Environ Health Perspect

DOI: 10.1289/EHP5862

Supplemental Material

Role of TET Dioxygenases and DNA Hydroxymethylation in Bisphenols-Stimulated Proliferation of Breast Cancer Cells

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Figure S3. Effects of bisphenol A (BPA) and bisphenol S (BPS) on the expression of ten-eleven translocation 2 (TET2) protein in MCF-7 cells transfected with shTET2 (A, B) and pcDNA3-FLAG-TET2 plasmids (C, D). Immunoblotting of TET2 protein in MCF-7 cells were carried out after 48 h of vehicle, BPA or BPS treatment. MCF-7 cells transfected with shNC and pcDNA3-FLAG plasmids were used as the control. Quantitative analysis of TET2 protein was carried out with Image J software. The relative intensity was calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with Student's paired t-test. #, $P < 0.05$ vs control.

Figure S4. Cell proliferation and 5-hydroxymethylcytosine (5hmC) level of MCF-7 cells transfected with the second shRNA constructs for ten-eleven translocation 1 (TET1), TET2 and TET3 upon bisphenol A (BPA) or bisphenol S (BPS) exposure. (A) Identification of TET protein expression in the MCF7 cells transfected with the second shRNA using western blot. (B) Cell proliferation assay by MTS of MCF7 cells transfected with the second shRNA upon exposure to BPA and BPS. (C) Relative level of genomic 5hmC measured by ELISA assay of genomic DNA from MCF-7 cells transfected with the second shRNA upon exposure to BPA and BPS. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA (Bonferroni posttest) (* for $P < 0.05$ and ** for $P < 0.01$ were indicated the differences relative to the control of NC).

Figure S5. Western blot assay of total and phosphorylated estrogen receptor α and β (ERα and ERβ) in MCF7 cells exposed to bisphenol A/S (BPA/BPS). (A) Changes of total and phosphorylated ERα proteins in MCF7 cells exposed to BPA and BPS. (B) Changes of total and phosphorylated ERβ proteins in MCF7 cells exposed to BPA and BPS. (C) Quantitative analysis of total and phosphorylated ERα proteins shown in (A) with Image J software. (D) Quantitative analysis of total and phosphorylated ERβ proteins shown in (B) with Image J software. The relative intensity was calculated by the ratio relative to the GAPDH intensity. The phosphorylated ratio was calculated by phosphor/total proteins. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA (Bonferroni posttest): * for $P < 0.05$ and ** for $P < 0.01$ were indicated the differences relative to the control.

Figure S6. Verification of $ERα^{-/-}$ and $ERβ^{-/-}$ MCF-7 cells by sequencing (A) and western blot (B).

Figure S7. Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of ten-eleven translocation 1 (TET1), TET2 and TET3 in ERα$^{-/-}$ (A) and ERβ$^{-/-}$ (B) MCF-7 cells at 48 h after vehicle, bisphenol A (BPA) or bisphenol S (BPS) treatment. Data represent mean ± SD of five independent experiments. Statistical analysis was performed with Student's paired t-test: * for $P < 0.05$ was indicated the differences relative to the control.

Figure S8. Western blot results of ten-eleven translocation 1 (TET1), TET2 and TET3 proteins in ERα$^{-/-}$ and ERβ$^{-/-}$ MCF-7 cells at 48 h of bisphenol A (BPA) or bisphenol S (BPS) treatment.

Figure S9. Interaction of ERα with ten-eleven translocation 2 (TET2) protein in bisphenol A/S (BPA/BPS) exposed MCF7 cells, showing no detectable interaction of ERα and TET2. Immunoprecipitation was performed using anti-ERα antibody followed by western blotting with indicated antibodies. Sp1 protein, the co-factor of ERα, was used as the positive control.

Figure S10. ERα-mediated DNA methyltransferases (DNMTs) expression in MCF7 cells under bisphenol A/S (BPA/BPS) exposure. (A) Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of DNMT1, DNMT3A and DNMT3B in MCF-7 cells after 48 h of vehicle, BPA, or BPS treatment. Data represent mean ± SD of five independent experiments. (B) Immunoblotting of DNMT1, DNMT3A and DNMT3B proteins in MCF-7 cells at 48 h after vehicle, BPA, or BPS treatment. (C) Quantitative analysis of DNMT1, DNMT3A and DNMT3B proteins shown in (B) with Image J software. The relative intensity was calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. (D) qRT-PCR analysis of the expression of DNMT1, DNMT3A and DNMT3B in ERα$^{-/-}$ MCF-7 cells at 48 h after vehicle, BPA, or BPS treatment. Data represent mean ± SD of five independent experiments. (E, F) Immunoblotting of DNMT1, DNMT3A and DNMT3B proteins in ERα$^{-/-}$ MCF-7 cells at 48 h after vehicle, BPA, or BPS treatment. The relative intensity was analyzed with Image J software and calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA (Bonferroni posttest). * for $P < 0.05$ and ** for $P < 0.01$.

Figure S11. Cell proliferation, ten-eleven translocation 2 (TET2) expression and promoter methylation of MCF-7 cells transfected with the second shRNA constructs for DNA methyltransferase 1 (DNMT1), DNMT3A and DNMT3B upon bisphenol A (BPA) or bisphenol S (BPS) exposure. (A) Identification of the MCF7 cells transfected with the second shRNA using western blot. (B) Cell proliferation assay by MTS of MCF7 cells transfected with the second shRNA upon exposure to BPA or BPS. (C) Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of TET2 in MCF-7 cells transfected with the second shRNA at 48 h of BPA or BPS treatment. (D, E) Immunoprecipitation of TET2 protein in MCF7 cells transfected with the second shRNA-DNMTs at 48 h of BPA or BPS treatment. The relative intensity was analyzed with Image J software and calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. (F) Quantitative DNA sequencing analysis of methylation specific PCR product of TET2 promoter in MCF7 cells transfected with the second shRNA for DNMTs at 48 h of BPA/BPS treatment. Data represent mean ± SD of five independent experiments. Statistical significance was evaluated by two-way ANOVA (Bonferroni posttest). *, $P < 0.05$ or **, $P < 0.01$ vs control of NC.

Additional File- Excel Document

Supplemental Tables

Table S1 Antibodies used in this work.

Antibodies	**Source**	**Identifier**
anti-TET1	EpiGenTek	A-1020-100
anti-TET2	Abcam	ab124297
anti-TET3	Abcam	ab139311
anti-ERα	Abcam	ab32063
anti-ERβ	Abcam	ab3577
anti-ERα (phospho S104/106)	Abcam	ab75753
anti-ERα (phospho S118)	Abcam	ab32396
anti-ERα (phospho S167)	Abcam	ab31478
anti-ERα (phospho S305)	Abcam	ab61049
anti-ERα (phospho T311)	Bethyl Laboratories	A701-101
anti-ERα (phospho Y537)	Sigma Aldrich	SAB4503910
anti-ERβ (phospho S87)	Sigma Aldrich	SAB4504683
anti-ERβ (phospho S105)	Abcam	ab62257
anti-DNMT1	Abcam	ab13537
anti-DNMT3A	Active Motif	39206
anti-DNMT3B	Abcam	ab79822
anti-RNA polymerase II	Abcam	ab817
anti-SP1	Abcam	ab13370
anti-5hmC	Active Motif	39791
anti-GAPDH	Abcam	ab8245
Alexa Fluor 555 conjugated goat anti-rabbit secondary antibody	Invitrogen	A32727
Goat anti-rabbit secondary antibodies (IRDye680)	Abcam	ab216777
Goat anti-mouse secondary antibodies (IRDye800)	Abcam	ab216776

Table S2 Primers for quantitative reverse transcript-PCR (qRT-PCR).

TET1	Forward: CAAGTGTTGCTGCTGTCAGG Reverse: AGCAATTGGACCCATGAC
TET2	Forward: GGTCCTAATGTGGCAGCTAT Reverse: CACTGCTGCTTCTGCGAAAC
TET3	Forward: AGCAAGACACCTCGCAAGTT Reverse: TGGTCACCTGGTTCTGATAG
DNMT1	Forward: TACCTGGACGACCCTGACCTC Reverse: CGTTGGCATCAAAGATGGACA
DNMT3A	Forward: TATTGATGAGCGCACAAGAGAGC Reverse: GGGTGTTCCAGGGTAACATTGAG
DNMT3B	Forward: GGCAAGTTCTCCGAGGTCTCTG Reverse: TGGTACATGGCTTTTCGATAGGA
β-ACTIN	Forward: ACAGAGCCTCGCCTTTGCCGAT Reverse: CTTGCACATGCCGGAGCCGTT

Table S3 Target sequences of short hairpin RNA (shRNA)

TET1	1-GCAGCTAATGAAGGTCCAGAA 2-CCCAGAAGATTTAGAATTGAT
TET2	1-GGGTAAGCCAAGAAAGAAA 2-AAACAAAGAGCAAGAGATT
TET3	1-AAACAAAGAGCAAGAGATT 2-GAACCTTCTCTTGCGCTATTT
DNMT1	1-GCCCAATGAGACTGACATCAA 2-GCCGAATACATTCTGATGGAT
DNMT3A	1-GCCTCAGAGCTATTACCCAAT 2-CCGGCTCTTCTTTGAGTTCTA
DNMT3B	1-AGGTAGGAAAGTACGTCGC 2-AGTGCCGACAGCTCTCCAATA
NC	GTTCTCCGAACGTGTCACGT

Table S4 Primers for chromatin immunoprecipitation-qPCR (ChIP-qPCR).

TET2	Forward: GGGCTCTTACGAGAGGCAAC Reverse: CGGCCCACCTTCTGTTTACT
DNMT1	Forward: CCTGAGGCCTTCACGTTCAA Reverse: ACTTGTGGGTGTTCTCAGGC
DNMT3A	Forward: CAGCGTCACACAGAAGCATATC Reverse: ACCACATTCTCAAAGAGCCAGA
DNMT3B	Forward: GGATGAAGATCAGAGCCGAGAA Reverse: CAGGCACTCCACACAGAAACAC
β-ACTIN	Forward: ACAGAGCCTCGCCTTTGCCGAT Reverse: CTTGCACATGCCGGAGCCGTT

Supplemental Figures

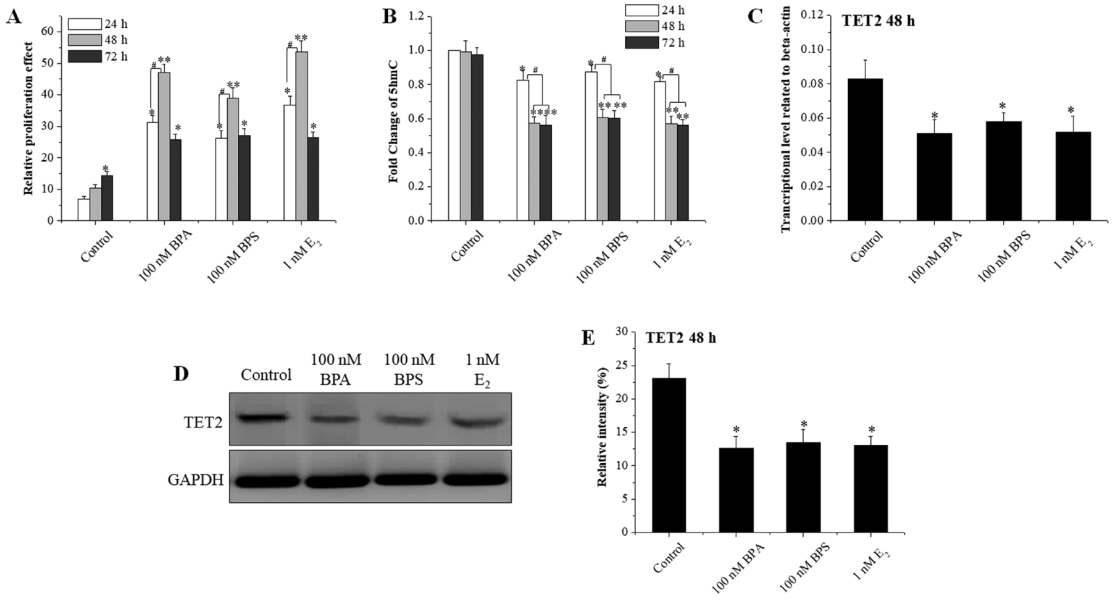


Figure S1 Cell proliferation, genomic DNA 5-hydroxymethylcytosine (5hmC) level and ten-eleven translocation 2 (TET2) expression of MCF-7 breast cancer cells upon 17-beta-estradiol (E_2) or bisphenol A/S (BPA/BPS) exposure. (A) MTS assay of MCF-7 proliferation induced by E_2 and BPA/BPS. (B) Relative level of genomic 5hmC measured by ELISA assay of genomic DNA from MCF-7 cells exposed to E_2 or BPA/BPS. Mean and corresponding ± SD (of three replicates) were shown for each treatment. (C) qRT-PCR analysis of the expression of TET2 in MCF-7 cells after 48 h of vehicle, E_2, or BPA/BPS treatment. (D, E) Immunoprecipitation and quantitative analysis of TET1, TET2 and TET3 proteins in MCF-7 cells at 48 h of E_2 and BPA/BPS treatment. The relative intensity was calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with two-way ANOVA (Bonferroni posttest). *, $P < 0.05$ or **, $P < 0.01$ vs control of 24 h (A, B) or vs control (C-E); #, $P < 0.05$ vs indicated samples (A, B).

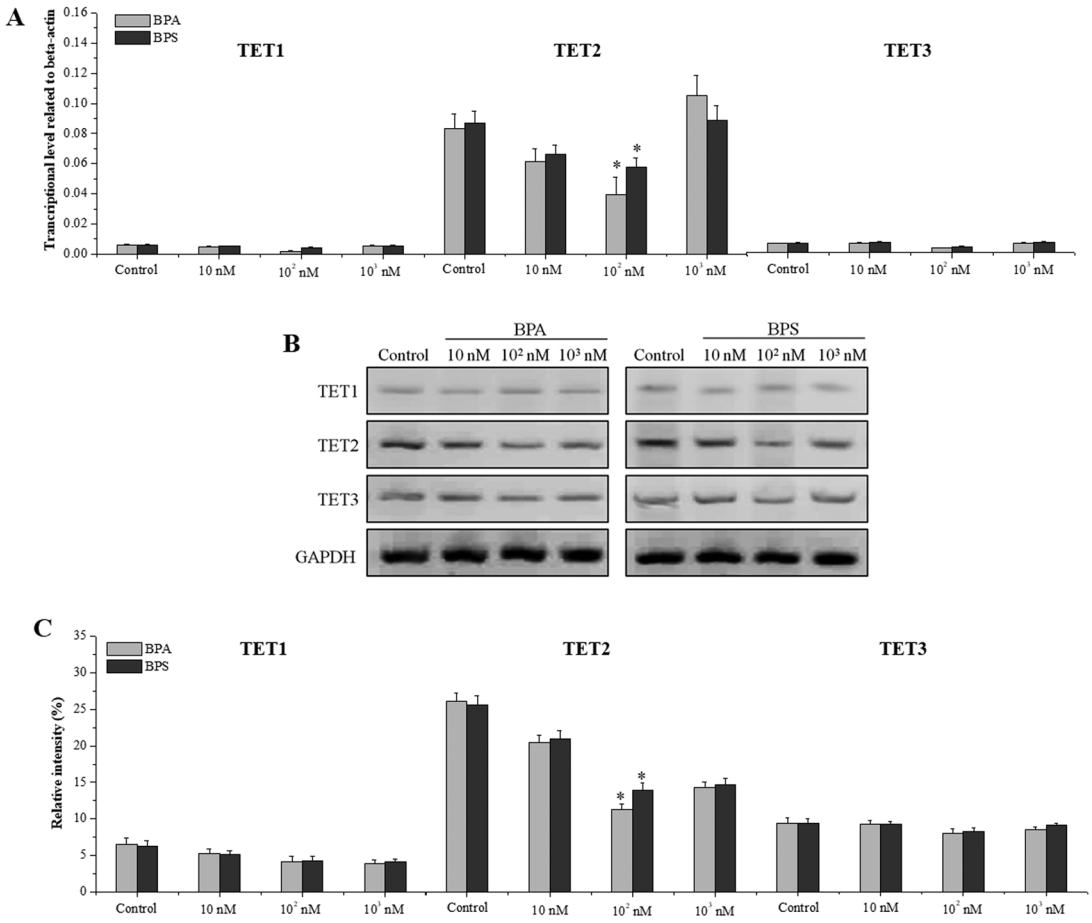
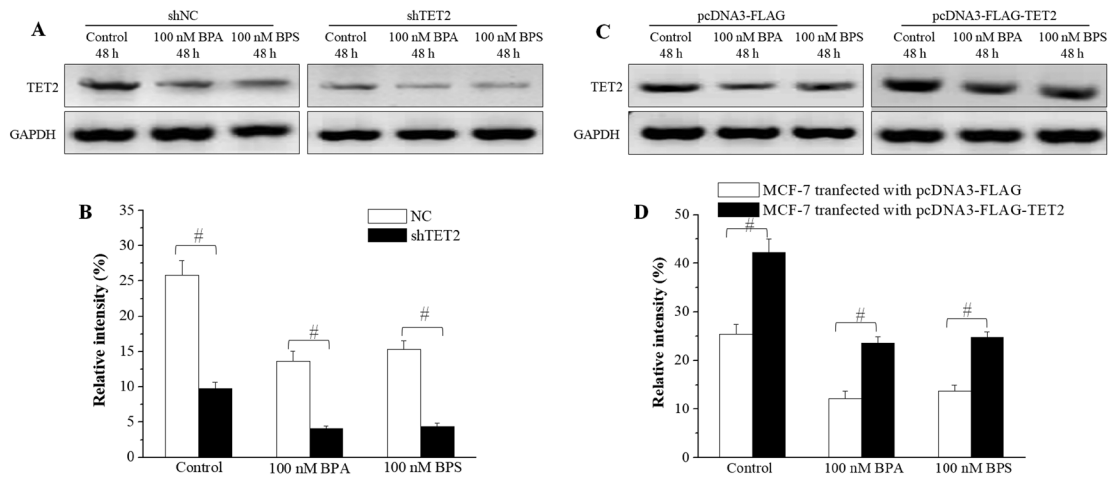


Figure S2 Ten-eleven translocation (TETs) expression in MCF-7 cells treated with bisphenol A/S (BPA/BPS). (A) Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of TET1, TET2 and TET3 in MCF-7 cells at 48 h of BPA and BPS treatment. (B) Western blot analysis of TET1, TET2 and TET3 proteins in MCF-7 cells after 48 h of BPA, BPS, or vehicle treatment. (C) Quantitative analysis of TET1, TET2 and TET3 proteins shown in (B) with Image J software. The relative intensity was calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. Statistical analysis was performed with Student's paired t-test, and asterisks indicated a significant difference from the control. *, $P < 0.05$.



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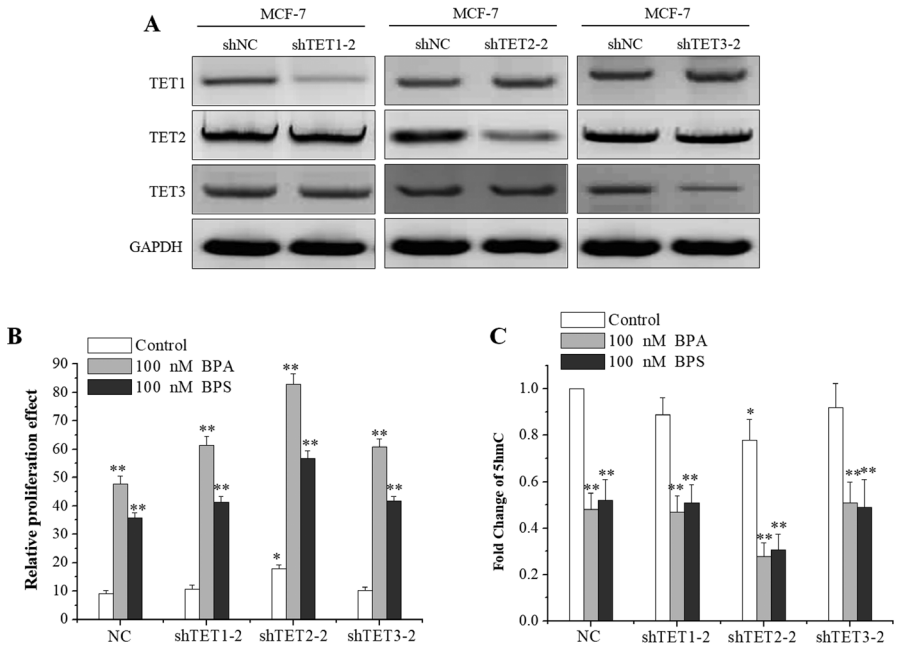

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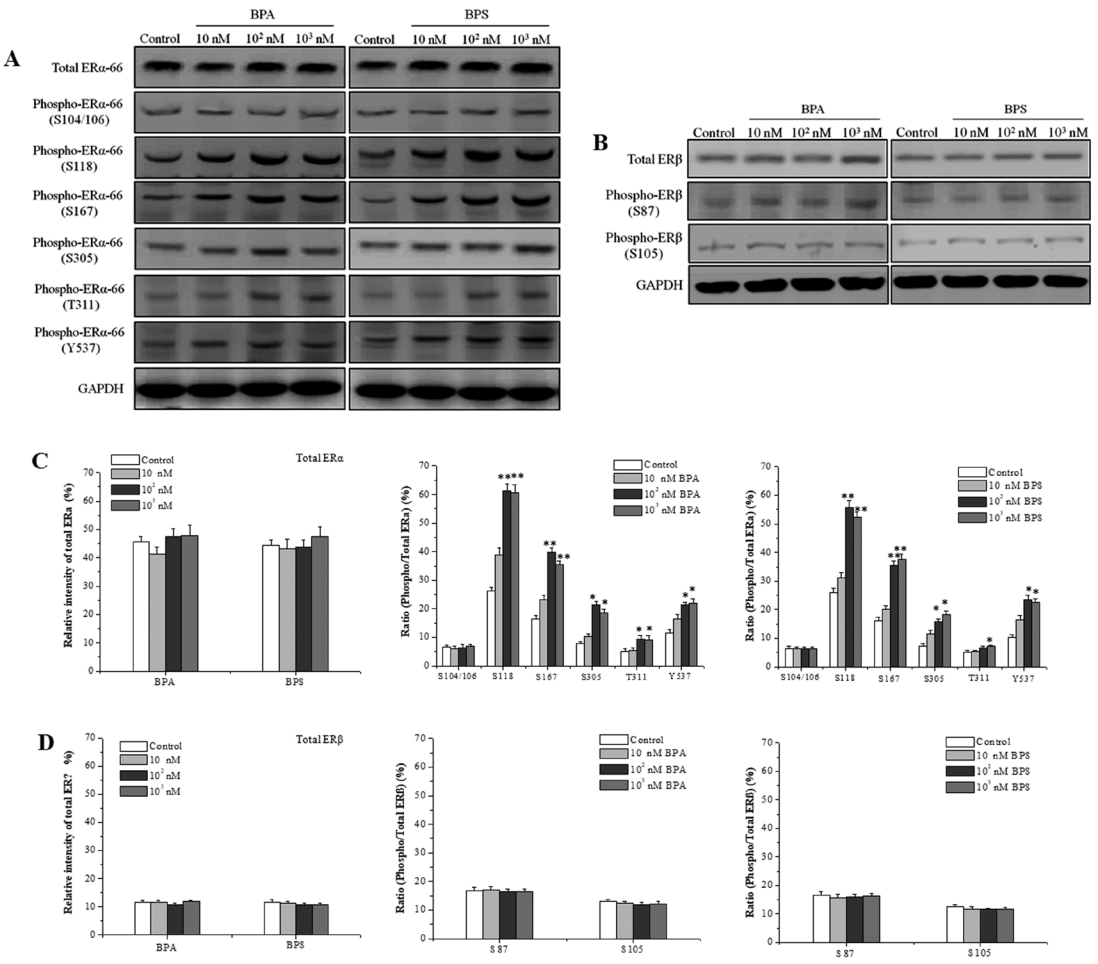

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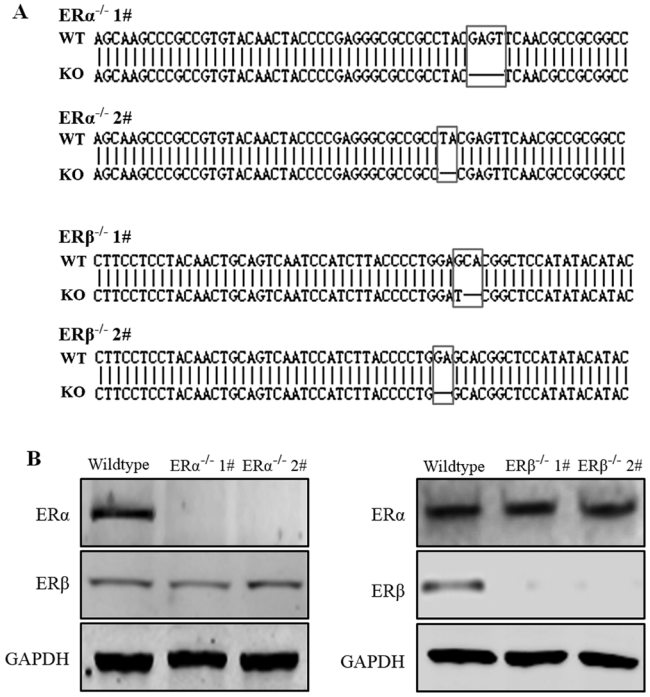


Figure S6 Verification of ERα$^{-/-}$ and ERβ$^{-/-}$ MCF-7 cells by sequencing (A) and western blot (B).

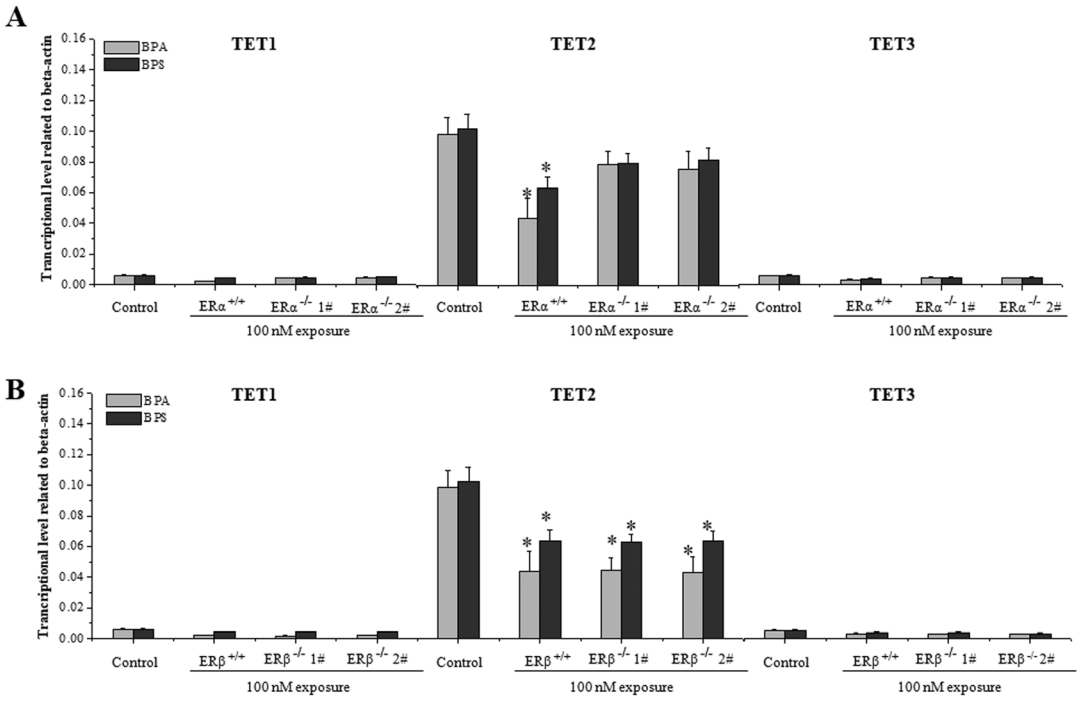
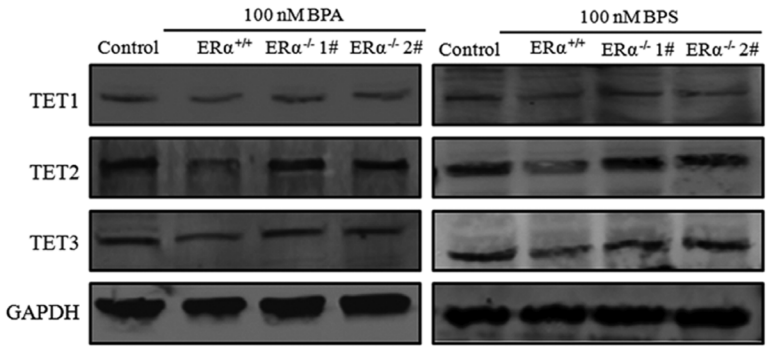


Figure S7 Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of ten-eleven translocation 1 (TET1), TET2 and TET3 in ERα$^{-/-}$ (A) and ERβ$^{-/-}$ (B) MCF-7 cells at 48 h after vehicle, bisphenol A (BPA) or bisphenol S (BPS) treatment. Data represent mean ± SD of five independent experiments. Statistical analysis was performed with Student's paired t-test: * for $P < 0.05$ was indicated the differences relative to the control.



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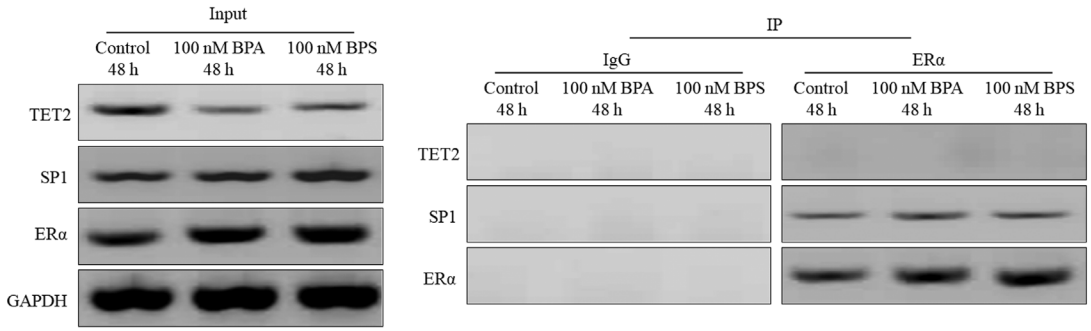

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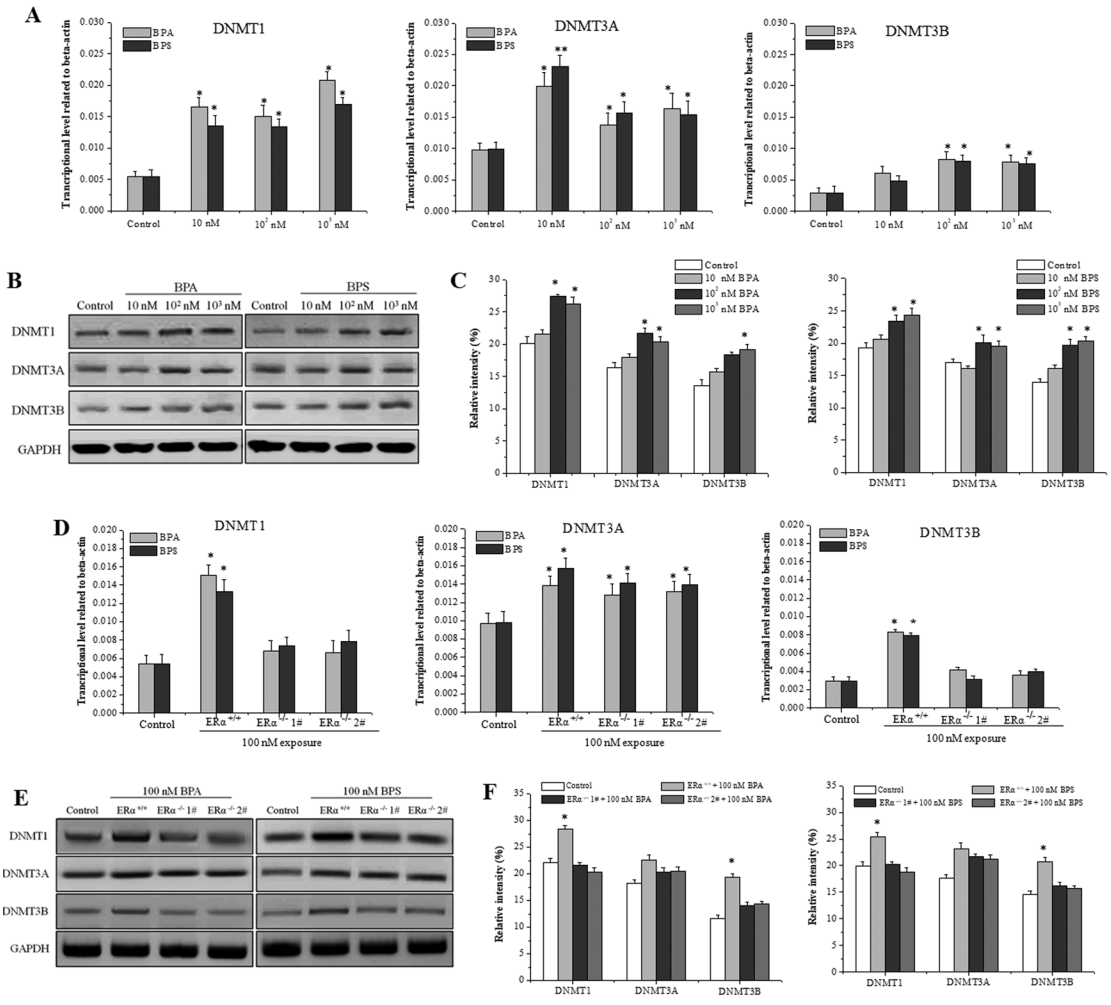

Figure S10 ERα-mediated DNA methyltransferases (DNMTs) expression in MCF7 cells under bisphenol A/S (BPA/BPS) exposure. (A) Quantitative reverse transcript-PCR (qRT-PCR) analysis of the expression of DNMT1, DNMT3A and DNMT3B in MCF-7 cells after 48 h of vehicle, BPA, or BPS treatment. Data represent mean ± SD of five independent experiments. (B) Immunoblotting of DNMT1, DNMT3A and DNMT3B proteins in MCF-7 cells at 48 h after vehicle, BPA, or BPS treatment. (C) Quantitative analysis of DNMT1, DNMT3A and DNMT3B proteins shown in (B) with Image J software. The relative intensity was calculated by the ratio relative to the GAPDH intensity. Data represent mean ± SD of three independent experiments. (D) qRT-PCR analysis of the expression of DNMT1,

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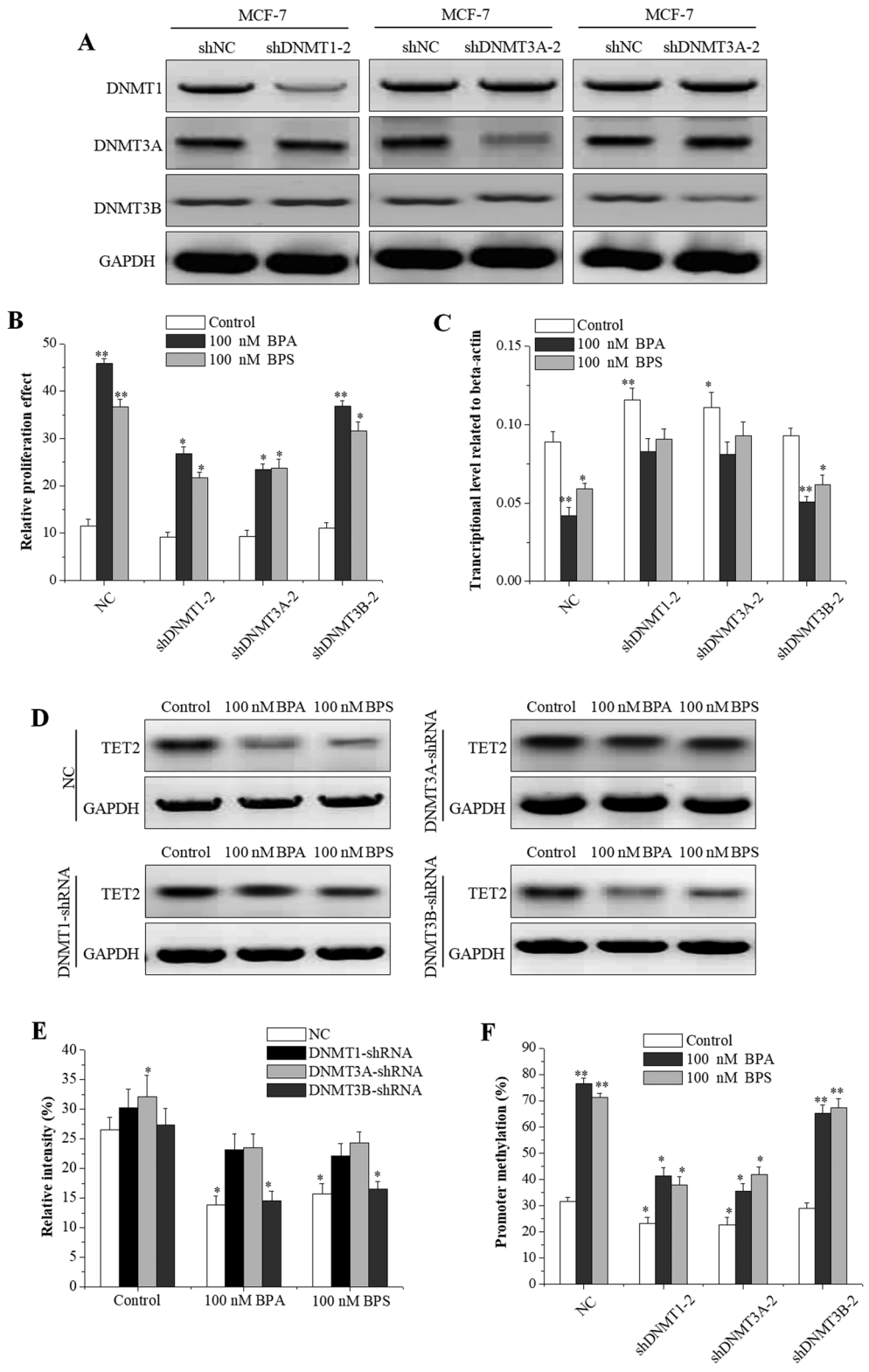

Figure S11 Cell proliferation, ten-eleven translocation 2 (TET2) expression and promoter methylation of MCF-7 cells transfected with the second shRNA constructs for DNA methyltransferase 1 (DNMT1), DNMT3A and DNMT3B upon bisphenol A

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