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Report

DNA Methylation Is Dispensable for the Growth and Survival of the Extraembryonic Lineages

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Summary

DNA methylation regulates development and many epigenetic processes in mammals [1], and it is required for somatic cell growth and survival [2, 3]. In contrast, embryonic stem (ES) cells can self-renew without DNA methylation [4–6]. It remains unclear whether any lineage-committed cells can survive without DNA-methylation machineries. Unlike in somatic cells, DNA methylation is dispensable for imprinting and X-inactivation in the extraembryonic lineages [7–12]. In ES cells, DNA methylation prevents differentiation into the trophectodermal fate [13]. Here, we created triple-knockout (TKO) mouse embryos deficient for the active DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b (TKO) by nuclear transfer (NT), and we examined their development. In chimeric TKO-NT and WT embryos, few TKO cells were found in the embryo proper, but they contributed to extraembryonic tissues. TKO ES cells showed increasing cell death during their differentiation into epiblast lineages, but not during differentiation into extraembryonic lineages. Furthermore, we successfully established trophoblastic stem cells (ntTS cells) from TKO-NT blastocysts. These TKO ntTS cells could self-renew, and they retained the fundamental gene expression patterns of stem cells. Our findings indicated that extraembryonic-lineage cells can survive and proliferate in the absence of DNA methyltransferases and that a cell's response to the stress of epigenomic damage is cell type dependent.

Results and Discussion

Ability of Dnmt TKO Cells to Develop into Embryonic and Extraembryonic Lineages In Vivo

We previously established mouse ES cells deficient for all three active DNA methyltransferases, Dnmt1/Dnmt3a/Dnmt3b triple knockout (TKO), in which DNA methylation is absent [6]. In the present study, we asked whether there is any lineage-committed cell type, including the extraembryonic lineages, into which TKO cells differentiate and then grow normally. Nuclear transfer (NT), the transfer of a nucleus from one cell into an enucleated oocyte, allows us to reconstruct totipotent embryos, which can develop to full term when normal somatic or ES cells are used as the source of the nucleus. Here we applied this technique by using TKO ES cells as the nucleus donor for NT to examine the role of DNA methylation in embryonic and extraembryonic differentiation.

We first examined the in vitro developmental potential of NT embryos derived from TKO ES cells. In preimplantation development, three lineages are generated in the blastocyst: the trophectoderm (TE), primitive endoderm (PE), and epiblast (Figure S1A available online) [14]. The epiblast forms the three germ layers for the future somatic cells and germ cells in the embryo proper, whereas the TE and PE form future extraembryonic tissues, the placenta, and yolk sac. Preimplantation development until the blastocyst stage occurred normally when either TKO or WT ES cells were used as the donor (Figures S1B and S1C). No apparent abnormalities, including increased developmental arrest or malformation of the preimplantation embryo, were found in the NT embryos derived from the TKO ES cells. All the NT blastocysts (n = 30) derived from the TKO ES cells expressed CDX2 (Figure S1D), an important transcription factor for the TE lineage. These rudimentary examinations revealed that preimplantation development occurred even in the absence of the three Dnmts.

We next attempted to induce embryonic and extraembryonic lineage differentiation from the TKO NT embryos in vivo. Because the NT embryos from TKO ES cells are predicted to show severe embryonic lethality based on phenotypes of Dnmt1 knockout or Dnmt3a/Dnmt3b double knockout mice [4, 15], the NT embryos, which we labeled with a GFP transgene, were rescued by aggregating them with WT embryos to form chimeras (Figure 1A).

At E10.5, and unlike the WT NT cells, the few TKO-derived GFP-positive cells found in the embryo (Figure 1B; Table S1) were mainly in the heart, suggesting that they were circulating cells that did not contribute to the embryonic lineages. Interestingly, TKO-derived GFP-positive cells were found in the placenta and yolk sac of the chimeric fetuses from aggregated TKO NT and WT embryos (Figure 1B; Figure S1E, Table S1). Later in development (E17.5 to E18.5), TKO-derived cells were found in the placenta and yolk sac, but not in the embryo (Figures S1F–S1L, Table S1). The inability of TKO cells to contribute to the embryonic lineages was supported by other in vivo approaches, including the generation of chimeric mice and teratoma formation via TKO ES cells (Figures S1M and S1N).

Notably, the initial integration of TKO ES cells into the inner cell mass (ICM, E4.5) and their contribution to the embryo proper (E8.5) were observed at early embryonic stages (Figure S1O), suggesting that the initial commitment of ES cells to the epiblastic lineage occurred in the absence of DNA methylation. Collectively, our results indicated that TE and PE differentiation occurs even in the absence of DNA methylation, whereas the maintenance of the embryonic cell lineages is highly dependent on DNA methylation.

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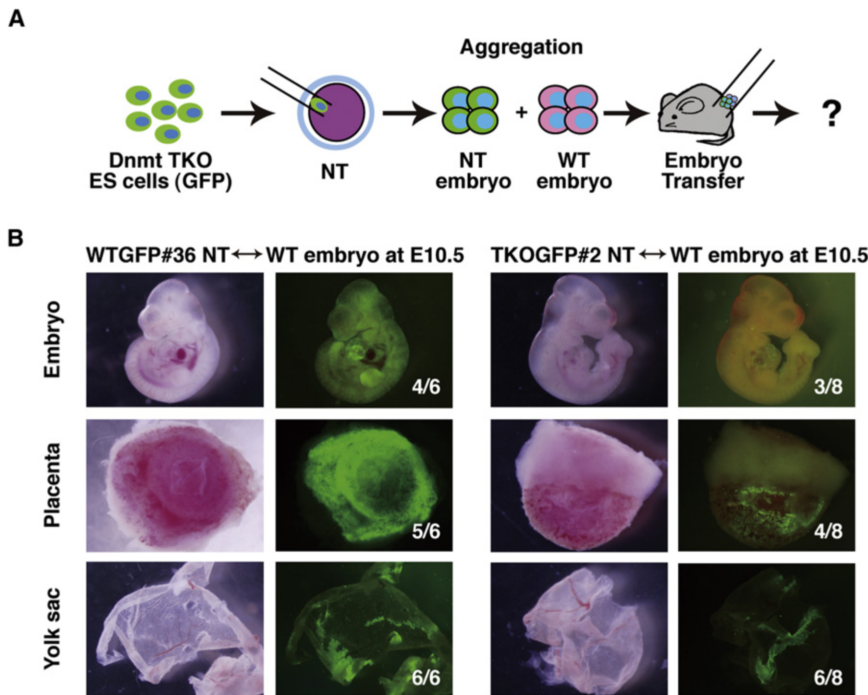

Figure 1. Nuclear Transfer of TKO ES Cells into Enucleated Oocytes

(A) Schematic representation of the experimental procedure for preparing NT embryos via Dnmt1, Dnmt3a, and Dnmt3b triple knockout (TKO) ES cells as a donor. To study in vivo development, NT embryos from WT or TKO ES cells that expressed a GFP transgene (WTGFP#36, TKOGFP#2) were aggregated with WT embryos and transferred to pseudopregnant females.

(B) In vivo development of NT embryos aggregated with WT embryos. Left: Chimeric WT NT embryos (WTGFP#36 NT↔WT embryos). Right: Chimeric TKO NT embryos (TKOGFP#2 NT↔WT embryos). Light and fluorescent micrographs of the embryo, placenta, and yolk sac at E10.5 are shown. Numbers of GFP-positive chimeras and contribution of NT-derived cells to embryonic and extraembryonic tissues are shown in each panel.

See also Figure S1 and Table S1.

Epiblastic Differentiation of TKO ES Cells and Their Associated Death

To elucidate the cellular process underlying the developmental defects of the TKO NT cells in the embryo proper, we analyzed the growth and differentiation properties of the TKO ES cells during epiblast lineage differentiation in vitro. Previous studies suggested that Dnmt1-deficient cells and TKO cells in embryoid bodies (EBs) have either a differentiation or a growth defect [4, 6]. Here we observed that the TKO ES cells initially formed ball-like cell aggregates as EBs, but these EBs failed to mature into cystic EBs and showed extensive apoptosis in their periphery, where few apoptotic cells were found in the WT EBs (Figure S2A). Interestingly, marker genes for primitive ectoderm and early mesoderm, which are epiblast derivatives, were upregulated in the TKO EBs to the same level as in WT EBs, whereas a pluripotent cell marker was similarly downregulated in the TKO and WT EBs (Figure 2A). These results indicated that TKO ES cells can initiate epiblastic differentiation in EBs. Consistent with this idea, we frequently observed a small number of beating TKO EBs, although they failed to form cysts, indicating differentiation into cardiomyocytes (data not shown).

We also induced the differentiation of TKO ES cells in monolayer culture with retinoic acid (RA), which drives the differentiation of ES cells into various cell types, including neuroectoderm. After RA-induced differentiation, both the WT and TKO cells expressed the neuroectoderm markers (Figure S2B). The RA-induced differentiation caused extensive apoptosis in the TKO cells, but only a few apoptotic cells were detected in the RA-treated WT ES cells (Figure 2B).

DNMT1 inactivation in human cancer cells leads to cell cycle arrest at G2/M and induces the DNA damage response [3]. Consistent with this report, we observed an increased cell population at G2/M (WT 18% versus TKO 41%) and a strong signal for γH2A.X, a DNA double-strand break marker, at multiple foci in the nuclei of the RA-treated TKO ES cells (Figures S2C and S2D and data not shown), suggesting that the DNA damage response was induced in these cells. Consistent with our in vivo chimera studies, these results indicated that specification of the epiblast lineage can occur in the absence of DNA methylation, although the survival of the epiblastic cells is highly dependent on DNA methylation.

Extraembryonic Differentiation and Survival of TKO ES Cells

The TKO NT cells were able to contribute to extraembryonic tissues, so we next investigated the differentiation and survival abilities of TKO ES cells in extraembryonic lineages in vitro. Mouse ES cells deficient for Dnmt1 can differentiate into trophectoderm, and the transcription factor Elf5 was identified as the DNA methylation-dependent gate-keeper to the trophectoderm lineage [13]. By morphological and marker analyses, we found that hypomethylated *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ ES cells and the nonmethylated TKO ES cells efficiently differentiated into the trophectoderm in a monolayer culture when leukemia inhibitory factor (LIF) was omitted from the ES maintenance medium (Figure 3A; Figures S2E and S2F; M. Oda and M. Okano, unpublished observation). Interestingly, morphological observations and the Annexin V apoptosis assay revealed no apparent apoptotic cell death in the TE-differentiated TKO cells (Figure 3A).

Next, we investigated the ability of the TKO ES cells to differentiate into another extraembryonic lineage, PE, by the forced activation of *Gata4,* a key transcription factor for PE differentiation [16]. As observed in the WT ES cells, the TKO ES cells differentiated into PE cells in response to *Gata4* activation (Figure 3B; Figure S2G). Notably, the TKO PE cells proliferated as actively as the WT PE cells even after differentiation and could be passaged a few times (data not shown). Furthermore, the TKO PE cells showed a cell cycle profile similar to that of differentiated WT PE cells (data not shown). Similar to the case for TE differentiation, apoptotic cell death was not apparent in the PE-differentiated TKO cells (Figure 3B).

Together, our in vitro differentiation studies demonstrated that, even in the absence of the DNA methylation machineries, mouse ES cells could differentiate into two extraembryonic

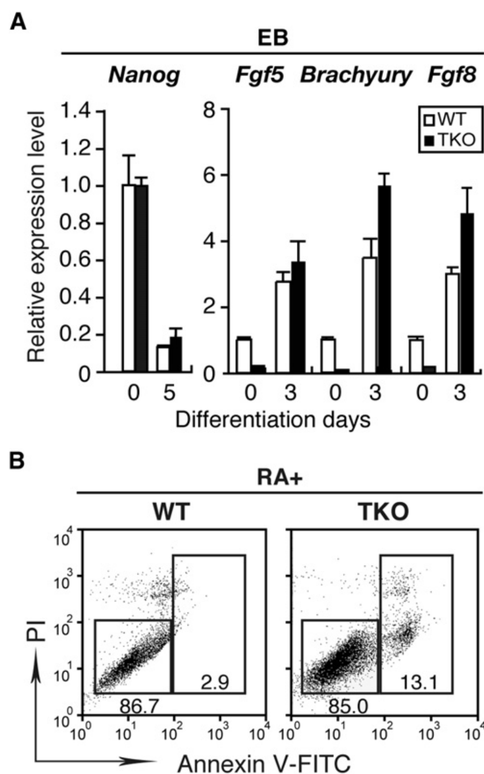

Figure 2. Initial Differentiation to the Epiblast Lineage and Associated Cell Death of TKO ES Cells

(A) ES cell differentiation by embryoid body (EB) formation. Expression of marker genes, *Nanog* (pluripotency), *Fgf5* (primitive ectoderm), *Brachyury*, and *Fgf8* (early mesoderm) in WT (open columns) or TKO (closed columns) EBs by quantitative reverse-transcribed PCR (qRT-PCR). RNA was prepared from undifferentiated ES cells (day 0) or EBs on day 3 or 5. Values indicate the expression level relative to that of WT ES cells. Error bars represent standard deviation (n = 3).

(B) ES cell differentiation induced by retinoic acid (RA). Quantification of the apoptosis of RA-treated ES cells by an Annexin V apoptosis assay via flow cytometry. WT or TKO ES cells differentiated by RA for 3 days were stained with Annexin V-FITC and propidium iodide (PI). The Annexin V-negative/PI-negative population (square gate) represents viable cells, and Annexin V-positive population (rectangular gate) represents cells in early and late apoptosis or necrosis. The percentage of each population is shown inside or below the gate.

See also Figure S2.

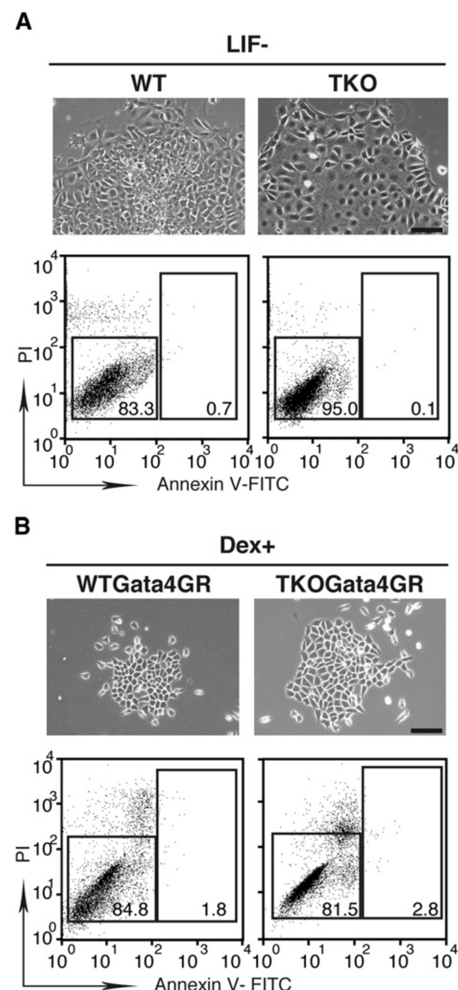

Figure 3. Extraembryonic Differentiation of TKO ES Cells

(A) Trophectoderm differentiation of TKO ES cells induced by the withdrawal of leukemia inhibitory factor (LIF). Morphologies of the WT and TKO ES cells induced to differentiate by the withdrawal of LIF for 4 days (top) and the quantification of apoptotic cells by the Annexin V apoptosis assay (bottom).

(B) Primitive endoderm (PE) differentiation of TKO ES cells by the dexamethasone (Dex)-induced activation of Gata4GR. Morphologies of the PE cells differentiated from WT or TKO ES cells (top) and the Annexin V apoptosis assay of these cells (bottom). Both the WTGata4GR and TKOGata4GR ES cells were induced to differentiate into PE by the addition of Dex for 3 days. Scale bars represent 100 μm. See also Figure S2.

lineages, without displaying growth abnormalities or apoptotic cell death.

Establishment and Characterization of TKO ntTS Cells

Finally, to examine whether DNA methylation is required for the self-renewal of lineage-committed stem cells, we sought to establish a trophoblastic stem (TS) cell line from the TKO NT blastocyst [17, 18]. After cultivating NT blastocysts in the presence of FGF4, we successfully established nuclear transfer TS (ntTS) lines (WT ntTS, TKO ntTS) from both WT and TKO NT blastocysts (21 and 7 ntTS cell lines from 68 WT and 32 TKO NT blastocysts, respectively). Of these, we selected two lines of each genotype that showed a normal karyotype, as determined by the chromosome number and SKY-FISH painting (Figure S3A, Table S2). Immunostaining revealed that CDX2 was reactivated and OCT3/4 was inactivated in the established TKO ntTS cell lines (Figure 4A). Southern blot analysis of IAP retrotransposons and bisulfite sequencing of major satellite repeats showed that no DNA methylation was detectable in these repetitive sequences in the TKO ntTS cell lines (Figure S3B and data not shown), confirming its absence.

The selected TKO ntTS cell lines were able to proliferate for more than 38 passages (approximately 5 months) without displaying any obvious growth abnormalities (Table S2). The SKY-FISH analysis suggested that about 30% of the TKO ntTS cells in the high-passage population retained the normal karyotype, while about 40% of this population showed a karyotype abnormality (Figure 4B; Table S2). Thus, it appeared that the self-renewal and chromosomal segregation of TS cells occurred normally, at least in some populations, even in the absence of DNA methylation.

To examine the differentiation ability of the TKO ntTS cells in vitro, we induced the differentiation of WT and TKO ntTS cells by withdrawing FGF4 from the TS maintenance medium

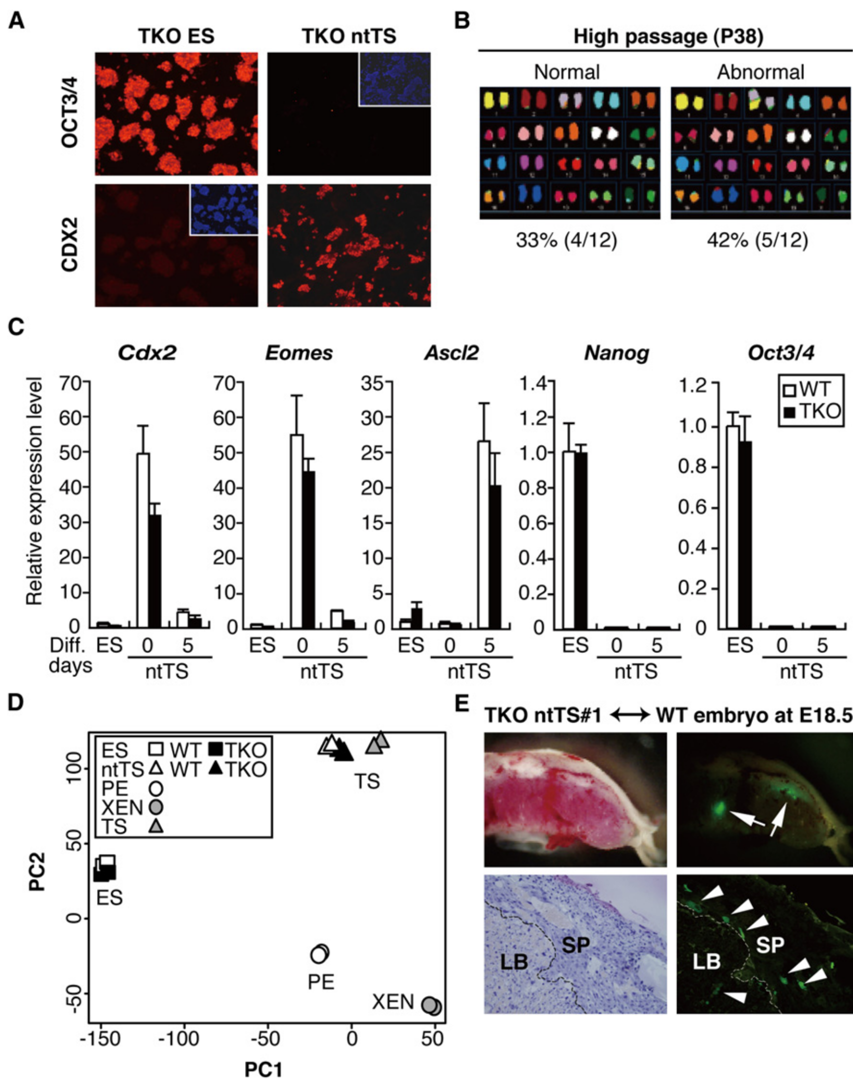

Figure 4. Self-Renewal and Differentiation of TKO ntTS Cells

(A) Immunofluorescence of TKO ES cells (TKOGFP#1) and ntTS cells (TKO ntTS#1) with OCT3/4 and CDX2 antibodies. TKO ntTS cells were negative for OCT3/4 and positive for CDX2. Insets show the cells stained with DAPI.

(B) Karyotype analysis of high-passage TKO ntTS#1 cells with SKY-FISH painting. About 40% of the population showed a karyotype abnormality ([40, XY, −3, +der(3), t(1q;3q)] or [40, XY, t(1q-;3q+)]). P38, passage 38.

(C) Expression of pluripotency and trophectoderm marker genes in undifferentiated and differentiated WT or TKO ntTS cells (WT ntTS#1, TKO ntTS#1) by qRT-PCR. RNA samples of WT and TKO ES cells were also used for comparison. The differentiation of ntTS cells was induced by the withdrawal of FGF4 under feeder-free conditions for 5 days. *Cdx2* and *Eomesodermin* (*Eomes*), trophoblast stem cell markers. Results are shown as in Figure 2A (n = 3).

(D) Principal component analysis via 16 microarray data sets. The data were obtained from WT (open squares) and TKO (closed squares) ES cells, WT (open triangles), and TKO (closed triangles) ntTS cells, TS cells derived from a WT blastocyst (gray-filled triangles), PE cells differentiated from WTGata4GR ES cells by Gata4 activation (open circles), and self-renewing extraembryonic endoderm (XEN) cells derived from a WT blastocyst (gray-filled circles). The PCA results were plotted in the 2D space according to the first and second principal components (PC1, PC2).

(E) E18.5 placenta injected with TKO ntTS#1 cells at the blastocyst stage. Top: Light and fluorescent photographs of a cross section of placenta at E18.5. Green fluorescence (arrows) indicates the contribution of donor TKO ntTS cells, which expressed GFP, to the host placenta. Bottom: Histological cross section of the placenta stained with hematoxylin (bottom left) and its fluorescent micrograph (bottom right). Arrowheads indicate GFP-positive cells. LB, labyrinth region; SP, spongiotrophoblast layer. Notably, the degree of contribution of TKO ntTS cells to the placenta was comparable to that of WT ntTS cells (data not shown).

See also Figure S3 and Tables S2–S4.

and examined the expression of marker genes. TS-specific marker genes *Cdx2* and *Eomes* were expressed in both the WT and TKO undifferentiated ntTS cells. After the withdrawal of FGF4, differentiation marker genes of giant trophoblasts (*Pl1*) and of spongiotrophoblasts (*Tpbp* and *Ascl2*) were induced in the WT and TKO ntTS cells at similar levels (Figure 4C; Figure S3C). These results indicated that the TKO ntTS cells were capable of differentiating in vitro.

To investigate the identity of the TKO ntTS cells from their expression profiles, we compared microarray data from the ntTS cells with those from five cell types that represent the three blastocyst-derived lineages (ICM, TE, and PE) via principal component analysis (PCA). The PCA plots from these data were separated into three distinct groups, consistent with the three origins of these cell lines (Figure 4D). The PCA results showed that the TKO ntTS cells globally retained the expression profile of TS cells, which was clearly different from those of their nuclear donor, TKO ES cells, or of the cells of the other two lineages. Notably, comparison between the TKO and WT ntTS cells showed that a substantial number of genes, such as prolactin-like proteins, *Rhox* genes, and several imprinted genes, were derepressed or inactivated in the TKO ntTS cells (Table S3), indicating that DNA methylation is functional as a transcriptional regulatory mechanism in TS cells.

Finally, we assessed whether the ntTS cells could contribute to placenta in the absence of DNA methylation in vivo. To this end, TKO ntTS cells were introduced into WT blastocysts and observed at E18.5. As shown in Figure 4E, Figure S3D, and Table S4, TKO ntTS cells were found in part of the labyrinth and the spongiotrophoblast layers of the host placenta, suggesting that the TKO ntTS cells differentiated not only in vitro but also in vivo.

Role of Dnmts in Extraembryonic Lineage Development

We showed that the NT embryos derived from TKO ES cells developed to the blastocyst stage at a similar frequency as those derived from WT ES cells (Figure 1), indicating that the zygotic DNA methyltransferases are dispensable for preimplantation development and for specification of the trophectoderm, primitive endoderm, and epiblast lineages. It

should be noted that mouse oocytes express Dnmt1o and Dnmt3a [19]; therefore, we could not exclude the possibility that these maternal Dnmts were sufficient for the lineage specification. However, TKO ES cells, which do not have maternal Dnmts, were able to initiate the differentiation of all three lineages in vitro (Figures 2 and 3). These results support the idea that DNA methylation is dispensable for the initial specification of the three lineages at the preimplantation stage.

Although we confirmed that TKO NT embryos or ntTS cells contributed to the placenta, it was still unknown whether the TKO cells were functional during the later development of the trophectoderm. *Igf2*, a paternally expressed imprinted gene that is required for proper placental growth [20], was almost completely repressed in the TKO ntTS cells (Table S3). The loss of placenta-specific imprinted genes *Peg10* or *Peg11/Rtl1*, or the maternal loss of Dnmt3L, a critical regulator of genome imprinting, leads to defects in the placenta [21–23]. Thus, transcriptional misregulation, including that of imprinted genes in the TKO cells, which had little impact in the early stage of trophectoderm development, may affect the differentiation and proliferation activities of later developmental processes in the TKO extraembryonic cells.

Self-Renewal of TKO ntTS Cells

Our establishment of TKO ntTS cells revealed that, in this cell type, DNA replication, chromosome segregation, and cell cycle progression occur properly without checkpoint activation in the absence of DNA methyltransferases. Along with pluripotent ES cells, this is the second mammalian cell type shown to self-renew in the absence of all three CpG methyltransferases. Importantly, not all stem cells are tolerant of the Dnmt deficiency, because the self-renewal and proliferation of hematopoietic stem cells/progenitors, epidermal progenitors, and germline stem (GS) cells derived from spermatogonial stem cells are sensitive to the loss of Dnmt1 [24–27].

It remains unclear why ES and TS cells are tolerant of the absence of DNA methyltransferases, given that the loss of DNA methylation or Dnmt1 protein, independent of its catalytic activity, leads to immediate growth arrest and cell death in somatic cells [28]. One possibility is that ES and TS cells are insensitive to putative damage signals triggered by epigenetic change or the loss of Dnmt1. Mouse ES cells have a unique cell cycle and checkpoint control system [29], which may underlie the epigenetic flexibility of this cell type. ES and TS cells are derived from the blastocyst, which is one of the most dynamically reprogrammed stages in the mammalian life cycle, with respect to epigenetic modification of the genome. A mechanism for stopping the cell cycle in response to the loss of Dnmts may be important for maintaining the epigenetic integrity of somatic cells. However, such a mechanism may need to be shut off during the developmental stage when the epigenetic signatures are subjected to change and need to be flexible.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at doi:10.1016/j.cub.2010.06.050.

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