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Brief Communication

AZGP1 deficiency promotes chronic kidney fibrosis in aging mice

Ru M Wen[1,2], Eric E Peterson[1], James D Brooks[1,3]

[1]Department of Urology, Stanford University School of Medicine, Stanford, CA 94305, USA; [2]Department of Biomedical Data Science, Stanford University School of Medicine, Stanford, CA 94305, USA; [3]Canary Center at Stanford for Cancer Early Detection, Stanford University School of Medicine, Stanford, CA 94305, USA

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Abstract: Objectives: Chronic kidney disease (CKD) affects 10-14% of the global population and is a leading cause of death. A hallmark of CKD is renal fibrosis, characterized by excessive extracellular matrix deposition. Although our understanding of fibrogenic mechanisms has advanced, effective antifibrotic therapies are lacking. Zinc-α2-glycoprotein 1 (AZGP1) regulates extracellular matrix remodeling in multiple organs, and we previously demonstrated that loss of AZGP1 induces fibrosis in the prostate. Methods: AZGP1 expression was analyzed in single-cell RNA sequencing data from the mouse kidney. Kidney tissues from wild-type and AZGP1$^{-/-}$ mice were analyzed by hematoxylin and eosin (H&E), immunohistochemistry, and picrosirius red staining. Results: AZGP1 is mainly expressed in the proximal tubule epithelial cell population. AZGP1-deficient (AZGP1$^{-/-}$) mice exhibited progressive renal fibrosis with age. While no interstitial fibrosis was observed at two months of age, prominent collagen deposition was detected by six months in the cortex and medulla of AZGP1$^{-/-}$ mice, persisting through ten months. Conclusions: These findings identify AZGP1 expressed in proximal tubular cells as protective against kidney fibrosis associated with aging and is a candidate therapeutic target.

Keywords: Zinc-α2-glycoprotein 1, AZPG1, renal fibrosis, chronic kidney disease

Introduction

Chronic kidney disease (CKD) affects 10-14% of the general population worldwide and is a common cause of death [1]. Kidney fibrosis is the final common pathway of CKD and is characterized by excessive extracellular matrix deposition leading to scarring [2]. Excessive deposition of extracellular matrix (ECM) proteins disrupts normal tissue architecture and impairs renal function.

The primary structural components of pathological ECM proteins include collagen, fibronectin, laminin, perlecan, and heparan, all of which modulate the activation and functioning of neighboring cells [3]. Matricellular proteins - such as tenascin-C, connective tissue growth factor (CTGF/CCN2), and fibrillin-1 (FBN1) - serve as binding scaffolds for these structural ECM elements and act as signal reservoirs by recruiting and concentrating growth factors and cytokines [4, 5]. Cells residing within this niche are activated through receptor-mediated interactions with ECM components [3]. Multiple signaling cascades are implicated in ECM-mediated renal fibrosis, including integrin signaling and TGF-β1/SMAD3 pathway [4], Despite significant advances in our understanding of these processes, effective antifibrotic therapeutic targets remain elusive.

Alpha-2-glycoprotein 1, zinc-binding (AZGP1) has been implicated in various biological process including lipid metabolism [6], immune cell function [7], angiogenesis [8], cell proliferation and migration [9, 10]. Recently, AZGP1 has been reported to be involved in ECM protein remodeling across several organs, including the retina, prostate, liver, and breast [8, 11]. We previously showed that AZGP1 deficiency drives fibrosis in the prostate [8]. Recombinant AZGP1 or AZGP1 overexpression attenuates collagen accumulation and downregulates fibrosis markers in retinal and hepatic cells [11,

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12]. Here, we report that AZGP1 deficiency drives fibrosis in the kidney.

Materials

Heterozygous AZGP1 knockout 4-week-old male mice were acquired from European Mouse Mutant Archive (EMMA, EM:02573, B6.129P2-Azgp1tm1.1Sbah/Orl) [6]. Mice were genotyped by clipping a small portion of the tail (2-4 mm) and placing the piece in 200 mL of PCR buffer in an Eppendorf tube [8]. Genotyping was performed by PCR using the Kapa Mouse Genotyping kit (catalog no. KK7301, Cliniscience, Nanterre, France) with AZGP1 primers (AZGP1-forward 5'-ACTCTGTGCCAGGCTCAGGTG-3', AZGP1-reverse 5'-ACCACAGGTCAGTCTGATTAC-3'). Heterozygous AZGP1 knockout female mice were obtained by breeding the heterozygous AZGP1 knockout male mice with wild type female mice on a C57BL/6 background (Jackson Laboratory). Back-crossing of male and female heterozygous AZGP1 knockout mice produced homozygous AZGP1 deficient mice.

Mice were housed in a temperature-controlled environment at the Veterinary Service Center of Stanford University. Mice had full access to food and water and had regular bedding/cage changes to maintain hygienic conditions. At the study endpoint, mice were sacrificed in a CO_2 chamber with controlled gas flow that was provided by the Stanford Veterinary Service Center. All animal experiments were conducted in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) at Stanford University (APLAC Protocol No. 30864).

Kidney tissues were harvested from mice at 2, 6, and 10 months of age. Tissue samples were dehydrated by a graded series of 70% ethanol, 95% ethanol, 100% ethanol, xylene, and paraffin solution at 56°C for two times (60 min each). The samples were then embedded in paraffin using a Leica EG1160 embedding station and sectioned with 5 μm thickness on a Leica RM2255 microtome.

Hematoxylin and Eosin staining of kidney tissue slides were performed as described previously [8, 13]. Specifically, tissue slides were baked at 60°C for 30 min, then rehydrated twice by placing them for 5 minutes in Clearify (SKU CACLELT, StatLab LLC, TX), 100% ethanol, 95% ethanol, and the 70% ethanol. Tissues were stained with Harris hematoxylin for 2 min, followed by five to six quick dips in 0.3% acid alcohol and immersion in Scott's tap water for 3 min. Tissues were counterstained with eosin for 30 seconds. The sections were then dehydrated in 95% ethanol, 100% ethanol, and xylene twice for 2 min per step, and finally mounted with a coverslip. Staining for collagen, picrosirius red (Abcam, cat. no. ab246832) was performed for 1 h. After two washes in 0.5% acetic acid, dehydration proceeded in 95% and 100% ethanol, followed by xylene (5 min, two cycles), slides were mounted with coverslips.

We obtained single-cell RNA sequencing (scRNA-seq) datasets from publicly available sources. Mouse kidney tissues from young (9-10-week-old; GSE129798 [14]) and aged (22-month-old; GSE285566 [15]) mice were analyzed.

We analyzed the single-cell RNA-seq data using Scanpy. To remove low-quality features, cells with fewer than 200 detected genes and genes present in fewer than 3 cells were excluded. The data were normalized to 10,000 total counts per cell and log transformed. We next identified the top 2,000 highly variable genes and used these genes for downstream analysis. PCA was performed for dimensionality reduction, and a neighborhood graph was computed using 10 nearest neighbors and 30 principal components.

Leiden cluster labels derived from the highly variable gene subset were assigned to the full filtered dataset. The corresponding Uniform Manifold Approximation and Projection (UMAP) coordinates were also transferred to ensure consistent embedding across analyses. This enabled visualization of both clustering structure and expression of AZGP1 not restricted to the highly variable gene set.

Cell-type annotation was performed based on cluster-specific marker genes. For each cluster, the top 10 differentially expressed genes were identified and compared with established canonical markers of known kidney cell populations [16], including proximal tubule epithelial cells, collecting duct cells, endothelial cells, fibroblasts, and immune cells. Cluster identi-

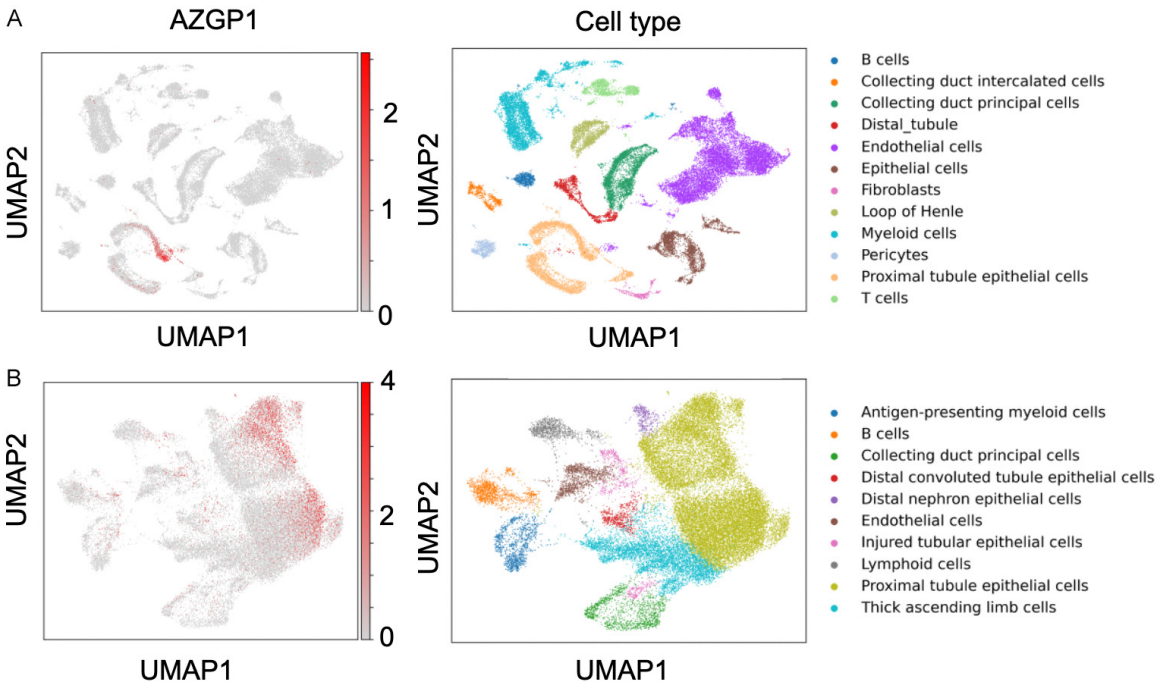

Figure 1. AZGP1 is mainly expressed in proximal tubule epithelial cells. Single-cell RNA sequencing analysis of AZGP1 expression in kidney tissues from (A) 9-10-week-old mice (GSE129798) and (B) 22-month-old mice (GSE285566). AZGP1 expression is mainly expressed in proximal tubule epithelial cells and largely absent from fibroblasts, immune and endothelial cells.

ties were first assigned by ChatGPT (GPT-5.3) and then reviewed by authors based on these marker genes.

Statistical analyses were performed by unpaired Welch's t test (two-tailed) for group comparison using GraphPad Prism (version 11.0). Data were expressed as mean ± standard error of the mean (S.E.M.), and statistical significance was defined as follows: ns: non-significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Results and discussion

AZGP1 has been previously shown to be expressed in the mouse kidney [17]. To better understand the distribution of AZGP1 expression in kidney cells, we analyzed publicly available single-cell RNA sequencing data from mouse kidney tissues for expression of AZGP1 in various cell types [14]. Using cell annotation schemes developed previously, cells were classified and displayed based on their UMAP coordinates. AZGP1 expression was observed predominantly in the proximal convoluted tubule epithelial cells, with minimal expression in immune cells, fibroblasts, or endothelial populations in both 2-month-old and 22-month-old mouse kidney tissues (**Figure 1**).

Based on previous data implicating AZGP1 in preventing fibrosis in several tissue types, we tested whether fibrosis differed with aging in wild-type and AZGP1 homozygous deficient mice ($AZGP1^{-/-}$) mice. Kidneys were harvested from $AZGP1^{-/-}$ mice and wild-type controls at 2-, 6-, and 10-months of age to evaluate age-dependent fibrosis. Paraffin sections were stained with Picrosirius Red, which selectively labels type I collagen. At 2 months, collagen deposition was negligible in renal cortex and medulla in both wild-type and $AZGP1^{-/-}$ mice (**Figure 2A** and **2B**). However, by 6 months, increased collagen deposition was observed in $AZGP1^{-/-}$ mouse kidneys, was prominent around the glomerular capsule, peritubular space and medullary interstitial space (**Figure 2C** and **2D**), and persisted at 10 months (**Figure 2E** and **2F**). In contrast, age-matched wild-type littermates exhibited little or no picrosirius-red staining in renal cortex and medulla at those time points (**Figure 2**). Specifically, AZGP1 knockout mice differed significantly from wild-type mice in the medulla in 6 month-old mice ($P = 0.047$) and 10 month-old mice ($P = 0.038$), and in the cor-

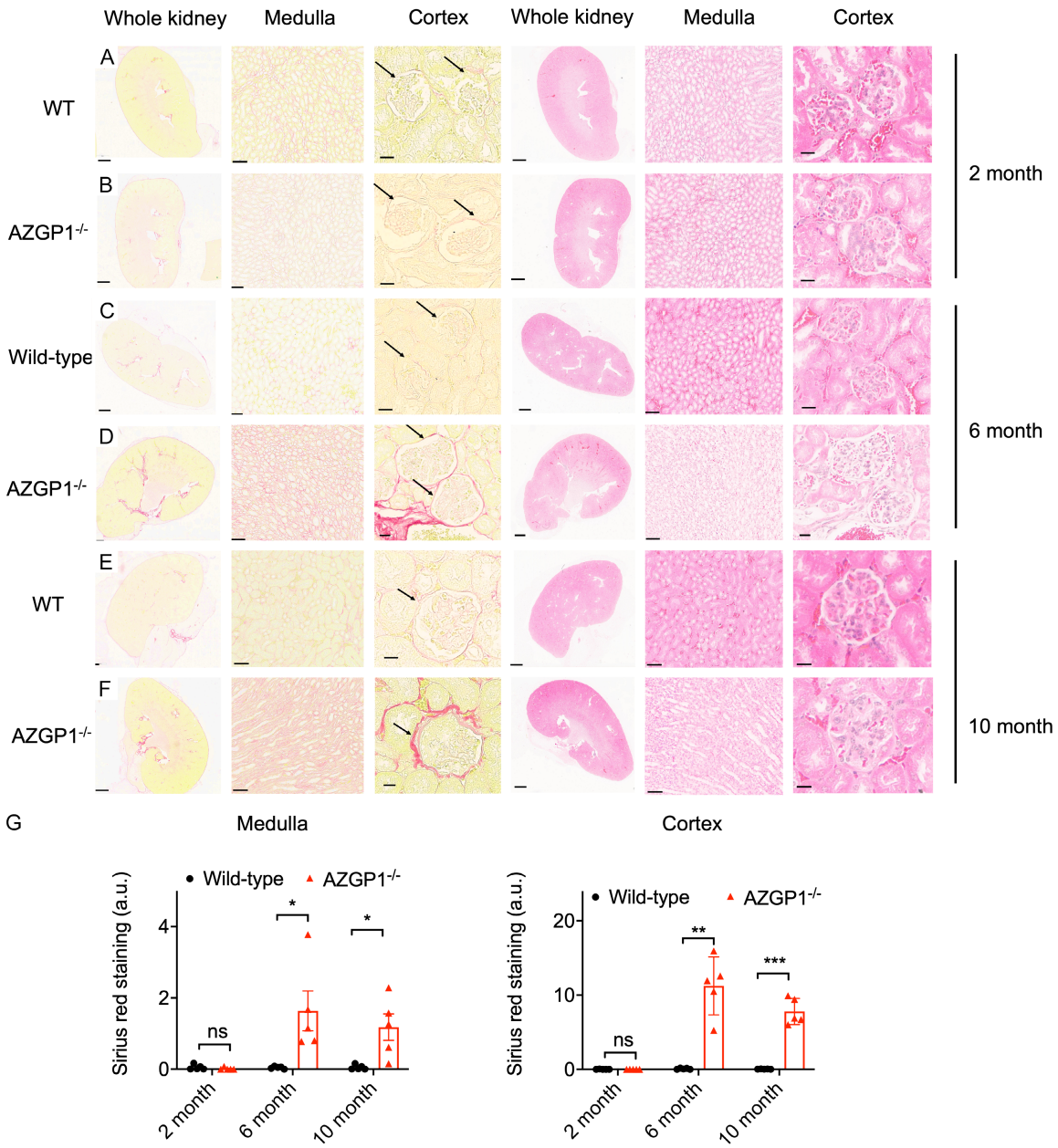

Figure 2. Knockout of AZGP1 induces fibrosis in medulla and cortex of kidney. Picosirius red, and H&E staining of whole kidney tissue derived from 2-month wild-type (A) and AZGP$^{-/-}$ mice (B); 6-month wild-type (C) and AZGP$^{-/-}$ mice (D), and 10-month-old wild-type (E) and AZGP1$^{-/-}$ mice (F). Scale bar: whole kidney image: 900 μm (4×), Medulla: 60 μm (20×), Cortex: 25 μm (40×). (G) The corresponding quantitation of picosirius red staining on medulla and cortex. ns: non-significant, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

tex in 6 month-old mice (P = 0.0031) and 10 month-old mice (P = 0.0006) (**Figure 2G**). Therefore, AZGP1 deficiency does not induce fibrosis in young mice, but fibrosis does become apparent as mice age, with increased fibrosis apparent by 6-months and continuing through 10 months. These results suggest AZGP1 protects against fibrosis in the kidney and that loss of expression AZGP1 potentiates renal fibrosis with aging.

AZGP1 has been implicated in fibrosis in retina, prostate, liver, breast and kidney tissues. AZGP1 is critical for protection from subretinal fibrosis (SRF), and AZGP1 expression levels are significantly decreased within SRF lesions.

Treatment with recombinant AZGP1 was associated with significantly lowered collagen I, α-SMA, and fibronectin in retinal pigment epithelia cells [11]. In the mouse prostate, we observed increased fibrosis and collagen deposition at base of the seminal vesicles in AZGP1-deficient mouse [8]. Similar findings have been reported in the liver, where forced overexpression of AZGP1 in hepatic a transgenic model results in significant decreases in fibrosis markers in hepatic stellate cells [5].

AZGP1 serum levels are increased in patients with renal failure [18] and genetic deletion of AZGP1 has been associated with increased renal fibrosis in unilateral ureteral obstruction (UUO) and aristolochic acid models of renal damage [19]. Our model demonstrates that AZGP1 null mice show increased collagen deposition in the kidney associated with aging alone. In wild-type C57Bl/6J mice, treatment with recombinant AZGP1 reduces collagen deposition and decreases the expression of fibrosis-associated markers in the kidney after obstruction. Similarly, mice engineered to overexpress AZGP1 in the proximal tubular cell epithelium show significantly lower renal damage and fibrosis after UUO [17]. Taken together, AZGP1 appears to protect against renal damage and scarring regardless of mechanism, including aging. AZGP1 should be explored as a potential therapeutic to mitigate renal damage.

Conclusions

AZGP1 is mainly expressed in proximal tubular cells and plays a protective role against aging-associated kidney fibrosis. AZGP1 is a promising candidate therapeutic target for kidney fibrosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ru M Wen, Department of Urology, Stanford University School of Medicine, 3172 Porter Dr., Palo Alto, CA 94305, USA. E-mail: r.wen@stanford.edu; Dr. James D Brooks, Canary Center at Stanford for Cancer Early Detection, Stanford University School of Medicine, Center of Academic Medicine, 453 Quarry Road, Urology 5656, Grant S287, Palo Alto, CA 94305, USA. Tel: 650-725-5746; E-mail: jdbrooks@stanford.edu

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