Albuminuria is associated with too few glomeruli and too much testosterone

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Normally, the glomerular filtration barrier almost completely excludes circulating albumin from entering the urine. Genetic variation and both pre- and postnatal environmental factors may affect albuminuria in humans. Here we determine whether glomerular gene expression in mouse strains with naturally occurring variations in albuminuria would allow identification of proteins deregulated in relatively 'leaky' glomeruli. Albuminuria increased in female B6 to male B6 to female FVB/N to male FVB/N mice, whereas the number of glomeruli/kidney was the exact opposite. Testosterone administration led to increased albuminuria in female B6 but not female FVB/N mice. A common set of 39 genes, many expressed in podocytes, were significantly differentially expressed in each of the four comparisons: male versus female B6 mice, male versus female FVB/N mice, male FVB/N versus male B6 mice, and female FVB/N versus female B6 mice. The transcripts encoded proteins involved in oxidation/reduction reactions, ion transport, and enzymes involved in detoxification. These proteins may represent novel biomarkers and even therapeutic targets for early kidney and cardiovascular disease.

Normally, macromolecules such as albumin are almost completely excluded from entering the filtrate by the glomerular filtration barrier consisting of endothelia, podocytes, and glomerular basement membrane.[1] Major barrier disruptions, as occur in individuals with mutations of slit diaphragm genes, cause massive protein leakage.[2,3] More moderate albumin excretion above the normal range, or 'microalbuminuria' (30–300 mg per 24 h), may also be clinically important, as may variations within the so-called normal range. In individuals with diabetes mellitus, microalbuminuria generally precedes, and may predict progression to nephropathy.[4,5] Indeed, filtered proteins, or bound molecules, may be tubulotoxic.[6,7] Microalbuminuria is an independent risk factor for cardiovascular mortality and morbidity not only in individuals with diabetes mellitus or systemic hypertension but also in the general population.[8] This association may be explained by increased albuminuria being just one manifestation of a generalized microvascular disturbance.[8]

In normotensive US adolescents, albumin excretion rate is higher in blacks than in whites.[9] In US adults, the prevalence of microalbuminuria is greater in non-Hispanic blacks and Mexican Americans as compared with non-Hispanic whites.[10] The importance of genetic background in determining albuminuria is supported by observations of inbred 'normal' mice.[11] Tsaih et al.[11] examined mice at advanced ages of 12–24 months, reporting up to a 100-fold difference of albuminuria between strains. Within certain strains (for example, A/J, C57BL/10J, and FVB/NJ), males excreted more albumin than females, whereas the opposite held in other strains (for example, BUB/BnJ and SJL/J). In healthy adults in the Netherlands, men had a higher average urinary albumin excretion rate than women (10 vs. 8 mg per 24 h), and the prevalence of microalbuminuria was twofold higher in males, even after controlling for smoking, hypercholesterolemia, and obesity.[12] In nondiabetic white UK adults, men had a higher albumin excretion rate than women; microalbuminuria in men was associated with short stature, whereas hypertension positively correlated with albumin excretion rate in women.[13]
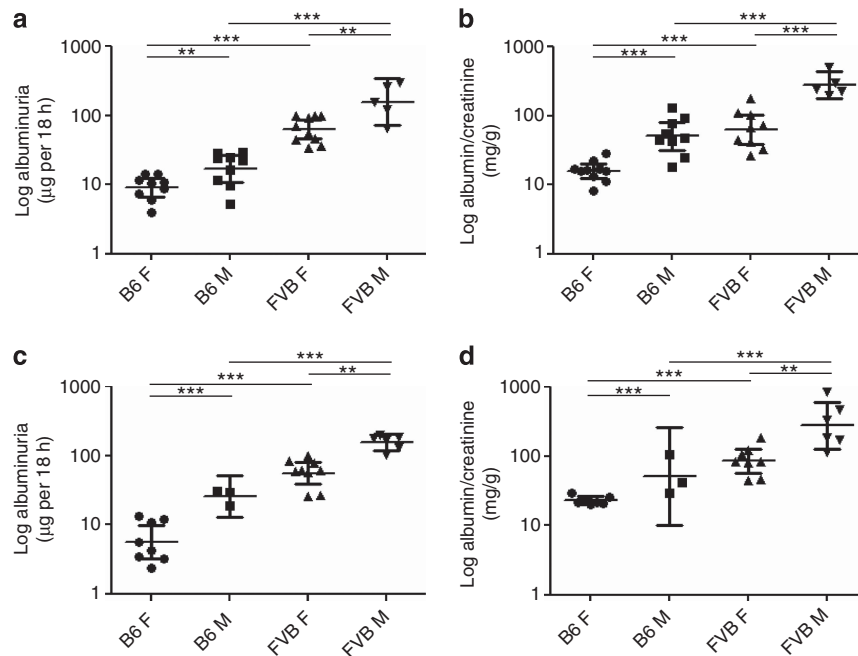
Figure 1 | Albuminuria in B6 and FVB/N mice. Overnight (**a**, **c**) albumin excretion and (**b**, **d**) albumin-to-creatinine ratios were evaluated in (**a**, **b**) 18-week-old and (**c**, **d**) 13-week-old adult mice. Data were log transformed before analysis and are presented as geometric means and confidence interval. There was a significant increase in urinary albumin in male (M) and female (F) FVB/N mice compared with sex-matched B6 animals. Within each strain, males had elevated albuminuria versus females (******$P < 0.01$, *******$P < 0.001$ between groups).

The antenatal environment to which an individual is exposed may influence urinary albuminuria. Adults gestated during the Dutch famine in World War II had an increased risk of microalbuminuria.[14] Possibly, maternal undernutrition led to birth of individuals whose kidneys contained fewer nephrons than normal and subsequent compensatory changes would have resulted in loss of filtration barrier functionality.[15,16] Indeed, rodent embryos exposed to maternal low-protein diet form kidneys with fewer glomeruli than normal.[17] Interestingly, within the general human population there exists considerable variation in the numbers of glomeruli per kidney,[18] with normotensives having about $1-2 \times 10^6$ glomeruli per kidney and adults with essential hypertension having approximately $0.5-1 \times 10^6$ glomeruli per kidney.

The above evidence is consistent with the contentions that genetic background and sex modify albumin excretion rate. We speculated such variations in albuminuria would relate to alterations in glomerular biology and/or numbers. We hypothesized that studying mice with naturally occurring variations in albuminuria would allow us to identify genes deregulated in 'leaky' glomeruli.

RESULTS
Urinary albumin excretion in B6 and FVB/N mice
The urinary albumin excretion rate in adult (18 weeks old) female and male FVB/NHanHsd (FVB/N) mice was, on average, eightfold more than age- and sex-matched C57BL/6JOlaHsd (B6) mice (Figure 1a). Within each strain, males had increased albumin excretion rates than females (twofold elevations in both B6 and FVB/N strains; Figure 1a). The

same patterns were apparent when albumin/creatinine concentration was measured (Figure 1b). Albumin excretion rates (Figure 1c) and albumin/creatinine (Figure 1d) were quantified in separate sets of mice aged 13 weeks, with the same patterns noted between the strains and sexes. There were no significant differences in albuminuria between mice of the same sex and strain at 13 versus 18 weeks.

Glomerular gene expression *in vivo*
Eighteen-week-old mice were perfused with magnetic beads that accumulate in glomerular capillaries (Figure 2a). Magnetically isolated glomeruli usually consisted of the tuft alone, although others also contained a capsule (Figure 2b). mRNA integrity was preserved in isolated glomeruli (Figure 2c). For each group, we undertook three sets of RNA microarrays, each from a separate mouse. We identified a common set of 39 genes significantly differentially expressed in each of the comparisons: female FVB/N versus female B6 mice; male FVB/N versus male B6 mice; male versus female B6 mice; and male versus female FVB/N mice. Expression levels of 34 transcripts positively correlated with albuminuria (Table 1), whereas levels of five others negatively correlated with albuminuria (Table 2). Several upregulated transcripts coded for proteins involved in oxidation/reduction reactions (*Aass*, *Aldh1l1*, *Cyp4a12a*, *Hsd3b2*, and *Ldhd*) or ion transport (*Slc22a2*, *Slc22a6*, *Slc5a8*, and *Slco1a1*). Other upregulated transcripts included: *Acy3* and *Tst*, coding for enzymes involved in detoxification; *Treh*, an enzyme hydrolyzing trehalose; *Aqp1*, coding for aquaporin-1; and *Hpn*, coding for hepsin, a serine protease. Downregulated transcripts
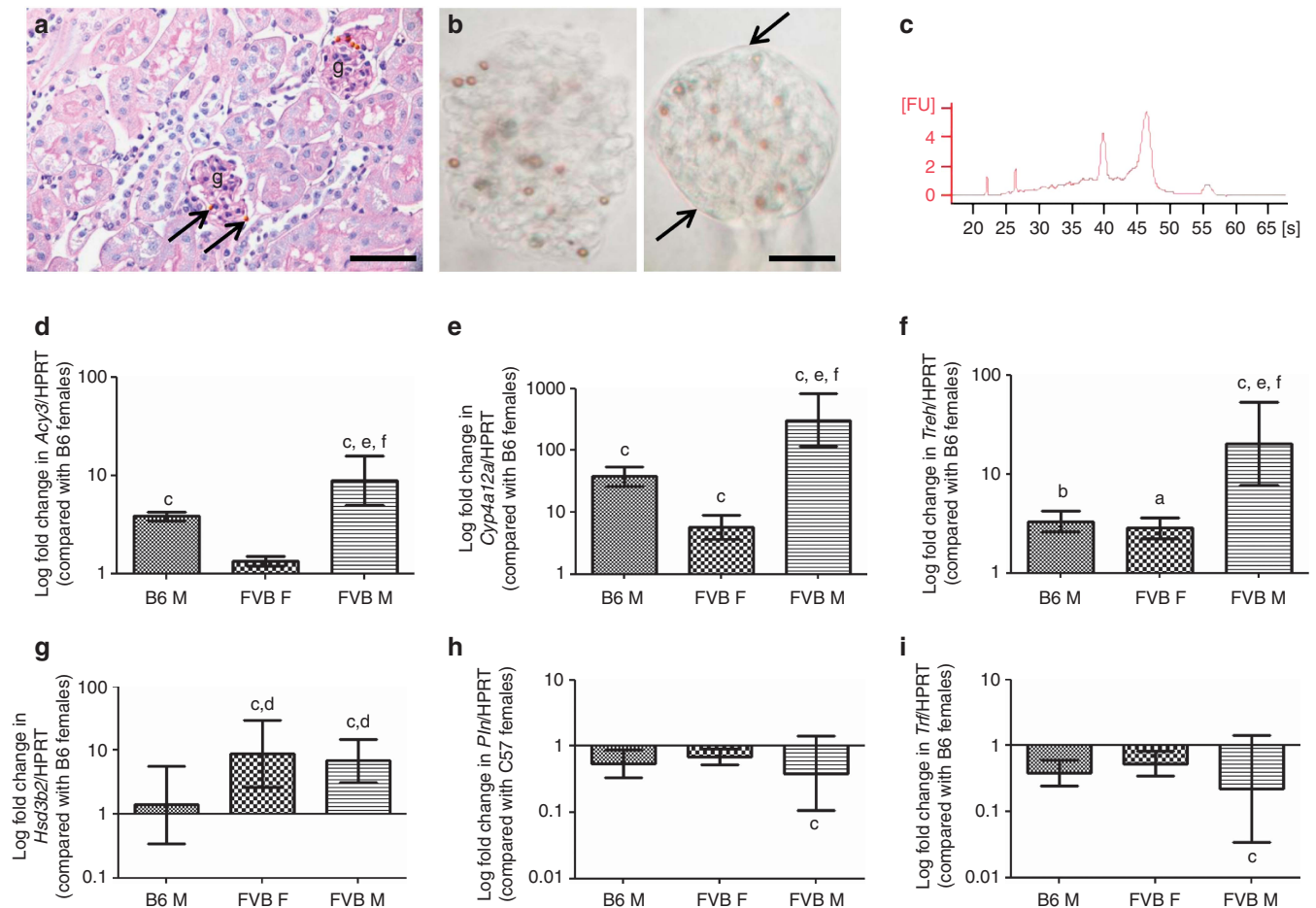
Figure 2 | Isolation of glomeruli and real-time PCR for selected genes. (a) Glomeruli (g) were isolated by perfusion of magnetic beads (arrows) that accumulated in the glomerular vessels. (b) Isolated glomeruli retain intact morphology with the majority simply comprising the tuft (left panel) whereas on occasions the tuft was surrounded by Bowman's capsule (arrows, right panel); (c) RNA integrity was preserved. FU, fluorescence units. Real-time quantitative reverse transcriptase–PCRs (qPCRs) for (d) *Acy3*, (e) *Cyp4a12a*, (f) *Treh*, and (g) *Hsd3b2*; transcripts positively correlating with albuminuria in array analyses. qPCRs for (h) *Pln* and (i) *Trf*, transcripts negatively correlating with albuminuria in array analyses. F, female; M, male. Hypoxanthine-guanine phosphoribosyltransferase (hprt) was used as a housekeeping gene. Fold changes in expression are expressed relative to B6 female mice where average expression was given an arbitrary value of 1 (a = $P < 0.05$, b = $P < 0.01$, and c = $P < 0.001$ compared with B6 female mice; d = $P < 0.01$ compared with B6 male mice; e = $P < 0.001$ compared with B6 male mice; f = $P < 0.001$ compared with FVB/N female mice, $n = 4$ in each group). Bar in **a** is 50 μm and in **b** is 20 μm. Data were log transformed before analysis and are presented as geometric means and confidence interval.

included: *Trf*, encoding transferrin; *Cldn11*, coding for the tight junction protein claudin 11; and *Pln*, encoding phospholamban, a membrane protein that regulates the calcium ion pump in muscle. Real-time quantitative reverse transcriptase–PCR (qPCR) was performed for selected upregulated (*Acy3*, *Cyp4a12a*, *Hsd3b2*, and *Treh*) and down-regulated (*Pln* and *Trf*) transcripts using independent samples. These results (Figure 2d–i) concurred with the directions indicated by the microarray. In the case of *Hsd3b2*, changes were seen between strains but not sex; this may be because of the microarray probes also detecting *Hsd3b3* and *Hsd3b6* that may contribute to the differences seen between male and female mice. Note that the relative fold change in the levels of any particular transcript as assessed by the array and qPCR analyses did not necessarily exactly correspond to the numerical fold change in albuminuria documented between sexes and also between strains.

Relation of candidate genes to mouse albuminuria loci

We compared the location of these genes with published genetic analyses of albuminuria loci in mice and rats.[19] *Acy3*, *Cyp4a12a*, and *Hsd3b2* were found to be within the confidence intervals of mouse and rat quantitative tract loci (QTLs) for albuminuria. We also interrogated the array data with respect to candidate genes previously implicated in albuminuria using a genome-wide association approach, namely *Aspa*, *Atic*, *Cyp24a1*, *Fdn4*, *Fn1*, *Mbp*, *Myo16*, *Negr1*, *Olfr381*, *Olfr389*, *Olfr392*, *Prdm5*, *Ripk2*, *Spata22*, *Trpv1*, *Trpv3*, and *Zfp236*.[11] There was no significant effect of sex on the expression levels of these genes. However, in FVB/N versus B6 glomeruli of either sex, we found decreased transcript levels of: *Negr1* (19.3- and 4.0-fold in males and females, respectively, $P < 0.01$), encoding a cell adhesion molecule; *Prdm5* (2-fold in both sexes, $P < 0.01$), encoding a transcription factor; and *Trpv1* (10.7- and 2.3-fold in male

Table 1 | Genes significantly upregulated in glomeruli by both strain and sex

Probe set	Gene symbol	Gene title	Mouse chromosomal location (chromosome (Chr):Mb)	Human chromosomal location (Chr:Mb)	Fold change compared with B6 female				
					B6 male	FVB/N female	FVB/N male	P-value (sex)	P-value (strain)
1450947_at	2610528J11Rik	RIKEN cDNA 2610528J11 gene	4:118.5	1:43.7	2.5	2.0	6.8	0.017	0.012
1431904_at	4933427G17Rik	RIKEN cDNA 4933427G17 gene	7:121.0	NA	4.8	2.8	8.0	0.024	0.045
1423523_at	Aass	Aminoadipate-semialdehyde synthase	6:23.1	7:121.7	3.0	1.6	6.8	0.013	0.043
1448539_a_at	Acy3	Aspartoacylase (aminoacylase) 3	19:4.0	11:67.4	4.8	1.7	12.8	0.001	0.009
1424400_a_at	Aldh1l1	Aldehyde dehydrogenase 1 family, member L1	6:90.5	3:125.8	2.7	2.0	6.1	0.041	0.042
1416203_at	Aqp1	Aquaporin 1	6:55.3	7:30.9	2.1	2.6	6.8	0.032	0.003
1418914_s_at	Bhmt2	Betaine-homocysteine methyltransferase 2	13:93.7	5:78.4	2.9	2.0	9.3	0.045	0.037
1451625_a_at	C8g	Complement component 8, γ polypeptide	2:25.5	9:139.8	2.7	2.5	9.5	0.020	0.006
1418976_s_at	Cideb	Cell death-inducing DNA fragmentation factor, α subunit-like effector B	14:55.8	14:24.8	2.2	1.6	6.8	0.013	0.012
1424811_at	Cml5	Camello-like 5	6:85.8	2:73.9	10.8	6.4	65.7	0.030	0.019
1423701_at	Coasy	Coenzyme A synthase	11:101.1	17:40.7	2.1	1.7	6.5	0.037	0.029
1424352_at	Cyp4a12a	Cytochrome P450, family 4, subfamily a, polypeptide 12a	4:115.3	1:47.4	28.6	2.2	359.8	0.003	0.022
1428738_a_at	D14Ertd449e/// EN-SMUSG00000072676	DNA segment, Chr 14, ERATO Doi 449, expressed/// predicted gene, EN-SMUSG00000072676	14:26.1	10:81.8	1.6	1.6	6.8	0.035	0.013
1417393_a_at	Fam132a	Family with sequence similarity 132, member A (also known as C1qdc2)	4:156.0	1:1.2	1.5	1.6	3.4	0.023	0.005
1417422_at	Gnmt	Glycine N-methyltransferase	17:46.8	6:42.9	3.0	2.3	8.9	0.037	0.024
1420712_a_at	Hpn	Hepsin	7:31.1	19:35.5	2.0	1.6	6.4	0.033	0.025
1460232_s_at	Hsd3b2/// Hsd3b3/// Hsd3b6	Hydroxy-δ-5-steroid dehydrogenase, 3 β- and steroid δ-isomerase 2/// hydroxy-δ-5-steroid dehydrogenase, 3 β- and steroid δ-isomerase 3/// hydroxy-δ-5-steroid dehydrogenase, 3 beta- and steroid δ-isomerase 6	3:98.7	1:120.0	4.4	4.5	11.5	0.025	0.005
1428614_at	Ldhd	Lactate dehydrogenase D	8:111.6	16:75.1	5.0	1.8	15.4	0.002	0.008
1419504_at	LOC100047046/// Mogat1	Similar to monoacylglycerol O-acyltransferase 1/// monoacylglycerol O-acyltransferase 1	1:78.5	2:223.5	8.3	6.0	69.4	0.005	0.002
1419520_at	Nat8	N-acetyltransferase 8	6:85.8	2:73.9	3.2	2.6	23.3	0.008	0.003
1455490_at	Pigr	Polymeric immunoglobin receptor,	1:130.8	1:207.1	3.9	2.4	25.5	0.004	0.003
1419117_at	Slc22a2	Solute carrier family 22 (organic cation transporter), member 2	17:12.6	6:160.6	2.5	1.8	9.0	0.029	0.023
1417072_at	Slc22a6	Solute carrier family 22 (organic anion transporter), member 6	19:8.6	11:62.7	3.7	1.7	16.8	0.020	0.046
1425606_at	Slc5a8	Solute carrier family 5 (iodide transporter), member 8	10:88.9	12:101.5	3.8	1.5	15.3	0.008	0.027
1436667_at	Slc6a20b	Solute carrier family 6, member 20b	9:123.6	3:45.8	2.1	2.1	6.5	0.036	0.009
1449301_at	Slc7a13	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 13	4:19.8	8:87.2	224.0	3.9	1267.6	<0.001	0.005
1420379_at	Slco1a1	Solute carrier organic anion transporter family, member 1a1	6:141.9	12:21.4	36.0	3.4	567.4	<0.001	0.002
1449409_at	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	17:53.8	2:108.9	1.5	2.5	5.9	0.036	0.001
1451528_at	Tmem82	Transmembrane protein 82	4:141.6	1:16.1	1.7	1.7	6.7	0.036	0.009
1430889_a_at	Tpmt	Thiopurine methyltransferase	13:47.0	6:18.1	2.1	1.8	13.7	0.020	0.007
1449430_a_at	Treh	Trehalase (brush-border membrane glycoprotein)	9:44.7	11:118.5	3.4	3.6	41.9	0.008	0.001
1448609_at	Tst	Thiosulfate sulfurtransferase, mitochondrial	15:78.4	22:37.4	2.2	2.1	7.3	0.015	0.004
1451373_at	Ugt3a1	UDP glycosyltransferases 3 family, polypeptide A1	15:9.3	5:36.0	3.1	1.9	13.7	0.028	0.022
1460244_at	Upb1	Ureidopropionase, β	10:75.4	22:24.9	1.8	1.6	5.2	0.048	0.030

All genes were significantly altered by both strain and gender ($P < 0.05$), followed the direction of albuminuria in the four groups assessed, and varied by >1.5-fold in individual comparisons between different sexes in both B6 and FVB/N mice and between mice of the same sex but from different strains. Fold changes are presented relative to B6 female mice (given a value of 1) that had the lowest albumin excretion rate. NA, not available.

Table 2 | Genes significantly downregulated in glomeruli by both strain and sex

Probe set	Gene symbol	Gene title	Mouse chromosomal location	Human chromosomal location	Fold change compared with B6 female				
					B6 male	FVB/N female	FVB/N male	P-value (sex)	P-value (strain)
1445938_at	5930427L02Rik	RIKEN cDNA 5930427L02 gene	NA	NA	−1.9	−1.5	−29.4	0.049	0.017
1416003_at	Cldn11	Claudin 11	3:31.1	3:170.1	−2.6	−2.2	−15.5	0.036	0.009
1450952_at	Pln	Phospholamban	10:53.3	6:118.9	−1.5	−1.6	−4.7	0.010	0.002
1425546_a_at	Trf	Transferrin	9:103.2	3:133.5	−1.8	−1.5	−3.4	0.046	0.042
1446477_at	Zfp622	Zinc-finger protein 622	15:26.0	5:16.5	−2.0	−2.1	−3.4	0.023	0.004

All genes were significantly altered by both strain and gender ($P < 0.05$), followed the direction of albuminuria in the four groups assessed, and varied by >1.5-fold in individual comparisons between different sexes in both B6 and FVB/N mice and between mice of the same sex but from different strains. Fold changes are presented relative to B6 female mice (given a value of 1) that had the lowest albumin excretion rate. NA, not available.
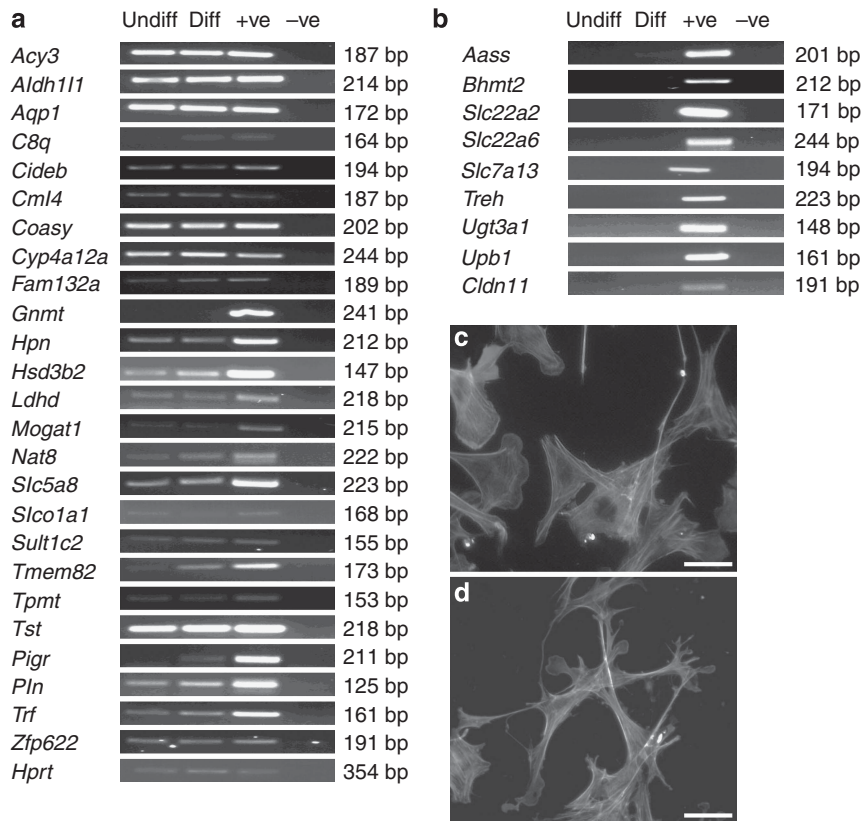
Figure 3 | Expression of candidate genes in cultured podocytes. (**a**, **b**) RNA was collected from undifferentiated (Undiff) and differentiated (Diff) podocytes and PCR performed for all annotated genes found to be differentially expressed in the microarray analysis. Positive controls (+ve) of reactions consisted of mouse whole kidney RNA. Negative controls (−ve) consisted of reactions without cDNA template. Panel **a** comprises genes that were found to be expressed in cultured podocytes and panel **b** comprises transcripts that were not detected using this methodology. Representative pictures of phalloidin-stained undifferentiated (**c**) and differentiated (**d**) podocytes showing the extensive process formation characteristic of the *in vivo* podocyte phenotype. (**c**, **d**) Bar = 50 μm.

and female mice, respectively, $P < 0.05$), encoding the vanilloid receptor-1 ion channel.

Glomerular expression of candidate molecules

Assessed by reverse transcriptase–PCR (RT–PCR) (Figure 3a), over half of the set of transcripts identified by the array were expressed in a podocyte line,[20] whereas others were not detected in the same cells (Figure 3b). Using protein lysates from isolated glomeruli, we undertook semiquantitative western blotting for CYP4A12A, a cytochrome *P*450, and aspartocyclase 3 (ACY3), respectively the proteins encoded by *Cyp4a12a* and *Acy3*. Reliable signals for HSD3B2 could not be obtained with available antibodies. Levels of CYP4A12A and ACY3 appeared markedly and reproducibly greatest in FVB/N males; lesser levels of these proteins were detected in B6 male glomeruli but they were barely detectable or undetectable in female glomeruli of either strain (Figure 4a). Immunohistochemistry of FVB/N male kidneys demonstrated CYP4A12A (Figure 4c–f) and HSD3B2 (Figure 4g–j) in the glomeruli; with some of the signal in podocytes as evidenced by double labeling with podoplanin. Furthermore, CYP4A12A was detected in western blots of both undifferentiated and differentiated cultured podocytes (Figure 4b). In

tissue sections, ACY3 was predominantly immunolocalized in parietal glomerular epithelia, with a faint signal in glomerular tufts (Figure 4k–n); we did not detect ACY3 in western blots of cultured podocytes.

Blood pressure, glomerular histology, and expression levels of other glomerular genes

Systolic and diastolic blood pressures were similar in male B6, male FVB/N, and female FVB/N mice, with female B6 mice tending to have higher pressures (Figure 5a and b). Glomerular histology was similar in the groups and sclerotic glomeruli were rare (Figure 5c–h). We interrogated microarray data, comparing transcript levels of genes implicated in the biology of podocytes (*Actn4, Fat1, Nck1, Nck2, Nphs1, Nphs2, Podxl, Synpo, Trpc6,* and *Wt1*), endothelia (*Ang1, Ang2, Ang3, Pecam1, Tie1, Tie2, Vegfa,* and *Vegfr2*), and glomerular basement membrane (*Agrn, Col4a1-5, Fn1, Hspg2, Lama1, Lama5, Lamb1, Lamb2,* and *Lamc1*). There was no significant effect of sex on their expression levels. However, in FVB/N versus B6 glomeruli of either sex, levels of the following transcripts were altered: *Col4a1* (decreased 1.4-fold; $P < 0.05$), *Nphs2* (decreased 2-fold; $P < 0.001$), *Podxl*
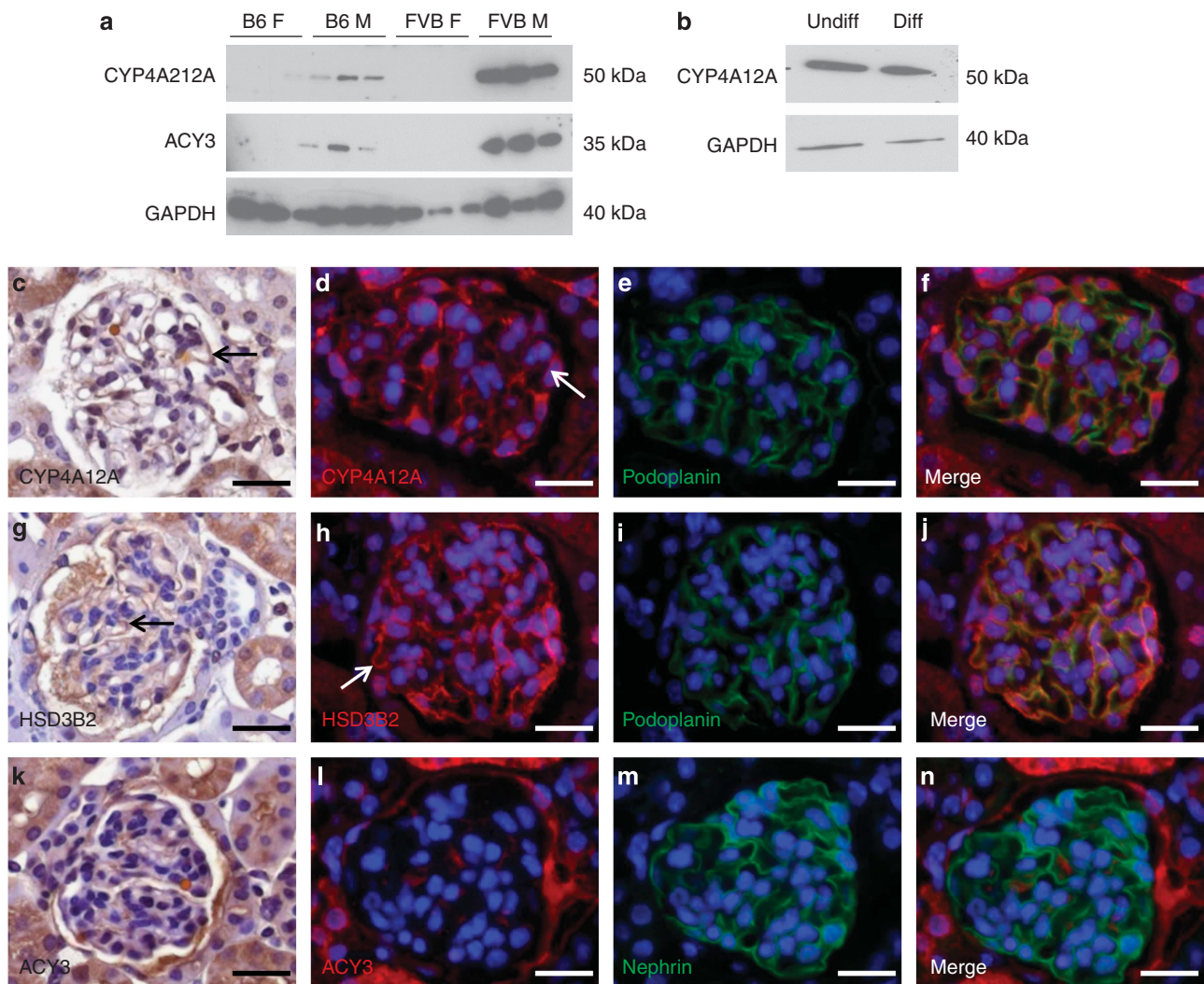
Figure 4 | Expression of candidate proteins in glomeruli. (a) Protein lysates from isolated glomeruli ($n = 3$ kidneys from each group) were used to immunoblot for CYP4A12A and ACY3. Levels of CYP4A12A and ACY3 appeared markedly and reproducibly greatest in FVB/N males; lesser levels of these proteins were detected in B6 male (M) glomeruli but they were barely detectable or undetectable in female (F) glomeruli of either strain; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to assess loading. (b) Protein isolates from both undifferentiated (Undiff) and differentiated (Diff) podocytes contained CYP4A12A protein. Using immunohistochemistry, CYP4A12A was immunodetected in the glomeruli of tissue sections obtained from FVB/N male kidneys; some of the signal was found in podocytes as evidenced by double labeling with podoplanin (c–f, arrows). HSDS3B2 was also detected in the podocytes of the glomeruli (g–j, arrows). ACY3 was predominately immunolocalized to parietal glomerular epithelia, with a faint signal observed in glomerular tufts (k–n). (c–n) Bars = 20 μm.

(decreased 2–3-fold; $P < 0.01$), and *Synpo* (decreased 2-fold; $P < 0.01$).

Testosterone administration in female mice

Sustanon 250, comprising testosterone esters, was administered to 5-week-old B6 and FVB/N females. At the start of the experiment, FVB/N tended to have higher excretion rates than B6 mice (18 ± 5 vs. 8 ± 2 μg per 18 h respectively, $P = 0.07$, $n = 12$ in each group). When evaluated at 9 weeks of age, Sustanon-exposed female B6 mice had increased serum testosterone than vehicle-administered controls (Figure 6a) and higher kidney/body weight ratios (Figure 6b). Compared with vehicle-treated mice, the Sustanon-exposed group had increases in albumin excretion rate ($373 \pm 71\%$, $P < 0.01$; Figure 6c) and albumin/creatinine ratios ($312 \pm 87\%$, $P < 0.05$; Figure 6d) but glomerular histology was similar (Figure 6e–g). Sustanon administration was associated with a 7-fold upregulation of *Acy3* and a 1072-fold upregulation of *Cyp4a12a* ($n = 4$ in each group, $P < 0.05$ in both cases). *In vitro*, addition of Sustanon to differentiating podocytes for 48 h caused significant elevation of *Cyp4a12a* transcripts (Figure 6h), with a tendency ($P = 0.09$) for *Acy3* levels to increase (Figure 6i). Sustanon administration to female FVB/N mice led to elevated serum testosterone levels (19 ± 3 vs. placebo 0.7 ± 0.1 ng/ml, $P < 0.001$, $n = 6$ in each group) and increased kidney/body weight ratios (1.3 ± 0.1 vs. vehicle alone $0.9 \pm 0.1 \times 10^{-2}$, $P < 0.001$), but no significant effect on albuminuria.
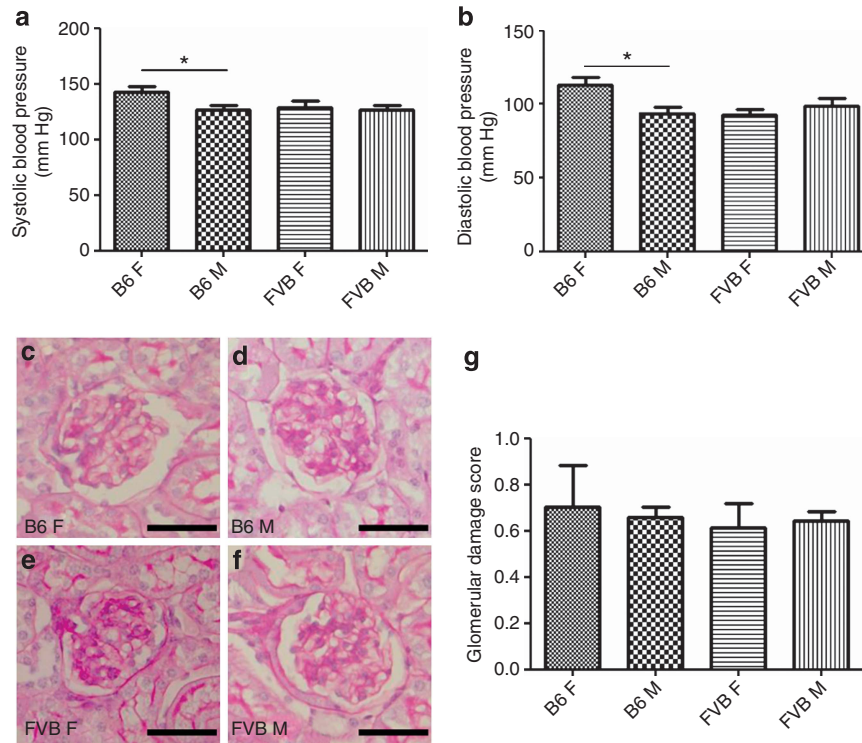
Figure 5 | **Blood pressure and glomerular histology in 18-week-old B6 and FVB/N mice.** (**a**) Systolic and (**b**) diastolic blood pressure was not positively associated with alterations in albuminuria, with the only differences found between B6 female (F) and male (M) mice, with the former having elevated pressures. Blinded analysis of glomerular histology revealed that there were no differences between (**c**) B6 female, (**d**) B6 male, (**e**) FVB/N female, and (**f**) FVB/N male mice; (**g**) quantification is shown. $*P < 0.05$, $n = 5$ for blood pressure analysis and $n = 3$ for glomerular analysis in each group. (**c–f**) Bars = 30 μm. All data are presented as means and s.e.m.

We considered the fact that exogenous testosterone increased albuminuria in B6 but not FVB/N females might be explained if the latter had higher endogenous testosterone levels. However, in 18-week-old mice there were no differences in testosterone levels between sex-matched animals of either the FVB/N or the B6 strain (B6 females, 0.5 ± 0.1; FVB/N females 0.4 ± 0.1; B6 males 5.1 ± 1.8; and FVB/N males 7.2 ± 3.4 ng/ml, $n = 9$ in each group).

Glomerular numbers in male and female FVB/N and B6 mice

We measured glomerular numbers/kidney in male and female 18-week-old B6 and FVB/N mice. Here, it should be noted that mouse glomerulogenesis starts a week before birth and continues through the first postnatal week; no new glomeruli can form after this period because the nephrogenic zone disappears.[21] Across the strains and sexes, the numbers of glomeruli (whether expressed as the total/kidney, or factored for either kidney or body weight) was inversely correlated with albuminuria. Therefore, glomerular numbers were: female B6 > male B6 > female > FVB/N > male FVB/N, whereas the degree of albuminuria was the exact opposite (Figure 7a–c).

DISCUSSION

Albuminuria is often quantified as urinary albumin/creatinine concentrations but up to one half of urinary creatinine in mice is derived from tubular secretion, and this contribution may vary between sexes.[22] Therefore, to compare albuminuria between sexes, a more robust measure might be to measure the absolute amount of albumin excreted per unit time. We found that, as assessed by either parameter, a characteristic and reproducible level of urinary albumin excretion had been established by early adulthood for each of the four groups of healthy mice. Specifically, we found increasing albuminuria in female B6 < male B6 < female FVB/ N < male FVB/N mice. Considering that a human is ~2000 times heavier than a mouse, the albumin excretion rate range between female B6 and male FVB/N mice (~15–150 μg/day) is similar to the range for human microalbuminuria (30–300 mg/day). Hence, our models may have relevance to humans with modestly elevated albuminuria.

Proximal tubules endocytose filtered macromolecules.[23] Although this will modify the amount of albumin in the final urine, we reasoned that because the glomerular filtration barrier is the main player in determining albuminuria, then the degree of albuminuria should reflect glomerular biology changes. Within whole glomeruli *in vivo*, across both strains and sexes, we identified reproducible changes in gene expression correlating with albuminuria. Most of these transcripts were also detected in cultured podocytes; perhaps those that were not would be expressed by other tuft cells such as endothelia and/or mesangial cells. Alternatively, some of them, such as genes encoding transporters, might have been
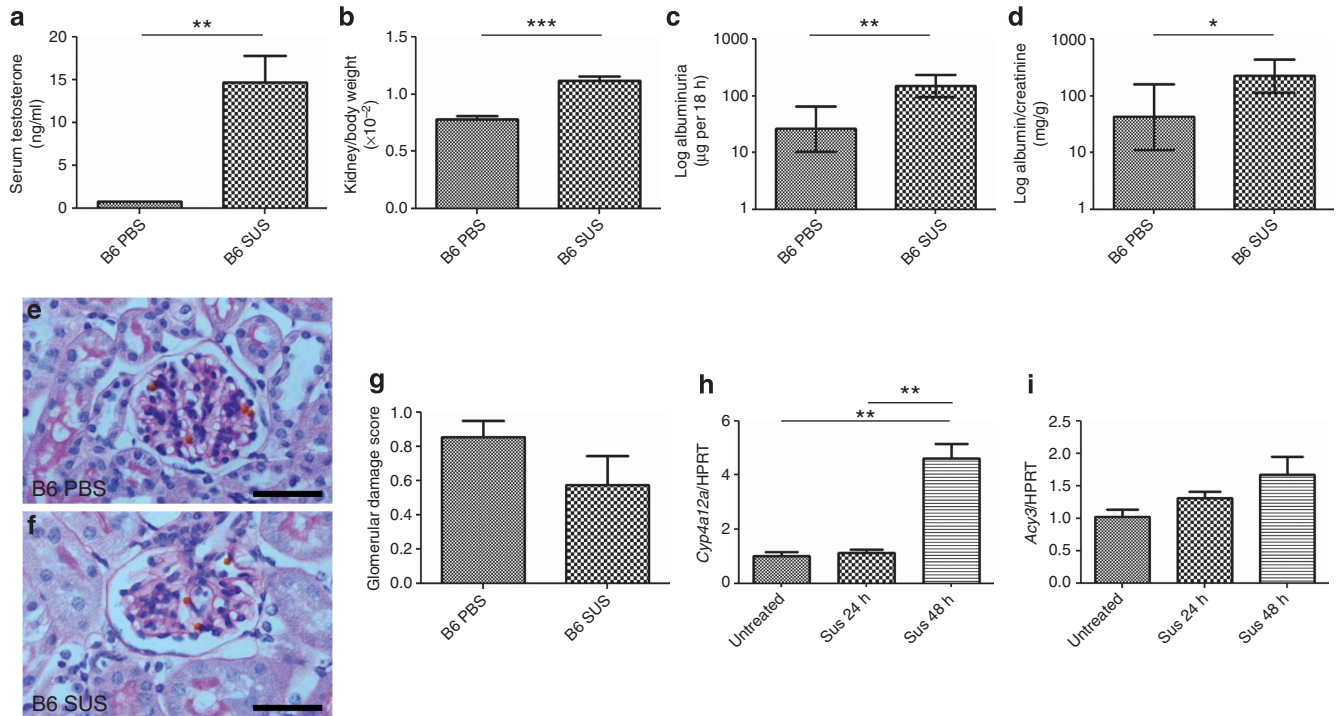
Figure 6 | Administration of testosterone to female B6 mice. Sustanon-250 injection for 4 weeks in 5-week-old B6 mice ($n = 6$ in each group) led to a significant increase in (**a**) serum testosterone, (**b**) kidney/body weight, (**c**) albumin excretion rate, and (**d**) albumin/creatinine ratio. Despite these changes, blinded analysis of glomerular histology showed that there was no difference between B6 female mice injected with either (**e**) phosphate-buffered saline (PBS) or (**f**) Sustanon (SUS); (**g**) quantification is shown. (**h**) Stimulation of mouse podocytes *in vitro* ($n = 3$ in each group) with Sustanon-250 led to a significant increase in *Cyp4a12a* mRNA levels; hypoxanthine-guanine phosphoribosyltransferase (hprt) was used as a housekeeping gene. (**i**) *Acy3* mRNA tended to be elevated following Sustanon-250 exposure but this was not significant *$P<0.05$, **$P<0.01$; ***$P<0.001$ between groups. (**e**, **f**) Bars = 30 μm. All data are presented as means and s.e.m. except **c** and **d** that were log transformed before analysis and are presented as geometric means and confidence interval.
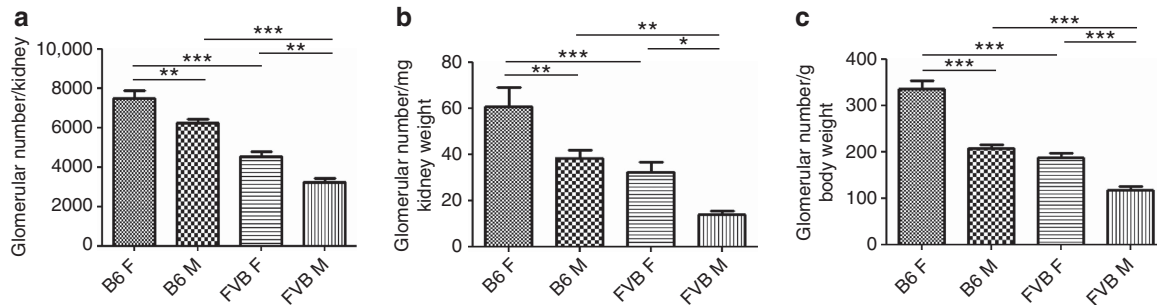

Figure 7 | Glomerular counts in B6 and FVB/N 18-week-old mice. (**a**) A reduction in glomerular number was observed in male (M) mice compared with strain-matched females (F); in addition, FVB/N had significantly lower number of glomeruli compared with sex-matched B6 mice. Similar changes were calculated when either (**b**) kidney or (**c**) body weight of the mice was taken into account (*$P<0.05$, **$P<0.01$; ***$P<0.001$ between groups, $n = 6$ in each group). All data are presented as means and s.e.m.

expressed by Bowman's capsule epithelia observed, adhering to tufts in some isolated glomeruli.

Cyp4a12a encodes a cytochrome *P*450 involved in degradation of long-chain fatty acids.[24] For arachidonic acid, this yields 20-hydroxyeicosatetraenoic acid, implicated in pathogenesis of systemic hypertension.[25] Thus, a possible explanation for differences in albuminuria between mouse sexes and strains were variations of systemic blood pressure, assuming higher pressures would increase hydrostatic pressure within glomerular capillaries and/or trigger physical damage, manifested for example by glomerulosclerosis.[15]

We found no evidence for a positive relation between albuminuria and blood pressure or glomerular dysmorphology. High glucose elevates CYP4A protein and 20-hydroxyeicosatetraenoic acid in podocytes, the effects ameliorated by pharmacological CYP4A inhibition.[26] In diabetic nephropathy mice, glomerular CYP4A is elevated, and blockade of this pathway attenuates albuminuria.[26]

Hsd3b2 encodes 3-β-hydroxysteroid dehydrogenase, essential for steroid synthesis.[27] *Hsd3b2* and related steroidogenic transcripts are expressed in fetal kidneys, although their specific localization was unreported.[28] Pezzi *et al.*[28]

speculated that low levels of the encoded proteins might produce local, intrarenal effects. HSD3B2 modulates biosynthesis of glucocorticoids that themselves alter glomerular biology.[29] Isolated glomeruli express the glucocorticoid receptor,[30] and here glucocorticoid exposure has antiapoptotic and anti-inflammatory effects.[31,32] In contrast, high-dose glucocorticoids in normal adult mice cause proteinuria, mesangial expansion, and glomerulosclerosis,[33] and exposure to developing murine kidneys retards organ growth[34] and glomerulogenesis.[35]

Acy3 encodes aspartoacylase 3, implicated in detoxifying xenobiotics[36] and metal ion binding.[37] ACY3 is upregulated during mycophenolate mofetil treatment of a collagen-4α3-deficient renal fibrosis model.[38] The enzyme binds cobalt and nickel[37] and, notably, anionic glycosaminoglycans in the glomerular basement membrane also bind divalent cations.[39] Pushkin *et al.*[36] reported expression of the protein in proximal tubules; in this study, we found that ACY3 was present in parietal glomerular epithelia. In cultured podocytes, RT–PCR detected *Acy3* transcripts but the encoded protein was not detectable on western blotting. As demonstrated in Figure 2, a subset of harvested glomeruli have a Bowman's capsule, and thus the most likely interpretation is that the association of levels of *Acy3* transcripts with albuminuria may predominantly reflect a secondary effect of ultrafiltered protein on parietal epithelia.

Our study indicated that male sex was associated with elevated albuminuria, and hence we hypothesized that androgens would increase albumin excretion. Testosterone administered to B6 females did so, accompanied by upregulated glomerular *Acy3* and *Cyp4a12a* transcripts; levels of the latter also increased in testosterone-exposed cultured podocytes. Indeed, Muller *et al.*[24] demonstrated that, in whole kidneys, *Cyp4a12a* transcript and protein levels in males exceeded those in females, and administration of testosterone increased the levels. They showed differences in expression between males of different strains correlating with the capacity of renal microsomes to hydroxylate arachidonic acid.[24] Supporting the contention that sex steroids affect expression of transcripts implicated in albuminuria, five genes identified in the array contain predicted androgen response elements (*Aass*, *Aqp1*, *D14Ertd449e*, *Fam132a*, and *Slc22a6*). The testosterone receptor has been shown to be expressed in isolated glomeruli[40] and cultured podocytes,[41] and hence these cells may be a direct target for sex steroids. Furthermore, incubation of cultured podocytes with testosterone increases apoptosis, which can be abolished by androgen receptor blockade.[41] *In vivo*, testosterone supplementation to ovariectomized B6 wild-type mice increases albuminuria, glomerulosclerosis, and podocyte apoptosis.[40,41] *Hsd3b2* is also essential for biosynthesis of testosterone[29] and perhaps increased levels of *Hsd3b2* within glomeruli increase local testosterone concentrations, with detrimental effects.

Sustanon increased albuminuria in B6 females but not in FVB/N females. From our microarray, we did not find changes in glomerular transcript levels of the androgen receptor between FVB/N and B6 mice of either sex. Another possible explanation for sex differences in albuminuria would be that estrogen is protective. Indeed, 14 of the genes implicated in our array analyses contain predicted estrogen response elements (*Acy3*, *Aqp1*, *Cideb*, *D14Ertd449e*, *Gnmt*, *Pigr*, *Slc5a8*, *Slc6a20b*, *Slc7a13*, *Slco1a1*, *Sult1c2*, *Tmem82*, *Tpmt*, and *Zfp622*). Estrogen receptors are expressed on podocytes[41,42] and estradiol attenuates podocyte apoptosis induced by puromycin or testosterone.[41,42] Furthermore, mice lacking estrogen receptor-α develop albuminuria and glomerulosclerosis.[40,41] Although circulating estrogen was not measured in this study, estrogen receptor 1 was increased in FVB/N versus B6 glomeruli (by 3.5- and 1.9-fold in male and females, $P < 0.01$). If estrogen effects were to be higher in FVB/N than B6 glomeruli, this may explain why the filtration barrier of FVB/N female animals was resistant to Sustanon-250 administration.

Our experiments using Sustanon were only partially consistent with the contention that androgens enhance albuminuria. Furthermore, male FVB/N mice had elevated albuminuria versus male B6 mice, although both had similar circulating testosterone levels. Therefore, we sought a different explanation for the variation in albuminuria between sexes and strains. Strikingly, numbers of glomeruli/kidney for the four experimental groups showed exactly the inverse pattern to the degree of albuminuria. It should be noted that we used acid dissociation[17] rather than a 'gold standard' sterology method[43] to assess glomerular numbers. However, the dissociation technique should allow valid relative comparisons between numbers of glomeruli/kidney between the groups studied, none of which had anatomically deranged or sclerotic kidneys. Recurrent observations have shown that low glomerular numbers per kidney correlate with systemic hypertension in white subjects,[18,44] but we did not observe an inverse relationship between blood pressure and glomerular counts in this study.

A glomerular deficiency could potentially lead to glomerular hyperfiltration that may cause microalbuminuria associated with activation of the renin–angiotensin system.[15,45] Although we found no positive correlation of albuminuria with systemic blood pressure in FVB/N compared with B6 mice, we noted increased glomerular transcript levels of *Ace1* (4.8-fold in males and 1.8-fold in females, $P = 0.02$) and *Ace2* (2.6-fold in males and 1.6-fold in females, $P = 0.02$) suggesting that renin–angiotensin system may be enhanced in the FVB/N strain of mice. There was no evidence of glomerular structural changes between sex- or strain-matched male and female B6 and FVB/N mice. However, there were some changes in the mRNA expression of several glomerular genes with loss of podocyte genes *Nphs2*, *Podxl*, *Synpo*, and the basement membrane gene *Col4a1*. These differences indicate that the molecular make-up of the filtration barrier may differ between FVB/N and B6 mice that potentially could lead to albuminuria.

We have demonstrated that low numbers of glomeruli and testosterone are potential mechanisms leading to albuminuria in mice. Several unsuspected genes have been identified that correlate with albuminuria, but further studies are needed to demonstrate whether these changes are simply reactions to albuminuria or may drive glomerular pathobiology. Some of these genes may also influence glomerulogenesis, although none have yet been functionally implicated in kidney development *per se*. Several of the genes coding for transcripts highlighted in this study are located within the mapped intervals for albuminuria QTL in mice and rats. For example, *Cyp4a12a* lies within the interval of an albuminuria QTL found in a cross between C57BL/6J and A/J[46] and a cross between C57BL/6J and NZM.[47] *Acy3* is located within albuminuria QTL found in crosses between C57BL/6J and A/J mice[46] and between MWF and SHR rats.[48] *Mogat1* maps within the albuminuria QTL found in a cross between MWF and SHR rats[48] and both *Hsd3b2* and *Mogat1* map within albuminuria QTL found from a cross between SS and SHR rats.[49] However, it is important to note that the QTL intervals are large and contain hundreds of genes. Expression analysis in the kidney for the above-mentioned genes in these mouse and rat strains would be necessary to establish whether these could be candidate genes for the QTL. Most interestingly, the human homolog of *Nat8* has been associated with estimated glomerular filtration rate in several human genome-wide association studies.[50,51] Furthermore, Juhanson *et al.*[52] resequenced the *NAT8* promoter and suggested that polymorphisms therein determined systolic blood pressure and glomerular filtration rate. Proteins encoded by these transcripts could be novel biomarkers and therapeutic targets for early kidney and vascular disease.

MATERIALS AND METHODS

Assessment of B6 and FVB/N mice

Reagents were obtained from Sigma Chemical Company (Gillingham, Dorset, UK) unless stated. C57BL/6JOlaHsd and FVB/NHanHsd male and female mice (Harlan, Oxfordshire, UK) were provided with Teklad 18% protein rodent diet (Harlan) and water *ad libitum* and used in protocols approved by the UK Home Office. Urine was collected from 13- and 18-week-old mice by housing them individually in metabolic cages for 18 h. Albumin concentrations were measured by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX). Urinary creatinine concentrations were assessed by a commercially available assay (Cusabio, Newark, DE). At 18 weeks, systolic blood pressure was assessed using a noninvasive blood pressure system (Kent Scientific, Torrington, CT[53]); measurements were taken in duplicate at the same time on subsequent days after animals had been trained every day for the prior two weeks. Mice were then killed and body and kidney weights measured. Blood was collected and testosterone levels assessed using a commercially available assay (Alpha Diagnostic International, San Antonio, TX[54]).

Microarray analysis of isolated glomeruli

Eighteen-week-old male and female B6 and FVB/N mice ($n = 3$ in each group) were anesthetized and perfused with 1×10^8 Dynabeads (Invitrogen, Paisley, UK) through the left ventricle of the heart.[55]

Kidneys were removed, decapsulated, minced, and digested,[55] and the glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and RNA prepared using RNeasy kit (Qiagen, Crawley, UK). RNA quality was assessed on the Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA); subsequent cDNA and cRNA synthesis was performed and hybridized to mouse MOE430 2.0 GeneChips. Functional categories for genes were assigned using the Database for Annotation, Visualization and Integrated Discovery software (http://david.abcc.ncifcrf.gov). For transcripts up- or downregulated with albuminuria, we used the ClustalW tool (http://www.ebi.ac.uk/Tools/clustalw/index.html) to determine whether the 1000 bp upstream of the transcription start site, in addition to either the complete 5′-untranslated region or the first exon of the 5′-untranslated region when this is split over several exons, contained sequences with at least 75% homology to the consensus sequence of the androgen response element (AGAACAnnnTGTTCT)[56] or estrogen response element (AGGTCAnnnTGACCT).[57] We compared our data with prior studies that have identified QTLs related to albumin excretion in the mouse[19] and candidate genes implicated in albuminuria using a genome-wide association approach.[11]

Quantitative real-time PCR

Independent glomerular extracts were obtained from 18-week-old male and female B6 and FVB/N mice ($n = 4$ in each group) for qPCR. cDNA was prepared and 1 μg used to examine *Acy3*, *Cyp4a12a*, *Hsd3b2*, *Pln*, *Treh*, and *Trf* using previously described methods,[58] with *Hprt* as a housekeeping gene. All measurements were performed in duplicate; primer details are available on request.

Western blotting of isolated glomeruli

Protein was extracted from isolated glomeruli using radioimmuno-precipitation assay buffer, quantified using bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL) and 10 μg separated on a 4–15% Mini-Protean gel and transferred to a nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK). Blots were probed with either rabbit anti-ACY3 (Abcam, Cambridge, UK) or rabbit anti-CYP4A12A.[24] Appropriate secondary antibodies were used and blots visualized using chemiluminescence. To confirm loading, blots were stripped and reprobed with mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Abcam).

Histological analysis and assessment of glomerular numbers

Kidneys were fixed in 4% paraformaldehyde, embedded, and 5 μm sections were then cut for staining with periodic acid–Schiff reagent. A total of 50 glomeruli were scored by a blinded observer using the following system: 0, normal glomerular structure; 1, glomeruli with mesangial expansion but some capillary loops still observed; and 2, sclerotic glomeruli with no capillary loops; an average score was obtained for each kidney. Immunohistochemistry was performed in FVB/N male mice as described[59] for ACY3 and CYP4A12A using the primary antibodies above and rabbit anti-HSD3B2; double labeling for these proteins was performed with either guinea-pig anti-nephrin (Progen, Heidelberg, Germany) or hamster anti-podoplanin antibodies (Abcam). Negative controls consisted of omission of primary antibodies or substitution with preimmune serum. Glomerular number was assessed from one kidney of 18-week-old male and female B6 and FVB/N mice using the acid digestion method.[17]

Sustanon administration

Five-week-old female B6 and FVB/N mice were administered either 10 μl of phosphate-buffered saline or Sustanon-250 (Organon Laboratories, Hoddesdon, UK) intramuscularly every 2 weeks ($n = 6$ in each group). After 4 weeks, overnight urine was collected and blood obtained to assess systemic testosterone levels. Animals were killed following Dynabead perfusion; half a kidney was embedded in 4% paraformaldehyde and the remaining material used to extract glomerular RNA for qPCR to examine *Cyp4a12a* and *Acy3*.

Cell culture

Podocytes isolated from 10-week-old H-2K^b-tsAJ58 mice[20] were cultured as described[60] and induced to differentiate for 7 days; at this time point, they formed extensive processes. Using TRI Reagent, 1 mg of RNA was isolated from proliferating and differentiating podocytes and cDNA prepared for RT–PCR of the genes identified in the microarray analysis. In addition, 30 μg of protein was extracted and western blotting performed for CYP4A12A and ACY3. Some cells were also plated onto chamber slides for phalloidin staining to visualize actin filaments. In other experiments, podocytes differentiated for 7 days were exposed to Sustanon-250 (250 μg/ml) for either 24 or 48 h and qPCR performed for *Cyp4a12a* and *Acy3*.

Statistical methods

Microarray. Signal values for transcripts on the Affymetrix array were calculated using the MAS 5.0 algorithm to generate .chp files that were exported to GeneSpring 9.0 (Agilent Technologies, Wokingham, Berkshire, UK) for further analysis. The MAS 5.0–generated values were log$_2$ transformed, normalized to the median within each array (to control for array loading), and these values were then baseline transformed to the median value of each transcript. Transcripts were filtered to exclude genes whose expression did not reach a threshold value for reliable detection (based on the relaxed Affymetrix MAS 5.0 probability of detection; $P \leqslant 0.1$) in at least 1 of the 12 chips assessed. To determine genes modified by strain and sex, a two-way analysis of variance analysis was performed. Transcripts that were shown to be significantly altered by both strain and sex ($P < 0.05$ after applying the Benjamini and Hochberg false discovery multiple testing correction[61]) were used in subsequent analysis. We then examined expression levels in female FVB/N versus female B6 mice, male FVB/N versus male B6 mice, male versus female B6 mice, and male versus female FVB/N mice, and genes were excluded if mean normalized expression levels did not vary by > 1.5-fold in all of these individual comparisons. Genes were also excluded if expression levels did not follow the degree of albuminuria observed in the four groups assessed. Expression differences are presented relative to B6 female mice (given a relative value of 1) that had the lowest albumin excretion rate.

Other statistical analyses. Blood pressure, glomerular histology, and number and qPCR were presented as mean ± s.e.m. and assessed by one-way analysis of variance with least significant difference correction. In experiments where testosterone administration was provided to female mice, the vehicle versus treated animals were assessed using unpaired two-tailed *t*-tests. In cases where the data were not normally distributed (albuminuria and some of the qPCR analysis; Figure 2d–i), the results were log transformed and presented as geometric mean (± 95% confidence intervals) and then analyzed by one-way analysis of variance with least significant difference correction. Significance was accepted at $P < 0.05$.

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