Gender-specific effects of endogenous testosterone: Female α-estrogen receptor-deficient C57Bl/6J mice develop glomerulosclerosis

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Young female mice on a C57Bl/6J (B6) background are considered glomerulosclerosis (GS)-resistant but aging B6 mice develop mild GS. Estrogen deficiency accelerates while estrogen replacement retards GS in young sclerosis-prone oligosyndactyly mutant mice on an ROP background.
To explore the effects of sex hormones on glomerular structure and function in the context of gender and genetic background, we studied mice in which the estrogen-receptor (ER) genes α- or -β were deleted (α- or βER knockout (KO) and crossed into the B6 background. We also studied ovariectomized (Ovx) B6 mice given testosterone. Male and female βERKO and male αERKO mice had no glomerular dysfunction at 9 months of age; however, αERKO female mice displayed albuminuria and GS. Ovx prevented glomerular dysfunction in αERKO female mice by eliminating endogenous testosterone production while exogenous testosterone induced GS in Ovx B6 mice. Androgen receptor (AR) expression and function was found in microdissected glomeruli and cultured mesangial cells. Testosterone compared to placebo increased both AR expression and TGF-β1 mRNA levels in glomeruli isolated from female B6 mice. Estrogen deficiency had no deleterious effects on the glomeruli in B6 mice. Our study shows that genetic traits strongly influence the GS-promoting effects of estrogen deficiency while testosterone induces GS in a gender-specific manner.

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Chronic kidney disease (CKD) affects about 20 million adults and is a cause of morbidity and mortality in the United States.[1] However, the role sex hormones play in the pathogenesis of CKD remains a matter of controversy. Some clinical studies have reported a more rapid progression of CKD in men than in women while others did not find a gender difference.[2–5] In contrast, the 'ACE Inhibition in Progressive Renal Disease Study Group' recently suggested that renal disease progression may be even faster in post-menopausal women.[3] Thus, the uncertainty concerning the effects of sex hormones on the development and progression of glomerulosclerosis (GS) in the context of gender, age, and genetic traits (genetic background) persists.

Studies in experimental models of renal disease have shown the detrimental effects of estrogen deficiency and conversely the benefits of 17β-estradiol (E_2) replacement.[6–9] We previously studied female ROP Os/+ mice which spontaneously develop GS at a young age.[8] The progression of GS in female ROP Os/+ mice is accelerated and worsened by experimental diabetes and unilateral nephrectomy. Most importantly, the deterioration of glomerular function and structure in female ROP Os/+ mice is dramatically hastened by estrogen deficiency after ovariectomy (Ovx). E_2 replacement not only delays the onset but nearly eliminates the progression of glomerular disease in ROP Os/+ mice.[10] On the other hand, female C57/Bl6 (B6) mice do not develop GS in response to noxious stimuli such as genetically induced nephron reduction, unilateral nephrectomy, or experimentally induced diabetes before menopause. Furthermore, female B6 mice only develop mild glomerular lesions consisting of glomerular hypertrophy, mesangial cell (MC) proliferation, and vascular pole sclerosis at 18–20 months of age in the early phase of anestrous.[11]

In contrast to the reports on the glomerular effect of estrogens, little data are available on androgen (testosterone)/androgen receptor (AR) action in the glomerulus. Among recently published studies, Ji *et al.*[12] showed that dihydrotestosterone administration contributed to renal disease induced by renal wrap hypertension in rats. Another study in aging spontaneously hypertensive rats reported that castration

prevented glomerular injury and reduction in glomerular filtration rate.[13] Testosterone has also been shown to increase apoptosis in human male proximal tubule kidney cells by activating caspase, Fas upregulation, and FasL expression.[14] While the above mentioned publications are not all inclusive, gender-specific response(s) of the glomerulus to testosterone have not been addressed in great detail. Interestingly, testosterone production by the ovaries persists in woman after menopause thereby changing the ratio of E_2 to testosterone in favor of the latter. This phenomenon may potentially contribute to the faster progression of CKD in women after menopause.

To further explore the role of sex hormones in the development and progression of GS in the context of gender and genetics, we studied male and female mice in which the respective genes of the estrogen receptor α (αERKO) or the ERβ (βERKO) were deleted by homologous recombination. The αERKO and βERKO mice were previously backcrossed for more than 10 generations onto a B6 genetic background. Interestingly, female αERKO mice have higher E_2 and testosterone levels than female wild-type B6 mice because of the activation of the hypothalamic–pituitary–ovarian axis because of the lack of the wild-type ERα. Thus, αERKO and βERKO mice are unique animal models to elucidate the role E_2, the respective ER subtypes, and testosterone play in the glomerulus, specifically in mice, which are apparently protected against GS by their genetic traits/background. We also studied female B6 mice supplemented with testosterone for comparison. Wild-type littermates and B6 mice served as controls in our experiments.

RESULTS

Urinary albumin excretion

Intact female αERKO mice have higher urinary albumin excretion (UAE) at 9 months of age compared to littermate controls (0.030 ± 0.002 vs 0.10 ± 0.018, $P < 0.001$, Figure 1a). There was no increase in UAE in male αERKO, male and female βERKO mice and their wild-type littermates (Figure 1b.) Interestingly, the increase in UAE did not follow a linear time/UAE relationship but developed at around 7–8 months of age (data not shown). UAE did not increase after unilateral nephrectomy in αERKO mice (0.13 ± 0.03 vs 0.10 ± 0.018) or wild-type littermate controls (0.012 ± 0.001 vs 0.03 ± 0.002).

Importantly, the increase in UAE was prevented in Ovx αERKO mice to a level observed in intact wild-type mice (0.13 ± 0.005 vs 0.03 ± 0.002, $P < 0.001$, Figure 2). This could have been due to decreased testosterone levels, since UAE was increased by testosterone supplementation of Ovx B6 mice compared to wild-type littermates (0.01 ± 0.007 vs 0.09 ± 0.02, $P < 0.001$, Figure 2).

Body, kidney, and heart weight

In intact mice, body weight was increased in female ERKO mice vs female littermates (Table 1). Body weight was increased in Ovx B6 mice, regardless of whether they were supplemented with placebo or testosterone. Kidney weight was only increased in testosterone-supplemented mice.

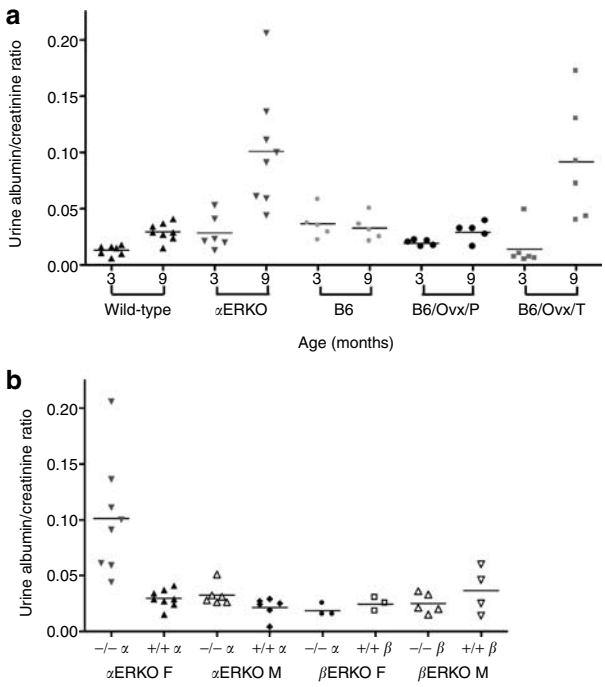

Figure 1 | Urinary albumin excretion. (**a**) Intact female αERKO mice and B6 mice substituted with testosterone (T) have albuminuria which increases with age. Urine was collected once a month for the duration of the study. Albumin/creatinine ratio (UAE) was determined as described in Materials and Methods. There was a significant increase in UAE in 9-month-old αERKO (▼) compared to 3-month-old αERKO mice and 9-month-old wild-type mice (▲, $^{**}P < 0.001$). There was no difference between 3-month-old αERKO and wild-type mice. T (■) increased UAE of Ovx B6 mice ($^{**}P < 0.001$). Each point represents an individual mouse. (**b**) UAE is increased only in Female αERKO mice. Female αERKO (−/−) had a significantly higher UAE compared to wild-type littermates (+/+), male αERKO, and male littermates ($^{**}P < 0.001$) compared to female and male βERKO and βERKO littermates ($^{*}P < 0.01$). Each point on the graph represents an individual mouse.

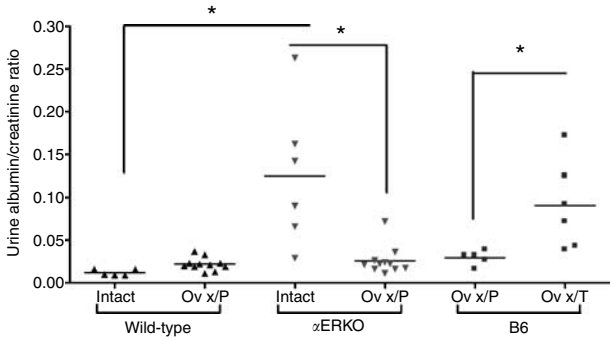

Figure 2 | Removal of testosterone by Ovx decreases UAE in 9-month-old female αERKO mice. Urine was collected once a month for the duration of the study. Albumin/creatinine ratio (UAE) was determined as described in Materials and Methods. Ovx (Ovx) reduced albumin/creatinine ratio in αERKO mice (▼, Intact vs Ovx, $^{*}P < 0.05$). Conversely Testosterone (T) treatment increased albumin/creatinine ratio compared to Ovx B6 (■, $^{*}P < 0.05$). Each point on the graph represents an individual mouse.

Table 1 | Group I (female mice without nephrectomy)

	Intact wild-type (9 months)	Intact αERKO (9 months)	Sham B6 (9 months)	B6 Ovx P (9 months)	B6 Ovx+T (9 months)
n	7	7	5	5	6
Body weight (g)	26.34±2.84	33.71±3.34***[a]	25.44±1.78	32.5±3.56**[b]	31.1±3.28**[b]
Kidney weight (g)	0.22±0.033	0.24±0.087	0.24±0.04	0.2±0.02	0.32±0.06**[c]
Kidney weight/ Body weight ratio (g)	0.008±0.0008	0.007±0.002	0.00±0.001	0.006±0.0002	0.01± 0.001
Uterus weight (g)	0.107±0.027	0.063±0.017***[d]	0.14±0.078	0.025±0.03*[e]	0.028±0.008*[e]
Serum E_2 concentrations pg/ml (±s.e.m.)	18.20±7.02	61.99±4.03	15±2.9	10.47±1.6	10.6±1.2
Serum T concentrations ng/ml (±s.e.m.)	0.18±0.03	1.56±0.17**[a]	0.16±0.03	0.08±0.02	0.34±0.09

E_2, 17β-estradiol; ERKO, estrogen receptor knockout; Ovx, ovariectomy, P, placebo; T, testosterone.
*$P<0.05$, ** $P<0.005$, *** $P<0.0005$.
[a]Vs wild-type.
[b]Vs intact B6.
[c]Vs intact b6.
[d]Vs intact wild-type.
[e]Vs intact B6.

As described previously, αERKO serum testosterone levels were approximately eight times higher than that of female littermates.[15] Testosterone supplementation of Ovx B6 mice increased testosterone to levels approximately threefold higher than those of Sham B6 mice.

Testosterone and E_2 levels were significantly decreased in Ovx αERKO mice. As expected, E_2 supplementation resulted in increased uterine weight in wild-type littermate mice but not in αERKO mice.

Histology

There was an increase in glomerular size in 9-month-old female αERKO mice compared to wild-type B6 mice and other ERKO groups (Figure 3a and b). In addition, diffuse mesangial matrix expansion was noted. Glomerular cell number was not increased and no arteriolar lesions were identified. Tubular or interstitial lesions were not present in any of the animals.

Glomerular area and volume

There was a conspicuous increase in glomerular size in αERKO mice (Figure 4). However, glomerular size was less prominent in Ovx αERKO mice (16191e+006± 1.8548e+006 vs 8988311±1223e+006, $P<0.001$). The glomerular volume of Ovx αERKO mice was similar to that of intact or Ovx wild-type or Ovx B6 mice. In contrast, testosterone supplementation increased glomerular volume in Ovx B6 mice (Figure 3, 1.0040e+006±1.1664e+006 vs 1.3735e+006±1.7987e+006, $P<0.005$). As expected, Ovx αERKO mice replaced with E_2 were not different than Ovx αERKO mice that received placebo (data not shown).

Extracellular matrix accumulation

There was an increase in laminin and collagen deposition in female αERKO mice (Figure 5b). This was less marked in Ovx αERKO mice (Figure 5d). Increased matrix deposition was also evident in B6 mice supplemented with testosterone (Figure 5f).

AR expression

Glomerular AR mRNA expression was twofold higher in female αERKO mice than intact female wild-type littermates

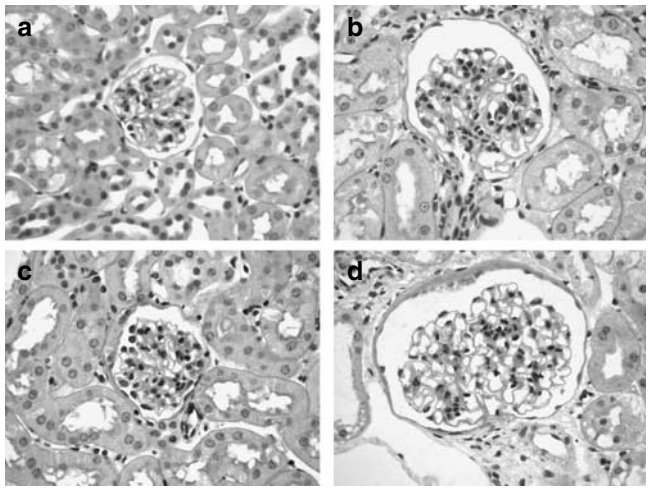

Figure 3 | Light microscopy. Methacrylate-embedded sections were stained with periodic acid-Schiff silver methanamine. (**c**) Sections from (**a**) a wild-type littermate mouse and (**b**) an intact female αERKO mouse. (**c**) Sections from an Ovx B6 mouse and a (**d**) testosterone (T)-supplemented Ovx B6 mouse. Original magnification ×400.

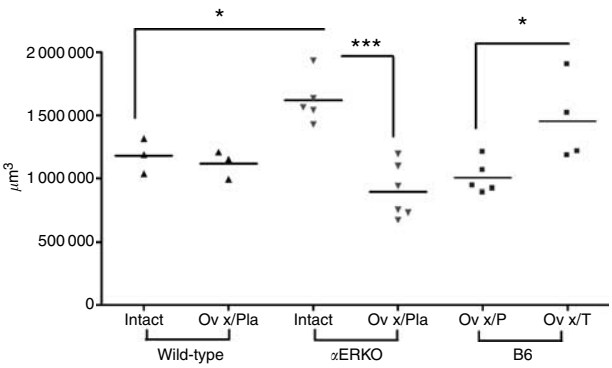

Figure 4 | Ovx reduces the expanded glomerular volume in αERKO mice. Glomerular volume was measured as described in Materials and Methods. The glomerular volume of Ovx αERKO (▼) mice is similar to that of intact or Ovx wild-type (▲) or Ovx B6 (■) mice. Testosterone (T) supplementation increases glomerular volume in Ovx B6 mice (■, *$P<0.05$, ***$P<0.005$). Each point on the graph represents individual mice.

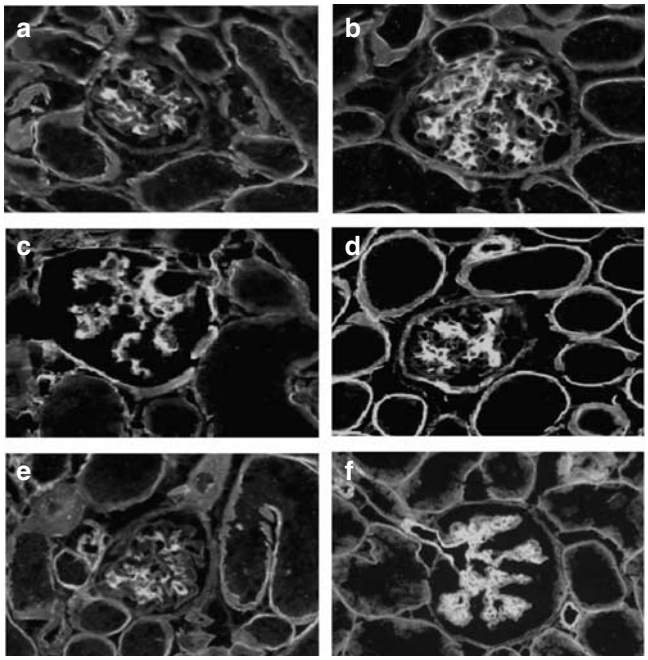

Figure 5 | Testosterone increases glomerular laminin accumulation. Immunofluorescence staining of laminin. (**a**) Wild-type littermate control, (**b**) 9-month-old αERKO female, (**c**) intact αERKO, (**d**) Ovx αERKO, (**e**) Ovx B6 mouse, and (**f**) testosterone-supplemented Ovx B6 mouse. Original magnification $\times 400$.

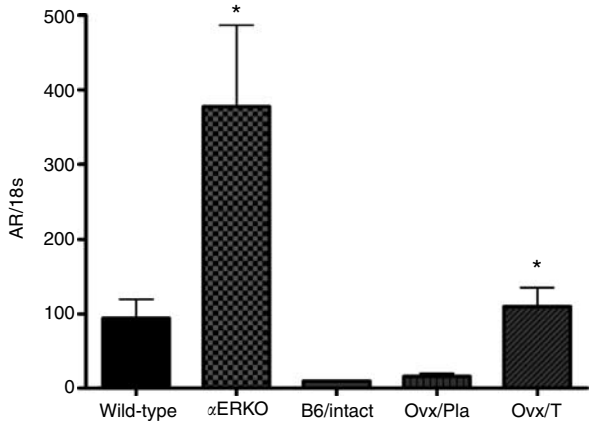

Figure 6 | Glomerular androgen (AR) mRNA expression is higher in intact αERKO than in intact B6 mice. Testosterone (T) supplementation increases AR mRNA levels in Ovx B6 mice. Real-time polymerase chain reaction was performed to assess mRNA expression of AR. Data are expressed as a ratio of AR/18s (*$P <$ 0.05), $n = 5$.

and B6 mice ($P < 0.05$, Figure 6). Testosterone treatment increased glomerular AR mRNA expression in Ovx B6 mice (Figure 6). There was no difference between female and male αERKO mice with respect to glomerular AR expression. The levels of AR mRNA were about twofold higher in the glomeruli of male αERKO mice compared to those of wild-type males (Figure 7a). The levels of AR protein expression were similar in the MCs of αERKO males and their male wild-type littermates as shown by Western analysis (Figure 7b).

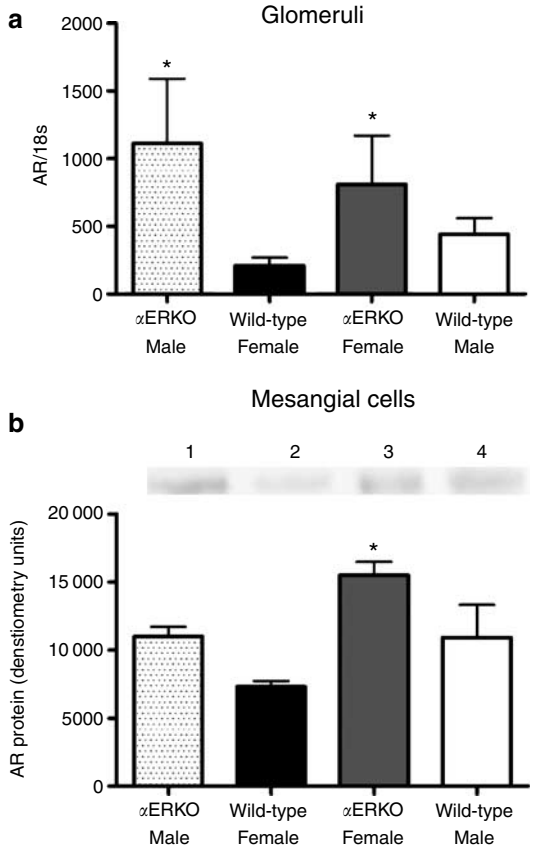

Figure 7 | Expression of glomerular AR mRNA and MC AR protein are higher in intact αERKO than in intact B6 mice. (**a**) AR mRNA expression was assessed in glomeruli by real-time reverse transcriptase-polymerase chain reaction. (**b**) AR protein was analysed in protein extracts isolated from MCs of wild-type littermates and αERKO male and female mice. (**a**) Data are expressed as the ratio of AR/18s, $n = 4$/group. *$P < 0.05$ wild-type male vs αERKO male, wild-type female vs αERKO female. (**b**). Inset depicts representative Western blot of MC AR. Protein extraction from MCs and Western blot analysis were performed as described in Materials and Methods. Lane 1: αERKO male, lane 2: wild-type female, lane 3: αERKO female, lane 4: wild-type male. Densitometry data from three experiments were graphed $\pm$ s.e.m, *$P < 0.05$ αERKO female compared to wild-type littemate female.

Androgen responsiveness of MCs

MCs isolated from female αERKO mice and wild-type littermates were transfected with a reporter gene under the transcriptional regulation of androgen response elements ($pARE_2$-tk-LUC). Testosterone increased reporter gene activity in MCs isolated from female αERKO mice (Figure 8b), but not in those from wild-type littermates (Figure 8a). The increased androgen responsiveness in female αERKO MC is, in part, mirrored by the higher glomerular AR expression (Figure 6).

Expression of glomerular ERα variants

Alternatively spliced ERα mRNA variants have been reported previously in the uterine tissue of female αERKO mice and have been termed E1 and E2.[16] We only detected the

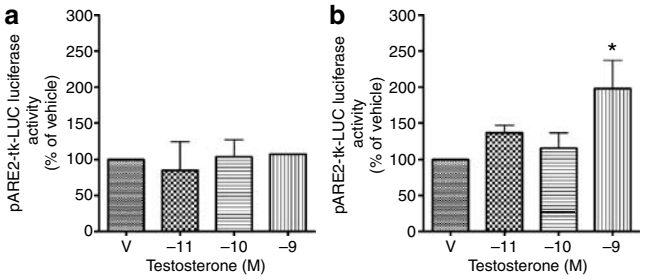

Figure 8 | MCs isolated from female αERKO mice exhibit increased androgen responsiveness. MCs isolated from (**a**) female wild-type littermates and (**b**) αERKO mice were transfected with a reporter gene under the transcriptional regulation of androgen response elements ($pARE_2$-tk-LUC). There was an increase in reporter gene activity in response to testosterone only in cells from αERKO mice (*$P<0.05$). Data are expressed as % of vehicle control. Mean ± s.e.m. of cell lysates collected from two individual cell lines obtained from each treatment group are shown. Triplicate wells were collected for each concentration of testosterone (*$P<0.05$ compared to control, $n=3$ individual collections).

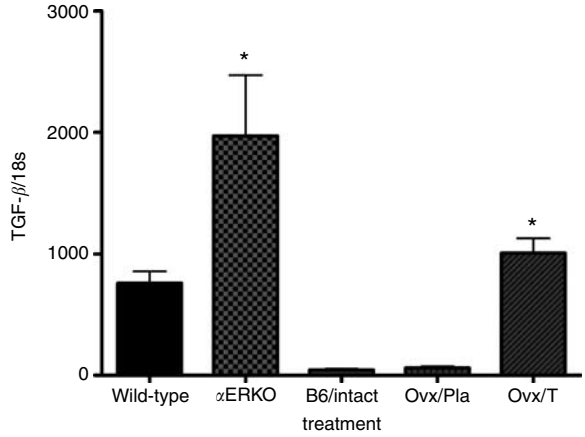

Figure 9 | The mRNA expression of TGF-β1 is higher in glomeruli isolated from intact female αERKO than in those from intact female or Ovx B6 mice (Ovx/P). Treatment with testosterone (Ovx/T) increases TGF-β1 mRNA expression in the glomeruli of Ovx B6 mice. Data are expressed as a ratio of TGF-β/18s (*$P<0.05$), $n=5$.

E2 variant in glomerular mRNA of αERKO mice, which does not translate into a mutant ER (data not shown). This is consistent with the earlier report by Couse *et al.*[17] who also did not detect the E1 variant in the brain, kidney and liver of αERKO mice.

Glomerular TGF-β1 expression

Transforming growth factor (TGF)-β signaling is widely viewed as an intracellular signaling pathway that promotes GS, especially in diabetes.[6,18–21] We and others have found that estrogen treatment decreases TGF-β1 expression in MCs and glomeruli.[9,10,22,23] Intact female αERKO have higher glomerular mRNA levels of TGF-β1 than wild-type littermates and B6 mice ($P<0.05$, Figure 9). Treatment of Ovx B6 mice with testosterone increased glomerular TGF-β1 expression to a level that was similar to that found in intact female αERKO mice (Figure 9). Ovx did not change glomerular TGF-β1 expression in B6 mice.

TGF-β1 signaling pathway

To test whether the intracellular TGF-β1 receptor/Smad signaling system was intact in MCs isolated from female αERKO and their littermate controls, we transfected cells with a TGF-β1 responsive luciferase reporter construct (p3TP-Lux). TGF-β1 increased transcription in MC isolated from both αERKO (Figure 10a) and intact wild-type mice (Figure 10b). There was no difference in the response to TGF-β1.

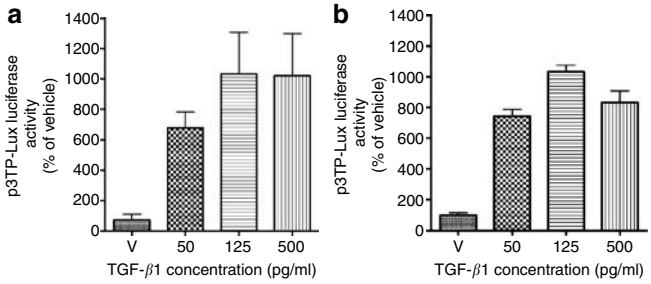

Figure 10 | TGF-β1 increased transcription in MC isolated from (a) αERKO and (b) intact wild-type mice. MC were transfected with a construct containing three smad-binding elements (p3TP-Lux, 0.3 μg/well) and cotransfected with the β-galactosidase gene (0.5 μg/well). MC were exposed to increasing concentrations of TGF-β1 (0–500 pg/ml) and collected as described in Materials and Methods. There was no difference in the response to TGF-β1. Data are expressed as % of vehicle control. Mean ± s.e.m. of cell lysates collected from two individual cell lines for each mouse treatment group are shown. Triplicate wells were collected for each concentration of TGF-β1 (*$P<0.05$, $n=2$ individual collections).

DISCUSSION

The paucity of controlled clinical studies in humans examining the role of E_2 and testosterone in the pathogenesis of GS, especially in the context of gender, age and genetic traits, is largely responsible for the controversy that still exists concerning the beneficial or unfavorable effects of sex hormones on the glomerulus. We previously showed that the glomerulus is an estrogen-target tissue and that estrogens via the ERα regulate genes, especially those involved in extra cellular matrix turnover, in a manner that is protective against GS.[24,25] We subsequently reported that estrogen deficiency accelerated the progression[8] and E_2-replacement retarded the development of GS in Ovx sclerosis-prone female ROP Os/+ mice.[10] In contrast, female B6 mice are sclerosis-resistant at a young age and only develop a mild form of GS at 15–20 months of age in early anestrus, that is rodent menopause.[11] These findings raised the questions whether estrogen deficiency has a detrimental effect on the glomerulus and whether testosterone promotes GS.

Thus, to examine the effects of estrogens and the distinct role of ERα and ERβ in the protection against GS in sclerosis-resistant mice, we first studied intact and Ovx female B6 mice as well as αERKO and βERKO, all on a B6 background, and their wild-type littermate controls. We also examined male

αERKO and βERKO mice to address potential gender-related differences in GS in response to ER deletions.

Intact female and Ovx B6 mice did not develop microalbuminuria, an early and sensitive marker of glomerular damage, over the 6-month study period. Similarly, UAE was normal in male αERKO and female and male βERKO mice at 9 months of age. This suggested that neither systemic estrogen deficiency over a 6-month period in young 9-month-old B6 mice nor the absence of functional ERα in male B6 (αERKO) nor the lack of ERβ in female and male B6 (βERKO) mice affected UAE.

On the contrary, intact female αERKO mice displayed increased UAE, increased glomerular size, and mesangial laminin accumulation at 9 months of age. The morphological changes, consistent with GS, correlated well with the albuminuria observed in intact female αERKO mice. However, these contrasting observations, such as the absence of UAE or glomerular pathology in estrogen-deficient B6 mice vs the pronounced glomerular dysfunction and histological changes in intact female αERKO mice, suggested an indirect rather than a direct effect of the lack of ERα on the glomerulus. In fact, disruption of the estrogen/ERα-mediated negative feed-back on the hypothalamic–pituitary–ovarian axis results in luteinizing hormone-driven ovarian hyperstimulation ultimately leading to increased gonadal synthesis of E_2 and testosterone in intact female αERKO mice.[15] Ectopic expression of the Hsd17b3 gene, which encodes the enzyme 17β-HSD III, is caused by chronic luteinizing hormone elevation and is responsible for the extraordinarily increased capacity of the αERKO ovaries to synthesize testosterone via conversion of androstendione.[17] Accordingly, the ovaries of intact female αERKO mice show large, hemorrhagic, and cystic follicles. As a result, serum testosterone concentrations rise in female αERKO mice to levels that are about eight times higher than those in female littermates. By comparison, female αERKO testosterone levels are only 1/3 of those in male wild-type littermates and about 1/5 of those of male αERKO mice, respectively.[15] Although endogenous E_2 levels are also elevated in intact female αERKO, glomerular estrogen action is impaired because of the absence of a functional wild-type ERα. Furthermore, male αERKO did not develop GS despite their higher testosterone levels compared to female αERKO mice and male wild-type littermates. These phenomena were observed despite similar levels of glomerular AR mRNA and mesangial AR protein expression in male and female αERKO mice. Absence of glomerular scarring in αERKO males was also observed despite the presence of about twofold higher glomerular AR mRNA concentrations and similar mesangial AR protein levels in αERKO males compared to wild-type males. These findings underscore the gender-specific effects of sex hormones on the glomerulus.

Taken together, these data suggested that endogenous testosterone, via direct glomerular androgen action, induced GS in female B6 mice which are resistant to the development of GS before anestrous (rodent menopause) in response to genetically induced nephron reduction, unilateral nephrectomy, or experimentally induced diabetes. To test the hypothesis that androgens induce GS in female B6 mice, we treated Ovx B6 mice with testosterone using subcutaneously implanted pellets. As predicted, Ovx B6 mice developed microalbuminuria and GS similar to what was observed in intact female αERKO mice. Reciprocally, Ovx of αERKO mice, which were simultaneously and unilaterally nephrectomized (Nx) as an additional renal injury, did not develop increased UAE or GS.

Expression of AR mRNA and protein was assessed in glomeruli and MCs by real-time reverse transcriptase-polymerase chain reaction and Western analysis. The functional activity of the AR in MCs was shown by T-testosterone mediated stimulation of a transfected androgen responsive luciferase-based reporter gene. These studies confirmed that the glomerulus is a direct target tissue for testosterone by demonstrating, for the first time to our knowledge, the expression of functional AR in glomerular cells. In female mice, AR expression in glomeruli and MCs was highest in intact αERKO compared to their wild-type littermates. Testosterone replacement of Ovx B6 mice increased glomerular AR expression. This suggests that glomerular AR expression in female mice is positively regulated by androgens, similar to ER expression in glomeruli.[24]

Interestingly, sex steroids have been previously shown to positively regulate AR in reproductive as well as in non-reproductive tissues other than the glomerulus. For instance, testosterone upregulates AR gene expression in primate ovaries, where AR are most abundant in the granulosa cells of healthy, growing follicles.[26] Bilateral orchiectomy caused a severe loss of both AR and ER staining in male excurrent ducts of goats.[27] On the contrary, testosterone increased AR expression in the Harderian glands of male hamsters[28] and Pelletier *et al.*[29] reported that estrogens exert positive regulation of AR mRNA expression in the uterus and vagina of adult mice.

Estrogen replacement of Ovx/Nx αERKO mice had no impact on UAE and was similar to that observed in placebo-treated Ovx/Nx αERKO mice. Thus, ERβ activation in female αERKO mice via elevated endogenous E_2 levels had no adverse effects or beneficial effects on glomerular function.

Involvement of the TGF-β1 system was also demonstrated. TGF-β1, a pleiotropic cytokine, is generally considered a progression factor for GS because of increased expression or activation of the TGF-β1 system in virtually every type of CKD in humans as well as in experimental kidney injury models.[8,10,30,31] Activation of the TGF-β1/Smad signaling pathway regulates glomerular gene expression in a manner that results in extra cellular matrix accumulation especially in the mesangium.[6,8,10,31–39] TGF-β1 expression was higher in the glomeruli of female αERKO mice than in those of wild-type littermates. The Ovx-induced fall in the elevated serum testosterone levels in female αERKO mice was associated with lower glomerular TGF-β1 mRNA levels. Conversely, testosterone supplementation increased glomerular TGF-β1

mRNA expression in female B6 mice. This appears to be in direct contrast to other androgen-sensitive organs. For example, in the prostate androgens suppress TGF-β1, most probably through direct binding of AR to Smad3. These data, in the context of previous studies, provide important evidence for the complex regulation of glomerular TGF-β1 expression by sex hormones. On the one hand, estrogen deficiency increased glomerular TGF-β1 mRNA expression in Ovx sclerosis-prone ROP Os/+ mice, a phenomenon that is reversed by E_2 supplementation.[8,10] In contrast, testosterone increases TGF-β1 mRNA levels in the glomeruli of female B6 mice. As the TGF-β1/Smad signaling pathway is functional and remains intact in the absence or presence of sex steroids in MCs, sex hormone regulation of TGF-β1 expression appears to be a crucial step in regulating the activation of the prosclerotic TGF-β1/Smad signaling pathway.

In summary, these data demonstrate a unique and gender-specific susceptibility to testosterone that is responsible for inducing GS in young female B6 mice which are resistant to GS in response to other pathological stimuli. The sclerosis-inducing effects of testosterone may be self-perpetuating via upregulation of AR in glomeruli and MCs and are potentially mediated via activation of the prosclerotic TGF-β1 signaling cascade. If such data are confirmed in humans, it may inject a note of caution against testosterone replacement in postmenopausal woman especially those with existing CKD. Furthermore, this study in conjunction with previous reports from our laboratory demonstrates the different, even contrasting, effects of sex hormones on the glomerulus.[8,10,24,40] These data expose the difficulties to draw conclusions on the effect of sex hormones on the glomerulus without knowledge of the specific genetic background of the individuals studied.

MATERIALS AND METHODS

ERKO mice: a murine model to study effects of sex hormones on the development and progression of GS

ERKO mice are transgenic mice engineered by homologous recombination of a neomycin-cassette leading to disruption of the ERα or ERβ gene, respectively.[17] Female αERKO have higher circulating E_2 and testosterone and male αERKO mice display elevated testosterone levels compared to wild-type littermates, respectively. Their progesterone and follicle-stimulating hormone levels remain in the normal range. In contrast, male and female βERKO mice have luteinizing hormone, E_2, and testosterone levels similar to those of wild-type mice.

Animals

Male and female ERKO mice and their wild-type littermates were obtained from Taconic farms (Germantown, NY). B6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Group 1: sham male and female ERKO mice which were aged and female sham B6 mice. B6 mice were Ovx at 3 months of age using the previously described procedure approved by the Committee For Animal Safety at the University of Miami School of Medicine[8] and replaced with 90-day time-release pellets either with placebo or testosterone pellets (12.5 mg/pellet) (Table 1). Group 2: female αERKO mice were either Nx or Nx and Ovx (Nx/Ovx) at 3–4 months of age. E_2 or placebo was administered to the mice via 90-day time-release pellets (Innovative Research of America, Sarasota, FL, USA) (Table 2). The E_2 pellets contained 0.05 mg/pellet, which maintained E_2 blood levels at 50–75 pg/ml. The 3 mm pellets were implanted subcutaneously into the back of the animals using a sterile trochar and forceps. These pellets were replaced every 90 days during the course of the experiment.

Animal killing

Mice were allowed free food and water and were killed at either 3 or 9 months of age. As described previously, the left kidney was perfused with a buffer solution containing collagenase and RNAse inhibitors for microdissection of glomeruli. The right kidney was perfused *in situ* with 6 ml of phosphate-buffered saline and 3 ml of 4% paraformaldehyde, post-fixed in 4% paraformaldehyde solution for at least 12 h and embedded in methacrylate. Sections, which were 4 μm thick, were stained with periodic acid–Schiff stain. Other kidney fragments were immediately frozen in OCT.

Measurements of urinary albumin and creatinine

Urine samples were collected once a month and at the time of killing. Albumin excretion was measured by enzyme-linked immunosorbent assay (Bethyl, Houston, TX, USA), corrected for creatinine concentration in the urine (kit using the Jaffe method, Stanbio, San Antonio, TX) and expressed as the urinary albumin/creatinine excretion ratio (UAE).

Measurements of E_2 and T

Serum E_2 and testosterone concentrations were measured in each group by a competitive enzyme immunoassay kit (Active Estradiol EIA kit DSL-10-4300 or Testosterone EIA kit DSL-10-4000; Diagnostic Systems Laboratories Inc,. Webster, TX).

Morphometry

A morphometric approach was employed to quantify the degree of GS.[41] Fifty cortical glomeruli, randomly selected from each mouse,

Table 2 | Group 2 (female mice with nephrectomy)

n	Wild-type 5	αERKO 4	Wild-type Ovx/P 12	Wild-type Ovx/ 17β-estradiol (E_2) 5	αERKO Ovx/P 10	αERKO Ovx/E_2 10
Body weight (g)	26.44±3.43	37.83±2.98**[a]	27.02±3.91	26.76±2.62	28.58±4.4	25.31±1.40
Uterus weight (g)	0.15±0.002	0.06±0.015	0.02±0.003	0.08±0.09*[c]	0.02±0.01	0.02±0.005
Serum E_2 concentrations (pg/ml±s.e.m.)	20±0.89**[b]	63±4.0	12.9± 0.57	15.88±2.9	10.59±1.4**[b]	27.04±9.5**[b]
Serum T concentrations (ng/ml±s.e.m.)	0.08±0.03**[b]	3.5±1.1	0.16±0.03	0.22±0.07	0.14±0.02**[b]	0.19±0.02**[b]

E_2, 17β-estradiol; ERKO, estrogen receptor knockout; Ovx, ovariectomy, P, placebo; T, testosterone.
[a]**$P<0.001$ a compared to all groups.
[b]**$P<0.001$ compared to ERKO intact.
[c]*$P<0.01$ compared to wild-type Ovx/P.

were recorded with an Olympus BH-2 microscope and Micro Image A209RGB color video camera. Glomerular volume (μm^3) and mesangial area (μm^2) were measured using MetaMorph 4.5.4 Imaging System computer program (Universal Imaging Corporation, West Chester, PA).

Immunofluorescence staining

Cryostat sections of 4-μm thickness were exposed to either rabbit anti-mouse collagen type IV (Biodesign, Saco, ME), rabbit anti-mouse laminin (Research Diagnostics, Flanders, NJ, USA), IgG, and IgM followed by goat anti-rabbit-conjugated fluorescein–isothiocyanate. The sections were examined and graded on a scale of 0–4 by a renal pathologist blinded to the origin of the kidney slides.

Isolation of RNA and quantitative analysis of RNA expression by real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from 100 glomeruli, microdissected from each animal, using the guanidinium thiocyanate–phenol–chloroform method as described.[24] Amplification and quantification of target RNAs were performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA).[25] TaqMan probes and primers for amplification of the specific transcripts were designed using the Primer Express 1.5 from Applied Biosystems. Primer sequences were identified with T_m values 10°C less than the probe. The TaqMan probes were labeled with the reporter dyes FAM, VIC, or TET at the 5′ end and with the quencher dye TAMRA at the 3′ end. The primers and probes for TGF-β1, AR, and 18s (endogenous controls) were synthesized commercially (Applied Biosystems, ABI Primer & Probes, Foster City, CA, USA) TaqMan.

Cell culture

Glomeruli were microdissected and outgrowing MCs were isolated propagated and identified as described previously.[24] Cell culture experiments were performed on cells between passages 3 and 10. All experiments were performed on two independent cell lines/group. Cells were maintained for experiments in phenol red-free Dulbecco's modified Eagle's medium F12 containing 20% charcoal-stripped serum as described previously.[24]

Western analysis

Protein (150 μg) was incubated overnight with AR antiserum and protein A-agarose. The immunoprecipitates were washed four times in phosphate-buffered saline and resuspended in 40 μl of phosphate-buffered saline. Western analysis was performed as described previously to determine the presence of AR protein.[24]

Determination of glomerular ERα variants in αERKO mice

The expression of potential ERα variants in the glomerulus of αERKO mice was determined by a polymerase chain reaction-based method as described previously.[17]

Transfection studies

MC were transfected with either a construct containing three Smad-binding elements (p3TP-Lux (0.3 μg/well), a gift from J Massague, Sloan Kettering, NY) or an androgen response element ($pARE_2$-tk-LUC (0.5 μg/well), a kind gift from Professor Janne, University of Helsinki, Finland) as described previously.[10,22]

Statistical analysis

All values were expressed as mean ± s.e.m. Significance of differences between experimental groups was determined by analysis of variance. The two-tailed unpaired Student's *t*-test was used to evaluate differences between means of corresponding sets of data obtained from 3- and 9-month-old mice.

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