PIK3R3 regulates PPARα expression to stimulate fatty acid β-oxidation and decrease hepatosteatosis

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Phosphatidylinositol 3-kinase (PI3K) signaling plays an important role in the regulation of cellular lipid metabolism and non-alcoholic fatty liver disease (NAFLD). However, little is known about the role of the regulatory subunits of PI3K in lipid metabolism and NAFLD. In this study, we characterized the functional role of PIK3R3 in fasting-induced hepatic lipid metabolism. In this study, we showed that the overexpression of PIK3R3 promoted hepatic fatty acid oxidation via PIK3R3-induced expression of PPARα, thus improving the fatty liver phenotype in high-fat diet (HFD)-induced mice. By contrast, hepatic PIK3R3 knockout in normal mice led to increased hepatic TG levels. Our study also showed that PIK3R3-induced expression of PPARα was dependent on HNF4α. The novel PIK3R3-HNF4α-PPARα signaling axis plays a significant role in hepatic lipid metabolism. As the activation of PIK3R3 decreased hepatosteatosis, PIK3R3 can be considered a promising novel target for developing NAFLD and metabolic syndrome therapies.

INTRODUCTION

The liver acts as the ‘metabolic integrator’ of lipid metabolism by regulating lipogenesis, fatty acid oxidation, and lipoprotein uptake and secretion.1 Non-alcoholic fatty liver disease (NAFLD) is currently the most common liver disease worldwide and has been estimated to occur in 30% of adults in North American and Asian populations. NAFLD is characterized by hepatic steatosis, which can subsequently progress to non-alcoholic steatohepatitis (NASH). Further progression from NASH to cirrhosis may cause liver failure and hepatocellular carcinoma (HCC).2 Moreover, fatty liver has also been linked to diabetes and other metabolic diseases. Although NAFLD has been associated with decreased fatty acid β-oxidation and increased lipogenesis, the molecular mechanisms for fatty liver development are not well understood.

Phosphatidylinositol 3-kinase (PI3K)/AKT signaling is involved in cell survival and proliferation.3 Recent reports suggest that PI3K/AKT activates transcriptional regulators of lipid metabolism, such as sterol regulatory element-binding proteins (SREBPs), and plays an important role in the regulation of cellular lipid metabolism.4–7 Class IA PI3Ks consist of a regulatory subunit and a catalytic subunit.4 It has been reported that the hepatic p110α catalytic subunit of PI3K-knockout mice causes hypolipidemia.8 However, little is known about the role of regulatory subunits of PI3K in lipid metabolism.

PIK3R3, a 62 kDa regulatory subunit of PI3K, can bind to the p110 catalytic subunit through the iSH2 domain.9 Previously, we reported that PIK3R3 was important for cell proliferation and tumor growth and was overexpressed in several types of cancer.10,11 Moreover, we showed that the inhibition of PIK3R3 blocked cell cycle progression, induced cell differentiation and inhibited tumor angiogenesis.10,12–14 However, the role of PI3R3 in physiology and lipid metabolism remains largely unknown.

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated nuclear receptor that is highly expressed in tissues with high FAO rates, such as the liver, skeletal muscle, heart and brown adipose tissue.2,15,16 PPARα is also a nuclear hormone receptor that functions as a central regulator of hepatic lipid metabolism. Many PPARα-targeted genes have been demonstrated to play key roles at different points of hepatic lipid metabolism, such as uptake, binding, oxidation of fatty acids, lipid droplet biology and ketogenesis.17–19 In addition, PPARα has emerged as a central transcriptional regulator involved in multiple metabolic processes, such as the metabolism of cholesterol, glucose, bile acids and amino acids in the liver.20,21
In this study, we have described a functional link between PIK3R3 and hepatic lipid metabolism and characterized the functional role of PIK3R3 in vivo under fasting and high-fat diet conditions. We showed that the overexpression of PIK3R3 promoted hepatic fatty acid oxidation via PIK3R3-induced expression of PPARα, thus improving the fatty liver phenotype in HFD-fed mice. By contrast, the knockdown of hepatic PIK3R3 in normal mice led to increased hepatic TG levels. Furthermore, PIK3R3-induced expression of PPARα was HNF4α-dependent. Collectively, PIK3R3 depends on the PIK3R3–HNF4α–PPARα axis to regulate lipid metabolism in the liver.

MATERIALS AND METHODS

Animals and experimental design

Eight-week-old male C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). They were housed and maintained on a 12 h light–dark cycle and received a regular unrestricted diet. Mice were fed either a normal chow (9% fat) or a high-fat diet (HFD) (45% fat) ad libitum and had free access to water. This study was conducted following the Animal Study Guidelines of Huazhong University of Science and Technology. Mice were injected with Ad-Pik3r3, Ad-Pparα, Ad-control, si-Pik3r3, si-Pparα or si-control (1.0 × 10⁶ active viral particles or 40 nmol siRNA in 100 μl of PBS) twice a week for 2 weeks. Five days after the last injection, the mice were fasted for 6 h, and their livers were collected for further analyses.

Preparation of expression plasmids, recombinant adenoviruses and RNA interference

The full-length human or mouse PIK3R3/PPARα (Pik3r3/Pparα) gene was amplified by polymerase chain reaction (PCR) from human liver cDNA or C57BL/6J mouse liver cDNA, respectively. Human PIK3R3 or PPARα was cloned into the pcDNA3.1 vector, and recombinant adenovirus expressing mouse Pik3r3 or Pparα were generated as previously described. RNA interference (targeting PIK3R3, PPARα, HNF4α, Pik3r3 or Pparα) and the siRNA negative control (si-control) were synthesized and purified by RiboBio (RiboBio Corporation, Guangzhou, China) (Supplementary Table 1).

Analytical procedures and chemicals

Concentrations of ketone bodies in serum or cell culture supernatants were determined using a colorimetric diagnostic kit (Cayman Chemical Company, Ann Arbor, MI, USA). Liver TG levels were measured using a colorimetric diagnostic kit (Jiancheng Bioengineering Institute, Nanjing, China).

Cell culture and transfection

HepG2 and L02 cell lines were cultured in DMEM containing 10% FBS and penicillin/streptomycin. Cells were transfected with expression plasmids at a final concentration of 4 μg or 100 nm siRNA using Lipofectamine 2000 (Invitrogen). Media was changed 6 h after transfection. The relative expression levels of genes were determined 48 h after transfection.

Quantitative real-time PCR

Total RNA was isolated from cells or pulverized liver using TRIzol (Invitrogen). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed using the SYBR Green I Q-PCR kit (Transgen Biotech) on an ABI 7300 system. Human or mouse gene expression data were normalized to Gapdh expression levels. All specific primer pairs for each gene are shown in Supplementary Table 2.

Western blot analysis

Protein was extracted from frozen organ samples or cultured hepatocytes in cell lysis buffer. Approximately 50 μg of protein was separated on 10% SDS–polyacrylamide gel (SDS-PAGE) gels and transferred to PVDF membranes. Western blot detection was performed using antibodies specific for PIK3R3, GAPDH (Santa Cruz Biotechnology, CA, USA), HNF4α (Cell Signaling Technology, MA, USA), ACADM, CPT1α (Proteintech Group, Chicago, IL, USA), PPARα or PPARγ (Boster, Wuhan, China), which were previously tested and studied using both human and mouse samples.

Histology and immunohistochemistry

For H&E staining, liver tissues were fixed, embedded and cut into sections as previously described. For Oil Red O staining, liver tissue was frozen in liquid nitrogen and cut into 10-μm sections. Sections were stained and analyzed at ×100 magnification using a microscope. Immunohistochemical examination was performed as previously described.

ChIP assay

ChIP assay kits purchased from Millipore were used according to the manufacturer’s protocol. ChIP assays were carried out using an anti-HNF4α antibody as previously described (Millipore #17-371, Burlington, MA, USA). RNA-polymerase II, which could bind the promoter region of GAPDH, was used as a positive control. Normal rabbit IgG was used as a negative control. The following primers for the PPARα promoter were used for the ChIP assays: forward 5′-TTGCGTAGGCAAAAGTCAG-3′ and reverse 5′-CCCAGGATGCAAATTAGGA-3′; the control primers used for GAPDH were provided in the kit.

Statistical analysis

All data are expressed as the mean ± s.e.m. (standard error of the mean). Intergroup and intragroup comparisons were conducted using Student’s t-test and ANOVA, respectively. P-values were calculated using an unpaired Student’s t-test.

RESULTS

Hepatic PIK3R3 expression is increased after fasting and is downregulated in a high-fat diet mouse model of fatty liver

We utilized a high-fat diet-induced fatty liver mouse model (HFD mice, C57BL/6j background). H&E staining showed that HFD mice had increased fatty liver, and Oil Red O staining showed that HFD mice had significantly increased hepatic intracellular triacylglycerol accumulation. We also observed that the expression levels of mitochondrial and peroxisomal fatty acid oxidation-related genes, such as carnitine palmitoyl transferase 1a (CPT1α) and medium-chain acyl-CoA dehydrogenase (ACADM), were regulated by fasting (Figure 1a).

Serum concentrations of TG were significantly lower in normal chow-fed mice than in the HFD-fed mice, while the levels of ketone bodies were lower in the HFD-fed mice than in the normal chow-fed mice (P<0.01) (Figure 1b). To identify the role of PIK3R3 in lipid metabolism, we examined the PIK3R3
mRNA and protein expression levels in HFD-fed mice. The results showed that PIK3R3 was downregulated over time in mice fed the HFD at both the protein and the mRNA levels (Figure 1c, Supplementary Figure 1). We further investigated whether hepatic PIK3R3 expression could be regulated by nutritional status during the fasting/re-feeding cycle. Indeed, prolonged fasting (24 h) increased hepatic PIK3R3 mRNA and protein expression levels, whereas refeeding decreased these levels to those found at baseline (Figure 1d). Taken together, these data revealed a strong correlation between PIK3R3 expression and both lipid metabolism and nutritional status in the liver.

Overexpression of PIK3R3 regulated increases in fatty acid β-oxidation in hepatic cells and reduced fatty liver in vivo

To identify the role of PIK3R3 in lipid metabolism, hepatic cells (HepG2 and LO2) were transfected with the PIK3R3 overexpression vector (PIK3R3) or the control vector (Vector). CPT1a and ACADM protein and mRNA expression levels were upregulated in cells with PIK3R3 overexpression vs cells that were transfected with the control vector (Figure 2a; Supplementary Figure 2A). We next measured the levels of ketone bodies in the media of the transfected cells. Overexpression of PIK3R3 increased the level of ketone bodies (Supplementary Figure 2B), suggesting that PIK3R3 plays an important role in fatty acid oxidation. Real-time PCR results suggested that the overexpression of PIK3R3 increased the expression levels of genes involved in mitochondrial and peroxisomal fatty acid oxidation, such as Cpt1a, Acadm, Cyp4a10 and Cyp4a14 (Figure 2b). To investigate the role of PIK3R3 in hepatic lipid metabolism in vivo, we injected HFD mice with the Pik3r3 recombinant adenovirus vector (Ad-Pik3r3) or control adenovirus (Ad-control) via the tail vein to induce the overexpression of Pik3r3 in the liver. Remarkably, Ad-Pik3r3-infected HFD mice had a significantly improved fatty liver phenotype, as revealed by gross morphological changes and histological analyses (H&E and Oil Red O staining) (Figure 2c). CPT1a and ACADM expression levels were also upregulated in Ad-Pik3r3-infected HFD mice (Figure 2c). Biochemical analyses also revealed a significant

Figure 1 PIK3R3 expression is regulated by nutritional status and is upregulated in HFD-induced fatty liver mice. (a) Representative morphology and H&E, Oil Red O, Acadm and Cpt1a stained liver sections from C57BL/6J mice fed a normal chow or high-fat diet (HFD) for 8 weeks (n=6/group). (b) Serum ketone body (top panel) and hepatic TG (bottom panel) levels of mice fed a normal chow diet or HFD. (c) Quantitative PCR (top panel) analysis and western blot (bottom panel) analysis showing the expression level changes of Pik3r3 in mice livers during HFD feeding. (d) Quantitative PCR (left) analysis and western blot (right) analysis showing expression levels of hepatic Pik3r3 in C57BL/6J mice subjected to ad libitum feeding, 24 h fasting and 24 h fasted/24 h re-fed conditions (n=2/group). Data are expressed as the means±s.e.m. *P<0.05; **P<0.01.
decrease in serum ketone bodies and hepatic TG levels in Ad-Pik3r3-infected HFD mice compared with control Ad-control-infected HFD mice (Figure 2d and e).

Knockdown of PIK3R3 impairs hepatic fatty acid β-oxidation in cell culture and in vivo
We then transfected the hepatic cell lines HepG2 and LO2 with PIK3R3 siRNA (si-PIK3R3) to knock down PIK3R3 expression and determined the protein expression levels of PIK3R3, CPT1a and ACADM by immunoblotting. When PIK3R3 expression was knocked down, CPT1a and ACADM protein levels were downregulated compared with those in the cells transfected with control siRNA (si-control) (Figure 3a). CPT1a and ACADM mRNA levels were also downregulated by si-PIK3R3 (Supplementary Figure 3A). The levels of ketone bodies in the cell media from cells treated with si-PIK3R3 or si-control showed that the downregulation of PIK3R3 decreased the levels of ketone bodies (Supplementary Figure 3B). These data further demonstrated that PIK3R3 plays an important role in fatty acid β-oxidation in hepatic cells. Real-time PCR results suggested that the downregulation of PIK3R3 also decreased the expression levels of genes involved in mitochondrial and peroxisomal fatty acid oxidation, such as Cpt1a, Acadm, Cyp4a10 and Cyp4a14 (Figure 3b). To examine the effects of the loss of PIK3R3 in vivo, mice were fed normal Chow and infected with si-Pik3r3 by tail vein injection to inhibit Pik3r3 expression in the liver. Interestingly, these normal Chow-fed mice infected with si-Pik3r3 developed a severe fatty liver phenotype (Figure 3c). Immunostaining showed that the Cpt1a and Acadm protein expression levels were also downregulated by si-Pik3r3 (Figure 3c). Biochemical analyses revealed a significant decrease in serum ketone bodies and an increase in hepatic TG levels in mice fed the normal Chow diet and treated with si-Pik3r3 compared with those treated with si-control (Figure 3d and e).

PIK3R3 regulates the expression of PPARα
PPARα and its target genes have been shown to play key roles in hepatic lipid metabolism. We thus examined the relationship between Pparα mRNA/protein expression levels in mice fed normal Chow and mice fed a HFD. Interestingly, Pparα mRNA and protein expression levels were downregulated in HFD-fed mice (Figure 4a), consistent with its important role in lipid metabolism. Moreover, adenovirus overexpression of PIK3R3 increased the expression of PPARα in the livers of HFD-fed mice at both the mRNA and the protein levels.
levels (Figure 4b and c, Supplementary Figure 4A), and downregulation of PIK3R3 decreased the expression of PPARα in normal chow-fed mice at both the protein and the mRNA levels (Figure 4d and e, Supplementary Figure 4B). Furthermore, siRNA-mediated knockdown of PIK3R3 in LO2 and HepG2 cells decreased the expression of PPARα but had no effect on PPARγ (Figure 4f, Supplementary Figure 4C). Adenovirus-mediated overexpression of PIK3R3 induced the expression of PPARα but had no effect on the expression of PPARγ (Figure 4g, Supplementary Figure 4D). Taken together, our data showed that PIK3R3 controls the expression of PPARα. Further supporting the role of PIK3R3 in the nutritional regulation of PPARα expression, mice were subjected to a fasting/feeding cycle. Hepatic Pparα mRNA and protein expression levels increased after prolonged fasting (24 h). These changes were reversed by refeeding (Supplementary Figure 4E).

**PPARα mediates the effects of PIK3R3 on cellular and hepatic lipid metabolism**

To test whether PPARα mediates the effects of PIK3R3 on hepatic lipid metabolism, we performed PPARα rescue and inhibition experiments in hepatic cells. The simultaneous overexpression of PIK3R3 and knockdown of PPARα expression by si-PPARα decreased the expression of PPARα and its target genes ACADM and CPT1a in LO2 cells (Figure 5a). The levels of ketone bodies in the cell supernatants were also reversed by co-treatment with PIK3R3 and si-PPARα in LO2 cells (Supplementary Figure 5A). The knockdown of PIK3R3 in HepG2 cells also decreased the expression of PPARα and its target genes ACADM and CPT1a. Transfection of the PPARα vector in the PIK3R3 knockdown cells restored the expression of ACADM and CPT1a (Figure 5b), as well as the levels of ketone bodies in the supernatants (Supplementary Figure 5B).

To further test whether PPARα mediates the effects of PIK3R3 on hepatic lipid metabolism in vivo, si-Pparα was injected via the tail vein into Ad-Pik3r3-infected HFD mice. si-Pparα significantly inhibited the Pik3r3 induction of Pparα and its target genes involved in hepatic fatty acid oxidation (Figure 5c and d, Supplementary Figure 5C) and almost completely abolished the ability of PIK3R3 to reduce hepatic TG content and to increase serum ketone bodies (Figure 5e and f). In addition, we also measured the body weights of the mice in each group; the data showed that the overexpression of Pik3r3 could reduce the body weights of HFD-fed mice, compared with the control group, and this body weight loss could be rescued by the knockdown of Pparα (Supplementary Figure 3B).
Conversely, the overexpression of Pparα in si-Pik3r3-treated mice receiving the normal chow diet and transfected with Ad-Pparα significantly reversed the inhibitory effects of si-Pik3r3 on Pparα expression and its fatty acid oxidation genes in the liver (Supplementary Figure 6A–C). The overexpression of Pparα also reversed the effects of si-Pik3r3 on hepatic TG content and serum ketone bodies (Supplementary Figure 6D and E). Taken together, these data show that PPARα signaling plays a critical role in mediating the effects of PIK3R3 on hepatic lipid homeostasis.

**PIK3R3 regulation of PPARα expression depends on HNF4α**

Our data showed that PIK3R3 induced both PPARα mRNA and protein levels, suggesting that PIK3R3 may regulate PPARα gene expression. HNF4α and NR2F2 were previously reported to be the main transcription factors that bound to the PPARα promoter and regulated the gene expression of PPARα. Accordingly, we examined the effects of PIK3R3 on HNF4α and NR2F2 expression in hepatic cells and found that HNF4α expression was upregulated in cells overexpressing PIK3R3 and downregulated in PIK3R3-deficient cells (Figure 6a, Supplementary Figure 7A). There was little change in NR2F2 expression under these conditions. To demonstrate the critical role of HNF4α in the PIK3R3 regulation of PPARα gene expression, we co-transfected LO2 cells with si-HNF4α and PIK3R3 vectors and found that knockdown of HNF4α expression abrogated the induction of PPARα expression due to PIK3R3 overexpression (Figure 6b, Supplementary Figure 7B). Furthermore, in LO2 cells co-transfected with the HNF4α vector and si-PIK3R3, the decreased PPARα expression due to PIK3R3 knockdown was rescued (Figure 6c, Supplementary Figure 7C). To further investigate the role of HNF4α in PIK3R3-regulated PPARα expression, we examined HNF4α binding to the PPARα promoter using chromatin immunoprecipitation (ChIP) assays. Interestingly, the overexpression of PIK3R3 promoted HNF4α binding to the PPARα promoter, whereas PIK3R3 knockdown inhibited the binding of HNF4α to the PPARα promoter (Figure 6d and e). These data suggest that PIK3R3 regulation of PPARα expression depends upon its induction of HNF4α.
DISCUSSION

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an important role in a variety of biological processes, such as cell metabolism, glucose transporter regulation, cell cycle regulation, cell growth, and cancer development. The activation of Akt suppresses the expression of genes involved in hepatic fatty acid β-oxidation and decreases hepatic gluconeogenesis. It was previously reported that sterol-regulatory element binding proteins (SREBPs), key players in fatty acid and cholesterol biosynthesis, regulate the expression of PIK3R3, a PI3K regulatory subunit, to help mediate metabolic responses to insulin and growth factors in various tissues. 

Previously, little was known about the downstream signaling pathway and role of PIK3R3 in hepatic lipid metabolism. In this study, we described a novel role and mechanism for PIK3R3 in hepatic lipid metabolism. In vivo and in vitro evidence suggested that PIK3R3 surprisingly promoted hepatic fatty acid β-oxidation rather than lipogenesis via the induction of PPARα expression and its target genes. In particular, tail vein injection of an adenoviral vector expressing Pik3r3 markedly reduced fatty liver and improved hepatic fatty acid β-oxidation in mice fed a HFD. By contrast, the transfection of Pik3r3 siRNA induced fatty liver and decreased hepatic fatty acid β-oxidation in mice fed a normal chow diet. Moreover, PIK3R3 mRNA and protein expression levels were decreased in the fatty livers from mice fed the HFD, suggesting that reduced PIK3R3 may contribute to the development of hepatosteatosis. These findings suggest that PIK3R3 expression may play an important compensatory role in opposing the lipogenic actions of insulin through its stimulation of fatty acid β-oxidation.

PPARα, PPARγ and PPARβ/δ are ligand-activated transcription factors belonging to the NR1C nuclear receptor subfamily. Many PPAR target genes are involved in fatty acid metabolism in tissues with high rates of oxygen consumption, such as the muscle, heart and liver. PPARα activation...
improves steatosis, inflammation and fibrosis in pre-clinical models of non-alcoholic fatty liver disease and has been identified as a new potential therapeutic area.2 Some PPARα agonists have been studied as potential candidate drugs for non-alcoholic fatty liver disease therapy in humans.28,29 Our study showed that PIK3R3 induced HNF4α expression, a transcription factor involved in the regulation of serum glucose and lipid levels. HNF4α is also a key transcription factor mediating PPARα expression; however, the precise mechanism by which PIK3R3 stimulates the gene expression of HNF4α is still not known. PPARα and HNF4α rescue and inhibition experiments in hepatic cells and in vivo provided further evidence for a PIK3R3-HNF4α-PPARα pathway that plays a significant role in hepatic lipid metabolism. A better understanding of this signaling pathway, particularly the regulation of PIK3R3 expression in different nutritional states, may help novel molecular targets for treating non-alcoholic fatty liver disease to be determined.

Currently, PI3K inhibitors are mainly used for anti-cancer therapy; however, only a subset of the many cellular processes regulated by the PI3K pathway is directly involved in cell proliferation.30 Recently, the pharmacological inhibition of PI3K has been shown to be an effective and safe anti-obesity intervention that could reverse the negative effects of metabolic syndrome in humans.5 Previously, we reported that PIK3R3 contained a unique NH2 terminus that mediated PIK3R3-specific functions that were different from other regulatory subunits.31 Some of the known functions of PIK3R3 are as follows. Our findings with Rb and PCNA, as well as earlier studies of PIK3R3 (Morris White), may help us to determine potential reasons why nutritional status might regulate PIK3R3. Our previous studies have suggested that targeting PIK3R3 regulatory subunits could potentially facilitate the development of a new class of drugs that may have potential advantages over present PI3K catalytic subunit inhibitors.12,31 In summary, we have identified and characterized an important role for PIK3R3 in hepatic lipid metabolism. We have also identified a novel PIK3R3–HNF4α–PPARα signaling pathway that appears to be a key contributor to hepatic fatty acid β-oxidation. As the induction of PIK3R3 improved the fatty liver phenotype by increasing fatty acid β-oxidation, targeting PIK3R3 may be an effective strategy for treating NAFLD, a condition for which there is currently no approved drug therapy.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Figure 6 PIK3R3 regulation of PPARα expression was dependent on HNF4α. (a) PIK3R3 was overexpressed in LO2 cells and knocked down in HepG2 cells; western blots were used to detect the protein levels of PIK3R3 and HNF4α. (b) PIK3R3 was overexpressed in LO2 cells with or without HNF4α knockdown; western blot analysis showing the expression levels of PIK3R3, HNF4α and PPARα. (c) PIK3R3 was knocked down in LO2 cells with or without HNF4α overexpression; western blot analysis showing the expression levels of PIK3R3, HNF4α and PPARα. (d) ChIP assay and quantitative PCR analysis showing the binding activity of HNF4α to the PPARα promoter after the overexpression of PIK3R3. (e) ChIP assay and quantitative PCR analysis showing the binding activity of HNF4α to the PPARα promoter after the downregulation of PIK3R3. Data are expressed as the means ± s.e.m. *P<0.05.
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