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Impacts of Gestational F-53B Exposure on Fetal Neurodevelopment: Insights from Placental and Thyroid Hormone Disruption

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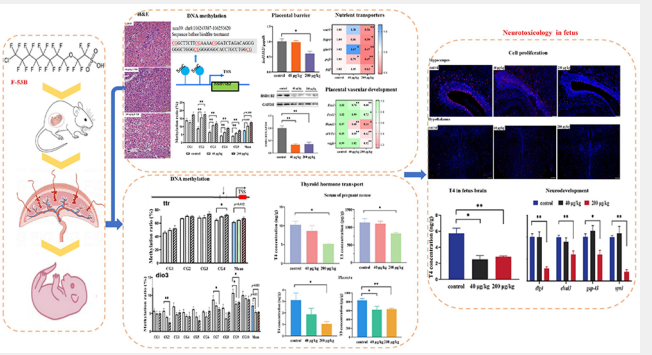
ABSTRACT: It has been evidenced that chlorinated polyfluoroalkyl ether sulfonic acids (Cl-PFESAs) have strong potential cross the placental barrier, but their adverse effects on offspring remain unclear. In this study, pregnant mice received daily intraperitoneal injections of chlorinated polyfluorinated ether sulfonate (Cl-PFESA; commercially known as F-53B, primarily comprising 6:2 Cl-PFESA and 8:2 Cl-PFESA) at dosages of 40 and 200 μg/kg from gestational days 6 to 17. Following gestational exposure, distinct accumulation of 6:2 and 8:2 Cl-PFESAs was observed in both the placenta and fetal brain, confirming their penetration across the placental and fetal blood-brain barriers. Maternal exposure to F-53B disrupted the placental 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*) barrier, characterized by hypermethylation of its promoter, decreased blood sinusoids in labyrinth layer, and downregulation of the nutrient transport genes, thereby severely impairing the placenta's protective and nutrient transfer functions. Concomitantly, significant fetal intrauterine growth restriction indicated by decreased fetal weight and crown-rump length was observed. Additionally, changes in thyroid hormones, along with transcriptional and DNA methylation alterations in the promoter regions of transthyretin (*ttr*) and deiodinase 3 (*dio3*) genes, were noted in the placenta. These epigenetic changes might affect the maternal-fetal transport of thyroid hormones, possibly leading to disrupted thyroid function in the F1 generation. With the decreased nutrient transport capacity of the placenta, T4 levels in the fetus are significantly reduced, resulting in significant fetal neurodevelopmental abnormalities, reduced nerve cell proliferation (Ki67), and damage to synaptic plasticity. This study reveals unveil the hidden dangers of F-53B, highlighting its neurotoxic effects on fetal development through the disruption of thyroid hormone transport across the placenta.



KEYWORDS: *F-53B, thyroid hormone, placental development, neurotoxicity, DNA methylation*

1. INTRODUCTION

Due to global regulations on perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), the most prevalent legacy per- and poly fluoroalkyl substances (PFASs) detected in diverse environmental compartments, biota, and human tissues, there has been a surge in the production and application of substitute compounds.[1] For instance, F-53B, a commercial mixture predominantly composed of 6:2 and 8:2 chlorinated polyfluorinated ether sulfonic acid (6:2 Cl-PFESA, 8:2 Cl-PFAES), has emerged as a PFOS replacement in China.[2] Initial reports documented 6:2 Cl-PFESA contamination in electroplating wastewater at concentrations exceeding 40 μg/L. Subsequently, widespread F-53B distribution has been observed in various environmental media, including municipal sludge (0.02−209 ng/L), surface water (2.0−44.2 ng/L), electroplating wastewater (43−112 μg/L), and river water (1.1−78.3 ng/L).[3−5] Additionally, F-53B has demonstrated a strong propensity for bioaccumulation, as evidenced by elevated 6:2 Cl-PFESA levels in organisms such as crucian carp (*Carassius carassius*),[6] black-spotted frog (*Pelophylax nigromaculatus*),[7] and even in Greenland marine mammals.[8] The pronounced binding affinity of 6:2 Cl-PFESA to human serum albumin results in a higher bioaccumulation factor than PFOS, coupled with a longer human elimination half-life (15.3 years versus 5.8 years for PFOS).[9] These findings underscore significant concerns regarding the potential adverse health impacts of F-53B on animals and humans.

Recent epidemiological and biomonitoring studies have reported median serum concentrations of 6:2 Cl-PFESA ranging from 4.78 to 93.7 ng/mL in general populations.[10−12] In contrast, electroplating workers exhibited significantly

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higher levels, with a maximum serum concentration of F-53B reaching 5.04 μg/mL.[12] Compared to the general population, pregnant women and newborns exhibit heightened vulnerability to PFAS exposure.[13] Given the fetus's susceptibility to xenobiotics, early life exposure can induce substantial and enduring adverse effects on normal child development.[14] Over the years, multiple studies have confirmed the efficient placental transfer of 6:2 Cl-PFAES during pregnancy, resulting in its detection in placental (0.34 ng/mL) and cord serum (0.6 ng/mL) samples.[15−17] Furthermore, this compound can traverse the blood-brain barrier, entering the cerebrospinal fluid.[18] Consequently, 6:2 Cl-PFAES has been associated with various adverse fetal health outcomes and birth defects, including low birth weight,[19] abnormal heart development,[20] and endocrine disorder,[21] which eventually leads to other health impacts in childhood.[22]

The placenta develops at the maternal-fetal interface[23] and performs numerous and diverse fundamental functions for proper growth and development of semiallogeneic fetus. In the murine model, the labyrinthine architecture becomes progressively complex to optimize nutrient and gas exchange with the developing embryo. Syncytiotrophoblast (STB) cells, formed through the fusion of chorionic trophoblast cells, constitute the primary exchange surface within the placental labyrinth, facilitating nutrient transport.[24] Epigenetic biomarkers have gained prominence in health risk assessments. Emerging evidence indicates that epigenetic regulation of both imprinted and nonimprinted genes in the placenta plays a crucial role in fetal and placental development.[25] DNA methylation, a well-characterized epigenetic mechanism, is essential for placental function and fetal growth.[26] Ye et al.[27] developed risk assessment models based on CpG site-specific methylation modifications to predict genetic damage in populations exposed to polycyclic aromatic hydrocarbons (PAHs), highlighting the potential of epigenetic modifications in human health risk assessment. Previous studies have reported that F-53B exposure can induce lipid metabolism disorders through epigenetic regulation (miRNA-gene interaction) in HepG2 and Huh-7 cells.[28,29] Given these findings, it can be speculated that the placenta is susceptible to F-53B-induced impairment, with DNA methylation potentially serving as a key mechanism underlying the adverse effects of 6:2 Cl-PFESA on placental health.

Thyroid hormones (THs) are indispensable for normal fetal brain development during pregnancy and the early postnatal period.[30] Since the fetal thyroid is not fully functional until midpregnancy (18−20 weeks), placental transfer of maternal thyroid hormones is essential during the early stages of gestation in human.[30] Abnormal maternal thyroid function during pregnancy can adversely affect fetal growth and neural development, leading to a range of severe health consequences, including impaired neurological development, cognitive and behavioral deficits, obesity, metabolic disorders, and cancer.[31] Prior epidemiological research has demonstrated evidence indicating an association between exposure to Cl-PFESAs and thyroid hormone levels in the general adult population.[32] Furthermore, a prospective cohort study has suggested a negative correlation between cord serum Cl-PFESA levels and TSH concentrations in newborns, with a particularly pronounced effect observed in male infants.[33] Recent research has demonstrated that Cl-PFESAs exhibit thyroid hormone-disrupting effects in zebrafish, potentially leading to alterations in offspring neurodevelopment.[34] However, there is a significant knowledge gap regarding the impact of F-53B on maternal metabolic processes and subsequent pregnancy outcomes. In this respect, the mechanisms underlying the transgenerational effects of F-53B on thyroid function and progeny neurodevelopment are still not fully explored. Given the placenta's crucial role in fetal development, it is necessary to investigate how placental factors influence neuroplasticity in offspring exposed to F-53B during gestation. Such research may provide valuable insights for assessing the safety of F-53B.

To address existing knowledge gaps, this study aimed to (1) evaluate the impact of F-53B exposure on placental structure and function; (2) explore the correlation between neurodevelopmental deficits in the fetus and thyroid hormone disorders; and (3) investigate the potential mechanisms associated with DNA methylation regulation in the placenta. Our findings provide some evidence of the adverse effects of early life F-53B exposure on the placenta during pregnancy, expanding our comprehension of the health risks and toxicity mechanisms of Cl-PFESAs in humans.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

A technical-grade F-53B product was obtained from Shanghai Synica Co. Ltd. As reported,[35] it comprises 91% 6:2 Cl-PFESA and 7% 8:2 Cl-PFESA. All other chemicals employed in this study were of chromatographic or analytical grade.

2.2. Animal Treatments

Eighteen nulliparous pregnant female CD-1 mice, aged 10−12 weeks, were obtained from Beijing Vital River (Beijing, China). Pregnancy was confirmed by the presence of a copulatory plug on gestational day (GD) 1. In mice, uterine receptivity for blastocyst implantation is established on GD 4, followed by blastocyst attachment on GD 5. Placental formation commences around embryonic day (E) 8.5.[24] All animal procedures were approved by the Laboratory Animal Ethics Committee of Nankai University and conducted in accordance with Chinese guidelines for animal care and use. Following a 5-day acclimation period, pregnant mice were randomly assigned to three groups. Daily intraperitoneal injections of F-53B solutions (40 and 200 μg/kg body weight) were administered to dams from GD 6 to GD 17. Dose selection was based on previous studies.[36,37] On GD 18, animals were euthanized under isoflurane anesthesia. Fetal parameters, including size and appearance, were recorded. Placentas and serum were collected for subsequent analysis. Detailed methodologies are provided in Supporting Information Text S1.

2.3. Measurement of 6:2 and 8:2 Cl-PFESA

Target compounds in the placenta, serum, fetal brain, and fetal liver samples were extracted using an ion-pair extraction method followed by solid-phase extraction (SPE) cleanup as previously described.[38] Detailed methodology is provided in Text S2. Identification and quantification of 6:2 and 8:2 Cl-PFESA in all samples were performed on a Waters Acquity ultrahigh-performance liquid chromatograph (UHPLC) coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer (MS/MS) (Waters, Milford, MA, USA) operating in negative electrospray ionization (ESI) mode.[35] All F0 serum samples, eight randomly selected placentae from each group, and four fetal brains and livers from each treatment group were analyzed for F-53B quantification.

2.4. Hematoxylin and Eosin (H&E) Staining, Immunohistochemical (IHC) Staining, Immunofluorescence (IF), and TUNEL Staining

Placentae from four mice and fetal brains from each treatment group were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Paraffin blocks were sectioned to a thickness of 4 μm. H&E staining, IHC staining, IF, and TUNEL staining were performed according to standard protocols (Text S3). Each group consisted of 3−6 samples.

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High-magnification images were analyzed using HistoQuest software (TissueGnostics, Vienna, Austria).

2.5. Real-Time PCR

Total RNA was extracted from mouse placenta, fetal brain, and fetal liver tissues using an RNA extraction kit (LS1040, Promega, Shanghai, China) according to the manufacturer's protocol. Genomic DNA was removed by RNase-free DNase treatment, followed by reverse transcription using a reverse transcriptase (11141ES60, Yeasen, Shanghai, China). Real-time PCR was conducted on a LightCycler 480 system with SYBR Green qPCR master mix (Takara, Dalian, China). Gene expression analysis was performed as previously described.[39] Detailed methodology is provided in Text S4. All primer sequences are listed in Table S1.

2.6. RNA-seq and Bioinformatic Analysis

Fetal brain RNA was extracted using Trizol reagent (Invitrogen, USA). Majorbio Co., Ltd. (Shanghai, China) constructed and sequenced RNA-Seq libraries on an Illumina HiSeq 4000 system, with sequencing data mapped to the mouse reference genome (GRCm39). Each RNA sample underwent library preparation with 1.5 μg of RNA as input. The FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) filtered raw RNA-Seq reads. Differentially expressed genes (DEGs) were identified using the R package DESeq2 with a fold change (FC) threshold $\geq$2 and adjusted *P*-value $\leq$0.05. The false discovery rate (FDR) was calculated using the Benjamini-Hochberg method.[40] Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on DEGs using custom R scripts with default parameters; KEGG pathways with *P*-value $\leq$0.05 were considered significantly enriched.

2.7. Western Blotting

Mouse placental and fetal brain tissues were homogenized and extracted using RIPA lysis buffer supplemented with a protease inhibitor. Protein concentration was determined using the BCA assay kit (Beyotime, Shanghai, China). Western blot analysis was conducted as previously described (Text S3) to evaluate protein expression.[41] Chemiluminescence detection was performed using an ECL kit (Advansta, Menlo Park, California, USA), and protein bands were quantified using Image-Pro Plus software on a digital imaging system (Tanon, Shanghai, China). Antibody sources and working concentrations are detailed in Table S2.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of general thyroxine (T4), 3,5,3′-triiodothyronine (T3) (E0452Ge, E0453Ge, EIAab Science, Wuhan, China), thyrotropin-releasing hormone (TRH), and thyroid-stimulating hormone (TSH) were determined in these samples via ELISA kits (EIAab Science and Elabscience Biotech, Wuhan, China) according to the manufacturer's protocols. Absorbance measurements were conducted at 450 nm using a multifunctional enzyme labeling instrument (Enspire2300, PerkinElmer, USA), with a minimum of four replicates per group.

2.9. DNA Methylation Analysis

The methylation status of *ttr*, *dio3*, and *hsd11b2* promoter CpG islands within the placenta was assessed using bisulfite pyrosequencing. PCR and pyrosequencing reactions were conducted with PyroMark products and reagents (Qiagen, Inc.) according to established protocols.[42] Methylation levels were quantified using PyroMark Q48 software (Qiagen, Inc.), and primer sequences, listed in Table S3, were designed with Pyrosequencing Assay Design Software v2.0 (Qiagen).

2.10. Statistical Analysis

All data were subjected to statistical analysis using SPSS version 26.0 and were presented as means $\pm$ standard error of the mean (SEM). To assess group differences, repeated measures ANOVA, one-way ANOVA followed by the Least Significant Difference (LSD) post hoc test, or nonparametric tests were employed as appropriate based on data distribution. Statistical significance was defined as a *P*-value less than 0.05 or 0.01.

3. RESULTS AND DISCUSSION

3.1. Accumulation of F-53B in F0 Mouse and Fetus

Following gestational exposure, F-53B was detected in maternal serum, placenta, fetal liver, and brain, signifying its transfer from mother to offspring during pregnancy. As illustrated in Figure 1A, 6:2 Cl-PFESA concentrations in the

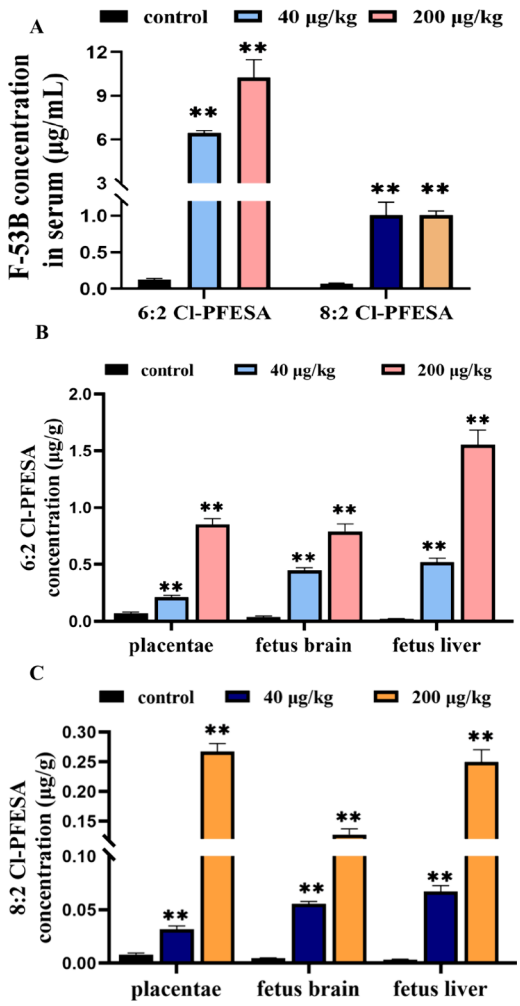

Figure 1. (A) Concentrations of 6:2 Cl-PFESA and 8:2 Cl-PFESA were analyzed in F0 serum of mouse after gestational F-5B exposure from GD 6 to GD 17 (n = 5−7). The level of 6:2 Cl-PFESA (B) and 8:2 Cl-PFESA (C) in placenta (n = 8 per group), the fetus brain (n = 4 per group), and liver (n = 4 per group). Results are presented as means $\pm$ SEM. Statistical differences are determined by $P < 0.05$ (*), $P < 0.01$ (**), respectively.

maternal serum of exposed mice were significantly elevated to 6.46 $\pm$ 0.15 and 10.27 $\pm$ 1.22 μg/mL in the 40 and 200 μg/kg exposure groups, respectively, compared to the control group (0.12 $\pm$ 0.01 μg/mL). Similarly, 8:2 Cl-PFESA levels were 1.01 $\pm$ 0.18 and 1.00 $\pm$ 0.06 μg/mL in both F-53B exposure groups, markedly surpassing the control (0.07 $\pm$ 0.008 μg/mL). Concurrently, placental 6:2 Cl-PFESA levels increased from 6.31 $\pm$ 0.92 μg/g in controls to 18.17 $\pm$ 2.25 and 63.11 $\pm$ 2.27 μg/g in the exposed groups (40 and 200 μg/kg), while 8:2 Cl-PFESA levels were 2.75 $\pm$ 0.40 and 20.09 $\pm$ 1.38 μg/g, respectively (Figure 1B). Notably, perinatal exposure to Cl-PFESAs enabled transplacental transfer, with 6:2 Cl-PFAES detected in maternal serum (1.54 ng/mL), cord serum (0.6

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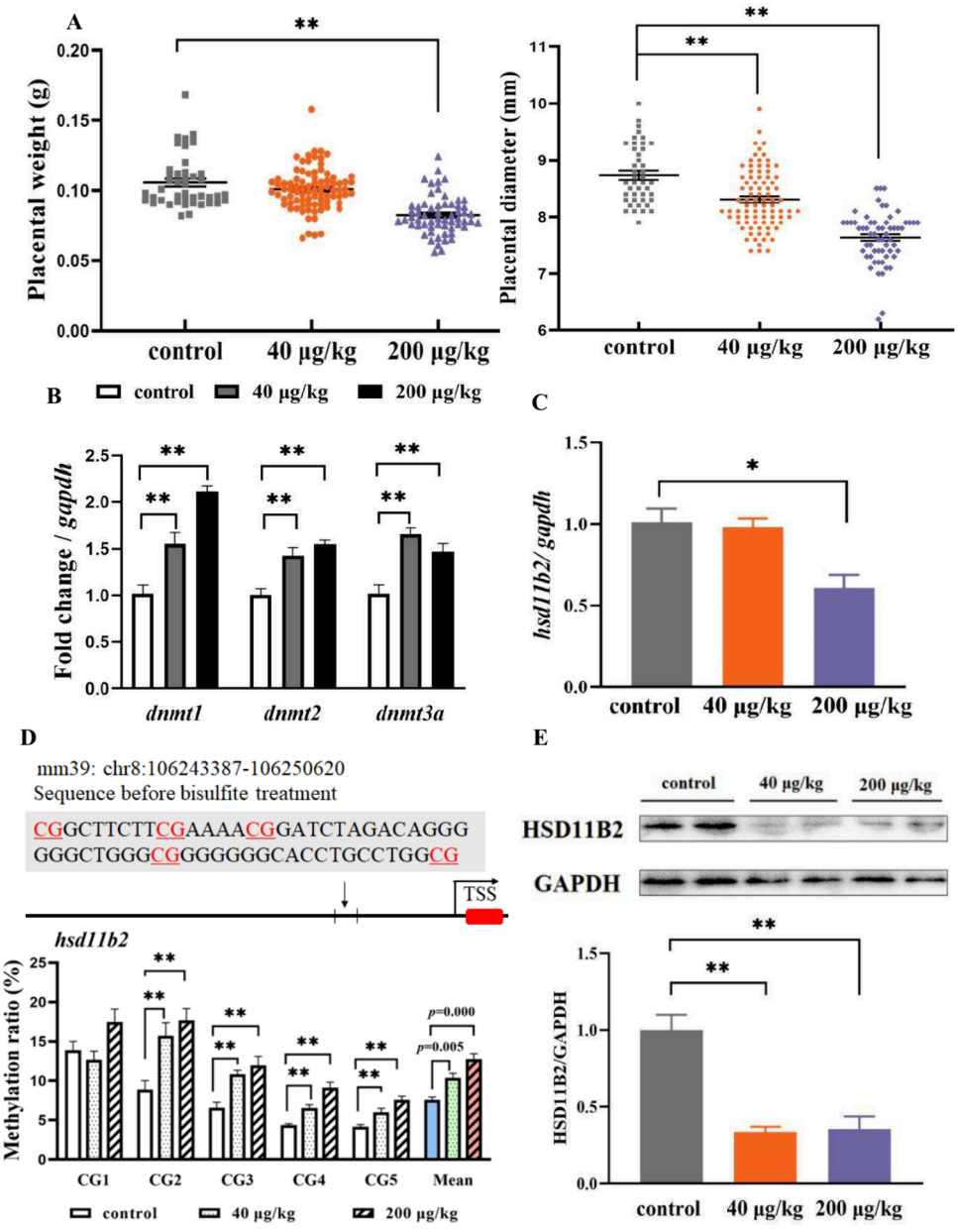

Figure 2. Effects of maternal F-53B exposure on placental *hsd11b2* barrier. (A) Placenta weight and diameter of all fetuses were measured. (B) The relative expression of *dnmt 1*, *dnmt 2*, and *dnmt 3a* analyzed in placentae (n = 6 per group). (C) Gene expression of hsd11b2 was determined by RT-PCR (n = 6 per group). (D) Representative pyrogram of analyzing methylation status of the *hsd11b2* gene in placentae using pyrosequencing (n = 5 per group). (E) The protein expression of HSD11B2 in placentae was verified by WB and relative protein expression of HSD11B2. All data were expressed as means ± SEM *P < 0.05; **P < 0.01. *hsd11b2*: 11β-hydroxysteroid dehydrogenase type 2; *dnmt*: DNA methyltransferase.

ng/mL), and placental tissue (0.34 ng/mL) among pregnant women in Wuhan.[43] While 6:2 Cl-PFESA concentrations were measured at 1.53 ng/mL in maternal serum, 0.59 ng/mL in cord serum, and 0.42 ng/g wet weight in both subchorionic and parabasal placental tissues from Guangzhou, China.[44] These findings demonstrate the ability of F-53B to traverse and accumulate within the placental barrier.

Notably, 6:2 and 8:2 Cl-PFESA were detected in both the fetal liver and brain (Figure 1C). Exposure to 40 μg/kg F-53B resulted in significantly elevated levels of 6:2 Cl-PFESA in the fetal brain (0.448 ± 0.022 μg/g) and liver (0.518 ± 0.037 μg/g) compared to the control group (0.036 ± 0.10 and 0.022 ± 0.002 μg/g, respectively). Similarly, 8:2 Cl-PFESA concentrations were significantly higher in the fetal brain (0.055 ± 0.002 μg/g) and liver (0.067 ± 0.005 μg/g) of the 40 μg/kg F-53B group compared to controls (0.004 ± 0.0004 and 0.0034 ± 0.0003 μg/g, respectively). In the 200 μg/kg F-53B group, 6:2 and 8:2 Cl-PFESA levels reached 0.789 ± 0.069 and 0.127 ± 0.01 μg/g in the fetal brain, respectively, and 1.555 ± 0.127 and 0.250 ± 0.02 μg/g in the fetal liver. The findings substantiate the capacity of F-53B to traverse both the placental and blood-brain barriers, resulting in the accumulation of Cl-PFESAs in fetal organs.

3.2. Epigenetic Regulation of Placental HSD11B2 Barrier and Placental Vascular Dysplasia

The placenta plays an important role in fetal growth and development.[45] In the murine uterus, implantation occurs 5 days after a fertile mating, a period known as the "window of

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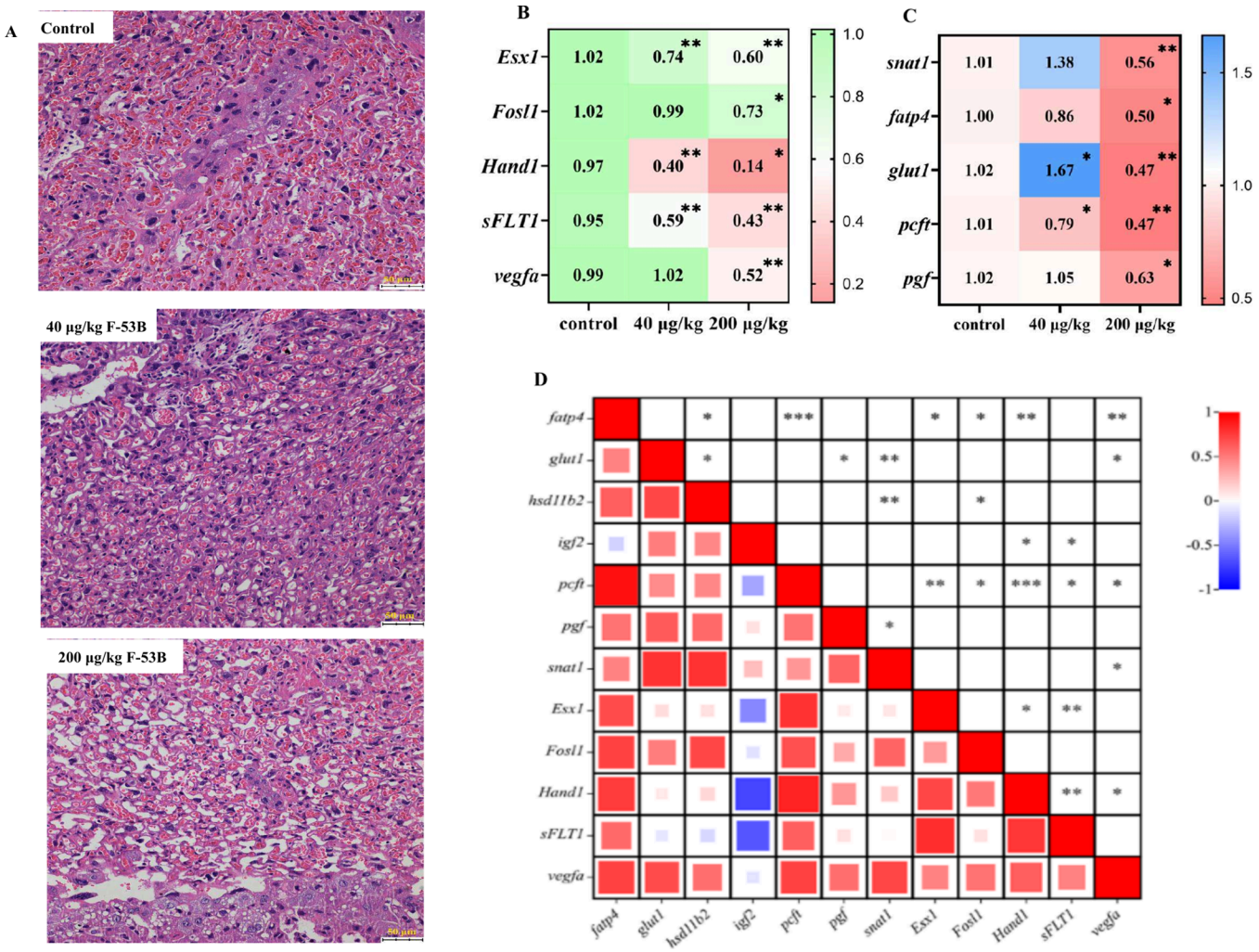

Figure 3. (A) Representative photos of placental morphology observed by hematoxylin-eosin (H&E) staining on GD 18 in labyrinth region. Scale bars: 50 μm. (B) Expression levels of *Esx1, Fosl1, Hand1, sFLT1,* and *vegfa* of placenta collected from mice (n = 6 per group). (C) Expression of nutrient transport pumps (*snat1, fatp4, glut1, pcft,* and *pgf*) in mouse placenta (n = 6 per group). (D) Pearson's correlation matrix visualized as a heat map plot with genes related to nutrient transport pumps in placenta and the development of placental vasculature tested in the study. * indicates significant difference from control at the level of α = 0.05.

acceptance". Placental development begins shortly after the blastocyst implants into the uterine wall, approximately at day 5 post conception.[24] Gestational day 6, coinciding with the stable organogenesis stage, was selected as the initiation point for exposure. As illustrated in Figure S1A, gestational exposure to F-53B significantly reduced placental weight and diameter compared to controls (Figure 2A), suggesting potential impairments in placental function. To further investigate the effects of gestational F-53B exposure on placental apoptosis and cell proliferation, additional analyses were performed. Figure S1B demonstrates that the proportion of TUNEL-positive cells in the placenta treated with 200 μg/kg F-53B was markedly higher than that in controls, indicating that F-53B induced apoptosis of placental cells. Ki67, a nuclear antigen indicative of cell proliferation, was assessed. While administration of 40 μg/kg F-53B did not influence placental cell proliferation, a dramatic decrease in the abundance of Ki67-positive cells was observed in the labyrinthine layer following treatment with 200 μg/kg F-53B (Figure S1C). The labyrinthine layer is the primary interface for oxygen and nutrient exchange between mother and fetus.[46] Taken together, our findings suggest that F-53B induces increased apoptosis while inhibiting cell proliferation in the placenta, and the observed decrease in placental weight is likely attributable to an imbalance between placental proliferation and apoptosis (Figure S1D).

Recent research has increasingly highlighted the role of epigenetic regulation in placental development.[26] DNA methylation, a critical process in gene expression and genomic imprinting, is essential for fetal growth and survival throughout gestation.[47] Abnormal placental DNA methylation has been linked to impaired placental function and fetal growth.[25] This study examined DNA methyltransferases (DNMTs), key mediators of active methylation, in mouse placenta. Gene expression analysis revealed significant upregulation of *dnmt1, dnmt2, and dnmt3a* following 40 and 200 μg/kg F-53B exposure (Figure 2B), suggesting altered DNA methylation in the exposed placenta, which may subsequently impact placental development and function. Furthermore, the DNA methylation rate of the *hsd11b2* promoter was significantly elevated in the placenta of mice exposed to 40 and 200 μg/kg F-53B compared to controls (Figure 2D). HSD11B2 acts as a placental glucocorticoid barrier, protecting the fetus from external stressors.[48] Previous studies have demonstrated an

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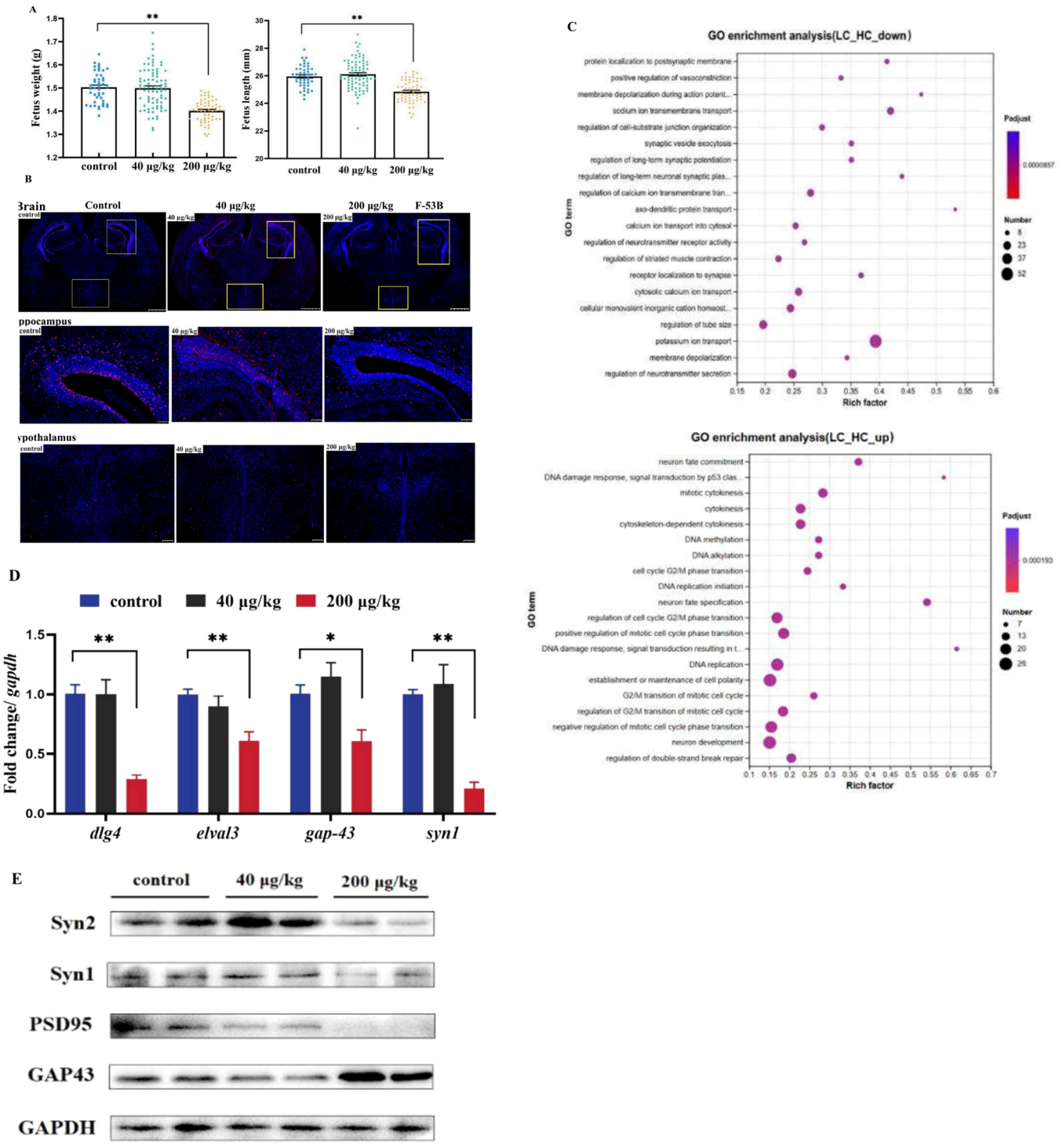

Figure 4. (A) Fetal weight and length were compared among different exposed fetuses and controls. Data were expressed as mean ± SEM from all fetus per group. *P < 0.05, **P < 0.01. (B) Immunofluorescence of Ki67 in fetal brain (n = 3 per group). Blue, DAPI-labeled nucleus; Red, iFluor 594-labeled Ki67. Scale bar: 500 and 50 μm. (C) Upregulated and downregulated gene enrichment in GO in fetal brain. The rich factor was used to identify the extent of enrichment. Target gene GO enrichment factor screened by the P-value. (D) The relative mRNA levels of *dlg4, elval3, gap43,* and *syn1* in the fetal brain, respectively (n = 6 per group). (E) Detection of protein (Syn1, 2, PSD95, GAP43) by immunoblot analysis in the fetal brain.

inverse correlation between *hsd11b2* DNA methylation and its expression, as well as newborn birth weight and length, in both humans and mice.[49] Consistent with these findings, our results showed that hypermethylation of the hsd11b2 promoter reduced *hsd11b2* mRNA and protein expression in the placenta of exposed mice (Figure 2C, E). The findings indicate that exposure to F-53B during gestation leads to the down-regulation of *hsd11b2* expression via DNA methylation, thereby compromising the protective function of the placenta.

As depicted in Figure 3A, gestational exposure to F-53B resulted in a reduction of maternal blood sinusoids within the labyrinthine region of the placenta, indicative of abnormal

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placental vascularization. Moreover, gene expression analysis revealed a significant decrease in genes associated with placental vasculature development (*Esx1, Fosl1, Hand1, sFLT1*, and *vegfa)* in the F-53B groups compared to controls (Figure 3B). Previous studies have demonstrated the critical roles of the X-linked homeobox gene (*Esx1*) and helix−loop−helix transcription factor (*Hand1*) in placental vascularization and labyrinthine development.[50,51] FOS-like 1 AP-1 transcription factor subunit (Fosl1) is essential for labyrinthine development,[52] while soluble vascular endothelial growth factor receptor 1 (sFLT1) maintains placental vascular integrity by sequestering excess maternal vascular endothelial growth factor (VEGF), a key angiogenesis regulator.[53] The VEGF/VEGFR signaling pathway is well-characterized in angiogenesis, and placental vascular network formation is crucial for nutrient and oxygen transfer to the fetus. Reduced placental blood vessel density diminishes placental blood flow, leading to intrauterine growth retardation (IUGR).[54] In the 200 μg/kg F-53B exposure group, a significant downregulation of VEGFA protein expression was observed in the mouse placenta (Figure S1E), suggesting F-53B-induced disruption of placental angiogenesis via inhibition of the VEGF signaling pathway. This finding aligns with a recent study demonstrating a correlation between altered placental angiopoietin-2 and vascular endothelial growth factor receptor-2 and adverse birth outcomes in humans exposed to PFOS and 8:2 Cl-PFESA.[44] Given the essential role of placental angiogenesis in fetal health, the observed downregulation of *Esx1, Fosl1, Hand1, sFLT1* mRNA, and VEGF protein following F-53B exposure may contribute to reduced placental blood sinusoids and subsequent adverse fetal growth and development.

3.3. Nutrient Transport Pumps in Placenta

Abnormal placental angiogenesis can adversely affect its function in supplying nutrients to the fetus, a process that relies on the involvement of placental nutrient transporters.[55] The placenta's capacity to deliver nutrients to the fetus is influenced by various factors, including size, morphology, blood flow, and transporter abundance. In mice exposed to 200 μg/kg F-53B, the expression levels of neutral amino acid transporter 1 (*snat1)*, fatty acid transporters 4 *(fatp4)*, glucose transporters 1 *(glut1)*, proton-coupled folate transporter *(pcft)*, and placental growth factor *(pgf)*mRNA were significantly lower than in the control group (Figure 3C). Snat1 is a primary contributor to amino acid transport between the fetus and the placenta.[56] Cellular membrane fatty acid transporters (*fatp1, 2, 4*), in conjunction with fatty acid translocase, facilitate fatty acid transfer from mother to fetus, providing energy for embryonic development.[57] Two primary glucose transporters, Glut1 and Glut3, are present in the placenta and transport glucose from the maternal circulation to supply energy to local endothelial cells and the fetus.[58] It has been reported that *pcft* is crucial for delivering adequate folate from maternal circulation to the fetus during pregnancy.[59] Numerous studies have reported that environmental pollutants, such as cadmium and oxidized soybean oil, impair placental nutrient transporters, leading to FGR.[60] As shown in Figure 3D, a significant positive correlation was observed between placental vasculature development and nutrient transporter expression, suggesting that reduced placental nutrient transport efficiency is an important factor contributing to fetal intrauterine growth restriction.

3.4. Fetal Intrauterine Growth (FGR) and Neurodevelopmental Toxicity in the Fetus

Maternal F-53B exposure during pregnancy did not significantly alter the body weight of F0 mice compared to controls (Figure S2A). No differences were observed in the number of fetal resorptions or live fetuses between the exposure and control groups (Table S4, Figure S2B), and no fetal malformations were detected (Figure S2C). However, a significant reduction in fetal length (crown-rump) and fetal weight was observed in the 200 μg/kg F-53B exposure group compared to the control group, indicating that maternal F-53B exposure induces fetal intrauterine growth restriction in mice (Figure 4A). Consistent with our findings, recent epidemiological studies have reported a negative association between birth weight and both legacy PFASs (PFOS and PFOA) and emerging PFASs (6:2 and 8:2 Cl-PFESAs).[44]

Importantly, we found alterations in fetal brain development following F-53B exposure. To elucidate the effects of F-53B on the fetal brain, cell proliferation and apoptosis were assessed in this study. A reduction in the rate of Ki67-positive cells (a marker of cell proliferation) was observed in the fetal brains of F-53B-exposed groups compared to controls (Figure 4B). No increase in TUNEL-positive cells was detected in the fetal brains of exposed groups (Figure S4A). The observed inhibition of cell proliferation in the fetal brain suggests that exposure to F-53B adversely impacts brain development.

Mechanistically, a transcriptomic analysis of fetal brain tissues was conducted following 40 and 200 μg/kg F-53B treatments, with gene expression changes illustrated in Figure S3A. A Venn plot was generated, revealing 4393 overlapping target genes between the 40 μg/kg F-53B and control groups, as well as the 200 μg/kg F-53B and control groups. Among these, 1993 genes were upregulated, while 2400 were downregulated (Figure S3B). GO analysis revealed that genes downregulated in both exposure groups were enriched in potassium transport and neurotransmitter secretion regulation, whereas upregulated genes were predominantly associated with the endocrine system, cellular growth and death, and signal transduction (Figure 4C, Figure S3C). Integrated Pathway (iPath) analysis indicated that differentially expressed genes following F-53B exposure were primarily involved in energy metabolism, nucleotide metabolism, and lipid metabolism (Figure S3D). The RNA-seq results collectively demonstrate that gestational F-53B exposure exerts a significant impact on fetal neurodevelopment.

To further elucidate the adverse effects on neurodevelopment in F1 offspring, the expression of several genes associated with central nervous system (CNS) development was examined. Proteins such as GAP43, synapsin (Syn), and postsynaptic density protein 95 (PSD-95) are integral to synaptic plasticity, which is crucial for the regulation and remodeling of the nervous system. Results indicated a significant downregulation of *dlg4, elavl3, gap43*, and *syn1* expression in fetal brains following 200 μg/kg F-53B treatment (Figure 4D). As depicted in Figure 4E and Figure S4B, protein levels of Syn1, Syn2, and postsynaptic density protein-95 (PSD95), biomarkers of synapse formation and postsynaptic function,[61] were significantly reduced in exposed fetal brains. Conversely, gap43, a marker of synapse modulation and axonal regeneration,[62] was upregulated in the 200 μg/kg group but downregulated in the 40 μg/kg exposure group. The observed downregulation of neurodevelopmental genes (*dlg4, elavl3*, and *syn1*) suggests maternal F-53B exposure adversely impacts

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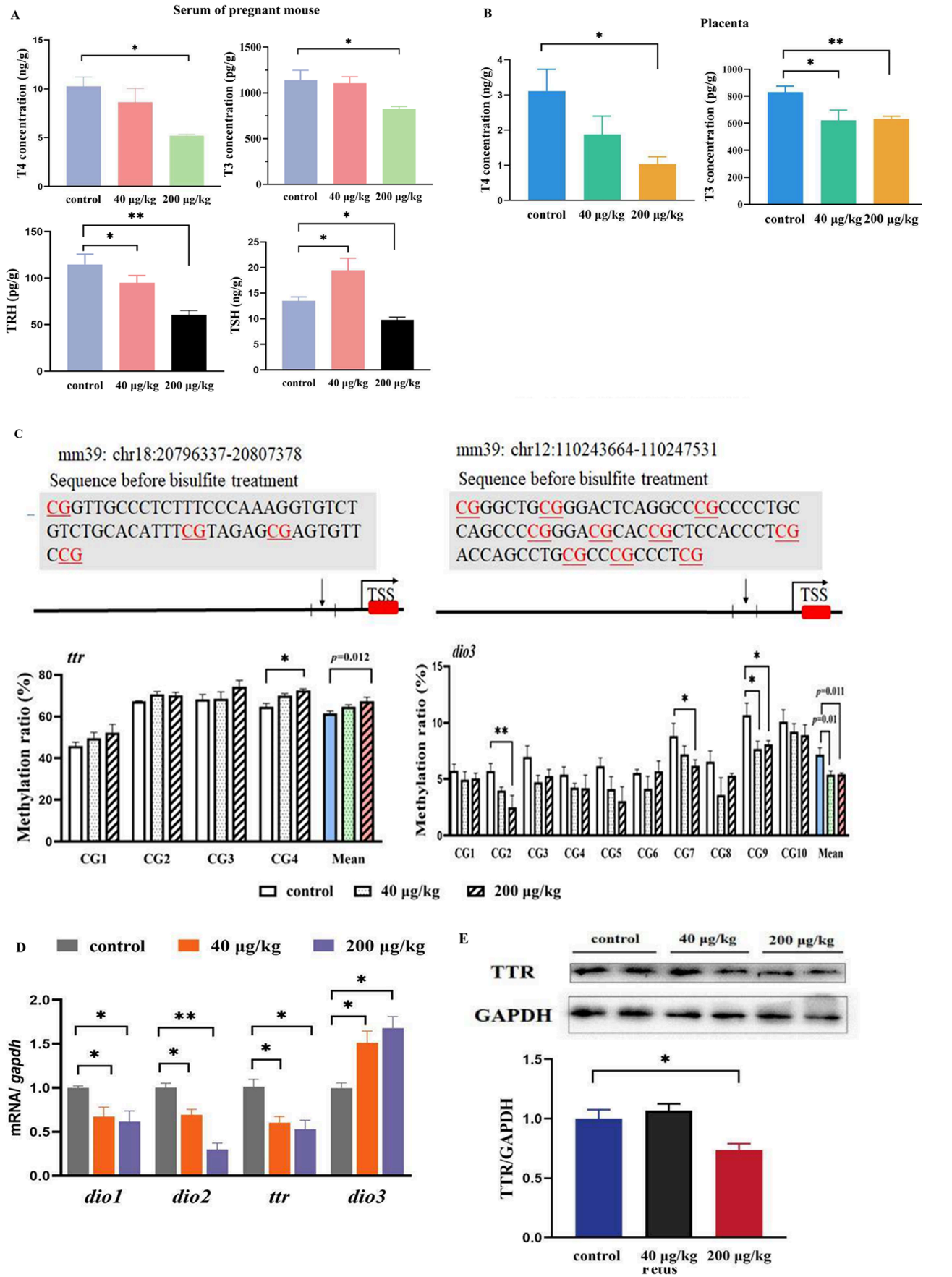

Figure 5. continued

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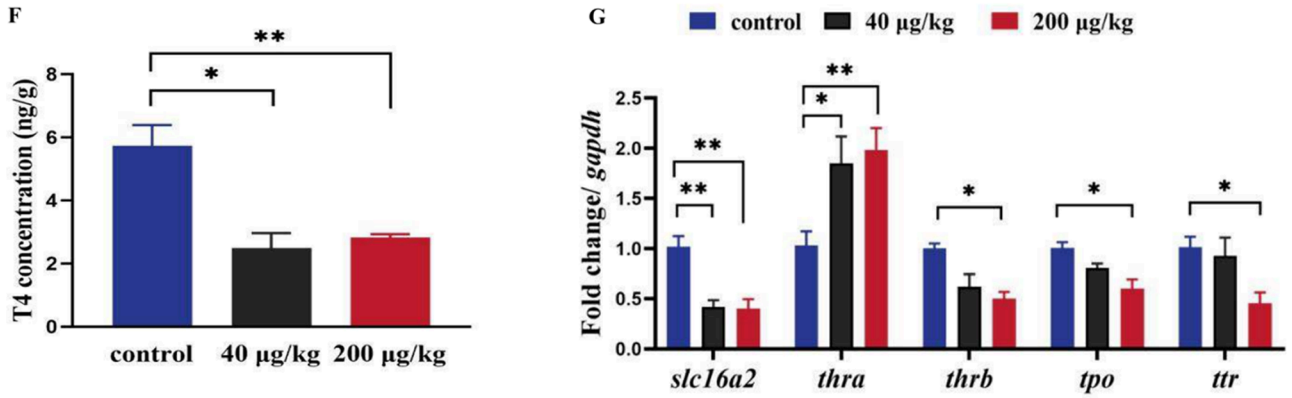

Figure 5. Effect of F-53B on thyroid hormone metabolism. (A) Total TRH, TSH, T4, and T3 levels in the serum of pregnant mice after exposed to different doses of F-53B (n = 5). (B) Placental T4 and T3 were measured (n = 6 per group). (C) Representative pyrogram of analyzing methylation status of the *ttr* and *dio3* genes using pyrosequencing (n = 5). (D) Quantification by quantitative RT-PCR of relative mRNA expression levels of *dio1*, *dio2*, *ttr*, and *dio3* in placentae (n = 6 per group). (E) Representative images of TTR protein expression detected by Western blot and relative protein quantification. (F) The T4 concentrations in fetus. The concentrations are displayed as ng/g. (G) Gene transcription of *slc16a2*, *thα*, *thrβ*, *tpo*, and *ttr* in the fetal liver (n = 6 per group). Values are expressed as means ± SEM *P < 0.05, **P < 0.01, significantly different from control group.

neuronal development and differentiation, leading to neurotoxicity in offspring. Previous studies have demonstrated that neurotoxicants, such as BDE-47 and microcystin-LR, inhibit axonal growth but can be compensated for by increased expression of genes like gap43.[63,64] The upregulation of gap43 in our study may represent a similar compensatory response to inhibited axonal growth induced by F-53B exposure. Immunohistochemistry staining revealed a decreased intensity of PSD95 protein expression in the 200 μg/kg F-53B group (Figure S4C), indicative of disrupted synaptic plasticity and memory formation. Additionally, a significant positive correlation was observed between *ttr* and *tpo* gene expression, and neurodevelopmental genes in fetuses (Figure S4D). Previous research has linked PFOS-induced brain development impairment to thyroid hormone disruption.[65] Similarly, 8:8 perfluoroalkyl phosphinic acid (8:8 PFPiA) has been shown to elicit developmental neurotoxicity through thyroid hormone disruption and inhibition of neuronal development.[66] Collectively, these findings suggest that F-53B exposure during pregnancy induces neurodevelopmental disorders in offspring through placental thyroid dysfunction. This represents a potential mechanism for the transgenerational neurotoxic effects of F-53B.

3.5. Thyroid Hormone Homeostasis in Two Generations

Thyroid hormones are essential for fetal development, particularly in the brain. The pituitary gland controls the HPT axis by releasing TRH, which triggers the thyroid gland to produce TSH. TSH regulates iodine uptake in the thyroid, where thyroglobulin is iodinated in follicular cells to synthesize thyroid hormones. When needed, thyroglobulin is processed into T3 and T4, with their levels managed by deiodinase enzymes. T3 and T4 then interact with thyroid hormone receptors.[67] Maternal TH homeostasis exerts a long-term, irreversible impact on fetal nervous system development. TH levels were measured in both F0 and F1 generations (Figure 5). In F0 serum, TRH levels decreased significantly in F-53B exposure groups, while TSH levels increased in the 40 μg/kg F-53B group but decreased markedly in the 200 μg/kg F-53B group. Total T4 and T3 levels decreased substantially by 49.2% and 27.4%, respectively, in the 200 μg/kg F-53B exposure group (Figure 5A, P < 0.05). Significant correlations were observed between T3, T4, and TRH concentrations in F0 serum and placental diameter and fetal weight (Figure S5). During early gestation, the fetus relies entirely on maternal thyroid hormone supply; fetal thyroid hormone synthesis commences around gestational day 17.[30] Therefore, placental TH levels should reflect maternal-origin fetal thyroid hormone levels. Compared to controls (3.11 ± 0.62 ng/g ww), exposure to F-53B (40 and 200 μg/kg) significantly reduced placental T4 levels (1.88 ± 0.52, 1.04 ± 0.21 ng/g ww, respectively). Similarly, placental T3 levels in both exposure groups were significantly lower (621.90 ± 74.91, 634.76 ± 18.31 pg/g ww, respectively) than controls (832.00 ± 43.82 pg/g ww, Figure 5B). These results demonstrate that F-53B not only affects maternal TH levels but also induces a reduction in T3 and T4 levels in the placenta.

Epigenetic modifications of genes within the TH pathway are implicated in placental TH abnormalities. Our study revealed a significant increase in DNA methylation of the *ttr* promoter and a concurrent decrease in the *dio3* promoter methylation ratio in the 200 μg/kg F-53B treatment group (Figure 5C). The specific methylation sites within these gene promoters are detailed in the Supporting Information (Figure S6). During early gestation, the fetus depends entirely on maternal T4, which crosses the placenta via specific transporters. Placental deiodinases convert maternal free T4 into T3 and reverse T3, allowing maternal T3 to reach fetal tissues before the fetal thyroid matures.[68] The trans-placental transfer of thyroid hormones from the mother to the fetus is critically dependent on the regulation of several placental genes.[69] These genes encompass iodothyronine deiodinases, such as deiodinase type 3 (DIO3), plasma membrane transporters, such as monocarboxylate transporter 8 (MCT8), and binding proteins within trophoblasts, such as transthyretin (TTR).[69] Prior research has indicated that prenatal exposure to environmental pollutants, specifically p,p′-dichlorodiphenyldichloroethylene (p,p′-DDE) and brominated diphenyl ether-47 (BDE-47), may alter the DNA methylation patterns of the dio3, mct8, and ttr genes in the placenta. Such epigenetic modifications could potentially impact the transport of thyroid

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hormones (THs) to target tissues, thereby partially contributing to the disruption of thyroid hormone function observed in neonates and infants.[70] During pregnancy, T4 and T3 are transported across the placenta via thyroid hormone transporters regulated by deiodinases (Dio 1, 2, 3).[71] Dio 3, primarily localized in the placenta, inactivates THs, while placental TTR facilitates T4 transfer to the fetal circulation.[72] Altered DNA methylation influenced the expression of *dio3* and *ttr*, with increased *dio3* and decreased *ttr* levels observed in placenta following gestational F-53B exposure (40 and 200 μg/kg) (Figure 5D, E). These findings suggest that F-53B-induced aberrant methylation of *ttr* (hypermethylation) and *dio3* (hypomethylation) in the placenta may disrupt TH transplacental transport.

Fetal brain T4 levels were significantly reduced by 43.5% and 49.2% in the 40 and 200 μg/kg F-53B exposure groups, respectively (Figure 5F). To assess the underlying mechanisms, gene expression profiles related to TH regulation, transport, combination, and metabolism were examined in F1 offspring (Figure 5G). TTR, a tetrameric protein primarily synthesized by the liver, is secreted into the plasma. Hepatic deiodinases regulate circulating and peripheral TH levels,[73] while thyroid hormone receptor β (THRβ) is the predominant liver-expressed THR isoform.[74] In the 200 μg/kg F-53B group, gene expression of TH transport and synthesis-related factors, including solute carrier family 16 member 2 (*slc16a2*, or monocarboxylate transporter 8, *mct8*), *ttr*, *tpo*, and *thrβ*, was markedly downregulated (0.4-, 0.46-, 0.5-, and 0.5-fold, respectively). Conversely, *thrα* transcription was significantly upregulated (1.85- and 2.0-fold) in both F-53B exposure groups. Mct8 is essential for TH transport across brain barriers, and its deficiency leads to brain hypothyroidism and severe neurological impairments. TTR facilitates T4 transport across the blood-brain barrier, crucial for fetal brain development.[75] TPO is vital for thyroid hormone generation.[76] THs exert their functions through binding to THRα and THRβ, and their critical role in brain development is well-established. Disruptions in TH homeostasis can lead to severe neurological deficits.[77,78] Our findings demonstrate that gestational F-53B exposure reduces fetal brain T4 levels and significantly alters gene expression related to TH transport and metabolism, potentially causing profound impacts on fetal brain development and function.

4. CONCLUSION

Our results from the mother-placenta-fetus triad in this study provide evidence that placenta is an important toxic target of F-53B. Disruptions to placental structure and function play a crucial role in the pathogenesis of fetal growth restriction (FGR) after gestational exposure to F-53B, manifesting as reductions in fetal weight and length. Our findings indicate that gestational F-53B exposure interferes with maternal-to-fetal thyroid hormone transfer. The underlying mechanism may involve the alteration of DNA methylation patterns of the *hsd11b2*, *ttr*, and *dio3* genes, along with changes in mRNA levels of these genes. The disruption of the placental barrier and the alteration of maternal-progeny thyroid hormone levels may adversely affect fetal brain development and function. These results unveil the hidden dangers of F-53B, exposing its neurotoxicity to fetal development through placental disruption of THs transport. This research highlights a critical environmental health concern, providing novel insights into the adverse effects of prenatal F-53B exposure on fetal growth. To further elucidate the long-term consequences of F-53B exposure, extensive investigation into the psychomotor behavior of children born to exposed mothers is warranted, with emphasis on potential associations with prenatal F-53B exposure.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.4c00158.

Additional experimental details, materials, methods, and results (PDF)

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Notes

The authors declare no competing financial interest.

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