Keratin 8 protection of placental barrier function

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The intermediate filament protein keratin 8 (K8) is critical for the development of most mouse embryos beyond midgestation. We find that 68% of K8\(^{-/-}\) embryos, in a sensitive genetic background, are rescued from placental bleeding and subsequent death by cellular complementation with wild-type tetraploid extraembryonic cells. This indicates that the primary defect responsible for K8\(^{-/-}\) lethality is trophoblast giant cell layer failure. Furthermore, the genetic absence of maternal but not paternal TNF doubles the number of viable K8\(^{-/-}\) embryos. Finally, we show that K8\(^{-/-}\) concepti are more sensitive to a TNF-dependent epithelial apoptosis induced by the administration of concanavalin A (ConA) to pregnant mothers. The ConA-induced failure of the trophoblast giant cell barrier results in hematoma formation between the trophoblast giant cell layer and the embryonic yolk sac in a phenocopy of dying K8-deficient concepti in a sensitive genetic background. We conclude that the lethality of K8\(^{-/-}\) embryos is due to a TNF-sensitive failure of trophoblast giant cell barrier function. The keratin-dependent protection of trophoblast giant cells from a maternal TNF-dependent apoptotic challenge may be a key function of simple epithelial keratins.

Introduction

The first epithelial cells of the mammalian embryo, trophectoderm, and later trophoblast derivatives form the cellular interface between maternal and embryonic environments. Type II keratin 8 (K8)* and type I keratin 18 (K18) are the first intermediate filament proteins expressed during embryogenesis and are diagnostic of the first epithelial cells (Brulet et al., 1980; Jackson et al., 1980). Subsequently, K8 and K18 are expressed in trophoblast derivatives, embryonic and extraembryonic endoderm (Jackson et al., 1981; Oshima, 1981), and simple epithelia of adult organs, such as liver, lung, kidney, pancreas, gastrointestinal tract, and mammary gland (Oshima et al., 1996). At least 49 keratins constitute a family of obligate, heteropolymeric intermediate filament proteins expressed in pairs in various epithelia (Moll et al., 1982; Hesse et al., 2001).

Although the function of epidermal keratins in providing mechanical strength to the epidermis is well documented (for reviews see Fuchs and Weber, 1994; Ma et al., 2001), the functions of the keratins of simple epithelium, K7, K8, K18, K19, and K20, have been more elusive. Tissue culture experiments have suggested a role of simple epithelium keratins in tissue culture experiments have suggested a role of simple epithelium keratins in invasion of extracellular matrix (Hendrix et al., 1996), drug resistance (Bauman et al., 1994; Parekh and Simpkins, 1995), and apoptosis (Caulin et al., 2000; Gilbert et al., 2001; Inada et al., 2001). K8, K18, and K19 knockout and dominant-negative K18 mutation mouse models have revealed functions during embryogenesis, in female reproduction, in colon homeostasis, and in providing protection against hepatotoxic drugs and other liver injuries (for review see Omary and Ku, 1997). The K8 knockout mutation results in embryonic lethality at midgestation in a C57BL/6; 129X1/SvJ (B6;129) hybrid genetic background (Baribault et al., 1993), whereas in a FVB/N genetic background, half of K8\(^{-/-}\) mice survive into adulthood (Baribault et al., 1995), and apoptosis (Caulin et al., 2000; Gilbert et al., 2001; Inada et al., 2001). K8, K18, and K19 knockout and dominant-negative K18 mutation mouse models have revealed functions during embryogenesis, in female reproduction, in colon homeostasis, and in providing protection against hepatotoxic drugs and other liver injuries (for review see Omary and Ku, 1997). The K8 knockout mutation results in embryonic lethality at midgestation in a C57BL/6; 129X1/SvJ (B6;129) hybrid genetic background (Baribault et al., 1993), whereas in a FVB/N genetic background, half of K8\(^{-/-}\) mice survive into adulthood (Baribault et al., 1994). These (FVB/N) K8\(^{-/-}\) mice develop a severe colorectal inflammation and hyperplasia. (FVB/N) K8\(^{-/-}\) mice are also more susceptible to liver injuries induced by porphyrogenic drugs (Zatloukal et al., 2000), microcystin-LR (Toivola et al., 1998), partial hepatectomy (Loranger et al., 1997), and hepatic apoptosis caused by concanavalin A (ConA) (Caulin et al., 2000). K8\(^{-/-}\) females are largely sterile. In contrast, K18\(^{-/-}\) and K19\(^{-/-}\) mice develop normally to adulthood and are fertile (Magin et al., 1998; Tamai et al., 2000). However, both K8\(^{-/-}\) and K18\(^{-/-}\) mice are more
sensitive to ConA-induced apoptosis in the liver where K8 and K18 are the only expressed intermediate filament proteins (Caulin et al., 2000). Similarly, transgenic mice expressing a dominant-negative K18 gene have an increased susceptibility to liver injury (Ku et al., 1995, 1996). The phenotypes of compound, simple epithelial keratin knockouts reflect overlapping functions for K18 and K19 and the obligate heteropolymeric stabilization of keratin subunits. K18+/–; K19−/− compound homozygotes die at E9.5 associated with a disruption of the trophoblast giant cell layer (Hesse et al., 2000). This was interpreted as increased fragility of K8−/− trophoblast cells. Similarly, K8−/−; K19−/− double mutants on an FVB/N background have trophoblast tissue abnormalities and develop hematoma beneath the yolk sac before death at about embryonic day E10.5 (Tamai et al., 2000).

We present three lines of evidence that support the view that K8 provides trophoblast giant cells resistance to a maternal challenge that results in failure of the trophoblast barrier function and eventually death of the embryo. The evidence includes the following: (1) the rescue of K8−/− lethality by tetraploid wild-type extraembryonic tissues; (2) the partial rescue of K8−/− embryos by the absence of maternal but not paternal TNF and TNF receptor (TNFR)2; and (3) the experimental induction of K8−/− trophoblast giant cell barrier function failure by treatment of mothers with ConA, a known nonspecific activator of the immune system. K8-deficient embryos die as a consequence of the failure of trophoblast giant cell barrier function.

**Results**

Nearly all (B6:129) K8−/− embryos die in utero by E12.5 with internal hemorrhages, which seem to originate from embryonic liver tissues (Baribault et al., 1993). However, these changes may be caused by primary failure of extraembryonic tissues, which separate the embryo from the maternal environment and also ensure nutrient supply. For example, placental malfunction in retinoblastoma-deficient mice causes multiple indirect embryonic defects (Wu et al., 2003). We have tested whether the K8−/− embryonic lethality originates from extraembryonic tissues by generating chimeric concepti in which the embryo proper was derived from k8-deficient embryos, and most extraembryonic tissues were derived from wild-type tetraploid cells.

Embryos resulting from heterozygous intercrosses were aggregated with tetraploid wild-type embryos and then reimplanted in the uteri of foster mothers. The concepti were allowed to develop to either E16.5 or to term. Tail samples were collected from E16.5 embryos and newborn pups for genotyping by PCR (Table I). 3 out of 19 E16.5 embryos (16%) were homozygous for the K8 knockout mutation, suggesting that the presence of K8 in extraembryonic tissues protected the embryos from dying at E12 (Table I). We confirmed the absence of K8 in all three E16.5 embryos proper, including in the liver and the intestine by immunofluorescence staining (unpublished data). Extraembryonic tissues, including the yolk sac and the placenta, stained positive for the presence of K8 (unpublished data). These three embryos appeared normal with no traces of internal bleeding. Three resorbing embryos were found in these experiments. These embryos were identified as wild-type and heterozygous by PCR. Thus, these resorptions appeared to be incidental to embryo manipulations rather than genotype related.

When the chimeric concepti were allowed to develop to term, 3 out of 17 newly born pups (18%) were homozygous mutants (Table I). A total of 6 out of 36 mice (16.7%) of the mice resulting from tetraploid-diploid aggregation were K8−/−. This represents 68% of the expected frequency of K8−/− embryos. This reflects an excellent rate of rescue because of the random distribution of donor cells to the trophoblast layer. These results confirmed that K8 expression in extraembryonic tissues is necessary for the development of (B6:129) concepti, whereas K8 expression in the embryo proper is dispensable for its development to birth. The (B6:129)K8−/− pups developed normally to adulthood without suffering anorectal prolapse as found for 88% of (FBV/N)K8−/− mice up to 1 yr old (Baribault et al., 1994). This is consistent with the lack of anorectal prolapse previously found in six rare, spontaneous (B6)K8−/− escape mice.

In control experiments, (B6:129)K8−/− embryos resulting from heterozygous mating were cultured overnight and transferred to pseudopregnant female recipient without previous aggregation with tetraploid embryos (Table I). No homozygous mutant was identified among the resulting progeny either at E16.5 or at birth. These results confirmed that the rescue of the (B6:129)K8−/− embryos was not due to the transfer to CD-1 recipients but to K8 expression in the extraembryonic tissues of chimeric concepti.

**Hematoma formation in (B6:129)K8−/− concepti**

The extraembryonic tissues of additional mutant concepti (B6:129) from heterozygous intercrosses were analyzed histologically. Consistent with the functional demonstration that K8−/− embryonic lethality originated from a defect in extraembryonic tissues, the dissection of the uterine wall of heterozygous females revealed partially coagulated blood between the decidua and the yolk sac attached to K8-deficient embryos starting at E10.5. The yolk sac, placenta, and em-

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**Table I. Genotype analysis of the progeny resulting from tetraploid-diploid aggregation experiments**

| Type of transplanted embryos | Stage of analysis | K8+/− | K8+/− | K8−/− |
|-------------------------------|------------------|-------|-------|-------|
| Tetraploid-diploid aggregates  | E16.5            | 4     | 12    | 3     |
|                               | Birth            | 4     | 10    | 3     |
| Diploid embryos               | E16.5            | 4     | 5     | 0     |
|                               | Birth            | 9     | 12    | 0     |

Preimplantation diploid embryos were collected from (B6:129)K8−/− intercrosses and aggregated with wild-type tetraploid four-cell stage embryos. Chimeric embryos were allowed to develop to the blastocyst stage and were then transferred to the uteri of foster mothers. E16.5 embryos and newly born pups were genotyped. In a control experiment, morulae collected from (B6:129) heterozygous intercrosses were cultured overnight without aggregation with tetraploid embryos. These nonchimeric blastocysts were transferred to the uteri of a pseudopregnant female recipient. The resulting E16.5 embryos and newborn pups were genotyped. The numbers of K8+/−, K8+/−, and K8−/− embryos are presented.
bryo proper appeared normal at this stage (Fig. 1, A and B). Histological examination of five homozygous mutant con-
cepti revealed the presence of massive hemorrhages of mater-
nal blood confined between the decidua capsularis and the
parietal yolk sac (Fig. 1, B and D). Resulting hematomas
consisted of nonnucleated maternal erythrocytes and fibrin
aggregates infiltrated with granulocytes. Hematomas in mu-
tant concepti were apparently due to the disruption of tro-
phoblast giant cell layer (Fig. 1 E) that normally forms a bar-
er between the maternal and embryonic compartments. At
the site of disruption, trophoblast giant cells undergo degen-
eration (Fig. 1 F). The overall structure of placenta and the
yolk sacs in K8<sup>−/−</sup> concepti (Fig. 1, D and E) and the struc-
ture of embryo proper (unpublished data) at E10.5 was mor-
phologically normal.

Figure 1. Morphological and histological analysis of (B6;129)
wild-type and K8<sup>−/−</sup> extraembryonic tissues. Wild-type (A) and
K8<sup>−/−</sup> (B) E10.5 concepti from which the uterine wall and part of
decidua are removed. Note the hematoma (h) located between the
decidual tissue (dec) and the yolk sac (ys) in the K8<sup>−/−</sup> concepti. In
mutant conceptus, the placenta (p) is hidden behind the hematoma.
(C and D) Sagittal histological sections of E10.5 wild-type (C) and
K8<sup>−/−</sup> (D) concepti. In the wild-type conceptus, the embryo proper
(em) is surrounded by the amnion (am), visceral yolk sac (vys), parietal
yolk sac (pys), the layer of trophoblast giant cells (arrowheads), and
maternal decidual tissue (dec). In the K8<sup>−/−</sup> conceptus, the hematoma
(h) is located between the decidual tissue (dec) and parietal yolk sac
(pys), p, placenta. Bar, 200 µm. (E and F) Trophoblast giant cells in
E10.5 wild-type (E) and K8<sup>−/−</sup> (F) concepti at higher magnification.
In wild-type conceptus, the continuous layer of trophoblast giant
cells (gc) divides maternal decidua (dec) from the parietal yolk sac
(pys). Note the disrupted layer of trophoblast giant cells (gc), which
undergo degeneration, maternal erythrocytes (er), and fibrin (fb),
infiltrated with granulocytes in K8<sup>−/−</sup> conceptus. Bar, 50 µm.

K8<sup>−/−</sup> death depends on maternal TNF

Some K8<sup>−/−</sup> epithelial cell lines are 100-fold more sensitive
to TNF-induced apoptosis (Caulin et al., 2000). To test geneti-
cally whether TNF may contribute to the death of K8<sup>−/−</sup>
embryos, we combined the K8 deficiency (FVB/N) with
TNF deficiency (C57Bl/6;129). The FVB/N genetic back-
ground was chosen for the K8<sup>−/−</sup> mice to efficiently obtain
adequate numbers of progeny for statistical analysis. Inter-
breeding of the two targeted alleles resulted in mixed back-
ground (FVB/N;B6;129) K8<sup>−/−</sup> parents with either TNF<sup>+/+</sup>
or TNF<sup>−/−</sup> (Fig. 2, G3). The recovery of K8<sup>−/−</sup> progeny from three different crosses was measured. If TNF and the
maternal immune system participated in the death of K8<sup>−/−</sup>
embryos, a maternal dependence on TNF was expected.
Approximately twice as many K8<sup>−/−</sup> progeny were recovered
when the mother was TNF<sup>−/−</sup> (C1 and C2) than when the
mother was TNF<sup>+/+</sup> (Fig. 3, A and B). The difference in
K8<sup>−/−</sup> recovery from the C1 cross was significantly different
from the reciprocal C3 cross in which the father, rather than the
mother, was TNF<sup>−/−</sup> (Fig. 3 B). TNF deficiency of both
the mother and father did not yield greater recovery of K8<sup>−/−</sup>
progeny than when only the mother was deficient. Combin-
ing the results of both crosses in which the mothers were
TNF<sup>−/−</sup> (C1 and C2) further reinforced the conclusion.
However, a full recovery of K8<sup>−/−</sup> mice was not obtained.
The recovery of K8<sup>−/−</sup> mice increased from ~25% to
>50% of the number expected for Mendelian inheritance
(Fig. 2 C). The recovery of K8<sup>−/−</sup> and K8<sup>+/+</sup> mice did not
differ significantly from the expected number. Survival of
TNF<sup>+/+</sup> and TNF<sup>−/−</sup> progeny in the C2 and C3 crosses
were not statistically different (46 TNF<sup>+/+</sup> versus 51 TNF<sup>−/−</sup>).
Thus, TNF deficiency of the embryo does not influence sur-
vival. These data indicate that maternally expressed TNF is
deleterious to the survival of K8<sup>−/−</sup> embryos.

(FVB/N) K8<sup>−/−</sup> mice develop inflammatory bowel disease.
Thus, it was of interest to determine if the deficiency in TNF,
a common mediator of inflammation, altered this phenotype.
60% (12 out of 20) of informative K8<sup>−/−</sup>;TNF<sup>−/−</sup> mice sur-
vived for 250 d. Five animals that died prematurely were
confirmed to have anoestral prolapse and colonic hyperplasia
with inflammation (unpublished data). Similarly, 50% of the
K8<sup>−/−</sup>;TNF<sup>+/+</sup> mice (two out of four) survived for >250 d,
and the inflammatory bowel disease was confirmed in the two that died prematurely. Inflammatory bowel disease can develop in the absence of TNF in K8\(^{-/-}\) mice.

**Maternal TNFR2 deficiency increases viability of K8\(^{-/-}\) embryos**

We also tested the influence of TNFR2 deficiency on K8\(^{-/-}\) viability. K8\(^{+/+}\) (FVB/N) mice were bred with TNFR2\(^{-/-}\) (B6;129), and progeny were backcrossed with TNFR2\(^{-/-}\) (B6;129) to generate K8\(^{+/+}\);TNFR2\(^{-/-}\) males and either K8\(^{+/+}\);TNFR2\(^{-/-}\) or K8\(^{+/+}\);TNFR2\(^{++}\) mothers (analogous to C1 and C3 crosses of Fig. 2 A). The absence of TNFR2 in mothers resulted in a more than fourfold increase in the frequency of K8\(^{-/-}\) progeny (Fig. 4 A). The difference between the recoveries of K8\(^{-/-}\) progeny was statistically significant (Fig. 4 B). However, TNFR2 deficiency also failed to completely rescue K8\(^{-/-}\) lethality (Fig. 4 C). TNFR2-deficient embryos were found as frequently as TNFR2 heterozygotes. Thus, both embryonic TNFR2 and TNF are dispensable for embryonic development. In the presence of maternal TNFR2,
examined both the livers and concepti for pathological changes. ConA treatment of pregnant K8⁺/⁻; TNF⁺/⁻ mothers resulted in the expected liver apoptosis. However, both the liver and concepti of a single available K8⁺/⁻; TNF⁺/⁻ mother were resistant to the effects of ConA (unpublished data) consistent with previous investigations of male mice.

ConA causes larger hematoma formation in K8⁻/⁻ concepti
(FVB/N; B6;129) K8⁺/⁻; TNF⁺/⁻ females were mated with (FVB/N; B6;129) K8⁻/⁻; TNF⁻/⁻ males (both from the experiment shown in Fig. 2) to generate K8⁺/⁻ and K8⁻/⁻ embryos within a TNF⁺/⁻ maternal environment. At E9.5, mothers were treated with ConA, and a portion of each embryo was dissected for PCR analysis, and the remaining concepti were fixed, bisected, and processed for paraffin sections. Embryos removed from the yolk sacs were all similar size and without obvious defects. Stained sections revealed hematoma formation in K8⁻/⁻ extraembryonic tissues very similar to the phenotype of the spontaneous K8⁻/⁻ concepti in the B6;129 genetic background at E10.5 (Fig. 5, A–F, and Fig. 1). Hematomas were commonly found between the trophoblast giant cell layer and the parietal yolk sac. Quantitation of bleeding in extraembryonic tissues revealed significantly increased bleeding in K8⁻/⁻ extraembryonic tissues (Fig. 5, G–I). The average hematoma area for 16 concepti from three litters was 4,578 arbitrary units compared with 1,607 for 14 K8⁺/⁻ concepti. (P = 0.007, two tailed t test). No significant correlation was found between the degree of bleeding and the TNF genotype of the embryos. Thus, K8⁻/⁻ concepti are more sensitive to a ConA-induced, maternal TNF-dependent loss of trophoblast giant cells barrier function. This phenotype is similar to that observed for K8⁻/⁻ concepti in a B6;129 genetic background without ConA treatment. ConA treatment of a single pregnant TNF⁻/⁻; K8⁺/⁻ mother of the same mixed genetic background did not result in visible hematoma formation. This suggests that both liver apoptosis and concepti hematoma formation caused by ConA treatment of pregnant mothers is dependent on maternal TNF.

Apoptotic death of trophoblast giant cells
Immunohistochemical analysis of extraembryonic tissues of ConA-treated mice confirmed the expression of K8 in trophoblast derivatives, including spongiotrophoblast, giant
cells, and epithelial cells, in the labyrinth region recognized by the presence of nucleated embryonic blood cells (Fig. 6 A). The placenta of E10 K8+/− embryos had a relatively normal structure but lacked K8 (Fig. 6 B). Staining nuclei for DNA nicks by the TUNEL method revealed apoptotic giant cells (Huen et al., 2002). The tissue shown in E and F represent an adjacent uterine wall region which has folded up against the placental region as revealed by tracing the folded yolk sac basement membrane. This results in the atypical juxtaposition of parietal yolk sac to delimit the hematomas and previous observations that extraembryonic endodermal cell formation and function from K8−/− ES cells was not impaired (Baribault and Oshima, 1991) suggests that the trophoblast giant cell layer, not the extraembryonic endoderm, is defective in K8−/− concepti. Trophoblast giant cells are derivatives of mural extraembryonic ectoderm (primary giant cells) and the ectlaplacental cone (secondary giant cells). They form an epithelial boundary between the maternal tissue and the embryonic environment (Cross et al., 1994; Cross, 2000; Ward and Devor-Henneman, 2000). Trophoblast giant cells also interact actively with maternal tissues, including the immune system, and produce several angiogenic factors and hormones (Cross, 2000).

The phenotype observed for dying (B6;129) K8−/− concepti is similar to that found when K8 and K19 or K18 and K19 deficiencies in FVB/N or mixed backgrounds are combined (Hesse et al., 2000; Tamai et al., 2000). These experiments indicate functional compensation between different simple epithelial keratin pairs (Oshima, 2002). Maternal TNF and TNFR2 deficiencies result in an increase in viable K8−/− progeny. This implicates TNF in the embryonic lethality of K8−/− embryos. Although maternal TNF deficiency results in a twofold increase in K8−/− progeny, it does not completely compensate. Other members of the TNF family or other cytokines may also be involved in the early death of K8−/− trophoblast cells. Alternatively, TNF and TNFR2 may be necessary for the activation of lymphoid cells or cytokine effectors. Circulating TNF and uterine natural killer cells are among possible direct effectors. Hepatic natural killer cells have been implicated in the ConA-induced liver pathology (Takeda et al., 2000). Natural killer cells also reside in the uterine environment of both rodents and humans (Moffett-King, 2002) where their cytolytic activity is inhibited. ConA stimulation may bypass the normal inhibition of uterine natural killer cells cytolytic activity (Muller et al., 1999). Our results are the first to associate the embryonic death of keratin-deficient concepti with TNF. The significant immunological differences between mice of different genetic backgrounds may contribute to the genetic background dependence of K8−/− embryonic lethality.

The molecular mechanism by which K8 provides protection to trophoblast cells from TNF remains to be determined. The protection that K8 and K18 heteropolymers provide cultured epithelial cell lines and hepatocytes against TNF- (Caulin et al., 2000; Inada et al., 2001) and fas- (Gilbert et al., 2001) mediated apoptosis may extend in vivo to derivatives of the first epithelial cells to form during development. The sequestration of the TRADD signal transduction adaptor protein by K18 provides an attractive model of keratin-dependent protection from apoptosis (Inada et al., 2001). It also remains to be determined if the absence of K8 alters the strength of desmosomal, intracellular adhesion of trophoblast cells (Huen et al., 2002).
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K18, and K19 are expressed in trophoblast derivatives. The expression of K8 and K18 keratins in the liver, whereas K7, K8, ConA-mediated hepatitis. Both K8 reflects the action of the host immune system, just as it does in the ConA sensitivity of the trophoblast barrier function re-

The use of ConA stimulation of pregnant TNF-positive mothers provided the opportunity of challenging K8−/− and K8−/− embryos in the same uterine environment. The ConA-induced hematomas of K8−/− concepti are similar to those observed for K8−/− concepti in the C57BL/6;129 genetic background and that reported for K18−/−, K19−/− and K8−/−; K19−/− compound homozygote concepti. ConA-induced liver apoptosis can be conferred by adoptive transfer of lymphoid cells and is dependent on TNF, TNFR1, TNFR2

GACCTG) and L6065 amplified the 601-bp mutant allele. For genotyping 887-bp wild-type fragment. Primers U5485 (CCTTAATATGCGAAGTG-

Materials and methods

ConA was purchased from Sigma-Aldrich. TROMA1 rat mAb against K8 was a gift from Rolf Kemler (Max-Planck-Institute für Immunbiologie, Freiburg, Germany).

Animal husbandry

Mice were housed under specific pathogen-free conditions with a 14/10 h light/dark cycle and standard temperature and humidity. They were fed standard laboratory chow and water ad libitum. The immunization was performed in the hypophyseal layer mice and the PCR genotyping procedure have been described previously (Baribault et al., 1993). The resulting hybrid progeny was back-

Motivation of hemorrhage size

Digital images of two hematoxylin and eosin–stained sections of individual uterine implantation sites were captured with a Spot digital camera and manipulated in Adobe Photoshop by using selective color replacement to enhance erythrocytes. After changing the picture to grayscale, a threshold was applied to produce a black and white picture. The pixel values were inverted, and the resulting black pixels, representing erythrocytes, were counted using NIH Image. Both halves of each implantation site were counted and averaged. All images of sections from the same litter were manipulated with the same threshold settings.

Generation of tetraploid-diploid aggregates

Super ovulated CD-1 females were mated with FVB/N males. E1.5 em-

Histology

Extraembryonic and embryonic tissues were dissected from individual utter-

ConA treatment

Mice were injected with ConA (30 mg/kg) in 200 μl pyrogen-free saline 10 d postcoitus. 5 h after injection, the mice were killed, and liver, spleen, and individual implantation sites within the uterine horns were fixed in 4% PFA or 1% acetic acid in ethanol, bisected, and both halves processed for embedding in paraffin. The cut sides of both halves of each implantation site were oriented to the cutting face so that increasing depths of both halves were included. The same site was sampled. 5-μm-thick sections were stained with hematoxylin and eosin for detection of apoptosis with the Apop Tag Kit (Intergen) according to the instructions of the manufacturer. Staining for K8 was performed using the Vectastain ABC Elite kit according to the in-

Collection of two-cell embryos and electro fusion

Super ovulated CD-1 females were mated with FVB/N males. E1.5 em-

Measurement of hemorrhage size

Digital images of two hematoxylin and eosin–stained sections of individual uterine implantation sites were captured with a Spot digital camera and manipulated in Adobe Photoshop by using selective color replacement to enhance erythrocytes. After changing the picture to grayscale, a threshold was applied to produce a black and white picture. The pixel values were inverted, and the resulting black pixels, representing erythrocytes, were counted using NIH Image. Both halves of each implantation site were counted and averaged. All images of sections from the same litter were manipulated with the same threshold settings.
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