCCACATTCCTGTGAGGT-3′; mPpia-ChIP reverse, 5′-AAGTCGGTGTTGGAGAC-3′.

Statistical Analysis—Quantitative data were presented as the mean ± S.E. Significance (p < 0.05) was assessed by two-tailed unpaired Student’s t test.

RESULTS

LDL-cholesterol Is Elevated in Hepatic Sirt6-deficient Mice—Sirt6 has been previously shown to regulate hepatic triglyceride metabolism and cholesterol biosynthesis (38, 43). To examine which lipoprotein-associated cholesterol might be modulated by Sirt6, we analyzed cholesterol in HDL and LDL/VLDL fractions of sera from control floxed (LoxpT6) and Sirt6 liver-specific knock-out mice (LKOT6). Whereas there was no significant difference in HDL-cholesterol, LDL/VLDL-cholesterol levels were increased 45% in the LKOT6 mice relative to the control LoxpT6 littermates (Fig. 1, A and B). VLDL secretion was also increased in the LKOT6 mice compared with the control mice (Fig. 1C). Microsomal triglyceride transfer protein (Mttp), an important factor for VLDL assembly and secretion, was moderately up-regulated in the LKOT6 livers (Fig. 1D).

Sirt6 Regulates LDL-cholesterol by Suppression of the Pcsk9 Gene Expression—Because hepatic deficiency of Sirt6 led to elevated LDL-cholesterol but not HDL-cholesterol, we decided to further investigate the underlying mechanisms. As Pcsk9 is critically involved in LDLR turnover and LDL-cholesterol homeostasis (5), we first analyzed Pcsk9 mRNA and protein levels in control and LKOT6 livers. The results showed that Pcsk9 mRNA was increased by ~3-fold in the LKOT6 mice as compared with the control mice (Fig. 2A). Consistent with an increase in Pcsk9 mRNAs, its protein level was also elevated in the LKOT6 liver (Fig. 2B). Because Pcsk9 targets LDLR for degradation, we also observed a decrease in LDLR in the LKOT6 liver (Fig. 2B). To verify the role of Pcsk9 in LDLR degradation, we performed Pcsk9 gene knockdown in mouse primary hepatocytes. As expected, knockdown of Pcsk9 led to a significant
increase in the LDLR proteins in both wild-type and LKOT6 hepatocytes (Fig. 2C).

To explore the regulatory mechanism for the Pcsk9 gene by SirT6, we first analyzed promoter sequences of human and mouse Pcsk9 genes. In addition to previously identified two cis-elements, sterol response element (SRE) and HNF1A binding site, we also found a consensus binding site for FoxO transcription factors (also called insulin response element (IRE)) (Fig. 2D). Interestingly, the insulin response element is completely embedded in the HNF1A site. This raised a question of whether FoxOs could affect HNF1A-activated Pcsk9 gene expression. We performed luciferase reporter assays for the proximal promoter region of mouse Pcsk9 gene, which also includes a part of the UTR containing the HNF1A binding site. To emphasize here, our nucleotide numbering (relative to the transcription start site) is different from the literature because most previous reports have numbered the Pcsk9 promoter constructs relative to the translation start site. The reporter assay data showed that HNF1A activated the reporter and FoxO3 gene expression. We performed a ChIP analysis. The data revealed a strong association between HNF1A and FoxO3 in the proximal promoter region of mouse Pcsk9 gene, which also includes a part of the UTR containing the HNF1A binding site. To verify that FoxO3 is associated with this region in the chromatin, we also performed a ChIP analysis (Fig. 2E). The data revealed a strong association between the 5'-UTR of the Pcsk9 gene and FoxO3 (Fig. 2F).

Interestingly, although insulin reduced the association of FoxO3 to the 5'-UTR of the Pcsk9 gene, the association of HNF1A was increased (Fig. 2G and H).

To further demonstrate that FoxO3 indeed regulates the Pcsk9 gene expression, we analyzed Pcsk9 mRNA and protein in the livers that were deficient in FoxO1 and FoxO3, or FoxO1/3/4 (LKO1, LKO3, and LTKO, respectively). The data indicated that knock-out of FoxO3 led to a significant increase in the Pcsk9 gene expression in the LKO3 livers, although SirT6 mRNA levels were not significantly changed (Fig. 3, A–C). As a result, hepatic LDLR protein was decreased in the liver of LKO3 mice relative to control mice (Fig. 3C). To confirm the correlation between Pcsk9 and LDLR, we also performed Pcsk9 gene knockdown in control and LKO3 mouse primary hepatocytes. As anticipated, LDLR protein levels were increased after the Pcsk9 gene was knocked down (Fig. 3D). Similar to LKOT6 mice, LKO3 and LTKO mice also had elevated LDL-cholesterol without any significant change in HDL-cholesterol (Fig. 3, E–H).
could interact with HNF1A and FoxO3 but not SREBP-2 (Fig. 4, A–D and G). We also observed an interaction between FoxO3 and HNF1A in HEK293 cells and mouse primary hepatocytes (Fig. 4, E and F). To examine whether Sirt6 has any effect on FoxO3 acetylation, we carried out immunoprecipitation and immunoblot analyses of FoxO3 acetylation in control and LKOT6 liver lysates. The data indicated that Sirt6 deficiency did not have any significant effect on the overall acetylation of FoxO3 (Fig. 4H).

To assess whether Sirt6 could bring about epigenetic changes to the \textit{Pcsk9} gene promoter, we overexpressed either GFP or Sirt6 in mouse primary hepatocytes and subsequently performed ChIP analysis of Sirt6 association and histone H3 acetylation. The results showed that Sirt6 was highly enriched at the 5'-UTR of the \textit{Pcsk9} gene, and H3K9 and H3K56 acetylation levels were dramatically decreased in Sirt6 overexpressed hepatocytes (Fig. 5, A and B). Conversely, those histone modifications were elevated in the liver of LKOT6 mice (Fig. 5C). These data suggest that Sirt6 may be involved in H3K9 and H3K56 modifications in the \textit{Pcsk9} gene because Sirt6 is known to deacetylate both sites (44–47). Because Sirt6 could interact with FoxO3, we also performed ChIP analysis of histone acetylation in FoxO3 overexpressed or knock-out hepatocytes. G, co-IP analysis indicates no interaction between Sirt6 and SREBP-2 in HEK293 cells. H, FoxO3 acetylation analysis of control and LKOT6 liver lysates by immunoprecipitation using FoxO3 antibodies and immunoblotting with anti-acetyl lysine antibodies.
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FIGURE 5. FoxO3 and Sirt6 modulate histone acetylation in the chromatin of the Pcsk9 gene. A and B, association of Sirt6 with the 5′-UTR chromatin of the Pcsk9 gene and histone H3 acetylation in the same region were analyzed by ChIP in mouse primary hepatocytes transduced with GFP and Sirt6 adenoviruses. Data are shown as -fold enrichment relative to the GFP control. C, the acetylation levels of H3K9 and H3K56 were analyzed by ChIP in the 5′-UTR of the Pcsk9 gene in control and LKO3 livers. Data are presented as -fold enrichment relative to the Loxp6 control. D, the effect of FoxO3 overexpression on histone acetylation in the 5′-UTR chromatin was analyzed using ChIP in mouse primary hepatocytes transduced with GFP or FoxO3-expressing adenoviruses. Data are expressed as -fold enrichment relative to the GFP control. E and F, ChIP analysis of Sirt6 association with the 5′-UTR of the Pcsk9 gene and histone H3 acetylation in control and LKO3 mouse primary hepatocytes using corresponding specific antibodies. Data in panel E are presented as -fold enrichment relative to the IgG control in the Loxp3 group, and data in panel F are shown as -fold enrichment relative to the Loxp3 control. Data are the mean ± S.E.; *, p ≤ 0.05 by t test.

DISCUSSION

In this work we have demonstrated that hepatic Sirt6 and FoxO3 have an important role in the regulation of LDL-cholesterol homeostasis. Because Sirt6 is decreased in the livers of obese animals and humans (38, 48), it implicates a potential consequence for the development of hypercholesterolemia, particularly high LDL-cholesterol. Previously, it was reported that systemic overexpression of Sirt6 in mice can lower LDL-cholesterol under conditions of either chow or high fat diet; however, the underlying mechanism is not clear (49). According to our data, we speculate that down-regulation of the Pcsk9 gene expression may be responsible for the low LDL-cholesterol phenotype in the Sirt6 transgenic mouse. Additionally, Sirt6 also significantly represses fatty acid and cholesterol biosynthetic genes and activates fatty acid oxidation genes (38, 43). Apparently, Sirt6 has a salutary effect on lipid homeostasis.

Because Sirt6 is an NAD-dependent deacetylase and mainly targets to histone H3, it may normally be recruited by transcription factors for regulation of specific genes. With regard to the Pcsk9 gene, SREBP-1/2 and HNF1A have been shown to play significant regulatory roles (29, 31, 33, 34, 36). Our data suggest that Sirt6 may be recruited by FoxO3 to the Pcsk9 gene promoter to suppress the gene expression. FoxO transcription factors are known to have both positive and negative effects on gene regulation. The negative effects of FoxOs can be mediated by several different mechanisms, including displacement of regulatory cofactors, recruitment of co-repressor or histone deacetylase, sequestration of other transcription factors, or promotion of associated protein degradation (50). In the case of Pcsk9 gene regulation, our data suggest that FoxO3 may suppress the HNF1A transcriptional activity on the Pcsk9 gene promoter by displacing this transcription factor and recruiting the histone deacetylase Sirt6 as well. This regulation may occur...
during starvation because under that condition Sirt6 and FoxO3 are both active. As a result of the Sirt6 recruitment, deacetylation of H3K9 and H3K56 by Sirt6 creates a repressive state in the chromatin of the Pcsk9 gene promoter to suppress the gene transcription. Additionally, reduced levels of SREBPs and HNF1A may also contribute to the down-regulation of the Pcsk9 gene during fasting (27, 29, 33). Upon feeding, the activity of FoxO3 and Sirt6 is decreased, and the levels of nuclear SREBPs are increased; the Pcsk9 gene transcription is thus activated. With regard to the involvement of FoxOs in the regulation of the Pcsk9 gene, some questions remain to be addressed in the future. First, why does FoxO3 play a major role rather than FoxO1, as FoxO1 is also highly abundant in the liver as well? In agreement with our data, previous reports have also shown that FoxO1 does not play a significant role in LDL-cholesterol regulation (36, 51, 52). Second, what is the role of FoxO3 in the regulation of the Pcsk9 gene in obese and diabetic conditions? Whereas several reports have shown that feeding or insulin can induce the Pcsk9 gene expression (23, 29, 53), another one has documented an increase in the Pcsk9 gene expression in the liver of insulin receptor knockout mice (36). In insulin-deficient type 1 diabetic rats, hepatic Pcsk9 mRNAs are dramatically decreased (53); however, in ob/ob leptin-deficient obese mice, hepatic Pcsk9 mRNAs are also decreased by 2-fold (36). Further investigation is needed to clarify what causes differential regulation of the Pcsk9 gene expression under those conditions.

Recently, we have reported that FoxO3 and Sirt6 also suppress the Srebp2 gene expression in the liver (43). This suggests that both factors may have a coordinated role in cholesterol homeostasis. By regulating the Srebp2 gene, the master regulator of cholesterol biosynthesis, FoxO3 and Sirt6 have an impact on total cholesterol levels in the circulation. With fine-tuning on the Pcsk9 gene expression, Sirt6 and FoxO3 enhance the salutary effects by lowering LDL-cholesterol levels. As Pcsk9 plays an important role in LDL-cholesterol homeostasis, proper regulation of the Pcsk9 gene expression by Sirt6 and FoxO3 may contribute to cardiovascular health of organisms. It is known that both Sirt6 and FoxO3 are associated with longevity in mammals (47, 54–59). Thus, it should be interesting to look into how Sirt6 and FoxO3 may influence longevity through regulation of LDL-cholesterol.

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