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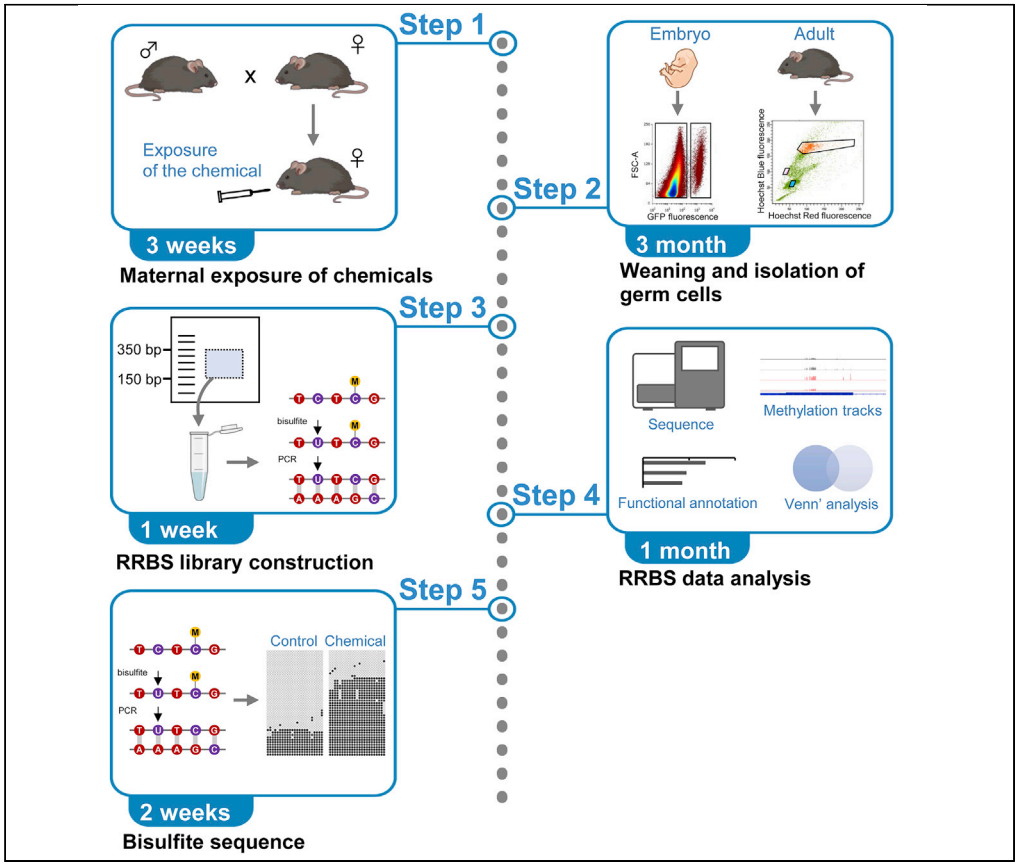

It is now recognized that maternal environmental factors, including chemical exposure and nutritional conditions, alter DNA methylation patterns in fetal germ cells, subsequently affecting germ cell development as well as offspring phenotypes. Here, we describe steps for detecting DNA methylation changes in mouse germ cells isolated from both embryonic and spermatogenic stages after maternal exposure to a chemical compound.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for maternal chemical exposure of mouse

Using flow cytometry to isolate fetal and adult germ cells after chemical exposure

Analysis of DNA methylation changes using RRBS and bisulfite sequencing

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Identification of spermatogenesis-associated changes in DNA methylation induced by maternal exposure to chemicals in male germ cells

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SUMMARY

It is now recognized that maternal environmental factors, including chemical exposure and nutritional conditions, alter DNA methylation patterns in fetal germ cells, subsequently affecting germ cell development as well as offspring phenotypes. Here, we describe steps for detecting DNA methylation changes in mouse germ cells isolated from both embryonic and spermatogenic stages after maternal exposure to a chemical compound.

For complete details on the use and execution of this protocol, please refer to Tando et al. (2021).[1]

BEFORE YOU BEGIN

The protocol below describes details of a method for detecting DNA methylation changes resulting from maternal chemical exposure in offspring fetal and adult mice (C57BL/6) germ cells. This protocol can be applied to various environmental exposure models, such as diet, with variations in the timing of exposure. To isolate germ cells from the embryonic stage, we used Oct4 (Pou5f1)-delta PE (proximal enhancer) -Green Fluorescent Protein (Oct4-GFP) transgenic mice,[2] but other germ cell–specific reporter transgenic lines as well as staining with germ cell–specific antibodies for cell sorting may also be applicable.

Institutional permissions

All animal experiments must be performed in accordance with the ethical guidelines of the institution. Animal protocols in this protocol were reviewed and approved by the Tohoku University Animal Studies Committee (approval number: 2019AcA-027-01).

Mating of males and females

Timing: 1 day

1. Select estrus females with red, swollen vulva from cohorts of C57BL/6J female mice.
   a. Place in the same cage with an Oct4-GFP transgenic male mouse and left 12–18 h at the night.

Note: If a vaginal plug is observed the next morning, noon on the day of mucus plug observation is defined as gestation day 0.5.

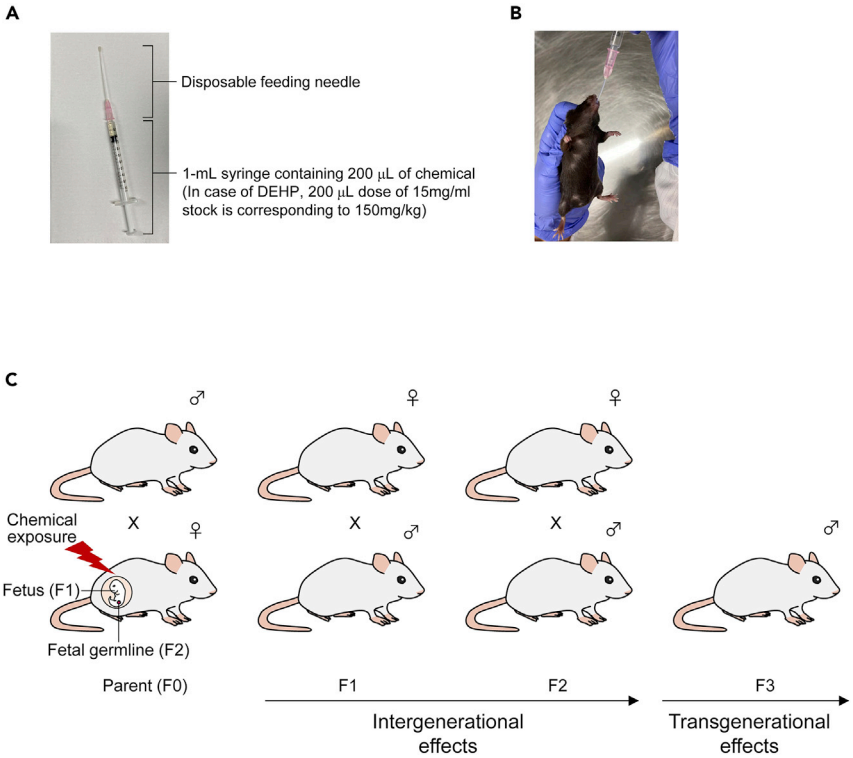

Figure 1. Oral administration of test chemical to mice

(A) Disposable feeding needle attached to a 1-mL syringe filled with chemical.

(B) Oral administration of the chemical to a mouse. Slowly insert the feeding needle into the esophagus from the animal's mouth. When slight resistance is felt, which means the feeding needle has reached the stomach, push out the contents of the syringe. In the case of C57BL/6J female mice at the age of 8–10 weeks, the length of the needle to be inserted is approximately 4 cm from the tip of the needle.

(C) Schemes of mice breeding. If the stress is applied to a pregnant female (F0), then the fetuses (F1) as well as their primordial germ cells, which give rise to F2 generation, are affected. Therefore influences by the stress in F1 and F2 are considered as intergenerational effects, and influences beyond F3 are transgenerational.

Exposing pregnant mice to an environmental stressor

Timing: variable, depending on the design of exposure period

Note: Because there are many different environmental stressors, including nutritional conditions and psychological stresses, this protocol focuses on chemical administration during pregnancy. The duration of administration could be the entire gestational period, a certain period of pregnancy, or periods including pre-pregnancy and lactation, depending on the experimental design. In addition, the method of administration, such as gavage or intraperitoneal injection, depends on the characteristics of the chemicals.

2. Pregnant mice are administered the chemical of interest for the desired duration of exposure according to your experimental design.

Note: In Tando et al.,[1] di(2-ethylhexyl) phthalate (DEHP) was administered to C57BL/6J female mice at 150 mg/kg once per day from gestational day 8 to day 18 via oral administration using a feeding needle (Figure 1). DEHP is diluted by corn oil from original stock. Diluted DEHP solution is kept at 15°C–25°C and used within a month. Corn oil was administrated as control. The dose of 150 mg/kg was determined with reference to a previous study.

Actually, we first administered pregnant mice with 300 mg/kg DEHP, which is the same dose as used in a previous study.[3] However, we obtained few pups under this condition, presumably reflecting embryonic lethality at this dose of DEHP. We then tested different doses of DEHP, and identified 150 mg/kg, at which we could obtain pups with previously reported germ cell abnormalities in F1 and F2 offspring and in F1 embryos.[4–6] Exposure of 150 mg/kg DEHP is higher than environmentally-relevant dose, and a previous report showed that administration of 500 mg/kg DEHP for 3 weeks to male mice increased the frequency of mutations in genomic DNA.[7] Although 150 mg/kg is lower than this dose, it may be important to verify whether DNA mutations occurs at 150 mg/kg in the maternally exposed mouse model.

Mouse breeding

Timing: at least 30 days (for step 3)

Note: In general, a pregnant female mouse exposed to the environmental stress is considered F0, and her litter pups are considered F1. Pups obtained by mating the F1 mice with unexposed mice are considered F2.

3. To isolate germ cells from offspring of the treated mother at adult stages, or to mate the mice, the pups are raised until the start of gametogenesis at least at about 8 weeks of age.[8]

Note: In Tando et al.,[1] F1 male mice were mated with unexposed C57BL/6J female mice at postnatal day (P) 60-P70 to produce F2 mice, and both F1 and F2 male mice were kept for isolation of germ cells at P100-P150.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
XL1-blue	Tando et al.[1]	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#11965-092
Fetal bovine serum	Biosera	Cat#FB-1380
Penicillin-Streptomycin Solution Hybri-Max	Sigma	Cat# P7539
10× trypsin/EDTA solution	Sigma	Cat#T4174
Gey's Balanced Salt Solution	Sigma	Cat#G9779
Collagenase type I	Sigma	Cat#C0130
DNase	Roche	Cat#11284932
Hoechst 33342	Thermo Fisher Scientific	Cat#H3570
Propidium iodide	BD	Cat#51-66211E
Proteinase K	NEB	Cat#P8107S
TE saturated phenol	Nippon Gene	Cat# 313-90091
Chloroform	FUJIFILM Wako	Cat# 038-02606
PureLink RNase A	Thermo Fisher Scientific	Cat# 12091021
Ethachinmate	Nippon Gene	Cat#312-01791
10× NEBuffer 2	NEB	Cat#B7002S
MspI	NEB	Cat#R0106S
Deoxynucleotide solution set	NEB	Cat#N0446S
Klenow Fragment (3′-5′ exo-)	NEB	Cat#M0212S
High Concentration T4 DNA Ligase	NEB	Cat#M0202M

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
10× T4 DNA Ligase Reaction Buffer	NEB	Cat#B0202S
KAPA HiFi HotStart Uracil+ Ready Mix (2×)	NIPPON Genetics	Cat#KK2801
NuSieve 3:1 agarose	Takara	Cat#50091
5× TBE	Nippon Gene	Cat#318-90041
Gel loading dye, purple (6×), no SDS	NEB	Cat#B7025S
100 bp DNA ladder	NEB	Cat#N3231
50 bp DNA ladder	NEB	Cat#N3236
Ethidium bromide	Sigma	Cat#E8751
Agentcourt AMpure XP	Beckman Coulter	Cat#A63880
EpiTaq HS	Takara	Cat#R110A
10× EpiTaq PCR Buffer (Mg^{2+} free)	Takara	Cat#SD3575
2.5 mM dNTPs	Takara	Cat#SD3576
25 mM $MgCl_2$	Takara	Cat#SD3577
rTaq DNA polymerase	TOYOBO	Cat# TAP-201
10× rTaq Buffer (Mg^{2+} free)	TOYOBO	Cat#TAP-2B
pGEM-T Easy Vector	Promega	Cat#A137A
2× Rapid Ligation Buffer	Promega	Cat#C671A
T4 DNA ligase	Promega	Cat#M180A
BD Difco Dehydrated Culture Media: LB Broth, Miller (Luria-Bertani)	BD	Cat#244620
Bacto-ager	BD	Cat#214010
Ampicillin	Sigma	Cat#A0166
Isopropyl-β-D-thiogalactopyranoside (IPTG)	FUJIFILM Wako	Cat#093-05011
5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-gal)	FUJIFILM Wako	Cat#021-07852
S.O.C. Medium	Thermo Fisher Scientific	Cat# 15544034
Big Dye terminator ver.1.1	Thermo Fisher Scientific	Cat#4337452
5× Sequencing buffer	Thermo Fisher Scientific	Cat#4336697
Critical commercial assays		
EZ DNA Methylation-Gold Kit	Zymo Research	Cat#D5005
Qiagen MinElute Gel Extraction Kit	Qiagen	Cat#28604
Qubit dsDNA HS Kit	Thermo Fisher Scientific	Cat#Q32851
D1000 Reagents	Agilent	Cat# 5067-5583
D1000 ScreenTape	Agilent	Cat#5067-5582
KAPA Library Quantification Kit	NIPPON Genetics	Cat#KK4824
Nucleospin Plasmid EasyPure	Macherey-Nagel	Cat#U0727Q
Experimental models: Organisms/strains		
Mouse: Oct4-delta PE-GFP TG male mice on a C57BL6/J background (8–20 weeks old age)	Yoshimizu et al.[2]	N/A
Mouse: C57BL6/J wild-type female mice (8–12 weeks old age)	SLC	N/A
Oligonucleotides		
RRBS Adaptor1: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T, C=5meC, *=phosphorothioate bond	Tando et al.[1]	N/A
RRBS Adaptor2: P-GATCGGAAGAGCACACGTCTGAACTCCAGTCA*C, P=phosphate, C=5meC, *=phosphorothioate bond	Tando et al.[1]	N/A
Primer: Arbp: Forward AGATTCGGGATATGCTGTTGGC	Tando et al.[1]	N/A
Primer: Arbp: Reverse TCGGGTCCTAGACCAGTGTTC	Tando et al.[1]	N/A
Primer: Gfra1: Forward TTTTACTGACAGTTGCGTCCAC	Tando et al.[1]	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer: Gfra1: Reverse TGAATGTGCTTCTGCTCAAAGTG	Tando et al.[1]	N/A
Primer: Sycp3: Forward AGCCAGTAACCAGAAAATTGAGC	Tando et al.[1]	N/A
Primer: Sycp3: Reverse CCACTGCTGCAACACATTCATA	Tando et al.[1]	N/A
Primer: Acrv1: Forward TCAGCAACTTTCAAGCGAGTAT	Tando et al.[1]	N/A
Primer: Acrv1: Reverse CTCCTGAAGAGTGCTCACCTG	Tando et al.[1]	N/A
Primer: Bisulfite sequence: H19 outer: Forward TAAGGAGATTATGTTTATTTTTGGA	Kim et al.[9]	N/A
Primer: Bisulfite sequence: H19 outer: Reverse CCCCCTAATAACATTTATAACCCC	Kim et al.[9]	N/A
Primer: Bisulfite sequence: H19 inner: Forward AAGGAGATTATGTTTATTTTTGGA	Kim et al.[9]	N/A
Primer: Bisulfite sequence: H19 outer: Reverse AAACTTAAATAACCCACAACATTACC	Kim et al.[9]	N/A
Primer: Bisulfite sequence: Hist1h2ba outer: Forward TATTAAGATTTAGAAAAAGGAGGGT	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Hist1h2ba outer: Reverse CAACTCTCTCAACAAAAAAAATAA	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Hist1h2ba inner: Forward TATTTATAAGGTGTTGAAATAAGTG	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Hist1h2ba inner: Reverse ACAAAAAAAATAAATAACTCTTAAAAAAAC	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Sycp1 outer: Forward GGGTTTTTAGGAGGTTTTGAGG	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Sycp1 outer: Reverse AACAATATAAAAAACTTTTACTTCTCCATA	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Sycp1 inner: Forward GGTTTTTAGGAGGTTTTGAGGG	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Sycp1 inner: Reverse ATAAAAAACTTTTACTTCTCCATACTACCT	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Taf7l outer: Forward TGTTTTTTTAAGGGAATTATTTTGTATAA	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Taf7l outer: Reverse AAAAATCTTCTAAAACTTAACCCTACC	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Taf7l inner: Forward GGGTTTTGGTGGAGGTTAAAGTTT	Tando et al.[1]	N/A
Primer: Bisulfite sequence: Taf7l inner: Reverse TATCACAAATATCCCCAACACCTTC	Tando et al.[1]	N/A
Primer: Sequence: SP6 GCTATTTAGGTGACACTATAG	This paper	N/A
Recombinant DNA		
Plasmid: pCpGL	Klug et al.[10]	http://www.ag-reh li.de/materials.html
Software and algorithms		
Bismark V0.10.1	Krueger and Andrews[11]	https://www.bioinformatics.babraham.ac.uk/projects/bismark/
MethPrimer	Li and Dahiya[12]	http://www.urogene.org/cgi-bin/methprimer/meth-primer.cgi
QUMA	Kumaki et al.[13]	http://quma.cdb.riken.jp/top/index.html
Other		
40-µm-pore cell strainer	BD Falcon	Cat#352340
1.5-mL DNA LoBind tube	Eppendorf	Cat#022431021
Disposable feeding needle	Fuchigami	Cat#FG7202

MATERIALS AND EQUIPMENT

Buffers for isolation of germ cells

Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin

Reagent	Final concentration	Amount
DMEM (Thermo Fisher Scientific, 11965-092)		44.5 mL
FBS (Biosera, FB-1380)	10% (v/v)	5 mL
Penicillin-streptomycin (Sigma, P7539)	1% (v/v)	0.5 mL
Total	**N/A**	**50 mL**

Store at 4°C, and do not store more than 3 months.

1× Trypsin/ethylenediaminetetraacetic acid (EDTA)

Reagent	Final concentration	Amount
Sterilized Phosphate-buffered saline (PBS)		90 mL
10× Trypsin/EDTA (Sigma, T4174)	1×	10 mL
Total	**N/A**	**100 mL**

Store at 4°C, and do not store more than 3 months.

DMEM containing 10% FBS

Reagent	Final concentration	Amount
DMEM		45 mL
FBS	10% (v/v)	5 mL
Total	**N/A**	**50 mL**

Store at 4°C, and do not store more than 3 months.

DMEM containing 2% FBS

Reagent	Final concentration	Amount
DMEM		49 mL
FBS	2% (v/v)	1 mL
Total	**N/A**	**50 mL**

Store at 4°C, and do not store more than 3 months.

10 mg/mL DNase

Reagent	Final concentration	Amount
DNase (Roche, 11284932)	10 mg/mL	100 mg
Sterilized Deionized distilled (dd) H_2O	N/A	10 mL
Total	**N/A**	**10 mL**

Aliquot 100 μL into 1.5-mL tubes and store at −20°C for up to 1 year.

Gey's Balanced Salt Solution (GBSS) containing 1% FBS

Reagent	Final concentration	Amount
GBSS (Sigma, G9779)		49.5 mL
FBS	1% (v/v)	0.5 mL
Total	**N/A**	**50 mL**

Store at 4°C, and do not store more than 3 months.

Collagenase/DNase		
Reagent	Final concentration	Amount
Collagenase (Sigma, C0130)	1.2 mg/mL	7.2 mg
10 mg/mL DNase	5 μg/mL	3 μL
GBSS		6 mL
Total	**N/A**	**6 mL**
Prepare immediately before use.		

Collagenase/DNase/Trypsin		
Reagent	Final concentration	Amount
Collagenase (Sigma, C0130)	1.2 mg/mL	7.2 mg
10 mg/mL DNase	5 μg/mL	3 μL
1× Trypsin/EDTA	0.025%	600 μL
GBSS		5.4 mL
Total	**N/A**	**6 mL**
Prepare immediately before use.		

Buffers for reduced representation bisulfite sequencing (RRBS)

Lysis buffer		
Reagent	Final concentration	Amount
5 M NaCl	150 mM	12 μL
1 M Tris-HCl (pH 8.0)	10 mM	4 μL
0.5 M EDTA (pH 8.0)	10 mM	8 μL
10% Sodium Dodecyl Sulfate (SDS)	0.1%	4 μL
Proteinase K (800 U/mL) (NEB Cat#8107S	10 U/mL	5 μL
Sterilized ddH_2O	N/A	367 μL
Total	**N/A**	**400 μL**
Prepare immediately before use.		

Phenol/Chloroform		
Reagent	Final concentration	Amount
TE Saturated Phenol (Nippon Gene, 313-90091)	50% (v/v)	50 mL
Chloroform (FUJIFILM wako, 131-038-02606)	50% (v/v)	50 mL
Total	**N/A**	**100 mL**
Mix well in the glass bottle and store at 4°C with shading against light for up to 1 year.		

TE (Adjust to pH 8.0)		
Reagent	Final concentration	Amount
1.0 M Tris-HCl	10 mM	5 mL
0.5 M EDTA (pH 8.0)	1 mM	1 mL
Sterilized ddH_2O	N/A	494 mL
Total	**N/A**	**500 mL**
Autoclave and store at 20°C–25°C for up to 1 year.		

3 M sodium acetate (Adjust to pH 5.2 by acetic acid)		
Reagent	Final concentration	Amount
Sodium acetate trihydrate	3 M	40.8 g
Sterilized ddH_2O	N/A	Up to 100 mL
Total	**N/A**	**100 mL**
Filter solution through a 0.22 μm filter and store at 20°C–25°C for up to 1 year.		

70% Ethanol		
Reagent	Final concentration	Amount
Ethanol	70% (v/v)	35 mL
Sterilized ddH_2O	N/A	15 mL
Total	**N/A**	**50 mL**
Prepare immediately before use.		

Nucleotide End-Repair mix (Use Deoxynucleotide Solution Set (NEB, N0446S))		
Reagent	Final concentration	Amount
100 mM ATP	1 mM	1 μL
10 mM CTP	0.1 mM	1 μL
10 mM GTP	0.1 mM	1 μL
Sterilized ddH_2O	N/A	97 μL
Total	**N/A**	**100 μL**
Make 10 mM CTP and 10 mM GTP by dilution of 100 mM original solution with sterilized ddH_2O. Store at −20°C for up to 1 year.		

Buffers for bisulfite sequencing

50 mg/mL Ampicillin		
Reagent	Final concentration	Amount
Ampicillin (Sigma, A0166)	50 mg/mL	2.5 g
Sterilized ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**
Aliquot 1 mL and store at −20°C for up to 1 year.		

Isopropyl-β-D-thiogalactopyranoside (IPTG)		
Reagent	Final concentration	Amount
IPTG (FUJIFILM WAKO, 093-05011)	0.1 M	0.24 g
Sterilized ddH_2O	N/A	10 mL
Total	**N/A**	**10 mL**
Aliquot 1 mL and store at −20°C for up to 1 year.		

5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-gal)		
Reagent	Final concentration	Amount
X-gal (FUJIFILM WAKO, 021-07852)	2%	200 mg
Dimethylformamide	N/A	10 mL
Total	**N/A**	**10 mL**
Aliquot 1 mL and store at −20°C for up to 1 year.		

Luria-Bertani (LB) ager plate		
Reagent	Final concentration	Amount
LB broth (BD, 244620)	2.5%	12.5 g
Bacto agar (BD, 214010)	1.5%	7.5 g
ddH_2O	N/A	500 mL
Total	**N/A**	**500 mL**

Add 500 μL of 50 mg/mL Ampicillin after autoclave, then pour 25 mL/10 cm dish, and store at 4°C for up to 3 months. LB needs to be cooled after autoclave a bit to body temperature to prevent denaturing of the Ampicillin.

LB medium		
Reagent	Final concentration	Amount
LB broth	2.5%	12.5 g
ddH_2O	N/A	500 mL
Total	**N/A**	**500 mL**

Add 500 μL of 50 mg/mL Ampicillin after autoclave, and store at 4°C for up to 3 months. LB needs to be cooled after autoclave a bit to body temperature to prevent denaturing of the Ampicillin.

STEP-BY-STEP METHOD DETAILS

Isolation of germ cells from fetal testis

Timing: 2–3 h. Time may vary depending on the number of embryos obtained

This step describes the method for isolating fetal male germ cells.

1. Isolation of embryonic testis.
   a. Sacrifice pregnant female mice on appropriate embryonic day.

   Note: Although GFP in the Oct4-GFP transgenic embryos is specifically detectable in primordial germ cells from embryonic day (E) 7.5 onwards (Yoshimizu et al., 1999[2]), this protocol is designed for germ cell isolation from later fetuses (E15.5-E19.5).

   b. Remove embryos from pregnant females using 8.5-cm dissecting scissors and place in DMEM containing 10% FBS and 1% penicillin/streptomycin in a 10-cm dish placed on ice.
   c. After confirming that the fetuses have stopped moving, transfer them to a new 10-cm dish.
      i. Remove the testes using forceps under a stereomicroscope (Figure 2A).
      ii. Transfer the testes into a 6-cm dish with DMEM containing 10% FBS and 1% penicillin/streptomycin using a 1-mL micropipette.

   Note: Cut the end of the micropipette tip using scissors to allow the testes to pass through.

   Note: It can be kept for 20–30 min on ice, but further storage is not recommended at this stage.

   d. Remove the epididymis using a curved surgical needle attached to a shaft (Figure 2B).
2. Preparation of single-cell suspension.
   a. Transfer the testes to a 15-mL centrifuge tube using a 1-mL micropipette.

   Note: Up to 20 testes from the same exposure group can be placed in a 15-mL tube.

   b. Remove as much of the medium as possible.
   c. Add 1 mL of 1× Trypsin/EDTA and mix by repeated pipetting.

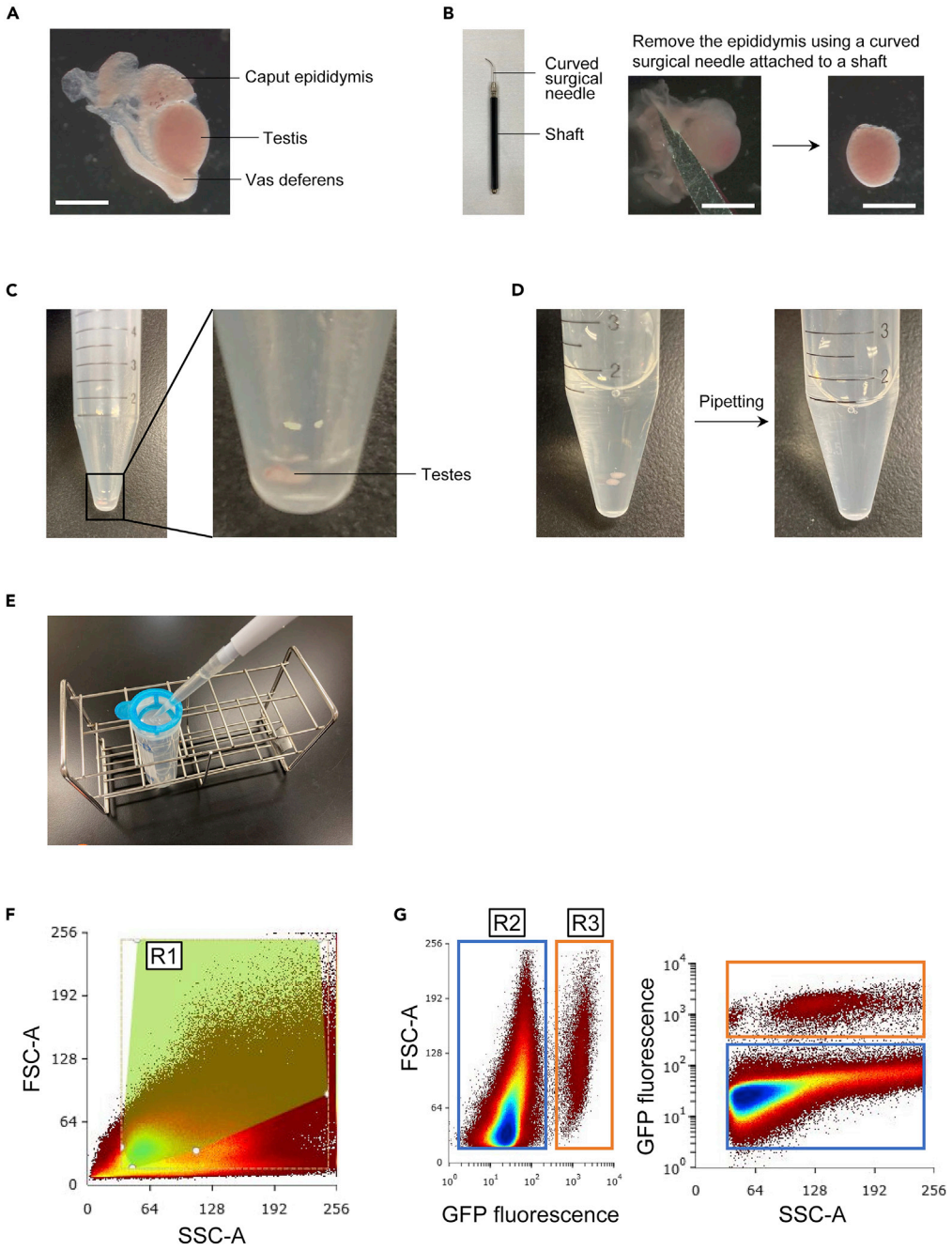


Figure 2. Isolation of fetal germ cells at the stage of E19.5

(A) Isolated fetal mouse testis. Bar = 1 mm.

(B) Removal of the epididymis from a testis. Bars = 1 mm.

(C) Collected testes after removal of liquid.

(D) Cell suspension of testes before and after pipetting.

(E) Cell filtering set-up using 40-μm cell-strainer and 50-mL tube.

(F) Gating the main population of fetal testicular cells (R1) at the stage of E19.5.

(G) Separation of germ cells (R3) and testicular somatic cells (R2) with GFP fluorescence intensity.

d. Remove the 1× Trypsin/EDTA as much liquid as possible using an aspirator after the testes have settled to the bottom of the tube.

⚠ CRITICAL: Remove as much of the liquid as possible, because residual FBS in the medium will interfere with the trypsin reaction (Figure 2C).

e. Add 1 mL of 1× Trypsin/EDTA and leave in a water bath at 37°C for 15 min with tapping in every 5 min.

Note: During this time, set a centrifuge to cool to 4°C. Also, fill a 1.5-mL tube with 1.5 mL of FBS and place the tube on ice to coat the tube with FBS. This 1.5-mL tube is used to collect cells using a cell sorter.

f. Pipet 20–30 times using a 1-mL micropipette until no cell aggregates are visible (Figure 2D).

⚠ CRITICAL: The presence of cell aggregates will result in decreased recovery of purified germ cells. If cell aggregates remain, repeat pipetting until aggregates disappear. Fragments of the membrane surrounding the testis may remain, but it is not a problem.

g. Add 4 mL of DMEM containing 10% FBS and mix by pipetting.
h. Set a 40-μm cell strainer upon a 50-mL tube (Figure 2E) and filter the cell suspension.
i. Count the cells once using a cell counting tool such as hemocytometer.
j. Centrifuge the 50-mL tube at 190 × *g* for 5 min at 4°C.
k. Remove the supernatant and suspend the pellet in DMEM containing 2% FBS at 1× 10^6–10^7/mL of all the tissue cells.

3. Purification of germ cells.
   a. Transfer the cell suspension into 5-mL tube and place the tube on ice.
   b. Remove the FBS from the 1.5-mL tube prepared in step 2 and add 0.5 mL of DMEM containing 10% FBS. Use this tube for collecting sorted cells.
   c. To purify germ cells using a cell sorter, the main cell population is gated on a scatter plot by forward scatter (FSC-A) and side scatter (SSC-A) to eliminate contaminated small materials including cell debris (Figure 2F, R1 gate) (https://www.bio-rad-antibodies.com/flow-cytometry-gates-regions.html?JSESSIONID_STERLING=3919B18883BCAC4B3C9CA7316CD6A8BC.ecommerce1&evCntryLang=JP-ja&cntry=JP&thirdPartyCookieEnabled=truehttps://expert.cheekyscientist.com/gating-strategies-get-your-flow-cytometry-data-published/).
   d. GFP-positive germ cells are then gated on a histogram of GFP fluorescence of the cells in the R1 gate (Figure 2G). The less-intense peak at the higher GFP fluorescence (R3) and more-intense peak at the lower GFP fluorescence (R2) represent GFP-positive germ cells and GFP-negative testicular somatic cells, respectively.

Alternatives: We used a Bio-Rad S3e Cell Sorter system. Other cytometers are likely suitable for this application once the proper filter/laser settings for detecting GFP have been implemented.

Note: The number of sorted testicular germ cells varies depending on the embryonic day of the fetus. In our experiments, 1,000–3,000 cells per embryo at E19.5 can be recovered.

   e. After cell sorting, centrifuge the sorted cells in the 1.5-mL tube at 190 × *g* for 20 min at 4°C.
   f. Remove the supernatant and store the pellet at −80°C until DNA isolation.

Isolation of germ cells from adult testis

Timing: 6–7 h

This step describes the method for isolating adult male germ cells in specific stages. This protocol refers to the previous papers.[14,15]

4. Isolation of testes from adult mice.
   a. Sacrifice adult male mice and remove the testes using 8.5-cm dissecting scissors (Figure 3A) and place in DMEM containing 10% FBS and 1% penicillin/streptomycin in a 10-cm dish.
5. Preparation of single-cell suspension and Hoechst staining.
   a. Remove tunica albuginea testis (membrane wrapping the testis) using forceps and place in a tube with Collagenase/DNase. Incubate the tube in a water bath at 32°C for 25 min and keep mixed by gently turning the tube upside down every 5 min.

      Note: This step allows for loosening of the seminiferous tubules. Thread-like loosened seminiferous tubules become visible (Figure 3B). During this time, pre-warm the tube containing Collagenase/DNase/Trypsin in the same water bath. Also, fill a 1.5-mL tube with 1.5 mL of FBS to coat the tube with FBS and place the tube on ice. This 1.5-mL tube will be used to collect cells purified using the cell sorter.

   b. Filter the testicular tubule suspension through a 40-μm cell strainer into a tube containing Collagenase/DNase/Trypsin (Figure 3C) and transfer the remaining testicular tubules on the strainer using a small medicine spoon.
   c. Incubate the tube containing the testicular tubule suspension in a water bath at 32°C for 25 min. In the middle of the incubation, pipet 10 times using a 10-mL pipettor.
   d. After incubation is complete, pipet the cell suspension 10 times using a 10-mL pipettor and filter through a 40-μm cell strainer.
   e. Centrifuge the cell suspension at 190 × *g* for 5 min at 4°C.
   f. Remove supernatant and suspend in 5 mL of ice-cold GBSS.
   g. Centrifuge at 190 × *g* for 5 min at 4°C.
   h. Remove the supernatant and suspend in 5 mL of ice-cold GBSS containing 1% FBS.
   i. Count the cells once using a cell counting tool such as hemocytometer.
   j. Add GBSS containing 1% FBS to adjust to 5×10^6 cells/mL, and transfer 2 mL of the cell suspension into a 15-mL tube.
   k. Add 5 μL of Hoechst33342 (Thermo Fisher Scientific, H3570) and mix by tapping the tube. Incubate in a water bath at 32°C for 1 h with shading from light. Invert the tubes by upside down to mix cell suspension in the middle of incubation.
6. Purification of germ cells.
   a. Transfer the stained cell suspension into a 5-mL tube and place the tube on ice.
   b. Remove FBS from the 1.5-mL tube prepared in step 5 and add 0.5 mL of DMEM containing 10% FBS. Use this tube for collecting sorted cells.
   c. Add 10 μL of propidium iodide (PI, BD, 51-66211E) and mix by tapping the tube just before sorting.
   d. Purify the germ cells using a cell sorter according to the previous reports.[14,15]
      i. Cells strongly positive for PI staining are first eliminated as dead cells (Figure 3D, P1 gate).
      ii. The main cell population in the P1 gate is further gated in a scatter plot by FSC-A and SSC-A as the P2 gate to eliminate cell debris (Figure 3E, P2 gate).
      iii. A histogram of Hoechst Blue-H fluorescence for the P2 gate reflects cellular DNA content, and the most-intense peak (arrow in Figure 3F) reflects spermatids (Figure 3F, haploid),

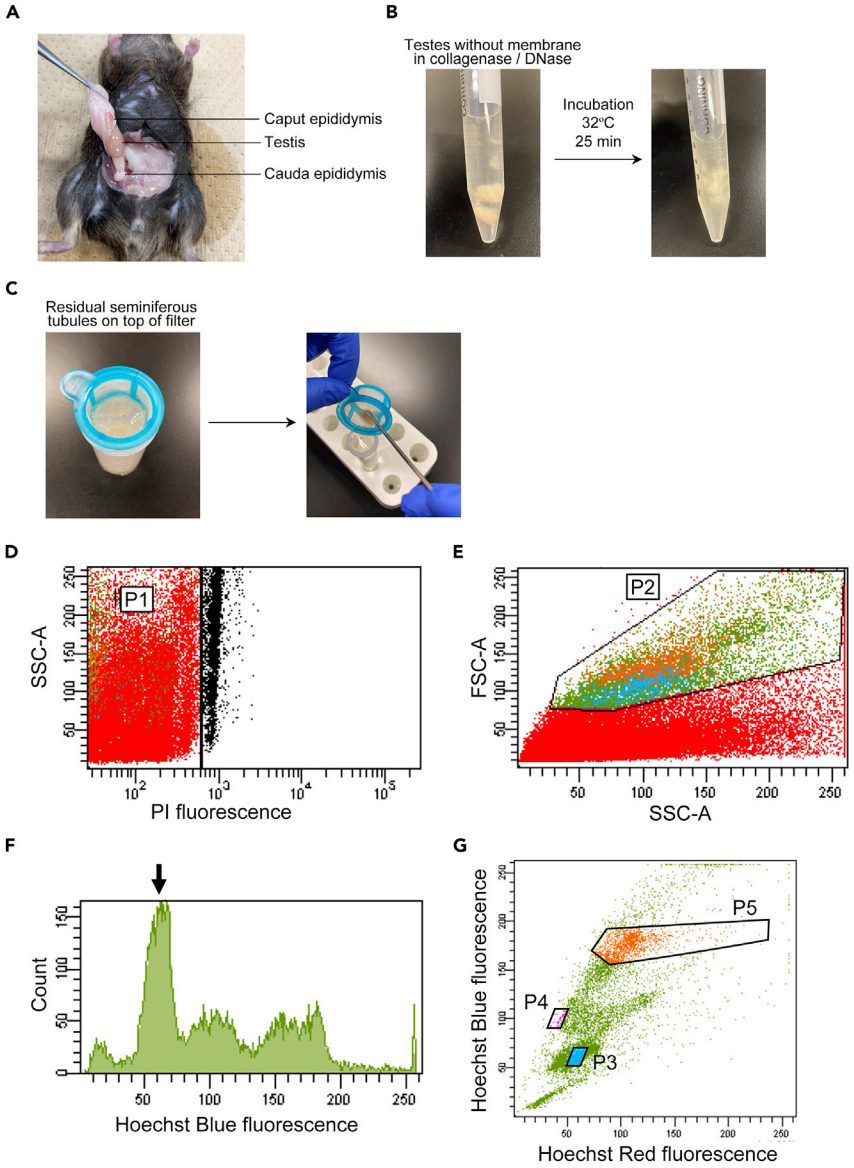


Figure 3. Isolation of adult-stage spermatogenic germ cells

(A) Identification of testis by pulling off the epididymal adipose tissue.

(B) Incubation of testes in collagenase/DNase solution after removal of the tunica albuginea.

(C) Transfer seminiferous tubules trapped by 40-μm cell strainer into a 15-mL tube.

(D) Elimination of dead cells by PI staining. P1 gate represents live testicular cells.

(E) Gating the main population in the P1 gate as the P2 gate.

(F) Histogram of Hoechst Blue fluorescence.

(G) Separation of spermatogenic cells using Hoechst33342 staining.

which should have the same fluorescence intensity by adjusting the voltage of Hoechst blue in each experiment.

Note: Under our experimental conditions, the most-intense peak is always positioned at around 65. Additional peaks at around 100 and 150–190 should represent diploid spermatogonia and tetraploid spermatocytes.

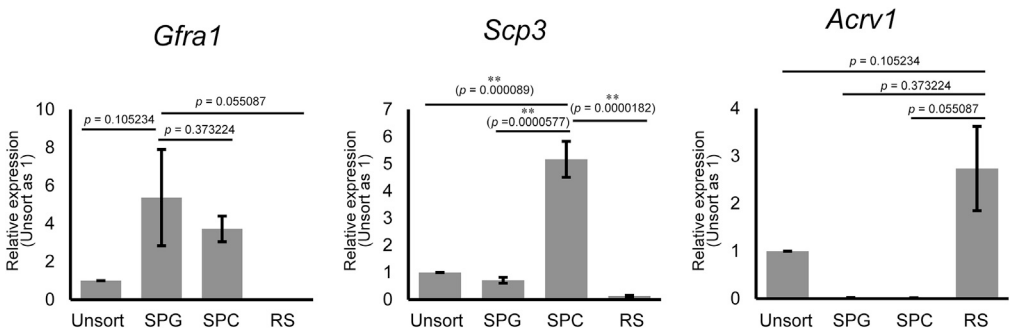


Figure 4. Expression of marker genes in purified spermatogenic cells

Relative expression of stage-specific germ cell marker genes in sorted spermatogenic cells as determined by RT-qPCR: *Gfra1* for spermatogonia, *Scp3* for spermatocytes, and *Acrv1* for spermatids. SPG; spermatogonia, SPC; spermatocytes, RS; round spermatids. This figure is modified form Tando et al.[1] Values were plotted as mean ± SEM of each cell sample from six F1 mice from two mothers. Statistical analysis was performed using the Student's *t* test.

iv. In a scatter plot of Hoechst Blue-H and Hoechst Red-H for the P2 gate, the population at the intersection of fluorescence intensity 65 of Hoechst Blue-H and Hoechst Red-H represents spermatids (Figure 3G, P3).

v. Diploid spermatogonia which include both differentiated and undifferentiated subtypes (Figure 3G, P4) and tetraploid meiotic cells (Figure 3G, P5) are assigned by the fluorescent intensity of Hoechst blue.

⚠ CRITICAL: To optimize horizontal widths of P3, P4 and P5 gates especially just getting started of experiment, the expression of typical marker genes is examined as described below for collected cells by different gating.

Alternatives: Here, we used a Becton Dickinson BD FACS Aria II system, in which 488 nm blue laser and 616/23 band-pass filter for PI, UV laser and 450/20 band-pass filter or 670LP filter for Hoechst blue and Hoechst red, respectively, are equipped. Other cytometers are likely suitable for this application once the proper filter/laser settings for detecting Hoechst33342 have been implemented.

Note: The spermatogonia fraction will have the lowest number of cells among the recovered cell fractions. We usually use mice at the age of P100-P200 and collect 1–5× 10^4 spermatogonia, 2–3× 10^5 spermatocyte, and over 1× 10^6 spermatid per animal.

e. After cell sorting, centrifuge the 1.5-mL tube with sorted cells at 190 × *g* for 20 min at 4°C.

f. Remove the supernatant and store the pellet at −80°C for DNA isolation.

Note: The purity of the cells should be confirmed by the expression of marker genes specific for each cell type in the recovered cell populations. We always aliquot and store one-fourth of the recovered cells for RNA extraction and RT-qPCR analysis for *Gfra1*, *Scp3*, and *Acrv1*, which are the marker genes of spermatogonia, spermatocyte, and spermatid (see Figure 4). Primer sequences are listed in the key resources table.

RRBS library construction

Timing: 4 days

This step describes a method for detecting DNA methylation in isolated germ cells by Reduced Representation Bisulfite Sequencing (RRBS), with particular attention to promoter regions.

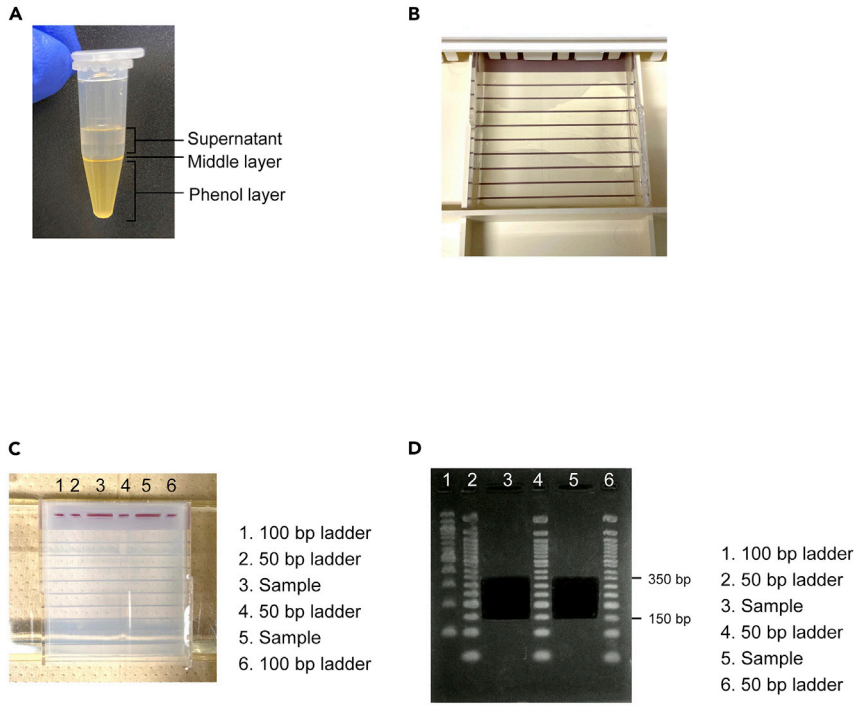


Figure 5. Fragment size selection by agarose gel electrophoresis

(A) A sample tube of phenol extraction after centrifugation. In this case, middle layer is not obvious because of small number of cells.

(B) Preparation of the gel.

(C) Gel after applying samples and ladder markers mixed with loading dye.

(D) Gel after staining with ethidium bromide and cutting a 150- to 350-bp region of sample lanes.

In our study, for E19.5 germ cells, the sorted cells were pooled until they reached more than 10,000 cells, which is a sufficient number of cells for DNA extraction, and two tools were used as two biological replicates. For spermatogonia, two biological replicates are composed of the cells from one F1 individual from different mother.

7. Genomic DNA isolation.

Alternatives: In Tando et al.,[1] DNA was purified by Phenol/Chloroform method described here to remove proteins and lipids. Other DNA isolation methods by using DNA purification kits may also be applicable.

a. Add 400 μL of Lysis buffer into a 1.5-mL tube containing a pellet of the stored frozen cells and mix by pipetting.
b. Incubate at 55°C in a heat block for 2–4 h. Mix the content in each tube by shaking in every 30 min.
c. Add 400 μL of Phenol/Chloroform (1:1) and mix vigorously for 1 min. This step is performed to isolate DNA by removing protein and other contaminants.
d. Centrifuge a 18,000 × *g* for 5 min at 25°C.
e. Transfer the supernatant to a new 1.5-mL tube, add 400 μL of Phenol/Chloroform (1:1), and mix vigorously for 1 min by shaking the tube.

⚠ CRITICAL: Never suck up the middle layer which contains denatured proteins, lipids and phenol (Figure 5A). It will result in contamination of proteins and lipids, and affect the quality of the DNA.

f. Centrifuge at 18,000 × *g* for 5 min at 25°C.
g. Transfer the supernatant to a new 1.5-mL tube, add 400 μL of Chloroform, and mix vigorously for 1 min.
h. Centrifuge at 18,000 × *g* for 5 min at 25°C.
i. Transfer the supernatant to a new 1.5-mL tube, add 5 μL of PureLink RNase A (Thermo Fisher Scientific, 12091021) and incubate at 37°C for 30–60 min.
j. Add 5 μL of 800 U/mL Proteinase K (NEB, P8107S) and incubate at 55°C for 60 min. This step is necessary to remove RNase A from the reaction solution.
k. Add 400 μL of Phenol/Chloroform (1:1) and mix vigorously for 1 min. This step is necessary to remove Proteinase K from the reaction solution.
l. Centrifuge at 18,000 × *g* for 5 min at 25°C.
m. Transfer the supernatant to a new 1.5-mL tube, add 400 μL of Chloroform, and mix vigorously for 1 min.
n. Centrifuge at 18,000 × *g* for 5 min at 25°C.
o. Transfer the supernatant to a new 1.5-mL tube and fill up to 400 μL by adding TE (pH 8.0).
p. Add 1 μL Ethachinmate (Nippon Gene, 312-01791), 40 μL 3 M sodium acetate and 800 μL 100% ethanol. Mix and leave for 8–16 h at −20°C (or 30 min at −80°C).
q. Centrifuge at 18,000 × *g* for 30 min at 4°C.
r. After discarding the supernatant, add 500 μL of chilled 70% ethanol and mix gently by vortex.

Note: 70% ethanol should be freshly made otherwise the ethanol can evaporate and create a solution with > 70% ethanol content.

s. Centrifuge at 18,000 × *g* for 10 min at 4°C.
t. After completely discarding the supernatant, air dry for 10 min (or about 1 min for 55°C heat block).
u. Dissolve in 10 μL of TE buffer and incubate at 55°C for 30 min.
v. Measure the amount of DNA with Qubit dsDNA HS kit (Thermo Fisher Scientific, Q32851) by manufacturer's instruction (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FBID%2FTechnical-Notes%2Fqubit-1x-dsdna-assays-simplified-workflow-tech-note.pdf).

Pause point: Isolated DNA can be stored at −20°C for several months.

8. MspI digestion.
   a. Prepare the following reaction solution in a 0.2-mL PCR tube.

Reagent	Amount
Genomic DNA	X μL (20 ng)
10× NEBuffer 2 (NEB, B7002S)	1.5 μL
Msp I (20 U/μL, NEB, R0106S)	1 μL
Sterilized ddH_2O	12.5 - X μL
Total	15 μL

   b. Incubate in a thermal cycler at 37°C for 16 h.
   c. Incubate in a thermal cycler at 80°C for 20 min to stop enzymatic activity and cool at 4°C.
9. End-repair and A-tailing.
   a. Prepare the following reaction solution in a 0.2-mL PCR tube.

Reagent	Amount
MspI-digested DNA (prepared in step 8)	15 μL
10× NEBuffer 2 (NEB, B7002S)	0.2 μL
Nucleotide End-Repair mix (1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP)	0.8 μL
Klenow Fragment exo- (5 U/μL) (NEB, M0212S)	1 μL
Total	17 μL

b. Incubate at the following temperatures in a thermal cycler.

Temperature	Time	Cycles
30°C	30 min	1
37°C	30 min	1
75°C	20 min	1
4°C	forever	

10. Preparation of adaptor mix.

⚠ CRITICAL: The sequence of Adaptors depends on the next generation sequencing platform. Before starting an experiment, determine the platform which you use. All cysteines of the adaptors should be methylated. The adapter sequences used in this protocol is listed in key resources table.

a. Prepare the following reaction solution in a 0.2-mL PCR tube.

Reagent	Amount
10× T4 DNA Reaction Buffer (NEB, B0202S)	5 μL
100 μM Adapter 1	0.5 μL
100 μM Adapter 2	0.5 μL
Sterilized ddH_2O	44 μL
Total	50 μL

b. Incubate at the following temperatures in a thermal cycler to anneal Adaptor 1 and Adaptor 2.

Note: Perform this step during the incubation time in step 9. The adaptor mix can be stored at −20°C. It can be stored for up to 1 year.

Temperature	Time	Cycles
95°C	5 min	1
70°C	1 min	1
60°C	1 min	1
50°C	1 min	1
40°C	1 min	1
30°C	1 min	1
25°C	forever	

11. Adapter annealing.
a. Prepare the following reaction solution in a 0.2-mL PCR tube.

Note: Reagents are added to the same tube from step 9.

Reagent	Amount
End-repair and A-tailed DNA (prepared in step 9)	17 μL
10× T4 DNA Reaction Buffer (NEB, B0202S)	0.3 μL
Pre-annealed 5mC adapter mix (prepared in step 10)	1 μL
T4 DNA ligase (2,000 U/μL) (NEB, M0202M)	1 μL
Sterilized ddH_2O	0.7 μL
Total	20 μL

b. Incubate in a thermal cycler at 16°C for 20 h and 65°C for 20 min and store at 4°C.

Note: When incubating at 16°C, set the temperature of the lid of the thermal cycler to 30°C to prevent evaporation of the reaction solution.

12. Fragment size selection.
    a. Prepare 3% Nusieve 3:1 agarose (Takara, 50091) in 0.5× TBE gel with large comb, in which 20 μL of the reaction product prepared in step 11 is added with 3.3 μL of Gel Loading Dye (Purple (6×), no SDS, NEB, B7025S) (total volume 23.3 μL) (Figure 5B).

⚠ CRITICAL: To prevent agarose from clumping, slowly add agarose while gently stirring a flask containing 0.5× TBE. Because of the high agarose concentration, bubbles are likely to appear during agarose melting, which may cause electrophoretic turbulence. In that case, bubbles should be removed by heating the agarose sufficiently.

b. Prepare makers for electrophoresis as the following mixture.

Reagent	Amount
10× NEBuffer 2 (NEB, B7002S)	0.6 μL
6× Dye, no SDS (NEB, B7025S)	1 μL
100 bp and 50 bp ladder (NEB, N3236 and N3231)	1 μL
Sterilized ddH_2O	3.4 μL
Total	6 μL

c. Apply the total annealed product in step 11 and the marker in step b to the gel, and carry out electrophoresis (50 mV, 90–120 min) (Figure 5C).

⚠ CRITICAL: The 50 bp ladder marker should be applied to both sides of the sample to serve as a marker when cutting out the gel in step f. The 100 bp ladder is also applied to make it easier to distinguish the 150 and 350 bp bands of the 50 bp ladder marker. Stain the gel by 1 μg/mL ethidium bromide in the clean container for 20–30 min.

Alternatives: Other types of DNA staining solutions (e.g., GelRed Nucleic Acid Stain) may be used.

d. Wash gel once with ddH_2O.
e. Cut out a 150- to 350-bp region of the gel using a razor blade (change for each sample) on a transilluminator (Figure 5D).
f. Transfer a piece of the cut-out gel to a 15 mL tube and weigh the gel. DNA cleanup and concentration can be performed using Qiagen MinElute Gel Extraction Kit (Qiagen, 28604)

by manufacture's instruction (https://www.qiagen.com/us/resources/resourcedetail?id=8f6b09b2-6dcd-4b55-bb4a-255ede40ca3b&lang=en) with minor modifications.

g. Add 6× volume of Buffer QG to the gel and incubate at 15°C–25°C for 20–30 min (until gel dissolves). Shake the tubes in every 10 min.
h. Add an equal volume of isopropanol to the gel and mix by pipetting.
i. Add dissolved gel to two MinElute spin columns, centrifuge (16,000 × *g*, 1 min at 25°C), and discard the filtrate. Repeat this step to process all solutions prepared in step i.

Note: Because of the large volume of dissolved gel, two columns can be used to process one sample and save time.

j. Add 500 μL of Buffer QG to the column, centrifuge (16,000 × *g*, 1 min at 25°C), and discard the filtrate.
k. Add 750 μL of Buffer PE to the column, centrifuge (16,000 × *g*, 1 min at 25°C), and discard the filtrate.
l. Centrifuge (16,000 × *g*, 1 min at 25°C) and transfer the column to a 1.5-mL low-binding tube.

Note: We usually use 1.5-mL DNA LoBind tubes supplied by Eppendorf throughout this protocol, but other low-binding tubes are also applicable.

m. Add 10.5 μL of Buffer EB pre-warmed at 65°C to the column, allow to stand for 1 min, centrifuge (16,000 × *g*, 1 min), elute and combine samples from two columns to one tube.

13. Bisulfite conversion.

Note: This step can be performed using EZ DNA Methylation-Gold Kit (Zymo Research, D5005) following manufacturer's instruction (https://fnkprddata.blob.core.windows.net/domestic/data/datasheet/ZYR/D5005.pdf) with minor modifications.

a. Add 900 μL of sterilized ddH_2O, 300 μL of M-Dilution Buffer, and 50 μL of M-Dissolving Buffer to the tube of CT Conversion Reagent in the Kit and mix with a vortex mixer for 10 min. If the mixture does not dissolve, heat it in a heat block at 64°C to dissolve it completely.
b. The following reaction solutions are added in PCR tube.

Reagent	Amount
Size-selected DNA (prepared in step 12)	20 μL
Bisuflite Mix (prepared in step a)	130 μL
Total	150 μL

c. Incubate in a thermal cycler.

Temperature	Time	Cycles
98°C	10 min	1
60°C	150 min	1
25°C	Forever (up to 20 h)	

d. Apply 600 μL of M-Binding Buffer, 1 μL of Carrier tRNA (1 μg/μL), and the entire Bisulfite reaction solution (150 μL) in step c to the Zymo-Spin IC column, and mix by inversion.
e. Centrifuge (20,000 × *g*, 1 min at 25°C), and discard the flowthrough.
f. Add 100 μL of M-Wash Buffer to the column.

g. Centrifuge (20,000 × *g*, 1 min at 25°C), and discard the flowthrough.
h. Add 200 μL of M-Desulfonation Buffer to the column and incubate at 15°C–25°C for 15 min.
i. Centrifuge at maximum for 1 min and discard the flowthrough.
j. Add 200 μL of M-Wash Buffer to the column, centrifuge at 20,000 × *g* for 1 min, and discard the flowthrough.
k. Add 200 μL of M-Wash Buffer to the column again, centrifuge at 20,000 × *g* for 1 min, and discard the flowthrough.
l. Transfer the column to a new 1.5-mL collection tube and centrifuge 20,000 × *g* for 1 min.
m. Transfer the column to a new 1.5-mL low-binding tube, add 20 μL of M-Elution Buffer, allow to stand for 1 min, and centrifuge (20,000 × *g*, 1 min) for elution.

14. PCR amplification.

⚠ CRITICAL: In the case of Illumina sequencers, multiple libraries are pooled and run at once, and to do this, the PCR primers corresponding to the adaptors in step 10 with different index sequences for each sample are needed to distinguish libraries. Before starting an experiment, determine the platform on which you will sequence your samples, the number of samples to sequence, and the appropriate primer sequences. If you use Illumina's TruSeq, the primers should include index sequences (https://support-docs.illumina.com/SHARE/AdapterSeq/Content/SHARE/AdapterSeq/OligoSeqsMultiplexOligo_obs.htm).

a. Prepare the following reaction solution in a 1.5-mL low-binding tube and is aliquoted into 10 μL per PCR tube.

Reagent	Amount
Bisulfite converted DNA (prepared in step 13)	18 μL
2× KAPA HiFi HotStart Uracil+ Ready Mix (NIPPON Genetics, KK2801)	40 μL
10 μM PCR primer X	2.4 μL
10 μM PCR primer Y	2.4 μL
Sterilized ddH_2O	17.2 μL
Total	80 μL

b. Perform the following PCR reactions in a thermal cycler.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	98°C	20 s	14 cycles (12–18 cycles)
Annealing	65°C	15 s	
Extension	72°C	30 s	
Final extension	72°C	1 min	1
Hold	4°C	forever	

c. Combine the PCR products of the same sample in multiple 0.2 mL tubes to a 1.5-mL low-binding tube, add 144 μL of well-mixed Agentcourt AMPure XP beads (Beckman Coulter, A63880, leave at 15°C–25°C for at least 30 min), vortex, and spin down.
d. Incubate at 15°C–25°C for 10 min.
e. Set tubes on a magnetic stand and allow to stand for 5 min.
f. Discard the supernatant, being careful not to aspirate the beads.
g. Add 500 μL of 70% ethanol (freshly prepared) into tubes on the magnetic rack.
h. Discard the supernatant, being careful not to aspirate the beads.

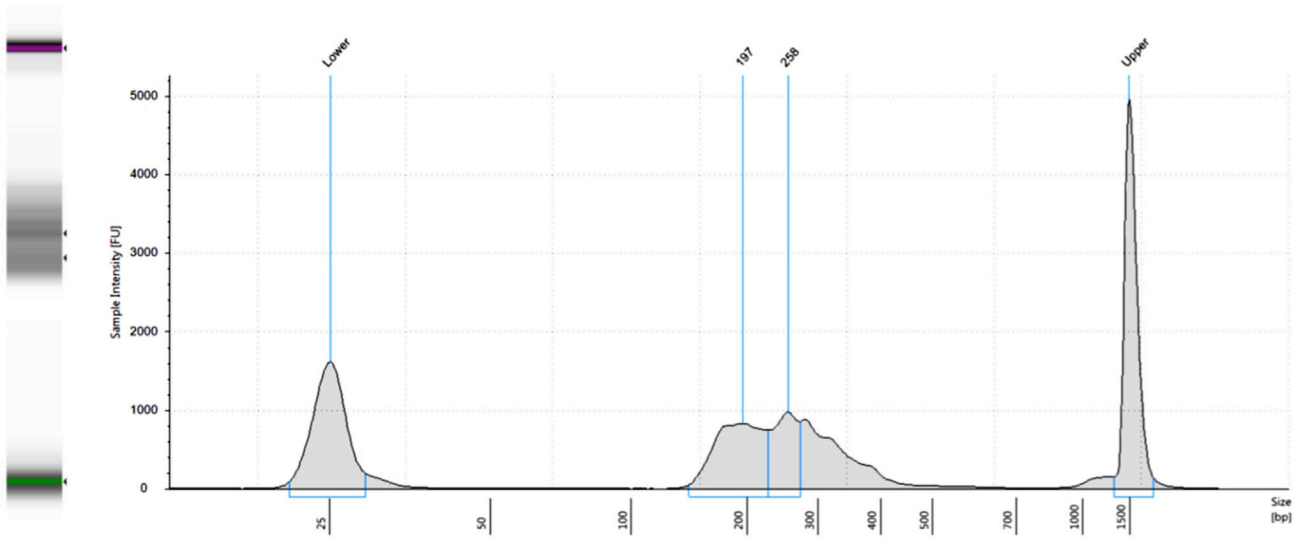


Figure 6. Bioanalyzer electropherogram of the RRBS library
Upper and lower peaks indicate the internal control.

i. Repeat the step g and h once more.
j. Remove the tube from the magnetic stand and dry beads in a heat block at 37°C within 5 min, until the beads turn white from gray.
k. Add 20 μL of Buffer EB, vortex and spin down.
l. Incubate at 15°C–25°C for 2 min.
m. Set tubes on a magnetic stand and leave for 5 min.
n. Collect the elution in a new 1.5-mL low-binding tube, being careful not to aspirate the beads.

Pause point: Eluted samples can be stored at −80°C for up to 1 year.

15. Quality check.
    a. Check the size distribution of the library using D1000 ScreenTape (Agilent, 5067-5582) and D1000 Reagents (Agilent, 5067-5583) by using the TapeStation according to manufacturer's instruction (https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D1000_QG.pdf).

Alternatives: A bioanalyzer may be used instead of a TapeStation. If using a bioanalyzer, a high-sensitivity DNA kit should be used.

Note: A main peak length of prepared RRBS library should be 150- to 350-bp (Figure 6). When the quality of the library is poor, the main peak is lower or invisible. If the main peak is detected over 400 bp (a problem of size selection by cutting the gel) or under 150 bp (adaptor contamination), the quality of the library is also poor.

    b. Quantify with KAPA Library Quantification Kit (NIPPON Genetics, KK4824) by manufacturer's instruction (https://rochesequencingstore.com/wp-content/uploads/2022/07/KAPA-Library-Quantification-Kit-Technical-Data-Sheet.pdf). In this step, the library is quantified by PCR using primers specific to the library. NGS can be performed after this step. Store the library at −80°C until use.

Note: From 20 ng of genomic DNA, which corresponds to about 4,300 cells, approximately 70 nM (approximately 1,400 nmol) of RRBS library is expected. Final library

concentration should be confirmed to be sufficient for your sequencing platform. In our experiment, libraries were sequenced on an HiSeq 2500 platform (Illumina) with 100 bp single-end reads using a TruSeq SR Cluster Kit v3-cBot-HS (Illumina GD-401-3001) and TruSeq SBS Kit v3-HS (Illumina FC-401-3001). However, paired-end leads are also acceptable.

c. The Illumina platform provides 270–300 Gb/ flow cell, and approximately one million mapped reads /sample are needed.

Bisulfite sequencing

Timing: 1 week

This step describes a method for confirming accuracy of DNA methylation levels of candidate individual genes detected by RRBS in isolated germ cells. The data were obtained from four F1 mice from different mothers in each treatment group.

16. Genomic DNA isolation.
    a. Isolate genomic DNA using the same protocol in step 7. If the purified genomic DNA for RRBS in step 7 remains, it is suitable for the following steps.
    b. Measure the amount of DNA with Qubit dsDNA HS kit by manufacturer's instruction as in step 7 and store at −20°C until use.
17. Bisulfite conversion.

Note: This step can be performed using EZ DNA Methylation-Gold Kit (Zymo Research, D5005) (https://fnkprddata.blob.core.windows.net/domestic/data/datasheet/ZYR/D5005.pdf). The protocol is mostly same as that in step 13, but there are some modifications such as incubation temperature, carrier RNA and elution volume.

a. Add 900 μL of sterilized ddH_2O, 300 μL of M-Dilution Buffer, and 50 μL of M-Dissolving Buffer to the tube of CT Conversion Reagent in the Kit and mix with a vortex mixer for 10 min. If the mixture does not dissolve, heat it in a heat block at 64°C to dissolve it completely.
b. The following reaction solutions are added in an PCR tube.

Reagent	Amount
Genomic DNA	20 μL (10 ng)
Bisulfite Mix (prepared in step a)	130 μL
Total	150 μL

c. Perform the following reactions in a thermal cycler.

Temperature	Time	Cycles
98°C	10 min	1
64°C	150 min	1
4°C	forever (up to 20 h)	

d. Apply 600 μL of M-Binding Buffer and the entire Bisulfite reaction solution (150 μL) to the Zymo-Spin IC column, and mix by inversion.

e. Centrifuge (20,000 × *g*, 1 min at 25°C), and discard the flowthrough.
f. Add 100 μL of M-Wash Buffer to the column.
g. Centrifuge (20,000 × *g*, 1 min at 25°C), and discard the flowthrough.
h. Add 200 μL of M-Desulfonation Buffer to the column and incubate at 15°C–25°C for 20 min.
i. Centrifuge at 20,000 × *g* for 1 min and discard the flowthrough.
j. Add 200 μL of M-Wash Buffer to the column, centrifuge at 20,000 × *g* for 1 min, and discard the flowthrough.
k. Again add 200 μL of M-Wash Buffer to the column, centrifuge at 20,000 × *g* for 1 min, and discard the flowthrough.
l. Transfer the column to a new 1.5-mL low-binding tube, add 11 μL of M-Elution Buffer.
m. Centrifuge at 20,000 × *g* for 1 min for elution.

Note: Store DNA after bisulfite modification at −20°C for short-term storage and at −80°C for long-term storage.

18. PCR amplification (1st round).

Note: We always design the primer sets for Bisulfite sequencing by using MethPrimer[12] (http://www.urogene.org/methprimer/). In Steps 18 and 19, the optimal annealing temperature in PCR depends on the primers.

a. Prepare the following reaction solution in an PCR tube.

Reagent	Amount
10× EpiTaq PCR Buffer (Mg^{2+} free) (Takara, SD3575)	2 μL
2.5 mM dNTPs (Takara, SD3576)	2.4 μL
25 mM $MgCl_2$ (Takara, SD3577)	2 μL
TaKaRa EpiTaq HS (5 U/μL) (Takara, R110A)	0.1 μL
10 μM outer primer (F)	0.8 μL
10 μM outer primer (R)	0.8 μL
Bisulfite modified DNA (prepared in step 17)	1 μL
Sterilized ddH_2O	10.9 μL
Total	20 μL

b. Perform the following PCR reactions in a thermal cycler.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	94°C	30 s	1
Annealing	55°C	30 s	30–40 cycles
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	forever	

c. Confirm a portion of PCR product by electrophoresis using 1.5% agarose gel. If the target PCR product is detected, the 2nd round PCR step is not necessary.

19. PCR amplification (2nd round).
a. Prepare the following reaction solution in PCR tube.

Reagent	Amount
10× EpiTaq PCR Buffer (Mg2+ free) (Takara, SD3575)	2 μL
2.5 mM dNTPs (Takara, SD357)	2.4 μL
25 mM $MgCl_2$ (Takara, SD3577)	2 μL
TaKaRa EpiTaq HS (5 U/μL) (Takara, R110A)	0.1 μL
10 μM inner primer (F)	0.8 μL
10 μM inner primer (R)	0.8 μL
1st round PCR product (prepared in step 18)	1 μL
Sterilized ddH_2O	10.9 μL
Total	20 μL

b. Perform the following PCR reactions in a thermal cycler.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	94°C	30 s	30–40 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	forever	

20. DNA extraction from gel.

Note: This step can be performed using MinElute Gel Extraction Kit by manufacture' s instruction (https://www.qiagen.com/us/resources/resourcedetail?id=8f6b09b2-6dcd-4b55-bb4a-255ede40ca3b&lang=en).

a. Measure concentration by Nano Drop. Typically, 20–30 ng/μL of DNA concentration, and 1.7–1.9 of A260/280 ratio is acceptable.

21. A (deoxyadenosine)-addition to gel extraction products.

Note: Although an A is added to the 3′ end of the PCR product amplified by EpiTaq, the A may be occasionally removed during the gel extraction process. To ensure 3′ overhung by A before ligation to the vector, we perform A-addition before TA-cloning of gel-extracted PCR products.

a. Prepare the following reaction solution in an PCR tube.

Reagent	Amount
10× rTaq Buffer (Mg^{2+} free) (TOYOBO, TAP-2B)	1.3 μL
2.5 mM dNTPs (Takara, SD3576)	1.04 μL
25 mM $MgCl_2$ (Takara, SD3577)	0.78 μL
rTaq DNA polymerase (5 U/μL) (TOYOB, TAP-201)	0.13 μL
Total	3.25 μL

b. Add 10 μL of gel-extracted DNA prepared in step 20 and mix by pipetting.
c. Incubate at 72°C for 30 min and place on ice after incubation.

Alternatives: Other Taq DNA polymerase which allow the addition of a single base of deoxyadenosine at the 3' end of PCR product may also be used for the A-addition.

22. TA cloning.

Note: TA cloning is a simple technique for cloning PCR products. A PCR product has an A overhung at its 3′ end, which is easily ligated to the vector with an T overhung at its 3′ end such as pGEM-T easy vector. This step is done to obtain single clones of the PCR product for sequencing.

a. Prepare the following pre-mixture in an PCR tube.

Reagent	Amount
2× Rapid ligation Buffer (Promega, C671)	5 μL
pGEM-T Easy Vector (50 ng) (Promega, A137A)	1 μL
T4 DNA ligase (Promega, M180A)	1 μL
Total	7 μL

b. Mix the Pre-mixture and the A-added gel extraction products.

Note: Calculate the amount of PCR product so that the molar ratio of vector-to-insert ratio is 1:3.

Reagent	Amount
Pre-mixture	7 μL
the A-added gel extraction product (prepared in step 21)	3 μL
Total	10 μL

c. Leave at 15°C–25°C for at least 1 h or 8–16 h at 4°C.
d. Thaw 100 μL of E. coli XL-1 Blue competent cells in a 1.5-mL tube and add 5 μL of ligation product.
e. Incubate on ice for 20 min.
f. Incubate at 42°C for 30 s, then place on ice for 2 min.
g. Add 900 μL of S.O.C. medium (Thermo Fisher Scientific, 15544034) and incubate at 37°C for 1 h.
h. Spread 40 μL of IPTG and 40 μL of X-gal on an LB agar plate.
i. Centrifuge the E. coli solution after incubation at 1,000 × *g* for 2 min.
j. Discard 800 μL of supernatant, suspend the pellet by pipetting, and spread on LB agar plate.
k. Incubate at 37°C for 18 h.

Pause point: LB agar plate with bacterial colonies can be stored at 4°C after sealing by film or tape up to 1 week.

23. Plasmid purification (Nucleospin Plasmid EasyPure (Macherey-Nagel, U0727Q)).
a. Pick a single colony from the LB agar plate with a toothpick and place them in a 15-mL tube containing 2 mL of LB medium and incubate at 37°C with shaking for 12–18 h. Sixteen colonies are usually picked. Background colonies can be checked by ligation and transformation with the vector alone.
b. Purify plasmid DNA using Nucleospin Plasmid EasyPure according to manufacturer's instruction (https://www.mn-net.com/media/pdf/0c/20/aa/Instruction-NucleoSpin-Plasmid-Easypure.pdf).

Alternatives: Other plasmid purification kits or systems can be used to prepare plasmids.

24. Sequence the TA cloned product.
    a. Prepare the following pre-mixture in an PCR tube. After preparation, the tube should be shaded from light because of the light sensitivity of Big Dye terminator.

Reagent	Amount
5× Sequence Buffer (ABI, AB4336697)	1.5 μL
Big Dye terminator ver. 1.1 (ABI, AB4336774)	1 μL
1.6 μM SP6 Sequencing Primer	0.5 μL
Sterilized ddH_2O	6 μL
Total	9 μL

Alternatives: T7 primers can be used for sequence reaction.

    b. Add 1 μL of plasmid DNA (100–300 ng) and mix by vortex.
    c. Perform the following PCR reactions in a thermal cycler.

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	96°C	10 s	25 cycles
Annealing	50°C	5 s	
Extension	60°C	4 min	
Hold	4°C	forever	

    d. Add 32.5 μL of the following mixed solution and mix by vortex.

Reagent	Amount
125 mM EDTA	2.5 μL
100% Ethanol	30 μL
Total	32.5 μL

    e. Leave at 15°C–25°C for 15 min, shaded from light.
    f. Centrifuge at 20,000 × *g* for 3 min at 15°C–25°C.
    g. Discard the supernatant, add 150 μL of freshly prepared 70% ethanol and wash gently.
    h. Centrifuge at 20,000 × *g* for 3 min at 15°C–25°C.
    i. Discard supernatant and incubate at 95°C for 2 min, shaded from light.
    j. Add 20 μL of HiDi Formamide, mix by vortex, and incubate at 95°C for 2 min, shaded from light. This product can be sequenced by appropriate sequencer for sanger sequence (e.g., ABI3130 Genetic Analyzer).

Pause point: Samples eluted in HiDi Formamide can be stored at −20°C with shedding the right up to 1 month.

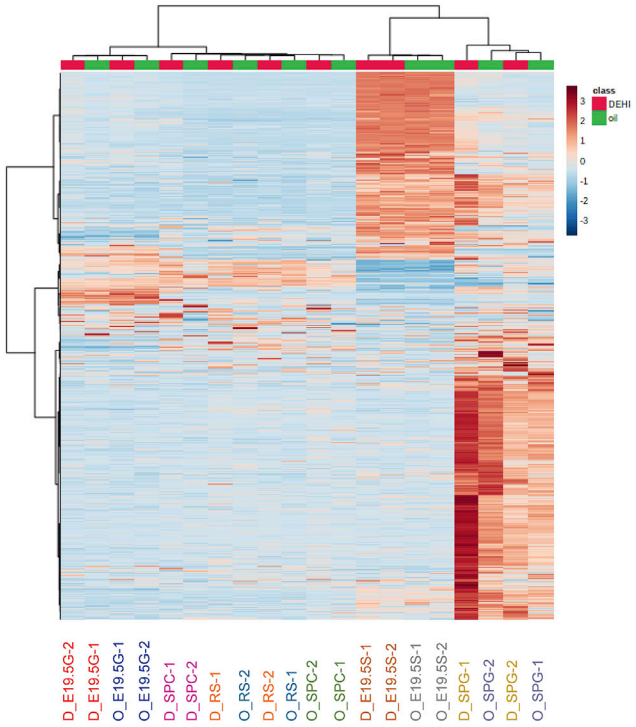
25. Data analysis.
    a. Sequence data are analyzed by QUMA (http://quma.cdb.riken.jp/top/index_j.html).

EXPECTED OUTCOMES

RRBS

Heatmap analysis of methylation levels showed moderate differences in promoter methylation profiles between the oil and the DEHP groups with correlation of the replicates (Figure 7A). In our study

A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O_E19.5G-2
D_SPC-1
D_SPC-2
D_RS-1
O_RS-2
D_RS-2
O_RS-1
O_SPC-2
O_SPC-1
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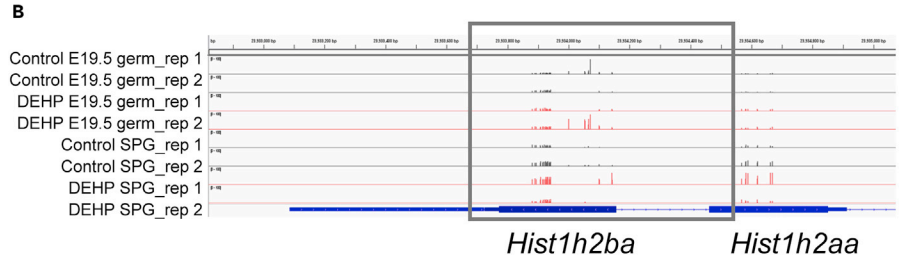
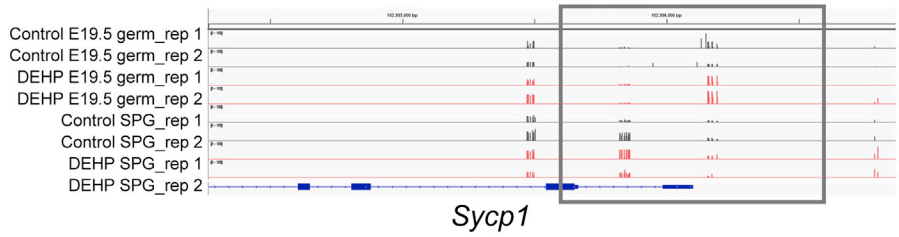
O_SPG-1

B


Control E19.5 germ_rep 1
Control E19.5 germ_rep 2
DEHP E19.5 germ_rep 1
DEHP E19.5 germ_rep 2
Control SPG_rep 1
Control SPG_rep 2
DEHP SPG_rep 1
DEHP SPG_rep 2
Hist1h2ba
Hist1h2aa


Control E19.5 germ_rep 1
Control E19.5 germ_rep 2
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Control SPG_rep 1
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Sycp1

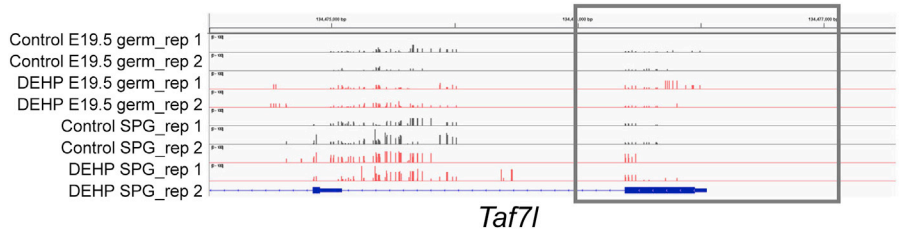
Control E19.5 germ_rep 1
Control E19.5 germ_rep 2
DEHP E19.5 germ_rep 1
DEHP E19.5 germ_rep 2
Control SPG_rep 1
Control SPG_rep 2
DEHP SPG_rep 1
DEHP SPG_rep 2
Taf7l

Figure 7. Representative examples of sequencing datasets obtained by RRBS

(A) Heatmap of promoter DNA methylation levels in the testicular cell populations prenatally exposed to oil as vehicle control (O) or DEHP (D) (clustering distance: Euclidean; clustering method: Ward). Each colored cell on the map corresponds to normalized value of methylation percentage. Reddish and bluish colors represent relatively hyper- and hypomethylation, respectively. E19.5G: E19.5 germ cell; E19.5S: E19.5 testicular somatic cells; SPG: spermatogonia; SPC: spermatocytes. This figure is modified form Tando et al.[1]

(B) Integrative Genome Viewer screenshots of control or maternally DEHP-exposed fetal (E19.5) germ cells and adult spermatogonia (SPG). Boxes denoted by gray lines indicate the TSS region ± 500 bp.

in Tando et al.,[1] we did not apply statistical analysis to the RRBS data due to two biological replicates, and changes in DNA methylation between control and DEHP exposure samples were calculated as described in the quantification and statistical analysis section. Figure 7B shows examples of Integrative Genome Viewer snapshots of sequencing datasets. Samples of germ cells were prepared with and without maternal exposure (E8-E18) to DEHP. Gene Ontology (GO) analysis is performed to identify functional terms enriched among the differentially methylated genes (Figures 3B and 3C in Tando et al.[1]). Venn diagram analysis is employed to identify genes commonly hyper- or hypomethylated in embryonic and adult germ cells (Figure 3D in Tando et al.[1]). Throughout these processes, candidate genes are selected for confirmation of methylation changes via bisulfite sequencing.

> ***Note:*** In Tando et al.,[1] nine hypermethylated spermatogenesis-related genes in both of embryonic germ cells and spermatogonia were identified. We then examined the expression of these genes by qPCR and identified three genes whose expression was significantly downregulated as epi-mutated genes (Figure 4A in Tando et al.[1]). Methylation levels of the three epi-mutated genes detected by RRBS are listed in Table 1. The analytical code is indicated in Figure 3—Source data 1 of Tando et al.[1]

Bisulfite sequencing

As a positive control for bisulfite sequencing of individual candidate genes in adult spermatogonia, an imprinted gene *H19*[16] is shown (Figure 8A). Primer sequences for *H19* are listed in Kim et al.[9]

> ***Note:*** We detected methylation changes in the promoter region of *His1h2ba*, *Sycp11*, and *Taf7l* by RRBS and then confirmed these results via bisulfite sequencing (Figure 8B, Tando et al.[1]). In this experiment, data were obtained from 6-18 clones of PCR products per mouse, and 4 mice were analyzed to ensure reproducibility. Primer sequences are shown in key resources table.

QUANTIFICATION AND STATISTICAL ANALYSIS

RRBS data

First, remove index and adapter sequences and trim the first and last four bases from the raw sequence data, then align the resulting reads to Mouse Genome Build 37 (mm10) using Bismark (v0.10.1) (Krueger and Andrews[11]) with default parameters. Calculate the methylation level of each cytosine using the Bismark methylation extractor. Annotations for Refseq genes and repeat sequences are downloaded from the UCSC Genome Browser (https://genome.ucsc.edu/). Exclude from the analyses Refseq genes encoding microRNAs and small nucleolar RNAs as well as

Table 1. Promoter DNA methylation change of *Hist1h2ba*, *Sycp1*, and *Taf7l* in fetal germ cells and adult spermatogonia after maternal DEHP exposure

	E19.5 germ cells			Adult spermatogonia		
Gene symbol	Oil (%)	DEHP (%)	DEHP-Oil (%)	Oil (%)	DEHP (%)	DEHP-Oil (%)
Hist1h2ba	9.35	14.89	5.54	19.53	31.18	11.65
Sycp1	11.77	25.65	13.88	17.34	28.73	11.39
Taf7l	5.44	14.73	9.29	12.04	18.52	6.48

Mean methylation % of two biological replicates in each group is indicated.

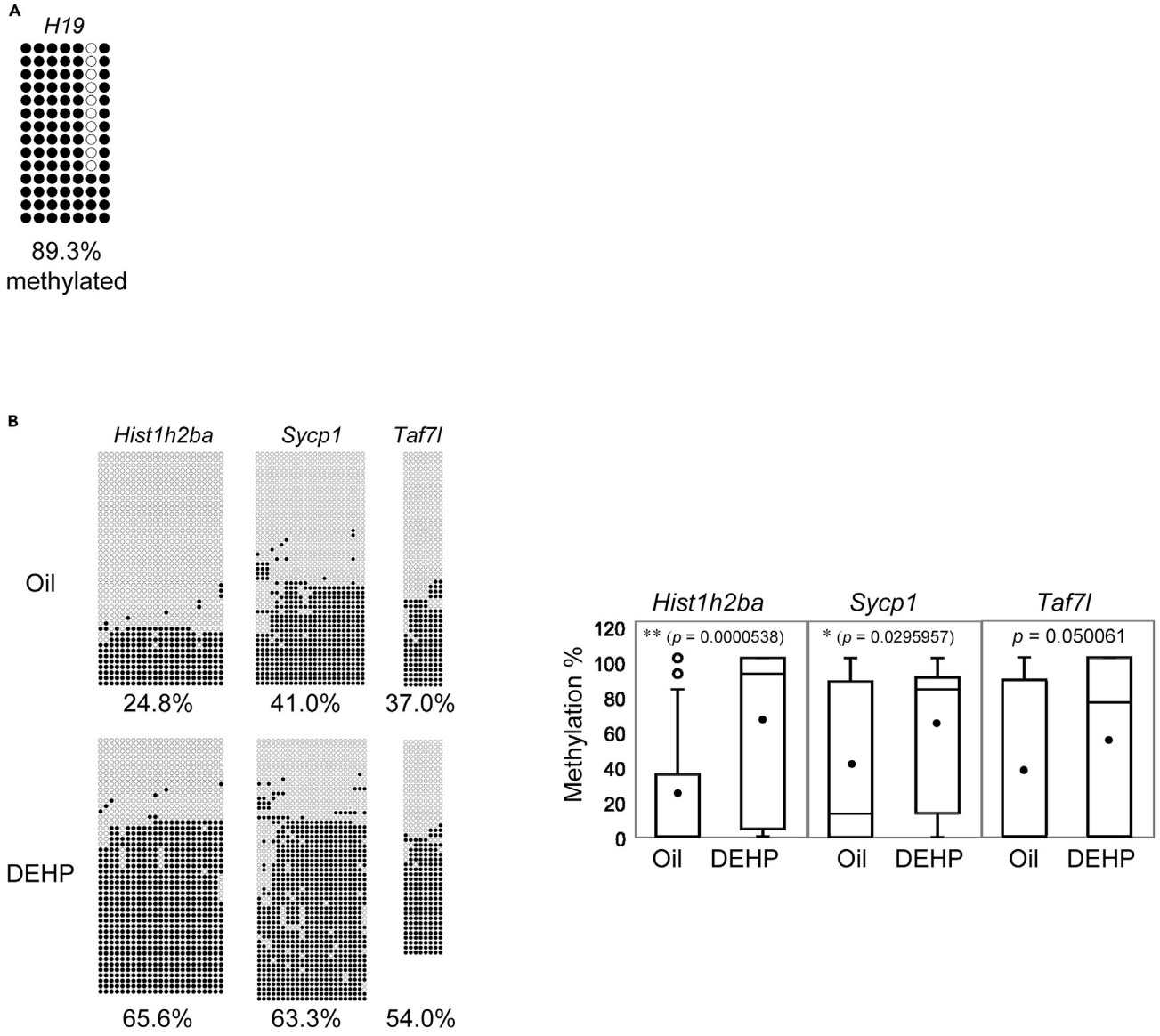

Figure 8. Bisulfite sequencing of candidate genes

(A) Methylation status of *H19* in untreated spermatogonia as a technical control. Methylated and unmethylated CpGs are presented as closed circles and open circles, respectively. The percentage of methylated CpGs is indicated. Paternally methylated *H19* is highly methylated in spermatogonia.

(B) Methylation status of *Hist1h2ba*, *Sycp1*, and *Taf7l* in control or maternally DEHP-exposed F1 spermatogonia are indicated in the right panel. The percentage of methylated CpGs is indicated. Box-whisker plots of the CpG methylation levels shown in the right panel. Lines inside boxes show medians. Whiskers indicate minimum and maximum values. Open and closed circles indicate outliers and mean value, respectively. This figure is modified form Tando et al.[1] The data were obtained from four F1 animals from different mother in each treatment group to evaluate statistically significant differences, and data for 6–18 clones in each animal were used for analysis. Statistical analysis was performed using the Mann-Whitney *U* test.

mitochondrial DNA. In our experiment, promoters were defined as regions 500 bp upstream and downstream from the TSSs (Transcription Start Sites) of Refseq genes. We analyzed only CpG cytosines covered with three reads. In addition, we defined the percentage of methylated CpG among all CpG sites in regions of interest as the methylation level in promoters containing $\geq$10 CpG cytosines with sufficient coverage for calculation of the methylation level. Methylation changes are calculated by subtracting the methylation percentage of the control group from that of the experimental group (Files S2 and S3 of Tando et al.[1]). In our experiment, we utilized the RRBS data to select candidate

differentially methylated spermatogenesis-related genes by DEHP for further validation analysis by bisulfite sequencing, and we did not perform statistical analysis because of two biological replicates. We selected the candidate genes whose promoter methylation levels increased over 5% by maternal administration of DEHP compared with those of control.

LIMITATIONS

At least 20 ng of genomic DNA is required for successful RRBS library construction. In our experience, more than 4,000 cells are needed to isolate ≥20 ng of DNA. Approximately 10,000 spermatogonia can be recovered from a male mouse, but several embryos are needed to obtain a sufficient number of germ cells at embryonic stages.

Spermatogonia are classified as either undifferentiated or differentiated.[17] By monitoring the expression of *Gfra1*, a marker of undifferentiated spermatogonia, we confirmed that the spermatogonia fraction isolated in this protocol contained undifferentiated spermatogonia. To determine how many differentiated spermatogonia are included in the isolated spermatogonia fraction, it is necessary to evaluate the expression of *Kit1*, a marker of differentiated spermatogonia.

TROUBLESHOOTING

Problem 1

Target spermatogenic cells cannot be recovered by cell sorting from adult testes in step 6.

Potential solution

The use of old Hoechst 33342 dye may result in poor separation during cell sorting, and long sorting time may lead to a poor survival rate for the collected cells due to increased temperature. Therefore, use fresh Hoechst 33342 dye, cool collecting tubes or replace with new collecting tubes within 1 h, and immediately place collected cells on ice.

Problem 2

Poor quality of RRBS libraries judged by TapeStation analysis in step 15.

Potential solution

If the amount of starting genomic DNA is <20 ng, library construction may be unsuccessful; therefore, use more than 20 ng of DNA. Increasing PCR cycles may result in amplification bias and is not recommended. The purity of the genomic DNA is also critical. The ideal A260/280 value of the DNA should be approximately 1.8.

Problem 3

Target PCR fragments are not amplified in bisulfite sequencing of individual genes in steps 18 and 19.

Potential solution

Redesign primers. Methprimer (http://www.urogene.org/methprimer/) is a useful website to design primers for bisulfite sequence. The amplification size should be <500 bp. After the bisulfite reaction, do not leave samples in the thermal cycler in order to avoid template DNA degradation.

Problem 4

Bisulfite sequencing results in clonal amplification at a high frequency in step 25.

Potential solution

More clones are sequenced. In addition, the number of PCR cycles should be kept to a minimum in order to prevent amplification bias. If the problem persists, change the primers.

Problem 5

The results of RRBS and bisulfite sequencing do not match.

Potential solution

If the methylation changes detected by RRBS are subtle, more clones for bisulfite sequencing may be required to detect methylation changes. Analysis of 20–30 independent clones is recommended if the RRBS methylation change in target genes is <10%. In our experience, if the methylation change is >5% in RRBS, the methylation changes can also be detected by bisulfite sequencing (Tando et al.[1]).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yasuhisa Matsui (yasuhisa.matsui.d3@tohoku.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets generated during this study are available at DDBJ: DRA012092.

Analytical code of RRBS is available in Figure 3—source data 1 of Tando et al.[1]

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.T., Y.M.; investigation, Y.T.; writing—original draft, Y.T.; writing—review and editing, Y.M.; funding acquisition, Y.T., Y.M.; supervision, Y.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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