0888-8809/08/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 22(10):2268–2277

doi: 10.1210/me.2008-0165

Prolactin Receptor-Associated Protein/17β-Hydroxysteroid Dehydrogenase Type 7 Gene (*Hsd17b7*) Plays a Crucial Role in Embryonic Development and Fetal Survival

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Our laboratory has previously cloned and purified a protein named PRAP (prolactin receptor-associated protein) that was shown to be a novel 17β-hydroxysteroid dehydrogenase (HSD) enzyme with dual activity. This enzyme, renamed HSD17B7 or PRAP/17β-HSD7, converts estrone to estradiol and is also involved in cholesterol biosynthesis. The major site of its expression is the corpus luteum of a great number of species including rodents and humans. To examine the functional significance of HSD17B7 in pregnancy, we generated a knockout mouse model with targeted deletions of exons 1–4 of this gene. We anticipated a mouse with a severe fertility defect due to its inability to regulate estrogen levels during pregnancy. The heterozygous mutant mice are normal in their development and gross anatomy. The females cycle normally, and both male and female are fertile with normal litter size. To our surprise, the breeding of heterozygous mice yielded no viable HSD17B7 null mice. However, we found HSD17B7 null embryo alive *in utero* on d 8.5 and d 9.5. By d 10.5, the fetuses grow and suffer from severe brain malformation and heart defect. Because the brain depends on *in situ* cholesterol biosynthesis for its development beginning at d 10, the major cause of fetal death appears to be due to the cholesterol synthetic activity of this enzyme. By ablating HSD17B7 function, we have uncovered, *in vivo*, an important requirement for this enzyme during fetal development. (*Molecular Endocrinology* 22: 2268–2277, 2008)

THE SYNTHESIS OF estradiol in the ovary requires the expression of two enzymes, P450 aromatase (CYP19) and 17β-hydroxysteroid dehydrogenases (17βHSD). Whereas CYP19 in rodents is found both in the follicle and corpus luteum, two different 17βHSDs are expressed in a cell-specific manner in the ovary (1). In the follicle, specifically the granulosa cells, 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1) converts estrone, generated by the conversion of androstendione by CYP19, to estradiol (1, 2). HSD17B1 also catalyzes the conversion of androstenedione to testosterone (3), which can be aromatized to estradiol directly. HSD17B1 was the first to be identified in the follicle characterized and crystallized (2, 4–7), yet no expression of this enzyme could be detected in the corpus luteum. Despite the fact that the rodent corpus luteum was shown to express CYP19 and able to synthesize estradiol (1, 8), the luteal 17βHSD responsible for converting estrone to estradiol and/or androstenedione to testosterone remained elusive for years. More by accident than by design, we discovered a protein in the rat corpus luteum (9) that was identified as a novel 17βHSD enzyme (10, 11). This enzyme, which has little homology to HSD17B1, was originally identified as a 32-kDa protein abundantly expressed in the large luteal cells of the corpus luteum (9). Subsequently, we cloned the cDNA (12), developed specific antibodies (13), and isolated and characterized the promoter region of this gene (14). We found this protein to be phosphorylated on tyrosine and to associate with the intracellular domain of the short form of the prolactin receptor (12) and therefore, originally named it PRAP (prolactin receptor-associated protein). Once it was established that PRAP was a novel 17βHSD (10, 11), it was consequently renamed PRAP/17βHSD-7 or HSD17B7. In contrast to HSD17B1 (1–3), HSD17B7 converts estrone to estradiol but not androstenedione to testosterone (10). It is found in the corpus luteum of every species investigated to date, including ruminants and humans (13), revealing the possibility that the corpus luteum has a universal capacity to convert estrone to estradiol. After luteinization, HSD17B1 dis-

First Published Online July 31, 2008

Abbreviations: CYP19, P450 aromatase; E0.5, embryonic d 0.5; ES cells, embryonic stem cells; HSD17B7, PRAP/17β-hydroxysteroid dehydrogenase type 7; HSD17B1, 17β-hydroxysteroid dehydrogenase type 1; 17βHSD, 17β-hydroxysteroid dehydrogenase; PFA, paraformaldehyde; PRAP, prolactin receptor-associated protein.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

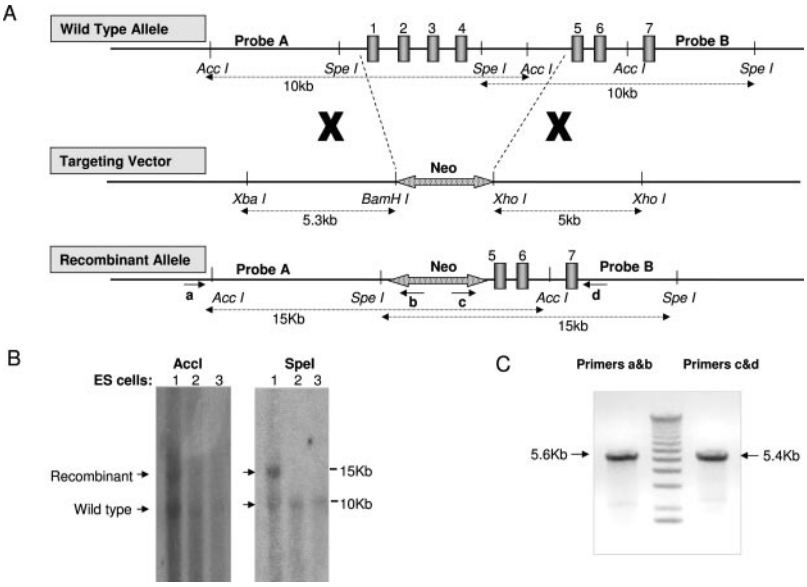

Fig. 1. Targeted Disruption of the HSD17B7 Gene

A, Partial restriction map of the mouse *Hsd17b7* genomic locus, the targeting vector, and the structure of the locus after combination. The targeting construct contains 5′-flanking coding region upstream of *Hsd17b7* and 3′-exons 5–7. Homologous recombination within the genomic sequence introduces the *neo* gene and eliminates exons 1–4. Restriction endonuclease sites used for cloning are shown in the panel. B, Southern blot analysis was performed on ES cells genomic DNA digested with either *Acc*I or *Spe*I before electrophoresis using probes to either the 5′- or 3′-end of *Hsd17b7.* Identification of recombinant alleles at 15.0 kb and wild-type alleles at 10.0 kb, are indicated by *arrows*. C, Genotyping of the targeted ES cells by PCR analysis confirmed the Southern blot results. Resulting PCR products of either 5.6 or 5.4 kb are indicative of the recombinant allele. Approximate position of PCR primers (a–d) are shown in the recombinant allele in panel A.

appears from the follicles and the luteal HSD17B7 becomes the enzyme responsible for the production of estradiol, especially during pregnancy (1, 13, 15). This luteal cell-derived estradiol is shown to play a key role in the hypertrophy, vascularization, and progesterone production by the corpus luteum in rodents and is considered to be, together with prolactin, a tropic hormone essential for corpus luteum survival and steroidogenesis (reviewed in Refs. 1, 16, and 17).

Recently, HSD17B7 has been described as possessing dual enzymatic activity. In addition to conversion of estrone to estradiol, it participates in postsqualene cholesterol biosynthesis and converts zymosterone to zymosterol *in vitro* (18, 19). There is great interest in this pathway because seven of 10 enzymes have been implicated in severe congenital malformation syndromes (20–22). Because our long-term interest is to define the role and importance of HSD17B7 in the normal progression of pregnancy and fetal development, we disrupted the coding sequence of *Hsd17b7* to generate HSD17B7 null mice.

RESULTS

Targeted Deletion of the Mouse HSD17B7 Gene

To investigate the function of HSD17B7 in mammalian development, we disrupted the *Hsd17b7* coding sequence by homologous recombination. The mouse HSD17B7 protein is encoded by 11 exons. To disrupt the NADH+/catalytic domain of HSD17B7, we inserted a neomycin resistance (*neo*) gene cassette into exons 1–4 (Fig. 1A). Of approximately 300 colonies resistant to both G418 and ganciclovir, we expanded 10 clones and screened for homologous recombination. Two probes, probe A and B, were used for Southern blot analysis of embryonic stem (ES) cells to identify the recombinant allele at 15-kb and wild-type allele at 10 kb (Fig. 1B). PCR primers for the flanking region outside both the 5′ and 3′ homologous recombination sites gave PCR products of either 5.6 kb or 5.4 kb corresponding to the recombinant allele (Fig. 1C). Of these ES cell clones, one clone had a legitimate homologous recombination event in one allele of the *Hsd17b7* gene as confirmed by both Southern blot (Fig. 1B) and PCR (Fig. 1C). To generate chimeric mice, we injected the HSD17B7 homologous recombinant ES cell clones into blastocysts from C57BL/6 mice and transplanted the injected blastocysts to pseudopregnant females. One of the clones transmitted to the germline, resulting in 16 male chimeric mice, which were subsequently backcrossed to the C57BL/6 background. The F_1 mice were genotyped by Southern blot (Fig. 2A) and PCR (data not shown).

Mice Lacking Functional HSD17B7 Are Lethal *in Utero*

Intercross of HSD17B7$^{+/-}$ mice gave offspring that were either wild type (HSD17B7$^{+/+}$) or heterozygous

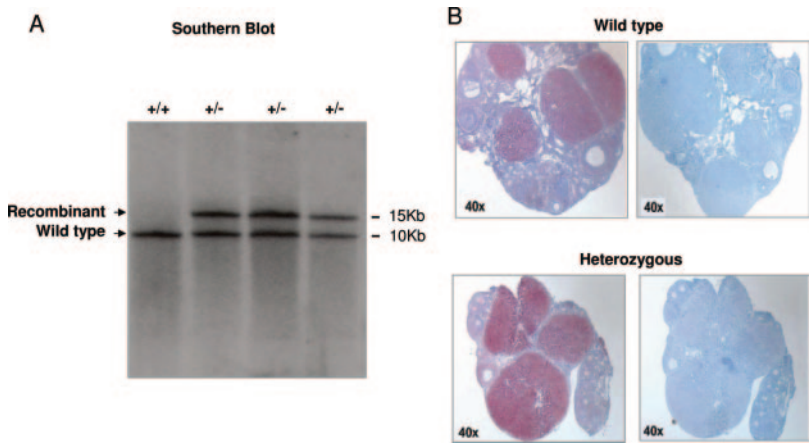

Fig. 2. Genotype of F_1 Offspring and Expression of HSD17B7 in +/+ and +/− Postpartum Ovaries

A, Representative Southern blot from F_1 offspring generated by intercross breeding of heterozygous mice. Mice from intercross breeding were genotyped using tail DNA digested with either *Acc*I or *Spe*I before electrophoresis using the probes in Fig. 1, A and B, Postpartum ovaries were obtained from wild-type and heterozygous females and subjected to immunohistochemistry as described in *Materials and Methods* using a HSD17B7 polyclonal antibody (1:150). Reactivity is shown in *red* and hematoxylin-counterstained in *blue*. Negative controls are displayed on the *right*.

(HSD17B7 $^{+/-}$). In total, of 314 offspring analyzed by Southern blot and PCR, 213 (68%) were HSD17B7$^{+/-}$ and 101 (32%) were HSD17B7$^{+/+}$. To our surprise, however, we did not obtain any homozygous null (HSD17B7$^{-/-}$) live-born offspring (Table 1). We examined whether the deletion of one allele is sufficient to silence the expression of HSD17B7 in HSD17B7$^{+/-}$ mice. Because this enzyme is highly expressed in the corpus luteum, we isolated postpartum ovaries from either wild-type or HSD17B7$^{+/-}$ females and subjected them to immunohistochemistry using a highly specific antibody generated in our laboratory (13). As shown in Fig. 2B, HSD17B7 is expressed similarly in the corpora lutea of the heterozygous and wild-type mice. Furthermore, HSD17B7$^{+/-}$ mice appear entirely normal in their development and gross anatomy. The females cycle normally, and both male and female are fertile with normal litter size. This indicates that one allele is sufficient for normal expression of HSD17B7 and for maintenance of pregnancy. The absence of newborn homozygous null mice suggests that targeted disruption of HSD17B7 may either prevent implantation or affect fetal survival during embryogenesis. To determine whether the absence of HSD17B7 homozygous null mice is due to a defect in implantation or embryonic lethality *in utero*, HSD17B7$^{+/-}$ mice were intercrossed, and the day a vaginal plug was found was considered d0.5 [embryonic d0.5 (E0.5)] of pregnancy. On d10.5 of pregnancy, the concepti were isolated, and embryos were dissected out from the yolk sac (Fig. 3, A and B). The yolk sac was used for genotyping by both Southern blot (Fig. 3C) and PCR (Fig. 3D). Of the ten E10.5 litters examined, 22 HSD17B7$^{+/+}$, 52 HSD17B7$^{+/-}$, and 12 HSD17B7$^{-/-}$ embryos were recovered. These results clearly indicate that implantation is unaffected by the deletion of HSD17B7 in embryos and that lethality occurs during development (Table 2).

Table 1. Intercross of HSD17B7$^{+/-}$ Mice Resulted in +/+ and +/− Offspring Only

	Total	+/+	+/−	−/−
Chimera	6			
Chimera × wt	48	23	25	0
F_1 (+/− × +/−)	105	36	69	0
F_2 (+/− × +/−)	129	41	88	0
F_3 (+/− × +/−)	80	24	56	0

wt, Wild type.

Timing of Fetal Lethality *in Utero*

To examine the exact time of embryonic death, we dissected embryos from timed pregnant HSD17B7$^{+/-}$ mice. Fetuses were isolated at different days of gestation between E7.5 and E18.5 and checked for survival. PCR analyses of the yolk sac confirmed the existence of the HSD17B7 mutation and loss of the HSD17B7 wild-type allele. At E8.5 and E9.5 $^{-/-}$ (Table 2), null embryos were recovered at Mendelian ratios with similar size to wild-type littermates, and no signs of resorption were noted. We found well-established anteroposterior axial structures and somites and a beating heart present in embryos. The earliest recognizable defects were detected in E10.5 HSD17B7$^{-/-}$ embryos (Fig. 4, *upper panel*). The fetuses appear distressed with no detectable blood in the heart and no heart beat. Lack of vascularization was also seen in both the fetus and the yolk sac, suggesting that the defect occurred between E9.5 and E10.5, a stage of major embryonic development. On E11.5, we found significant embryo resorption with severe fetal disintegration (Fig. 4, *lower panel*). When we isolated E10.5 embryos,

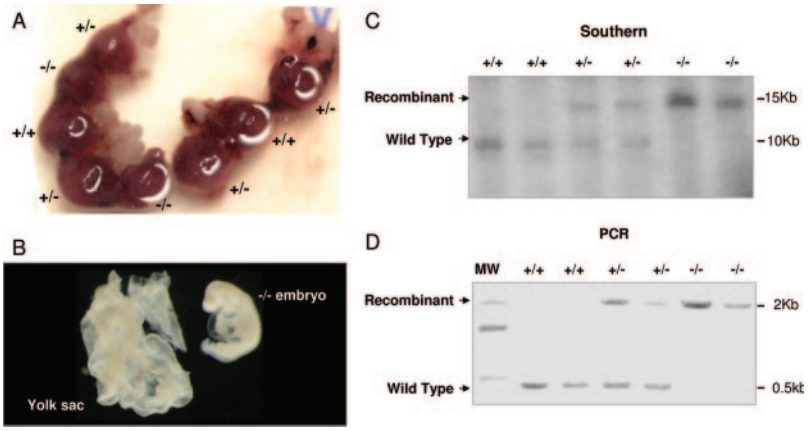

Fig. 3. Deletion of *Hsd17b7* Causes Embryonic Lethality *in Utero*

A, On d 10.5 of pregnancy, the concepti of HSD17B7$^{+/-}$ pregnant mice intercrossed with +/− males were numbered and isolated from the uterus (n = 10). B, The yolk sacs were dissected from the embryos and were used for genotyping by both Southern blot (C) and PCR (D) showing the correct size of wild-type and that of recombinant alleles. MW, Molecular weight.

we found the null embryos to be smaller in size than their wild-type littermates (Fig. 5). Pathology report of the HSD17B7$^{-/-}$ whole embryos indicates that the major defects occur in the development of the nervous system. In normal brain development, there are major cell migration, cell differentiation, and invaginations specifically in the region of forebrain, midbrain, and hindbrain. This stage of embryo development is normally accompanied by an increase in the volume of the forebrain and the fourth ventricle in the hindbrain region. A drastic reduction in size of these regions was found in the null embryos as compared with the wild-type and heterozygous littermates (Fig. 5, *upper panel*, *yellow arrows*). Histological studies of the brain region of wild-type and null HSD17B7 embryos at E10.5 (Fig. 5, *lower panel*) confirmed the pathological studies and show a marked underdevelopment and reduced size and volume in the forebrain and midbrain (shown by *arrowhead* and *arrow*, respectively). Furthermore, the boundaries between each of these regions are very poorly defined in null embryo as compared with the wild type. In addition, null embryos also had a heart defect (Fig. 5, *upper panel*, *black arrows*) with abnormal accumulation of fluid in the pericardial cavity.

Tissue-Specific Expression of HSD17B7 Gene in the Placenta

We examined the tissue-specific expression of HSD17B7 enzyme in the fetus, because pathological analysis of the HSD17B7$^{-/-}$ fetuses revealed major defects in brain and heart development. We performed whole-mount *in situ* hybridization on wild-type embryos using specific HSD17B7 probe (Fig. 6). Indeed, we found HSD17B7 expression not only in the brain and heart, but in the eye and ear as well. We also examined the localization of HSD17B7 in the placenta by immunohistochemistry (Fig. 7). This enzyme was found to be expressed in the ectoplacental cone and in the spongiotrophoblast, confirming the previous report by Vihko and co-workers (24). However, we also clearly detected HSD17B7 in the giant cells. Moreover, HSD17B7 is expressed in the maternal deciduas; however, the expression of this enzyme in the mother could not rescue the embryo.

DISCUSSION

To better define the role of HSD17B7 in pregnancy and/or in fetal development, we disrupted HSD17B7 coding sequence by homologous recombination. Because this enzyme is abundantly expressed in the corpus luteum where it converts estrone to the potent estradiol, and because estradiol was shown to act locally to stimulate the growth, vascularization, and progesterone production of the corpus luteum (reviewed in Refs. 1, 16, and 17), we expected severe defects in the development and steroidogenic capac-

Table 2. Timing of Fetal Lethality *in Utero*

No. of Heterozygous Matings	Embryonic Development	Wild-Type Embryos	Heterozygous Embryos	Null Embryos	Resorbed Embryos
40	Newborn	101	213	0	0
5	E 11.5	10	20	4	7
10	E 10.5	22	52	12	0
4	E 9.5	6	20	8	0
4	E 8.5	11	14	6	0

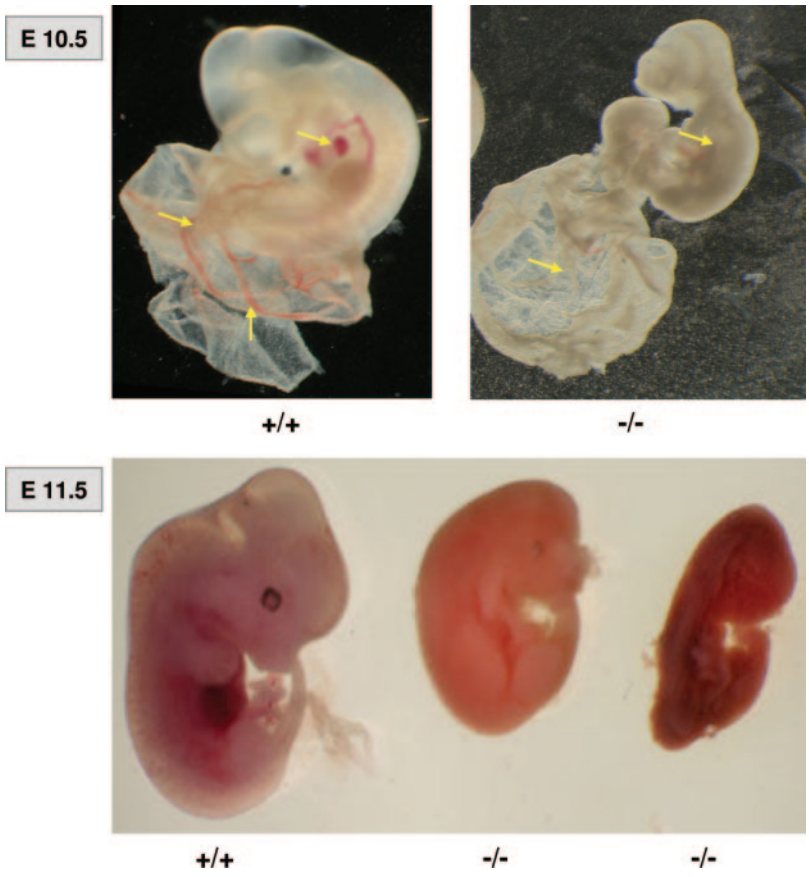

Fig. 4. Morphological Defects in HSD17B7$^{-/-}$ Embryo

E10.5 embryos were isolated and identified as wild type, heterozygous, and null after the yolk sac was genotyped by PCR. The null embryos were smaller in size and appeared distressed with no detectable blood in the heart and in the yolk sac of null embryos (*arrows* in the *upper panel*). Further examination of embryos at E11.5 revealed a dramatic decrease in size and severe fetal disintegration (*lower panel*).

ity of this gland leading to abortion. In addition, our finding that this enzyme is not expressed in the granulosa cells led us to doubt the accepted theory that the large luteal cells originate from the follicular granulosa cells (reviewed in Ref. 1). Instead, we proposed that estradiol formed in the large luteal cells was responsible for the hypertrophy and increase in size of these cells. We therefore expected the large luteal cells to remain the same size as the small luteal cells in the HSD17B7 null mice. However, to our surprise, we found that deletion of *Hsd17b7* gene leads to embryonic lethality after d 9.5 of pregnancy.

The detection of this enzyme in the fetal forebrain, midbrain, and hindbrain regions of wild-type embryos and its absence in null mice strongly suggests that HSD17B7 plays a key role in the normal formation of the brain *in utero*. Estradiol was shown to be required for normal brain maturation (reviewed in Refs. 25 and 26). A recent investigation (27) indicates a crucial role for estradiol/estrogen receptor-α during the initial phases of differentiation of brain cells that sequentially involves both glia and neurons. Estradiol was shown to promote proliferation of embryonic cortical progenitor cells *in vitro*, whereas blockade of estrogen receptors *in utero* decreases proliferation (28). CYP19, the enzyme responsible for the formation of estrone that serves as substrate for HSD17B7, is present in the embryonic neocortex. These findings suggest a functional role for estradiol as a proliferative agent during critical stages of cerebral cortex development. Because HSD17B7 converts estrone to estradiol and CYP19 is essential for estrone biosynthesis, one would expect to see fetal death in the CYP19 null mice similar to that seen in the HSD17B7 null mice if estradiol was essential for embryo survival. Nevertheless, the finding that female and male mice lacking functional CYP19 are born and grow to adulthood (29) indicates that estradiol is not essential for fetal brain development and fetal survival. Therefore, the reason of the lethality seen in our mice appears not to be due to the lack of conversion of estrone to estradiol, but rather due to a different activity of this enzyme. Indeed, Adamski and co-workers (18, 19) have reported the intriguing finding that in addition to converting estrone to estradiol, HSD17B7 is the last unknown enzyme in mammalian cholesterol biosynthesis and has 3-keto-steroid reductase activity. It can convert zymosterone to zymosterol, an important step in cholesterol biosyn-

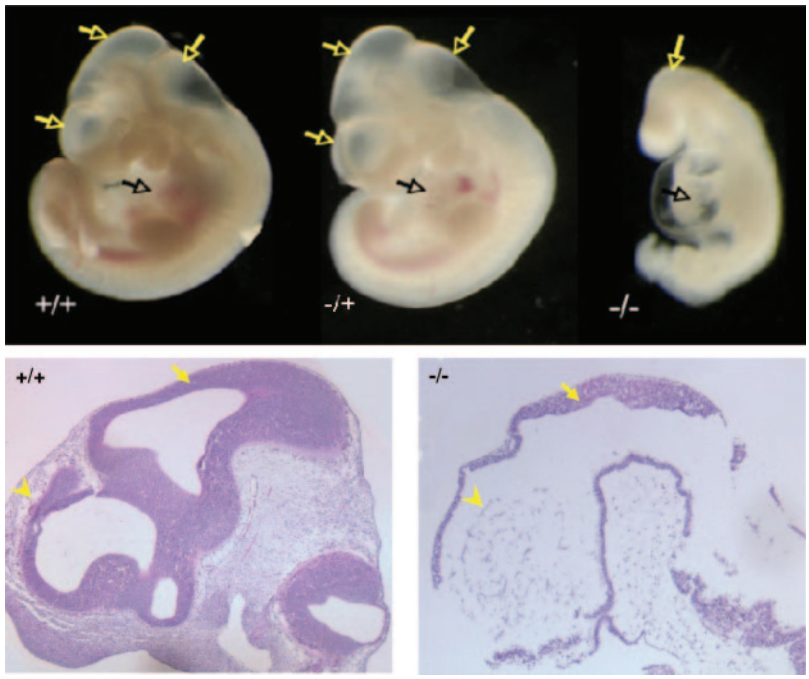

Fig. 5. Deletion of HSD17B7 Causes Severe Brain and Heart Defect

Pathology report of the whole HSD17B7$^{-/-}$ embryos (*upper panel*) indicates that the major defect in these embryos was the absence of forebrain, midbrain, and hindbrain regions (*yellow arrows*) and pericardial effusion (*black arrows*). Wild-type and null HSD17B7 embryos at E10.5 were serially sectioned and hematoxylin-eosin stained (*lower panel*). A marked underdevelopment of the different brain regions is shown in the null embryo in comparison with the wild type. The telencephalic vesicle and midbrain region in the HSD17B7$^{-/-}$ embryo is reduced in size and volume (shown by *arrowhead* and *arrow*, respectively). This stage of embryo development is normally accompanied by increased differentiation and invaginations in these brain regions, which are lacking in HSD17B7$^{-/-}$ embryos.

thesis. Unlike nonneuronal cells, the viability of neurons depends on the intracellular cholesterol content (30). Originally, they demonstrated that the ortholog of the yeast 3-ketosteroid reductase, Erg27p, converts zymosterone to zymosterol *in vitro*. Expression of human and murine *Hsd17b7* in an Erg27p-deficient yeast strain and in a HSD17B7 mammalian deficient cells complemented the 3-ketosteroid reductase deficiency of the cells and restored growth in cholesterol-deficient medium (31, 32), further establishing the role of HSD17B7 in the cholesterol biosynthesis pathway and demonstrating that this enzyme is the last undiscovered enzyme in this pathway. Promoter analysis substantiated the finding that HSD17B7 is involved in

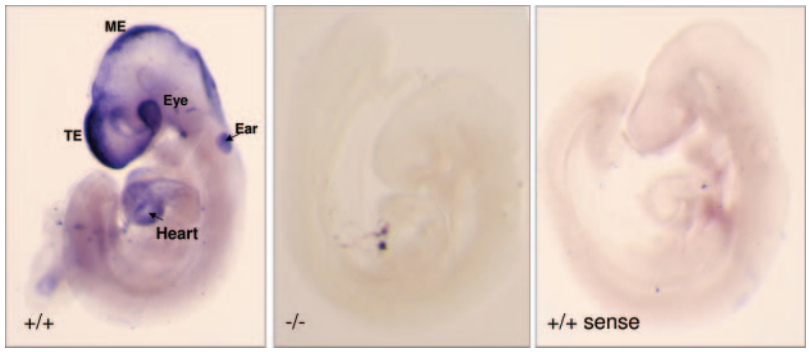

Fig. 6. Expression of HSD17B7 in the Embryo by *in Situ* Hybridization

Wild-type (*left*) and null (*middle*) HSD17B7 embryos at E9.5 were hybridized with HSD17B7 antisense riboprobe. Whole-mount hybridization with wild-type E9.5 embryo shows specific high expression of HSD17B7 in the brain, heart, eye, and ear. Expression of HSD17B7 was undetectable in the null embryo. Sense probe on wild-type E9.5 embryo was used as a negative control to show probe specificity (*right*). ME, Mesencephalon; TE, telencephalon.

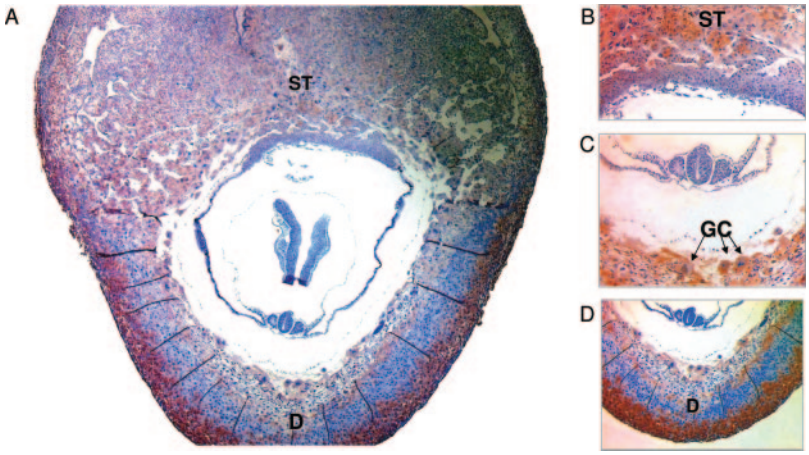

Fig. 7. Expression of the HSD17B7 in the Conceptus and Deciduas

Concepti were isolated from wild-type females at d 9.5 of pregnancy and immunostained with a HSD17B7 polyclonal antibody (1:150). Reactivity is shown in *red* (*yellow arrows*) and hematoxylin counterstained in *blue*. HSD17B7 is expressed most abundantly in the ectoplacental cone, specifically in the spongiotrophoblast (ST), giant cells (GC) and the peripheral area of the decidua capsularis (D).

postsqualene cholesterol biosynthesis in both humans and mice (33).

We originally thought that the involvement of HSD17B7 in cholesterol biosynthesis in the corpus luteum is of little importance because the cholesterol used by the ovary originates from circulating lipoprotein (34, 35). However, the marked expression of this enzyme in the fetal brain and the fact the viability of neurons depends on the intracellular cholesterol content and not on the intermediate nonsterol isoprenoid products (30) made us reconsider the importance of this activity in brain formation. The demands for cholesterol in the fetus are high, but whereas maternal cholesterol substantially contributes to fetal cholesterol during early pregnancy, fetal cholesterol biosynthesis rather than cholesterol transfer from maternal lipoproteins seems to be the main mechanism for satisfying fetal requirements (36, 37). From E10 the blood-brain barrier is formed and the brain rapidly becomes the source of almost all of its own cholesterol (36, 37). Interestingly, E10 is the stage where severe defects in brain formation are most apparent in the HSD17B7 null embryos. In humans, genetic defects in the enzymes of the cholesterol biosynthetic pathway lead to abnormal brain development *in utero* causing mental retardation (38, 39). Similar brain developmental anomalies and embryonic lethality to the *Hsd17b7*$^{-/-}$ fetuses were observed in mice with deletion of other proteins involved in cholesterol synthesis (20, 40, 41). The most studied enzyme is squalene synthase that catalyzes the first committed step in cholesterol biosynthesis. The complete knockout displays an early embryonic lethal phenotype similar to that of *HSD17B7* (20). The squalene synthase null fetuses have undeveloped brain and also die after d 9.5 at a time when the brain barrier is formed and the brain becomes dependent on its own synthesis of cholesterol. These fetuses are also smaller the day they die. Subsequent investigations generated conditional mouse lines, with cell-type deletion of squalene synthase and thus cholesterol production in specific cells in the brain. When squalene synthase was eliminated only in myelinating glia cells (41, 42), mutant animals were born but showed severe hypomyelination, and one third of the newborn died. When squalene synthase was deleted embryonically in neuronal and glial precursors, no pups were born (41, 42), highlighting the importance of brain cholesterol synthesis during development for fetal survival. Our deletion of *Hsd17b7*, the last enzyme in the cholesterol biosynthetic pathway to be discovered, further establishes the importance of cholesterol synthesis in brain development.

In addition to the expression of HSD17B7 in the brain, this enzyme is also found in the heart. Interestingly, the pathological analysis of the *Hsd17b* $^{-/-}$ fetuses indicates a defect in the heart with pericardial effusion. Whether this pathology is responsible for the lack of vascularization found in the E10.5 fetus remains to be seen. Deletion of HSD17B7 should not deplete heart cholesterol because the heart receives its cholesterol from the maternal circulation. This defect may be due to accumulation of the precursors of zymosterol or to a yet not defined HSD17B7 activity involved in proper vascular development. Generation of HSD17B7 conditional knockout should unravel the importance of this enzyme in the brain *vs*. vascular system in fetal survival.

Interestingly, HSD17B7 is expressed in the trophoblast, yet deletion of this enzyme did not affect the apparent morphology of the placenta, most probably due to the fact that the placenta obtains its cholesterol from circulating lipoprotein (reviewed in Ref. 43). In addition, because of the absence of CYP19 (43, 44), the placenta is unable to produce estrone that is converted to estradiol by HSD17B7, and estradiol is gen-

erated during pregnancy by the maternal ovary. This further substantiates the specific and important role of the cholesterol biosynthetic activity of HSD17B7 in fetal development.

In summary, we have successfully generated mice lacking HSD17B7, an enzyme with dual activity involved in both estradiol and cholesterol biosynthesis. We have expanded the functional repertoire for this enzyme *in vivo* and shown its importance in fetal development.

MATERIALS AND METHODS

Animals were kept under conditions of controlled light (0700–1900 h) and temperature (22–24 C) with free access to standard rodent chow and water.

Experimental Animals

All experimental procedures were performed in accordance to the Guidelines of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Generation of HSD17B7 Targeting Construct and Identification of Homologous Recombinant ES Cell Clones

We isolated *Hsd17b7* by screening a mouse 129/SvJ genomic library (Stratagene, La Jolla, CA) using a probe containing mouse HSD17B7 exon 1 and exon 2. Twenty phage clones were selected and sequenced. Of the 20 clones selected, four clones contained fragments of different sizes encoding the 5′-upstream region and exons 1–7. These clones were subsequently subcloned into either pBS (Stratagene) or pUC18 (Invitrogen, Carlsbad, CA) vectors, resequenced, and confirmed as part of the mouse *Hsd17b7* gene by BLAST analysis. The HSD17B7 targeting vector was constructed by using a positive-negative selection vector, pOSDUPDEL. To generate the site of 5′-homologous recombination in the targeting vector, a 5.3-kb fragment containing 5′-upstream sequences of the *Hsd17b7* gene was cloned into pOSDUPDEL at the *Xba*I-*Bam*HI cloning site upstream of the neomycin phosphotransferase gene (*neo*) cassette. Orientation of this fragment was confirmed by restriction enzyme digestion and sequencing. Exons 1–4 were replaced by the *neo* cassette. To generate the 3′-homologous region, a 5.0-kb fragment, containing a part of intron 4 and exons 5–7, was isolated and inserted into pOSDUPDEL at the *Xho*I cloning site (Fig. 1A). Orientation of this fragment was confirmed by restriction enzyme digestion and sequencing. In addition to neomycin, pOSDUPDEL contains a thymidine kinase cassette distal to the 5′-homologous region that is used as a secondary selection marker. *Not*I-linearized targeting vector was introduced into CJ7 ES cells by electroporation. Positively transfected cells were selected in 0.5 mg/ml G418 and 0.2 μM ganciclovir. Two probes, probe A (0.7 kb) and probe B (0.8 kb), were used for Southern blot analysis of ES cells, after either *Acc*I or *Spe*I digestion. To further confirm homologous recombination in ES cells, PCR was used to analyze the regions outside both the 5′- and 3′-homologous recombination sites extending into the *neo* cassette region (Primers a–d shown in third diagram of Fig. 1A). In addition, PCR products were cloned into pGEM-Teasy (Promega Corp., Madison, WI) and sequenced, to confirm the sequence of the recombinant allele. Both PCR and Southern blot confirmed successful homologous recombination (Fig. 1, B and C). After positive identification of homologous recombinant ES cell clones, recombinant ES cells were microinjected into C57BL/6 blastocysts and transplanted into uteri of pseudopregnant C57BL/6 mice. Chimeric males resulting from the microinjections were crossed to C57BL/6 females, and agouti pups were screened for germ line transmission of the mutant allele. The genotypes from these matings and all subsequent matings were determined by both Southern blot and PCR analysis of tail DNA.

Genotyping of Mouse Embryos

Southern Blot. Genomic DNA was isolated and digested with either *Acc*I or *Spe*I overnight. Samples were run on a 0.7% agarose gel and blotted on Hybond-n + (GE Healthcare, Piscataway, NJ). The blots were prehybridized for 4 h and hybridized overnight in a buffer containing 50% formamide, 5× standard sodium citrate, 5× Denhardt, 0.05 M phosphate, 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 250 μg/ml fish sperm. External probes located 5′ and 3′ of the HSD17B7 gene were radioactively labeled using the Rediprime II Random Primer Labeling Kit (GE Healthcare). The blots were exposed to KODAK Biomax MS (Sigma, St. Louis, MO) film with an intensifier screen overnight at −80 C. The Southern blots were visualized by autoradiography.

PCR. Offspring were genotyped by PCR for HSD17B7$^{+/-}$ mice using genomic DNA extracted from mice tails (Direct PCR lysis buffer, Viagen Biotech, Los Angeles, CA). To identify the wild-type allele, primers to exon 2, were used: P1, 5′-CAC ATA ATG TTT GGG TAT TTT CTA-3′; and P2, 5′-AGC AAT CTA TGA CAA AGA GAC ATA-3′. Two sets of primers were used to identify the HSD17B7 recombinant allele: set 1: a, 5′-TGT ATG AGC GTT TGG TAG TTA CCA-3′ and b, 5′-AGT TCT TCT GAG GGG ATC AAT TCA-3′, and set 2: c, 5′-ATC TTT TCC CTT TGT TTC TGG TCA-3′ and d, 5′-TCG ACG AAG CTA ATT CAT AAC TTC-3′ (*third diagram* in Fig. 1A). PCR was performed in 50 mM Tris (pH 9.0), 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 1 mM deoxynucleotide triphosphates, and 40 U *KOD Taq* polymerase (Novagen, Gibbstown, NJ). The cycling parameters were 94 C for 15 sec, 63 C for 30 sec, and 72 C for 2.5 min for 35 cycles. Each PCR product was cloned in pGEM-Teasy (Promega) vector, and each sequence was confirmed as part of mouse HSD17B7 gene by BLAST analysis. For embryos of midgestational stage, genomic DNA was isolated from the yolk sac. Recombinant primers to give around 2 kb piece for genotyping of yolk sac were 5′-GGG ATA ACG ATT ATA ACA GC-3′ and 5′-GAT GTG CAA CTC ACA TAA AG-3′. PCR was performed in 1× PCR buffer, 1.75 mM $MgSO_4$, 1 mM deoxynucleotide triphosphates, 20 pmol primers, and 10 U *KOD Taq* polymerase (Novagen). The cycling parameters were 94 C for 315 sec, 61 C for 30 sec, and 72 C for 1.75 min for 35 cycles. Each PCR product was cloned in pGEM-Teasy (Promega) vector, and each sequence was confirmed as part of mouse HSD17B7 gene by BLAST analysis.

Whole-Mount *in Situ* Hybridization

In situ hybridization probes were generated by PCR using cDNA of ovaries at d 15 of pregnancy as a template (19). PCR product was cloned in pGEMT-easy vector (Promega), sequenced, and confirmed to be part of the mouse HSD17B7 gene [bp 213–806; GenBank NM_010476]. HSD17B7 cDNA was digested with either *Spe*I/Sp6 (antisense) or *Nco*I/T7 (sense) and subjected to *in vitro* transcription (Roche Diagnostics Corp., Indianapolis, IN) to generate cRNA probes. Antisense probes used in this study were specific for HSD17B7, and sense probes served as negative control. *In situ* hybridization was performed essentially as described (23). Briefly, embryos were fixed in 4% paraformaldehyde (PFA) at 4 C, dehydrated in a graded methanol series, and stored at −20 C in 100% methanol until *in situ* hybridization

was performed. Embryos were then rehydrated, bleached in methanol/H_2O_2 (4:1) for 1 h, washed in PBS-Tween 20, treated with proteinase K (4–5 min E9.5), postfixed in 4% PFA/0.2% glutaraldehyde, and hybridized with digoxigenin-labeled cRNA probes. Hybridized cRNA probes were detected with sheep antidigoxigenin AP Fab antibody (Roche Diagnostics Corp.). After BCIP/NBT (Roche Diagnostics Corp.) reaction to detect signal, embryos were dehydrated through a graded methanol series to develop the purple colored precipitate, rehydrated, and cleared in 50% glycerol before imaging.

Immunohistochemistry

For histological analysis, postpartum ovaries were dissected and fixed in Bouin's fixative (Sigma). Concepti from wild-type timed matings were removed from the uteri and fixed in their surrounding membranes in 4% PFA. Both postpartum ovaries and concepti were serially sectioned (5 μm) and stained with hematoxylin and eosin using standard procedures. To examine whether postpartum ovaries and wild-type concepti express PRAP/17βHSD-7, sections were incubated overnight at 4 C with a primary polyclonal antibody PRAP/17βHSD-7 (1:150 dilution) (13) and then incubated with a secondary biotinylated goat antirabbit IgG according to manufacturer's instructions (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity was developed with Nova Red solution (Vector Laboratories) and sections were counterstained with hematoxylin (Vector Laboratories).

Acknowledgments

We thank Dr. Michal Lahav for helpful suggestions, Dr. Susan Ball-Kell and Dr. Marteen Brandon for the pathological analysis of the embryos, and Patricia Mavrogianis for technical support. We especially thank Dr. Gail S. Prins, Dr. Liwei Huang, and Jackson Hoffman for their help in the development of the whole mount *in situ*.

Received May 22, 2008. Accepted July 23, 2008.

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This work was supported by National Institutes of Health Grants HD11119 and HD12356 (to G.G.) and R01-HD38085 and CA100204 (to H.K.).

These results were presented in part at the Annual Meeting of the Society for the Study of Reproduction in 2007.

Disclosure Summary: The authors have nothing to disclose.

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