Single-cell transcriptomics and chromatin accessibility profiling elucidate the kidney protective mechanism of mineralocorticoid receptor antagonists

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Title: Single-cell transcriptomics and chromatin accessibility profiling elucidate the kidney protective mechanism of mineralocorticoid receptor antagonists

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Abstract

Mineralocorticoid excess commonly leads to hypertension and kidney disease. In our study, we employed single-cell expression and chromatin accessibility tools to characterize the mineralocorticoid target genes and cell types. We demonstrated that mineralocorticoid effects are established through open chromatin and target gene expression, primarily in principal and connecting tubule cells, and to a lesser extent, in segments of the distal convoluted tubule cells.

We examined the kidney-protective effects of steroidal and non-steroidal mineralocorticoid antagonists (MRAs), as well as amiloride, an epithelial sodium channel inhibitor, in a rat model of deoxycorticosterone acetate, unilateral nephrectomy, and high salt consumption-induced hypertension and cardiorenal damage. All antihypertensive therapies protected from cardiorenal damage. However, finerenone was particularly effective in reducing albuminuria and improving gene expression changes in podocytes and proximal tubule cells, even with equivalent blood pressure reduction.

There was a strong correlation between the accumulation of injured/profibrotic tubule cells expressing Spp1, Il34, and Pdgfb and the degree of fibrosis in rat kidneys. This gene signature also showed potential for classifying human kidney samples. Our multi-omics approach provides fresh insights into the possible mechanisms underlying hypertension associated kidney disease, the target cell types, and the protective effects of steroidal, non-steroidal MRAs, and amiloride.
Introduction

Chronic kidney disease (CKD) is a serious and growing public health issue, affecting approximately 14% of the population in the United States (1). It is the 9th leading cause of death globally and one of the fastest growing causes of mortality (2, 3). While medications including angiotensin-converting-enzyme inhibitors (ACEIs), angiotensin-receptor-blockers (ARBs), and more recently, sodium-glucose cotransporter 2 inhibitors have been shown to slow the progression of CKD (4-7), there is still a significant need for treatments and interventions to prevent this disease. There are currently half a million people in the United States with end stage kidney disease (8).

Mineralocorticoids, such as aldosterone, play a key role in maintaining blood volume, sodium, and potassium balance (9). The primary mechanism by which aldosterone exerts its effects is through binding to the mineralocorticoid receptor (MR or Nr3c2). When aldosterone binds to MR in the cytosol, the complex travels to the nucleus, binds to DNA, and regulates gene transcription (10). While both aldosterone and cortisol can bind to MR, in mineralocorticoid-sensitive tissues, an enzyme called 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) degrades glucocorticoids, allowing MR to be exclusively regulated by mineralocorticoids (11). The kidney is the primary target organ of mineralocorticoids (12).

Excess aldosterone can cause a range of health problems including hypertension (HTN), cardiovascular disease, and kidney disease (13, 14). The exact mechanisms by which aldosterone and HTN cause kidney damage are not fully understood (15). At high blood pressure, the afferent glomerular arteries must constrict to protect the glomerulus from high physical pressure, but glomerular vasoconstriction can lead to interstitial and tubule hypoxia, as the efferent artery plays...
a critical role in supplying blood for the tubules (16). In addition to the hemodynamic effects of mineralocorticoids, aldosterone may also directly regulate gene expression to induce kidney disease. The mineralocorticoid receptor (MR) is expressed by various cell types in the body, including the brain, heart, blood vessels, and immune cells (17). Some studies propose a direct role of MRs in regulating collagen accumulation and inflammation through the action of transforming growth factor beta (TGFβ) and NFκB (18, 19). There is also evidence that MR may directly affect immune cells, inducing the production of inflammatory cytokines such as IL-1 in macrophages (20).

Mineralocorticoid receptor antagonists (MRAs) are a group of medications that inhibit the effect of aldosterone (21). MRAs can be classified into two groups: steroidal MRAs such as spironolactone and nonsteroidal MRAs such as finerenone (22). Spironolactone use is often associated with hyperkalemia and gynecomastia, which likely in part caused by the nonspecific binding of spironolactone to the sex hormone receptors (23). In several small trials, spironolactone treatment on top of standard of care, have resulted in blood pressure and proteinuria reduction in CKD patients (21, 22). Unfortunately, in these studies the risk of potential life-threatening hyperkalemia was about three-fold higher when compared to ACEIs or ARBs alone.

Finerenone is a novel highly specific nonsteroidal MRA (24). Finerenone demonstrated kidney and heart protection in animal models, but compared to steroidal MRAs it had fewer effects on potassium homeostasis (24). Finerenone significantly reduced renal composite outcomes (time to onset of kidney failure, sustained decrease of eGFR > 40% from baseline or renal death) and cardiovascular outcomes (time to cardiovascular death, nonfatal MI or stroke, or hospitalization for heart failure) on top of maximized ACEi or ARB compared to placebo in at risk patients (5).
The incidence of hyperkalemia-related discontinuation of the drug was lower than spironolactone when used on top of ACEi and ARB (5). The exact molecular mechanism of finerenone action is however not fully understood.

The kidney contains more than 30 different cell types, and bulk gene expression analysis can be strongly confounded by changes in cell fractions (25). Single cell and nucleus RNA-sequencing (scRNA-seq and snRNA-seq) enables the unbiased transcriptomic characterization of kidney cells (26-28) including cell-type specific changes in disease state (29, 30). Single nucleus profiling of chromatin accessibility, which is called single nucleus Assay for Transposase-Accessible Chromatin using sequencing (snATAC-seq) has recently been developed to identify open chromatin regions in the genome where transcription factors can bind (26, 30). This is particularly important for transcription factors such as MR, which can directly bind to the DNA to regulate gene transcription.

In this study, we employed snRNA-seq and snATAC-seq to elucidate mineralocorticoid-induced HTN and the development of hypertensive kidney disease. We define DOCA-sensitive cells and genes, and the mechanism of finerenone-afforded kidney protection, and made a direct comparison with changes induced by spironolactone and amiloride.
Results

Finerenone ameliorates cardiac and renal injury in the DOCA/Salt rat injury model

We modelled mineralocorticoid-induced HTN and cardio-renal syndrome by performing uninephrectomy followed by deoxycorticosterone acetate (DOCA) injection and high salt intake in rats. Groups of rats were treated with 10 mg/kg of finerenone, 50 mg/kg of spironolactone or 20 mg/kg amiloride (24, 31-33). Two rats from each group were sacrificed after 3 weeks to evaluate short term changes (HTN), while the majority were sacrificed at 6 weeks to assess hypertensive organ damage (Fig. 1A). Rats treated with DOCA-salt developed severe hypertension with 181 mmHg SBP compared to 115 mmHg SBP in sham control rats by the end of the study (Fig. 1B). Finerenone, spironolactone, and amiloride resulted in a similar reduction in systolic and diastolic blood pressure (Fig. 1B). Serum blood urea nitrogen (BUN) levels were higher in the DOCA salt group compared to controls, and it was lowered by finerenone and spironolactone treatment. Anemia, a common complication of chronic kidney disease, was ameliorated by treatment with finerenone, spironolactone, and amiloride (Supplementary Fig. 1A). DOCA rats had developed severe proteinuria, which was reduced by all treatments, but the reduction only reached statistical significance in the finerenone group ($P = 0.04$) (Fig. 1B). As the UACR variance was high the study was not powered to detect differences in other groups. Plasma renin levels were lower in DOCA-treated rats when compared to controls but showed marked variation (Supplementary Fig. 1A).

We observed cardiac hypertrophy in DOCA treated rats, likely secondary to the hypertension. The heart to body weight ratio was lower in finerenone, spironolactone and amiloride treated rats, consistent with drugs’ effect on blood pressure (Supplementary Fig. 1A). The kidney to body
weight ratio was markedly increased in DOCA rats, but was reduced by treatment with finerenone, spironolactone or amiloride (**Fig. 1B and Supplementary Fig. 1A**).

Histological analysis revealed severe global and segmental glomerulosclerosis, proteinaceous casts, tubulointerstitial fibrosis, and extensive cardiac fibrosis (**Fig. 1C, D, Supplementary Fig. 1A**). Sirius red staining was used to measure the extent of fibrosis in kidneys and hearts (**Supplementary Fig. 1B**). Rats treated with MRAs or amiloride showed less fibrosis and damage.

We analyzed several phenotypic outcomes, including, blood pressure, biochemical parameters (such as proteinuria, BUN, blood electrolytes) and structural damage (such as glomerulosclerosis and fibrosis) in rats. To evaluate overall phenotypic similarities among the samples, we used unbiased principal component analysis (PCA) and hierarchical clustering of the outcomes (**Supplementary Fig. 2**). The results showed that the DOCA-treated rats were distinct from the control rats, as well as those treated with finerenone, spironolactone, and amiloride. Control rats were distinct from the finerenone, spironolactone, and amiloride-treated groups. The finerenone, spironolactone, and amiloride-treated groups did not separate, indicating that hypertension played a key role in the development of the phenotype.

In summary, we observed severe hypertension and renal failure in the DOCA-salt rat model. MRA and ENaC inhibition lowered blood pressure and similarly improved phenotypes indicating the key role of hypertension driving cardiorenal damage. Within the same blood pressure range, finerenone was more successful in reducing proteinuria.
Integrated single cell multi-omics atlas of the healthy and diseased rat kidney

We sought to identify genes, cell types, pathways and transcription factors affected by DOCA, hypertension, hypertensive kidney disease and the response to MRAs and amiloride. Therefore, we conducted a single-cell multi-omics analysis, which included snRNA-seq, snATAC-seq and bulk RNA-seq of kidneys of control and experimental groups at both timepoints (Supplementary Fig. 3).

SnRNA-seq was conducted on 22 whole rat kidney samples from the 5 different groups. Quality control (QC) metrics, including gene counts, reads, and mitochondrial gene percentages, are shown in Supplementary Fig. 4. DOCA administration, in combination with a high salt intake, has been reported to increase proximal tubular cell size (34). Therefore, we compared the mean RNA counts per cell and sample between the groups. We observed a trend towards higher count numbers in DOCA animals compared to controls, but this did not reach statistical significance (Supplementary Fig. 4D). After filtering low quality cells, 310,218 cells were used in the final analysis. We identified 41 clusters after batch effect correction using Harmony (35) (Supplementary Fig. 5). We next performed cell type specific differential expression analysis and identified all previously described kidney cell types (28, 36, 37) (Fig. 2A and Supplementary Table 1). The key marker genes used to identify different cell clusters in the snRNA-seq are shown in Fig. 2D. As this is one of the first rat kidney snRNA-seq dataset (38), we analyzed cell-type consistency (39) and specific gene markers in rat, mouse (28) and human (36, 37) kidneys. We observed that most cell-type specific markers were conserved between the different species (Supplementary Fig. 6, 7)
To understand gene regulatory changes induced by DOCA and those influenced by MRAs, we performed snATAC-seq on nine samples. Supplementary Fig. 8 and 9 show the QC parameters of the snATAC-seq libraries. After filtering low quality cells, clustering was performed on 53,298 nuclei. After batch effect correction by Harmony (35), we identified 20 clusters (Supplementary Fig. 10). We next examined chromatin accessibility around the transcription start site and gene body regions of the known cell type-specific marker genes using Signac (40) (Fig. 2B). Key marker genes showed cell type-specific accessibility, and we were able to identify all major kidney cell types (Fig. 2E). Chromatin accessibility-based marker gene activity is shown in Supplementary Fig. 10E. Supplementary tables 2 and 3 provide the full list of cluster-specific differentially accessible peaks and list of gene activity calculated based on differentially expressed genes, respectively.

We performed label transfer to understand the relationship between cell type-specific gene expression and open chromatin in the snRNA-seq and snATAC-seq datasets (41). We found strong consistency (0.78 mean of maximum prediction score) between these two datasets (Supplementary Fig. 11A, B, C and Fig. 2B). To further quantify the consistency between cluster assignment in the ATAC-seq and RNA-seq data, we extracted gene activities of top 3,000 highly variable genes of snATAC-seq and snRNA-seq and run a Pearson’s correlation test between gene expression (snRNA-seq) and gene activity (snATAC-seq) (Supplementary Fig. 11D). Integration of the snRNA-seq and snATAC-seq data also yielded highly consistent results (Fig. 2C and Supplementary Fig. 12). The list of marker genes for the integrated dataset can be found in Supplementary Fig. 12B and Supplementary table 4.
Overall, our snRNA-seq, snATAC-seq and integrated datasets captured all kidney cell types, including endothelial cells, fibroblast, myofibroblast, podocytes, different types of proximal tubules (PT), loop of Henle (LOH), distal convoluted tubule (DCT), principal cells of collecting duct (PC), intercalated cells (IC), and diverse immune cells. Cell-type-specific markers were consistent with prior publications for mouse (28) and human (27, 36, 37) kidneys such as *Emcn* for endothelial cells, *Coll1a1* for fibroblast, *Synpo2* for myofibroblast, *Nphs1* for podocyte, *Mki67* for proliferating tubule, *Slc12a1* for LOH, *Slc12a3* for DCT, *Aqp2* for PC, *Slc4a1* for IC_A, *Slc26a4* for IC_B, *Cd96* for lymphocyte, *Lyz2* for monocyte, and *Csf1r, C1qa* for macrophage. We captured different types of PT cells including proximal convoluted tubules (PCT) and proximal straight tubule (PST) expressing *Cubn* and *Slc7a13*, respectively (Supplementary Fig. 13). We also captured PT cells positive for *Haver1, Vcam1*, and *Pdgfb* well-known markers of tubular injury. We labelled this cluster as injured or profibrotic PT (iPT) (Supplementary Fig. 12, 13).

To further understand key cell type transcription factors in determining the cellular gene expression, we used chromVAR to analyze motif activity and predicted key gene expression-driving transcription factors (42) in individual cell clusters (Fig. 2F). This was based on the presence of binding motifs in the open chromatin area of each cell type. The complete list of differentially activated chromVAR motifs is shown in Supplementary table 5. Our predicted gene expression driving transcription factors in each cluster were consistent with prior reports; including *Hnf1a* and *Hnf4g* (26, 27) in proximal tubules and *Wt1* (26, 43) in podocytes among others.

In summary, we have generated one of the first comprehensive single cell expression and gene regulatory atlas for healthy and diseased rat kidneys, which is now available to the public on the Susztak lab website [www.susztaklab.com](https://susztaklab.com/genemap_rat).
Mineralocorticoid receptor target cell types and gene regulatory network in the rat kidney

Our primary objective was to understand mineralocorticoid and glucocorticoid action; target genes and cell types. This has traditionally been a challenging task, as cells cultured in vitro demonstrate marked differences in their gene expression. First, we examined open chromatin regions at the mineralocorticoid receptor (MR, \(Nr3c2\)) and glucocorticoid receptor (GR, \(Nr3c1\)), loci in the entire dataset. We observed accessible chromatin for MR in several cell types, the highest being in PC cells, but also in DCT and IC cells. In contrast, GR open chromatin exhibited almost an inverse pattern, with a lack of open regions in the distal nephron, but accessibility in most other cell types (Fig 3A). These results suggest that the cell-type specific expression of GR and MR plays key role in their target actions (44). MR expression, as measured in the snRNA-seq data was the highest in PC cells (Fig 3B) (45). GR expression was much lower than MR in the kidney and was expressed by multiple cell types except PC cells.

Next, we examined the activity of the MR (\(Nr3c2\)) motif in open chromatin areas within our rat kidney atlas. We found open chromatin for MR motifs in almost all cell types (Fig. 3A). As the binding motif for \(Nr3c2\) and \(Nr3c1\) is the same, we did not observe any difference in open motif activity between MR and GR. We then investigated the open chromatin and gene expression of previously published MR and GR target genes (46-54) (Supplementary Table. 6). The results showed a consistent pattern with MR and GR expression, respectively (Fig. 3B, C, Supplementary Fig. 14).
Further sub-clustering of DCT and PC cells, led us to identify the connecting tubule (CNT) and two subtypes of DCT cells (DCT1 and DCT2). DCT2 cells expressed \textit{Slc12a3} (classic DCT marker) as well as the classic PC markers (ENaC) (55) (Fig. 3C, Supplementary Fig. 15). MR sensitivity is established by MR expression and by the expression of \textit{Hsd11b2} (11β-hydroxysteroid dehydrogenase type 2) an enzyme that degrades glucocorticoids. In cells that do not express 11-beta-HSD2, NR3C2 is effectively a second glucocorticoid receptor. As expected, \textit{Hsd11b2} expression was the highest in PC cells, however sub-clustering indicated expression in DCT2 and CNT cells. The expression of classic MR target genes ENaC (\textit{Scnn1a}, \textit{Scnn1b}, \textit{Scnn1g}), and \textit{Sgk1} was the highest in PC cells, but CNT cells also expressed ENaC, while its expression in DCT2 cells were lower. In summary, the effect of MR effect appeared to be controlled by multiple sequential mechanisms, including chromatin accessibility of MR, cell type expression of MR, \textit{HSD11B2} and target genes.

Next, we focused on genes upregulated by DOCA but lowered by MRA. We identified a number of MRA-sensitive genes in snRNA-seq and bulk RNA-seq (Supplementary Fig. 16, 17) (6 weeks data). In bulk RNA-seq data the expression of key MR target genes, including the Na/K ATPase (\textit{Atp1a1}) and PI3K (\textit{Pik3r3}) were higher in DOCA-treated rats and returned to baseline after MRA treatment (Fig. 3D, Supplementary Fig. 17B). MRA-sensitive expression changes in PC cells were consistent with the bulk data and showed that amongst other genes, the expression of \textit{Atp1a1} was lower in finerenone treated animals. A full list of MRA-sensitive genes in PC, CNT and DCT2 can be found in Supplementary Table 7. Genes encoding the corticosteroid hormone-induced factor (CHIF), a negative regulator of Na/K ATPase (\textit{Fxyd2} and \textit{Fxyd4}) exhibited lower levels in DOCA treated rats. \textit{Hsd11b2} expression was also lower in DOCA-treated rats and returned to baseline after treatment with finerenone (Supplementary Fig. 16, 17). \textit{Aqp2} expression was also
lower in DOCA rats, but normalized by MRA (Fig. 3D, Supplementary Fig. 16, 17). snATAC-seq results were consistent with snRNAseq (Supplementary Fig. 15D) and changes in Na/K/ATPase, ENaC, and PI3K was observable in bulk RNA-Seq as well.

In summary, unbiased snRNA-and ATAC-seq analysis highlighted that PC cells are the primary mineralocorticoid-sensitive cell type with minor role of DCT2 and CNT cells. Mineralocorticoid sensitivity is achieved by cell-type selective gene expression and open chromatin.

Target genes, cell types and pathways by MRAs and amiloride

To investigate specific cell types impacted by MRAs and ENaC inhibitors and to differentiate between the effects of hypertension and mineralocorticoids, we conducted a cell-type specific gene expression analysis on samples from control, DOCA, and drug-treated groups, both at the time of hypertension (3 weeks) and at the time of hypertensive kidney disease (6 weeks). Cells obtained from kidneys at the onset of hypertension (3 weeks) demonstrated comparable changes in gene expression levels across various cell types, suggesting that hypertension leads to significant functional changes in these cells. By contrast, PT cells from kidneys collected at the time of hypertensive kidney damage (6 weeks) showed much larger changes in gene expression (approximately 2,700 differentially expressed genes, compared to approximately 200 DEG in the other cell types). (Fig. 4A, Supplementary Tables 7, 9). This is congruent with previous research emphasizing the critical role of PT cell plasticity in the development of fibrosis (56, 57). These differences in gene expression were likely due to the presence of injured/profibrotic proximal tubules in the kidneys exhibiting hypertensive nephrosclerosis. These tubules formed a distinct
cluster that expressed higher levels of Havcr1, Spp1, and Il34, and lower levels of classic PT genes Slc7a13 and Kap (Fig. 4D). The proportion of iPT cells in the kidney correlated with the degree of fibrosis across samples, suggesting a robust link between iPT cells and fibrosis (Fig. 4D). The same pattern was observed in the snATACseq data (Supplementary Fig. 18, Supplementary Table. 8).

To understand the specific changes in kidney cells that were brought about by different medications, we conducted gene expression analysis on each treatment group and cell type (at 6 weeks of treatment). As shown in Figure 4C, the expression levels of most genes that were altered in the DOCA rats reverted to baseline following treatment with MRAs or amiloride. This is likely due to the blood pressure-lowering effects of these medications (Fig 4.C, Supplementary Fig. 19, 20). Amiloride exerted marginally greater impact on immune cells and fibroblasts, although the absolute number of DEGs affected in these groups was minimal. Finerenone was found to be more effective in podocytes and PT cells, as more genes in these cell types returned to healthy levels following treatment. This is consistent with the observed clinical change in proteinuria (Fig. 4C).

In PT cells, the genes normalized by finerenone were mainly involved in metabolic processes, including Wfde2, Ass1, and Assc3. In podocytes, finerenone reduced the levels of Tgfb2, Nfkbiz, and Fn1 that had been induced by DOCA, as well as genes associated with cell adhesion. The complete list of genes affected by each drug can be found in Supplementary Table 10.

To summarize, we observed a robust correlation between PT and iPT genes and the development of hypertension-induced fibrosis. All the drugs tested were able to normalize gene expression changes induced by DOCA, with finerenone being particularly effective in podocytes and PT cells.
Unbiased tensor decomposition of key phenotype driving cell types and pathways

To identify cell types that drive phenotypic changes, first we generated bulk RNA-seq data from whole kidney tissue samples (Supplementary Fig. 21) (6 weeks treatment data). Differentially expressed gene (DEG) analysis revealed changes in the expression levels of 3,219 genes in kidneys between control and DOCA rats (Fig. 5A, Supplementary Table. 11). Gene ontology pathway analysis by DAVID (58), indicated enrichment for inflammation and metabolic processes in the DOCA-treated group (Supplementary Fig. 21 B, C). Clustering analysis of the gene expression matrix of the bulk RNA-seq indicated co-clustering of the control and finerenone groups (Supplementary Fig. 21D). We subsequently examined the expressions of the DEGs identified by bulk RNAseq analysis in different cell types in the entire snRNA-seq and snATAC-seq datasets. We found that genes that had lower expression in DOCA samples were enriched in the PST segment (Fig. 5C, D, E), while genes with higher expression in DOCA samples were enriched in the iPT cluster (Fig. 5 D, E). Overall, DEGs from bulk RNA-seq were enriched in PT cells, which was consistent in the results from the snRNA-seq and snATAC-seq (Supplementary Fig. 22). Weighted correlation network analysis (WGCNA) (59) of the bulk RNA-seq data identified various modules that correlated with different phenotypic outcomes, such as SBP, DBP and fibrosis (Supplementary Fig. 22). The primary module driving the phenotype in the DOCA models were enriched in PST. Genes whose expression returned to baseline in the finerenone group were also enriched in PST and injured PT cells potentially indicating the key role of these cell types (Supplementary Fig. 23).
Given the complex experimental design, with multiple treatment groups, cell types, and phenotypic outcomes, we used tensor decomposition to identify the key cell types, genes, and pathways that were driving these phenotypes (on the entire snRNA-seq dataset) (60) (Fig. 6). In this analysis, we identified five factors. The first factor was found to be associated with multiple important phenotypic outcomes, including blood pressure, serum sodium, BUN, and creatinine. Changes in expression of MR target genes, including Scnn1a, Scnn1b, Scnn1g, and Atp1a1 and DCT and PC were found to be driving this factor (Fig. 6C). Interestingly, PST and iPT cells also exhibited enrichment in this factor (Fig. 6C). Factor 4 was correlated with diastolic BP and included the genes such as Ren, Cox1, Plce1, Cxcl12. Most variations in this factor were observed in the DOCA group, and it was enriched in PC cells, although we again noted effect in PST cells (Fig. 6C). The genes identified in factors 1 and 4 by this analysis are presented in Supplementary Table 12. In summary, tensor decomposition indicated PST, iPT, and PC cells as key phenotype driving cell types.

After identifying key phenotype-associated cell types, we performed unbiased machine learning gene expression analysis using weighted gene co-expression network analysis (WGCNA) on PC, PST, and iPT cells (in the entire dataset) (59, 61, 62) (Fig. 6D). We found a specific gene module in PC cells containing MR and its target genes. This module had the highest score in DOCA and amiloride group, and the lowest in the finerenone group. We identified a specific gene module in PST cells. This module expressed typical PST markers such as Hnf4a and Slc5a12 as well as Zeb1 a marker for epithelial to mesenchymal transition. This module had the highest score in DOCA and normalized best by finerenone. Furthermore, we identified an iPT-specific module, which showed enrichment for inflammatory markers such as Il34, Spp1, and Nfkbi.
highest score in DOCA and was normalized by finerenone. The complete list of the genes in these three identified modules is presented in Supplementary Table 13.

Overall, the bulk RNA-seq, snRNA-seq, and snATAC-seq data suggested a critical association between PC and PT cells as the primary cell types driving phenotypes. WGCNA identified a core MR target network in PC cells and a proinflammatory profibrotic gene module in iPT cells.

**Finerenone protects from maladaptive differentiation of injured PT cells**

Our bulk RNA-seq and single cell RNA-seq analyses revealed a key association between PT cells and hypertensive organ damage. To identify factors that might be responsible for the maldifferentiation of injured PT cells, we used Monocle3 to conduct a cell trajectory analysis (63, 64) (Fig. 7). This analysis identified a path of differentiation of injured PT cells from PST cells (Fig. 7A). Injured PT cells were enriched in the diseased state (27) (Supplementary Fig. 24). Gene ontology analysis revealed an enrichment of genes associated with metabolism and development along the trajectory (Supplementary Fig. 24, Supplementary Table. 14). We identified *Spp1, Il34, and Pdgfb* as top differentially expressed genes, showing higher levels in the injured PT pseudotime trajectory (Fig. 7A, Supplementary Fig. 24B) and *Hnf4a*, higher at the start of trajectory (PST) and lower in injured PT (at the end) (Supplementary Fig. 24). Our snATAC-seq data recapitulated changes observed by snRNA-seq (Fig. 7B) including changes in metabolic and developmental genes along the trajectory (Supplementary Fig. 24, Supplementary Table 15). Similar to the snRNA-seq, chromatin accessibility at *Spp1* and *Il34* increased at the end of the trajectory (injured PT), indicating that chromatin remodeling might be
responsible for the gene expression changes (Supplementary Fig. 24D). To identify transcription factors that drive expressions of Spp1, Il34, and Pdgfb, we performed SCENIC (65) analysis. We identified Bcl3, Runx1, Fosl2, and Cebpz, as likely driver transcription factors (Supplementary Fig. 25).

Bulk gene expression data showed consistent changes in Spp1 and Il34 (Supplementary Fig. 24E). In situ hybridization further confirmed Spp1 and Il34 expression in PT cells in the DOCA group, and lower level in the control group (Supplementary Fig. 26). Additionally, we were able to validate the co-expression of HAVCR1, VCAM1, SPP1, and IL34 as the markers for iPT in spatial transcriptomics data obtained from patients with CKD in region with high extracellular matrix (ECM) production score (fibrosis) (37) (Supplementary Fig. 27).

Both Spp1 and Il34 are secreted molecules. To understand their effects on other cell types, we employed CellChat to conduct ligand-receptor interaction analysis (66, 67) (Fig. 7C, Supplementary Fig. 28). The receptors for Spp1, Itgav and Cd44, were expressed by immune cells (Fig. 7C). The predicted interaction between Spp1 and its receptors was weak in control and finerenone groups but much stronger in DOCA and spironolactone groups (Fig. 7C, Supplementary Fig. 28). We observed a similar pattern for Il34 and its receptor Csf1r (Fig. 7C and Supplementary Fig. 28). This cell-cell communication analysis revealed intense interactions emanating from iPT cells, which were markedly lower in the finerenone group (Supplementary Fig. 29). Pdgfb, a profibrotic factor, was highly expressed in DOCA samples by iPT cells and was found to interact with its receptor in the fibroblasts (Fig. 7C). Genes involved in ECM production and fibrosis, such as Col1a1, Col3a1, and Acta2, were expressed at the highest level in the DOCA treated group and lower levels in the finerenone treated group (Supplementary Fig. 30).
In summary we identified maladaptive differentiation of PT cells in a rat model of hypertensive kidney disease as well as changes in expression of proinflammatory molecules that occur during maldifferentiation.

**Conserved changes in Human Kidney Disease Samples**

Finally, we sought to determine the relevance of gene expression changes observed in injured PT cells in the DOCA rat model to human kidney disease. To do this, we analyzed 991 microdissected human kidney tubule samples from healthy individuals and patients with varying degrees of diabetic and hypertensive kidney disease. The demographics, clinical and histological characteristics of the human kidney samples are shown in Supplementary Table. 16.

The expressions levels of *SPP1*, *IL34*, and *PDGFB* were found to positively correlate with fibrosis (Fig. 8A). We then used the injured PT gene signature to perform hierarchical clustering of the human kidney samples (Fig. 8B and C). This signature clustered the 991 human kidney samples into three subgroups. Although the clinical information was not used for the clustering, the samples in these groups showed differences in their clinical characteristics. For instances, samples in cluster 2 had the lowest eGFR and highest degree of interstitial fibrosis. In this cluster, the expressions of *SPP1*, *IL34*, and *PDGFB* were the highest compared to the two other clusters, which exhibited better kidney function (Supplementary Fig. 31).

Overall, the extensive human kidney dataset from patients with hypertensive and diabetic kidney disease demonstrated changes consistent with those observed in the rat kidney dataset.
Additionally, the injured PT signature, which includes \textit{SPP1, IL34, and PDGFB} was capable of identifying diseased human kidney samples.

**Discussion**

Mineralocorticoids are hormones that play important roles in the conservation of sodium in land animals, a vital process for maintaining blood volume in environments where sodium is scarce (68). Mineralocorticoid excess can cause hypertension as well as kidney and heart disease (69). MRAs have been shown to protect against kidney and heart disease, however, the exact mechanism of action is poorly understood. In this study we used single-nucleus transcriptomics and open chromatin atlasing to examine the effects of mineralocorticoids in the kidneys of rats and analyzed the effects of two different MRAs and a direct blocker of ENaC side-by-side. Our 'omics' data, alongside various bioinformatics analyses, enabled us to identify the primary cell types targeted by mineralocorticoids, as well as the cell types indirectly affected by these hormones, and the target genes and networks involved. Our results demonstrate the central role of changes in PC cells in hypertension and mineralocorticoid action, and the importance of PST and injured PT cells in driving hypertensive kidney fibrosis in the DOCA/uninephrectomy/high salt intake rat model. Moreover, the injured PT gene signature was able to classify kidneys from patients with hypertensive and diabetic kidney disease.

Hypertension is the most common chronic disease (70), affecting nearly half of the population in the US (70). Hypertensive kidney disease is responsible for close to 25% of all CKD and ESRD (8), but the underlying disease mechanisms are not fully understood. Recent clinical reports
indicate the important role of mineralocorticoids excess in the development of HTN and CKD (12, 71). In this study, we performed detailed phenotypic and gene expression and gene regulation analyses at the bulk and single-cell levels in a rat model of mineralocorticoid-induced hypertension and hypertensive end-organ damage (72). We also compared our results to those obtained from patient kidney samples.

We present one of the first kidney single-nucleus transcriptomics and chromatin accessibility datasets for rat kidneys. Our results demonstrate that cell types and cell-type specific gene expressions changes are mostly conserved between mouse (28), human (37) and rat kidneys, although further studies are needed to explore species-specific differences. Using gene expression and chromatin accessibility information, we confirmed the mineralocorticoid-sensitive segment of the distal nephron, which includes PC cells as well as CNT and DCT2 cells (73). We also identified the MR target gene network, which will be important for understanding the actions of mineralocorticoid in the future (74, 75). Our findings show that mineralocorticoid sensitivity in the kidney is established through multiple sequential mechanisms, including changes in chromatin openness and the expression of MR, Hsd11b2, and MR target genes. We also observed that the expression of GR in the kidney is lower and follows an almost inverse pattern compared to that of MR.

Our work demonstrates the utility of single cell expression analysis for the understanding of disease driving pathways, identifying drug targets and assessing molecular outcomes for drug effectiveness. As clinical interventional studies are often costly, these intermediate molecular analyses could pave the way for novel clinical trial designs (76). We compared the effectiveness of spironolactone, finerenone, and a direct ENaC inhibitor in this study. All of these drugs
effectively lowered blood pressure and protected against kidney damage. When we analyzed gene expression changes at the single-cell level, we found that these drugs effectively normalized DOCA-induced gene expression changes. Our data highlights the importance of blood pressure-driven changes in kidney-specific genes, and the usefulness of single-cell gene expression in detecting such molecular outcomes. We also observed that finerenone had a greater benefit in reducing proteinuria for the same blood pressure change and had consistent effectiveness on podocytes and PT cells.

In this study, we used various orthogonal bioinformatic approaches to identify the cell types and gene expression changes driving disease development. Our results showed that hypertension is associated with changes in gene expression in multiple cell types. At the time of hypertensive nephrosclerosis, we observed that changes in PT cells were particularly important in driving the development of the disease phenotype. This was evident at both the bulk gene expression and single-cell levels. Unbiased tensor decomposition analysis revealed the crucial role of PC cells, as well as PT cells, especially injured PT cells, in driving the development of the disease phenotype. The presence of injured/profibrotic PT cells strongly correlated with fibrosis in all analyzed samples. Our analysis also identified Spp1, Il34, and Pdgfb as some of the top genes showing differences in both the bulk and single-cell datasets. Their levels correlated with disease severity, and improved following treatment with finerenone.

SPP1 is a phosphoprotein produced by the kidney, playing a role in cell adhesion and migration (77). Prior studies have shown the causal role of SPP1 in kidney disease in the unilateral ureteral obstruction (UUO), diabetic and LPS-induced kidney disease models (77, 78). Recently, SPP1 was proposed to be a hub gene for DKD progression by microarray analysis (79). Here, we show
that \textit{Spp1} is one of the key genes along PST to injured PT differentiation. \textit{Spp1} is influenced by finerenone. The receptors of \textit{Spp1}, such as \textit{Cd44} and \textit{Itgav} were expressed by stromal and immune cells and finerenone altered this interaction.

IL34 is another gene we found to correlate with PST to injured PT differentiation and influenced by finerenone. IL34 is a key cytokine stimulating the influx of macrophages and monocytes via binding to its receptor colony stimulating factor receptor-1 (CSF1R) (80). Prior studies have established the key role of PT derived IL34 and macrophage influx in the AKI and UOO models of kidney disease (80, 81). We demonstrated that \textit{Il34} is highly expressed in DOCA treated rats especially in injured PT cells and its expression is lower in finerenone treated animals. PDGFB is a well-known secreted molecule involved in the activation of fibroblasts and has been shown to play a role in kidney fibrosis in mice (57, 82). We observed high expression of \textit{PDGFB} in our iPT or profibrotic PT cells, and high expression of its receptor, \textit{PDGFRB}, in stromal cells, which could contribute to kidney fibrosis in hypertensive kidney disease. We also observed high production of extracellular matrix in the DOCA-treated group.

In this study, we observed that changes in a rat model of hypertensive kidney disease, including the expression of iPT cells, \textit{SPP1}, \textit{IL34}, and \textit{PDGFB}, strongly correlated with renal fibrosis in human kidney samples obtained from patients with hypertensive and diabetic kidney disease.

There are several limitations to our study. Although we incorporated earlier DOCA time points into our analysis to investigate a stage less affected by long-term hypertension effects, it can be challenging to distinguish between gene expression changes related to direct DOCA-MR effects and secondary effects due to hypertension. Additionally, our rat model incorporates three interventions: DOCA, unilateral nephrectomy, and salt intake, collectively inducing hypertension.
Therefore, the data does not only reflect DOCA-induced changes but changes in response to uninephrectomy and high-salt intake. Most importantly the observed changes are more consistent with the long-term hypertension and DOCA effect rather than the acute and short-term activation of MR in the kidney. While we employed a multi-omics approach to evaluate the mechanisms of action of MRAs, computational analysis can be challenging, and some findings necessitate further experimental validation. Some cell types are difficult to capture in snRNA-seq or snATAC-seq, which limits our ability to identify MR targets. We used a rat model and supraphysiological doses of DOCA (an aldosterone precursor), which are likely higher than aldosterone concentrations in CKD patients. Additional validation using samples from patients will be an important next step.

In summary, we have provided expression and chromatin accessibility maps for the rat kidney, defined MR target genes, cell networks, and the effects of steroidal and non-steroidal MRAs as well as ENaC inhibition and validated these findings in patient samples. Our study has delineated the mechanisms of MRA-mediated kidney protection at a cellular and molecular level.
Methods:

Animals

Male Sprague-Dawley rats weighing 250–300 g were obtained from Charles River. Animals were housed in an environment with controlled temperature and humidity, light/dark cycle at 22-24°C, and access to water and food ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

DOCA-Salt Hypertension Model

Rats were randomly divided into 5 groups. Group 1 underwent sham operation, whereas group 2-4 were uninephrectomized. Rats were anesthetized under isoflurane and buprenorphine (0.03mg/kg) and subjected to left nephrectomy. One week after the operation, groups 2-5 received weekly subcutaneous injections of deoxycorticosterone acetate (DOCA, 50 mg/kg; Sigma) suspended in sesame oil and were supplied 1% NaCl in the drinking water. Daily gavage was performed: group 2 received vehicle (10% ethanol, 40% solutol, 50% water); group 3 received finerenone (10mg/kg/d); group 4 received spironolactone (50mg/kg/d, Sigma); group 5 received amiloride (20mg/kg/d). To evaluate the early effects of DOCA and hypertension, two rats from each group were sacrificed 3 weeks after the start of the intervention, with the remaining rats sacrificed at the conclusion of the six-week period following the initiation of DOCA injections. Sham animals were subjected to the same surgical procedure where the kidney was exposed but not removed and received sesame oil injections and tap water.
**Blood Pressure Measurement**

Systolic and diastolic blood pressure were measured in conscious animals using the standard tail-cuff method (Blood Pressure Analysis System, BP2000-series, Visitech). All measurements were performed between 9:00 am and 1:00 pm, after 3–5 days of training.

**Plasma and Urine samples analyses**

Blood samples were analyzed by Abbott i-STAT portable clinical analyzer (Abbott Point-of-Care, East Windsor, NJ) and iSTAT 8+ cartridges (Abbott Laboratories) to determine sodium (Na), potassium (K), chloride (Cl), TCO₂, Anion Gap, Ionized Calcium (iCa), Urea Nitrogen (BUN), Creatinine (Cre), glucose (Glu), hematocrit (Hct), and hemoglobin (Hb).

Urine albumin was determined using rat albumin specific ELISA (Bethyl Laboratories) and creatinine by reagent set (Diazyme, DZ072b-KY1), per manufacturer’s protocol. Plasma renin sample was measured using ELISA (Sigma Aldrich, #RAB1162-1KT) per manufacturer’s protocol.

**Histological Analysis**

For histological analysis, kidneys and hearts were fixed in 4% paraformaldehyde overnight, dehydrated, embedded into paraffin blocks and sectioned onto glass slides. Sections were stained with H&E and picrosirius red.

**In situ hybridization**

*In situ* hybridization was performed using formalin-fixed paraffin-embedded kidney tissue samples and the RNAScope 2.5 HD Duplex Detection Kit (bio-techne, 322430) following
manufacturer’s original protocol. The following probes were used for the RNAScope assay: Rn-Ill34-C2 (Cat No. 1056011-C2), Rn-Spp1-C2 (Cat No. 405441-C2), and Rn-Haver1 (Cat No. 519081).

**Single nuclei RNA and ATAC sequencing**

A detailed description of the single-nuclei RNA and ATAC protocols can be found in the supplemental methods (83).

**Rat kidney Bulk RNA sequencing**

The total RNA from 10 mg of each frozen rat kidney tissues was isolated using Qiagen RNeasy kit (catalog #74106) according to manufacturer’s instructions. To check RNA quality, Agilent Bioanalyzer RNA 6000 Pico kit (Agilent Technologies, 5067-1513) was used. All samples with RNA integrity number (RIN) at least 6 were used for cDNA preparation. Strand specific RNA-seq libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (catalog #E7530L) following manufacturer’s protocol and the RNA seq libraries were sequenced to a depth of 20 million 2x150 pair end reads.

**Human Sample Procurement**

The collection of human kidney tissue was approved by the University of Pennsylvania institutional review board (IRB). The study was IRB exempt because the samples were considered medical discard and they were permanently deidentified and no personal identifier were collected. We used an honest broker to gather clinical information. Part of the collected tissues was formalin-fixed and paraffin-embedded and sectioned and stained with periodic acid–Schiff and scored by a local renal pathologist. Human kidney tissues were microdissected under dissecting microscope.
RNA isolation, quality control, cDNA library for human microdissected tubules were performed as described for rat bulk RNA-seq. The data files were already deposited into GSE115098 and GSE173343.

**Bioinformatic analysis**

A detailed summary of the performed bioinformatic analysis and applied computational tools can be found in the supplemental methods (83-89).

**Statistics**

Data were expressed as means ± SEM. One-way ANOVA was used to compare the continuous parameters between more than two groups. The student’s t test (2-tailed) was used to compare each group to DOCA treatment. DOCA group was the reference in by-comparison analyses. UACR values were log transformed before comparing between groups. The Chi-square test was used to compare the fractions and frequencies between groups. The correlation analysis between gene expressions and clinical characteristics in human samples was done using Spearman’s test. A P value less than 0.05 was considered significant.

**Study approval**

For the rat study, the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. The collection of human kidney tissue was approved by the University of Pennsylvania institutional review board (IRB). The study was IRB exempt because
the samples were considered medical discard and they were permanently deidentified and no personal identifier were collected.

Data Availability

Raw data, processed data, and metadata from the snRNA-seq, snATAC-seq, and bulk RNA-seq of different experimental groups of rats have been deposited in Gene Expression Omnibus (GEO) with the accession code of GSE183842. The human kidney RNA-seq data are available under following accession numbers: GSE115098 and GSE173343. All the data supporting the graphs and tables are provided in the file named "Supporting Data Values." The snRNA-seq and snATAC-seq data are visualized in susztaklab.com (https://susztaklab.com/genemap_rat).

Accession code

All the codes used for the analysis were deposited on GitHub (https://github.com/amin69upenn/DOCA_Rat_Kidney).

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Competing interests

HC and PK are employees of BAYER AG, Pharmaceuticals.

Author Contributions

ASN, JW, ZM, AA, and JF performed experiments and analyzed data. AA, ZM, MSB, HL, SV, HC, KAK and PK performed computational analysis. HC and PK offered experimental suggestion. KS was responsible for overall design and oversight of the experiments. KS supervised the experiment. AA and KS wrote the original draft. All authors contributed to and approved the final version of the manuscript.
References


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**A**

<table>
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<th>DOCA</th>
<th>Finerenone 10mg/kg</th>
<th>Spironolactone 50mg/kg</th>
<th>Amiloride 20mg/kg</th>
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</tr>
</tbody>
</table>

1% NaCl drinking water

DOCA, 50mg/kg, s.c. weekly

**B**

- **SBP (mmHg)**
- **DBP (mmHg)**
- **BUN (mg/dL)**
- **Cr (mg/dL)**
- **UACR (mg/mg)**
- **Kidney Weight/Body Weight (mg/g)**

**C**

- **Heart**
- **Kidney**

**D**

- **Kidney**

**Fig. 1.**
Fig. 1. Mineralocorticoid receptor antagonists protect from DOCA-salt induced cardio-renal damage.

(A) Study overview. Rats were divided into five groups: (i) Sham, (ii) DOCA+Vehicle, (iii) DOCA+Finerenone, (iv) DOCA+Spironolactone, (v) DOCA+Amiloride.

(B) Clinical and biochemical parameters in experimental groups, including systolic and diastolic blood pressure, (SBP, DBP), blood urea nitrogen (BUN), creatinine (Cr), urinary albumin creatinine ratio (UACR), kidney to heart weight ratio, and renal fibrosis. The x-axis shows the weeks following the surgery, y-axis shows the measurement values. Black lines represent sham, red lines DOCA salt group, blue lines DOCA salt rats treated with finerenone, green lines DOCA salt group treated with spironolactone, and yellow lines DOCA salt group treated with amiloride. One-ANOVA test were used to compare all groups and student’s t-test (2-tailed) was used to compare each group with DOCA salt group. For statistical comparison log-transformed data was used for the UACR. Asterisks indicate significant differences in measure parameters between sham, finerenone, spironolactone, and amiloride treated compared to the DOCA group as follows: *$P<0.05$, **$P<0.01$. Bars indicate SEM.

(C) Representative H&E-stained kidney and heart section from experimental groups (20X magnification).

(D) Representative Picrosirius Red staining of kidney sections of experimental groups (20X magnification).

NaCl; Sodium Chloride, DOCA; deoxycorticosterone acetate, UNX; uni-nephrectomized, BUN; blood urea nitrogen, UACR; Urine Albumin to Creatinine Ratio.
Fig. 2.

- **Motifs**
  - Sox13
  - Meis1
  - Zbtb7c
  - Wt1
  - Hnf1a
  - Hnf4a
  - Hnf4g
  - Gli3
  - Tapp2b
  - Nr3c2
  - Foxp1
  - Tfec

- **Open Chromatin**

- **Gene Expression**
Fig. 2. The single cell multi-omics landscape of the healthy and diseased rat kidney.

(A) UMAP of 310,218 rat kidney single nuclei RNA-seq data.

(B) UMAP of 53,298 rat kidney single nuclei ATAC-seq data.

(C) UMAP of integrated snRNA-seq and snATAC-seq of rat kidneys.

(D) Bubble dot plots of marker genes used for cell type annotation in the snRNA-seq. The size of the dot indicates the percentage of positive cells, and the darkness of the color indicates the average expression.

(E) Fragment coverage (frequency of Tn5 insertion) in each snATAC-seq cluster at the cell type marker gene promoter site.

(F) Heatmap of average chromVAR motif activity for each cell type (left panel). The color scale shows a z-score scaled by row. Chromatin accessibility and gene expression of representative motifs of each cluster are shown in middle and right panel, respectively. The color scheme of the heatmap is based on z score distribution. Each row is a gene, and each column is a cell type.

Endo; endothelial cells, MyoFib; myofibroblast, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, iPT; injured proximal tubule cells, Prolif_Tubule; proliferative tubule cells, LOH; loop of Henle, DCT; distal convoluted tubule; PC; principal cells of collecting ducts, IC; intercalated cells, IC_A; Type A intercalated cells, IC_B; Type B intercalated cells, Mono; monocyte, Macro; macrophage.
Fig. 3.
Fig. 3. Mineralocorticoid receptor target cell types and gene regulatory network in rat kidneys

(A) Feature plots of open chromatin, motif in open chromatin, and gene expression of Nr3c2 (mineralocorticoid receptor: MR), and Nr3C1 (glucocorticoid receptor: GR), mineralocorticoid receptor target genes, and glucocorticoid receptor target genes. MR target genes and GR target genes expression are shown based on the mean expression in each cell type.

(B) Bubble plots of open chromatin, motif, and gene expression of Nr3c2 (MR) and Nr3C1 (GR), including their target genes, with the mean expression displayed for each cell type.

(C) Bubble dot plots of mineralocorticoid target genes and the glucocorticoid receptor (Nr3c1) in the snRNA-seq dataset before and after subclustering of DCT and PC. The size of the dots indicates the percentage of positive cells, and the darkness of the color indicates average expression.

(D) A schematic of MR target genes impacted by DOCA in the DOCA salt rat nephropathy model. Genes are colored blue (lower), red (higher), or white (unchanged). Notably, Atp1a1 and Atp1b1 show increased expression, while Hsd11b2 is lower in all cells. Pik3r3 is higher in PC cells. ENaC genes (Scnn1a, Scnn1b, Scnn1g), Wnk1, and Aqp2 show decreased expression.

Endo; endothelial cells, MyoFib; myofibroblast, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, iPT; injured proximal tubule cells, Prolif_Tubule; proliferative tubule cells, LOH; loop of Henle, DCT; distal convoluted tubule; PC; principal cells of collecting ducts, IC; intercalated cells, IC_A; Type A intercalated cells, IC_B; Type B intercalated cells, Mono; monocyte, Macro; macrophage, ROMK; renal outer medullary potassium channel, CHIF; corticosteroid hormone-induced factor, Nox4; NADPH oxidase 4.
CNT; connecting tubule cells, DCT1; Type 1 distal convoluted tubule, DCT2; Type 2 distal convoluted tubule.
Fig. 4.
Fig. 4. MRA and amiloride target genes, cell types and pathways

(A) The number of differentially expressed genes between DOCA treated and control groups in all kidney cell types 6 weeks and 3 weeks after DOCA administration.

(B) Volcano plot of differentially expressed genes between DOCA and the control group in PC cells at 6 weeks on DOCA.

(C) The number of DEGs between control and DOCA in each cell type. The number of genes normalized by all drugs or by specific drugs; finerenone, spironolactone, or amiloride. The color indicates a heatmap, more DEGs are in red, fewer in blue. Asterisks (*) indicates the significant DEG differences (normalized genes) calculated using chi-square test (p<0.05).

(D) Volcano plot of differentially expressed genes between DOCA and control groups in PT cells at 6 weeks on DOCA (upper panel). The correlation between injured proximal tubule fractions and renal fibrosis in all samples using Pearson’s correlation.

(E) Gene ontology analysis of the genes affected by finerenone in PT and podocyte using DAVID. The enriched pathway is shown by the -Log (FDR) of each pathway.

Endo; endothelial cells, MyoFib; myofibroblast, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, iP; injured proximal tubule cells, Prolif_Tubule; proliferative tubule cells, LOH; loop of Henle, DCT; distal convoluted tubule; PC; principal cells of collecting ducts, IC; intercalated cells, IC_A; Type A intercalated cells, IC_B; Type B intercalated cells, Mono; monocyte, Macro; macrophage.
Fig. 5.
Fig. 5. Genome-wide gene expression changes in whole kidney samples of DOCA rats treated with MRAs.

(A) The number of differentially expressed genes between DOCA treated and control group by bulk RNAseq analysis.

(B) The enrichment of genes showing lower expression by DOCA in PST in snRNA-seq (upper panel) and snATAC-seq dataset (lower panel). The color scheme of the heatmap is based on z-score distribution. Each row is a gene, and each column is a cell type. Yellow indicates cell type enriched genes.

(C) The expression of 25 genes showing higher or lower levels in DOCA vs. control groups in bulk RNA-seq dataset. The color scheme of the heatmap is based on z-score distribution. Each row is a gene, and each column is a rat sample. Black and red colors indicate control and DOCA treated rats, respectively.

(D) The volcano plot of DEGs between DOCA and control in bulk RNA-seq data.

(E) The cell type expression (snRNA-Seq and snATAC-Seq) of top up-regulated DEGs in DOCA vs. control groups identified in the bulk analysis. The color scheme of the heatmap is based on z score distribution, yellow is higher while blue is lower. Each row is a gene, and each column is a cell type.

DOCA; deoxycorticosterone acetate, Endo; endothelial cells, MyoFib; myofibroblast, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, iPT; injured proximal tubule cells, Prolif_Tubule; proliferative tubule cells, LOH; loop of Henle, DCT; distal convoluted
tubule; PC; principal cells of collecting ducts, IC; intercalated cells, IC_A; Type A intercalated cells, IC_B; Type B intercalated cells, Mono; monocyte, Macro; macrophage.
**A**

Tensor Decomposition

**B**

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<th>Factor 3</th>
<th>Factor 4</th>
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</table>

**C**

Fig. 6.

**D**

PC → PST → iPT → Single Cell WGCNA → Phenotype-Associated Genes

- **PC** 2,352 Genes
  - PC-M: Nr3c2/Kcnq1/Scnn1b
  - Expression

- **PST** 2,268 Genes
  - PST-M: Hnf4a/Slc5a10/Zeb1
  - Expression

- **iPT** 2,828 Genes
  - iPT-M: Il34/Spp1/Nfkbi
  - Expression

Fig. 6.
Fig. 6. Principal cells and proximal tubule cells are the main target of mineralocorticoids and MRAs

(A) Overview of the tensor decomposition analysis. All rat samples were included in the analysis.

(B) Sample score heatmap for the decomposition of the snRNA-seq data showing each sample and its loading scores for each factor. Colors on the right indicate sample groups. Each identified factor and its association with phenotypes. Each row is a phenotype and the color indicates the p value of factor and phenotype association using univariate linear model F-tests (upper panel). Two samples filtered by the analysis.

(C) Heatmaps showing the cell type gene loading scores of genes in factors 1 and 4. Some of the genes are highlighted.

(D) Single nuclei WGCNA on PC, PST, and iPT identified gene expression modules in cell types. Top representative genes in each cell type specific module are highlighted. Bubble plots indicate gene expression level of the genes in each module in each cell type (middle panel) calculated by the average expression of all genes in a module in a specific cell type. The lower panel shows the module scores per condition in each cell type.

Endo; endothelial cells, MyoFib; myofibroblast, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, iPT; injured proximal tubule cells, Prolif_Tubule; proliferative tubule cells, LOH; loop of Henle, DCT; distal convoluted tubule; PC; principal cells of collecting ducts, IC; intercalated cells, IC_A; Type A intercalated cells, IC_B; Type B intercalated cells, Mono; monocyte, Macro; macrophage.
Fig. 7.
Fig. 7. Cellular Trajectory of PST and injured PT cells highlights Spp1, Il34 and Pdgfb

(A) UMAP representation of PT cell sub-clustering and injured PT differentiation trajectory from PST in snRNA-seq (Left panels). Cells are colored by pseudotime and the arrow indicates the direction of the pseudotime. Right panel shows the UMAP representation of gene expressions of Spp1, and Il34, Pdgfb during trajectory (Red dots indicate the expression of each gene in the cells).

(B) UMAP representation of PT sub-clustering and injured PT differentiation trajectory from PST in snATAC-seq (Left panels). Cells are colored by pseudotime and arrow indicates the direction of the pseudotime. Right panel shows the UMAP representation of gene activity of Spp1, Il34, Pdgfb during trajectory (Red dots indicate the gene activity calculated based on chromatin accessibility).

(C) Bubble dot plot of expressions of Spp1, Il34, Pdgfb and their receptors Itgav, Cd44, Csf1, and Pdgfrb in different cell types and groups. The size of the dot indicates the percentage of positive cells and the darkness of the color indicates average expression.

Endo; endothelial cells, Podo; podocyte, PCT; proximal convoluted tubule, PST; proximal straight tubule, Injured_PT; injured proximal tubule cells, Prolif_PT; proliferative proximal tubule cells, LOH; loop of Henle, DCT; distal convoluted tubule; PC; principal cells of collecting ducts, IC; intercalated cells, Spp1; secreted phosphoprotein, Il34; interleukin 34.
Fig. 8.

**A**

![Graphs showing correlations between SPP1, IL34, and PDGFB expressions and fibrosis](image)

**B**

**RNAseq for 991 human kidneys**

DOCA rat Injured PT Gene Signature

**C**

Cluster 1
N=473

Cluster 2
N=238

Cluster 3
N=280

- **Cluster 1**
  - p-values: 0.77, 0.46, 0.04, 0.54, 0.03, 0.0001

- **Cluster 2**
  - Age: 30, Female: 60, African American: 40, Hypertension: 80, Diabetes Mellitus: 80, eGFR: 50, Proteinuria: 20
  - p-values: 0.77, 0.46, 0.04, 0.54, 0.03, 0.0001

- **Cluster 3**
  - p-values: 0.77, 0.46, 0.04, 0.54, 0.03, 0.0001
Fig. 8. Injured PT signature can classify disease severity in human diabetic and hypertensive kidney tissue samples

(A) Correlations between SPP1, IL34, and PDGFB with fibrosis in microdissected human kidney tubule samples. X-axis represents normalized (logTMP) gene expression and y-axis represents fibrosis score (log transformed). Each dot indicates one sample. Spearman’s test and correlation coefficient (r) as well as regression line are shown in each plot. *P<0.05, **P<0.01, ***P<0.0001

(B) Overview of the experiments. The homologous genes for the injured PT gene signature in rats were used to cluster 991 human kidney microdissected tubules.

(C) Three distinct human kidney clusters were identified based on the injured PT signature using hierarchical clustering. Three main clusters in the dendrogram were shown by different colors. The graphs represent the clinical information of samples in the three clusters. Chi-square test for non-parametric and one-way ANOVA test for parametric data was used for statistical comparison. Bars indicate SD.

SPP1; secreted phosphoprotein, IL34; interleukin 34, PDGFB; platelet derived growth factor subunit b. AA; African American, HTN; hypertension, DM; diabetes mellitus, eGFR; estimated glomerular filtration rate.