Single-Cell DNA-Methylation Analysis Reveals Epigenetic Chimerism in Preimplantation Embryos

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Single-Cell DNA-Methylation Analysis Reveals Epigenetic Chimerism in Preimplantation Embryos

Chanchao Lorthongpanich,[1]*† Lih Feng Cheow,[2]† Sathish Balu,[1] Stephen R. Quake,[2,3] Barbara B. Knowles,[1,4] William F. Burkholder,[2,5,6]‡ Davor Solter,[1,7] Daniel M. Messerschmidt[1,8]‡

Epigenetic alterations are increasingly recognized as causes of human cancers and disease. These aberrations are likely to arise during genomic reprogramming in mammalian preimplantation embryos, when their epigenomes are most vulnerable. However, this process is only partially understood because of the experimental inaccessibility of early-stage embryos. Here, we introduce a methodologic advance, probing single cells for various DNA-methylation errors at multiple loci, to reveal failed maintenance of epigenetic mark results in chimeric mice, which display unpredictable phenotypes leading to developmental arrest. Yet we show that mouse pronuclear transfer can be used to ameliorate such reprogramming defects. This study not only details the epigenetic reprogramming dynamics in early mammalian embryos but also suggests diagnostic and potential future therapeutic applications.

Genomic imprinting ensures adequate monoallelic, parent-of-origin–specific gene expression patterns in mammals (*1*). The importance of this process is reflected in increasing recognition of human syndromes related to imprinting defects (*2*, *3*). Because genomic imprinting relies on locus-specific differentially methylated regions (DMRs), these defects can arise from both genetic and epigenetic mutations. These epimutations often simultaneously affect multiple loci, causing complex and little-understood phenotypes (*2*, *3*).

Recent studies suggest that imprints are established in a two-step process of DNA methylation in the germline and subsequent, site-specific DNA-methylation maintenance during reprogramming in the preimplantation embryo (*4*, *5*). DNA methyl transferase 1 (DNMT1), primordial germ cell protein 7 (PGC7)/STELLA, zinc finger protein 57 (ZFP57), and tripartite motif–containing 28 (TRIM28) are each required for DMR protection in the face of global DNA demethylation (*6*–*13*). It has been proposed that detrimental epimutations most likely occur when these maintenance mechanisms fail (*2*, *4*). ZFP57, which only binds methylated imprinted alleles, mediates specific DNA-methylation maintenance, recruiting DNMT1 through the scaffolding protein TRIM28 (*10*, *14*, *15*). Active targeting of DNMT1 is thought to be required because nuclear DNMT1 levels are very low in the preimplantation embryo facilitating genome-wide DNA demethylation (*16*). Disrupting this targeting complex very early in development, by eliminating maternal *Trim28*, causes stochastic DMR demethylation, which results in the phenotypic variability proposed to be caused by epigenetic chimerism (*11*, *12*). Similarly, loss of the oocyte-specific DNMT1 variant results in mosaic defects in postimplantation embryos, whereas full deletion of DNMT1 causes complete loss of imprinting (*6*, *7*, *13*). Although it has been inferred that epigenetic mosaicism could emerge during the early preimplantation phase of development (*6*, *12*), how such chimerism is established in time and space has not been conclusively demonstrated. To address these dynamics, we combined methylation-sensitive restriction digestion (*17*) and multiplexed quantitative real-time polymerase chain reaction (PCR) in a microfluidics device (Fig. 1A, detailed description in supplementary information) to analyze, simultaneously, six imprinted loci in single cells. The assay was validated by addressing DMR methylation in control oocytes (*Trim28*^{f}). Attesting to its robustness, we found that paternally imprinted regions were indeed unmethylated (Fig. 1B, *Trim28*^{f}), whereas maternally imprinted loci were reliably shown to be methylated (Fig. 1C, *Trim28*^{f}). We next used a *Zp3-cre* knock-out strategy (*11*) to genetically remove *Trim28* from oocytes (*Trim28*^{matΔ}) and addressed the effect of its absence on DMR methylation. DMR-methylation patterns remained unchanged compared with those of controls (Fig. 1, B and C, *Trim28*^{matΔ}), demonstrating that TRIM28 is not required for imprinting maintenance in growing and mature oocytes.

In contrast to the stable environment in the mature oocyte, epigenetic reprogramming initiates shortly after fertilization (*18*). Because the oocyte and embryo are transcriptionally silent until zygotic gene activation (ZGA) at the late two-cell stage, early embryonic processes, such as protection of

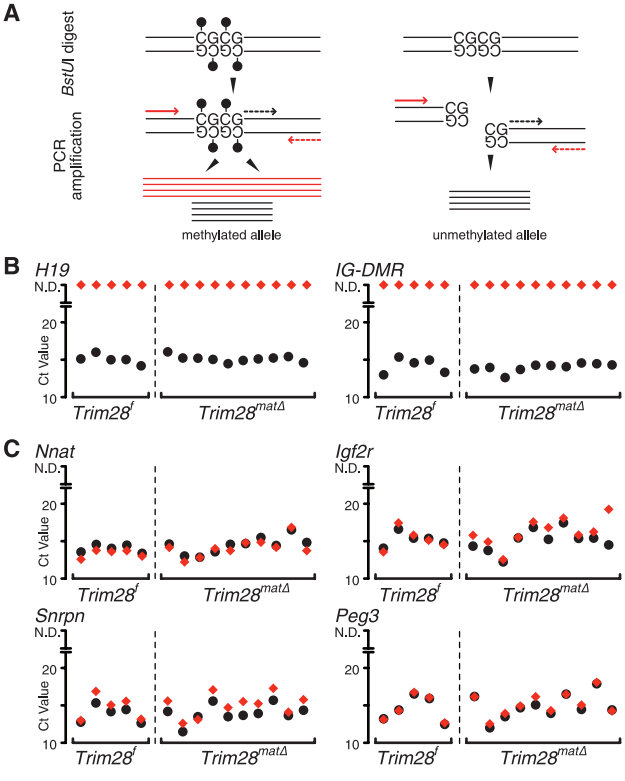

Fig. 1. Single-cell DNA-methylation analysis. (**A**) Illustration of the single-cell DMR-methylation assay. DNA methylation (lollipops) prevents *BstUI* digest; both primer combinations will generate PCR products (red/black). If unmethylated, *BstUI* cuts and the large amplicon (red) cannot be generated. Analysis of (**B**) paternally methylated and (**C**) maternally methylated DMRs in *Trim28*-null oocytes. Black dots represent cycle threshold (Ct) values of the short amplicon (positive control); red diamonds represent Ct values for the amplicon spanning the *BstUI* restriction site. N.D., not detectable.

[1]Mammalian Development Group, Institute of Medical Biology (IMB), A*STAR, Singapore. [2]Microfluidics Systems Biology Lab, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore. [3]Department of Bioengineering and Department of Applied Physics, Stanford University and Howard Hughes Medical Institute, Stanford, CA, USA. [4]The Jackson Laboratory, Bar Harbor, ME, USA. [5]Genome Institute of Singapore, A*STAR, Singapore. [6]Nanyang Technological University, Singapore. [7]Duke–National University of Singapore, Graduate Medical School, Singapore. [8]Developmental Epigenetics and Disease, IMCB, A*STAR, Singapore.

*Present address: Siriraj Center of Excellence for Stem Cell Research (SISR), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

†These authors contributed equally to this work.

‡Corresponding author. E-mail: danielm@imcb.a-star.edu.sg (D.M.M.); wfburkholder@gmail.com (W.F.B.)

inherited imprints, rely on maternal gene products. Mutation, reduction, or lack of maternal factors; developmental noise; or even environmental cues can result in epimutations at imprinted gene loci. Maternal deletion of *Trim28* causes such a defect, which cannot be rescued by *Trim28* reexpression from the paternal allele at ZGA (*11*). We and others have previously proposed that a combination of DNA-methylation–dependent targeting of TRIM28/DNMT1 through ZFP57, maternal absence, and paternal reexpression of TRIM28 could cause stochastic and mosaic imprinting defects in these embryos (*12*).

To conclusively demonstrate the occurrence and frequency of such defects, we used the single-cell assay to examine imprinted DMR states in maternal *Trim28*-null ($Trim28^{matΔ/+}$) and control ($Trim28^{f/+}$) eight-cell embryos. Single-cell resolution was achieved by mechanically separating embryos into eight blastomeres (*14*), which were individually analyzed (Fig. 2A). Nearly all control blastomeres (99.3%, $n = 288$ DMRs) carried methylated alleles for each examined locus (Fig. 2B and fig. S1A), demonstrating very robust imprint maintenance during ongoing epigenetic reprogramming. However, blastomeres derived from maternal *Trim28*-null embryos displayed highly variable degrees of hypomethylation at all tested loci (Fig. 2B and fig. S1B). Loss of DNA methylation was not uniform across analyzed DMRs, embryos, or even among blastomeres of a given embryo. A histogram of the number of demethylated loci per cell, observed in 121 blastomeres from 16 maternal mutant embryos (Fig. 2B and fig S1B), followed a Poisson distribution, showing that the loss of DNA methylation occurred randomly and independently at similar average rates across all cells (Fig. 2D). In agreement with observations in postimplantation maternal-null *Trim28* embryos (*11*), we noted that the *H19* locus had a significantly higher incidence of demethylation (44/116 blastomeres) compared with the demethylation rate averaged across all loci (Fisher's exact test, $P = 6.2 \times 10^{-5}$). However, the degree of demethylation is notably lower than predicted assuming full penetrance of the maintenance defect and semiconservative DNA replication. This scenario should result in six demethylated and two hemimethylated blastomeres at the eight-cell stage, which is only occasionally observed (fig S1B), considering that *BstU*I is inhibited by DNA hemimethylation (*19*). This incomplete penetrance can be explained by methylation protection, mediated by factors such as PGC7/STELLA and possibly by rare ZFP57/TRIM28-independent binding of DNMT1. It is important to note that ZFP57 can interact with a hemimethylated target sequence (*20*), which can attract paternal TRIM28 translated after ZGA (*11*) to restore full methylation. However, ZFP57 cannot interact with demethylated loci, which are not restored.

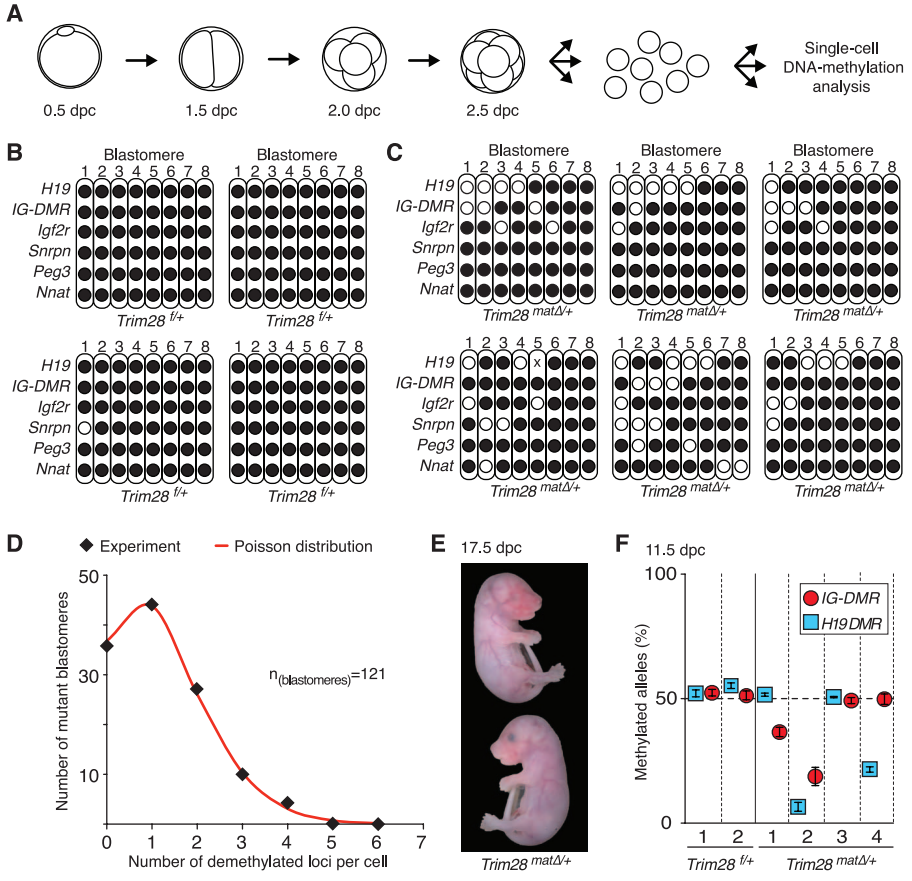

Fig. 2. Mosaic DMR-demethylation defects in maternal *Trim28* mutants. (**A**) Eight-cell embryos were dissected, and blastomeres were subjected to analysis. The methylation state for six DMRs in single blastomeres of (**B**) four $Trim28^{f/+}$ and (**C**) six $Trim28^{matΔ/+}$ eight-cell embryos is shown. Black dot indicates DNA methylation, both amplicons were detected after *BstU*I digest; white dot, no DNA methylation, only the small amplicon was detected; X, neither amplicon was detected. (**D**) The number of demethylated loci per cell follows a Poisson distribution (Poisson parameter λ equals the experimental mean, 1.2; data were pooled across six loci for maternal *Trim28*-null 121 blastomeres). (**E**) Both missing and developing eye of an 18.5–days postcoitus (dpc) maternal *Trim28*-null fetus is shown. (**F**) Control and maternal-mutant 11.5-dpc embryos were tested for *H19*/IG-DMR methylation by bisulfite conversion/pyrosequencing. Methylation levels in mutants range between normal (50%) and fully unmethylated, indicating chimeric imprinting defects. Error bars, standard deviation of three technical replicates.

Despite incomplete penetrance, examining only 6 of 21 known germline DMRs reveals the prodigious potential for imprinting defect combinations, the phenotypic outcome of which will further depend on blastomere viability, and their contribution to the embryo proper. This mosaicism may account for phenotypic traits, such as occasional hemi-anophthalmia in maternal-null *Trim28* fetuses (Fig. 2E), which are hard to explain by simple genetics. Additionally, this chimerism translates into incomplete demethylation patterns when analyzing DMR methylation of DNA from whole-embryo lysates (Fig. 2F).

Although highly expressed in oocytes (*11*), the role of TRIM28 in imprint maintenance is restricted to postfertilization stages. To further define the temporal requirement for TRIM28, we created two different embryonic scenarios by pronuclei transfer (*21*). Transferring control ($Trim28^{f/+}$) pronuclei into enucleated mutant ($Trim28^{matΔ/+}$) zygotes should create a temporal reduction of maternal TRIM28, phenocopying the maternal *Trim28*-null defects. On the other hand, placing maternal *Trim28*-null pronuclei into enucleated control zygotes should provide TRIM28 to the mutant genome much earlier than achieved by ZGA, thus rescuing the defects. Control transfers (control pronuclei placed into control recipients) generated 64% viable weanlings (Fig. 3A). Yet, when control pronuclei were transferred into *Trim28*-null recipients, this survival was strongly reduced (25%, Fig. 3B), supporting our premise that maternal TRIM28 is required immediately postfertilization, before, and possibly beyond ZGA. However, 25% survival contrasts with no survival of unmanipulated maternal *Trim28*-null embryos (Fig. 3D). Carry-over of DNA-bound TRIM28 in control pronuclei may increase the yield of viable offspring.

Transferring the genetic material from a defective into a healthy, enucleated recipient egg or zygote can overcome mitochondrial disease (*22*–*24*). We asked whether early epigenetic defects

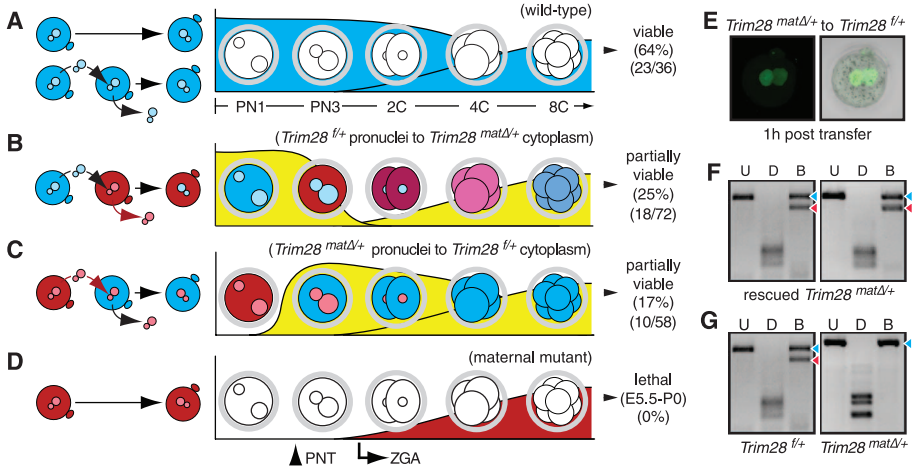

Fig. 3. Phenocopy and rescue of the maternal *Trim28*-null phenotype. (**A**) Control pronuclear transfer (64% viability) and schematic representation of early preimplantation development depicting maternal/zygotic *Trim28* expression. Mutant and control-derived embryos, pronuclei, and nuclei are shaded red and blue, respectively. PN, pronuclear stage; C, cell embryo; PNT, pronuclear transfer. (**B**) Phenocopy by transfer of control pronuclei into maternal *Trim28*-null zygotes (25% viability). (**C**) Partial rescue by transfer of mutant pronuclei into enucleated control zygotes (17% viability). (**D**) Zygotic expression of TRIM28 alone is not sufficient to support development (0% viability). E5.5, embryonic day 5.5; P0, postnatal day 0. (**E**) Immunostaining of TRIM28 in maternal *Trim28*-mutant pronuclei 1 hour posttransfer. (**F**) *H19* DMR methylation in tail biopsies from rescued mice displaying methylated (red arrowhead) and unmethylated (blue arrowhead) alleles comparable to a [(**G**), left] control transfer animal and in contrast to a [(G), right] maternal-null 12.5-dpc embryo with hypomethylated *H19* DMR. *Dra*I digestion shows efficiency of bisulphite conversion; the *Bst*UI restriction site is protected from bisulphite mutagenesis if methylated. U, undigested; D, *Dra*I; B, *Bst*UI.

caused by maternal deficiencies could also be rescued by this approach (Fig. 3C). Maternal *Trim28*-null pronuclei, when placed into enucleated control zygotes, displayed nuclear TRIM28 within 1 hour of transfer (Fig. 3E). Indeed, early TRIM28 presence allowed 17% of these embryos to develop into pups, in contrast to the total lack of viable pups obtained from unmanipulated maternal *Trim28*-null zygotes (Fig. 3D). These "rescue pups" became fertile adults and showed normal *H19* DMR methylation (the most frequently affected imprinted locus in maternal-null *Trim28* mutants) in tail biopsies (10/10), comparable to controls and in contrast to maternal-null embryos (Fig. 3, F and G). The rescue again shows that *Trim28* is not necessary during oocyte maturation because *Trim28* null-derived pronuclei do support normal development. The incompleteness of the rescue is most likely explained by a very early, postfertilization requirement for TRIM28. Simultaneous transfer of both pronuclei requires their close proximity, which is reached only at the late pronuclear stage 3, when reprogramming and replication is well under way (*25*). Prolonged exposure to a maternal *Trim28*-null environment could cause sufficient, irreversible damage to some pronuclei and thus reduce rescue efficiency.

During early embryonic development, DNA methylation at imprinted gene loci is robustly maintained by several maternal factors, including TRIM28. By analyzing preimplantation embryos on a single-cell level, we show that absence of maternal *Trim28* causes highly asynchronous, aberrant demethylation. This creates complex chimeras, providing an explanation for the innumerable permutations of defects and composite phenotypes previously described (*11*). Similar scenarios are likely to apply to other maternal factors but also to human imprinting syndromes. The variable degree of DMR hypomethylation observed in patients (*2*) likely reflects pools of normally and aberrantly imprinted cells, similar to the case of the maternal-null *Trim28* embryos (Fig. 2F). This type of mosaicism has been proposed for several imprinting syndromes, including transient neonatal diabetes (TND) (*26*). Moreover, other rare human syndromes or embryonic defects resulting in molar pregnancies and abortion could also have their yet-unrecognized basis in the random multiplicity of epimutations (*2*). The single-cell DNA-methylation assay is a powerful tool to address such defects and is well suited for accurate diagnosis in these patients or to address the occurrence of rare, random imprinting defects suspected to result from assisted reproductive technology (*27*). Last, we provide initial evidence for a potential therapeutic approach. In mice, pronuclear transfer into healthy enucleated zygotes can ameliorate epimutations caused by the absence of maternal *Trim28*, a scenario that might apply to other maternal epigenetic regulators or to any maternal cytoplasmic defect. Homozygous mutations in *ZFP57* have been described in some TND pedigrees (*28*), yet it remains to be seen whether homozygous female patients are fertile and their (maternal-null) progeny display imprinting defects. A pronuclear transfer approach, as it is already explored in humans to prevent mitochondrial disease (*22*–*24*), may be developed to prevent the development of epimutation-based imprinting syndromes.

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Supplementary Materials

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Materials and Methods
Fig. S1
Table S1
References

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