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Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR- β

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Classical endocrine studies have shown that steroid hormones are required for the maintenance of pregnancy and placental viability. The oestrogen-receptor-related receptor β (ERR- β) is an orphan member of the superfamily of nuclear hormone receptors¹. Although ERR- β is homologous to the oestrogen receptor and binds the oestrogen response element², it is not activated by oestrogens¹. Expression of ERR- β during embryogenesis defines a subset of extra-embryonic ectoderm that subsequently forms the dome of the chorion, suggesting that ERR- β may be involved in early placental development. Homozygous mutant embryos generated by targeted disruption of the *Estrrb* gene have severely impaired placental formation, and die at 10.5 days post-coitum. The mutants display abnormal chorion development associated with an overabundance of trophoblast giant cells and a severe deficiency of diploid trophoblast. The phenotype can be rescued by aggregation of *Estrrb* mutant embryos with tetraploid wild-type cells, which contribute exclusively to extra-embryonic tissues. Our results indicate that ERR- β has an important role in early placental development, and suggest that an inductive signal originating from or modified by the chorion is required for normal trophoblast proliferation and differentiation.

During early embryonic development in the mouse, expression of the gene encoding ERR- β is restricted to extra-embryonic tissues (Fig. 1). ERR- β transcripts are first detected in a subset of cells in

extra-embryonic ectoderm at 5.5 days post-coitum (d.p.c.) and become more prominently expressed at 6.0 d.p.c. (Fig. 1a–d). At 6.5 d.p.c., ERR- β is expressed by ectodermally derived regions of the amniotic fold (Fig. 1e, f); these cells then form the chorion, where ERR- β expression is present at 7.5 d.p.c. (Fig. 1g, h). ERR- β expression diminishes as the chorion fuses with the ectoplacental cone, and by 8.5 d.p.c. ERR- β transcripts are present only at the free margin of the chorion (Fig. 1i, j). ERR- β transcripts are not detected in extra-embryonic tissue at subsequent stages of development. This highly specific expression pattern suggests that ERR- β may be important for the formation or function of the chorion.

To assess the role of ERR- β in placental development, we have created homozygous null *Estrrb* mutants by homologous recombination. Targeted disruption of the *Estrrb* gene was achieved in R1 embryonic stem (ES) cells by replacement of the second *Estrrb* exon with a neomycin phosphotransferase cassette (Fig. 2a). Two independent clones containing the predicted mutant allele produced male chimaeric mice that transmitted the mutation through the germ line (Fig. 2b). Mice heterozygous for the mutation were viable and were bred to generate homozygous mutants. No homozygotes were found in 62 live-born progeny of heterozygous intercrosses, indicating that the *Estrrb* mutation caused embryonic lethality. Embryos were genotyped at various stages of gestation to determine when fetal death occurred; viable homozygous mutants were not detected later than 10.5 d.p.c. (Fig. 2c and Table 1). Embryos examined at 9.5 d.p.c. demonstrated growth failure as well as absence of chorioallantoic fusion (Fig. 3a). At age 10.5 d.p.c., *Estrrb*^{−/−} embryos had no detectable heartbeat and had started to undergo resorption (Fig. 3b). These results indicate that the *Estrrb* mutation was lethal at 9.5–10.5 d.p.c., and that *Estrrb* ablation was associated with abnormal placental development.

Histological examination showed that embryonic and extra-embryonic tissues in *Estrrb* homozygous mutants were normal at 6.5 d.p.c. (Fig. 3c, d). In particular, development of the ectoplacental cone and the extra-embryonic ectoderm appeared normal in the mutant embryos. At 7.5 d.p.c., the chorion of the *Estrrb* homozygous mutants was either absent or hypoplastic and the embryos displayed varying amounts of growth retardation (Fig. 3d, f). In all cases, the extra-embryonic tissues appeared viable. At 8.5 d.p.c., abnormalities were seen in the development of early placenta and the ectoplacental cone, as the *Estrrb* mutants showed a marked increase in the number of secondary giant cells accompanied by a near absence of diploid trophoblast cells. Further, chorioallantoic fusion did not occur, and the placenta was absent or markedly decreased in size (Fig. 3g–j). Although most of the mutant embryos at 8.5 d.p.c. did not show obvious abnormalities, the mutants with the most marked placental abnormalities tended to be smaller and to have disorganized internal structure. By 9.5 d.p.c., the mutant placentas contained multiple layers of giant cells with no labyrinthine trophoblast or spongiotrophoblast (Fig. 3k–n). Mice derived from two independent targeted ES cell lines studied in C57BL, CD1 and 129/Sv genetic backgrounds displayed identical phenotypes (data not shown).

To confirm the histological findings in *Estrrb* mutant trophoblast tissues, expression of several markers of trophoblast differentiation

Table 1 Genotype of offspring from ERR- β heterozygous parents

Stage	Total	Genotype		
		+/+	+/-	-/-
E7.5	81	20	41	20
E8.5	134	33	64	37
E9.5	40	7	24	9*
E10.5	44	14	22	8*
E11.5	46	14	32	0
E12.5	10	3	7	0
Term	8	4	4	0
P28	54	19	35	0

E, embryonic day number; P, postnatal day number.

* Abnormal embryos.

Figure 1 Expression of *Estrrb* in trophoblast tissues. Bright-field images of normal embryos at 5.5 (a), 6.0 (c), 6.5 (e), 7.5 (g) and 8.5 (i) d.p.c. RNA *in situ* hybridization was performed using a 320-bp riboprobe specific for *Estrrb* exon 2. *Estrrb* transcripts are present in the extra-embryonic ectoderm at 5.5 (b), 6.0 (d) and 6.5 (f) d.p.c. At 7.5 d.p.c., *Estrrb* transcripts are exclusively detected in chorion (h), and at 8.5 d.p.c. are present only at its free margin (j). *Estrrb* expression was not detected in the ectoplacental cone at any stage of development, nor was placental expression detected after 8.5 d.p.c. Arrows indicate regions of specific hybridization; ch, chorion; ee, extra-embryonic ectoderm; ma, maternal decidua.

Figure 2 Targeted disruption of the *Estrrb* gene and heterozygote inbreeding analysis. **a**, Schematic representation of the ERR- β locus (wild type, top), targeting vector (middle), and targeted allele (recombinant, bottom). Arrows indicate the positions of primers P1, P2 and P3 used to genotype the offspring of heterozygous matings. Shaded rectangles represent the 5' and 3' probes used for Southern analysis of ES cell colonies. P, *Pst*I; X, *Xho*I. **b**, Southern blot analysis of targeted ES clones. DNA from parental ES cells (R1) and targeted clones (81 and 118) was digested with *Pst*I and hybridized to the 5' probe. The positions of bands corresponding to the wild-type allele (WT, 6.2 kb) and the mutant allele (KO, 2.8 kb) are indicated. **c**, PCR analysis of genotypes of embryos at 8.5 d.p.c. from a heterozygote intercross. **d**, ERR- β mutant embryos are present among the progeny at 12.5 d.p.c. of heterozygous intercross rescued by tetraploid aggregation.

was examined by RNA *in situ* hybridization. Expression of the *Mash-2* gene, which is a marker of spongiotrophoblast, labyrinthine trophoblast cells and their precursors³, was examined in *Estrrb* mutant embryos. At 7.5 d.p.c., *Mash-2* expression was not changed in the trophoblast region of *Estrrb*^{-/-} embryos (data not shown). Diploid trophoblast cells expressing the *Mash-2* gene were reduced in number at 8.5 d.p.c. (Fig. 4a–d) and absent at 9.5 d.p.c. in the *Estrrb*^{-/-} trophoblasts (Fig. 4g–j). Similar results were obtained with the markers 4311 (ref. 4) and Flt-1 (ref. 5), which have expression restricted to the spongiotrophoblast and its precursors (data not shown). In contrast, expression of transcripts for the trophoblast giant-cell markers placental lactogen-1 (ref. 6) and proliferin (ref. 7; data not shown) were markedly increased at both 8.5 d.p.c. (Fig. 4e, f) and 9.5 d.p.c. (Fig. 4k, l). To determine when diploid

trophoblast proliferation in *Estrrb* mutants became impaired, we examined the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA in trophoblast cells at different stages of development. At 7.5 d.p.c., the fraction of diploid trophoblast cells incorporating BrdU was similar in wild-type and mutant ectoplacental cones (approximately 85% of cells). However, at 8.0 and 8.5 d.p.c. the fraction of *Estrrb*^{-/-} diploid trophoblast cells incorporating BrdU was less than half that of normal trophoblast (between 30% and 40% of cells). Taken together, these results confirmed the histological observations and demonstrated that reduced proliferation of diploid trophoblast cells first occurred a few hours after failure of chorion development.

By 9.5 d.p.c., *Estrrb*^{-/-} embryos displayed impaired development, as would be expected if placental function were significantly

Figure 3 Morphology of homozygous mutant embryos and trophoblast tissues. **a, b**, Control and *Estrrb* mutant embryos at 9.5 (**a**) and 10.5 (**b**) d.p.c. At 9.5 d.p.c., the allantois of the *Estrrb* mutant has not fused to form the placenta. By 10.5 d.p.c., the *Estrrb* mutant embryo is dead and undergoing resorption. **c, d**, Wild-type (**c**) and mutant (**d**) embryos at 6.5 d.p.c. Development of the embryonic and extra-embryonic structures appear normal. **e, f**, Wild-type (**e**) and mutant (**f**) embryos at 7.5 d.p.c. The growth of the mutant embryo is retarded, and the chorion is diminished in size. **g, h**, Wild-type (**g**) and mutant (**h**) embryos at 8.5 d.p.c. The chorion and ectoplacenta of the mutant embryo are dramatically reduced in size with increased numbers of secondary giant cells. **i, j**, Higher power views of **g** and **h**, respectively. **k, l**, Wild-type (**k**) and mutant (**l**) trophoblast tissue at 9.5 d.p.c. In the mutant, diploid trophoblast cells are absent and secondary giant cells form multiple layers. **m, n**, Higher power views of **k** and **l**. Abbreviations: ac, amniotic cavity; al, allantois; ch, chorion; ee, extraembryonic ectoderm; gi, giant cells; la, labyrinthine trophoblast; ma, maternal decidua; pc, proamniotic cavity; pl, placenta; sp, spongiotrophoblast; tr, trophoblast.

impaired. Expression of ERR- β during early fetal development was present in specific regions of extra-embryonic ectoderm, although no ERR- β transcripts were detected in the embryo. This suggested that embryonic growth failure resulted from changes in placental function, a hypothesis that could be tested by performing tetraploid embryo rescue experiments^{8,9}. Diploid morulae from *Estrrb*^{+/-} intercrosses were aggregated with four-cell-stage wild-type tetraploid embryos, and the resulting chimaeras were transferred to CD1 foster mothers. In these experiments, wild-type tetraploid cells cannot contribute to embryonic cell lineages, but can contribute to trophoblast lineages, where they might compensate for defects present in the *Estrrb*^{-/-} offspring. Because *Estrrb*^{-/-} embryos die by 10.5 d.p.c., the pregnancies were allowed to continue to 12.5 d.p.c., when the embryos were genotyped (Fig. 2d). The offspring of four independent rescue experiments included eight *Estrrb*^{-/-} embryos (19% of the recovered embryos). In one experiment, the tetraploid component was marked by ubiquitous expression of the *Escherichia coli* β -galactosidase gene. This provided visual confirmation that all of the trophoblast cells were wild type, and therefore blue, in rescued -/- embryos. The *Estrrb*^{-/-} embryos were externally indistinguishable from their *Estrrb*^{+/+} littermates. These experiments indicate that the early embryonic growth retardation and lethality results solely from abnormal placental development in the *Estrrb*^{-/-} embryos.

We have shown that the nuclear hormone receptor ERR- β is essential for normal placental formation. Ablation of ERR- β in extra-embryonic ectoderm results in abnormal chorion formation followed by failure of diploid trophoblast self-renewal, increased formation of giant cells, placental failure and embryonic death. Impaired diploid trophoblast proliferation has been observed when ectoplacental cone fragments are cultured *in vitro*¹⁰ or grown ectopically¹¹. Trophoblast proliferation could be at least partly restored by transplanting ectoplacental cone fragments into the amniotic cavities of embryos at 7.5 d.p.c.; however, these growth conditions incompletely suppressed the formation of giant cells, suggesting that additional signals from extra-embryonic tissues were required for normal trophoblast development^{12,13}. ERR- β is expressed in only a subset of diploid trophoblast cells making up the chorion, and disruption of its function causes widespread failure of

diploid trophoblast proliferation, which occurs only after impaired formation of chorion. It is therefore possible that ERR- β mediates some critical function of the chorion in producing or modulating a diffusible signal that is necessary to support proliferation and block terminal differentiation of the diploid trophoblast. Common methods of influencing fertility have used drugs that target the oestrogen and progesterone receptors^{14,15}. As the classic targets of these hormonal agents do not seem to play a unique role in regulating implantation and placentation^{16,17}, identification of more specific means of influencing fertility could significantly increase the reproductive health of women. Pharmacological modulation of the activity of ERR- β , a nuclear hormone receptor that plays an essential role in early placentation, may provide means by which this may be accomplished. □

Methods

Targeted disruption of the *Estrrb* locus. The targeting vector was constructed using genomic DNA from a 129/Sv mouse strain. The 1.8-kilobase upstream region and the 4.1-kb downstream region of exon 2 were included in the targeting vector. The targeting vector replaces the first zinc-binding motif of the DNA-binding domain of the ERR- β protein with a *neo* cassette. The linearized construct was electroporated into R1 ES cells¹⁸, which were then selected with G418 (150 μ g ml⁻¹) and gancyclovir (2 μ M). Selected ES colonies were screened by probing *Pst*I-digested ES cell DNA with the 5' external probe, which hybridized to a 6.2-kb restriction fragment in the wild-type allele and 2.8-kb fragment in the knockout allele. We obtained 12 correctly targeted clones from 165 double-resistant colonies. Single integration of the targeting construct in these ES cell clones was confirmed by reprobing the Southern blots with a *neo* probe (data not shown). Appropriate integration of the 3' end of the targeting construct was verified using Southern blot analysis of *Xho*I-digested DNA using the 3' external probe (data not shown). Three clones were injected into CD1 blastocysts to generate chimaeras. Chimaeric males from two independent clones (81 and 118) passed the mutation to their offspring. PCR analysis of genotypes of embryos from a heterozygote intercross was performed using primers located in *Estrrb* intron 1 (P1, 5'-TCTTGCCATCCTTAGGCCAGC-3'), the 5' region of *Estrrb* exon 2 (P2, 5'-AACTAGAGCCCAGGTGCCTGT-3') and the *neo* cassette (P3, 5'-CCTCTGAGCCCAGAAAGCGAA-3'). DNA was obtained from embryonic tissue scraped from histological sections or from the yolk sac. Primers P1

Figure 4 Expression of cell-type-specific markers in trophoblast tissues. Bright-field images of normal placentas at 8.5 (a) and 9.5 (g) d.p.c., and the corresponding sections for *Estrrb* mutants (b and h). RNA *in situ* hybridization was performed on near-adjacent sections using probes for Mash-2 (c, d, i and j) and PL-1 (e, f, k and l). Expression of the placental markers confirms a progressive reduction in the number of diploid trophoblast cells accompanied by a marked increase in the number of trophoblast giant cells between 8.5 and 9.5 d.p.c.. Abbreviations: al, allantois; gi, giant cells; ma, maternal decidua; pl, placenta; tr, trophoblast.

and P2 amplify a fragment of 291 base pairs from the wild-type allele; primers P1 and P3 amplify a product of 440 bp, indicating the presence of the targeted allele.

Histological and *in situ* hybridization studies. Embryos and trophoblast tissues from heterozygous intercrosses were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (7 µm) were stained with haematoxylin and eosin¹⁹ or processed for *in situ* hybridization with radio-labelled riboprobes²⁰. Genotypes of embryos were identified by PCR using DNA from embryonic tissues scraped from histological sections.

BrdU labelling of trophoblast tissues. BrdU (100 µg per gram of body weight) was injected intraperitoneally into pregnant female mice. The mice were killed 1 h later, and the embryos and trophoblast tissues were removed and fixed in 4% paraformaldehyde and processed for immunohistochemistry. The genotype of each embryo was identified by PCR using DNA from embryonic tissues scraped from sections. Sections were incubated with an anti-BrdU monoclonal antibody²¹ (BROMO-2), and antibody binding was visualized with anti-mouse immunoglobulin-alkaline phosphatase (Boehringer Mannheim). Sections were stained with haematoxylin to visualize nuclei and allow cells to be counted.

Generation of tetraploid aggregation chimaeras. Electrofusion of two-cell-stage blastomeres collected from CD1 female mice or CD1 females crossed with male homozygous for the ROSA-26 gene trap insertion²² was used to produce wild-type tetraploid embryos. The fused embryos were cultured overnight in embryo culture medium in 5% CO₂ at 37 °C. Eight-cell-stage diploid embryos were recovered from the oviduct of embryonic-day (e)2.5 pregnant ERR-β heterozygote females mated to an ERR-β heterozygote male. Two four-cell tetraploid embryos were sandwiched around a diploid eight-cell-stage embryo from the heterozygous cross and aggregated overnight. Successfully aggregated embryos were transferred to e2.5-pseudopregnant CD1 recipients. Recipients were killed at e12.5 and embryos were genotyped and examined. Those aggregates made with ROSA-26 tetraploid embryos were also stained for β-galactosidase activity in the placenta, yolk sac and embryonic components²³.

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Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells

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Dendritic cells have the remarkable property of presenting any incoming antigen¹. To do so they must not only capture antigens with high efficiency and broad specificity, but must also maximize their capacity to load class II molecules of the major histocompatibility complex (MHC) with antigenic peptides in order to present a large array of epitopes from different proteins, each at a sufficient copy number. Here we show that formation of peptide–MHC class II complexes is boosted by inflammatory stimuli that induce maturation of dendritic cells. In immature dendritic cells, class II molecules are rapidly internalized and recycled, turning over with a half-life of about 10 hours. Inflammatory stimuli induce a rapid and transient boost of class II synthesis, while the half-life of class II molecules increases to over 100 hours. These coordinated changes result in the rapid accumulation of a large number of long-lived peptide-loaded MHC class II molecules capable of stimulating T cells even after several days. The capacity of dendritic cells to load many antigenic peptides over a short period of initial exposure to inflammatory stimuli could favour presentation of infectious antigens.

A key feature of dendritic cells is that they can capture antigens in peripheral tissues and migrate to secondary lymphoid organs where they acquire the ability to stimulate naive T cells¹. This maturation process was originally noted in freshly isolated Langerhans cells which, upon culture *in vitro*, lost acidic organelles and the capacity to synthesize class II molecules and to present soluble antigen, while acquiring the ability to stimulate T cells^{1–6}. Although the final results of the maturation process is loss of antigen-capturing and -processing capacity, it is not known whether the generation of peptide–MHC class II complexes is enhanced during the transition from immature to mature dendritic cells.

We previously described an *in vitro* culture system suitable for analysing the maturation process of dendritic cells. Human monocytes cultured in medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 develop into a homogeneous population with the characteristics of immature dendritic cells, namely high endocytic activity and a low capacity for stimulating T cells. When challenged by inflammatory stimuli such as tumour-necrosis factor (TNF)-α, IL-1 and lipopolysaccharide (LPS), these cells can no longer endocytose, they upregulate adhesion and co-stimulatory molecules, and become mature stimulatory dendritic cells^{7,8}. We used this system to study the effect of dendritic cell maturation on class II-restricted antigen presentation.

Cell-surface and total class II molecules were quantified in intact and permeabilized cells. As shown in Fig. 1a, immature dendritic cells express high levels of total class II molecules, but only a small fraction is present on the cell surface. During the first 24 hours after