

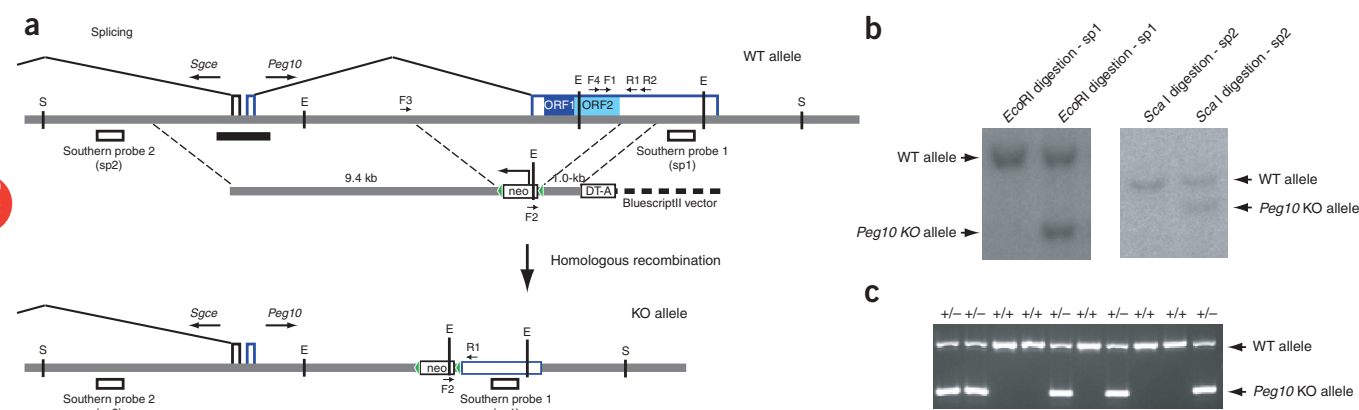
# Deletion of *Peg10*, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality

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By comparing mammalian genomes, we and others have identified actively transcribed Ty3/gypsy retrotransposon-derived genes with highly conserved DNA sequences and insertion sites<sup>1–6</sup>. To elucidate the functions of evolutionarily conserved retrotransposon-derived genes in mammalian development, we produced mice that lack one of these genes, *Peg10* (paternally expressed 10)<sup>1–3,7</sup>, which is a paternally expressed imprinted gene on mouse proximal chromosome 6. The *Peg10* knockout mice showed early embryonic lethality owing to defects in the placenta. This indicates that *Peg10*

is critical for mouse parthenogenetic development and provides the first direct evidence of an essential role of an evolutionarily conserved retrotransposon-derived gene in mammalian development.

Recently, we and others discovered the evolutionarily conserved retrotransposon-derived gene *Peg10* (refs. 1–3,8). Mouse *Peg10* is a single-copy gene located in an imprinted gene cluster on proximal chromosome 6, and its human homologue *PEG10* is in the syntenic imprinted region with identical gene order on human



**Figure 1** Targeted disruption of the *Peg10* locus. Schematic representation of the complete *Peg10* locus, the targeting vector and the targeted *Peg10* allele. Relevant restriction sites are indicated: E: *EcoRI*; S: *ScaI*. Primers are indicated by small arrows. (a) In the targeted allele, ORF1 and ORF2 were completely replaced by a neomycin resistance gene cassette. The *loxP* sites are shown as green triangles. WT, wild-type; KO: knockout. Black bar represents position of differentially methylated region (DMR). (b) DNA blot analysis of targeted ES cell clones. Genomic DNA was digested with *EcoRI* or *ScaI* and hybridized with Southern Probe 1 (SP1) or SP2. Left: wild-type ES cells; right: ES cells heterologous for the *Peg10* knockout allele. (c) Genotype analysis of 9.5-d.p.c. embryos by PCR using the *Peg10* F1, F2 and R1 primers. The results for each wild-type and Pat-KO embryo derived from mating a *Peg10* knockout chimera male with a wild-type female are shown.

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**Table 1** Lethality of *Peg10* knockout (Pat-KO) fetuses and neonates

Dissection stage (d.p.c.)	<i>Peg10</i> <sup>+/+</sup> total	Pat-KO total (dead)	Percentage death in Pat-KO
Embryos from male chimeras			
8.5	9	13 (0)	0
9.5	12	12 (0)	0
10.5	8	4 <sup>a</sup> (4)	100
11.5–12.5	2	2 (2)	100
12.5–term	3	12 (12)	100
Born	53	0 (nd)	100
Embryos from F2 males			
8.5	14	5 (0)	0
9.5	37	35 (0)	0
10.5	12	10 <sup>a</sup> (10)	100
11.5–12.5	13	17 (17)	100
12.5–term	12	10 (10)	100
Born	16	0 (nd)	100

No *Peg10* Pat-KO pups were born as the result of natural mating of two male chimeras and three F2 males with normal C57BL/6 females. *In utero*, viable Pat-KO embryos were present in numbers consistent with normal mendelian inheritance before 9.5 d.p.c.

However, all of these embryos died until 10.5 d.p.c. nd, not detected.  
<sup>a</sup>Embryos that died before 10.5 d.p.c. were severely growth retarded and apparently dead, as no heartbeat was detectable and their bodies were resorbed.

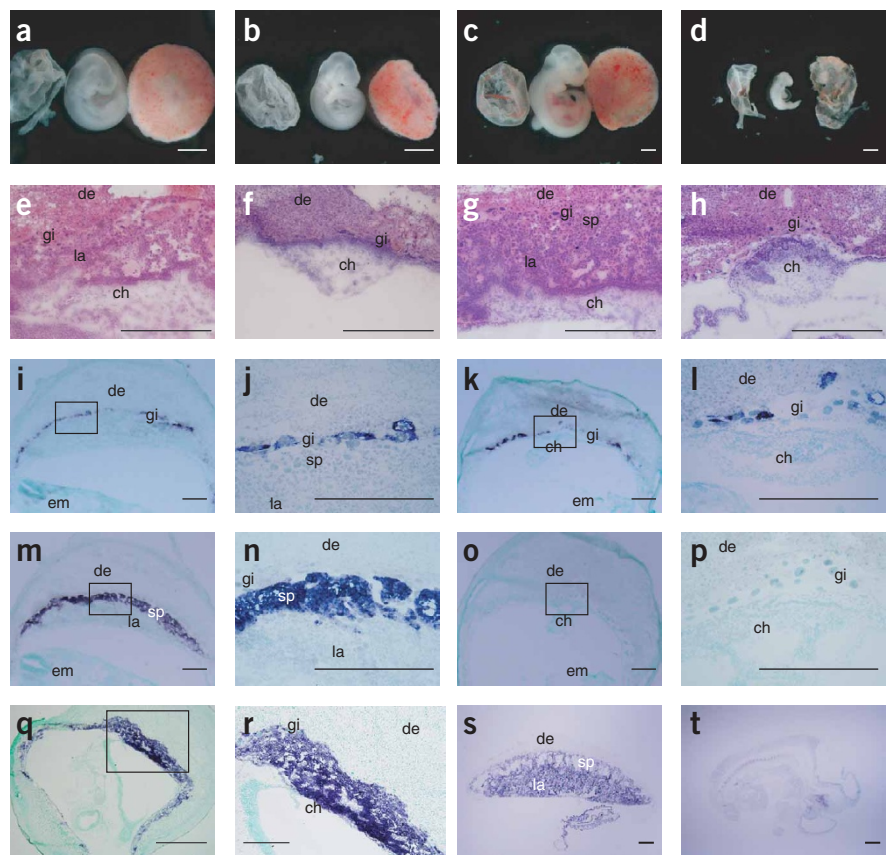
chromosome 7q21. Structural analysis of *Peg10* clearly showed that it was derived from a Ty3/gypsy long terminal repeat (LTR) retrotransposon, as is the Sushi-ichi retrotransposon<sup>9</sup> (with which it shows most similarity), but it does not function as an active retrotransposon. It is expressed in some embryonic tissues and extensively in the placenta as an endogenous gene<sup>1,8</sup>. Notably, *Peg10*

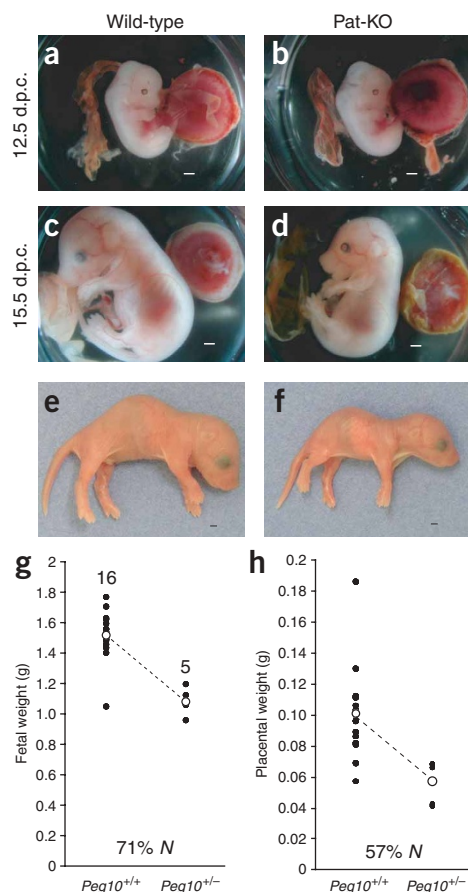
is highly conserved across mammalian species. As far as we know, *Peg10* orthologues are present in all mammals, suggesting that it acquired some essential function as an endogenous gene after losing its transposition capabilities<sup>1,8</sup>.

We attempted to generate *Peg10* knockout mice to explore this hypothesis. As we first isolated *Peg10* as a candidate imprinted gene for an early embryonic lethal phenotype of mice with maternal duplication of proximal chromosome 6 (ref. 10), it is possible that *Peg10* knockout mice might show parent of origin-specific lethality. A targeting vector was designed to remove the entire coding regions of ORF1 and ORF2 while retaining the promoter region, as it overlaps a differentially methylated region (DMR; Fig. 1a). Using targeted embryonic stem (ES) cell clones (Fig. 1b), we produced two male chimeric mice whose progeny inherited recombinant ES cell-derived alleles at a very high rate. However, none of the pups had the knockout alleles (Table 1). We dissected pregnant females at various embryonic stages and found that all *Peg10*<sup>+/−</sup> embryos died *in utero* by 10.5 d post coitus (d.p.c.; Table 1 and Fig. 1c), although they were detected at approximately the expected mendelian frequency before 9.5 d.p.c. (Table 1).

Morphologically, the *Peg10*<sup>+/−</sup> embryos and yolk sacs appeared normal at 9.5 d.p.c. as they reached the major development steps, such as functional chorioallantoic fusion, rotation, formation of head structures, initiation of cardiac contraction and vascularization of the yolk sac (Fig. 2a,b). However, they became severely growth retarded and had no detectable heartbeat at 10.5 d.p.c. (Fig. 2c,d). Their placentas were slightly smaller than those of the normal wild-type at 9.5 d.p.c. and became severely depleted at 10.5 d.p.c. The labyrinth layer was not developed and no spongiotrophoblast cells were observed at 9.5–10.5 d.p.c. (Fig. 2e–h). However, there were no

**Figure 2** *Peg10* knockout embryos show severe growth retardation and placental defects. Wild-type (a,c) and Pat-KO (b,d) embryos (em) are shown, together with their placentas (right) and yolk sacs (left) at 9.5 d.p.c. (a,b) and 10.5 d.p.c. (c,d) (scale bars: 1 mm). The Pat-KO embryos have poorly developed placentas. Hematoxylin and eosin-stained histological sections of 9.5-d.p.c. (e,f) and 10.5-d.p.c. (g,h) wild-type (e,g) and Pat-KO (f,h) littermates (scale bars: 500 μm). The trophoblast giant cells (gi), spongiotrophoblast (sp), labyrinthine (la), chorionic plate (ch) and maternal decidua (de) are normal in the wild-type placentas, whereas poorly developed placentas are observed in the Pat-KO mice. The *in situ* hybridization of 10.5-d.p.c. wild-type (i,j,m,n) and Pat-KO (k,l,o,p) (scale bars: 500 μm) using the trophoblast-specific marker gene *PL-1* (i–l) for trophoblast giant cells and the *4311* probe (m–p) for spongiotrophoblast cells, clearly demonstrates that the giant cells are normal, whereas the spongiotrophoblast cells, which are derived from the ectoplacental cone, are completely missing (o,p). Higher magnifications of the boxed regions of i,k,m,o are shown as j,l,n,p, respectively. *In situ* hybridization with the antisense *Peg10* probe of the wild-type conceptus at 9.5 d.p.c. (q; scale bar: 500 μm) and 12.5 d.p.c. placenta (s) and embryo (t; scale bar, 1 mm) demonstrates that *Peg10* is strongly expressed in all the extraembryonic tissues at these stages (q,s), and relatively well expressed in the brain and vertebral cartilage (t). (r) Higher magnifications of the boxed regions shown in q (scale bar: 200 μm).





**Figure 3** Rescue of *Peg10* Pat-KO embryos by tetraploid wild-type extraembryonic tissues. Wild-type and Pat-KO embryos with their placentas (right) and yolk sacs (left) rescued by tetraploid wild-type extraembryonic tissues. Weights in **a,b** are as follows (mean  $\pm$  s.d.): *Peg10<sup>+/+</sup>* embryo,  $0.092 \pm 0.003$  ( $n = 2$ ); *Peg10<sup>+/-</sup>* embryo,  $0.091$  ( $n = 1$ ); *Peg10<sup>+/+</sup>* placenta,  $0.051 \pm 0.005$  ( $n = 2$ ); *Peg10<sup>+/-</sup>* placenta,  $0.057$  ( $n = 1$ ). Weights in **c,d** are as follows: *Peg10<sup>+/+</sup>* embryo,  $0.548 \pm 0.065$  ( $n = 3$ ), *Peg10<sup>+/-</sup>* embryo,  $0.411 \pm 0.027$  ( $n = 3$ ),  $P = 0.028$ ; *Peg10<sup>+/+</sup>* placenta,  $0.113 \pm 0.003$  ( $n = 3$ ); *Peg10<sup>+/-</sup>* placenta,  $0.122 \pm 0.005$  ( $n = 3$ ),  $P = 0.049$ . Scale bar: 1 mm. **(e,f)** Wild-type and Pat-KO newborn pups rescued by tetraploid wild-type extraembryonic tissues (scale bars: 1 mm). **(g)** Fetal weights of *Peg10* Pat-KO and wild-type littermates rescued by tetraploid wild-type extraembryonic tissues at 19.5 d.p.c. Each filled circle represents a single embryo. Number at top represents total number of weighed embryos. Open circles represent average weight. The degree of growth deficiency of the rescued Pat-KO embryos is shown as a percentage of wild-type fetal weight ( $N$ ). Fetal weights (mean  $\pm$  s.d.) are as follows: *Peg10<sup>+/+</sup>*,  $1.526 \pm 0.159$  ( $n = 16$ ); *Peg10<sup>+/-</sup>*,  $1.089 \pm 0.088$  ( $n = 5$ );  $P = 1.306 \times 10^{-55}$ . **(h)** Placental weights of *Peg10* Pat-KO and wild-type littermates rescued by tetraploid wild-type extraembryonic tissues at 19.5 d.p.c. The data are from the same litters as in **g**. Placental weights (mean  $\pm$  s.d.) are as follows: *Peg10<sup>+/+</sup>*,  $0.102 \pm 0.029$  ( $n = 16$ ); *Peg10<sup>+/-</sup>*,  $0.058 \pm 0.014$  ( $n = 5$ );  $P = 0.004$ .

morphological differences in the placentas at 7.5 d.p.c. and 8.5 d.p.c. (Supplementary Fig. 1 online). *In situ* hybridization of 8.5- to 10.5-d.p.c. placentas with trophoblast-specific marker genes (*PL-1* for trophoblast giant cells and *Tpbpa* for spongiotrophoblast cells) clearly demonstrated that although the giant cells were normal (Fig. 2i-l and Supplementary Fig. 1), the spongiotrophoblast cells, which are derived from the ectoplacental cone, were completely missing (Fig. 2m-p and Supplementary Fig. 1). Therefore, we conclude that these placentas are abnormal compared with typical three-layered placentas. In light of these defects, it is conceivable that the severe defect in placenta formation was the cause of the growth retardation and early embryonic lethality of the *Peg10<sup>+/-</sup>* embryos. The *Peg10* expression profile supports this assumption: high expression in all the extraembryonic tissues at 9.5 d.p.c. and 12.5 d.p.c. (Fig. 2q-s)<sup>1,8</sup> and low-level expression in the embryonic brain and vertebral cartilage at 12.5 d.p.c. (Fig. 2t).

To confirm these findings, we attempted to rescue the phenotype by aggregating *Peg10<sup>+/-</sup>* embryos with tetraploid embryos derived from wild-type fertilized eggs<sup>11</sup>. As expected, 12.5- and 15.5-d.p.c. knockout embryos were recovered with normal-looking placentas, although the latter showed growth retardation in both embryos and placentas (Fig. 3a-d). As a result, half of the knockout embryos (18/37) developed to term, demonstrating that the early embryonic lethality of *Peg10<sup>+/-</sup>* results from incomplete placenta formation (Fig. 3e,f). Recovered *Peg10<sup>+/-</sup>* embryos showed growth retardation in most cases (Fig. 3g,h). However, the fetal-to-placental weight ratios of *Peg10<sup>+/-</sup>* embryos were slightly higher than those of the wild-type embryos, suggesting the mutant placenta, although small, is relatively more efficient than the wild-type placenta (Supplementary Fig. 2 online).

Although the *Peg10* knockout mice had approximately 30% lower weight at birth than their wild-type littermates (Fig. 3g), they gained weight, and the *Peg10* knockout F<sub>1</sub> females delivered F<sub>2</sub> pups normally with or without the knockout allele (Mat-KO; Fig. 4a). These F<sub>2</sub> pups matured normally, *Peg10* knockout F<sub>2</sub> females consistently had normal deliveries, and F<sub>3</sub> pups with the knockout allele grew normally. Conversely, all the F<sub>3</sub> embryos with the knockout allele died by 10.5 d.p.c. in paternal transmission (Pat-KO; Table 1 and Fig. 4a).

Quantitative RT-PCR of the entire region showed normal expression of fifteen genes, which included at least six imprinted genes, in 9.5-d.p.c. Pat-KO embryos, whereas *Peg10* itself was not expressed (Fig. 4b). We also confirmed that the differential DNA methylation status of the *Peg10*-*Sgce* DMR remained normal<sup>8</sup>, using polymorphisms between JF1 (*Mus musculus molossinus*) and *Mus musculus musculus* (Fig. 4c). The early embryonic lethal phenotype was also observed in this genetic background (data not shown). The expression of *Ppp1r9a* (*Neurabin*) was apparently reduced in affected 9.5- and 10.5-d.p.c. Pat-KO placentas (Fig. 5) but normal in the normal-looking 8.5-d.p.c. Pat-KO placentas, which suggests that the *Peg10* knockout does not affect the expression levels of nearby placental genes (Fig. 5). All the basic phenotypes of the *Peg10* knockout embryos were confirmed in *Peg10 loxP* knockout mice without a *neo* expression cassette (Supplementary Figs. 3-5 and Supplementary Table 1 online). Thus, we conclude that *Peg10* is essential for placenta formation and is responsible for the early embryonic lethality seen during mouse development. Our results also suggest that *Peg10* is one of the lethal target genes in mice with maternal duplication of proximal chromosome 6, which bears the *Peg10* locus<sup>10</sup>.

Deletion of the maternally expressed imprinted gene *Ascl2* (also known as *Mash2*)<sup>12</sup> leads to early embryonic lethality (death by 10.5 d.p.c.) owing to similar placental morphological defects to *Peg10* Pat-KO embryos. Therefore, we examined the expression of *Ascl2* in *Peg10* knockout embryos using quantitative RT-PCR (Supplementary Fig. 6) and found that *Ascl2* expression was normal at 8.5 d.p.c. but heavily reduced at 9.5 d.p.c., probably owing to a lack of spongiotrophoblasts and labyrinth cells. These results clearly indicate that *Peg10* is not situated upstream of *Ascl2*.

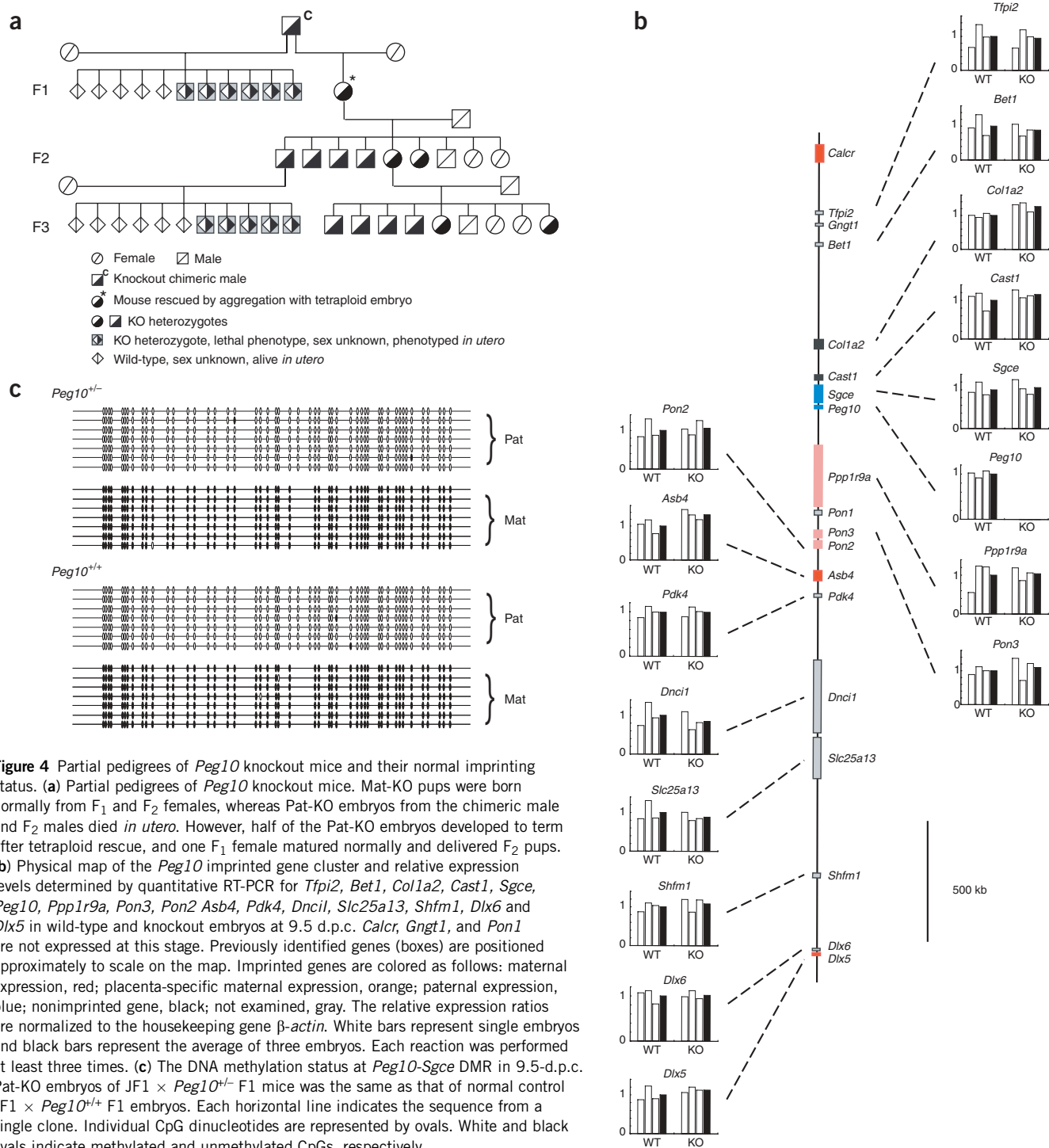
Our present study also indicates that *Peg10* could be critical for parthenogenetic development in mice because parthenogenetic

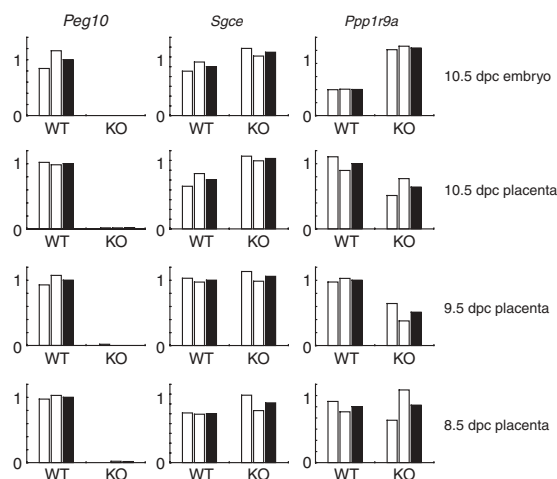


embryos that contain two maternally derived genomes lack expression of all paternally expressed imprinted genes. Parthenogenetic embryos die before 9.5 d.p.c. and show early embryonic lethality with poorly developed extraembryonic tissues<sup>13,14</sup>. Morphological defects of the most developed parthenotes are very similar to those of *Peg10*-Pat KO embryos; they lack the diploid trophoblast cells of the labyrinth layer and the spongiotrophoblast but have some giant cells and some chorion at days 9 and 10 (ref. 15). However, the majority of parthenotes show more severe phenotypes;

the diploid trophoblast cells of the ectoplacental cone almost completely fail to develop, leading to lack of extraembryonic ectoderm, and therefore chorion, so that at 6.5 d.p.c. the conceptus is abnormal, despite the vigorous embryonic ectoderm (S. Barton and M.A. Surani, personal communication). Therefore, it is clear that some other genes could also contribute to the parthenogenetic phenotypes<sup>13,14</sup>.

The genetic conflict hypothesis predicts that genes that promote embryonic and placental growth have become paternally expressed,





**Figure 5** Relative expression levels of *Peg10*, *Sgce* and *Ppp1r9a* at different stages, as determined by quantitative RT-PCR. The relative expression levels of 8.5- to 10.5-d.p.c. Pat-KO placentas and 10.5-d.p.c. Pat-KO embryos were determined by quantitative RT-PCR (as in Fig. 4b). Each reaction was performed at least three times. White bars represent single embryos and black bars represent the average of two embryos.

whereas genes that inhibit these growth have become maternally expressed during mammalian evolution<sup>16</sup>. Our results clearly show that *Peg10* conforms well to this hypothesis, as *Peg10* is paternally expressed and essential for the formation of the placenta, which functions to promote nutrient transfer from mother to fetus, as shown for the *Igf2* P0 transcript<sup>17</sup>. Therefore, we provide further evidence that the functional bias predicted by this hypothesis exists among imprinted genes.

Recently, RAG (the V(D)J recombinase)<sup>18</sup> and telomerase<sup>19</sup> have been shown or suggested to be derived from transposons and retrotransposons, respectively, indicating that some of these transposable elements have contributed to the acquisition of several new important genomic functions during evolution<sup>20</sup>. There are other reports that the rat IgE-binding protein contains a partial coding sequence of the retrotransposon intracisternal A particle (IAP)<sup>21</sup> and that the human *syncytin* gene<sup>22</sup>, which functions in syncytiotrophoblast formation in placental tissues, is derived from a human endogenous retrovirus (HERV-W). Recently, mouse *syncytin-A* and *syncytin-B* genes have been isolated and, notably, have been found to be derived from similar but different retroviruses derived from the human HERV-W<sup>23</sup>. These findings also provide evidence that various species-specific retrotransposons play roles in different species. However, as far as we know, this is the first demonstration that an evolutionarily conserved mammal-specific, retrotransposon-derived gene has an essential function in development, at least in mice.

Recently, it has been reported that human PEG10 may have a carcinogenic function in affecting cell cycle progression<sup>24</sup> or inhibiting apoptosis mediated by SIAH1 (ref. 25), and/or inhibiting TGF- $\beta$  signaling by interacting with the TGF- $\beta$  receptor ALK1 (ref. 26). Therefore, PEG10 may have a wide variety of functions such as the regulation of cell growth and differentiation as well as placental function<sup>1,8</sup>.

It is of great interest to learn the role of *Peg10* in the acquisition of the placenta during mammalian evolution, as it is highly conserved in eutherian mammals. Based on database searches, *Peg10* is not present in the *Fugu rubripes* (fish) or chicken genomes (Supplementary Fig. 7). Ancestral mammals might have developed this new organ,

the placenta, from newly acquired, retrotransposon-derived genes, or endogenous genes present in oviparous animals might have been modified for placenta formation some time after the divergence of mammals and birds, more than 92 million years ago<sup>27</sup>. Further comparative genome analyses among eutherians, marsupials, and monotremes may help to uncover the origin of *Peg10* in mammalian evolution. We found another ten homologues to the Sushi-ichi retrotransposon (called *Sirh* family genes) in the mouse and human genomes, including another paternally expressed gene, *Rtl1* (also called PEG11 in sheep)<sup>3-6</sup>, which is located on the mouse distal chromosome 12, and *Ldoc1* (refs. 3,28) on the X chromosome. Similar conclusions have recently been reported by other researchers<sup>29</sup>. It will be fascinating to discover the functions of these evolutionarily conserved retrotransposon-derived genes, as well as those of *Peg10*.

## METHODS

**Deletion of the *Peg10* gene.** We obtained 9.4-kb (nucleotides 6903–16254; AC084315) and 1.0-kb (nucleotides 20116–21135; AC084315) genomic fragments by screening the 129SvJ lambda genomic library (Stratagene). We used these fragments as the right- and left-arm sequences of a construct in which both *Peg10* ORFs were replaced with the neomycin resistance gene. After a 2-week incubation under G418 selection followed by electroporation of the linearized DNA into ES cells (CCE) of 129/SvEv mouse origin, we obtained 120 colonies. The genomic DNA of these colonies was checked by DNA blot analysis using DNA fragments of nucleotides 5300–6525 and nucleotides 21271–21766 as 5'-end and 3'-end probes, respectively.

The *Peg10*-targeted ES cells that resulted from homologous recombination of the construct were used to generate chimeric mice by blastocyst injection. From two male chimeras, germline transmission of the knockout allele was confirmed when pregnant female mice that had mated with a *Peg10* male chimera were dissected and their embryos analyzed.

**PCR.** Genomic DNA and total RNA samples were prepared from embryos and placentas at various stages using ISOGEN (Nippon Gene), as described previously<sup>8</sup>. The cDNA was synthesized from 1  $\mu$ g of RNA using Superscript II reverse transcriptase (Life Technologies) with oligo-dT primer. For RT-PCR, 10 ng cDNA in a 100- $\mu$ l reaction mixture containing 1 $\times$  ExTaq buffer (TaKaRa), 2.5 mM dNTP mixture, primers and 2.5 units (U) ExTaq (TaKaRa) was subjected to 25–30 PCR cycles of 96  $^{\circ}$ C for 15 s, 65  $^{\circ}$ C for 30 s and 72  $^{\circ}$ C for 30 s in a Perkin Elmer GeneAmp PCR System 2400. Gene expression profiles were deduced from agarose gel electrophoresis of RT-PCR products with ethidium bromide staining. The primer sequences are available upon request.

**Quantitative RT-PCR.** The expression levels of 15 genes in the *Peg10* cluster and *Ascl2* were measured with the ABI PRISM 7700 using SYBR Green PCR Core Reagents (Applied Biosystems), which were designed to detect these cDNAs. The target cDNA fragments were cloned into plasmids to be used as standards in the quantitative analysis of gene expression. The relative expression ratios were normalized to the housekeeping gene  $\beta$ -actin. The primers for *Dncl1* have been described previously<sup>30</sup>. The other primer sequences and conditions for their use are available upon request.

**DNA methylation analyses of the *Peg10-Sgce* DMR.** Genomic DNA samples were isolated from 9.5-d.p.c. embryos of JF1  $\times$  *Peg10*<sup>+/-</sup> F1 mice using ISOGEN, as described in the RT-PCR section. Purified genomic DNA was treated with a sodium bisulfite solution, and *Peg10-Sgce* DMR was amplified by PCR<sup>8</sup>.

**Generation of tetraploid aggregation chimeras.** Electrofusion of two-cell-stage blastomeres collected from B6D2 F1 females was used to produce wild-type tetraploid embryos. The fused embryos were cultured overnight in embryo culture medium in 5% CO<sub>2</sub> at 37  $^{\circ}$ C. Each eight-cell-stage diploid *Peg10*<sup>+/-</sup> embryo was aggregated overnight with a four-cell tetraploid embryo. Successfully aggregated embryo pairs at the morula or blastocyst stage were transferred to 2.5-d.p.c. pseudopregnant ICR recipients.

**Histology.** Pregnant mice that had mated with a *Peg10* knockout male were killed at 7.5, 8.5, 9.5, 10.5 and 11.5 d.p.c. Whole embryos were collected and immediately embedded in OCT. Sections from embedded embryos were cut in 7- $\mu$ m sections. For hematoxylin and eosin staining or *in situ* hybridization, the sections were fixed in 4% paraformaldehyde before the standard staining procedures. The nuclei of the cell samples for *in situ* hybridization were stained with 2% methyl green.

Note: Supplementary information is available on the Nature Genetics website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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