Disabling de novo DNA methylation in embryonic stem cells allows an illegitimate fate trajectory

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Genome remethylation is essential for mammalian development but specific reasons are unclear. Here we examined embryonic stem (ES) cell fate in the absence of de novo DNA methyltransferases. We observed that ES cells deficient for both Dnmt3a and Dnmt3b are rapidly eliminated from chimeras. On further investigation we found that in vivo and in vitro the formative pluripotency transition is derailed toward production of trophoblast. This aberrant trajectory is associated with failure to suppress activation of Ascl2. Ascl2 encodes a BH3L transcription factor expressed in the placenta. Misexpression of Ascl2 in ES cells provokes transdifferentiation to trophoblast-like cells. Conversely, Ascl2 deletion rescues formative transition of Dnmt3a/b mutants and improves contribution to chimeric epiblast. Thus, de novo DNA methylation safeguards against ectopic activation of Ascl2. However, Dnmt3a/b-deficient cells remain defective in ongoing embryogenesis. We surmise that multiple developmental transitions may be secured by DNA methylation silencing potentially disruptive genes.

DNA methylation | embryonic stem cells | pluripotency

The mammalian genome is characterized by widespread methylation of cytosine residues. After fertilization, however, both maternal and paternal genomes undergo extensive de methylation, reaching a low point in the blastocyst (1–4). The embryo genome is then remethylated by the activity of de novo DNA methylation enzymes (5). Mouse embryonic stem (ES) cells exhibit global hypomethylation, similar to the in vivo blastocyst profile (6–8). Methylation is gained during the formative pluripotency transition to lineage competence, recapitulating early postimplantation development in vivo (9, 10).

Mammals have three DNA methyltransferases (DNMTs). Dnmt1 maintains methylation during DNA replication, while Dnmt3a and Dnmt3b are responsible for de novo methylation. DNA methylation is not required for general cell viability, and with the exception of imprint control regions, largely occurs at seemingly nonfunctional regions of the genome (11). Nonetheless, knockout of Dnmt1 in mice results in embryonic lethality around embryonic day 9.5 (E9.5) (12). Dnmt3a mutants die during puberty, but Dnmt3b mutant embryos fail from E9.5 onwards, exhibiting multiple abnormalities (13). When both de novo DNMTs are inactivated, development is disrupted by E8.5 with defective somitogenesis and abnormal morphogenesis. Seemingly normal progress to late gastrulation suggests that remethylation in the early postimplantation embryo is not critical for epiblast transition or germ layer formation. However, the reason(s) for the subsequent catastrophic failure is unclear. It has also been found that ES cells doubly deficient for Dnmt3a and Dnmt3b show a progressive genome-wide reduction in DNA methylation and loss of ability to form teratomas after long-term culture (14).

We previously showed that depletion of Dnmt3a and Dnmt3b delays timely exit from naïve pluripotency in vitro (15). Here we investigate the functional consequences of the lack of de novo methylation in ES cells for pluripotency progression and lineage potential at the cellular level.

Results

Chimera Colonization and Lineage Potential of Dnmt3a/3b-Deficient ES Cells. We examined the ability of Dnmt3a and Dnmt3b double knockout (Dnmt3dkO) ES cells (15) to contribute to chimeric embryos. Compound Dnmt3a/b mutant embryos are reportedly normal until somitogenesis (13). However, after blastocyst injection of Dnmt3dkO cells genetically labeled with constitutive mKO2, we found very sparse contributions in presomite-stage embryos at E7.5 (Fig. 1L). Even at E6.5 contributions were reduced compared with typical ES cell chimeras (Fig. 1B). Furthermore, some mutant donor cells were located in the extraembryonic ectoderm, a rare occurrence with wild-type (WT) ES cells (16). To improve survival of Dnmt3dkO ES cells in chimeras, we introduced a constitutive BCL2 transgene (17). In E6.5 embryos we observed higher contributions to epiblast, comparable to wild-type ES cells (Fig. 1C). However, we also saw donor cells in extraembryonic regions. We confirmed localization in the extraembryonic ectoderm and ectoplacental cone in five out of six chimeras examined by confocal microscopy (SI Appendix, Fig. S14).

Significance

Mammalian DNA is widely modified by methylation of cytosine residues. This modification is added to DNA during early development. If methylation is prevented, the embryo dies by midgestation with multiple abnormalities. In this study we found that stem cells lacking the DNA methylation enzymes do not differentiate efficiently into cell types of the embryo and are diverted into producing placental cells. This switch in cell fate is driven by a transcription factor, Ascl2, which should only be produced in the placenta. In the absence of DNA methylation, the Ascl2 gene is misexpressed. Removing Ascl2 redirects embryonic fate but not full differentiation potential, suggesting that methylation acts at multiple developmental transitions to restrict activation of disruptive genes.

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Fig. 1. Dnmt3a/b deficiency compromises chimera contribution and somatic lineage commitment. (A–C) Chimeric embryos obtained with parental, Dnmt3dKO (A and B), and hBCL2-expressing (C) mKO2 reporter ES cells. The 30% opacity brightfield images are overlaid onto fluorescent images. (Scale bars, 1 mm in A and 500 μm in B and C.) (D) qRT-PCR analysis of marker during culture in trophoblast medium (20). (E) qRT-PCR analysis of undifferentiated ES cells, Rex1::GFPLow sorted AFK cells, and further differentiated cells. (F) Cell morphology of WT and Dnmt3dKO cells in trophoblast stem cell (TS) cell medium (20) for 8 d. (Scale bars, 50 μm.) (G) DNA content quantified by propidium iodide (PI) staining at day 8 in TS cell medium. qRT-PCR data were normalized to beta-actin. Error bars are SD from technical duplicates.
We inspected blastocyst-stage chimeras to test whether Dmnt3a/b deficiency or BCL2 expression enabled primary trophectoderm colonization. However, donor cells were correctly localized to the inner cell mass (ICM) and did not contribute to trophectoderm (SI Appendix, Fig. S1H). Thus, the presence of Dmnt3dKO cells in postimplantation trophoblast likely arises by displacement from the epiblast rather than differentiation via trophectoderm.

The poor and aberrant colonization behavior of Dmnt3dKO cells prompted us to investigate in vitro differentiation competence. In response to mesendoderm induction, mutant cells up-regulated T and FoxA2 but to a lower level than parental cells (SI Appendix, Fig. S1C). In permissive conditions for neural induction (18), Dmnt3dKO cells showed only weak up-regulation of Sox1 and Pax6 (SI Appendix, Fig. S1D) but displayed substantial and sustained up-regulation of Ascl2, Hand1, and Tphbpa, genes associated with the trophoblast lineage (SI Appendix, Fig. S1 C and D). To assess whether Dmnt3dKO cells adopt trophoblast identity, we applied two culture conditions for trophoblast cells (19, 20). Unlike parental cells, Dmnt3dKO cells showed no or low up-regulation of formative pluripotency factors Oct2 and Oct6, but instead gained expression of trophoblast markers (Fig. 1D and SI Appendix, Fig. S1E).

We also subjected single Dmnt KO ES cells (15) to lineage induction (SI Appendix, Fig. S1F). Expression of germ layer markers was reduced in both mutants, and in neural conditions trophectoderm genes were up-regulated (SI Appendix, Fig. S1F). The phenotype was more marked in Dmnt3b KO cells, with higher trophoblast gene induction and lower neural and mesendodermal gene activation. We introduced expression constructs for Dmnt3a and Dmnt3b into Dmnt3dKO cells (SI Appendix, Fig. S1G). Rescued cells displayed normal differentiation with suppression of trophoblast genes (SI Appendix, Fig. S1H).

Cultured in activin, FGF2, and KSR (AFK) induces ES cell conversion into postimplantation formative epiblast-like cells (EpiLCs) (21). Dmnt3dKO cells showed delayed morphological change on day 1 but by day 2 appeared similar to parental cells with a comparable increase in cell number (SI Appendix, Fig. S1 I and J). The Rex1;GFPm2 (RGd2) reporter allows reliable monitoring of ES cell exit from naive pluripotency (9). Dmnt3dKO cells showed delayed down-regulation of the reporter in AFK (SI Appendix, Fig. S1K), consistent with findings in N2B27 (15). We sorted the GFP low (GFP−;⁴) fraction that has exited naive pluripotency and saw that trophoblast genes Ascl2 and Tphbpa were misexpressed in mutant cells (Fig. 1E). Levels increased further upon continued culture in N2B27 (Fig. 1E) while neural markers, Sox1 and Pax6, were very lowly expressed (SI Appendix, Fig. S1L). GFP+⁴ cells transferred into activin and CH1 gained only modest up-regulation of T and FoxA2, though they did not express most trophoblast markers.

After ongoing culture of dKO cells in N2B27, we observed additional trophoblast markers. Strikingly, however, the sequence of marker appearance differed from the in vivo developmental program. Ascl2 and Hand1, evident after 48 h in AFK, are characteristic of postimplantation trophoblast, whereas Cdx2 and Elf5, associated with primary trophectoderm, showed appreciable expression only at later stages (Fig. 1E). These results also differ from Dmnt1 mutants where up-regulation of Elf5 is thought to drive trophoblast transdifferentiation (22).

Finally, we transferred Dmnt3dKO cells from AFK into trophoblast medium (20). In contrast to parental cells, mutant cells flattened and some became very large with prominent nuclei (Fig. 1F). Propidium iodide staining showed a fraction with greater than 4N DNA content, consistent with polyploid trophoblast giant cell formation (Fig. 1G).

Transcriptome Analyses of ES Cell Differentiation Trajectory in the Absence of Dmnt3a/b. We examined the initial misregulation of gene expression by single-cell qRT-PCR. We used Nanog (naive), Oct2 (formative), and Ascl2 (trophoblast) as representative markers (Fig. 2A and SI Appendix, Fig. S2A). The majority of parental cells down-regulated Nanog and gained Oct2 after 48 h in AFK. Dmnt3dKO cells similarly gained Oct2 but generally retained higher Nanog levels. Most strikingly, Ascl2 was up-regulated in more than half of the Dmnt3dKO cells and many of these were also positive for both Nanog and Oct2. Unexpectedly, we also detected a fraction of triple positive cells among parental cells (Fig. 2A and SI Appendix, Fig. S2A).

We extended this analysis using the 10x Genomics platform for single-cell RNA-sequencing (scRNA-seq). Uniform manifold approximation and projection (UMAP) analysis clustered cells by culture condition in the first dimension and by genotype in the second dimension (Fig. 2B). We inspected markers for pluripotency states, germ layers, and trophoblast (SI Appendix, Fig. S2B). In ES cells, expression was not significantly altered between parental and mutant. However, in mutant 48-h AFK cells we observed persistent expression of multiple naive genes, lower up-regulation of formative genes, and ectopic expression of several trophoblast genes, though not Elf5 or Cdx2, which were detected in only 0.1 and 0.7% of cells, respectively. We examined Nanog, Oct2, and Ascl2 using a unique molecular identifier (UMI) count above zero to classify expression. Nanog+Ascl2−, Oct2−Ascl2−, and Nanog−Oct2−Ascl2− cells were present in both parental and Dmnt3dKO cells at 48 h (Fig. 2C). However, the combined proportion of dual and triple positive cells involving Ascl2 was three times higher in the mutants, consistent with single-cell qRT-PCR.

Pseudotime analysis using Monocle 2 (23) indicated a branchpoint in differentiation trajectory (Fig. 2D). We arbitrarily partitioned cells at and after the branchpoint into five groups (a–e). Parental cells were predominantly distributed along path 1, whereas mutant cells were almost exclusively located on path 2. Notably, however, 6.4% of parental cells initiated path 2, although very few reached the endpoint. Differentially expressed genes in parental cells featured formative markers on path 1 and trophoblast genes on path 2 (SI Appendix, Fig. S2C). Differential expression analysis without considering genotypes substantiated these alternative fates (Fig. 2E).

We investigated relatedness between path 2 and in vivo trophoblast. From published data (24) we identified genes up-regulated in E3.5 trophoderm or E6.5 trophoblast relative to ICM and postimplantation epiblast, respectively. Correlation was low for E3.5 trophoblast-enchriched genes with either pathway. In contrast, many E6.5 trophoblast-enriched genes were up-regulated in E3.5 trophoblast or E6.5 trophoblast relative to ICM and postimplantation epiblast, respectively. Correlation was low for E3.5 trophoblast-enchriched genes with either pathway. Novel DNA Methylation. Chromatin is remodelled during formative transition (25). We used assay for transposable accessible chromatin coupled to deep sequencing (ATAC-seq) (26) to survey chromatin accessibility in the absence of de novo DNA methylation. We analyzed ES cells, 48-h GFPH⁴ transitional cells, and GFPH⁴ posttransition cells and identified loci that are more open after 48 h in Dmnt3dKO cells than parental cells (Log2fold change >0.7, P value <0.05). We classified four groups according to opening in transitional (GFPH⁴) groups I and III) or posttransition populations (GFPH⁴ groups II and IV), and presence or absence of a CpG island ( CGI), annotated by the University of California Santa Cruz (UCSC) genome browser (Fig. 3A). The strongest peaks were associated with CGIs, which were mostly methylated at all stages (Fig. 3B and SI Appendix, Fig. S3 A and B). Non-CGI peaks were weaker and within regions that are methylated in parental cells, although a short stretch of reduced methylation was apparent at many group IV peaks (Fig. 3B and SI Appendix, Fig. S3 A and B). As expected, CGI peaks are mainly associated with annotated promoters and non-CGI peaks with enhancers (SI Appendix, Fig. S3C).
The analysis revealed chromatin regions that opened transiently in parental cells but remained accessible in Dnmt3dKO cells (groups II and IV, Fig. 3 A and SI Appendix, Fig. S3). Example genome browser tracks are shown in Fig. 3 C. Genes associated with these peaks showed high correlation with differentially expressed genes in Dnmt3dKO GFP Lo cells (SI Appendix, Fig. S3 D). We also found a positive correlation with the set of E6.5 trophoblast-enriched genes (SI Appendix, Fig. S3 E).

Thus, during exit from naive pluripotency, Dnmt3dKO ES cells fail to close down loci that normally transiently during transition. These regions encompass promoters and proximal...
Ascl2 locus opens during formative transition and remains open in Dnmt3a/3b mutants. (A) Heatmaps of ATAC-seq intensity distributions grouped according to increased accessibility in Dnmt3dKO GFP\textsuperscript{Hi} (I and III) or GFP\textsuperscript{Lo} (II and IV) presence or absence of CGI. (B) Heatmap of CpG methylation across ATAC-seq peaks in ES cells and EpiLCs (10). (C) Genome browser examples of genes that are more open in Dnmt3dKO cells. (D) Expression of Ascl2 in pseudotime. (E) Ascl2 gene locus methylation pattern during ES-to-EpiLC transition (data from ref. 10). Dashed line marks the TSS. (F) Bisulfite Sanger sequence analysis of Ascl2 TSS upstream sequence. Region 1 is within CGI and region 2 is within the CGI shore. Filled circles represent methylated cytosine and open circles represent unmethylated cytosine. At least eight clones each were sequenced.

Fig. 3. Ascl2 locus opens during formative transition and remains open in Dnmt3a/3b mutants.
enhancers for a subset of E6.5 trophoblast genes that become misexpressed.

Transcription factor motif enrichment analysis across ATAC peaks at CGI loci in Dnmt3dKO GFP+ cells identified, among others, Ascl2 (SI Appendix, Fig. S3F). Ascl2 was not expressed in any of the samples studied, but the motif is shared with Ascl2, which, as noted above, is rapidly up-regulated in transitioning mutant cells (Fig. 3D). The Ascl motif was present in 531 of 609 promoter regions (~2,000 to +500 bp around the transcription start site [TSS]) of E6.5 trophoblast-enriched genes. In parental ES cells the Ascl2 locus opened during formative transition (SI Appendix, Fig. S3G) but in Dnmt3dKO cells, Ascl2 promoter accessibility increased further posttransition (SI Appendix, Fig. S3G).

Inspection of published bisulfite sequencing data (10) revealed as expected that the Ascl2 CGI is not methylated in ESCs or during naive-to-EpiLC transition. However, the flanking CGI shore gained methylation during transition (Fig. 3E). We confirmed gain of CGI shore methylation in parental cells in AFK that did not occur in dKO cells (Fig. 3F). CGI shores are thought to contribute to regulation of CGI genes (27). The CGI shore sequence (1,807 bp, within 2 kb upstream of the TSS) contains five Ascl2 motifs identified by JASPAR (28), consistent with autoactivation potential as reported in intestinal stem cells (29).

Surprisingly it was found that ES cell deficient for Dnmt3a/b gradually lost DNA methylation and after multiple passages could no longer form teratomas (14). That study was performed on ES cells cultured in serum, which have a hypermethylated genome compared to the early embryo. In 2iL medium used here the genome is hypomethylated, similar to the embryo (6–8). To examine the acute effect of Dnmt3a/b depletion we used expression vectors for integration of gRNA and Cas9 expression vectors to achieve rapid enrichment for targeted cells. We transfected two ES cell lines and after 3 d, cells were transferred to AFK for a further 48 h. qRT-PCR with primers spanning the deletion registered a reduction in Dnmt3a and Dnmt3b transcripts indicating effective genome editing. The actual mutation frequency is likely to be higher due to small indels which are not detected. In the mixed populations, assayed almost immediately after Dnmt3a/b depletion, we detected up-regulation of Rfx6, a gene previously reported to be regulated by DNA methylation (30), together with Ascl2 (SI Appendix, Fig. S3H). We carried out bisulfite sequencing of the Ascl2 locus following Dnmt3a/b depletion as above. We detected gain of methylation in the CGI shore in parental cells after 48 h in AFK, that was reduced in the Dnmt3a/b mutant population (SI Appendix, Fig. S3I). These findings indicate a direct effect of Dnmt3a/b depletion on both expression and methylation of Ascl2.

Imprinting silenced of the paternal allele of Ascl2 in placenta does not involve DNA methylation (31). Instead, the imprinted lncRNA Kcnq1ot1 suppresses transcription in cis (32). We inspected levels of Kcnq1ot1 and found no change in Dnmt3dKO cells, indicating that loss of imprinting is not responsible for up-regulation of Ascl2 (SI Appendix, Fig. S3J).

We then investigated whether expression of Ascl2 is sufficient to impose trophoblast-like differentiation. For this we introduced an Ascl2−estrogen receptor (ER) fusion construct into parental ES cells. Upon tamoxifen (Tx) treatment in serum and LIF, cells changed morphology within 2 d, becoming larger and flattened (Fig. 4A). Oct4 was down-regulated and trophoderm genes Elf5, Cdx2, and Tphb2 were up-regulated (Fig. 4B). Thus, misexpression of Ascl2 can initiate trophoblast-like differentiation.

Finally, we tested whether activation of Ascl2 was necessary for trophoblast-like differentiation of Dnmt3dKO cells. We deleted Ascl2 to create Dnmt3dKOΔA2 cells (SI Appendix, Fig. S4A) and saw that the misregulation of trophoblast genes was abolished (Fig. 4C). Furthermore, at 24 h, Dnmt3dKOΔA2 cells expressed Otx2, Oct6, and Fig5 formative markers (SI Appendix, Fig. S4B). However, neural genes were not subsequently up-regulated and mesendodermal differentiation remained inefficient (Fig. 4D), indicating later differentiation defects unrelated to Ascl2.

### Dnmt3a/b Deficient Cells Are Outcompeted by Wild-Type Cells

We investigated whether deletion of Ascl2 may restore contribution to chimeric epiblasts. We saw substantial colonization by Dnmt3dKOΔA2 cells in 5 of 10 epiblasts at E6.5 (Fig. 5A and SI Appendix, Fig. S5A). Moreover, we observed no donor cells in extraembryonic regions. At E7.5, contributions were reduced and appeared biased to the posterior region (Fig. 5B and SI Appendix, Fig. S5B). Immunostaining for Stella and Tflp2c showed no enrichment for primordial germ cells (PGCs) (SI Appendix, Fig. S5C and D). By E9.5, we observed only sparse contributions in 4 of 11 embryos (Fig. 5C and SI Appendix, Fig. S5E).

Progressive dilution of mutant cells in chimeras suggested a competitive disadvantage with wild-type host epiblast cells (33). To examine this possibility, we used formative pluripotent stem (FS) cells related to E6.0 epiblasts (34). We were unable to establish FS cells from Dnmt3dKO cells, likely due to disruptive effects of Ascl2 misexpression. However, Dnmt3dKOΔA2 ES cells could be converted into stable FS cell lines that expressed the core pluripotency factor Oct4 together with formative markers (Fig. 5D and E). We cocultured equal numbers of GFP expressing wild-type and mKO2 expressing Dnmt3dKOΔA2 FS cells. Proportions of each genotype were quantified by flow cytometry. We observed reduction in the fraction of mutant cells by passage 0 that was further increased by passage 1 (Fig. 5F). In cocultures Annexin V staining was higher for Dnmt3dKOΔA2 FS cells during self-renewal and mesendoderm induction (P < 0.05) though not neural induction (P > 0.05) (Fig. 5G and SI Appendix, Fig. S5G).

Relative level of cMyc is a key determinant in cell competition (35–37). By immunostaining we saw that Dnmt3dKOΔA2 FS cells have lower levels of myc protein than wild-type cells (Fig. 5H). Immunoblotting confirmed that cMyc expression was reduced in mutant cells (SI Appendix, Fig. S5F). These observations suggest that cMyc-based cell competition may cause elimination of Dnmt3dKO cells mixed with wild-type cells (36) and that inactivation of Ascl2 avoids only the first round of competition. The mechanism that reduces cMyc is unclear, but we surmise is secondary to other perturbations in the absence of de novo methylation.

We introduced BCL2 into Dnmt3dKOΔA2 ES cells. Under in vitro differentiation conditions BCL2 transfectants showed up-regulation of Sox1, Pax6, T, and Foa2, although still below the levels observed for parental transfectants (Fig. 5J). After blastocyst injection we recovered five chimeras among seven embryos at E9.5. Contributions were variable, but substantial in three of the five (Fig. 5J). We also collected embryos at E12.5 and detected mKO2 fluorescence in two of the four specimens, with donor-derived cells in neural tissues and mesenchyme (Fig. 5K and SI Appendix, Fig. S5H). These contributions were noticeably lower than at E9.5, however, suggesting ongoing loss of Dnmt3a/b-deficient cells even in the presence of a strong survival factor.

### Discussion

Our findings indicate that de novo DNA methylation safeguards the formative pluripotency transition to somatic lineage competence. In the absence of Dnmt3a and Dnmt3b, cells exiting naive pluripotency are liable to adopt a deviant fate trajectory and develop features of postimplantation trophoblast. Notably, a small fraction of parental ES cells initiates the trophoblast transdifferentiation trajectory but they do not continue on this path because de novo DNA methylation prevents full activation of the trophoblast program. Our study identifies Ascl2 as a trophoblast determination gene that is potentially directly regulated by de novo DNA methylation. Misexpression of Ascl2 can provoke ES cell transdifferentiation. Conversely, removal of Ascl2...
Fig. 4. Ascl2 propels transdifferentiation to trophoblast-like cells. (A) Images of ES cells cultured in serum/LIF with or without Tx induced activation of Ascl2–ER. (Scale bars, 100 μm.) (B) qRT-PCR analysis of marker expression during Ascl2–ER induction. (C) qRT-PCR analysis of trophoblast marker expression in neural differentiation culture by two clonal lines of Dnmt3dKOΔA2 cells. (D) Analysis of somatic lineage marker expression by Dnmt3dKOΔA2 cells in neural and mesendoderm induction protocol. Error bars represent SD from technical triplicates (B) or duplicates (C and D).

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Fig. 5. Dnmt3a/b-deficient cells are outcompeted by wild-type cells and do not persist in chimeras. (A) Maximum projection confocal images of chimeric contributions to E6.5 embryos. Red, mKO2; blue, Eomes immunostaining. (Scale bar, 100 μm.) (B) Dnmt3dKOΔA2 ES cell chimeras at E7.5. The 25% opacity brightfield images are overlaid onto fluorescent image. (Scale bar, 250 μm.) (C) Dnmt3dKOΔA2 ES cell chimeras at E9.5. (Scale bar, 1 mm.) (D) FS cells established from parental and Dnmt3dKOΔA2 ES cells. (Scale bar, 100 μm.) (E) qRT-PCR analysis of FS cell markers. Error bar represents SD from technical duplicates. (F) Percentage of GFP-labeled WT FS cells and mKO2-labeled dKO-A2 FS cells in the mixed coculture. Passage zero (p0) is 2 d after plating. Error bar represents SD from two cultures. (G) Annexin V positive cells quantified by flow cytometry after 24 h in indicated coculture. Error bars represent SD from six experiments. P < 0.05. (H) Immunofluorescence images of cMyc parental FS cells (GFP), Dnmt3dKOΔA2 FS cells (mKO2), and coculture for 24 h. Dashed regions highlight mKO2 positive cells in coculture. (I) qRT-PCR analysis of marker expression during neural and mesendoderm differentiation of BCL2-transfected ES cells. Error bar represents SD from technical duplicates. (J) BCL2-Dnmt3dKOΔA2 ES cell chimeras at E9.5. The 20% opacity brightfield images are overlaid onto fluorescent images. (Scale bar, 1 mm.) (K) BCL2-Dnmt3dKOΔA2 ES cell chimeras at E12.5. (Scale bar, 1 mm.)
elaborates the aberrant fate trajectory in Dnmt3a/3b mutant cells and restores pluripotency progression. Dnmt3a/3b-deficient cells remain compromised in later differentiation, however, and are unable to compete with wild-type cells in the chimera context.

Deletion of Dnmt3a/b in ES cells generated in serum and LIF culture was previously shown to result in progressive erosion of methylation at repetitive sequences and, after long-term culture, a failure to produce teratomas (14). Effects of continuous culture on transcription or lineage commitment were not characterized. ES cells in serum are subject to heterogeneous and dynamic hypermethylation (38) whereas in 2i/LIF the genome is hypomethylated, similar to the inner cell mass (7, 39). The requirement for de novo methylation may be more acutely apparent in ground state ES cells in 2i/LIF due to the lower basal level of methylation. Crucially, the phenotype of Ascl2 misregulation and trophoblast-like differentiation is specifically associated with absence of the de novo Dnmts and is eliminated by their restoration. The CGI shore adjacent to the Ascl2 promoter is subject to de novo DNA methylation during formative transition, suggestive of a potential direct silencing effect. Alternatively, an upstream activator of Ascl2 may be silenced by de novo methylation.

Regardless of the mechanism, our findings are consistent with detection of Ascl2 misexpression in Dnmt3a/b dKO embryos at E9.5 (30). Inspection of recent transcriptome data from Dnmt3a/3b-deficient embryos at E8.5 (40) also revealed low but significant (P < 0.01) up-regulation of Ascl2. Nonetheless, ectopic trophoblast-like differentiation is not the major cause of embryonic lethality. Heightened susceptibility of ES cells to this fate alteration may reflect adaptation to the in vitro environment or the absence of constraints operative in the embryo. It is also important to note that deregulation of Ascl2 does not instruct normal trophoblast lineage development but triggers a deviant differentiation process and generation of an aberrant cell phenotype. Our findings serve as a cautionary note for interpretation of lineage potential and differentiation from pluripotent stem cells, in particular claims of expanded potency after exposure to epigenome modifying agents or other perturbations.

Overall, our results demonstrate that in the absence of de novo DNMTs the ability to execute a cell state transition is compromised due to misexpression of a gene with fate switching potency. The example of Ascl2 illustrates how genome-wide methylation may have been coopted to constrain expression of a pivotal requirement for DNA methylation at critical loci to safeguard transcriptome and lineage commitment trajectories. In this context it is of interest that mutations in DNMT3A and DNMT3B are associated with tumorigenesis in various tissues (41, 42), possibly relating to corruption of cellular transitions.

Materials and Methods

Cell Culture. ES cells were maintained in 2iL medium on gelatin-coated plates as described (18). 2iL medium consists of 10 ng/mL of mouse LIF, 1 μM of Mek inhibitor, 30 μM of GSK3 inhibitor CHIR99021 in N2B27 basal medium. EpiliCs were induced by plating 2 × 10^4 ES cells in 20 ng/mL Activin A, 12.5 ng/mL bFGF, and 1% knockout serum replacement (KSR), in N2B27 medium on fibronectin-coated six-well plates. GFP high or low fractions were selected with G418. Ascl2 misexpression in Ascl2−/− ES cells was associated with tumorigenesis in various tissues (41, 42), possibly relating to corruption of cellular transitions.
TOPO cloning vector (Thermo Fisher Scientific). The sequence results were analyzed with QUMA (44). The primers are listed in Table S1.

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