<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Single-Cell DNA-methylation analysis reveals epigenetic chimerism in preimplantation embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Lorthongpanich, Chanchao; Cheow, Lih Feng; Balu, Sathish; Quake, Stephen R.; Knowles, Barbara B.; Burkholder, William F.; Solter, Davor; Messerschmidt, Daniel M.</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>2013</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10220/19858">http://hdl.handle.net/10220/19858</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>© 2013 American Association for the Advancement of Science. This is the author created version of a work that has been peer reviewed and accepted for publication by Science, American Association for the Advancement of Science. It incorporates referee’s comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<a href="http://dx.doi.org/10.1126/science.1240617">http://dx.doi.org/10.1126/science.1240617</a>].</td>
</tr>
</tbody>
</table>
Single-cell DNA-methylation analysis reveals epigenetic chimerism in preimplantation embryos

Chanchao Lorthongpanich\textsuperscript{1,2\textasciitilde}, Lih Feng Cheow\textsuperscript{3\textasciitilde}, Sathish Balu\textsuperscript{1}, Stephen R. Quake\textsuperscript{4,5}, Barbara B. Knowles\textsuperscript{1,6}, William F. Burkholder\textsuperscript{3,7,8\*}, Davor Solter\textsuperscript{1,9}, Daniel M. Messerschmidt\textsuperscript{1,10\*}

\textsuperscript{1}Mammalian Development Group, Institute of Medical Biology (IMB), A*STAR, Singapore
\textsuperscript{2}Current address: Siriraj Center of Excellence for Stem cell Research (SISR), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand
\textsuperscript{3}Microfluidics Systems Biology Lab, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore
\textsuperscript{4}Departments of Bioengineering and Applied Physics, Stanford University and Howard Hughes Medical Institute, USA
\textsuperscript{5}Visiting Investigator, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore
\textsuperscript{6}Adjunct and emeritus staff at The Jackson Laboratory, Bar Harbor, ME USA
\textsuperscript{7}Adjunct Senior Research Scientist, Genome Institute of Singapore (GIS), A*STAR, Singapore
\textsuperscript{8}Adjunct Associate Professor, Nanyang Technological University (NTU), Singapore
\textsuperscript{9}Duke-NUS, Graduate Medical School, Singapore
\textsuperscript{10}Developmental Epigenetics & Disease, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore

\textasciitilde These authors contributed equally to this work

\* Corresponding authors: danielm@imcb.a-star.edu.sg, wfburkholder@gmail.com
Abstract:

Epigenetic alterations are increasingly recognized as cause 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 marks 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 potentially future therapeutic applications.
Main Text:

Genomic imprinting ensures adequate mono-allelic, 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). Since 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). DNMT1, PGC7/STELLA, ZFP57, and TRIM28 are each required for the 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, ZFP57 mediates specific DNA-methylation maintenance, recruiting DNMT1 through the scaffolding protein TRIM28 (10, 14, 15). Active targeting of DNMT1 is thought to be required as 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 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<sup>f</sup>). Attesting to its robustness we found that paternally imprinted regions were indeed unmethylated (Fig. 1B, Trim28<sup>f</sup>), whereas maternally imprinted loci were reliably shown to be methylated (Fig. 1C, Trim28<sup>f</sup>). We next used a Zp3-cre-knock-out strategy (11), to genetically remove Trim28 from oocytes (Trim28<sup>mat∆</sup>) and addressed the effect of its absence on DMR-methylation. Remarkably, DMR-methylation patterns remained unchanged compared to controls (Fig. 1B-C, Trim28<sup>mat∆</sup>), 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). Since the oocyte and embryo are transcriptionally silent until zygotic gene activation (ZGA) at the late 2-cell stage, early embryonic processes, such as protection of 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 re-expression 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 re-expression of TRIM28 could cause stochastic and mosaic imprinting defects in these embryos (12).
To conclusively demonstrate the occurrence and frequency of such defects, we employed the single-cell assay to examine imprinted DMR-states in maternal Trim28-null (Trim28<sup>mαtΔ/+</sup>) and control (Trim28<sup>f/+</sup>) 8-cell embryos. Single-cell resolution was achieved by mechanically separating embryos into eight blastomeres (14), which were individually analyzed (Fig. 2A). Virtually 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). Notably, 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 to the demethylation rate averaged across all loci (Fisher’s exact test, p=6.2x10<sup>-5</sup>). However, the degree of demethylation is notably lower than predicted assuming full penetrance of the maintenance defect and semi-conservative DNA-replication. This scenario should result in six demethylated and two hemimethylated blastomeres at the 8-cell stage, which is only occasionally observed (fig S1B), considering that BstUI 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.

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 of 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.

Interestingly, 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 post-fertilization stages. To further define the temporal requirement for
TRIM28 we created two different embryonic scenarios by pronuclei transfer (21).

Transferring control (Trim28+/+) pronuclei into enucleated mutant (Trim28\(\text{mat}^-/+\)) 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 post-
fertilization, prior to, and possibly beyond, ZGA. However, 25% survival contrasts with no
Lorthongpanich et al.

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 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 one hour of transfer (Fig. 3E). Indeed, early TRIM28 presence allowed 17% of these embryos to develop into pups, a clear 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. 3F, G). The rescue again shows that Trim28 is not necessary during oocyte-maturation as Trim28 null-derived pronuclei do support normal development. The incompleteness of the rescue is most likely explained by a very early, post-fertilization 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 underway (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 importantly, 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 be based on the random multiplicity of epimutations, yet unrecognized (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). Finally, 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, which might apply to other maternal epigenetic regulators or indeed to any maternal cytoplasmic defect. Homozygous mutations in ZFP57 have been described in some TND pedigrees (28), yet it remains to be seen if 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 in future be developed to prevent the development of epimutation-based imprinting syndromes.
References and Notes:


15. X. Zuo et al., *jbc.org*.


29. We thank H. Wollmann for scientific input and proofreading the manuscript. This work was supported by IMB, IMCB, and a Visiting Investigator Program grant from the Joint Council Office, A*STAR to S.R.Q. (project-0921100080). S.R.Q. is a founder, consultant, and member of the advisory board and equity holder of Fluidigm.
Figure Legends:

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

**Fig. 2.** Mosaic DMR-demethylation defects in maternal Trim28 mutants. (A) 8-cell embryos were dissected and blastomeres subjected to analysis. The methylation state for six DMRs in single blastomeres of (B) four Trim28^{fl/+} and (C) six Trim28^{matΔ/+} 8-cell embryos is shown. (Black dot: DNA-methylation, both amplicons were detected after BstUI 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 18.5dpc maternal Trim28-null fetus is shown. (F) Control and maternal-mutant 11.5dpc embryos were tested for H19/IG-DMR-methylation by bisulfite conversion/pyro-sequencing. Methylation levels in mutants range between normal (50%) and fully unmethylated indicating chimeric imprinting defects. (dpc, days post-coitus)

**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. (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). (E) Immunostaining of TRIM28 in maternal Trim28-mutant pronuclei 1h post-transfer. (F) H19 DMR-methylation in tail biopsies from rescued mice displaying methylated (red arrowhead) and unmethylated alleles (blue arrowhead) comparable to a (G, left) control transfer animal and in contrast to a (G, right) maternal-null 12.5dpc embryo with hypomethylated H19 DMR. DraI digestion shows efficiency of bisulphite conversion; The BstUI restriction site is protected from bisulphite mutagenesis if methylated. (Mutant/control-derived embryos, pronuclei, and nuclei are shaded red/blue, respectively. PN-pronuclear stage; C-cell embryo; PNT-pronuclear transfer; ZGA-zygotic gene activation; U- undigested; D-DraI; B-BstUI)
Supplementary Materials:

www.sciencemag.org

Materials and Methods

Figure S1

Table S1