Genetic Analysis of Mesangial Matrix Expansion in Aging Mice and Identification of Far2 as a Candidate Gene

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ABSTRACT

Aging of the kidney is associated with renal damage, in particular mesangial matrix expansion (MME). Identifying the genes involved in this process will help to unravel the mechanisms of aging and aid in the design of novel therapeutic modalities aimed at prevention and regression. In this study, structural changes in glomeruli of 24 inbred mouse strains were characterized in male mice at 6, 12, and 20 months of age. Haplotype association mapping was used to determine genetic loci associated with the presence of MME at 20 months. This analysis identified a significant association with a 200-kb haplotype block on chromosome 6 containing Far2. Sequencing revealed that mouse strains with MME contain a 9-bp sequence in the 5′ untranslated region of Far2 that is absent in most of the strains without MME. Real-time PCR showed a two-fold increase in the expression of Far2 in the kidneys of strains with the insert, and subsequent experiments performed in vitro with luciferase reporter vectors showed that this sequence difference causes differential expression of Far2. Overexpression of Far2 in a mouse mesangial cell line induced upregulation of platelet activating factor and the fibrotic marker TGF-β. This upregulation of MME-promoting factors may result, in part, from the FAR2-catalyzed reduction of fatty acyl-coenzyme A to fatty alcohols, which are possible precursors of platelet activating factor. Overall, these data suggest the identification of a novel pathway involved in renal aging that may yield therapeutic targets for reducing MME.


Renal aging is associated with a decline in renal structure and function, making the elderly more vulnerable for superimposed stress, such as hypertension, diabetes, or AKI.1,2 Eventually, renal aging may lead to CKD, and, ultimately, treatment with dialysis or transplantation might be needed. CKD is a major health problem, especially for the growing geriatric population.3

The aging kidney shows functional changes, such as decreased GFR; reduced sodium homeostasis; and morphologic changes in glomeruli, tubuli, and interstitium.4 A characteristic feature of glomerular aging is the mesangial accumulation of extracellular matrix (ECM) proteins, which usually precedes glomerulosclerosis.5,5 The mesangial cell is cardinal for glomerular function through its close interaction with both endothelial cells and podocytes.6 Mesangial matrix expansion (MME) might be caused by nephron loss and subsequent hyperfiltration in the functional nephrons. This may result in local glomerular hypertension and compensatory...
hypertrophy, which are thought to lead to cytokine and growth factor-mediated MME and, eventually, glomerulosclerosis.3

The normal mesangium contains several ECM proteins, including collagen type IV, V, and VI; fibronectin; and proteoglycans.6,7 MME is believed to result from an imbalance between synthesis of ECM components and decreased ECM degradation by matrix metalloproteinases that are under the control of specific inhibitors.8 Several growth-promoting factors are involved in this process, but an important promoter of ECM accumulation is TGF-β.9,10 An age-related increase in TGF-β has been shown in the rat kidney, along with an increase in age-related structural changes such as glomerulosclerosis, MME, and interstitial fibrosis.11 Different pathways seem to play a role in age-related kidney damage, and although sex and genetic background seem to be of high importance, specific genes that contribute to age-related damage of the kidney still remain to be identified.4

Mice are an ideal species for studying the genetics of aging because they have a relatively short lifespan and share 99% of their genes with humans.12,13 With the availability of large numbers of mouse inbred strains, haplotype association mapping (HAM) can be readily performed to identify associations between the phenotype and the haplotypes of mouse inbred strains.14 Recently, several genes involved in the age-related susceptibility for albuminuria have been identified in various strains of mice based on the albumin-to-creatinine ratio.15 This ratio, however, has limitations because it is a quantitative phenotype with an unequal distribution among individuals and is far downstream of the disease cascade. In this study we characterized MME in the kidneys of 24 inbred strains in male mice at 20 months of age, using HAM to identify genes associated with MME in these aged mice.

RESULTS

Strains with Mesangial Matrix Expansion

Histologic analysis was performed for males of all strains from The Jackson Laboratory Shock Center cross-sectional study (agingmice.jax.org/) that survived until 20 months of age and for which kidneys were available. On periodic acid-Schiff staining, 50 glomeruli were scored for the presence or absence of MME for each animal (Figure 1). The threshold for accounting a strain as positive for MME was set at 10% (5 of 50) of affected glomeruli. Analysis of glomeruli at 6 months of age in strains that did not develop MME at later time points also resulted in an average of one or two affected glomeruli using the criteria described above. We considered these glomeruli as false positive; the three-dimensional structure of the glomerulus may always account for some false-positive scoring in tissue sections. On the basis of this we decided to set the relatively high threshold of 10% to prevent inclusion of false-positive strains that would obscure our genetic analysis and to include only the strains that we considered had moderate to severe MME. Strains with an average of fewer than five affected glomeruli at 20 months of age were considered negative for MME. Twelve of 24 strains showed MME at 20 months of age (Table 1). Strains that were positive for MME or above the threshold of 10% at 20 months were also evaluated at 12 months of age. At 6 months of age, only the five strains that were positive for MME or close to the threshold at 12 months of age were evaluated. Figure 2 demonstrates the variation of MME between the strains and the variation within a strain over time.

HAM Identified Far2

A panel of 623,124 single-nucleotide polymorphisms (SNPs) was used for the HAM. After removing the noninformative SNPs, analysis was carried out with a total of 274,648 SNPs. For each SNP the association between genotype and MME phenotype was analyzed individually and a P value was recorded as the strength of the genotype-phenotype association (Figure 3). To identify the most significant association with MME, we focused on the peak with the lowest P value and...
identified a 200-kb haplotype block (between rs32310749 and rs30560187) on chromosome 6, which is different between strains with MME and strains without MME. According to the Ensembl Genome Browser (NCBI m37 assembly), Far2 is the only gene within this haplotype block (Figure 4A). By comparing genome sequencing within this region using the Sanger Institute Mouse Genomes Project database (www.sanger.ac.uk/resources/mouse/genomes/), we identified a 9-bp sequence (CTCGAGTGC) in the 5' untranslated region (UTR) Far2 (encoded by exon 1 [ENSMUSE00000860810] according to Ensembl) that is present in all strains with MME but absent in most strains without MME, such as DBA/2J and C3H/HeJ (Figure 4A). We designed primers flanking this region and sequenced all strains used in our study. All strains with MME contain the 9-bp sequence insertion, while 7 of 12 of the strains without MME do not (Table 1).

Far2 Expression Associates with MME
To determine whether this polymorphism affects gene expression, we designed primers for real-time PCR and measured the renal expression of Far2 in five strains without MME and three strains with MME. We observed a two-fold higher expression of Far2 in the strains with MME compared with the strains without MME (Figure 4B).

The 9-bp Indel in the 5’UTR of Far2 Causes the Difference in Far2 Expression
Both alleles of the Far2 5’UTR were synthesized and cloned into pGL4.10 in front of the luc2 luciferase gene (Figure 5A). The plasmids were co-transfected with pGL4.73 containing the hRluc luciferase into SV40 MES-13 (mesangial) cells and NMu3Li (mouse liver) cells. The luc2 luciferase expression was divided by the hRluc luciferase expression. Luciferase activity was measured as an indicator of expression. Comparing the construct containing the deletion to the construct with the insertion, a significant increase in the relative luciferase expression was demonstrated for the allele with the 9-bp insert in both cell types (Figure 5B).

Far2 Overexpression Causes Upregulation of Platelet-Activating Factor and TGFβ
Transfection of a mouse mesangial cell line (MES 13) with the entire C57BL/6J Far2 cDNA (ENSMUST00000111607) (containing the insert) cloned into a pCMV6 vector was performed to determine whether Far2 overexpression leads to an increase

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**Table 1. Characteristics of the 24 mouse inbred strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Presence of MME</th>
<th>Far2 Insertion/Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cByJ</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>C57BL/10J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>C57BR/cdJ</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>CBA/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>KK/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>NOD.B10S-H2F/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>NON/LtJ</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>NZW/LacJ</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>P/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>SM/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>MRL/J</td>
<td>Yes</td>
<td>Ins</td>
</tr>
<tr>
<td>129S1/SvlmJ</td>
<td>No</td>
<td>Ins</td>
</tr>
<tr>
<td>PWD/PHJ</td>
<td>No</td>
<td>Ins</td>
</tr>
<tr>
<td>RI1S/J</td>
<td>No</td>
<td>Ins</td>
</tr>
<tr>
<td>C57L/J</td>
<td>No</td>
<td>Ins</td>
</tr>
<tr>
<td>WS8/EiJ</td>
<td>No</td>
<td>Ins</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>C57BLKS/J</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>FVB/NJ</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>LP/J</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>SWR/J</td>
<td>No</td>
<td>Del</td>
</tr>
<tr>
<td>BTBR T+ tf/J</td>
<td>No</td>
<td>Del</td>
</tr>
</tbody>
</table>

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**Figure 2.** Strain and age-dependent increase in matrix accumulation. For each strain the mean percentage and SD of affected glomeruli are shown at 20 (gray bar), 12 (white bar), and 6 (black bar) months of age. Scoring at a younger time point was performed only if the threshold of 10% was reached at the 20-month time point. At each time point, n=6, except for P at 12 months (n=3). The results represent the mean ± SD.
in production of platelet-activating factor (PAF) and fibrosis-promoting factor TGF-β. The production of PAF ($P<0.05$) and TGF-β ($P<0.02$) was significantly upregulated in the media of cells transfected with the Far2 plasmid compared with the media of cells transfected with the empty control plasmid (Figure 6).

**DISCUSSION**

Using HAM, this study identified a small locus on chromosome 6 that is associated with age-related mesangial matrix expansion and contains the Far2 gene. All strains with MME contained a 9-bp sequence insertion in the 5'UTR of Far2.
the reduction of fatty acyl-CoA to fatty alcohols. We hypothesize that these fatty alcohols are the precursors of PAF, a potent inflammatory mediator that activates platelets, neutrophils, eosinophils, and macrophages. PAF also participates in the pathogenesis of proteinuria and glomerular damage, probably through changing the glomerular permeselectivity, although its exact role is still unknown. A study using PAF receptor–deficient mice showed the involvement of PAF in folic acid–induced renal injury. These mice showed amelioration of interstitial fibrosis and reduced macrophage infiltration, indicating the involvement of PAF in the pathogenesis of chronic interstitial fibrosis. In response to exogenous stimulations, PAF is produced by a variety of cells, such as neutrophils, eosinophils, monocytes, mesangial cells, and renal medulla and cortex. Exposure of cultured rat and human mesangial cells to PAF stimulates the gene expression and synthesis of the matrix proteins fibronectin and type IV collagen. To test our hypothesis we transfected mouse MES-13 mesangial cells with a plasmid overexpressing Far2 cDNA and could demonstrate that PAF production was upregulated. We also show that Far2 overexpression is causing a significant upregulation of TGF-β. TGF-β is a profibrotic cytokine that greatly stimulates the accumulation of extracellular matrix by several mechanisms. It directly stimulates the production of matrix proteins such as fibronectin, collagens, and proteoglycans. On the other hand, it also inhibits degradation of matrix by inhibiting the production of MMPs and stimulating the production of tissue inhibitors of metalloproteinases.

Upregulation of PAF and TGF-β in response to Far2 overexpression suggests that intervening in the expression or activation of Far2 would reduce MME and consequently delay renal aging. However, it must be mentioned that little is known about the specific functions of Far2 in mammals. Therefore, blocking or inhibiting the expression of Far2 might have unknown consequences. Interventions farther downstream of Far2 might influence matrix accumulation more specifically. However, the exact pathway through which Far2 increases PAF and TGF-β is unknown.

By unraveling the mechanism of kidney aging, we aim to develop novel strategies to intervene in the process of renal aging. To our knowledge this is the first study to identify a novel gene involved in MME through induction of PAF and TGF-β, both of which are involved in the pathogenesis of sclerosis. Further experiments focusing on the modulation of the Far2/PAF/TGF-β system are needed to verify these findings.

**Figure 5.** The 9-bp indel in the promoter region of Far2 influences gene expression. (A) Both alleles of the 5’UTR of Far2 were cloned in front of the luciferase gene of pGL4.10 and co-transfected with 4.73 into MES 13 mesangial cells and NMu3Li liver cells. (B) Relative luciferase expression in the allele with the 9-bp insert is significantly higher compared with the allele without the insert. The results represent the mean ± SEM.
Periodic acid-Schiff staining was performed on 3-μm paraffin sections. This staining was used for morphologic evaluation. Histologic scoring was performed in a blinded manner. For each animal, 50 glomeruli were scored for the presence or absence of MME. A glomerulus was marked as positive for mesangial matrix expansion if the mesangium was broadened more than twice. For each individual animal a score was given of percentage of affected glomeruli. Per strain, the mean of six animals was calculated, representing the percentage of affected glomeruli and the SD to depict the variation within one strain. The threshold for accounting a strain as positive for MME was set a 10% of affected glomeruli. This cutoff point was chosen because the three-dimensional structure of the glomerulus always accounts for some false-positive scoring in tissue sections.

Haplotype Association Mapping
Association mapping for MME was performed using the free R add-on package EMMA (Efficient Mixed-Models Association; http://mouse.cs.ucla.edu/emma), which uses a linear mixed-model algorithm to control for population structure and genetic relatedness.20 The strain mean was used as phenotype input and the analysis was conducted using a panel of 623,124 SNPs from the Mouse Diversity Genotyping Array, a high-density mouse genotyping array that has captured the known genetic variation present in the laboratory mouse.21 Noninformative SNPs (i.e., SNPs that were not polymorphic between the strains used in our study) were removed from this data set, which resulted in a total of 274,648 informative SNPs. Each SNP was evaluated individually and a P value was recorded as the strength of the genotype-phenotype association. All P values were transformed using $-\log_{10}(P)$ value in the scan plot. Genome sequences within the candidate regions were compared between the different strains based on their haplotype distribution using the Sanger Institute’s mouse genome databases.

Real-Time PCR of Far2
RNA was isolated from kidney samples using the Trizol method. Samples were diluted and 2 μg was used for cDNA synthesis using the QuantiTect RT kit (Qiagen). Renal mRNA levels for Far2 were determined using a primer set designed by Primerdesign Ltd (Southampton, United Kingdom) (forward: 5'-GGGTGGTGATAATC-TAAATTG-3', reverse: 5'-GGATCATCTGCCACTGC-3'). Real-time PCR was performed using the 7900 HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA) with SYBR green. mRNA levels were expressed relative to those of the β2 microglobulin gene (B2m).

Cloning of the Far2 5’UTR Indel into pGL4.10 and Transfection of Mesangial Cells
To clone the 5’UTR 9-bp indel of Far2, restriction site–containing 5’UTR regions of Far2 were synthesized by Aldevron for both the 9-bp insertion and the 9-bp deletion. Synthesized oligos were subsequently restriction digested and ligated into separate pGL4.10 vectors (named pGL4.10-ins and pGL4.10-del). Mesangial cells (MES-13) isolated from (C57Bl/6J x SJL/J)F1 mice transgenic for the early region of simian virus 40 and mouse liver NMuLi cells were obtained from ATTC.22 A total of 1.0 × 10^6 MES-13 cells/ml were seeded into 1 ml of serum containing 3:1 DMEM-Ham F12 media and incubated for 24 hours at 37°C. Dual transfection was done with pGL4.10-ins or pGL4.10-del and pGL4.73 using FuGene HD transfection reagent (Promega) in a 3.0 μl per 2.0 μg plasmid ratio. After a 48-hour incubation in serum free media, cells were lysed via Dual Luciferase Reporter Assay System (Promega) and cell extract was collected for each sample. Each cell extract was transferred into a separate well and activated
using a luciferase assay substrate (Promega). Firefly luciferase and Renilla luciferase counts per second were determined on a Victor 3 multilabel plate reader. The relative luciferase expression was calculated by dividing firefly luminescence counts per second by Renilla luminescence counts per second.

Cloning of Far2 cDNA into pCMV6 and Transfection of Mesangial Cells
The Far2 cDNA pCMV6 vector construct was kindly provided by Dr. D.W. Russell.16 The plasmid was prepared for transfection into mesangial cells. A total of 1.0x10^6 MES-13 cells/ml were seeded into 1 ml of serum containing 3:1 DMEM–Ham F12 media and incubated for 24 hours at 37°C. After incubation, a transient transfection was done using pCMV6-mFar2 plasmid and FuGene HD transfection reagent at a 2.0 µg per 3 µl ratio. A similar transfection was done using pCMV6-XL control plasmid. After incubation, media were collected and processed by scraping into RIPA buffer, and tissues were collected in lysis buffer for RNA studies. Samples were collected at 24-, 48-, and 72-hour incubation times. PAF levels were measured using a K-assay Mus musculus ELISA kit (USCN), and immunoactive TGF-β1 levels were measured using a mouse TGF-β1 DuoSet ELISA development kit (R&D biosystems).

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DISCLOSURES

None.

REFERENCES