

RESEARCH ARTICLE**The renoprotective effects of soy protein in the aging rat kidney****Authors**

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Abstract

Aging is a risk factor for chronic kidney disease (CKD) and is itself associated with alterations in renal structure and function. There are no specific interventions to attenuate age-dependent renal dysfunction and the mechanism(s) responsible for these deficits have not been fully elucidated. In this study, male Fischer 344 rats, which develop age-dependent nephropathy, were fed a casein- or soy protein diet beginning at 16 mon (late life intervention) and renal structure and function was assessed at 20 mon. The soy diet did not significantly affect body weight, but was renoprotective as assessed by decreased proteinuria, increased glomerular filtration rate (GFR) and decreased urinary kidney injury molecule-1 (Kim-1). Renal fibrosis, as assessed by hydroxyproline content, was decreased by the soy diet, as were several indicators of inflammation. RNA sequencing identified several candidates for the renoprotective effects of soy, including decreased expression of Twist2, a basic helix-loop-helix transcription factor that network analysis suggest may regulate the expression of several genes associated with renal dysfunction. Twist2 expression is upregulated in the aging kidney and the unilateral ureteral obstruction of fibrosis; the expression is limited to distal tubules of mice. Taken together, these data demonstrate the renoprotective potential of soy protein, putatively by reducing inflammation and fibrosis, and identify Twist2 as a novel mediator of renal dysfunction that is targeted by soy.

Keywords: aging, chronic kidney disease, fibrosis, inflammation, soy, Twist2

Background

The percentage of the U.S. population over 65 is now 12%, by the year 2040, it is expected to rise to 21% [1]; this will likely be accompanied by an increase in age-related disease [2]. The normal kidney loses about 20-25% of its mass during aging, with the loss involving both glomeruli and tubules [3]. In the Baltimore Longitudinal Study of Aging, an average decrease of 1 ml/min/yr in glomerular filtration rate was shown in humans over time. However, 30% of the patients had no change in GFR, suggesting that age-related decreases in kidney function are not inevitable [4]. Although not identical, aging and CKD share common features [5] and age is a risk factor for CKD [2,6]. Structurally, many of the changes observed in the aging human kidney are seen in rats, including thickening of the glomerular basement membrane and degenerative changes in the proximal tubule; the most notable functional changes are proteinuria and reduced urine concentrating ability [7,8]. The male Fischer 344 develops severe glomerulosclerosis and tubulointerstitial fibrosis similar to human CKD [9].

The renoprotective effects of a soy-based diet were demonstrated twenty-five years ago using the male Fischer 344 rat model of CKD. In 1988, Kalu *et al.* demonstrated that life-long feeding of a soy-based diet attenuated the late-life (21 months and older) increase in serum creatinine [10]. Iwakai *et al.* used a similar protocol and demonstrated that median life span of the control rats was 730 days, compared to 844 in the soy-fed rats [11]. In the control group, 41% of the rats that spontaneously died exhibited end-stage renal disease, which was reduced to 7% by the soy diet. In the Pcy mouse model of polycystic kidney disease (PKD), a soy diet attenuates cyst volume and renal dysfunction [12-14]. This effect has been verified in the Han-SPRD rat

model of PKD^{3,102}. The renoprotective effects of soy protein have also been shown in metabolic models of renal dysfunction, including Zucker diabetic fatty and high-fructose fed rats [15-17].

The renoprotective effects of soy have also been demonstrated in humans. In an early clinical study, Azadbakht *et al.* used a randomized, crossover study (7 weeks of dietary intervention, 4 week washout, 7 weeks of diet) and showed a reduction in proteinuria and blood urea nitrogen (BUN), but not serum creatinine, in 14 patients with proteinuria between 300-1000 mg/day [18]. In a 4 year trial, a soy-based diet was shown to decrease proteinuria and levels of C-reactive protein, a circulating biomarker of inflammation, in 41 diabetic nephropathy patients [19]. In male patients with type 2 diabetic nephropathy, a diet containing isolated soy protein was shown to significantly reduce albuminuria in a crossover study [20].

In these studies, we used a well-characterized model - the male Fischer 344 rat - to determine if a late-life intervention with a soy-based diet was renoprotective against a naturally occurring, progressive model of renal dysfunction. In addition, we used RNA sequencing and network analysis to identify novel targets of soy that may mediate the renoprotective effect.

Results

Our laboratory has been investigating age-dependent CKD and has identified several mechanisms that contribute to renal dysfunction [21-24]. Importantly, we have shown that renal dysfunction is detectable at 16 months in male Fischer 344 rats [25]. Our initial studies used a dietary intervention with soy - a low-fat, semi-purified diet containing 14% by weight soy protein - beginning at 16 months (Table 1). Rats were fed the casein or soy diet for the next four months; the soy diet contained 916

mg/g diet of isoflavones (diadzin, glycitin, genistin, daizein, glycitein, genistein, formononetin) and 283 mg/g of phytosterols (campesterol, stigmasterol, β -sitosterol). We assessed renal function every month, and the animals (n=6 animals/group for control; 5

for soy) were sacrificed at 20 months for histological evaluation of the kidney. No difference in body weight or renal:body weight ratio was seen in the soy fed animals compared to the control (**Figure 1**).

Figure 1

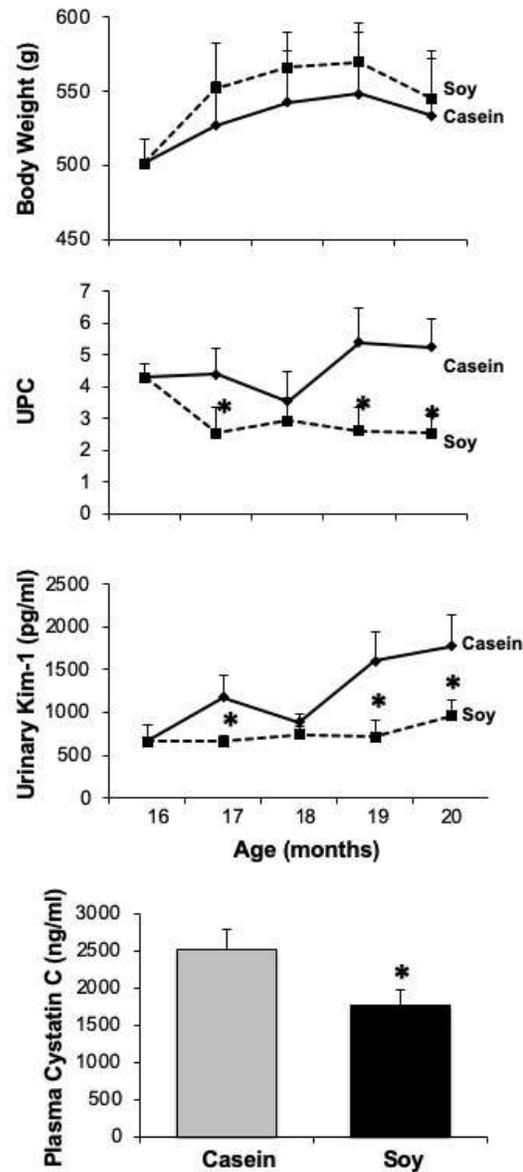


Figure 1: Soy Attenuates Age-Dependent Renal Dysfunction. Male Fischer 344 rats were fed a soy-based diet for 4 months, beginning at 16 months of age. Soy did not significantly affect body weight over the 4 month study, but did decrease proteinuria (UPC), urinary Kim-1 levels and the age-dependent loss of GFR at 20 months as assessed by serum cystatin C. Each data point represents the mean+SD of 6 (control) or 5 (soy) animals; * indicates a significant difference from control (p < 0.05).

Proteinuria, as assessed by measuring the ratio of protein:creatinine (UPC) in the urine, is a highly predictive measure of renal dysfunction. The soy diet completely prevented age-dependent increases in proteinuria (**Figure 1**). Interestingly, within the first month of soy-based intervention, a reduction in proteinuria was observed, suggesting a rapid effect of the soy diet. Importantly, in a second independent study using the same soy diet we have replicated the finding that the soy protein diet decreased UPC as compared to the control diet after two months of intervention (16-18 months); control 3.0 + .3 versus soy 2.0 + .20 (n=6). These results demonstrate that the renoprotective effect of soy is reproducible in our rat model. Kim-1 (*Havcr1*) is a type 1 transmembrane protein that has previously been shown to be a urinary biomarker for acute tubular injury in patients [26]. We

have shown that Kim-1 levels are elevated in the aging kidney, suggesting that Kim-1 may also have value as a chronic kidney disease biomarker [27,28]. Recent data have demonstrated that overexpression of Kim-1 contributes to disease progression through fibrosis [29], further suggesting a role in CKD. In our studies, soy reduced gene expression of Kim-1 (80% reduction as compared to controls; data not shown) as well as urinary Kim-1 levels (**Figure 1**). GFR is a functional assessment of the kidney and, in fact, a decrease in GFR is the definition of renal failure. Plasma cystatin C levels are proposed to be a more sensitive measure of GFR as compared to the commonly used plasma creatinine value [30]. In our model, soy attenuated age-dependent increase in plasma cystatin C (**Figure 1**), suggesting that GFR was maintained by soy.

Figure 2

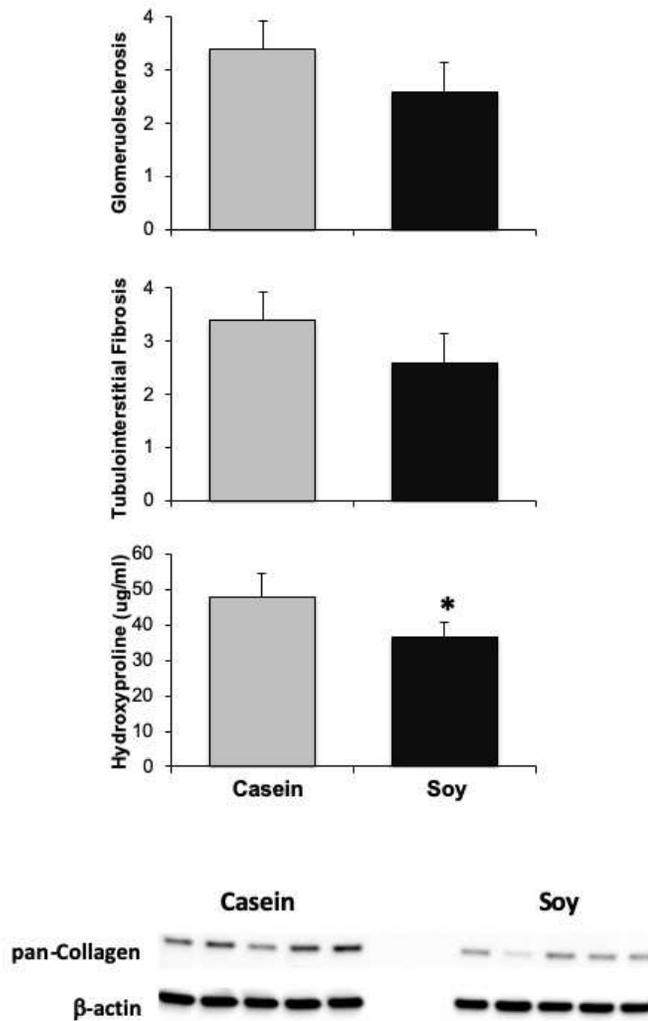


Figure 2: Soy Attenuates Age-Dependent Fibrosis. Blinded histological evaluation of glomerulosclerosis and tubulointerstitial demonstrates a non-significant trend toward a reduction by the soy diet; samples were scored on a 1-4 scale; 1=none, 2=mild, 3-moderate, 4=severe. Hydroxyproline content was reduced by the soy diet. The reduction in collagen 1 was confirmed by western blot analysis, each lane represents an individual animal. Each data point represents the mean+SD of 6 (control) or 5 (soy) rats; * indicates a significant difference from control ($p<0.05$).

The impact of soy on fibrosis was also examined. Blinded histological evaluation demonstrated that while glomerulosclerosis and tubulointerstitial fibrosis were lower in the soy diet, the results were not significant. Hydroxyproline content, an indicator of collagen content, was significantly reduced by the soy diet at 20 months. (Figure 2). The reduction in type 1 collagens by the soy diet was also confirmed by western blot with

a pan-Collagen I antibody (Figure 2). Blinded evaluation of inflammation in the kidney (1=normal 4=severe interstitial inflammation), showed that soy significantly decreased tubulointerstitial inflammation (Figure 3). Tissue myeloperoxidase content, an indicator of inflammation [31], was also decreased by the soy diet, as was the nuclear level of the p65 subunit of NF-kB (Figure 3).

Table 1: Diet Composition

Ingredient	Casein Diet (g/kg)	Soy Diet (g/kg)
Cornstarch	465.9	463.83
Casein	140	
Lo-fat Soy Protein*		140
L-cystine	1.78	1.78
L-methionine		1.78
Dextrose	155	155
Sucrose	100	100
Fiber (a-cellulose)	50	50
Mineral Mix (#210050)**	35	
Mineral Mix (#210057)**		35
Vitamin Mix (#310025)**	10	10
Choline Bitartrate	2.5	2.5
Soybean oil	40	40
Tert-butylhydroquinone	0.008	0.008
Food Dye (green)		0.1

*Solae 1b.1.2 UN30CA

** Dyets, Inc. custom-formulated mixes

Figure 3

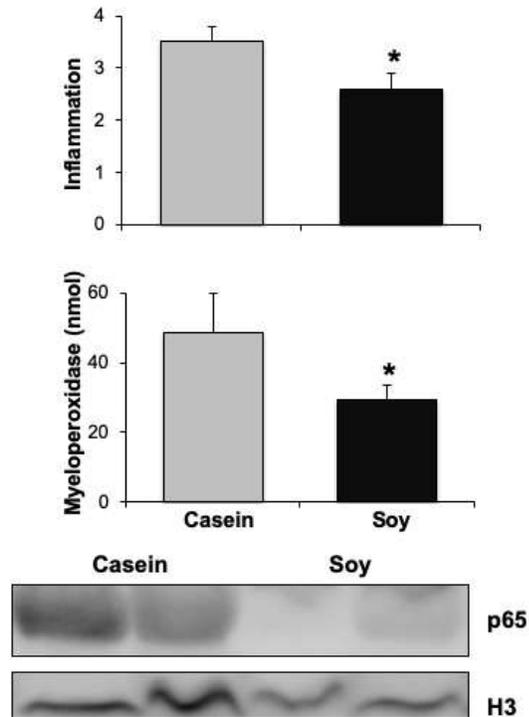


Figure 3: Soy Attenuates Age-Dependent Inflammation. The anti-inflammatory effect of soy was shown by blinded histological evaluation of inflammatory cell infiltration in the kidney; samples were scored on a 1-4 scale; 1=none, 2=mild, 3-moderate, 4=severe inflammation. Tubulointerstitial fibrosis is shown by the arrows. Soy also attenuates age-dependent increases in tissue myeloperoxidase. Each data point represents the mean+SD of 6 (control) or 5 (soy) rats; * indicates a significant difference from control ($p < 0.05$). A reduction of NF- κ B was demonstrated by blotting nuclear fractions of renal tissue from control and soy diet rats at 20 month with anti-p65 and histone H3 antibodies. Each lane represents a protein from an individual rat.

RNAseq analysis was performed on RNA harvested from cortical tissue from control and soy diet animals (**Table 2**). Statistically significant reductions in expression of several genes that our laboratory had previously correlated with dysfunction in the aging kidney were found,

including MMP-7 [23,27] and Kim-1 [27,28]. Using network analysis of the RNAseq data and modeling/network analysis, Twist2, a basic helix-loop-helix transcription factor [32], was identified as a potential regulator of several genes that were downregulated by soy, including Kim-1,

collagen (Col) 4a1, chemokine (C-C- motif 5 (CCL5), CCL19 and CCL 21 (**Figure 4**). Importantly, expression of Twist2, but not the highly related Twist1, is reduced by the soy diet (**Figure 4**). Gene expression of kidneys from aging rats indicates that Twist1 expression is stable over their lifespan, while the expression of Twist2 is significantly increased at 16-24 months (**Figure 5**). The increased expression is prevented by caloric restriction, suggesting a

role in the pathogenesis of age-related renal dysfunction as caloric restriction prevents age-dependent fibrosis and renal dysfunction. Increased Twist2, but not Twist1, expression is also seen in aged C57Bl/6 mouse kidneys (26 months) and in 4 month old Zucker obese rats, other models of renal fibrosis and dysfunction (data not shown).

Table 2: Genes Significantly Changed by Soy Diet

<i>Adamts 1</i>	0.26	<i>Col4a1</i>	0.45	<i>Lcp1</i>	0.47	<i>Slc7a7</i>	0.42
<i>Adra1d</i>	0.28	<i>Col5a2</i>	0.48	<i>Lgals3bp</i>	0.48	<i>Slfn8</i>	0.44
<i>Atf3</i>	0.40	<i>Col6a1</i>	0.44	<i>Lsp1</i>	0.41	<i>Slpi</i>	0.26
<i>Axl</i>	0.38	<i>Col6a2</i>	0.52	<i>Ltbp2</i>	0.33	<i>Trpv5</i>	0.20
<i>B4galnt4</i>	0.29	<i>Coro1a</i>	0.43	<i>Ltbp2</i>	0.26	<i>Ttk</i>	0.17
<i>Birc3</i>	0.44	<i>Cr2</i>	0.18	<i>Mgp</i>	0.41	<i>Tubb6</i>	0.32
<i>Blk</i>	0.25	<i>Crfl1</i>	0.22	<i>Mmp12</i>	0.33	<i>Twist2</i>	0.25
<i>C6</i>	0.27	<i>Cxcr4</i>	off	<i>Mmp7</i>	0.16	<i>Tyrobp</i>	0.45
<i>Calb2</i>	0.08	<i>Dcdc2</i>	0.48	<i>Mpeg1</i>	0.48	<i>Ubd</i>	0.32
<i>Car15</i>	0.54	<i>Dpp7</i>	0.53	<i>Myo1f</i>	0.42	<i>Vim</i>	0.39
<i>Car3</i>	0.22	<i>Ephb6</i>	0.37	<i>Myo9b</i>	0.29		
<i>Cbln2</i>	0.11	<i>Fbn1</i>	0.43	<i>Nkg7</i>	0.30	<i>Akr1b1</i>	2.50
<i>Ccl17</i>	0.09	<i>Fcrls</i>	0.34	<i>Olr1</i>	0.35	<i>Aqp4</i>	6.33
<i>Ccl19</i>	0.42	<i>Fgb</i>	0.29	<i>Pcsk2</i>	0.19	<i>Atp1b2</i>	7.59
<i>Ccl21</i>	0.42	<i>Gja1</i>	0.36	<i>Plac8</i>	0.54	<i>Cacna1d</i>	On
<i>Ccl22</i>	0.17	<i>Gpnmb</i>	0.48	<i>Psmb8</i>	0.50	<i>Coll3a1</i>	On
<i>Ccl5</i>	0.31	<i>Gpx2</i>	0.51	<i>Ptgs2</i>	0.28	<i>Cryab</i>	1.92
<i>Cd163L1</i>	0.24	<i>Gzma</i>	0.26	<i>Ptprc</i>	0.48	<i>Fam43a</i>	1.84
<i>Cd2</i>	0.16	<i>Gzmk</i>	0.25	<i>Ptprz1</i>	0.25	<i>Fbfbp1</i>	4.23
<i>Cd276</i>	0.44	<i>Gzmm</i>	0.30	<i>Rac2</i>	0.43	<i>Fxyd4</i>	1.90
<i>Cd44</i>	0.41	<i>Havcr1</i>	0.23	<i>Rcn1</i>	0.5	<i>Matn1</i>	2.49
<i>Cd53</i>	0.48	<i>IL24</i>	0.29	<i>Reg3b</i>	0.05	<i>Slc22a13</i>	2.01
<i>Cdh17</i>	0.25	<i>Iqgap3</i>	0.24	<i>Reg3g</i>	0.07	<i>Slc23a3</i>	1.82
<i>Cenpf</i>	0.21	<i>Itgad</i>	0.28	<i>Rfxfp2</i>	Off	<i>Slc2a1</i>	2.15
<i>Clefl</i>	0.40	<i>Itgal</i>	0.44	<i>Sectm1b</i>	0.36	<i>Stc2</i>	3.21
<i>Clec10a</i>	0.42	<i>Itgax</i>	0.34	<i>Slc2a2</i>	0.46	<i>Wnt9b</i>	2.98
<i>Col1a1</i>	0.36	<i>Knng1</i>	0.23	<i>Slc34a2</i>	0.45		
<i>Col1a2</i>	0.50	<i>Kgn1L1</i>	0.23	<i>Slc4a4</i>	0.52		
<i>Col3a1</i>	0.37	<i>Laptm5</i>	0.47	<i>Slc5a12</i>	0.42		

The full list of annotated genes whose expression was significantly different in casein and soy fed rats. Genes in italics were identified in network analysis as candidates for regulation by Twist2.

Figure 4

Transcription factor:
[ENSRNOG00000020355](https://www.ncbi.nlm.nih.gov/gene/?term=ENSRNOG00000020355): Twist2 twist basic helix-loop-helix transcription factor 2
 [*Rattus norvegicus* (Norway rat)]
<http://www.ncbi.nlm.nih.gov/gene/?term=ENSRNOG00000020355>

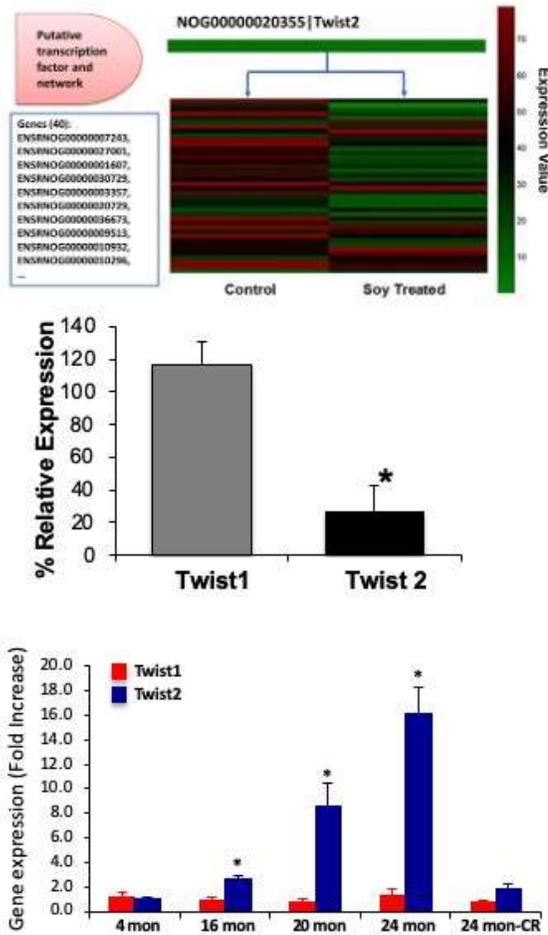


Figure 4: Identification of Twist2 as a Novel Soy Target. The decision tree illustrates how the transcription factor (ENSRNOG00000020355/Twist2) may regulate the cluster of co-expressed genes; each row denotes a gene listed in the bottom left box and each column denotes one of 2 samples (Control and Soy Treated). The levels of expression values were represented by different colors ranging from lowest (green) to highest (red). Soy reduces Twist2, but not Twist 1, expression at 20 months, as demonstrated by qPCR; each data point represents the mean+SD of 6 (control) or 5 (soy) rats, * indicates a significant different from young ($p < 0.05$). Twist2, but not Twist1 expression is increased in the aging rat kidney, and the increase is attenuated by caloric restriction.

Figure 5

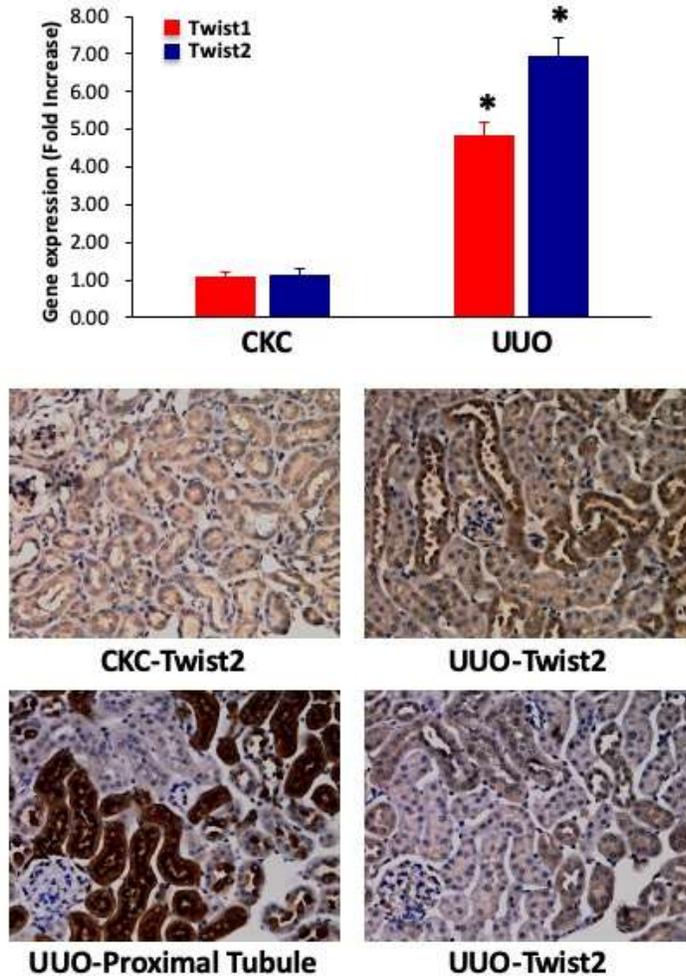


Figure 5: Twist2 Overexpression in the Mouse UO Model. Twist2, and Twist1, gene expression is increased in the obstructed kidney at day 10; each data point represents the mean+SD of 3 animals; * indicates a significant difference from 4 mon ($p < 0.05$). Immunohistochemistry reveals an increase in Twist2 staining, which does not overlap with proximal tubules.

Using samples generously provided by obtained from Dr. Benjamin Humphreys (Washington University), we have shown that gene expression of Twist2 is increased in the unilateral ureteral obstruction model 10 days following obstruction (**Figure 5**). Interestingly, in contrast with the other models we have examined, Twist1 is elevated. Twist2 staining was increased in the kidney; positive staining of Twist2 was heterogenous, suggesting that Twist2 overexpression in renal fibrosis was segment-specific. The staining of Twist2 and phytohemagglutinin E (PHA-E), a marker of proximal tubules, does not colocalize (**Figure 5**), suggesting that Twist2 overexpression is confined to distal tubules; similar results were seen in the aging rat kidney (data not shown). Taken together, these data demonstrate that overexpression of Twist2 is correlated with renal dysfunction in multiple models, and a reduction in Twist2 expression is associated with interventions (soy diet and caloric restriction) that maintain renal function, suggesting a pathogenic role for Twist2.

Discussion

In this study, we demonstrate that soy protein has renoprotective effects as a late-life intervention in the male Fischer 344 rat model. Mechanistically, the data suggest that soy has anti-inflammatory and anti-fibrotic effects in the kidney. Given that CKD is a prototypical example of progressive fibrosis leading to organ failure [33], it is not surprising that the renoprotective effects of soy correlate with reduced fibrosis. In animal models [15,34], and clinical studies [19,35,36], soy has been shown to have an anti-inflammatory impact, including a reduction in NF- κ B activity [15,16]. Given the emerging link between inflammation and renal fibrosis [37], this suggests that the anti-inflammatory effect of soy may underlie the reduction in fibrosis

observed. Importantly, RNA sequencing and network analysis identified a novel target of soy in the kidney, Twist2. Twist2 (dermo-1) is a basic helix-loop-helix (bHLH) transcription factor which recognizes the 5'-nCA_nTTG_n-3' consensus sequence - the E-box (3). Twist2 has a high degree of homology (66% identical) and an overlapping pattern of cellular expression with the more studied Twist1 [32]. Twist2 inhibits osteoblast and myoblast differentiation [32,38] and mutations in Twist2 are linked to ablepharon macrostomia, Barber-Say syndrome and Setleis syndrome [39-41]. Network analysis suggests that Twist2 regulates both pro-inflammatory and pro-fibrotic gene expression. We have shown that Twist2 is elevated in the aging kidney [42] and these data, which correlate decreased expression with improved renal function, suggest that it may be a novel mediator of renal aging.

Given the positive effects of soy in several animal models, and limited clinical studies, efforts have been made to identify the mechanism of the soy-based protection – both the specific soy constituent and the molecular/cellular pathways affected by soy. Beneficial effects have been seen in several of the animal models using a low-isoflavone diet [43,44], suggesting that the isoflavones are not responsible for renoprotection. This is supported by the finding that genistein did not have a protective effect in the Pcy mouse [13]. However, genistein alone did reduce inflammation and albuminuria and increased creatinine clearance in a high fructose model [16]. In a second study, the renoprotective effect of soy, as assessed by decreased proteinuria, was recapitulated using the same diet; we also compared a low- and high-lunasin diet; lunasin is a soy peptide that has been linked to beneficial cardiovascular effects via an anti-inflammatory mechanism [45]. In this study, lunasin did not influence soy-based

renoprotection (data not shown). As such, we have yet to identify a specific soy constituent(s) that is responsible for renoprotection.

Twist2 (dermo-1) is a basic helix-loop-helix (bHLH) transcription factor which recognizes the 5'-nCAATGn-3' consensus sequence - the E-box (32). Twist2 has a high degree of homology (66% identical) and an overlapping pattern of cellular expression with the more studied Twist1 (46). A role for Twist in renal pathophysiology is emerging. Overexpression of Twist induces EMT and increases migration in MDCK cells (47). Twist is increased in distal tubules, but also in proximal tubules and glomeruli at day 3 of UO (48). It is co-expressed with FSP1 in some tubular epithelial cells and has high expression in α SMA-positive myofibroblasts. Direct evidence for a role of Twist (Twist1) in renal fibrosis is from a study that demonstrated that overexpression of Twist2 in the proximal tubular epithelium induced a partial EMT that is linked with the development of fibrosis (49).

Limitations of this study include the small sample size (5 rats per group); this is exemplified by the fact that for several indices there was tendency for renoprotective effects – most notably for the qPCR verification of target genes – but differences were not significant and would likely benefit from additional animal numbers. However, we have observed the protective effects in two independent feeding studies. In addition, the NIH-31/NIA diet that is used at the NIA already contains soy products (5% by weight soybean meal that is 47.5% protein; other protein sources are fish meal, alfalfa meal and corn gluten meal) and does not contain casein. Therefore, using casein as the control protein likely accelerated age-dependent renal dysfunction in the control rats, as shown in previous studies [11]. However,

we have identified a novel renoprotective mechanism of soy - the reduction in Twist2 expression. The renoprotective effect is not simply due to a plant-based diet as we test an equi-nutritive diet with a lycopene-enriched tomato powder in a casein-protein base and did not see the same attenuation of age-dependent kidney dysfunction (data not shown).

In summary, we have shown a late-life renoprotection of a soy-based diet in a progressive model of renal dysfunction. As expected, we have correlated the positive renal function effects with reduced inflammation and fibrosis. Importantly, we have identified a novel pathway – Twist2 – that may mediate the age-dependent increase in renal inflammation and fibrosis and have shown that reduction of Twist2 expression by soy is a potential intervention to attenuate progressive nephropathy.

Methods

Animals

Male Fisher 344 (F344) rats were purchased from the NIA colony at 15-16 months of age. Animals were weighed and allowed to acclimate for 1 week on a standard rat chow diet. In the first feeding study, rats were randomly grouped and placed on one of three separate diets for 16 weeks: AIN-93M a casein control diet (n=6), a 14% tomato protein diet or a 14% soy diet (n=5) (**Table 1**); in the second study only the control (n=6) and 14% soy diet (n=6) were used over 8 weeks. Diets were made by (Dyets Inc., Bethlehem, PA). Animals were weighed weekly over the course of the two studies. Heparinized plasma was collected prior to the start of each study via the tail vein. Urine was also collected prior to the start of each study and once a month thereafter via overnight housing in metabolic cages (Tecniplast USA). Animals were euthanized at the conclusion of week 16 in the first study and week 8 in the

second study. On the day of the experiment, rats were anesthetized by ketamine (80-120 mg/kg)/xylazine (5-10 mg/kg) intraperitoneal (IP) injection, the chest cavity was opened and blood obtained by cardiac puncture prior to a bilateral pneumothorax. All experimental procedures and animal care were approved (Protocol 8125, Interventions for Chronic Kidney Disease) and in accordance with the NIH guidelines.

Renal function assays

Cystatin C (KT-545, Kamiya Biomedical Company, Tukwila, WA), Kim-1 (RKM100, R&D Systems, Minneapolis, MN), Hydroxyproline and the urinary protein creatinine ratio (UPC) were all measured as described previously [25]. Myeloperoxidase was measured in kidney lysates using an ELISA kit per the manufacturer's instructions (K744-100, BioVision, Milpitas, CA).

Histological evaluation

Kidney samples were fixed, embedded, and sliced into 5 μ m thick sections. Mayer's Hematoxylin and Eosin (H&E) staining was done on unstained slides, and then slides were scored on a scale of 0-4 for tubulointerstitial inflammation, glomerulosclerosis, and tubulointerstitial fibrosis as described previously [25].

Western blot/Immunohistochemistry

Nuclear fractions of tissue were isolated from kidney tissue using a commercially available nuclear extract kit (Active Motif, Carlsbad, CA). Total cell lysates were prepared by homogenizing tissue in 10 mM Tris-HCl, 1% SDS. Protein concentration was determined by NanoDrop 2000c Spectrophotometer at 280 nm. Blots were probed with a monoclonal antibody against NF-kB p65 (D14E12) and a rabbit polyclonal against histone H3 (Cell

Signaling Technology, Danvers, MA), or a pan-collagen 1 (Ab903595, AbCam) or β -actin (Sigma). Blots were developed using West Femto (ThermoFischer Scientific) and imaged using the ChemiDoc imaging system (Bio-Rad, Hercules, CA).

Unstained slides were deparaffinized and rehydrated according to the following: 12 min xylenes, 5 min 95% EtOH, 5 min 80% EtOH, 5 min 70% EtOH, 5 min 50% EtOH and 10 min 1X tris-buffered saline (TBS). Slides were stained for Twist2 using a rabbit polyclonal anti-Twist2 antibody (LSBio; LS-C205233) or the HRP-conjugated peanut agglutinin (USBiological). All slides were viewed on an Olympus IX51 microscope and images were taken using cellSense Dimension software with an automatic exposure time and white balance on all slides.

RNA Sequencing

RNA was extracted from snap-frozen tissues using the NucleoSpingRNA kit with on-column DNA digestion and was submitted for high-throughput sequencing. A mRNA-focused, barcoded library was generated using the TruSeq kit (Illumina, San Diego, CA) and analyzed using the HiSeq 2000 platform from Illumina at the DNA Core Facility. The sequencing reaction yielded approximately 7.5Gb of data, corresponding to around 30 million 50-base reads per sample across the whole transcriptome. The Informatics Research Core Facility aligned the reads against the rat genome (*Rattus norvegicus* RGSC3.4, Ensemble, Hinxton, UK) and analyzed them using Bowtie [50], TopHat and Cufflink [51] software. Differential expression values defined as fragments per kilobase of transcript per million mapped reads (FPKM) with a false-discovery corrected p-value equal or lower than 0.05 were considered significant. The raw data from our Illumina High-throughput sequencing has been deposited in the

Sequence Read Archive (SRA) with the National Center for Biotechnology Information (Bethesda, MD) under the project GSE103167 – the renoprotective effects of soy protein in the aging kidney.

Network Modeling

We predicted the gene regulatory network that controlled the expression of 145 differentially expressed genes with p-value less than 0.05. Following the method in the previous work [52-54], we initially clustered all 145 genes into 5 groups based on the similarity of their expression profiles across both control and soy treated samples, and the differentially expressed transcription factors were assigned to each co-expression group to construct a regulatory decision tree such that the expression of transcription factors in the tree could potentially induce the expression pattern of genes in the group with the maximum likelihood. The regulatory trees were then used to re-cluster the 145 genes into new groups, which were then used to update the regulatory trees. The process was iterated until it converged. Each group of genes and its regulatory tree formed a regulatory module. All the regulatory modules constitute the gene regulatory network. **Figure 4** illustrates how the transcription factor (ENSRNOG00000020355/Twist2) regulates the expression of 40 differentially expressed genes across the control and soy treated samples in one gene regulatory module.

Real-time PCR

RNA was extracted from snap frozen kidney tissue samples using the NucleoSpin RNA kit with on-column DNA digestion (740955.50, Macherey-Nagel, Bethlehem, PA). cDNA was made from the extracted RNA using the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA). Real-Time PCR was performed in duplicate

using 50 ng cDNA/reaction using the TaqProbe reagents; the following TaqMan primer sets (ThermoFisher) were used: rat Twist1 (Rn00585479_s1), rat Twist2 (Rn0057248_m1); rat cancer susceptibility candidate gene 3 (Casc3; Rn00595941_m1); mouse Twist1 (Mm04208233_g1); mouse Twist2 (Mm00492147_m1); mouse Casc3 (Mm01296308_m1). Relative fold change expression was calculated using the Pfaffl method normalized to Casc3.

Statistics

Data is expressed as the mean +/- standard error. A two-tailed t-test, assuming two-sample equal variance, was used to determine significance. P-values of <0.05 are considered significant.

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