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Female mice deficient in steroid 5α-reductase type 1 have a decreased litter size. The average litter in homozygous deficient females is 2.7 pups *vs.* 8.0 pups in wild type controls. Oogenesis, fertilization, implantation, and placental morphology appear normal in the mutant animals. Fetal loss occurs between gestation days 10.75 and 11.0 commensurate with a midpregnancy surge in placental androgen production and an induction of 5α-reductase type 1 expression in the decidua of wild type mice. Plasma levels of androstenedione and testosterone are 2- to 3-fold higher on gestation day 9, and estradiol levels are chronically elevated by 2- to 3-fold throughout early and midgestation in the knockout mice. Administration of an estrogen receptor antagonist or inhibitors of aromatase reverse the high rate of fetal death in the mutant mice, and estradiol treatment of wild type pregnant mice causes fetal wastage. The results suggest that in the deficient mice, a failure to 5α-reduce androgens leads to their conversion to estrogens, which in turn causes fetal death in midgestation. These findings indicate that the 5α-reduction of androgens in female animals plays a crucial role in guarding against estrogen toxicity during pregnancy. (Molecular Endocrinology 11: 917–927, 1997)

INTRODUCTION

The androgens androstenedione and testosterone are substrates for two metabolic pathways that produce potent and antagonistic sex steroids (Fig. 1). In one pathway, they are converted into 5α-reduced androgens by steroid 5α-reductase isozymes (1). In the other, they are converted into estrogens by the aromatase enzyme, a cytochrome P450 of the endoplasmic reticulum (2). Conversion to these end products is irreversible and mutually exclusive in that 5α-reduced androgens are not aromatase substrates and estrogens are not 5α-reductase substrates (Fig. 1). The actions of 5α-reduced androgens and estrogens oppose one another: 5α-reduced androgens masculinize while estrogens feminize. Under physiological conditions in each sex, a delicate balance is established in which appropriate amounts of androstenedione and testosterone are converted into one or the other class of hormones. The set point of this fulcrum is differentially sustained in the two sexes by regulating the production of testicular androgens in males and the expression of the aromatase enzyme in females.

Occasional individuals have an imbalance in the 5α-reduced androgen-estrogen ratio due to genetic defects in the enzymes that determine these metabolic fates (Fig. 1). For example, mutations in the aromatase gene cause an increase in the androgen-estrogen ratio leading to virilization and polycystic ovarian disease in affected women (3, 4). Mutations in the 17β-hydroxysteroid dehydrogenase type 3 gene decrease the androgen-estrogen ratio and cause gynecomastia in affected men (5). Two different 5α-reductase genes encode the type 1 and type 2 isozymes in several mammalian species (2). Mutations in the human 5α-reductase type 2 gene, which is normally expressed in the urogenital tract and liver, cause male pseudohermaphroditism but do not alter the 5α-reduced androgen-estrogen ratio in men or women (6, 7). This outcome is presumably due to the ability of the type 1 isozyme, which is expressed in the liver and skin (8), to compensate for the absence of the type 2 isozyme in affected individuals.

No mutations in the 5α-reductase type 1 gene have yet been identified in humans, perhaps because of the presence of the type 2 isozyme in multiple tissues. However, the female mouse is an ideal animal model in which to study the type 1 isozyme because very little 5α-reductase type 2 is expressed in the tissues of this mammal (9). To take advantage of this expression pattern, and to explore the physiological role of the type 1 isozyme, a line of mice with a mutation in the 5α-reductase type 1 gene was developed (9). The absence of this isozyme had no obvious effect in males but caused a parturition defect and a reduction in litter size in females. The parturition defect was

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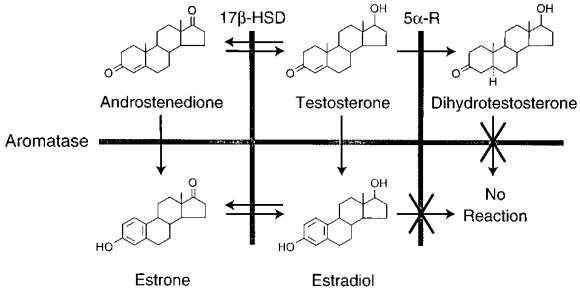

Fig. 1. Biosynthesis of Androgens and Estrogens

The pathways leading to the conversion of androgenic precursors into dihydrotestosterone and estrogens are shown together with the enzymes that catalyze individual biosynthetic steps. *Arrows* indicate reaction directions. The abbreviations are: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 5αR, steroid 5α-reductase.

traced to a failure of the uterus to synthesize 5α-reduced androgens in late gestation (9), a result that implicated the type 1 isozyme as playing an anabolic role in steroid hormone metabolism.

In the current study, we show that an imbalance in the ratio of 5α-reduced androgens to estrogens underlies the reduction in litter size in the 5α-reductase type 1-deficient mice. Approximately half of embryos die between gestation days 10.75 and 11 in animals homozygous for a null mutation in the type 1 gene (*Srd5a1*$^{-/-}$ mice). Fetal loss correlates with a transient increase in the plasma levels of androstenedione and testosterone and with a chronic elevation of plasma estradiol. The increase in the latter hormone is shown to cause fetal death. The results indicate that the type 1 isozyme plays an important catabolic role in sex steroid homeostasis by controlling the availability of substrate for estrogen synthesis.

RESULTS

A Fecundity Defect in *Srd5a1*$^{-/-}$ Mice

The average litter size of wild type mice with a mixed strain background (C57BL/6J/129Sv) was 8.0 pups (Table 1). Similarly, the litter size of *Srd5a1*$^{+/-}$ heterozygotes was 8.5. In contrast, the average number of pups in the litters of *Srd5a1*$^{-/-}$ homozygous mice was only 2.7. Essentially equal percentages of male and female pups were born to homozygous mothers (51% male, 49% female, n = 78). When homozygous mutant males were mated with wild type females, the litters were of normal size ($\bar{x}$ = 8.0), whereas crosses between wild type males and homozygous mutant females produced small litters ($\bar{x}$ = 3.0). Thus, the 5α-reductase type 1 genotype of the mother, and not that of her mate or pups, determined litter size.

Table 1. Fecundity Defect in *Srd5a1*$^{-/-}$ Mice

Parental 5αR1 Genotypes		No. of Matings	Litter Size ($\bar{x}$)[a]
Male	Female		
+/+	+/+	44	8.0
+/−	+/−	16	8.5[b]
−/−	−/−	15	2.7[c]
+/+	−/−	6	3.0[c]
−/−	+/+	2	7.5[b]

[a] Mean litter size (number of live pups born/number of litters) in homozygous knockout animals was determined from the 33% of mice who delivered at term (see Ref. 9 for an explanation of the parturition defect in 5α-reductase type 1-deficient mice).

[b] Litter size not significantly different from +/+ × +/+ matings (P = 0.6, Student's t test; P = 0.53, Wilcoxon rank sum test).

[c] Litter size significantly different from +/+ × +/+ matings ($P < 0.0001$, Student's t test or Wilcoxon rank sum test).

Fetal Death in Midgestation

Fecundity defects can arise from a failure of oogenesis, fertilization, implantation, or embryo survival during gestation. Inspection of the uteri of *Srd5a1*$^{-/-}$ mice revealed an increased number of embryo resorbtions, which suggested a postimplantation defect. To determine the time of embryo death during gestation, pairs of wild type or *Srd5a1*$^{-/-}$ mice were allowed to mate *ad libitum* over a 3-h time period and then separated. Pregnant females arising from these interludes were killed at various times thereafter, and the number of live embryos were counted as described in *Materials and Methods*. The percentage of living embryos at each time point is shown in Fig. 2. In both wild type and *Srd5a1*$^{-/-}$ females, about 80% of embryos survived through gestation day 10.75. However, approximately half of the surviving embryos in the mutant mice died between day 10.75 and 11.0, whereas no subsequent embryo loss occurred in the wild type mice (Fig. 2).

Tissue-Specific Expression of 5α-Reductase Type 1

To determine whether the time of embryo death in the knockout animals correlated with expression of 5α-reductase type 1 in wild type mice, RNA was isolated from the placentas and surrounding decidua, ovaries, livers, and brains of pregnant and nonpregnant animals and subjected to blot hybridization. The steady state levels of type 1 mRNA did not change during pregnancy in the ovaries (data not shown); however, the levels of type 1 mRNA transiently increased in the placenta/decidua between days 6 and 10 (Fig. 3A). In the brain, the level of type 1 mRNA was low in nonpregnant animals but substantially increased upon pregnancy (Fig. 3B). In the liver, a transient and modest increase in type 1 mRNA was detected on days

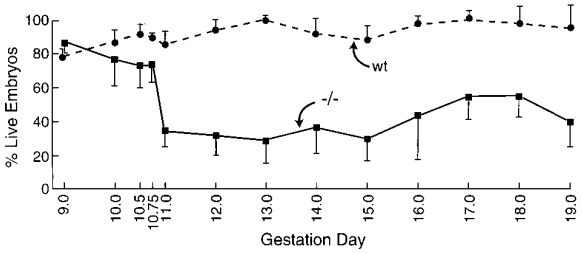

Fig. 2. Fetal Death in Wild Type and *Srd5a1*$^{-/-}$ Mice

The percentage of living embryos present in wild type (●—●) or knockout mice (■—■) was determined as described in *Materials and Methods* and is plotted as a function of gestation day. Three to seven animals were examined on each day. Points represent the means of embryo survival on a given day, and error bars represent mean ± SD. In wild type animals, approximately 80–90% of embryos survive to term, whereas in the −/− mice, survival rates are normal until day 10.75 ($P = 0.1$, Mantel-Haenszel χ^2 test) after which a significant number (50%) of embryos die suddenly ($P < 0.0001$). The rise in embryo survival detected on days 16–19 in the −/− mice is an experimental artifact caused by an underestimate of the number of resorbed fetuses present in late gestation.

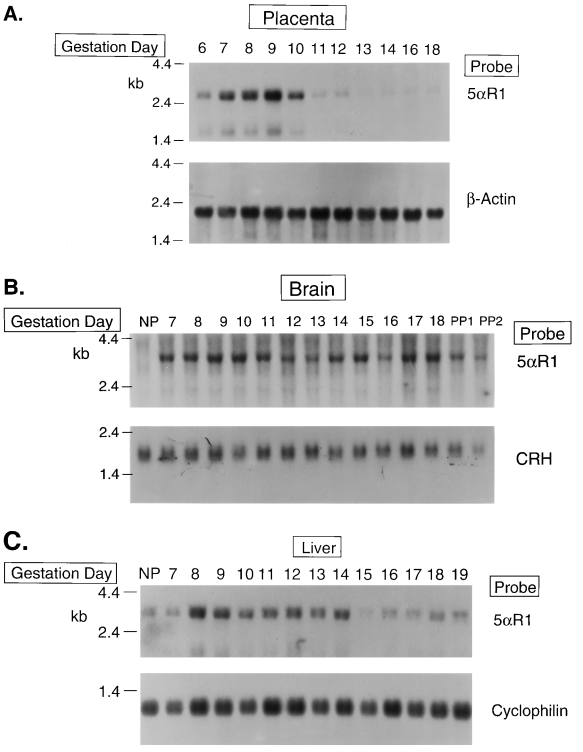

Fig. 3. Expression of 5α-Reductase Type 1 mRNA in Placenta/Decidua, Brain, and Liver of Wild Type Mice

A, Poly (A)$^+$-enriched RNA was isolated from placenta/decidua on the indicated day of gestation, and aliquots (5 μg) were subjected to blot hybridization with radiolabeled 5α-reductase type 1 (5αR1) or β-actin cDNA probes. The positions to which size standards migrated are shown on the *left* of the autoradiograms. Exposure times were 16 h for 5αR1 and 1.5 h for β-Actin. B, Poly (A)$^+$-enriched RNA was isolated from brain tissue of nonpregnant (NP) animals of the indicated gestation day, postpartum day 1 (PP1), and postpartum day 2 (PP2) mice, and aliquots (25 μg) were subjected to blot hybridization with radiolabeled 5α-reductase type 1 (5αR1) or CRH cDNA probes. The positions to which size standards migrated are shown on the *left* of the autoradiograms. Exposure times were 6 days for 5αR1 and 16 h for CRH. C, Poly (A)$^+$-enriched RNA was isolated from livers as above and analyzed for 5α-reductase type 1 (5αR1) and cyclophilin mRNA content. Size standards are indicated on *left*. Exposure times were 5 days for 5αR1 and 4 h for cyclophilin.

9–14 of gestation (Fig. 3C). The levels of control mRNAs (β-actin in the placenta/decidua, CRH in the brain, and cyclophilin in the liver) remained constant during these times (Fig. 3).

To confirm that the observed induction of 5α-reductase type 1 mRNA in the placenta/decidua led to an increase in enzyme mass, immunoblotting assays were performed (Fig. 4). A protein of approximately 22 kDa that comigrated with a recombinant 5α-reductase type 1 standard was induced in the tissue on gestation days 6 through 11 coincident with the observed increase in mRNA. The induction of 5α-reductase type 1 protein in the uterus was monitored as a control. In agreement with previous results (9), the content of uterine 5α-reductase type 1 increased in late but not midgestation (Fig. 4).

5α-Reductase Type 1 Expression in the Decidua

In situ mRNA hybridization was used to determine which cell types in the placenta/decidua express 5α-reductase type 1 on gestation day 8. An antisense probe revealed type 1 transcripts in decidual cells of the tissue (Fig. 5, A and B), whereas a sense probe produced no specific hybridization pattern (Fig. 5, C and D). Cells containing the type 1 mRNA were concentrated in the decidua basalis, which is the region of the decidua opposed to the mesometrial aspect of the uterus. No specific hybridization was detected in the decidua capsularis, which is located on the antimesometrial side of the uterus, or in any of the extraembryonic membranes of the fetal-placental unit (Fig. 5, A and B). Cells of the decidua basalis differentiate from endometrial connective tissue cells of the uterus (10). Thus, the finding that maternal decidual cells express 5α-reductase type 1 is consistent with results from the breeding experiments (Table 1), which showed that the fecundity defect was maternal in origin.

Expression of Placental Markers

The time of embryo death in gravid *Srd5a1*$^{-/-}$ females correlates with a switch in the source of lactogenic hormones required for pregnancy maintenance. In early gestation, PRL secretion from the anterior pituitary subserves both lactogenic and luteotrophic function in the establishment and maintenance of pregnancy (11). During midgestation, the sources and

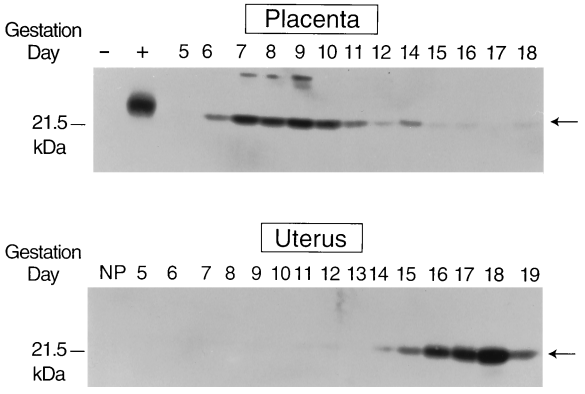

Fig. 4. Detection of 5α-Reductase Type 1 Enzyme in Reproductive Tissues

Whole cell extracts (100 μg protein) were isolated from either placentae/decidua of the indicated gestation day (*upper panel*) or uteri (*lower panel*) and subjected to immunoblotting as described in *Materials and Methods*. The positions to which the type 1 isozyme migrated are indicated by *arrows* on the *right* of the autoradiograms. The lane marked − contained extract (5 μg protein) isolated from mock-transfected Chinese hamster ovary cells. The lane marked + contained extract (5 μg protein) isolated from Chinese hamster ovary cells transfected with an expression plasmid for the human 5α-reductase type 1 (41). The lane marked NP contained extract isolated from the uterus of a nonpregnant mouse.

hormones required to continue pregnancy switch to lactogens secreted by the placenta (11). To determine whether fetal death in 5α-reductase type 1-deficient mice was associated with misexpression of one or more of the placental lactogens, the expression of placental lactogen 1 and 2 and proliferin were compared in wild type and knockout placentas in RNA blotting experiments. No differences in the induction patterns of these peptide hormone mRNAs were detected (data not shown). In agreement with these findings, no obvious morphological differences were apparent at gross or microscopic levels in the day 11 placentas associated with living or dead embryos in the mutant animals, nor were the patterns of desmin expression, a product of maternal decidual cells (12), different between these two tissue groups (data not shown).

Serum Steroid Hormone Levels

The lack of a gross placentation defect in the mutant mice suggested that fetal death might be due to the absence of an essential steroid produced by 5α-reductase type 1 or to the presence of a toxic metabolite that was normally catabolized by the enzyme. To test these hypotheses, plasma levels of androstenedione, testosterone, dihydrotestosterone, and estradiol were measured by RIA in pregnant wild type and knockout mice. The data of Fig. 6 show that in wild type females serum levels of the three androgens were low on days 6 and 7 of gestation, transiently increased between days 8 and 10, with a peak on day 9, and then returned to baseline levels. In knockout females, similar temporal increases in androstenedione and testosterone, but not dihydrotestosterone, were observed. However, the mean serum concentrations of androstenedione and testosterone were as much as 5-fold higher in the mutant animals during this period (Fig. 6). A statistical analysis indicated that the androstenedione levels on day 9 in knockout mice were not significantly different from those in wild type animals ($P = 0.1$, Wilcoxon rank sum test), whereas the testosterone and dihydrotestosterone levels were significantly different ($P = 0.03$ and 0.01, respectively).

Estradiol levels in early gestation were 2- to 3-fold higher in the mutant mice *vs.* wild type counterparts. This difference was statistically different (*e.g.* $P = 0.02$, day 8). Estradiol levels in the knockout mice remained high in midgestation, but the observed difference was not statistically significant (*e.g.* $P = 0.05$, day 13). Late gestation levels of estradiol appeared higher in wild type mice; however, the standard deviations in these measurements were substantial (Fig. 6), which led to a statistically significant difference on day 18 ($P = 0.04$) and a nonsignificant difference on day 19 ($P = 0.8$).

Together these data suggested that plasma levels of both estrogen precursors (androstenedione and testosterone) and estradiol itself were higher before and during the time of fetal death in the mutant mice.

A Bioassay for Fetal Death

To determine whether excess androgens or estradiol caused fetal death, we developed a bioassay in which pellets containing different amounts of individual steroids were subcutaneously implanted on day 5 of gestation. The pregnancies were allowed to proceed until gestation day 11 or 12, at which time the animals were killed and the living embryos were counted. The results obtained in wild type and *Srd5a1*$^{-/-}$ mice are summarized in Table 2. Pellets containing 0.5 mg of androstenedione killed approximately half of the embryos in wild type mice and appeared to increase the frequency of embryo death in the knockout animals. Testosterone had no statistically significant effects in two wild type mice in this assay, and in the mutant mice this hormone may have conferred a mild protective effect (Table 2). Estradiol at doses between 0.5 and 0.005 mg killed essentially all embryos in mice of both genotypes. When the estradiol dose was decreased to 0.0025 mg, the frequency of embryo survival in the wild type animals (~50%) was approximately the same as that seen in the untreated knockout mice (Table 1). This dose did not decrease embryo survival in the mutant mice. Ten additional 5α-reduced steroids or combinations of steroids, which are listed in *Materials and Methods*, were tested in the assay and did not affect embryo survival in animals of either genotype (data not shown).

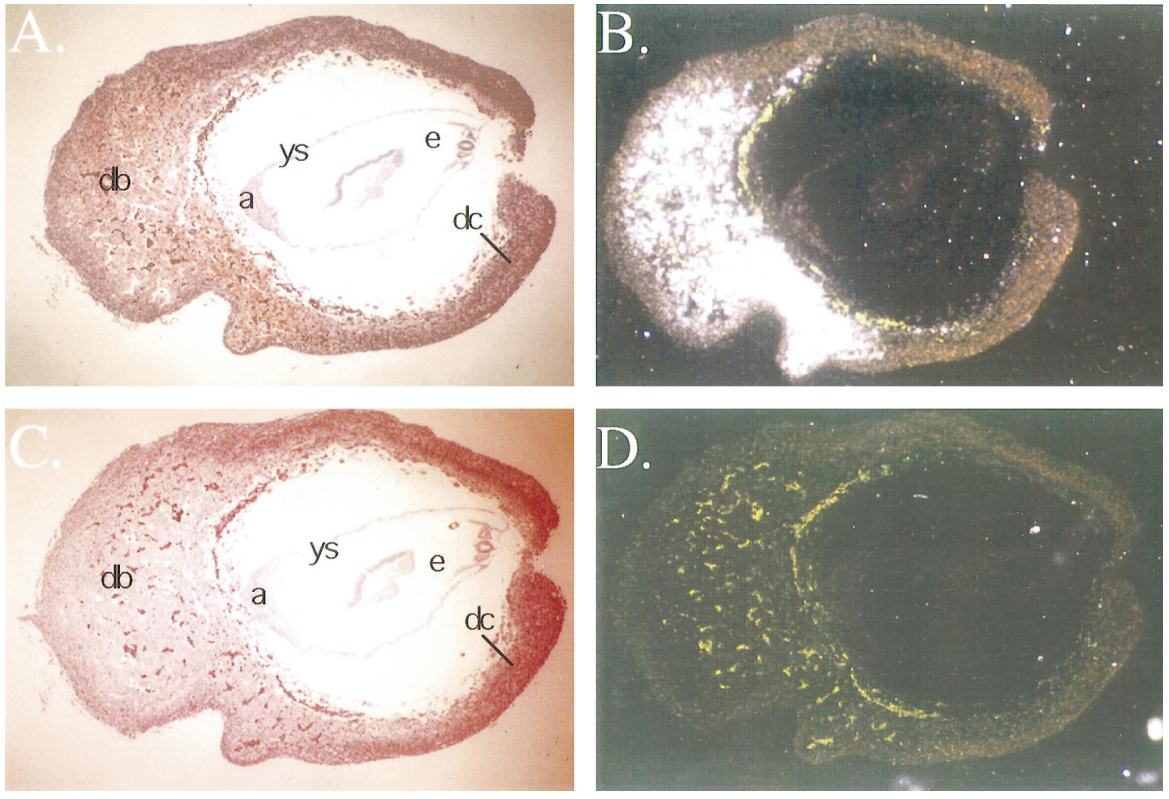

Fig. 5. Detection of 5α-Reductase Type 1 mRNA in Decidual Cells

Placenta/decidua tissue from gestation day 8 wild type mice was isolated and subjected to mRNA *in situ* hybridization analyses using an antisense strand probe to detect 5α-reductase type 1 transcripts (A and B) or a sense strand probe as a negative control (C and D). Exposure times were 21 days. Hybridized sections were stained with hematoxylin and eosin and photographed using lightfield and darkfield optics on a Leitz microscope. The fold-magnification was 9×. A, Lightfield photograph, antisense probe. B, Darkfield photograph of panel A, antisense probe. C, Lightfield photograph, sense probe. D, Darkfield photograph of panel C, sense probe. Labels are: a, allantois; db, decidua basalis; dc, decidua capsularis; e, embryo; ys, yolk sac. Red blood cells marking maternal and fetal blood vessels exhibit a *yellow-green* birefringence in the darkfield exposures of panels B and D.

In a preliminary attempt to determine the cause of embryo death in estrogen-treated mothers, uteri from control and experimental animals were examined at a gross morphological level. These experiments revealed that estrogen treatment caused hemorrhaging in the uteri of gravid wild type and knockout females (Fig. 7). Bleeding did not occur in the abdominal space, but rather was limited to the uterine lumen and the encircled fetal-placental units.

Estrogen Antagonists Increase Embryo Survival In *Srd5a1*$^{-/-}$ Mice

The results from the steroid pellet studies suggested that excess androstenedione or estradiol caused fetal death in both wild type and knockout mice. Since androstenedione is readily converted to estrone by the aromatase enzyme and thereafter to estradiol (Fig. 1), estrogen was thought to be the more likely hormone responsible for fetal death. If this interpretation is correct, then compounds that inhibit aromatase or block estrogen binding to the estrogen receptor should restore normal embryo survival to *Srd5a1*$^{-/-}$ mice. The data of Table 3 show that injection of aromatase inhibitors (4-hydroxyandrostenedione or Arimidex) on gestation days 6 through 10 prevented excess fetal death in the mutant mice. Approximately 79% of the knockout embryos survived in these experiments, which was a frequency identical to that observed in untreated wild type mice (Fig. 2). Similar results were obtained when pellets containing either 5.0 or 7.5 mg of the estrogen receptor antagonist tamoxifen were implanted on day 5 of gestation: administration of this drug led to more than 70% embryo survival frequencies (Table 3).

DISCUSSION

The absence of 5α-reductase type 1 in mice causes a fecundity defect of maternal origin. The pregnancies of these animals are normal until day 10.75, but between gestation day 10.75 and 11, approximately half of the embryos die. In wild type mice, expression of 5α-reductase type 1 increases to high levels in the decidua between gestation days 6 and 11. This increase

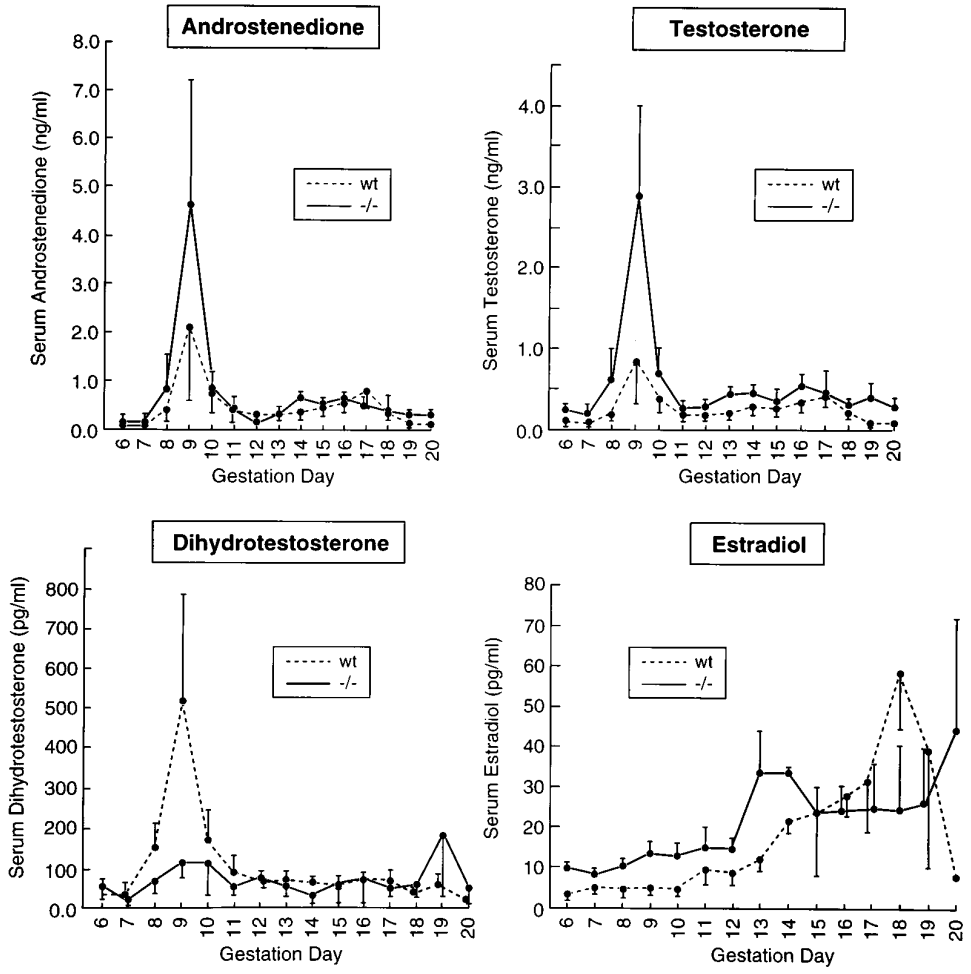

Fig. 6. Steroid Hormone Levels in Wild Type and *Srd5a1*$^{-/-}$ Mice

The plasma levels of the indicated steroid hormones were measured in a single RIA assay in wild type (wt) and 5α-reductase type 1 knockout mice ($-/-$) on the indicated days of gestation. Days 19 and 20 in wild type mice refers to values measured in postpartum animals. Day 19 and 20 values in the mutant mice were measured in animals exhibiting delayed parturition (9). Hormone levels were measured in three to seven animals for each day except for day 20, wild type, for which only a single animal was used. Points indicate mean hormone concentrations on a given day. Error bars represent mean ± SD.

correlates with a burst of androgen synthesis by the placenta that leads to elevated plasma levels of androstenedione, testosterone, and dihydrotestosterone. In the knockout mice, serum levels of androstenedione and testosterone increase during this same time period, whereas plasma dihydrotestosterone levels remain unchanged. The serum estradiol levels are elevated throughout early and midgestation in the *Srd5a1*$^{-/-}$ mice. Antagonists of estrogen synthesis and action correct the fecundity defect in knockout animals, a finding consistent with the concept that fetal death in midpregnancy is caused by estradiol toxicity. This scenario is further supported by the observation that exogenous estradiol causes fetal death in the offspring of wild type mothers. Given the known pathways of androgen and estrogen biosynthesis (Fig. 1), the simplest interpretation of these results is that 5α-reductase type 1 normally plays an important catabolic role in pregnant females by converting androgens to their nonaromatizable forms and that prevention of this action causes estradiol levels to increase and cause fetal demise.

Steroid 5α-reductase occurs in two isozymic forms called type 1 and type 2 that were initially postulated to fulfill distinct physiological roles. The type 1 isozyme was predicted to play a catabolic role in steroid hormone metabolism based on a high level of expression in tissues that break down steroids such as the liver and kidney and a micromolar affinity for steroid substrates (13). Conversely, a preferential expression of the type 2 isozyme in androgen target tissues of the male reproductive tract, coupled with a nanomolar affinity for steroid substrates, implied an anabolic role for the type 2 isozyme. Men that lack the type 2 isozyme exhibit symptoms of androgen deficiency, which confirms the anabolic role of this isozyme in humans (6).

Table 2. Effect of Steroid Hormones on Embryo Survival

Steroid[a]	Dose (mg)[b]	Wild Type Females			*Srd5a1*$^{-/-}$ Females		
		No. of Litters	Live Embryos ($\bar{x}$)	Dead Embryos ($\bar{x}$)	No. of Litters	Live Embryos ($\bar{x}$)	Dead Embryos ($\bar{x}$)
No pellet[c]		4	8.0	0.4	8	3.2	5.1
Placebo pellet[d]		3	9.6	0.67	3	4.3	5.0
Androstenedione[e]	0.5	4	3.3	4.5	5	1.0	7.8
Testosterone[f]	0.5	2	8.8	1.5	5	6.2	3.2
Estradiol[g]	0.5	2	0	7.0	5	0	8.4
	0.08	5	0	7.4	6	0	7.8
	0.02	2	0	5.0	6	0.3	6.3
	0.01	2	0	11.0	6	0	6.8
	0.005	2	0	8.5	5	0	5.0
	0.0025	2	4.5	4.0	4	2.8	5.5

[a] A single pellet containing the indicated amount of steroid was subcutaneously implanted in wild type or knockout females on day 5 of gestation. Animals were killed on day 11 or 12 of gestation, and the number of live and dead embryos was determined as described in *Materials and Methods*.
[b] Total amount of steroid per pellet. All pellets were 21-day release.
[c] Numbers in this line are derived from the day 11 experiments reported in Fig. 2.
[d] Animals received placebo pellets containing vehicle alone.
[e] For wild type animals, embryo survival significantly different from no pellet controls ($P = 0.009$, Fisher's exact test). For knockout animals, embryo survival significantly different from no pellet controls ($P = 0.018$).
[f] For wild type animals, embryo survival not significantly different from no pellet controls ($P = 1.0$). For knockout animals, embryo survival significantly different from no pellet controls ($P = 0.001$).
[g] For 0.0025 mg dose, embryo survival in knockout animals not significantly different from no pellet controls ($P = 1.0$).

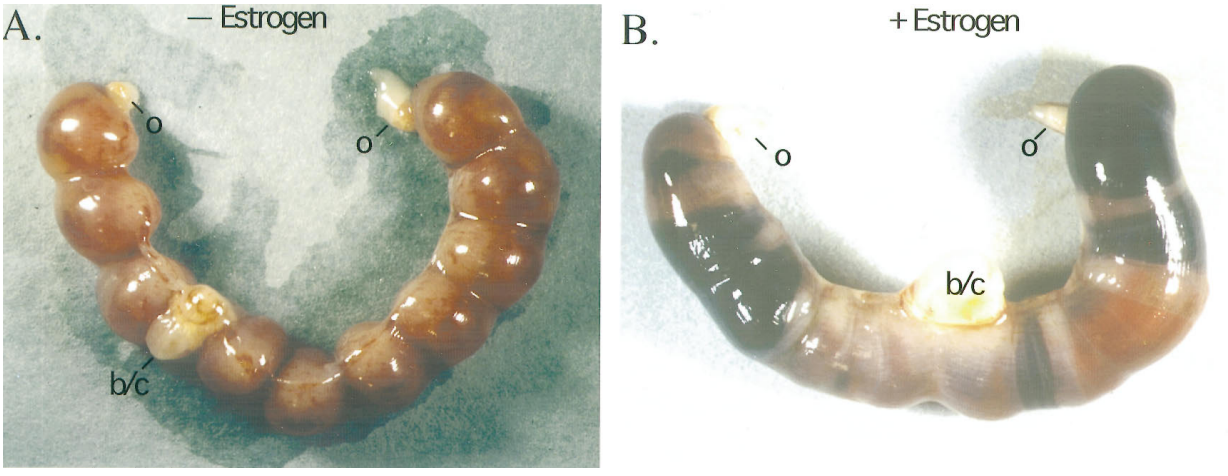

Fig. 7. Uteri of Control and Estrogen-Treated Mice

A, Gestation day 12 uterus from wild type control mouse containing 10 embryos. Note *pink* color and turgid fetal-placental units within both uterine horns. Labels are: o, ovary; b/c, bladder/cervix. B, Gestation day 11 uterus from *Srd5a1*$^{-/-}$ mouse treated with 10 μg estradiol pellet. Note sectors of hemorrhage corresponding to fetal-placental units. Some are *black*, one is a *deep red* indicative of more recent bleeding. Labels are same as panel A. Pronounced bleeding was observed in both wild type and knockout females at all tested doses of estradiol with the exception of wild type mice treated with 2.5 μg estradiol pellets.

The physiological role of the type 1 isozyme has been assessed in mice containing a null allele at the *Srd5a1* locus. The analysis of these animals reveals that the function of the type 1 isozyme is more complex than originally proposed in that it plays both anabolic and catabolic roles in pregnant female mice. In an anabolic capacity, the type 1 isozyme synthesizes a 5α-reduced androgen, most likely 5α-androstane-3α,17β-diol, which is required for the delivery of young at term (9). In a catabolic capacity, the current studies show that the type 1 isozyme breaks down androgens and, in so doing, prevents their conversion to estrogens.

Male and female mice do not appear to require 5α-reductase type 1 for phenotypic sexual differentiation or for the maintenance of steroid hormone homeostasis under normal conditions (9). The adverse consequences of enzyme loss are not realized until

Table 3. Estrogen Blockers Reverse Fecundity Defect in *Srd5a1*$^{-/-}$ Mice[a]

Drug	Dose (mg)	No. of Litters	Live Embryos ($\bar{x}$)	Dead Embryos ($\bar{x}$)	% Living
No pellet[b]		8	3.2	5.1	39%
Placebo pellet[c]		3.0	4.3	5.0	46%
Tamoxifen[d,e]	5.0	9	6.0	2.6	70%
	7.5	6	6.7	2.0	77%
4-Hydroxyandrostenedione[e,f]	1.5	5	7.0	2.4	74%
Arimidex[e,f]	0.15	4	8.0	1.5	84%

[a] *Srd5a1*$^{-/-}$ animals were administered the indicated drug in early pregnancy and killed on day 11 or 12. The number of live embryos was determined as described in *Materials and Methods*.
[b] Numbers in this line are derived from the day 11 experiments reported in Fig. 2.
[c] Numbers in this line are derived from experiments reported in Table 2.
[d] The indicated amounts of tamoxifen were administered by subcutaneous implantation of a 21-day release pellet on gestation day 5.
[e] Embryo survival significantly different from no pellet controls ($P < 0.0001$, Fisher's exact test).
[f] The indicated amounts of 4-hydroxyandrostenedione or Arimidex were administered on days 6–10 of gestation by daily subcutaneous injections as described in *Materials and Methods*.

pregnancy, when the endocrine system of the female changes drastically in ways that reveal essential catabolic and anabolic functions of the type 1 isozyme. The first requirement is one of catabolism and is manifest in midpregnancy at a time when circulating levels of several androgens are transiently increased to very high levels. These increases have been noted before (14–17) and appear to be peculiar to the mouse in that the rat does not exhibit midpregnancy androgen surges (18). The source of the murine androgens is the placenta (17), and we show here that 5α-reductase type 1 increases in the associated decidua with the same time course as androgen biosynthesis (Figs. 3–5). The physiological role of androgens at midpregnancy is not known.

The serum levels of estradiol that accumulate in the knockout mice are consistently at least 2-fold higher throughout days 6–14 of gestation, which span the time of fetal death (Fig. 6). Estrogen is among the most powerful of steroid hormones in mammals (19), and even slight changes in the estrogen response system can affect reproduction (20). This potency is further demonstrated by the ability of 21-day release pellets containing as little as 5 μg of hormone to kill all fetuses in a wild type animal (Table 2). The identification of estradiol as the toxic steroid in the type 1-deficient mice is further supported by the ability of an estrogen antagonist and aromatase inhibitors to reverse the fecundity defect in the mutant animals (Table 3). We conclude that the most likely culprit causing fetal death in the knockout animals is estrogen excess.

The aromatase mRNA (and presumably protein) are not expressed in the murine placenta (our unpublished observations); thus the placenta is not the source of excess serum estradiol in the mutant mice. Rather, the androgenic precursors produced by this tissue and others must be converted into estrogens in another organ, such as the ovary, in which aromatase is actively expressed throughout gestation (21, 22), or in some unidentified extraglandular location. The levels of aromatase mRNA in the ovary during gestation were similar between wild type and knockout females (data not shown), suggesting that a supraphysiological level of this enzyme in the mutant mice was not the cause of increased plasma estradiol. The absence of 5α-reductase in the liver may contribute to higher estradiol levels since circulating androgens would be expected to have a longer half-life and thus a greater chance of conversion into estrogens. In addition, the induction of 5α-reductase type 1 mRNA in the brain with pregnancy (Fig. 2B) may serve to regulate estrogen levels in wild type mice through classical endocrine feedback mechanisms involving the hypothalamic-pituitary-gonadal axes.

There are two apparent inconsistencies in the data reported here. First, we did not observe a direct correlation between serum estradiol and androgen levels in the knockout mice. Thus, on gestation day 9, when serum levels of androstenedione and testosterone doubled or tripled in the knockout mice, there was no corresponding spike of estradiol (Fig. 6). A brief elevation of estradiol was observed on days 12–15, but this rise was several days after the day 9 peak of androgens. Second, the time of fetal death (day 10.75–11) did not correlate with the largest observed increase in plasma androgens (day 9), as would be expected if these precursors were converted into circulating estrogens.

Several possible explanations exist for these inconsistencies. First, chronic exposure of the fetuses to excess estradiol before day 10.75 may lead to death shortly thereafter. Second, the embryos may become hypersensitive to estrogen around day 10.75 as a consequence of a developmental change in the fetal-placental unit. Third, death may be caused by an acute accumulation of estradiol in the placenta/decidua before the time of death that is not reflected in plasma hormone levels. This latter explanation is supported by the observation that 5α-reductase type 1 is induced in the decidua on gestation days 6–11 (Fig. 3). In normal

mice, this induction would protect the fetus from perilous estrogen accumulation. Little is known concerning the turnover of estrogens and androgens in wild type mice, much less in mutant animals; thus we can not interpret these results from the standpoint of kinetics. Despite these overall uncertainties, the ability of excess estradiol to recapitulate the knockout phenotype in wild type mice and of estrogen antagonists to restore fecundity in the mutant mice argues in favor of an estrogen excess theory of fetal death. Finally, a mild protective effect was observed upon testosterone administration to knockout mice (Table 2). This result may be a consequence of the ability of activated androgen receptor to oppose the deleterious effects of excess estradiol.

The mechanism by which estradiol brings about loss of fetal life in midgestation has not been explored. Inspection of the uteri of estrogen-treated animals revealed evidence of hemorrhage, which was limited to the fetal-placental units within the organ (Fig. 7). Similar observations were made in some, but not all, untreated knockout mice. Estradiol may thus alter the permeability or development of the maternal and fetal blood vessels, causing the fetuses to bleed to death. Estradiol affects the production of nitric oxide by the endothelium (reviewed in Ref. 23), which suggests the testable hypothesis that aberrant production of this potent vasodilator may lead to excess bleeding and fetal death in the *Srd5a1*$^{-/-}$ mice. The observation that tamoxifen blocks estrogen action in the knockout mice (Table 3) suggests that the hormone is acting through a receptor-based mechanism; however, the target tissue and the mechanism by which tamoxifen exerts this protective effect remain to be determined. No differences in the level of estrogen receptor mRNA were detected in the uteri and ovaries of mutant mice *vs.* wild type controls (data not shown). Thus, it is unlikely that enhanced estrogen sensitivity brings about fetal death in the mutant animals.

The administration of estrogens during early, mid, and late gestation has previously been shown to disrupt implantation (24), cause fetal death and resorbtion (25), or delay parturition (26). In the knockout mice studied here, implantation appears normal; however, fetal death occurs in midgestation as reported (25). The delay in parturition seen when ovarian extracts were administered late in gestation (26) is similar to the parturition defect described in the type 1-deficient mice (9). However, tamoxifen did not reverse the parturition defect in these animals (data not shown), whereas 5α-reduced androgens did (9).

Finally, we can ask whether the current results have any bearing on reproduction in women. There are well-documented cases of recurrent miscarriage occurring in midgestation (27), which ostensibly could be due to estrogen excess caused by the absence of the 5α-reductase type 1 isozyme. However, unlike the rodent placenta, the human placenta is laden with aromatase, which produces very high levels of estrogen in the amniotic fluid (28). Thus, the human fetal-placental unit must have developed a mechanism that protects the fetus from the toxic effects of estrogen that we observe in mice.

MATERIALS AND METHODS

Mice

Animals were housed under a 12-h light cycle (0400–1600 h) at 22 C. All mice were of mixed strain (C57BL/6J/129Sv) and were either wild type at the *Srd5a1* locus on chromosome 13 (29) or contained an induced null allele at this locus [deletion of proximal promoter and exon 1 (9)]. Timed matings were carried out by placing one male with four female mice in a cage from 0900–1200 h, after which the male was removed and females were checked for the presence of vaginal plugs. Gestation day 0 was defined by the presence of a plug.

Embryo survival in wild type and homozygous knockout mice was determined as follows. Pregnant females arising from timed matings were killed on gestation days 9–19 and their uteri were removed. The number of fetuses was determined by counting implantation sites. Fetal-placental units were dissected from the uteri and scored for the presence or absence of a beating heart in the embryo with the aid of a low-power microscope.

Organs were dissected from timed pregnant females on the indicated days of gestation. Placental tissues removed on gestation days 6–8 refer to fetal-placental units and the immediately opposed decidua. After gestation day 8, embryos were removed from this tissue before RNA or protein isolation.

All animal experiments were carried out using protocols approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee.

RNA Blotting

RNA isolation and blotting were performed as previously described (9). Radiolabeled cDNA probes were prepared by random hexanucleotide priming or by the PCR (30). Complementary DNA probes included mouse 5α-reductase type 1 (9), human β-actin (31), mouse CRH (32), mouse placental lactogen 1 (33), mouse placental lactogen 2 (34), mouse proliferin (35), mouse estrogen receptor (36), rat aromatase (37), and rat cyclophilin (38). These were obtained from individual investigators (β-actin, CRH, proliferin, aromatase) or by the PCR using published cDNA sequences. Each RNA blotting experiment was repeated two to three times with samples isolated from different sets of animals.

Immunoblotting

Immunoblotting of 5α-reductase type 1 protein was carried out as previously described (8). The primary antibody used in these experiments was raised against a multiantigen peptide (Bio-Synthesis, Lewisville, TX) composed of the amino acid sequence RAKEHHEWYLRKFEEYPKSRKILI, which corresponds to residues 233–256 of the human type 1 isozyme (2). Before use, the antiserum was affinity-purified on a Sepharose 4B column to which a peptide (LRKFEEYPKFRKIIIP) was coupled (39). This sequence is a hybrid derived from the carboxy termini of the rat and human type 1 isozymes (2). The purified antiserum was used at a concentration of 2 μg/ml in the blotting reactions. Antigen-antibody complexes were detected by enhanced chemiluminescence. The placental expression of the intermediate filament protein desmin was followed by immunoblotting with an antibody from Sigma

(D-8281, St. Louis, MO). Each immunoblotting experiment was repeated two or more times using tissues isolated from different animals.

In Situ mRNA Hybridization

Transcripts of the 5α-reductase type 1 gene were detected by *in situ* hybridization in 5 μm sections of day 8 placenta/decidua as described previously (40). [^{33}P]-Radiolabeled RNA probes in sense and antisense orientation were transcribed *in vitro* from a cDNA encoding amino acids 1–94 of the murine type 1 enzyme. Exposure times were 21 days. After development, tissue sections were photographed under lightfield and darkfield illumination on a Leitz Labrolux S Photomicroscope (Rockleigh, NJ) outfitted with a Bunton low magnification darkfield illuminating condenser.

Hormone Measurements

Blood was drawn from the inferior vena cava of pregnant animals between days 6 and 19 of gestation and 1 day postpartum (labeled as d20). Blood samples were collected from three to seven wild type or *Srd5a1*$^{-/-}$ females for each time point with the exception of day 20, wild type, for which steroids in only one animal were measured. Serum was collected and stored at −20 C until steroid analyses were performed.

Estradiol, androstenedione, testosterone, and dihydrotestosterone levels were quantified in serum by RIA after chromatographic separation of steroids on Sephadex LH-20 columns (Pharmacia, Inc., Piscataway, NJ). Steroid measurements were performed at the Oregon Regional Primate Research Center (Beaverton, OR). All samples were analyzed in a single, large experiment. The intraassay coefficients were: estradiol, 5.8%; androstenedione, 12%; testosterone, 1.0%; dihydrotestosterone, 9.8%. The average blank values were 1.2 pg for estradiol, 3.8 pg for androstenedione, 2.8 pg for testosterone, and 7.0 pg for dihydrotestosterone.

Steroid Pellet Studies

On day 5 of pregnancy (plug day = 0) animals were anesthetized by intraperitoneal injection of 1.6 mg Nembutal (Abbott Laboratories, North Chicago, IL) dissolved in approximately 100 μl saline. A 1-cm incision was made through the back skin, one or more steroid pellets (Innovative Research of America, Sarasota, FL) were inserted, and the incision was closed with wound clips. Animals were thereafter killed on day 11 or 12 of gestation, and embryo survival was determined by the presence of a beating heart. Twenty-one-day time release pellets were employed that contained the steroids or drugs indicated in Tables 2 and 3.

Several additional 5α-reduced steroids were tested for their ability to reverse the fecundity defect in *Srd5a1*$^{-/-}$ mice. Pellets were inserted as described above on gestation day 5. Pregnant females were then killed on gestation day 17 or 18, and the number of live and resorbed fetuses was determined. None of the following 5α-reduced steroids were effective in reversing the fecundity defect: dihydroprogesterone, 1.5 or 25 mg, 30-day release; dihydrotestosterone, 0.5 or 1.5 mg, 21-day release; 5α-androstan-3α,17β-diol, 0.13 or 0.91 mg, 14-day release; 5α-androstan-3β,17β-diol, 0.13 mg, 14-day release; androstanedione, 0.13 mg, 14-day release; androsterone, 0.22 mg, 21-day release; 5α-androstan-3α,17β-diol together with 5α-androstan-3β,17β-diol, 0.13 mg each, 14-day release; epiandrosterone, 1 mg, 14-day release; allodihydrocortisone, 7.5 mg, 14-day release.

Aromatase Inhibitor Studies

The aromatase inhibitors 4-hydroxyandrostenedione (Sigma, St. Louis, MO) and Arimidex [ZD1033, 2,2′-(5-(IH-1,2,4-triazol-1-ylmethyl)-1,3-phenylene)-bis(2-methylpropiononitrile), Zeneca Pharmaceuticals, Maccelsfield, England], were administered on days 6–10 of gestation by daily subcutaneous injections. 4-Hydroxy-androstenedione (1.5 mg) was injected daily in 75 μl propylene glycol. Arimidex (0.15 mg) was injected daily in 50 μl triolene (Sigma, St. Louis, MO). On gestation day 11 or 12, the treated female was killed, and the number of live and dead embryos was determined by observation of a heartbeat.

Statistical Analysis

The Students *t* test and Wilcoxon rank sum test were used to determine significant differences in litter sizes reported in Table 1. The significance of changes in embryo survival with drug/hormone treatments (Tables 2 and 3) was determined using Fisher's exact test. The Mantel-Haenszel χ^2 test with continuity correction was used to compare significance of embryo survival data in wild type *vs.* *Srd5a1*$^{-/-}$ animals from day 9 to day 10.75 and from days 11 through 19 (Fig. 2). Steroid hormone profiles of wild type and *Srd5a1*$^{-/-}$ animals were compared using the Wilcoxon rank sum test. For all statistical tests employed, $P < 0.05$ indicates significance.

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