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Ding Ai,1 Chiyuan Chen,2 Seongah Han,1 Anjali Ganda,1 Andrew J. Murphy,1 Rebecca Haeusler,1 Edward Thorp,1 Domenico Accili,1 Jay D. Horton,2 and Alan R. Tall1

1Department of Medicine, Columbia University, New York, New York, USA. 2Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

Individuals with type 2 diabetes have an increased risk of atherosclerosis. One factor underlying this is dyslipidemia, which in hyperinsulinemic subjects with early type 2 diabetes is typically characterized by increased VLDL secretion but normal LDL cholesterol levels, possibly reflecting enhanced catabolism of LDL via hepatic LDLRs. Recent studies have also suggested that hepatic insulin signaling sustains LDLR levels. We therefore sought to elucidate the mechanisms linking hepatic insulin signaling to regulation of LDLR levels. In WT mice, insulin receptor knockdown by shRNA resulted in decreased hepatic mTORC1 signaling and LDLR protein levels. It also led to increased expression of PCSK9, a known post-transcriptional regulator of LDLR expression. Administration of the mTORC1 inhibitor rapamycin caused increased expression of PCSK9, decreased levels of hepatic LDLR protein, and increased levels of VLDL/LDL cholesterol in WT but not Pcsk9−/− mice. Conversely, mice with increased hepatic mTORC1 activity exhibited decreased expression of PCSK9 and increased levels of hepatic LDLR protein levels. Pcsk9 is regulated by the transcription factor HNF1α, and our further detailed analyses suggest that increased mTORC1 activity leads to activation of PKCδ, reduced activity of HNF4α and HNF1α, decreased PCSK9 expression, and ultimately increased hepatic LDLR protein levels, which result in decreased circulating LDL levels. We therefore suggest that PCSK9 inhibition could be an effective way to reduce the adverse side effect of increased LDL levels that is observed in transplant patients taking rapamycin as immunosuppressive therapy.

Introduction

Dyslipidemia is an important factor underlying the increased atherosclerosis risk of diabetic patients and is typically characterized by increased VLDL and reduced HDL, but surprisingly no change in LDL cholesterol levels (1). Metabolic studies have shown that increased hepatic VLDL lipid and apoB secretion are characteristics of type 2 diabetes and metabolic syndrome (2, 3), but that LDL fractional catabolism is increased at least in early type 2 diabetes when subjects are hyperinsulinemic (4). There was a strong correlation between hyperinsulinemia and the fractional catabolic rate (FCR) of LDL particles. Only in advanced diabetes, when plasma insulin levels were reduced, did LDL FCRs decrease (4). Since LDL catabolism is primarily mediated through the hepatic LDL receptor (LDLR) (5), this suggests that in early diabetes and metabolic syndrome hyperinsulinemia is associated with upregulation of hepatic LDLR, compensating for increased VLDL secretion and leading to no overall change in LDL levels.

Studies in cell culture and animal models have also linked insulin signaling to upregulation of LDLR (6, 7). Early studies in fibroblasts showed that insulin treatment increased LDLR numbers independent of cell proliferation (8). Regulation of the LDLR by insulin in HepG2 cells is PI3K dependent (9), but relevant pathways downstream of PI3K such as mTOR, FoxOs, or GSK3 (10) have not been elucidated. Decreased hepatic LDLR protein was clearly seen in LIRKO (liver-specific insulin receptor knockout) mice (11) and in mice with insulin receptor (InsR) knockdown (12). Based on these findings, we suggested that genetically restricted hepatic insulin signaling leads both to reduced VLDL lipid secretion and to decreased levels of hepatic LDLR (12). However, the mechanisms linking chronic changes in hepatic InsR to LDLR levels have not been elucidated.

Results

InsR knockdown suppresses hepatic LDLR expression via PCSK9. Knockdown of the hepatic InsR by shRNA in chow-fed, 5-hour-fasted C57BL/6 mice resulted in an 85% reduction in InsR and a 57% reduction in LDLR protein levels compared with controls (Figure 1A), as described previously (12), as well as decreased mTOR activity as shown by reduced phosphorylation of p70 ribosomal protein S6 kinase at T389 (p-S6KThr) (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI61919DS1). However, there was no change in Ldlr mRNA levels, suggesting that the change in LDLR protein was due to a post-transcriptional regulatory event. Plasma insulin levels were reduced in InsR knockdown mice, but plasma glucose levels were unchanged (Supplemental Figure 1B). The mRNA levels of Srebp-1c and its target genes fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (Sd1) were decreased (Figure 1B), but expression of Srebp-2 mRNA and its targets HMGCoa reductase (HMGCoaRed) and HMGCoa synthase (HMGCoaSyn) were unchanged, consistent with the lack of change in Ldlr mRNA. The post-transcriptional regulation of the LDLR may be mediated by proprotein convertase subtilisin/kexin type 9 (PCSK9) (13, 14) or LXR/inducible degrader of LDLR (IDOL) signaling pathways (15). Interestingly, in InsR knockdown mice Pcsk9 mRNA and protein levels were increased 2- and 2.4-fold, respectively (Figure 1, A and B), while Mylip (encoding IDOL) mRNA was unchanged (Figure 1B). These findings suggested that increased PCSK9 might be responsible for the reduction in LDLR that results from decreased insulin signaling.
To further assess the significance of PCSK9 in the regulation of LDLR by insulin signaling, we treated WT or Pcsk9–/– mice with InsR shRNA. As expected (16), LDLR levels were higher in Pcsk9–/– mice (Figure 1C). Although the InsR was reduced by more than 80% in both strains and the LDLR was decreased by 59% in WT mice, there was no change in LDLR levels in Pcsk9–/– mice (Figure 1C). There was no change in Ldlr or Mylipp mRNA among the groups (Supplemental Figure 1C). We also employed a genetic InsR knockdown model, L1B6Ldlr–/– mice (12). In L1B6Ldlr–/– mice, InsR knockdown resulted in decreased mTOR activity (p-S6K T389) and increased Pcsk9 expression (Supplemental Figure 1, D and E). These data show that PCSK9 mediates the decrease in LDLR protein caused by reduced insulin signaling.

LDLR is regulated downstream of AKT independent of FoxO1 or GSK3β signaling. To further evaluate the role of the insulin signaling pathways in the regulation of LDLR, we injected adenovirus expressing constitutively active myristoylated AKT (myrAKT) or control adenovirus into the tail vein of C57BL/6 mice (Supplemental Figure 2A). This resulted in a decrease in Pcsk9 expression and an increase in LDLR protein (Supplemental Figure 2, A and B). There were no significant changes in mRNA of Srebp2 or its target genes including Ldlr (Supplemental Figure 2, A and B). We also evaluated
a possible role of PCSK9 in regulation of hepatic LDLR in ob/ob mice, in which we previously described increased levels of hepatic LDLR (12). LDLR protein levels were increased in livers of ob/ob mice, but there was no significant change in Ldlr mRNA levels (Figure 2A and B). Pcsk9 levels were decreased by 60% in livers of ob/ob mice (Figure 2B). Thus, ob/ob mice that were hyperinsulinemic and showed increased AKT phosphorylation in the fasting state and increased lipogenic gene expression had a phenotype opposite to that in mice in which insulin signaling is restricted by InsR knockout (17).

We next considered the potential involvement of each of the 3 major signaling pathways acting downstream of AKT: mTORC1, FoxO1, and GSK3β (10). AKT phosphorylates and leads to nuclear exclusion and inactivation of FoxO1; thus, if decreased insulin signaling by InsR shRNA and activation of FoxO1 were to mediate decreased LDLR expression, the reduction of LDLR by InsR shRNA and activation of FoxO1 were to mediate decreased LDLR expression, following InsR knockdown LDLR levels were even more markedly reduced (Figure 2C) than in control mice. There were no changes in Srebp-1c, Srebp-2, Fas, Scd1, Hmgcr, Hmgcs, Ldlr, Pcsk9, or Idol mRNA levels in control compared with L-FoxO1 mice (Supplemental Figure 2C). AKT signaling also leads to phosphorylation and inactivation of GSK3β (10). Thus, if this pathway was involved, a dominant negative form of GSK3β should mimic the effect of AKT and lead to increased LDLR expression. We thus used an adenoviral vector expressing a dominant negative form of GSK3β (GSK-KM) in C57BL/6 mice but did not see an increase in LDLR expression (Figure 2D). There were no changes in Srebp-1c, Srebp-2, Fas, Scd1, Hmgcr, Hmgcs, Ldlr, Pcsk9, or Idol mRNA levels between control and GSK-KM mice (Supplemental Figure 2D). These data suggest that hepatic FoxO1 and GSK3β signaling pathways are not directly involved in the regulation of Pcsk9 and LDLR.

Hepatic mTORC1 regulates LDLR levels. After excluding the other two pathways, we determined whether activation of mTORC1 by AKT could change LDLR levels. To this end, we administered the mTOR inhibitor rapamycin (2 mg/kg/d) to C57BL/6 mice for 1 week. Phosphorylation of p70 ribosomal protein S6 kinase (p70sk) was blocked by this treatment (Figure 3A), indicating effective inhibition of mTOR signaling. Rapamycin caused a reduction in LDLR protein levels and an increase in plasma cholesterol levels (Figure 3, A and B), whereas there was no significant change in the mRNA levels of Ldlr, Srebp-2, or its target genes (Supplemental Figure 3A). However, there was an increase in the mRNA and protein levels of Pcsk9. There was no difference between rapamycin-treated and control mice in the mRNA levels of Srebp-1c and its target genes (Supplemental Figure 3A). These findings suggest that regulation of Pcsk9 and LDLR by insulin signaling occurs via the mTORC1 pathway. To further assess the role of mTORC1 in the regulation of Pcsk9 and LDLR, we treated WT and Pcsk9 knockout mice with rapamycin or vehicle. The ability of rapamycin to reduce LDLR levels was abolished in Pcsk9−/− mice (Figure 3C). Moreover, while rapamycin caused an increase in VLDL/LDL cholesterol in WT mice, it had no effect on VLDL/LDL cholesterol in Pcsk9−/− mice (Figure 3D). Total cholesterol was increased in WT and Pcsk9−/− mice treated with rapamycin (Figure 3D), reflecting an increase in HDL caused by rapamycin treatment (data not shown). This indicates that the ability of rapamycin to decrease LDLR and increase VLDL/LDL cholesterol requires the presence of Pcsk9.

To determine whether increased mTORC1 activity would be associated with reduced Pcsk9 expression and increased levels of hepatic LDLR, as predicted by our model, we used mice with genetically increased mTORC1 activity, involving liver-specific knockout of the upstream inhibitor Tsc1, i.e., Li-Tsc1−/− mice (19). These mice displayed an increase in hepatic mTORC1 activity (Figure 3E). Hepatic LDLR protein levels were increased 1.6-fold in Li-Tsc1−/− mice, Ldlr mRNA levels were unchanged, while Pcsk9 mRNA levels were reduced by 48% in Li-Tsc1−/− mice (Figure 3, E and F).
mTORC1 induces transcription of PCSK9 in an HNF1α-dependent manner. We carried out further studies to explore potential mechanisms underlying the mTORC1-mediated increase in Pcsk9 mRNA levels. Recently, HNF1α was shown to mediate the transcriptional induction of Pcsk9 (20, 21) via a highly conserved cis-acting element 28 bp upstream of the sterol regulatory element (SRE) in the Pcsk9 promoter (20, 21). Consistent with a role of HNF1α in the induction of PCSK9 in the two mouse models with decreased hepatic mTORC1 activity, the protein level of HNF1α was increased in both InsR knockdown and rapamycin-treated mice (Figure 4, A and B), while the cognate mRNA levels were increased 2.5-fold and 2.4-fold (Figure 4, A and B). Furthermore, ChIP analysis showed increased occupancy by HNF1α of its binding site in the Pcsk9 promoter region, which included the identified HNF1α binding site (20) (proximal) rather than a control region that is 903 bp from the binding site (distal), following rapamycin treatment (Figure...
4C). Also consistent with a role of altered HNF1α expression in the regulation of PCSK9, we found reduced hepatic expression of HNF1α in ob/ob mice (Supplemental Figure 4A) and Li-Tsc1+/− mice (Supplemental Figure 3B), and the binding of HNF1α to the Pck9 promoter was decreased in Li-Tsc1+/− mice (Supplemental Figure 3C). Furthermore, adenoviral overexpression of HNF1α in livers of ob/ob mice led to increased mRNA and protein levels of PCSK9, decreased LDLR protein but not mRNA levels (Figure 4, D and F), and increased plasma cholesterol levels (Figure 4E), while there were no changes in the expression of hepatic Srebp-1c, Srebp-2, or their target genes (Supplemental Figure 4B).

mTOR has been shown to activate PKCδ (22, 23), and PKCδ has been reported to play an important role in hepatic insulin resistance, glucose intolerance, and hepatosteatosis in obese humans and mice (24). Consistent with a role of mTORC1 in its activation, Li-Tsc1+/− mice exhibited markedly enhanced serine 662 phosphorylation of hepatic PKCδ. Since activated PKCs can induce nuclear exclusion of HNF4α (25) and HNF4α activates HNF1α (26), we evaluated the nuclear levels of HNF4α in Li-Tsc1+/− mice. Consistent with a role of HNF4α upstream of HNF1α, nuclear HNF4α was decreased by 51% (Figure 5A) and total HNF1α by 44% compared with control mice (Figure 5A). To more directly assess the role of

Figure 4
HNF1α is involved in the regulation of LDLR by insulin signaling. (A and B) Immunoblot and real-time qPCR analysis of HNF1α in InsR- or scrambled shRNA–injected mice and vehicle- or rapamycin-treated mice. (C) Pcsk9 promoter ChIP assay in the liver of vehicle- or rapamycin-treated mice using the HNF1α or IgG antibody. (D) ob/ob mice were killed 4 days after HNF1α adenovirus (Ad-HNF1α) injection and following a 6-hour fast. Immunoblot analysis of the HNF1α, LDLR, PCSK9, and β-actin. (E) Plasma cholesterol and triglyceride concentrations. (F) Hepatic mRNA levels of Ldlr and Pcsk9 in the mice. n = 5 mice/group. *P < 0.05.
PKCδ in the regulation of PCSK9 and LDLR, we transfected Tsc1-null mouse embryonic fibroblasts (MEFs), which have increased mTORC1 activity (27), with control or PKCδ siRNA. Total HNF1α and nuclear HNF4α were both increased following PKCδ knockdown; Pcsk9 mRNA was increased; and protein but not mRNA levels of Ldlr were decreased in response to PKCδ knockdown in the Tsc1–/– MEFs (Figure 5, B–D). Furthermore, ChIP analysis showed increased occupancy by HNF1α of its binding site in the Pcsk9 promoter region, which includes the identified HNF1α binding site (proximal) rather than the region that is 903 bp from the binding site (distal), following PKCδ knockdown in Tsc1–/– MEFs (Supplemental Figure 5). These findings are consistent with the model shown in Figure 5E, in which PCKδ activation downstream of increased mTORC1 activity leads to repression of HNF4α/1α, reduced PCSK9, and increased LDLR.

Discussion

In hyperinsulinemic obese mouse models, the liver is insulin resistant in terms of gluconeogenesis but remains insulin sensitive for lipogenesis (17), VLDL secretion, and LDLR expression (11, 12). Insulin signaling via AKT2 and mTORC1 has emerged as having a key role in the regulation of hepatic lipogenesis in obese mice (28, 29), likely with involvement of additional pathways/effects downstream of AKT (30, 31). Interestingly, our studies also implicate AKT/mTORC1 signaling in the regulation of hepatic LDLRs. The underlying mechanism involves induction of PCSK9 via mTORC1, leading to post-transcriptional downregulation of hepatic LDLR. The relationship between mTORC1 activity and regulation of LDLR via PCSK9 was demonstrated in two mouse models with reduced hepatic mTORC1 activity, rapamycin- and InsR shRNA-treated mice, and in two models with increased mTORC1 activity, ob/ob and Li-Tsc1–/– mice. We provide evidence for involvement of PCSK9 in the first two models by demonstrating that the effects of rapamycin or InsR knockdown on LDLR were abolished in Pcsk9–/– mice. Consistent with recent evidence (21), our data suggest that HNF1α is responsible for the transcriptional regulation of Pcsk9 gene expression in these models, with increased HNF1α levels shown in rapamycin- and InsR shRNA-treated mice and reduction in LDLR levels in ob/ob mice by HNF1α overexpression. Further analysis of potential signaling pathways in Li-Tsc1–/– mice and fibroblasts suggested activation of PKCδ downstream of mTORC1, leading to nuclear exclusion of HNF4α, decreased HNF1α, and decreased Pcsk9 expression (Figure 5E). Interestingly, PKCδ is induced by a high-fat diet in C57BL/6 mice, with adverse effects on hepatic lipogenesis and gluconeogenesis, possibly mediated through effects on p70 S6K, a known mTORC1 effector (24). mTORC1 represents a central hub integrating information not only from insulin signaling but also from nutritional stimuli and cytokines (32). Thus, it is likely that the prominent induction of LDLR in obese mice reflects multiple chronic inputs to mTORC1 in addition to effects of insulin/AKT signaling.

PCSK9 is a member of the mammalian subtilisin family of pro-protein convertases (33) that regulates circulating LDL cholesterol levels primarily by diverting recycling LDLRs into the endosomal-lysosomal pathway, leading to LDLR degradation. PCSK9 was also identified as a SREBP-2– and dietary cholesterol–regu-
labeled gene in livers of mice (34, 35). Humans with loss-of-function mutations (36) and Pcsk9 knockout mice have significantly reduced circulating LDL cholesterol levels, reflecting increased LDLR protein levels (16, 36). Loss-of-function mutations in Pcsk9 are also associated with a marked reduction in cardiovascular disease (13, 37). Several different approaches to inhibit PCSK9 function for the treatment of hypercholesterolemia have been undertaken. PCSK9 antisense oligonucleotides administered to mice increased hepatic LDLR expression and lowered plasma cholesterol (38). Administration of RNAi against PCSK9 delivered using lipidoid nanoparticles to cynomolgus monkeys reduced plasma LDL cholesterol by approximately 60% (39). Antibody inhibition of PCSK9 in cynomolgus monkeys also reduced LDL cholesterol levels by as much as 80% (39, 40).

Previous studies in cultured cells have shown that rapamycin can reduce LDLR levels independent of changes in SREBP-2 expression (41–43). However, the in vivo significance and mechanism of this effect were unknown. Our studies suggest that the post-transcriptional downregulation of hepatic LDLR protein levels via PCSK9 is responsible for the increased LDL levels induced by rapamycin, implying that this adverse effect of rapamycin treatment could be overcome by therapeutic inhibition of PCSK9. As an mTORC1 inhibitor, rapamycin is a potent immunosuppressive agent used in transplantation medicine because of its low renal toxicity. However, a major side effect of rapamycin is hypercholesterolemia and increased LDL levels, likely contributing to cardiovascular disease, a leading cause of mortality after kidney transplantation (44). In addition, dyslipidemia has been implicated as a risk factor for chronic allograft loss (45). It is important to note that statin treatment upregulates transcription of both LDLR and its natural inhibitor PCSK9. Following depletion of the intracellular cholesterol pool, a processed mature form of SREBP-2 enters the nucleus, where it binds to the SRE of both the LDLR and PCSK9 promoters, activating transcription. The protein product of PCSK9 gene transcription reduces LDLR protein levels, and this intrinsic regulatory loop has been recognized as an undesirable limitation of statin-related LDL cholesterol–lowering capacity (20).

In a recent systematic review of randomized controlled trials that attempted to characterize mTOR inhibitor dyslipidemia in kidney transplant patients, approximately 60% of mTOR inhibitor–treated patients received lipid-lowering agents (2-fold greater than non-mTOR inhibitor–treated patients) (28). Given our finding that post-transcriptional downregulation of LDLR protein levels via PCSK9 is directly responsible for the increased circulating LDL induced by rapamycin, it follows that statin therapy, which affects LDLR at the transcriptional level, would be expected to lower LDL levels independent of changes in SREBP-2 expression (39). Inhibition of mTORC1 and consequent induction of autophagy would lead to increased cholesterol efflux from macrophage foam cells (47). While the overall effects of rapamycin on atherogenesis in humans remains unknown, it seems likely that our studies are relevant to the mechanisms by which rapamycin raises LDL levels in humans and that this effect is likely pro-atherogenic. Our studies imply that therapeutic inhibition of PCSK9 might effectively treat hypercholesterolemia in transplant patients receiving rapamycin, and could potentially have a beneficial effect in cardiovascular disease, allograft loss, and mortality in this population.

**Methods**

*Animals.* C57BL/6, L-FoxO1, and Pcsk9 knockout mice were killed using CO2 11 days after tail injection of Insr shRNA or GSK-KM or myrAKT adenovirus or their control adenovirus and following a 5-hour fast. C57BL/6 mice or Pcsk9 knockout mice received 400 μl PBS/0.1% DMSO supplemented with rapamycin (2 mg/kg body weight; LC Laboratories) through intraperitoneal injection every day. After 1-week injections, mice were anesthetized by CO2, and blood and liver tissues were collected from non-fasted mice. Mice carrying a floxed allele of Tiel (Tielflo/flo) (48) were purchased from The Jackson Laboratory. Adenovirus expressing Cre (adenovirus-Cre; 5 × 109 PFU/ml, 100 μl) or control adenovirus (adenovirus-empty) was administered via tail vein injection under isoflurane anesthesia to mice at 8 weeks in age, and the mice were killed on the seventh day after injection following a 5-hour fast. ob/ob mice, 8–10 weeks old, were killed using CO2 4 days after mouse HNF1αt or control adenovirus injection and following a 6-hour fast. Mice had free access to food and water and were housed in a pathogen-free facility according to animal welfare guidelines established by the Office of Laboratory Animal Welfare (OLAW) of the NIH. All experiments were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee and were conducted according to that committee’s guidelines.

*Plasma triglyceride and cholesterol determination.* Blood samples were collected by retroorbital venous plexus puncture. Plasma was separated by centrifugation and stored at −70°C until analysis. Total plasma cholesterol and triglycerides were measured with Wako enzymatic kits and Infinity (Thermo Scientific) according to the manufacturers’ instructions.

*Immunoblot analysis.* Western blot analysis was carried out using the following primary antibodies: anti-p-AKT (Cell Signaling Technology Inc.); anti-InsR (Santa Cruz Biotechnology Inc.); anti–p-S6K (Cell Signaling Technology Inc.); anti–S6K (Cell Signaling Technology Inc.); anti-HNF4α (R&D Systems); anti–lamin B (Abcam); anti–PCSK9 (gift from Jan L. Breslow’s laboratory, the Rockefeller University, Inc.); anti–LDLR-related protein (anti-LRP) (14); anti–HNF1α (Santa Cruz Biotechnology Inc.); anti-LDLR-related protein (anti–LRP) (14); anti–LIP (14); anti–LDLR (Abcam); anti–PCSK9 (gift from Jan L. Breslow’s laboratory, the Rockefeller University, New York, New York, USA) and anti–β-actin antibody (Sigma-Aldrich). Nuclear extract was isolated by NE-PER (Thermo), and liver lysate was prepared with T-PER (Thermo). Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed separately with antibodies as described in the respective figure legends. Following incubation with horseradish peroxidase–conjugated secondary antibodies, proteins were visualized with SuperSignal West Pico Chemiluminescent reagents (Pierce; Thermo Scientific) on x-ray films. The band intensity was quantified using scanning densitometry of the autoradiogram with NIH ImageJ software (http://rsb.info.nih.gov/ij/).

*Real-time quantitative PCR analysis.* Liver tissues were homogenized and total RNA was isolated using RNAzol B (Tel-Test) according to the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed at 42°C with SuperScript II (Life Technologies). Gene expression was measured using SYBR Green PCR core reagents (Applied Biosystems). Transcript levels of β-actin were used for normalization across samples. The sequences of PCR probes and primers used are described in Supplemental Table 1.

**CNP assays.** Chromatin lysates from mouse livers were cross-linked in 1% formaldehyde for 15 minutes, and then liver nuclei were isolated by NE-PER kit, followed by sonication. After being precleared with protein G agarose beads, chromatin lysates were immunoprecipitated using antibod-
ies against HNF1α (Santa Cruz Biotechnology Inc.) or control rabbit IgG in the presence of BSA and salmon sperm DNA. Beads were extensively washed before reverse cross-linking. Sample DNA was isolated from the immunoprecipitates and then amplified by quantitative PCR (qPCR) using primers flanking the enhancer (distal, 903 bp from the identified HNF1α binding site; ref. 20) or the proximal promoter (proximal, including the identified HNF1α binding site) (Supplemental Table 1).

siRNA transfection. Tsc1−/− MEFs were cultured as previously described (27). Mouse PKCδ and control siRNA were supplied by Ira Tabas (Columbia University Medical Center, New York, New York, USA). Lipofectamine RNAiMAX (Invitrogen) was used. Transfection was performed according to the manufacturer’s instructions.

Statistics. Results are expressed as mean ± SEM (n is indicated in the figure legends or figures). Results were analyzed using a 2-tailed Student’s t test or 1-way ANOVA where appropriate, using GraphPad Prism software. A P value less than 0.05 was considered statistically significant.

Study approval. All animal experiments were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee and were conducted according to that committee’s guidelines.

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Address correspondence to: Ding Ai, Columbia University Medical Center, P&S8-401, 630 West 168th Street, New York, New York 10032, USA. Phone: 212.305.5789; Fax: 212.305.5052; E-mail: da2424@columbia.edu.

Seonghan Han’s present address is: Merck & Co. Inc., Whitehouse Station, New Jersey, USA.