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Supplementary Information for

Loss of p57^{KIP2} expression confers resistance to contact inhibition in human androgenetic trophoblast stem cells

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Supplementary methods

Immunostaining

Cells were fixed with 4% PFA for 10 minutes and permeabilized with 0.3% Triton X-100 for 5 minutes. After blocking with 2% FBS/PBS, the cells were incubated with primary antibodies for >6 hours at 4°C. The following primary antibodies were used: anti-GATA3 (1:100; Clone D13C9; Cell Signaling Technology, Danvers, MA), anti-TFAP2C (1:200; Clone 6E4/4; Santa Cruz Biotechnology, Dallas, TX), anti-KRT7 (1:100; Clone SP52; Abnova, Taipei, Taiwan), anti-$p57^{KIP2}$ (1:1000; Clone 57P06; Neomarkers, Fremont, CA). Alexa Fluor 555- or Alexa Fluor 488-labeled antibody (Cell Signaling Technology) was used as a secondary antibody, and Hoechst 33258 (Dojindo, Tokyo, Japan) was used for nuclear staining. The images were obtained using a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan).

CNV analysis

Genomic DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel, Düren, Germany) and was subjected to genome-wide SNP genotyping using Japonica Array v2 (Toshiba, Tokyo, Japan) (1). The obtained data were processed according to the Best Practice Workflow protocol using the software Axiom Analysis Suite (ver.1.1.1.66), and CNV analysis was performed with the software Axiom CNV Tool (ver.1.1).

RNA-seq

Primary CT cells isolated from CHMs and ~80% confluent TS^{mole} cells were used for RNA-seq. Total RNA was extracted using the RNeasy Mini Kit and RNase-free DNase (QIAGEN, Hilden, Germany). RNA integrity (RINe) values were measured using TapeStation 2200 (Agilent, Santa Clara, CA) and all samples had RINe values of >9. RNA-seq libraries were constructed from the total RNA employing the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. These libraries were sequenced on Hiseq 2500 (Illumina) with 101-base paired-end reads. The reads were mapped to the human genome build 38 (hg38) using TopHat (2) with the RefSeqGene annotation. The expression levels (FPKM) of genes were calculated using Cufflinks (v2.1.0) (2).

Real-time PCR

Total RNA was extracted as described above, and first-strand cDNA was synthesized using the PrimeScriptTM II 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan). Oligo dT primer was used for reverse transcription. Real-time PCR was performed using TB Green Premix Ex TaqTM II (Takara) and StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative expression levels were calculated by ΔΔCT method using *GAPDH* and *B2M* as the

internal control gene. Sequences of the primers are shown in Dataset S3.

WGBS

WGBS was performed by the post-bisulfite adaptor-tagging (PBAT) method (3, 4). Briefly, genomic DNA was extracted from TS^{mole} cells with phenol/chloroform extraction and ethanol precipitation, and treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). PBAT libraries were sequenced on the Illumina HiSeq 2500 (Illumina) with 101-bp single-end reads. The sequenced reads were mapped to hg38 using Bismark (v0.9.0) (5), and methylation levels were calculated with the Bismark methylation extractor. Reads from both strands were combined to calculate the methylation level of each CpG site. CpGs covered with more than 5 reads were considered.

Flow cytometry

For staining of $p57^{KIP2}$, cells were fixed with 70% EtOH for >12 hours at 4°C and permeabilized with 0.1% Triton X-100 for 10 minutes. After blocking with 2% FBS/PBS, the cells were incubated with an anti-$p57^{KIP2}$ antibody (1:200; Neomarkers) for >6 hours at 4°C. Then, the cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:500; Cell Signaling Technology), 20 μg/ml RNase (Thermo Fisher Scientific), and 50 μg/ml PI (Dojindo) for an hour at room temperature. For cell cycle analysis, cells were incubated with 10 μM BrdU (Wako) for an hour and collected using TrypLE. The collected cells were fixed and permeabilized. After blocking with 2% FBS/PBS, the cells were incubated with an anti-BrdU antibody (1:100; Clone BU1/75 (ICR1); Abcam, Cambridge, UK) for >6 hours at 4°C. Then, the cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rat IgG (1:500; Cell Signaling Technology), 20 μg/ml RNase (Thermo Fisher Scientific), and 50 μg/ml PI for an hour at room temperature. Flow cytometry was carried out using a FACSAria II (BD Biosciences, Franklin Lakes, NJ), and the data were analyzed using FlowJo (BD Biosciences).

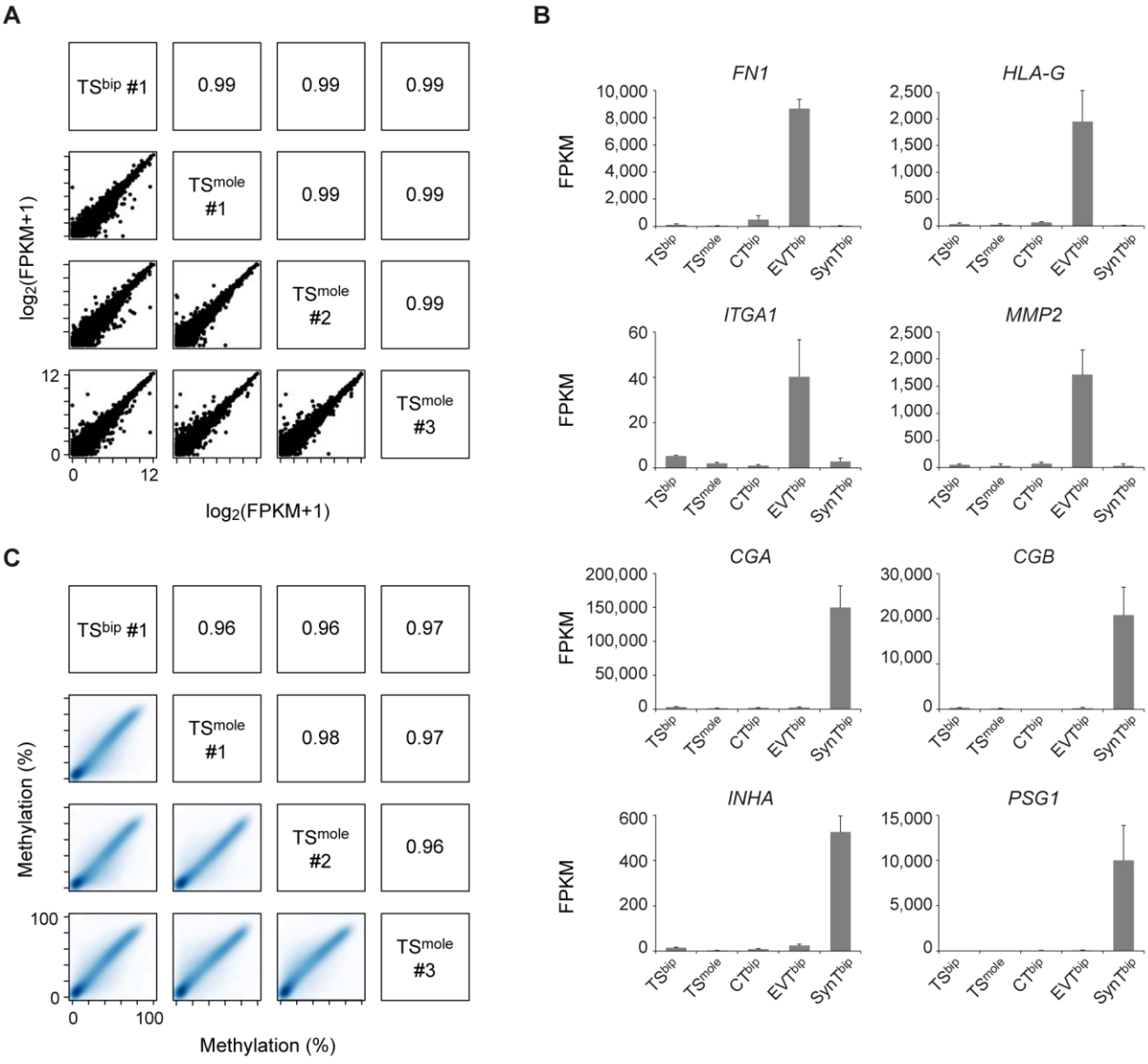

A
TSbip #1
0.99
0.99
0.99
TSmole #1
0.99
0.99
TSmole #2
0.99
TSmole #3
log2(FPKM+1)
12
0
0
12
log2(FPKM+1)
B
FN1
HLA-G
ITGA1
MMP2
CGA
CGB
INHA
PSG1
FPKM
TSbip
TSmole
CTbip
EVTbip
SynTbip
C
TSbip #1
0.96
0.96
0.97
TSmole #1
0.98
0.97
TSmole #2
0.96
TSmole #3
Methylation (%)
100
0
0
100
Methylation (%)

Fig. S1. Transcriptome and methylome profiling of TS^{mole} cells.

(*A*) Scatterplots and Pearson correlation coefficients for comparison of gene expression levels between TS^{bip} and TS^{mole} cells. TS^{bip} and TS^{mole} cells had similar transcriptome profiles. (*B*) Expression levels of differentiation markers in TS^{bip} and TS^{mole} cells. *FN1*, *HLA-G*, *ITGA1*, and *MMP2* are the markers of EVT cells, and *CGA*, *CGB*, *INHA*, and *PSG1* are the markers of syncytiotrophoblast (SynT) cells. These genes showed very low expression levels in TS^{bip} and TS^{mole} cells. CT^{bip}, EVT^{bip}, and $SynT^{bip}$ indicate primary trophoblast cells isolated from bi-parental placentas, and their RNA-seq data were obtained in our previous studies (6, 7). Expression levels are shown as means + SDs (n = 3). (*C*) Scatterplots and Pearson correlation coefficients for the comparison of DNA methylation between TS^{bip} and TS^{mole} cells. 10-kb windows were used for the comparison. TS^{bip} and TS^{mole} cells had similar methylome profiles.

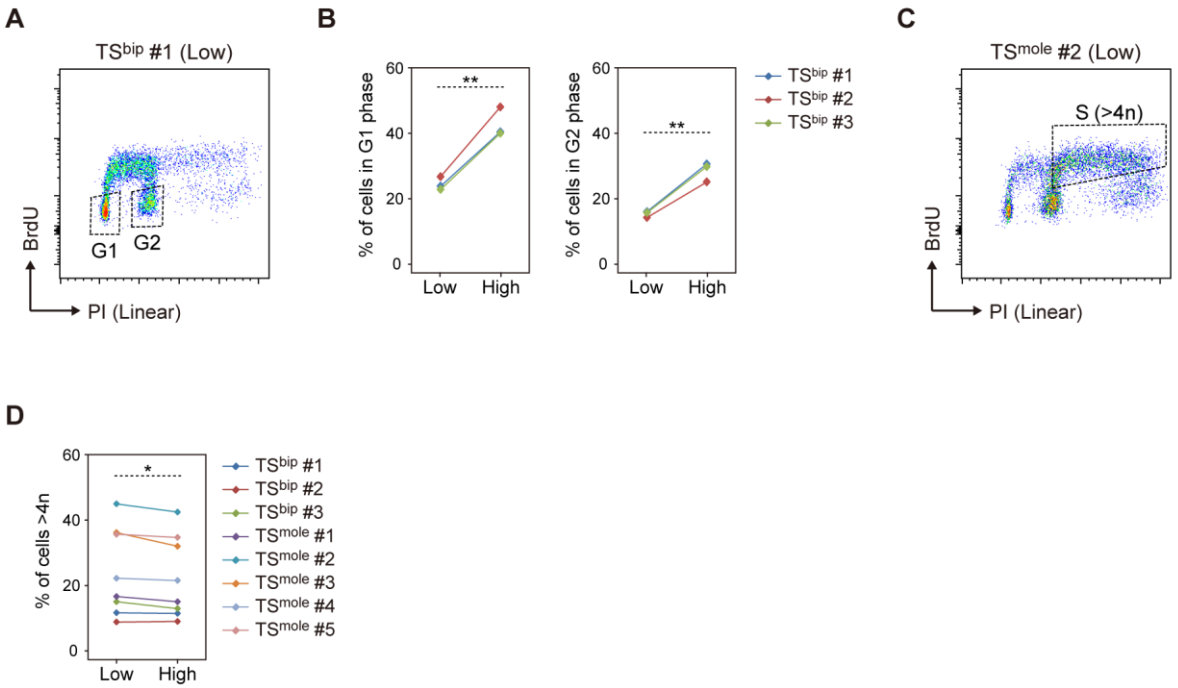


Fig. S2. Cell cycle analysis of TS^{bip} and TS^{mole} cells.

(*A*) Definition of cells in G1 and G2 phases. Cells were seeded at a low density (5,000 cells/cm^2). After 2 days of culture, the cells were labeled with BrdU and analyzed by flow cytometry. Nuclei were stained with PI. Note that the density plot is identical to that shown in Fig. 3C. (*B*) Summary of the cell cycle analysis in (A). Three TS^{bip} cell lines were analyzed. We did not analyze TS^{mole} cells because of the high proportions of cells >4n, which made it difficult to define cell cycle phases. Statistical analysis was performed by Student's paired *t*-test. **, $P < 0.01$. (*C*) Cell cycle analysis of TS^{mole} #2. Cells were seeded at a low density (5,000 cells/cm^2). After 2 days of culture, the cells were labeled with BrdU and analyzed by flow cytometry. Nuclei were stained with PI. Cells >4n in S phase are indicated. Note that most cells >4n are BrdU-positive. (*D*) Proportions of cells >4n in TS^{bip} and TS^{mole} cells. Cells were seeded at a density of 5,000 (Low) or 100,000 cells/cm^2 (High). After 2 days of culture, the cells were analyzed by flow cytometry. The proportion of cells >4n was marginally reduced by increased cell density. Statistical analysis was performed by Student's paired *t*-test. *, $P < 0.05$.

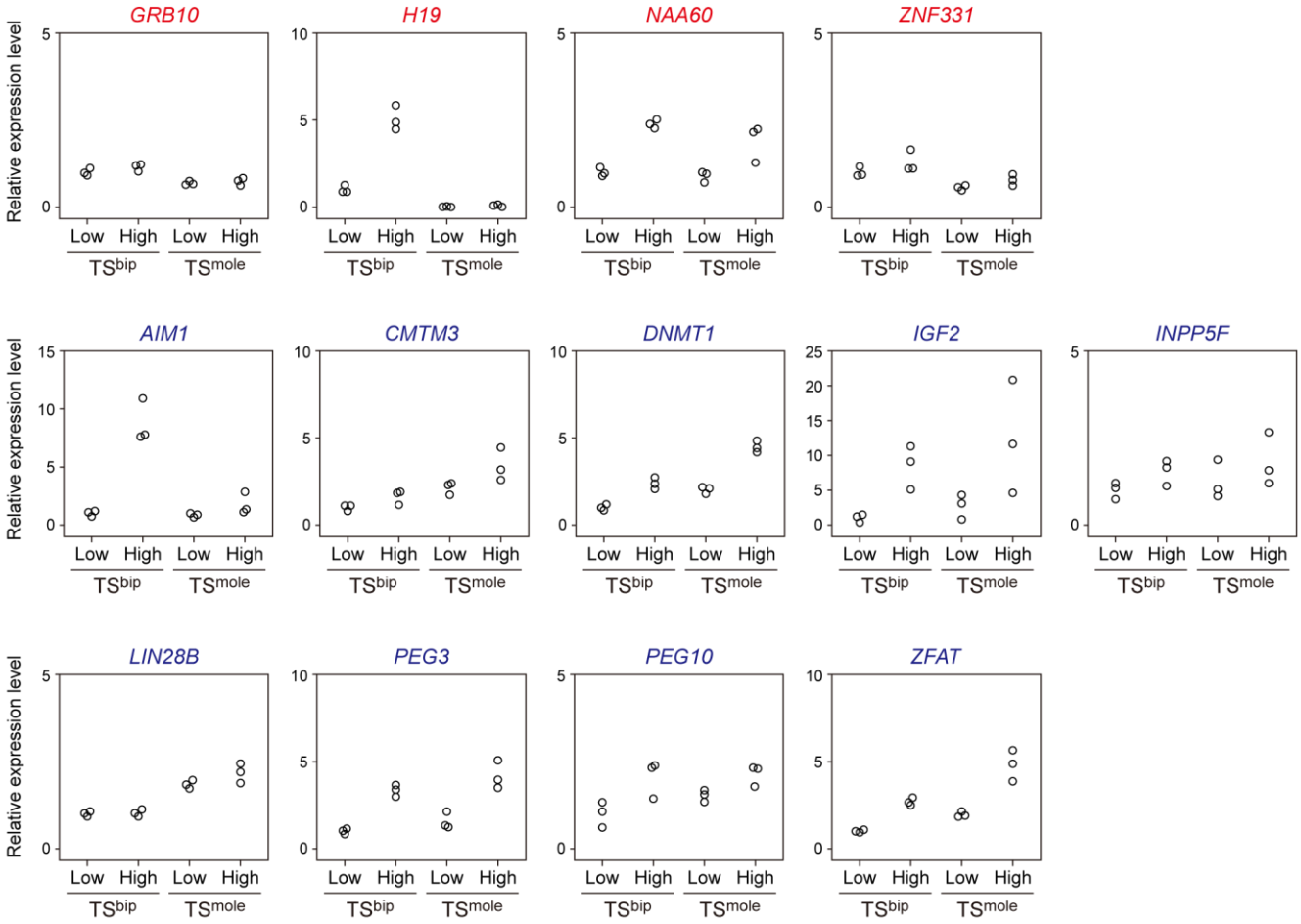
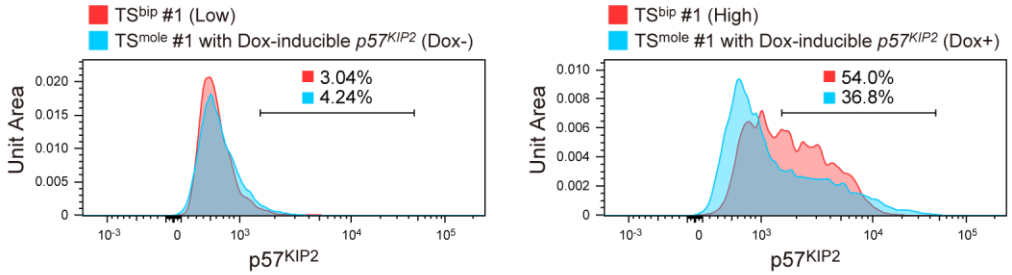


Fig. S3. Effects of cell density on the expression levels of imprinted genes

Three TS^{bip} and three TS^{mole} cell lines were seeded at a density of 5,000 (Low) or 100,000 cells/cm^2 (High) and cultured for two days. Gene expression levels were analyzed by quantitative real-time PCR. The 14 imprinted genes labeled in Fig. 2A, which had >10 FPKM in TS^{bip} cells, were analyzed (the data for *p57^{KIP2}* are shown in Fig. 4D). Maternally and paternally expressed genes are represented in red and blue, respectively. In TS^{bip} cells, high cell density increased the expression levels of some imprinted genes, such as *H19*, *NAA60*, *AIM1*, *IGF2*, *p57^{KIP2}*, and *PEG3*. Among them, *p57^{KIP2}* was most strongly induced (Fig. 4D).

A



B

CGTCCCTCCGCAGCACACATCCACG **ATG** GAG CGT CTT GTC GCC CGT GGG ACC

gRNA

C

p57KIP2(+) clone #1

A

CGTCCCTCCGCAGCACACATCCACG **ATG** GAG CGT CTT GTC GCC CGT GGG ACC

CGTCCCTCCGCAGCACACATCCACG ---------- CC CGT GGG ACC

p57KIP2(+) clone #2

CGTCCCTCCGCAGCACACATCCACG **ATG** GAG CGT CTT GTC GCC CGT GGG ACC

CGTCCCTCCGCAGCACACATCCACG **ATG** GAG CGT CTT GTC GCC CGT GGG ACC

p57KIP2(-) clone #1

CGT ---------- CTT GTC GCC CGT GGG ACC

CGTCCCTCCGCAGCACACATCCACG ---------- GT GGG ACC

p57KIP2(-) clone #2

CGTCCCTCCGCAGCACACATCCACG **A** ------- G C - T CTT GTC GCC CGT GGG ACC

CGTCCCTCCGCAGCACACATCCACG **A** --- GAG CGT CTT GTC GCC CGT GGG ACC

TCTTGTCGCCCGTGGGACCTTCCCAGTG

p57KIP2(-) clone #3

CGTCCCTCCGCAGCACACATCCACG --- **G** GAG CGT CTT GTC GCC CGT GGG ACC

CGTCCCTCCGCAGCACACATCCACG **ATG** GA ---------- CC

CA

p57KIP2(-) clone #4

CGTCCCTCCGCAGCACACATCCACG **A** ------ AG CGT CTT GTC GCC CGT GGG ACC

CGTCCCTCCGC ---------- GT CTT GTC GCC CGT GGG ACC

Fig. S4. Characterization of TS^{mole} cells with Dox-inducible *p57^{KIP2}* and *p57^{KIP2}* knockout clones.

(*A*) Expression levels of p57^{KIP2} induced by Dox. TS^{mole} cells with Dox-inducible *p57^{KIP2}* were seeded at a high density (100,000 cells/cm^2) and cultured for 24 hours. Then, the cells were treated with 20 ng/ml Dox for 24 hours, and p57^{KIP2} expression was analyzed by flow cytometry. Nuclei were stained with PI. The proportions of p57^{KIP2}-positive cells are indicated. For comparison, we analyzed TS^{bip} cells that were seeded at a density of 5,000 (Low) or 100,000 cells/cm^2 (High) and cultured for two days. The fluorescence intensity of p57^{KIP2} induced by Dox was comparable to that observed in TS^{bip} cells cultured at the high density, which predominantly ranged from 10^3 to 10^4. Similar results were obtained with three independent TS^{mole} cell lines. (*B*) The gRNA target sequence for *p57^{KIP2}*. The expression pattern and genomic structure of the *p57^{KIP2}* gene are shown on top. The start codon is in red and the position of the gRNA is indicated by a solid line. (*C*) Sanger sequencing of genomic DNA from p57^{KIP2}(+) and (-) clones. Insertions are shown in blue and deletions are indicated by dotted lines. The parental alleles were indistinguishable due to the lack of available SNPs.

Table S1. Summary of TS^{mole} and TS^{bip} cell lines

Cell line	Gestational age	Sex*	Maternal age	Gravida	Para	Proportion of cells >4n (%)**
TS^{mole} #1	8 weeks	F	38	2	0	15.1
TS^{mole} #2	8 weeks	F	28	4	3	42.6
TS^{mole} #3	8 weeks	F	47	3	1	32.1
TS^{mole} #4	8 weeks	F	33	NA	NA	21.6
TS^{mole} #5	8 weeks	F	26	4	2	34.8
TS^{bip} #1	6 weeks	F	NA	NA	NA	11.5
TS^{bip} #2	7 weeks	M	NA	NA	NA	9.0
TS^{bip} #3	7 weeks	F	NA	NA	NA	13.0
TS^{bip} #4	7 weeks	F	NA	NA	NA	NA

*Determined by real-time PCR of *EIF1AY*; **Measured by flow cytometry. NA, not available.

Dataset S1. Transcriptome profiling of TS and primary CT cells

Expression levels of Refseq genes are shown as $\log_2$ (FPKM+1). The data of TS^{bip}, CT^{bip}, EVT^{bip}, and $SynT^{bip}$ cells were obtained in our previous studies (6, 7).

Dataset S2. DNA methylation levels of known DMRs in TS^{bip} and TS^{mole} cells

Methylation levels of known DMRs (8) were calculated based on the WGBS data. The data of TS^{bip} cells were obtained in our previous study (7). M, maternal DMR; P, paternal DMR; Pla, placenta-specific DMR.

Dataset S3. List of primer sequences used in this study

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