

Oral Exposure to Polystyrene Nanoplastics Disrupts Gut–Testis Metabolic Homeostasis and EGR1-Associated Steroidogenic Regulation in Male Mice

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ABSTRACT: Polystyrene nanoplastics (PS-NPs) may impair male reproduction through gut–testis metabolic disruption. ICR mice received 100 nm PS-NPs (1 mg/mouse/day) orally for 28 days; fecal microbiota transplantation, metabolomics, sodium butyrate (NaB) supplementation, and TM3-cell assays were used. PS-NPs impaired intestinal barrier integrity and testicular steroidogenesis. Following antibiotic pretreatment, these abnormalities were partially reproduced in exposed-donor recipients relative to control-donor recipients. Untargeted metabolomics showed an FDR-significant decrease in relative testicular butanoic acid levels, while serum butyrate decreased in PS-NP-exposed mice and exposed-donor recipients. In vivo NaB supplementation partially improved reproductive and steroidogenesis-related outcomes in PS-NP-exposed mice. Exploratory analyses and validation experiments implicated a NaB-responsive EGR1–*Cyp11a1* regulatory relationship in Leydig cells. These findings suggest that gut-associated butyrate disturbance contributes to PS-NP-induced steroidogenic dysfunction.

KEYWORDS: polystyrene nanoplastics, testicular steroidogenesis, butyrate, gut–testis axis

INTRODUCTION

Nanoplastics (NPs) are commonly defined as plastic particles smaller than 1 μm .¹ Global plastic releases into the environment are estimated at 10–40 million tons per year, and continued plastic production and disposal are expected to further increase environmental plastic burdens by 2040. Owing to their small size and high surface area, NPs can enter organisms through ingestion, inhalation, and other exposure routes, and may translocate across biological barriers, raising concerns about their systemic toxicity.² Growing evidence indicates that NPs can impair male reproductive function by damaging testicular structure, disrupting spermatogenesis, endocrine homeostasis, and steroidogenic signaling, and inducing oxidative stress.^{3–5} Because orally ingested NPs first interact with the gastrointestinal tract, intestinal injury and gut-associated systemic responses may represent important links between oral NP exposure and distal reproductive dysfunction.

The gut microbiota is a complex microbial community that contributes to host metabolism, immune regulation, endocrine homeostasis, and intestinal barrier maintenance.^{6,7} Increasing evidence suggests that gut microbial dysbiosis and intestinal barrier disruption are associated with impaired male reproductive function through the gut–testis axis.^{8,9} Microbial enzymes and metabolites may influence androgen metabolism, inflammatory homeostasis, and systemic metabolic status, thereby affecting spermatogenesis and Leydig cell steroidogenesis.^{10,11} Among these metabolites, short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are major products of microbial fermentation and important mediators of host–microbiota metabolic communication.^{12,13} Butyrate contributes

to intestinal barrier integrity, immune regulation, and metabolic homeostasis.^{14–17} Emerging preclinical evidence further suggests that butyrate may modulate spermatogenesis, testosterone production, and Leydig cell steroidogenesis, particularly under metabolic or inflammatory stress.^{18,19} However, whether oral PS-NP exposure perturbs butyrate-related metabolic homeostasis and whether such disturbances contribute to impaired testicular steroidogenesis remain unclear.

Leydig cells are the principal testosterone-producing cells in the testis.²⁰ Testosterone biosynthesis begins with the delivery of cholesterol to mitochondria, a process that requires StAR and intact mitochondrial organization.^{21,22} At the inner mitochondrial membrane, CYP11A1 converts cholesterol to pregnenolone, which is subsequently processed through a series of steroidogenic reactions involving enzymes such as CYP17A1. Consistent with the importance of this pathway, disruption of CYP17A1 expression in Leydig cells markedly reduces testosterone production and impairs spermatogenesis.²³ Transcription factors regulate the expression of steroidogenic enzymes and thereby contribute to the control of testosterone synthesis in Leydig cells.²⁴ Early growth response 1 (EGR1) is a rapidly inducible zinc-finger transcription factor that converts extracellular stimuli into context-dependent transcriptional

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Table 1. Primers Used for Vector Construction

Primer	Primer sequence (5' → 3')
U6-shEgr1-F	gatccTCTGACATCGCTCTGAATAATcaagagATTATTACAGAGCGATGTCTAGAttttg
U6-shEgr1-R	aattcaaaaaTCTGACATCGCTCTGAATAATctcttgaATTATTACAGAGCGATGTCTAGAG

69 responses.²⁵ In the reproductive endocrine system, recent
70 studies support the involvement of EGR1 in gonadotropin-
71 responsive transcription: GnRH-induced EGR1 contributes to
72 regulation of the luteinizing hormone β -subunit gene in pituitary
73 gonadotropes, whereas hCG-induced EGR1 mediates transcrip-
74 tional responses in ovarian granulosa cells.^{26,27} Recent single-cell
75 and functional studies have also revealed substantial transcrip-
76 tional plasticity in Leydig cells during differentiation, aging, and
77 changes in steroidogenic function.^{21,28,29} Against this back-
78 ground, exploratory single-cell transcriptomic analysis nomi-
79 nated EGR1 as a candidate regulator associated with Leydig cell
80 dysfunction following PS-NP exposure, prompting further
81 cellular and molecular investigation.

82 In this study, we examined whether oral exposure to 100 nm
83 polystyrene nanoplastics (PS-NPs) impairs male reproductive
84 function and whether gut-associated metabolic disturbances
85 contribute to this effect. We characterized intestinal barrier
86 injury, testicular pathology, and reproductive dysfunction
87 following PS-NP exposure and used fecal microbiota trans-
88 plantation to compare intestinal and testicular phenotypes
89 between recipients of fecal material from control and PS-NP-
90 exposed donors. Gut microbiota profiling and fecal and
91 testicular metabolomics were used to characterize microbial
92 alterations and prioritize candidate metabolic signals. Explor-
93 atory single-cell transcriptomic analysis was used to prioritize
94 candidate transcriptional regulators within Leydig cells. Sodium
95 butyrate (NaB) supplementation was subsequently used to
96 evaluate the in vivo functional relevance of the butyrate-related
97 disturbance. Finally, TM3 Leydig-like cells, shRNA-mediated
98 *Egr1* silencing, and ChIP-qPCR were used to examine the
99 involvement of EGR1 in NaB-responsive steroidogenic
100 regulation and its enrichment at a predicted binding region of
101 the *Cyp11a1* promoter. Together, these experiments evaluated a
102 putative NaB-responsive EGR1-*Cyp11a1* regulatory relation-
103 ship in PS-NP-associated testicular steroidogenic dysfunction.

104 ■ MATERIALS AND METHODS

105 Animal Experimental Design

106 Healthy 6-week-old male ICR mice (Chengdu Dashuo Laboratory
107 Animal Co., Ltd.) were acclimatized for 1 week and randomly assigned
108 to the CON or NPS group ($n = 10$ per group). Mice received saline or
109 PS-NPs (1 mg/mouse/day) by oral gavage in a volume of 100 μ L for 4
110 weeks, after which intestinal and testicular tissues were collected. The
111 number of animals analyzed for each assay is specified in the
112 corresponding Methods subsection and figure legend. When an assay
113 was performed using a subset of animals, the animals were selected
114 before outcome assessment using the predefined randomization
115 procedure. Each mouse represented one biological replicate, and
116 technical replicate measurements were averaged to obtain a single value
117 per animal. No animals or samples were excluded from the analyses.
118 Histological assessment and image quantification were performed using
119 blinded group codes. All procedures were approved by the Animal
120 Welfare and Ethics Committee of Northwest A&F University
121 (IACUC2026-0105).

122 In Vivo Sodium Butyrate Supplementation

123 Twenty-four male ICR mice were randomly assigned to CON, NPS,
124 NaB, and NPS + NaB groups ($n = 6$ per group). The NPS and NPS +
125 NaB groups received PS-NPs by daily oral gavage at 1 mg/mouse/day,

whereas the other groups received saline. The NaB and NPS + NaB
groups received 2 g/L NaB in drinking water for 28 days. Drinking
solutions (150 mL per group) were replaced every 2 days. The CON
and NPS groups received regular drinking water.

Fecal Microbiota Transplantation

A separate cohort of SPF male ICR mice comprised CON and NPS
donors and their corresponding Re-CON and Re-NPS recipients ($n = 6$
per group). Donors and recipients were housed separately, with all six
mice within each donor or recipient group housed together in a single
cage. Recipient mice received 200 μ L of an antibiotic cocktail
containing ampicillin (1 g/L), neomycin sulfate (0.5 g/L), vancomycin
hydrochloride (0.5 g/L), and metronidazole (1 g/L) by oral gavage
once daily for 3 consecutive days, followed by a 48 h washout period.
On each transplantation day, 20–30 mg of freshly collected feces from
each donor were individually suspended in 200 μ L of sterile PBS,
homogenized, and centrifuged at 500g to remove particulate matter.
Fecal samples were not pooled, and each donor was assigned to one
designated recipient before the first transplantation. This donor-
recipient pairing was maintained throughout the experiment. Each
recipient received 100 μ L of the supernatant prepared from its
designated donor by oral gavage four times per week for 4 weeks. Mice
were euthanized 2 days after the final transplantation for sample
collection.

Vector Construction

The sh-*Egr1* and nontargeting control (shNC) oligonucleotides listed
in Table 1 were annealed and cloned into the *Bam*HI/*Eco*RI-digested
U6-shRNA-EF1 α -copGFP-T2A-Puro lentiviral vector. Recombinant
plasmids were propagated in *Escherichia coli* DH5 α and verified by
Sanger sequencing. The sequence-verified plasmids were used for
lentiviral production, and the resulting viral particles were used to
transduce TM3 cells.

Establishment of Stable sh-*Egr1* TM3 Cells

Lentiviruses carrying *Egr1*-targeting or nontargeting control shRNA
were packaged in HEK293T cells. Filtered viral supernatants were used
to transduce TM3 cells in the presence of Polybrene, followed by
puromycin selection. Stable knockdown was confirmed by qRT-PCR
and Western blotting.

NaB Treatment of TM3 Cells

TM3 cells were seeded into culture plates and grown to approximately
50% confluence before treatment. NaB was dissolved in sterile PBS to
prepare a stock solution, filter-sterilized, and diluted into complete
medium immediately before use. Cells were treated with NaB at final
concentrations of 0.5, 1, or 2 mM for 12, 24, or 48 h. Cells were then
collected for subsequent analyses of *Egr1* and steroidogenic gene
expression.

ChIP-qPCR

ChIP-qPCR was performed using a Chromatin Immunoprecipitation
Kit (Bes5001; BersinBio) according to the manufacturer's instructions.
Approximately 2×10^7 TM3 cells were cross-linked with 1%
formaldehyde for 10 min, and chromatin was sonicated to 300–
1000-bp fragments. Chromatin was immunoprecipitated overnight at 4
°C with 3 μ g of ChIP-grade anti-EGR1 antibody (A20H11; Selleck) or
normal rabbit IgG and captured using protein A/G magnetic beads.
Following washing, elution, reverse cross-linking, and DNA purifica-
tion, qPCR was performed using primers spanning the predicted EGR1-
binding region of the *Cyp11a1* promoter. Enrichment was calculated
using the percent-input method.

Single-Cell RNA Sequencing

Testicular tissues from one control (CON) mouse and one PS-NP-
exposed (NPS) mouse were subjected to scRNA-seq; because each

Table 2. Primers Used for qPCR

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
<i>Star</i>	TCGCTACGTTCAAGCTGTGT	ACGTCGAACCTGACCCATCC
<i>Cyp11a1</i>	TCCGGGCTTCTTTCCCAATC	GATGGTCATCTCCAGCTCCG
<i>Cyp17a1</i>	TGGAGGCCACTATCCGAGAA	CTGACAACATGGGCTGCCTCTG
<i>Egr1</i>	TATACTGGCCGCTTCTCCCT	AGAGGTCGGAGGATTGGTCA
<i>β-actin</i>	TTTCCAGCCTTCTTCTTGGG	TCTTTACGGATGTCAACGTCA
<i>Zo-1</i>	AAGTTGGCAAGAGAGGAGCC	CAACCGCATTGGCGTTACA
<i>Ocludin</i>	CCTCCACCCCATCTGACTA	GCTTGCCATTCACTTTGCCA
<i>Cyp11a1-ChIP1</i>	CTTCCAGAGGATCCAGGTTCA	TCTTTGAAAATGTTCTGACGAGCA

186 condition included only one biological sample, all single-cell analyses
187 were considered exploratory. Single-cell suspensions were prepared
188 using the Singleron PythoN system, and libraries were generated using
189 the GEXSCOPE platform and sequenced on an Illumina NovaSeq 6000
190 platform. The CON and NPS libraries yielded 17,995 and 17,280
191 putative cell barcodes, respectively, with mean sequencing depths of
192 15,194 and 16,013 reads per cell. Raw sequencing data were processed
193 using CeleScope v1.9.0, aligned to the mouse reference genome
194 (GRCm38) using STAR v2.6.1a,³⁰ and quantified using featureCounts
195 v2.0.1.³¹ Downstream analyses were performed using Scanpy v1.10.3.³²
196 Removal of low-quality cells and predicted doublets yielded 34,175 cells
197 for downstream analyses. Principal component analysis (PCA) was
198 performed using normalized expression matrices. Harmony integration
199 was performed on the first 50 principal components using sample
200 identity (CON or NPS) as the grouping variable, with all other
201 parameters kept at default settings. The Harmony-integrated
202 representation was used exclusively for unsupervised clustering,
203 UMAP visualization, and cell-type annotation. Cell types were
204 annotated based on the expression of established marker genes. Cell
205 clusters that could not be reliably annotated were excluded from
206 downstream analyses, resulting in 33,462 annotated cells retained for
207 further analysis. Exploratory differential expression analysis was
208 performed using Wilcoxon rank-sum tests based on pre-Harmony
209 normalized expression matrices. Gene Ontology (GO) and Kyoto
210 Encyclopedia of Genes and Genomes (KEGG) enrichment analyses
211 and regulatory-network analyses were performed using clusterProfiler
212 v4.10.1 and pySCENIC v0.12.1,³³ respectively. Detailed procedures
213 and parameters are provided in the [Supporting Methods](#).

214 16S rRNA Gene Sequencing and Analysis

215 Fecal microbial DNA was extracted using the CTAB method, and
216 amplicons were sequenced on an Illumina NovaSeq platform. Reads
217 were processed using QIIME2/DADA2 to generate amplicon sequence
218 variants, which were annotated against the SILVA 138 database.
219 Microbial composition and group separation were evaluated using
220 relative-abundance analysis and PCA. Differential taxa were identified
221 using the Mann–Whitney U test and LefSe ($P < 0.05$; LDA > 3.0).

222 Untargeted Metabolomics Analyses

223 Untargeted LC–MS/MS metabolomics of fecal and testicular samples
224 was performed on a quadrupole-Orbitrap platform with interspersed
225 quality-control samples. After feature filtering, missing values were
226 imputed as half the minimum detected value and intensities were
227 normalized to internal standards. PCA and OPLS–DA were used to
228 assess metabolic variation and derive VIP scores, respectively.
229 Differences between NPS and CON were evaluated using two-sided
230 Welch’s t -tests, followed by Benjamini–Hochberg correction. Nominal
231 candidates met $VIP \geq 1$, $P < 0.05$, and $|\log_2 FCI| > 0.58$, whereas FDR-
232 supported metabolites additionally met $Q < 0.05$. Fold changes were
233 calculated as NPS/CON, and shared metabolites with $P < 0.05$ in both
234 data sets were assessed using Fisher’s combined P value. Butanoic acid
235 effects across compartments were expressed as Hedges’ g with 95%
236 confidence intervals without pooling. Metabolites were annotated by
237 accurate mass, retention time, and MS/MS spectra.

238 Hematoxylin and Eosin (H&E) Staining

239 Testes and 2-cm segments from the middle ileum were fixed in 4% PFA
240 at 4 °C for 24 h, paraffin-embedded, sectioned at 5 μ m, and stained with

H&E. Testicular morphology and ileal villus height and crypt depth
241 were evaluated by light microscopy. 242

Biochemical Measurement 243

To assess biochemical changes, serum testosterone and LPS levels were
244 measured using ELISA kits purchased from Elabscience (China), and
245 serum butyrate concentrations were measured using a Butyric Acid
246 (BA) Enzyme-Linked Immunosorbent Assay Kit purchased from ELK
247 Biotechnology (China). 248

Transmission Electron Microscopy (TEM) Analysis of Mouse
Testicular Tissues 249 250

For TEM analysis, mouse testicular tissues were fixed in 2%
251 glutaraldehyde followed by 1% osmium tetroxide, dehydrated through
252 a graded ethanol series, and embedded in Epikote resin. Ultrathin
253 sections (50 nm) were prepared, stained with lead citrate, and examined
254 using a JEM-1400FLASH transmission electron microscope (JEOL,
255 Japan). 256

Quantitative Real-Time PCR 257

Total RNA from testes and cultured cells was extracted using TRIzol
258 (TaKaRa), reverse-transcribed into cDNA, and analyzed by SYBR
259 Green qRT–PCR on a CFX96 system. Relative expression was
260 calculated using the $2^{-\Delta\Delta Ct}$ method with the indicated reference
261 gene. Primers are listed in [Table 2](#). 262 12

Western Blotting 263

Detailed Western blotting procedures have been described in our
264 previous study.³⁴ Total protein lysates from testes and cultured cells
265 were separated by 10% SDS–PAGE, transferred to PVDF membranes,
266 and blocked with 5% BSA. Membranes were incubated with antibodies
267 against β -Actin (1:50,000; Boster), Star (1:1000; Proteintech),
268 CYP11A1 (1:1000; Proteintech), CYP17A1 (1:1000; Abcam), and
269 EGR1 (1:1000; Selleck). EGR1 was measured in total rather than
270 nuclear protein extracts. 271

Immunofluorescence Staining 272

Tissue sections and TM3 cells were fixed, permeabilized with 0.5%
273 Triton X-100, and blocked with 5% BSA. Samples were incubated
274 overnight at 4 °C with antibodies against E-cadherin, ZO-1, Star,
275 CYP11A1, CYP17A1, or EGR1 (all 1:200), followed by fluorophore-
276 conjugated secondary antibodies for 1 h and DAPI counterstaining.
277 Images were acquired using an EVOS FL microscope. 278

Statistics 279

Data are presented as box-and-whisker plots showing median,
280 interquartile range, and min–max values. Two-group comparisons
281 were performed using unpaired two-tailed Student’s t -tests or Welch’s t -
282 tests, as specified. Multiple-group experiments were analyzed using one-
283 way ANOVA followed by Tukey’s multiple-comparisons test. Analyses
284 were performed in GraphPad Prism. Statistical significance is indicated
285 as * $P < 0.05$ and ** $P < 0.01$. 286

RESULTS 287

PS-NP Exposure Is Associated with Testicular Injury and
Intestinal Barrier Impairment in Male Mice 288 289

Male ICR mice were orally administered saline or 100 nm PS-
290 NPs at 1 mg/mouse/day for 28 consecutive days ([Figure S1A](#)). 291

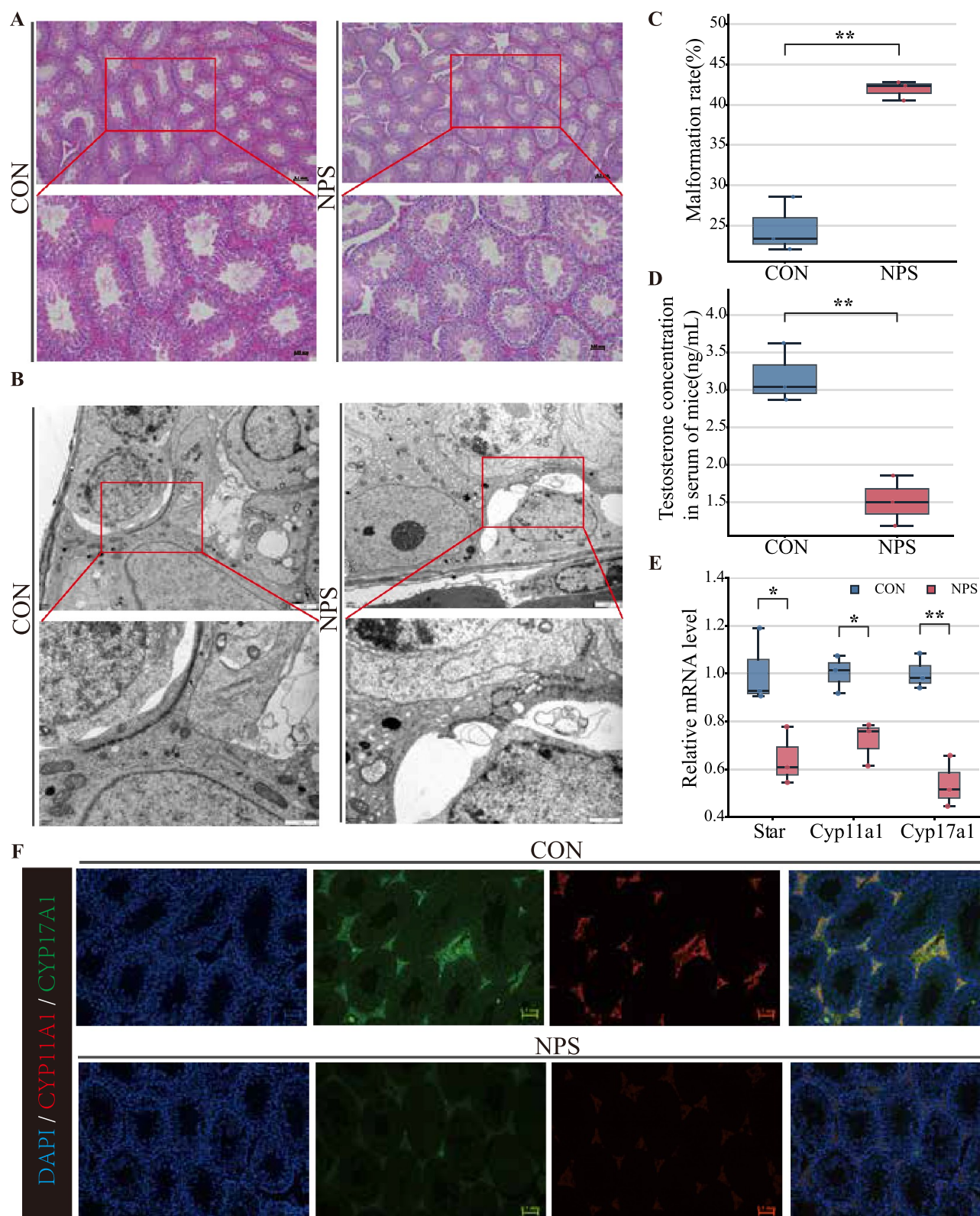


Figure 1. Oral exposure to 100 nm PS-NPs is associated with testicular injury and impaired reproductive function in male mice. (A) Representative H&E-stained testicular sections. Scale bars, 0.1 and 0.05 mm. (B) Representative transmission electron microscopy images of testicular ultrastructure. Scale bars, 2 and 1 μ m. (C) Sperm malformation rate. (D) Serum testosterone concentration. (E) Relative mRNA levels of *Star*, *Cyp11a1*, and *Cyp17a1* in testicular tissue. (F) Immunofluorescence staining of CYP11A1 and CYP17A1 in testicular tissue. $n = 3$ for panels C–E. Panels C–E were analyzed using unpaired two-tailed Student's t tests. * $P < 0.05$, ** $P < 0.01$.

292 Compared with CON mice, NPS mice exhibited a significantly
293 lower testis index (Figure S1B). Gross examination also showed

apparent differences in the size of the testes and epididymides 294
between the two groups (Figure S1C). Testicular histological 295

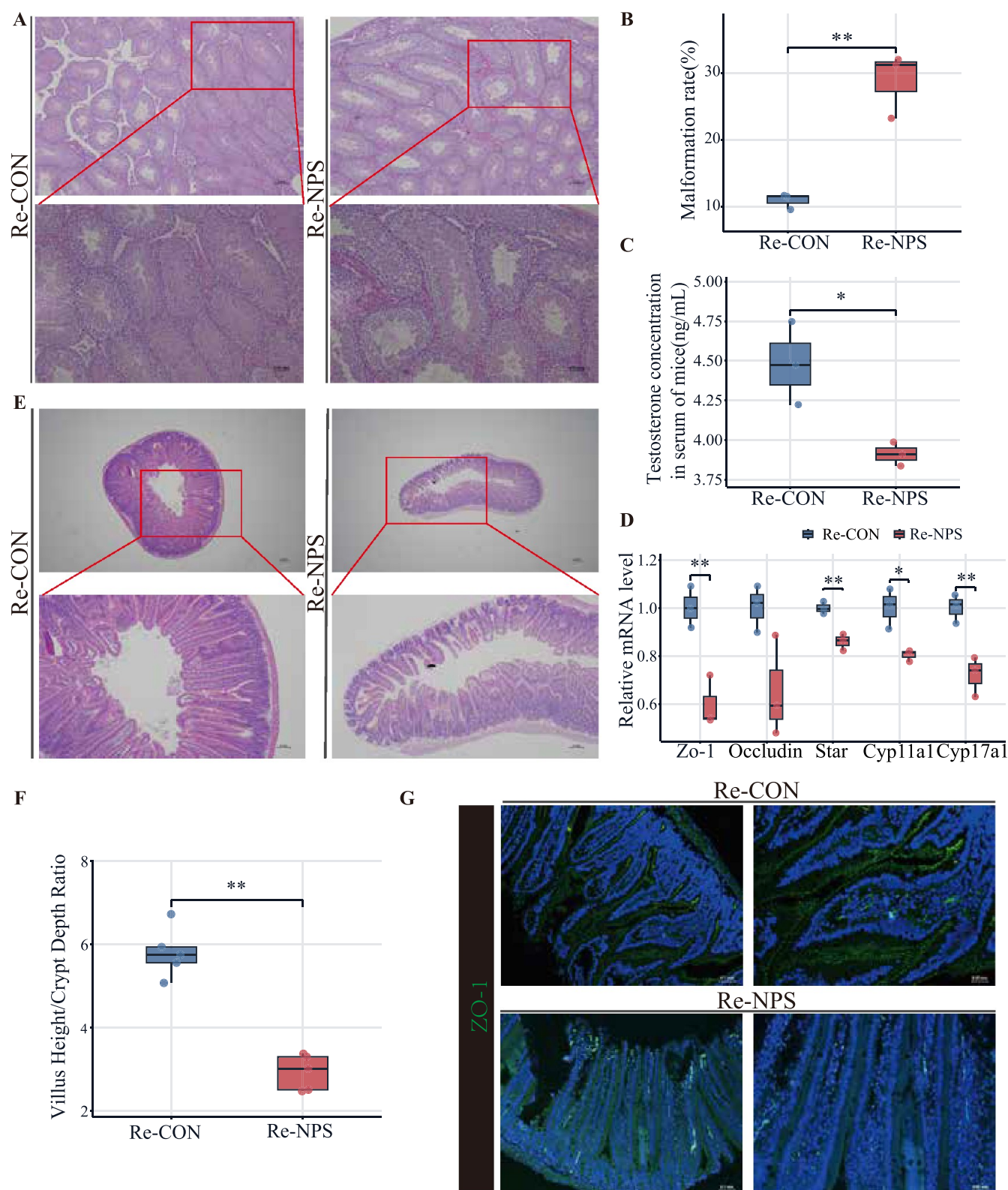


Figure 2. Recipients of fecal material from PS-NP-exposed donors exhibit testicular and intestinal alterations. (A) Representative H&E-stained testicular sections. Scale bars, 0.1 mm and 0.05 mm. (B) Sperm malformation rate. (C) Serum testosterone concentration. (D) Relative mRNA levels of *Zo-1*, *Occludin*, *Star*, *Cyp11a1*, and *Cyp17a1* in recipient mice. (E) Representative H&E-stained intestinal sections. Scale bars, 0.2 mm and 0.1 mm. (F) Villus height-to-crypt depth ratio. (G) Immunofluorescence staining of ZO-1 in intestinal tissue. Scale bars, 0.1 and 0.05 mm. $n = 3$ for panels B–D and $n = 6$ for panel F. Panels B–D and F were analyzed using unpaired two-tailed Student's t tests. * $P < 0.05$, ** $P < 0.01$.

examination revealed disorganized seminiferous tubules, an apparent reduction in spermatogenic cell layers, and loosening

of the seminiferous epithelium in NPS mice (Figure 1A). Transmission electron microscopy further showed widened

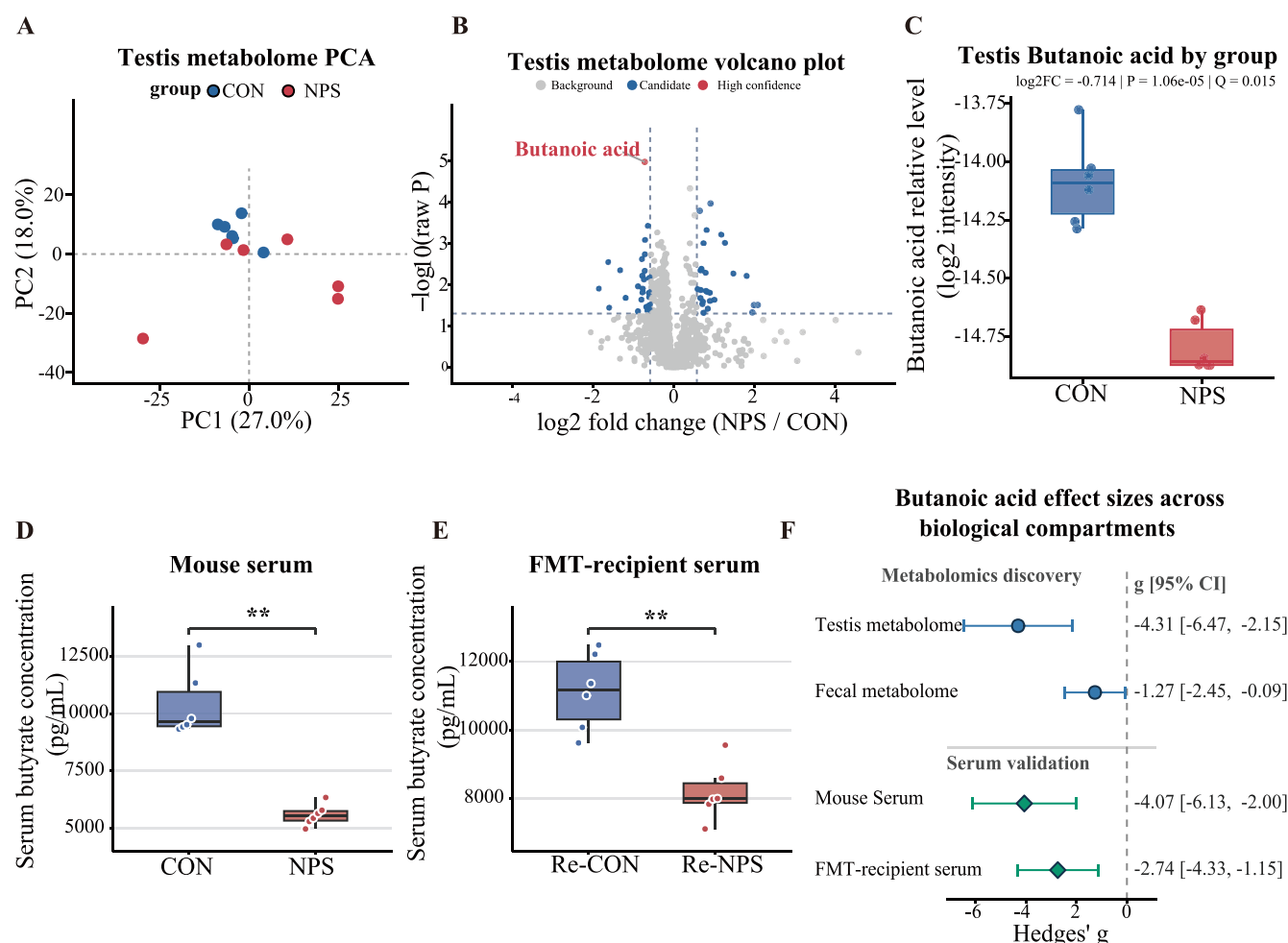


Figure 3. PS-NP exposure reduces testicular butanoic acid and circulating butyrate in mice. (A) PCA of the testicular metabolome. (B) Volcano plot showing background features (gray), candidate metabolites (blue; VIP ≥ 1 , raw $P < 0.05$, and $|\log_2 \text{FC}| > 0.58$), and high-confidence metabolites (red; VIP ≥ 1 , BH-adjusted $Q < 0.05$, and $|\log_2 \text{FC}| > 0.58$). (C) Relative testicular butanoic acid levels. (D, E) Serum butyrate concentrations in CON versus NPS mice and Re-CON versus Re-NPS mice, respectively. (F) Hedges' g with 95% confidence intervals across testicular, fecal, serum, and FMT-recipient serum data sets; negative values indicate lower levels in NPS or Re-NPS mice. In panels C–E, each dot represents one biological replicate ($n = 6$ per group). Panel C was analyzed using Welch's t test, whereas panels D and E were analyzed using unpaired two-tailed Student's t tests. * $P < 0.05$, ** $P < 0.01$.

intercellular spaces, less distinct cell–cell contacts and junctional regions, and cytoplasmic vacuolization in the testes of NPS mice (Figure 1B). Representative sperm images showed more morphologically abnormal spermatozoa in NPS mice (Figure S1D), consistent with the significantly increased sperm malformation rate (Figure 1C). Serum testosterone concentrations were significantly lower in NPS mice than in CON mice (Figure 1D). In parallel, the testicular mRNA levels of *Star*, *Cyp11a1*, and *Cyp17a1* were significantly decreased (Figure 1E). Immunofluorescence staining further showed weaker CYP11A1 and CYP17A1 signals in the testes of NPS mice (Figure 1F), together with weaker StAR and ZO-1 staining (Figure S1E). These qualitative observations were consistent with the RT-qPCR and ultrastructural findings.

Given the oral route of exposure, intestinal morphology and barrier-related indicators were also evaluated. Histological examination showed villus injury in NPS mice (Figure S2A), accompanied by a significantly lower villus height-to-crypt depth ratio (Figure S2B). Serum LPS concentrations were significantly elevated in NPS mice (Figure S2C). Representative immunofluorescence images also showed visually weaker E-cadherin and

ZO-1 staining in the intestinal epithelium of NPS mice (Figure S2D,E).

Collectively, under the present experimental conditions, PS-NP-exposed mice exhibited concurrent testicular injury, reproductive alterations, and intestinal barrier-related abnormalities. These observations provided the phenotypic basis for subsequent investigation of exposure-associated gut–testis metabolic alterations.

Recipients of Fecal Material from PS-NP-Exposed Donors Exhibit Testicular and Intestinal Alterations

To determine whether recipient phenotypes differed according to the source of the transplanted fecal material, all recipient mice underwent the same antibiotic pretreatment and transplantation schedule. Following antibiotic pretreatment, mice received fecal material from either CON or NPS donors, generating the Re-CON and Re-NPS groups, respectively (Figure S3A). Gross examination indicated that the testes and epididymides of Re-NPS mice appeared smaller than those of Re-CON mice (Figure S3B), and the testis index was significantly lower in the Re-NPS group (Figure S3C). Histological examination of the testes revealed disorganized seminiferous tubules and fewer spermato-

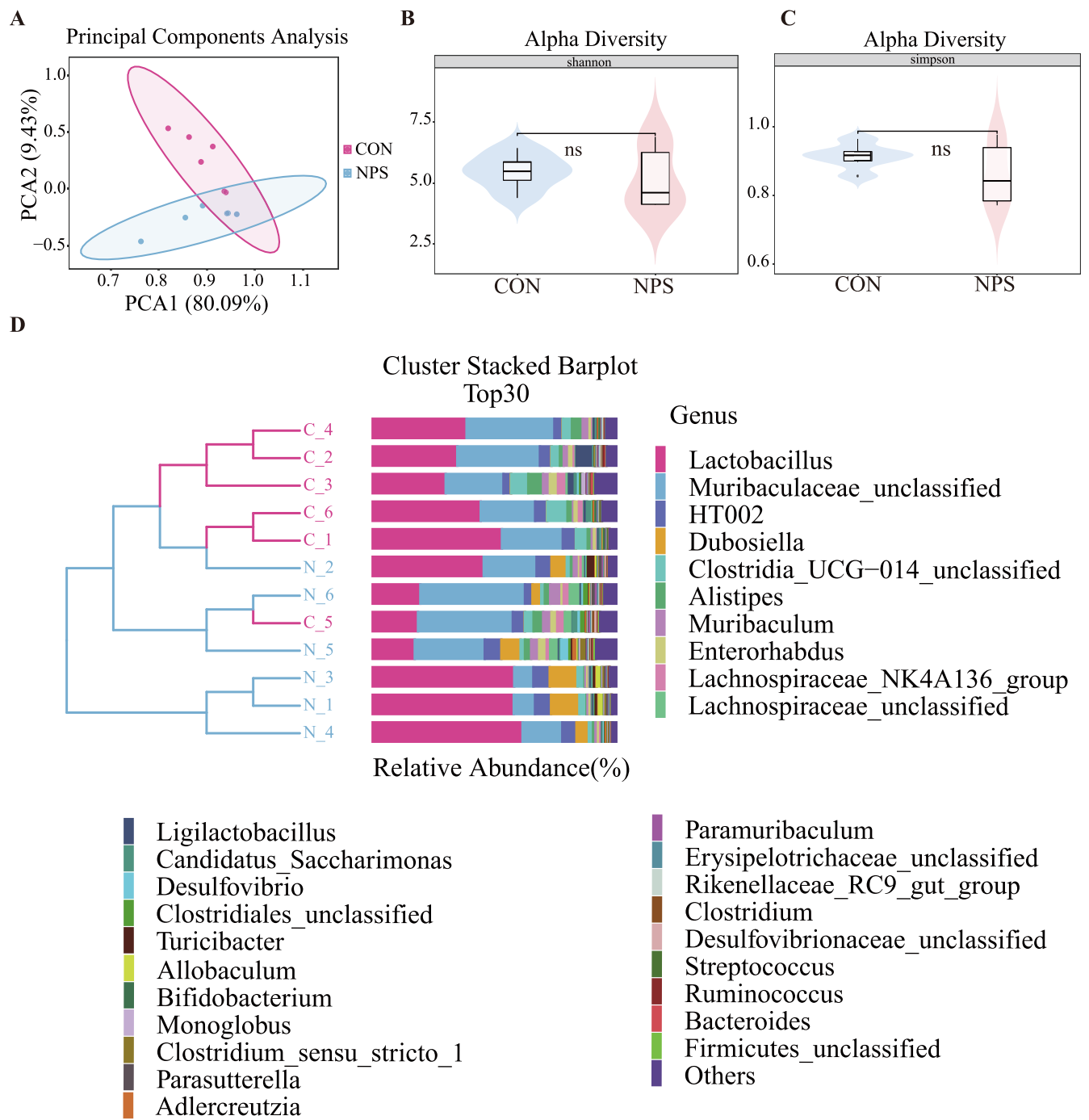


Figure 4. PS-NP exposure is associated with gut microbiota remodeling. (A) Principal component analysis of fecal microbial community structure in CON and NPS mice ($n = 6$ per group). (B, C) α -diversity analysis based on the Shannon and Simpson indices. (D) Hierarchical clustering and stacked bar plot showing the relative abundance of the top 30 genera. Differences in α -diversity indices between groups were evaluated using two-sided Mann–Whitney U tests.

genic cell layers in Re-NPS mice (Figure 2A). Consistent with these structural alterations, the sperm malformation rate was significantly higher in Re-NPS mice than in Re-CON mice (Figure 2B). Representative images of sperm morphology are shown in Figure S3D. Serum testosterone concentrations were also significantly lower in Re-NPS mice (Figure 2C), accompanied by reduced testicular mRNA expression of the steroidogenesis-related genes *Star*, *Cyp11a1*, and *Cyp17a1* (Figure 2D).

In the intestine, *Zo-1* mRNA expression was significantly lower in Re-NPS mice, whereas *Occludin* expression showed a nonsignificant downward trend (Figure 2D). Histological examination further revealed intestinal villus injury in Re-NPS mice (Figure 2E), together with a significantly reduced villus height-to-crypt depth ratio (Figure 2F). Immunofluorescence staining also showed weaker ZO-1 signals in intestinal tissue of Re-NPS mice (Figure 2G).

Collectively, under the same antibiotic-pretreated background, recipients of fecal material from NPS donors exhibited

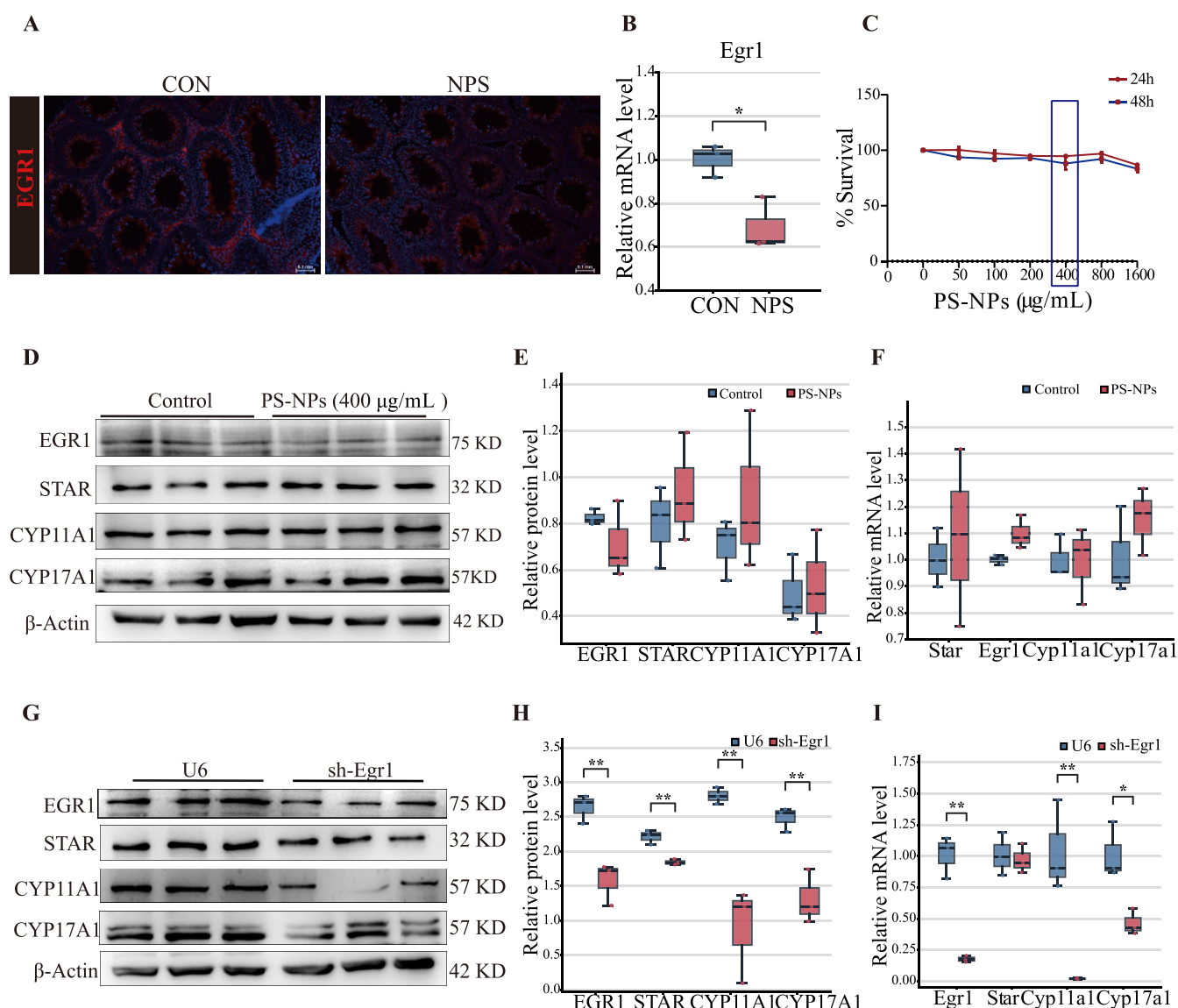


Figure 5. EGR1 is prioritized as a candidate regulator of Leydig cell steroidogenesis-related marker expression. (A) Immunofluorescence staining of EGR1 in testicular tissue from CON and NPS mice. Scale bar, 0.1 mm. (B) Relative *Egr1* mRNA level in testicular tissue. (C) Viability of TM3 Leydig-like cells after PS-NP exposure at the indicated concentrations and time points. (D) Representative Western blot images of EGR1, STAR, CYP11A1, and CYP17A1 in TM3 cells treated with PS-NPs (400 µg/mL). (E) Quantification of protein levels shown in panel D. (F) Relative mRNA levels of *Star*, *Egr1*, *Cyp11a1*, and *Cyp17a1* in TM3 cells after PS-NP exposure. (G) Representative Western blot images in U6 and sh-*Egr1* TM3 cells. (H) Quantification of protein levels shown in panel G. (I) Relative mRNA levels of *Egr1*, *Star*, *Cyp11a1*, and *Cyp17a1* in U6 and sh-*Egr1* TM3 cells. Data ($n = 3$ per group) were analyzed using unpaired two-tailed Student's *t*-tests. * $P < 0.05$, ** $P < 0.01$.

alterations in testicular structure, sperm morphology, steroidogenesis-related indicators, and intestinal barrier integrity compared with recipients of CON-donor fecal material. These recipient phenotypes were associated with donor fecal source and partially resembled those observed after direct PS-NP exposure.

Reduced Relative Testicular Butanoic Acid Levels and Serum Butyrate Concentrations Following PS-NP Exposure

PCA showed partial separation between CON and NPS mice in both the testicular and fecal metabolomic profiles (Figure 3A and Figure S5A, respectively), suggesting group-associated differences in global metabolite patterns. OPLS-DA score plots also showed discrimination between the two groups, and permutation tests were performed to assess model performance (Figure S4A–D).

In the testicular metabolome, 52 metabolites met the prespecified screening criteria (Figure 3B). Among these candidates, butanoic acid was the only metabolite that remained significant after Benjamini–Hochberg correction, with a lower relative level in NPS mice than in CON mice ($\log_2\text{FC} = -0.714$, $P = 1.06 \times 10^{-5}$, $Q = 0.015$; Figure 3C). In the fecal metabolome, 420 metabolites met the prespecified screening criteria (Figure S5B). The relative level of fecal butanoic acid was nominally lower in NPS mice but did not remain significant after multiple-testing correction ($\log_2\text{FC} = -1.308$, $P = 0.0386$, $Q = 0.273$; Figure S5C). Serum butyrate concentrations were significantly lower in NPS mice than in CON mice (Figure 3D). Serum butyrate concentrations were also significantly lower in Re-NPS recipients than in Re-CON recipients (Figure 3E), indicating that circulating butyrate differed according to the source of the

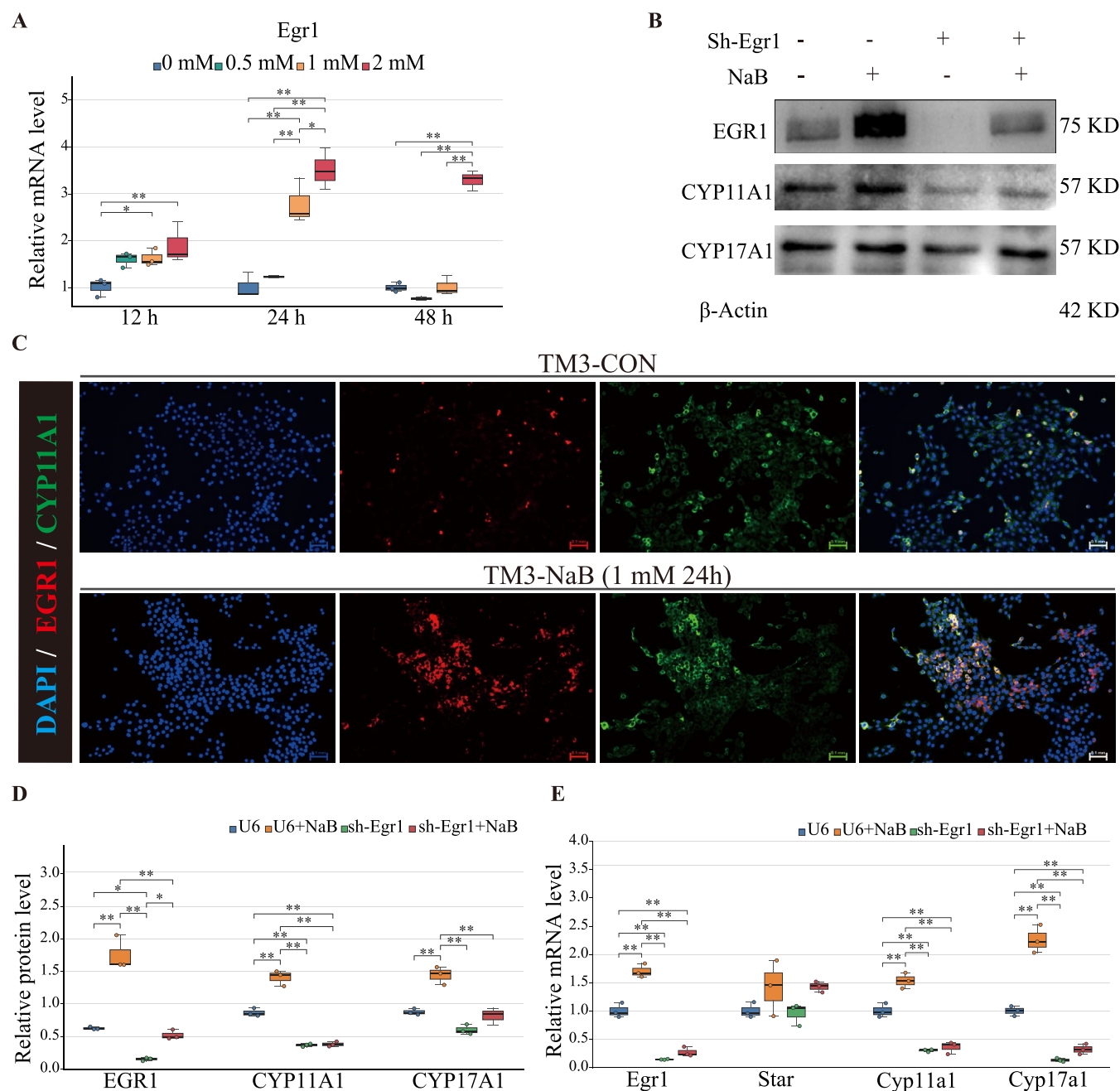


Figure 6. NaB promotes EGR1-associated steroidogenesis-related marker expression in TM3 Leydig-like cells. (A) Relative *Egr1* mRNA levels following NaB treatment. (B) Representative Western blots of EGR1, CYP11A1, and CYP17A1 in U6, U6 + NaB, sh-*Egr1*, and sh-*Egr1* + NaB cells. (C) Immunofluorescence staining of EGR1 and CYP11A1 in untreated and NaB-treated TM3 cells (1 mM, 24 h). Scale bar, 300 μ m. (D) Quantification of the protein levels shown in panel B. (E) Relative mRNA levels of *Egr1*, *Star*, *Cyp11a1*, and *Cyp17a1* in the indicated groups. U6 denotes the control-vector group. Data ($n = 3$ per group) were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$.

transplanted fecal material within the common antibiotic-pretreated background.

Standardized effect-size estimates were negative for testicular butanoic acid relative level, fecal butanoic acid relative level, serum butyrate concentration in PS-NP-exposed mice, and serum butyrate concentration in FMT recipients, with all 95% confidence intervals below zero (Figure 3F). Collectively, the FDR-significant reduction in testicular butanoic acid relative level, the nominal reduction in fecal butanoic acid relative level, and the concordant decreases in serum butyrate concentrations

provided the rationale for prioritizing the butyrate signal for subsequent functional evaluation using NaB.

PS-NP Exposure Is Associated with Changes in Gut Microbial Community Composition

Fecal 16S rRNA gene sequencing was performed to characterize gut microbial alterations associated with PS-NP exposure. PCA showed partial separation between the microbial communities of CON and NPS mice (Figure 4A), suggesting group-associated differences in overall community composition. In contrast, neither the Shannon nor the Simpson index differed

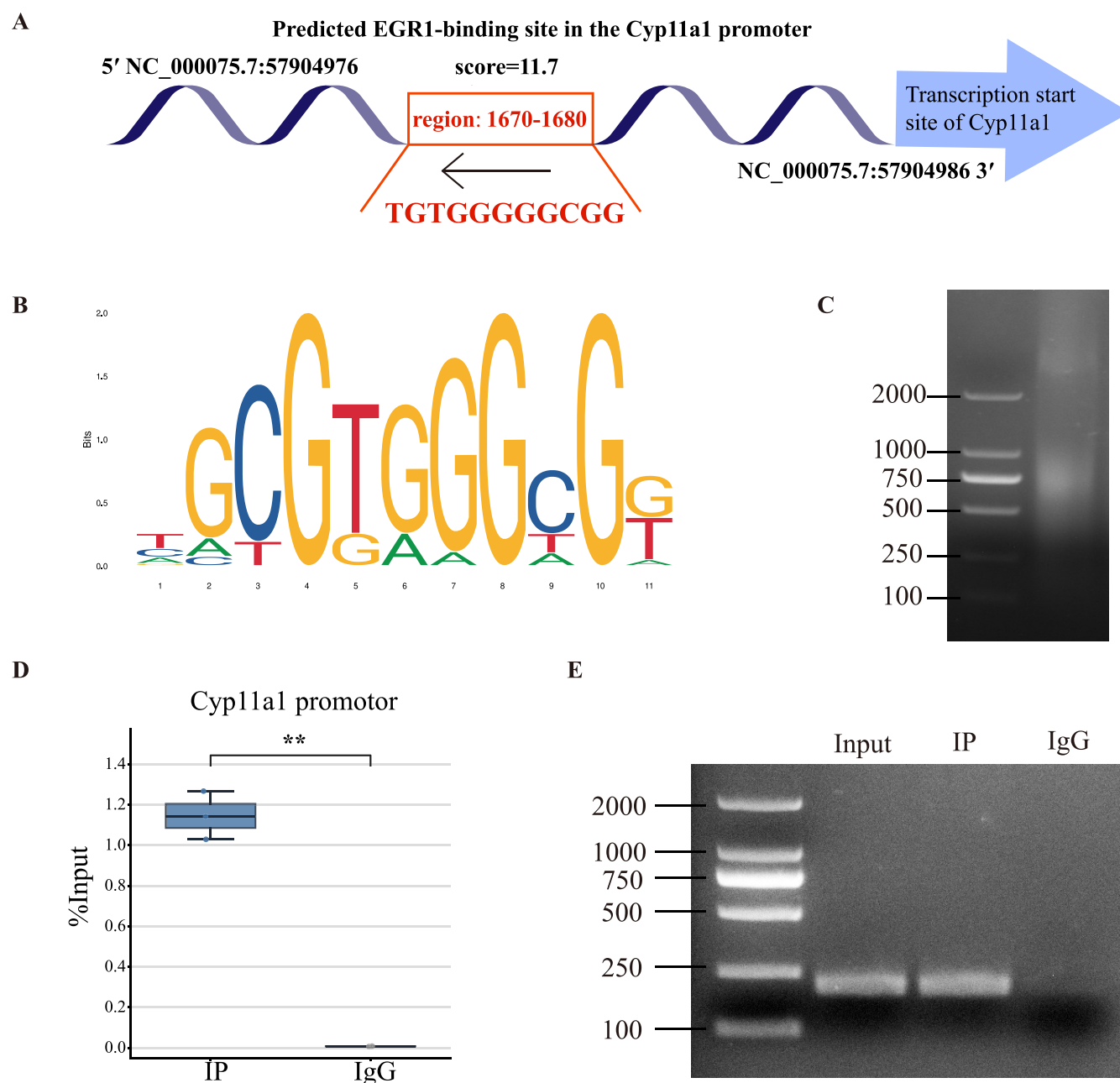


Figure 7. EGR1 enrichment at a predicted binding region of the *Cyp11a1* promoter. (A) Schematic diagram of the predicted EGR1-binding site in the *Cyp11a1* promoter, showing the predicted motif position and score. (B) Sequence logo of the predicted EGR1-binding motif. (C) Agarose gel electrophoresis showing chromatin shearing efficiency before ChIP analysis. (D) ChIP-qPCR analysis of EGR1 enrichment at the predicted *Cyp11a1* promoter region, shown as % input, with IgG used as a negative control. (E) Agarose gel electrophoresis of ChIP-PCR products from the Input, EGR1-IP, and IgG groups. Data ($n = 3$ per group) were analyzed using paired two-tailed Student's t -test. $*P < 0.05$, $**P < 0.01$.

significantly between the groups (Figure 4B,C), indicating no detectable difference in α diversity.

Genus-level profiling of the 30 most abundant taxa showed substantial interindividual variation as well as group-associated differences in relative abundance patterns (Figure 4D). Hierarchical clustering indicated that several samples with similar taxonomic profiles clustered together, although the CON and NPS samples were not completely separated.

LEfSe analysis further identified group-associated differences across multiple taxonomic levels (Figure S7A,B). At the family level, Erysipelotrichaceae, Clostridiaceae, and Sutterellaceae were relatively enriched in NPS mice. At the genus level,

Dubosiella, *Allobaculum*, *Turicibacter*, *Clostridium sensu stricto* 1, and *Parasutterella* were relatively enriched in NPS mice. In comparison, genus-level features assigned to *Streptococcus*, *Paraprevotella*, *Enterorhabdus*, *Alistipes*, and *Candidatus Saccharimonas* were relatively enriched in CON mice.

Collectively, these findings indicate that PS-NP exposure was associated with differences in fecal microbial community composition and the relative enrichment of specific bacterial taxa.

EGR1 Is Prioritized as a Candidate Regulator of Leydig Cell Steroidogenesis-Related Marker Expression

Given the reductions in serum testosterone concentration and testicular expression of steroidogenesis-related genes observed in NPS mice, we next examined the transcriptional features of Leydig cells, the principal testosterone-producing cells in the testis. Exploratory scRNA-seq followed by unsupervised clustering and marker-based annotation identified the major testicular cell populations, including spermatogonia, spermatocytes, round and elongating spermatids, Sertoli cells, Leydig cells, fibroblasts, and macrophages (Figure S6A,B).

Because the scRNA-seq data set comprised one mouse per condition, individual cells were not treated as independent biological replicates, and the data were used for cell-type characterization and candidate prioritization rather than population-level inference. Subsequent descriptive analyses focused on the Leydig cell profiles. Pathway analysis of genes showing lower expression in the NPS Leydig cell profile highlighted cholesterol metabolism and steroidogenesis-related pathways (Figure S6C). Regulatory network analysis further prioritized EGR1 as a candidate regulator associated with the Leydig cell transcriptional profile (Figure S6D), providing a cell-type-specific rationale for its subsequent evaluation in independent tissue samples and TM3 cells.

EGR1 expression was subsequently examined in independent biological samples. Testicular sections from NPS mice showed weaker EGR1 immunofluorescence staining than those from CON mice, and testicular *Egr1* mRNA expression was significantly reduced following PS-NP exposure (Figure 5A,B). To determine whether direct exposure of Leydig-like cells could reproduce these changes, TM3 cells were treated with PS-NPs. Exposure to 400 $\mu\text{g/mL}$ PS-NPs did not significantly affect cell viability or alter the protein or mRNA expression of EGR1, StAR, CYP11A1, or CYP17A1 under the tested conditions (Figure 5C–F).

The potential involvement of EGR1 in steroidogenesis-related marker expression was further evaluated using stable *Egr1*-knockdown TM3 cells (Figure S8A,B). *Egr1* knockdown markedly reduced EGR1 protein expression and was accompanied by lower StAR, CYP11A1, and CYP17A1 protein levels (Figure 5G,H). At the mRNA level, *Egr1*, *Cyp11a1*, and *Cyp17a1* expression was significantly reduced, whereas *Star* expression was not significantly changed (Figure S1).

Collectively, the tissue observations and cell-based loss-of-function experiments support EGR1 as a candidate regulator of Leydig cell steroidogenesis-related marker expression. Direct PS-NP exposure did not reproduce the *in vivo* suppression of EGR1 and steroidogenic markers in TM3 cells under the tested conditions, whereas *Egr1* knockdown reduced multiple steroidogenic markers.

NaB Promotes EGR1-Associated Steroidogenesis-Related Marker Expression in TM3 Cells

Because PS-NP exposure was associated with a reduced relative level of testicular butanoic acid and lower serum butyrate concentrations, and EGR1 was prioritized as a candidate regulator of Leydig cell steroidogenesis-related marker expression, we next examined the effects of NaB in TM3 cells. *Egr1* mRNA expression varied across NaB concentrations and treatment durations. Because 1 mM NaB was the lowest concentration that produced robust *Egr1* induction at 24 h, this condition was selected for subsequent experiments (Figure 6A). Under this condition, NaB-treated cells showed stronger EGR1

and CYP11A1 immunofluorescence signals than untreated cells (Figure 6C).

To evaluate the involvement of EGR1 in the NaB response, stable control-vector (U6) and *Egr1*-knockdown TM3 cells were treated with vehicle or NaB. In U6 cells, NaB significantly increased EGR1, CYP11A1, and CYP17A1 at both the protein and transcript levels (all Tukey-adjusted $P < 0.01$; Figure 6B,D,E). Compared with U6 + NaB cells, sh-*Egr1* + NaB cells showed significantly lower levels of EGR1, CYP11A1, and CYP17A1 protein and lower *Egr1*, *Cyp11a1*, and *Cyp17a1* mRNA expression. Nevertheless, NaB induced a smaller increase in EGR1 protein in sh-*Egr1* cells. No significant differences in *Star* mRNA expression were detected among the indicated groups (Figure 6E).

Collectively, these findings show that NaB enhances EGR1 and multiple steroidogenesis-related markers in TM3 cells, whereas these responses are less pronounced in *Egr1*-knockdown cells, supporting the involvement of EGR1 in NaB-responsive steroidogenesis-related marker expression.

EGR1 Is Enriched at a Predicted Binding Region of the *Cyp11a1* Promoter

To further examine the regulatory relationship between EGR1 and *Cyp11a1*, potential EGR1-binding sites in the *Cyp11a1* promoter region were predicted by motif analysis. A putative EGR1-binding motif was identified within the analyzed *Cyp11a1* promoter sequence, with the predicted motif located approximately -1680 to -1670 bp upstream of the transcription start site and showing a prediction score of 11.7 (Figure 7A,B). Before ChIP-qPCR analysis, chromatin shearing efficiency was evaluated by agarose gel electrophoresis. The sheared DNA fragments were mainly distributed within the expected range of approximately 300–1000 bp, indicating that the chromatin preparation was suitable for subsequent ChIP analysis (Figure 7C).

ChIP-qPCR was then performed to determine whether EGR1 was enriched at the predicted *Cyp11a1* promoter region. Compared with the IgG control, the EGR1 immunoprecipitation group showed significant enrichment of the *Cyp11a1* promoter fragment (Figure 7D). Agarose gel electrophoresis of the ChIP-PCR products further showed a detectable promoter fragment in the Input and EGR1-IP samples, whereas only a weak or undetectable band was observed in the IgG control (Figure 7E). These results demonstrate EGR1 enrichment at the examined motif-containing region of the *Cyp11a1* promoter and support a potential EGR1–*Cyp11a1* regulatory relationship that requires further functional validation.

In Vivo Sodium Butyrate Supplementation Partially Attenuates PS-NP-Induced Testicular Injury and Impaired Steroidogenesis in Male Mice

To evaluate the functional relevance of butyrate-related metabolic disturbance in PS-NP-induced reproductive toxicity, male ICR mice were assigned to the CON, NPS, NaB, and NPS + NaB groups and treated for 28 consecutive days (Figure S9A). Compared with CON mice, NPS mice exhibited a significantly lower testis index, whereas NaB supplementation significantly increased the testis index in NPS-exposed mice (Figure S9B). Representative sperm images showed more morphologically abnormal spermatozoa in the NPS group and comparatively fewer abnormalities in the NPS + NaB group (Figure S9C). Testicular histological examination revealed disorganized seminiferous tubules, reduced spermatogenic cell layers, and loosening of the seminiferous epithelium in NPS mice, whereas

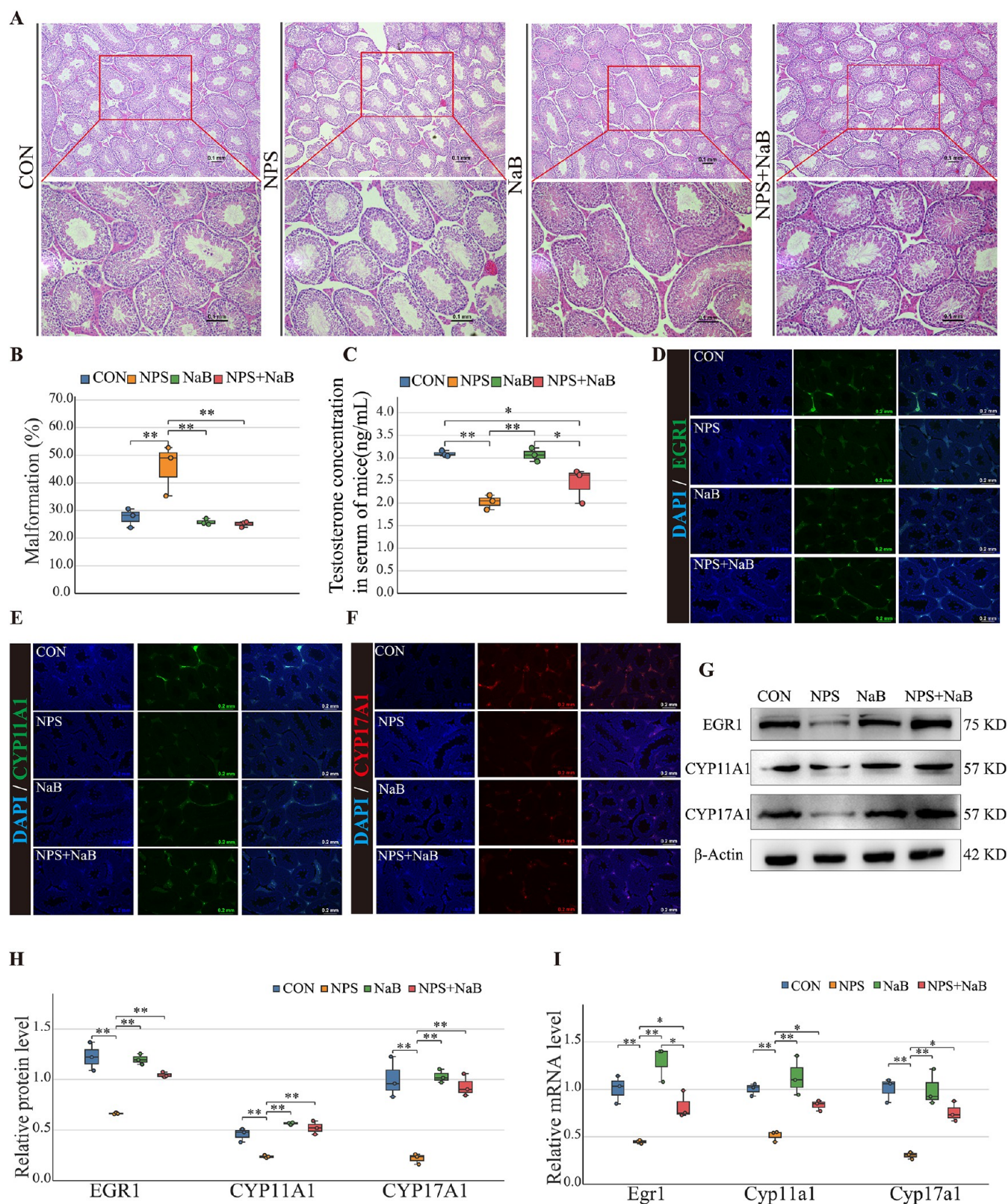


Figure 8. In vivo NaB supplementation partially improved reproductive and steroidogenesis-related outcomes in PS-NP-exposed mice (A) Representative H&E-stained testicular sections. Scale bar, 0.1 mm. (B) Sperm malformation rate. (C) Serum testosterone concentration. (D–F) Immunofluorescence staining of EGR1, CYP11A1, and CYP17A1 in testicular tissue. Scale bars, 0.2 mm. (G) Representative Western blot images of EGR1, CYP11A1, and CYP17A1 in testicular tissue. (H) Quantification of protein levels shown in panel G. (I) Relative mRNA levels of *Egr1*, *Cyp11a1*, and *Cyp17a1* in testicular tissue. Data ($n = 3$ per group) were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. $*P < 0.05$, $**P < 0.01$.

the NPS + NaB group showed comparatively preserved seminiferous tubule architecture (Figure 8A). Consistent with these observations, NaB supplementation significantly reduced the sperm malformation rate and increased the serum testosterone concentration in NPS-exposed mice (Figure 8B,C). Immunofluorescence staining showed weaker EGR1, CYP11A1, and CYP17A1 signals in the testes of NPS mice than in CON mice, whereas stronger signals were observed in the NPS+NaB group than in the NPS group (Figure 8D–F). Western blotting and protein quantification similarly showed that EGR1, CYP11A1, and CYP17A1 protein levels were decreased following PS-NP exposure and significantly increased following NaB supplementation (Figure 8G,H). At the transcriptional level, testicular *Egr1*, *Cyp11a1*, and *Cyp17a1* mRNA levels were significantly lower in NPS mice than in CON mice but significantly higher in the NPS + NaB group than in the NPS group, indicating partial recovery following NaB supplementation (Figure 8I). Comparisons among the four groups were performed using one-way ANOVA followed by Tukey's multiple-comparisons test.

Collectively, under the present experimental conditions, NaB supplementation partially improved testicular histology and reproductive outcomes and restored steroidogenesis-related marker expression in PS-NP-exposed mice. Together with the observed reduction in butyrate levels following PS-NP exposure, these findings provide functional evidence that reduced butyrate availability may contribute to PS-NP-induced male reproductive toxicity.

DISCUSSION

PS-NPs are increasingly recognized as potential male reproductive toxicants.^{4,35} However, how oral PS-NP exposure disrupts testicular steroidogenesis, particularly through gut-associated alterations, remains incompletely understood. A key finding of the present study was that the suppression of EGR1 and steroidogenesis-related markers observed in vivo was not reproduced by direct PS-NP exposure in TM3 cells under the tested conditions. In contrast, the FMT, metabolomic, and NaB supplementation results collectively pointed to the involvement of transferable gut-associated factors and altered butyrate availability. These findings suggest that gut-associated metabolic alterations may connect oral PS-NP exposure with impaired testicular steroidogenesis, with EGR1–*Cyp11a1* representing a putative downstream regulatory relationship.

Leydig cells are the principal testosterone-producing cells in the testis and are essential for male reproductive function.^{21,23} In the present study, PS-NP exposure reduced serum testosterone levels and testicular expression of steroidogenesis-related markers, including *Star*, *Cyp11a1*, and *Cyp17a1*, consistent with impaired steroidogenic function. Because the scRNA-seq analysis included only one mouse per condition, it was used solely as an exploratory tool to identify candidate cell types and regulators for subsequent investigation, rather than to infer exposure-induced differences in gene expression or cell-type abundance. Subsequent analyses of individual animal samples showed reduced testicular *Egr1* expression, lower expression of steroidogenesis-related genes and proteins, and weaker immunofluorescence signals for EGR1, CYP11A1, CYP17A1, and StAR in PS-NP-exposed mice. Collectively, these animal-level findings support impaired testicular steroidogenesis and identify Leydig cells as a plausible cell population affected by PS-NP exposure.

The TM3-cell exposure and FMT experiments provided complementary information on direct cellular responses and potentially transferable gut-associated factors. Direct exposure of TM3 Leydig-like cells to PS-NPs did not significantly affect cell viability or alter EGR1 or steroidogenesis-related marker expression at either the mRNA or protein level under the tested conditions. Thus, the suppression of these markers observed in vivo was not reproduced by direct PS-NP exposure in this cellular model, although this finding does not exclude direct effects under other exposure conditions or in vivo. In the FMT experiment, all recipient mice underwent the same antibiotic pretreatment and transplantation schedule and received donor-specific, unpooled fecal preparations from either control or PS-NP-exposed donors.³⁶ Recipients of fecal material from PS-NP-exposed donors exhibited reproductive, steroidogenesis-related, and intestinal alterations relative to recipients of control-donor fecal material. Together, these findings suggest that transferable gut-associated factors may contribute to the intestinal and testicular alterations associated with oral PS-NP exposure.

Metabolomic analyses further identified a butyrate-related metabolic disturbance associated with PS-NP exposure. This prioritization is biologically plausible because microbiota-derived butyrate can influence intestinal homeostasis and gene regulation, while intestinal butyrate-related changes have been linked to circulating metabolism and biological effects in distal organs.^{37–40} The relative level of butanoic acid in testicular tissue was significantly reduced after FDR correction, whereas the decrease in fecal samples was directionally consistent but did not remain significant after FDR correction. Serum butyrate concentrations were also lower in PS-NP-exposed mice and in recipients of fecal material from PS-NP-exposed donors, providing a circulating measurement consistent with the testicular metabolomic finding. Together, the significant testicular reduction after FDR correction, the concordant serum changes, and the directional fecal trend supported the prioritization of butyrate for subsequent functional evaluation.

The functional relevance of this butyrate-related metabolic disturbance was subsequently evaluated by supplementing PS-NP-exposed mice with NaB in the drinking water. Compared with PS-NP exposure alone, NaB supplementation improved testicular histological injury and sperm abnormalities and partially restored serum testosterone concentrations and testicular expression of EGR1, CYP11A1, and CYP17A1. These results indicate that exogenous NaB counteracted multiple reproductive and steroidogenesis-related alterations associated with PS-NP exposure. Consistent with our findings, recent mouse studies showed that NaB enhanced testosterone synthesis and improved spermatogenic parameters under hyperuricemic conditions,¹⁸ while ameliorating spermatogenic histopathology and improving sperm quality and serum testosterone levels in a humanized FMT model of asthenozoospermia.⁴¹ Together with the reduced relative level of butanoic acid in testicular tissue and the lower serum butyrate concentrations, the attenuation observed following NaB supplementation provides in vivo functional support for a contribution of butyrate-related metabolic disturbance to PS-NP-associated testicular steroidogenic dysfunction.

The NaB-associated restoration of EGR1 expression in vivo prompted us to examine whether EGR1 participated in the NaB response in Leydig-like cells. NaB increased EGR1 and steroidogenesis-related marker expression in control-vector cells, whereas *Egr1* knockdown reduced basal EGR1 expression. Compared with U6+NaB cells, sh-*Egr1* + NaB cells showed

lower CYP11A1 and CYP17A1 expression at both the transcript and protein levels, indicating that the NaB-associated responses were less pronounced following *Egr1* knockdown. Motif prediction identified a putative EGR1-binding site in the *Cyp11a1* promoter, and ChIP–qPCR demonstrated EGR1 enrichment at the examined motif-containing region. Together, these findings are consistent with the involvement of EGR1 in the NaB response and support a putative EGR1–*Cyp11a1* regulatory relationship in TM3 cells.

Several limitations should be acknowledged. The scRNA-seq analysis included only one mouse per condition and was therefore used solely for exploratory candidate prioritization. The absence of an antibiotic-only control prevented independent assessment of the effects of antibiotic pretreatment in the FMT experiment. In addition, TM3 cells do not fully recapitulate primary Leydig-cell physiology, and ChIP–qPCR enrichment alone does not establish direct transcriptional regulation of *Cyp11a1* by EGR1. Because NaB has pleiotropic effects, its beneficial effects do not establish reduced butyrate availability as the sole mediator or resolve the upstream gut–testis signaling pathway. Finally, the single-dose design precluded dose–response assessment. Further studies using additional controls, primary cells, and multiple exposure doses are needed to validate the proposed mechanism.

In summary, oral PS-NP exposure induced intestinal and testicular abnormalities accompanied by gut microbiota remodeling and butyrate-related metabolic alterations. Under the shared antibiotic-conditioned setting, the FMT experiment identified donor-source-associated differences in recipient phenotypes. The NaB supplementation experiment provided in vivo functional support for a contribution of altered butyrate availability to impaired testicular steroidogenesis, while the TM3-cell experiments supported a putative NaB-responsive EGR1–*Cyp11a1* downstream regulatory mechanism. The upstream mechanisms linking PS-NP exposure to butyrate-related metabolic disturbance and the routes by which butyrate-related signals reach the testis remain to be determined. Nevertheless, these findings provide a testable framework for understanding how gut-associated metabolic disturbance may participate in PS-NP-associated male reproductive toxicity.

■ ASSOCIATED CONTENT

Data Availability Statement

Source data underlying the figures are provided with this paper. Raw sequencing and metabolomics data have been deposited in public repositories as described below. (1) 16S rRNA sequencing and single-cell RNA sequencing data have been deposited in the Genome Sequence Archive (GSA) of the National Genomics Data Center (NGDC), China National Center for Bioinformation (CNCB): 16S rRNA sequencing, Accession No. CRA030907; single-cell RNA sequencing, Accession No. CRA031513. (2) Non-targeted metabolomics data have been deposited in Metabolomics Workbench: fecal metabolome, Study ID ST005013; testicular metabolome, Study ID ST005012.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.6c10448>.

Additional experimental materials; oral exposure to 100-nm PS-NPs is associated with testicular and intestinal injury and impaired reproductive function in male mice (Figure S1); oral PS-NP exposure is associated with

intestinal morphological injury and barrier impairment in male mice (Figure S2); FMT from PS-NP-exposed donors partially recapitulates testicular and intestinal injury in recipient mice (Figure S3); OPLS-DA score plots and permutation tests of fecal and testicular metabolomic profiles (Figure S4); fecal metabolomic profiling of PS-NP-exposed mice (Figure S5); exploratory prioritization of EGR1 in Leydig cells by scRNA-seq (Figure S6); PS-NP exposure is associated with gut microbiota remodeling (Figure S7); construction and validation of stable *Egr1*-knockdown TM3 cells (Figure S8); experimental design and additional reproductive phenotypes following in vivo NaB supplementation (Figure S9) (PDF)

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Y.W. and L.F. contributed equally to this work. Conceptualization: Y.W. and L.F. Investigation: Y.W., K.Q., and W.C.

800 Visualization: Y.W., C.Y., and C.W. Writing—original draft: Y.W.
801 and J.Y. Writing—review and editing: E.A.K., L.G., N.L., M.L.,
802 and J.H.

803 Notes

804 The authors declare no competing financial interest.

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