Single-Cell DNA-methylation analysis reveals epigenetic chimerism in preimplantation embryos

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Supplementary Materials for

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Materials and Methods:

Single-cell DNA-methylation analysis:

Sample preparation and methylation sensitive restriction digest

For single-cell analysis 8-cell embryos were mechanically separated into individual blastomeres. These, or individual oocytes were harvested directly into 5 µL CellsDirect (Invitrogen, 10:1 Resuspension buffer:Lysis enhancer) and lysed at 75˚C for 10 min.

DNA-methylation sensitive restriction digest was performed by adding 5 µL of reaction mix (0.5 U *BstU*I (NEB), methylated and unmethylated control templates (1 ag each) in 2X NEBuffer 4) and incubating at 60˚C for 2 h. To inactivate *BstU*I, 1 µL Proteinase K (10 mg/mL) was added and allowed to incubate at 50˚C for 2 h followed by 10 min heat inactivation at 95˚C.

Primer design and controls

Three primers were designed for each analyzed locus and each control template (Table S1): A forward primer upstream of the *BstU*I restriction site (Long Forward, LF), a forward primer downstream of the *BstU*I restriction site (Short Forward, SF), and a common reverse primer (R) (see Figure 1A). Methylation or hemimethylation of the *BstU*I site prevents cleavage of the template, allowing PCR amplification the long (LF-R) fragment, while an unmethylated *BstU*I site will be cleaved, preventing PCR amplification of the long (LF-R) fragment. DNA-methylation of individual CpGs within a given germline DMR is representative for the entire locus. All CpGs analyzed by *BstU*I restriction digestion in this study lie within these very well defined differentially methylated regions and are therefore considered diagnostic. Amplification of the short (SF-R) fragment is independent of the methylation status at the *BstU*I site and serves as control.

Control templates were obtained by PCR amplification of lambda DNA (Fermentas) using the primer pairs λU LF+λU R (to amplify the region used as the unmethylated lambda template) and λM LF+λM R (to amplify the region used as the methylated lambda template). The latter was DNA-methylated in vitro with CpG Methytransferase (M.SssI, NEB) overnight following the manufacturer's instruction.

Post-digest pre-amplification

We performed a pre-amplification step for 6 imprinted loci and two (methylated and unmethylated respectively) control DNA templates on the restriction-digested single-cell DNA. 15 µL PreAmp master mix containing a pool of all 24 primers (including 6 control primers) with a final concentration of 500 nM each were added to the digested DNA (PreAmp master mix: 5 µL GoTaq 5X buffer (Promega), 2.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 2.5 µL of 500 nM primer pool, 0.125 µL of 5 U/µL GoTaq DNA Polymerase, 4.375 µL dH_2O).

Pre-amplification was then performed by initial denaturation at 95˚C for 10 min, followed by 27 cycles of 30 sec denaturation at 95˚C and 4 min annealing/extension at 60˚C. 2 µL of 4U/µL Exonuclease I (Enzymatics) were added to 5 µL of pre-amplified products and incubated at 37˚C for 30 min to remove unincorporated primers. Exonuclease I was heat inactivation at 80˚C for 15 min. The final product was diluted 5-fold with TE Buffer for site-specific amplification.

Site-specific real-time amplification

Single-cell DNA-methylation analysis was performed on 48.48 Dynamic Arrays using the Biomark System (Fluidigm). Assay inlets were loaded with 5 µL of combined forward and reverse primers for each assay at a final concentration of 5 µM in the provided assay loading reagent (Fluidigm). Testing 6 imprinted loci and 2 control templates required 16 assays (SF+R and LF+R for each template), therefore each 48.48 Dynamic Array allowed three technical replicates of the 16 assays to be carried out simultaneously. Each sample inlet was loaded with 5 µL sample premix (3 µL 2X SsoFast EvaGreen Supermix (Bio-Rad), 0.3 µL 20X DNA binding Dye Sample Loading Reagent (Fluidigm) and 2.7 µL 5-fold diluted ExoI treated samples). A chip run consisted of 30 cycles of on-chip qPCR followed by melting curve analysis.

Data analysis

Ct values were calculated using Fluidigm Real-Time PCR Analysis software. The data was first filtered by eliminating wells displaying unspecific amplification i.e. incorrect melting temperatures. The melting profile of each reaction was used to confirm that the right PCR product was amplified (within +/- 1.5°C of expected melting temperature). The lowest valid Ct value of the three technical replicates of each assay was used. A locus in a single cell was considered normally methylated if the Ct value was <30 for both the short and long amplicon. It was considered hypomethylated if the Ct value was <30 for the short and >30 for the long amplicon. A data point was scored as not amplified if it had a wrong melting temperature and Ct >19. A data point was considered indeterminate and removed from analysis if it had a wrong melting temperature and Ct <19. Data points where both the short and long PCR reaction failed to amplify were removed from analysis.

Mice and embryos

Trim28$^{f/f}$ (or *Tif1β*$^{L2/L2}$) mice and the transgenic line C57BL/6-Tg(Zp3-cre)93Knw/J were used to breed maternal *Trim28*-null embryos (*11*). Time of the vaginal plug was taken as E0.5 of embryonic development. Mouse-keeping and procedures were strictly performed according to IACUC (Singapore) regulations.

Pronuclear transfer and embryo transfer

Pronuclear transfer experiments were performed according to McGrath et al. (*21*). Cumulus cells were removed from cumulus-oocyte-complexes 16 h post-hCG by gentle pipetting in 10 mg/mL hyaluronidase. The zygotes were placed into M2 holding medium supplemented with 5 µg/mL cytochalasin B. Manipulations were performed using an inverted microscope (Olympus-IX 71) equipped with a micromanipulation system (Olympus). Zygotes were immobilized with a holding pipette. For enucleation the pronuclei and surrounding cytoplasm were gently aspirated into the biopsy pipette as a membrane-bound karyoplast. The donor pronuclei were aspirated from the donor zygote by using the same procedure. While the donor pronucleus remained in the biopsy pipette a small amount of lentivirus envelope was aspirated before both were released underneath a zona-pellucida of the enucleated recipient zygote. The manipulated zygotes were incubated at 37ºC for 30 min until successful fusion was observed. Successfully fused zygotes were transferred to oviducts of E0.5 pseudopregnant ICR females and developed to term.

Immunofluorescence staining

Embryos were fixed in 4% PFA for 20 min at room temperature before permeabilization in 0.5% PBT for 5 min. Embryos were then blocked 1 h in 0.1% PBT with 10% FBS. Primary antibody incubation was performed at 4˚C over night (KAP1, Abcam, 1:100). Embryos were washed in blocking solution 2x for 1 h. Secondary antibody incubation was performed for 1 h at room temperature.

Bisulphite Conversion and COBRA:

For DMR methylation state analysis of tail biopsies, 1 μg of genomic DNA was used for conversion according to the manufacturer's protocol (Sigma, MOD50-1KT). Converted DNA was eluted in 20 μl and 1 μl of converted DNA was used in a 25 μL PCR reaction (Hotstar, Qiagen 203205). Nested PCR was conducted for COBRA of the *H19* DMR. For primer sequences see Table S1. PCR products were digested for COBRA analysis (*Dra*I, Promega; *BstU*I, NEB).

Additional Author notes:

Author contribution: C.L., L.F.C. and D.M.M. performed the experiments; L.F.C. designed the DNA-methylation analysis assay; S.B., D.M.M. and B.B.K. performed animal work; L.F.C. and S.R.Q. performed statistical analysis; D.M.M. and D.S. designed the study; B.B.K., D.S., W.F.B., S.R.Q. assisted in experimental design and supervised the study; D.M.M. wrote the manuscript.

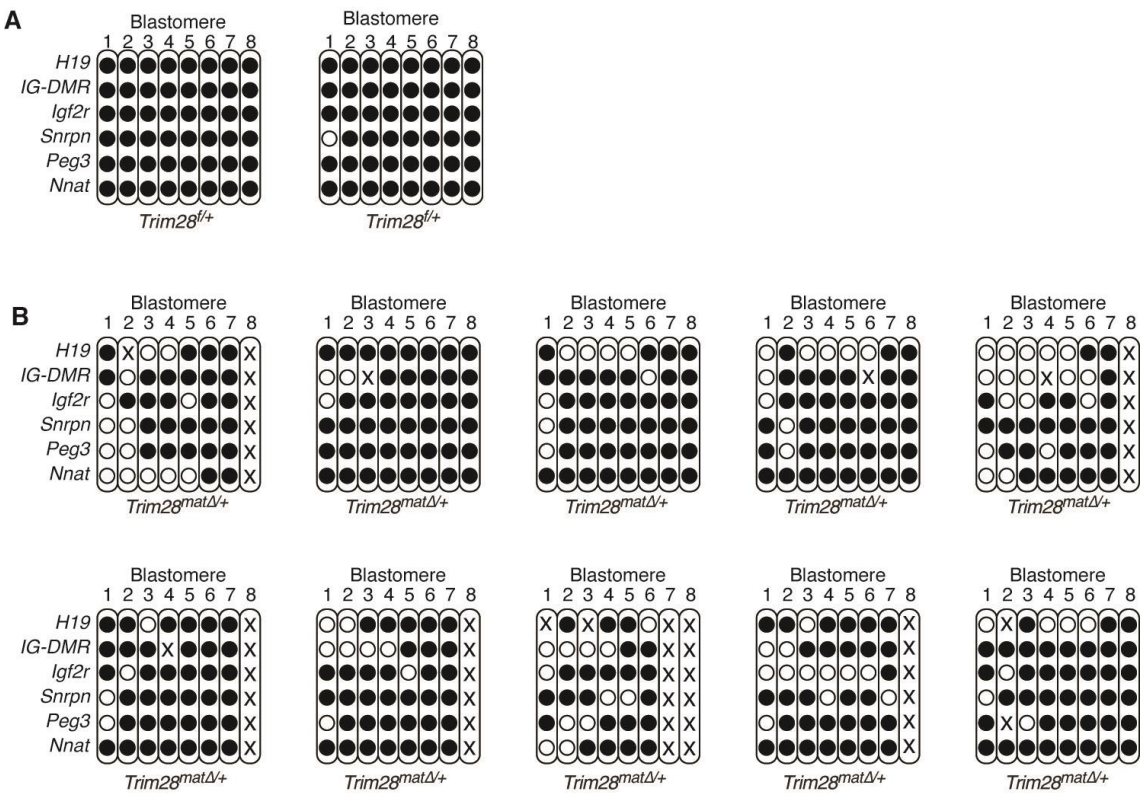

Fig. S1

Additional 8-cell-stage embryos analyzed for DMR-methylation defects.

(A) The methylation state for six DMRs in all blastomeres of two additional *Trim28*$^{f/+}$ 8-cell-stage embryos is shown. (**B)** Same analysis as in (**A**) showing the methylation state for six DMRs in blastomeres of ten additional *Trim28*$^{mat\Delta/+}$ 8-cell-stage embryos. (Black dot: DNA-methylation, i.e. large and short amplicons were detected after *BstU*I digest; White dot: no DNA-methylation, i.e. only the small PCR fragment was detected; x: neither amplicon was detected.)

Table S1.

Oligonucleotides used in this study.

Primer	Symbol	Sequence
Peg3 Long Forward	Peg3 LF	GTGCGTAGAGTGCTGTGCTC
Peg3 Short Forward	Peg3 SF	GCTCCCAAGGGTAACTGACA
Peg3 Reverse	Peg3 R	CGAGGCCTGGACCTATAGAA
H19 Long Forward	H19 LF	GACCATGCCCTATTCTTGGA
H19 Short Forward	H19 SF	GATTGCGCCAAACCTAAAGA
H19 Reverse	H19 R	GTCCACGAGGTACCAGCCTA
Snrpn Long Forward	Snrpn LF	CCGCAGTAGGAATGCTCAA
Snrpn Short Forward	Snrpn SF	ACTAGCGCAGAGAGGAGAGG
Snrpn Reverse	Snrpn R	ATCCACAAGCCCAGCTGAC
Igf2r Long Forward	Igf2r LF	CCCTTTGAACTCTCCCTTTG
Igf2r Short Forward	Igf2r SF	CTTTTGAGCTTGCCTCTCTTG
Igf2r Reverse	Igf2r R	AGGATTCGAAGGGTTCTGTG
Nnat Long Forward	Nnat LF	AAACAGCTGGCATGGGTTTA
Nnat Short Forward	Nnat SF	GTGTGTCGAACCAAGAGGCT
Nnat Reverse	Nnat R	CGGTGTAAAATGGAGGGAAG
IG-DMR Long Forward	IGDMR LF	CTATGGACTGGTGCCAAGGT
IG-DMR Short Forward	IGDMR SF	GCCGCTATGCTATGCTGTTT
IG-DMR Reverse	IGDMR R	TTTAGCCATCCCCTGTGCT
Methylated Lambda Long Forward	λM LF	TACCCATTGCTCACGAAAAA
Methylated Lambda Short Forward	λM SF	AGGCATCACCGAAAATTCAG
Methylated Lambda Reverse	λM R	TTTAGCCATCCCCTGTGCT
Unmethylated Lambda Long Forward	λU LF	ACAGGCAGTTTCGATTACGG
Unmethylated Lambda Short Forward	λU SF	TGCCCACACAAGTGGTTTAA
Unmethylated Lambda Reverse	λU R	GGCTGTACCGGACAATGAGT
Cobra H19 DMR outer forward	H19_OF	GAGTATTTAGGAGGTATAAGAATT
Cobra H19 DMR outer reverse	H19_OR	ATCAAAAACTAACATAAACCCCT
Cobra H19 DMR inner forward	H19_IF	GTAAGGAGATTATGTTTATTTTTGG
Cobra H19 DMR inner reverse	H19_IR	CCTCATTAATCCCATAACTAT