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Differential Effects of Continuous and Intermittent 17β-Estradiol Replacement and Tamoxifen Therapy on the Prevention of Glomerulosclerosis

Modulation of the Mesangial Cell Phenotype in Vivo

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Female ROP Os/+ mice are partially protected by endogenous estrogens against progressive glomerulosclerosis (GS) during their reproductive period; however, ovariectomy accelerates GS progression. We examined the effects of continuous and intermittent 17β-estradiol (E_2) replacement and tamoxifen therapy on the development of GS in ovariectomized (Ovx) ROP Os/+ mice. Continuous E_2 replacement (CE_2) throughout 9 months prevented microalbuminuria and excess extracellular matrix accumulation in Ovx ROP Os/+, not only compared to placebo-treated Ovx mice but also in comparison to intact female ROP Os/+. Tamoxifen had a similar effect, but of lesser magnitude. Intermittent 3-month on-off-on E_2 did not reduce the kidney changes. Mesangial cells (MCs) from CE_2 mice maintained their estrogen responsiveness. E_2 *in vitro* prevented transforming growth factor-β1 stimulation of a Smad-responsive reporter construct and increased MMP-2 expression and activity in MCs isolated from CE_2 mice. MCs from mice on either placebo or intermittent E_2 treatment did not respond to added E_2, consistent with a stable alteration of their estrogen responsiveness. Tamoxifen protection against GS was less pronounced in ROP Os/+ mice. Thus, prolonged estrogen deficiency promotes GS and renders MCs insensitive to subsequent estrogen treatment. This underscores the importance of continuous estrogen exposure for maintaining glomerular function and structure in females susceptible to progressive GS. *(Am J Pathol 2006, 169:351–361; DOI: 10.2353/ajpath.2006.051255)*

The glomerulus is a direct estrogen target tissue that predominantly expresses the estrogen receptor (ER) subtype α.[1] Estrogens regulate genes involved in extracellular matrix (ECM) turnover in a manner leading to the prevention of the accumulation of ECM in the mesangium in mouse strains that do not develop glomerulosclerosis (GS) in response to renal injuries such as nephron reduction or experimentally induced diabetes (sclerosis-resistant mouse models).[1–5] In comparison, the level of glomerular ER expression is inversely correlated with the susceptibility to GS among different mouse strains.[3] Furthermore, in other rodent models estrogen replacement therapy has also been shown to decrease glomerular damage in uninephrectomized, spontaneously hypertensive rats, to attenuate GS in aging Dahl salt-sensitive rats, and to normalize the decreased renal functional reserve in ovariectomized (Ovx) Wistar rats.[6–8]

Female ROP Os/+ mice develop GS during their reproductive period of life and are, therefore, considered sclerosis-prone.[9] However, estrogen deficiency, induced by ovariectomy, accelerates glomerular dysfunction and scarring in young ROP Os/+ mice.[9] This suggests that endogenous estrogens only partially protect this mouse strain from the development of GS. Thus, female ROP Os/+ mice represent a model of progressive GS that specifically resembles those women who develop chronic kidney disease (CKD) in midlife with rapidly deteriorating kidney function after the onset of menopause.

To address the question of the efficacy of estrogen replacement in this model of progressive GS, we examined the effects of continuous 17β-estradiol (E_2) replacement throughout a 9-month period in ROP Os/+ mice directly initiated after ovariectomy at 3 months of age. In

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another group of mice, E_2 replacement was interrupted after 3 months of estrogen therapy and reinstated after a 3-month estrogen-deficient period. This part of our study was conducted to determine whether the time of initiation and the duration of estrogen replacement therapy were crucial for preventing the progression of GS. Because intact female ROP Os/+ mice already have mild glomerular lesions at 3 months of age, the animals on the interrupted replacement schedule received E_2 for the first 3 months after ovariectomy to allow for a potential improvement of glomerular lesions and recovery of glomerular function before estrogen withdrawal. We also asked whether estrogen replacement and/or deficiency modulated the phenotype of mesangial cells (MCs), which play an important role in the development of GS.[10–12] We further expanded our studies to examine the effects of tamoxifen, a selective estrogen receptor modulator (SERM), on the progression of GS because this medication is taken by an increasing number of women for the prevention and treatment of breast cancer.

We found that continuous estrogen exposure preserved glomerular function and structure. The mechanism may be that an estrogen-sensitive phenotype was maintained in MCs isolated from these sclerosis-prone mice. Tamoxifen also decreased albuminuria and reduced glomerular ECM expansion, although to a lesser magnitude than that of continuous E_2. On the contrary, chronic lack of estrogens or interruption of estrogen exposure for an extended period of time appeared to alter the MC phenotype to that of a sclerosis-permissive and/or inducing mode that was not reversible by subsequent estrogen treatment.

Materials and Methods

Experimental Design

Female ROP Os/+ (ROP/Le-*Os* Es^{1b}/+ Es^{1a}) mice obtained from Jackson Laboratories (Bar Harbor, ME) were Ovx at 3 months of age using a previously described procedure that was approved by the committee for animal safety of the Leonard M. Miller School of Medicine at the University of Miami.[9] The mice received E_2, tamoxifen (Tam), or placebo via 90-day time-release pellets (E_2, 0.05 mg/pellet; Tam, 0.5 mg/pellet) (Innovative Research of America, Sarasota, FL). The 3-mm pellets were implanted subcutaneously into the back of the animals using a sterile trochar and forceps. Pellets were replaced every 90 days during the course of the experiment. Animals were fed a soy-free diet to avoid potential ER stimulation by exogenous phytoestrogens. Mice were allowed free food and water for 9 months and were sacrificed at 12 months of age, as previously described.[9] One group of mice was given E_2 continuously throughout the entire experiment (CE_2), and another group of mice was given E_2 in an intermittent manner consisting of 3 months on-off-on periods (IE_2, Figure 1).

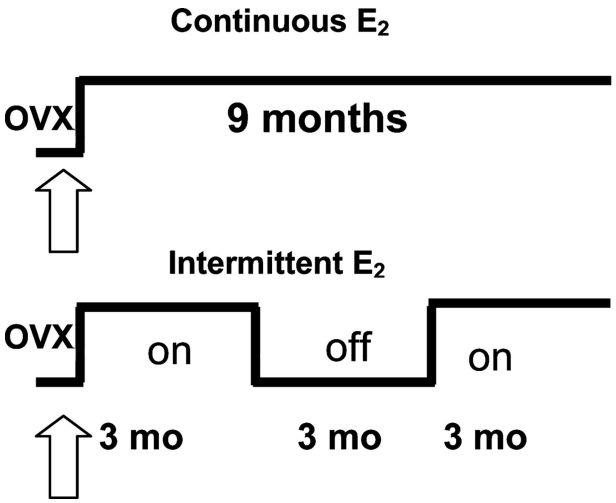

Figure 1. Treatment scheme for mice receiving E_2. **Arrow** indicates time of ovariectomy (Ovx). **Top** of figure denotes 9-month continuous treatment with E_2 and **bottom** denotes 9-month intermittent treatment with E_2.

Tissue Preparation

Briefly, the left kidney was perfused with a buffer solution containing collagenase and RNase inhibitors for microdissection of the glomeruli. The right kidney was fixed *in situ* with 4% paraformaldehyde and postfixed in 4% paraformaldehyde solution for at least 12 hours then embedded in methacrylate. Four-μm-thick sections were stained with periodic acid-Schiff. Other kidney fragments were immediately frozen in OCT.

Measurement of E_2

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

Urine Albumin and Creatinine

Freshly voided spot urine samples were collected once a month and at the time of sacrifice. Urinary albumin was measured by enzyme-linked immunosorbent assay (ELISA) (Bethyl, Houston, TX). Urine creatinine concentrations (Stanbio, San Antonio, TX) were measured in the same urine sample, and the urinary albumin excretion (UAE) rate was expressed as the ratio of albumin to creatinine.

Morphometry

All cortical glomeruli (~50) in a section of methacrylate-embedded kidney tissue were recorded with an Olympus U-SDO2 microscope and MicroPublisher 3.3 RTV photocamera. Glomerular volume (μm^3) and percent measurement of patent capillary lumina percentage of vascular space, as a surrogate for the estimation of sclerosis, were measured using the MetaMorph 6.2 Imaging System

computer program (Universal Imaging Corp., West Chester, PA).

Immunofluorescence Staining

Approximately 4-μm cryostat sections were exposed to either rabbit anti-mouse collagen type IV (Biodesign, Saco, ME) or rabbit anti-mouse laminin (Research Diagnostics, Flanders, NJ) followed by fluorescein-isothiocyanate-conjugated goat anti-rabbit antibody. The sections were examined by a renal pathologist blinded to the origin of the kidney slides.

Cell Culture

After microdissection of the glomeruli, outgrowing MCs were isolated and propagated as previously described.[13] MCs were identified by their morphology and positive staining with an antibody to α-smooth muscle cell actin. Cell culture experiments were performed on cells between passages 3 to 10. All experiments were performed on two independent cell lines/group derived from individual animals. Cells were maintained during the experiments in phenol red-free Dulbecco's modified Eagle's medium:F12 containing 20% charcoal-stripped serum, as previously described.[1]

Transforming Growth Factor (TGF)-β1 ELISA and Real-Time Polymerase Chain Reaction (PCR)

TGF-β1 protein was measured in the supernatants of MCs with a TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN). MCs were plated in 24-well plates (35,000 cells/well) and maintained in phenol red-free medium containing 20% estrogen free serum, 3 days before the start of the experiment. On day 0, cells were placed in phenol red-free medium containing 0.1% bovine serum albumin and collected after 48 hours. On the day of collection, supernatants were centrifuged to remove cell debris. MCs in the cell layers were counted with a Z Coulter counter (Beckman Coulter, Inc., Fullerton, CA). Real-time reverse transcriptase (RT)-PCR (Applied Biosystems, Foster City, CA) was performed on isolated glomeruli as previously described.[9]

Transfection Studies

MCs were plated as for ELISA and transfected in phenol red-free medium containing 10% charcoal-stripped serum with 0.3 μg/well of p3TP-Lux, a luciferase-based reporter construct containing three Smad-binding elements in its promoter region (a gift from J. Massague, Memorial Sloan Kettering, New York, NY) or 0.5 μg/well of a human MMP-2-promoter-luciferase reporter gene construct (kindly provided by Dr. Hugh Watkins, John Radcliffe Hospital, Oxford, UK). To control for transfection efficiency, MCs were co-transfected with the β-galactosidase gene (0.5 μg/well). After transfection, MCs were treated with E_2 (0 to 10 nmol/L) or recombinant TGF-β1 (0 to 500 pg/ml) and collected 48 hours later. Luciferase and β-gal were measured as previously described.[3]

ER-α Western Analysis

Cell lysates were extracted from MCs isolated from mice continuously treated with E_2 and from those on placebo. Western analysis of ER-α was performed as previously described.[1]

Zymography

Gelatin zymography was performed as previously described.[9] Briefly, 100,000 cells were plated into each well of a 24-well plate and maintained in phenol red-free medium containing 0.1% bovine serum albumin for 24 hours. After treatment with either vehicle (V), E_2 (0.1 and 1 nmol/L), the complete ER antagonist ICI 182,780 (ICI,1 μmol/L), or ICI (1 μmol/L) + E_2 (1 nmol/L) for 24 hours, cell supernatants were collected, and cell layers counted. Cell supernatants were applied to a sodium dodecyl sulfate-polyacrylamide gel co-polymerized with 10% gelatin. Gels were rinsed twice after electrophoresis in 2.5% Triton X-100 and then incubated for 18 hours at 37°C in incubation buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 0.05% NaN3). The gelatin gels were stained with Coomassie brilliant blue and destained with10% acetic acid and 10% isopropanol and dried. Densitometry was performed using NIH image J (version 1.34).

Statistical Analysis

All *in vitro* experiments were performed in triplicate, and triplicate wells were collected. All values were expressed as mean ± SEM. One-way analysis of variance and Tukey's multiple comparison post hoc tests were performed for the statistical analysis (GraphPad Prism, San Diego, CA). A value of $P < 0.05$ was considered significant.

Results

Body, Kidney, and Uterus Weight and Serum Estrogen Levels

Ovx ROP Os/+ mice on either continuous or intermittent E_2 replacement, tamoxifen, or placebo were sacrificed at 12 months of age (Table 1). Body and kidney weight did not differ among the groups except for Ovx mice on continuous E_2 replacement (CE_2). As expected, circulating E_2 concentrations were very low in the Ovx mice on placebo (5.35 ± 1.4 pg/ml). Serum E_2 levels were 20.39 ± 6.2 pg/ml in mice receiving continuous E_2 and 29.82 ± 7.9 pg/ml in animals on the intermittent E_2 replacement schedule at the time of sacrifice. Uterine weight was lower in mice on placebo compared to animals on continuous or intermittent E_2 therapy (Table 1,

Table 1. Body, Kidney, and Uterus Weight, and Serum Estrogen Levels

Treatment	*N*	Body weight (g)	Kidney weight (g)	Kidney weight/ body weight ($\times 10^3$)	E_2 (pg/ml)	Uterine weight (mg wet weight)
Sham	5	24.7 ± 0.1	0.13 ± 0.01	4.3 ± 0.2	12.32 ± 4.0	0.25 ± 0.03†
Placebo	6	25.88 ± 1.9	0.13 ± 0.03	5.2 ± 0.8	5.35 ± 1.6	0.09 ± 0.02
CE_2	6	24.3 ± 2.2	0.16 ± 0.02*	6.5 ± 0.5*	20.39 ± 6.8*	0.15 ± 0.05*
IE_2	5	24.8 ± 2.6	0.14 ± 0.02	5.6 ± 0.8	29.82 ± 7.9†	0.17 ± 0.02*
Tam	5	24.32 ± 1.3	0.11 ± 0.01	4.5 ± 0.3	ND	0.04 ± 0.02

CE_2, continuous 17β-estradiol treatment; IE_2, intermittent 17β-estradiol treatment; Tam, tamoxifen; ND, not done.
*$P < 0.05$, $^{\dagger}P < 0.005$ compared to placebo.

$P < 0.05$) and similar to the tamoxifen-treated group. There was no difference in uterine weight between mice receiving continuous or intermittent E_2 replacement at the time of sacrifice when animals on the intermittent E_2 treatment schedule had been restarted and had received E_2 for the last 3 months.

UAE

UAE was determined in all groups of mice once a month throughout the study and at the time of sacrifice (Figure 2, A and B). After the initial decline of albuminuria, which had been observed during the first 3 months of E_2 replacement (Figure 1A; UAE 3 versus 6 months, 0.14 ± 0.011 versus 0.10 ± 0.02), UAE increased in the mice on the intermittent E_2 replacement schedule. This coincided with the removal of E_2 during the second phase of the treatment schedule during 6 to 9 months of age (0.10 ± 0.02 versus 0.25 ± 0.04). UAE of these mice continued to increase despite the reintroduction of E_2 during the third phase, of the on-off-on schedule, initiated at 9 months of age (0.25 ± 0.04 versus 0.36 ± 0.058, 9 months versus12 months of age *$P < 0.05$). In contrast those mice on CE_2 and tamoxifen treatment had an initial decline in UAE at 4 months of age that was maintained throughout the entire treatment period.

As previously reported,[9] Ovx mice on placebo had 2.5-fold higher UAE than intact sham-operated ROP Os/+ mice at sacrifice (Figure 2B; 0.41 ± 0.04 versus 0.17 ± 0.01, $P < 0.005$). At the time of sacrifice, Ovx mice on CE_2 had lower UAE than placebo-treated animals (0.01 ± 0.02 versus 0.41 ± 0.05, $P < 0.0005$) and, importantly, less albuminuria than intact sham-operated ROP Os/+ mice (0.01 ± 0.02 versus 0.17 ± 0.01, $P < 0.05$). There was no difference in UAE between placebo-treated mice and animals on the intermittent E_2 replacement schedule at the time of sacrifice (0.41 ± 0.04 versus 0.38 ± 0.07). Tamoxifen-treated Ovx ROP Os/+ mice had lower UAE than animals on placebo (0.15 ± 0.02 versus 0.40 ± 0.05, $P < 0.005$). UAE did not differ between Ovx mice on Tamoxifen and intact sham-operated ROP Os/+ mice (0.15 ± 0.02 versus 0.17 ± 0.01).

Histology

The glomeruli of Ovx ROP Os/+ mice on placebo and on the intermittent E_2 replacement schedule were enlarged and showed prominent glomerular lesions represented by mesangial matrix expansion and segmental sclerosis. In contrast, the glomeruli of mice that had received CE_2 or Tamoxifen therapy were smaller than those of the placebo group and had less mesangial matrix deposition (Figure 3).

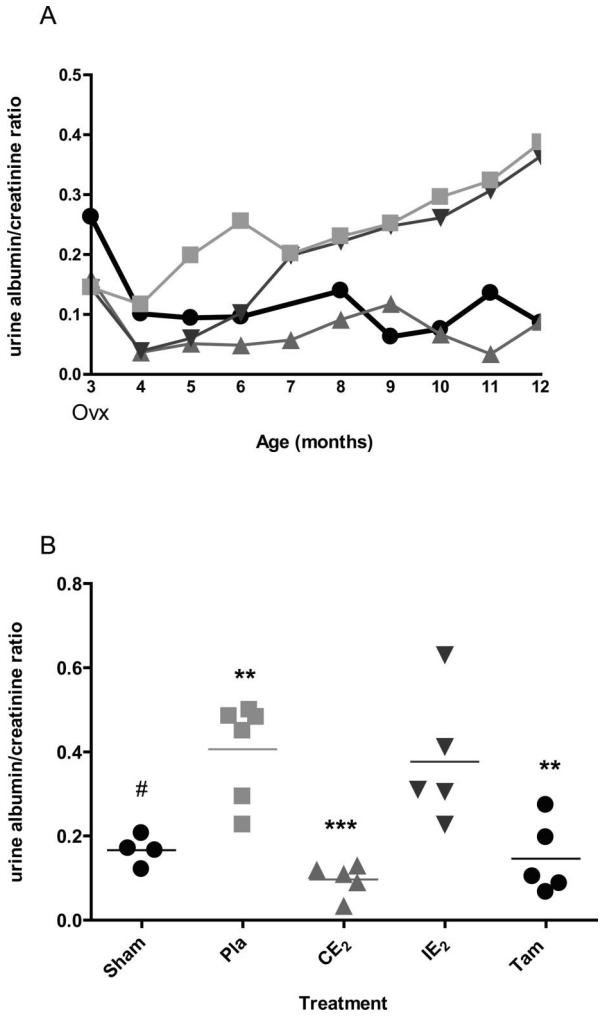

Figure 2. Effect of continuous and intermittent E_2 replacement and tamoxifen therapy on UAE. ROP Os/+ mice were Ovx at 3 months of age and subsequently received either continuous or intermittent E_2 (0.05 mg/pellet), tamoxifen (Tam, 0.5 mg/pellet), or placebo (Pla) via subcutaneously implanted 90-day release pellets as described in the Materials and Methods section. **A:** Time course of UAE of Ovx mice on placebo (Pla, square), continuous E_2 (CE_2, triangle), intermittent E_2 (IE_2, inverted triangle), and tamoxifen (Tam, circle) replacement. Data are the mean ± SEM of each group of animals from 3 months of age until sacrifice at 12 months of age. **B:** UAE at the time of sacrifice of Ovx ROP Os/+ mice on placebo (Pla, square), continuous E_2 (CE_2, triangle), intermittent E_2 (IE_2, inverted triangle), and tamoxifen (Tam, circle) replacement. Compared to Pla-treated mice: ***$P < 0.001$, **$P < 0.01$. Each point on the graph represents an individual mouse.

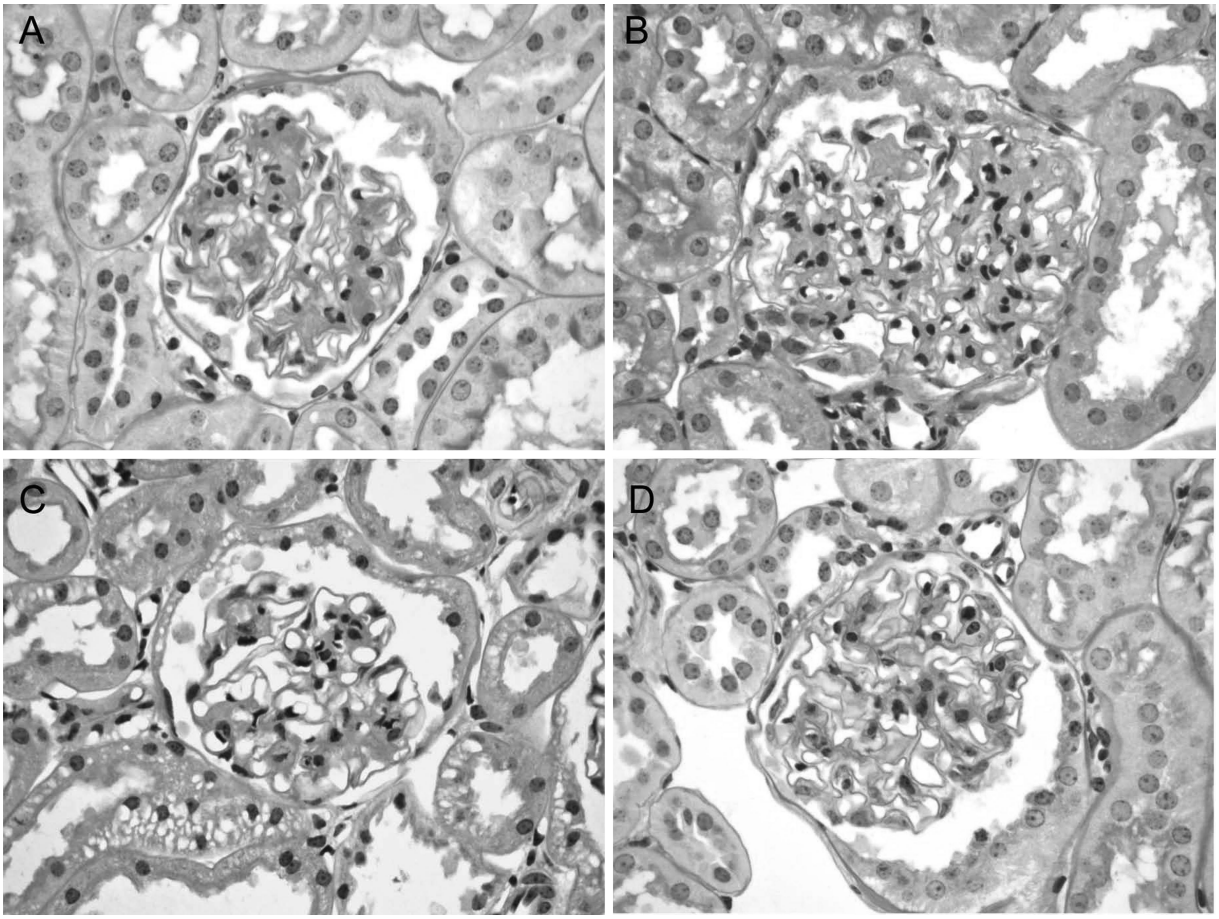

Figure 3. Histology: methacrylate-embedded sections were stained with periodic acid-Schiff silver methanamine. Renal cortex sections of 12-month-old female ROP Os/+ mouse are shown after mice underwent sham operation (**A**) or ovariectomy at 3 months of age and subsequently received placebo (**B**), CE_2 (**C**), or tamoxifen (**D**). Original magnifications, ×200.

Morphometry

There was a conspicuous decrease in glomerular area (14,170 ± 503 versus 17,350 ± 860 μm^2; $P < 0.05$) and glomerular volume (2.18 ± 0.10 versus 2.9 ± 0.21 × 10^6 μm^3; $P < 0.05$) in Ovx ROP Os/+ mice on CE_2 compared to mice on placebo (Figure 4, A and B). Intermittent E_2 replacement did not result in a decrease in glomerular size as compared to Ovx mice on placebo (2.6 ± 1.3 versus 2.5 ± 2.4; Figure 4). Tamoxifen therapy had no effect on glomerular area or volume in comparison to mice on placebo. The percentage of vascular space, used as an estimated inverse measure of the degree of sclerosis, was increased in mice on CE_2 (41.03 ± 1.13% versus 15.57 ± 1.37%, $P < 0.0001$) and tamoxifen therapy (33.77 ± 2.25% versus 15.57 ± 1.37%, $P < 0.0001$) compared to mice on placebo (Figure 4C).

ECM Accumulation (Immunofluorescence Microscopy)

Laminin deposition was increased in the mesangial areas of mice receiving placebo (Figure 5A) compared to those on CE_2 (Figure 5B). Type IV collagen increased in a similar manner (data not shown). Mice treated with tamoxifen also had less mesangial laminin deposition than animals on placebo, although the reduction was not as pronounced as in mice on CE_2 therapy (Figure 5C).

TGF-β1 Synthesis by MCs

TGF-β1 protein was measured in the supernatants of MCs isolated from mice that had received placebo or CE_2 *in vivo*. TGF-β1 protein levels were twofold lower in MCs isolated from mice on CE_2 compared to those from animals on placebo (7.31 ± 1.41 pg/ml versus 13.28 ± 0.71 pg/ml, $P < 0.005$). Similarly, TGF-β1 mRNA expression assessed by real-time RT-PCR was lower in glomeruli of mice on CE_2 than in those on placebo (TGF-β/18s ratio 257 ± 27 versus 443 ± 72, $P < 0.05$).

TGF-β1 Responsiveness in MCs

MCs were transfected with p3TP-Lux, a TGF-β1-responsive luciferase reporter construct that contains three Smad-binding elements in its promoter region, and were exposed to TGF-β1 in the range of 0 to 500 pg/ml. TGF-β1 stimulated the reporter construct and increased luciferase activity in a dose-dependent manner in MCs

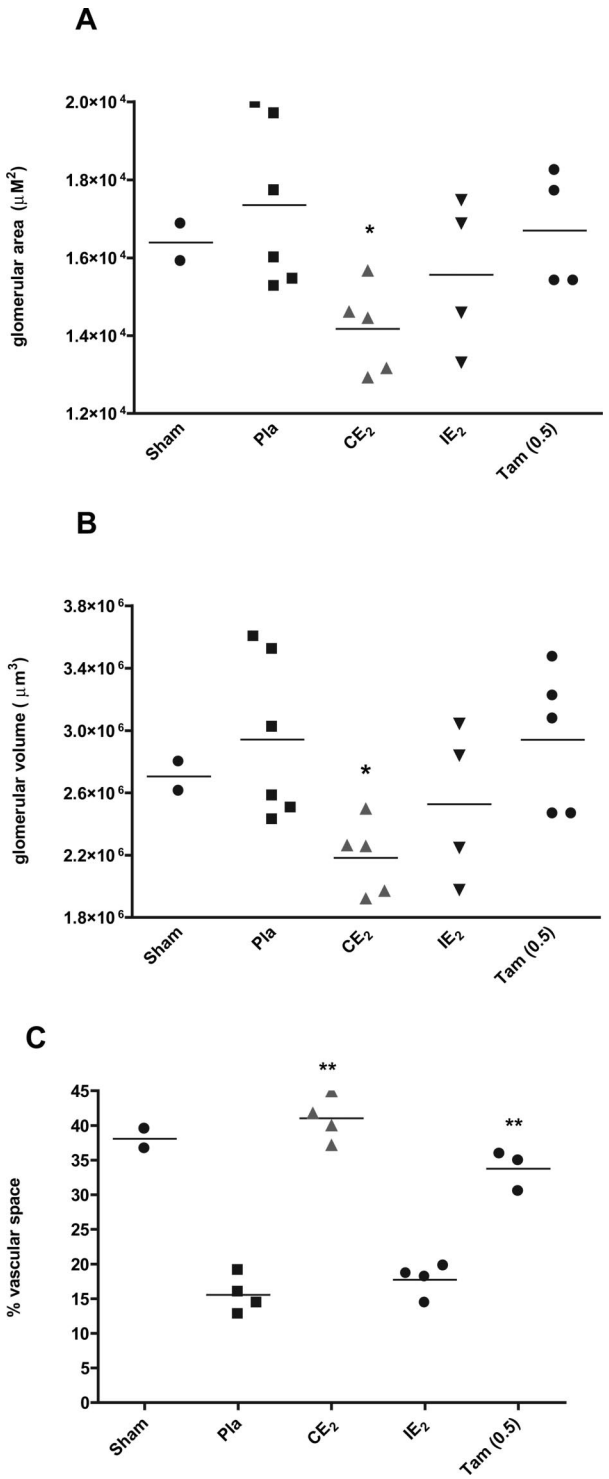

Figure 4. Morphometric analysis. Glomerular area (**A**) and volume (**B**) were smaller in mice on CE_2 (triangles) than in animals receiving placebo (Pla, squares) ($P < 0.005$) or sham-operated mice. Intermittent E_2 replacement (IE_2, inverted triangles) or tamoxifen (Tam, circles) did not reduce either of these parameters. Percentage of vascular space (C) increased with CE_2 and Tam therapy. Each point on the graph represents an individual mouse. $^*P < 0.05$, $^{**}P < 0.005$.

isolated from mice that had received placebo or had been on CE_2 *in vivo* (Figure 6, A and B, white bars; $P < 0.05$). This confirmed that the TGF-β1 signaling pathway was intact in MC lines isolated from both placebo and E_2-treated mice. E_2 (10 nmol/L) prevented the TGF-β1-induced response but only in those MCs that had been derived from mice on CE_2 *in vivo* (Figure 6B, black bars). ICI 182,780 (ICI), a complete ER antagonist, blocked this E_2 effect and restored the TGF-β1 response on the reporter construct (data not shown). This suggested that the estrogen-induced blunting of the TGF-β1 response was mediated via mesangial ERs. E_2 had no effect on TGF-β1 signaling in MCs derived from placebo-treated mice (Figure 6A, black bars).

Expression of ER-α in MCs

At baseline ER-α protein expression was ~1.5-fold higher in MCs isolated from mice on CE_2 compared to those from animals that had received placebo.

Matrix Metalloproteinase-2 (MMP-2) Promoter Activation in MCs

MCs were transfected with a construct containing an MMP-2 promoter linked to a luciferase-based reporter gene and were stimulated with E_2 (0 to10 nmol/L). E_2 had no effect on the promoter activity in cell lines isolated from Ovx mice that had received placebo *in vivo*. In contrast, E_2 increased luciferase activity in a dose-dependent manner in MCs isolated from mice that had been on CE_2 *in vivo* (Figure 7B, black bars). The presence of 500 pg/ml of TGF-β1 in the medium completely abrogated the estrogen-induced activation of the MMP-2 promoter (Figure 7B, white bars). In contrast, addition of anti-TGF-β1 antibodies abolished the blocking effect of TGF-β1 on the estrogen-mediated MMP-2 promoter activation, thereby providing evidence for the importance of TGF-β1 in interfering with the anti-sclerotic actions of E_2 (data not shown).

MMP-2 Activity in MCs

Baseline MMP-2 activity, measured in the absence of exogenous hormone or cytokine stimulation, was higher in MCs isolated from mice on CE_2 than in those derived from Ovx mice on placebo (Figure 8A). E_2 increased MMP-2 activity in a dose-dependent manner but only in MCs isolated from mice that had received CE_2 *in vivo* (Figure 8C, $P < 0.05$). This was an ER-mediated effect because treatment with the complete ER antagonist ICI 182,780 blocked the E_2-induced increase in MMP-2 activity. In contrast, E_2 had no effect on MMP-2 activity in MCs isolated from placebo-treated mice (Figure 8B).

Discussion

No randomized prospective studies have been reported that address the question of whether estrogens protect women against chronic kidney disease (CKD), particularly progressive GS. Interestingly, estrogens have been shown in animal models to have protective effects on

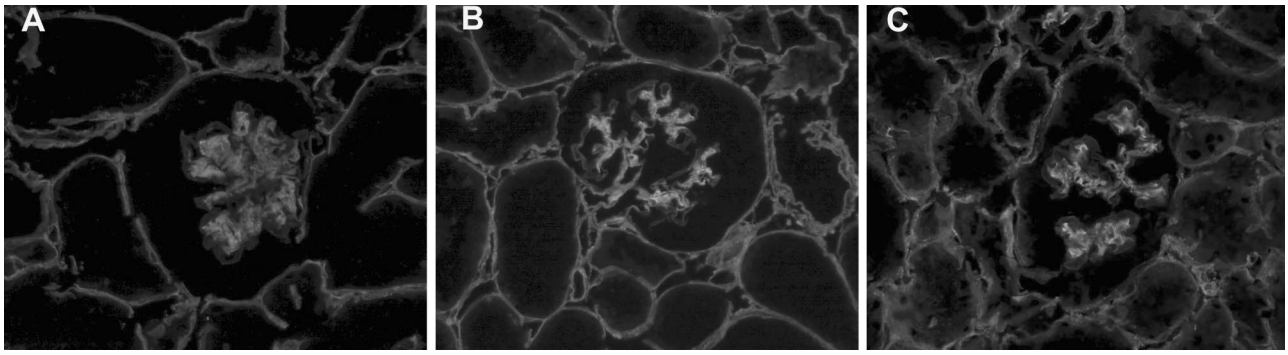

Figure 5. Immunofluorescence staining of laminin. Representative kidney sections of Ovx ROP Os/+ mouse on placebo (**A**), CE_2 (**B**), or tamoxifen (**C**) at the time of sacrifice at 12 months of age. Original magnifications, ×400.

various forms of glomerular and interstitial kidney disease.[7,9,14,15] However, the questions of whether the time of initiation and the duration of estrogen replacement therapy are crucial parameters for preventing the progression of GS have not been resolved. Furthermore, the effects of estrogens on MCs, which play an important role in the pathogenesis of GS because of their ability to secrete and digest ECM components, have not been fully elucidated.

Our group previously reported that female ROP Os/+ mice have mild GS, manifest by increased ECM and diffuse glomerular expansion, at 3 months of age.[16] As they develop more severe GS, their MC phenotype is stably altered.[10–12] We recently found that glomerular disease progressed and rapidly accelerated in ROP Os/+ mice after ovariectomy at 3 months of age and resulted in severe progressive GS in 9-month-old animals.[9]

In this study, continuous estrogen replacement throughout a 9-month period, initiated at the time of ovariectomy, prevented microalbuminuria and reduced glomerular size and volume. It also decreased glomerular ECM deposition especially of laminin, an ECM component we had previously shown to accumulate in the mesangium of Ovx ROP Os/+ mice.[9] Similarly, an estrogen-mediated decrease of mesangial laminin has also been reported in the aging Dahl salt-sensitive rat.[7]

Importantly, continuous E_2 treatment preserved glomerular function and structure not only compared to Ovx ROP Os/+ mice on placebo but also in comparison to intact sham-operated female ROP Os/+ mice. In other words, continuous E_2 exposure caused regression of the

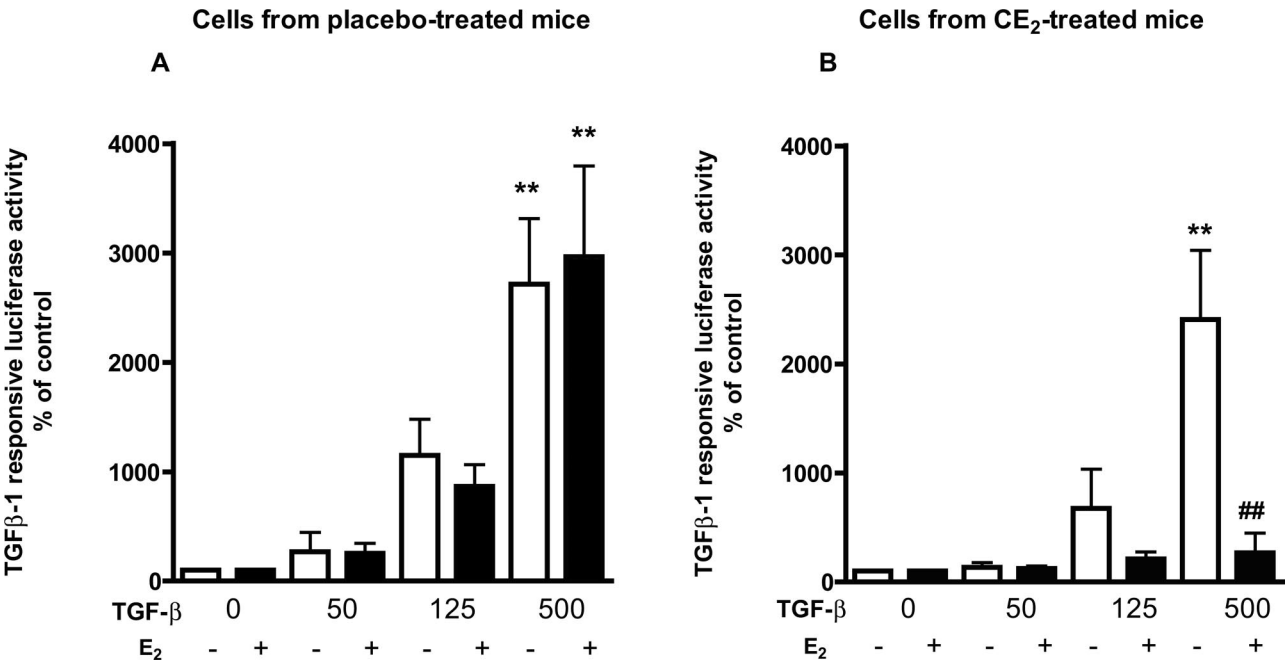

Figure 6. TGF-β1 responsiveness. MCs were isolated and propagated from Ovx ROP Os/+ mice that had received either placebo (**A**) or CE_2 therapy (**B**) *in vivo*. Cells were transfected with the TGF-β1-responsive luciferase-based reporter construct p3TP-Lux, which contains three Smad-binding elements in the promoter region. After 24 hours, MCs were treated with vehicle or TGF-β1 (50, 125, and 500 pg/ml) in the absence (white bars) or presence of 10 nmol/L E_2 (black bars). Data are expressed as percentage of vehicle control. Shown are means ± SEM of cell lysates collected from two individual cell lines obtained from each treatment group. Triplicate wells were collected for each concentration of TGF-β1 ($^{**}P < 0.05$ compared to control of same treatment, $^{\#\#}P < 0.05$ compared to untreated at the same dose, $n = 3$ individual collections).

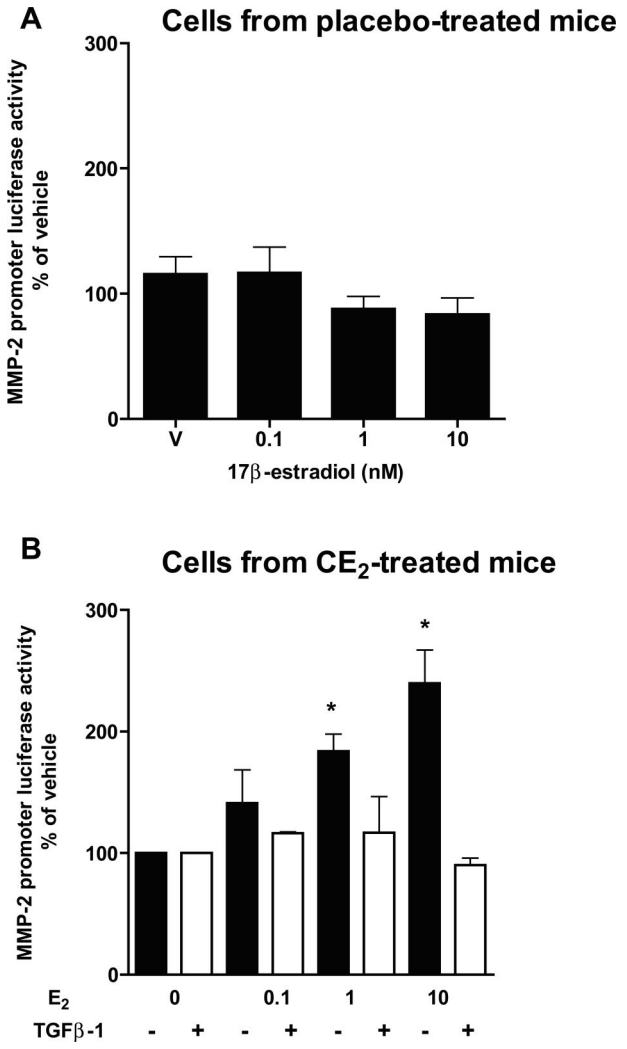

Figure 7. MMP-2 promoter activation. MCs were isolated from mice that had received either placebo (**A**) or CE_2 (**B**) and were transfected with an MMP-2 promoter linked to a luciferase reporter gene as described in Materials and Methods. After 24 hours, MCs were treated with vehicle or E_2 (0.1, 1, or 10 nmol/L). E_2 elicited a dose-dependent increase in luciferase activity but only in MCs isolated from mice that were on CE_2 *in vivo* (**B**, black bars). TGF-β1 (500 pg/ml) abolished the estrogen-mediated increase in luciferase activity in MCs derived from mice on CE_2 *in vivo* (**B**, white bars). Data are expressed as percentage of vehicle control. Shown are means ± SEM of cell lysates collected from two individual cell lines for each mouse treatment group. Triplicate wells were collected for each concentration of E_2 ($^*P < 0.05$, $n = 4$ individual collections).

mild glomerular lesions already observed in 3-month-old intact female ROP Os/+ mice, which naturally have cyclical changes in their endogenous serum estrogen levels.

When E_2 was given in an intermittent manner consisting of 3-months on-off-on E_2, glomerular function and structure deteriorated during the 3-month period of estrogen deficiency. The reinstitution of E_2 replacement for 3 months did not halt or reverse the deterioration of glomerular function and structure that had developed during the 3-month hiatus in estrogen exposure. This lack of glomerular response to estrogens occurred despite the initial 3-month E_2 treatment that had been directly instituted after ovariectomy. This first-phase E_2 replacement was given to potentially improve glomerular lesions and function and in the absence of potentially interfering ovarian androgens before the subsequent estrogen withdrawal.

Uterine weight did not differ among mice receiving either continuous or intermittent E_2 replacement at the time of sacrifice. This is consistent with the fact that the uterus remained estrogen responsive and that the final 3 months of E_2 replacement on the intermittent schedule were ample time to elicit this response. These data also show that the serum E_2 levels after reinstitution of E_2 replacement were sufficient to stimulate uterine smooth muscle cell growth and that myometrial smooth muscle cells maintain their estrogen responsiveness even after a prolonged period of estrogen deprivation.

Taken together, these findings establish that estrogen status is critically important for glomerular function and structure. We postulate that endogenous estrogens, albeit in a cyclical manner, and continuous E_2 exposure maintain glomerular cells in an estrogen-responsive or estrogen-sensitive mode with all of the subsequent beneficial effects on glomerular function and structure.

Interestingly, continuous E_2 exposure of Ovx mice appears to be superior in its ability to protect intact animals from GS compared to endogenous estrogens, which are secreted in an undulating and cyclical manner during the estrous cycle consisting of estrogen peaks and troughs. However, ovariectomy not only abolishes ovarian estrogen production but also eliminates progesterone and testosterone secretion by the ovaries. Previously, we found no evidence for progesterone receptor expression in MCs or glomeruli consistent with the notion that progesterone is an unlikely antagonist of estrogen action in the glomerulus. Accordingly, Nielsen and colleagues[8] did not report a difference between E_2 alone and E_2 + progesterone replacement in normalization of the decreased renal functional reserve including the glomerular filtration rate in Ovx Wistar rats. Future studies will need to determine whether the protective effects of endogenous estrogens are antagonized by the small amounts of ovarian-secreted testosterone or whether endogenous estrogen-mediated protection is limited because of the relatively short but cyclical reoccurring periods of physiological estrogen deficiency during met-estrous.[17]

We also studied tamoxifen, a SERM that is used with increasing frequency in women for breast cancer treatment and prevention. SERMs could have either protective or deleterious effects on glomerular function and structure depending on their tissue-specific agonistic and antagonistic effects.[18] Although this could also have important implications for women with chronic kidney disease, data regarding the glomerular-specific effects of SERMs in women are sparse. We found that tamoxifen reduced UAE in Ovx animals to a degree comparable to intact ROP Os/+ mice. Tamoxifen had similarly beneficial effects on glomerular morphology although not of the same magnitude as seen in mice receiving continuous E_2. Similarly, Neugarten and colleagues[19] found that tamoxifen treatment *in vitro* reduced MC synthesis of type I and type IV collagens. In contrast, Cohen and Rosenmann[20] reported that tamoxifen ameliorated GS in diabetic Cohen rats but the same was true for Ovx animals on placebo,

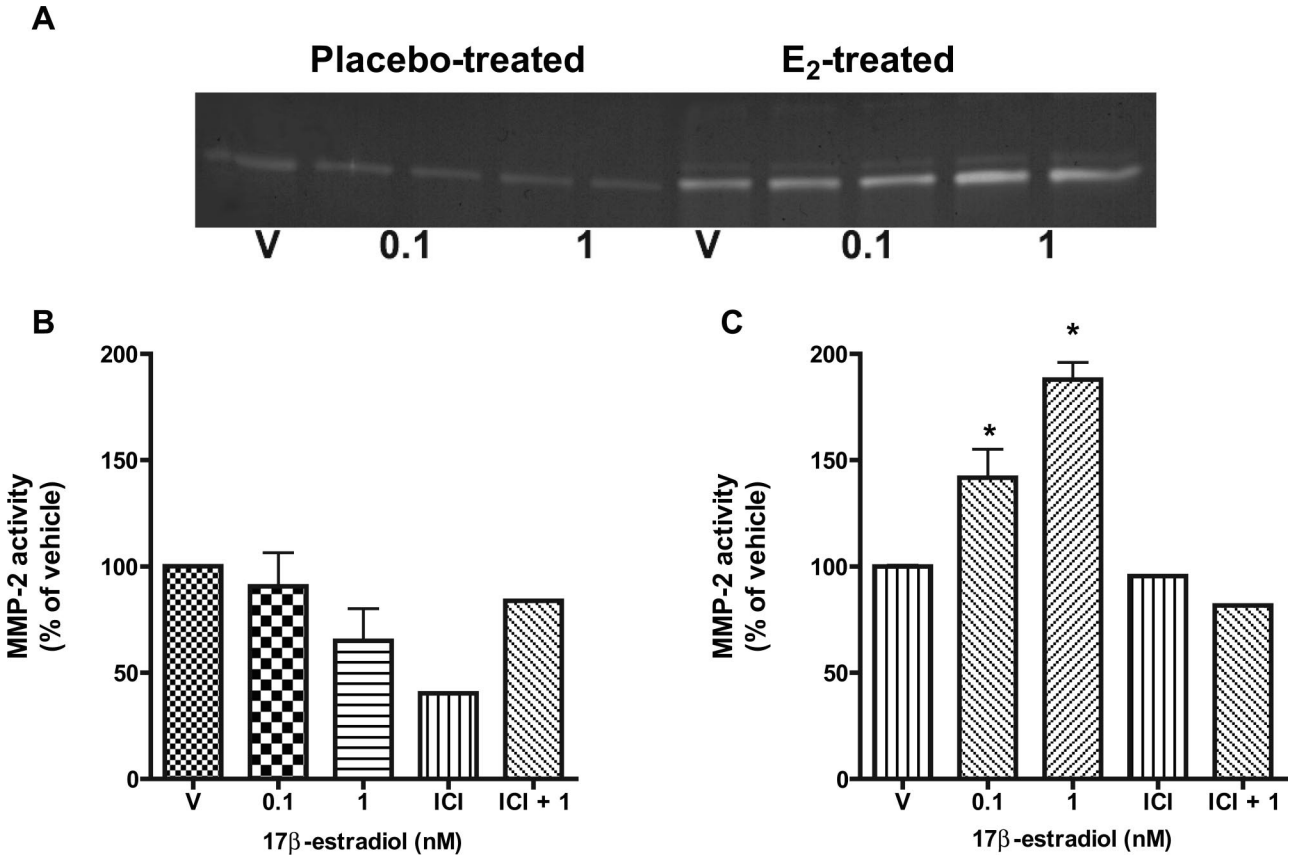

Figure 8. MMP-2 activity. Supernatants of MCs isolated from mice on placebo or CE_2 were collected for zymography as described in Materials and Methods. **A:** Representative zymogram of supernatants of MCs that were treated with vehicle (V), E_2 (0.1 and 1 nmol/L), ICI (1 μmol/L), or ICI + E_2 (1 nmol/L). **B:** Graph depicting the mean ± SEM of duplicate experiments of MMP-2 activity in the supernatant of MCs isolated from mice on placebo after *in vitro* E_2 stimulation. **C:** Graph depicting the mean ± SEM of duplicate experiments of MMP-2 activity after *in vitro* E_2 stimulation of MCs isolated from mice on CE_2. Data are expressed as percentage of vehicle control. $^*P < 0.05$.

suggesting that estrogen deficiency or estrogen antagonism were responsible for this effect. In agreement with our data, tamoxifen decreased renal inflammation and reduced proteinuria in NZB/W mice, a model of lupus nephritis.[21] In addition, raloxifene, a third generation SERM, has recently been shown to improve diabetic GS in db/db mice (C57BLKsJ-*db/db*).[22] Although tamoxifen has reno-protective effects in estrogen-deficient mouse models, including Ovx ROP Os/+ mice, the contrasting data on the Cohen rat model warrants further examination in humans to determine whether the effects of SERMs on the glomerulus are species-specific.

MCs play an important role in the development and progression of GS. We have previously shown that the phenotype of MCs changes after the onset of type 1 and type 2 diabetes in mouse models susceptible to developing diabetic GS and that this diabetes-induced, disease phenotype is preserved *in vitro* even in the absence of a high glucose/diabetic milieu.[23,24] In addition, GS can be transmitted into healthy animals via cells carrying the diseased phenotype.[10] Furthermore, phenotype modulation is not only restricted to MCs but has also been shown to occur in vascular smooth muscle cells affected by atherosclerosis.[10,25]

Based on our *in vivo* data, we hypothesized that continuous exposure to estrogens are critical for maintaining MCs in an estrogen-sensitive mode. In other words, we proposed that estrogens also modulate the phenotype of MCs. However, this concept of hormonal regulation of the MC phenotype remained to be examined in greater detail.

The first *in vitro* data supporting our hypothesis were obtained by examining MMP-2 activity and TGF-β1 levels in the supernatant of MCs isolated from Ovx ROP Os/+ mice. We focused on MMP-2 because it is one of the major enzymes involved in the degradation of ECM matrix components, thereby providing protection against GS. We targeted TGF-β1 because TGF-β1 signaling is widely viewed as an intracellular signaling pathway that promotes GS.

At baseline, in the absence of exogenous hormonal or cytokine stimulation, MMP-2 activity was higher and the TGF-β1 concentration was lower in the supernatant of MCs isolated from Ovx ROP Os/+ mice, which received continuous E_2 *in vivo*, than in those of animals given placebo. ER-mediated E_2 stimulation increased MMP-2 activity *in vitro* but only from those MCs that were derived from mice treated with continuous E_2 *in vivo*. In contrast, there was no increase in MMP-2 activity in MCs of Ovx mice that had received placebo. Similarly, E_2 stimulated luciferase activity from a transfected reporter gene under the regulatory control of an MMP-2 promoter, which con-

tains at least three incomplete or half-palindromic ER-binding sites,[26] but only, as previously seen, in those MCs derived from mice that were treated with continuous E_2 during the *in vivo* study. Interestingly, in the presence of TGF-β1, E_2 could not further elicit a response from the MMP-2 promoter in these MCs.

In contrast, exogenous TGF-β1 was able to stimulate a TGF-β/Smad-responsive luciferase-based reporter gene in MCs derived from mice that had received either continuous E_2 or placebo. This suggested that the TGF-β1 signaling system is intact in MCs of female ROP Os/+ mice and that estrogen treatment *in vivo* did not affect the *in vitro* responsiveness of MCs to TGF-β1. However, in the presence of E_2, the TGF-β1-mediated response was blunted or abolished but again only in those MCs that were isolated from mice that had been treated with CE_2 *in vivo*. This difference in the estrogen-mediated response is, in part, explained by the higher levels of ER-α in the MCs isolated from mice on CE_2 and appears to be one of the characteristics of the estrogen-modulated phenotype. The estrogen-mediated blunting of the TGF-β1 response occurs most likely via direct interactions of estrogen-activated ERs with Smad molecules that are part of the TGF-β1 signaling pathway in a manner similar to what has been previously described for other steroid hormone receptors.[15,27–29]

Taken together, these data demonstrate the intricate relationship between the estrogen/ER and the TGF-β1 signaling system in MCs, demonstrating major implications for the development or prevention of GS. Estrogens down-regulate TGF-β1 expression and interfere with and diminish the prosclerotic TGF-β1 signaling response but only in those MCs that were exposed to estrogens *in vivo*. This clearly demonstrates that estrogens modulate the phenotype of MCs by maintaining them in an estrogen-responsive condition characterized, most likely among other attributes, by the ability of estrogens to increase MMP-2 expression and activity, to decrease TGF-β1 expression, and to interfere with the TGF-β1 signaling pathway. To maintain this estrogen-sensitive phenotype MCs need to be exposed to estrogens *in vivo*. MCs irreversibly revert into an estrogen-insensitive phenotype during a period of chronic estrogen deficiency that cannot be modified with subsequent estrogen treatment. Further studies will be necessary to determine whether this phenomenon induced by chronic estrogen deficiency is caused by epigenetic gene silencing.[30]

However, it should be noted that estrogens may exert their effects on MCs not only via ER-dependent mechanisms. Our study in MCs isolated from db/db mice showed that E_2 increased IGF-I protein in an ER-independent manner.[24] Recent *in vivo* and *in vitro* studies have reported ER-independent renoprotective effects of E_2 and its metabolites, 2-hydroxyestradiol and 2-methoxyestradiol, on rat and human MC proliferation and ECM synthesis.[31–33] Blockade of tubulin polymerization by catechol- and methoxy-estradiols, which interferes with cell migration, proliferation, and collagen synthesis, has been evoked as an important mechanism underlying glomerular protection. However the antioxidant effects of E_2 and its metabolites have also been proposed to play a role in preserving glomerular structure and function. For instance, estrogen molecules that have a hydroxyl group in the C3 position of their phenolic A ring can serve as antioxidants by scavenging hydroxyl radicals,[34,35] leading to the formation of nonphenolic quinols that have no affinity to ERs.[34] At the present time, we do not know to what extent the ER-independent mechanisms contribute to the protection against GS in our model. However, staining of glomeruli with Ki67, a marker of MC proliferation, did not reveal a difference between sclerotic glomeruli of estrogen-deficient and the preserved glomeruli of continuously estrogen-replaced mice. It remains to be seen whether this is attributable to the late stage of GS in our current study. Experiments are underway to determine the extent of the ER-independent effects of estrogens *in vivo*.

In summary, CE_2 prevented the development of microalbuminuria and maintained normal glomerular structure in Ovx ROP Os/+ mice, a model of progressive GS. The protective effects of continuous estrogen exposure were superior to those of endogenous estrogens that are secreted in a cyclical manner. Glomerular function and structure deteriorated during phases of chronic estrogen deficiency regardless of the timing of its onset, and the ensued glomerular dysfunction and morphological changes could not be prevented by subsequent estrogen replacement. The *in vivo* findings are mirrored in the MC phenotype. MCs remain estrogen-sensitive if exposed to continuous or cyclical levels of estrogens *in vivo* and irreversibly regress into an estrogen-insensitive, diseased cell type after prolonged estrogen deficiency. Another important finding was that tamoxifen reduced microalbumin excretion, and prevented the deterioration of the glomerular structure although not to the same extent as continuous E_2. The implications of these findings could be far reaching if similar observations are made in women, especially those with a propensity to develop CKD.

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