Significant contributions of the extraembryonic membranes and maternal genotype to the placental pathology in heterozygous Nsdhl deficient female embryos

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Mutations in the gene encoding the cholesterol biosynthetic enzyme NSDHL are associated with the X-linked male-lethal bare patches (Bpa) mouse. Mutant male embryos for several Nsdhl alleles die in midgestation with placental insufficiency. We examined here a possible role of the maternal genotype in such placental pathology. Pre-pregnancy plasma cholesterol levels were similar between wild-type (WT) and Bpa1H/1 dams fed a standard, cholesterol-free diet. However, there was a marked decrease in cholesterol levels between embryonic day (E)8.5 and E10.5 for both genotypes. Further, there was a significant lag between E11.5 and E13.5 (P = 0.0011) in the recovery of levels in Bpa1H/1 dams to their pre-pregnancy values. To investigate possible effects of the maternal genotype on fetal placentation, we generated transgenic mice that expressed human NSDHL and rescued the male lethality of the Bpa1H null allele. We then compared placenta area at E10.5 in WT and Bpa1H/+ female embryos where the mutant X chromosome was transmitted from a heterozygous mother or a rescued mutant father. In mutant conceptuses, placental areas were 50% less than WT. Surprisingly, expression of Nsdhl in trophoblast lineages of the placenta and yolk sac endoderm, which occurs only from the maternally inherited allele in a female embryo, had the largest effect on placental area (−0.681 mm²; P < 0.0001). The maternal genotype had a smaller effect, independent of the fetal genotype (−0.283 mm²; P = 0.024). These data demonstrate significant effects of the mother and fetal membranes on pregnancy outcome, with possible implications for cholesterol homeostasis during human pregnancy.

INTRODUCTION

NSDHL is a sterol dehydrogenase (EC 1.1.1.170) involved in the removal of C-4 methyl groups in one of the later steps of cholesterol biosynthesis. Mutations in the murine Nsdhl gene are associated with the X-linked dominant, male lethal mutations bare patches (Bpa) and striated (Str) (1). Human NSDHL mutations cause CHILD syndrome (congenital hemidysplasia with ichthyosis and limb defects), a rare X-linked dominant malformation syndrome that is often characterized by unilateral ichthyosiform skin lesions with a sharp demarcation at the midline [(2) and reviewed in (3)].

Heterozygous Bpa females have a skeletal dysplasia and are dwarfed compared with normal littermates. They develop a hyperkeratotic skin eruption on postnatal days 5–7 that resolves, producing a striping of the adult coat consistent with random X-inactivation [reviewed in (3)]. Str females appear normal in size and cannot be distinguished from their wild-type (WT) littermates until postnatal days 12–14, when striping of their coat becomes apparent. We have identified

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several distinct Nsdhl mutations that provide an allelic series (1,4). All of the known Nsdhl alleles are lethal prenatally in affected male embryos (5). Recently, we demonstrated that the male lethality for moderate (Bpa\textsuperscript{Bpa1H}) and mild (Str\textsuperscript{Str10}) alleles occurs at midgestation and is associated with a thin and poorly vascularized fetal placental labyrinth (5). In addition, the yolk sacs of many of these mutant male embryos were pale with fewer and/or narrower vessels. No consistent abnormalities were observed in the embryos themselves. Subsequently, we noted defective hedgehog signaling in placental allantoic mesoderm in affected Bpa\textsuperscript{Bpa1H} male embryos following chorioallantoic fusion at ~E8.5 (6).

To analyze further the role of the NSDHL protein in lipid metabolism and during mammalian development, we report here the generation of transgenic mice that contain a human BAC expressing the NSDHL gene. Rescue of the male lethality for the Bpa\textsuperscript{Bpa1H} and Bpa\textsuperscript{Bpa8} alleles demonstrates that the human gene functions in the mouse. Further, experiments in which a mutant Nsdhl allele was transmitted to female embryos by a rescued male demonstrate significant contributions of the extraembryonic membranes and maternal genotype to the placental pathology.

**RESULTS**

Generation of transgenic mice expressing the human NSDHL gene

We obtained a 168 kb human BAC, 11G21, from the RPC11 library (7). It contains the entire NSDHL gene, the centromeric CETN2 gene that is transcribed from a dual promoter in the opposite direction from NSDHL (8), and the S' end of the ZNF185 gene that is telomeric to NSDHL (8,9). We expected that all necessary regulatory sequences would be present on this BAC for proper in vivo expression of NSDHL. We employed a human rather than a mouse BAC, since screening for the presence and expression of the transgene would be greatly simplified across species. Although it was possible that the human protein would not complement the murine deficiency, we considered this unlikely since the two proteins share 82.9% amino acid identity (1). Further, the murine protein can rescue the lethality of a Saccharomyces cerevisiae strain (erg26) that lacks the yeast ortholog of NSDHL (4,10).

The major difference between the predicted human and mouse proteins is a 12 amino acid insertion near the N-terminus of the former. In fact, the antibody that we generated against the human NSDHL protein was designed to include these species-specific amino acids and does not cross-react with the murine NSDHL protein (Material and Methods).

Purified BAC DNA was injected into fertilized FVB pronuclei by our institutional Transgenic Core. Five founder animals were recovered, of which three transmitted an intact transgene to their offspring at expected ratios (~50%). Two of the transgenic lines were expanded and employed for the studies described below. Heterozygous animals for both of the lines appeared normal and were fertile. Dosage analysis by Southern blotting confirmed that four to five copies of the transgene were present in each of the two lines (not shown). Expression of human NSDHL was demonstrated by RT–PCR from selected tissues of adult mice using human specific primers (not shown), as well as by western blotting and immunohistochemistry (Fig. 1A–E). We were able to generate homozygous transgenic mice for one of the lines (#4418), while none were obtained after several rounds of intercrossing and progeny testing for the other (#4419), suggesting that the integration site in this line may have disrupted an essential gene.

**Rescue of the lethality of Bpa/Y embryos using transgenic mice expressing human NSDHL**

We next examined whether mice containing the human BAC could rescue the male lethality of two of our mutant Nsdhl alleles, Bpa\textsuperscript{Bpa1H} and Bpa\textsuperscript{Bpa8}. The Bpa\textsuperscript{Bpa1H} allele results from a nonsense mutation to amino acid 103 of the murine protein (K103X) and is predicted to be a null allele (1). The majority of affected Bpa\textsuperscript{Bpa1H} male embryos die by embryonic day (E)7.5, and none survive beyond E10.5 (5). The Bpa\textsuperscript{Bpa8} allele results from a missense mutation (A94T) in a highly conserved amino acid residue (4). It is associated with a moderate phenotype in affected heterozygous females; affected males for this allele die between E10.5 and E12.5, secondary to placental insufficiency (5).

Males heterozygous for the BAC transgene were mated to heterozygous Bpa\textsuperscript{Bpa1H} females (see schematic Fig. 1F). As shown in Figure 1H, the human protein complements the murine deficiency and rescues the lethality of Nsdhl deficient Bpa\textsuperscript{Bpa1H} male embryos. Similar results were obtained for the Bpa\textsuperscript{Bpa8} allele (not shown). Further, the affected Nsdhl\textsuperscript{Bpa1H} X chromosome and autosomal transgene segregate as expected upon breeding of a rescued male (see schematic Fig. 1G). Specifically, in three litters from a mating of a B6CBA female by a rescued male carrying the human transgene, there were 11 female and 11 male offspring. Eleven of the 22 pups (four females and seven males; 50% total) carried the transgene. All of the female and none of the male pups received a Nsdhl\textsuperscript{Bpa1H} X chromosome, as expected for an X-linked locus. Seven of these females demonstrated the Bpa phenotype with hyperkeratotic eruptions, smaller size, and later striping of the coat; upon genotyping, they did not receive the human NSDHL transgene (Fig. 1I). Four female offspring without a striped coat received both the mutant X chromosome and the human transgene.

**Determination of total plasma cholesterol levels in pregnant dams**

Recently, there has been considerable interest in understanding contributions of the maternal environment and genotype to fetal development, birth weight and pregnancy outcomes, and risks for later disease in the offspring [reviewed in (11,12)]. In the mouse, significant cholesterol transport from the mother to the fetus occurs throughout gestation (13–15). Thus, we reasoned that heterozygosity for Nsdhl in a pregnant dam might contribute to the phenotype in affected embryos. To assess possible contributions of the maternal genotype to the embryonic phenotype, we first examined plasma cholesterol levels in non-pregnant WT and Bpa\textsuperscript{Bpa1H}+ females. Plasma total cholesterol levels were not significantly different
between WT B6CBA and \textit{Bpa}^{III/+} females, with mean values of 111.5 ± 14.1 and 111.0 ± 26.7 mg/dl, respectively (Fig. 2).

Studies of cholesterol levels in pregnant dams have not been reported (see http://phenome.jax.org), so we next performed a series of total cholesterol determinations on pregnant WT and \textit{Bpa}^{III/+} females from E6.5 to E17.5. We noted a precipitous drop in total plasma cholesterol in pregnant WT B6CBA females between E8.5 and E10.5 (Fig. 2). A similar drop in maternal cholesterol levels was observed in pregnant \textit{Bpa}^{III/+} females. The greater variability in the cholesterol levels at some time points in the \textit{Bpa}^{III/+} females compared with WT dams may result from differences in patterns of X-inactivation in individual animals, although we cannot exclude other mechanisms, such as effects of variation in litter size. Interestingly, whereas cholesterol levels in WT mice rose to their pre-pregnancy values by E13.5, there was a lag and...
slower rise from the nadir at E10.5 in the Bpa1H/+ dams. Combining the values for the total cholesterol levels at E11.5–13.5, there was a significant difference between levels in WT compared with Bpa1H/+ dams (P < 0.001, by two group t-test). Comparisons of cholesterol levels between genotypes by single embryonic days were significant for E12.5 (P = 0.027) and E13.5 (P < 0.0001) and borderline for E15.5 (P = 0.06). No other stages showed significant differences in cholesterol levels between mutant and WT females (P > 0.1).

Assessment of the role of the maternal genotype and extraembryonic membranes on the placental phenotype of female Nsdhl-deficient embryos

The generation of NSDHL transgenic lines that could rescue the lethal phenotype of Nsdhl-deficient embryos provided a unique opportunity for us to examine the possible contributions of the maternal genotype and trophoblast lineages to the fetal pathology. In our previous studies, we measured the placental thickness at E10.5 in conceptuses for a moderate (Bpa1Hi) and two mild (Str) Nsdhl alleles (5). Although differences in fetal placental thickness between WT and mutant male embryos were highly significant (P ≤ 0.001), only borderline (P = 0.027, 0.037) or no significance (P = 0.20) was found between similar classes of female embryos. In these experiments, the mutant allele was always inherited from a heterozygous dam, and effects of Nsdhl expression in extraembryonic lineages could not be separated from those of the mutant fetus itself.

Here we used NSDHL transgenic males and a series of Nsdhl mutant crosses to examine possible effects of differential Nsdhl expression in the mother and in extraembryonic and embryonic tissues. Specifically, we examined contributions of the maternal genotype to placental size by comparing transmission of the mutant X chromosome from the mother or the father. In addition, we also examined effects of a lack of Nsdhl expression in trophoblast-derived extraembryonic lineages, due to the unique features of X-inactivation in these tissues. Specifically, in rodent female embryos, X-inactivation is not random in most extraembryonic lineages, such as the allantois, amnion and mesothelium overlying the visceral endoderm of the yolk sac (16,17). Thus, a maternally inherited, deficient Nsdhl allele in trophoblast-derived cells of the placenta (syncytiotrophoblasts, spongiotrophoblasts or giant cells) or yolk sac endoderm would be expected to have identical consequences in affected male and heterozygous female embryos. The fact that most affected female Bpa1Hi and Str embryos demonstrate placental defects and survive led us to hypothesize that the primary defect contributing to the male prenatal lethality must occur in the allantoic mesodermal lineage (5). Supporting this assumption, we subsequently demonstrated defective hedgehog signaling in this lineage in male Bpa1Hi embryos, using Ptch1-lacZ reporter mice (6).

Since a rescued male transmits his mutant X chromosome to all of his female, and to none of his male offspring, in the current study, we could only examine placentas from female embryos. We studied crosses where the affected X chromosome came from the mother, father or both. Further, as described above and as shown in Figures 3 and 4, in affected Bpa1+ female embryos, if the affected X chromosome is transmitted by the mother, expression of Nsdhl in yolk sac endoderm and trophoblast lineages occurs only from the mutant allele (is not mosaic), whereas it is WT if the affected X comes from the father. The genotype in these extraembryonic lineages is always the same, with an Nsdhl-deficient X chromosome and a WT chromosome. Only the parent-of-origin, and, hence, the expression of Nsdhl changes.

Figure 2. Maternal plasma cholesterol levels of B6CBA and Bpa1Hi/+ females during pregnancy. Total plasma cholesterol levels were determined using a commercial colorimetric assay (see Material and Methods). Data are presented as the Mean ± SD (standard deviation). The numbers at the bottom of each bar represent the number of females of each genotype tested at that time point. Bpa1Hi/+ females were not analyzed at E6.5.
We compared areas of the fetal placenta using the most severe \(Bpa^{H1}\) null allele. Mutant males for this allele could not be examined in our original study (5) because the majority of them die prior to formation of a functional fetal placenta. We chose to examine the \(Bpa^{H1}\) allele here reasoning that any differences noted in heterozygous females would be most marked in a null allele, and we were concerned with inherent biologic variability in heterozygous embryos. A summary of our data are presented in Table 1 and Supplementary Material, Figure S1. Comparisons of the crosses (maternal genotype, embryonic genotype and extraembryonic expression) by ANOVA demonstrated a significant difference in placental area \((F4,61 = 17.05; P < 0.001)\). Post hoc testing showed, as expected, that there were significant differences in area between WT and affected (mosaic) female embryos in a \(Bpa^{H1/+}\) dam in Cross 2 (size reduction 47%; \(P < 0.001)\). The placental area of mosaic female embryos in Cross 2 was also different from the placental area of each of the other crosses in post hoc pair-wise testing \((P < 0.05 \text{ for all comparisons})\).

We then performed linear regression analysis on these data. This allowed us to control for each of the other variables while independently assessing the effect of the maternal genotype, extraembryonic expression or embryonic genotype. Each contributed to overall placental area, with the largest change in placental size resulting from non-random \(Nsdhl\) expression in extraembryonic lineages with paternal X-inactivation \([-0.681 \text{ mm}^2 (95\% \text{ CI } -0.971 \text{ to } -0.391); \ P < 0.0001]\). The maternal genotype had a smaller, but significant, effect \([-0.283 \text{ mm}^2 (95\% \text{ CI } -0.529 \text{ to } -0.038); \ P = 0.024]\), whereas the fetal genotype had a small, non-significant effect on placental size \([-0.168 \text{ mm}^2 (95\% \text{ CI } -0.415 \text{ to } +0.080); \ P = 0.18]\).

DISCUSSION

The placenta is essential for the growth and development of all Eutherian mammals. Its primary function is to serve as a surface for the exchange of nutrients and waste products.
between the developing fetus and its mother. It also acts as a barrier to fetopaternal antigens, and produces hormones for the establishment and maintenance of the pregnancy (18,19). In the mouse, the labyrinth is the site of placental nutrient exchange. The mature fetal labyrinth contains mononuclear and syncytial trophoblast cells, as well as mesenchymal (stromal) and endothelial cells of mesodermal origin. Nutrient exchange occurs across three cell layers of syncytiotrophoblasts and cytotrophoblasts that separate endothelial lined fetal vessels from sinuses of maternal blood (18,20).

Following fusion of the fetal allantois with the placental chorion at ~E8.5 (21,22), there is rapid proliferation and differentiation of both trophoblast and fetal-derived cells of the placental labyrinth, the latter originating in allantoic mesoderm. This is also a critical time for the fetus, with substantial growth as organogenesis proceeds. Large amounts of cholesterol are necessary for the production of membranes for this fetal and placental growth. Unlike the adult where cholesterol is in steady state, there is net accrual in the fetus [reviewed in (23)]. Cholesterol is also necessary for the synthesis of steroid

Table 1. Fetal placental area

<table>
<thead>
<tr>
<th>Cross</th>
<th>Female × Male</th>
<th>Number</th>
<th>Nsdhl Genotypea</th>
<th>Maternal Embryonic</th>
<th>Nsdhl Expressionb</th>
<th>Maternal Embryonic</th>
<th>Placental areac (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. WT × WT</td>
<td>11</td>
<td>+/-</td>
<td>+/-</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>1.90 ± 0.07</td>
</tr>
<tr>
<td>2. Bpa1H × WT</td>
<td>8</td>
<td>+/-</td>
<td>+/-</td>
<td>Mosaic</td>
<td>WT</td>
<td>WT</td>
<td>1.64 ± 0.15</td>
</tr>
<tr>
<td>3. WT × TgNsdhl/Bpa1H</td>
<td>17</td>
<td>+/-</td>
<td>+/-</td>
<td>WT</td>
<td>WT</td>
<td>Mosaic</td>
<td>0.78 ± 0.10</td>
</tr>
<tr>
<td>4. Bpa1H × TgNsdhl/Bpa1H</td>
<td>12</td>
<td>+/-</td>
<td>+/-</td>
<td>Mosaic</td>
<td>Mosaic</td>
<td>Mosaic</td>
<td>1.45 ± 0.11</td>
</tr>
</tbody>
</table>

aThe Nsdhl genotypes of the mother and female embryos are designated as (+) for the WT allele and (–) for the mutant Bpa1H allele. The maternally inherited allele is always presented first; i.e. in a (+/-) embryo, the WT allele was maternal in origin and the mutant allele was paternal. In Crosses 3 and 4, only the female genotypes shown were included in the analyses. For example, in Cross 4, one out of eight embryos has the genotype shown.

bDenotes which Nsdhl is expressed in the animal or tissues shown. WT = wild-type allele expressed; Mosaic = random expression of WT or mutant Bpa1H allele in heterozygous female; ExEmbry = expression in yolk sac endoderm and trophoblast lineages of the placenta. There is no mosaicism in female embryos in these lineages due to preferential paternal X-inactivation.

cAreas of fetal placentas were measured as described (see Material and Methods) and listed as the mean ± standard error (SEM).
hormones, bile acids and oxysterols. The latter are involved in lipid homeostasis through their regulation of orphan nuclear receptors, such as LXRαs (24,25). Lipid rafts, which are involved in signaling at the plasma membrane, are enriched in cholesterol. Finally, a cholesterol molecule is covalently bound to hedgehog proteins during their processing. Secreted hedgehog proteins act as dose-dependent morphogens and play important roles in numerous processes throughout embryogenesis (26,27).

There are two sources of fetal cholesterol, endogenous synthesis and transport from the mother (13,23). In the mouse, significant cholesterol transport from the mother to the fetus occurs throughout gestation. On the basis of a recent study (14), prior to E12.5, most fetal cholesterol is maternal in origin. Thereafter, endogenous synthesis contributes up to ~50% of the total fetal cholesterol in peripheral tissues. However, by midgestation in the mouse, most cholesterol found in the developing brain is synthesized in situ due to the presence of a functional blood-brain-barrier (14,15).

We reasoned that heterozygosity for Nsdhl in a pregnant dam might contribute to the phenotype in affected embryos: affected males die before E12.5 at a time when most cholesterol is maternal in origin. To assess possible contributions of the maternal genotype to the embryonic phenotype, we first examined plasma cholesterol levels in non-pregnant and gravid WT and Bpa+/+ females fed our institutional cholesterol-free breeder chow. The significant decrease in serum cholesterol levels that we noted in pregnant dams beginning at ~E8.5 (Fig. 2) corresponds with the phase of rapid growth following choioallantoic fusion and likely reflects increased demands for cholesterol in both the fetus and placenta. We believe that the lag in recovery of cholesterol levels in Bpa+/+ dams reflects less cholesterol stores in the mutant females and/or a lower capacity for de novo synthesis, although further experiments will be necessary to prove this hypothesis.

The generation of mice expressing a human NSDHL transgene enabled us to ask whether the maternal and extraembryonic environments make significant contributions to the overall fetal placental pathology by examining female Bpa+/+ embryos. Surprisingly, although the genotype of the embryo and the mother contributed to the overall placental size and area, the major contributing factor was the presence or absence of Nsdhl expression in the fetal membranes (Table 1). The maternal effect could come from the maternal decidua of the placenta, a source of cholesterol-derived steroid hormones, or lipids, such as cholesterol, transported from the pregnant dam. The apparent delay in recovery of maternal plasma cholesterol levels in Bpa-/- dams (Fig. 2), possibly related to a lower capacity for de novo synthesis, could accentuate effects of the physiologic decrease found at midgestation. Thus, the increased demands of the growing fetus and placenta for maternal cholesterol and the reduced capacity of a Bpa+/+ dam to produce cholesterol could create a bottleneck between ~E9.5 and E11.5, where cholesterol is limiting for growth.

The murine placenta becomes functional after ~E10.5 (18). On the basis of the much higher expression of Nsdhl in the yolk sac than the fetal placenta at this time in gestation (Fig. 4), we believe that the former is likely to be the important determinant in the extraembryonic tissues. In support of this hypothesis, we previously demonstrated that yolk sacs from E9.5 to E10.5 mutant Nsdhl embryos are pale, with possible defects in vascular remodeling (5). However, it is not clear how a primary yolk sac defect would result in a smaller placenta. It is possible that the yolk sac defects result in overall slower growth with suboptimal transport of nutrients to the fetus. Alternatively, we have demonstrated the migration of small numbers of cells from yolk sac visceral endoderm into placental mesoderm following choioallantoic fusion (6). It is possible that this migration, and signaling by one or more pathways between the two extraembryonic tissues, is compromised. Unfortunately, we cannot separate effects of trophoblast-derived placental lineages from those of yolk sac endoderm using the genetic approaches employed here. It would require a conditional Nsdhl allele with inactivation in specific extraembryonic tissues using cre recombinase or similar technology.

Although these studies were performed using a mutant mouse model, they could have implications for human pregnancy and placental function. Rare, inherited human disorders of cholesterol biosynthesis, such as Smith–Lemli–Opitz syndrome (SLOS), demonstrate that cholesterol deficiency is highly teratogenic to the developing human fetus [reviewed in (28,29)]. The presence of detectable cholesterol levels in SLOS fetuses carrying two null alleles further demonstrates that there is maternal cholesterol transport during human pregnancy, although the extent and duration are not known. Further, the maternal, but not the paternal, ApoE genotype influences the severity of SLOS in the offspring, suggesting an effect of maternal cholesterol levels (30).

It should be noted that there are many similarities, but also some important differences, between human and rodent placentas (18,19,23). Many genes and proteins, including transcription factors and those involved in cellular signaling pathways, originally described in one system have subsequently been found to play a similar role in the other (31,32). Both share similar trophoblast cell lineages, such as giant cells and syncytiotrophoblasts, although some nomenclature and morphologic or molecular details differ. However, the human placenta is fully functional earlier in gestation, at 8–10 weeks post-conception, whereas the murine placenta becomes fully functional only in midgestation after E10.5 (18). This difference in timing is due in large part to the presence of the yolk sac and active choriovitelline circulation that persists throughout gestation in the rodent embryo. The human yolk sac functions only during the first trimester. Further, the rodent yolk sac is inverted, with endodermal cells facing out, enabling substantial uptake from maternal tissues and plasma. Finally, although some skewing of X-inactivation can occur in human female placentas, extreme skewing (>85%) is rare (33). Expression of NSDHL and other cholesterol biosynthetic enzymes in human fetal membranes has not been well studied.

Interest in understanding lipid metabolism in the human fetus and placenta has increased recently with the recognition that maternal hypercholesterolemia during pregnancy is a predictor of cardiovascular disease in offspring, an example of ‘fetal programming’ (11,34). Effects of maternal hypocholesterolemia on the fetus are not well studied, although Edison
et al. (35) have recently demonstrated that lower maternal plasma cholesterol is associated with lower birth weight, and there was a trend with microcephaly. Further, Steffen et al. (36) noted suggestive associations for SNPs at several loci involved in cholesterol metabolism with prematurity and birth weight. It is likely that risks of low maternal cholesterol in pregnancy will become better defined as more studies are performed.

**MATERIAL AND METHODS**

**Mouse strains and crosses**

The Bpa<sup>III</sup> and Bpa<sup>8th</sup> alleles have been described (5) and are maintained by mating heterozygous females to C57BL/6J<sup>wt</sup> × CBA (B6CBA) F<sub>1</sub> hybrid males (The Jackson Laboratory). All of the mice were fed ad libitum our institutional irradiated breeder chow that contains 9% fat as soybean oil, 19% vegetable protein, and no added cholesterol (Teklad 2919).

Transgenic mice were generated by pronuclear microinjection of fertilized one cell FVB/N embryos by an institutional core facility using standard methods (16). Human NSDHL transgenic line #4418 is currently being backcrossed onto the B6CBA background used to maintain our Nsdhl alleles. For experiments using the transgenic line to examine fetal pathology, animals were at the N6 or N7 backcross generation (<2% FVB/N). The backcross matings were established such that the X chromosome always contains the B6 genotype and X<sup>xe</sup> allele [see (5) for further discussion].

All mice were maintained in an AALAC-approved SPF animal facility within The Research Institute at Nationwide Children’s Hospital, and all experiments were conducted under an institutionally approved animal care and use protocol.

**Molecular studies**

Screening of potential founder NSDHL transgenic mice was performed by PCR on tail tip DNA using human specific primers 5'-TTCAACTTTGGGCAAGTGGA-3' and 5'-CTCC ATAGCATCATTCAATGG-3' that amplify a 350 bp product from intron 7 and exon 8 of NSDHL. Amplification conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s 60°C for 30 s and 72°C for 30 s. PCR genotyping for the presence of the mutant Bpa<sup>III</sup> or Bpa<sup>8th</sup> allele was performed as described (4) and confirmed in rescued mutant males by direct sequencing of DNA amplified from the exon containing the mutation. Southern blotting to determine transgene copy number was performed on HindIII digested genomic DNA using standard techniques (37). Southern blots were hybridized to 32P-labeled probes generated from a 700 bp PCR product from human NSDHL exon 8 or an 800 bp PCR product from exon 8 of mouse Nsdhl.

**Immunologic studies**

Polyclonal rabbit antisera were raised commercially (Genemed Synthesis, San Francisco, CA, USA) against a C-terminal mouse peptide (DEAVERTQSFHHLRKDK) or human-specific peptide (NADIEKVNQNQA) from the respective predicted NSDHL proteins. The underlined 11 amino acids in the predicted human peptide sequence are found near the N-terminus and comprise part of a unique 12 amino acid, human-specific insertion (1). Antisera were affinity purified using purified peptide and a Sulfolink kit (Pierce, Rockford, IL, USA). Western blots were prepared as previously described (38) using total protein extracts of livers from transgenic animals and a WT littermate. The blots were probed with the anti-mouse and anti-human NSDHL antibodies at dilutions of 1:4000 and 1:2000, respectively. Antibody binding was detected using an HRP-conjugated anti-rabbit IgG and chemiluminescent substrate as previously described (39). For immunohistochemical analysis, embryos were fixed in Bouin’s solution, sectioned, probed with anti-human or anti-mouse NSDHL antibody at a 1:1000 dilution, and counterstained with hematoxylin.

**Lipid analyses**

Total cholesterol determinations were performed on plasma following a 4 h fast using an enzymatic colorimetric assay as recommended by the manufacturer (Chol E Wako kit 439–1750; Wako Chemicals, Richmond, VA, USA). All animals were 8–12 weeks of age. Determinations were performed in gravid females during their first pregnancy. We had previously determined that total cholesterol values obtained using this method were reproducible and consistent with those obtained following extraction of the lipids into CHCl<sub>3</sub> by the Folch method (40) or following Folch extraction, saponification in ethanolic KOH (41) and analysis by gas chromatography and selected-ion mass spectroscopy (42).

**Measurement of fetal placental area**

Embryos and placentas from timed matings were dissected under a Nikon SMZ-10A microscope equipped with a SPOT digital camera as described (5,6). Genotyping of individual embryos was performed on yolk sac DNA with primers for the Scm locus to determine sex (43), for Nsdhl alleles (4,44) and for the presence of the human transgene (see above). The embryos and placentas were fixed in a solution of 0.2% glutaraldehyde, 2% formalin, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 100 mM potassium phosphate buffer, pH 7.3, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin/eosin using standard techniques. Placental sections that included the umbilical cord as an indicator of the central location of the section were photographed and the area of the fetal placenta was measured using SPOT Version 3.5 software. The fetal placenta was defined as the region bounded by the single layer of extraembryonic giant cells and the chorionic plate at the base of the placenta (Fig. 4).

**Statistical analyses**

Descriptive and analytic statistical testing was performed using Stata version 10.1. Cholesterol values during gestation were analyzed at individual time points or grouped for analysis (Not Pregnant; E6.5 and 7.5; E8.5, 9.5, and 10.5; E11.5, 12.5
and 13.5; E15.5 and 17.5) and compared using two-group t-test with equal variances. Placental areas for the various crosses and resulting female embryos were analyzed by ANOVA followed by post-hoc pair-wise comparisons using the Tukey-Kramer test (45). Linear regression analysis of placental area was performed for the variables of maternal and embryonic genotypes and extraembryonic Nsdhl expression.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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