# Transactivating function (AF) 2–mediated AF-1 activity of estrogen receptor α is crucial to maintain male reproductive tract function

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**Estrogen receptor alpha (ERα) is a ligand-dependent transcription factor containing two transcriptional activation function (AF) domains. AF-1 is in the N terminus of the receptor protein, and AF-2 activity is dependent on helix 12 of the C-terminal ligand-binding domain. We recently showed that two point mutations converting leucines 543 and 544 to alanines in helix 12 (AF2ER) minimized estrogen-dependent AF-2 transcriptional activation. A characteristic feature of AF2ER is that the estrogen antagonists ICI182780 and tamoxifen (TAM) act as agonists through intact AF-1, but not through mutated AF-2. Here we report the reproductive phenotype of male AF2ER knock-in (AF2ERKI) mice and demonstrate the involvement of ERα in male fertility. The AF2ERKI male homozygotes are infertile because of seminiferous tubular dysmorphogenesis in the testis, similar to ERα KO males. Sperm counts and motility did not differ at age 6 wk in AF2ERKI and WT mice, but a significant testis defect was observed in adult AF2ERKI male mice. The expression of efferent ductal genes involved in fluid reabsorption was significantly lower in AF2ERKI males. TAM treatment for 3 wk beginning at age 21 d activated AF-2–mutated ERα (AF2ER) and restored expression of efferent ductule genes. At the same time, the TAM treatment reversed AF2ERKI male infertility compared with the vehicle-treated group. These results indicate that the ERα AF-2 mutation results in male infertility, suggesting that the AF-1 is regulated in an AF-2–dependent manner in the male reproductive tract. Activation of ERα AF-1 is capable of rescuing AF2ERKI male infertility.**

antagonist reversal | tubule dilation | domain function

The essential role of estrogen in male reproductive function has been demonstrated in the estrogen receptor α (ERα) gene knockout (αERKO) mouse model (1–4). ERα in the male reproductive system is expressed predominantly in the efferent ducts, proximal epididymis, and Leydig cells. αERKO males are infertile and have dilated seminiferous tubules resulting from compromised efferent ductule fluid reabsorption. Loss of expression of ion transport-related proteins, such as sodium–hydrogen exchanger 3 (SLC9A3) and carbonic anhydrase 2 (CAR2) (5, 6), results in altered luminal fluid pH and osmolality in the epididymis, reduced motility, and abnormal sperm morphology (7, 8) as a consequence of loss of ERα activity in male reproductive tract somatic cells.

Transplantation of germ cells from αERKO homozygote males into germ cell-depleted WT testes has been shown to restore fertility in the recipient WT males (9). Further studies have provided evidence of ERα involvement in the regulation of serum testosterone (T) levels. T is produced in the testis by Leydig cells, and αERKO males have significantly higher serum T levels than WT males (10, 11). These results demonstrate that ERα is not required in male germ cells, but is required by somatic cells of the male reproductive tract, including efferent ductal epithelial cells and testicular Leydig cells, which provide the proper environment for male gamete development and maturation.

ERα is a transducer of estrogenic activity and functions as a ligand-dependent transcription factor (12–14). ERα has two transcriptional activation function (AF) domains, AF-1 and AF-2. AF-1 is localized in the N-terminal region, and AF-2 is positioned in the C-terminal ligand-binding domain (LBD) of the receptor protein. Although in vitro studies have demonstrated that AF-1 function is regulated in a cell type-specific and ligand-independent manner (15–17), the physiological role and in vivo tissue actions of AF-1 remain unclear.

Previous in vitro studies have demonstrated the activity of AF-2 disruption mutant (AF2ER) that is introduced by L543A and L544A mutations on helix 12 of the ERα LBD and retains intact AF-1 in the N-terminal region (18–20). Despite these mutations, however, binding of the mutated ERα to estrogen-responsive DNA sequences and affinity for estradiol (E2) were unaffected (21). Transcriptional activity was markedly reduced in the presence of E2 compared with the WT ERα, owing to a failure to recruit the p160 transcriptional coactivators (18). Furthermore, the L543A and L544A mutations of ERα resulted in antagonist reversal, with ICI182780 (ICI) and tamoxifen (TAM) acting as agonists. Removal of the N-terminal region of the AF2ER mutant results in loss of activity with ICI or TAM, suggesting that the N-terminal dependent transactivation function (AF-1) is required for this activity (19, 20).

We recently reported the generation of a knock-in mouse model with L543A and L544A mutations (AF2ERKI) (20). The phenotype of AF2ERKI female mice was similar to αERKO females in showing a lack of E2 stimulation; however, it also was consistent with the in vitro data, because ICI can activate uterine growth and gene expression similarly to E2 in the WT (20). Given the importance of ERα in male reproduction, we evaluated the physiological role of ERα AF-1 and AF-2 in male reproductive functions by characterizing the AF2ERKI male reproductive phenotypes.

AF2ERKI male homozygotes were infertile owing to the seminiferous tubular degeneration within the testis and defects in sperm motility. The expression of efferent ductal ion transporter genes was also diminished. AF2ERKI male mice were treated with TAM to test the antagonist reversal activity. Treatment with TAM recovered the gene expression in the efferent ducts and the fertility of AF2ERKI homozygote males by activation of the AF2ER mutant. This study demonstrates that the AF-2–mediated AF-1 activation of ERα stimulates efferent duct gene expression to support male reproductive function and fertility.

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**Table 1. Infertility in AF2ERKI homozygote males**

| Genotype | No. fertile/total no. | Mean no. of litters | Mean no. of offspring |
|---|---|---|---|
| +/+ | 5/5 | 2.6 | 5.6 |
| KI/KI | 0/9 | 0 | 0 |

Three-month-old male littermates were used for this 3-mo continuous breeding study.

## Results

**AF2ERKI Homozygote Males Are Infertile.** To determine the fertility of AF2ERKI male mice, 10- to 12-wk-old littermates were used in a 3-mo continuous breeding study. All WT control animals were fertile. In contrast, the AF2ERKI homozygous ($AF2ER^{KI/KI}$) males sired no offspring during this period (Table 1). Given the infertility phenotype of $AF2ER^{KI/KI}$ male, we analyzed sperm count (Fig. 1*A*) and sperm motility (Fig. 1*B*) in 6-wk-old and 4-mo-old (adult) WT and $AF2ER^{KI/KI}$ male mice. Sperm count and motility were not different at 6 wk in the two genotypes, but a significant defect was observed in the adult $AF2ER^{KI/KI}$ male mice. Histological assessment of morphology revealed dilated and degenerated seminiferous tubules and rete testis in the $AF2ER^{KI/KI}$ males (Fig. 1*C*). This phenotype is similar to that reported previously in ERα null mutant male mice (1, 11).

**Male Reproductive Organs Express AF2ER Protein.** Because the $AF2ER^{KI/KI}$ male phenotypes were similar to αERKO male mice, we assessed the expression of ERα protein in the male reproductive tissues by immunohistochemistry (Fig. 1*D*). ERα expression was detected in the WT and $AF2ER^{KI/KI}$ efferent ductal epithelial cells and testicular Leydig cells, but not in the αERKO tissues. These findings indicate that $AF2ER^{KI/KI}$ male phenotypes are associated with disrupted ERα AF-2 function.

**Seminiferous Tubules Are Dilated in AF2ERKI Homozygotes, but Less Severely than in αERKO Mice.** We examined seminiferous tubule structure in 10-d-old, 20-d-old, 6-wk-old, and adult 4-mo-old (adult) WT, $AF2ER^{KI/KI}$, and αERKO animals. The lumen of the seminiferous tubule of 10-d-old $AF2ER^{KI/KI}$ male was not different from that of WT, whereas all tubules from the mice aged 20 d and older showed detectable dilation in $AF2ER^{KI/KI}$ males (Fig. 1*E*). Varying degrees of dilation were seen in the seminiferous tubules of 10-d-old αERKO mice, and all tubules exhibited distinguishable dilation in 20-d-old αERKO mice. At age 6 wk, the seminiferous tubules were dramatically dilated in $AF2ER^{KI/KI}$ and αERKO mice compared with WT mice. In the adult animals, some differences were seen among the genotypes; namely, sperm tails were observed in the lumen of dilated seminiferous tubules in $AF2ER^{KI/KI}$ males, but not in αERKO males, even those with similar levels of tubule dilation.

We analyzed serum T and luteinizing hormone (LH) levels in the adult mice and found differences among the genotypes (Table 2). The $AF2ER^{KI/KI}$ males had a 10-fold higher mean T level than the WT males and a similar level as the αERKO males but, conversely, a lower mean serum LH level than the αERKO males and a similar level as the WT males.

**Expression of Testicular Fluid Reabsorption-Related Genes Is Down-Regulated in Efferent Ducts of AF2ERKI Homozygotes.** The repressed expression of certain membrane proteins in the efferent ducts of αERKO mice is related to seminiferous tubule dilation and male infertility (5). Based on that relationship, we analyzed the expression of SLC9A3 and aquaporin 9 (AQP9) by immunohistochemistry in WT, $AF2ER^{KI/KI}$, and αERKO efferent ducts. SLC9A3 and AQP9 signals were detected at the apical borders of efferent ductal epithelial cells in the WT males and at much lower levels in the $AF2ER^{KI/KI}$ and αERKO males (Fig. 2*A*). We then assessed the steady-state mRNA levels of *Slc9a3*, *Aqp9*, *Car2*, and *Aqp1* genes related to testicular fluid reabsorption in efferent ducts using

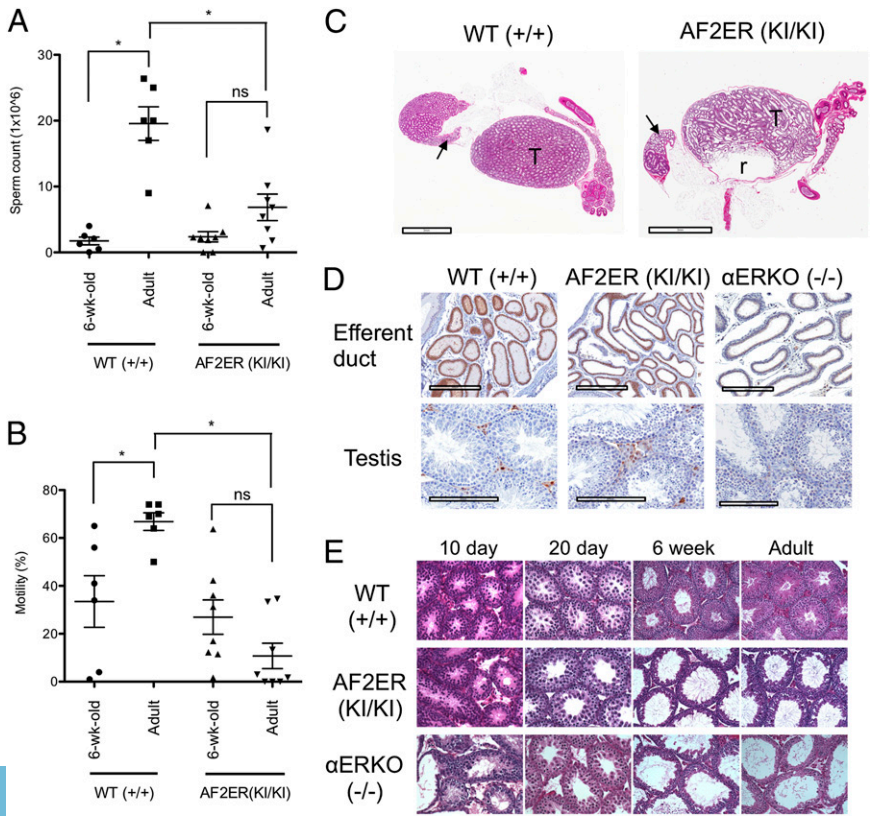


**Fig. 1.** AF2ERKI homozygote males are infertile. (*A*) Sperm counts from the cauda epididymis of 6-wk-old and adult WT and AF2ERKI mice. Data are expressed as mean ± SEM. Statistical analysis was done by ANOVA.*$P < 0.05$; ns, not significant. (*B*) Motility of sperm from the cauda epididymis of 6-wk-old and adult WT and AF2ERKI mice, determined with a computer-assisted sperm analyzer after a 60-min incubation in M2 medium. Data are expressed as mean ± SEM. Statistical analysis was done by ANOVA. *$P < 0.05$; ns, not significant. (*C*) Representative longitudinal sections from adult WT and AF2ERKI testes and epididymis. Arrow indicates efferent duct; T indicates testis; r indicates rete testis. (Scale bars: 3 mm.) (*D*) Representative ERα expression levels in the efferent ducts and testes of adult WT, AF2ERKI, and αERKO mice. Immunohistochemical studies were performed as described in *Materials and Methods*. Signals were developed by DAB chromogen, and the slides were counterstained in modified Harris hematoxylin. (Scale bars: 0.3 mm for efferent duct; 0.2 mm for testis). (*E*) Representative histology of testes from 10-d-old, 20-d-old, 6-wk-old, and adult WT, AF2ERKI, and αERKO mice. Sections were stained with H&E.

**Table 2. Serum hormone levels in WT, AF2ERKI, and αERKO males**

| Hormone | WT (+/+) | AF2ER (KI/KI) | αERKO |
|---|---|---|---|
| T, ng/dL | 75.6 ± 47.1 | 800.2 ± 199.7* | 1,102 ± 75.0* |
| LH, ng/mL | 2.38 ± 0.51 | 2.28 ± 0.42 | 4.91 ± 0.38* |

Values are mean ± SEM; $n = 10$ for WT (+/+) and AF2ER (KI/KI), $n = 3$ for αERKO. *$P < 0.05$ in one-way ANOVA against WT.

quantitative PCR (qPCR). The mRNA levels of these genes were decreased in the AF2ER$^{KI/KI}$ efferent ducts, as was seen in αERKO males (Fig. 2*B*).

We previously demonstrated that estrogen antagonists (TAM and ICI), but not the agonist (E2), activate AF2ER function in the AF2ER$^{KI/KI}$ uterus (20). To examine whether TAM activates AF2ER function in the efferent duct, we treated AF2ER$^{KI/KI}$ and WT mice with TAM for 3 d. As expected, the expression of *Slc9a3*, *Aqp9*, and *Aqp1* in WT mice was down-regulated by the antagonist effect of TAM; however, *Car2* gene expression was not down-regulated (Fig. 2*C*). In contrast, TAM induced expression of *Slc9a3*, *Car2*, and *Aqp1* in AF2ER$^{KI/KI}$ efferent ducts to the WT levels, indicating activation of the AF2ER mutant receptor by TAM in AF2ER$^{KI/KI}$ efferent ducts. Expression of *Aqp9* was not up-regulated by TAM. In addition, we treated αERKO male mice with TAM to evaluate the ERα dependency of TAM-mediated gene regulation in efferent ducts in this strain. The expression of *Slc9a3*, *Car2*, *Aqp9*, and *Aqp1* was not changed by TAM treatment in the αERKO efferent ducts, supporting the hypothesis that TAM-mediated activation of *Slc9a3*, *Car2*, and *Aqp1* in AF2ER$^{KI/KI}$ efferent ducts is regulated in an ERα-dependent manner.

**Male Fertility Was Restored by TAM Treatment.** Based on our finding that TAM treatment activated the AF2ER mutant receptor and stimulated gene expression in efferent ducts, we investigated whether such treatment had an effect on fertility. We treated the 21-d-old males with TAM or placebo for 3 wk, then, starting at 1 wk after the end of treatment, used these mice in a 2-mo continuous breeding study. Three of four placebo-treated WT males (75%) were fertile during this period. Under these experimental conditions, five of six TAM-treated WT males were fertile (83%). In contrast, none of the five placebo-treated AF2ER$^{KI/KI}$ males sired litters (0%); however, three of the eight TAM-treated AF2ER$^{KI/KI}$ males sired offspring (38%), suggesting that TAM activated the AF2ER to restore fertility.

After the breeding study, sperm numbers and motility were assessed. TAM treatment had no effect on sperm counts in WT and AF2ER$^{KI/KI}$ males compared with placebo treatment (Fig. 3*A*); however, sperm from placebo-treated AF2ER$^{KI/KI}$ males were immotile. In contrast, all TAM-treated AF2ER$^{KI/KI}$ males had motile sperm (Fig. 3*B*). Of particular interest is our finding that the dysmorphology seen in the testis of AF2ER$^{KI/KI}$ mice was not seen after TAM treatment (Fig. 3*C*).

To analyze the effect of TAM treatment on gene regulation in the efferent ducts, we collected tissue samples from several animals in each group at 1 wk after treatment. We found that mRNA expression levels of the efferent ductal genes (*Slc9a3*, *Aqp1*, *Aqp9*, and *Car2*) in AF2ER$^{KI/KI}$ mice were induced to the WT levels by TAM treatment (Fig. 4*A*). Moreover, immunohistochemistry showed that TAM treatment resulted in recovery of SLC9A3 and AQP9 protein expression in the AF2ER$^{KI/KI}$ efferent ducts. The expression of AQP9 protein varied in different regions of the efferent ducts (Fig. 4*B*).

## Discussion

In this study, we compared phenotypes of AF2ERKI mice with αERKO and WT mice to determine the role of AF-1 and AF-2 in male reproductive tract function and male fertility. AF2ERKI males were infertile, and the morphology of the AF2ERKI testis was quite similar to that of the αERKO testis (Fig. 1*C*). Comparing the progression of seminiferous tubule degeneration in AF2ERKI

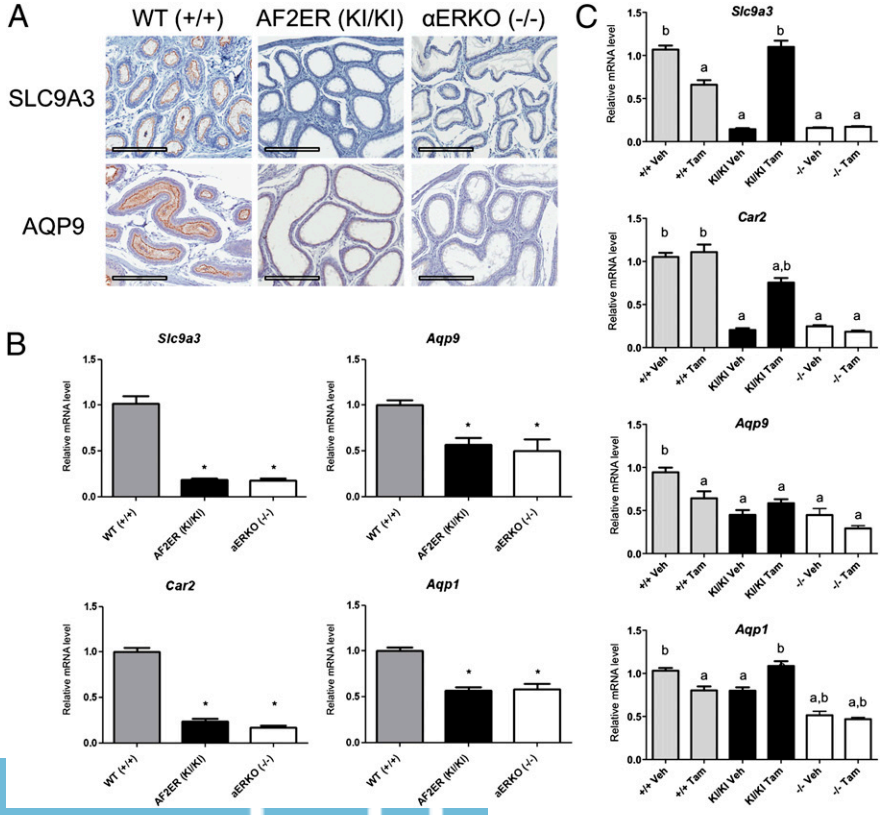


**Fig. 2.** Efferent ductal gene expression in AF2ERKI and αERKO mice. (*A*) Representative expression levels of efferent duct SLC9A3 and AQP9 in adult WT, AF2ERKI, and αERKO mice. Immunohistochemical studies were performed as described in *Materials and Methods*. Signals were developed by DAB chromogen. The slides were counterstained in modified Harris hematoxylin. (Scale bars: 0.3 mm.) (*B*) mRNA levels of *Slc9a3*, *Car2*, *Aqp1*, and *Aqp9* in efferent ducts from WT, AF2ERKI, and αERKO mice, quantified by qPCR. mRNA levels are presented as fold change vs. WT. Values are mean ± SEM. Statistical analysis was done by ANOVA. *$P < 0.05$ against WT. (*C*) mRNA levels of *Slc9a3*, *Car2*, *Aqp1*, and *Aqp9* in vehicle (Veh)- and TAM (Tam)-treated efferent ducts from WT (+/+), AF2ERKI (KI/KI), and αERKO (–/–) mice, quantified by qPCR. mRNA levels are presented as fold change vs. vehicle-treated WT mice. Values are mean ± SEM. Statistical analysis was done by ANOVA. a indicates $P < 0.05$ against WT (+/+) vehicle-treated mice; b indicates $P < 0.05$ against AF2ERKI (KI/KI) vehicle-treated mice.

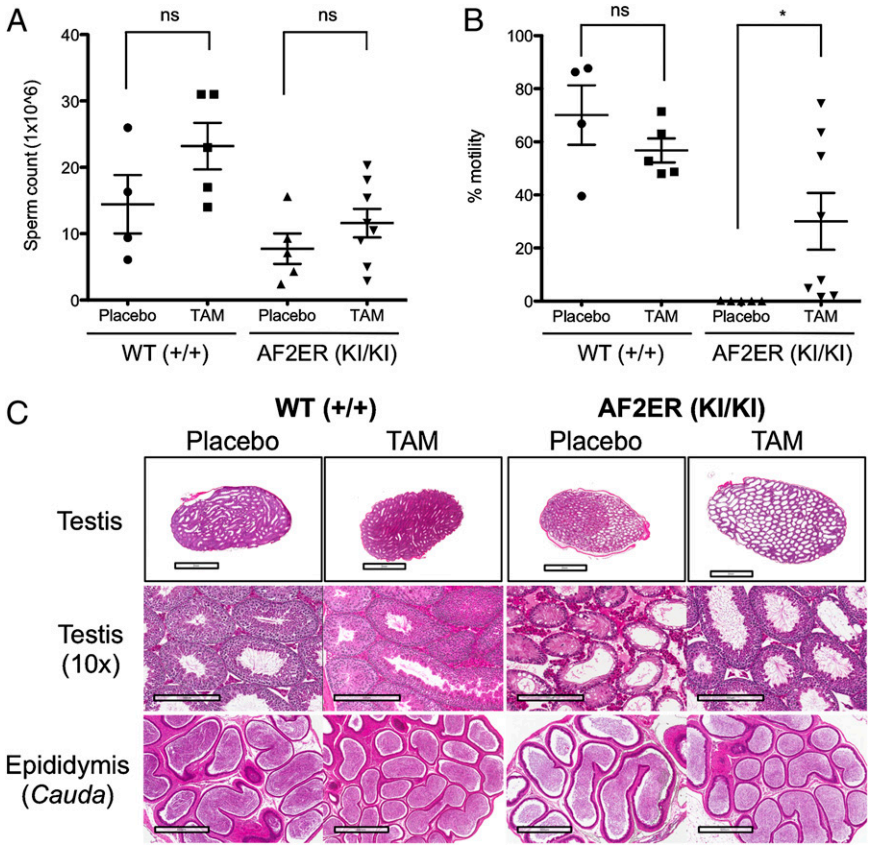


**Fig. 3.** AF2ERKI homozygote male fertility was recovered by TAM treatment. (*A*) Sperm counts from the cauda epididymis of WT and AF2ERKI mice after breeding. Data are expressed as mean ± SEM. Statistical analysis was performed using the Mann–Whitney *U* test between placebo and TAM. ns, not significant. (*B*) Sperm motility from the cauda epididymis of WT and AF2ERKI mice after 60 min of incubation in M2 medium. Data are expressed as mean ± SEM. Statistical analysis was performed using the Mann–Whitney *U* test between placebo and TAM. $*P < 0.05$; ns, not significant. (*C*) (*Top*) Representative low-magnification view of sections for placebo- and TAM-treated WT and AF2ERKI testes. The sections were stained with H&E. (Scale bars: 2 mm.) (*Middle*) Representative histology of testes from placebo- and TAM-treated WT and AF2ERKI. (Scale bars: 0.3 mm.) (*Bottom*) Representative histology of cauda epididymis from placebo- and TAM-treated WT and AF2ERKI. (Scale bars: 0.6 mm.)

and αERKO testes revealed that although seminiferous tubule dilation occurred in both genotypes, sperm were observed in the seminiferous tubules of adult (4-mo-old) AF2ERKI males, but not in adult αERKO males (Fig. 1*E*). This finding suggests that the phenotype of AF2ERKI males is similar to, but less severe than that of αERKO males.

We analyzed serum LH and T levels in adult WT, AF2ERKI, and αERKO males (Table 2). Serum T level was elevated in AF2ERKI males, as was reported previously in αERKO males (10, 11). In contrast, however, serum LH level in AF2ERKI males was not elevated as in αERKO males (10, 22) and was equivalent to that in WT males. The phenotypic differences might be related to the lack of ERα protein expression in αERKO males, whereas a mutated protein is expressed in AF2ERKI animals. This mutant protein may mediate some receptor activities through AF-1 function, thereby resulting in a less severe phenotype. Mice with a deleted AF-1 or AF-2 region of the ERα have been generated, but the male reproductive phenotype was not reported in those studies (23, 24).

To evaluate gene regulation in the reproductive tract of AF2ERKI males, we subjected these mice to short-term and long-term treatment with TAM. For short-term treatment, TAM was injected s.c. for 3 d, and samples were collected 24 h after the last injection. For long-term treatment, a timed-release pellet was implanted s.c. for 3 wk, and samples were collected on the fourth week after withdrawal of TAM. Without TAM treatment, the expression levels of *Slc9a3*, *Aqp1*, *Aqp9*, and *Car2* in the efferent duct of AF2ERKI mice were reduced to the same low levels seen in αERKO males. However, the expression of *Slc9a3*, *Aqp1*, and *Car2* was induced by both short- term (Fig. 2*C*) and long-term (Fig. 4*A*) TAM treatment of AF2ERKI males, indicating that those genes are likely directly regulated by ERα.

As reported previously, AF-1 activity is involved in TAM-dependent AF2ER activation (20). This suggests that *Slc9a3*, *Aqp1*, and *Car2* are regulated by AF-1 in an AF-2–dependent manner in the efferent ducts, as demonstrated by our finding that the expression of these genes is significantly lower in the nontreated animals and activated by TAM in AF2ERKI males. Previous in vitro experiments suggested that AF-1 can be activated by phosphorylation independent of ligand (15, 16); however, our AF2ERKI animal model suggests that AF-2 is necessary to regulate AF-1 function properly in vivo. Although *Aqp9* expression was not induced in short-term TAM-treated AF2ERKI males, *Aqp9* mRNA levels were restored by long-term TAM treatment (Fig. 4*A*). This may suggest that the regulation of *Aqp9* expression is an indirect ERα mechanism or can occur independent of the AF-1–mediated regulation. Long-term TAM treatment restored efferent ductal gene expression in AF2ERKI mice and the fertility of AF2ERKI males simultaneously. These findings strongly support the idea that ERα functionality involves the regulation of ion and fluid transporter genes in the efferent ducts critical to maintaining male reproductive function (5).

The seminiferous tubule dilation and testicular dysmorphogenesis seen in AF2ERKI males have been suggested to be related either to increased fluid production in the seminiferous tubules or to reduced fluid reabsorption in the efferent ductules (1). The latter possibility is the more likely explanation, given that TAM treatment supports the expression of genes related to fluid reabsorption in efferent ducts and prevents the development of dysmorphogenic testicular morphology (Fig. 3*C*). The epididymal fluid of αERKO mice is of higher pH and lower osmolality than that of WT mice (7, 8). The lower osmotic pressure of luminal fluid in the epididymis of αERKO mice has been implicated in abnormal flagellar coiling, which could be rescued by a WT-like osmotic environment (8). The down-regulated expression of ion transporter genes in the efferent ducts in AF2ERKI mice suggests that the pH and osmolality of efferent duct fluid also might be abnormal in these mice. Furthermore, TAM treatment restored the expression levels of these genes in the efferent ducts and maintained sperm motility at WT sperm levels. These findings are consistent with the

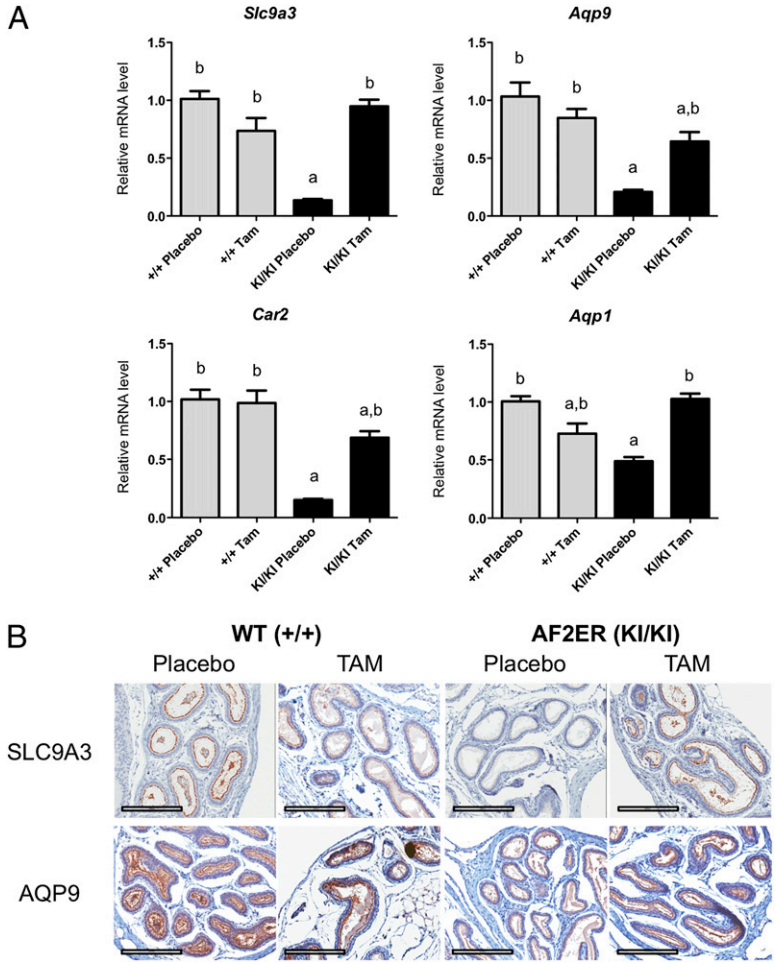


**Fig. 4.** Expression of efferent ductal genes was recovered by TAM treatment in AF2ERKI mice. (*A*) mRNA levels of *Slc9a3*, *Car2*, *Aqp1*, and *Aqp9* in the placebo- and TAM (Tam)-treated efferent ducts from WT (+/+) and AF2ERKI (KI/KI) mice, quantified by qPCR. mRNA levels are presented as fold change vs. placebo-treated WT. Values are mean ± SEM. Statistical analysis was done by ANOVA. a indicates $P < 0.05$ against WT (+/+) placebo-treated mice; b indicates $P < 0.05$ against AF2ERKI (KI/KI) placebo-treated mice. (*B*) Immunohistochemical results of placebo- and TAM-treated WT and AF2ERKI efferent ducts. Representative results of SLC9A3 and the highest AQP9 expression are shown. Immunohistochemistry was performed as described in *Materials and Methods*. Signals were developed by DAB chromogen, and the slides were counterstained in modified Harris hematoxylin. (Scale bar: 0.2 mm.)

previous findings characterizing the role of ERα function in male reproduction, namely that ERα is not required by male germ cells, but is needed by somatic cells of the male reproductive system to provide a suitable environment for normal male fertility (25).

Several previous studies have used ERα mutant knock-in mice to evaluate ERα function in male reproduction (26, 27). The E207A, G208A mutated ERα knock-in (NERKI) mice were found to lack ERE-mediated transcription, but allowed E2-dependent transcription by ERα tethering with AP-1 (28). NERKI male mice had normal sperm counts and sperm motility and AQP9 expression at the WT level in the efferent duct, but did not express SLC9A3 (26). These observations are consistent with our results showing that *Slc9a3*, but not *Aqp9*, is directly regulated by ERα. Because AF2ER does not activate tether-mediated gene expression (20), these results suggest that *Aqp9* expression might be regulated by a tether-mediated transcription mechanism.

Another ERα mutant mouse model, the G525L-mutated ERα knock-in mouse (ENERKI), was shown to prevent endogenous E2- mediated activation owing to the reduction of E2 binding affinity to the LBD (27). ENERKI mice are subfertile and show normal expression of ion and fluid transporters (SLC9A3, AQP1, and CAR2) in the efferent ducts (27). The authors speculated that the ligand-independent AF-1 function might involve restoring efferent duct gene regulation (27). The ENERKI mutation disrupts the AF-2 function of ERα similar to the AF2ER mutation, but the ENERKI has an intact helix 12, which might be associated with the less severe phenotype of ENERKI compared with AF2ERKI. This provides additional evidence suggesting that helix 12, a core of AF-2, is the critical domain for regulating proper AF-1 functionality.

In summary, this study provides insight into the ERα AF-1– and AF-2–mediated male reproductive functions. Our findings suggest that the AF2ERKI mouse model can elucidate the roles of genes regulated directly and indirectly by ERα and evaluate AF-2–dependent or AF-2–independent functions of AF-1 in tissues. These properties will allow an expansion of the previous phenotype characterizations made using the αERKO model, which were limited to defining the overall role of ERα without defining more precise domain functions of ERα involved in hormonally mediated physiological functions.

## Materials and Methods

**Animals.** All experiments involving animals were carried out in accordance with US Public Health Service guidelines, and this study was approved by the National Institute of Environmental Health Sciences Institutional Animal Care and Use Committee. The generation of AF2ERKI mice has been described previously (20), as has the generation of Ex3αERKO mice (29).

**Fertility Studies.** Fertility of 10- to 12-wk-old $AF2ER^{KI/KI}$ males ($n = 9$) and their WT littermates ($n = 5$) was determined by rotated mating for 3 mo with WT C57BL/6 females. The females were monitored for pregnancy during and after the mating periods, and the numbers of litters and offspring were recorded.

**Sample Collection.** The animals were euthanized by $CO_2$. The organs were weighed, and sperm counts and motility were analyzed. Blood for serum hormone measurements was collected from the descending aorta at the time of euthanasia. Testes were preserved in Bouin fixative, washed in PBS, dehydrated in increasing concentrations of ethanol, and then embedded in paraffin. The sectioned tissues were stained with H&E for histological examination. For immunohistochemistry, the testis and epididymis were fixed in 10% neutral buffered formalin, dehydrated in 70% ethanol, and then

embedded in paraffin. For RNA extraction, the excised tissues were frozen in liquid $N_2$ and stored at −70 °C.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections were used for immunohistochemical studies. Endogenous peroxidase was quenched using 3% (vol/vol) $H_2O_2$, after which heat-induced epitope retrieval was performed using citrate buffer. Nonspecific sites were blocked with 10% donkey serum (Jackson ImmunoResearch) for NHE3 (Slc9a3) and AQP9 staining or mouse-specific IgG-blocking reagent (Vector Laboratories) for ERα staining. The sections were incubated with anti-NHE3 rabbit polyclonal antibody (1:250; Millipore), anti-AQP9 rabbit polyclonal antibody (1:400; Alpha Diagnostic Intl.), or anti-ERα mouse monoclonal antibody (clone ER1D5, 1:50; Immunotech) for 1 h at room temperature. The sections were incubated further with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch) for NHE3 (1:500), AQP9 (1:500), or biotinylated horse anti-mouse IgG for ERα (1:500; Vector Laboratories) for 30 min at room temperature, and finally incubated with the Vectastain Kit (Vector Laboratories) for 30 min at room temperature. The antigen–antibody complex was visualized using 3-diaminobenzidine (DAB) chromogen (Dako) for 6 min. The sections were counterstained with modified Harris hematoxylin.

**Sperm Motility Assays and Counts.** The cauda epididymis was collected in PBS ($Ca^{2+}$/$Mg^{2+}$-free) at room temperature and transferred to 500 μL of M2 medium (Chemicon). Several cuts were made in the epididymis to allow sperm to move out into the medium for 10 min at room temperature. For analysis of sperm motility, sperm tracks (1.5 s, 30 frames) were evaluated with a computer-assisted sperm analyzer (HTM-IVOS version 12.2L; Hamilton Thorne Biosciences). The collected sperm were counted under a microscope with a hemocytometer.

**Serum Steroid Hormone and Gonadotropin Assay.** All hormone assays were performed in serum collected from individual animals. Serum was processed from whole blood and stored at −70 °C until analysis. Serum LH levels were measured in a 20-μL sample by a sensitive dissociation-enhanced lanthanide fluoroimmunoassay. Serum free testosterone levels were assayed in individual animals in the National Institute of Environmental Health Sciences Clinical Pathology Group Laboratory. Testosterone levels were measured with an RIA kit (Coat-A-Count; Siemens Medical Solutions).

**qPCR Assay.** The frozen tissues were pulverized individually and homogenized in TRIzol (Invitrogen), and RNA was prepared as recommended by the manufacturer (Invitrogen). Analysis was performed by qPCR with the ABI Prism 7900 Sequence Detection System (Applied Biosystems) as described previously (29). Primer sequences were as follows: *Slc9a3*, forward: 5′-TCC CTC TAT GGT GTC TTC CTC AGT GGC TT-3′, reverse: 5′-TTC CTC AAA CAC AGC CAG CAC AGC C-3′; *Car2*, forward: 5′- TTT CAC TTT CAC TGG GGC TCA TCT G-3′, reverse: 5′-GGC AGG TCC AAT CTT CAA AAA AAT ACC-3′; *Aqp9*, forward: 5′- CTT TGC CAT CTT TGA CTC CAG AAA CC-3′, reverse: 5′-CCC ACG ACA GGT ATC CAC CAG AAG-3′; *Aqp1*, forward: 5′-TTC CCC TTT GGT CTG ACT TAC C-3′, reverse: 5′-CTC AGC ACA GGG ACA ATT CCA-3′; *Rpl7*, forward: 5′-AGC TGG CCT TTG TCA TCA GAA-3′, reverse: 5′-GAC GAA GGA GCT GCA GAA CCT-3′. Relative expression levels were determined using the mathematical model described by Pfaffl (30). Samples were analyzed in triplicate, and the expression of *Rpl7* was used as an internal control for all analyses.

**TAM Treatment Studies.** Intact adult male mice (six animals of each genotype) were treated with TAM (2 mg/kg s.c.; Sigma-Aldrich) or 50 μL of corn oil as a vehicle in WT, AF2ERKI, and αERKO for 3 d. The mice were euthanized by $CO_2$ at 24 h after the last injection, and efferent ducts were collected for gene expression analysis. For breeding studies, males were genotyped at age 19 d and separated into experimental groups. Placebo or TAM pellets (0.5 mg/21-d release; Innovative Research of America) were implanted s.c. in WT and AF2ERKI males at age 21 d. The males were bred for a 2-mo period, after which they were euthanized by $CO_2$ and organs were collected. Sperm counts and motility were determined as described above. One testis was fixed in Bouin solution, and the other testis and epididymis were fixed in formalin. For gene expression studies, the pellet implanted mice were euthanized at 4 wk after implantation and efferent ducts were collected.

**Statistical Analysis.** Statistical analyses were performed by one-way ANOVA with the Bonferroni test and Mann-Whitney *U* test using GraphPad Prism. $P < 0.05$ was considered to indicate statistical significance.

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