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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 Bpa^{1H}/+ 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 Bpa^{1H}/+ 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 Bpa^{1H} null allele. We then compared placenta area at E10.5 in WT and Bpa^{1H}/+ 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 mm^2; $P < 0.0001$). The maternal genotype had a smaller effect, independent of the fetal genotype (-0.283 mm^2; $P = 0.024$). 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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seven 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^{8H}) and mild (Str^{1H}, Str^{1Or}) 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^{8H}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^{1H} and Bpa^{8H} 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 5′ 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^{1H} and Bpa^{8H}. The Bpa^{1H} allele results from a nonsense mutation at amino acid 103 of the murine protein (K103X) and is predicted to be a null allele (1). The majority of affected Bpa^{1H} male embryos die by embryonic day (E)7.5, and none survive beyond E10.5 (5). The Bpa^{8H} 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^{1H} 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^{1H} male embryos. Similar results were obtained for the Bpa^{8H} allele (not shown). Further, the affected $Nsdhl^{Bpa1H}$ 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 $Nsdhl^{Bpa1H}$ 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 $Bpa^{1H}/+$ females. Plasma total cholesterol levels were not significantly different

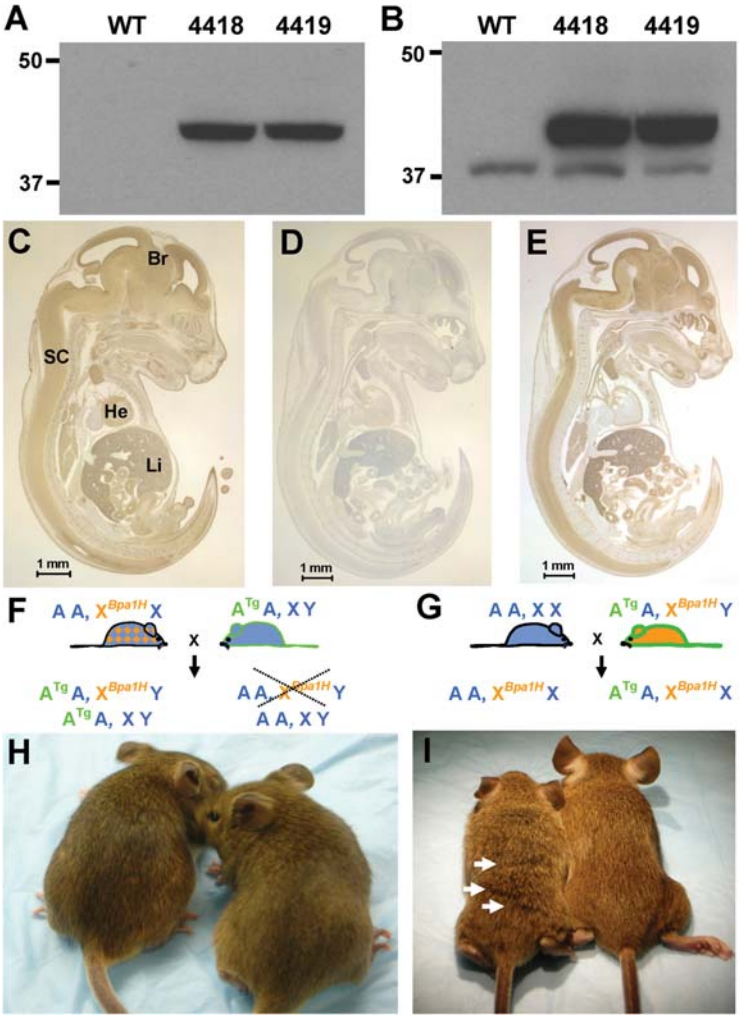

Figure 1. Protein expression and rescue of *Bpa*^{1H} male lethality in *NSDHL* transgenic mice. (**A**, **B**) Western blotting of liver extracts prepared from a 4-week-old non-transgenic wild-type (WT) mouse or from transgenic mice from lines 4418 and 4419. About 20 μg of protein was loaded per lane. Blots were hybridized with polyclonal rabbit antibodies prepared against an N-terminal epitope of human NSDHL (A) or C-terminal epitope from the murine protein (B) (see Material and Methods). The antibody against the human protein is species-specific, whereas the antibody prepared against mouse NSDHL cross-reacts with the human protein, as predicted based on the amino acid sequences of the orthologous proteins. The predicted molecular weight of the murine and human proteins are 40.7 kDa (362 amino acids) and 41.9 kDa (373 amino acids), respectively. Immunohistochemistry of sagittal sections prepared from E14.5 transgenic (**C**) or non-transgenic (**D**, **E**) embryos treated with antibody prepared against human (C, D) or mouse (E) NSDHL. Note similar staining patterns of mouse and human proteins and lack of background staining with anti-human antibody in the control non-transgenic embryo (D). Br = brain, He = heart, Li = liver, SC = spinal cord. (**F**) Schematic of cross to generate rescued males. A heterozygous *Bpa*^{1H}/+ female is mated to a male carrying a heterozygous *NSDHL* transgene (Tg) on an unknown autosome (A). One-half of the male offspring will receive an affected X^{Bpa1H} from their mother. One-half of those males will receive the transgene from their father (A^{Tg}), since there is independent assortment. The dotted lines through the non-transgenic, mutant male indicate that this class of progeny is lost prenatally. The genotypes of female offspring are not shown. (**G**) Schematic of cross to show transmission of affected X^{Bpa1H} by a rescued male. A WT female is mated to a rescued male generated from the cross shown in (F). All of the daughters will receive the mutant X^{Bpa1H} from their father, and 50% will receive the transgene. Genotypes of resulting males are not shown, 50% of whom will receive the transgene and none of whom will receive the mutant paternal X. (**H**) Eight week old rescued *Bpa*^{1H} transgenic male carrying human BAC (right). The rescued male cannot be distinguished from his WT male littermate (left). (**I**) Progeny of a cross between the rescued male in (H) and a WT B6CBA female. An affected *Bpa*^{1H}/+ female (left) received an X^{Bpa1H} chromosome from her father, but not the autosomal BAC transgene. Note striping of the coat in the affected female following lines of X-inactivation (white arrows). A 'WT' female (right) received the mutant X^{Bpa1H}, but also the transgene, from her father.

between WT B6CBA and *Bpa*^{1H}/+ females, with mean values of 111.5 $\pm$ 14.1 and 111.0 $\pm$ 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 *Bpa*^{1H}/+ 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 *Bpa*^{1H}/+ females. The greater variability in the cholesterol levels at some time points in the *Bpa*^{1H}/+ 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

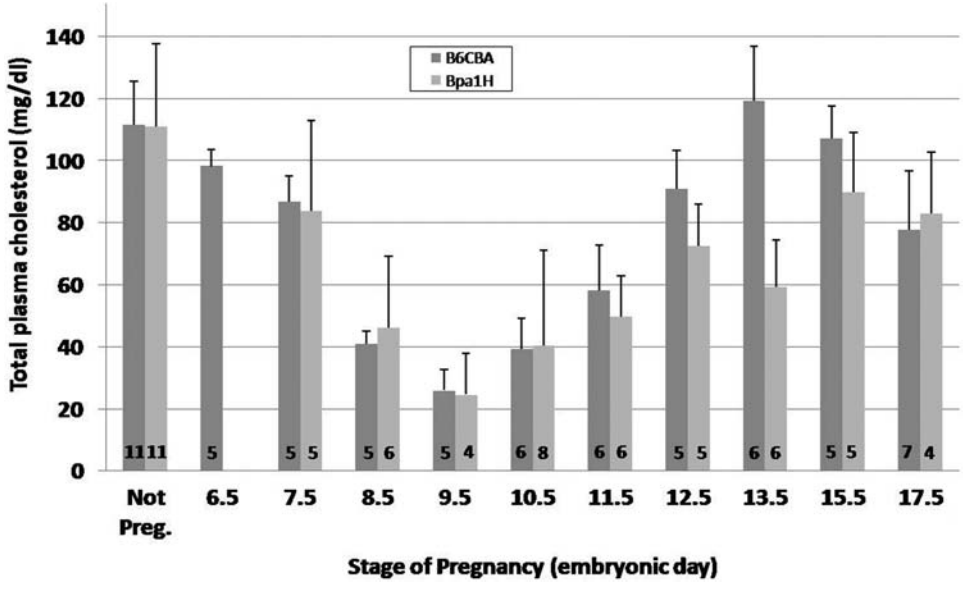

Figure 2. Maternal plasma cholesterol levels of B6CBA and $Bpa^{1H}/+$ females during pregnancy. Total plasma cholesterol levels were determined using a commercial colorimetric assay (see Material and Methods). Data are presented as the Mean ± SD (standard deviation). The numbers at the bottom of each bar represent the number of females of each genotype tested at that time point. $Bpa^{1H}/+$ females were not analyzed at E6.5.

slower rise from the nadir at E10.5 in the $Bpa^{1H}/+$ 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 $Bpa^{1H}/+$ 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 (Bpa^{8H}) and two mild (*Str) Nsdhl* alleles (5). Although differences in fetal placental thickness between WT and mutant male embryos were highly significant ($P \leq 0.001$), 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: The paternal X chromosome is preferentially inactivated in trophoblast-derived cells. Random X-inactivation occurs only in mesodermal 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 Bpa^{8H} 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 Bpa^{8H} 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 *Bpa*/+ 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.

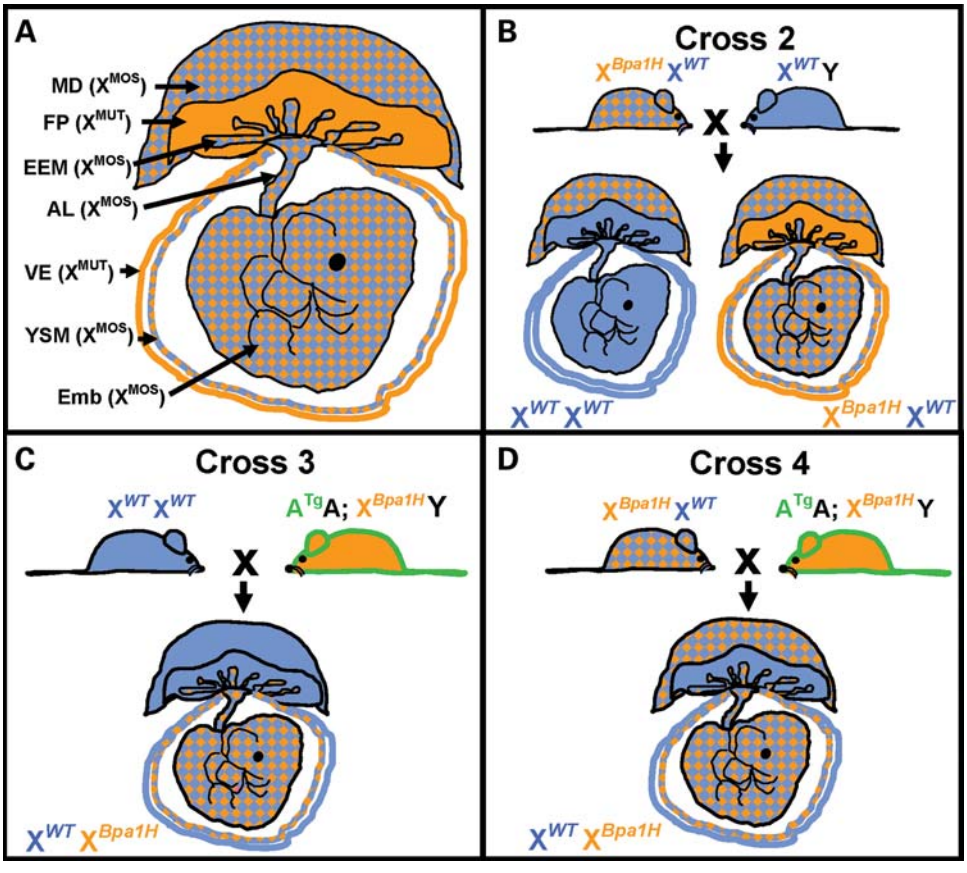

Figure 3. Schematics of patterns of X-inactivation in extraembryonic membranes. (**A**) Representation of a heterozygous *Bpa*^{1H}/+ female conceptus at E10.5 where the mutant X chromosome is inherited from the mother and the WT X chromosome from the father (Cross 2). Orange denotes expression of the mutant, maternal X chromosome and blue represents expression of the WT paternal X. Note mosaic (X^{MOS}; orange and blue hatched pattern) expression in the embryo (Emb) proper, maternal decidua (MD), and extraembryonic mesodermal lineages of the allantois (AL), yolk sac mesoderm (YSM), and fetal vessels in the labyrinth (EEM). Trophoblast derived lineages of yolk sac visceral endoderm (VE) and the fetal placenta (FP) express only the maternal (orange) mutant X chromosome (X^{MUT}). The latter includes trophoblast-derived mononuclear and syncytial cells of the labyrinth, as well as spongiotrophoblasts, and giant cells. (**B**–**D**) Schematic representations of relevant genotypes for Crosses 2–4 in Table 1. Parental genotypes for each cross are listed as in Table 1. Only the genotypes of relevant female embryos and placentas analyzed in Table 1 are shown. Orange and blue patterns of *Nsdhl* expression are as in (A). As in Table 1, the X chromosome inherited from the mother is always shown first, followed by the X inherited from the father. A = unspecified autosome; A^{Tg} = unspecified autosome with transgenic insertion.

We compared areas of the fetal placenta using the most severe *Bpa*^{1H} 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*^{1H}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*^{1H}/+ 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 ($P < 0.05$ 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 mm^2 (95% CI -0.971 to -0.391); $P < 0.0001$]. The maternal genotype had a smaller, but significant, effect [-0.283 mm^2 (95% CI -0.529 to -0.038); $P = 0.024$], whereas the fetal genotype had a small, non-significant effect on placental size [-0.168 mm^2 (95% CI -0.415 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

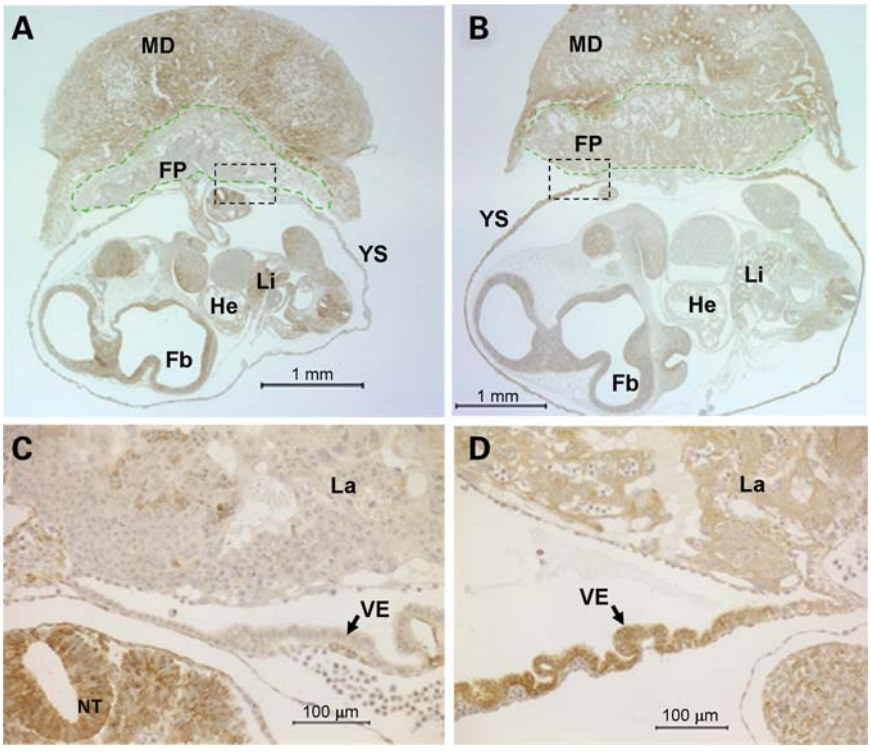

Figure 4. *Nsdhl* expression in extraembryonic membranes. Immunohistochemistry of cross-sections of E10.5 conceptuses (embryo and fetal membranes) using polyclonal antibody against murine NSDHL. The embryo in (**A**) is a heterozygous $Bpa^{1H}/+$ female from Cross 2, with the mutant X chromosome inherited from the mother. In (**B**), the embryo is a $Bpa^{1H}/+$ female from Cross 3 where the mutant X is inherited from the father. The region outlined in green represents the measured area of the fetal placenta (see Material and Methods). (**C, D**). Higher magnification of boxed areas in (A) and (B), respectively. Note mosaic *Nsdhl* expression within the embryo proper in both samples. However, in the yolk sac and placenta, there is *Nsdhl* expression only in mesodermally derived lineages in (A) and (C), whereas both mesodermal and trophoblast lineages demonstrate *Nsdhl* expression in (B) and (D). MD = maternal decidua; FP = fetal placenta; La = fetal placental labyrinth; YS = yolk sac; Li = fetal liver; He = heart; Fb = developing forebrain; VE = visceral endoderm; NT = developing neural tube.

Table 1. Fetal placental area

Cross Female × Male	Number	*Nsdhl* Genotype[a] Maternal	Embryonic	*Nsdhl* Expression[b] Maternal	ExEmbry	Embryonic	Placental area[c] (mm^2)
1. WT × WT	11	+/+	+/+	WT	WT	WT	1.90 ± 0.07
2. Bpa^{1H} × WT	8	−/+	+/+	Mosaic	WT	WT	1.64 ± 0.15
	18	−/+	−/+	Mosaic	Mutant	Mosaic	0.78 ± 0.10
3. WT × Tg^{Nsdhl}/Bpa^{1H}	17	+/+	+/−	WT	WT	Mosaic	1.75 ± 0.13
4. Bpa^{1H} × Tg^{Nsdhl}/Bpa^{1H}	12	−/+	+/−	Mosaic	WT	Mosaic	1.45 ± 0.11

[a]The *Nsdhl* genotypes of the mother and female embryos are designated as (+) for the WT allele and (−) for the mutant Bpa^{1H} 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.
[b]Denotes which *Nsdhl* is expressed in the animal or tissues shown. WT = wild-type allele expressed; Mosaic = random expression of WT or mutant Bpa^{1H} 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.
[c]Areas of fetal placentas were measured as described (see Material and Methods) and listed as the mean ± standard error (SEM).

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

hormones, bile acids and oxysterols. The latter are involved in lipid homeostasis through their regulation of orphan nuclear receptors, such as LXRs (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 $Bpa^{1H}/+$ 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 chorioallantoic 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 $Bpa^{1H}/+$ 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 $Bpa^{1H}/+$ 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^{1H} 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 chorioallantoic 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^{1H} and Bpa^{8H} alleles have been described (5) and are maintained by mating heterozygous females to C57BL/6J$A^{w\text{-}J}$ × CBA (B6CBA) F_1 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 *Xce* 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′-TTCAACTTTGGGCAGGTGGG-3′ and 5′-CTCC ATAGCATCATCCATGG-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^{1H} or Bpa^{8H} 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 *Hin*dIII digested genomic DNA using standard techniques (37). Southern blots were hybridized to ^{32}P-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 (NADIEKVNQNQAKR) 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 $CHCl_3$ 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 *Scmx* 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 $MgCl_2$, 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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REFERENCES

1. Liu, X.Y., Dangel, A.W., Kelley, R.I., Zhao, W., Denny, P., Botcherby, M., Cattanach, B., Peters, J., Hunsicker, P.R., Mallon, A.M. *et al.* (1999) The gene mutated in bare patches and striated mice encodes a novel 3beta-hydroxysteroid dehydrogenase. *Nat. Genet.*, **22**, 182–187.
2. Konig, A., Happle, R., Bornholdt, D., Engel, H. and Grzeschik, H. (2000) Mutations in the NSDHL gene, encoding a 3β-hydroxysteroid dehydrogenase, cause CHILD syndrome. *Am. J. Med. Genet.*, **90**, 339–346.
3. Herman, G.E. (2000) X-Linked dominant disorders of cholesterol biosynthesis in man and mouse. *Biochim. Biophys. Acta*, **1529**, 357–373.
4. Lucas, M.E., Ma, Q., Cunningham, D., Peters, J., Cattanach, B., Bard, M., Elmore, B.K. and Herman, G.E. (2003) Identification of two novel mutations in the murine Nsdhl sterol dehydrogenase gene and development of a functional complementation assay in yeast. *Mol. Genet. Metab.*, **80**, 227–233.
5. Caldas, H., Cunningham, D., Wang, X., Jiang, F., Humphries, L., Kelley, R.I. and Herman, G.E. (2005) Placental defects are associated with male lethality in bare patches and striated embryos deficient in the NAD(P)H Steroid Dehydrogenase-like (NSDHL) Enzyme. *Mol. Genet. Metab.*, **84**, 48–60.
6. Jiang, F. and Herman, G.E. (2006) Analysis of Nsdhl-deficient embryos reveals a role for hedgehog signaling in early placental development. *Hum. Mol. Genet.*, **15**, 3293–3305.
7. Osoegawa, K., Mammoser, A.G., Wu, C., Frengen, E., Zeng, C., Catanese, J.J. and de Jong, P.J. (2001) A bacterial artificial chromosome library for sequencing the complete human genome. *Genome Res.*, **11**, 483–496.
8. Heiss, N.S., Rogner, U.C., Kioschis, P., Korn, B. and Poustka, A. (1996) Transcription mapping in a 700-kb region around the DXS52 locus in Xq28: isolation of six novel transcripts and a novel ATPase isoform (hPMCA5). *Genome Res.*, **6**, 478–491.
9. Levin, M.L., Chatterjee, A., Pragliola, A., Worley, K.C., Wehnert, M., Zhuchenko, O., Smith, R.F., Lee, C.C. and Herman, G.E. (1996) A comparative transcription map of the murine bare patches *(Bpa)* and striated *(Str*) critical regions and human Xq28. *Genome Res.*, **6**, 465–477.
10. Gachotte, D., Barbuch, R., Gaylor, J., Nickel, E. and Bard, M. (1998) Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. *Proc. Natl. Acad. Sci. USA*, **95**, 13794–13799.
11. Barker, D.J. (2007) The origins of the developmental origins theory. *J. Intern. Med.*, **261**, 412–417.
12. Yajnik, C.S. and Deshmukh, U.S. (2008) Maternal nutrition, intrauterine programming and consequential risks in the offspring. *Rev. Endocr. Metab. Disord.*, **9**, 203–211.
13. Woollett, L.A. (2005) Maternal cholesterol in fetal development: transport of cholesterol from the maternal to the fetal circulation. *Am. J. Clin. Nutr.*, **82**, 1155–1161.
14. Tint, G.S., Yu, H., Shang, Q., Xu, G. and Patel, S.B. (2006) The use of the Dhcr7 knockout mouse to accurately determine the origin of fetal sterols. *J. Lipid Res.*, **47**, 1535–1541.
15. Yoshida, S. and Wada, Y. (2005) Transer of maternal cholesterol to embryo and fetus in pregnant mice. *J. Lipid Res.*, **46**, 2168–2174.
16. Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
17. Hemberger, M. (2002) The role of the X chromosome in mammalian extra embryonic development. *Cytogenet. Genome Res.*, **99**, 210–217.
18. Malassine, A., Frendo, J.L. and Evain-Brion, D. (2003) A comparison of placental development and endocrine functions between the human and mouse model. *Hum. Reprod. Update*, **9**, 531–539.
19. Carter, A.M. and Enders, A.C. (2004) Comparative aspects of trophoblast development and placentation. *Reprod. Biol. Endocrinol.*, **2**, 46.
20. Rossant, J. and Cross, J.C. (2001) Placental development: lessons from mouse mutants. *Nat. Rev. Genet.*, **2**, 538–548.
21. Downs, K.M. and Gardner, R.L. (1995) An investigation into early placental ontogeny: allantoic attachment to the chorion is selective and developmentally regulated. *Development*, **121**, 407–416.
22. Downs, K.M. (2004) Extraembryonic Tissues. Stern, C.D. (Ed), *Gastrulation, from cells to embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 449–459.
23. Woollett, L.A. (2008) Where does fetal and embryonic cholesterol originate and what does it do? *Annu. Rev. Nutr.*, **28**, 97–114.
24. Soccio, R.E. and Breslow, J.L. (2004) Intracellular cholesterol transport. *Arterioscler. Thromb. Vasc. Biol.*, **24**, 1150–1160.
25. Gill, S., Chow, R. and Brown, A.J. (2008) Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised. *Prog. Lipid Res.*, **47**, 391–404.
26. Mann, R.K. and Beachy, P.A. (2004) Novel lipid modifications of secreted protein signals. *Annu. Rev. Biochem.*, **73**, 891–923.
27. Varjosalo, M. and Taipale, J. (2008) Hedgehog: functions and mechanisms. *Genes Dev.*, **22**, 2454–2472.
28. Herman, G.E. (2003) Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. *Hum. Mol. Genet.*, **12** (Spec No 1), R75–R88.
29. Porter, F.D. (2003) Human malformation syndromes due to inborn errors of cholesterol synthesis. *Curr. Opin. Pediatr.*, **15**, 607–613.
30. Witsch-Baumgartner, M., Gruber, M., Kraft, H.G., Rossi, M., Clayton, P., Giros, M., Haas, D., Kelley, R.I., Krajewska-Walasek, M. and Utermann, G. (2004) Maternal apo E genotype is a modifier of the Smith–Lemli–Opitz syndrome. *J. Med. Genet.*, **41**, 577–584.
31. Cross, J.C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D.G., Yamamoto, H. and Kingdom, J.C. (2003) Genes, development and evolution of the placenta. *Placenta*, **24**, 123–130.
32. Cross, J.C. (2005) How to make a placenta: mechanisms of trophoblast cell differentiation in mice–a review. *Placenta*, **26** (Suppl. A), S3–S9.
33. Zeng, S.M. and Yankowitz, J. (2003) X-inactivation patterns in human embryonic and extra-embryonic tissues. *Placenta*, **24**, 270–275.
34. Palinski, W., Yamashita, T., Freigang, S. and Napoli, C. (2007) Developmental programming: maternal hypercholesterolemia and immunity influence susceptibility to atherosclerosis. *Nutr. Rev.*, **65**, S182–S187.
35. Edison, R.J., Berg, K., Remaley, A., Kelley, R., Rotimi, C., Stevenson, R.E. and Muenke, M. (2007) Adverse birth outcome among mothers with low serum cholesterol. *Pediatrics*, **120**, 723–733.
36. Steffen, K.M., Cooper, M.E., Shi, M., Caprau, D., Simhan, H.N., Dagle, J.M., Marazita, M.L. and Murray, J.C. (2007) Maternal and fetal variation in genes of cholesterol metabolism is associated with preterm delivery. *J. Perinatol.*, **27**, 672–680.
37. Faust, C.J. and Herman, G.E. (1991) Physical mapping of the loci Gabra3, DXPas8, CamL1, and Rsvp in a region of the mouse X chromosome homologous to human Xq28. *Genomics*, **11**, 154–164.
38. Caldas, H. and Herman, G.E. (2003) NSDHL, an enzyme involved in cholesterol biosynthesis, traffics through the Golgi and accumulates on ER membranes and on the surface of lipid droplets. *Hum. Mol. Genet.*, **12**, 2981–2991.
39. Cunningham, D., Spychala, K., McLarren, K.W., Garza, L.A., Boerkoel, C.F. and Herman, G.E. (2009) Developmental expression pattern of the

cholesterogenic enzyme NSDHL and negative selection of NSDHL-deficient cells in the heterozygous Bpa1H/+ mouse. *Mol. Genet. Metab.*, **98**, 356–366.
40. Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.*, **226**, 497–509.
41. Morin, R.J. and Elms, N.J. (1975) Rapid microanalysis of cholesterol in bile and serum by gas chromatography. *Ann. Clin. Lab. Sci.*, **5**, 52–56.
42. Kelley, R.I. (1995) Diagnosis of Smith–Lemli–Opitz syndrome by gas chromatography/mass spectrometry of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. *Clin. Chim. Acta*, **236**, 45–58.
43. Mroz, K., Carrel, L. and Hunt, P.A. (1999) Germ cell development in the XXY mouse: evidence that X chromosome reactivation is independent of sexual differentiation. *Dev. Biol.*, **207**, 229–238.
44. Angel, T., Faust, C., Gonzales, J.C., Kenwrick, S., Lewis, R.A. and Herman, G.E. (1993) Genetic mapping of the X-linked dominant mutations striated (*Str*) and bare patches (*Bpa*) to a 600-kb region of the mouse X Chromosome: implications for mapping human disorders in Xq28. *Mamm. Genome*, **4**, 171–176.
45. Kramer, C.Y. (1956) Extension of multiple range tests to group means with unequal number of replications. *Biometrics*, **12**, 307–310.