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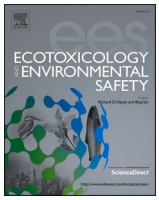

# Environmental chemical TCPOBOP disrupts milk lipid homeostasis during pregnancy and lactation

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ABSTRACT

Humans are exposed to different kinds of environmental contaminants or drugs throughout their lifetimes. The widespread presence of these compounds has raised concerns about the consequent adverse effects on lactating women. The constitutive androstane receptor (CAR, Nr1i3) is known as a xenobiotic sensor for environmental pollution or drugs. In this study, the model environmental chemical 1, 4-bis [2-(3, 5-dichloropyridyloxy)] benzene, TCPOBOP (TC), which is a highly specific agonist of CAR, was used to investigate the effects of exogenous exposure on lactation function and offspring health in mice. The results revealed that TC exposure decreased the proliferation of mammary epithelial cells during pregnancy. This deficiency further compromised lobular-alveolar structures, resulting in alveolar cell apoptosis, as well as premature stoppage of the lactation cycle and aberrant lactation. Furthermore, TC exposure significantly altered the size and number of milk lipid droplets, suggesting that TC exposure inhibits milk lipid synthesis. Additionally, TC exposure interfered with the milk lipid metabolism network, resulting in the inability of TC-exposed mice to efficiently secrete nutrients and feed their offspring. These findings demonstrated that restricted synthesis and secretion of milk lipids would indirectly block mammary gland form and function, which explained the possible reasons for lactation failure and retarded offspring growth.

## 1. Introduction

Humans are exposed to different environmental pollutants or drugs in various ways throughout their lifetimes. These compounds are now considered serious and imminent threats to public health and might become a major global environmental risk. Although numerous descriptive studies regarding the harmful roles of foreign chemical exposure, little is known about the relationship between the persistence of effects and overall biological outcomes and their underlying mechanisms. Most recently, the evidence linking environmental chemicals and xenochemical receptors is increasing (Baldwin and Roling, 2009; Hernandez et al., 2009; Kublbeck et al., 2020). The fact that constitutive androstane receptor (CAR) is structurally activated by multiple environmental chemicals from pesticides, pharmaceuticals, consumer products, and industrial processes (Ashrap et al., 2017; Baldwin and Roling, 2009; Chang and Waxman, 2006; DeKeyser et al., 2011; Ito et al., 2012; Laurenzana et al., 2016; Oshida et al., 2015; Ren et al., 2010; Rooney et al., 2019). As reported, this receptor is regarded as a ligand-activated transcription factor with high constitutive activity, as well as a xenobiotic sensor that participates in the regulation of drug

*Abbreviations:* CAR, Constitutive androstane receptor; TC, TCPOBOP,1, 4-bis [2-(3, 5-dichloropyridyloxy)] benzene; MG, Mammary gland; LD, Lipid droplet; MFG, Milk fat globule; TG, Triglyceride; PND, Postnatal day; PPAR, Peroxisome proliferator-activated receptor; FA, Fatty acid; KEGG, Kyoto encyclopedia of genes and genomes; GSEA, Gene set enrichment analysis; XOR, Xanthine oxidoreductase.

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metabolic enzymes, detecting and responding to foreign chemicals (Forman et al., 1998; Honkakoski et al., 1998). In addition, CAR has also been characterized as an important participant in disrupting endocrine pathways and other adverse pathway outcomes, and some of its ligands are common endocrine disruptors (Eveillard et al., 2009; Kublbeck et al., 2020; Lukowicz et al., 2018). According to hundreds of publications, most in vivo studies aiming at CAR-related metabolism or endocrine effects were performed using the directly potent mouse CAR activator TC. It is a lipophilic polyhalogenated heterocyclic chemical compound that was originally isolated as a pesticide contaminant (Baldwin and Roling, 2009). It has an extremely long half-life in the liver and adipose tissue (Skoda et al., 2022). After a single TC treatment, it continues to accumulate in liver and adipose tissue after 30 days, with elevated levels of liver microsomal enzymes for up to 140 days (Poland et al., 1980; Smith et al., 1993). Accordingly, given the persistence of TC in vivo, it may act as a metabolic disruptor. TC treatment induces multiple pathogenic responses, including hepatomegaly (Bhushan et al., 2019) and hepatocarcinogenesis (Dragani et al., 1985). The persistent lipophilic chemical TC administered long before pregnancy has been reported to be transmitted through milk and cause health-related phenotypes in F1 offspring (Dietrich et al., 2018). This finding provides a prime example of how such phenotypes might arise through the accumulation of environmental chemicals and their transfer to offspring. However, activation of CAR by TC affects lipid metabolism in the liver and adipose tissue (Yan et al., 2015). Currently, controversies surrounding the effects of TC-induced CAR activation on human health remain.

Studies have demonstrated that various environmental pollutants can interfere with mammary gland development in humans and experimental animals (Macon and Fenton, 2013; Rudel et al., 2007, 2011). The mystery of the mammary gland is that its growth and development occur throughout a woman's life (Russo and Russo, 1996). During puberty, the mammary glands develop from a rudimentary tree into a branching epithelial network of ducts, which further undergo alveolar formation during pregnancy to generate lactation-competent glands (Brisken and Rajaram, 2006; Macias and Hinck, 2012; Pang and Hartmann, 2007). Mammary gland development during pregnancy determines the number of alveolar epithelial cells during lactation and subsequent lactation function (Macias and Hinck, 2012). In addition, the growth and differentiation of the mammary gland epithelium are tightly regulated by diverse hormonal and metabolic signals throughout development (Hennighausen and Robinson, 2005), suggesting that there are critical periods in which the mammary gland is susceptible to developmental programming through environmental stressors. Previous studies by our laboratory showed that TC-induced CAR activation affected mammary gland development and accelerated collagen formation and fibrosis in the mammary fat pad of adolescent female mice (Xu et al., 2018). Pregnancy and lactation are recognized as important windows for the susceptibility of the developing mammary gland to environmental exposures (Rudel et al., 2011; Vandenberg, 2021). However, there is limited information on the morphological and functional events of the mammary gland during this sensitive period.

The main function of the mammary gland is to breastfeed and provide the ideal nutrition for infants. Among the components of milk, lipids are an important source of energy, essential fatty acids (FAs), nutrients, and signaling molecules for offspring of most mammals (Yang et al., 2018). Decreased milk lipid production and secretion of mammary glands during lactation are associated with poor neonatal growth, development, health, and survival (Beigneux et al., 2006; Boxer et al., 2006; Cunnick et al., 2015; Ogg et al., 2004; Ogra, 2020; Smith et al., 2000; Vorbach et al., 2002; Yang et al., 2006; Zhao et al., 2020). Numerous studies have contributed to demystifying milk lipid regulation in the mammary gland during lactation. The milk protein and lipid biosynthesis capacity increase dramatically in alveolar epithelial cells upon parturition (Anderson et al., 2007; Brisken and Rajaram, 2006; Pang and Hartmann, 2007). A key event in the initiation of milk secretion is the release of mature lipid droplets (LDs) from alveolar epithelial cells into luminal spaces (Anderson et al., 2007; Brisken and Rajaram, 2006; Pang and Hartmann, 2007). Lipid secretion from the mammary glands occurs through a specialized apocrine-like mechanism, in which elements of the apical plasma membrane encapsulate the intact LDs to generate membrane-bound secretory products termed milk fat globules (MFGs) (McManaman, 2014; Russell et al., 2007; Shi et al., 2015). The interaction between xanthine oxidoreductase (XOR), butyryl granulocyte (Btn1a1), and perilipin 2 (Plin2) is critical for the regulation of milk lipid secretion in lactating mammary glands (Anderson et al., 2007; Chong et al., 2011; Ogg et al., 2004). The patterns of LD accumulation have indicated that milk lipid formation entails sequentially regulated modes of lipid production, transportation, and secretion that are functionally associated with the maturation of alveoli into milk secretory structures (McManaman, 2009; Russell et al., 2007). Defects in milk lipid formation or secretion may disrupt MFG assembly or secretion in late pregnancy and lactation. Apoptosis of alveolar cells is frequently observed in mouse models with defective secretion activation or initiation (Akhtar et al., 2016; Vorbach et al., 2002; Watkin et al., 2008). Thus, milk lipid synthesis and secretion in the mammary gland are regulated by a strict and complex network during the transition from pregnancy to lactation (Anderson et al., 2007; Rudolph et al., 2007). These functions are linked to exogenous lipid secretion activation and availability. However, the role of TC-induced CAR activation in lipid metabolism is controversial, and researchers have not yet determined whether TC affects milk lipid synthesis and secretion.

Taken together, this study aimed to determine the effects of maternal exposure to the chemical model substance TC during pregnancy and lactation on the histological morphology and function of mammary gland development and the associated alterations in the regulation of milk lipid production and secretion. In addition, we linked maternal TC exposure to the potential risks of subsequent growth in offspring. Collectively, this study presented the detrimental pathological effects of exposure to exogenous environmental pollutants on mammary gland development and offspring growth.

## 2. Materials and methods

### 2.1. Animal care and experimental design

Specific pathogen-free (SPF)-grade 8-week-old female CD1 (ICR) mice were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All experimental procedures involving mice included ad libitum access to food and water. The mice were housed in a standard laboratory environment (humidity 50–60% and temperature 25 ± 2 °C) with a 12-h light-12-h dark cycle throughout the study. All experimental protocols describing these procedures were approved by the Ethics and Animal Welfare Committee of Beijing Normal University (Approval No. CLS-EAW-2020–004).

Mice were randomly divided into control (Con) and TC-exposed groups after 1 week of adaptive feeding. The experimental scheme is shown in Supplementary Fig 1. Female mice in the TC-exposed group were injected intraperitoneally with TC at a dose of 0.5 mg/kg twice a week, and females in the Con group were injected intraperitoneally with the corresponding DMSO (Xu et al., 2018). After 2 weeks of treatment, females and males were mated, and females were checked for the presence of vaginal plugs the next morning. The day when the vaginal plug was observed was recorded as 0.5 d of pregnancy (P0.5 d). The pregnant females were then housed in a single cage for the remainder of our study. The birth day of offspring was judged as day 0 of lactation (L0 d), and the number of newborn offspring was adjusted to 10. Throughout the experimental period, female mice were exposed to TC until sacrifice (n = 10), but male mice were not treated with TC. At the corresponding experimental endpoints (14.5 days, 18 days of pregnancy and 1, 5, 10, 15 days of lactation), females fasted for 12 h, and then euthanized with $CO_2$. Mouse blood and tissues, including the mammary gland from the fourth pair and the liver, were collected and prepared for

further analysis.

### 2.2. Milk yield and milk sample collection

Milk yield was estimated by recording pup body weights during lactation days 1–7 (Sampson and Jansen, 1984). To exclude possible defects in pups, pups born to Con mothers were cross-fostered by TC mothers. The litter size was standardized to 10 pups per litter for control and TC mice. The pups were weighed daily at 10:00 am (W1) and separated from their mothers for 4 h. At 2:00 pm, the pups were weighed (W2), returned to their mothers, and allowed to suck for 1 h. They were weighed once more (W3) at 3:00 pm. Daily milk yields were adjusted for weight loss due to metabolic processes in pups during lactation. The milk yield value in units of g/litter/h was defined as (W3-W2) + (W1-W2)/4.

In addition, milk samples were collected from control and TC female mice on lactation day 10 (L10 d) according to previously reported methods (Wang et al., 2012; Willingham et al., 2014). Briefly, the pups were removed for 3–5 h at L10. Then, 10 IU of oxytocin was given by intraperitoneal injection, and milking was started after 10 min. Milk was ejected by manually massaging the mammary glands and immediately collected into a 1.5-ml tube with a P-200 Pipetman. Milk samples were frozen and stored for further analysis (n = 10).

### 2.3. Histological analysis

To produce paraffin sections, the mammary glands from the fourth pair were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Hematoxylin and eosin (H&E) staining was performed according to standard methods (Xu et al., 2017). Briefly, hematoxylin solution was added for 3 min, and eosin solution was added for 1 min. The average alveolar luminal area and volume fraction of the average alveolar luminal area were analyzed using ImageJ software.

### 2.4. Gene expression analysis

For qPCR, total RNA was extracted using TRIzol reagent (Life Technologies) and then subjected to cDNA synthesis using FastKing gDNA Dispelling RT SuperMix (Tiangen, China). qPCR was performed in triplicate using SYBR Green qPCR SuperMix (Transgen Biotech, Beijing, China) with an ABI Q6 instrument (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. Fold changes in expression were calculated by the comparative CT method using mouse 18S as the housekeeping gene for mRNA expression. All fold changes are expressed normalized to the control. The primers used (all for mouse genes) are shown in Supplementary Table S1.

### 2.5. Western blot analysis and immunofluorescence

Equal portions of tissue protein lysates (10 μg) were electrophoresed by 10% SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon®-P transfer membranes (Millipore, Billerica, MA, USA). Then, western blot analysis was performed using standard procedures that we previously established (Pan et al., 2021). The following antibodies were used: XOR, Plin2, CAR, and GAPDH. The band intensity was quantified using ImageJ software. For immunofluorescence, sections were incubated overnight in a humidity chamber at 4 ℃ with antibodies against Ki67 and Bax, coimmunostained with the corresponding secondary antibodies (anti-mouse 488- or 549-conjugated secondary antibody), and subsequently counterstained with DAPI. Information on the antibodies used could be found in Supplementary Table S2.

### 2.6. RNA-seq analysis and bioinformatics analysis

The gene expression profiles of mammary gland tissues from control and TC female mice on lactation Day 10 were analyzed by RNA-seq. We used three independent sets of biological replicates of mammary gland samples. Total RNA was extracted, and a complementary DNA library was constructed. The library preparations were sequenced on the Illumina platform. Biomarker Technologies Co., Ltd. (Beijing, China) performed RNA-seq data analysis using a bioinformatics pipeline tool, the BMKCloud online platform (www.biocloud.net). DESeq2 was used for differential expression analysis between the two groups. Genes with adjusted P values $< 0.01$ identified by DESeq2 were designated as differentially expressed.

Gene set enrichment analysis (GSEA) was performed using the OmicStudio tools at https://www.omicstudio.cn/tool. For each Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway, the identified genes were characterized as a gene set, and then an ordered list and a "gene set" permutation were generated. Gene sets with $P < 0.05$ and false discovery rates $< 0.25$ were considered to be statistically significant.

### 2.7. Quantification and statistical analysis

We used Prism 9.0 software for statistical analysis (GraphPad Software Inc., San Diego, CA, USA). Experimental data are presented as the mean ± standard error (SEM). Differences between groups were assessed by using a two-tailed Student's t-test. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant, and significance is expressed as *$p < 0.05$, * *$p < 0.01$ and * **$p < 0.001$.

## 3. Results

### 3.1. TC exposure inhibited mammary epithelial cell proliferation in pregnant mice

To investigate the effects of TC exposure on mammary gland development during pregnancy, we first generated a TC-activated mouse model (Fig. 1A). As shown in Fig. 1B, TC-induced CAR activation robustly suppressed average mammary gland weight at 14.5 days of pregnancy (P14.5 d). As expected, TC exposure significantly enlarged the liver and reduced fat accumulation in gonadal adipose (Gon-WAT), with no difference in spleen and kidney weights, as represented by the tissue/body weight ratio (Fig. 1B). Moreover, TC exposure had little effect on body weight (Fig. 1C). Given that the most striking feature of the mammary gland during pregnancy is the rapid and global proliferation of mammary epithelial cells in preparation for alveolar development (Richert et al., 2000), we asked about the effects of TC exposure on mammary morphology. We found that alveolar buds and epithelial surface area were markedly restricted after TC treatment (Fig. 1D-E), suggesting TC exposure inhibited the proliferation of mammary epithelial cells. Furthermore, the expression of target genes associated with mammary gland proliferation (*Igf 2*, *Ccnd 1*, *Rankl*, and *Nfkb1*) was considerably decreased after TC exposure (Fig. 1 F). Interestingly, the reduced nuclear localization of Ki67 observed in mammary epithelial cells was consistent with changes in the histomorphology of mammary glands (Fig. 1G-H). Collectively, our findings revealed that TC exposure could repress mammary epithelial cell proliferation in pregnant mice, which may lead to subsequent defects in alveologenesis and lactation.

### 3.2. TC exposure accelerated alveolar cell apoptosis

Massive tissue remodeling of the mammary gland during pregnancy operates the secretory lobuloalveolar units switch to initiate subsequent lactation events. Therefore, we next sought to identify whether TC exposure affected alveolar morphology, resulting in lactation failure. As shown in Fig. 2A, H&E staining revealed that cell shedding was seen in the mammary alveolar lumen of TC-treated mice at lactation 15 days (L15 d), suggesting a significant increase in apoptotic cells. In addition,

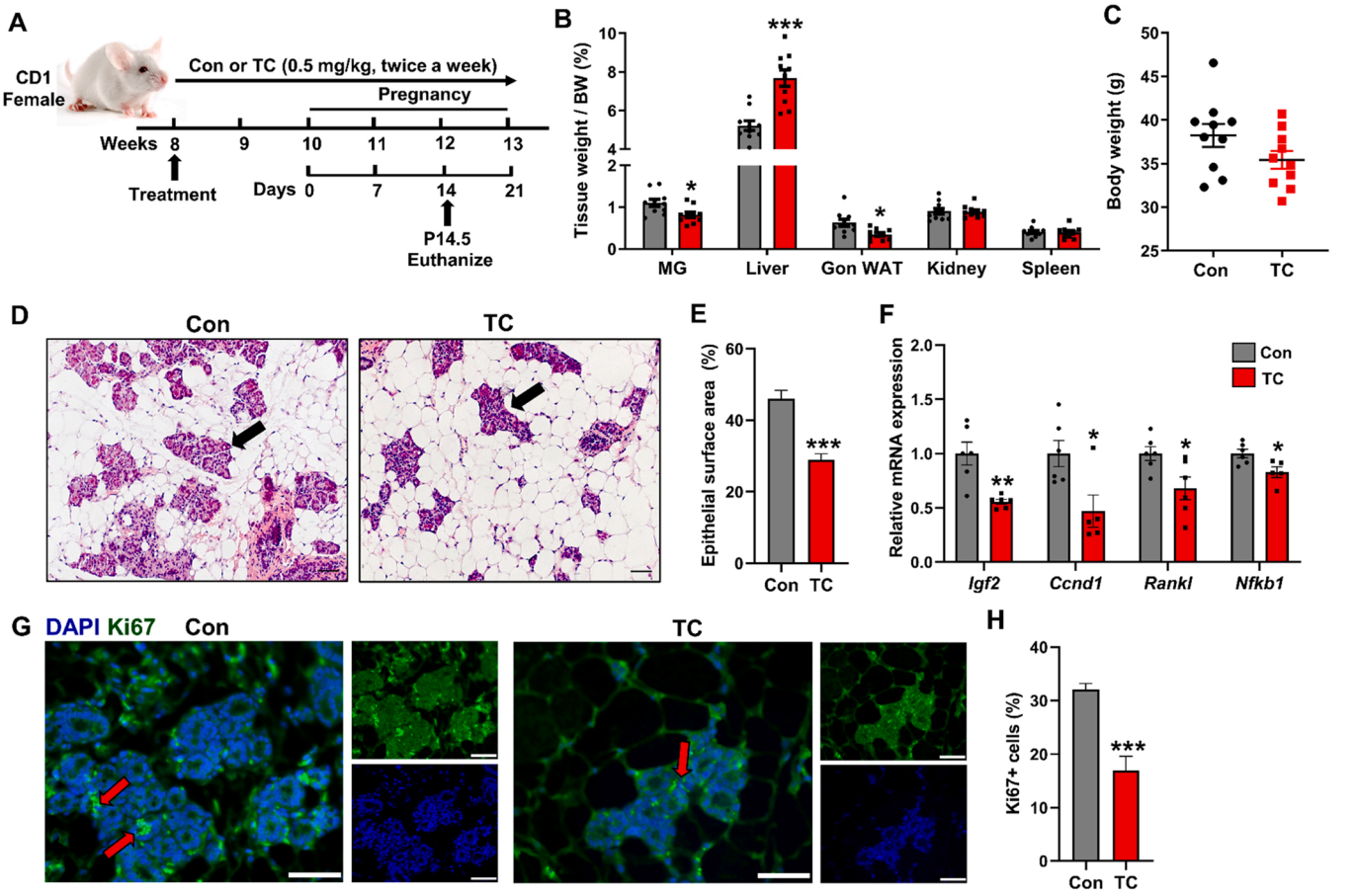


**Fig. 1.** TC exposure inhibited mammary epithelial cell proliferation in pregnant mice. (A) Schematic of the experimental procedures. (B) The relative tissue index at P14.5 d (n = 10). (C) Body weight at P14.5 d (n = 10). (D) Representative H&E staining of the mammary gland at P14.5 d. Black arrows represent alveolar buds. Scale bar = 50 μm. (E) Quantification of the area fraction of the mammary epithelial surface at P14.5 d. (F) qPCR analysis for *Igf 2*, *Ccnd 1*, *Rankl* and *Nfkb1* in the mammary gland at P14.5 d (n = 5-6). (G) Immunofluorescence of Ki67 (green) and d DAPI (blue) in the mammary gland at P14.5 d. Scale bar = 50 μm. Red arrows represent Ki67-positive cells. (H) Statistical analysis of the proportion of Ki67-positive cells in mammary epithelial cells. MG, mammary gland. Values are shown as the means ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

the expression levels of marker genes associated with cell apoptosis in the mammary gland were examined to further validate the phenotype of abnormal apoptosis observed by histological morphology. Strikingly, a marked increase in mRNA levels of *Casp3*, *Bax*, *Cebpd*, *Socs3,* and *Ctsk* was detected in the mammary gland of TC-exposed mice (Fig. 2B-F), suggesting that TC exposure during lactation resulted in abnormal activation of the apoptotic signaling pathway. In line with this observation, the number of cells positive for Bax was markedly upregulated in TC-exposed mammary glands (Fig. 2G-H). Taken together, these findings indicated that TC exposure accelerated apoptosis of alveolar cells, which may lead to premature termination of the lactation cycle, ultimately manifesting as a lactation defect.

### 3.3. TC exposure induced lactation failure

The well-developed morphological structure of the mammary gland at different developmental stages is essential for mammary gland lactation function. As mentioned above, TC exposure resulted in impaired mammary gland structure. Accordingly, whether TC exposure interferes with lactation performance is our next concern. Given that the body weight changes of pups could be used to characterize the milk production of mothers, we monitored the body weight of pups. Results showed that in the early lactation period (L1-L7 d), the milk production per hour of the mice exposed to TC was significantly lower than that of the control group, suggesting that TC exposure may have altered lactation capacity (Fig. 3 A). To exclude possible defects in pups, we conducted crossfostering experiments. Control (Con) pups were nursed by their biological mother (Con mother) and cross-nursed by their foster mother (TC mother) in order to assess lactation capacity. Pup's weight gain was decreased in litters nursed by TC mother, which suggested that TC treatment may be defective in milk production (Fig. 3B-C). Moreover, compared to pups born to TC mother but fostered by Con mother, pups born to Con mother but fostered by TC mother exhibited significantly reduced growth (Fig. 3B-C). The growth retardation in pups nursed by TC mother indicated that the growth-restricted phenotype was mainly a feeding-mediated (postnatal) rather than a transplacental (intrauterine) mechanism of induction transmission. This result was consistent with a previous study by Dietrich et al. (Dietrich et al., 2018). In addition, TC-F1 survival in PND21 was remarkably lower in TC-exposed mothers than in controls (Fig. 3D). Taken together, the relatively lower body weight and survival rate of offspring were associated with mammary lactation failure following maternal TC exposure.

### 3.4. TC exposure suppressed lactation capacity in RNA-sequencing analysis

To explore the mechanism of lactation failure in TC-exposed mice, we performed RNA sequencing (RNA-seq) to determine the molecular processes in Con and TC-exposed mice at day 10 (L10 d) of peak lactation. Similarly, exposure to TC significantly suppressed the mammary weight/body weight ratio at L10 d (Supplementary Fig. 2). As shown in Fig. 4A, the principal components analysis (PCA) plot indicated that

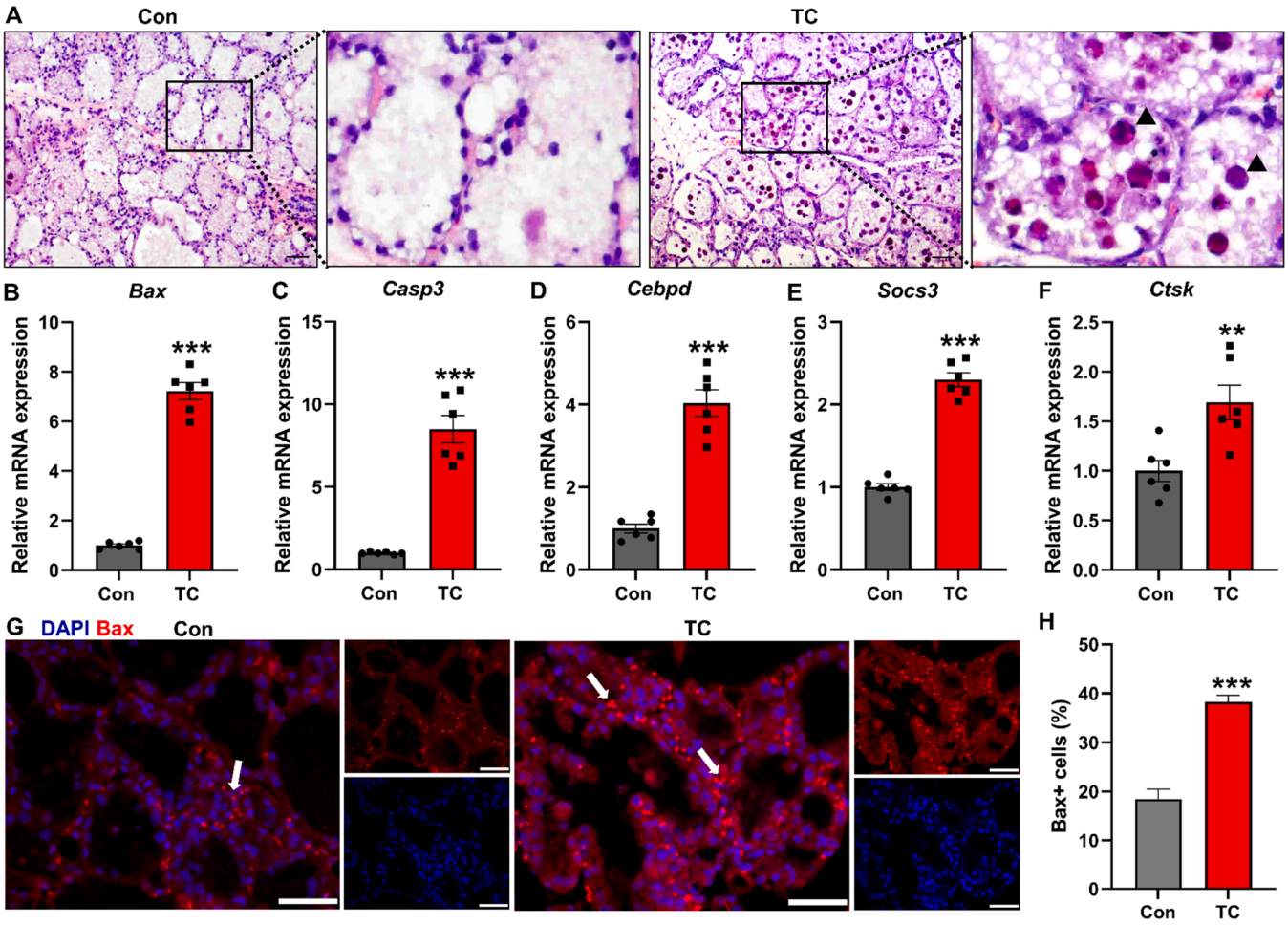


**Fig. 2.** TC exposure accelerated alveolar cell apoptosis. (A) Representative H&E sections of mammary glands at L15 d. Arrowhead shows shedding cells. Scale bar = 50 μm. (B-F) qPCR analysis of *Bax* (B), *Casp3* (C), *Cebpd* (D), *Socs3* (E), and *Ctsk* (F) in the mammary gland at L15 d, n = 6. (G) Immunofluorescence for Bax in mammary epithelial cells at L15 d. White arrows represent positive cells. Scale bar = 50 μm. (G) Statistical analysis of the proportion of Bax-positive cells in mammary epithelial cells. Data are presented as the mean ± SEM. ** represents $p < 0.01$, and *** represents $p < 0.001$.

there were separations between the Con and TC exposure groups. Additionally, a total of 2543 genes were significantly different in the mammary gland between the Con and TC-exposed groups (Fig. 4B). Among them, the levels of 1287 genes were found to be remarkably upregulated and 1256 were downregulated in the TC group compared with the Con group (Fig. 4B). As expected, RNA-Seq results showed that TC exposure resulted in significant differences in genes associated with drug-metabolizing enzymes in the mammary gland (Fig. 4C). qRT-PCR results further supported the differential expression of representative genes (*Cyp2b10*, *Cyp2d22*, *Cyp2e1*, *Cyp2d9* and *Sult2b1*) obtained from RNA-sequencing (Fig. 4D). Notably, KEGG analyses revealed that these differential genes were mainly enriched in some lipid metabolism, such as steroid biosynthesis, FA biosynthesis, and glycerolipid metabolism, as shown in Fig. 4E. GO analysis also revealed differential genes enriched in multiple pathways, particularly in lipid metabolism and alveolar development signaling pathway (Supplementary Fig. 3). Together, our results underscored several pathways and genes that were extensively affected by TC exposure as compared with the controls. TC exposure was capable of repressing lactation capacity at transcriptional levels, including lipid metabolism, alveolar development, apoptosis, and other biological processes.

### 3.5. TC exposure blocked the synthesis of milk lipid

It has been reported that TC is also involved in lipid homeostasis. Moreover, RNA-Seq results revealed that the differential genes were mainly enriched in multiple lipid metabolism pathways. These findings imply TC exposure would induce lipid disorder in the mammary gland. To further investigate the effect of TC on milk lipids, neutral lipids were analyzed by oil red O staining to assess the secretion or accumulation of lipid droplets (LDs) in the alveolar lumen during the stage of alveolar morphogenesis. As shown in Fig. 5, LDs were observed in mammary epithelial cells and alveolar lumen on day 18 of late pregnancy and days 1, 5, and 10 of lactation. Of note, the mammary glands of TC-exposed dams had a decreased accumulation of LDs. Consistently, LDs in the samples from the TC-exposed dams revealed a decreased size during these days (Fig. 5A-H). Besides, the total area occupied by LDs in TC-exposed dams was significantly lower than that in the control group (Fig. 5A-H). Bodipy staining also supported the above findings (Supplementary Fig. 4). Collectively, these results demonstrate that milk lipids synthesis in the mammary gland could be modified by maternal TC exposure.

### 3.6. TC exposure reduced MFG membrane protein expression

Evidence that milk lipids are present as MFG, which are directly derived from LDs in mammary epithelial cells. To explore whether compromised milk lipid synthesis in the TC- exposed mice was associated with the downstream events of the altered assembly or secretion of MFG, the mRNA and protein levels of the MFG membrane proteins Xor,

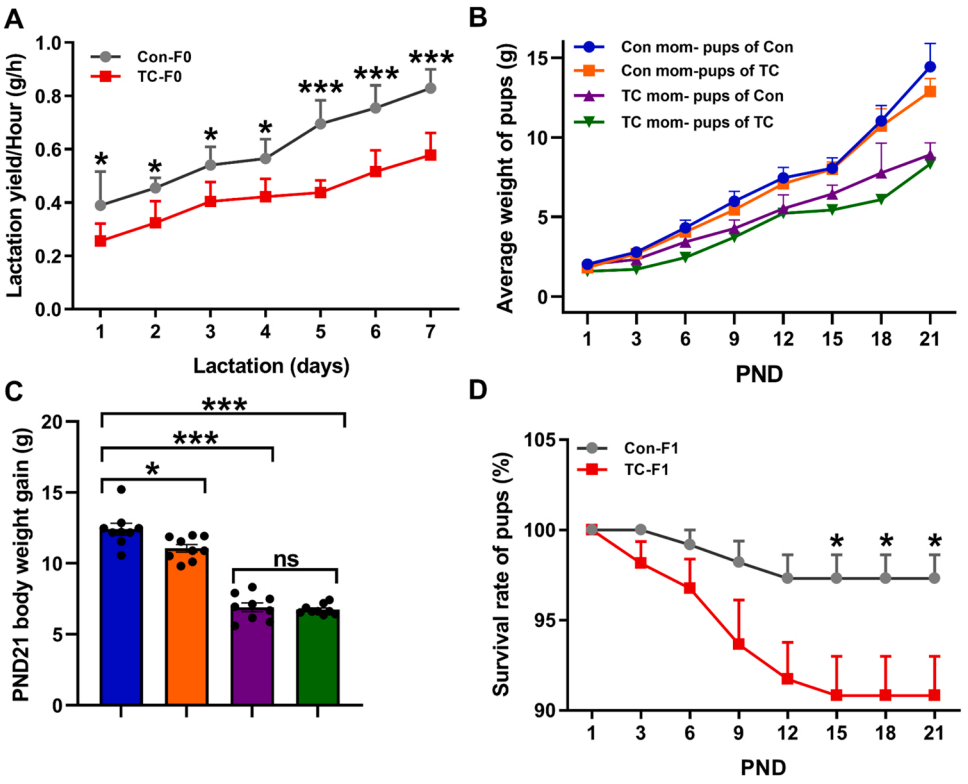


**Fig. 3.** TC exposure induced lactation failure. (A) Lactation yield per hour of dams. (B) Average body weight and body weight gain (C) of offspring from Con and TC mothers. Pups fed by their biological mothers (Con or TC) and cross-fed by their foster mothers (TC or Con) were recorded, with each dam with 9 pups beginning at L1 d. (D) The survival rate of offspring. F0, control or dam directly exposed to TC. F1, F0 offspring. PND, postnatal day. Data are presented as the mean ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

Plin2, and Btn1a1 were assessed at P18, L1, L5, and L10 d. qPCR results revealed that the significant decrease in *Xor* expression in the TC-exposed mammary gland at P18, L1, L5, and L10 (Fig. 6A-D). Moreover, *Plin2* was markedly decreased on these days, while *Btn1a1* was differentially expressed at P18, L5, and L10 (Fig. 6A-D). We therefore focused on the Xor and Plin2 protein levels. As expected, the protein expression of Xor was lower in TC-mediated CAR activation at P18, L1, L5, and L10 (Fig. 6E-L). In addition, Plin2 protein expression was significantly reduced at L1, L5, and L10, whereas it was comparable in TC-exposed mice at P18 compared with the Con mice (Fig. 6E-L). These findings indicated that TC-induced CAR activation would affect the expression patterns of the MFG membrane proteins Xor, Plin2, and Btn1a1. This may disrupt the assembly and secretion of MFG, resulting in impaired lipid homeostasis.

### 3.7. TC exposure affected milk lipid synthesis gene network

The synthesis and secretion of milk lipids appear to play a crucial role in the morphology and function of the mammary gland, and that interference with these biological processes may lead to lactation failure (Beigneux et al., 2006; Cases et al., 2004; Chen et al., 2008; Nagle et al., 2008; Ogg et al., 2004; Russell et al., 2011; Smith et al., 2000; Vorbach et al., 2002). To understand the molecular mechanisms by which TC affects milk lipid synthesis, we analyzed the transcriptome data from the mammary gland at L10 d of peak lactation. We screened the TC-regulated KEGG pathways through combined GSEA. Results revealed that TC exposure extensively suppressed the pathways related to lipid metabolism, including FA metabolism, FA biosynthesis, biosynthesis of unsaturated FA, and peroxisome proliferator-activated receptor γ (PPARγ) signaling (Fig. 7A-D). These findings suggested that milk lipid synthesis and secretion may be attributable to the regulation of multiple genes or proteins in the mammary gland. To further determine the mechanism of restricted milk lipid metabolism in TC exposure, we focused on the key regulators involved in milk lipid synthesis and secretion at lactation. A heat map exhibited that the expression of critical regulators in the milk lipid synthesis gene network was unbalanced by TC exposure (Fig. 7E). The key findings of the RNA-Seq data set were further demonstrated by qPCR analysis (Fig. 7F-G). These results showed that TC exposure may affect the processes of milk lipid metabolism, including LCFA import, de novo FA synthesis, FA desaturation, TAG synthesis, lipid droplets formation, transcriptional regulation, mitochondrial FA synthesis, and ceramide synthesis, ultimately triggering corresponding phenotypic differences. Combined with our data, we further mapped the milk lipid production gene network, as shown in Fig. 7H. Together, these results demonstrated that exposure to TC dysregulated milk lipid biosynthesis and secretion, resulting in the inability of TC-exposed mice to efficiently secret nutrients to support the survival and growth of their offspring.

## 4. Discussion

With the development of modern society, people are inevitably exposed to a variety of environmental endocrine disruptors, such as pesticides, detergents, cosmetics, and packaging materials in various ways. Most of these disruptors are agonists of the nuclear receptor CAR, which disrupts endocrine signaling and affects metabolic activities, posing a serious threat to human health (Ashrap et al., 2017; Baldwin and Roling, 2009; Chang and Waxman, 2006; DeKeyser et al., 2011; Ito et al., 2012; Laurenzana et al., 2016; Oshida et al., 2015; Ren et al., 2010; Rooney et al., 2019). In our study, we used the environmental chemical TC, which is a specific activator of CAR, to investigate the effects of exogenous exposure on mammary gland development, lactation function, and offspring health in mice. We found that TC-induced CAR activation resulted in early involution and atrophy of mammary gland during pregnancy and lactation It was mainly through inhibiting the proliferation of mammary epithelial cells during pregnancy, which in turn affected the functional differentiation of mammary secretory cells (alveolar epithelial cells). This deficiency ultimately resulted in insufficient milk lipid secretion and poor offspring health outcomes.

Acute exposure to environmental endocrine disruptors is less

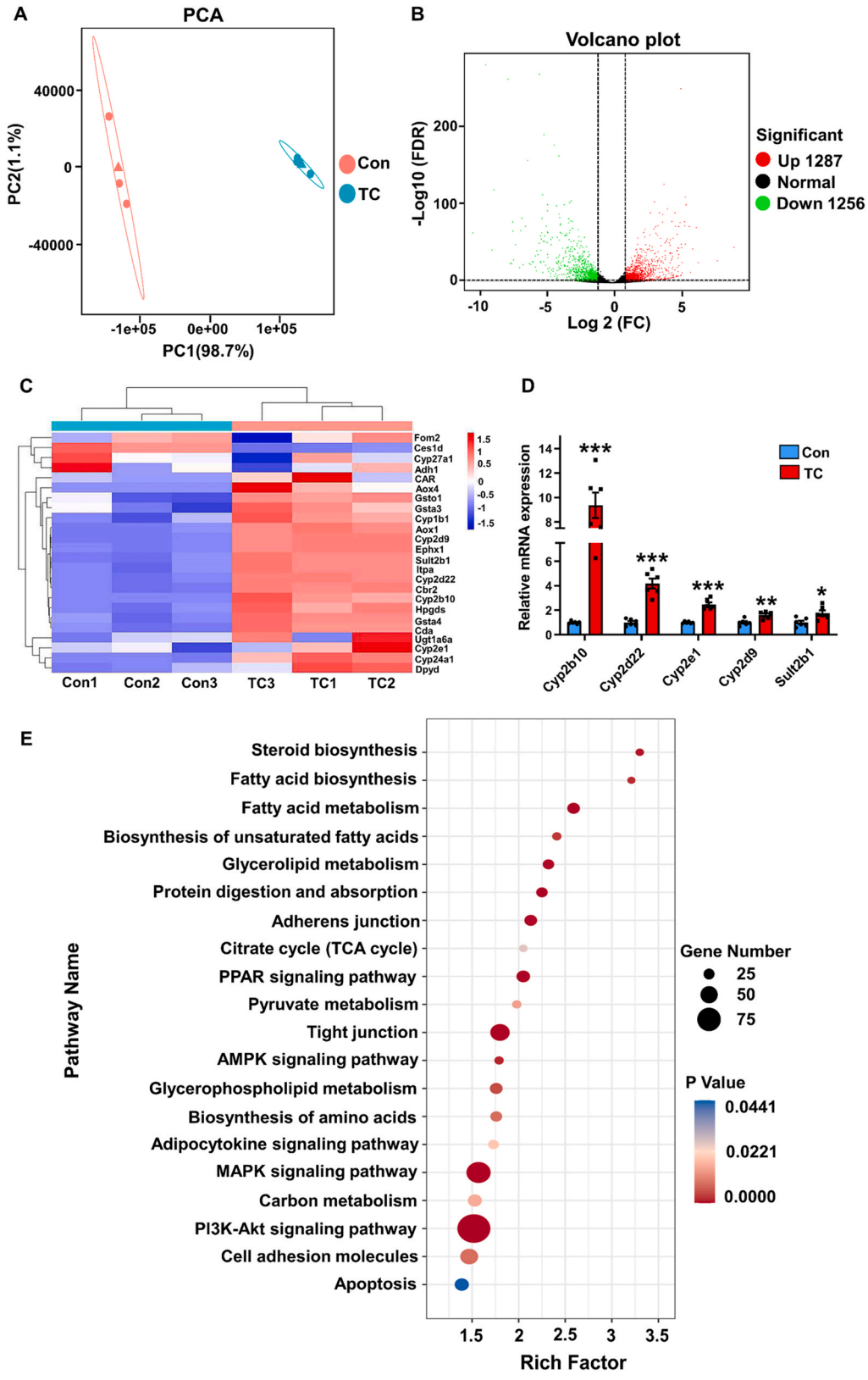


**Fig. 4.** TC exposure suppressed lactation capacity in RNA-Sequencing Analysis at L10. (A) Principal component analysis (PCA) score plot depicting the distribution profiles in mouse mammary gland samples (n = 3). Arrowheads show the mean values. (B) Volcano plot depicting differential genes in two groups. Signature genes were underlined in red (upregulated) and green (downregulated). n = 3. (C) Heat map depicting the representative gene expression profiles associated with drug-metabolizing enzymes. n = 3. (D) Relative mRNA levels of drug-metabolizing enzymes–related genes (*Cyp2b10*, *Cyp2d22*, *Cyp2e1*, *Cyp2d9*, and *Sult2b1*) in the mammary gland of indicated mice. n =5-6. (E) KEGG enrichment results depicting the enrichment pathways in the RNA-Seq data set. Data are presented as the mean ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

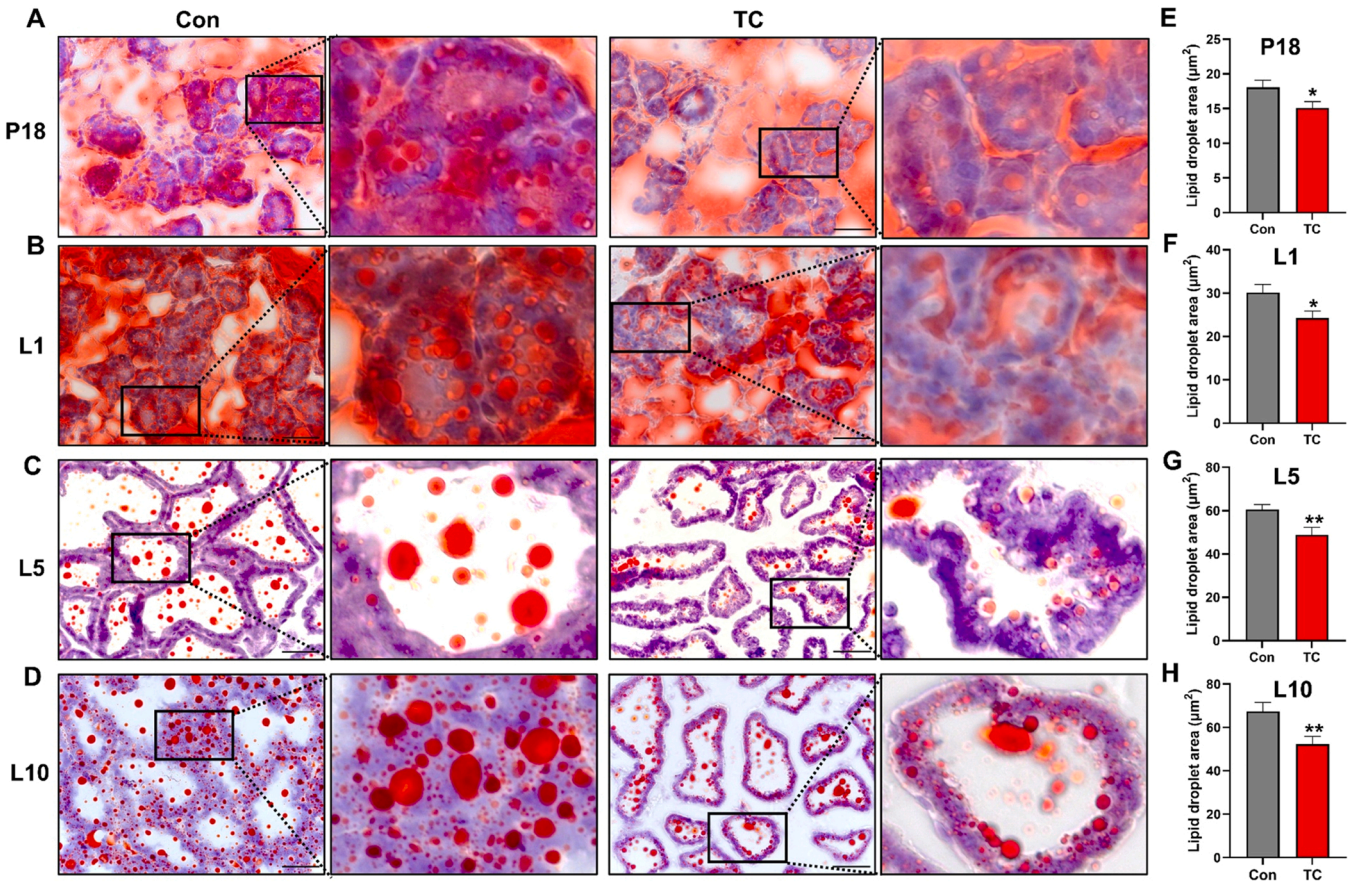


**Fig. 5.** TC exposure blocked the synthesis of milk lipids. (A-D) Oil red O staining of neutral lipids (red) in frozen sections of mammary glands on day 18 (A) of pregnancy and day 1 (B), day 5 (C), and day 10 (D) of lactation. Scale bar = 50 μm. (E-H) Quantification of LDs area within mammary epithelial cells and alveolar lumen on day 18 (E) of pregnancy and day 1 (F), day 5 (G), and day 10 (H) of lactation. Data are presented as the mean ± SEM. * represents $p < 0.05$, and * * represents $p < 0.01$.

common today, but long-term low-dose exposure is currently a serious public health concern. Summarizing the relevant research findings on TC that have been reported, the treatment mode of TC is 3 mg/kg single dose (Baskin-Bey et al., 2006; Bhushan et al., 2019; Chen et al., 2012; Shin and Waxman, 2022), or 0. 5 mg/kg multiple administration dose (Cai et al., 2021; Gao et al., 2009; Masuyama and Hiramatsu, 2012a; b). Notably, several studies have reported the association of CAR agonist TC treatment (0.5 mg/kg, intraperitoneal injection) with offspring health (Masuyama and Hiramatsu, 2012a; b). In addition, several studies have also used TC (0.5 mg/kg) to study the effect of CAR on lipid metabolism (Cai et al., 2021; Feng et al., 2022; Gao et al., 2009; Mo et al., 2016). Previous studies by our group have shown that TC (0.5 mg/kg, intraperitoneally, twice a week) affects mammary gland development in adolescent female mice, as evidenced by the inhibition of adipocyte size in the mammary fat pad and accelerated collagen fibrosis (Xu et al., 2018). Pregnancy and lactation stages are considered to be critical windows of vulnerability to environmental exposures during mammary gland development (Rudel et al., 2011; Vandenberg, 2021). Therefore, we established experimental doses of TC based on previous work that has been performed to reveal the toxic effects of TC on the mammary gland during pregnancy and lactation. This exposure approach is unique and informative, as it could better simulate the realities of environmental chemical exposure nowadays. Our data show that exposure to TC during pregnancy and lactation affects mammary gland development in mice, which could interfere with mammary gland lactation function and offspring health. However, as a limitation of this study, we were unable to reflect the residual levels of TC in the mouse mammary gland. We found that TC exposure led to hepatomegaly and activation of CAR-related target genes (such as Cyps enzymes) in liver and mammary tissue, combined with TC lipophilicity and persistence, suggesting that TC played a role in this study. The mammary gland is known to have multiple cell types such as adipocytes, fibroblasts, blood vessels and immune cells. More importantly, the histological evidence obtained from mammary biopsy is valuable, but only presents a static snapshot of the mammary gland at a specific time point and does not allow a better analysis of the dynamic patterns of multiple cell renewal in the mammary gland. It is unclear which mammary cell types the toxicity of TC-activated CAR acts on. Future work involving mammary cell turnover in response to TC exposure will provide a broader understanding of the harmful effects on the mammary gland.

During lactation, the ability of the mammary gland to synthesize milk depends on mammary epithelial cell activity and number, the latter being driven by dynamic changes in cell proliferation and apoptosis (Capuco et al., 2002, 2003). Our finding suggested that TC exposure significantly affects these processes. Milk lipid is known to be synthesized in mammary epithelial cells (Lemay et al., 2007). Interestingly, lack of milk lipid secretion and synthesis is closely related to changes in alveolar maturation of mammary gland (McManaman, 2009; McManaman et al., 2002, 2007). Downregulation of XOR and Btn1a1, or reduction of their interaction with Plin2, have been characterized to disrupt MFG secretion(McManaman, 2009; McManaman et al., 2002; McManaman et al., 2007). Our results suggested that TC exposure not only impaired alveolar maturation, but also affected the mRNA and protein expression of Btn1a1, XOR and Plin2, thereby disrupting MFG assembly and secretion. The inability of TC-exposed mice to adequately nourish their pups, could be a direct consequence of poor upregulation

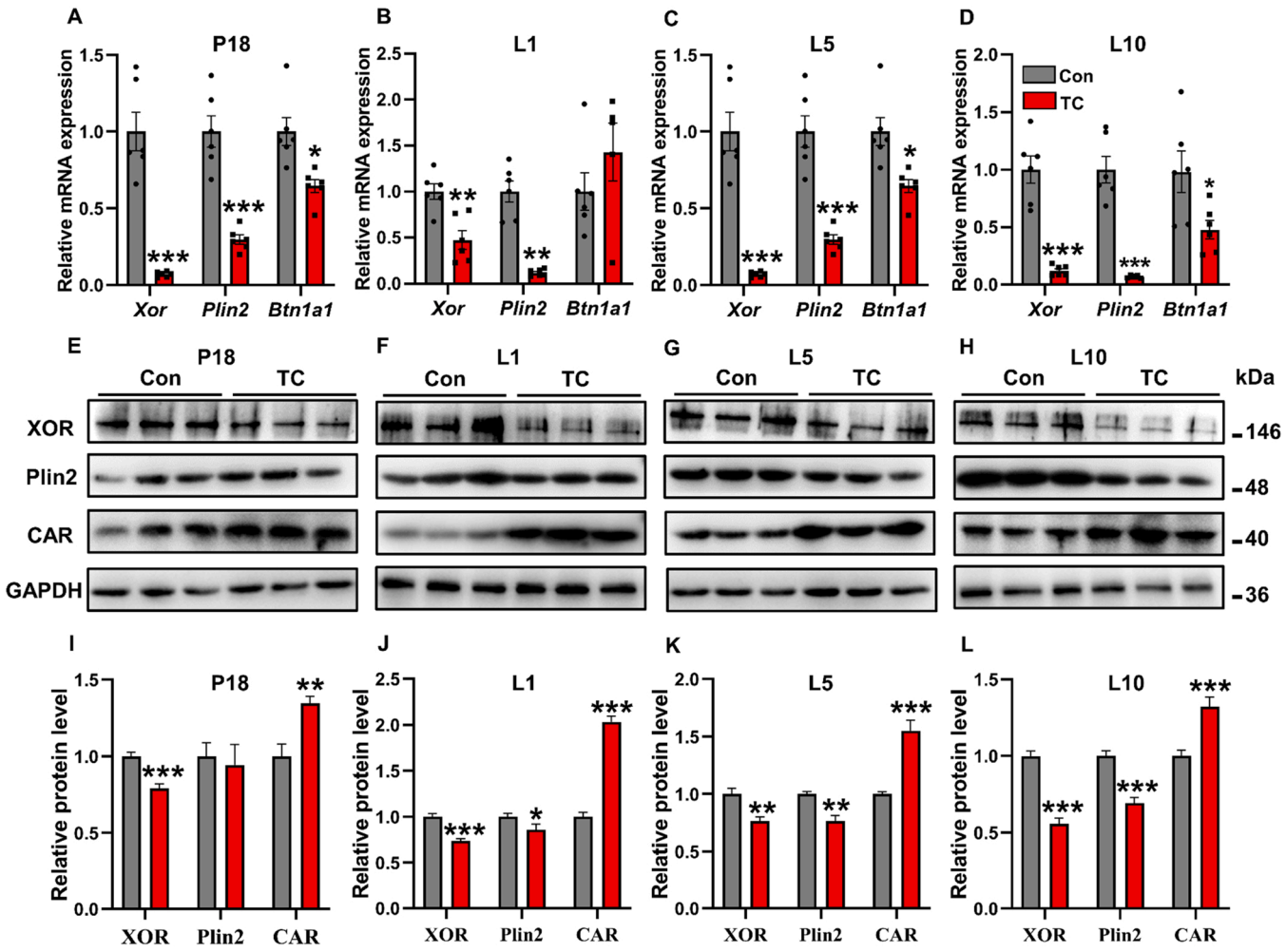


**Fig. 6.** TC exposure reduced MFG membrane protein expression. (A-D) qPCR analysis of *Xor*, *Plin2*, and *Btn1a1* in Con and TC-exposed mammary glands at P18 d (A), L1 d (B), L5 d (C), and L10 d (D). n = 5-6. (E–H) Western blotting for XOR, Plin2, and CAR in mammary glands at P18 d (E), L1 d (F), L5 d (G), and L10 d (H). GAPDH was used as a loading control. n = 3. (I–L) Quantification of the corresponding protein intensities at P18 d (I), L1 d (J), L5 d (K), and L10 d (L). The results are expressed as the means ± SEM. * represents $p < 0.05$, * * represents $p < 0.01$, * ** represents $p < 0.001$.

of XOR and Btn1a1 during lactation, whose activity is critical for milk LDs secretion. A similar expression patterns of Plin2 mRNA and protein levels were observed in the TC-exposed group during lactation. ADPH has been reported to interfere with LDs growth by preventing lipase from entering the triglyceride (TG) core of LDs and affecting alveolar maturation, suggesting that one of the mechanisms by which TC exposure may alter MFG homeostasis in the mammary gland is by regulating Plin2 expression. These results highlight the adverse role of environmental exposure TC in milk lipid secretion, causing the inability of pups to obtain adequate milk.

From our RNA-seq data, many genes participating in lipid synthesis were significantly reduced in TC treatment. This may be attributed to feedback from impaired lipid secretion. Of note, TC is also known to be involved in systemic lipid homeostasis (Gao et al., 2009; Miao et al., 2006; Skoda et al., 2022). The mammary gland is a highly dynamic tissue and nutrient organ for lipid metabolism, particularly during lactation. However, it is not clear whether TC exposure affects lipid metabolism in mammary gland development during lactation. In this study, we found that TC exposure in the mammary gland led to lipid accumulation in mammary epithelial cells, which induced failure of milk lipid secretion and poor neonatal survival. These results demonstrated that environmental chemical TC may play a broader role than previously expected in the regulation of lipid homeostasis in biology.

There is growing evidence that exposure to environmental pollutants and drugs could disrupt endocrine homeostasis and mammary gland function in a persistent manner (Diamanti-Kandarakis et al., 2009; Fenton, 2006). Our study suggests that exposure to environmental chemical TC affects mammary lactation function. It is now crucial to decipher the mechanisms and functional consequences of structurally diverse environmental chemicals-mediated CAR activation in lactation performance, especially for normal growth and development of offspring.

## 5. Conclusion

In our study, the model environmental chemical TC, was used to explore the effects of exogenous exposure on mammary gland development and offspring health in mice. It has been discovered that TC exposure could reduce mammary epithelial cell proliferation during pregnancy, and create a shortage of secretory (alveolar) cells during lactation. Next, it restricts alveolus secretion and activation restricted. This deficiency might lead to alveolar cell apoptosis, as well as premature stoppage of the lactation cycle and aberrant lactation. Consistently, TC exposure caused decreases in the maternal milk amount and pup weight, implying that TC exposure may be linked to lactation function downregulation. Furthermore, the results of RNA-seq analysis of the mammary gland during the peak lactation phase revealed that TC exposure has an impact on mammary gland gene expression. These

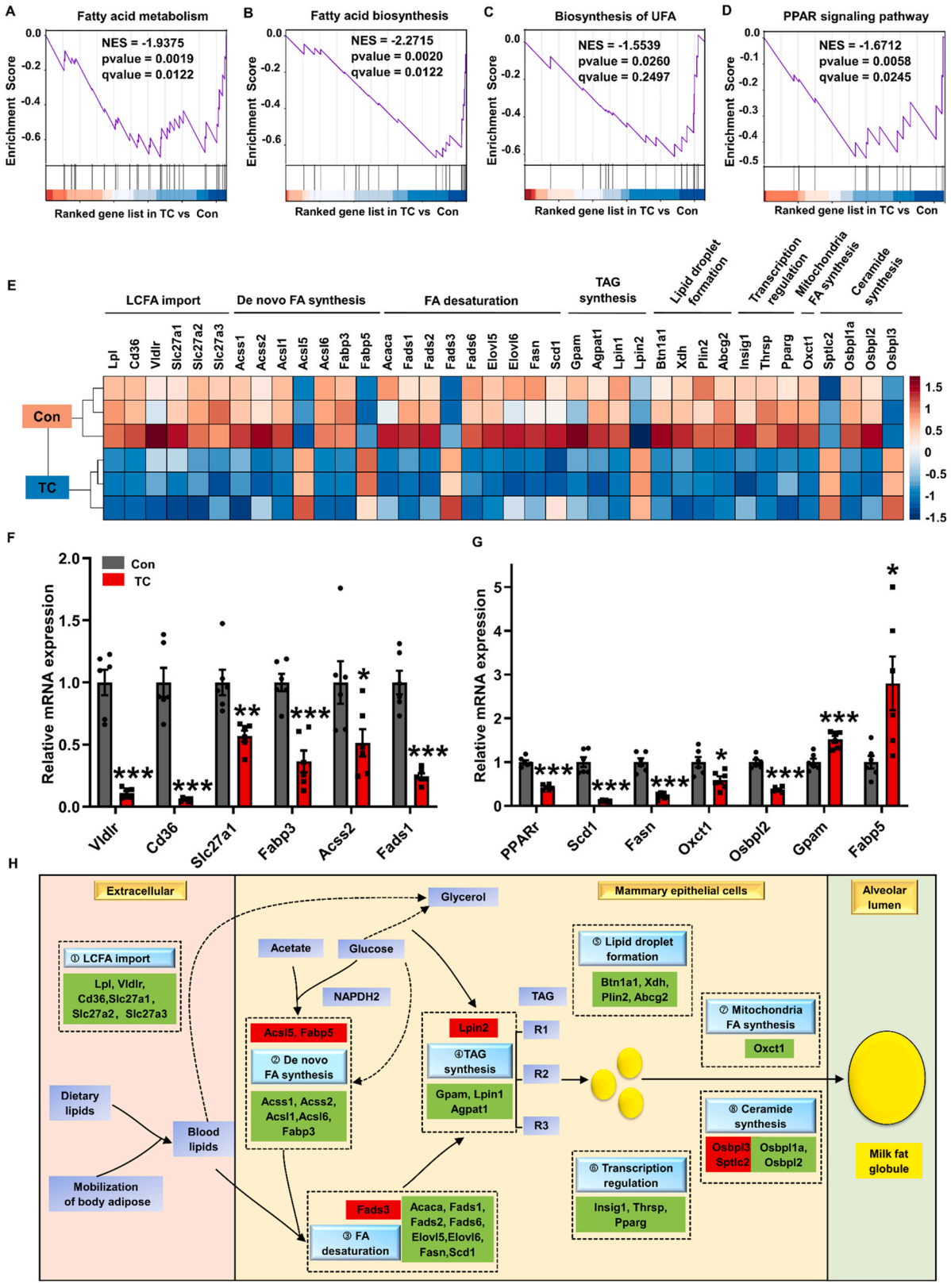


**Fig. 7.** TC exposure affected the milk lipid synthesis gene network. (A-D) GSEA analyses of genes regulating fatty acid metabolism (A), fatty acid biosynthesis (B), biosynthesis of unsaturated fatty acid (UFA) (C), and PPARγ signaling (D) in TC-treated mammary gland compared to the Con mice at L10. (E) Heat map showing the representative gene expression profiles related to milk lipid metabolism. n = 3 per group. (F) qPCR analysis of *Vldlr*, *Cd36*, *Slc27a1*, *Fabp3*, *Acss2,* and *Fads1* in Con and TC-exposed mammary glands at L10 d. n = 6. (G) qPCR analysis of *PPARγ*, *Scd1*, *Fasn*, *Oxct1*, *Osbpl2*, *Gpam,* and *Fabp5* in Con and TC-exposed mammary glands at L10 d. n = 6. (H)Schematic of networks among genes involved in milk lipid synthesis. Increased expression is marked with red boxes, while decreased expression is marked with green boxes. The results are expressed as the means ± SEM. * represents $p < 0.05$, * * represents $p < 0.01$, and * ** represents $p < 0.001$.
Gene networks adapted from Free Fatty Acids in Milk: Origin and Effects on Milk Quality by AG Marangoni et al. (2018).

differential genes could be the key factors leading to the regulation of lactation function. In addition, TC exposure could disrupt the regulatory network of milk lipid metabolism, which explained why lactation function was downregulated and retarded offspring growth. These findings have suggested a link between maternal model environmental chemical TC exposure and changes to mammary gland lactation function and offspring health. From a public health perspective, changes in lactation function caused by structurally diverse environmental chemicals should be considered, as they might reprogram organ developmental trajectories and affect the normal growth and development of offspring.

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## CRediT authorship contribution statement

**Shijia Pan:** Conceptualization, Methodology, Project administration, Writing – review & editing, Supervision. **Yuan Guo:** Data curation, Investigation. **Wen Yu:** Conceptualization, Methodology. **Fan Hong:** Investigation, Visualization. **Xiaoxiao Qiao:** Formal analysis, Visualization. **Jia Zhang:** Investigation, Resources. **Pengfei Xu:** Writing – review & editing, Supervision. **Yonggong Zhai**: Validation, Investigation, Resources.

## 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.

## Data Availability

Yes.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114463.

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