MOLECULAR AND CELLULAR BIOLOGY, Jan. 2002, p. 525–535 Vol. 22, No. 2
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.2.525–535.2002

Genetic Ablation of the Steroid Receptor Coactivator-Ubiquitin Ligase, E6-AP, Results in Tissue-Selective Steroid Hormone Resistance and Defects in Reproduction

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Received 13 April 2001/Returned for modification 6 June 2001/Accepted 5 October 2001

The E6-associated protein (E6-AP), although originally identified as a ubiquitin ligase, has recently been shown to function as a coactivator of steroid receptor-dependent gene expression in in vitro assays. In order to determine whether E6-AP acts as a coactivator in vivo, physiological parameters associated with male and female sex steroid action were assessed in the E6-AP null mouse. Gonadal size was reduced in E6-AP null male and female mice in comparison to wild-type controls in conjunction with reduced fertility in both genders. Consistent with this observation, defects in sperm production and function, as well as ovulation were observed. In comparison to wild-type controls, induction of prostate gland growth induced by testosterone and uterine growth by estradiol were significantly reduced. In contrast, estrogen and progesterone-stimulated growth of virgin mammary gland was not compromised by E6-AP ablation despite E6-AP expression in this tissue. This latter finding contrasts with the impaired estrogen and progesterone-induced mammary gland development observed previously for steroid receptor coactivator type 1 (SRC-1) and SRC-3 female knockout mice. Taken together, these results are consistent with a role for E6-AP in mediating a subset of steroid hormone actions in vivo. Nevertheless, differences observed between SRC and E6-AP knockout phenotypes indicate that these two families of steroid receptor coactivators are not functionally equivalent and supports the hypothesis that coactivators contribute to tissue-specific steroid hormone action.

Steroid receptors are ligand-regulated members of a large family of transcription factors that control many important biological processes including growth, differentiation, homeostasis, and reproduction. These proteins exert many of their effects through binding to specific DNA response elements in the promoter region of target genes and recruitment of coactivator proteins. Many coactivators have been identified, and among these, the steroid receptor coactivator (SRC) family of coactivators (SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2/SRC-2, and p/CIP/ACTR/RAC3/AIB1/TRAM-1/SRC-3) has been most extensively characterized (12, 42, 52). These proteins possess multiple receptor interaction motifs that enable them to bind to receptor superfamily members (16, 41). In addition, they also possess or interact with proteins that possess histone acetyltransferase activity (5, 6, 47, 57). These activities, in combination with the ability of steroid receptors to interact with general transcriptional factors and other coactivators, results in increased target gene transcription through their ability to promote the formation of a functional preinitiation complex, chromatin remodeling, and the reinitiation of transcription (12, 23, 30, 36).

We recently identified a novel coactivator for the steroid receptor superfamily called E6-associated protein (E6-AP [46]), whose expression has been documented in the heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, mammary gland, uterus, ovary, and testis (56, 60). Like other coactivators, this protein binds to steroid receptors in a ligand-dependent fashion and enhances the transcriptional activity of receptors for estrogen, progesterone, androgen, glucocorticoid, retinoic acid, and thyroid hormone in transient-transfection assays (46). However, E6-AP is a dual-function protein originally identified as an E3 ubiquitin ligase (18, 19). In this role, E6-AP participates in the transfer of ubiquitin to target proteins, thereby directing them to the 26S proteasome for degradation. The ability of E6-AP to contribute to steroid receptor transcriptional activity is particularly intriguing in view of the importance of proteasome activity for estrogen receptor (ER), progesterone receptor (PR), and thyroid hormone receptor transcriptional activity (37). Moreover, the proteasome is responsible for the hormone-dependent downregulation of both PR and ER expression (1, 11, 35, 45, 61).

Loss of appropriate E6-AP gene expression has been identified as the causative factor for Angelman syndrome, a human genetic disorder characterized by severe motor dysfunction, seizures, and mental retardation (25, 28, 50, 68). Interestingly, it is the loss of the expression of the maternal copy of the E6-AP gene, arising from genetic deletion, paternal uniparental disomy, or mutation of the maternal allele, that gives rise to Angelman syndrome in most cases (25, 28). The paternal E6-AP allele is silenced by imprinting in regions of the brain that include the hippocampal neurons and Purkinje cells of the cerebellum (2, 53, 65), and mice lacking appropriate maternal E6-AP expression therefore experience a complete or near-complete loss of E6-AP expression in these areas (24). In contrast, animals lacking paternal expression of the gene ex-

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hibit E6-AP expression patterns in these regions similar to those of wild-type animals (24). However, this imprinting is tissue specific, and both alleles of the E6-AP gene appear to be expressed throughout other regions of the brain (e.g., the cerebral cortex) and other tissues (24, 44). Humans with loss-of-function mutations for E6-AP on the paternal chromosome do not exhibit symptoms of the Angelman syndrome.

Recently, an E6-AP knockout mouse was generated as a mouse model for Angelman syndrome (24). The maternal-deficiency heterozygote (m^-/p^+) animals exhibit significant defects such as motor dysfunction, inducible seizures, and deficiencies in context-dependent learning and long-term potentiation. Furthermore, the abundance of the tumor suppressor gene p53, a target of the E6-AP ubiquitination pathway, was increased in postmitotic neurons in the cerebellum (Purkinje cells) and in pyramidal neurons within the CA region of the hippocampus. Thus, these animals embody many of the traits ascribed to Angelman syndrome patients. Although the reproductive capabilities of heterozygous male and female mice are not compromised in comparison to wild-type controls, no studies have been performed on homozygous null mutant animals.

Numerous transient-transfection assays and other in vitro studies reveal a pattern of ligand-dependent coactivator interaction with steroid receptors and stimulation of receptor-dependent gene expression and, in most instances, little receptor-coactivator specificity has been detected. However, studies of the limited number of coactivator knockout mice studied to date indicate that coactivators play distinct functional roles in vivo (22, 63, 70, 71, 73). For instance, SRC-1 knockouts exhibit normal growth patterns, while SRC-3 null mice exhibit body weights approximately 20 to 50% lower than those of wild-type controls (66, 70, 71). While this may reflect, at least in part, differences in coactivator expression patterns, virgin mammary gland growth and differentiation is dissimilar between SRC-1 and SRC-3 knockouts, even though both coactivators are expressed in this steroid-dependent tissue (70, 71), supporting the notion that coactivators fulfill specific functions in vivo. In contrast, null mutations of the more general coactivator proteins CBP, p300, and TRAP220 result in embryonic lethality (22, 63, 64, 73). Thus, different coactivators play critical but distinct roles in vivo. Therefore, in order to characterize the impact of ablation of E6-AP expression on reproductive functions associated with sex steroid action, we examined the consequences of an E6-AP null mutation on reproductive function and steroid-dependent responses in male and female knockout mice.

MATERIALS AND METHODS

Animal care and genotyping. Animals were examined on a hybrid (C57BL/6 and 129/SvEv) genetic background and were bred for these studies by crossing heterozygous E6-AP males and females. Animals were weaned at 3 weeks, and tail cuts were made for DNA isolation. E6-AP genotype screening was accomplished by Southern blot analysis or PCR as described previously (24). For Southern blot analysis, genomic DNA was digested by *Bam*HI and probed with a radiolabeled 5′ external probe (24). The primers used for PCR-based genotyping were as follows: P1/genomic forward, 5′-ACTTCTCAAGGTAAGCTGAGCTTGC-3′; P2/reverse, 5′-GCTCAAGGTTGTATGCCTTGGTGCT-3′; and P3/HPRT forward, 5′-TGCATCGCATTGTCTGAGTAGGTGTC-3′. PCR cycling conditions were 92°C for 5 min, 92°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 35 cycles, followed by 72°C for 5 min. The distribution of E6-AP genotypes for the animals at weaning was 30.0% wild type, 49.1% heterozygous, and 20.9% homozygous null. All procedures involving animals were performed in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved in advance by the Animal Use and Care Committee of Baylor College of Medicine.

Northern blot analysis. Tissues from wild-type male and female mice were harvested immediately after animals were euthanized and stored at −80°C until further use. TRIzol extraction reagent was used to isolate total RNA according to the manufacturer's recommendations (Life Technologies). Poly(A)$^+$ RNA was isolated from total seminal vesicle RNA by using the Micro-FastTrack 2.0 kit (Invitrogen). A total of 25 μg of total RNA or 1 to 5 μg of poly(A)$^+$ RNA was electrophoresed on a formaldehyde–1% agarose gel and was subsequently transferred to a nitrocellulose membrane. The membrane was hybridized with a 435-bp *Xho*I-*Xho*I fragment of mouse E6-AP cDNA radiolabeled with [^{32}P]dCTP by using the RadPrime DNA Labeling kit (Life Technologies). After being washed, the blot was subjected to autoradiography with Biomax MS film (Kodak).

Western blot analysis. Extracts of tissues isolated from wild-type or E6-AP null mice were prepared by homogenization in 20 mM HEPES (pH 7.5) buffer containing 50 mM NaCl, 5 mM KCl, 10% glycerol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1.25 μg of leupeptin per ml, and 1 μg of pepstatin A/ml). Protein concentrations were estimated by the Bradford reagent (Bio-Rad Laboratories, Hercules, Calif.). Then, 50 μg of total protein or ~15 ng of purified E6-AP was denatured in a sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and probed with an E6-AP specific polyclonal antibody at a 1:10,000 dilution. E6-AP protein was visualized by using the ECL Detection system (Amersham).

Breeding studies. Male mice (wild type and −/−) were housed individually with two wild-type females each for 8 weeks. During that time, the breeding cages were checked daily for pups. At the conclusion of the breeding period, male and female mice were separated and the births of any pups in the following 4 weeks were recorded. Similarly, female continuous breeding studies were conducted over a 2-month period, during which 10 wild-type or E6-AP null virgin mice were housed with wild-type males (two females per male per cage) for 2 months and monitored for 1 month after the completion of the breeding period. The numbers of pups and litters were recorded.

Testes and prostate measurements. Testes were dissected from 12-week-old male mice (day 0), and their wet weights were recorded and corrected for body weight. Nine days after castration, testosterone slow-release pellets, delivering 119 μg of testosterone/day, were surgically implanted. Animals were sacrificed 7 days thereafter (day 16), the male reproductive tract dissected, and the prostate and urethra were isolated from the seminal vesicles, bladder, vas deferens, and ampillary gland. The wet weights of the prostate and a section of the urethra between the bladder and penis and the seminal vesicles were recorded.

Sperm analyses. Male mice were individually housed for at least 1 month prior to the analyses. Blood samples were collected from anesthetized animals for subsequent measurement of serum testosterone levels by radioimmunoassay (Diagnostic Systems Laboratory, Webster, Tex.). Testes were dissected, and the epididymis was isolated. The epididymis was finely minced, sperm was collected, and the concentration was determined by using a hemocytometer. Sperm motility was assessed microscopically and assessed as a percentage of motile sperm relative to the total concentration (69). The forward progression was estimated microscopically by using the basic procedure established for routine human semen analyses (69). An optimized sperm penetration assay was performed to assess the fertilization potential of sperm (26). Briefly, epididymal sperm were allowed to capacitate by mixing with Test yolk buffer [4.83% *n*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 1.16% tris(hydroxymethyl)aminoethane, 0.2% dextrose, and 20% chicken fresh egg yolk] at 4°C for 42 h. After rapid warming to 37°C, the sperm were incubated with zona-free hamster oocytes, and the number of penetrations per oocyte was scored. The results are expressed as a sperm capacitation index that indicates the mean number of penetrations per ovum. Oocytes for this experiment were collected from Golden Syrian hamsters that had been injected with 30 IU of pregnant mare serum gonadotropin (PMSG; Calbiochem) and 30 IU of human chorionic gonadotropin (hCG; Sigma) to induce superovulation (72) and then processed as previously described (26).

Ovarian and uterine analyses. Ovaries were removed from 8-, 10-, 12-, and 14-week-old virgin female mice and dissected free from fat; the wet weights were recorded. To assess oocyte production, wild-type and E6-AP null female littermates (age, 21 days) were treated with 4 IU of PMSG (Biosynth B.V.), followed 44 h later with 5 IU of hCG (Organon). Animals were sacrificed 24 h after the second injection, and the ovaries and oviduct were surgically removed. Oocytes and associated cumulus mass were physically extracted from the oviduct, and the oocytes were counted under a dissecting microscope. Blood samples were col-

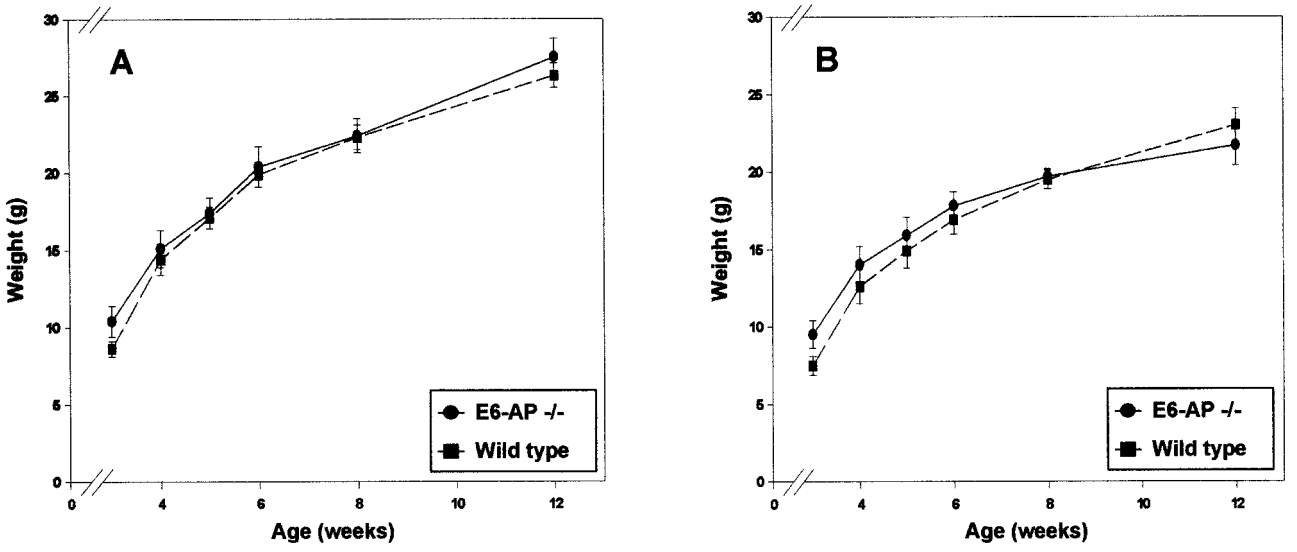

FIG. 1. Growth curves for male and female E6-AP wild-type and knockout animals. Male (A) and female (B) E6-AP null (●) and wild-type (■) mice were weighed 3, 4, 5, 6, 8, and 12 weeks after birth. Two-way ANOVA revealed no significant difference between wild-type and E6-AP null mice weights. Values represent the average ± the standard error of the mean (SEM) of 5 to 18 animals for each time point.

lected from anesthetized, randomly cycling, virgin female mice at 2 to 8 months of age for measurement of the estradiol levels in serum by radioimmunoassay (Diagnostic Systems Laboratory kit DSL-4800). Uterotropic responses to estrogen stimulation were examined in virgin female mice ovariectomized at 8 weeks of age. Animals were allowed to recover for 2 weeks to reduce endogenous estrogen levels and were then injected subcutaneously with 0.8 μg of 17β-estradiol (E2)/day for 3 days (days 15 to 17). On day 18, the mice were sacrificed, the uteri were dissected, and the uterine wet weights were recorded.

Histology. Testes were dissected and placed in CHO fixative (3% paraformaldehyde, 0.2% glutaraldehyde, and 2% sucrose in phosphate-buffered saline), while the ovaries were fixed in formalin-buffered saline. Both tissue types were embedded in paraffin, and 7-μm-thick sections were cut and stained with hematoxylin and eosin. Micrographs of stained sections were obtained using a Zeiss Akioskop microscope linked to a Hamamatsu Color Chilled 3CCD camera.

Mammary gland studies. Virgin mammary glands were obtained from 8-week-old female mice. To assess mammary gland responses to estrogen and progesterone stimulation, 12- or 14-week-old virgin female mice were ovariectomized (day 0) and allowed to recover for 2 weeks to reduce endogenous hormone levels. Thereafter, the animals were implanted subcutaneously with beeswax pellets (containing ~20 μg of E2 and 20 mg of progesterone per pellet) designed to deliver estrogen and progesterone at levels sufficient to stimulate mammary gland growth and differentiation (43). After 21 days of treatment, animals were sacrificed, and their inguinal mammary glands were collected and fixed in 10% formalin for subsequent whole-mount staining with hematoxylin solution (39). Micrographs of stained glands were obtained by using a Zeiss Stemi 2000-C dissecting microscope linked to a Hamamatsu Color Chilled 3CCD camera.

RESULTS

Growth of E6-AP-deficient mice. Previous analyses of maternal deficiency, but not paternal deficiency, E6-AP mice revealed that total body weights varied by approximately 10% at 18 days of age and that this difference disappeared by 4 months of age (24). Since body weights were to be used as a standardizing factor throughout our analyses, we sought to determine whether mice in which both alleles of E6-AP had been deleted exhibited any difference in body mass during weeks 3 to 12 of postnatal development. Although E6-AP null male and female mice tended to be slightly larger than their wild-type controls at 3, 4, 5, 6, 8, and 12 weeks of age, this apparent difference in weight between genotypes was not significant (Fig. 1). Body weights were used to standardize our measurements of reproductive tissue sizes and/or growth in all subsequent experiments.

Expression of E6-AP in reproductive tissues. Although E6-AP expression has been previously demonstrated in many tissues, including the placenta, prostate, testis, ovary, and mammary gland (56, 60), its expression in other reproductive tissues, such as the uterus and seminal vesicle, has not been extensively characterized. Therefore, prior to assessing the impact of E6-AP on male and female reproduction, E6-AP mRNA expression was characterized in male and female reproductive tissues. As expected, Northern blot analysis of total RNA demonstrated that E6-AP mRNAs of approximately 4.5, 5.5, and 10 kb are expressed in the ovary, uterus, mammary gland, testis, and prostate; however, no expression was detected for seminal vesicle total RNA (data not shown). Anal-

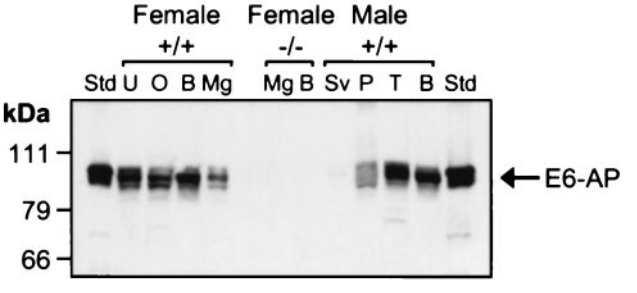

FIG. 2. Tissue analysis of E6-AP expression. Western blot analysis of 50 μg of protein extracts prepared from uterus (U), ovary (O), brain (B), mammary gland (Mg), seminal vesicle (SV), prostate (P), and testis (T) obtained from wild-type (+/+) or E6-AP null (−/−) mice. Purified E6-AP protein obtained from HeLa cells (Std) is shown on the left and right extreme lanes. Molecular size standards are shown on the left.

TABLE 1. E6-AP null males are less fertile than wild-type controls

Mouse group	No. of litters	Total no. of pups	Mean no. of pups/ litter ± SEM
Wild type	13	100	7.7 ± 0.7
E6-AP$^{-/-}$	9	46	5.1 ± 0.6[a]

[a] $P < 0.05$ in comparison to wild-type males as determined by one-way ANOVA.

ysis of 5 μg of poly(A)$^+$ RNA isolated from the seminal vesicle did reveal a weak E6-AP signal, indicating that this gene is expressed at very low levels in this tissue or in a small subpopulation of cells. To confirm this finding, cellular extracts prepared from these tissues were examined for E6-AP protein expression (Fig. 2). A strong E6-AP signal was readily detectable for the uterus, ovary, brain, testis, mammary gland, and prostate, and very low expression of E6-AP protein was detected in seminal vesicle extracts. No E6-AP expression was detected in mammary gland or brain extracts prepared from a E6-AP null mouse, confirming that E6-AP expression is lost in the knockout mice and that the antibody used in this experiment is detecting authentic E6-AP protein. Based on this expression pattern, we predicted that ablation of E6-AP gene expression may result in reproductive abnormalities in both male and female mice.

Reduced male reproductive function. To characterize the effects of E6-AP ablation on male reproduction, the fertility of male mice was examined in a continuous breeding study with wild-type female mice. A total of 8 wild-type or knockout male mice were housed with 15 wild-type females (generally two females were bred with each male) for 8 weeks. The numbers of pups born both during the breeding period and the subsequent 4 weeks were scored. The total number of offspring sired by the knockout mice was lower than those produced by wild-type males, and the number of pups per litter was reduced by ~34% ($P < 0.05$; Table 1). Sperm analyses indicated that the average epididymal sperm counts were ~38% lower in E6-AP knockouts ($P < 0.05$; Table 2). Functional assays were also performed to assess the fertilization potential of the male animals lacking E6-AP expression. In these studies, neither the motility nor the forward progression of sperm was significantly different between knockout and wild-type animals. However, sperm obtained from E6-AP null animals showed a significantly lower ability to penetrate oocytes in a standardized sperm penetration assay ($P < 0.01$) compared to their wild-type controls, and this finding is consistent with the lower in vivo fertility observed for the knockout males. Because this assay is standardized to assess a constant concentration of sperm, this test indicates a functional deficit in the ability of the sperm to penetrate and fertilize the egg.

TABLE 2. Results of male in vitro fertility assessment

Mouse group	Mean ± SEM (n)[a]			
	Sperm (10^6/ml)	% Motile sperm	FP	SCI
Wild type	13.9 ± 1.4 (20)	49.1 ± 5.7 (20)	1.7 ± 0.2 (10)	2.7 ± 0.6 (8)
E6-AP$^{-/-}$	8.6 ± 1.4[b] (19)	41.6 ± 5.3[d] (19)	1.4 ± 0.2[d] (9)	0.5 ± 0.1[c] (8)

[a] FP, grade forward progression; SCI, sperm capacitation index.
[b] $P < 0.05$ in comparison to wild-type males by one-way ANOVA.
[c] $P < 0.01$ in comparison to wild-type males by pairwise multiple comparison (Tukey test).
[d] No significant difference between wild-type and E6-AP null males.

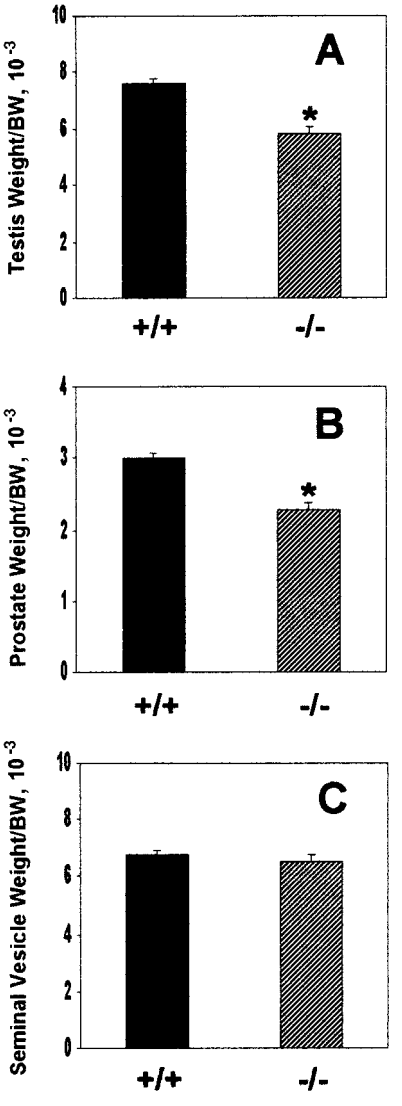

FIG. 3. Impaired response to testosterone in E6-AP null mice. (A) Smaller testes in E6-AP null mice. The ratio of testes to body weight was calculated for 12-week-old wild-type (+/+; $n = 23$) and knockout (−/−; $n = 15$) male mice. Values are significantly different as assessed by one-way ANOVA (*, $P < 0.001$). (B) Reduced prostate size in E6-AP null males. Twelve-week-old wild-type (+/+; $n = 17$) or knockout (−/−; $n = 14$) male mice were castrated on day 0 and treated with testosterone (119 μg/day) from days 9 to 15. On day 16, the prostate and urethra were dissected, and the ratio of prostate and urethra to body weight was calculated. One-way ANOVA showed a significant difference (*, $P < 0.001$) between +/+ and −/− mice. (C) E6-AP does not influence seminal vesicle size. The weights of seminal vesicles from the same animals assessed in panel B were determined. No significant difference was observed between +/+ and −/− males. Values represent the average ± the SEM.

Assessment of testis size in 12 week-old mice (Fig. 3A) revealed that the gonadal weight was reduced by ~24% in E6-AP null mice compared to wild-type controls ($P < 0.001$). Histological analyses of the testes revealed grossly normal

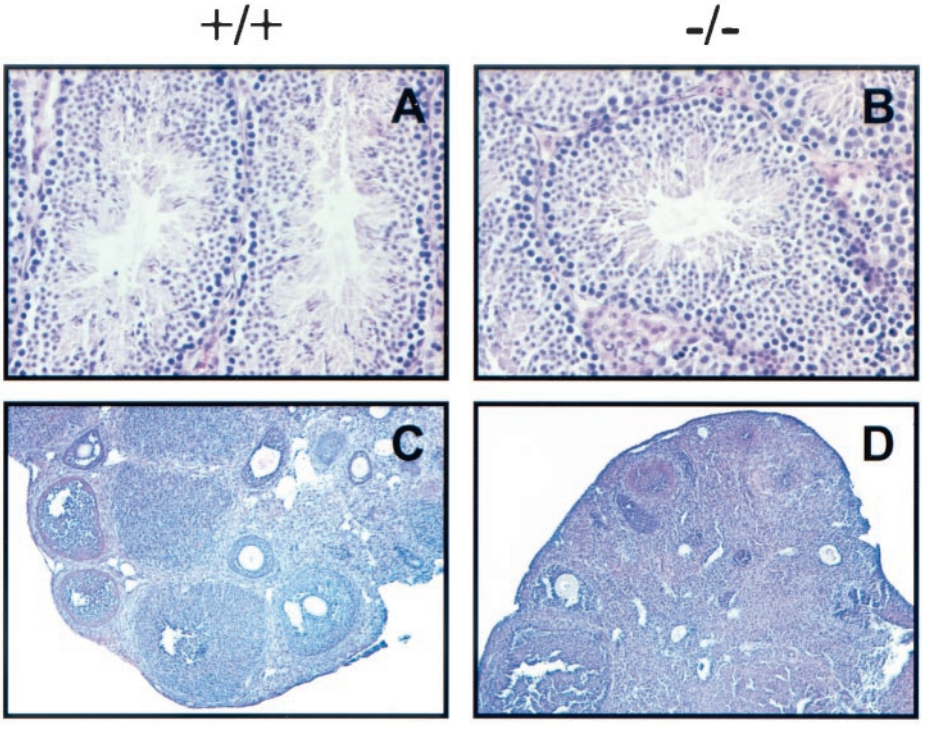

FIG. 4. Gonadal histology of wild-type and E6-AP null mice. Testes sections were obtained from 12-week-old male wild-type (A) and E6-AP null (B) mice. Sections of ovarian tissue from PMSG- and hCG-treated, 3-week-old wild-type (C) and E6-AP null (D) female mice are also shown.

morphology with good cellularity, normal tubule sizes, and a normal spectrum of the stages of spermatogenesis (Fig. 4A and B), suggesting that lower sperm counts are reflective of diminished testicular size rather than an intrinsic spermatogenic defect. No difference in serum testosterone measurements between wild-type and E6-AP null mice was observed (711 ± 282 [$n = 5$] versus 708 ± 147 [$n = 13$] ng/dl, respectively).

Reduced androgen-stimulated prostate growth. Prostate gland growth is androgen dependent (34, 58). Therefore, to determine whether E6-AP plays a role in the ability of male tissues to respond to androgen signaling, we assessed prostate growth in castrated mice implanted with testosterone slow-release pellets. After 1 week of hormone stimulation, the prostate and a section of the urethra between the bladder and penis was dissected, and wet weights were determined. As shown in Fig. 3B, the weight of the prostate in the E6-AP null animal was ~24% smaller than in the wild-type control ($P < 0.001$). However, the weights of the seminal vesicle, another androgen-dependent male reproductive tissue (27), were similar for E6-AP knockout and wild-type littermates (Fig. 3C). Taken together, these data indicate that E6-AP plays a role in androgen-dependent growth in a tissue-specific manner consistent with its expression pattern.

Effect of E6-AP ablation on female fertility. We next examined the fertility of wild-type and E6-AP knockout females. Ten animals of each genotype were bred with wild-type males over a period of 8 weeks and monitored thereafter for an additional 4 weeks; the date of birth, the number of litters, and the number of pups per litter were recorded. The litter size was ~48% lower in knockout females than from wild-type controls ($P < 0.001$), indicating that E6-AP null female are subfertile (Table 3). We therefore characterized in more detail various aspects of ovarian and uterine function.

Ovarian weights were characterized from wild-type and knockout littermates in animals 8 to 14 weeks of age. Two-way analysis of variance (ANOVA) demonstrated a difference ($P < 0.001$) between wild-type and E6-AP homozygous null ovarian weights. However, the magnitude of the difference varied with age. Significant reductions (~30%) in ovarian weight were observed for knockout ovaries in comparison to wild-type controls at 8 and 10 weeks of age ($P < 0.001$ and $P < 0.05$, respectively), but not at later time points (Fig. 5). We next assessed the ability of ovaries to produce oocytes in 3-week-old mice subjected to a standard regimen of PMSG followed by hCG to induce superovulation. At 24 h after hCG treatment, ovaries and oviducts were dissected, and oocytes were collected manually and counted. These studies were performed blindly since mice were genotyped after the superovulation procedure. As shown in Fig. 6, oocyte production by heterozygous females tended to be lower compared to wild-type controls (~27% less), although these differences did not reach

TABLE 3. E6-AP null females are less fertile than wild-type controls

Mouse group	No. of litters	Total no. of pups	Mean no. of pups/litter ± SEM
Wild type	9	67	7.4 ± 1.0
E6-AP$^{-/-}$	7	27	3.9 ± 1.5[a]

[a] $P < 0.001$ in comparison to wild-type females as determined by one-way ANOVA.

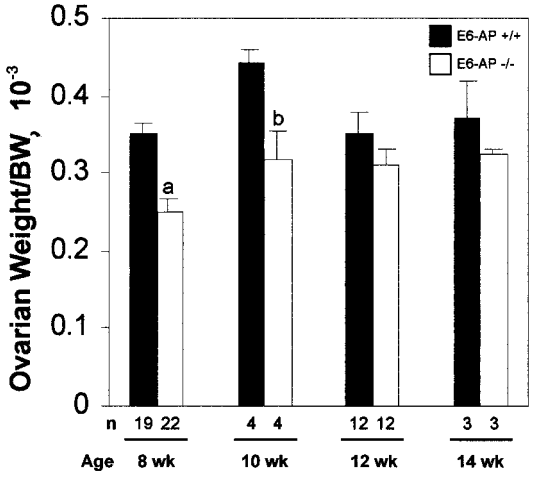

FIG. 5. Decreased ovarian size in E6-AP null virgin females. Body and ovarian weights for 8-, 10-, 12-, and 14-week-old mice were measured, and the ratio of ovarian to body weight was calculated. Two-way ANOVA indicates that E6-AP null mice (□) had significantly smaller ovaries in comparison to wild-type (■) controls at 8 (a, $P < 0.001$) and 10 (b, $P < 0.05$) weeks of age. No significant differences were observed at 12 and 14 weeks. Values represent the mean ± the SEM. The number (n) of ovaries per datum point are shown on the x axis.

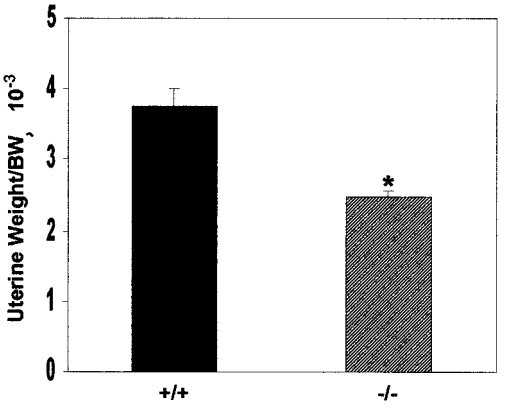

FIG. 7. Impaired uterotropic response to estrogen in E6-AP null mice. Eight-week-old wild-type (+/+; $n = 10$) or E6-AP null (−/−; $n = 11$) female mice were ovariectomized (day 0) and subsequently treated with 0.8 μg of E2/day on days 15 to 17. On day 18, uteri were dissected, and the wet weights were recorded. The ratios of uterine to body weight were calculated. One-way ANOVA revealed a significant difference (*, $P < 0.001$) between genotypes. Values represent the mean ± the SEM.

statistical significance ($P = 0.077$). However, ooctye numbers in E6-AP null animals were significantly lower than in either wild-type (~68% less) or heterozygous (~57% less) female littermates ($P < 0.001$ and $P < 0.05$, respectively). Distinct morphologic differences were observed between ovaries obtained from 3-week-old PMSG- and hCG-treated wild-type and E6-AP null females (Fig. 4C and D). While ovaries from wild-type females revealed many corpora lutea and developing follicles, E6-AP null ovaries were comparatively deficient in leuteinized cells and had fewer developing follicles, a finding consistent with a phenotype of compromised oocyte production.

To characterize the ability of the uterus to respond to hormone stimulation, ovariectomized female mice were treated with 0.8 μg of E2/day for 3 days. The resulting uterine wet weights of E6-AP null mice were smaller than those of wild-type controls by ~35% ($P < 0.001$), indicating that lack of E6-AP in this tissue impairs estrogenic response (Fig. 7). No difference in estradiol levels in serum between wild-type and E6-AP null mice was observed (9.73 ± 1.81 pg/ml [$n = 9$] versus 7.65 ± 1.39 pg/ml [$n = 8$], respectively).

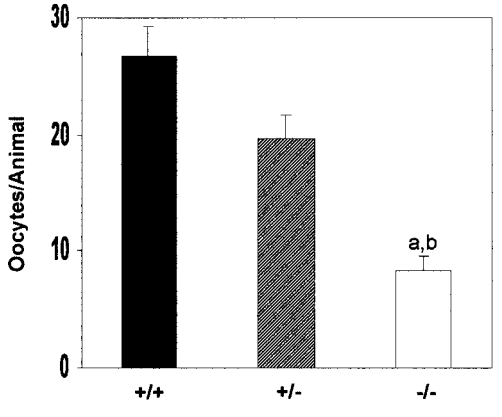

FIG. 6. Reduced ooctye maturation in superovulated E6-AP null mice. Three-week-old wild-type (+/+; $n = 15$), heterozygous (+/−; $n = 18$), or E6-AP null (−/−; n = 7) female mice were injected with 4 IU of PMSG, followed by 5 IU of hCG. After 24 h the ovaries were dissected, and the oocytes were counted. The numbers of oocytes recovered from E6-AP null mice were significantly lower than for heterozygotes (a, $P < 0.05$) and wild-type (b, $P < 0.001$) females, as assessed by pairwise multiple comparison (Tukey test). Values represent the mean ± the SEM.

Effect of E6-AP ablation on the mammary gland. The ovarian hormones estrogen and progesterone are critical for mammary gland development (40). We therefore examined the mammary glands of wild-type and E6-AP null females to determine whether ablation of this coactivator influences development of this tissue. Whole-mount analyses of inguinal mammary glands obtained from virgin 8-week-old wild-type and E6-AP null females revealed significant breast development for both genotypes (Fig. 8A and B). In both groups of animals, the mammary gland ducts grew extensively throughout the fat pad. The extent of ductal branching and the number of ducts were also grossly similar. Occasionally, some E6-AP null animals appeared to have more mammary gland development (e.g., increased ductal branching and lobuloaveolar development), but this phenotype was not consistently observed. Western blot analysis revealed similar levels of SRC-1 expression in wild-type and E6-AP null mammary gland extracts, indicating that the lack of an E6-AP ablation effect is not due to a compensatory increase in SRC-1 expression (data not shown). Because mammary gland ductal growth in virgin mice depends on the estrogen-signaling pathway (29), these results suggest that E6-AP is not required to mediate estrogen-induced growth in the breast.

To circumvent any potential differences in systemic estrogen (E) and progesterone (P) levels that could influence mammary

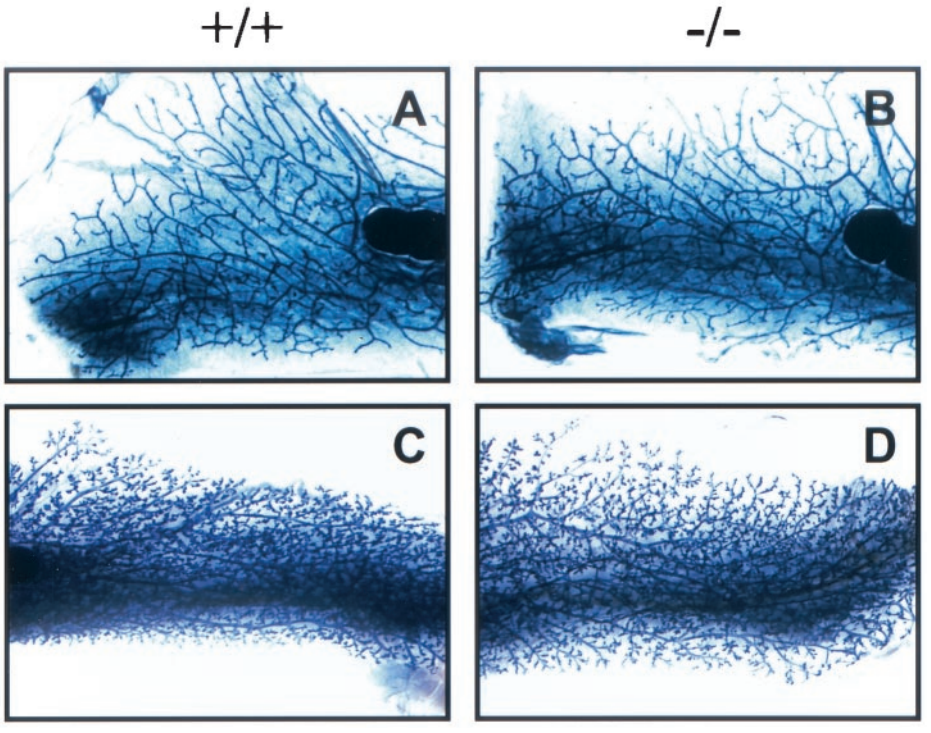

FIG. 8. Mammary gland development in E6-AP null mice. Whole mounts of inguinal mammary glands were obtained from 8-week-old female virgin wild-type (A) and E6-AP null (B) mice or from 14-week-old ovariectomized wild-type (C) or E6-AP homozygous null (D) mice implanted with E+P pellets for 21 days.

gland growth, whole-mount analysis of glands obtained from E+P-treated ovariectomized females were also performed. This treatment also mimics an early stage of pregnancy-induced mammary gland differentiation and therefore facilitates assessment of pregnancy-induced mammary gland development in subfertile animals (21, 54). Virgin mice were ovariectomized and implanted subcutaneously with E+P pellets to deliver hormone for 21 days. Similar to the results obtained for virgin animals, the mammary glands of 12-week-old (data not shown) and 14-week-old E6-AP null animals were at least as developed as those of wild-type littermate controls (Fig. 8C and D). The mammary gland ductal network filled the fat pad, and lobuloalveolar development was noted. As with the virgin mammary gland, some E6-AP knockout animals also showed increased ductal branching and lobuloaveolar development in comparison to wild-type controls, but the penetrance of this phenotype was inconsistent. Accordingly, these data indicate that neither estrogen nor progesterone responses in the mouse mammary gland required E6-AP expression.

DISCUSSION

Biological roles of E6-AP. E6-AP is widely expressed in many organ systems. Although the consequences of absent or mutated E6-AP expression on neurological function have been characterized with respect to Angelman syndrome, much less is known about the biological role of E6-AP at other sites of the body. Initial reports suggested that the E6-AP protein, in association with the E6 protein of papillomavirus, functions as a ubiquitin ligase targeting the tumor suppressor p53 for degradation by the proteasome (19, 55, 62). Thus, in papillomavirus-infected cervical cells, E6-AP contributes to the loss of p53 expression and the deregulation of growth control (62). Evidence on whether E6-AP targets p53 for degradation in the absence of E6 protein has been conflicting (8, 18, 19, 20, 55, 62). However, in E6-AP maternal deficiency heterozygote females, elevated levels of p53 expression are detected in postmitotic neurons, such as Purkinje cells in the cerebellum and some pyridimal cells of the CA1, CA2, and CA3 regions of hippocampus (24), suggesting that E6-AP contributes to the regulation of p53 expression in at least these cell types in vivo. In more recent in vitro studies, E6-AP also has been implicated in the targeted degradation of the product of the DNA repair gene HHR23A (33), a subunit of the replication licensing factor M, Mcm7 (32), and possibly Src kinases (13). In addition to this, E6-AP possesses intrinsic transcriptional activity and coactivates steroid receptor function in transient *trans*-activation assays (46). Furthermore, it reverses steroid receptor squelching and is found in limiting concentrations in the cell, thus fulfilling criteria defining steroid receptor coactivators. Molecular studies therefore suggest that E6-AP is a bifunctional molecule that targets proteins for degradation by the 26S proteasome, as well as coactivates the transcriptional activity of steroid receptors.

Collectively, the results implicate E6-AP as a molecule important in various aspects of reproduction and therefore extend the physiological role of this protein to tissues outside the central nervous system. Furthermore, the phenotype of the E6-AP null mice is consistent with E6-AP playing a role in mediating steroid receptor function in vivo and is therefore

supportive of the assertion that E6-AP is a steroid receptor coactivator. However, the cumulative evidence to date indicates that E6-AP is a dual-function protein in vivo. Whether the effects of E6-AP deletion on steroid hormone action in vivo result from a lack of coactivator function, a lack of ubiquitin ligase activity, or a combination of the two factors cannot be unequivocally resolved at the present time. However, a recent report (49) demonstrates that the ubiquitin conjugating activity of the $TAF_{II}250$ coactivator is required for optimal gene expression in *Drosophila melanogaster*. Furthermore, when considered in the context of intrinsic enzymatic functions demonstrated for other steroid receptor coactivators (e.g., histone acetyltransferase for SRC-1 and SRC-3 [5, 57] and arginine methyltransferase for CARM1 [4]), it is not surprising that E6-AP may utilize both its intrinsic transcriptional and ubiquitin ligase activities to stimulate gene expression.

The nuclear receptors for estrogen, progesterone, and androgens also require proteasome activity for efficient stimulation of gene expression (37). The precise mechanism(s) by which receptors and their associated proteins are targeted to the proteasome is unclear, but receptor-interacting molecules such as E6-AP that also possess ubiquitin ligase activity may be poised to do so since they possess both activities. Although an E6-AP mutant lacking ubiquitin ligase activity retained coactivator activity in in vitro transactivation assays (46), it is possible that a relationship between coactivator and ubiquitin ligase activity may be apparent only in the context of a chromatin template and/or endogenous genes in specific cell types. Resolution of this question will require the development of new models in which the coactivator and ubiquitin ligase activities of E6-AP can be individually assessed in vivo.

Effect of E6-AP ablation on reproduction. Ablation of E6-AP gene expression negatively impacts reproduction in the mouse. Male subfertility correlates with a reduction in sperm production and diminished sperm function, as assessed by measurement of the ability of the sperm to penetrate oocytes. The sperm penetration assay provides important information concerning the ability of the sperm to penetrate a zona-free egg, and for human in vitro fertilization (IVF) a positive score on this clinical test is highly predictive of a positive outcome in IVF. In contrast to the sperm penetration assay in which a constant number of sperm are analyzed, the relative natural fertility potential of an animal in vivo may be increased or diminished by the presence of high or low sperm counts or other alterations in the functional characteristics of sperm (e.g., motility, survival, capacitation, and/or penetration of the zona pellucida). The E6-AP null mice exhibited lowered sperm counts, as well as reduced ability to penetrate an ova, suggesting that these two parameters contributed to the smaller number of litters and the reduction of litter size observed.

Circulating testosterone levels are similar in wild-type and E6-AP null animals, indicating that reduced sperm number and function are not due to defects in low androgen production by the testis. Indeed, the reduced sperm count is likely a reflection of the smaller testes size of E6-AP null mice. However, studies of hypophysectomized males demonstrate that normal androgen action is required for the progression of meiosis to the round spermatid stage and completion of the wave of spermatogenesis (7, 59), and it is possible that the loss of E6-AP expression may compromise this process. More detailed studies of Sertoli cells and spermatogenesis staging are required to determine whether the lack of E6-AP influences these parameters. Estrogen action also is required for the production of normal levels of mature sperm. Targeted deletion of ERα results in a disruption of spermatogenesis with increasing age and degeneration of the seminiferous tubules (9), secondary in part to deficient fluid resorption in the rete testis and efferent ducts, as well as to developmental abnormalities of the epididymis, efferent ducts, and rete testis (17). In addition, sperm from the ERKO mice have lower motility and, like the E6-AP mice, exhibit a reduced ability to penetrate and fertilize eggs, in this case as assessed by IVF (9).

Testis size also is reduced in E6-AP null animals, and this is consistent with the reduced testicular weights observed in other models of compromised steroid production and/or action, such as some of the human androgen receptor mutations (see the Androgen Receptor Gene Mutations Database [http://ww2.mcgill.ca/androgendb/]) and the mouse aromatase, ERα, and SRC-1 knockout models (38, 51, 71). The lack of effect of E6-AP ablation on the seminal vesicle weight is interesting in view of the role of androgens in regulating the size of this organ (48). However, our Northern and Western blot analyses indicate that E6-AP mRNA and protein expression, respectively, is low in the seminal vesicle and suggest that E6-AP may not be present at sufficient levels to significantly contribute to androgen action. In contrast, prostate growth, which is dependent on androgen action (34, 58), is reduced in animals lacking E6-AP expression even though testosterone levels are comparable to those of wild-type controls, and this finding is consistent with the relatively abundant expression of E6-AP protein and its ability to contribute to steroid hormone action in this tissue.

Female fertility also was negatively impacted by ablation of E6-AP gene expression, and this was reflected in the reduced number of oocytes obtained in superovulation protocols. It is not clear why ovaries in young (≤10 weeks) female E6-AP knockout mice are smaller than in their wild-type counterparts, but this may be a reflection of the reduced ovulatory capacity of the E6-AP null ovaries. After 10 weeks, wild-type ovarian size decreases, probably as a result of follicular loss associated with normal aging (10), and E6-AP null and wild-type ovarian sizes become indistinguishable. Steroid hormone action is critical for ovarian function and female fertility, and it is clear from knockout models that both ERα and ERβ are required for appropriate ovarian size, follicular growth and ovulation (31, 38). In addition, PR is important for these processes, as evidenced by the complete infertility of PR knockout mice and the severely reduced number of oocytes produced by superovulated PR_A knockout animals (39, 43). Thus, the ovarian phenotype of E6-AP ablation also is consistent with loss of steroid receptor coactivation. Intriguingly, genetic ablation of the expression of the putative nuclear receptor corepressor, Nrip1/RIP140, leads to anovulation characterized by failure to release mature oocytes at ovulation (67). These observations suggest that positive- and negative-acting nuclear receptor coregulators are critical for the control of steroid receptor activities necessary for ovarian function.

Although it is not possible to examine prostate function in male mice lacking the androgen receptor, androgen receptor antagonists or androgen ablation inhibit prostate growth and

clearly implicate the receptor in this process (34, 58). Similarly, the role of estrogens and ERα in stimulating uterine growth is well established. The growth of the prostate, uterus, and mammary gland in response to testosterone, estradiol or estrogen, and progesterone, respectively, was therefore examined to determine the impact of loss of E6-AP expression on the ability of these tissues to respond to a steroid signal. Both prostatic and uterine size was reduced in comparison to wild-type controls after hormonal stimulation, suggesting that these tissues also utilize E6-AP to mediate steroid hormone action. Decreases in hormone-induced growth of these two tissues have also been noted in SRC-1 null mice (71), indicating that multiple coactivators contribute to steroid responsiveness within a given tissue. Further studies are required to determine whether E6-AP and SRC-1 can coactivate the expression of the same steroid target genes or whether they are even expressed in the same cells within either the uterus or the ovary.

In contrast to the prostate and uterus, mammary gland growth in response to exogenous estrogen and progesterone administration is not compromised in E6-AP null females in comparison to wild-type controls, nor is virgin mammary gland development. The importance of ERs and PRs for mammary gland development has been firmly established by the phenotype of each receptor's mouse knockout model; both ERα and PR null animals exhibit severely retarded mammary gland development and growth in response to hormone treatment (3, 39). In addition, genetic ablation of the expression of the SRC-1 and SRC-3 coactivators also results in defects in mammary gland development (70, 71). The lack of a mammary gland phenotype in E6-AP null mice indicates that E6-AP is not required to mediate estrogen or progesterone action in this tissue. This is not, however, simply due to a lack of E6-AP expression, since we detected E6-AP mRNA and protein expression in our analyses (our data and reference 56). Furthermore, we have localized E6-AP expression to mammary epithelial cells in a spontaneous mouse mammary tumor model (56), and it is therefore likely that E6-AP is coexpressed in at least some cells with ERα and/or PR (14, 15). Taken together, these results suggest that the role of E6-AP with respect to steroid hormone action is tissue specific. Interestingly, Sivaraman et al.(56), have shown by Northern blot analyses that tumorigenesis in a spontaneous, hormone-independent mouse model of breast cancer is accompanied by overexpression of a 10-kb E6-AP mRNA. Although the significance of that finding is not clear, it is consistent with our inability to observe a relationship between E6-AP expression and estrogen or progestin action in the mammary gland. Indeed, it has been postulated that E6-AP may exert ubiquitin ligase activity in the mammary gland, and cell extracts prepared from mammary gland can induce the degradation of a known E6-AP ubiquitin ligase target, HHR23A, in in vitro assays (56).

Tissue-selective coactivator function. Mouse null models for SRC-1 (71), SRC-3 (66, 70), TRAP220 (22), CBP (63), p300 (73), and E6-AP (this study and reference 24) have been generated and characterized, but only SRC-1, SRC-3, and E6-AP null mice are viable and have been assessed for reproductive phenotypes. Analyses of the E6-AP null phenotype reveals that a second class of coactivators can modulate steroid hormone action in vivo and therefore provides compelling evidence that multiple coactivators are required to mediate the full range of steroid hormone action. It is clear that the loss of coactivator expression influences steroid hormone action in a tissue-specific fashion. For instance, SRC-3 and E6-AP, but not SRC-1, knockout females exhibit fertility defects, even though all three coactivators are expressed in reproductive tissues. Similarly, all three coactivators are expressed in mammary gland, but only SRC-1 and SRC-3 null animals show a phenotype in this tissue. These results support the hypothesis that coactivators contribute to the specificity of steroid receptor action and provide a strong rationale for more detailed mechanistic investigations, including studies of coactivator expression patterns and the underlying gene, tissue, and potential cell or promoter specificity of the coactivator action.

ACKNOWLEDGMENTS

The technical assistance of Carol Nolan, Lata Murthy, Josie Beck, Claire Lo, and Kate Ramsayer is gratefully acknowledged. We thank Franco DeMayo, JoAnne Richards, and Barbara Foster for useful suggestions and technical expertise, John Lydon for providing E&P beeswax pellets, and Norman Maitland for E6-AP antibody and purified E6-AP protein.

This work is supported by NIH grants to C.L.S., D.J.L., A.L.B., and B.W.O.

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