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DNA methylation and its role in the trophoblast cell lineage

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ABSTRACT DNA methylation functions as cellular memory beyond generations of cells and is involved in many biological processes. Because of its relatively stable nature compared with the transcriptome, the DNA methylation profile of cells can also be used to evaluate developmental similarity and cellular phenotypes. Recent insights into 5-hydroxymethylcytosine have started to reshape our view of the epigenetic regulation of mammalian development. Both global DNA methylation and hydroxymethylation levels change dynamically during preimplantation embryogenesis. It is known that DNA methylation plays an essential role in embryonic cell fate restriction, whereas its role in trophoblast development requires further research. Two distinct blastocyst-derived stem cell lines, embryonic stem (ES) cells and trophoblast stem (TS) cells, are used to study the epigenetic mechanisms underlying cell lineage maintenance and the regulation of cell differentiation. Such studies will allow us to understand the details of the epigenetic landscape of trophoblast development, which should offer valuable information for managing pregnancy-related diseases in humans.

KEY WORDS: *DNA methylation, blastocyst, ICM, TE, TS cell*

Introduction

Starting from a single totipotent cell (i.e., a fertilized egg), the process of mammalian development generates more than 200 different types of cells (Alberts *et al.*, 2007), almost all of which possess an identical genomic DNA sequence. In addition to the networks of transcription factors, by changing the chromatin structure, epigenetic systems play a fundamental role in the cell type-specific use of genetic information stored in genomic DNA. DNA methylation is one of the best-studied epigenetic modifications and is involved in many biological processes such as repression of transcription, genome imprinting, suppression of retrotransposons, and X chromosome inactivation (Bird, 2002; Jaenisch and Bird, 2003; Smith and Meissner, 2013).

DNA methylation at cytosine bases has long been regarded as the only covalent modification of mammalian DNA. However, recent discoveries of enzymes that catalyze the oxidation of methylated cytosine to produce 5-hydroxymethylcytosine (5hmC) have started to reshape our view of the epigenetic landscape of mammalian development. The first cell differentiation in mammalian development segregates the trophoblast cell lineage from embryonic cell lineage, resulting in the formation of the trophectoderm (TE) and inner cell mass (ICM) at the early blastocyst stage. After implantation of the blastocyst to the uterus, the TE produces trophoblast cells that constitute most of the placenta on the fetal side and an outermost membrane that surrounds the developing fetus. The ICM gives rise to three germ layers and germ cells. In mice, stem cell lines have been derived successfully from these two tissues, thereby recapitulating their developmental potency: trophoblast stem (TS) cells from TE and the embryonic stem (ES) cells from the ICM (Evans and Kaufman, 1981; Martin, 1981; Tanaka *et al.*, 1998). Much of the knowledge about the epigenetic status of the trophoblast and embryonic cell lineages has been obtained from research comparing these two distinct stem cells. In this review, we first summarize basic information about DNA methylation and hydroxymethylation. We then focus on recent insights into these epigenetic modifications obtained from ES and TS cells and early embryos, and we discuss the possible involvement of these epigenetic modifications in the development and function of the trophoblast cell lineage.

DNA methylation and further oxidation

In mammals, DNA methylation occurs predominantly on cytosine bases in 5'–CG–3' dinucleotide (CpG) sequences to produce

Abbreviations used in this paper: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; Dnmt, DNA methyltransferase; Dnmt TKO, triple knockout of Dnnt1, Dnmt3a and Dnmt3b; Dnmt TKO-NT, nuclear transfer from Dnmt TKO cell; ES cell, embryonic stem cell; ICM, inner cell mass; NT, nuclear transfer; TE, trophectoderm; TS cell, trophoblast stem cell; T-DMR, tissue-dependent and differentially methylated region; Tet, ten-eleven translocation.

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5-methylcytosine (5mC). Non-CpG methylation has also been detected at some specific loci (Ichiyanagi *et al.*, 2013; Imamura *et al.*, 2005; Nishino *et al.*, 2011), but its biological significance in mammals is not known. Non-CpG methylation appears to be more enriched in the genome of germ cells and ES cells (Ramsahoye *et al.*, 2000; Shirane *et al.*, 2013). Although CpG methylation is associated mostly with repression of gene expression (Weber *et al.*, 2007; Yagi *et al.*, 2008, 2012), the presence of methylated promoters with low CpG content at transcriptionally active genes has also been noted by genome-wide DNA methylation and gene expression analyses (Weber *et al.*, 2007). This suggests that sparse CpG methylation does not interfere with the transcription machinery and/or that CpG methylation in a particular sequence context activates transcription. The latter is not totally improbable because it has been shown that the CpG methylation within CRE sequences (TGACGTCA) creates a binding site for the transcription factor C/EBPα and results in activation of CRE sequence-associated tissue-specific genes (Rishi *et al.*, 2010).

Cytosine to 5mC conversion is catalyzed by three members of the DNA methyltransferase (Dnmt) family. Dnmt1, referred to as "maintenance methyltransferase," prefers hemimethylated DNA to nonmethylated DNA as its substrate *in vitro* (Gruenbaum *et al.*, 1982) and localizes at replication fork through interaction with its chaperone protein Np95/Uhrf1 (Sharif *et al.*, 2007). This ensures immediate and accurate copying of the CpG methylation pattern from a parent strand to the newly synthesized daughter strand during replication. Disappearance of Dnmt1 (or inactivation of its enzymatic activity) therefore leads to a gradual dilution of DNA methylation in dividing cells (theoretically, 1/2 per cell cycle) in a replication-dependent manner, which is called "passive demethylation."

By contrast, two other members of the Dnmt family, Dnmt3a and Dnmt3b, are essential for methylation of nonmethylated DNA (Okano *et al.*, 1999) and are thus called "*de novo* methyltransferases." There are at least two isoforms of Dnmt3a in the human and mouse: a long isoform DNMT3A1/Dnmt3a1 and a short isoform DNMT3A2/Dnmt3a2. Both isoforms have enzymatic activity, but show different localization patterns in the nucleus. Dnmt3a1 is more concentrated at the densely DAPI-stained heterochromatic region of the nucleus, whereas Dnmt3a2 appears to be excluded from the heterochromatic region, which suggests that these Dnmt3a isoforms have distinct genomic targets (Chen *et al.*, 2002). Dnmt3L, another member of the Dnmt family, does not contain a catalytic domain conserved among other members and shows no Dnmt activity on its own, but it is also essential for *de novo* DNA methylation in germ cells (Hata *et al.*, 2002, 2006). Dnmt3L has been shown to interact with both Dnmt3a and Dnmt3b, and to stimulate their activity *in vitro* (Gowher *et al.*, 2005, Suetake *et al.*, 2004). It has been reported that Dnmt3L physically interacts with Dnmt3a2, but not with Dnmt3a1 or Dnmt3b, in ES cells (Nimura *et al.*, 2006).

Conversion of 5mC back to unmodified cytosine independent of DNA replication, so-called "active demethylation," takes place on the genome-wide scale during mammalian development. Very recently, this was proven experimentally to occur in primordial germ cells (Kawasaki *et al.*, 2014). Another massive active demethylation had been thought to occur soon after the fertilization on the paternal genome, although this is now somewhat controversial (see below). Identification of genuine DNA demethylase in mammals has been a long-standing issue in the field of epigenetic research. It was found recently that 5mC can be successively oxidized to 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) by the ten-eleven translocation (Tet) family of Fe(II) and 2-oxoglutarate-dependent DNA dioxygenases (He *et al.*, 2011; Ito *et al.*, 2010; Ito *et al.*, 2011; Tahiliani *et al.*, 2009). Three members of the Tet family, Tet1–3, have been identified and shown to play essential roles in diverse biological processes. Because 5fC and 5caC can be excised and repaired to regenerate unmodified cytosine by the thymine DNA glycosidase and base excision repair pathways, 5hmC is now regarded as an essential intermediate of active demethylation (Kohli and Zhang, 2013; Wu and Zhang, 2014).

DNA methylation profile of cells as an identifier of cell type

Although the DNA methylation pattern of cells is stably transmitted from parent cell to daughter cells, a certain portion of CpGs change their methylation status as the cells differentiate, resulting in a renovation of the DNA methylation pattern of the genome, or DNA methylation profile, which is unique to each cell type. Initial attempts to identify such CpGs were pursued by restriction landmark genomic scanning (RLGS) using a methylation-sensitive restriction enzyme *Not*I as a methylation sensor (Ohgane *et al.*, 1998, 2002). By comparing ES cells, embryonic germ (EG) cells, TS cells, germ cells, and several somatic tissues, RLGS identified >200 tissue-dependent and differentially methylated regions (T-DMRs) out of ~1,500 analyzable *Not*I sites (Shiota *et al.*, 2002). Although *Not*I sites tend to locate within CpG islands or CpG-rich regions, T-DMRs were distributed disproportionately in the non-CpG island loci (Sakamoto *et al.*, 2007). Interestingly, even with fewer *Not*I sites compared with recent deep sequencing-based methylome analyses, hierarchical clustering of the methylation profiles could be used to define developmental similarity and cellular phenotypes (Sakamoto *et al.*, 2007), demonstrating that the DNA methylation profile of cells is a powerful index for evaluating the relatedness of different cell types.

This concept was expanded further and confirmed by showing that EG cells and iPS cells show a DNA methylation profile very similar to but still distinctive from that of ES cells (Sato *et al.*, 2010). A series of studies has identified T-DMRs that are differentially methylated between TS cells and ES cells (TS–ES T-DMRs) (Nakanishi *et al.*, 2012; Shiota *et al.*, 2002). One example is the T-DMRs at the pluripotency-related *Pou5f1* (*Oct4*) gene locus that are heavily methylated in TS cells when the gene is silent but are hypomethylated in ES cells (Hattori *et al.*, 2004). Treatment with the Dnmt inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) and Dnmt1 deficiency caused the ectopic expression of *Oct4* in TS cells and in placental tissue, respectively, showing that DNA methylation plays an essential role in the suppression of *Oct4* expression (Hattori *et al.*, 2004). Another pluripotency-related gene, *Nanog*, has also been reported to have T-DMRs and to be regulated by DNA methylation (Hattori *et al.*, 2007). Thus, DNA methylation of T-DMRs around some specific genes should play important roles in restricting cell potency.

DNA methylation dynamics during embryogenesis

The mammalian genome undergoes dynamic changes in the global DNA methylation level during preimplantation embryogenesis (Fig. 1), which can be visualized by immunostaining of mouse

embryos at sequential stages using an anti-5mC antibody (Santos *et al.*, 2002). Soon after the fertilization, the 5mC signal on the paternal pronucleus decreases markedly before the onset of the first replication, whereas the 5mC signal on the maternal genome gradually decreases and is lowest at the morula stage (Dean *et al.*, 2003; Morgan *et al.*, 2005; Santos and Dean, 2004). The loss of the 5mC signal from the paternal genome has been regarded as active demethylation because of its rapid and replication-independent nature. It has been suggested, however, that it reflects the rapid conversion of 5mC to 5hmC (and further oxidation products) by Tet3 and that the 5hmC on the paternal genome is thereafter diluted gradually by replication and cell division similar to the passive demethylation of the maternal genome (Gu *et al.*, 2011; Inoue *et al.*, 2011; Inoue and Zhang, 2011; Iqbal *et al.*, 2011; Kohli and Zhang, 2013).

A global *de novo* DNA methylation takes place in the mouse blastocyst in which higher levels of 5mC signal were observed in the ICM compared with the TE (Santos *et al.*, 2002). This suggested the idea that the hypomethylation status of the genome might have some advantage for trophoblast regulation. It should be mentioned, however, that the pattern of global DNA methylation dynamics observed in mouse embryogenesis does not appear to be common among mammalian species (Ma *et al.*, 2012). Instead, immunostaining of embryos from different mammalian species gives rather puzzling results. For example, the intensity of 5mC signal does not decrease in the male pronucleus of the pig zygote, and the global DNA methylation level appears to be maintained throughout the cleavage stages. In addition, differences in the intensity of the 5mC signal between the TE and ICM of the pig blastocyst are not as obvious as those observed in the mouse blastocyst (Jeong *et al.*, 2007). 5mC signal of almost equal intensity has also been observed in the TE and ICM in the goat blastocyst (Park *et al.*, 2010). Even more intriguing is the rhesus monkey blastocyst, in which the TE stains more brightly with anti-5mC antibody than does the ICM (Yang *et al.*, 2007). In the human blastocyst, one report showed more intense staining in the TE than in the ICM (Fulka *et al.*, 2004), but another report showed the opposite (Santos *et al.*, 2010). The reason for this peculiar difference is unknown. Although 5mC immunostaining in preimplantation embryos appears to be influenced by the experimental conditions (Li and O'Neill, 2012, 2013), these results argue against the importance of the globally hypomethylated state of the genome to the specification of the TE. However, it does not exclude the possibility that the hypomethylated status is essential for the further development and function of trophoblast cells.

To take a closer look at the DNA methylation dynamics in mouse embryogenesis, Nakanishi *et al.,* performed bisulfite sequencing analyses of some selected TS-ES T-DMRs in isolated TE and ICM cells (Nakanishi *et al.*, 2012). The bisulfite sequencing allows base-resolution readout of the DNA methylation status of specific loci. Although this method cannot distinguish 5hmC from 5mC (Huang *et al.*, 2010), for simplicity, here the result is explained as if it detects only 5mC. The ratio of 5hmC/5mC in TS cells and E7.5 trophoblastic tissue was < 0.005 and ~0.03, respectively, in a mass spectrometry study by Senner *et al.,* (2012). Nakanishi *et al.,* revealed the nearly unmethylated status of the TS-ES T-DMRs at three loci (*Elf5*, *Pou5f1*, and the one identified by this study) both in the TE and ICM of E3.5 blastocysts. Near-complete demethylation of eight more TS-ES T-DMRs, including one at the *Nanog* locus in E3.5 whole blastocysts, was also demonstrated (Nakanishi *et al.*, 2012). Because these TS-ES T-DMRs exhibited cell lineage-dependent differential DNA methylation status in E6.5 embryos, it was proposed that the cell lineage-specific DNA methylation profile is superimposed on the epigenome after the morphological segregation of the TE and ICM (Fig. 1). It was also suggested by Nakanishi *et al.,* that the TS and ES cells are distinct from their tissue of origin in terms of DNA methylation status and that the DNA methylation profile would be acquired and stabilized during derivation of stem cell lines from E3.5 embryos.

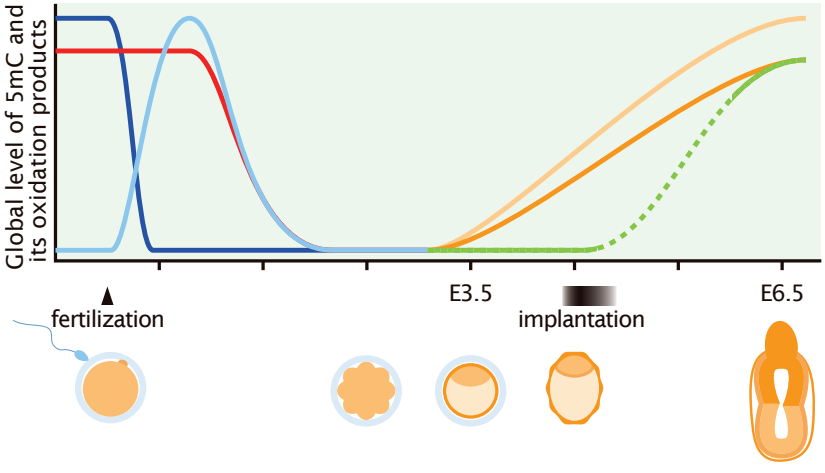

Fig. 1. Asymmetric dynamics of cytosine modification in early embryogenesis. *Changes in the level of 5mC and its oxidation products are illustrated schematically. The global 5mC level of the paternal genome (darker blue line) declines rapidly after the fertilization, and the level of 5hmC/5fC/5caC of the paternal genome increases (lighter blue line). Both paternal and maternal (red line) genomes then gradually lose the modified cytosines during cleavage stages in a replication-dependent manner.* De novo *methylation begins in blastocysts in which the TE has a lower 5mC level (darker orange line) than the ICM (lighter orange line). Methylation of T-DMRs occurs after morphological segregation of trophoblast and embryonic cell lineages (green line, see text). The exact timing of the methylation of these T-DMRs is not known. Note that the curves do not show the exact amount, and the axes are not to scale.*

In agreement with the concept that the cell lineage-specific DNA methylation profile is set up after cell lineage specification, genome-wide DNA methylation analysis by methylated DNA immunoprecipitation (MeDIP) using anti-5mC antibody followed by unbiased amplification and promoter array analysis has revealed that the major *de novo* methylation is mediated mainly by Dnmt3b and occurs between E3.5 and E6.5 in the embryonic cell lineage (Borgel *et al.*, 2010). Another genome-wide DNA methylation analysis using reduced representation bisulfite sequencing also revealed that the largest increase in 5mC (most likely including 5hmC to some degree) occurs between the early ICM and E6.5 epiblast (Smith *et al.*, 2012). Moreover, the embryos developed from reconstituted oocytes after transfer of the nuclei of Dnmt1/3a/3b-null (Dnmt TKO) ES cells (Dnmt TKO-NT embryos), so that they contained no Dnmt activity, could develop to the blastocyst stage and could be used to derive TS

cells (TKO ntTS), indicating that DNA methylation is dispensable for specification of the TE and ICM (Sakaue *et al.*, 2010). These reports imply that DNA methylation at specific genomic regions is likely to be important for the maintenance of cell-lineage identity but not for the specification of extraembryonic and embryonic cell lineages, at least in the mouse.

At exactly what stage is the cell lineage-specific DNA methylation profile established? By what kind of cue is this *de novo* methylation ignited? And what kind of mechanisms underlie establishment of cell lineage- and region-specific DNA methylation? These questions should be addressed in future to understand the regulation of the trophoblast lineage by epigenetic systems including DNA methylation.

Mouse ES cells can be converted to TS-like cells by manipulating the expression of a single transcription factor, such as the induced depletion of Oct4 or the induced activation of Cdx2 or Eomes (Niwa *et al.*, 2000, 2005). These systems offer the opportunity to analyze the mechanisms underlying the establishment of the trophoblast cell lineage-specific epigenome. For example, the inducible Oct4-depletion system was used to understand why the mouse trophoblast genome shows global hypomethylation compared with ES and somatic cells (Oda *et al.*, 2013). This analysis found reduced expression of Np95 and failure of Dnmt1 to localize at replication foci in induced trophoblast cells. Although the localization pattern of Dnmt1 was restored by overexpression of Np95, DNA hypomethylation was maintained. From these results, it was concluded that the trophoblast cells (at least the induced cells) might have a mechanism to resist a genome-wide increase in DNA methylation. This resistance should be region-specific because, as mentioned above, some TS-ES T-DMRs show the hyper- and hypomethylated state in TS and ES cells, respectively (Hattori *et al.*, 2004, 2007, Nakanishi *et al.*, 2012). Transcription factors confer such region specificity at least in part. Carey *et al.,* used Cdx2-inducible ES cells and showed that *de novo* DNA methylation on *Oct4* T-DMRs follows transcriptional repression by direct binding of Cdx2 and changes in histone acetylation around the promoter region. Although *Oct4* and *Nanog* were silenced 48 hours after the induction of Cdx2, only a slight increase in DNA methylation was observed at 72 hours. This increase reached a similar level to that in TS cells at 120 hours after induction (Carey *et al.*, 2014). This result suggests that the *de novo* methylation of TS-ES T-DMRs occurs autonomously as a consequence of transcription repression. It should be noted, however, that T-E T-DMRs, including *Nanog* T-DMR, were barely methylated in diapause blastocysts (4 days in the diapause state after E3.5 when *Nanog* is already shut off in the TE) and that some of the T-E T-DMRs are not located near any known promoters (Nakanishi *et al.*, 2012). Thus, the *de novo* methylation in trophoblast cells cannot be explained simply by transcription factor-directed stepwise mechanisms.

Cell fate restriction in embryonic cell lineage by DNA methylation

The restriction of embryonic cell lineage fate by DNA methylation has been elucidated. Insufficient DNA methylation in ES cells caused by *Dnmt1* deficiency and/or *Dnmt3a/3b* deficiency causes the ectopic expression of trophoblast-specific genes such as *Pl1* (*Prl3d1*) and *Tpbpa* when the cells are differentiated *in vitro*, whereas expression of these genes was very low in the wild-type control (Jackson *et al.*, 2004; Ng *et al.*, 2008). Ectopic expression of trophoblast marker genes was also detected in embryonic tissue of E9.5 *Dnmt1*$^{-/-}$ embryos (Ng *et al.*, 2008). Moreover, derivatives of Dnmt TKO-NT embryos contributed predominantly to the placenta in the context of the chimeric conceptus between wild-type embryos (Sakaue *et al.*, 2010). A genome-wide screen for promoter methylation by MeDIP array hybridization identified a promoter region of the *Elf5* locus encoding a transcription factor of the Ets family that is essential for trophoblast lineage development (Donnison *et al.*, 2005), as a target of DNA methylation in ES cells (Ng *et al.*, 2008). *Elf5* is not expressed and its promoter is heavily methylated (90%) in wild-type ES cells, whereas the gene is expressed and is hypomethylated (9.3%) in TS cells. The DNA methylation level of the *Elf5* promoter region was decreased to 41.5% in *Dnmt1*$^{-/-}$ ES cells, which resulted in ectopic activation of *Elf5* gene in differentiating *Dnmt1*$^{-/-}$ ES cells. The forced expression of *Elf5* in wild-type ES cells induced expression of other transcription factors Cdx2 and Eomes (Ng *et al.*, 2008) both of which can provoke wild-type ES cells to adopt the trophoblast cell fate (Niwa *et al.*, 2005). Thus, DNA methylation works to fix cell lineage restriction through the regulation of the *Elf5* locus.

Besides *Elf5*, another gene might also be involved in DNA methylation-mediated cell lineage restriction. It was recently reported that the forced expression of the noncoding RNA, *H19*, induces expression of trophoblast lineage markers in ES cells under differentiation conditions (Fujimori *et al.*, 2013). *H19* is a well-known imprinted gene, and the DNA methylation of the *Igf2–H19* imprinting control region (ICR) on paternally derived chromosomes suppresses transcription of *H19*, allowing the maternal allele-specific expression of this gene (Kurukuti *et al.*, 2006). Hypomethylation of the ICR and induction of *H19* is evident in *Dnmt1*$^{-/-}$ ES cells (Biniszkiewicz *et al.*, 2002). Therefore, it is possible that upregulation of *H19* also increases the transdifferentiation of *Dnmt1*$^{-/-}$ ES cells toward the trophoblast cell fate. It would be interesting to determine whether *Dnmt1*$^{-/-}$ ES cells efficiently differentiate into trophoblast cells even when *Elf5* and/or *H19* is depleted.

A causal role of *H19* in ES-to-trophoblast transdifferentiation is also speculated in *poly(ADP-ribose) polymerase-1* (*Parp1*)-deficient ES cells (Fujimori *et al.*, 2013). *Parp1*$^{-/-}$ ES cells differentiate into trophoblast derivatives *in vitro* and in ES cell-derived tumors (Hemberger *et al.*, 2003; Nozaki *et al.*, 2013; Nozaki *et al.*, 1999; Ogino *et al.*, 2007). Significant upregulation of *H19* in *Parp1*$^{-/-}$ ES was detected by a microarray analysis and was then validated by RT-PCR (Ogino *et al.*, 2007). Given that *H19* can unleash the trophoblast cell fate in ES cells (Fujimori *et al.*, 2013), it is also possible that increased expression of *H19* leads to differentiation of *Parp1*$^{-/-}$ ES cells toward the trophoblast cell fate. However, the involvement of DNA methylation remains equivocal. Parp1 is thought to inhibit Dnmt1 activity (Caiafa *et al.*, 2009). Parp activity is also suggested to be involved in the active demethylation process in primordial germ cells (Ciccarone *et al.*, 2012; Kawasaki *et al.*, 2014). Based on these reports, one may expect hypermethylation of the genome in *Parp1*-deficient ES cells in contrast to *Dnmt1*-deficient ES cells, although this has not been reported yet. Thus, it is unclear why *H19* is upregulated in *Parp1*$^{-/-}$ ES cells. Interestingly, transient treatment of preimplantation mouse embryos with the Parp inhibitor 3-aminobenzamide for 24 hours around the eight-cell to morula stages completely

blocked blastocyst formation (Imamura *et al.*, 2004). This suggests that Parp activity plays pivotal role in the specification or maintenance of the TE.

Gene regulation by DNA methylation in the trophoblast cell lineage

Compared with the role of DNA methylation in the embryonic cell lineage, the role in the trophoblast lineage has not been explored deeply. Dnmt TKO-NT embryos show normal development until the blastocyst stage (Sakaue *et al.*, 2010). Cells derived from either Dnmt TKO-NT embryos or TKO ntTS cells contributed to and survived in the placenta in the context of the chimeric conceptus between wild-type embryos. This indicates that DNA methylation is dispensable for specification of the TE, but it does not show conclusively whether the Dnmt-TKO trophoblast cells are functionally normal. As mentioned above, ectopic expression of *Oct4* has been detected by RT-PCR in the *Dnmt1*-deficient E10.5 placenta (Hattori *et al.*, 2004). Oct4 alone has been shown to reprogram TS cells into ES-like cells, although this occurs at a low efficiency (Wu *et al.*, 2011b). It is therefore possible that the ectopic expression of *Oct4* in trophoblast cells compromises their identity and causes yet-to-be unrevealed abnormalities. Loss of DNA methylation should also affect the regulation of imprinted genes. It has been shown that the loss of maternal imprint because of a lack of Dnmt3L during oogenesis results in placental defects even in heterozygous conceptuses (Arima *et al.*, 2006), which suggests that *Dnmt*-deficient trophoblast cells also show abnormalities because of the deregulated expression of imprinted genes. In addition, insufficient DNA methylation has been implicated in pregnancy-associated diseases in humans (Novakovic and Saffery, 2012). Thus, the evidence points to an essential role of DNA methylation in the normal development and function of trophoblast cells. Further examination of mouse models such as conditional knockout of *Dnmt* genes in trophoblast cells should unveil the roles of DNA methylation in trophoblast cell lineage.

An irregular increase in DNA methylation may also be harmful for trophoblast development and placental function. The genes of two human endogenous retroviruses, *Syncytin-1* (*ERVWE1*) and *Syncytin-2* (*ERVFRDE1*), contribute to the formation of the multinucleated syncytiotrophoblast, which forms a physical barrier to maternal blood in the chorionic villi of the human placenta (Mi *et al.*, 2000). Expression of these fusogenic proteins is tightly restricted to placental trophoblast cells. It has been shown that these genes are silenced in somatic cells by DNA methylation of CpGs within 5' long terminal repeats (LTRs) (Matousková *et al.*, 2006; Trejbalová *et al.*, 2011). Aberrant methylation of these 5' LTRs in trophoblast cells might lead to the inadequate formation of the syncytiotrophoblast and a malfunctioning placenta. DNA methylation is also implicated in the differentiation state-dependent control of gene expression in mouse trophoblast cells. For example, the *dimethylarginine dimethylaminohydrolase 2* (*Ddah2*) gene is suppressed in TS cells with a hypermethylated enhancer both *in vivo* and *in vitro*, and the enhancer is demethylated in differentiated trophoblast cells in which the gene is expressed. Treatment with 5-aza-dC induces ectopic expression of *Ddah2* in undifferentiated TS cells. Reporter assay analyses showed that the methylation of the *Ddah2* enhancer diminished activity of the *Ddah2* promoter, which suggests that DNA methylation of the enhancer suppresses *Ddah2* expression *in vivo* (Tomikawa *et al.*, 2006). Again, aberrant methylation of the *Ddah2* enhancer in differentiated trophoblast cells might cause reduced expression of this gene. In a classical RLGS analysis, 30 T-DMRs were detected through a comparison between TS cells and differentiated TS cells. One half of the T-DMRs showed greater methylation in undifferentiated cells, and the other half showed greater methylation in differentiated cells, which suggests that the fine control of DNA methylation accompanies even trophoblast differentiation (Shiota *et al.*, 2002). Overall, DNA methylation should play a pivotal role in the regulation of trophoblast differentiation and function.

Possible role of ten-eleven translocation (Tet) in trophoblast regulation

A growing body of evidence now suggests that 5hmC is not just a transient intermediate of active demethylation but that it plays a unique role as an epigenetic mark (Inoue and Zhang, 2011; Iqbal *et al.*, 2011; Ruzov *et al.*, 2011; Salvaing *et al.*, 2012). Possible contribution of *Tet* genes in the regulation of trophoblast development has been suggested by loss-of-function analyses of *Tet* genes. In mouse blastocysts, immunostaining using anti-5hmC antibody revealed slightly higher content of 5hmC in the ICM than in the TE (Ruzov *et al.*, 2011). Tet1 protein also appears to be enriched in the ICM compared with the TE (Ito *et al.*, 2010). Similar asymmetry was also reported between ES and TS cells. The expression levels of *Tet1* and *Tet2* mRNAs as well as the global 5hmC level analyzed by mass spectrometry are significantly lower in TS cells than in ES cells (Ito *et al.*, 2010; Senner *et al.*, 2012). Reduction of *Tet1* expression in one blastomere of mouse two-cell stage embryos biased the blastomere's cell fate toward the TE (Ito *et al.*, 2010). These findings imply that *Tet1* plays an essential role in the first cell-fate decision during mouse embryogenesis. However, it has been reported that *Tet1* and *Tet2* are dispensable for blastocyst formation and for postnatal development (Dawlaty *et al.*, 2013; Dawlaty *et al.*, 2011), which refutes the idea of an essential role of *Tet1* and *Tet2* in the specification of the TE and ICM. It is possible that the asymmetry in the global level of 5hmC between two blastomeres of two-cell stage embryos caused by *Tet1* knockdown in one blastomere somehow skewed the equivalence of blastomeres, whereas other mechanisms governed the cell-fate decision without such asymmetry in *Tet1*/*Tet2*-deficient embryos. It is also possible that the loss of *Tet1* and *Tet2* was compensated by induction of *Tet3* in early embryos, as seems to be the case at least in part in tissues of *Tet1*/*Tet2*-deficient adult mice (Dawlaty *et al.*, 2013).

Tet1-deficient and *Tet1*/*Tet2*-deficient ES cells show induction of TS cell marker genes such as *Cdx2*, *Eomes*, and *Elf5 in vitro* and form hemorrhagic teratomas with trophoblast-like cells, which suggests that 5hmC also plays a pivotal role in cell-fate restriction in the embryonic cell lineage (Dawlaty *et al.*, 2013, Dawlaty *et al.*, 2011, Koh *et al.*, 2011). However, it should be kept in mind that Tet1 protein is implicated in the repression of Polycomb-targeted developmental regulators in ES cells independent of its enzymatic activity (Wu *et al.*, 2011a), which makes it less likely that 5hmC plays a major role in cell-fate restriction. Nevertheless, the importance of *Tet1* in the trophoblast lineage has been suggested. Some of the *Tet1*/*Tet2*-deficient embryos show midgestation lethality with a wide variety of abnormalities including a smaller fetus compared with normal littermates at E10.5 (Dawlaty *et al.*, 2013). *Tet1*-deficient

embryos also show a mild developmental delay at E12.5, and the mutant pups are smaller in size and weight. A placental defect was suggested for *Tet1*$^{-/-}$ mice because the tetraploid complementation rescued these phenotypes (Dawlaty *et al.*, 2011). Taken together, these data suggest that Tet proteins and 5hmC are likely to be involved in the development and function of the trophoblast cell lineage.

Perspective

Loss-of-function analyses in mice have revealed insights into the genetic regulation of the development, differentiation, and function of trophoblast cells (Cross, 2005, Watson and Cross, 2005), but our knowledge about epigenetic regulation remains limited. Genetic ablation of epigenetic factors including members of the Dnmt and Tet families in a TE-specific or trophoblast subtype-specific manner will be required for further understanding of the epigenetic regulation of trophoblast cells. Genome-wide analyses of DNA methylation and hydroxymethylation in the context of gene expression and histone modification patterns should be also performed in trophoblast cells of different subtypes and at various differentiation stages. Even with recent technical advances, it is still difficult to perform such epigenome analyses with *in vivo* materials because of the small amount in early pregnancy and the complexity at mid to late pregnancy. Mouse TS cells will provide a useful tool for circumventing such difficulties. Development of culture conditions that direct the differentiation of TS cells toward specific subtypes will be the next challenge for this purpose. This type of research will help us elucidate the details of the epigenetic landscape of trophoblast development, which should supply valuable information for understanding the epigenetic mechanisms underlying pregnancy-related diseases in humans.

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