Rex1/Zfp42 as an epigenetic regulator for genomic imprinting

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Zfp42/Rex1 (reduced expression gene 1) is a well-known stem-cell marker that has been duplicated from YY1 in the eutherian lineage. In the current study, we characterized the in vivo roles of Rex1 using a mutant mouse line disrupting its transcription. In contrast to the ubiquitous expression of YY1, Rex1 is expressed only during spermatogenesis and early embryogenesis and also in a very limited area of the placenta. Yet, the gene dosage of Rex1 is very critical for the survival of the late-stage embryos and neonates. This delayed phenotypic consequence suggests potential roles for Rex1 in establishing and maintaining unknown epigenetic modifications. Consistently, Rex1-null blastocysts display hypermethylation in the differentially methylated regions (DMRs) of Peg3 and Gnas imprinted domains, which are known to contain YY1 binding sites. Further analyses confirmed in vivo binding of Rex1 only to the unmethylated allele of these two regions. Thus, Rex1 may function as a protector for these DMRs against DNA methylation. Overall, the functional connection of Rex1 to genomic imprinting represents another case where newly made genes have co-evolved with lineage-specific phenomena.

INTRODUCTION

Rex1 (reduced expression 1) or Zfp42 is a well-known stem-cell marker, which was originally identified due to a dramatic decrease in its expression levels during the differentiation of the mouse F9 teratocarcinoma cells by retinoic acid (1). Since then, the expression of this gene has been observed mainly in many different types of stem cells, including embryonic and adult stem cells (2). The expression levels of Rex1 are very high in the preimplantation-stage embryos, and modest levels of expression are also detected during spermatogenesis and in the placenta (3). According to several previous and recent genome-wide chromatin immunoprecipitation (ChIP) studies, Rex1 is indeed controlled by the master gene set of stem cells, Oct3/4, Sox2 and Nanog (4–7).

Rex1 encodes a 37 kDa zinc finger protein, which functions as a DNA-binding transcription factor. The protein sequence of Rex1 shares high levels of sequence identity with another C2H2 zinc finger protein YY1, indicating these two genes are evolutionarily related to each other. Despite the sequence similarity, however, the two genes display quite different exon structures: the entire protein-coding region of Rex1 is localized within a single exon, whereas the coding region of YY1 is split into five individual exons. Furthermore, Rex1 is found only in placental mammals in contrast to the evolutionary conservation of YY1 in all vertebrates and even in flying insects. This suggests the duplication of Rex1 from YY1 via retroposition during mammalian evolution (8). Also, the recent formation of Rex1 within eutherian mammals further posits that Rex1 may have co-evolved with some unknown mammal-specific genes or phenomena.

Recently, the mouse Rex1 gene has been mutated through knockout (KO) experiments to test its potential contribution to the self-renewal and pluripotency functions of the embryonic stem (ES) cells (9,10). Despite the high expectation, mutant mice lacking Rex1 were viable and fertile, suggesting that Rex1 is dispensable for the core functions of the ES cells. According to the results of breeding experiments, however, about half of both homozygous (Rex1−/−) and heterozygous (Rex1−/+ or +/−) mice were shown to die during the late gestation and neonatal stages (9). This result indicates that although Rex1 is expressed in the early stages of development, the actual contributions by Rex1 are required for the survival of the later-stage embryos or neonates.

Consistent with the predictions described above, recent genome-wide cDNA expression analyses indeed identified

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genes with mammal specificity as potential Rex1-downstream genes based on their up- or down-regulation in Rex1-null ES cells (9,10). The list of these genes includes several members of the keratin family (Krt7, Krt8, Krt9, Krt18, Krt19) and imprinted genes (Gtl2, Tsix, Xist, Igf2, Peg10). Among these potential downstream genes, imprinted genes are regarded as a set of candidate genes with special interest since genomic imprinting is found only within mammals and also involves epigenetic modifications, such as DNA methylation and histone modifications (11,12). To test whether Rex1 is involved in the epigenetic control of these imprinted genes, we have carried out a series of experiments with a mutant mouse line targeting Rex1 in the current study. According to the results, Rex1 is indeed required for maintaining the allele-specific DNA methylation of the Peg3 and Gnas domains. Also, the other data agree with our initial prediction that Rex1 may have co-evolved with mammal-specific genes and phenomena.

RESULTS
Generation of a mutant mouse line targeting Rex1
To characterize the in vivo functions of Rex1, we have generated a mutant mouse line with one gene trap ES clone (XB238; http://baygenomics.ucsf.edu/overview/welcome.html). After we established this mutant line, we first characterized the insertion position of the gene trap vector within a genomic contig of Mmu8 (GenBank accession no. NC_000074). (B, C) A litter of 18.5 dpc embryos were genotyped and also analyzed with RT-PCR using total RNA from their corresponding placenta. The PCR products derived from P1–P3 and P2–P3 primer sets represent a wild-type (275 bp) and knockin (500 bp) allele, respectively. (D) The protein extracts prepared from the placenta were analyzed using western blotting with polyclonal anti-Rex1 and β-Actin antibodies.

Figure 1. A knockin allele disrupting mouse Rex1. (A) The genomic locus of mouse Rex1 is indicated by exons (rectangles) and introns (lines). The gray and black rectangles indicate the exons and open reading frame, respectively. The gene trap vector (β-Geo) has been inserted into the third intron, and three primers (arrows) were used for genotyping. The numbers indicate the exact insertion position of the gene trap vector within a genomic contig of Mmu8 (GenBank accession no. NC_000074). (B, C) A litter of 18.5 dpc embryos were genotyped and also analyzed with RT-PCR using total RNA from their corresponding placenta. The PCR products derived from P1–P3 and P2–P3 primer sets represent a wild-type (275 bp) and knockin (500 bp) allele, respectively. (D) The protein extracts prepared from the placenta were analyzed using western blotting with polyclonal anti-Rex1 and β-Actin antibodies.
three primers that can be used for genotyping the embryos derived from the breeding of this mutant line. As shown in Figure 1B, the 18-day embryos from the crossing of two heterozygotes [female (+/−) × male (+/−)] showed all different categories of genotypes [wild-type (+/+), heterozygotes (+/−) and homozygotes (−/−)]. To test the truncation of Rex1 transcription by the β-Geo vector, we also performed reverse transcriptase–polymerase chain reaction (RT–PCR) assays using the total RNA isolated from the placenta since Rex1 is expressed in the placenta (3). According to the results from this survey (Fig. 1C), the placenta of the wild-type embryos displayed the highest levels of expression, that of the heterozygotes showed the second highest levels and that of the homozygotes showed no expression of Rex1. We also confirmed this through western blotting (Fig. 1D). This survey confirmed the proper truncation of Rex1 transcription by the gene trap vector (β-Geo).

### Rex1 gene dosage is critical for the survival of the embryos and neonates

We have performed two series of breeding experiments to test potential Rex1 roles for the normal development and survival of the mouse. First, we performed the following three breeding experiments: male or female heterozygotes with their littermates and an intercrossing between two heterozygotes (Table 1). The results revealed that the litter sizes (about 6) of these breeding were consistently smaller than those arising in control breedings between two normal littermates (average 9.6). Also, in litters resulting from intercrosses between two heterozygotes, some fractions of the homozygotes and heterozygotes had died during the gestation period based on the deviation from the expected Mendelian ratio (−/−:−/+:+/+ ratio of 27:57:35 versus 1:2:1). A similar observation was also been reported from another KO model targeting Rex1 (9). It is interesting to note that the number of homozygous males is smaller than homozygous females (9 to 18). Second, this initial result was followed up through performing a more detailed series of breeding experiments (Table 2). Overall, in these breedings, litter sizes correlated well with the combined gene dosage of Rex1 in the parents. Specifically, the loss of one Rex1 allele in either parent resulted in similar reduction from an average of 9.6 to 5.2 for F(+/−) × M(+/+) and to 5.9 for F(+/) × M(−/−). The loss of two alleles caused further reduction with some variations: 6.8 for F(+/−) × M(+/+), 6.3 for F(−/−) × M(+/+) and 4.7 for F(+/+ × M(−/−)). The most dramatic reduction was observed in the breeding with male homozygotes, 4.7 for F(+/+ × M(−/−)), suggesting that Rex1 dosage is more critical during spermatogenesis than during oogenesis. This result is consistent with the fact that Rex1 expression is mainly detected in spermatogenesis (3). Further reduction in the litter size was observed in breeding pairs with the combined loss of gene dosage 3 or 4 within the two parents. In summary, the observed gradual decrease in the litter size suggests that Rex1 may play critical roles for the survival of the mouse at multiple stages of germ cell and embryo development.

### Spatial expression profiles of Rex1 in the testis and placenta

As the Rex1 gene locus in the mutant line has been targeted by the promoterless gene trap vector (β-Geo), we took advantage of the β-Geo reporter system for analyzing the spatial expression profiles of mouse Rex1 (Fig. 2). First, we performed β-Gal staining with cryo-sectioned testes that were harvested from Rex1(−/−). Rex1 expression was mainly detected in all layers of spermatogonia, which were located in the outer edge of seminiferous tubules. In contrast, Rex1 expression was not detected at all in spermatids and mature sperm that were located in the inner layer of the tubules (Fig. 2A). Second, we also repeated β-Gal staining with 10.5 dpc conceptuses that were derived from the crossing

### Table 1. Genotype analysis of offspring from several crosses of Rex1 mutants

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<tr>
<th>Cross</th>
<th>Gender</th>
<th>−/−</th>
<th>+/−</th>
<th>Total no.</th>
<th>Litters</th>
<th>Average litter size</th>
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<td></td>
<td>F</td>
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<td>F</td>
<td>38</td>
<td>38</td>
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### Table 2. Average litter sizes from all available crosses with Rex1 mutants

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<th>Average litter size</th>
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<td>129</td>
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<td>19</td>
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<td>13</td>
<td>4.3</td>
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</tbody>
</table>

*Each t-test compares against wild-type cross.
Expression level change in the Rex1-null embryos

As a potential DNA-binding transcription factor, the in vivo roles of Rex1 are likely manifested in the functions of its downstream genes that are presumably controlled by Rex1. As an initial step for identifying these Rex1 target genes, we examined two independent sets of the cDNA expression analysis results derived from Rex1-null ES cells (9,10). Both studies derived a similar conclusion that only a handful of genes were affected in their expression levels by the Rex1-null mutation. Nevertheless, two groups of mouse genes were immediately noticeable due to their over-representation within this small-size affected gene pool: imprinted genes and Keratin (Krt) gene family members. To test whether these genes are indeed controlled by Rex1, we performed RT–PCR analyses using total RNA isolated from the two groups of blastocyst-stage embryos: normal (+/+) and null (−/−) embryos derived from the cross between F(+/+) × M(+/+) and between F(−/−) × M(−/−), respectively (Fig. 3). We grouped 50–60 blastocysts as one pool for each trial and repeated three different trials.

Among the genes analyzed, the following genes showed consistently some differences in their expression levels between the two groups of blastocysts. First, both Peg3 and Igf2 displayed up-regulation in their expression levels in the Rex1-null embryos, whereas Nespas showed down-regulation. However, changes in other imprinted genes, for instance, H19 and Gtl2, were either marginal or not reproducible. Second, the three members of the Krt family displayed up-regulation in the Rex1-null embryos: Krt8, 18 and 19. Beside these two groups of genes, we also detected changes in the expression levels of two retrotransposon families: up-regulation for MUERV (Mus Endogenous RetroVirus) and down-regulation for IAP (intracisternal A-particle). It is interesting to note that YY1 was originally identified as a binding factor to IAP (13). Among the genes involved in epigenetic modifications, we also observed slight up-regulation of one of the DNMT family members, DNMT1, but the functional significance of this change remains to be investigated. Overall, the changes in the expression levels of the two groups of genes, some imprinted genes and Krt members, appear to be reproducible and consistent with those seen in the other previous studies (9,10), suggesting that these genes are most likely the downstream genes of Rex1.

In vivo binding of REX1 to several imprinting control regions of imprinted domains

To further follow up the above results, we performed a series of ChIP experiments using a polyclonal antibody raised against the 100-amino acid-long peptide derived from the N-terminal portion of the mouse Rex1 protein (10). As Rex1 is expressed in ES cells and placenta, two sets of the cross-linked chromatin prepared from ES cells and placenta were used to assay in vivo binding of REX1 to potential
downstream genes (Fig. 4A). In ES cells, we were able to confirm the in vivo binding of REX1 to Peg3 and Tsix, but REX1 binding to Nr3c1 (glucocorticoid receptor), Nespas and Xist was inconclusive due to relatively low levels of the enrichment compared with the negative control (Fig. 4A, IgG lane). This was further confirmed through a quantitative PCR approach, revealing that the highest levels of the enrichment were detected at Tsix (Fig. 4B). In the placenta, we were able to confirm the in vivo binding of REX1 to Peg3, Nespas and Nr3c1. Each of these regions is known to have an unusual cluster of YY1 binding sites (14). In fact, similar ChIP experiments have already demonstrated that these regions are indeed in vivo binding sites of YY1 (14). This implies that Rex1 may share its DNA-binding sites with YY1 for these genes. Consistent with this, many genes with YY1 binding sites were shown to be bound by REX1 according to the results derived from genome-wide ChIP analyses using ES cells (7). The list of these REX1-bound genes indeed includes Peg3 and Nespas, which is also in a good agreement with our own ChIP results.

Two of the REX1- and YY1-bound loci, Peg3 and Nespas, are imprinted genes that display differential methylation between maternal and paternal alleles, and yet YY1 is known to bind to only the unmethylated, paternal alleles of these genes (14,15). Thus, we also tested potential allele-specific binding of REX1 to these genes (Fig. 4C). We performed bisulfite conversion-based DNA methylation analyses on the REX1-immunoprecipitated DNAs from placenta as shown in the Input lane. However, the immunoprecipitated DNAs by both antibodies were mainly derived from one type, the unmethylated form.

DNA methylation changes in the Rex1-mutant mouse
As described earlier, the REX1-binding regions within the Peg3 and Nespas loci are differentially methylated between two parental alleles, thus called differentially methylated regions (DMRs). The allele-specific DNA methylation of these DMRs is established during gametogenesis and maintained throughout somatic cells, even against the genome-wide resetting of DNA methylation during early embryogenesis (16).
As Rex1 is expressed during spermatogenesis, we first tested whether Rex1 is involved in establishing the allele-specific DNA methylation at these DMRs (Fig. 5). For this test, we have prepared several genomic DNAs isolated from the sperm of the wild-type (+/+) and Rex1-null (−/−) mice. These DNAs were treated with the bisulfite conversion protocol (17), and the converted DNA was amplified with PCR. The PCR product from each locus was analyzed with COBRA (combined bisulfite and restriction digestion analysis) (18). This analysis revealed that the DMRs of Peg3 and Nespas are unmethylated in both the wild-type and Rex1-null mice, indicating no major effects by the Rex1 mutation on the DNA methylation of these two DMRs. This is also the case for the DMRs of Snrpn, H19, IG-DMR, Xist and Tsix, showing no changes in the methylation status between the wild-type and Rex1-null samples.

Second, since Rex1 is highly expressed during early embryogenesis, we also tested whether Rex1 is involved in maintaining the allele-specific DNA methylation at the DMRs of Peg3 and Nespas. For this test, we have prepared three pools of genomic DNAs isolated from the blastocyst-stage embryos of the following crosses: F(+/+) × M(+/+), F(+/+) × M(−/−) and F(−/−) × M(−/−). Each pool of genomic DNAs was derived from about 50 blastocysts. These DNAs were also treated similarly as described above, and some of the PCR products were further analyzed by sequencing. This series of experiments was repeated three times, and one representative set of results is shown in Figure 6. This analysis revealed significant changes in the DNA methylation levels at the DMRs of Peg3 and Nespas. These two loci were hypermethylated in the Rex1-null embryos, but not in the Rex1-heterozygous embryos (data not shown), suggesting that these two regions require at least half-dosage of Rex1 for maintaining their proper DNA methylation levels. In contrast, we did not detect any major changes in the DNA methylation levels of the other imprinted loci, including the DMRs of H19, Snrpn, IG-DMR, Xist, Peg10 and Igf2. We also did not detect any major changes in the DNA methylation levels of IAPs, indicating no major effects on the genome-wide DNA methylation levels by the Rex1 mutation. This indicates that the observed DNA hypermethylation on the DMRs of Peg3 and Nespas is target-specific and that this is likely a direct outcome of the Rex1 mutation, given the observed in vivo binding of Rex1 to these two regions. We also tested the DNA methylation levels of the two DMRs using DNAs isolated from 14.5 dpc embryos and placentas, but we have not obtained any surviving conceptuses with hypermethylation at these two DMRs (Supplementary Materials 2 and 3). This suggests that the blastocysts with hypermethylation at these DMRs may not have survived up to the 14.5 dpc stage.

Overall, the result described above suggests that Rex1 may serve a protective role for these two DMRs, shielding the paternal alleles against DNA methylation during early embryogenesis.

**DISCUSSION**

In the current study, the in vivo roles of Rex1 have been investigated using a mutant mouse line disrupting its transcription. Rex1 most likely plays important roles in epigenetic setting during spermatogenesis and early embryonic stages based on the lethality observed from the later-stage embryos and neonates. The ChIP experiments identified two imprinted genes, Peg3 and Nespas, as its downstream genes. Furthermore, detailed analyses suggest that Rex1 may act as a protector against DNA methylation during early embryogenesis. Overall, this predicted role of Rex1 in genomic imprinting is consistent with the mammal-specific formation and epigenetic connection of Rex1.

Although Rex1 is expressed only during spermatogenesis and early embryogenesis, the gene dosage of Rex1 appears to be very critical for the survival of the late-stage embryos and neonates (Tables 1 and 2). A similar observation was also been reported from another KO model targeting Rex1: some fraction of the homozygotes and heterozygotes are lethal during gestation and neonatal stages (9). It is also noteworthy that the paternal transmission of the Rex1-mutant allele (F(+/+) × M(−/−)) causes higher levels of lethality than the maternal transmission (F(−/−) × M(+/+)). As the offspring from both breeding schemes should be the same in terms of Rex1 gene dosage (heterozygosity), the different levels of lethality observed between the two breeding experiments is likely an outcome of some unknown Rex1-mutation-driven defects during spermatogenesis being carried over to fertilized eggs. This is also consistent with the fact that Rex1 is expressed only during spermatogenesis, but not during oogenesis. In this regard, it is prudent to note that YY1, the original gene of Rex1, has an epigenetic role during spermatogenesis, establishing the H3K9me3 mark in the pericentromeric repeats of chromosomes in primary spermatocytes (19). Interestingly, Rex1 is also expressed in the same stage of spermatocytes according to the results (Fig. 2A). This coincidence may be an indication for similar roles played by Rex1 and YY1 in epigenetic setting during spermatogenesis. Although this possibility remains to be tested, the delayed phenotypes observed...
from the breeding experiments suggest potential roles for Rex1 in epigenetic setting during spermatogenesis and early embryogenesis.

The ChIP experiments demonstrated that two imprinted genes, Peg3 and Nespas, are downstream target genes of Rex1 (Fig. 4). This further implies that the two similar proteins, YY1 and Rex1, may share their DNA-binding sites for the transcription and imprinting control of Peg3 and Nespas. According to the data presented in the current and previous studies, however, these two proteins probably have opposite roles for the epigenetic setting of the Peg3 imprinted domain. YY1 has been shown to be involved in setting up DNA methylation on the maternal allele of Peg3-DMR during oogenesis (20). In contrast, Rex1 may act as a protector for the unmethylated, paternal allele of the Peg3-DMR against DNA methylation during early embryogenesis (Fig. 6). Then, what would be the mechanistic basis for these opposite roles played by the two similar proteins? Compared with the protein domain structure of Rex1, YY1 has one additional subdomain, called REPO, which is responsible for recruiting polycomb complexes (21). Thus, these two proteins most likely have at least one difference in recruiting polycomb complexes, which might have different consequences in epigenetic setting (histone modifications). The opposite roles played by YY1 and Rex1 might be further feasible due to their different expression patterns. Rex1 (protector) is not expressed during oogenesis, and thus, YY1 could recruit epigenetic machineries, such as de novo DNA methyltransferases, to the Peg3-DMR. On the other hand, Rex1 expression during spermatogenesis and early embryogenesis might out-compete YY1 and subsequently hinder YY1’s recruiting of de novo DNA methylation machineries to the Peg3-DMR. Overall, the opposite effects by the depletion of YY1 and Rex1 on the DNA methylation levels of the Peg3-DMR clearly suggest that these two transcription factors have functionally non-redundant, unique roles for the Peg3 imprinted domain.

The ChIP experiments also demonstrated that Tsix is another downstream gene of Rex1 (Fig. 4). This result is consistent with the following data sets. First, according to DNA-binding motif studies (8,14), one evolutionarily conserved motif found within Tsix exhibits much higher levels of DNA-binding affinity to Rex1 than YY1, suggesting that this motif likely has been selected for recruiting Rex1 to the Tsix locus. Second, a recent study also demonstrated functional involvement of Rex1 in Tsix transcription. According to this study (22), RNAi-based depletion of REX1 protein in ES cells resulted in a dramatic decrease in the transcriptional levels of Tsix. However, the RT–PCR survey described in the current study did not detect similar levels of decrease in Tsix transcription, although the analysis revealed slight down- and up-regulation of Tsix and Xist in Rex1−/− blastocysts. Although this discrepancy requires further exploration, the data suggest that Rex1 and YY1 might play different roles in regulating Tsix transcription.
investigation in the near future, a similar conclusion, Rex1’s involvement in Tsix transcription, could also be inferred from the results of the breeding experiments described in the current study. According to the results (Table 1), males are underrepresented in the homozygous population for the Rex1 knockin allele. This is likely caused by potential X chromosome inactivation on male embryos with Rex1–/– due to the failure of Tsix-driven repression of Xist transcription. Similar gender-specific effects were also observed in the mutant mice targeting Tsix, in which the survival of the male population was severely compromised (23). Although we need to study more, the results described in this study and others strongly suggest critical roles played by Rex1 for Tsix transcription during early embryogenesis.

The spatial expression patterns of Rex1 in the placenta are noteworthy: Rex1’s expression is detected only on the surface of the parietal yolk sac adjacent to where the embryo is positioned (Fig. 2B). Parietal yolk sacs, along with visceral yolk sacs, are known to be transient: both membranes disappear upon the completion of placenta development, around the 13.5 dpc stage in the mouse (24). Interestingly, these membranes are found only in placental mammals, suggesting that these membranes are newly invented structures in the eutherian animals during mammalian evolution (25). Given this evolutionary context, Rex1’s expression in the parietal membranes appears to be unique and intriguing: a eutherian-specific gene is expressed in a eutherian-specific tissue. This situation may be an indication for eutherian-specific roles for Rex1. The parietal and visceral yolk sacs are known to be very critical for early fetal–maternal interaction, controlling the rates of nutritional uptake as well as maternal influence (25). Therefore, it is reasonable to think that Rex1 may control a set of genes that are involved in early fetal–maternal interactions. In that regard, it is important to note that Peg3, a downstream gene of Rex1, is well known for its involvement in controlling fetal growth rates and also its high-level expression in the placenta (26). Rex1 might exert its unknown eutherian-specific roles in the parietal yolk sacs via Peg3. In summary, the spatial expression profiles of Rex1 in the placenta are consistent with the idea that Rex1 may have co-evolved with the lineage-specific biology of the eutherian animal.

MATERIALS AND METHODS

Generation and breeding of Rex1 knockin mice

The gene trap clone, XB238 (strain 129/OlaHsd), from BayGenomics, was injected into mouse blastocysts to generate chimeric mice. Injection of these cells into C57BL/6 blastocysts was performed by The Darwin Transgenic Mouse Core Facility (Baylor College of Medicine, Houston, TX, USA). The male chimeric mice were further bred with female C57BL/6 mice to obtain F1 mice with the germine transmission of the Rex1-mutant allele.

Genotyping of the mouse was determined by using two parallel PCRs. The first PCR used one forward primer located in the third intron of Rex1 gene, P1 (5’-GTTCCAGTATCCTTT AATCCCTCCAAGAC-3’), whereas the second PCR used the other forward primer located in the gene trap vector (pGT0pfs), P2 (5’-GCTTCCCGAAGGAGAAAGCGGAC AGG-3’). These two PCRs used the same reverse primer from the third intron of Rex1 gene, P3 (5’-GTCCTCTAG GCAGGGGATGGCTCAC-3’). These PCRs yielded a 275 bp wild-type product and a 500 bp gene trap product. PCR conditions were 33 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Also, the gender of neonatal mice and embryos was determined by one primer set targeting the mouse Sry gene under the same PCR conditions described above: mSry-F (5’-GTCCCGTGGTGAAGGCGACAA-3’) and mSry-R (5’-GCAGCTCTACCAGTCTTGGCC-3’). To prepare genomic DNAs, each tissue from either clipped tails or ears was incubated overnight at 55°C in the lysis buffer (0.1 M Tris–Cl, pH 8.8, 5 mM EDTA, pH 8.0, 0.2% SDS, 0.2 M NaCl, 20 μg/ml proteinase K), and then the lysed extract was diluted 1/30th for PCR amplification.

β-Galactosidase staining

Testes were isolated from 2-month-old Rex1(–/–) male mice, and 10.5 dpc conceptuses were isolated from the pregnant Rex1(–/–) female mice that had been time-mated with male Rex1(–/–) mice. The isolated testes were treated with the fixing solution (0.2% paraformaldehyde, 0.1 M PIPES buffer, pH 6.9, 2 mM MgCl2, 5 mM EGTA). The fixed testes were then cryo-protected in the PBS buffer containing 30% sucrose and 2 mM MgCl2 at 4°C overnight. These samples were further embedded in OCT and frozen at −80°C. The embedded sections were sectioned on a cryotome (Leica CM1850) to 50 μm thickness and placed onto poly-L-lysine-coated slides. The sections were further immobilized in the fixing solution for 10 min. After rinsing in PBS for 10 min, they were placed in detergent rinse solution for 10 min. The sections were then placed at 37°C overnight in the staining solution containing 1 mg/ml of bromo-chloro-indolyl-galactopyranoside (X-gal) (24). For whole-mount staining of the isolated conceptuses, the samples were fixed in 4% paraformaldehyde for 2 h and stained overnight at 37°C in the staining solution containing 1 mg/ml of X-gal. The testis sections and whole-mount conceptuses were visualized using a dissecting stereomicroscope (Stemi 2000C, Zeiss). Images were captured with a digital camera (Infinity USB2.0, Lumenera).

Reverse transcriptase–polymerase chain reaction and quantitative reverse transcriptase–polymerase chain reaction

Total RNA was isolated from neonates and embryos, placentas and blastocysts with the Trizol (Invitrogen) and RNeasy mini kits (Qiagen). The isolated RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). PCR amplifications were performed with a series of gene primer sets using the Maxime PCR Premix Kit (Intron Biotech). The information regarding individual primer sequences and PCR conditions is available as Supplementary Material 1. Quantitative real-time PCR was also performed with the iQ SYBR green supermix (Bio-Rad) using the iCycler qTMT multicolor real-time detection system (Bio-Rad). All quantitative RT–PCRs (qRT–PCRs) were
carried out for 40 cycles under the standard PCR conditions. The analyses of the results derived from qRT–PCR were described previously (27).

Western blot analysis

For our western blot analysis, the tissues were first homogenized using the lysis buffer (0.25 M Tris–HCl, pH 7.8, plus 0.1% NP-40). Cellular debris was removed by centrifugation for 10 min. Protein concentrations were determined by the Bradford Assay Kit (Pierce). Twenty micrometers of lysate were separated on 10% SDS–PAGE gels and transferred to PVDF membranes (Hybond-P, Amersham) using a Mini Trans-Blot transfer cell (Bio-Rad). Membranes were blocked for 1 h in the Tris-buffered saline containing 5% skimmed milk and 0.05% Tween 100 and incubated at 4°C overnight with anti-REX1 (Cat. No. ab28141, Abcam, Inc.) or anti-β-ACTIN (Cat. No. sc-1615, Santa Cruz Biotechnology) antibodies. These blots were incubated for an additional 1 h with the secondary antibody linked to horseradish peroxidase (Sigma). The blots were developed using a western blot detection system according to the manufacturer’s protocol (Intron Biotech).

ChIP experiments

The ChIP experiments described in this study used two samples: 1 × 10^7 ES cells and 14.5 dpc placentas. The actual ChIP experiments were performed according to the protocol provided by Upstate Biotechnology with some modification as described previously (15). Briefly, the placentas were homogenized in 10 ml of PBS. These samples were treated with formaldehyde to the final concentration of 1% and incubated at 37°C for 10 min. The ES cells were also treated similarly with formaldehyde. The treated samples were sheared by sonication and immunoprecipitated with anti-REX1 antibodies (Cat. No. ab28141, Abcam, Inc.). Precipitated DNA and protein complexes were reverse-cross-linked and purified through phenol/chloroform extraction. Purified DNAs were used as template DNAs for PCR amplification. PCRs were carried out for 40 cycles using standard PCR conditions. The resulting PCR products were run on 1.6% agarose gels containing ethidium bromide. All ChIP assays were performed independently at least three times. The oligonucleotide sequences used for this study are available as Supplementary Material.

Isolation of sperm and blastocyst-stage embryos

Sperm was isolated from the epididymis of male mice according to the protocol established previously (28). In brief, the retrieved epididymis were incubated at room temperature for 1 h with gentle rocking in the sperm elution buffer (130 mM NaCl, 20 mM Tris, pH 7.8, 2 mM EDTA, pH 8.0). After a spin for 3 min at 800 rpm on a tabletop centrifuge (Cat. No. Centrifuge 5415D, Eppendorf), the supernatant was transferred into new tubes, and then the sperm was precipitated with 30 min centrifugation. The precipitated sperm was washed once more with the same buffer. The isolated sperm was examined under a microscope to measure the total number and purity of each sperm pool. The sperm was subsequently used for isolating DNAs.

For the isolation of blastocyst-stage embryos, we followed the superovulation protocol (24,29). In brief, the female mice were first injected intraperitoneally with 5 IU of the pregnant mare’s serum (Cat. No. G4877, Sigma), and the same mice were injected again with 5 IU of the human chorionic gonadotropin (hCG) hormone (Cat. No. C1063, Sigma) 48 h after the initial injection. After the hCG injection, these female mice were put together with male littermates. The female mice were sacrificed 3 days after the breeding setup, and the embryos were isolated from their uterus (24). The isolated blastocysts were further used for the analyses of DNA methylation and transcriptional levels of several genes.

Bisulfite conversion and COBRA

The DNAs isolated from embryos, sperm and blastocysts were treated with the bisulfite conversion reaction according to the manufacturer’s protocol (EZ DNA Methylation Kit, Zymo Research). The converted DNAs were used as templates for the PCR using specific primers that were designed for amplifying each imprinting control region. The oligonucleotide sequences for this study are available upon request. To determine the DNA methylation levels of target regions, we used the following two approaches: (i) the restriction enzyme digestion-based COBRA (18) and (ii) subcloning and sequencing of each PCR product. For the COBRA analysis, each PCR product was digested with a series of restriction enzymes that are shown in Figures 5 and 6. Some of the PCR products were also individually subcloned into the pGEM T-Easy Vector (Promega), and the purified DNAs from 10 to 20 clones were sequenced for the survey of DNA methylation levels of a given PCR product.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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