Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipid adrenal hyperplasia

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ABSTRACT An essential component of regulated steroidogenesis is the translocation of cholesterol from the cytoplasm to the inner mitochondrial membrane where the cholesterol side-chain cleavage enzyme carries out the first committed step in steroidogenesis. Recent studies showed that a 30-kDa mitochondrial phosphoprotein, designated steroidogenic acute regulatory protein (StAR), is essential for this translocation. To allow us to explore the roles of StAR in a system amenable to experimental manipulation and to develop an animal model for the human disorder lipid congenital adrenal hyperplasia (lipoid CAH), we used targeted gene disruption to produce StAR knockout mice. These StAR knockout mice were indistinguishable initially from wild-type littermates, except that males and females had female external genitalia. After birth, they failed to grow normally and died from adrenocortical insufficiency. Hormone assays confirmed severe defects in adrenal steroids—with loss of negative feedback regulation at hypothalamic–pituitary levels—whereas hormones constituting the gonadal axis did not differ significantly from levels in wild-type littermates. Histologically, the adrenal cortex of StAR knockout mice contained florid lipid deposits, with lesser deposits in the steroidogenic compartment of the testis and none in the ovary. The sex-specific differences in gonadal involvement support a two-stage model of the pathogenesis of StAR deficiency, with trophic hormone stimulation inducing progressive accumulation of lipids within the steroidogenic cells and ultimately causing their death. These StAR knockout mice provide a useful model system in which to determine the mechanisms of StAR's essential roles in adrenocortical and gonadal steroidogenesis.

A critical component of regulated steroidogenesis is the induction of steroid biosynthesis by pituitary trophic hormones. This hormonal induction is divided temporally into acute and chronic effects: acute effects reflect increased mobilization and delivery of cholesterol precursor to the inner mitochondrial membrane, whereas chronic effects result from increased transcription of genes that encode the steroidogenic enzymes and other key components of the steroidogenic complex (reviewed in ref. 1). Protein synthesis inhibitors block the acute response, suggesting that a newly synthesized, labile protein facilitates cholesterol translocation from the outer to the inner mitochondrial membrane where the cholesterol side-chain cleavage enzyme P450_{sc} catalyzes the initial reaction in steroidogenesis (2–4). Recently, a mitochondrial phosphopro-

tein was purified and cloned that met many of the criteria predicted for this labile mediator (5); this protein was designated steroidogenic acute regulatory protein (StAR). Evidence linking StAR with the acute induction of steroidogenesis includes its selective expression in the mitochondria of steroidogenic cells of the adrenal cortex, testis, and ovary, and its rapid induction within these cells by trophic hormone. Moreover, transient transfection of MA-10 Leydig cells with a StAR expression plasmid enhanced their ability to convert cholesterol precursor to pregnenolone.

Compelling evidence for StAR's essential role in regulated steroidogenesis came from analyses of patients with congenital lipid adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder characterized by impaired adrenal and gonadal steroidogenesis and male pseudohermaphroditism coupled with characteristic lipid deposits within the steroidogenic tissues (refs. 6 and 7; reviewed in ref. 8). Three unrelated patients with lipoid CAH were shown to carry mutations in the *StAR* gene causing production of truncated, nonfunctional StAR proteins (9).

Although the genetic cause of lipoid CAH has been identified, many questions regarding its clinical manifestations and pathogenesis remain unanswered. We have generated StAR knockout mice to allow us to analyze the role of StAR within an intact endocrinological milieu amenable to experimental manipulation. Using this model system, we establish essential roles of StAR in regulated steroidogenesis in mice and demonstrate sex-specific differences in the gonads that support a two-stage model of lipoid CAH.

METHODS

Generation of StAR Knockout Mice. The mouse *StAR* gene was cloned previously from a 129/SvJ genomic library (10). The embryonic stem cell line E14TG2a was cultured and electroporated with linearized targeting plasmid. A positive/negative selection strategy using neomycin and gancyclovir was employed to facilitate the isolation of homologous recombinants (11), which were verified by a PCR-based strategy, using one primer derived from sequences within the neomycin cassette (5'-TGTGGTTTTCGAAGAGGAAGC-3') and a second primer containing sequences from the 5' flanking region outside of the targeting construct (5'-TTCCCTGCCTGGTGTGTCTGG-3'). Clones that gave the PCR pattern

Abbreviations: StAR, steroidogenic acute regulatory protein; lipoid CAH, congenital lipid adrenal hyperplasia; ACTH, corticotropin; CRH, corticotropin-releasing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone.

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predicted for homologous recombination events were identified and injected into C57BL/6 blastocysts to produce chimeric mice. One of the chimeric founders transmitted to offspring the disrupted allele, allowing us to produce StAR $+/-$ mice. These $+/-$ mice were then mated to produce potential StAR knockout mice. For developmental analyses of StAR knockout mice, timed matings of $+/-$ mice were established, with the morning after mating considered embryonic day 0.5. At the indicated days postcoitus, the mothers were anesthetized and embryos were isolated for histological analyses.

Histological Analyses. Histological analyses were carried out with either paraformaldehyde-fixed sections (hematoxylin/eosin staining) or frozen sections (oil red O staining with hematoxylin counterstaining).

Immunoblot Analyses of StAR Protein. Levels of StAR protein in the wild-type and StAR knockout mice were measured by immunoblot analysis with a rabbit anti-peptide antiserum specific for StAR as described (5). Positive controls included whole-cell and mitochondria-enriched extracts from MA-10 Leydig cells, and negative controls included extracts from liver and spleen.

Rescue of Knockout Mice with a Corticosteroid Replacement Regimen. Mice were rescued with a glucocorticoid/mineralocorticoid replacement regimen as described (12). Stock solutions of dexamethasone 21-phosphate (4 mg/ml in H_2O) and fludrocortisone acetate (5 mg/ml in 100% dimethyl sulfoxide) were prepared and stored at 4°C. A corticosteroid mixture was made by diluting both stock solutions 1 to 10,000 in water. All newborn pups were injected with 0.1 ml subcutaneously once daily until StAR knockout pups were identified by PCR analysis. Steroid injections were then continued in StAR knockout pups until weaning, when the mice were provided *ad libitum* with 0.9% sodium chloride as drinking solution.

Hormonal Analyses. Hormonal assays were performed with commercially available radioimmunoassay kits for corticosterone (ICN), aldosterone (Diagnostic Products, Los Angeles), progesterone (Pantex, Costa Mesa, CA), and testosterone (Pantex) according to the manufacturers' instructions. The values for aldosterone were obtained in single analyses of pooled serum samples from 10 ($+/+$), 9 ($+/-$), and 12 ($-/-$) mice. All other determinations were made on samples from individual mice. Corticotropin-releasing hormone (CRH) assays used a kit purchased from Phoenix Pharmaceutical (Mountain View, CA). Corticotropin (ACTH) and follicle-stimulating hormone (FSH) assays were done with radioimmunoassay kits for rat ACTH and FSH developed and provided by A. Parlow (Harbor–University of California, Los Angeles, Medical Center, Torrance, CA) and distributed by the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases). [^{125}I]ACTH and [^{125}I]FSH were purchased from COVANCE Labs (Vienna, VA). Synthetic gonadotropin-releasing hormone (GnRH) was iodinated and the RIA was done as described (13). Using previously described methods, pituitaries (13) and the preoptic area including the hypothalamus (14) were dissected from StAR knockout and wild-type pups and sonicated; tissue supernatants following sonication were used for CRH, ACTH, FSH, and GnRH assays as described above.

RESULTS

To analyze more precisely StAR function *in vivo*, and to elucidate the mechanisms underlying the lipoid CAH phenotype, we used targeted gene disruption to produce StAR knockout mice. The targeting vector, following homologous recombination in embryonic stem cells, disrupted the *StAR* gene by deleting part of exon 2 and all of exon 3 (see Fig. 1A), thereby leading to the synthesis of StAR protein lacking C-terminal residues essential for function (8). Ultimately,

heterozygous $+/-$ StAR knockout mice were mated, and the resulting litters were analyzed, with a typical litter shown in Fig. 1B. StAR knockout pups comprised $\approx 25\%$ of the offspring (72/286), indicating that StAR is not required for survival *in utero*. Moreover, as expected for an autosomal recessive trait, there were equal numbers of male and female StAR knockout pups. StAR knockout mice looked normal, except that both genetic males and females had female external genitalia.

Like lipoid CAH patients—who exhibit considerable variability in their age at clinical presentation (8)—StAR knockout mice were heterogeneous in their clinical onset. Most mice died either within 1–2 days after birth ($\approx 40\%$ of StAR knockout mice) or between 8–10 days (also $\approx 40\%$). Fewer than 10% survived beyond 10 days, with a maximum survival of 16 days without therapy. Of note, the pups that died within 1–2 days of birth appeared cyanotic and had labored breathing. Similar findings were observed in mice with targeted disruption of the glucocorticoid receptor (15), suggesting that the low glucocorticoid levels in StAR knockout mice may impair lung maturation and lead to respiratory distress. By day 6–8, surviving StAR knockout mice were appreciably smaller than wild-type littermates (mean weights of 3.5 ± 0.5 g for StAR knockout pups versus 5.5 ± 0.7 g for wild-type $+/+$ and $+/-$ pups). Thereafter, they became increasingly lethargic and eventually died, with physical findings consistent with hypovolemia secondary to adrenocortical insufficiency. In support of this model, treating StAR knockout mice with a corticosteroid replacement regimen kept some of them alive—with the oldest surviving StAR knockout mouse now 14 weeks old—proving that at least a subset die from adrenocortical insufficiency.

To verify that the StAR knockout mice truly were deficient in StAR, immunoblot analyses were used to examine levels of StAR protein. Adrenal extracts from StAR knockout mice lacked the 30-kDa band that represents mature StAR protein (Fig. 1C), whereas the appropriate band was readily visualized in adrenal extracts from $+/+$ littermates.

StAR knockout mice exhibited multiple hormonal abnormalities (Table 1). Levels of corticosterone—the predominant glucocorticoid in mice—and aldosterone were depressed, whereas levels of ACTH and CRH were elevated. These findings are consistent with impaired production of adrenal steroids and consequent loss of negative feedback inhibition at hypothalamic and pituitary levels. In contrast, levels of hormones related to the gonadal axis [e.g., testosterone, pituitary FSH, and hypothalamic GnRH (Table 1) and progesterone (K.M.C., unpublished observation)] did not differ significantly from those normally found in prepubertal mouse pups, consistent with the low levels of gonadal steroidogenesis before puberty.

Grossly, the adrenal glands of the StAR knockout were smaller than those of the wild-type littermates. Microscopic examination of adrenal sections revealed no abnormalities in the structure of the medulla (Fig. 2C and D). In contrast, the adrenal cortex of StAR knockout mice was markedly abnormal, with disrupted fascicular structure and a foamy, vacuolated cytoplasm (Fig. 2B). Staining with oil red O revealed florid lipid deposits in the adrenal cortex of StAR knockout mice (Fig. 2D) that exceeded considerably the staining with wild-type adrenal glands (Fig. 2C), indicating that these vacuoles most probably reflect lipid deposition within the steroidogenic compartment of the adrenal gland.

The male-to-female sex reversal of external genitalia—indicative of impaired androgen production *in utero*—suggested that the testes might also reveal abnormalities. The testes looked grossly normal. Moreover, at the prepubertal times examined, the testes of the StAR knockout mice were indistinguishable histologically from those of wild-type littermates, with normal appearing seminiferous tubules and interstitial region (compare Fig. 3A

FIG. 1. Generation of StAR knockout mice by gene targeting in embryonic stem cells. (A) Strategy to disrupt the StAR locus. A diagram of the mouse StAR gene is shown. Upon homologous recombination, the targeting vector will delete part of exon 2 and all of exon 3. The targeting vector was electroporated into embryonic stem cells and clones screened by PCR analysis as described. (B) Pedigree analysis from mating of heterozygous $+/-$ mice. Genomic DNA was isolated from pups born from a mating of StAR $+/-$ mice, and genotypes were determined by Southern blot analysis with the probe shown in A. The smaller (4.5 kb) band results from the wild-type locus, and the larger (5.1 kb) band results from the disrupted locus. (C) Immunoblot analysis of StAR protein in StAR knockout mice. Tissue extracts from the indicated sources were prepared and 6 μ g of the MA-10 mitochondrial extract or 15 μ g of all other extracts were analyzed by immunoblotting with an antibody specific for StAR. Numbers on the left indicate the positions of molecular weight markers. Mature StAR protein, which has an apparent M_r of 30,000, is absent in the extract from the StAR knockout adrenals but readily detected in the extract from wild-type adrenals.

and B). However, as in the adrenal sections, lipid deposits again were visualized within the steroidogenic interstitial region (Fig. 3A), documenting abnormal testicular steroidogenesis and consequent lipid deposition. The clustering of staining in discrete areas of the interstitium and the fact that adult Leydig cells have not yet initiated steroidogenesis at this time (16) suggest that the staining represents residual lipid deposits in fetal Leydig cells. In

parallel studies (Fig. 3 C and D), the ovaries appeared completely normal histologically and lacked abnormal lipid deposits. Thus, at this time point, the ovary is spared relative to the testis and adrenal cortex.

To explore in greater detail the basis for the apparent inability to make androgens in levels sufficient to virilize the external genitalia *in utero*, we examined the testes and ovaries

Table 1. Hormone levels in StAR knockout mice

	StAR		
	+/+	+/-	-/-
Corticosterone, ng/ml serum	11.6 $\pm$ 3.9 (n = 5)	9.6 $\pm$ 6.5 (n = 5)	4.5 $\pm$ 2.4 (n = 13)
Aldosterone*, pg/ml serum	425	275	57
Testosterone, ng/ml serum	0.47 $\pm$ 0.28 (n = 8)	0.33 $\pm$ 0.03 (n = 3)	0.40 $\pm$ 0.23 (n = 7)
Pituitary ACTH, ng/mg protein	1,820 $\pm$ 159 (n = 8)	1,795 $\pm$ 199 (n = 16)	2,472 $\pm$ 351 (n = 8)
Pituitary FSH, ng/mg protein	215 $\pm$ 22 (n = 19)	258 $\pm$ 43 (n = 16)	155 $\pm$ 18 (n = 8)
Hypothalamic CRH, pg/mg protein	366 $\pm$ 23 (n = 19)	453 $\pm$ 36 (n = 16)	922 $\pm$ 111 (n = 8)
Hypothalamic GnRH, pg/mg protein	339 $\pm$ 37 (n = 19)	425 $\pm$ 39 (n = 16)	386 $\pm$ 40 (n = 8)

Hormone levels in StAR knockout mice and wild-type littermates were measured as described. Values are reported as means $\pm$ standard errors of the means, except for *, which represents single determinations from pooled samples.

FIG. 2. Histology of the adrenal gland from wild-type and StAR knockout mice. Adrenal glands were isolated from wild-type and StAR knockout mice 6 days after birth. (A) Hematoxylin/eosin staining of wild-type adrenal section. (B) Hematoxylin/eosin staining of StAR knockout adrenal section. (C) Oil red O staining of section from wild-type adrenal. (D) Oil red O staining of section from StAR knockout adrenal.

of mouse embryos following the known onset of *StAR* gene expression (10). At embryonic day 14.5, the testes of StAR knockout embryos (Fig. 4 B and D), but not wild-type embryos (Fig. 4 A and C), contained lipid deposits denoting impaired cholesterol delivery into the mitochondria. In contrast, the fetal ovaries, which do not express StAR and which are not hormonally active *in utero*, appeared normal in both wild-type and StAR knockout embryos (K.M.C., unpublished observation). Collectively, our studies in embryonic and newborn StAR knockout mice demonstrate a histological sparing of StAR-deficient ovaries *in utero* and before puberty that presumably permits the production of estrogens by XX lipoid CAH patients at the onset of puberty (17, 18).

Previously, Strauss and colleagues (19) reported that StAR is expressed in the human kidney, raising the possibility that

StAR facilitates the transfer of precursors to the mitochondria where 1α -hydroxylase makes 1,25-dihydroxyvitamin D. The StAR knockout mice did not exhibit any renal abnormalities, consistent with a previous observation that StAR is not expressed in mouse kidney (K.M.C., unpublished observation). Similarly, although it has been reported that StAR can modulate the delivery of cholesterol to sites of bile acid synthesis, the livers of StAR knockout mice exhibited no obvious abnormalities (K.M.C., unpublished observation).

DISCUSSION

In this report, we have generated and characterized knockout mice that are deficient in StAR. These mice exhibit generalized defects in steroid hormone biosynthesis, with consequent male

FIG. 3. Sex-dependent differences in gonadal effects of StAR deficiency. Testes and ovaries were isolated from wild-type and StAR knockout mice 7 days after birth, and frozen sections were stained with oil red O. (A) StAR knockout testis. (B) Wild-type testis. (C) StAR knockout ovary. (D) Wild-type ovary.

FIG. 4. Developmental analysis of lipid deposition in the testes of StAR knockout embryos. Embryos from timed matings were collected at embryonic day 14.5, and transverse sections were analyzed by staining with oil red O. (A) Lower power magnification of section from wild-type testis. (B) Lower power magnification of section from StAR knockout testis. (C) Higher power view of section from wild-type testis. (D) Higher power view of section from StAR knockout testis. Arrows point to regions of lipid deposition. t, Testis; m, metanephros.

pseudohermaphroditism. The absence of StAR is lethal, with some animals dying shortly after birth—most probably from respiratory distress—and others dying approximately 1 week after birth—presumably from fluid and electrolyte abnormalities secondary to adrenocortical insufficiency. In support of the latter, treatment of StAR knockout mice with a corticosteroid replacement regimen kept some of them alive. Finally, the StAR knockout mice exhibit histopathological changes consistent with a two-stage model for the pathogenesis of lipid CAH. The ovaries, which do not produce steroid hormones in significant amounts before puberty, do not exhibit detectable abnormalities, whereas the adrenal cortex and testes are clearly abnormal. These findings, coupled with the clinical observation that some XX lipid CAH patients manifest unequivocal signs of estrogen production at puberty [i.e., they develop female secondary sexual characteristics and undergo menarche (17, 18)], argue that some capacity for steroidogenesis is initially present in StAR-deficient cells, and that total abrogation of steroidogenesis does not occur until sufficient lipid has accumulated to kill the steroidogenic cells. Thus, it will be very interesting to follow the disease progression in female StAR knockout mice that are kept alive by corticosteroid replacement. Presumably, when trophic hormone stimulation commences at puberty, they will experience a transient period of increased ovarian steroidogenesis, followed by progressive histological evidence of lipid deposition and deteriorating ovarian function. Similarly, if steroidogenic cell death is caused by the toxic accumulation of lipids and oxidation products, it may be possible to ameliorate the progression of ovarian dysfunction by treating the mice with anti-oxidants such as vitamin E and vitamin C.

Based on a compilation of multiple studies (8), males comprise $\approx 70\%$ of the ≈ 100 lipid CAH patients reported to date—an unexpected distribution for an autosomal recessive condition. This finding suggests that StAR-deficient females die *in utero* or postnatally before diagnosis, or that sperm carrying an X chromosome and the lipid CAH StAR allele fertilize less efficiently. Despite the male predominance in lipid CAH patients, our data are entirely consistent with an autosomal recessive condition (i.e., 25% StAR knockout mice with a 1:1 male/female ratio). Thus, although species-specific differences may underlie this apparent discrepancy between

mice and humans, our data suggest that the skewed ratio in lipid CAH may result from excessive loss of affected females after birth but prior to diagnosis.

Although mice lacking StAR exhibit severe defects in adrenal and gonadal steroidogenesis, consistent with the proposed essential role of StAR in regulated steroidogenesis, they nonetheless maintain some capacity for steroid biosynthesis, as indicated by their apparently normal epididymis and vas deferens. The apparent discrepancy between appropriate development of internal genital structures (i.e., the epididymis and vas deferens) and sex reversal of the external genitalia may reflect differing thresholds for paracrine actions in the immediate vicinity of the fetal testis versus more distal, endocrine actions on the developing external genitalia. The effect of the putative androgen deficiency *in utero* may be exacerbated by the need to convert testosterone to dihydrotestosterone to obtain full virilization of the external genitalia (20).

The StAR knockout mice provide a powerful system for investigating StAR's function within an intact endocrine milieu. For example, analyses in transfected COS cells suggest that the NH₂-terminal 60 residues of StAR are dispensable for its function, and that StAR can facilitate steroidogenesis without being imported into the mitochondria (21). The ability to use transgenic reconstitution studies in StAR knockout mice affords an excellent way to delineate mechanisms of StAR action within *bona fide* steroidogenic cells. Similarly, we should be able to use transgenic oncogenes to generate immortalized steroidogenic cell lines deficient in StAR (22), again providing a superior system for analyzing StAR's structure–function relationship within an homologous system. Such cell lines also may facilitate studies of potential interactions between StAR and other components that have been implicated in the acute steroidogenic response (reviewed in ref. 23). Finally, the StAR knockout mice may enable us to determine the effect of severe steroid deficiency on selected aspects of neuroendocrine development and function. For example, it will be interesting to examine the production of neurosteroids in the brains of StAR knockout mice, as well as the degree to which their brains become sexually dimorphic. Through studies such as these, we hope to increase our understanding of how StAR makes its essential contributions to adrenocortical and gonadal steroidogenesis and endocrine development.

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