Preserved tissue structure of efferent ductules in aromatase-deficient mice

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Abstract

Estrogen receptor α (*Esr1*) is proposed to play a critical role in the regulation of testicular fluid reabsorption at efferent ductules, and disruption of the *Esr1* gene (*Esr1*^{-/-}) resulted in marked dilation of the lumens of efferent ductules. This study was aimed to clarify whether disruption of the gene for aromatase (*Ar*), an enzyme responsible for estrogen biosynthesis, results in morphological and transcriptional alterations at efferent ductules as observed in *Esr1*^{-/-} mice. Histology demonstrated structural preservation of the ducts in aromatase-deficient (*Ar*^{-/-}) mice. Electron microscopic examinations reveal that endocytic apparatus and tubule-cisternal endoplasmic reticulum are present in non-ciliated cells irrespective of the genotypes. However, electron-dense and acid phosphatase-negative granules and apical tubules,

which are components thought to be related to membrane recycling of endosomes, are observed only in wild-type (WT) and *Ar*^{-/-} mice. By contrast, the Golgi complex is highly developed in *Esr1*^{-/-} mice when compared with WT and *Ar*^{-/-} mice. RT-PCR analysis reveals no significant differences in the expression levels of a subset of genes involved in ion transportation. Thus, from the structural and transcriptional points of view, the efferent ductules of *Ar*^{-/-} mice are indistinguishable from those of WT mice. Moreover, data from electron microscopic examinations indicate the possible involvement of *Esr1* in the regulation of vesicle recycling processes.

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Introduction

Testicular development and the maintenance of spermatogenesis are controlled mainly by gonadotropins and androgens (Holdcraft & Braun 2004). However, besides the well-known negative effect of estrogens on the secretion of gonadotropins, they have been shown to directly regulate testicular functions (Simpson *et al.* 1994, Carreau *et al.* 2006, Ebling *et al.* 2006). Estrogens are synthesized by an enzyme complex, aromatase, through the conversion of androgens to estrogens in Sertoli cells in immature animals and in Leydig cells in adults (Rommerts *et al.* 1982, Simpson *et al.* 1994, O'Donnell *et al.* 2001). Furthermore, germ cells were proposed to be one of the major sites for estrogen synthesis in adult mouse testis (Nitta *et al.* 1993).

Estrogens are thought normally to modulate the transcription of specific genes in estrogen target tissues through binding to estrogen receptors of either α - or β -subtype (Couse & Korach 1999, Nilsson *et al.* 2001). Both subtypes have been reported to be expressed abundantly in the efferent ductules (Zhou *et al.* 2002), where a large part of testicular luminal fluid is reabsorbed to concentrate spermatozoa (Clulow *et al.* 1998, Hess 2000). *Esr1* gene knockout (*Esr1*^{-/-}) mice display dilation of the lumens of the efferent ductules, of which the diameter becomes more than double

the size of wild-type (WT) males (Hess *et al.* 1997), indicating that luminal fluid is not removed causing accumulation of fluids in the lumen of the efferent and seminiferous tubules. As *Esr2*^{-/-} mice did not display apparent morphological abnormalities in the efferent ductules (Krege *et al.* 1998, Antal *et al.* 2008) and the phenotype of *Esr1*^{-/-}*Esr2*^{-/-} mice was similar to that of *Esr1*^{-/-} mice (Couse *et al.* 1999), *Esr1* rather than *Esr2* plays a major role in normal fluid reabsorption at the efferent ductules. Nevertheless, the requirement of aromatase activity for *Esr1* to function at the ducts has not been established.

Because estrogens are synthesized by aromatase in the testis, aromatase gene knockout (*Ar*^{-/-}) mice are a useful animal model to assess the testicular function of estrogens *in vivo*. Three lines of *Ar*^{-/-} mice have been generated independently (Fisher *et al.* 1998, Honda *et al.* 1998, Toda *et al.* 2001a). However, the testicular phenotypes are variable among the lines. The *Ar*^{-/-} mice generated by us were nearly infertile and showed no disruptions in spermatogenesis until 10 months of age, although an apparent reduction in seminiferous epithelial height was observed at that age (Toda *et al.* 2001b). The *Ar*^{-/-} males generated by other group are reportedly fertile at 12–14 weeks of age and one of four *Ar*^{-/-} mice examined displayed grossly dysmorphic seminiferous tubules

Table 1 Primer pairs used for RT-PCR

	5' Primer primer	3' Primer primer	Size (bp)
Gene			
<i>Atp1a1</i>	GGGGATTGTTGGCTCTGATG	TTTGTCCGTTTGGGGTTTC	328
<i>Cfr</i>	CTGGAGGCGAAATGGTTGTC	TTGGTATGTTATGGGGTCTA	530
<i>Slc9a1</i>	GGCCAACATCTCCACAAAT	GCCTGCTTCATCTCCATCTT	651
<i>Slc9a3</i>	GGATGAAAAGCAGGACAAGG	AGGGGAGAACACGGGATTAT	343
<i>Gapdh</i>	CGGATTTGGTCGTATTGG	TCTTGAAGATGGTGATG	210

and disrupted spermatogenesis at 4·5 months of age (Robertson *et al.* 1999, 2001). This phenotypic heterogeneity in spermatogenesis between the *Ar*^{-/-} lines seems to be attributable to differences in genetic backgrounds rather than in the genomic region used for the gene inactivation, as the *neo* gene was inserted at the EcoRV site in exon 9 of *Cyp19a1* for the disruption of the gene in both *Ar*^{-/-} lines (Fisher *et al.* 1998, Toda *et al.* 2001a). We thus generated fully congenic *Ar*^{-/-} mice with a C57BL/6J background by repeated backcrossing to C57BL/6J mice. This study was conducted using *Ar*^{-/-} mice with a C57BL/6J genetic background to examine the effects of aromatase inactivation on spermatogenesis and the efferent ductules with special reference to the changes observed in the tissue sites of *Esr1*^{-/-} mice.

Materials and Methods

Experimental animals

The animal experiments were carried out according to the Guidelines of our Institutional Animal Regulations. All animals were maintained on a 12 h light:12 h darkness cycle at 22–25 °C and given water *ad libitum* and a chow, NIH-07PLD, which was developed by the National Institute of Health (USA) in order to lower phytoestrogen contents in the chow (Oriental Yeast Co., Tokyo, Japan; Yamasaki *et al.* 2002). *Cyp19a1* was disrupted by homologous recombination (Toda *et al.* 2001a) and the genetic background was unified to C57BL/6J by repeated backcrossing. *Esr1*^{+/-} mice (Lubahn *et al.* 1993) were purchased from Taconic Farms, Inc. (Hudson, NY, USA) and bred in the animal facility of Kochi University to yield *Esr1*^{+/-} mice with a C57BL/6J genetic background. *Esr1*^{-/-} mice were generated by

Table 2 Summary of spermatogenic phenotype of mice with the C57BL/6J genetic background. Testes of WT, *Ar*^{-/-}, and *Esr1*^{-/-} mice at 5 or 10 months of age were histologically examined.

	WT	<i>Ar</i> ^{-/-}	<i>Esr1</i> ^{-/-}
Age			
5 M	0 ^a /44 ^b (0%)	13/20 (65%)	14/14 (100%)
10 M	0/8 (0%)	9/13 (69%)	ND

^aNumber of mice showing spermatogenic impairment.
^bNumber of mice examined.

crossing of *Esr1*^{+/-} mice. Mice at 5 or 10 months of age were used for this study.

Histological examination

Testes and efferent ductules were fixed in a solution of 10% (v/v) buffered formalin for 24 h, dehydrated in graded ethanol, and then embedded in paraffin. Samples were cut into 3 µm thick sections and stained with hematoxylin–eosin. The luminal areas of the efferent ductules were quantified using MacScope (ver. 2.5.9) software.

Electron microscopic examination

The efferent ductules were fixed in modified Karnovsky's fixative containing 2% paraformaldehyde, 2% glutaraldehyde, and 0·05% CaCl₂ in 0·1 M cacodylate buffer (pH 7·2) for 1 h at 4 °C. The specimens were post-fixed with 1% osmium tetroxide in 0·1 M cacodylate buffer (pH 7·2) containing 0·8% potassium ferrocyanide for 2 h in the dark to preserve the structure of tubule–cisternal endoplasmic reticulum (TCER; Möller *et al.* 1983). After rinsing, the specimens were exposed to en bloc stain in 1% uranyl acetate in 0·05 M sodium maleate (pH 6·0) for 1 h in the dark. After rinsing in 0·05 M sodium maleate (pH 5·0) for 1 h, the specimens were dehydrated in a graded series of alcohol (Karnovsky 1967), and embedded in Spurr's resin. Thin sections were observed using a Hitachi H-7000H electron microscope. Acid phosphatase activity was detected by means of a cerium-based cytochemical method to identify lysosomes (Robinson *et al.* 1986).

Western blot analysis

The efferent ductules were kept in RNAlater (Ambion, Austin, TX, USA) at –20 °C until use. After removing the fat and connective tissue under a microscope, the tissues were minced using scissors and homogenized in an ice-cold homogenization buffer consisting of 50 mM Tris–HCl buffer (pH 7·4), 0·4 M NaCl, 0·1 mM EDTA, 0·1 mM EGTA, and protease inhibitor cocktail (Roche). The protein concentration of the homogenate was measured with a BCA protein assay reagent (Pierce, Rockford, IL, USA). Each sample (40 µg) was subjected to SDS–PAGE in a 10% gel. The separated polypeptides were then transblotted onto a polyvinylidene difluoride (PVDF) membrane. After treatment with blocking agent, the membrane was incubated with anti-*Esr1* antibody at a dilution of 1:2000 (MC-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

After washing, the membrane was subsequently incubated with horseradish peroxidase (HRP)-anti-rabbit antibody and the enzyme activity of HRP was detected with an ECL western blotting detection kit. After detaching the anti-*Esr1* antibody, the same membrane was reprobed with an HRP-anti- β -actin monoclonal antibody (Sigma-Aldrich, Inc). The results were quantified using Quantity One (ver. 3.0) software (PDI Inc., Huntington Station, NY, USA).

RNA preparation and RT-PCR analysis

Total RNA was extracted from pooled efferent ductules kept in RNA later as described (Zarlenga & Gamble 1987) after removing fat and connective tissue under a microscope. Total RNA (1 µg) was reverse transcribed using oligo-dT primers (Ambion) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a total volume of 25 µl per

Figure 1 Histological examination of the testes of *Ar*^{-/-} and *Esr1*^{-/-} mice with a C57BL/6J genetic background. (A) Each circle represents an individual mouse, of which both testes were examined histologically. Vertical numerals indicate the sum of the numbers of seminiferous tubules showing impaired spermatogenesis per section in each mouse examined. As no abnormality was found in the testes of WT mice with a C57BL/6J background, the data were not plotted in this figure. (B and C) Representative photographs of the testis of an *Ar*^{-/-} mouse at 5 months of age displaying various degrees of impairment in spermatogenesis. The seminiferous tubules indicated by arrowhead in (C) are judged to be impaired. (D) The image shown at higher magnification is from the area indicated by a box in (B). Scale bar, 200 μ m.

reaction, according to the manufacturer's instructions. The expression levels of genes for ATPase, Na^+/K^+ transporting, α -1 polypeptide (*Atp1a1*), cystic fibrosis transmembrane conductance regulator homolog (*Cfr*), solute carrier family 9 (sodium/hydrogen exchanger) member 1 (*Slc9a1*), and solute carrier family 9 (sodium/hydrogen exchanger) member 3 (*Slc9a3*) were examined. PCR was carried out with 1.5 μ l of the RT samples in a 30 μ l reaction volume.

Cycling parameters were as follows: an initial melting step of 94 °C for 1 min, amplification by 28 cycles at 94 °C for 30 s, 60 °C for 30 s, and 74 °C for 45 s and then a final 5 min at 74 °C for extension. The amplified DNA products were resolved on 5% polyacrylamide gels. After staining the gels with ethidium bromide, the banding pattern of amplified DNA fragments was recorded using a charged-coupled device camera (Sony Corp., Tokyo, Japan). The results were

Figure 2 Photomicrographs of the testis and efferent ductules in the distal region of two individual $Ar^{-/-}$ mice at 5 months of age. (A and B) Testis from one $Ar^{-/-}$ mouse at 5 months of age showed spermatogenic impairment and (D and E) the testis from the other $Ar^{-/-}$ mouse displayed morphologically normal spermatogenesis. (B and E) The images shown at higher magnification are from the areas indicated by boxes in (A) and (D) respectively. (C and F) Both $Ar^{-/-}$ mice reveal similar morphology in the distal efferent ductules. Scale bars, 200 μ m.

quantified using Quantity One (ver. 3.0) software. The intensity values for each transcript were normalized to the respective expression level of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and expressed as fold difference over the value of WT mice. The nucleotide sequences of primers and the sizes of amplified fragments are listed in Table 1.

Statistical analysis

Data are expressed as the means $\pm$ S.E.M. The significance of differences was analyzed using Student's *t*-test employing InStat software (GraphPad Software, Inc., San Diego, CA, USA, 2003). $P < 0.05$ was considered significant.

Results

Histological examination of the testicular phenotype of $Ar^{-/-}$ mice with a C57BL/6J genetic background

Spermatogenesis was evaluated morphologically at 5 and 10 months of age by measuring the numbers of dysmorphic seminiferous tubules per section. None of the WT males

displayed spermatogenic impairment at either age (Table 2). By contrast, variable degrees of aberrant morphological features were observed in the seminiferous tubules of $Ar^{-/-}$ mice with a fully congenic C57BL/6J genetic background at 5 months of age (Fig. 1A). Out of 20 $Ar^{-/-}$ mice, 2 displayed grossly dysmorphic seminiferous tubules and disrupted spermatogenesis, and 11 mice showed variable degrees of spermatogenic impairment (Fig. 1B–D). As reported previously (Eddy *et al.* 1996), all of the $Esr1^{-/-}$ mice examined showed spermatogenic impairment (Fig. 1A; Table 2). When analyzed at 10 months of age, 70% of $Ar^{-/-}$ mice (9 out of 13 mice) showed histologically severe impairment of spermatogenesis.

Morphology of efferent ductule of $Ar^{-/-}$ mice

The morphology of the efferent ductules of an $Ar^{-/-}$ mouse showing impairment in spermatogenesis was indistinguishable from that of an $Ar^{-/-}$ mouse showing relatively normal spermatogenesis at 5 months of age (Fig. 2). When measuring the luminal areas of the efferent ductules at the distal region, the luminal area of $Esr1^{-/-}$ mice at 5 months of age was twofold larger than that of WT mice ($Esr1^{-/-}$, $92.6 \pm 6.5 \mu\text{m}^2$; WT, $42.5 \pm 2.9 \mu\text{m}^2$; $P < 0.001$; Fig. 3), which is consistent with

Figure 3 Photomicrographs of the distal region of the efferent ductules and the luminal areas of the efferent ductules. Distal efferent ductules of (A) WT and (B) $Ar^{-/-}$ mice showed similar morphology, but those of (C) $Esr1^{-/-}$ mice were dilated. (D) The luminal area of the $Esr1^{-/-}$ efferent ductules was significantly larger than those of WT and $Ar^{-/-}$ mice. Scale bar, 200 μm .

the previous study (Hess *et al.* 1997). By contrast, no significant difference was detected between $Ar^{-/-}$ and WT mice ($Ar^{-/-}$, $41.0 \pm 2.6 \mu\text{m}^2$; WT, $42.5 \pm 2.9 \mu\text{m}^2$; Fig. 3). In addition, there was no difference in the area at 10 months of age between WT and $Ar^{-/-}$ mice (WT, $59.2 \pm 7.8 \mu\text{m}^2$; $Ar^{-/-}$, $54.5 \pm 6.6 \mu\text{m}^2$), while the area was significantly enlarged in $Esr1^{-/-}$ mice ($93.9 \pm 5.6 \mu\text{m}^2$, $P < 0.001$). Furthermore, the height of epithelial cells was similar between WT and $Ar^{-/-}$ mice at 5 months of age (data not shown), while the height was decreased in $Esr1^{-/-}$ mice (Fig. 4A and B).

Electron microscopic examinations

The lumen of the efferent ductule is covered with a single layer of columnar epithelium that is composed of ciliated and non-ciliated cells. Non-ciliated cells had many electron-dense granules at the supranuclear and basal cytoplasm in WT and $Ar^{-/-}$ but not in $Esr1^{-/-}$ mice (Fig. 4A and B). Cytochemical analysis for detecting acid phosphatase activity demonstrated that the granules were totally negative for the activity, indicating that they were not lysosomes (Fig. 4C).

Figure 4 Transmission electron micrograph analysis of the efferent ductule. Electron micrographs of the epithelium of (A, C, D, and G) WT, (E and H) $Ar^{-/-}$, and (B, F, and I) $Esr1^{-/-}$ efferent ductules. (A) Non-ciliated epithelial cells of the WT efferent ductule have many electron-dense granules and vacuolar endosomes (asterisk). (B) Non-ciliated epithelial cells of the $Esr1^{-/-}$ efferent ductule have well-developed Golgi complex (G) but do not have electron-dense granules. N and C indicate non-ciliated and ciliated epithelial cells respectively. (C) Acid phosphatase activity was detected cytochemically in epithelial cells of the WT efferent ductules. The reaction products of acid phosphatase are observed at the Golgi complex of the ciliated cells (G) and lysosomes of the non-ciliated cells (large arrow). (D) Electron-dense granules are totally negative for staining. Endosomes are indicated by asterisks. (E) The apical part of a non-ciliated cell of the WT efferent ductules reveals the presence of a well-developed apical tubule (AT) (representatives indicated by arrows), vacuolar endosomes (asterisks), intermicrovillous membrane invaginations (open arrows), and a tubule-cisternal endoplasmic reticulum (TCER) (arrowheads). An insert shows endosomes connecting to the AT. (F) The apical part of a non-ciliated cell of the $Ar^{-/-}$ efferent ductules reveals a similar ultrastructure to that of WT mice. (G) The apical part of a non-ciliated cell of the $Esr1^{-/-}$ efferent ductules reveals vacuolar endosomes (asterisks) and TCER (arrowheads). The Golgi complex (G) is well developed but the AT is not in a non-ciliated cell of the $Esr1^{-/-}$ efferent ductule. (H) The apical part of a ciliated cell of the WT efferent ductule reveals the presence of well-developed TCER (arrowheads). C indicates coated invagination. (I) The apical part of a ciliated cell of the $Ar^{-/-}$ efferent ductule reveals well-developed TCER. C indicates coated invagination. NC indicates a non-ciliated cell. Scale bars, (A–C) 1 μm and (D–I) 0.5 μm .

Three types of tubule-vesicular components were observed by transmission electron microscopy. One is a component belonging to an endocytic apparatus including intermicrovillous membrane invaginations that are decorated with clathrin coat lining over the cytoplasmic surface of the invaginating region, clathrin-coated vesicles, and vacuolar early sorting endosomes (Hatae *et al.* 1986a). This component was observed in the WT, $Ar^{-/-}$, and $Esr1^{-/-}$ efferent ductules (Fig. 4B–I), while the sizes of the invaginations and early sorting endosomes were smaller in non-ciliated cells of $Esr1^{-/-}$ mice relative to those in WT and $Ar^{-/-}$ mice (Fig. 4D–F). The second component was TCER that is well developed in ciliated cells (Fig. 4G–I) but not so prominent in non-ciliated cells (Fig. 4D–F). The third component was referred to as an apical tubule (AT), which had a cylindrical structure with a diameter ranging from 60 to 80 nm and its lumen was filled with moderately electron-dense materials (Fig. 4D and E). Furthermore, it appeared to be covered with smooth membranous materials and occasionally connected to endocytic vesicles with a diameter ranging from 140 to 200 nm (Fig. 4D, insert). The AT was present densely in the apical part of non-ciliated cells of WT and $Ar^{-/-}$ mice, but completely absent in $Esr1^{-/-}$ mice (Fig. 4D–F). Another noticeable feature was the Golgi complex, which was highly developed in the non-ciliated cells of $Esr1^{-/-}$ mice but was not prominent in those of WT and $Ar^{-/-}$ mice (Fig. 4B and F).

Western blot and RT-PCR analyses

The expression level of *Esr1* in the efferent ductules was examined by western blotting. The antibody against *Esr1* recognized a 67 kDa protein as a main band in the extracts of efferent ductules of WT and $Ar^{-/-}$ mice, but not in $Esr1^{-/-}$ mice. In addition to the band, the antibody detected several minor bands, which might represent truncated forms of *Esr1*, degradation products, or non-specific bands. Nonetheless, the patterns of the bands were essentially the same between the samples of WT and $Ar^{-/-}$ mice (Fig. 5A). Furthermore, the expression level of the main band was not different between WT and $Ar^{-/-}$ efferent ductules (Fig. 5B). These findings demonstrated that ESR1 protein was not overexpressed in $Ar^{-/-}$ efferent ductules. Suppression of gene expressions related to ion transportation including *Slc9a3* has been reported to be one of the reasons for impaired testicular fluid reabsorption at the efferent ductules in $Esr1^{-/-}$ mice (Lee *et al.* 2001, Zhou *et al.* 2001). Therefore, we examined the expression levels of a subset of genes involved in ion transportation by a semiquantitative RT-PCR procedure. As shown in Fig. 6, no significant differences were found in the expression levels of genes for *Atp1a1*, *Cjfr*, *Slc9a1*, and *Slc9a3* between WT and $Ar^{-/-}$ efferent ductules. By contrast, the expression levels of *Cjfr* and *Slc9a1* respectively increased 1.8- and 2.4-fold and those of *Slc9a3* decreased to 30% in $Esr1^{-/-}$ efferent ductules when compared with those in WT mice (Fig. 6).

Figure 5 Western blot analysis of ESR1 protein. (A) *Esr1* expression was analyzed in the efferent ductules of WT, $Ar^{-/-}$, and $Esr1^{-/-}$ mice at 5 months of age. Gels were loaded with 40 μ g protein per lane. Filters were stained with anti-*Esr1* antibody (upper panel) or with anti- β -actin antibody (lower panel). In addition to the main band (indicated by an arrow), several minor bands were detected by the antibody. (B) Bar graph illustrates fold differences in the expression level of the main band detected by anti-*Esr1* antibody, which is indicated by an arrow in (A), in comparison with that of the WT efferent ductules. Normalization of the band was done using the signals obtained with the anti- β -actin antibody.

Discussion

$Ar^{-/-}$ mice with a fully congenic C57BL/6J genetic background were infertile and displayed progressive disruption in spermatogenesis. The $Ar^{-/-}$ mice used in the previous study displayed a more moderate spermatogenic phenotype than that of the present line (Toda *et al.* 2001b). This difference might reflect an effect of estrogenic compounds in the diet as reported (Robertson *et al.* 2002), since the current mice were fed a phytoestrogen-lowered diet (NIH-07PLD) instead of a conventional chow diet (NMF; Oriental Yeast Co.) Alternatively, the current strain with a C57BL/6J genetic background might contain subsets of genes that are critical for maintaining normal spermatogenesis and are highly susceptible to aromatase inactivation. The data presented here indicate that aromatase activity is involved in maintaining spermatogenesis in mice; nevertheless, the molecular basis has not been established. It was reported that round spermatids showing apoptosis were detected more frequently in the seminiferous tubules of $Ar^{-/-}$ than WT mice (Robertson *et al.* 1999, 2001), suggesting that aromatase inactivation results in an increase in apoptosis-inducing molecules. Instead, estrogens might act as a potent inhibitor of apoptosis (Ebling *et al.* 2000, Pentikainen *et al.* 2000, Thuillier *et al.* 2003, Wang *et al.* 2004, Wahlgren *et al.* 2008), but a number of studies reported that estrogens or estrogenic compounds act as an apoptotic inducer on male germ cells (Koji 2001).

We provided experimental evidence to show that the morphological features of the efferent ductules of $Ar^{-/-}$

Figure 6 Semi-quantitative RT-PCR analysis of *Atp1a1*, *Cfr*, *Slc9a1*, and *Slc9a3* mRNA expression in the efferent ductules. Whole efferent ductules were collected from WT (W), $Ar^{-/-}$ (A), and $Esr1^{-/-}$ (E) mice at 5 months of age, and total RNA was prepared for gene expression analysis. (A) Representative gel patterns of RT-PCR analysis. (B) Bar graphs illustrate the fold difference compared with the expression level in the WT efferent ductules. Differences in the amount of template cDNA input were normalized by the signals obtained with specific primers for *Gapdh*. Data were expressed as mean $\pm$ S.E.M. * $P < 0.05$ versus WT mice.

mice are indistinguishable from those of WT mice at 5 and 10 months of age, indicating that morphometric alterations of the efferent ductules are not associated with the abnormalities of spermatogenesis caused by estrogen insufficiency. Electron microscopic examinations demonstrated that TCER is well developed in ciliated cells of the WT, $Ar^{-/-}$, and $Esr1^{-/-}$ efferent ductules. The TCER has been described to exist selectively in epithelial cells that actively transport ions (Møllgård & Rostgaard 1978, Forge 1982). RT-PCR analysis with total RNA prepared from efferent ductules, which consist of both ciliated and non-ciliated cells, demonstrated that the transcription levels of genes related to ion transportation were regulated in an aromatase-independent manner. Therefore, our findings might indicate that ions are normally transported at least in ciliated cells of WT and $Ar^{-/-}$ efferent ductules.

Membrane invagination and endocytotic tubular-vesicle apparatus are present with similar abundance in non-ciliated cells of WT, $Ar^{-/-}$, and $Esr1^{-/-}$ mice, although the sizes of

these cellular structures appeared to be smaller in $Esr1^{-/-}$ than WT and $Ar^{-/-}$ mice. These observations are somewhat inconsistent with the previous studies, in which endocytic apparatus including apical vesicles was markedly reduced in $Esr1^{-/-}$ mice (Hess *et al.* 2000). The most striking feature is the almost complete absence of the AT in the efferent ductules of $Esr1^{-/-}$ mice. The AT is frequently observed in epithelial cells of tissues such as kidney proximal tubule and visceral yolk sac (Hatae *et al.* 1986b) where fluids are actively absorbed. Although the roles of the AT have not been established, it was proposed that the components may be related to a membrane recycling process, by which intracellular membrane components are returned to the plasma membrane (Hatae *et al.* 1986b). Thus, the present findings indicate that membrane recycling activity might be compromised in the $Esr1^{-/-}$ efferent ductules leading to the low activity of fluid reabsorption. The well-developed Golgi complex observed in the $Esr1^{-/-}$ efferent ductules might reflect the aberrant activity of membrane recycling. Involvement of *Esr1*/estrogen in maintaining the apical cytoarchitecture of non-ciliated epithelial cells of the efferent ductules was pointed out previously (Zhou *et al.* 2001).

Several lines of evidence provide clues regarding the less profound effects of aromatase inactivation on the efferent ductule, namely 1) ER ligands other than estrogens might be produced endogenously in an aromatase-independent manner (Weihua *et al.* 2002, McCarthy *et al.* 2007), 2) *Esr1* is activated estrogen independently by other signaling pathways triggered by growth factors or cAMP (Julie *et al.* 2001, Aguirre *et al.* 2007). It is also of interest to speculate that exposure of $Ar^{-/-}$ mice to maternal estrogen during development in the uterus or during weaning through milk might affect the architecture of the efferent ductules or that aromatase inactivation might cause less active fluid secretion. Fluid reabsorption could be mediated by various aquaporins. Expressions of aquaporin 1, 9, and 10 were detected in the efferent ductules (Huang *et al.* 2006), of which the former two might be regulated by estrogen in the tissue (Ruz *et al.* 2006), suggesting that fluid reabsorption activity is affected by the expression levels of the aquaporins. Further studies will clarify the causative molecular events occurring in the $Ar^{-/-}$ mice, which could promote our understanding of the actions of estrogenic compounds in the male gonadal tissues.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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