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Syngeneic Immune-Dependent Abortions in Mice Suggest Paternal Alloantigen-Independent Mechanisms

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Abstract

Problem—Recurrent immune-associated miscarriages in humans are thought to result from maternal immune responses to paternal alloantigens. We investigated the role of paternal alloantigens in a mouse model of immune-dependent abortion.

Method of study—Sib-crosses of C57Bl/6J (haplotype b/b) mice heterozygous for a targeted hypomorphic allele of the *thp* gene (*thp*^{ΔN/+}) resulted in selective mid-gestational abortion of 88% of the *thp*^{ΔN/ΔN} fetuses. In dams lacking mature lymphocytes (*rag1*^{−/−}), nearly all *thp*^{ΔN/ΔN} fetuses survived to birth, indicating abortions were immune-dependent. Allogeneic pregnancies bearing *thp*^{ΔN/ΔN} fetuses were established by either hybridizing the paternal lineage to BALB/cJ (haplotype d/d) and mating hybrid *thp*^{ΔN/+} sires to haplotype b/b *thp*^{ΔN/+} C57Bl/6J dams, or by transfer of haplotype b/b zygotes from *thp*^{ΔN/+} × *thp*^{ΔN/+} matings into pseudopregnant wild-type CByD2F1/J dams (haplotype d/d).

Results—Neither hemizygous paternal allogeneic loci nor homozygous allogeneic loci, including a haplotype-mismatched major histocompatibility complex (MHC), increased abortion frequencies.

Conclusion—Results suggested that mechanisms for maternal tolerance of paternal alloantigens, including mismatched MHC antigens, were intact in these pregnancies, yet maternal immune-dependent paternal antigen-independent abortion of mutants occurred. These data indicate that, in some cases of immune-mediated abortions, the presence of paternal alloantigens can be coincidental and superfluous to the compromising rejection response.

Keywords

Inbred mice; major histocompatibility complex; pregnancy; semi-allogeneic; spontaneous recurrent abortion

Introduction

Most eutherian pregnancies expose the maternal immune system to paternal alloantigens; successful pregnancy requires maternal tolerance of these antigens.^{1–5} Immune-associated

early post-implantation abortions are common in humans and are thought to result from a failure in normal maternal tolerance to paternal alloantigens.^{4,6,7}

The mechanisms underlying maternal tolerance are not well understood. In humans, a strong correlation has been identified between immune-mediated abortion and a failure of the zygote-derived cells of the placenta (trophoblasts) to express genes encoding the non-classical major histocompatibility class I (MHC-I) molecules human leukocyte antigen-G (HLA-G) and HLA-E.^{8–10} An analogous system has not yet been clearly delineated in mice; however genes encoding classical MHC-I molecules and the gene encoding β 2-microglobulin (β 2m) are down-regulated in mouse trophoblasts.¹¹ Whereas this should restrict presentation of paternal antigens by trophoblasts, haplotype-mismatched pregnancies do result in maternal sensitization to paternal antigens.^{6,12,13} Moreover, in matings between certain haplotype-mismatched strains of inbred mice, some fetuses are lost to immune-associated abortions.^{14–16} Thus, tolerance mechanisms do not prevent maternal detection of paternal alloantigens, yet it has been suggested that maternal responses to paternal antigens can cause abortions in mice.^{17–20}

We previously developed a line of mice that exhibit hallmark characteristics of having a disruption in a fetal component of the maternal/fetal immune interaction.²¹ These mice bear a targeted hypomorphic mutation that replaces the endogenous *TATA-binding protein* (*tbp*) gene with a version, entitled *tbp* ^{Δ N}, lacking sequences encoding most of the vertebrate-specific N-terminus of this protein.²² Molecular studies on homozygous mutant (*tbp* ^{Δ N/ Δ N}) and wild-type (*tbp*^{+/+}) primary embryonic fibroblast cultures showed no gross differences in fundamental cellular properties.²³ Moreover, adult homozygous mutants are obtained, albeit at a very low frequency (~1%), and these generally exhibit no overt phenotypic abnormalities.²¹

In immune-normal dams, most but not all *tbp* ^{Δ N/ Δ N} fetuses die in mid-gestation.²¹ This effect is selective; all wild-type and most heterozygous litter-mates are unaffected. Mid-gestational survival can be achieved by providing the mutant embryo with wild-type tetraploid trophoctoderm at embryonic day 2.5 (E2.5), indicating the lethal defect is, at least partially, manifested in extra-embryonic tissues.²¹ Survival can also be achieved by rearing homozygous mutants with their own trophoctoderm in severely immune-compromised dams,²¹ suggesting that a maternal immune response participates in the abortions. Moreover, an increase in survival is measured when the homozygous-mutant zygote is also homozygous-null for β 2m.²¹ Surprisingly, however, the mice in these studies were purely haplotype-b and extensively back-crossed onto a C57Bl/6J background,²¹ suggesting that these immune-mediated abortions were occurring in syngeneic pregnancies.

At least one other genetically engineered mouse model of immune-dependent mid-gestational abortion that of fetuses lacking indoleamine 2,3-dioxygenase, an enzyme involved in tryptophan catabolism, is dependent on paternal alloantigens.^{24–26} Thus, we suspected that syngeneic immune-dependent abortions of *tbp* ^{Δ N/ Δ N} fetuses likely involved minor antigens, for example trophoblast-specific gene expression products that would still be present in syngeneic pregnancies. Moreover, we posited that if we provided stronger placental antigens, we should augment the rejection response. In this study, we tested whether introduction of paternal alloantigens exacerbated the rejection response. Results showed that fetal loss was independent of paternal allogeneic determinants. This study suggests that spontaneous immune-mediated abortions, even in situations where strong paternal alloantigens exist, can involve an immune response that is independent of paternal alloantigens.

Materials and methods

Animals and Care Conditions

The *thp*^{ΔN} mutation (on the proximal end of chromosome 17 at 8.25 cM) was derived in strain 129XI ES cells (haplotype b/b) and has been back-crossed into C57Bl/6J (haplotype b/b) for >10 generations.²¹ Mice bearing a targeted null mutation of the *rag1* locus on a C57Bl/6J background (stock #002096) and strains C57Bl/6J (stock #000664), BALB/cJ (stock #000651), and CByD2F1/J (stock #100015) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mutated alleles were followed by PCR-based analyses of genomic DNA using the primers and reaction conditions described previously.²¹ Fetal survival rates were determined at E14.5 ± 1 by PCR-based genotyping of genomic DNA from three independent tissue isolates (typically two limbs and the tail) from each fetus. All animals were housed under aseptic specialized care conditions, including continuous HEPA-filtered air-flow cages (Techniplast, Exton, PA, USA) and sterilized bedding, feed, and water. All mouse lines and other renewable resources developed for this study are available for unrestricted non-profit research use unless specifically regulated by other parties. Animal protocols for this study were approved by the Montana State University Institutional Animal Care and Use Committee.

Embryo and Ovary Transfers

For embryo transfers, donor dams (*thp*^{ΔN/+} on a C57Bl/6J × BALB/cJ hybrid background) were induced to superovulate using intraperitoneal (I.P.) injections of pregnant mare's serum gonadotrophin (5 I.U./animal, Calbiochem, La Jolla, CA, USA) followed 2 days later by human chorionic gonadotrophin (5 I.U./animal, Calbiochem, La Jolla, CA, USA.) and were then housed overnight with fertile sires (*thp*^{ΔN/+} on a C57Bl/6J × BALB/cJ background). Fertilized eggs were harvested from plugged females the following morning and were transferred into the oviducts of time-matched pseudopregnant wild-type CByD2F1/J dams (pseudopregnancy was achieved by coordinated natural matings of virgin CByD2F1/J females with vasectomized sires).

For ovary transfers, ovaries were harvested immediately upon killing from donor dams (*thp*^{ΔN/+}) and were transferred into both sides of ovariectomized recipient dams of genotype *thp*^{+/+;rag1}^{-/-}.²⁷ Recipients were allowed to recover for 3–4 weeks prior to timed matings with sires of genotype *thp*^{ΔN/+;rag1}^{+/+}. Fetuses were harvested at E14.5 ± 1 and three independent samples from each fetus were analyzed for both the presence of the *thp*^{ΔN} allele and the *rag1*⁻ allele (fetuses from transferred ovaries would be *rag1*^{+/+}; if residual recipient eggs or ovarian tissue contributed to a pregnancy, the resultant fetuses would be *rag1*^{-/+}).

Statistical Analyses

Founding studies²³ and subsequent analyses (A.L. Sealey and E.E. Schmidt, unpublished data) showed that matings of *thp*^{ΔN/+} sibs results in Mendelian embryonic and fetal ratios until E9.5, indicating that there is no loss of *thp*^{ΔN} gametes from either parent. Therefore, 1:2:1 Mendelian genotype distributions were predicted from numbers of *thp*^{+/+} fetuses, and percent survival was based on this prediction. Deviation from this Mendelian prediction was evaluated using a chi-squared test. To test for rescue or exacerbation of fetal loss, the percent survival of heterozygous and homozygous mutant fetuses for the control genotype set (typically *thp*^{ΔN/+} × *thp*^{ΔN/+} matings, Tables I and II, group A) was multiplied by the predicted Mendelian genotype distribution for the experimental genotype set to obtain a normalized control set. For example in Table I, the control group A (genotype ratio: 232:441:28) showed 95% survival of heterozygous fetuses and 12% survival of homozygous mutants. Experimental group B (genotype ratio: 29:59:26) had 29 wild-type fetuses, so our normalized control ratio for statistical analysis was 29:55:3.5. Deviation between experimental sets and the normalized control sets were then evaluated using the chi-squared test.

Results

Maternal Role in Mid-gestational Loss of $tbp^{\Delta N/\Delta N}$ Fetuses

Matings between $tbp^{\Delta N/+}$ parents result in 88% loss of $tbp^{\Delta N/\Delta N}$ fetuses at mid-gestation (reference²¹ and Table I, group A). Similarly, transfer of E0.5 zygotes from such matings into wild-type surrogates results in roughly 90% loss of $tbp^{\Delta N/\Delta N}$ fetuses.²¹ Conversely, nearly all $tbp^{\Delta N/\Delta N};ragI^{-/+}$ fetuses reared in $tbp^{\Delta N/+};ragI^{-/-}$ dams survive to birth (reference²¹ and Table I, group B), suggesting the maternal immune system participates in the selective mid-gestational abortions of $tbp^{\Delta N/\Delta N};ragI^{+/+}$ fetuses in $tbp^{\Delta N/+};ragI^{+/+}$ dams. To ensure that neither the zygotic heterozygosity at the *ragI* locus nor the maternal heterozygosity at the *tbp* locus in a *ragI*^{-/-} background contributed to this increased survival, ovaries from $tbp^{\Delta N/+};ragI^{+/+}$ donors were transferred into ovariectomized $tbp^{+/+};ragI^{-/-}$ surrogates and, after allowing 3–4 weeks for residual eggs to clear, these were mated to $tbp^{\Delta N/+};ragI^{+/+}$ sires. Results showed that all $tbp^{\Delta N/\Delta N};ragI^{+/+}$ fetuses survived past mid-gestation in the $tbp^{+/+};ragI^{-/-}$ surrogates (Table I, group C). This verified that abortions of $tbp^{\Delta N/\Delta N}$ fetuses in immune-competent dams were dependent on the maternal immune system.

Survival of Syngeneic Versus Allogeneic $tbp^{\Delta N/\Delta N}$ Fetuses

The $tbp^{\Delta N}$ mutation was generated on strain 129X1 ES cells (haplotype b/b) and has been back-crossed >10 generations onto C57Bl/6J (also haplotype b/b).²¹ Thus, in natural matings between $tbp^{\Delta N/+}$ animals, the dams possessed all autosomal and X-linked alleles at all loci in the genome that were found in their fetuses. Nevertheless, we measured only 12% survival of $tbp^{\Delta N/\Delta N}$ fetuses to E14.5 in these matings (Table I, group A).

To test whether paternal allogeneic determinants could exacerbate the rejection response, we hybridized only the paternal lineage to BALB/cJ and bred F1 or F2 BALB/cJ $\times$ C57Bl/6J hybrid $tbp^{\Delta N/+}$ males to C57Bl/6J $tbp^{\Delta N/+}$ females. Progeny from these matings would have, on average, half of their paternal loci derived from a source that was allogeneic to the mother. Results showed that 74% of the $tbp^{\Delta N/\Delta N}$ fetuses in these pregnancies were lost at mid-gestation (Table II, group D). This indicated that the presence of paternal alloantigens did not exacerbate the maternal rejection response; the slight increase in survival was not significant ($P > 0.05$), but may reflect a subtle hybrid vigor effect from hybridization of the fetal genetic background.

These results showed that, in reference to the maternal genetic composition (C57Bl/6J), the presence of most BALB/cJ-derived fetal allogeneic alleles did not influence the probability of immune rejection of $tbp^{\Delta N/\Delta N}$ fetuses. However, these studies could not assess the role of *tbp*-linked alloantigens because homozygous mutants were homozygous for all alleles on chr. 17. Importantly, this would include the MHC complex, which is near *tbp* on the proximal arm of chr. 17. Because MHC-dependent haplotype determinants are the dominant antigenic determinants of graft tolerance versus rejection and have been implicated in triggering immune-mediated abortion in mice, we wished to test whether rearing $tbp^{\Delta N/\Delta N}$ fetuses in surrogate mothers homozygous for a haplotype-mismatched chr. 17 would exacerbate the rejection response.

Synchronized matings were set up between fertile C57Bl/6J $\times$ BALB/cJ $tbp^{\Delta N/+}$ hybrid sires and dams (donors) and between vasectomized sires and fertile virgin CByD2F1/J dams (surrogates). Although the donor parents and their offspring were hybrid for most loci, all homozygous mutant pups were homozygous for the $tbp^{\Delta N}$ -bearing haplotype b chr 17. Fertilized eggs were harvested at E0.5 and were transferred into the oviducts of pseudopregnant virgin CByD2F1/J surrogates (haplotype d/d). Results showed 78% loss of the $tbp^{\Delta N/\Delta N}$ fetuses (Table II, group E), indicating that homozygous allogeneic MHC loci did not exacerbate loss

of the *thp*^{ΔN/ΔN} fetuses. We conclude that the presence of allogeneic loci, including severe MHC-haplotype mismatching, was inconsequential to the probability of immune-mediated abortion of *thp*^{ΔN/ΔN} fetuses.

Discussion

As a result of natural genetic variation between individuals within populations, nearly all eutherian pregnancies might be considered allografts, wherein half of the genetic constitution of the developing fetus is of paternal origin and potentially recognizable as ‘non-self’ to the mother.^{1,5} Spontaneous recurrent abortions are commonly associated with maternal immune manifestations including the appearance of antibodies that recognize paternal antigens.^{29–32}

Inbred lines of laboratory mice provide a system in which either allogeneic or syngeneic pregnancies can be established to test the roles of paternal alloantigens in the maternal–fetal interaction. Classical studies on gestational survival of hybrid fetuses in matings between CBA/J (haplotype k/k) dams and DBA/2J (haplotype d/d) sires showed an increase in spontaneous mid-gestational abortion (18–30% as compared to 0–9% in either CBA/J × CBA/J, DBA/2J × DBA/2J, or DBA/2J-dam × CBA/J-sire matings).^{14–16,30} Nevertheless, because, with the exception of gender differences, all fetuses in the CBA/J-dam × DBA/2J-sire matings are genetically identical F1 hybrids (haplotype k/d) between the strains, it is unclear why 18–30% are rejected while their sibs survive. Necessarily, some overarching epigenetic or stochastic mechanism must participate in determining which fetuses are selectively culled.

Previous studies showed increased numbers of natural killer (NK) cells at resorption sites in CBA/J × DBA/2J matings and correlative analyses suggested that NK cell accumulation at a subset of implantation sites (presumably those destined to abort) precedes resorption.^{33,34} More recent studies show that CBA/J dams mated to DBA/2J sires exhibit reduced levels of splenic and circulating CD25⁺;FoxP3⁺ regulatory T cells (T-reg).³⁵ It is conceivable that differential T-reg and NK homing might participate in determining which implantation sites will survive and which will be targeted for rejection. However, it remains unclear what might direct such differential homing to a subset of implantation sites in litters of genetically identical F1 fetuses. Interestingly, replacement of the DBA/2J sires with BALB/cJ (also haplotype d/d) sires results in only baseline abortion rates (matching syngeneic matings),^{14,16} indicating the haplotype mismatch, alone, is not sufficient to trigger the rejection response. Moreover, although abortion frequencies can be reduced by immunizing the CBA/J dams,^{14,36} this effect can be achieved using non-specific (antigen-independent) immunizations.^{37,38} Thus, although paternal alloantigens exist in the CBA/J × DBA/2J F1 fetuses, it is not yet clear what, if any, role these might play in the selective abortion process.

Homozygous genetic disruption of fetoplacental expression of the complement inhibitor Crry results in 100% penetrant mid-gestational abortions in both allogeneic and syngeneic pregnancies.^{39–41} Recently, antibody-independent complement activation has been shown to play a causal role in CBA/J × DBA/2J abortions,⁴² which suggests that the rejection response in these matings might be triggered by a failure of trophoblasts to inhibit complement activation.⁴³ In this case, we might predict that the selectivity of the abortion process among genetically identical F1 sibs would result from epigenetic differences between sibs in expression of a complement inhibitory pathway in this mating. Moreover, the ‘parent-of-origin’ effect (DBA/2J-mated CBA/J dams are abortion-prone; CBA/J-mated DBA/2J dams are not¹⁴) suggests the likelihood of survival versus abortion for each fetus might be more dependent on strain-specific differences in epigenetic patterning of the gametes⁴⁴ than on strain-specific antigenic differences.

The study presented here is based on a line of mice we previously generated in which most of the vertebrate-specific N-terminal domain of TBP was removed.²¹ The predominant phenotype is that of selective immune-dependent mid-gestational abortion of most homozygous mutant fetuses. Wild-type and heterozygous littermates are largely unaffected, and genetic elimination of maternal lymphocytes by establishing pregnancies in either *rag1*^{-/-} dams resulted the mutant fetuses surviving gestation.²¹ This suggested that, in immune-normal mothers, *thp*^{ΔN/ΔN} fetuses were inappropriately presenting antigen, leading to rejection by maternal lymphocytes. Unexpectedly, however, these abortions occurred when both parents were on a highly homogeneous C57Bl/6J background.²¹ Thus, the question arose: if the pregnancies were syngeneic, what antigens were the maternal lymphocytes reacting to? Preliminary studies investigated whether male fetuses, which differ genetically from their mother by the presence of the Y chr, were preferentially aborted, and they were not (S. Iverson and Edward E. Schmidt, unpublished data). In the study presented here, we tested whether paternal alloantigens from any chromosome could participate in these immune-associated abortions. Our results showed that abortion of the *thp*^{ΔN/ΔN} fetuses, although immune-dependent, was entirely independent of, and insensitive to, paternal allogeneic determinants. Thus, antigens involved in this response must be of syngeneic origin, for example, a strictly placenta-specific gene product or an antigen arising from placenta-specific processing or presentation of an immunogenic macromolecule.

This study indicates that critical aspects of the maternal–fetal immune interaction differ fundamentally from alloantigen-based models of host-graft interactions. Specifically, we conclude that, in some cases of immune-dependent abortion, both the presence of paternal alloantigenic determinants and the maternal immune response to these antigens are superfluous to the abortion process. These findings also lead us to suspect that, in some cases of recurrent miscarriages that manifest as immune-associated in humans, the maternal responses to paternal alloantigens may be only coincidental to the abortion process as well. Our ongoing studies address the physiological processes that are skewed in *thp*^{ΔN/ΔN} placentas, which will lend insights into the nature of the defect that is triggering the selective maternal rejection response.

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Abbreviations

chr	chromosome
E	embryonic day
HLA	human leukocyte antigen
I.P	intraperitoneal
I.U	international unit

MHC

major histocompatibility complex

rag1

recombinase activating gene 1

TBP

TATA-binding protein

 β 2m β 2-microglobulin**References**

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Table 1
Post-Mid-Gestational Fetal Survival in *rag I^{+/+}* and *rag I^{-/-}* Dams On a C57Bl/6J Background

Group	Zygote production		Maternal environment ^d	E14.5 ± 1 fetal <i>thp</i> genotypes		
	Sire	Ovary-source		<i>thp</i> ^{+/+}	<i>thp</i> ^{ΔN/+}	<i>thp</i> ^{ΔN/ΔN}
A	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	232	441	28 (12%) ^b
B	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{-/-}	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{-/-}	29	59	26 (90%) ^c
C	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	<i>thp</i> ^{ΔN/+} ; <i>rag I</i> ^{+/+}	<i>thp</i> ^{+/+} ; <i>rag I</i> ^{-/-}	5	17	6 (120%) ^d

Values in parentheses are percentages.

^aIn groups A and B, where the maternal environment matches the ovary-source genotype, results are from natural matings. In group C, where the maternal environment differs from the ovary-source genotype, fresh ovaries from *thp*^{ΔN/+};*rag I*^{+/+} donors were transferred into ovariectomized *thp*^{+/+};*rag I*^{-/-} females. Ovary recipients were allowed 3–4 weeks to recover from surgery and to clear any pre-existing eggs, and then were mated to *thp*^{ΔN/+};*rag I*^{+/+} sires.

^bPercent survival based on the number of *thp*^{+/+} animals and Mendelian ratios of zygote genotypes. Fetal genotype ratio differed significantly from Mendelian distribution, *P* < 0.001.

^cFetal genotype ratio of group B was not significantly different from Mendelian, *P* > 0.05; it was significantly different from group A, *P* < 0.001.

^dFetal genotype ratio of group C was not significantly different from Mendelian, *P* > 0.05 or from group B, *P* > 0.05; it was significantly different from group A, *P* < 0.001.

Table II
Post-Mid-Gestational Fetal Survival as a Function of Paternal Allogeneic Contribution

Group	Zygote production			E14.5 ± 1 fetal <i>thp</i> genotypes ^b		
	Sire (haplotype)	Dam (haplotype)	Maternal environment ^d (haplotype)	<i>thp</i> ^{+/+} (haplotype)	<i>thp</i> ^{ΔN/+} (haplotype)	<i>thp</i> ^{ΔN/ΔN} (haplotype)
A	<i>thp</i> ^{ΔN/+} (b/b)	<i>thp</i> ^{ΔN/+} (b/b)	<i>thp</i> ^{ΔN/+} (b/b)	232 (b/b)	441 (b/b)	28 (12%) ^c (b/b)
D	<i>thp</i> ^{ΔN/+} (b/d) ^d	<i>thp</i> ^{ΔN/+} (b/b)	<i>thp</i> ^{ΔN/+} (b/b)	23 (b/d)	47 (b/b or b/d)	6 (26%) ^e (b/b)
E	<i>thp</i> ^{ΔN/+} (b/d) ^d	<i>thp</i> ^{ΔN/+} (b/d) ^d	<i>thp</i> ^{+/+} (d/d) ^f	23 (d/d)	33 (b/d)	5 (22%) ^e (b/b)

Values in parentheses are percentages.

^aIn groups A and D, where the maternal environment genotype matched that of the zygote production dam, results were from natural matings. In group E, where the maternal environment genotype differed from that of the zygote production dam, zygotes from natural matings of the zygote production sire and dam were harvested at E0.5 and transferred into the oviducts of pseudopregnant *thp*^{+/+} CB γ D2F1/J surrogates.

^bFetal genotype ratios for all three groups differed from Mendelian distributions calculated based on the number of wild-type fetuses, $P < 0.001$ for group A; $P < 0.01$ for groups D and E.

^cPercent survival based on the number of *thp*^{+/+} animals and Mendelian ratios of zygote genotypes; group A is the same data as Table I group A.

^dThe *thp*^{ΔN} allele is very tightly linked to the MHC region on a haplotype b chr 17. In groups D and E, where hybrid haplotypes were used for zygote production, all animals were F1 or F2 crosses between C57BL/6J (haplotype b/b) and BALB/cJ (haplotype d/d), and all *thp*⁺ alleles were on a BALB/cJ-derived haplotype d chr 17. Therefore, all *thp*^{+/+} progeny were haplotype d/d; all *thp*^{ΔN/+} progeny were haplotype b/d; and all *thp*^{ΔN/ΔN} progeny were haplotype b/b.

^eFetal genotype ratios of group D and group E did not differ significantly from group A, $P > 0.05$.

^fThe maternal environment in group E was provided by surrogates of strain CB γ D2F1/J, which were haplotype d/d.