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Research article

The P300-ARRDC3 axis participates in maternal subclinical hypothyroidism and is involved in abnormal hepatic insulin sensitivity in adult offspring

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ABSTRACT

Numerous studies have suggested potential associations between maternal subclinical hypothyroidism (SCH) and adverse metabolic outcomes in offspring, however, the underlying mechanism remains unclear. In this study, we generated a maternal SCH mouse model by administering 50 ppm 6-propyl-2-thiouracil (PTU) in the drinking water of pregnant mice until delivery. This model was used to investigate the mechanisms influencing glucose metabolism in offspring. RNA sequencing (RNA-seq) revealed a substantial increase in ARRDC3 expression in the livers of the offspring of the SCH model mice, which may contribute to insulin resistance. Additionally, the phosphorylation levels of key proteins in the insulin signalling pathway, such as protein kinase B (Akt), glycogen synthase kinase 3 beta (GSK-3β), and Forkhead box protein O1 (FoxO1), were correspondingly reduced in the SCH offspring. Moreover, overexpression of ARRDC3 in Hepa1−6 cells suppressed the Akt/GSK-3β/FoxO1 signalling pathway and increased the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), which was consistent with the molecular changes observed in SCH offspring. Our results also indicated that the upregulation of ARRDC3 in SCH offspring may result from increased H3K27 acetylation of the ARRDC3 promoter region, driven by elevated expression of P300. Importantly, adequate L-T4 supplementation during pregnancy improved insulin sensitivity and reversed the molecular alterations in the insulin signalling pathway observed in SCH offspring. In conclusion, exposure to intrauterine SCH resulted in altering the P300-ARRDC3 axis in offspring and impaired insulin sensitivity by disrupting the Akt/GSK-3β/FoxO1 signalling pathway. Timely L-T4 supplementation during pregnancy is an effective strategy to prevent insulin resistance in offspring of SCH mothers. This study elucidates potential molecular mechanisms behind insulin resistance in SCH offspring and suggests novel therapeutic targets for treating metabolic disorders related to maternal SCH.

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1. Introduction

Dr. David Barker proposed the Foetal Origins of Adult Disease (FOAD) theory, which posits that foetuses and neonates are vulnerable to adverse intrauterine or early developmental environments, increasing their susceptibility to chronic diseases later in life [1]. A detrimental maternal metabolic environment during pregnancy can have lasting effects on the offspring's health, including an elevated risk of developing chronic metabolic conditions such as obesity, type 2 diabetes (T2DM), and cardiovascular disease [2,3]. Maternal subclinical hypothyroidism (SCH)—characterized by elevated serum thyroid-stimulating hormone (TSH) levels with normal serum free T4 levels—occurs in 3.5 % to 18 % of pregnancies, depending on the diagnostic criteria used, and is more common than overt hypothyroidism [4]. Recent evidence suggests that maternal hypothyroidism can induce the metabolic phenotype in offspring. Studies in rodents have shown that offspring exposed to maternal hypothyroidism exhibit increased weight, hyperglycaemia, hyperinsulinaemia, insulin resistance, hypertriglyceridaemia, hypercholesterolaemia, and hyperleptinaemia [5–9], these conditions may worsen when the offspring are fed a hypercaloric diet [10,11]. However, relatively few studies have explored the long-term effects of maternal SCH on the metabolic parameters of offspring. Therefore, this study aimed to investigate the impacts of maternal SCH on glucose metabolism in adult progeny and explore the molecular mechanisms underlying these metabolic changes in SCH offspring.

The liver plays a critical role in glucose homeostasis, producing glucose through glycogenolysis and gluconeogenesis during fasting and converting glucose into glycogen in response to feeding [12]. Insulin insufficiency disrupts glucose homeostasis, resulting in elevated blood glucose levels during fasting and the depletion of glycogen stores in the liver [13,14]. The insulin signalling pathway consists primarily of two classical branches: the Ras/MAP kinase pathway, which regulates cell growth and differentiation, and the PI3-kinase/Akt pathway, which mediates most metabolic effects [15,16]. Activation of the insulin receptor (INSR) by insulin triggers phosphorylation of insulin receptor substrates (IRS) on tyrosine residues, which serve as binding sites for the p85 regulatory subunit of PI3K [17]. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PI45P2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), while PIP3 is a key molecule that recruits and activates Akt [18]. Activated Akt phosphorylates Forkhead box protein O1 (FoxO1), leading to its exclusion from the nucleus and a consequent reduction in the transcriptional activity of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [19]. Moreover, AKT-mediated phosphorylation of glycogen synthase kinase 3 beta (GSK-3β) at Ser9 alleviates its inhibitory effect on glycogen synthase, promoting glycogen synthesis [20].

Recently, a novel group of arrestins known as α-arrestins has been identified [21,22]. These proteins play important roles in maintaining membrane protein homeostasis and global cellular metabolism [23]. The α-arrestin family includes arrestin domain-containing proteins (ARRDC) 1 through 5 and thioredoxin-interacting protein (TXNIP) [21,22]. Among these, ARRDC3 represents a newly discovered α-arrestin involved in inflammation, cancer, obesity, and glucose and lipid metabolism [24,25]. ARRDC3-null rodents exhibit increased insulin sensitivity, a lower propensity to gain weight, and reduced hepatic steatosis [25]. In hyperinsulinaemic states, ARRDC3 expression in the liver is upregulated and is dependent on insulin receptor signalling [26]. Studies have shown that ARRDC3 likely interacts with phosphatidylinositol 4,5-bisphosphate (PI45P2) [27], which is involved in the recruitment and activation of Akt. While it has been shown that ARRDC3 reduces insulin sensitivity by binding directly to the insulin receptor in the liver [26], the precise role of ARRDC3 in regulating the insulin signalling pathway and inducing insulin resistance in SCH offspring remains unclear [24].

Epigenetics refers to the modification of heritable gene expression patterns without altering the underlying DNA sequence [28]. Numerous studies have suggested that exposure to environmental factors such as chemicals, drugs, stress, or infections during specific sensitive periods of intrauterine development or early childhood can influence gene expression and increase susceptibility to adult diseases [29–31]. Epigenetic modifications include DNA methylation, histone modification, and noncoding RNAs [32,33]. Among these, histone acetylation is a reversible post-translational modification that regulates gene expression by altering chromatin structure, making it more accessible for transcription factors to bind [34]. A well-studied epigenetic mark is the acetylation of histone H3 at lysine 27 (H3K27Ac), which has been extensively characterized for its role in gene regulation. Chen et al. reported that a chronic maternal high-fat diet (HFD) leads to changes in the methylation and acetylation of H3K27, thereby influencing the expression of genes involved in osteoblast differentiation [35].

In this study, we established a mouse model of maternal SCH to investigate the alterations in glucose metabolism and the underlying epigenetic regulatory mechanisms in offspring. The primary objectives were to elucidate the impact of maternal SCH on insulin sensitivity and identify key molecules involved in the induction of insulin resistance in offspring. Our findings offer new insights and potential therapeutic targets for the prevention and treatment of metabolic abnormalities in offspring affected by maternal SCH.

2. Materials and methods

2.1. Animals

C57BL/6J mice were obtained from the Experimental Animal Center at Soochow University. All animal experiments were approved by the Soochow University Institutional Animal Care and Use Committee (approval number: SYXK-2022-0043). The mice were housed at 22 ± 2 °C in a room with a 12-h light/dark cycle, with chow and water ad libitum. Appropriate anaesthesia was administered prior to all invasive procedures. Pregnant mice were randomly divided into two groups: the SCH group, receiving 6-propyl-2-thiouracil (PTU) at 50 μg/ml (50 ppm, P3755, Sigma, USA) in drinking water, and a control group receiving standard water [36,37]. PTU supplementation was ceased at pup birth. Pups' body weights were measured weekly from weaning. Levels of serum TSH, free T3

(FT3), and free T4 (FT4) were determined via ELISA kits (YJ063200, YJ301038, YJ057763, Elabscience, China). Given that estrogen may contribute to sex-specific differences in glucose tolerance related to fetal hypothyroidism [38], only male offspring were selected for this study.

2.2. LT4 treatment

L-T4 is a standard treatment for hypothyroidism. The pregnant mice were randomly assigned to three groups: the CON + VEH, SCH + VEH, and SCH + LT4 groups. LT4 (0.1 μg/ml, S2599, Selleck Chemicals, USA) or vehicle (VEH) was added to their drinking water on the basis of previously reported dosing until delivery [39].

3. Metabolic analyses

3.1. Glucose, insulin, and pyruvate tolerance test

Metabolic assessments were conducted at 16 weeks of age. For the intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal pyruvate tolerance test (IPPTT), mice were fasted overnight (>16 h) and injected intraperitoneally with glucose (ST1228, Beyotime, China, 2 g/kg body weight) [40] or pyruvate (P4562, Sigma, USA, 2 g/kg body weight) [41]. For the intraperitoneal insulin tolerance test (IPITT), mice were fasted for 2 h and injected intraperitoneally with insulin (P3376, Beyotime, China, 1 IU/kg body weight) [42]. Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min. Blood samples were collected from the tail. A single droplet of blood was applied onto a glucose test strip and analysed via glucometer (Onetouch, Johnson & Johnson). The area under the curve (AUC) was calculated.

3.2. Plasma insulin measurement

Blood samples were collected from the abdominal aorta. The measurement of the serum insulin level was conducted via an enzyme-linked immunosorbent assay (ELISA) kit (F2579A, Fankew, China).

3.3. Tissue morphological analysis

Liver tissues were fixed in 4 % formaldehyde and embedded in paraffin. Paraffin blocks were cut into 6 μm slices and subjected to hematoxylin and eosin (HE) staining and periodic acid-Schiff (PAS) staining. Images were captured with a TE300 microscope (Nikon, Japan). Histopathological analysis of whole liver sections was performed in a blinded manner. The extent of hepatic steatosis was calculated as a percentage of the total hepatic steatosis area in HE-stained liver sections via ImageJ software [43]. PAS staining was performed according to the manufacturer's instructions (C0142M, Beyotime, China), with purple-stained cells identified as positively stained cells. Hepatic glycogen was quantified as the percentage of the PAS-positive area relative to the total section area by ImageJ [44].

3.4. RNA sequencing (RNA-seq)

Total RNA was extracted from liver tissues of two groups of mice (n = 5/group) using TRIzol reagent (A33251, TaKaRa, Japan). RNA was quantified via a NanoDrop spectrophotometer (NanoDrop, USA) and an Agilent 2100 bioanalyzer (Agilent, USA). RNA-seq was conducted via the Illumina HiSeq 4000 platform (Genewiz, China). GRCm38 was used as the reference genome. Expression levels of each transcript were determined via the fragments per kilobase of transcript sequence per million base pairs (FRKM) approach. The DEseq2 program was used for the analysis of differentially expressed genes (DEGs). DEGs between two groups were determined by a log2-fold change <-1 or >1 and a P value ≤ 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs was performed with the "clusterProfiler" R package [45].

3.5. Real-time quantitative RT-PCR

Total RNA was prepared with TRIzol reagent (A33251, TaKaRa, Japan) and used for cDNA synthesis via the PrimeScript RT reagent kit (K1622, Thermo Scientific, USA). Real-time PCR was performed via SYBR Green PCR Master Mix (CN830A, TaKaRa, Japan), and relative gene expression was calculated via the $2^{-\Delta\Delta CT}$ method, with 18S as an internal control. The primer sequences are listed in Supplementary Table 1.

3.6. Cell culture and treatment

The Hepa1-6 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (SH30243, HyClone, USA) supplemented with 10 % foetal bovine serum (FBS) (SV 30208, HyClone, USA) and 100 μg/mL penicillin–streptomycin solution (SV30010, Cytiva, USA) at 37 °C in a 5 % CO2 atmosphere. Transfection of pcDNA3.1 (empty vector) or pcDNA3.1-ARRDC3 was performed via Lipofectamine 2000 Transfection Reagent (11668, Invitrogen, USA) according to the reagent protocol. Following a 6-h incubation with the transfection mixture, cells were returned to fresh complete medium for gene expression. After overnight starvation in low glucose-

DMEM (SH30021, HyClone, USA) containing 0.2 % bovine serum albumin (ST025, Beyotime, China), the cells were treated for 40 min with insulin (25 nM) in high-glucose DMEM (a schematic diagram of the protocol used to study ARRDC3 overexpression-induced insulin resistance is shown in Supplementary Fig. 1).

3.7. Antibodies and immunoblot analysis

The liver tissue and Hepa1-6 cells were dissolved in lysis buffer (P0013C, Beyotime, China) containing 1 % PMSF (P001, NCM Biotech, China). After homogenization, the lysates were clarified by centrifugation (16,000×*g*, 30 min, 4 °C). The resulting supernatant was then collected and used for immunoblotting. The protein concentration was quantified via a BCA protein assay kit (P0010, Beyotime, China). Protein samples were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes (Millipore, Germany). The membranes were incubated with 5 % nonfat milk (P0216, Beyotime, China) in TBST for 2 h at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After three washes with TBST, the membranes were incubated with a secondary antibody conjugated to HRP for 1 h at room temperature. An enhanced chemiluminescence (ECL) system (Tanon, China) was used to visualize the immunoreactive bands, and the ImageJ software was used to quantify the results. The primary antibodies were as follows: ARRDC3 (1:1000, DF3517, Affinity Biosciences, China), PEPCK (1:1000, 16754-1-AP, Proteintech, China), G6Pase (1:1000, 22169-1-AP, Proteintech, China), p-Akt (Thr308) (1:1000, #2965, Cell Signaling Technology, USA), Akt (1:1000, #4691, Cell Signaling Technology, USA), p-FoxO1 (Ser253) (1:500, AF2305, Affinity Biosciences, China), FoxO1 (1:4000, 18592-1-AP, Proteintech, China), p-GSK3β (Ser9) (1:1000, #9322, Cell Signaling Technology, USA), GSK3β (1:4000, A5007, Bimake, USA), H3K27Ac (1:1000, #39133, Active Motif, China), H3 (1:1000, #9717, Cell Signaling Technology, USA), and P300 (1:1000, AF5360, Affinity Biosciences, China).

3.8. ChIP assays

Chromatin immunoprecipitation (ChIP) assays were conducted with a SimpleChIP Plus Enzymatic Chromatin IP Kit (#9005, Cell Signaling Technology, USA) according to the manufacturer's instructions. Liver tissue fragments were fixed with 37 % formaldehyde (8.18708, Sigma, USA) solution for 30 min at room temperature to facilitate chromatin cross-linking. The crosslinking reaction was quenched by adding glycine at room temperature. Subsequently, the samples were washed three times with precooled PBS buffer (SH30256, HyClone, USA) containing a protease inhibitor cocktail (PIC). After washing, tissue fragments were homogenized into single-cell suspensions with a homogenizer. Each sample was supplemented with 0.5 μL of micrococcal nuclease and incubated at 37 °C for 20 min to fragment the DNA into 150–900 bp fragments. After digestion, sonication was used to disrupt the nuclear membrane (20 s on/30 s off for 13 cycles). Next, the fragmented chromatin samples were centrifuged at 16,000×*g* for 10 min at 4 °C. The supernatant was carefully collected and appropriately diluted with ChIP buffer. A total of 10 μL of the diluted supernatant was saved as input DNA, while the remainder was incubated at 4 °C overnight on a rotator with anti-H3K27Ac (#39133, Active Motif, China). Rabbit IgG (#2729, Cell Signaling Technology, USA) served as the isotype control. Protein A/G was added, and the mixture was incubated for 2 h at 4 °C with rotation. Afterward, elution buffer was added, and the mixture was incubated at 65 °C for 30 min to elute the protein-DNA complexes from the beads. The samples were then incubated at 65 °C with 40 μg/mL proteinase K for 2 h and subsequently purified via spin columns following the manufacturer's protocol. Finally, the purified DNA was assayed via quantitative polymerase chain reaction (qPCR). The primer sequences can be found in Supplementary Table 1.

3.9. DNA isolation and targeted bisulphite sequencing assay

Genomic DNA from liver tissue was extracted using the conventional phenol/chloroform method. Sodium bisulphite treatment was carried out on the DNA samples via the EZ DNA Methylation-GOLD Kit (D5006, Zymo Research, USA), following the methods recommended by the manufacturer. The DNA concentration was measured and then diluted to 20 ng/μL in preparation for bisulphite amplicon sequencing (BSAS). The CpG island situated in the proximal promoter region of ARRDC3 was chosen based on the following criteria: (1) a GC content of more than 50 %, (2) a minimum length of 200 base pairs, and (3) an observed/expected ratio over 60 %. BSAS primers were meticulously constructed and are listed in Supplementary Table 1. Following the completion of PCR amplification and library creation, the products were sequenced on the Illumina MiSeq platform. The methylation level at the CpG site was determined by calculating the ratio of methylated cytosines to the total number of cytosines that were tested.

3.10. Statistical analysis

SPSS 20.0 (IBM SPSS, Armonk, NY, USA) was used to perform all the data analyses. All the data are presented as the means ± SEMs. Student's t-test was used for comparisons between two groups. Comparisons among multiple datasets were performed by one-way analysis of variance (ANOVA), and Bonferroni correction was used to adjust P values for multiple comparisons. Significant differences are displayed as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Statistical analysis was performed with GraphPad Prism 8 (GraphPad, San Diego, California, USA). The sample size (n) for statistical tests representing biological replicates.

4. Results

4.1. Maternal subclinical hypothyroidism alters body weight, liver profile, and plasma insulin levels in offspring

To assess the impact of maternal SCH on offspring development, we established an SCH mouse model during pregnancy. Thyroid hormone testing confirmed significantly elevated TSH concentrations in the SCH mice, while serum FT4 and FT3 levels remained unchanged on the day of delivery (Fig. 1A), indicating the successful establishment of the maternal SCH model. To explore this further, we measured thyroid hormones in 16-week-old offspring, finding similar concentrations of TSH, FT4, and FT3 between the SCH and control groups (Fig. 1B). Additionally, the SCH offspring exhibited similar food and water intake (Supplementary Figs. 2A and B) but had higher body weights compared to controls at 15 and 16 weeks (Fig. 1C). Histological analysis revealed that SCH offspring exhibited mild but noticeable hepatic steatosis and reduced hepatic glycogen content compared to normal offspring (Fig. 1D). Moreover, fasting serum insulin concentrations were significantly higher in the SCH offspring (Fig. 1E). In summary, while maternal SCH does not affect the thyroid hormone levels of offspring, it has negative effects on their development, particularly on body weight, liver profile, and insulin levels.

4.2. Maternal subclinical hypothyroidism alters glucose metabolism in offspring

Previous studies have demonstrated that maternal hypothyroidism can lead to hyperglycaemia, hyperinsulinaemia, and insulin resistance in offspring. However, limited research has been conducted on the metabolic effects of maternal SCH. To investigate this further, we examined glucose metabolism and insulin sensitivity in SCH offspring. During the IPGTT, SCH offspring exhibited a higher blood glucose response at 60 and 90 min, indicating significantly reduced glucose tolerance compared to the control group (Fig. 2A). Insulin resistance and hepatic gluconeogenesis were evaluated using IPITTs and IPPTTs, respectively. In both tests, SCH offspring showed elevated blood glucose levels at 60 and 90 min (Fig. 2B and C). Additionally, the expression levels of key gluconeogenic enzymes, G6Pase and PEPCK, were significantly higher in SCH offspring (Fig. 2D). Overall, these results indicate that maternal SCH causes a decrease in glucose tolerance and insulin sensitivity, and increases gluconeogenesis in progeny.

4.3. Maternal subclinical hypothyroidism decreases insulin sensitivity in offspring via the ARRDC3-Akt/GSK-3β/FoxO1 pathway

To explore the molecular mechanisms underlying glucose metabolism dysregulation caused by maternal SCH, we performed RNA sequencing to identify differences in gene expression in liver tissues of SCH offspring. We found 41 differentially expressed genes (DEGs) between the SCH offspring and control groups, including ARRDC3, which showed significantly increased expression in the

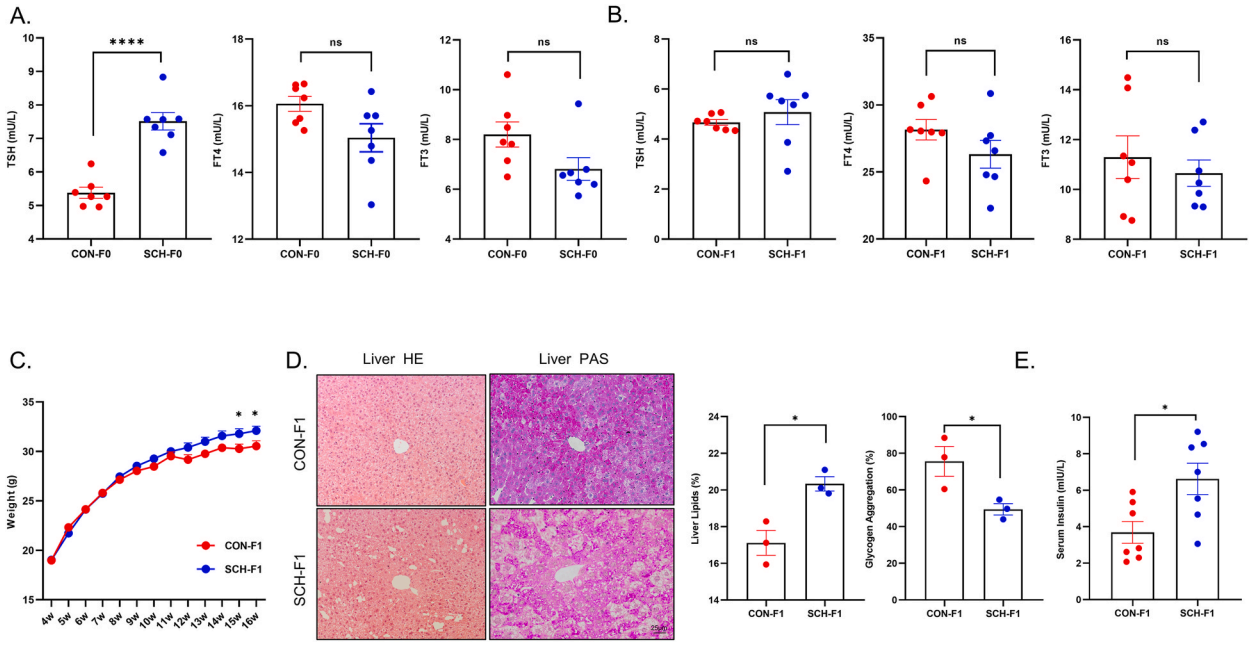

Fig. 1. Maternal subclinical hypothyroidism alters body weight, liver profile, and plasma insulin levels in offspring. (A) Serum levels of TSH, FT4, and FT3 of F0 on the day of delivery, (B) and F1 at postnatal 16 weeks (n = 7 per group). (C) Postnatal growth curve of offspring from weaning to 16 weeks (CON-F1: n = 15; PTU-F1: n = 22). (D) Representative images of hematoxylin-eosin (HE) and periodic acid Schiff staining (PAS) of the liver (magnification: × 400, scale bar = 25 μM) and the quantitative analysis of HE and PAS staining. (E) Serum insulin levels of CON-F1 and SCH-F1 mice were measured after fasting overnight (or 16h) (n = 7 per group). *p < 0.05, ***p < 0.001, ****p < 0.0001. Data are mean ± SEM.

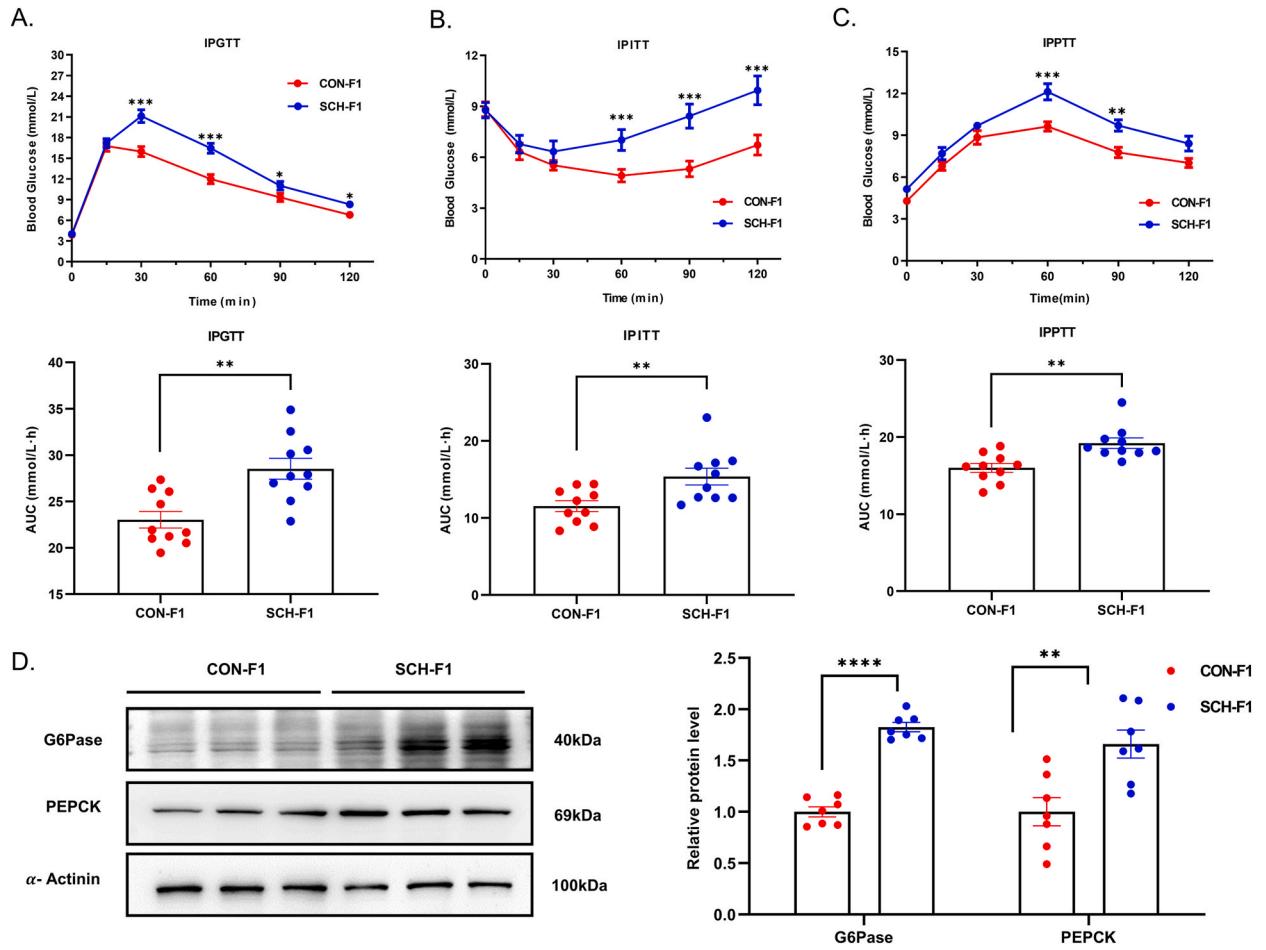

Fig. 2. Maternal subclinical hypothyroidism alters glucose metabolism in offspring. (A) Glucose tolerance test (2 g/kg body weight) and AUC of CON-F1 and SCH-F1 mice (n = 10 per group). (B) Insulin tolerance test (1 IU/kg body weight) and AUC of CON-F1 and SCH-F1 mice (n = 10 per group). (C) Pyruvate tolerance test (2 g/kg body weight) and AUC of CON-F1 and SCH-F1 mice (n = 10 per group). (D) G6pase and PEPCK protein expression in liver tissue from CON-F1 and SCH-F1 mice was detected by Western blot analysis (n = 7 per group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are mean ± SEM.

SCH-F1 (SCH offspring) group (Fig. 3A). A heatmap of DEGs (Fig. 3B) illustrates changes in gene expression, with red indicating increased and blue indicating decreased expression. Gene Ontology (GO) analysis indicated that maternal SCH may affect liver development in terms of biological processes (Fig. 3C). The KEGG pathway enrichment analysis revealed that the predicted target genes were involved mainly in the PI3K-Akt and MAPK signalling pathways (Fig. 3D). RT−PCR and Western blot analysis confirmed the RNA-seq findings, showing elevated ARRDC3 expression in SCH-F1 liver tissues (Fig. 3E−F). Since ARRDC3 is known to regulate insulin receptor signalling [26] and the PI3K-Akt pathway is essential for insulin signalling [46], we examined key genes in the Akt pathway in liver tissues of both groups. SCH-F1 exhibited lower phosphorylation levels of Akt (Thr308), FoxO1 (Ser256), and GSK-3β (Ser9), although total Akt, FoxO1, and GSK-3β levels remained unchanged (Fig. 3G). Similar reductions in phosphorylation were observed during fasting and refeeding in the SCH group compared to controls (Supplementary Fig. 3A). These findings suggest that the reduction in insulin sensitivity in SCH offspring may be linked to the ARRDC3-Akt/GSK-3β/FoxO1 pathway.

4.4. ARRDC3 overexpression inhibits Akt/GSK-3β/FoxO1 signalling in Hepa1-6 cells

To further investigate whether ARRDC3 contributes to abnormal glucose metabolism in SCH offspring through the Akt/GSK-3β/FoxO1 signalling pathway, we measured protein expression in Hepa1-6 cells overexpressing ARRDC3. The results showed that overexpression of ARRDC3 significantly increased the levels of G6Pase and PEPCK (Fig. 4A). Additionally, ARRDC3 overexpression significantly reduced the phosphorylation of Akt (Thr308), FoxO1 (Ser256), and GSK-3β (Ser9) in Hepa1-6 cells(Fig. 4B). These findings suggest a negative correlation between ARRDC3 expression and Akt/GSK-3β/FoxO1 signalling.

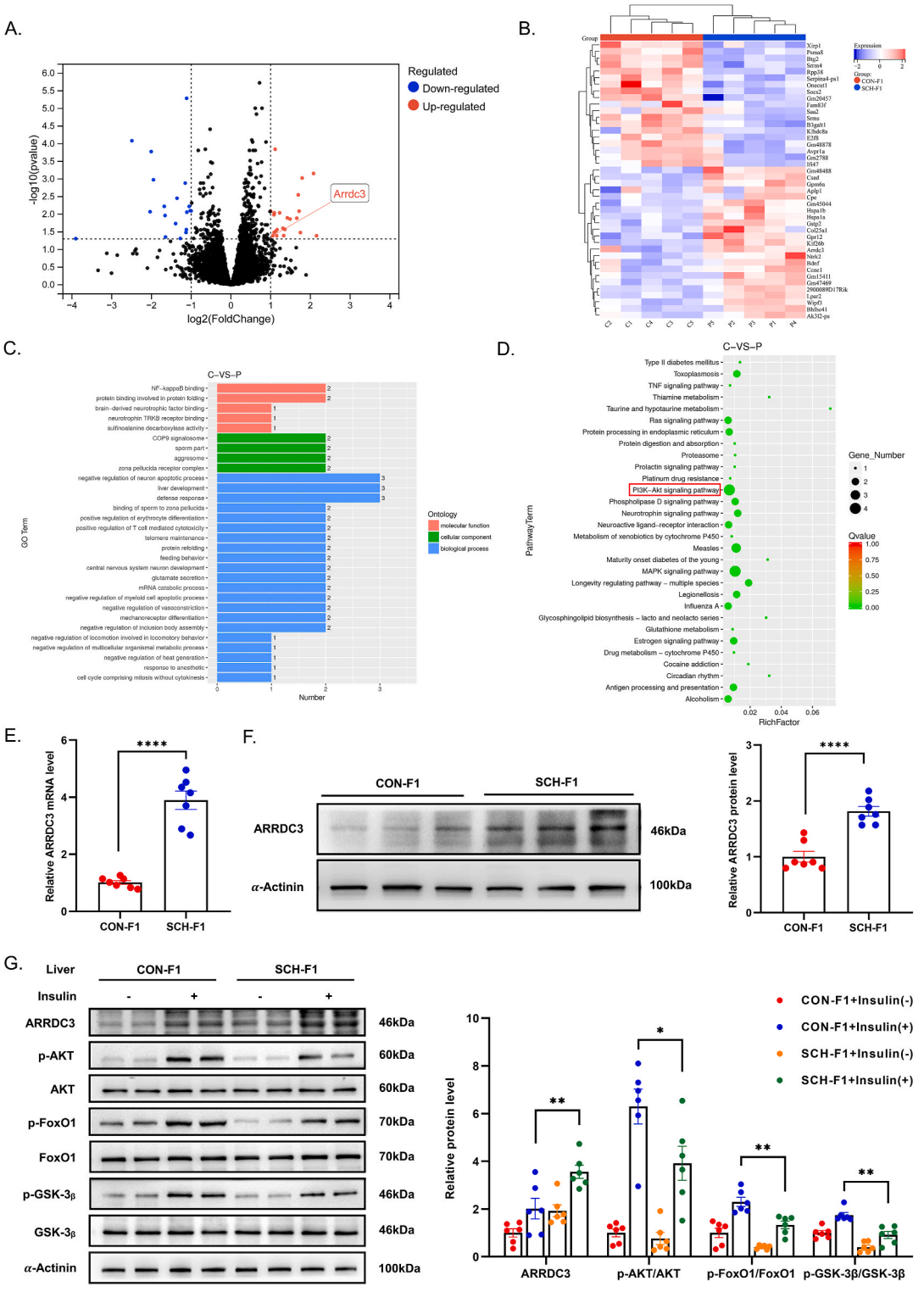

Fig. 3. Maternal subclinical hypothyroidism downregulated insulin sensitivity in offspring via ARRDC3-Akt/GSK-3β/FoxO1 pathway. (A)Volcano plot showing the differentially expressed genes between CON-F1 and SCH-F1 mice, red points denote the upregulated genes and blue points indicate the downregulated ones in SCH-F1 compared to CON-F1 (n = 5 per group). (B) Heat map of differential expression genes across all 10 samples. (C) Gene Ontology (GO) classification for predicted target genes in three categories (cellular component and molecular function and biological process). (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis for the predicted target genes. (E) ARRDC3 mRNA and (F) protein expression levels in liver from 16-week-old CON-F1 and SCH-F1 mice (n = 7 per group). (G) CON-F1 and SCH-F1 mice were fasted overnight

and then injected intraperitoneally with either insulin (2 IU/mouse) or PBS (phosphate-buffered saline) for 5 min. Representative western blots and the associated quantifications show phosphorylation of Akt (Thr308), FoxO1 (Ser256), and GSK-3β (Ser9) levels in liver (n = 6 per group). *p < 0.05, **p < 0.01, ****p < 0.0001. Data are mean ± SEM.

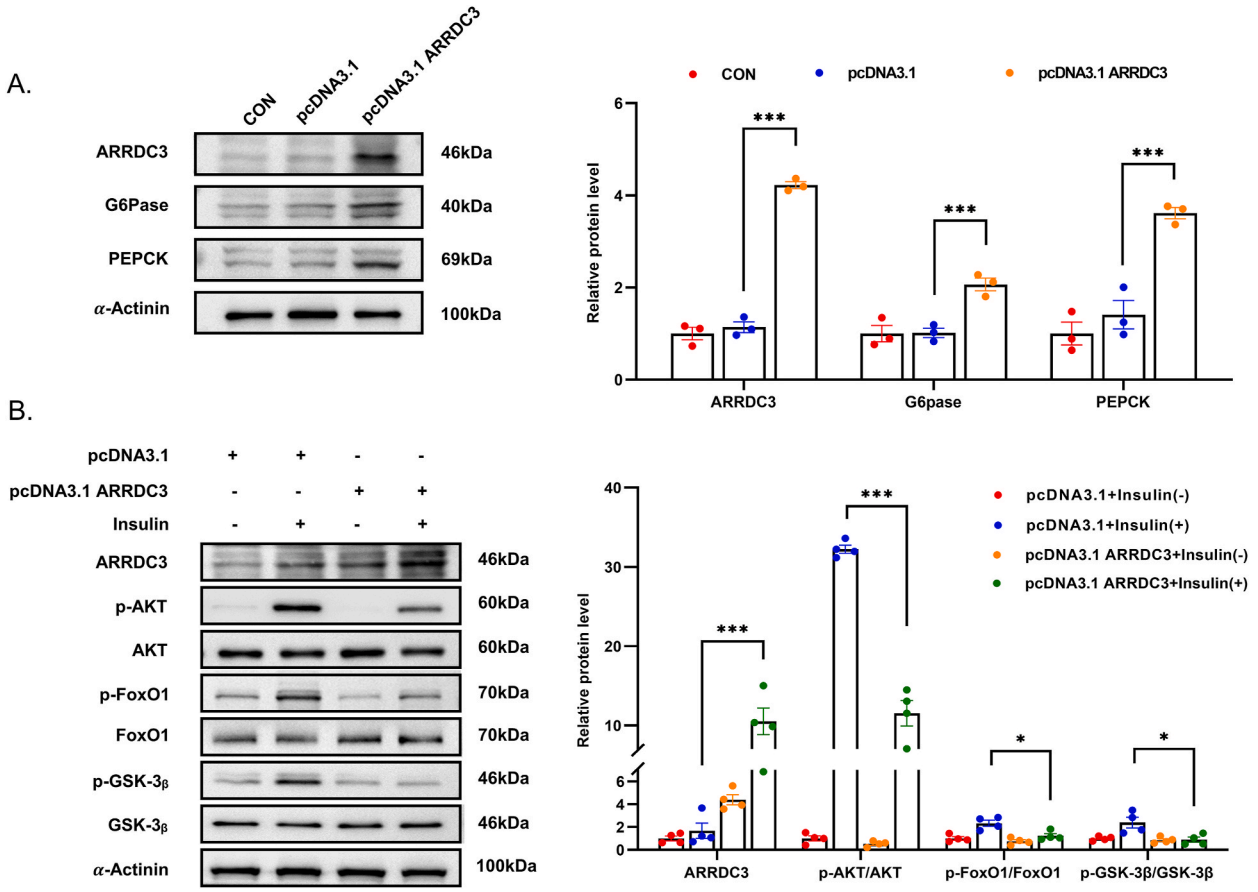

Fig. 4. ARRDC3 overexpression inhibits Akt/GSK-3β/FoxO1 signalling in Hepa1-6 cells. (A) Representative Western blots and the associated quantifications show ARRDC3, G6pase, and PEPCK levels in hepatocyte lysates (n = 3 per group). (B) Representative Western blots and the associated quantifications show phosphorylation of Akt (Thr308), FoxO1 (Ser256), and GSK-3β (Ser9) levels in hepatocyte lysates (n = 4 per group). *p < 0.05, ***p < 0.001. Data are mean ± SEM.

4.5. Maternal subclinical hypothyroidism promotes ARRDC3 expression via increased promoter H3K27 acetylation

Growing evidence suggests that epigenetic modifications play a crucial role in how adverse pregnancy factors affect offspring health [47]. To determine whether the upregulation of ARRDC3 in SCH offspring is regulated by epigenetic modifications, we used the Cistrome Data Browser (DB) to predict that ARRDC3 may be regulated by H3K27Ac (http://cistrome.org/db). Additionally, P300, a well-known histone acetyltransferase (HAT), is primarily responsible for promoting H3K27 acetylation [48]. We quantitatively determined the protein expression of H3K27Ac and P300 in the liver and found significant increases in H3K27Ac and P300 in the SCH offspring compared with the normal offspring (Fig. 5A–C). Chromatin immunoprecipitation assays revealed higher enrichment of H3K27Ac in the promoter region of ARRDC3 in SCH offspring (Fig. 5D). We also evaluated the DNA methylation status of the CpG islands of ARRDC3 between the two groups. We selected the CpG island containing 18 CpG sites located within 1000 bp upstream of the transcription start site (TSS) of the ARRDC3 gene (Supplementary Fig. 4A). Quality control procedures were implemented to validate the data quality (Supplementary Fig. 4B). Bisulphite sequencing revealed that DNA methylation in the promoter region of ARRDC3 in the liver did not significantly differ between the two groups. (Supplementary Figs. 4C and 4D). These findings suggest that maternal SCH promotes the H3K27 acetylation of the ARRDC3 promoter region by increasing P300.

4.6. Levothyroxine treatment decreases ARRDC3 expression and improves insulin sensitivity in SCH offspring

Levothyroxine (LT4) therapy is a common treatment for SCH and has been shown to improve pregnancy outcomes of patients with

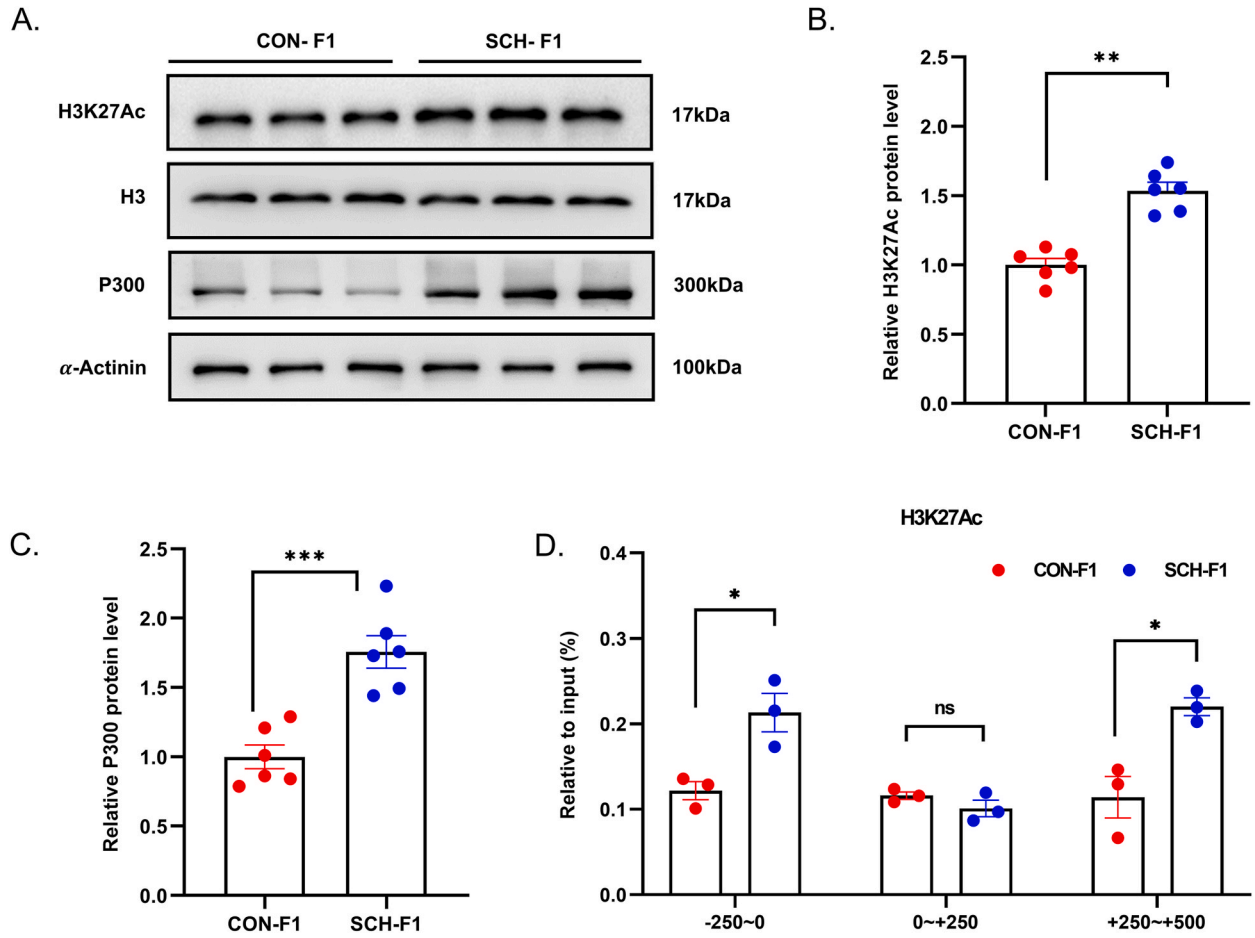

Fig. 5. Maternal subclinical hypothyroidism promotes ARRDC3 expression via increased promoter H3K27 acetylation. (A–C) The protein expression of H3K27Ac and p300 in the liver of 16-week-old CON-F1 and SCH-F1 mice (n = 6 per group). (D) Enrichment of H3K27Ac in the ARRDC3 promoter region of liver by ChIP-PCR (n = 3 per group). *p < 0.05, **p < 0.01, ***p < 0.001. Data are mean ± SEM.

SCH [49]. To assess whether LT4 supplementation could improve glucose metabolism in SCH offspring, pregnant mice were divided into three groups: the CON + VEH, SCH + VEH, and SCH + LT4 (LT4 0.1 μg/ml/day) groups. Results showed that serum TSH levels in the SCH-F0 group were significantly higher than in the SCH + LT4-F0 group, while FT3 and FT4 levels remained unchanged (Supplementary Figs. 5A and 5C), confirming that LT4 supplementation restored thyroid function in SCH-F0 mice. LT4 treatment also improved glucose tolerance and insulin sensitivity in SCH offspring (Fig. 6A and B), as serum insulin levels were significantly lower in the SCH + LT4-F1 mice compared to SCH + VEH-F1 mice (Fig. 6C). Moreover, H3K27Ac and P300 levels were significantly reduced in SCH + LT4-F1 mice (Fig. 6D), and ARRDC3 expression was also significantly lower in this group (Fig. 6E). LT4 supplementation increased the phosphorylation levels of Akt (Thr308), FoxO1 (Ser256), and GSK-3β (Ser9) (Fig. 6E), indicating enhanced Akt signalling and improved insulin sensitivity in SCH offspring. These results indicate that LT4 alleviates glucose intolerance and insulin resistance in SCH offspring through the P300-ARRDC3-Akt/GSK-3β/FoxO1 pathway.

5. Discussion

Gestational subclinical hypothyroidism is more common than overt hypothyroidism and autoimmune thyroid disease during pregnancy, with its effect on foetal outcomes and long-term offspring health remaining controversial [50]. To investigate this, we established a maternal SCH mouse model by administering PTU during pregnancy, which resulted in elevated serum TSH concentrations without significantly affecting serum FT4 and FT3 levels in the pregnant mice. Accumulating evidence suggests that maternal hypothyroidism may disrupt abnormal glucose metabolism in offspring. For instance, Lucaccioni L et al. reported that children born to mothers with hypothyroidism are more likely to develop metabolic syndrome, have higher body mass indices (BMI), and exhibit greater waist circumferences [10]. Offspring of hypothyroid mothers may be at higher risk for type 2 diabetes mellitus (T2DM), although potential confounding factors, such as preterm birth or low birth weight, cannot be entirely excluded [7]. Previous studies in rodents have also shown that maternal hypothyroidism leads to glucose intolerance in offspring, increasing their susceptibility to

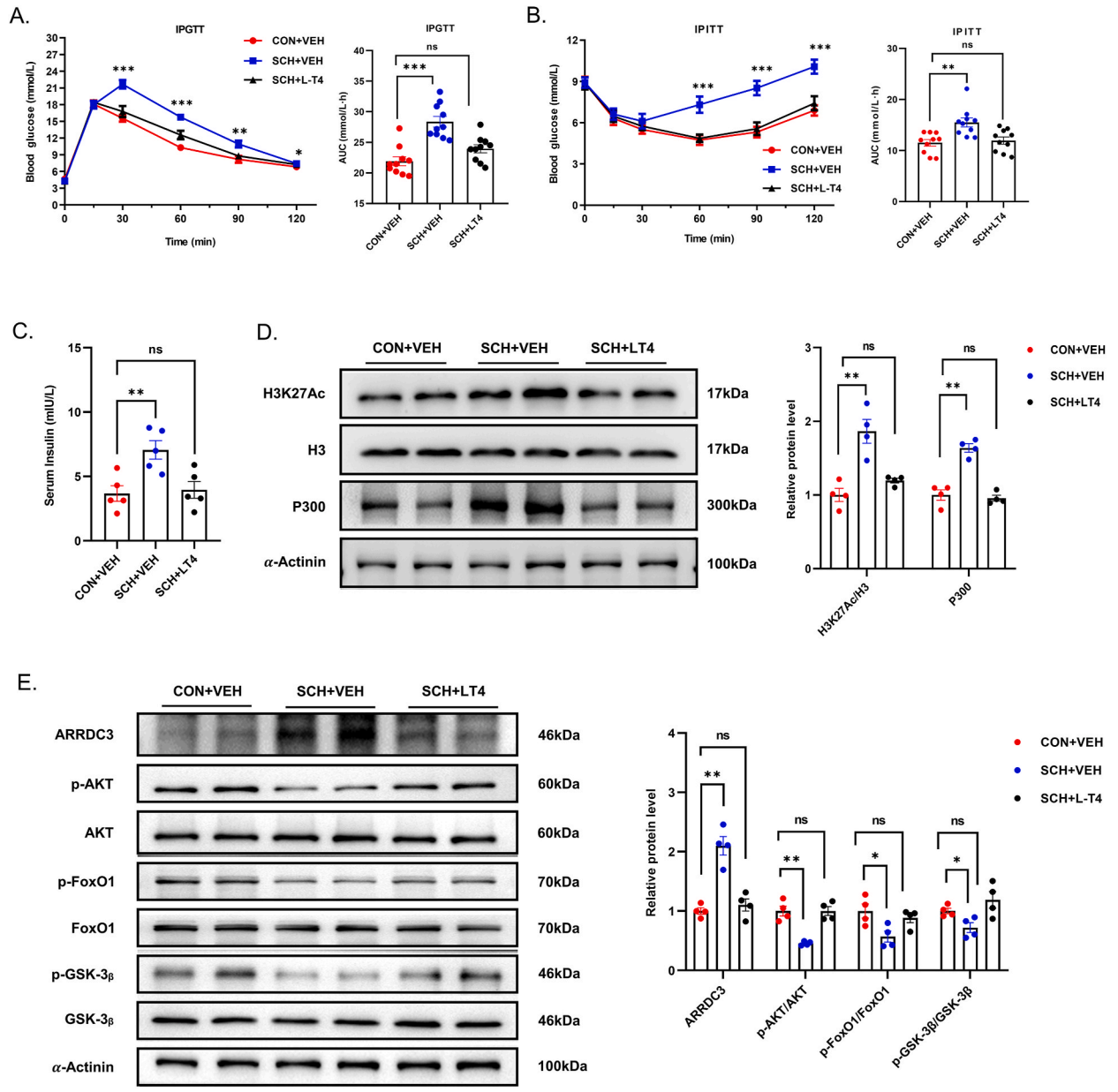

Fig. 6. Levothyroxine treatment decreases ARRDC3 expression and improves insulin sensitivity in SCH offspring. (A) Blood glucose levels during GTT and (B) ITT were detected in CON + VEH, SCH + VEH, PTU + LT4 mice. The area under the curve (AUC) is shown (n = 10 per group). (C) Serum insulin levels in CON + VEH, SCH + VEH, PTU + LT4 mice under fasting conditions (n = 5 per group). (D) The expression of H3K27Ac and P300 in the liver of three groups was detected by the Western bolt (n = 4 per group). (E)Protein levels of ARRDC3, phosphorylated and total Akt, FoxO1, GSK3β in liver of three groups after insulin injection (2 IU/mouse, 5min) (n = 4 per group). *p < 0.05, **p < 0.01, ***p < 0.001. Data are mean ± SEM.

diabetes in adulthood [9,38,51]. In our study, we found that maternal SCH predisposes offspring to glucose intolerance, insulin resistance, and impaired liver glucose regulation compared to offspring from euthyroid conditions. Early supplementation with L-T4 mitigated glucose intolerance and reduced insulin resistance in offspring of SCH mothers, indicating that even mild intrauterine thyroid hormone deficiency has significant long-term metabolic implications for offspring.

However, the molecular mechanisms by which maternal SCH impairs glucose metabolism and induces insulin resistance in offspring remain poorly understood. To explore this, we conducted RNA sequencing of liver tissues from two groups and identified *ARRDC3* as one of the most differentially expressed genes. ARRDC3 is closely associated with glucose and lipid metabolism. A previous study demonstrated that ARRDC3 (also known as thioredoxin-binding-protein-2-like inducible membrane protein [TLIMP]) regulates peroxisome proliferator-activated receptor (PPAR) activity, which is involved in glucose metabolism [52]. In another study, global

ARRDC3 deletion in rodents prevented age-related adiposity, alleviated hepatic steatosis, and improved insulin sensitivity [25]. Adipocyte-specific *ARRDC3* deletion also enhanced glucose tolerance. However, the effects of *ARRDC3* deletion in adipocytes alone did not fully explain the phenotypes observed in these mice, suggesting that other tissues may also contribute to the observed outcomes [25]. Recent studies have reported that *ARRDC3* is one of the top upregulated transcripts in the liver during a hyperinsulinemic-euglycemic clamp, and its loss in the liver increases insulin sensitivity [26]. Based on these findings, we speculate that the upregulation of *ARRDC3* may be a key mechanism underlying the abnormal glucose metabolic phenotype observed in SCH offspring.

The Akt signaling pathway, which is activated by INSR, plays a central role in liver metabolism, particularly in balancing processes like gluconeogenesis and glycogen synthesis [53]. Our results demonstrated that overexpression of ARRDC3 inhibited the insulin-induced activation of Akt at Thr308, GSK-3β at Ser9, and FoxO1 at Ser256. This led to increased expression of key gluconeogenic enzymes, G6Pase and PEPCK, which are known to promote hepatic glucose production, further suggesting that ARRDC3 plays a negative regulatory role in insulin sensitivity and glucose homeostasis. Recent studies have proposed that ARRDC3 may interact with phosphatidylinositol 4,5-bisphosphate (PI45P2) [27], a phospholipid that plays a crucial role in the recruitment and activation of Akt at the plasma membrane [54], which is essential for insulin-mediated signalling [55]. By interacting with PI45P2, ARRDC3 could potentially disrupt Akt's recruitment to the membrane, thereby attenuating its activation. This would inhibit downstream signaling required for insulin's effects on glucose metabolism.

Maternal hypothyroidism has been shown to influence epigenetic mechanisms, such as DNA methylation and histone modifications, which in turn alter the expression of various genes and affect processes like embryonic astrocyte maturation and cerebellar function in rodents [56,57]. In a similar context, elevated H3K27Ac levels have been reported in the offspring of mothers fed a high-fat diet (HFD) and treated with methimazole (MMI) [58]. Consistent with these findings, we observed increased H3K27Ac expression in the liver tissue of SCH offspring. The ChIP results demonstrated that increased enrichment of H3K27Ac in the promoter region of ARRDC3 in the liver tissue of the SCH offspring result in the upregulation of ARRDC3. Moreover, the upregulation of P300 further

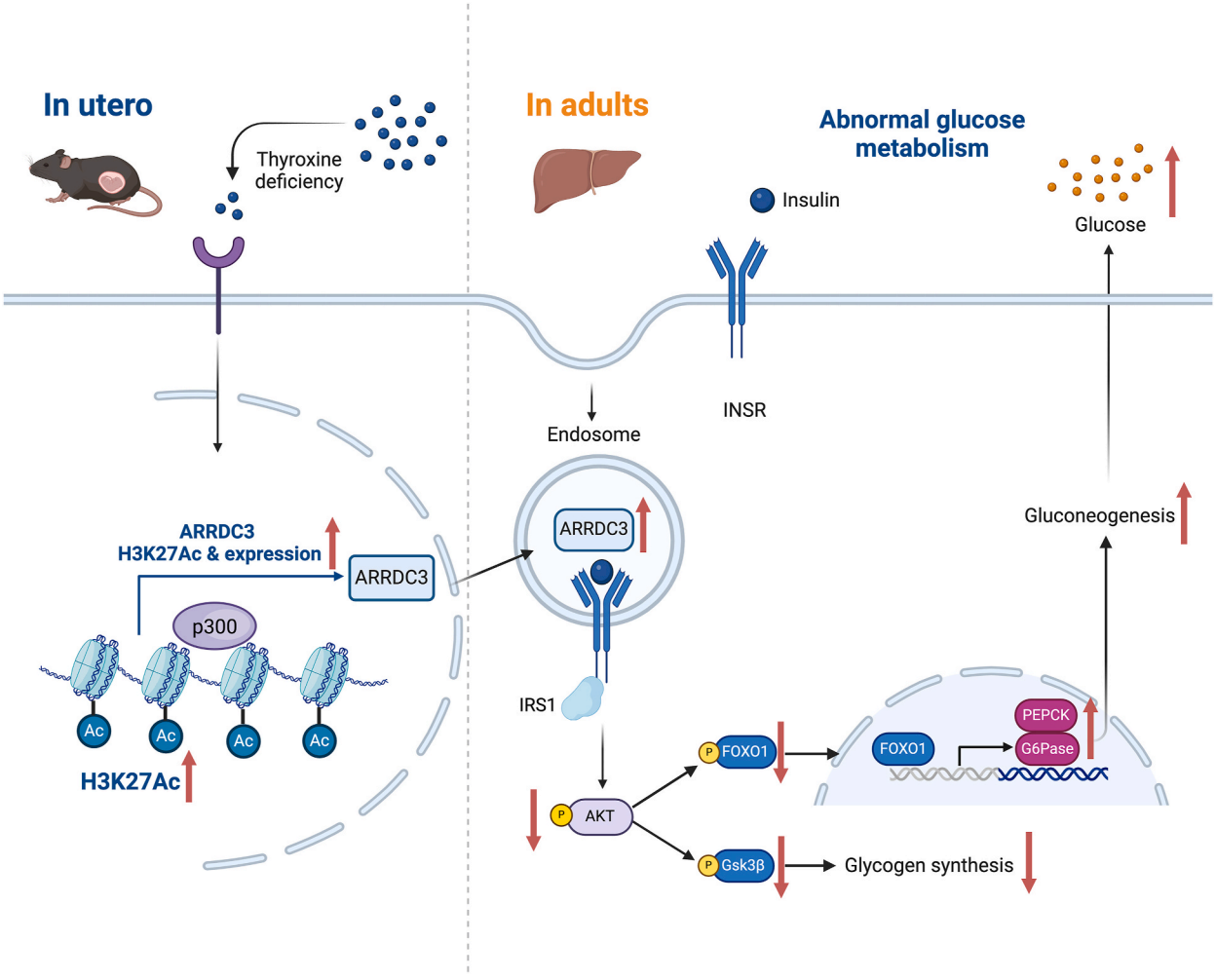

Fig. 7. Fig 7.

supports the role of H3K27Ac in regulating *ARRDC3* expression. In addition, our study found that this upregulation did not result from changes in the methylation status of the *ARRDC3* promoter region (Supplementary Fig. 3).

The results of this study provide valuable insights into the long-term metabolic health risks associated with mild intrauterine thyroid hormone deficiency. Nevertheless, there are several limitations to consider. We observed slight lipid accumulation in the livers of the SCH offspring, suggesting that ARRDC3 is involved in lipid metabolism. However, we did not investigate the specific effects of *ARRDC3* on lipid metabolism, nor could we elucidate the relationship between abnormal lipid metabolism and insulin resistance in SCH offspring. Further studies are needed to explore these connections. Additionally, we focused exclusively on the role of *ARRDC3* in the liver, but given its multiple physiological functions, this molecule may also contribute to abnormalities in other organs of SCH offspring. Regarding the regulation of ARRDC3, although we concentrated on P300 as a HAT, we recognize that other HATs may also play a role in its acetylation. Future studies should explore a broader range of HATs and their regulatory mechanisms to provide a more comprehensive understanding of acetylation in the ARRDC3 regulatory network.

In summary, our study revealed that maternal SCH causes insulin resistance and glucose intolerance in adult offspring. The potential molecular mechanism might involve the upregulation of P300-ARRDC3 axis, which suppresses the Akt pathway, eventually resulting in increased hepatic glucose production and the suppression of gluconeogenesis (Fig. 7). Notably, when mild in utero thyroid hormone deficiency was corrected, both the insulin resistance phenotype and related molecular alterations, such as *ARRDC3* expression, were reversed in SCH offspring. This suggests that timely L-T4 supplementation during pregnancy is crucial for ensuring the metabolic health of offspring exposed to maternal SCH. Our findings contribute to a deeper understanding of the prolonged detrimental effects of maternal SCH on metabolic health and provide a foundation for preventing abnormal metabolic function in SCH offspring.

CRediT authorship contribution statement

Ming Huo: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Data curation. **Xi Yu:** Formal analysis, Data curation. **Xianbin Yuan:** Software, Investigation, Data curation. **Jun Guo:** Software, Methodology. **Bin Wei:** Formal analysis, Data curation. **Yajun Shi:** Methodology, Investigation. **Yannan Gu:** Data curation. **Xuehong Zhang:** Visualization, Validation, Supervision. **Miao Sun:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition.

Ethics approval statement

The experiment was approved by the Committee and Animal Ethics Committee of the Soochow University (approval number SYXK-2022-0043).

Ethics approval and consent to participate

All animal experiments were performed in accordance with the guidelines of the University Committee on Animal Care of Soochow University.

Consent for publication

All authors have approved for publication.

Data availability statement

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39259.

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