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Trophoblast-specific DNA methylation occurs after the segregation of the trophectoderm and inner cell mass in the mouse periimplantation embryo

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Key words: DNA methylation, trophoblast, blastocyst, trophectoderm, placenta

Abbreviations: E, embryonic day; ES, embryonic stem; EPC, ectoplacental cone; Epi, epiblast; ExE, extraembryonic ectoderm; FBS, fetal bovine serum; ICM, inner cell mass; MSRE-qPCR, methylation-sensitive restriction enzyme-quantitative PCR; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone; RLGS, restriction landmark genomic scanning; T-DMRs, tissue-dependent and differentially methylated region; TE, trophectoderm; TS, trophoblast stem

The first cell differentiation in the mammalian development separates the trophoblast and embryonic cell lineages, resulting in the formation of the trophectoderm (TE) and inner cell mass (ICM) in blastocysts. Although a lower level of global DNA methylation in the genome of the TE compared with ICM has been suggested, the dynamics of the DNA methylation profile during TE/ICM differentiation has not been elucidated. To address this issue, first we identified tissue-dependent and differentially methylated regions (T-DMRs) between trophoblast stem (TS) and embryonic stem (ES) cells. Most of these TS-ES T-DMRs were also methylated differentially between trophoblast and embryonic tissues of embryonic day (E) 6.5 mouse embryos. Furthermore, we found that the human genomic regions homologous to mouse TS-ES T-DMRs were methylated differentially between human placental tissues and ES cells. Collectively, we defined them as cell-lineage-based T-DMRs between trophoblast and embryonic cell lineages (T-E T-DMRs). Then, we examined TE and ICM cells isolated from mouse E3.5 blastocysts. Interestingly, all T-DMRs examined, including the *Elf5*, *Pou5f1* and *Nanog* loci, were in the nearly unmethylated status in both TE and ICM and exhibited no differences. The present results suggest that the establishment of DNA methylation profiles specific to each cell lineage follows the first morphological specification. Together with previous reports on asymmetry of histone modifications between TE and ICM, the results of the current study imply that histone modifications function as landmarks for setting up cell-lineage-specific differential DNA methylation profiles.

Introduction

DNA methylation is one of the epigenetic modifications in mammals and affects the functional state of the genome. In the developmental process, cells arising from the single cell, i.e., fertilized zygotes, give rise to various types of cells via epigenetic drive, and cell differentiation is closely linked to the renovation of the epigenome. The mammalian genome contains tissue-dependent and differentially methylated regions (T-DMRs) that exhibit cell-type-specific DNA methylation levels.^{1,2} Thus, the sum of DNA methylation status of T-DMRs is equivalent to cell-type-specific DNA methylation profiles,³ i.e., cell differentiation is accompanied by the construction of new DNA methylation profiles.

The first cell differentiation in mammalian development segregates the trophoblast from the embryonic cell lineages. In mice,

these two cell lineages are specified by embryonic day 3.5 (E3.5), which results in the formation of the trophectoderm (TE) and inner cell mass (ICM) of blastocysts. The TE gives rise exclusively to extraembryonic tissues, such as the placenta,⁴ whereas the ICM gives rise to the embryo proper, germ cells and some extraembryonic membranes. As each cell type has its own distinctive DNA methylation profile, it is assumed that cells of the TE and ICM also have their unique DNA methylation profiles, which suggests the presence of T-DMRs between the TE and ICM (TE-ICM T-DMRs).

Differences in DNA methylation levels between extraembryonic and embryonic tissues were reported in the 1980s in studies using postimplantation-stage embryos. Hypomethylation in extraembryonic tissues compared with embryonic tissues was first found in the rabbit;⁵ subsequently, it was confirmed in the

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Submitted: 10/21/11; Revised: 12/02/11; Accepted: 12/06/11

<http://dx.doi.org/10.4161/epi.7.2.18962>

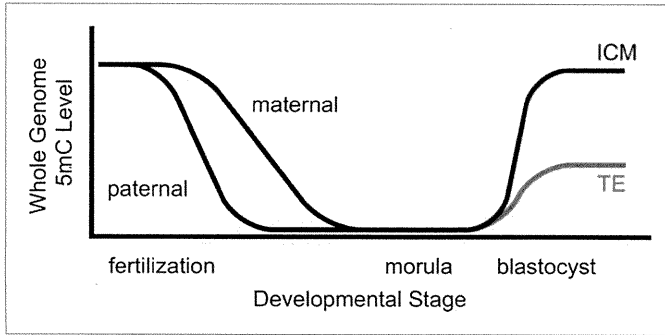


Figure 1. Schematic diagram of the dynamic change in the 5-methylcytosine levels of the whole genome in mouse preimplantation embryos. The x-axis (time) and the y-axis (relative 5-mC levels of genomic DNA) are not to scale. Modified from Dean et al.¹⁰

mouse at the level of not only total DNA, but also at repetitive elements and some unique genes.⁶⁻⁸ However, the process of the establishment of such differences in DNA methylation remained unclear because it was technically difficult to analyze the DNA methylation state in trophoblast and embryonic tissues separately using preimplantation embryos. Currently, this issue is explained by differences in the extent of de novo methylation in the preimplantation stage, which was shown in a recent report that examined changes in DNA methylation levels via immunostaining of mouse embryos at sequential stages using an anti-5-methylcytosine (5-mC) antibody.⁹ After fertilization, there is a drastic decrease in the content of 5-mC in genomic DNA.¹⁰⁻¹² Thus, 5-mC is barely detected at the morula stage, which is followed by de novo methylation that results in detectable levels of 5-mC in E3.5 blastocysts. In the blastocyst, higher levels of the methylated signal were observed in the ICM compared with the TE.⁹ Based on these findings, the wave of DNA methylation dynamics shown in **Figure 1** is currently well accepted.¹⁰ It should be mentioned, however, that this wave represents the global change in the methylation level of the whole genome. In fact, some individual loci and repetitive elements, in addition to imprinted gene loci, do not follow this wave.¹³⁻¹⁶ In addition, recent reports suggest that the conversion of 5-mC to 5-hydroxymethyl-cytosine accounts for rapid reduction of 5-mC in male pronuclei in zygotes.^{17,18}

Cultured stem cell lines have been derived from the TE and ICM: trophoblast stem (TS) cells¹⁹ and embryonic stem (ES) cells,^{20,21} respectively. Because their developmental potentials correspond well with their tissues of origin, these cells have been used as in vitro models. It was reported that some lineage-specific genes are regulated via DNA methylation in TS and ES cells. ELF5 is expressed specifically in extraembryonic tissues in mouse embryos and is required for the maintenance of TS cells.²² In ES cells, ELF5 expression is repressed by the highly methylated status of the upstream region, preventing a trophoblastic cell fate. In contrast, the *Elf5* locus is not methylated in TS cells, which express ELF5.²³ Conversely, we have reported that the pluripotency-related genes *Pou5f1* and *Nanog*²⁴⁻²⁷ exhibit an expression pattern that is opposite to that of *Elf5*.^{28,29} Namely, the *Pou5f1* and *Nanog* loci contain T-DMRs that are hypermethylated in TS cells and hypomethylated in ES cells. However, whether such

T-DMRs between TS and ES cells (TS-ES T-DMRs) represent faithfully TE-ICM T-DMRs has not been elucidated. It was reported previously that the *Pou5f1* and *Nanog* loci are scarcely methylated in E3.5 embryos,^{30,31} but TE and ICM were not analyzed separately in these reports; thus, whether these loci are T-DMRs remains unclear.

DNA methylation profiles reflect the multilayered regulation of tissue-specific gene function.^{32,33} In addition, the analysis of the methylation profile of 18 different tissues and cells revealed that DNA methylation profiles reflect developmental similarity and cellular phenotypes.³⁴ Thus, TS-ES T-DMRs potentially represent the prime difference between trophoblast and embryonic cell lineages, and are considered as good candidates for TE-ICM T-DMRs. In this study, we elucidated the DNA methylation status of TS-ES T-DMRs in trophoblast and embryonic lineages in the mouse embryo to determine definitively whether T-DMRs defined between TS cells and ES cells are established as TE-ICM T-DMRs in the developmental process.

Results

Identification of novel TS-ES T-DMRs. In our effort to identify T-DMRs in the mouse genome, we sampled more than 200 *NotI* recognition sites as potentially informative loci and examined their DNA methylation status using real-time quantitative methylation-sensitive restriction enzyme PCR (MSRE-qPCR) in various cells and tissues.³⁴ Through this effort, we also identified several *NotI* sites as potential TS-ES T-DMRs showing differential methylation levels between TS and ES cells (data not shown). Some of these sites were used in our previous reports to verify the epigenetic integrity of TS and ES cells derived from somatic cell nuclear-transferred embryos.^{35,36}

In this study, we selected 14 potential TS-ES T-DMRs from the MSRE-qPCR data set based on the criteria mentioned in the "Materials and Methods." All of these loci were hypermethylated in TS cells and hypomethylated in ES cells. We performed bisulfite sequencing analysis of the regions around these *NotI* sites to elucidate the regional DNA methylation status. Among them, seven regions (*Ff46*, *Bd22*, *Ef40*, *Hd20*, *Eb41*, *Ff36* and *Hf01*; Group A) exhibited a markedly hypermethylated status in TS cells (at least 40% higher in TS than in ES cells); thus, these were confirmed as definite TS-ES T-DMRs (**Fig. 2 and Table 1**). Five regions (*Gc06*, *Eg01*, *Ef30*, *Ca37* and *Gb20*; Group B) also showed higher DNA methylation status in TS vs. ES cells, but with a moderate difference (30–40%). The other two regions (*Ec23* and *He06*) showed only a modest difference (<15%; Group C). Thus, we successfully identified TS-ES T-DMRs that are good candidates for T-DMRs between trophoblast and embryonic cell lineages in vivo.

DNA methylation status of TS-ES T-DMRs in the extra-embryonic ectoderm (ExE) and epiblast (Epi) of E6.5 embryos and in E14.5 placenta. Having identified TS-ES T-DMRs, we then analyzed their DNA methylation status in the ExE and Epi of E6.5 postimplantation embryos to ascertain whether they were methylated differentially in vivo. The ExE and Epi originate from the TE and ICM, respectively. TS cell lines can also be derived

from the ExE of E6.5 embryos;^{19,37} thus, it is implied that stem cells of the trophoblast lineage are maintained in E6.5 ExE. At the *Ddah2* gene locus, the epigenetic properties of the ExE were reflected in that of TS cells.³⁸

Among the TS-ES T-DMRs identified above, all seven loci from Group A and four loci (*Gc06*, *Eg01*, *Ca37* and *Gb20*) from Group B showing <5% methylation in ES cells were selected for analysis. The ExE and Epi from a single embryo were used for single-locus analysis. Bisulfite sequencing analysis revealed three types of methylation pattern. First, eight loci (*Ff46*, *Hd20*, *Eb41*, *Ff36*, *Hf01*, *Gc06*, *Eg01* and *Ca37*) were hypermethylated in ExE and hypomethylated in Epi ($p < 0.01$), as expected from the methylation trend observed in TS and ES cells (Fig. 3A and Table S1). Second, *Ef40* was also methylated differentially between ExE and Epi, but the methylation trend was opposite to that observed in cells in vitro (Fig. S1 and Table S1). Finally, there was very little methylation difference at the *Gb20* and *Bd22* loci (<8%) between ExE and Epi (Fig. S1 and Table S1).

We also examined the methylation status of the three TS-ES T-DMRs reported previously (*Elf5*, *Pou5f1* and *Nanog*),^{23,28,29} and observed that all three loci were hypermethylated or hypomethylated in ExE and Epi in the same manner as that observed in TS cells and ES cells (Fig. 3B and Table S1); namely, the *Elf5* locus was hypomethylated in ExE and hypermethylated in Epi, and converse findings were obtained at the *Pou5f1* and *Nanog* loci. Although the tendency of the methylation pattern was consistent throughout every experiment, the extent of methylation varied among embryos at some loci. Most conspicuously, between two embryos analyzed, the extent of methylation of *Pou5f1* in ExE was lower in one embryo than in the other. This may indicate that differential methylation is still in the process of being established at E6.5; thus, some embryos are a little more advanced than others. Nevertheless, these loci were confirmed as T-DMRs between trophoblast and embryonic lineages in vivo at E6.5 (ExE-Epi T-DMRs), as reflected in TS and ES cells.

Collectively, most of the TS-ES T-DMRs were ExE-Epi T-DMRs and can be defined as trophoblast-embryonic T-DMRs (T-E T-DMRs) in vivo. No common features were found among these T-E T-DMRs regarding chromosomal location, relative position to the nearest genes and CpG islands (Fig. S2).

We also checked whether T-E T-DMRs were hypermethylated in the placenta. Bisulfite sequencing of the eight T-E T-DMR loci was performed in mouse E14.5 placenta and showed that the T-E T-DMRs were also hypermethylated in the placenta, almost equally to that observed for E6.5 ExE, with only one exception: *Hd20* was scarcely methylated (Fig. S3). The methylation rates in placenta were lower than those observed in TS cells, which is consistent with our previous analysis using the MSRE-qPCR method (unpublished data and ref. 34). In the present study, ExE also showed a lower extent of hypermethylation compared with that of TS cells. Previously, it was shown that the methylation extent at the *Pou5f1* and *Ddah2* loci in the placenta and ExE was lower than that observed in TS cells, respectively.^{28,38} This may reflect the heterogeneity of in vivo tissue samples.

DNA methylation status of T-E T-DMRs in human placenta and ES cells. To investigate whether the cell-lineage-based

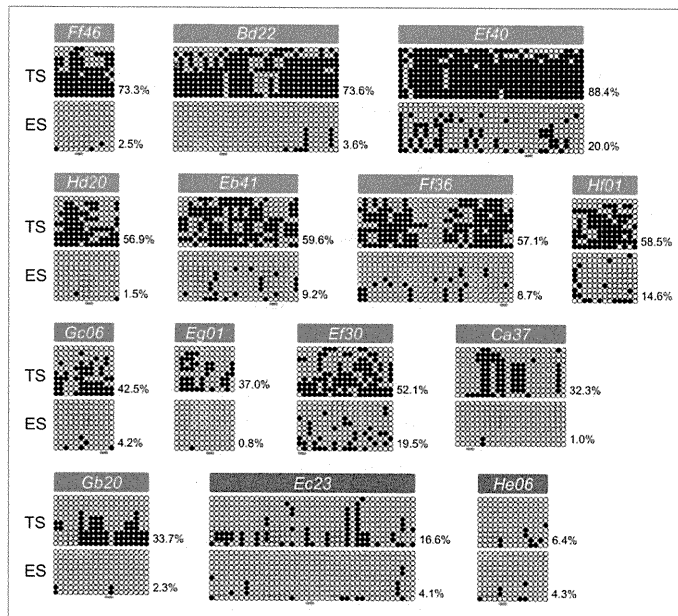


Figure 2. DNA methylation status of the TS-ES T-DMRs around the *NotI* site in TS and ES cells. The DNA methylation status of potential TS-ES T-DMRs around the *NotI* site were determined via bisulfite sequencing. Open and filled circles represent unmethylated and methylated cytosines, respectively. X, not read by sequence analysis. Overall methylation percentage (the number of methylated CpGs per number of total CpGs) is shown to the right side of each part. The red bars under each part indicate CpGs at the *NotI* site. For *Hf01*, the region upstream of the *NotI* site was analyzed in this study. TS-ES T-DMRs were classified into three groups according to the extent of differences between TS and ES cells: seven loci as Group A (shown in the pink box); five loci as Group B (shown in the purple box); and two loci as Group C (shown in the gray box).

differential DNA methylation pattern at T-E T-DMR loci is also present in other mammalian species, we analyzed the DNA methylation status in human samples. Because it is ethically difficult to use human early-stage embryos, we used later-stage placental tissues.

The sequences of the eight loci confirmed as T-E T-DMRs in mice (*Ff46*, *Hd20*, *Eb41*, *Ff36*, *Hf01*, *Gc06*, *Eg01* and *Ca37*) are conserved in the human genome (Table S2 and Fig. S4). We performed bisulfite sequencing analysis of these homologous regions using human cells and tissues. Placental villus samples from gestational stages under 14 weeks (u14wks) of pregnancy and term placenta, and human ES (hES) cells were used as trophoblastic and embryonic samples, respectively. The bisulfite PCR amplicons were designed to cover the conserved genomic regions (Table S2). Consequently, all regions examined were hypermethylated in u14wks and/or term placental tissues and hypomethylated in ES cells ($p < 0.01$) (Fig. 4). These results demonstrate that trophoblast cell-lineage-specific hypermethylation is conserved between mice and humans at these T-E T-DMRs.

DNA methylation status of T-E T-DMRs in E3.5 blastocysts. As shown above, cell-lineage-based differences between trophoblast and embryonic cell lineages regarding DNA methylation exist at T-E T-DMR loci in E6.5 mouse embryo. To assess whether this difference exists in E3.5 blastocysts, we collected

Table 1. DNA methylation rate of TS-ES T-DMRs in TS and ES cells

Locus	TS*	(%)	ES*	(%)	TS-ES (%)	p value**	Group
<i>Ff46</i>	88/120	73.3	3/120	2.5	70.8	2.2×10^{-5}	A
<i>Bd22</i>	242/329	73.6	12/330	3.6	70.0	1.1×10^{-5}	A
<i>Ef40</i>	327/370	88.4	74/370	20.0	68.4	1.1×10^{-5}	A
<i>Hd20</i>	74/130	56.9	2/130	1.5	55.4	1.7×10^{-4}	A
<i>Eb41</i>	143/240	59.6	22/240	9.2	50.4	1.1×10^{-5}	A
<i>Ff36</i>	177/310	57.1	27/310	8.7	48.4	1.1×10^{-5}	A
<i>Hf01</i>	76/130	58.5	19/130	14.6	43.9	2.3×10^{-3}	A
<i>Gc06</i>	51/120	42.5	5/120	4.2	38.3	9.3×10^{-4}	A
<i>Eg01</i>	40/108	37.0	1/120	0.8	36.2	2.3×10^{-4}	A
<i>Ef30</i>	99/190	52.1	37/190	19.5	32.6	1.0×10^{-3}	B
<i>Ca37</i>	71/220	32.3	2/198	1.0	31.3	2.2×10^{-5}	B
<i>Gb20</i>	64/190	33.7	4/171	2.3	31.3	1.5×10^{-2}	B
<i>Ec23</i>	68/410	16.6	17/410	4.1	12.4	4.8×10^{-3}	C
<i>He06</i>	9/140	6.4	6/140	4.3	2.1	7.4×10^{-1}	C

*Number of methylated CpGs/total number of CpGs analyzed using the bisulfite sequencing illustrated in Figure 2. **p values were calculated using the nonparametric two-tailed Mann-Whitney test.

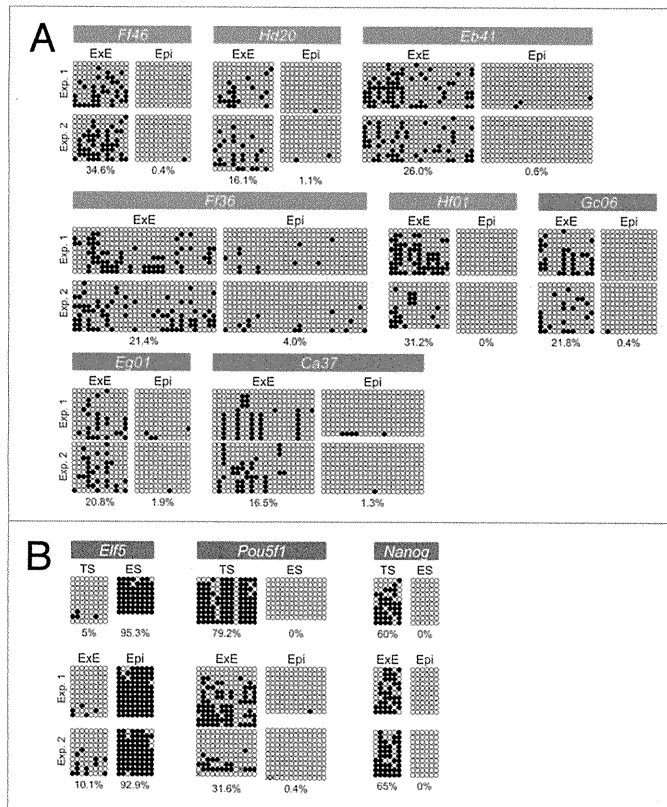


Figure 3. DNA methylation status of TS-ES T-DMRs in the ExE and Epi. (A) DNA methylation status of eight TS-ES T-DMRs around *Not1* sites in the ExE and Epi, as determined using bisulfite sequencing. Samples for experiments 1 (Exp. 1) and 2 (Exp. 2) were collected independently. The color of boxes indicates the group defined by the methylation status of TS and ES cells (see Fig. 2 and Table 1). (B) DNA methylation status of *Elf5*, *Pou5f1* and *Nanog* in TS cells, ES cells, ExE and Epi, as determined using bisulfite sequencing. Open and filled circles represent unmethylated and methylated cytosines, respectively. Overall methylation percentage (the number of methylated CpGs per number of total CpGs) is shown under each part.

E3.5 blastocysts and isolated TE and ICM cells enzymatically, to analyze their DNA methylation status separately.

Because the number of TE and ICM cells available after completion of this separating method was very limited, we assessed the fidelity of the bisulfite sequencing analysis using as few as 100 cells before the analysis of the TE and ICM cells. We designed two experiments with ~100 cells in which the methylation status would be ~50% theoretically. First, we examined the DNA methylation status of the *H19* locus using two E3.5 blastocysts. *H19*, an imprinted locus, is methylated only at the paternal allele in E3.5 embryos.³⁹ As shown in Figure S5A, both methylated and unmethylated clones emerged, although the methylation rate was not exactly 50%. Next, DNA methylation status was analyzed at the *Elf5* and *Pou5f1* using a 1:1 mixture of TS and ES cells. Trypsinized cells were counted and collected under a dissecting microscope, and 50 cells of each type were combined to obtain 100 cells in total per analysis of each locus. The T-DMR at the *Elf5* locus was methylated at ~100% in ES cells and at ~0% in TS cells, whereas that at the *Pou5f1* locus exhibited the opposite methylation pattern (reviewed in refs. 23 and 28 and this study). Theoretically, the 1:1 cell mixture should exhibit ~50% methylation at these two loci. In our study, although the methylation level was not exactly 50%, methylated and unmethylated DNA strands were detected at both loci and the methylation rate was around 50% (Fig. S5B). Therefore, we concluded that the method adopted here provides the methylation status in samples of ~100 cells, although its accuracy did not reach the predicted methylation value exactly.

The results of the bisulfite sequencing analysis using TE and ICM cells demonstrated that the three T-E T-DMRs *Elf5*, *Pou5f1* and *Hf01* were remarkably hypomethylated in both the TE and ICM, and that there was no difference in DNA methylation rate between the TE and ICM ($p > 0.05$) (Fig. 5A). The T-DMRs at the *Elf5* and *Pou5f1* loci were almost completely unmethylated, which is consistent with the results of a previous study based

on whole blastocysts.^{30,40} *Hf01* also showed a hypomethylated status; however, there were more methylated CpGs at this locus than at *Elf5* and *Pou5f1* in both the TE and ICM. To summarize, all three T-E T-DMRs were in a state of hypomethylation in both E3.5 TE and ICM.

Because the three T-E T-DMRs examined above appeared to be barely methylated and showed no apparent difference between the TE and ICM, we explored whether there was any DNA methylation at other T-E T-DMRs in E3.5 blastocysts. Thus, we evaluated the DNA methylation status of the other eight T-E T-DMRs (*Ff46*, *Hd20*, *Eb41*, *Ff36*, *Gc06*, *Eg01*, *Ca37* and *Nanog*) in whole blastocysts. As shown in **Figure 5B**, we found that these loci exhibited little or no methylation in E3.5 blastocysts, which led us to conclude that all T-E T-DMRs examined here were almost totally hypomethylated in E3.5 blastocysts, whether in the TE or ICM.

DNA methylation status of T-E T-DMRs in E4.5 and diapause blastocysts. Having demonstrated that all T-E T-DMRs were scarcely methylated in blastocysts at E3.5, we then examined the methylation status of T-E T-DMRs in E4.5 blastocysts flushed out from uteri. The following four loci were selected for examination: *Ff46*, *Eb41* (especially highly methylated in E6.5 ExE and in E14.5 placenta), *Nanog* (the most methylated locus in E6.5 ExE) and *Elf5* (the only locus that was hypermethylated in the embryonic cell lineage examined in this study). We observed that all four loci examined were hypomethylated in E4.5 blastocysts, to the same extent as that observed at E3.5 (**Fig. 6A**).

We also examined the DNA methylation status of the four loci in diapause blastocysts obtained via experimentally induced delayed implantation. A slight increase in methylation rate was observed in diapause blastocysts compared with E4.5 blastocysts; however, this difference was not significant, with the exception of the results obtained for *Elf5* ($p < 0.01$) (**Fig. 6B**).

Discussion

Here, we identified T-E T-DMRs that were methylated differentially between trophoblast and embryonic cell lineages both in vitro and in vivo. Regarding the correspondence between the DNA methylation patterns in vitro and in vivo, this epigenetic feature observed at our T-E T-DMRs is likely an inherent property of each cell lineage. Using these T-E T-DMRs, we traced and delineated the trophoblast/embryonic cell lineage differentiation from the viewpoint of the DNA methylation profile and observed that the T-E T-DMRs *Elf5*, *Pou5f1* and *Hf01* were scarcely methylated in both the TE and ICM. The nearly unmethylated status observed in whole blastocysts was also present in all other T-E T-DMR loci. Collectively, all T-E T-DMR loci examined were almost unmethylated in E3.5 mouse blastocysts. This is of

interest because the TE and ICM are specified distinctly to each cell lineage; thus, each of these structures was expected to exhibit a different methylation pattern at the T-E T-DMRs.

It is known that the expression of *Elf5*, *Pou5f1* and *Nanog* is regulated via DNA methylation in TS and ES cells.^{23,28,29} However, in this study, these genes showed little methylation both in TE and ICM, which exhibit a differential expression pattern.^{22,41} This result indicates that the transcription of these genes is not regulated in a DNA-methylation-dependent manner in the blastocyst. In a recent study, an *Elf5* transgene lacking the differentially methylated region did not show ectopic expression in blastocysts.⁴² Moreover, nuclear-transferred embryos with no functional DNA methyltransferase can reach the blastocyst stage.⁴³ Based on these observations, DNA methylation is considered dispensable in regulating the expression of the factors necessary for TE/ICM differentiation.

The present study provides an additional epigenetic insight into trophoblast/embryonic cell differentiation. We demonstrated clearly that the trophoblast and embryonic cell-lineage-specific DNA methylation patterns of T-E T-DMRs are set up by E6.5,

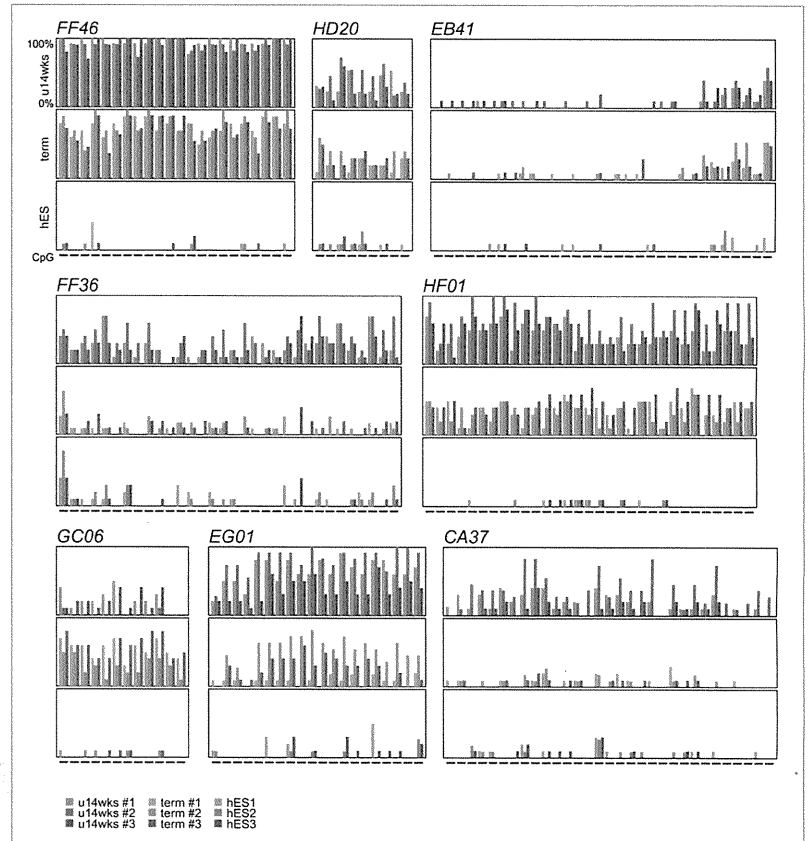


Figure 4. DNA methylation status of T-E T-DMRs in human placenta and ES cells. Graphic representation of the DNA methylation level at human genomic regions homologous to mouse T-E T-DMRs in human placental tissue from under 14 weeks of gestation (u14wks), from term pregnancy (term), and from human ES cells (hES). Three independent samples or cell lines were examined for each tissue/cell type. CpG sites are indicated by short horizontal bars at the bottom of each part. The percentage ratio of methylated cytosines at each CpG site, which was determined from the data of clone-based bisulfite sequencing, is indicated by vertical columns, with the height of each column representing the degree of methylation.

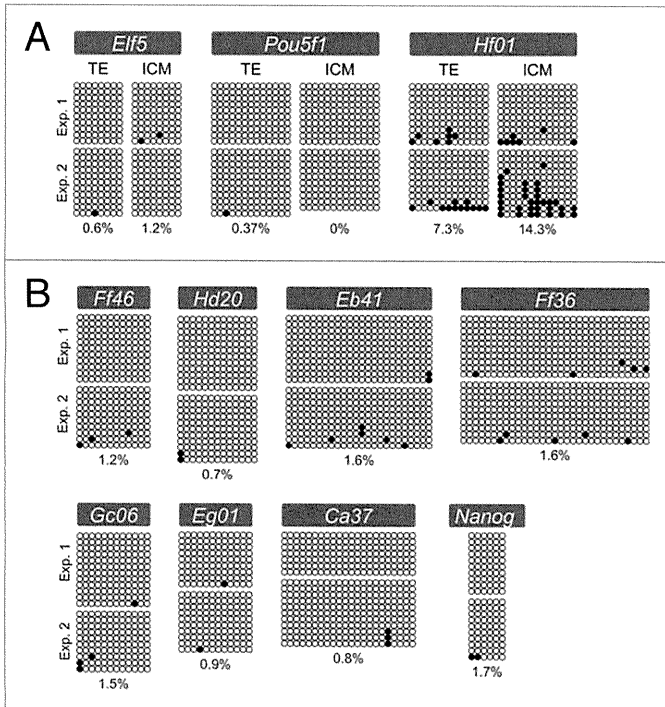


Figure 5. DNA methylation status of T-E T-DMRs in E3.5 blastocysts. (A) DNA methylation status of *Eif5*, *Pou5f1* and *Hf01* in the TE and ICM of E3.5 blastocysts. (B) DNA methylation status of the T-E T-DMRs *Ff46*, *Hd20*, *Eb41*, *Ff36*, *Gc06*, *Eg01*, *Ca37* and *Nanog* in E3.5 blastocysts, as determined using bisulfite sequencing. Open and filled circles represent unmethylated and methylated cytosines, respectively. Overall methylation percentage (the number of methylated CpGs per number of total CpGs) is shown under each part.

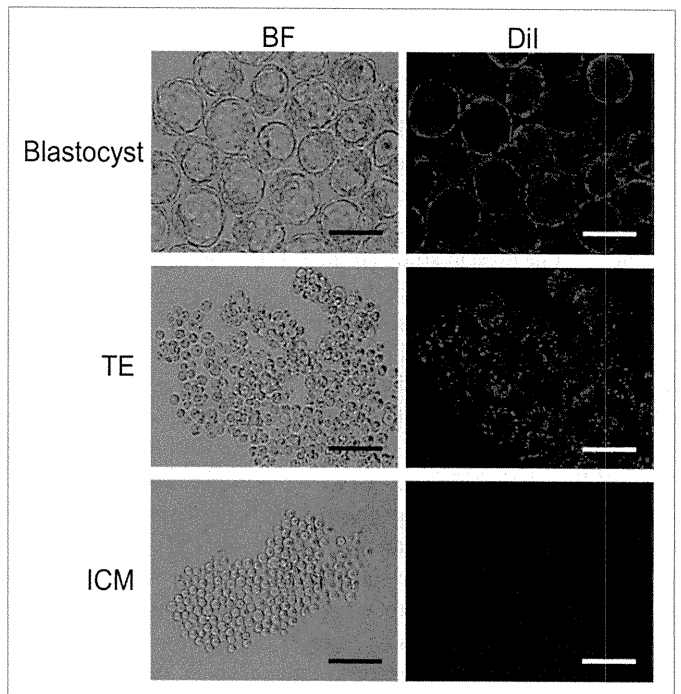


Figure 7. Separation of TE and ICM cells. Top, intact blastocysts stained with Dil at the outer surface. Middle, cells with Dil fluorescence (TE). Bottom, cells without Dil fluorescence (ICM). Scale bars, 100 μ m. BF, bright field.

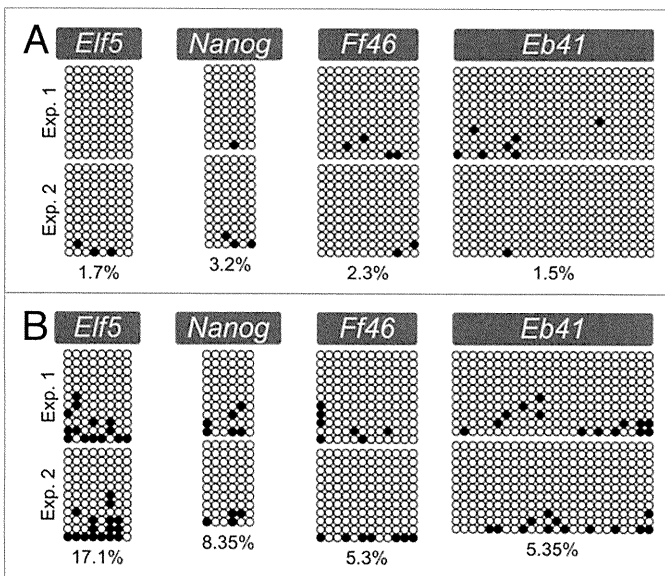


Figure 6. DNA methylation status of T-E T-DMRs in E4.5 and diapause blastocysts. DNA methylation status of selected T-E T-DMRs (*Eif5*, *Nanog*, *Ff46* and *Eb41*) in E4.5 (A) and diapause (B) blastocysts. Open and filled circles represent unmethylated and methylated cytosines, respectively. Overall methylation percentage (the number of methylated CpGs per number of total CpGs) is shown under each part.

but not at E3.5. The comparison of E4.5 and diapause blastocysts revealed the presence of a slight, yet significant, increase in the DNA methylation level at the *Eif5* locus in diapause blastocysts. It is possible that de novo methylation gradually progresses in the ICM during the few days of diapause. Alternatively, gradual de novo methylation may have started in the advanced, already implanted embryos at E4.5, which were not recovered by uterine flushing. Nevertheless, our results demonstrate that de novo methylation of T-E T-DMRs occurs after TE/ICM specification.

Our observations suggest that factors other than DNA methylation lead TE/ICM differentiation. In addition to the balance of key transcription factors and apical/basal cell polarity, epigenetic modification on histone tails is another potential differentiation-leading factor. The histone modification status of some genes in the TE and ICM was analyzed previously in reference 44. In the ICM, the promoter regions of *Pou5f1* and *Nanog* are enriched in H4K16 acetylation, which is a permissive modification, whereas repressive modification of H3K9 dimethylation is depleted. In contrast, the converse is true in the TE. These modification patterns correspond to the transcriptional states of these genes. Furthermore, at an earlier stage after the first cleavage, it is suggested that histone modification asymmetry in two blastomeres functions as an epigenetic marker for cell allocation in extraembryonic or embryonic lineages.⁴⁵ Together with our present results, these data imply that preceding histone modifications may be landmarks for the establishment of trophoblast-specific DNA methylation profiles during trophoblast and embryonic cell-lineage segregation, because interactions between

histone modification and DNA methylation have been observed in many studies,^{46,47} although whether this is true remains an open question.

Our results also showed that TS and ES cells are distinct from their corresponding tissues of origin in blastocysts. Based on transcriptome analysis, some reports showed that the global gene expression patterns of ES cells and the ICM are significantly different.^{48,49} The present study confirmed this notion epigenetically in TS cells and in the TE from the viewpoint of DNA methylation. There are two possible explanations for this: one is that the culture conditions and microenvironment enhanced de novo DNA methylation; the other is that there is a very small subset of cells in blastocysts with a highly methylated status that are selected to survive and expand in culture. Drastic changes during the cell-line derivation process were reported recently in studies of other aspects of the epigenome. One heterochromatic histone modification is acquired during culture for ES cell derivation, and culture conditions have some effect on this phenomenon.⁵⁰ The stem cell derivation process from blastocysts is accompanied by revisions of the epigenome.

Our observations suggest that, when TS cells are derived from E3.5 TE, a TS cell-specific DNA methylation profile is acquired during the cell culture process. In our previous study, TS cells derived from blastocysts via nuclear transfer (ntTS cells) exhibited a DNA methylation status that was very similar to that of TS cells derived from normally fertilized blastocysts,³⁶ although an aberrant methylation status has been reported for nuclear-transferred preimplantation embryos.^{51,52} The present results raise a hypothesis to explain this observation: the DNA methylation profile is processed as TS cell-specific during cell-line derivation, even if somatic cell-specific DNA methylation marks remain in the TE of nuclear-transferred blastocysts.

Although TS cells can be derived from both types of tissue, TS cells are distinct from the TE and are rather similar to the ExE, from the viewpoint of the DNA methylation status of T-E T-DMRs. Previously, it was reported that TS cell lines can be derived from E6.5 ExE, but not from E3.5 TE in MAP3K4 (MEKK4)-deficient mice,⁵³ suggesting a different capacity to generate TS cells between MAP3K4-deficient TE and ExE. This phenotype possibly involves differences in epigenetic status between the TE and ExE.

In our MSRE-qPCR screening for TS-ES T-DMRs, we did not find T-DMRs that are hypermethylated specifically in ES cells. The seeming inconsistency between the present study and previous reports describing a hypomethylated genome in trophoblast cells⁵⁻⁸ may be explained by the fact that we focused on CpGs of *NotI* sites, in contrast with the *HpaII* sites used in other reports. *NotI* and *HpaII* have a different tendency regarding the distribution of their recognition sites in the genome;⁵⁴ thus, the TS-ES T-DMRs tended to be located in CpG-rich portions of the genome (Table S3), where *NotI* sites are preferentially located. It has been suggested that 5-mC is converted to thymine (T) via spontaneous deamination.⁵⁵ Due to 5-mC-to-T conversion, CpG content might have gradually decreased over generations at genomic regions methylated in the epiblast that give rise to germ cells, which enriches the regions methylated in extraembryonic

tissues among CpG-rich portions. This may underlie the unique epigenetic profile of trophoblast cells.

The eight T-E T-DMRs identified here were hypermethylated in trophoblast cells. Again, this is in contrast with the conventional understanding that global DNA methylation levels in extraembryonic tissues are lower than those of embryonic tissues. Previous reports indicated the hypomethylated status of placenta and extraembryonic tissues,^{7,56} as was the case for TS cells (Fig. S6 and Table S4). Therefore, the significance of DNA methylation in trophoblast tissues has been downplayed. However, this study showed clearly the presence of genomic regions that were hypermethylated specifically in a trophoblast cell lineage, independently from the bulk genomic DNA methylation levels. A genome-wide survey of the promoter methylome in TS and ES cells via immunoprecipitation analysis using an anti-5-mC antibody identified several promoters that are hypermethylated in TS cells, and hypomethylated in ES cells.³¹ Moreover, a genome-wide DNA methylation analysis using a methylation-sensitive restriction enzyme, restriction landmark genomic scanning (RLGS), revealed that methylation and demethylation occur to change the DNA methylation profile during the differentiation of rat trophoblastic cell lines.⁵⁷ In mouse embryos and TS cells, the DNA methylation status of the *Ddah2* gene in the trophoblast changes during the progression of the differentiation process.³⁸ As indicated by these findings, the establishment and modification of DNA methylation profiles clearly constitute the identity of trophoblast cells.

We found that the regions in the human genome that are homologous to mouse T-E T-DMRs are also hypermethylated in human placental tissues. A previous study of DNA methylation dynamics showed that the methylation level appeared higher in the TE than in the ICM in human blastocysts, as assessed using immunostaining;⁵⁸ this is in contrast with observations performed using mouse blastocysts.⁹ Despite the presence of such differences between species, we identified genomic regions that were hypermethylated in trophoblastic tissues/cells and hypomethylated in embryonic tissues/cells, both in mouse and human samples. The fact that the epigenetic marks specific to the trophoblastic cell lineage were conserved across species raises the possibility that these epigenetic marks are somewhat meaningful for trophoblast identity.

In conclusion, the trophoblast or embryonic cell-lineage-based DNA methylation profile is constructed during native development after the segregation of the TE and ICM and during stem cell derivation. The T-E T-DMRs identified here will be used as an epigenetic indicator to advance the understanding of cell differentiation between trophoblast and embryonic cell lineages.

Materials and Methods

Mice. C57BL/6N and C57BL/6J mice were purchased from Japan CLEA and Charles River Japan, respectively. Both strains gave equivalent results and the data in the present study are a sum of the results obtained in these two strains. Animals were maintained under a 12 h light cycle. The experiments were performed according to the guidelines for the care and use of laboratory

animals (Graduate School of Agriculture and Life Sciences, University of Tokyo).

Embryo collection and manipulation. Preimplantation embryos were obtained from naturally mated or superovulated mature female mice. Copulation was determined by the presence of a vaginal plug and E0 was assumed to be midnight. E3.5 and E4.5 blastocysts were collected according to the standard protocol.⁵⁹ Briefly, blastocysts were collected by flushing uteri. After the removal of the zona pellucida using acid Tyrode's solution, blastocysts were incubated in KSOM (ARK Resource) containing Vybrant CM-DiI (1:200 dilution; Invitrogen, V22888) for 15–20 min at 37°C and 5% CO₂. Labeled blastocysts were washed in 0.25% PVP/PBS(-) several times. To segregate TE from ICM cells, washed blastocysts were then incubated at room temperature in 1.25% pancreatin (Wako Pure Chemicals, 163-00142)/0.25% trypsin (Invitrogen)/1 mM EDTA/PBS(-). Five minutes after the start of the incubation, blastocysts were pipetted in enzyme solution through a fine glass pipette (~50 μm diameter) to dissociate blastomeres. The enzyme reaction was stopped with 10% FBS/PBS(-) before the enzyme treatment exceeded 10 min. Cells with and without DiI fluorescence were collected as TE and ICM cells, respectively, under a fluorescence microscope equipped with micro-manipulators (Olympus) (Fig. 7).

Induction of delayed implantation was performed according to the standard protocol,⁵⁹ with a slight modification. Briefly, pregnant mice were ovariectomized in the morning of E3.5 and injected daily with progesterone (2 mg per mouse). Diapause blastocysts were recovered by flushing uteri 4 d after ovariectomy.

E6.5 postimplantation embryos were obtained via natural mating. The embryos were dissected from decidua in 10% FBS/PBS(-). Reichert's membrane and the primitive endoderm were removed surgically using fine forceps. The ectoplacental cone (EPC) was cut off and embryos were separated into extraembryonic ectoderm (ExE) and epiblast (Epi).

The cells and tissues collected were stored at -80°C until use.

Cell culture. TS cell (established in our laboratory) and ES cell (MS12),⁶⁰ lines of C57BL/6 background were used in this study. Cells were maintained as described previously in reference 61 and 62.

Human samples. Human tissues were collected with permission from the Local Research Ethics Committees of the National Center for Child Health and Development. Written informed consent was obtained from all subjects. Chorionic villi were obtained after elective termination of normal pregnancies under 14 weeks of gestation. Placental tissues were obtained from normal-term pregnancies after delivery. Samples were snap-frozen for RNA and DNA isolation.

Genomic DNA samples of human ES cell lines (khES1, khES2 and khES3) were provided by Dr. Nakatsuji (Kyoto University).

Screening of TS-ES T-DMRs from the MSRE-qPCR data set. *NotI* sites to be analyzed by bisulfite sequencing in TS and ES cells were selected from the MSRE-qPCR data set (unpublished

data and ref. 34). The detailed procedure used for MSRE-qPCR analysis was described previously in reference 34. Briefly, genomic DNA was incubated in the presence or absence of the methylation-sensitive restriction enzyme *NotI* prior to genomic qPCR flanking a *NotI* site of interest. The methylation level at each *NotI* site was estimated by the ratio of the amount of PCR product from *NotI*-treated DNA to that from the *NotI*-untreated DNA. In this study, potential TS-ES T-DMRs were selected using the following criteria: >35% methylation in undifferentiated/differentiated TS cells and ≤35% methylation in ES and EG cells, or vice versa. All resulting *NotI* sites appeared to be hypermethylated in TS cells. Among these *NotI* sites, we were able to design primer sets for bisulfite sequencing analysis for 14 loci.

Bisulfite sequencing analysis. With the exception of TS and ES cells, bisulfite treatment of genomic DNA was performed using the EZ DNA Methylation-Direct kit (Zymo Research, D5020). Genomic DNA from TS and ES cells was purified and then subjected to the bisulfite reaction using the kit. The procedures were performed according to the manufacturer's instructions, with a slight modification. Briefly, the last centrifugation step before elution was performed twice, to dry the column completely, and the elution solution was preheated to 50–60°C. PCR was performed using BIOTAQ HS DNA Polymerase (BIOLINE, BIO-21040) with the following parameters: 95°C for 10 min, 43 cycles of 95°C for 30 sec, 55°C (or 58°C) for 30 sec, and 72°C for 60 sec, followed by a step of 72°C for 2 min. The sequences of the PCR primers and annealing temperatures used are listed in Table S5. Bisulfite-treated DNA equivalent to approximately 100 TE or ICM cells, two blastocysts or 2.5–10 ng of genomic DNA was used as a template. For human samples, genomic DNA extracted from human ES cells and placenta was bisulfite treated using the EpiTect Bisulfite kit (QIAGEN, 59104), and an aliquot corresponding to 50 ng of untreated genomic DNA was used in one PCR.

The PCR products were purified, cloned into the pGEM-T Easy vector (Promega, A1360), and sequenced. Sequence analyses and statistical comparisons (nonparametric two-tailed Mann-Whitney test) were performed using the QUMA web service (quma.cdb.riken.jp).⁶³

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Norio Nakatsuji for providing the genomic DNA of human ES cell lines. We also thank Dr. Shintaro Yagi for valuable discussions. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [20062003 (S.T.)].

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/epi/article/18962

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Imprinted DNA methylation reprogramming during early mouse embryogenesis at the *Gpr1-Zdbf2* locus is linked to long *cis*-intergenic transcription

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ARTICLE INFO

Article history:

Received 12 January 2012

Revised 27 January 2012

Accepted 31 January 2012

Available online xxx

Edited by Ned Mantei

Keywords:

DNA methylation

Genomic imprinting

Imprinting control region

Differentially methylated region

Non-coding RNA

ABSTRACT

The paternally-expressed imprinted genes *Gpr1* and *Zdbf2* form a gene cluster wherein the imprinted-methylated regions of these two genes differ. We identified a novel, paternally expressed, long intergenic non-coding *Zdbf2* variant (*Zdbf2linc*) transcribed from maternally methylated *Gpr1* DMR during early embryogenesis in the mouse. While the *Gpr1* DMR displayed biallelic hypermethylation, *Zdbf2linc* expression was rarely observed in the post-gastrulation, despite a positive correlation between the methylation of *Zdbf2* DMRs and the mono-allelic transcription of the original *Zdbf2* coding variant. Furthermore, lack of the maternal methylation imprint resulted in the biallelic expression of both coding and non-coding *Zdbf2* transcripts as well as complete methylation of *Zdbf2* DMRs. Globally, our findings suggest the role of *Zdbf2linc* in the establishment of secondary epigenetic modifications after implantation.

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1. Introduction

Functional non-equivalence between parental genomes in mammals is caused by parental origin-specific mono-allelic expression of certain genes, termed “imprinted” genes [1]. To date, over one hundred imprinted genes have been identified in humans and mice (MRC Mammalian Genetics Unit, Harwell, UK, http://www.har.mrc.ac.uk/research/genomic_imprinting), most of which form clusters in specific genomic regions responsible for the abnormal phenotypes of uniparental disomies. Imprinted gene clusters are marked epigenetically and imprinted differentially in the parental germline, depending on the parent. Differentially methylated regions (DMRs), which exhibit parent-of-origin-dependent DNA methylation patterns, have been identified within or near imprinted genes. Two classes of DMRs have been identified: germline DMRs (primary DMRs), which acquire methylation during gametogenesis, and somatic DMRs (secondary DMRs), which estab-

lish their allelic methylation patterns after fertilization, most likely through the influence of the germline DMRs at each gene cluster [2–6]. Gene knockout experiments in mice point to the involvement of DNA methylation in this process. De novo methyltransferase *Dnmt3a* and de novo methyltransferase-related protein *Dnmt3L* are required for the establishment of primary methylation imprints in paternal and maternal germlines, as well as the mono-allelic expression patterns of imprinted genes in the embryo proper [7–9]. The targeted deletion of some germline DMRs in mice has also been found to result in the aberrant expression of single or several associated imprinted genes, as well as the loss of the allelic methylation of secondary DMRs [10–19]. Such germline DMRs are called imprinting control regions (ICRs), and act as long-range *cis*-acting regulatory elements.

An imprinted gene cluster containing the *Gpr1* (G protein-coupled receptor 1) and *Zdbf2* (zinc finger, DBF-type containing 2) genes has been identified in mouse chromosome 1; both are specifically expressed from the paternal allele [20–22]. *Zdbf2* was found to have imprinted expression in various embryonic and adult tissues, whereas *Gpr1* showed kidney-specific imprinted expression (Fig. 1A). However, functional roles of these genes in biological processes remain undetermined. Multiple methylome analyses have identified two kinds of imprinted methylated regions in this cluster: maternal allele-specific methylation at a CpG island in *Gpr1* intron 2 (*Gpr1* DMR), and paternal allele-specific

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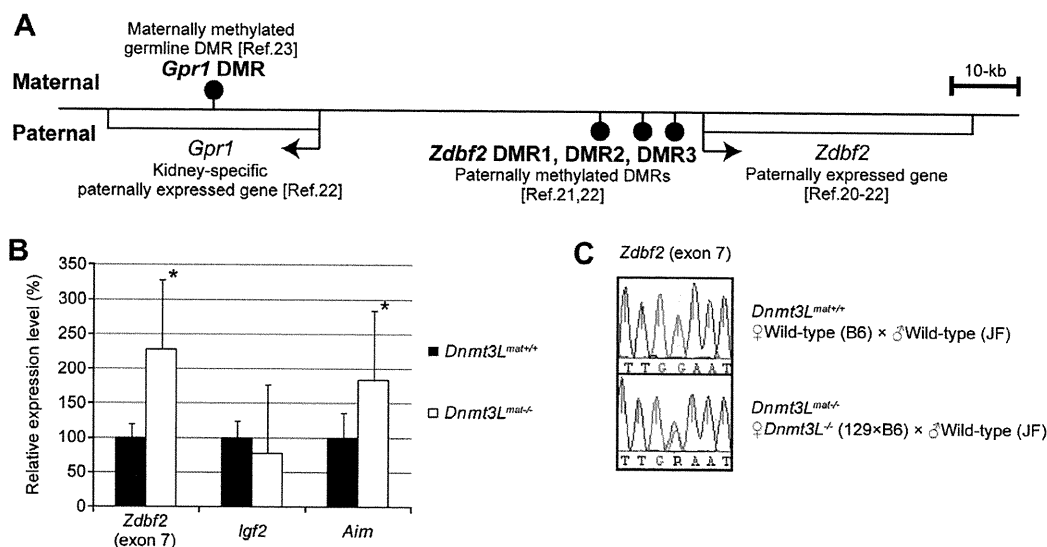


Fig. 1. Comparison of *Zdbf2* gene expression in wild-type and *Dnmt3L^{mat-/-}* embryos. (A) Schematic representation of the imprinted *Gpr1-Zdbf2* domain on mouse chromosome 1C2. Open boxes represent the locations of the two paternally expressed genes; arrows denote the transcriptional direction of these genes. The positions of the differentially methylated regions (DMRs) are indicated by filled pins on the methylated alleles. (B) Quantitative real-time RT-PCR expression analysis of paternally expressed genes *Zdbf2* (exon 7), *Igf2*, and *Aim* in E9.5 mouse embryos from *Dnmt3L^{mat+/+}* and *Dnmt3L^{mat-/-}* mice ($n \geq 3$). Expression levels of each gene were normalized to those of the housekeeping gene *Gapdh*. Error bars represent SEM ($n = 3$), while asterisks indicate $P < 0.01$ (Student's *t*-test). *Igf2* and *Aim* are regulated by paternal (*H19* DMR) and maternal (*Igf2r* DMR2) methylation imprints, respectively. (C) Allelic expression analysis of *Zdbf2* was performed using F₁ hybrid crosses between wild-type C57BL/6 (B6) or *Dnmt3L*-deficient 129SvJae × C57BL/6N (129 × B6) females and JF1/Msf (JF) males. Maternal and paternal alleles were distinguished by an SNP (A/G at chr1.63,361,080; highlighted in yellow).

methylation at the intergenic regions between the *Gpr1* and *Zdbf2* genes (*Zdbf2* DMR1, DMR2, and DMR3) [21–23]. However, the identity of the DMR that acts as a true ICR on the *Gpr1-Zdbf2* locus remains unknown.

Similar to the *Gpr1-Zdbf2* gene cluster, three other imprinted gene clusters, *Gnas-Nespas*, *Kcnq1ot1*, and *Igf2r-Airn*, have been found to have contradicting imprinted methylation. In these clusters, maternal allele-specific methylations act as ICRs, while the long non-coding RNAs transcribed from them have a role in silencing genes in *cis* through gene- and lineage-specific repressive chromatin modifications [24–26]. Other DMRs may additionally become methylated on the paternal allele after fertilization as somatic DMRs. Furthermore, recent studies have shown that transcription through *Gnas* DMRs is required for their allele-specific methylation to be established during pre- or post-fertilization [26,27]. It is important to elucidate the relationship between imprinted methylation and gene expression profiles on a gene cluster in order to understand the mechanisms by which imprinted expression is regulated.

In this study, we identified a novel, long (>100 kb), imprinted non-coding *Zdbf2* variant transcribed from the *Gpr1* DMR in mice, which showed paternal-allele-specific expression in early embryonic cells. Its expression was found to be associated with the methylation of the *Gpr1* DMR in *cis*. Our results indicate that maternal-allele-specific methylation of the *Gpr1* DMR may directly regulate the imprinted expression of this long non-coding RNA. The transcription of the *Zdbf2* variant may be essential for the establishment of the somatic intergenic differential methylation of *Zdbf2* DMRs that regulates imprinted *Zdbf2* expression after implantation.

2. Materials and methods

2.1. Sample preparation

Mouse early embryos, embryonic stem (ES) cells, and adult tissues were prepared as described previously [6,21,28]. To use DNA polymorphisms for allele discrimination, wild-type reciprocal F₁

hybrids (*Dnmt3L^{mat+/+}*) were obtained by crossing C57BL/6 (B6; Clea Japan, Tokyo, Japan) and JF1/Msf (JF) mice. *Dnmt3L^{mat-/-}* embryos were obtained by crossing *Dnmt3L*-deficient female (129SvJae × B6 hybrid genetic background) and wild-type JF mice. ES cells were derived from B6 mice (Clea Japan).

2.2. Real-time RT-PCR and allelic expression analyses

Total RNA from E3.5 blastocysts, E5.5 whole embryos, E6.5, E7.5 embryonic tissues (extra-embryonic tissues were removed), and E9.5 whole embryos (yolk sacs and amnions were removed) [6], and ES cells was isolated using the Allprep DNA/RNA micro kit (Qiagen, Valencia, CA) and TRIzol reagent (Invitrogen, Carlsbad, CA). Genomic DNA-free total RNA was reverse transcribed to cDNA with SuperScript III (Invitrogen). Quantitative analysis for *Igf2*, *Airn*, and *Zdbf2* gene expression was performed in a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). Relative expression levels of each gene were normalized to those of the *Gapdh* or *Actb* housekeeping genes. RT-PCR and direct sequencing for *Zdbf2* variants were performed using an ABI PRISM 3730xl genetic analyzer (Applied Biosystems), as described earlier [21]. Primer sequences and PCR conditions are listed in Table 1.

2.3. Rapid amplification of cDNA ends analysis

The 5'-region of the mouse *Zdbf2* gene was obtained using the GeneRacer Kit (Invitrogen). Total RNA was prepared from ES cells, and two rounds of PCR were carried out using TaKaRa EX Taq (TaKaRa) under the following conditions: 25 cycles of 30 s at 94 °C, 30 s at 64 °C, 1 min at 72 °C for the first PCR; and 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C for the second PCR. The *Zdbf2* gene-specific primer sets used for the nested PCR were as follows: anti-sense 5R8: 5'-AGCTGAGGACCCGGAATCCTCACAT-3' for the first PCR and anti-sense 5R9: 5'-TGAGGACCCGGAATCCTCACATGGT-3' for the second PCR. The amplified products were sequenced directly, after purification.

Table 1
PCR primer information.

Zdbf2 exon A&B	chr1: 63,247,197–63,247,248 63,254,089–63,254,143	5'-TTGAAGTGGCTTACCAGTGTGT-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[21]			
		5'-TTTCTCAAGGTGAAACAGGGAGT-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40				
Zdbf2 exon 5	chr1: 63,337,249–63,337,356	5'-CAGACCCCTACTAAAGCAAAACT-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[21]			
		5'-CTGTCATTTCTTCTACTTACTTC-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40				
Zdbf2 exon 7	chr1: 63,360,906–63,361,130	5'-TGATCCACAGTTATGGACTT-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40	[21]			
		5'-TCAAACTCACATCAGATGG-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40				
Igf2	chr7: 149,839,164–149,839,745	5'-GGAGATGTCCAGCAACCATC-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40	[21]			
Aim	chr17: 12,935,735–12,935,961	5'-CTGAAGCAATACATGCCAC-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40				
Gapdh	chr6: 125,112,864–125,113,058 125,113,157–125,113,201	5'-GTGGATTTCAGGTTTCATG-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40	[21]			
		5'-GTCCTGAGTCTACTGGTGTGTC-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40				
Actb	chr5: 143,664,856–143,665,043	5'-GAGCCCTCCCAATGCCAAA-3'	94 °C, 1 min	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[22]			
		5'-AGGTGACACCATTTGCTTC-3'	95 °C, 10 min	95 °C, 15 s	60 °C, 1 min	72 °C, 30 s	72 °C, 5 min	40				
Gpr1 DMR	chr1: 63,246,988–63,247,117	5'-GCTGCTCAACCCCTCAAC-3'	94 °C, 1 min	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[22]			
		5'-AGATTAGTTAGTTTGAATT-3'	94 °C, 1 min	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40				
Zdbf2 DMR1	chr1: 63,306,143–63,306,507	5'-AACACTAATCCAAATAATTC-3'	94 °C, 1 min	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[22]			
		5'-TGTGTATAGTTTCTATGGTTTGT-3'	94 °C, 1 min	94 °C, 30 s	57 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40				
Zdbf2 DMR2	chr1: 63,311,117–63,311,450	5'-ATAATCAACTACACAAAAACCTAA-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[22]			
		5'-TAAATAAATATAAAGGGTTGTTAGTT-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40				
Zdbf2 DMR3	chr1: 63,314,651–63,314,991	5'-AAATCAAACTACAACCTCAACTA-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40	[21]			
		5'-ATTAAGATATTTAGATTGGATAGAT-3'	94 °C, 1 min	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s	72 °C, 5 min	40				
		5'-AAAATAAACTCTCCAAAACGAAAA-3'										

2.4. Bisulfite sequencing analysis

Genomic DNA samples were isolated from *Dnmt3L^{mat+/+}* (F₁ hybrid of B6 and JF) and *Dnmt3L^{mat-/-}* embryos at the E3.5–E9.5 stages, as well as from the kidneys and hearts of 9-week-old female *Dnmt3L^{mat+/+}* mice. Genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen). The bisulfite-treated DNA was amplified by PCR with TaKaRa Taq Hot Start Version (TaKaRa) for *Gpr1* and *Zdbf2* DMRs. The primers and PCR conditions for the amplification are listed in Table 1. Subcloning and sequencing analyses were performed as described in a previous study [21]. Visualization and quantification of bisulfite sequence data for CpG methylation was performed using the QUMA web-based tool [29].

3. Results

3.1. Allelic expression analysis of *Zdbf2* in mouse embryos lacking maternal methylation imprints at the post-gastrula stage

To determine if *Zdbf2* is regulated by maternal or paternal methylation imprints, we investigated *Zdbf2* expression patterns using real-time PCR and allelic expression analyses in mouse embryos at the E9.5 post-gastrula stage. The fetuses derived from wild-type and *Dnmt3L*-deficient female (*Dnmt3L^{mat-/-}*) mice, which lack maternal methylation imprints, were examined. Interestingly, an analysis of the 3'-UTR at exon 7 of the *Zdbf2* transcript showed biallelic expression and approximately twofold increased expression in *Dnmt3L^{mat-/-}* embryos when compared with wild-type embryos, which showed paternal-allele specific expression (Fig. 1B and C). This concurs with what has been observed to occur with the *Aim* gene (Fig. 1B), which is paternally expressed under the control of the maternal methylation imprint (*Igf2r* DMR2), suggesting that *Zdbf2* expression is regulated by a *Dnmt3L*-mediated maternal methylation imprint, whereby the maternally methylated *Gpr1* DMR may act as an ICR for this imprinted locus. However, bisulfite sequencing analysis surprisingly showed that the *Gpr1* DMR was biallelically hypermethylated in E9.5 embryos (Fig. 2A), whereas its maternal-allele-specific methylation was maintained until at least the blastocyst stage [23].

3.2. Loss of differential methylation at *Gpr1* DMR during embryonic development

To reveal the timing and progression of differential methylation changes over the course of embryonic development, the allelic methylation pattern of the *Gpr1* DMR was examined between the blastula and gastrula stages in mouse embryos at E3.5 (blastocyst), E5.5, E6.5, E7.5 (early- to late-gastrulation), and E9.5 (post-gastrulation). Although complete maternal-allele-specific methylation (i.e., paternal-allele-specific hypomethylation) at the *Gpr1* DMR was maintained during the E3.5–E5.5 stages, the methylation of the paternal allele was significantly increased at subsequent stages (Fig. 2B). Hence, both maternal and paternal alleles were almost completely methylated at the E9.5 stage in wild-type (and even *Dnmt3L^{mat-/-}*) embryos (Fig. 2A). Adult somatic tissues, including the kidney, also showed biallelic hypermethylation of the *Gpr1* DMR (Fig. 2A), demonstrating that the kidney-specific imprinted expression of *Gpr1* is not associated with *Gpr1* DMR methylation. These results show that *Gpr1* DMR gains biallelic methylation after implantation, and that the methylation status of this DMR is not associated with the imprinted expression of *Zdbf2* or *Gpr1*, at least after gastrulation.

3.3. Allelic expression analysis of *Zdbf2* locus

Whereas our results showed that imprinted *Zdbf2* expression was controlled by maternal methylation imprint, maternally meth-

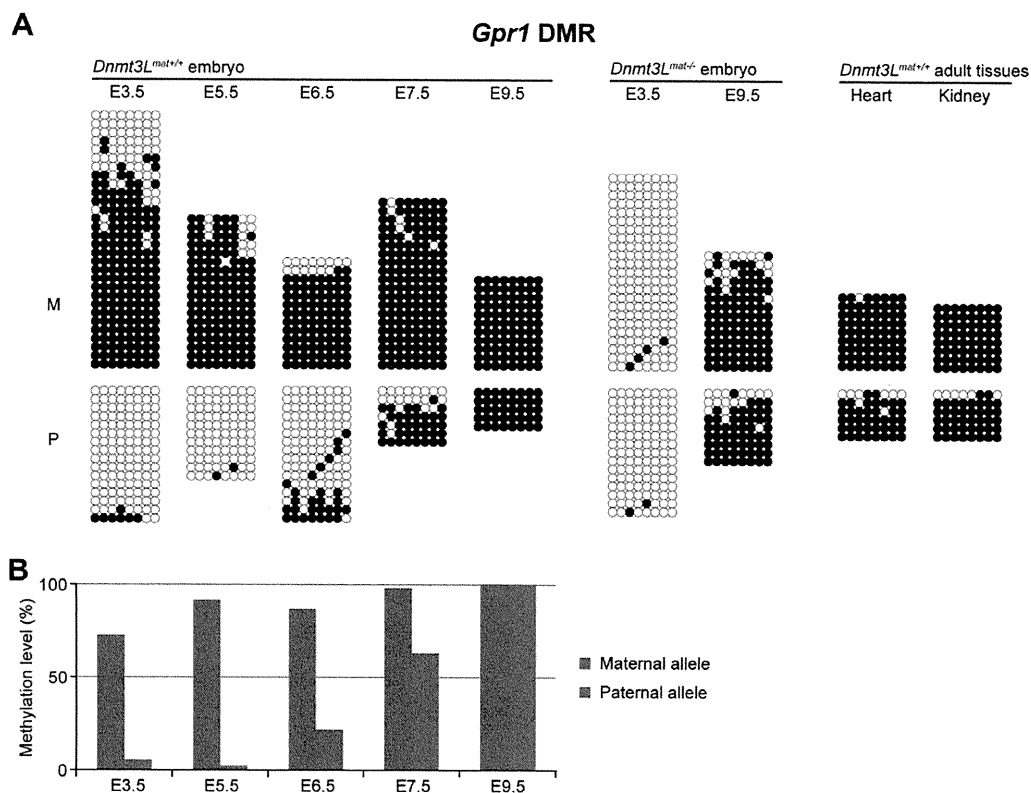


Fig. 2. DNA methylation profile of the *Gpr1* DMR. (A) Bisulfite sequencing analysis of the *Gpr1* DMR in wild-type and *Dnmt3L^{mat-/-}* mouse embryos and adult tissues. Each row of circles represents the results of an independent sequencing reaction. The open and closed circles denote unmethylated and methylated CpGs, respectively. M and P indicate the maternally inherited B6 or 129 × B6 alleles and the paternally inherited JF allele, respectively. Both alleles were discriminated by three polymorphisms [23]. (B) Differential methylation status between M and P alleles during embryogenesis. CpG methylation levels of maternal and paternal alleles are represented by red and blue bars, respectively. These histograms were obtained from bisulfite sequencing analyses shown in (A).

ylated *Gpr1* DMR lost its mono-allelic methylation during gastrulation. To explain the apparent contradiction of the *Gpr1* DMR methylation status being unassociated with the imprinted expression of either the *Zdbf2* or *Gpr1* genes, we focused on a long intergenic non-coding RNA (lincRNA) located within the *Gpr1-Zdbf2* locus (Fig. 3A). This 114-kb lincRNA, which we named *Zdbf2linc*, had previously been identified in mouse ES cells by massively parallel cDNA sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) [30]. *Zdbf2linc* is a splice variant of *Zdbf2* that contains seven exons, including three unique exons within intron 2 of the *Gpr1* gene (exon A and B), the *Gpr1/Zdbf2* intergenic region (exon C), and exons 3, 4, 6, and 7 of the *Zdbf2* gene (according to NCBI RefSeq). A long-range splice form of *Zdbf2linc* lacking exon C was also identified in ES cells by 5' rapid amplification of cDNA ends (RACE) (Fig. 3A). Interestingly, the transcription start site of *Zdbf2linc* overlaps the *Gpr1* DMR. To determine if *Zdbf2linc* is detectable in vivo and is imprinted, we performed RT-PCR and allelic expression analysis in early to mid-term mouse embryos. *Zdbf2linc* expression was detected from E3.5 to E7.5 (Fig. 3B and C), during which time paternal-allele-specific expression was observed (Fig. 3D). Quantitative RT-PCR showed higher expression of *Zdbf2linc* than that of the original *Zdbf2* coding variant (including exon 5) in blastocysts and ES cells (Fig. 3C). Although *Zdbf2linc* was undetectable at E9.5, the expression of the original *Zdbf2* variant was clearly detected (Fig. 3B). Interestingly, the timing of *Zdbf2linc* repression was nearly identical to that of the loss of the differential methylation of the *Gpr1* DMR. Furthermore, *Dnmt3L^{mat-/-}* embryos showed biallelic expression of *Zdbf2linc*. These findings indicate

that methylation at *Gpr1* DMR may directly repress *Zdbf2linc* expression in *cis*.

3.4. Erasure and re-establishment of paternal-allele-specific methylation at *Zdbf2* DMRs

While the methylation of *Gpr1* DMR and *Zdbf2linc* expression were correlated in *cis*, the epigenetic regulation of the original *Zdbf2* variant, which is expressed exclusively from the paternal allele after the E9.5 stage (Fig. 3B), remains unclear. We therefore investigated the methylation patterns of the *Zdbf2* DMRs present in the intragenic region of *Zdbf2linc*. All DMRs showed male-germ-cell specific hypermethylation in previous studies [21–23] and paternal-allele-specific hypermethylation in E9.5 embryos in this study (Fig. 4 middle); however, our previous DNA methylome analyses have shown that *Zdbf2* DMRs are deeply undermethylated in blastocysts [23]. Allelic methylation analyses performed in this study further confirmed that both the paternal and maternal alleles are unmethylated at the *Zdbf2* DMR3 in blastocysts (Fig. 4 left). Taken together, our results demonstrate that paternal-allele-specific methylation of the *Zdbf2* DMRs established in germ cells are erased after fertilization and re-established after implantation. Interestingly, biallelic hypermethylation of the *Zdbf2* DMRs was observed in *Dnmt3L^{mat-/-}* embryos at E9.5 (Fig. 4 right), which is similar to the methylation pattern observed for the somatic DMR (*Nesp* DMR) in the imprinted *Gnas* cluster [17]. This suggests that not only are the *Zdbf2* DMRs in the intragenic region of *Zdbf2linc* somatic (secondary), but that the methylation of *Zdbf2* DMRs is

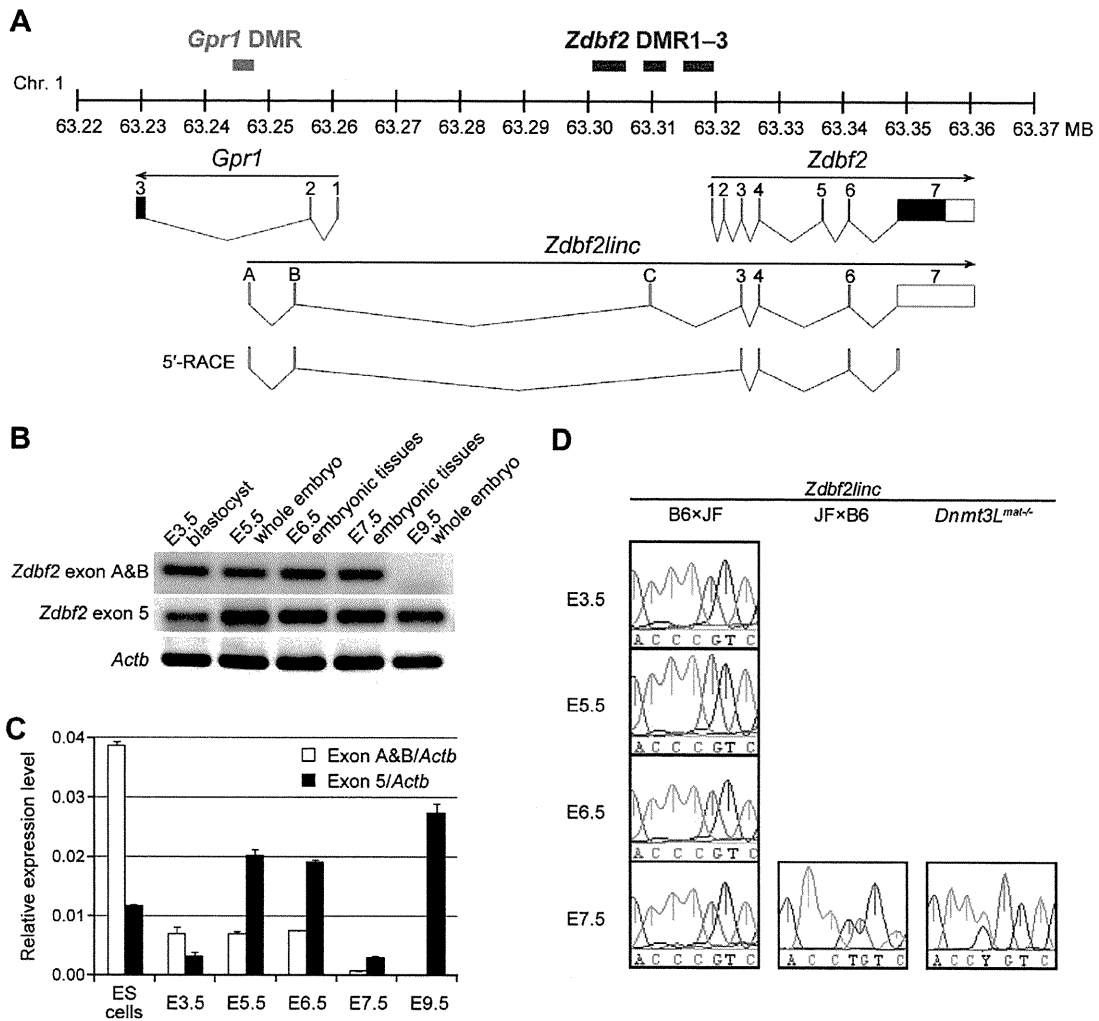


Fig. 3. Quantitative and allelic-specific expression analyses of *Zdbf2linc*. (A) Schematic physical map of the imprinted *Gpr1-Zdbf2* domain showing three paternally expressed transcripts and the 5'-RACE sequence. Red and blue bars represent maternally or paternally methylated DMRs, respectively. (B) Expression of two variants of *Zdbf2* during embryonic development. Forty cycles of RT-PCR were carried out for exon A/B and exon 5 of the *Zdbf2* gene (no SNPs were present) and *Actb* (as a control). (C) Quantitative RT-PCR analysis using the same primers as for (B) in ES cells and individual stage embryos. Expression levels of transcripts were normalized to those of the *Actb* gene. Error bars represent SEM ($n = 3$). (D) Allelic expression analysis of *Zdbf2* was performed using reciprocal F₁ hybrid embryos (B6 × JF and JF × B6), and embryos produced by mating between *Dnmt3L*-deficient females and JF males (*Dnmt3L^{mat-/-}*). Maternal and paternal alleles were distinguished by a SNP in exon A (C/T at chr1. 63,247,248; highlighted in yellow).

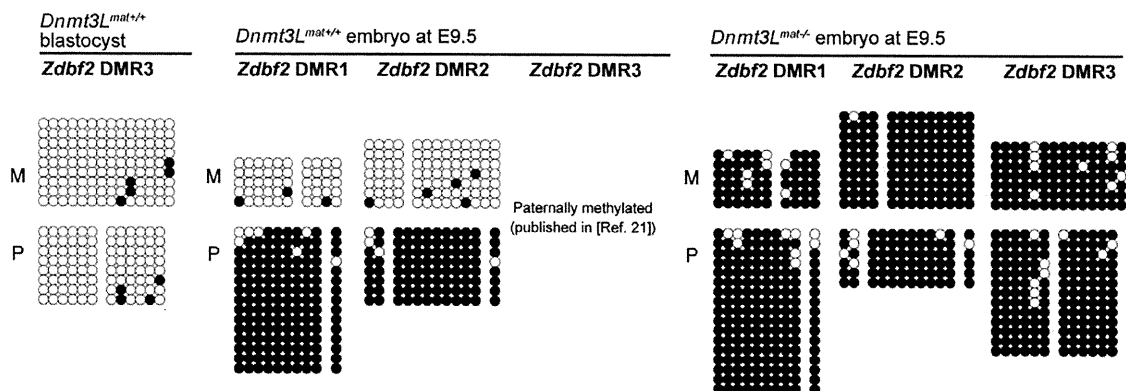


Fig. 4. DNA methylation profiles of the *Zdbf2* DMRs. Bisulfite sequencing analysis of *Zdbf2* DMR1, DMR2, and DMR3 in mouse embryos. M and P indicate the maternally inherited (B6 or 129 × B6) and paternally inherited (JF) alleles, respectively.

Please cite this article in press as: Kobayashi, H., et al. Imprinted DNA methylation reprogramming during early mouse embryogenesis at the *Gpr1-Zdbf2* locus is linked to long *cis*-intergenic transcription. FEBS Lett. (2012), doi:10.1016/j.febslet.2012.01.059

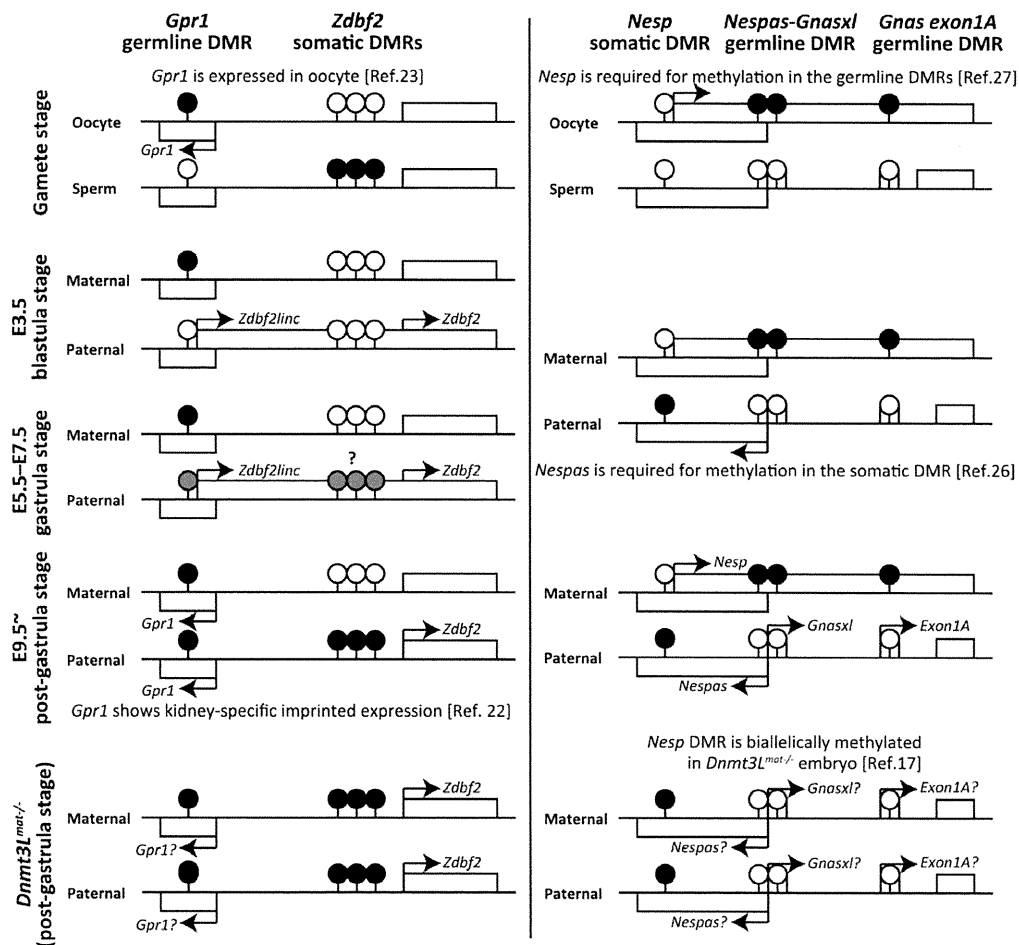


Fig. 5. Summary of allele-specific epigenetic and gene expression differences within the *Gpr1-Zdbf2* (left) and *Gnas* (right) loci. Open boxes represent the locations of the individual imprinted genes (or alternative promoter/first exons); arrows represent the transcriptional direction of these genes. The positions of the DMRs are indicated by filled and unfilled pins on the methylated and unmethylated alleles, respectively. Grey-filled pins indicate the partial methylated CpGs.

positively correlated to the expression of the original *Zdbf2* variant in *cis* after gastrulation.

4. Discussion

We identified a novel, long, imprinted, non-coding variant of the *Zdbf2* gene, *Zdbf2linc*, which is transcribed from the *Gpr1* DMR during the blastula and gastrula stages of mouse embryonic development. Furthermore, we found the paternal-allele-specific expression of *Zdbf2linc* to be negatively correlated with germline-derived maternal methylation at the *Gpr1* DMR. The *Gpr1* DMR was also shown to display complete biallelic methylation, while *Zdbf2linc* was rarely expressed in the post-gastrulation. It is unknown why the *Gpr1* DMR becomes hypermethylated after gastrulation; however, our results indicate that methylation at this DMR may repress the transcription of the *Zdbf2linc* variant in *cis*. The *Zdbf2* DMRs located within the transcribed region of *Zdbf2linc* were also shown to be secondary DMRs, with paternal-allele-specific methylation established after implantation. The imprinted *Gpr1-Zdbf2* locus therefore has two similarities with the *Gnas-Nespas*, *Kcnq1ot1*, and *Igf2r-Airn* imprinted clusters: (i) two differential states of imprinted methylation; namely, maternal-allele-specific methylation at gametogenesis and paternal-allele-specific methylation after fertilization, and (ii) transcription of long non-coding

RNAs from each maternally methylated primary DMR (Fig. 5). Recently, lincRNAs have become a new paradigm for gene regulation via chromatin remodeling in a variety of biological processes, including events during embryonic development such as X chromosome dosage compensation [31,32], regulation of *Hox* genes [33], or genomic imprinting. The non-coding RNAs of the well-studied imprinted clusters *Nespas*, *Kcnq1ot1*, and *Airn* repress neighboring genes in *cis* [24–26], suggesting that the transcription of imprinted *Zdbf2linc* may also have a similar *cis*-regulating effect on flanking genes.

Allele-specific expression analyses using the heterozygous offspring of homozygous *Dnmt3L*-deficient females demonstrated that the imprinted expression of both *Zdbf2linc* and the original *Zdbf2* variant are subject to maternal methylation imprinting, whereby the original *Zdbf2* variant maintains paternal-allele-specific expression even after the gastrula stage, and *Zdbf2linc* and *Gpr1* DMR imprinting loss occurs. It is also possible that another mono-allelic epigenetic modification may become established via long-range transcription of *Zdbf2linc*, or that paternal-allele-specific methylation of *Zdbf2* DMRs may arise through the methylation of a transcribed region of *Zdbf2linc*. Some studies have also suggested that histone modifications at imprinted domains are a prerequisite for DNA methylation. Transcription across DMRs has been shown to be associated with histone methylation changes and the acquisition of genomic imprinting [26,27,34]. For example,

Williamson et al. have shown that paternally expressed non-coding *Nespas* transcription is required for the demethylation of trimethylated histone H3 lysine 4 (H3K4), followed by DNA methylation at the somatic *Nesp* DMR [26]. *Zdbf2linc* has also been identified as a lincRNA on the basis of the trimethylation states of H3K4 at its promoter region and histone H3 lysine 36 (H3K36) along its transcribed region, at least in ES cells [30]. Finally, the interaction of DNMT3A with chromatin is inhibited by H3K4 methylation [35], but promoted by H3K36 methylation [36]. Histone modifications may therefore control imprinted gene expression prior to allele-specific DNA methylation [5].

The imprinted *Gpr1-Zdbf2* locus also has features distinct from those of other imprinted loci. For instance, the primary *Gpr1* DMR does not maintain its mono-allelic methylation after fertilization, while the secondary *Zdbf2* DMRs are differentially methylated, even during gametogenesis. Furthermore, only paternally expressed transcripts were identified in the *Gpr1-Zdbf2* cluster, whereas the majority of imprinted clusters comprise both maternally and paternally expressed genes. Although the true functional roles of the *Gpr1* and *Zdbf2* genes remain unclear, we have herein identified a novel imprinted lincRNA variant of *Zdbf2* expressed exclusively from the paternal allele in the early embryo. Further investigations would elucidate the roles of the imprinted *Gpr1-Zdbf2* locus and its lincRNA variants in the control of embryonic growth and development.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (Grant Nos. 222228004, 20062009, 221S0002, S0801025).

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