Profound phenotypic variation among mice deficient in the maintenance of genomic imprints

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BACKGROUND: An alteration in the mechanism that maintains the monoallelic, imprinted expression of genes can result in their biallelic expression and lead to disruptions in fetal development. Here, we examined the consequences of a loss of maintenance methylation at one specific stage of preimplantation, induced by a deficiency of the oocyte-derived Dnmt1o protein and known to produce biallelic expression of imprinted genes. METHODS: Phenotypes of mid-gestation Dnmt1o-deficient mouse embryos were assessed by a scoring system based on the developmental stage of 17 anatomical features and by magnetic resonance microscopy. RESULTS: Many mid-gestation embryos developing without Dnmt1o protein exhibited significant developmental delays of multiple organ systems \((P<0.05)\) and a wide variety of morphologic anomalies compared with wild-type embryos. Most of the remaining mid-gestation Dnmt1o-deficient embryos appeared normal. CONCLUSIONS: These findings indicate that a profound range of gestational phenotypes can be induced by the loss of a single protein at a specific preimplantation developmental stage. This is best explained by the formation of epigenetic mosaic early embryos, composed of somatic cells with different spectra of normal intact genomic imprints. These findings have important implications for understanding the types of embryonic phenotypes related to the disruption of inherited imprints, and thus may provide a model of altered imprinting in humans. In particular, because Dnmt1o functions in the preimplantation embryo, a complete or partial loss of Dnmt1o function may play a role in epigenetic abnormalities seen in assisted reproduction technology births.

Keywords: imprinting; embryogenesis; phenotype; birth defect; epigenetic

Introduction

Genomic imprinting is a molecular process in which the parental alleles of imprinted genes are distinguished by epigenetic marks (imprints) and by differences in expression (Reik, 2007). The accurate establishment (in germ cells) and maintenance (during embryogenesis) of genomic imprints is necessary for normal fetal development. Phenotypes related to the abnormal or biallelic expression of normally imprinted genes are generated in many different ways. For example, androgenones and parthenogenones, which contain only paternal alleles or only maternal alleles respectively and therefore fail to express certain imprinted genes, have severe developmental impairments and perish during early embryogenesis (McGrath and Solter, 1984; Surani et al., 1984). Many uniparental disomic mice, which fail to express a smaller set of imprinted genes, show less severe abnormalities, such as defects in their growth and behavioral changes (Cattanach and Kirk, 1985). Individual imprinted genes, when mutated, can lead to abnormal phenotypes (DeChiara et al., 1991; Bartolomei and Tilghman, 1997; http://www.mgu.har.mrc.ac.uk/research/imprinting/). Lastly, perturbations in the general mechanism of establishing epigenetic imprints in the female and male germ lines also result in abnormal phenotypes. For example, null mutations in either the Dnmt3a or Dnmt3L gene in the female germ line preclude the establishment of maternal imprints and consistently result in embryos that perish before day 10 of gestation (Bourc’his et al., 2001; Hata et al., 2002; Kaneda et al., 2004). All of these alterations in normal imprinting represent deviations, some greater than others, from a
complete complement of maternal and paternal imprints at the
time of zygote formation.

The abnormal phenotypes described above are all due to
imbalance in the inheritance of a haploid genome from each
parent. In each case, all cells of the organism possess the
same imprinting abnormality. For example, every cell of a
mouse with an inherited maternal disomy, paternal nullisomy
for chromosome 11 contains this uniparental disomy (Cattan-
ach and Kirk, 1985). It is the uniformity of a cellular imprint-
ing abnormality that likely explains the reproducible phenotype
of reduced birth size in association with this chromosome-11
disomy/nullisomy. Germline imprinting defects such as these
are contrasted to embryonic imprinting defects, which disrupt
the mechanism that maintains imprints in the early mouse
embryo. Depending on the degree and extent of the disruption,
embryonic sources can produce embryos composed of cells
with different imprinted abnormalities.

The loss of Dnmt1o cytosine methyltransferase from the
developing embryo best exemplifies the consequences of
failing to maintain methylation on inherited genomic imprints.
Dnmt1o is a variant Dnmt1 protein of Mr 175 000 (compared
with the somatic Mr 190 000 form of Dnmt1) that is synthesized
and stored in the maturing oocyte, and functions in preimplanta-
tion embryos to maintain methylation patterns on differentially
methylated domains (DMDs) of imprinted loci (Mertineit
et al., 1998; Howell et al., 2001). Embryos developing in the
absence of Dnmt1o lose methylation on one-half of the normally
methylated parental alleles within all DMDs examined. This
pattern of methylation loss most likely occurs because of an
absence of maintenance methylation during one embryonic
cell cycle (Howell et al., 2001). These findings can only be
explained by cellular mosaicism, resulting from the loss, on
average, of maintenance methylation for one cell cycle.
Because of this, for one-half of the embryo’s cells, a given
DMD will be normally methylated in a parent-specific manner,
whereas for the other one-half, the DMD will have no
methylation. Because the Dnmt1o protein is localized to nuclei
of only the 8-cell embryonic stage, Dnmt1o’s function is
likely the maintenance of imprinted methylation patterns at the
fourth S phase of embryogenesis. Moreover, because of the in-
dependent assortment of chromatids following the loss of main-
tenance methylation at a single cell cycle, and the presence
of imprinted genes on at least 12 of the 19 mouse autosomes
(http://www.mgu.har.mrc.ac.uk/research/imprinting/), cells
of the 16-cell embryo (and all embryonic stages thereafter)
would be expected to be epigenetically variable (mosaic), and
any given 16-cell embryo highly likely to be epigenetically dis-
tinct from other Dnmt1o-deficient 16-cell embryos.

There are also less well-defined, but equally important, ways
of disrupting imprints in the embryo. Significant perturbations
in inherited genomic imprints are more likely to occur under
certain in vitro culture conditions for mouse preimplanta-
tion embryos (Doherty et al., 2000; Khosla et al., 2001). For
example, when H19 gene expression and DNA methylation
were measured following in vitro culture of preimplantation
embryos, it was observed that culturing in Whitten’s medium
led to many more abnormalities in the methylation of H19
DMD sequences compared with culturing in synthetic
oviductal medium enriched in potassium (KSOM) plus amino
acids (Doherty et al., 2000). An effect that may mirror this
embryo culture effect is seen in some individuals conceived
by assisted reproduction technologies (ART), in which
human preimplantation embryos are cultured for different
lengths of time before transferring them to the uterus. The
rare appearance of congenital anomalies such as the Beck-
with–Weidemann syndrome, in association with a loss of
DNA methylation on imprinted alleles, may be due to an
adverse effect of human ART culture conditions on the main-
tenance of imprints (Maher et al., 2003; Lucifero et al.,
2004; Paoloni-Giacobino and Chaillet, 2004).

Here, we explored the phenotypes associated with the loss of
Dnmt1o maintenance methyltransferase activity in the early
mouse embryo. Our previous studies suggested that the effect
of Dnmt1o deficiency on embryonic phenotypes was variable;
although many Dnmt1o-deficient embryos were dead at mid-
gestation, we identified rare survivors that developed into
adults (Howell et al., 2001). These observations suggested
that there may be a wide range of abnormal phenotypes due
to the loss of embryonic Dnmt1o protein, and perhaps this
range of abnormalities might be due to stochastic differences
among genomic DNA methylation patterns on imprinted
genes. Such stochastic differences would result in mosaic
mice, each mouse developing from a set of epigenetically het-
ergyous embryo stem cells. On the basis of our previous
findings and the notion that abnormalities due to the mosaic
composition of the embryo’s stem cells would develop early
in embryogenesis, we analyzed both pre- and post-implantation
embryo loss, and recorded morphological abnormalities and
developmental delays in mid-gestation embryos. In order to
elucidate the full range of embryonic and extraembryonic
membrane abnormalities, mid-gestation Dnmt1o-deficient
embryos were removed and scored by a revised rodent
scoring system and by three-dimensional (3D) magnetic reso-
nance microscopy (MRM).

Materials and Methods

Mice

The mutant Dnmt1Δ1o allele was produced by a targeted deletion of
exon 1o of the mouse Dnmt1 gene (Howell et al., 2001), and
maintained in the inbred 129/Sv background. Dnmt1Δ1o mice were geno-
typed using a PCR assay as previously described (Howell et al.,
2001). PCR primers for this assay flank exon 1o: 5’-GTC CT 3’
ACA GTG GAG GAA AC-3’.

Embryos from fertile crosses between wild-type 129/Sv and homozygous
Dnmt1Δ1o male mice were crossed to wild-type 129/Sv male mice. Copulation was deter-
mined by the presence of a vaginal plug and embryonic age zero
(E0) was assumed to be midnight, the midpoint of the dark portion
of the dark-light cycle. Reproductive organs of the female mice
were collected at various times after mating to determine pre- and
post-implantation embryo losses, and to collect embryos for inspec-
tion and for imaging. Wild-type male and female 129/Sv mice were
purchased from Charles River Canada (St Constant, Quebec,
Canada). Mouse embryonic fibroblasts (MEFs) were isolated from
crosses between wild-type 129/Sv and homozygous Dnmt1Δ1o/Δ1o
Mice

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maintained in the inbred 129/Sv background. Dnmt1Δ1o mice were geno-
typed using a PCR assay as previously described (Howell et al.,
2001). PCR primers for this assay flank exon 1o: 5’-AGG AAA
ACA GTG GAG GAA AC 3’ and 5’-TAC TTG TTC CAC AGG GCT
GTC CT 3’. The Dnmt1Δ1o allele PCR product is 120 bp in size and
the wild-type PCR product is 380 bp in size.

Both wild-type 129/Sv and homozygous Dnmt1Δ1o female mice
were crossed to wild-type 129/Sv male mice. Copulation was deter-
mimed by the presence of a vaginal plug and embryonic age zero
(E0) was assumed to be midnight, the midpoint of the dark portion
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purchased from Charles River Canada (St Constant, Quebec,
Canada). Mouse embryonic fibroblasts (MEFs) were isolated from
crosses between wild-type 129/Sv and homozygous Dnmt1Δ1o/Δ1o
mice.
female mice and male mice of the inbred *M. mus castaneus* strain CAST/Ei.

**Allele-specific DNA methylation**

Cell clones of both wild-type and Dnmt1o-deficient MEFs were obtained by plating MEFs at low density on tissue culture dishes. Clones that reached large sizes (>400 cells) were scraped off the dish. DNA was extracted from the isolated clones, treated with sodium bisulfite, and DNA methylation patterns of parental alleles of the Snurf/Snrpn and H19 genes (within their DMDs) were determined as previously described (Howell et al., 2001).

**Morphological scoring and microscopy**

The rat morphological scoring system (Brown and Fabro, 1981) was modified for mouse studies using developmental parameters outlined elsewhere (Theiler, 1972; Kaufman, 1992). The morphological scoring system (Supplementary Table I) incorporates 17 morphologic features that are readily observed under a dissection microscope, and the system is most appropriate for assessing the development of mouse embryos at embryonic age 8–11 days (late presomite to 40-somite stage). Conceptuses were explanted and examined fresh and without fixation in cold 1 x phosphate-buffered saline (PBS) under a Leica MZ2.5 dissecting microscope (Wetzlar, Germany). Photographs were taken with a Leica MZFLIII dissecting microscope (Wetzlar, Germany) using Spot RT Software, v3.2.4 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The crown-rump length (the maximum length of the embryo in its natural position) was recorded for each embryo using an eyepiece micrometer. Embryonic features often appeared to be intermediate between stages and in these cases were assigned a half mark between the two scores. Flexion was examined prior to opening the yolk sac. Each of the 17 features was individually scored and the sum of the 17 scores was the embryo’s morphological score (MS).

**Table I.** Effects of Dnmt1o removal in mice on the total morphological score (MS) and on the development of specific morphological features.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Wild type</th>
<th>Dnmt1o-deficient</th>
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<tbody>
<tr>
<td>Total morphological score</td>
<td>32.74 ± 2.59</td>
<td>18.98 ± 3.03*</td>
</tr>
<tr>
<td>Yolk sac circulation</td>
<td>2.45 ± 0.16</td>
<td>2.13 ± 0.13*</td>
</tr>
<tr>
<td>Allantois</td>
<td>2.25 ± 0.14</td>
<td>1.74 ± 0.09*</td>
</tr>
<tr>
<td>Flexion</td>
<td>2.82 ± 0.22</td>
<td>1.23 ± 0.31*</td>
</tr>
<tr>
<td>Heart</td>
<td>2.52 ± 0.18</td>
<td>1.49 ± 0.30*</td>
</tr>
<tr>
<td>Caudal Neural Tube</td>
<td>2.82 ± 0.16</td>
<td>1.51 ± 0.33*</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>2.52 ± 0.19</td>
<td>1.48 ± 0.30*</td>
</tr>
<tr>
<td>Midbrain</td>
<td>2.52 ± 0.15</td>
<td>1.50 ± 0.27*</td>
</tr>
<tr>
<td>Forebrain</td>
<td>2.08 ± 0.24</td>
<td>1.30 ± 0.25*</td>
</tr>
<tr>
<td>Otic System</td>
<td>2.38 ± 0.21</td>
<td>1.48 ± 0.34*</td>
</tr>
<tr>
<td>Optic System</td>
<td>2.16 ± 0.27</td>
<td>1.23 ± 0.23*</td>
</tr>
<tr>
<td>Olfactory System</td>
<td>2.12 ± 0.25</td>
<td>1.05 ± 0.24*</td>
</tr>
<tr>
<td>Branchial Bars</td>
<td>1.99 ± 0.15</td>
<td>1.13 ± 0.19*</td>
</tr>
<tr>
<td>Maxillary Process</td>
<td>0.30 ± 0.15</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>Mandibular Process</td>
<td>0.63 ± 0.35</td>
<td>0.14 ± 0.07*</td>
</tr>
<tr>
<td>Forelimb</td>
<td>0.91 ± 0.39</td>
<td>0.22 ± 0.12*</td>
</tr>
<tr>
<td>Hindlimb</td>
<td>0.14 ± 0.14</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>Somites</td>
<td>2.13 ± 0.18</td>
<td>1.11 ± 0.26*</td>
</tr>
<tr>
<td>Quantitative measures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somite number</td>
<td>18.35 ± 1.04</td>
<td>9.88 ± 1.89*</td>
</tr>
<tr>
<td>Crown-Rump length (mm)</td>
<td>2.37 ± 0.18</td>
<td>2.24 ± 0.19</td>
</tr>
</tbody>
</table>

*1Morphological features (Mean ± SEM) were scored according to the methods of Brown and Fabro (1981).

*2Significantly different from wild type according to Mann–Whitney rank sum test (*P < 0.05*).**

**Analysis of pregnancy outcome**

Analysis of pregnancy outcome was carried out as previously described (Trasler et al., 1986). Briefly, copulation was ascertained by the presence of a vaginal plug and noon on the day a vaginal plug was discovered was designated embryonic day 0.5 (E0.5). Females were sacrificed at noon on E9.5 and their ovaries were excised and the number of corpora lutea counted. The uteri were opened and the numbers of resorption sites, implantation sites, and live fetuses were determined. Preimplantation loss, representing lack of fertilization or embryo death prior to endometrial implantation, was determined by calculating the difference between the number of eggs ovulated (corpora lutea) and the implantations for each female (Robaire et al., 1984). Post-implantation loss, referring to later impairment of embryo viability indicated by increased fetal resorption, was determined by calculating the difference between the number of implantation sites and the number of live embryos on E9.5. Post-implantation loss was further divided into early and late post-implantation loss. Here, early post-implantation loss considers embryos necrotic if the yolk sac or decidual mass was completely void of any embryonic material at E9.5. Late post-implantation loss considers embryos dead if their MS was less than 10 on E9.5. An MS less than 10 on E9.5 correlates with a developmental delay >24 h; embryos with such scores are decaying and in the process of being resorbed.

**Magnetic resonance microscopy**

High-resolution 3D volume images were acquired from 9 E9.5 and 29 E11.5 Dnmt1o-deficient embryos using MRM (Ahrens et al., 2006). Each imaged embryo was carefully excised while keeping the yolk sac intact, fixed overnight in 4% paraformaldehyde, and stored in 1 x PBS at 4°C prior to imaging. E11.5 embryos without yolk sacs were not imaged and were discarded. Prior to imaging, each embryo was transferred to a nuclear magnetic resonance sample tube and embedded in 1% agarose to prohibit sample movement. Embryos were positioned in an 11.7T, Bruker AVANCE Micro-imaging system and temperature regulated at 12°C for scanning. Scans were performed using near-isotropic voxels of size 50–100 µm. The E9.5 embryos were scanned using a 3D fast spin echo (FSE) pulse sequence with acquisition parameters echo time (TE)/repetition time (TR) = 15/1000 ms, matrix size 256 × 128 × 128, four echoes per excitation, and four averages. The E11.5 embryos were also scanned with an FSE sequence, using the modified parameters TE/TR = 45/1500 ms and 512 × 256 × 128 image points. Alternatively, E11.5 embryos were imaged using a steady-state free precession echo pulse sequence with parameters TE/TR = 5/10 ms, 512 averages, and a matrix size of 512 × 128 × 128. Each 3D volume image was imported into the 3D rendering software package Amira (Mercury Computer Systems, Chelmsford, MA, USA) and intensity thresholded to remove image noise. In Amira, embryos were digitally rotated to align with axial, sagittal and coronal axes, and serial image stacks were digitally sectioned along each axis. The software then created 3D density and surface renderings of each embryo for visualizing key embryonic features. Finally, the software was used to make accurate distance measurements of key embryonic features in both two-dimensional (2D) axis-aligned digital sections and 3D surface renderings in order to screen for abnormalities and to better determine Carnegie stages of development (O’Rahilly, 1979). Severely abnormal embryos that could not be classified by the Carnegie method were classified as growth disorganized (GD) or growth retarded (GR). GD types were defined according to Harris et al. (1981). GD Type 1 (GD1) is an empty sac. GD Type 2 (GD2) is an intact sac containing embryonic tissue with no organized structure. GD Type 3 (GD3) is a severely disorganized embryo with cephalic and caudal poles distinguishable.
A GD Type 4 (GD4) embryo contains multiple, often nonspecific, anomalies including growth retardation, stubby limb buds and misshapen areas of retinal pigment. A GR embryo is one in which growth is retarded but morphology is normal.

**Statistical evaluation**

Data were analyzed with the aid of Sigma Stat computer program (Sigma Stat v2.03, Statistical Package for the Social Sciences, Chicago, IL, USA). The frequency data (total MS, number of somites, crown-rump length, individual morphological features, number of corpora lutea, number of resorptions, number of implantations) were analyzed using the Mann–Whitney rank sum test or the t-test as indicated in the text and figure legends. The proportional data (numbers of specific malformations, preimplantation loss and post-implantation loss) were compared by Fisher’s exact test or the chi-square test. In all cases, a value of \( P \leq 0.05 \) was considered significant. Data are presented as mean \( \pm \) SEM. Statistical differences in methylation of CpG dinucleotides (percent methylated on maternal versus paternal alleles) of imprinted genes were evaluated using a paired Student’s \( t \)-test.

**Results**

**Evidence for the hypothesis that Dnmt1o-deficient embryos are somatic epigenetic mosaics**

To investigate the notion that embryos developing in the absence of the Dnmt1o protein are epigenetic mosaics, we studied the extent of DNA methylation on maternal and paternal alleles of the normally imprinted \( H19 \) and \( Snurfp/\)

\( Snrpn \) genes in individual cell clones from mid-gestation embryos. As shown in Fig. 1A, a representative MEF clone (Clone 1) from a wild-type E14.5 embryo has normal paternal-specific \( H19 \) methylation and normal maternal-specific \( Snurfp/\)

\( Snrpn \) methylation, whereas two Dnmt1o-deficient MEF clones have abnormal DNA methylation patterns. Dnmt1o-deficient Clone 19 lacks paternal-specific \( H19 \) methylation and Clone 21 lacks both paternal-specific \( H19 \) methylation and maternal-specific \( Snurfp/\)

\( Snrpn \) methylation. Similar abnormalities were documented in a number of other Dnmt1o-deficient MEF clones (Fig. 1B). Such variable loss of parent-specific DNA methylation in individual cells (clones) of an embryo supports the idea that a transient absence of maintenance methylation during preimplantation development can lead to epigenetically mosaic embryos.

**Asynchronous and abnormal development in Dnmt1o-deficient embryos**

Dnmt1o-deficient embryos were first examined by two easily obtained quantitative measures of developmental progress, namely length (specifically the crown-rump length) and the number of somites (Fig. 2, Table I). These two measurements were obtained on 37 wild-type embryos from 9 litters and 63 Dnmt1o-deficient embryos from 11 litters. All embryos were examined at 9.5 days post coitum (E9.5) and Dnmt1o-deficient embryos were obtained from homozygous \( Dnmt1^{+/+} \) females crossed to wild-type males. The genetic background of all mice was the inbred 129/Sv strain. For each litter, the average of the measured crown-rump lengths and the average of the embryos’ somite numbers was calculated, and the collection of averages for the nine wild-type litters compared with the collection of averages for the 11 Dnmt1o-deficient litters is shown in Fig. 2A. Dnmt1o embryos have a slightly but not significantly shorter crown-rump length than the wild-type embryos (2.24 ± 0.19 versus 2.37 ± 0.18 mm). The reason for the small difference between the two groups was probably because of the difficulty in accurately identifying the rump and/or crown, and thus in determining the distance between them in many anatomically distorted Dnmt1o-deficient embryos. A clear difference between the two embryo groups is apparent when the average number of embryo somites per litter is plotted for the two groups (Fig. 2B, Table I). The average litter somite number in the Dnmt1o-deficient embryos was 9.88 ± 1.89, compared with an average litter somite number of 18.35 ± 1.04 in the wild-type control mice. The markedly lower number of somites in the Dnmt1o-deficient group suggests that the mutant mice are either developmentally delayed compared with the wild-type controls and/or anatomical abnormalities have developed in the mutant mice due to the absence of Dnmt1o protein.

To better address the possibility that the mutant embryos were developmentally delayed relative to the wild-type control embryos at the same gestational time, the structure of the E9.5 embryos was analyzed in greater detail using a morphological scoring system. This method is a modification of a scoring system devised for assessing the extent of teratogen-induced damage in mid-gestation rat embryos (Brown and Fabro, 1981). The system as adapted to mouse development is described in Supplementary Table S1. Seventeen different anatomical features, including the quantitative trait of somite number, are examined, and the developmental progression of each feature is determined and scored on an integer scale from 0 to 5. Because of the difficulty in accurately measuring the crown-rump length in dissected E9.5 embryos (see above), this quantitative trait is excluded from a calculation of a MS. Seven measurements of central nervous system development are acquired. In addition, various features of branchial arch, limb, circulatory system, embryo flexion and extraembryonic tissue development are assessed. For each embryo, the 17 features were individually scored, and the sum of scores provided the MS of the embryo.

The 37 embryos from 9 wild-type litters and 63 embryos from 11 Dnmt1o-deficient litters were scored for the 17 different anatomical features at E9.5, and a MS determined for each embryo. The MS values for embryos within a litter were plotted together, along with the calculated average MS for that litter (Fig. 2C). Considering all litters, the average MS was significantly decreased in Dnmt1o-deficient embryos derived from homozygous \( Dnmt1^{+/+} \) females compared with control embryos (Table I). At E9.5, wild-type embryos had an average MS of 32.74 ± 2.59. In contrast, Dnmt1o-deficient embryos of the same embryonic age had an average MS of 18.98 ± 3.03. The average scores of individual anatomical features that comprise the MS are listed in Table I for both Dnmt1o-deficient embryos and controls. For all individual morphologic features that were assessed, the average score of the wild-type group of embryos was higher than for the Dnmt1o-deficient group. The average scores of individual
features among Dnmt1o-deficient litters were consistently between a half day (feature score difference of 1) and a full day (feature score difference of 2) behind that of their control counterparts (Table I). Fifteen of 17 individual parameters were significantly different between the two groups (Table I). No significant difference was observed for the development of the maxillary process in the facial region or for the growth of the hindlimb bud; the reason for this is that these features become prominent in embryos only toward the end of this embryonic period (Supplementary Table S1).

In addition to the significantly lower average MS for the Dnmt1o-deficient group of E9.5 embryos, there was also a much greater variation in the MS among the mutant embryos compared with the wild-type embryos (Table I). This difference between the two groups was particularly evident when the MSs of the individual embryos, grouped by litters, were plotted (Fig. 2C). In two Dnmt1o-deficient litters at E9.5 (litter numbers 9 and 10 in Fig. 2C), there was exceptional size variability among littermates with some conceptuses demonstrating advanced growth and others developmental delay, ranging across 48 h in developmental stage between E8.5 and E10.5 (see Supplementary Table S1; individual feature score of 1.0 represents ~0.5 days of embryonic development). This contrasted sharply with the relative consistency of developmental potential observed among wild-type littersmates that typically scored within half a day of their actual age (Fig. 2C). In many Dnmt1o-deficient litters, embryos with MSs near the wild-type average developed along with embryos with very low MSs. These observations are consistent both with the previous observation that, in rare instances, Dnmt1o-deficient embryos survive to term (Howell et al., 2001) and with independent in utero development of each Dnmt1o-deficient embryo.

**Pre- and post-implantation losses in Dnmt1o-deficient embryos**

In addition to identifying morphological defects and developmental delays, the approach to analyzing the effect of Dnmt1o removal on pregnancy outcome included an assessment of pre- and post-implantation embryo loss. Control embryos were derived from wild-type 129/Sv females crossed with wild-type 129/Sv males. Preimplantation loss was assessed by comparing the number of ovarian corpora lutea with the number of uterine implantation sites; the excess of corpora lutea to implantation sites was used to calculate a fraction of ovulated oocytes lost prior to implantation.
This implantation loss was similar in both control and Dnmt1o-deficient litters (0.09 ± 0.04 and 0.08 ± 0.03, respectively) and not statistically significant (Table II). Pre-implantation loss among embryos derived from homozygous Dnmt1o−/− females, however, was far more severe. At E9.5, 64 implantation sites were observed in 10 pregnancies from control females (mean of 6.4 ± 0.7 implantations per female, scoring only those females with at least one implantation site) (Table II). For heterozygous Dnmt1o-deficient E9.5 embryos (derived from homozygous Dnmt1o−/−/− females crossed with wild-type 129/Sv males), a total of 88 implantation sites were observed in 11 pregnancies at E9.5 (7.5 ± 0.7 implantations per female). In contrast to control embryos in which 53/64 (82.8%) of implantations were associated with the development of live embryos, only 42/88 (47.7%) of implantations observed in Dnmt1o-deficient females were associated with the development of live embryos. Moreover, 23/88 (26.1%) of implantation sites in homozygous Dnmt1o−/−/− females at E9.5 were necrotic sites. These findings indicate that Dnmt1o-deficient embryos underwent much higher post-implantation attrition than did wild-type embryos.

To better appreciate the time at which implanted Dnmt1o-deficient embryos were lost, post-implantation loss was divided into an early and a late period of loss. Early post-implantation loss considers embryos dead if the yolk sac is completely void of any embryonic material at E9.5. Late post-implantation loss considers embryos dead if they exhibit a composite MS less than 10 on E9.5 according to the criteria in Supplementary Table S1; such embryos demonstrate >24 h of developmental delay and are in the process of being resorbed. These embryos are decaying, highly fragmented and lack a beating heart rudiment when one is present (Fig. 3A–F). Control litters demonstrated a fractional early post-implantation loss of 0.05 ± 0.03, compared with 0.24 ± 0.06 in Dnmt1o-deficient litters (Table II). Fractional late post-implantation loss was also significantly different between the two groups: control embryos had a late post-implantation loss of 0.02 ± 0.02 in contrast to 0.21 ± 0.07 exhibited by the Dnmt1o-deficient embryos. The high percentage of post-implantation embryo loss (both early and late) among Dnmt1o-deficient embryos compared with the low percentage of preimplantation loss (which is indistinguishable from the low level of preimplantation loss in wild-type embryos) is consistent with the importance of imprinted gene expression during fetal development, rather than during preimplantation development.

**Developmental delays and specific embryonic malformations induced by Dnmt1o deficiency**

Two observations suggested that specific embryonic defects might be associated with the absence of Dnmt1o and death of Dnmt1o-deficient fetuses: the highly variable MSs among E9.5 Dnmt1o-deficient embryos (Fig. 2) and the range (early and late) among Dnmt1o-deficient embryos compared with the low percentage of preimplantation loss (which is indistinguishable from the low level of preimplantation loss in wild-type embryos) is consistent with the importance of imprinted gene expression during fetal development.

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Litters examined</th>
<th>Number of corpora lutea</th>
<th>Number of resorption sites</th>
<th>Number of implantations</th>
<th>Preimplantation loss</th>
<th>Early post-implantation loss</th>
<th>Late post-implantation loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10</td>
<td>7.0 ± 0.6</td>
<td>0.9 ± 0.4</td>
<td>6.4 ± 0.7</td>
<td>0.09 ± 0.04</td>
<td>0.05 ± 0.03</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Dnmt1o−/−/−</td>
<td>11</td>
<td>8.7 ± 0.3*</td>
<td>0.2 ± 0.1</td>
<td>7.5 ± 0.7</td>
<td>0.08 ± 0.03</td>
<td>0.24 ± 0.06*</td>
<td>0.21 ± 0.07*</td>
</tr>
</tbody>
</table>

*Values are presented as Mean ± SEM.

Preimplantation loss was calculated as (Number corpora lutea − Number implantations)/Number corpora lutea.

Post-implantation loss was calculated as (Number implantations − Number live embryos)/Number implantations. Early post-implantation loss considers embryos dead if the yolk sac is completely void of any embryonic material at E9.5.

Late post-implantation loss considers embryos dead if they exhibit a composite MS < 10 according to the criteria in Table I. MS < 10 at E9.5 indicates >24 h of developmental delay. These embryos are decaying and in the process of being resorbed.

Significantly different from wild type according to t-test or Mann–Whitney rank sum test (P < 0.05).

Significantly different from wild type according to Chi-square test (P < 0.05).
particular, we focused on those Dnmt1o-deficient embryos that were considered significantly delayed in their embryonic development based on a MS of less than 25 at E9.5. According to this definition, 38 out of 63 (60.32%) E9.5 embryos derived from homozygous Dnmt1o<sup>D10/D10</sup> females, compared with 5 out of 37 (13.51%) of wild-type 129/Sv embryos, were developmentally delayed (Table III).

Although the average scores of all individual anatomical categories were lower for the Dnmt1o-deficient embryos than for the wild-type embryos (Table I), most individual developmentally delayed Dnmt1o-deficient embryos exhibited low scores in just a subset of the 17 different anatomical categories. The commonly affected embryo features included delayed heart morphogenesis, failure to engage in axial rotation (embryonic turning), retarded somitogenesis and anterior closure defects (Table III). The vasculature of the yolk sac was also delayed among Dnmt1o-deficient embryos. Even though the average yolk sac circulation score was significantly different between control and Dnmt1o-deficient embryos, 2.45 ± 0.16 and 2.13 ± 0.13, respectively (Table I), the percentage of embryos with defects in their yolk sac circulation was not significantly different between control and Dnmt1o-deficient embryos (Table III). Abnormalities in the vasculature of Dnmt1o-deficient embryos included a reduction in fetal red blood islands (data not shown).

Table III. Numbers (percentages) of specific malformations induced by Dnmt1o-deficiency on mouse embryos

<table>
<thead>
<tr>
<th>Wild type</th>
<th>Dnmt1o-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of embryos scored&lt;sup&gt;1&lt;/sup&gt;</td>
<td>37</td>
</tr>
<tr>
<td>Delayed development&lt;sup&gt;2&lt;/sup&gt;</td>
<td>38 (60.32)*</td>
</tr>
<tr>
<td>Poor yolk sac circulation&lt;sup&gt;3&lt;/sup&gt;</td>
<td>10 (15.87)</td>
</tr>
<tr>
<td>Retarded turning&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4 (10.81)</td>
</tr>
<tr>
<td>Heart formation delay&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2 (5.41)</td>
</tr>
<tr>
<td>Open Anterior Neuropore&lt;sup&gt;3&lt;/sup&gt;</td>
<td>34 (53.97)*</td>
</tr>
</tbody>
</table>

<sup>1</sup>Morphological features were scored according to the methods of Brown and Fabro (1981).
<sup>2</sup>Developmental delay was a MS < 25 at E9.5 equivalent to >12 h delay.
<sup>3</sup>The remaining malformations examined the individual scores of the following: yolk Sac, Flexion, Heart and Hind Brain. Primordia were considered developmentally retarded if they received an individual score <2 according to the criteria in Table I equivalent to >12 h developmental delay.
<sup>4</sup>Somitogenesis was considered retarded if embryos exhibited <13 pairs of somites at E9.5 equivalent to a score <2 and a developmental delay >12 h.
<sup>5</sup>Significantly different from WT according to Fisher exact test (P < 0.05).

There was a striking effect of a loss of Dnmt1o protein on heart formation; 32/63 (50.79%) of Dnmt1o-deficient embryos had a heart score <2, indicating a >12 h delay in heart patterning. In contrast, 5/37 (13.51%) of control embryos scored <2 for heart development (Table III). Heart rudiments in Dnmt1o-deficient mutant embryos were consistently undersized and, in some instances, failed to undergo looping morphogenesis. In contrast to wild-type control embryos, mutant rudimentary hearts were seldom found to be beating at the time of dissection. We conclude from this analysis that the large percentage (~51%) of Dnmt1o-deficient embryos with significant heart malformations could be interpreted as a specific adverse effect of Dnmt1o deficiency on heart formation.
heart development, which in turn could lead to a series of more generalized development anomalies.

Low E9.5 somite numbers and low flexion scores suggested that there might be frequent turning defects in the Dnmt1o-deficient embryos. Most of the Dnmt1o-deficient embryos had fewer pairs of somites and were comparable to E8.5 or younger embryos in terms of overall somite number (Fig. 2B). Indeed, based on criteria defined in Table III, only 2/37 (5.41%) of wild-type E9.5 embryos had severely delayed somitogenesis (fewer than 13 pairs) compared with 34/63 (53.97%) of those embryos developing without Dnmt1o. A similar incidence of flexion abnormalities was seen in the Dnmt1o-deficient embryos (57.14% compared with 10.81% in the wild-type controls). We conclude from this analysis that the high incidence of these two specific abnormalities may indicate that one or both of these defects could be the cause(s) of the high rate of post-implantation embryonic loss in the Dnmt1o-deficient population.

Finally, examination of anterior neuropores via evaluation of the developing hindbrain was carried out to determine the frequency of significant neural closure defects in Dnmt1o-deficient E9.5 embryos. Although a fair percentage of wild-type E9.5 embryos still have an open anterior neuropore (6/37 or 16.22%), more than half of Dnmt1o-deficient embryos had open anterior neuropores at the same gestational age (34/63 or 53.97%) (Table III and Fig. 3, bottom row). Such a significant percentage of closure defects in Dnmt1o-deficient embryos suggests that this fundamental defect could be the primary cause of much of the embryonic loss among the population of Dnmt1o-deficient embryos. However, because neurulation defects can be a consequence of developmental retardation and degenerative processes (Copp et al., 2003), mid-gestation embryonic lethality may be manifested by neural tube defects (NTDs). Such a mechanism likely explains the variety of NTDs due to the teratogenic effects of ethanol; excessive cell death due to ethanol exposure can disrupt cranial neurulation by providing insufficient numbers of normal cells for morphogenesis (Kotch and Sulik, 1992). On the basis of this, it is difficult to conclude that Dnmt1o deficiency is specifically associated with NTDs, or whether the defects reflect a more generalized lack of embryonic cell viability.

**Specific Dnmt1o-deficient abnormalities detected by MRM**

To more thoroughly define the types of abnormalities in Dnmt1o-deficient embryos, we examined their anatomy in detail with MRM. Nine E9.5 and 29 E11.5 embryos were imaged in their protective yolk sacs, which permitted the distinction between true developmental anomalies and abnormalities induced by the trauma of dissection. Embryos were classified as either GD, GR or were otherwise sufficiently developed to allow for anatomical analysis using the Carnegie stage of development classification method (O’Rahilly, 1979; Harris et al., 1981). This method was devised to assess development of the larger human embryo, based on the presence of developmental landmarks. Because the same landmarks are also present in the mouse (but at an earlier absolute time of in utero development), the method can also be applied to the developing mouse embryo. E9.5 embryos span Carnegie stages 12 and 13, corresponding to 26–31 human days, whereas E11.5 embryos span Carnegie stages 14–16, corresponding to 32–37 human days.

Four out of nine (4/9) E9.5 and 6 out of 29 (6/29) E11.5 embryos were unsuited for Carnegie staging, being severely GR or GD. Of the four GR or GD E9.5 embryos (embryos #6–#9), one was GD1 (embryo #6), one was GD2 (embryo #7), one was GD3 (embryo #8) and the other embryo was not well enough visualized by MRM to assess the GD type (embryo #9). Of the six GR or GD E11.5 embryos (embryos #24–#29), two were GD2 (embryos #25 and #29), three were GD4 (embryos #26, #27 and #28) and the other (embryo #24) was GR but otherwise morphologically normal. Embryos #28 and #29 are twin embryos (Supplementary Fig. S1). On the basis of the MS method, these GR and GD embryos would likely be classified as early or late post-implantation losses (Table II).

Five out of nine (5/9) E9.5 and 23 out of 29 (23/29) E11.5 embryos were sufficiently developed to permit Carnegie classification. The MRM serial sections and 3D renderings of each E9.5 Dnmt1o-deficient embryo were inspected for a number of expected developmental landmarks, based on the Carnegie classification and the easily recognizable features of the morphological scoring system (Supplementary Table S1). A summary of this analysis is shown in Supplementary Table S2. Three of the imaged E9.5 embryos (embryos #1–#3) appeared normal, whereas the other two (embryos #4 and #5) exhibited major anatomical anomalies. Prominent among these were the delayed or abnormal development of pharyngeal arches and clefts, the absence of a closing caudal neuropore, and indented lens discs. These two embryos, as well as embryos #1 and #3, exhibited features of delayed limb formation. The conclusion from this analysis is that a range of phenotypes were observed by MRM, ranging from those that were indistinguishable from normal to those with features that indicated either a profound developmental delay and/or an occurrence of specific defects. Such a conclusion is entirely consistent with that of the MS method applied to a larger group of E9.5 Dnmt1o-deficient embryos.

Close examination of the MRM images of the morphologically abnormal E9.5 Dnmt1o-deficient embryos revealed important details of their anomalies. Exemplary MRM images of normal appearing and highly abnormal E9.5 Dnmt1o-deficient embryos are shown in Fig. 4, Supplementary Fig. S1 and Supplementary Movies 1–4. The abnormal E9.5 embryo in Fig. 4 (E9.5 embryo #5 in Supplementary Table S2) was most notable for a large NTD. The developmentally delayed embryos, some shown in Supplementary Fig. S1 (E9.5 embryos #1, #3 and #4 in Supplementary Table S2) contained a variety of abnormalities. We conclude from this that a wide range of developmental anomalies can be clearly distinguished by maintaining the E9.5 embryo within the protective yolk sac and imaging this undisturbed embryo by the MRM technique.

The MRM serial sections and 3D renderings of each E11.5 Dnmt1o-deficient embryo were also inspected for a number of expected developmental landmarks, based on the Carnegie method. Excluding the six E11.5 embryos that were classified as GR or GD, the results of inspecting the remaining 23 E11.5 embryos.
embryos is shown in Supplementary Table S3. Features of the imaged embryos were compared with those expected for normally developing embryos at embryonic day 11.5. Four out of the 23 imaged embryos (#1, #14, #17 and #20) exhibited significant anatomical defects. A 3D rendering of one of these (E11.5 embryo #20) is shown in Fig. 5, and it was compared with the 3D rendering of a normal appearing Dnmt1o-deficient embryo (E11.5 embryo #4). The most compelling anomaly found in this embryo is the large NTD extending from the floor of the fourth ventricle to the upper segment of the lumbar spinal cord. This anomaly is also seen in a 2D virtual section (Fig. 5). Close examination of the MRM images of the morphologically abnormal E11.5 Dnmt1o-deficient embryos revealed important details of their anomalies (Fig. 4, Supplementary Fig. S1 and Supplementary Movies 5–8). MRM images of additional normal appearing and highly abnormal E11.5 Dnmt1o-deficient embryos are shown in Fig. 5 and Supplementary Fig. S1. We conclude from this that a wide range of developmental anomalies can be clearly distinguished by maintaining E11.5 embryos within their protective yolk sacs while imaging by the MRM technique.

Because a visual inspection MS method was not applied to E11.5 embryos, we could not directly compare these MRM findings of anomalies in E11.5 to those obtained by a visual inspection (MS-like) method. Nevertheless, it is apparent that there was a range of phenotypes observed by MRM, ranging from those that are indistinguishable from normal to those with features that indicated either a profound developmental delay and/or the occurrence of specific defects. Such a conclusion is similar to that obtained by examining Dnmt1o-deficient embryos at two earlier days of development, although it is interesting that a smaller percentage of E11.5 Dnmt1o-deficient embryos were in the GD categories. Possibly, those embryos that perished soon after implantation (early post-implantation losses) had become more thoroughly resorbed by E11.5, and therefore were discarded.

Discussion

**Loss of preimplantation Dnmt1o results in post-implantation embryo losses**

Evidence to date suggests that the oocyte-derived Dnmt1o cytosine methyltransferase functions to maintain DNA methylation on imprinted genes during 1-cell cycle. This maintenance probably occurs at the fourth embryonic S phase, because the Dnmt1o protein is present in the nuclear compartment only in blastomeres of 8-cell embryos (Howell et al., 2001). Despite the time of Dnmt1o function, no difference was observed between Dnmt1o-deficient and control embryos in the percentage of lost preimplantation embryos. These findings suggest that imprinted genes have little, if any, role in the viability of preimplantation embryos. This is consistent with the complete development of parthenogenetic, gynogenetic and androgenetic embryos through preimplantation development to the blastocyst.
stage, the viability and pluripotency of gynogenetic and androgeneric embryonic stem cells, and the absence of preimplantation phenotypes in mice with targeted mutations in individual imprinted genes (McGrath and Solter, 1984; Surani et al., 1984; Mann et al., 1990; Lucifero et al., 2004).

In contrast to the absence of an effect of Dnmt1o deficiency on development of preimplantation embryos, the lack of Dnmt1o leads to significant loss of post-implantation embryos. It was previously reported that Dnmt1o-deficient embryos probably die between Days 14 and 21 of gestation, because many live embryos can be recovered in mid-gestation, few are born and even fewer survive after birth (Howell et al., 2001). The careful analysis performed here shows that a significant amount of embryo loss has occurred by E9.5. There are three reasons to suspect that Dnmt1o deficiency actually results in embryo losses well before E9.5, most likely near the time of gastrulation. First, there is a marked difference in early post-implantation loss between wild-type and Dnmt1o-deficient embryos (Table II). Second, the large number of necrotic conceptuses observed among Dnmt1o-deficient embryos by E9.5 suggests that these mice die around the time of gastrulation (E6.5 to E7.5). Lastly, E9.5 Dnmt1o-deficient mice with MSs < 10 were likely delayed in their development by >24 h (Supplementary Table S1), and were likely undergoing resorption at the time of inspection (Fig. 3, bottom row). It is interesting to note that these early post-implantation Dnmt1o-deficient phenotypes are reminiscent of the severe developmental abnormalities seen in parthenogenotes or gynogenotes (McGrath and Solter, 1984; Surani et al., 1984). This similarity suggests that Dnmt1o-deficient embryos dying soon after implantation are composed of cells with widespread disruptions in imprinted gene expression. Moreover, it suggests that developmental abnormalities in Dnmt1o-deficient embryos are largely, if not solely, due to imprinting defects.

There were many Dnmt1o-deficient embryos that appeared normal or nearly normal at E9.5 and E11.5 (Figs 3–5 and Supplementary Fig. S1). The large percentage of normal appearing E11.5 Dnmt1o-deficient embryos that we observed in MRM studies is likely due, in part, to the exclusion of E11.5 embryos without yolk sacs in this analysis. Those E11.5 Dnmt1o-deficient embryos without yolk sacs probably represented a combination of E9.5 GR or GD embryos whose phenotype persisted at E11.5, and anatomically better developed E9.5 Dnmt1o-deficient embryos that were significantly developmentally impaired at E9.5 such that by 2 days later they had died and were resorbed. Taken together with our documented instances of early post-implantation losses (Table II), these findings point to a highly variable effect of Dnmt1o deficiency on embryo development.

**Etiology of abnormalities in Dnmt1o-deficient embryos**

The marked variation in embryonic phenotype, evident in the differences in time of death and in the range of MSs, suggests that embryos lacking Dnmt1o protein die from many different causes following implantation. Unfortunately, because of the existence of widely variable phenotypes among Dnmt1o-deficient embryos, no single phenotype can be deliberately reproduced using Dnmt1<sup>Δ1o</sup> mutant mice. Stated differently, it is not possible to determine the cause of any one of the mutant
phenotypes by examining that particular phenotype at many defined time points in embryogenesis. Therefore, as an alternative to identifying potential lethal effects due to Dnmt1o deficiency, we studied the occurrence of certain embryonic features (such as yolk sac vasculature, heart morphogenesis, axial rotation (turning) and closure of the anterior neuropore), with the goal of determining whether certain embryonic defects alone might account for post-implantation losses. Because defects in cardiac morphogenesis, axial rotation or neural development were not observed at a high enough frequency among the E9.5 Dnmt1o-deficient embryos, we concluded that they were not likely to be the primary causes of embryonic lethality in Dnmt1o-deficient embryos.

**Epigenetic variation as a potential cause of phenotypic variation**

The assumption that the Dnmt1o methyltransferase is required for 1-cell cycle of maintenance methyltransferase activity in the preimplantation embryo establishes a theoretical source of epigenetic variation in mice developing in the absence of Dnmt1o protein. Dnmt1o deficiency should generate cells within a single preimplantation embryo that contain either normal methylation of imprinted alleles or an abnormal lack of methylation (Howell et al., 2001). These embryos would be epigenetic mosaics, a conclusion supported by the data shown in Fig. 1. Because random chromosome segregation occurs in every cell of a small embryo founder cell population during the cell cycle following the Dnmt1o deficiency, there is much variety in the types of epigenetic mosaics that can form. In any given embryo, only a few of the many varieties of epigenetically distinct cells that can be produced would actually be produced. We propose that such a two-step process of Dnmt1o removal and random chromosome segregation is the root of the phenotypic variation we observe in offspring of homozygous Dnmt1o female mice. Because this two-step process occurs before formation of the blastocyst, both embryonic and extraembryonic tissues would be expected to have this type of mosaicism.

The variety of epigenetically distinct cells that can be found in embryos derived from homozygous Dnmt1o/Dnmt1o mothers is theoretically quite large because there are 12 autosomes in mice that contain imprinted genes (http://www.mgu.har.mrc.ac.uk/research/imprinting/) and, assuming Dnmt1o functions in the 8-cell embryo, $2^{12}$ or >4000 possible epigenetically distinct cell types would be present at the fifth embryonic S phase. There is potential for even greater diversity of cell types if one considers the occurrence of mitotic recombination between imprinted genes on the same chromosome. In the event that maintenance methylation is faithfully maintained in the cell cycle following the Dnmt1o-associated one, the epigenetically distinct cell types would be maintained throughout subsequent development. Given that there are >4000 possible epigenetically distinct cell types following the fifth S phase, no mutant embryos are likely to possess the same array of cell types. Each mosaic embryo produced from Dnmt1o deficiency at the fourth S phase of preimplantation development, therefore, should be composed of 32 epigenetically distinct cell types. It is this form of early embryonic epigenetic mosaicism that likely explains the lack of a consistent, discernible mutant phenotype among Dnmt1o-deficient embryos.

**Supplementary Data**

Supplementary data are available at http://humupd.oxfordjournals.org/.

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**References**


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