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The genome-defence gene Tex19.1 suppresses LINE-1 retrotransposons in the placenta and prevents intra-uterine growth retardation in mice

Judith Reichmann†, James P. Reddington†, Diana Best, David Read, Rupert Öllinger‡, Richard R. Meehan∗ and Ian R. Adams∗

MRC Human Genetics Unit, MRC Institute of Genetics and Molecular Medicine, Western General Hospital, University of Edinburgh, Crewe Road, Edinburgh EH4 2XU, UK

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DNA methylation plays an important role in suppressing retrotransposon activity in mammalian genomes, yet there are stages of mammalian development where global hypomethylation puts the genome at risk of retrotransposition-mediated genetic instability. Hypomethylated primordial germ cells appear to limit this risk by expressing a cohort of retrotransposon-suppressing genome-defence genes whose silencing depends on promoter DNA methylation. Here, we investigate whether similar mechanisms operate in hypomethylated trophectoderm-derived components of the mammalian placenta to couple expression of genome-defence genes to the potential for retrotransposon activity. We show that the hypomethylated state of the mouse placenta results in activation of only one of the hypomethylation-sensitive germline genome-defence genes: Tex19.1. Tex19.1 appears to play an important role in placenta function as Tex19.1−/− mouse embryos exhibit intra-uterine growth retardation and have small placentas due to a reduction in the number of spongiotrophoblast, glycogen trophoblast and sinusoidal trophoblast giant cells. Furthermore, we show that retrotransposon mRNAs are derepressed in Tex19.1−/− placentas and that protein encoded by the LINE-1 retrotransposon is upregulated in hypomethylated trophectoderm-derived cells that normally express Tex19.1. This study suggests that post-transcriptional genome-defence mechanisms are operating in the placenta to protect the hypomethylated cells in this tissue from retrotransposons and suggests that imbalances between retrotransposon activity and genome-defence mechanisms could contribute to placenta dysfunction and disease.

INTRODUCTION

Retrotransposons are mobile genetic elements that amplify and integrate into new genomic locations through RNA intermediates. These parasitic DNA sequences are highly abundant in mammalian DNA and typically account for 40–70% of sequenced mammalian genomes (1–4). To limit the activity of these elements, mammals possess numerous genome-defence mechanisms that can suppress retrotransposon activity at transcriptional and post-transcriptional levels in different cell types (5–7). DNA methylation, histone modification and polycomb-mediated repression have all been implicated in transcriptional repression of retrotransposons in mouse embryonic stem (ES) cells (8–10). DNA methylation also appears to play an important role in repressing retrotransposons in differentiated embryonic tissue, particularly in the repression of intracisternal A particle (IAP) retrotransposons (9,11,12). Indeed, transcriptional repression of retrotransposons has been proposed to be the primary function of DNA methylation in mammalian genomes (13).
At a genome-wide level, DNA methylation is high in most differentiating embryonic tissues, with the notable exception of the developing germline (14). Primordial germ cells undergo extensive loss of DNA methylation during their development as part of a wider epigenetic reprogramming event that resets imprints and other epigenetic marks (15,16). However, global DNA hypomethylation has the potential to induce retrotransposon activity and, therefore, genome instability, in the developing germline (17). We have previously shown that DNA hypomethylation in primordial germ cells induces expression of a group of hypomethylation-sensitive genome-defence genes (Tex19.1, Piwil2, Mov10l1, Dazl and Asz1) that function to protect the germline DNA from retrotransposon activity (17). Mutations in Tex19.1, Piwil2, Mov10l1 or Asz1 result in derepression of retrotransposons in testicular germ cells and male sterility (18–22), whereas Dazl is required for efficient translation of retrotransposon-suppressing genes Tex19.1 and Movh (23,24).

The DNA methylation-dependent silencing of these genome-defence genes appears to represent a developmental mechanism that helps to suppress retrotransposon activity during periods of global DNA hypomethylation and epigenetic reprogramming in the developing germline when the potential for retrotransposon activation is high (17).

Like primordial germ cells, the placenta is globally hypomethylated relative to other mouse tissues, and the hypomethylated state of the placenta extends to retrotransposons (14,25–27). Approximately, 40–50% of cytosines in a CpG context are methylated within long terminal repeat (LTR), long interspersed nuclear element (LINE) and short interspersed nuclear element (SINE) classes of retrotransposon in mouse placentas, compared with ~75–80% in the embryo (27). Specific types of retrotransposon, such as IAP and LINE-1 elements, have also been shown to be hypomethylated in the placenta relative to the embryo (25,28). The hypomethylated epigenetic state of placental DNA could potentially generate a transcriptionally permissive environment for retrotransposon expression (25), and numerous studies report expression of retrotransposons in this tissue (reviewed in 29). However, it is not known whether mechanisms operate in the placenta, as they do in the germline, to couple transcriptional activation of hypomethylation-sensitive genome-defence genes to the potential for retrotransposon activation (17).

In this study, we show that the hypomethylated epigenetic state of the placenta is associated with expression of specific retrotransposons, but does not result in widespread activation of retrotransposon expression in general. We find that only one of the hypomethylation-sensitive germline genome-defence genes, Tex19.1, is hypomethylated and expressed in mouse placenta and that Tex19.1<sup>−/−</sup> embryos exhibit intrauterine growth retardation. Tex19.1<sup>−/−</sup> placentas are small with thinner junctional zones and a reduced abundance of multiple trophctoderm-derived cell types when compared with littermate controls. Microarray expression profiling of Tex19.1<sup>−/−</sup> placentas shows that loss of Tex19.1 results in increased expression of LINE-1 retrotransposons in this tissue, and immunohistochemistry suggests that LINE-1 derepression is occurring in the hypomethylated trophctoderm-derived cell types that normally express Tex19.1. Our data extend to the placenta the associations between DNA hypomethylation, Tex19.1 and post-transcriptional genome-defence against retrotransposons that we have previously described in the developing germline (17) and suggest that imbalances between retrotransposon activity and host genome-defence mechanisms might be associated with impaired placenta function in mammals.

RESULTS

Retrotransposon expression in the mouse placenta

DNA methylation is associated with transcriptional repression of retrotransposons in embryos and embryo-derived cell lines (9,11,12,30,31). Therefore, we investigated whether the hypomethylated state of the mouse placenta (25–27) permits widespread retrotransposon expression in this tissue. We used our recently developed microarray repeat-annotation methodology (10) to extract information about placental expression of hundreds of different types of retrotransposon from mouse multiple-tissue gene expression data (32). Repeat annotation of gene expression microarray data from E16 placentas and E16 embryos from within this dataset showed that, in general, retrotransposon classes (LTR, LINE and SINE) of repeat probes appeared to be no more differentially expressed between placenta and embryo than non-repeat probes (Fig. 1A and B): although 51% of non-repeat probes are differentially expressed (<i>P</i> < 0.01) between placenta and embryo, only 39% of retrotransposon probes are differentially expressed (<i>P</i> < 0.01) between these tissues. Furthermore, similar numbers of retrotransposon probes are upregulated and downregulated in the placenta [1850 retrotransposon probes significantly (<i>P</i> < 0.01) upregulated, 1740 retrotransposon probes significantly (<i>P</i> < 0.01) downregulated, <i>x</i>²-test <i>P</i> = 0.2], suggesting that the placenta is not any more permissive than embryonic tissues for retrotransposon derepression, despite its hypomethylated epigenetic state. Multiple families of LTR retrotransposons (endogenous retroviruses) are reported to be hypomethylated in placenta (~40% DNA methylation) relative to the embryo (~75% DNA methylation) (27). Although LTR retrotransposons in general do not appear to be any more differentially expressed between placenta and embryo than non-repeat probes, we also investigated whether the five LTR retrotransposons that are upregulated in mouse ES cells in response to hypomethylation (9) might be specifically upregulated in the placenta. Interestingly, this group of methylation-sensitive LTR retrotransposons showed divergent behaviour in the placenta, with expression of the IAP<sub>Ez</sub> subclass of IAP elements increased relative to embryonic tissues, MMERGLN and some RLTR45 elements decreased and RLTR45 and some RLTR1B elements not changing (Fig. 1C). Thus, there does not appear to be a strong correlation between the retrotransposons upregulated in mouse ES cells in response to DNA hypomethylation and retrotransposons expressed in the hypomethylated placenta.

IAP and LINE-1 elements are both strongly repressed by DNA methylation in the developing germline (11,33) and have each been shown to be hypomethylated in mouse placenta (25,28). Therefore, we examined whether the hypomethylated status of these elements in the placenta would...
correlate with their increased expression in this tissue. Interestingly, although LINE-1 elements do not show increased expression in the placenta, IAP elements (endogenous retrovirus type K family of LTR retrotransposons) do (Fig. 1D and E). Consistent with previous observations using hypomethylated ES cells (9,10), some IAP probes were more strongly upregulated than others in the hypomethylated placenta. The bulk IAP probe population is upregulated around 2-fold in the placenta; however, some groups of IAP probes are upregulated more than 10-fold (Fig. 1E). Quantitative reverse
transcription-polymerase chain reaction (qRT-PCR) for IAP and LINE-1 expression in embryonic and placental tissue confirmed that IAP elements are upregulated in the placenta relative to the embryo, whereas LINE-1 elements are expressed at a similar level in these tissues (Fig. 1F). Thus, expression of IAP elements is associated with their hypomethylated state in the placenta, but expression of LINE-1 elements is not.

The placenta contains cells derived from the mother, and from the extraembryonic mesoderm and the trophectoderm of the embryo, but only trophectoderm-derived cells are expected to contribute to the decidua basalis, junctional zone and labyrinth layers of the mature placenta (34). To determine whether the hypomethylated trophectoderm-derived cell types in the placenta are responsible for the IAP expression in this tissue, we performed immunohistochemistry for IAP-gag protein. Anti-IAP gag immunostaining showed that parietal trophoblast giant cells in the decidua basalis, and spongiotrophoblast cells in the junctional zone of the placenta, are expressing IAP (Fig. 1G). Low-level expression of IAP gag protein is also detectable in sinusoidal trophoblast giant cells in the labyrinth layer of the placenta (Fig. 1G).

The distribution of IAP gag-expressing cells in the placenta correlates well with the distribution of trophectoderm-derived cells (34). Thus, as proposed in the original study on DNA methylation at repetitive sequences in extraembryonic tissues (25), the hypomethylated state of the placenta does appear to allow expression of certain retrotransposons such as IAP. However, hypomethylation does not appear to cause widespread upregulation of retrotransposon expression in general in this tissue. This suggests that mechanisms other than DNA methylation are contributing to transcriptional and/or post-transcriptional repression of LINE-1 and other retrotransposons in the placenta.

DNA hypomethylation in the placenta is associated with expression of the hypomethylation-sensitive genome-defence gene Tex19.1

We have recently shown that expression of a cohort of germline genome-defence genes is activated by DNA hypomethylation in primordial germ cells as part of a mechanism that can protect germline DNA from the mutagenic activity of retrotransposons (17). The hypomethylated epigenetic state of placental DNA affects gene promoters in addition to retrotransposons (27); therefore, we investigated whether this cohort of hypomethylation-sensitive germline genome-defence genes is also expressed in the hypomethylated placenta. Tex19.1 has been previously reported to be expressed in extraembryonic tissues (17,36), but analysis of multiple-tissue microarray data (32) suggests that no other known member of this group of genome-defence genes is expressed in the placenta (Fig. 2A). We verified by qRT-PCR that Tex19.1 is the only known hypomethylation-sensitive germline genome-defence gene highly expressed in the placenta (Fig. 2B). The differential expression of the germline genome-defence genes in the placenta suggests that Tex19.1 may be functioning independently of the remaining germline genome-defence genes in this tissue.

We next tested whether the differential expression of the genome-defence genes reflects differences in promoter DNA methylation. Bisulphite sequencing of placental and embryonic DNA showed that Tex19.1 is more extensively hypomethylated in placental DNA than the other germline genome-defence genes (Fig. 2C). Interestingly, individual clones in the Tex19.1 bisulphite sequencing data were either highly methylated or unmethylated suggesting that two epigenetically distinct populations of Tex19.1 DNA molecules are present in the placenta. Furthermore, the proportion of hypomethylated Tex19.1 clones (~60%) correlates reasonably well with the contribution of hypomethylated trophectoderm-derived cells in mid-gestation placentas (35). In contrast to Tex19.1, Dazl is highly methylated in both placenta and embryo, and although there is a small drop in methylation levels at Mov10l1 and Aszl promoters in the placenta relative to the embryo, these genes are each highly methylated in both tissues (Fig. 2C). A small number of highly hypomethylated Aszl clones are present in the placenta samples (Fig. 2C), which could reflect some Aszl expression in a small proportion of cells in the placenta (Fig. 2B). Piwil2 is not as highly methylated as any of the other genome defence genes in the embryo and also appears to be less methylated in the placenta (Fig. 2C). However, the placental hypomethylation of Piwil2 is not as extensive as Tex19.1. The absence of robust Piwil2 expression in the placenta (Fig. 2A and B) could reflect the activity of the residual ~40% methylation at this promoter, or the DNA methylation-independent component of Piwil2 repression that is evident upon differentiation of Dnmt3a−/− Dnmt3b−/− ES cells (17). Regardless, with the exception of Tex19.1, the germline genome-defence genes do not appear to be as sensitive to placental DNA hypomethylation as they are to the DNA hypomethylation event that occurs in the developing germline (17). Thus, any methylation-sensitive feedback loop to protect the placenta from retrotransposons either involves only Tex19.1 or a completely different set of genes altogether.

Dazl has been reported to be required for efficient translation of Tex19.1 mRNA in germ cells (24); therefore, the absence of Dazl expression in the placenta (Fig. 2A and B) could impair TEX19.1 protein expression, despite the presence of Tex19.1 mRNA in this tissue. Anti-TEX19.1 immunohistochemistry in E18.5 (Fig. 2D) and E9.5 (Supplementary Material, Fig. S1) placentas suggests that TEX19.1 protein is present in the placenta, despite the absence of Dazl expression. The anti-TEX19.1 immunostaining appears to be specific as it is not present in Tex19.1−/− placenta (Fig. 2D). In wild-type embryos, anti-TEX19.1 immunostaining is present in trophoblast cells in each of the decidua basalis, junctional zone and labyrinth layers of the placenta, but was not detected in the endothelial cells or the chorionic plate (Fig. 2D). This suggests that Tex19.1 is expressed in the hypomethylated trophectoderm-derived components of the placenta, but not in methylated extraembryonic mesoderm-derived components (25,34) and is consistent with mRNA in situ hybridization data (36). There is also considerable overlap between IAP expression (Fig. 1G) and TEX19.1 expression (Fig. 2D) with parietal trophoblast giant cells, spongiotrophoblasts and sinusoidal trophoblast giant cells expressing both of these proteins. Taken together, these data suggest that Tex19.1 is the only hypomethylation-sensitive germline genome-defence gene that is hypomethylated and expressed in the placenta.
Figure 2. *Tex19.1* is the only known germline genome-defence gene that is hypomethylated and expressed in the placenta. (A) Microarray expression data showing expression of the hypomethylation-sensitive genome-defence genes in E16 placenta and adult testis relative to E16 embryo for each gene. Error bars indicate standard error. *n* = 3. (B) qRT-PCR for expression of the hypomethylation-sensitive genome-defence genes in E18.5 placenta and embryo relative to adult testis. Error bars indicate standard error. *n* = 2. (C) Bisulphite sequencing of the promoters of the hypomethylation-sensitive genome-defence genes in two independent placental and embryonic DNA samples. Each line represents a sequenced clone, filled circles represent methylated CpGs and open circles represent unmethylated CpGs. The percentage of total methylation in each sample is indicated. (D) Immunohistochemistry (brown precipitate) with anti-TEX19.1 antibody on wild-type and *Tex19.1*<sup>−/−</sup> E18.5 placenta. Sections were counterstained with haematoxylin. Approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) are indicated. Scale bar, 500 μm.
**Tex19.1 is required for normal placental development**

We next investigated if Tex19.1 expression has any functional consequences for placental development. Defects in placental function would be expected to manifest as embryonic lethality and/or reduced embryo weight, depending on the severity of the defect. Tex19.1<sup>−/−</sup> embryos have been reported to be born at a sub-Mendelian frequency from heterozygous crosses (19,37), with lethality affecting females more strongly than males (37). The reduced viability of Tex19.1<sup>−/−</sup> embryos could reflect a function for Tex19.1 in the placenta, or in pluripotent cells, where it is also expressed. In contrast to previously published data (37), although loss of Tex19.1 was associated with embryonic lethality, this lethality did not appear to affect females more strongly than males, despite our analysis including almost 1000 genotyped pups (Supplementary Material, Table S1). However, we noticed that the requirement for Tex19.1 during embryonic development was much stronger when mothers are lactating and nursing a pre-existing litter during pregnancy (20% of Tex19.1<sup>−/−</sup> embryos die without concurrent lactation, 66% die with concurrent lactation) and that loss of Tex19.1 was affecting viability of female embryos more strongly than male embryos specifically in the concurrent pregnancies (Tex19.1<sup>−/−</sup> homozygotes born with a 1:1 male:female sex ratio without concurrent lactation, 4.7:1 male:female sex ratio with concurrent lactation) (Supplementary Material, Table S2). Concurrent lactation has been reported to affect the viability of embryos in a sex-specific manner in some mouse strains (38), and this phenomenon appears to be exacerbating the Tex19.1<sup>−/−</sup> embryonic lethality phenotype. Differences between breeding schedules, which significantly influence the magnitude and sex-specificity of the effects of Tex19.1<sup>−/−</sup> on embryonic lethality, are likely to account for differences between the data in Supplementary Material, Table S1 and previously published observations (37). Therefore, we performed subsequent analyses on pregnancies where mothers were not concurrently lactating to avoid this issue.

The reduced viability of Tex19.1<sup>−/−</sup> embryos could be indicative of defects in placenta function. Therefore, we assessed the size and weight of Tex19.1<sup>−/−</sup> placentas and embryos during development. Tex19.1<sup>−/−</sup> embryos dissected at E18.5 appeared visibly smaller than their heterozygous and wild-type littermates (Fig. 3A). We measured this effect by weighing E18.5 embryos and observed a 20% reduction (P < 0.01, Mann–Whitney U-test) in embryo weight in both male and female Tex19.1<sup>−/−</sup> embryos (Fig. 3C). Tex19.1<sup>−/−</sup> placentas are also smaller than wild-type and heterozygous littermates at E18.5: female and male Tex19.1<sup>−/−</sup> placentas are 26% (P < 0.01, Mann–Whitney U-test) and 30% (P < 0.01, Mann–Whitney U-test) lighter than littermate controls, respectively (Fig. 3A and D). Although Tex19.1<sup>−/−</sup> embryos are smaller than their littermates during embryonic development, male Tex19.1<sup>−/−</sup> animals reach normal weights during post-natal growth (Fig. 3E). In contrast, adult female Tex19.1<sup>−/−</sup> animals remain slightly smaller (8% reduction in weight, Mann–Whitney U-test, P < 0.01) than their littermate controls (Fig. 3B and E). However, the reduction in Tex19.1<sup>−/−</sup> female weight is less severe in adults than in embryos. Taken together, the reduction in placental weight and intra-uterine growth defect present in Tex19.1<sup>−/−</sup> embryos suggest that Tex19.1 is required for normal placenta function during development.

**Tex19.1<sup>−/−</sup> placentas have defects in the junctional zone and labyrinth**

We next examined whether any defects in Tex19.1<sup>−/−</sup> placental function might be caused by abnormalities in placenta structure or development. Histology of E18.5 placenta sections revealed that Tex19.1<sup>−/−</sup> placentas possess the main structural layers: decidua basalis, junctional zone and labyrinth (Fig. 4A). However, the junctional zone appears to be thinner in Tex19.1<sup>−/−</sup> placenta sections when compared with littermate controls. To further investigate any potential defects in the junctional zone, we used periodic-acid Shiff’s (PAS) stain to label the carbohydrate-rich junctional zone and decidua basalis layers (Fig. 4B) and measured the area of the junctional zone relative to the total placenta area in sections (Fig. 4E). This confirmed that there is a ~40% reduction in the amount of junctional zone in Tex19.1<sup>−/−</sup> placentas relative to littermate controls (P < 0.05, t-test).

The junctional zone comprises two main cell types, spongiotrophoblasts and glycogen trophoblasts, each of which is derived from the trophectoderm (34). To investigate whether loss of Tex19.1 affects one or both of these cell types in the junctional zone, we counted the number of glycogen trophoblasts and spongiotrophoblasts in histological sections of E18.5 placentas (Fig. 4C and F). Interestingly, the abundance of both spongiotrophoblasts and glycogen trophoblasts was reduced by 40% (P < 0.05, t-test) and 80% (P < 0.05, t-test), respectively in Tex19.1<sup>−/−</sup> placentas. Thus, the reduction in the thickness of the junctional zone in Tex19.1<sup>−/−</sup> placentas is presumably caused by reduced numbers of spongiotrophoblast and glycogen trophoblast cells in this tissue.

In addition to the histological defects in the junctional zone of Tex19.1<sup>−/−</sup> placentas, we also noticed a difference in the labyrinth layer, which appeared to have fewer sinusoidal trophoblast giant cells, another trophectoderm-derived cell type (34), than their littermate controls (Fig. 4D). Cell counts confirmed that this cell type is 40% (P < 0.05, t-test) less abundant in Tex19.1<sup>−/−</sup> placentas (Fig. 4F). Thus, loss of Tex19.1 affects trophectoderm-derived cells in both the junctional zone and labyrinth of the placenta.

As loss of Tex19.1 appears to be affecting multiple cell types in the placenta, we performed qRT-PCR for cell type markers to confirm and extend our histological analysis. Tek, a marker of extraembryonic mesoderm-derived endothelial tissues (39), does not show any statistically significant change in mRNA abundance in Tex19.1<sup>−/−</sup> placentas when compared with littermate controls (Fig. 5A). Similarly, mRNAs encoding markers of chorion-derived trophoblast cells in the labyrinth (Dlx3, Nrla1) (40), and markers of the two syncytiotrophoblast layers (Syna and Synb) (40), do not significantly change abundance in Tex19.1<sup>−/−</sup> placentas either (Fig. 5A). However, mRNA for the sinusoidal
trophoblast giant cell marker \textit{Ctsq} (40) is significantly depleted (≏4-fold, \(P < 0.05\), \(t\)-test) in \(Tex19.1^{2/2}\) placentas, and levels of \textit{Hand1} mRNA, a marker for trophoblast giant cells (40), were also reduced (≏2-fold, \(P < 0.05\), \(t\)-test) (Fig. 5A). This is consistent with our histology data showing that \(Tex19.1^{2/2}\) placentas have fewer sinusoidal trophoblast giant cells. Similarly, ≏2-fold reductions (\(P < 0.05\)) in the levels of junctional zone markers \([\text{Tpbpa}, \text{glycogen trophoblasts and spongiotrophoblasts} (41); \text{Pcdh12} \text{and} \text{Gjb3}, \text{glycogen trophoblasts} (41,42); \text{Prl8a8} \text{and} \text{Prl3a1}, \text{spongiotrophoblasts} (41)]\) in \(Tex19.1^{2/2}\) placentas are consistent with the reduced thickness of this layer and the reduction in the number of glycogen trophoblasts and spongiotrophoblasts we observed histologically.

We performed a more objective analysis of gene expression changes in \(Tex19.1^{2/2}\) placentas using Illumina WG-6v2.0 Beadchip microarrays to profile gene expression at a genome-wide level. Microarray analysis shows that mRNAs for only 2 genes increase in abundance by more than 3-fold in \(Tex19.1^{2/2}\) placentas (\(P < 0.01\)) and that mRNAs for 22 genes decrease in abundance by the same amount (\(P < 0.01\)) (Fig. 5B). A number of placenta-associated gene families, such as placental lactogen genes (\textit{Prl}), pregnancy-specific glycoprotein genes (\textit{Psg}), cathepsin genes (\textit{Cts}) and caudal homeobox genes (\textit{Cdx}) (41,43–45), had multiple genes whose mRNAs were at least 1.5-fold lower in \(Tex19.1^{2/2}\) placentas (\(P < 0.05\)) [microarray data are available in GEO repository (46) accession GSE41823]. Expression levels of seven placental lactogen genes (\textit{Prl7c1}, \textit{Prl4a1}, \textit{Prl5a1}, \textit{Prl7b1}, \textit{Prl8a6} and \textit{Prl8a9}), six pregnancy-specific glycoprotein genes (\textit{Psg17}, \textit{Psg18}, \textit{Psg19}, \textit{Psg23}, \textit{Psg25} and \textit{Psg29}), three cathepsin genes (\textit{Ctsm}, \textit{Cts3}, \textit{Cts6}) and two caudal homeobox genes (\textit{Cdx1} and \textit{Cdx2}) were all reduced in the \(Tex19.1^{2/2}\) placentas by these criteria. We verified by qRT-PCR that mRNAs for candidate genes belonging to each of these families are less abundant in \(Tex19.1^{2/2}\) placentas than littermate controls (\(P < 0.05\), \(t\)-test) (Fig. 5C). Taken together, the microarray and qRT-PCR data are consistent with our histological analyses and show that the amount of junctional zone in the placenta and the abundance of specific trophoblast cell types are reduced in the absence of \textit{Tex19.1}. 

Figure 3. \(Tex19.1^{2/2}\) embryos have small placentas and intrauterine growth retardation. (A) Photograph of E18.5 embryos and their placentas in a single litter from a cross between \(Tex19.1^{+/+}\) heterozygotes. The \(Tex19.1\) genotype is indicated. Scale bar, 500 μm. (B) Photograph of 6–8 week old female adult mice. The \(Tex19.1\) genotype is indicated. (C–E) Boxplots showing the weights of littermate E18.5 embryos (C), E18.5 placentas (D) and adult animals (E) derived from heterozygous crosses, grouped according to sex and \(Tex19.1\) genotype. * indicates \(P < 0.01\) (Mann–Whitney \(U\)-test).
A number of imprinted genes are important for placenta development and function, and some imprinted genes have different requirements for their silencing in hypomethylated placental tissue than in the embryo-derived somatic cells (47,48). Defective silencing of the imprinted allele of imprinted genes would result in a gene expression change that falls below the 3-fold cutoff used in the microarray analysis in Figure 5B, but these changes can be sufficient to cause a placenta phenotype (49,50). However, even in the absence of a fold-change threshold, imprinted genes are no more likely to be differentially expressed in Tex19.1−/− placentas than non-imprinted genes (Supplementary Material, Fig. S2). Although we cannot exclude the possibility that differences in cell composition between Tex19.1−/− and control placentas are masking the detection of derepression of imprinted genes, loss of Tex19.1 does not appear to be causing widespread defects in imprinted gene silencing in the placenta.

Figure 4. Tex19.1−/− placentas have reduced numbers of spongiotrophoblasts, glycogen trophoblasts and sinusoidal trophoblast giant cells. (A) Haematoxylin and eosin staining of E18.5 Tex19.1+/+, Tex19.1−/+ and Tex19.1−/− placenta sections. The approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) layers are indicated. Scale bar, 500 μm. (B) PAS staining of E18.5 placenta sections highlighting the carbohydrate-rich decidua basalis and junctional zone layers in the placenta. Scale bar, 500 μm. (C and D) High magnification images of placenta sections stained with PAS and haematoxylin. Regions of the junctional zone (C) and labyrinth (D) are shown. Scale bars, 50 μm. (E) Contribution of the junctional zone to the placenta in PAS-stained sections. Error bars indicate standard error, * indicates P < 0.05 (t-test). (F) Abundance of trophoblast cell types in Tex19.1−/− and control littermate placenta sections. n = 3, error bars indicate standard error, * indicates P < 0.05 (t-test). spT, spongiotrophoblast; glyT, glycogen trophoblast; S-TGC, sinusoidal trophoblast giant cell.
We have previously shown that $Tex19.1^{+/+}$ represses the LTR retrotransposon $MMERVK10C$ in spermatocytes (19). However, any derepression of retrotransposon expression in $Tex19.1^{+/+}$ placentas would not be detected in the gene expression microarray analysis as any retrotransposon probes in the microarray were excluded from the gene expression analysis. Therefore, we re-analyzed the $Tex19.1^{+/+}$ placenta microarray data using our recently developed microarray repeat-annotation technique (10) and found that a number of microarray probes corresponding to the LINE and LTR classes of retrotransposon are upregulated between 2- and
4-fold in Tex19.1\(^{-/-}\) placenta relative to littermate controls. Interestingly, 11 of the 13 retrotransposon probes that are upregulated more than 2-fold in Tex19.1\(^{-/-}\) placenta (P < 0.01) correspond to LINE-1 elements (Fig. 6A), one of the most abundant retrotransposons in the mouse genome (2). An LTR retrotransposon probe corresponding to MMVL30 LTR retrotransposons is also among the upregulated retrotransposon probes in Tex19.1\(^{-/-}\) placenta (Fig. 6A).

The large number of significantly upregulated LINE-1 probes in the Tex19.1\(^{-/-}\) placenta microarray data prompted us to look more closely at LINE-1 expression in this tissue. Overall, LINE-1 probes were differentially distributed relative to non-repeat probes within the microarray dataset (Mann–Whitney U-test, P < 0.01), and a subset of LINE-1 probes showed a ~2-fold upregulation in Tex19.1\(^{-/-}\) placenta relative to littermate controls (Fig. 6B). However, some LINE-1 probes appear to be more strongly affected by loss of Tex19.1 than others (Fig. 6B). We verified the microarray repeat-annotation data by performing qRT-PCR on Tex19.1\(^{-/-}\) placenta and littermate controls. Consistent with the microarray data, MMERVK10C and IAP retrotransposons are not upregulated in Tex19.1\(^{-/-}\) placenta by qRT-PCR (Fig. 6C); however, MMVL30 retrotransposons are modestly upregulated ~1.7-fold (t-test, P < 0.05). Generic primers to the ORF2 region of LINE-1 and primers designed to detect the copies of LINE-1 that correspond to the upregulated microarray probes, all show a modest but statistically significant ~1.6–2-fold (P < 0.05) upregulation in Tex19.1\(^{-/-}\) placenta by qRT-PCR (Fig. 6C). We confirmed that the upregulation of LINE-1 mRNA in Tex19.1\(^{-/-}\) placenta that we detected by microarray and qRT-PCR represents a bona fide increase in LINE-1 element expression by western blotting for LINE-1 ORF1p (Fig. 6D). Tex19.1\(^{-/-}\) placenta showed a modest, but consistent increase in LINE-1 ORF1p protein abundance commensurate with the ~2-fold increase in LINE-1 mRNA levels detected by qRT-PCR and microarray (Fig. 6D).

The increase in LINE-1 mRNA and protein abundance that occurs in Tex19.1\(^{-/-}\) placenta could be caused by the difference in cell composition between wild-type and knockout placenta, or by upregulation of LINE-1 in the trophoblast cell types that normally express Tex19.1. Immunohistochemistry for LINE-1 ORF1p showed that LINE-1 is primarily expressed in the junctional zone trophoblast cells in both Tex19.1\(^{-/-}\) and control littermate placenta, with some low level expression also detectable in the trophoblast giant cells in the labyrinth (Fig. 6E). The cell types primarily expressing LINE-1 in Tex19.1\(^{-/-}\) placenta are, therefore, the trophobectoderm-derived cell types that normally express Tex19.1. Furthermore, as there are fewer junctional zone trophoblast cells in Tex19.1\(^{-/-}\) placenta (Fig. 4), the ~2-fold increases in LINE-1 expression observed by qRT-PCR and western blotting likely underestimate the increase in LINE-1 expression that occurs in the junctional zone cells that remain in Tex19.1\(^{-/-}\) placenta (Fig. 6D and E). Taken together, these data suggest that expression of the genome-defence gene Tex19.1 is linked to the epigenetic state of the placenta and suppresses LINE-1 retrotransposons in the hypomethylated trophoblast-derived cells in this tissue.

**DISCUSSION**

Suppressing retrotransposons in the hypomethylated placenta

The mammalian placenta has been associated with retrotransposon expression in a number of studies (reviewed in 29), and retrotransposon-derived proteins have even been co-opted to fulfil placental functions on multiple occasions during mammalian evolution (51–54). However, our microarray analysis of retrotransposon expression suggests that, although a number of retrotransposons are differentially expressed between placenta and embryo, the murine placenta is in general no more permissive for retrotransposon expression than the rest of the embryo. This contrasts strongly with, for example, ES cells knocked down for the histone methyltransferase Eset that show widespread upregulation of many different types of retrotransposon in microarray repeat annotation (10). Studies assessing the behaviour of specific types of retrotransposons in the human placenta also suggest that the association between retrotransposon expression and the placenta reflects expression of a subset of hypomethylated genomic copies of these elements (55). Thus, the strong associations between retrotransposon expression and the placenta might represent expression of specific types of retrotransposon, or even expression of specific genomic copies of a retrotransposon, rather than a widespread general activation of retrotransposon expression in this tissue.

Multiple retrotransposon families and classes have been reported to be DNA hypomethylated in the placenta relative to the embryo (25,27,28). At least for LINE-1, this hypomethylation is present in trophobectoderm-derived rather than extraembryonic mesoderm-derived components of the placenta (25). IAP elements appear to be repressed by DNA methylation in multiple embryo-derived cell types (11,30,31) and is one of the hypomethylation-sensitive genes strongly activated in mouse somatic cells in multiple models of DNA hypomethylation (17). Thus, although the hypomethylated state of the placenta does not appear to cause widespread derepression of retrotransposons in general, the derepression of IAP elements in the placenta likely represents a direct consequence of its hypomethylated state. A number of other retrotransposons that have been shown to be repressed by DNA methylation in ES cells or germ cells, including LINE-1 (9,10,33), do not appear to be strongly upregulated in the placenta relative to the embryo. However, it is still the hypomethylated derivatives of the trophobectoderm that are the primary source of LINE-1 ORF1p expression in this tissue. Thus, although differences in transcription factor expression, and/or differences in additional retrotransposon suppression mechanisms, could all contribute to the differential sensitivity of retrotransposons to DNA hypomethylation between placenta and embryo, the hypomethylated epigenetic state of the trophobectoderm derivatives within the placenta does appear to make these cell types vulnerable to retrotransposon activity.

We have recently shown that the DNA hypomethylation that occurs in developing primordial germ cells induces expression of a cohort of germline genome-defence genes that can act to protect the genome from the activity of any retrotransposons that might be derepressed during this period of
epigenetic reprogramming (17). Interestingly, although this cohort of genes all become hypomethylated and expressed during the extensive epigenetic reprogramming event that occurs in the developing germline, only Tex19.1 is hypomethylated and expressed in the placenta. DNA hypomethylation in developing germ cells has been proposed to occur through multiple active mechanisms (27,56), whereas DNA hypomethylation in the placenta might be a consequence of reduced de novo DNA methylation in trophectoderm-derived cells (57,58), possibly in combination with additional events. Differences between the molecular mechanisms responsible for DNA hypomethylation could potentially account for the differences in genome-defence gene promoter methylation between the placenta and the germline. However, our finding that Tex19.1 functions to repress retrotransposons in the placenta, where the remaining genome-defence genes are not expressed, suggests that at least some genome-defence genes are able to function independently of each other, possibly in a modular manner. Deploying a group of genome-defence genes in hypomethylated germ cells that are each independently able to target multiple retrotransposons at different stages of the retrotransposon life cycle would be expected to provide an effective multi-layered defence against these elements.

**Tex19.1 function in the placenta**

Our study shows that Tex19.1 is required for normal placenta development and function. In the absence of Tex19.1, trophectoderm-derived spongiotrophoblasts, glyco- gen trophoblasts and sinusoidal trophoblast giant cells are all reduced in abundance, or have altered gene expression patterns in the placenta. TEX19.1 protein is expressed in the trophoblast giant cells, extraembryonic ectoderm and chorion at E6.5 and E7.5 (17), and the defects that we describe here
in E18.5 placenta may arise from defects at much earlier stages of placenta development. We have previously shown that Tex19.1 plays a role in suppressing a specific LTR retrotransposon, MMERVK10C, in the developing germline, but has no effect on the abundance of LINE-1 mRNA in that tissue (10,19). Our findings here extend the range of retrotransposons that this genome-defence mechanism is targeting and highlight the complex interplay that is operating between genome-defence mechanisms in different tissues. The insensitivity of LINE-1 to the loss of Tex19.1 in developing germ cells presumably reflects the existence of multiple overlapping defence mechanisms operating against this element in the germline. Analysis of retrotransposon mRNAs might be a relatively indirect approach to detect the activity of Tex19.1, and the increase in retrotransposon mRNA that we have identified in the placenta (this study) and in the testis (19) could reflect loss of a genome-defence mechanism operating at any point in a retrotransposon life cycle as increased flux through a retrotransposon life cycle will generate an increase in the genomic copy number of that element that can then produce more retrotransposon mRNA. Differences in the types of retrotransposon derepressed in Tex19.1−/− testes and Tex19.1−/− placentas may simply reflect the presence or absence of mechanisms that are operating alongside Tex19.1 to suppress different retrotransposons at different stages of their life cycle in each of these tissues.

The increase in LINE-1 retrotransposon mRNA that is present in Tex19.1−/− placentas has the potential to cause increased retrotransposition and could also reflect increased LINE-1 retrotransposition that has already occurred in this tissue. Emerging technologies that identify and map de novo LINE-1 retrotransposition events in human tissue (59,60) might allow the extent of any de novo LINE-1 retrotransposition in these mouse tissues to be assessed in future studies. As Tex19.1 is expressed from early stages of placental development, there is plenty of opportunity for any increased LINE-1 expression in these mutant tissues to cause de novo retrotransposition and for these de novo retrotransposition events to accumulate and cause genetic instability. We have not yet determined whether the reduction in placental cell types and changes in mRNA abundance that we describe in Tex19.1−/− placentas are caused by increased apoptosis, decreased proliferation or impaired differentiation. In addition, although Tex19.1 has a role in suppressing retrotransposons in the placenta, we cannot exclude the possibility that Tex19.1 also has a role in modulating other aspects of cell function and that the placental defects in Tex19.1−/− mice are not directly caused by an increase in retrotransposition. However, it is also possible that the defects that we observe in Tex19.1−/− placentas are a direct consequence of increased retrotransposon activity causing high levels of insertional mutagenesis. As some combinations of de novo LINE-1 integration events are likely to be more deleterious than others, the somatic variation between cells in Tex19.1−/− placentas could result in sporadic occurrences of cell death, proliferation defects or developmental abnormality in trophoblast-derived cell types throughout placenta development. LINE-1 retrotransposition has been proposed to generate somatic variation in human neural tissue (61) and to drive tumourigenesis in some cases of human colorectal cancer (60). Thus, although a small amount of LINE-1 retrotransposition has been proposed to be important for generating somatic variation and normal brain function, too much retrotransposition is likely to be deleterious. It would appear that mutating Tex19.1 might be sufficient to this balance in the developing placenta.

The exacerbating sex-biased effect that concurrent lactation has on Tex19.1−/− embryonic lethality is intriguing, but the molecular basis for this phenomenon is not clear at present. The preferential loss of XX Tex19.1−/− embryos during concurrent lactation could indicate defects in X-inactivation under these conditions. However, the Tex19.1−/− placental phenotype that we describe in this work occurs in both XX and XY placentas suggesting that Tex19.1 is not required for X-inactivation in the absence of concurrent lactation and that X-inactivation defects are not causing the placental phenotype characterized here. In addition, there is no evidence for activation of X-linked genes in the microarray gene expression profiles of XX Tex19.1−/− placentas (Supplementary Material, Fig. S3). Interestingly, there are differences in gene expression between XX and XY placentas, and gene expression in XX placentas is more sensitive to changes in maternal diet than gene expression in XY placentas (62,63). Perhaps the increased nutritional demand of concurrent pregnancy and lactation is similarly altering placenta gene expression, and these changes could be increasing the requirement for Tex19.1 function in this tissue. Given the findings presented here, it would be of interest to determine whether retrotransposon expression or activity is altered in the placenta during concurrent lactation and pregnancy.

Although placenta development is compromised in the absence of Tex19.1, Tex19.1 does not appear to be absolutely required for development or survival of any specific terminally differentiated placenta cell type. Nevertheless, Tex19.1−/− embryos exhibit intra-uterine growth retardation suggesting that loss of Tex19.1 impairs placenta function. The reduction in the number of trophoblast cells in Tex19.1−/− placentas is likely to result in lower amounts of some placenta-derived hormones entering the maternal circulatory system and reduced communication across the maternal–fetal interface. mRNAs encoding a number of secreted hormones such as placental lactogens, which are secreted into the maternal blood to influence maternal metabolism (41), and pregnancy-specific glycoproteins, one of the most abundant group of fetal-derived proteins present in the maternal blood during pregnancy (44), are all reduced in abundance in Tex19.1−/− placentas. Pregnancy-specific glycoproteins have been proposed to have a role in modulating maternal immune responses during pregnancy, possibly through inducing expression of anti-inflammatory cytokines (44). It is, therefore, noteworthy that the two genes whose mRNAs were more than 3-fold higher in Tex19.1−/− placentas, Npy and Ceacam1, have been reported to be upregulated in response to inflammation and to promote angiogenesis (64,65). Reduced levels of placental lactogens and pregnancy-specific glycoproteins in the maternal blood have both been reported to be associated with impaired placenta function and conditions such as intra-uterine growth retardation and pre-eclampsia in humans (66). The placental defects in Tex19.1−/− mice, therefore, raise the intriguing possibility that deregulation of retrotransposons could contribute to impaired placenta function in humans.

The placental defects in Tex19.1−/− placentas has the potential to cause increased retrotransposition that has already occurred in this tissue
In this respect, the generally hypomethylated epigenetic state of the placenta might make retrotransposons in this tissue particularly sensitive to any additional epigenetic disruption or sequence variation that would remove another layer of suppression from these elements. Although there are a number of differences between the retrotransposons present in human and mouse genomes (1,2), and presumably also between genome-defence mechanisms operating between these species, it is possible that imbalances between retrotransposon suppression and genome-defence mechanisms might contribute to placenta dysfunction and disease in both mice and humans.

MATERIALS AND METHODS

Animals

The Tex19.1 mutation used in this study is a complete replacement of the Tex19.1 open reading frame (19), back-crossed three times to C57BL/6 mice (Charles River). For timed matings, noon on the day the vaginal plug was found was termed E0.5. Genotyping was performed essentially as described (19). All animal experiments were performed in concordance with local ethical guidelines and national regulations under the authority of UK Home Office Project Licence PPL 60/3785.

qRT-PCR

RNA was extracted from tissues using TRIzol (Invitrogen), DNase-treated and used as a template for random-primed cDNA synthesis with Superscript III (Invitrogen). Quantitative PCR was performed using this cDNA as a template, primers listed in Supplementary Material, Table S3, Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) and a CFX96 Real-Time PCR Detection System (Bio-Rad). Quantification was performed relative to a standard curve, and β-actin was used to normalize gene expression between samples. Data were tested for statistical significance using a two-tailed t-test. Six Tex19.1−/− placentas and six Tex19.1+/− or Tex19.1+/+ control littermate placentas were used for qRT-PCR assays to investigate changes in mRNA abundance in Tex19.1−/− placentas (four XX and two XY for each group).

Bisulphite sequencing

Genomic DNA was extracted from tissues by SDS/proteinase K lysis and ethanol precipitation. Five hundred nanograms of genomic DNA were bisulphite treated with the EZ DNA Methylation Gold kit (Zymo Research), then used as a template for nested PCR using primers listed in Supplementary Material, Table S3. PCR products were gel purified using a Nucleospin Extract II kit (Macherey-Nagel) and cloned into pGEM-T Easy (Promega). Plasmid DNA from individual colonies was isolated, sequenced using the SP6 sequencing primer, and the methylation status of each CpG in the plasmid insert was determined and plotted using QUMA (67). Sequences were excluded from the analysis, if the CpH conversion rate was less than 95%, or if identical sequences were already present in the sample.

Microarray

RNA was isolated from six Tex19.1−/− and six Tex19.1+/− or Tex19.1+/+ control littermate placentas (four XX and two XY for each group) using TRIzol (Invitrogen) and biotin-labelled using the Illumina TotalPrep RNA Amplification Kit (Ambion). RNA quality was assessed using an Agilent Bioanalyzer (RNA Integrity Number ≥8) and labelled RNA hybridized to Illumina Mouse WG-6 v2.0 Expression Beadchips. For analysis of gene expression in this dataset, the raw sample probe and control probe data from Illumina Beadstudio were read into R (68), background-adjusted (force Positive method), log-transformed and quantile-normalized using limma (69). Poorly performing probes were removed from the dataset as described (70), and probe level data summarized to gene level using limma. Differences between Tex19.1−/− and control littermate datasets were identified by linear modelling with limma (71), using false discovery rate-adjusted P-values to determine statistical significance. The Tex19.1−/− placenta microarray data are available in the GEO repository (46), accession GSE41823. Gene expression analysis of publicly available multiple-tissue microarray data (32) (GEO accession GSE9954) was performed similarly, except that the rna method in affy (72) rather than limma was used for pre-processing. Probe level repeat-annotation of microarray data (GEO accessions GSE9954 and GSE41823) was performed as previously described (10).

Histology and immunohistochemistry

Tissue was fixed with freshly depolymerized 4% paraformaldehyde in PBS at 4°C overnight, washed with PBS, then dehydrated through ethanol, xylene and embedded in paraffin wax. Microtome sections (6 µm) were collected on glass slides, dewaxed with xylene and re-hydrated through ethanol to dH2O. For histology, sections were then stained with either haematoxylin and eosin, PAS or PAS and haematoxylin. For immunohistochemistry, antigen retrieval was performed by boiling the slides in 0.1 M citrate buffer pH 6.0 in a microwave for 15–20 min. Sections were treated with 3% H2O2 in methanol for 30 min, blocked with PBS containing 10% goat serum and 0.1% Tween, then incubated with primary antibodies [rabbit anti-Tex19.1 (19), 1:300; rabbit anti-IAP gag, 1:300 (73); rabbit anti-LINE-1 ORF1p, 1:500 (74)] diluted in blocking buffer at 4°C overnight. Sections were washed with PBS, bound primary antibody was detected using the Envision HRP Rabbit DAB+ system (Dako) and sections were counterstained with haematoxylin. Stained sections were dehydrated through ethanol and xylene, mounted with DPX mounting media (CellPath) and photographed using an Olympus BX51 upright or Zeiss Axioplan 2 microscope equipped with a digital camera.

For measurement of the contribution of junctional zone to the placenta, shapes were drawn around the junctional zone and the entire placenta on digital images of PAS-stained sections for each placenta, and areas calculated using the Olympus dotSlide system. For measurement of cell type
abundance, three separate rectangular segments were drawn on digital images of PAS and haematoxylin-stained sections of each placenta, with each segment spanning the entire width of the placenta section. The numbers of spongiotrophoblast, glycogen trophoblast and sinusoidal trophoblast cells in each segment were counted, and the area of the placenta within each segment was calculated using the Olympus dotSlide system.

Western blotting
Placenta was homogenized in Laemmli sample buffer (60 mm Tris pH 6.8, 2% SDS, 10% glycerol, 0.1 m dithiothreitol and 0.01% bromophenol blue), boiled for 5 min and then sonicated to disrupt genomic DNA. Western blotting was performed using an Invitrogen precast gel system according to the manufacturer’s instructions. Rabbit anti-TEX19.1 polyclonal antibodies (Abcam) at 1:5000 and rabbit anti-LINE-1 ORF1p (74) at 1:2000. Bound primary antibodies were detected with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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