

MMP-9 contributes to glomerulosclerosis by causing profibrotic changes in podocytes and glomerular endothelial cells

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Research article

Keywords: Matrix metalloproteinase 9, glomerulosclerosis, podocyte, endothelial cell

Posted Date: September 10th, 2019

DOI: <https://doi.org/10.21203/rs.2.14205/v1>

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Abstract

Background Glomerulosclerosis is characterized by progressive (myo)fibroblast accumulation and collagen deposition involving profibrotic changes of podocytes and endothelial cells. A profibrotic role of MMP-9 in interstitial fibrosis has been reported. Whether MMP-9 plays a role in glomerulosclerosis is not clear yet.

Methods A mouse glomerulosclerosis model [adriamycin nephropathy (AN)] was produced in MMP-9^{-/-} and wild-type control BALB/c mice. All animals were sacrificed at 4 weeks after injection. Albuminuria (albumin to creatinine ratio) and calculated GFR were measured. Gomori Trichrome (GT) and Sirius Red (SR) staining were used for assessment of glomerular fibrosis. Profibrotic changes of podocytes or glomerular endothelial cells were examined by confocal microscopy using immunofluorescence staining (IF) of desmin or α -SMA with P-cadherin or VE-cadherin.

Results Albuminuria was reduced while GFR was increased in MMP-9^{-/-} AN mice compared with those of wild-type mice. Confocal microscopy showed a significant decrease in podocytes double-stained with P-cadherin and desmin and decrease in glomerular endothelial cell co-staining with VE-cadherin and α -SMA, demonstrating that MMP9^{-/-} AN mice were protected from profibrotic changes in podocytes and glomerular endothelial cells. Glomerulosclerosis was significantly reduced in MMP-9^{-/-} AN mice compared to that of WT, as demonstrated by GT and SR staining.

Conclusions MMP-9 contributes to profibrotic changes in podocytes and glomerular endothelial cells and thereby glomerulosclerosis.

Background

Glomerulosclerosis is a hallmark of chronic kidney disease (CKD) [1, 2]. It is characterized by the accumulation of myofibroblasts and excessive deposition of extracellular matrix components. Myofibroblasts are the key effectors in glomerulosclerosis. Both epithelial-mesenchymal transition (EMT) of podocytes and endothelial-mesenchymal transition (EndoMT) are major sources of myofibroblasts formation in kidney fibrosis [3, 4].

Podocytes are terminally differentiated visceral epithelial cells that are critical components of the glomerular barrier and play an important role in selective permeability of the glomerular filtration barrier. Previous studies proved that podocyte depletion leads to glomerulosclerosis [5]. Accumulating evidence indicates that in response to injurious stimuli, podocytes may undergo EMT, lose their epithelial surface markers such as P-cadherin, and express mesenchymal markers such as desmin. Podocytes are rendered motile after EMT, resulting in detachment from the glomerular basement membrane and podocyte loss,

without apoptosis, and finally leading to a defective glomerular filtration, proteinuria and glomerulosclerosis [6].

Renal endothelial cells, especially glomerular endothelial cells, contribute to fibroblast formation in kidney by EndoMT [7]. EndoMT may be a major source of activated fibroblasts or myofibroblasts [8]. During EndoMT, endothelial cells lose endothelial markers, such as vascular endothelial–cadherin (VE-cadherin), and acquire mesenchymal markers, such as α -smooth muscle actin (α -SMA) [9]. Previous studies have indicated that glomerular sclerosis is related to EndoMT, and inhibition of EndoMT can prevent glomerular sclerosis [10].

Matrix metalloproteinase 9 (MMP-9) has been proven to cause kidney interstitial fibrosis [11]. However, whether it plays a role in glomerulosclerosis is not clear yet. We have demonstrated that MMP-9 induced EndoMT in mouse peritubular endothelial cells downstream of TGF- β 1 [11], indicating that it may also contribute to glomerulosclerosis. In the present study, we aimed to investigate the role of MMP-9 in the development of glomerulosclerosis. We hypothesized that MMP-9 may induce EMT of podocytes and EndoMT of glomerular endothelial cells, thereby leading to glomerular sclerosis. Animal model of Adriamycin nephropathy, an analogue of human fragmental glomerular sclerosis, was used to test the hypothesis.

Methods

Animals and adriamycin-induced nephropathy model

Mouse adriamycin nephropathy (AN) was induced by a single injection of adriamycin (10.2 mg/kg, with physiological saline for controls) through tail vein in MMP-9 knockout (MMP-9^{-/-} mice crossed into a BALB/c background, obtained from Prof Zena Werb of University of California San Francisco) and wild-type control (BALB/c mice crossed with MMP-9^{-/-} mice of BALB/c background, the MMP-9^{+/+} phenotype was chosen as control mice. Both MMP-9^{-/-} and control mice, male healthy with body weight between 20 to 25 gram, are randomly selected control (saline) and adriamycin injection group, 5 mice each group, total 20 miced, standard 4 mice each standard filtered PVC cage, with standard light/dark cycle, temperature control, free access to water and standard normal food. Injection was performed in specific pathogen free PC2 operation room of animal housing facility. All animals were euthanased by CO₂ at 4

weeks after injection, to collect tissue samples. Experiments were carried out in accordance with the protocols approved by the Animal Ethics Committee of Western Sydney Local Health District.

Urinary proteinuria concentration and kidney function

Urinary albumin to creatinine ratio was used to evaluate proteinuria. Urinary albumin concentration was determined by nephelometric method as reported [12]. Urinary creatinine concentration was determined by enzymatic method. Blood samples were taken from mice before sacrifice.

For calculation of GFR, mice were acclimatised to the metabolism cages for 48 h prior to 24 h urine collection. Urine samples were collected in metabolism cages 24 h before sacrifice. Serum creatinine levels were determined using a creatinine assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Calculated GFR was evaluated by creatinine clearance using the standard formula.

Histological analysis

Four weeks after adriamycin treatment, paraffin-embedded kidney sections (4 μm) were deparaffinised with xylene and rehydrated through a descending ethanol gradient. Histological sections were examined following Sirius red (SR) or Gomori trichrome (GT) staining. Quantification of pulmonary and kidney fibrosis was performed as we described previously [13]. All scoring was performed in a blinded manner.

Immunofluorescence analysis

Frozen kidney blocks were cut into 7 μm sections and fixed with ice-cold acetone for 10 min at -20°C and blocked with 2% BSA for 1 h. Double immunofluorescence staining was performed using combinations of antibodies against P-cadherin and desmin, VE-cadherin and α -SMA, respectively. Tissue sections were then incubated with its corresponding fluorescence-conjugated secondary antibody. After washing with PBS, sections were counterstained with DAPI for 5 min before mounting with fluorescence mounting

medium. Images were obtained using a confocal microscope (Olympus FV1000) at $\times 40$ magnification. For quantitative analysis, the percentage of the area stained positive for P-cadherin and desmin, or VE-cadherin and α -SMA, were counted on high power fields (HPFs) in a blinded manner.

Statistical analysis

Results were expressed as means \pm SEM. Statistical significance was evaluated using unpaired two-tailed *t*-test for comparison between two groups. The level of significance was set at $p < 0.05$.

Results

MMP-9 KO reduces albuminuria and improves kidney function in mice

The urinary albumin/creatinine ratio and GFR were not different between wild-type control mice and MMP-9^{-/-} controls. Adriamycin injection resulted in massive proteinuria in MMP-9 wild-type mice. However, it induced less pronounced albuminuria in MMP-9^{-/-} mice (Figure 1). Mice treated with adriamycin had markedly decreased GFR compared with normal animals. MMP-9^{-/-} mice were protected from developing renal impairment; their GFR was significantly higher than wild-type AN mice. (Figure 2).

Effect of MMP-9 knockout on the progression of glomerulosclerosis

Kidney injury is characterized by glomerulosclerosis in AN. We showed here that in both wild-type and MMP-9^{-/-} mice, adriamycin induced glomerular changes in contrast to the normal glomeruli of control mice. MMP-9^{-/-} AN mice showed significantly less glomerulosclerosis than wild-type AN mice. These results indicate that knockout of MMP-9 gene attenuated the progression of glomerulosclerosis (Figure 3A and B).

Effect of MMP-9 knockout on podocytes and glomerular endothelial cells

Mesenchymal transition of podocytes (EMT) and endothelial cells (EndoMT) have been implicated in glomerulosclerosis [14] [15]. We hypothesized that MMP-9 plays a role in both of these. To test our hypothesis, double staining for P-cadherin (a podocyte marker) and desmin (a podocyte injury marker), or VE-cadherin (an endothelial marker) and α -SMA (a myofibroblast marker) was performed on kidney sections. P-cadherin and desmin, VE-cadherin and α -SMA were not significantly different in MMP-9^{-/-} control kidneys compared with wild-type controls (Figure 4A and C, 5A and C). Immunofluorescence staining showed that P-cadherin was primarily localized in cell-cell junctional sites of differentiated podocytes in wild-type and MMP-9^{-/-} mice (Figure 4A and C). P-cadherin was significantly reduced and desmin significantly increased after adriamycin treatment in both wild-type and MMP-9^{-/-} mice (Figure 4B and D). In wild-type mice and MMP-9^{-/-} controls mice, VE-cadherin was highly expressed in the whole glomerular tuft, whereas α -SMA was present along the capillary loop (Figure 5A and C). In contrast, α -SMA was strongly expressed in the whole glomerular tuft of AN mice (Figure 5B and D). Of note, MMP-9^{-/-} AN had significantly decreased the levels of desmin with increased P-cadherin (Figure 4D), and decreased α -SMA with increased VE-cadherin (Figure 5D) when compared with wild-type AN mice (Figure 4A and 5A). These results demonstrate that MMP-9 contributes to EMT of podocytes and EndoMT, and thus to development of glomerulosclerosis.

Discussion

Glomerulosclerosis is the final pathological process common to CKD [1]. MMP-9 plays a key role in kidney interstitial fibrosis [16], but little is known about its behaviour in glomerulosclerosis. In the present study, we investigated the effect of MMP-9 on glomerulosclerosis in murine AN, a model of human focal segmental glomerulosclerosis [17]. We found that glomerular fibrosis in MMP-9^{-/-} mice was less severe than in wild-type controls, indicating that MMP-9 is involved in glomerulosclerosis. Although an inducible knockout of MMP-9 would be necessary to distinguish effects on induction versus development and progression of AN, this was not possible as AN could not be induced in inducible knockout mice which are of a non-BALB/c background.

Podocyte is regarded as a key player in glomerular health and disease [18]. Recent studies indicate that podocyte injury is a common trigger leading to the disruption of the filtration barrier and protein

leakage [19], and ultimately results in glomerulosclerosis. Podocytes are involved in glomerulosclerosis, at least in part, by EMT [20]. We examined the expression of P-cadherin, a predominant podocyte marker, and desmin, a mesenchymal marker, in glomeruli in AN mice. Our results showed that podocytes had decreased expression of P-cadherin protein and instead showed increased expression of desmin, which indicates that podocytes undergo EMT in both MMP-9^{-/-} and wild-type control AN mice. However, podocyte EMT was significantly decreased in MMP-9^{-/-} compared to control mice, suggesting that MMP-9 plays an important role in podocyte EMT.

Emerging evidence indicates a critical role for EndoMT in tissue fibrogenesis [21]. It is reported that EndoMT of glomerular endothelial cells is also involved in the development of glomerulosclerosis [22]. During EndoMT, endothelial cells lose endothelial markers, such as cluster of differentiation 31 (CD31) and VE-cadherin, and acquire mesenchymal markers, such as fibroblast-specific protein 1 and α -SMA [9, 10]. We showed here that AN injection increased VE-cadherin expression, and decreased the expression of α -SMA in both MMP-9^{-/-} mice and wild-type controls, suggesting that an EndoMT program is activated in glomerular endothelial cells after AN treatment. The EndoMT was more predominant in wild-type than MMP-9^{-/-} mice, indicating that MMP-9 plays an important role in EndoMT of glomerular endothelial cells. Given the solid evidence for EMT and EndoMT in kidney fibrosis in previous studies including ours, their respective contribution to glomerulosclerosis was not the focus of the current study.

Conclusions

We found that MMP-9 plays an important role in kidney fibrosis, at least in part through podocyte EMT and glomerular endothelial cell EndoMT. Pharmacological inhibition of the MMP-9 could be a therapeutic approach for treating glomerular renal disease.

Abbreviations

CKD, chronic kidney disease; AN, Adriamycin nephropathy; MMP-9, matrix metalloproteinase 9; GFR, Glomerular filtration rate; EMT, epithelial-mesenchymal transition; EndoMT, endothelial-mesenchymal transition.

Declarations

Ethics approval

Animal experiments were carried out in accordance with the protocols approved by the Animal Ethics Committee of Western Sydney Local Health District.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

There are no competing interests except author Stephen I Alexander being the associate editor of the BMC nephrology

Funding

The study was supported by an NHMRC project grant (APP632688 & APP1046647). NHMRC does not play a role in the design of the study, nor in collection, analysis, and interpretation of data and writing the manuscript.

Authors' contributions

XQ, JG performed experiments, analyzed data, wrote the manuscript. ML, LL, PR, YZ, QC, YPW helped with experiment design and analyzed data. YMW, VL, SIA helped with experiments and analyzed data. DCH and GZ conceived and supervised the study, analyzed and interpreted data, wrote, revised and approved the final version of the manuscript.

Acknowledgements

Not applicable

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Figures

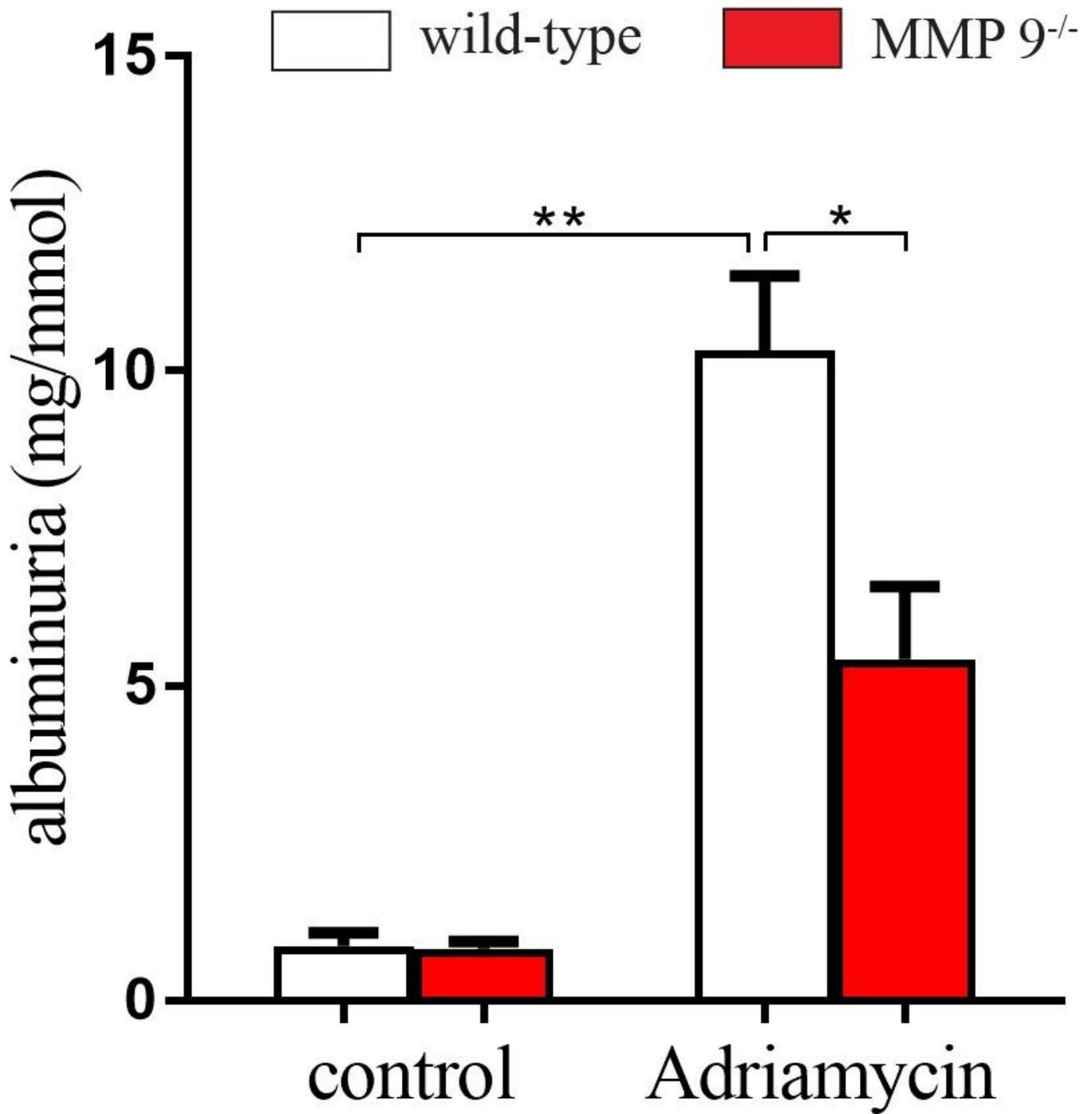


Figure 1

Effects of MMP-9 on albuminuria in mouse adriamycin nephropathy. Mouse adriamycin nephropathy was induced by a single tail vein injection of adriamycin (10.2 mg/kg) or saline as control in MMP-9^{-/-} and wild-type mice. Quantitation of albuminuria, using unpaired two-tailed t test. Results are shown as \pm SEM (n=5 for each group). *P<0.05, **P<0.01.

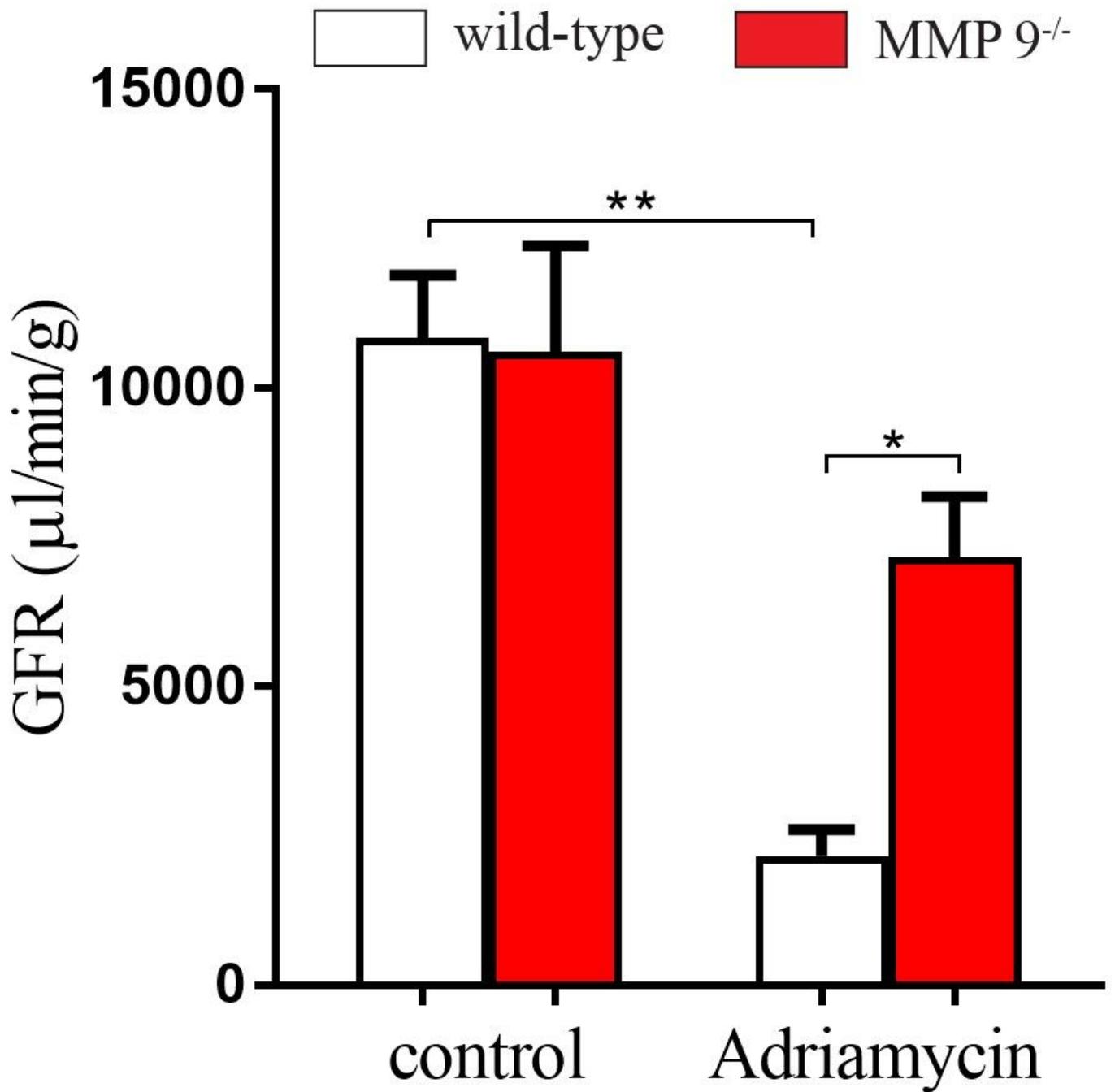


Figure 2

Effects of MMP-9 on GFR in adriamycin nephropathy. Quantitation of GFR, using unpaired two-tailed t test. Results are shown as \pm SEM (n=5 for each group). *P<0.05, **P<0.01.

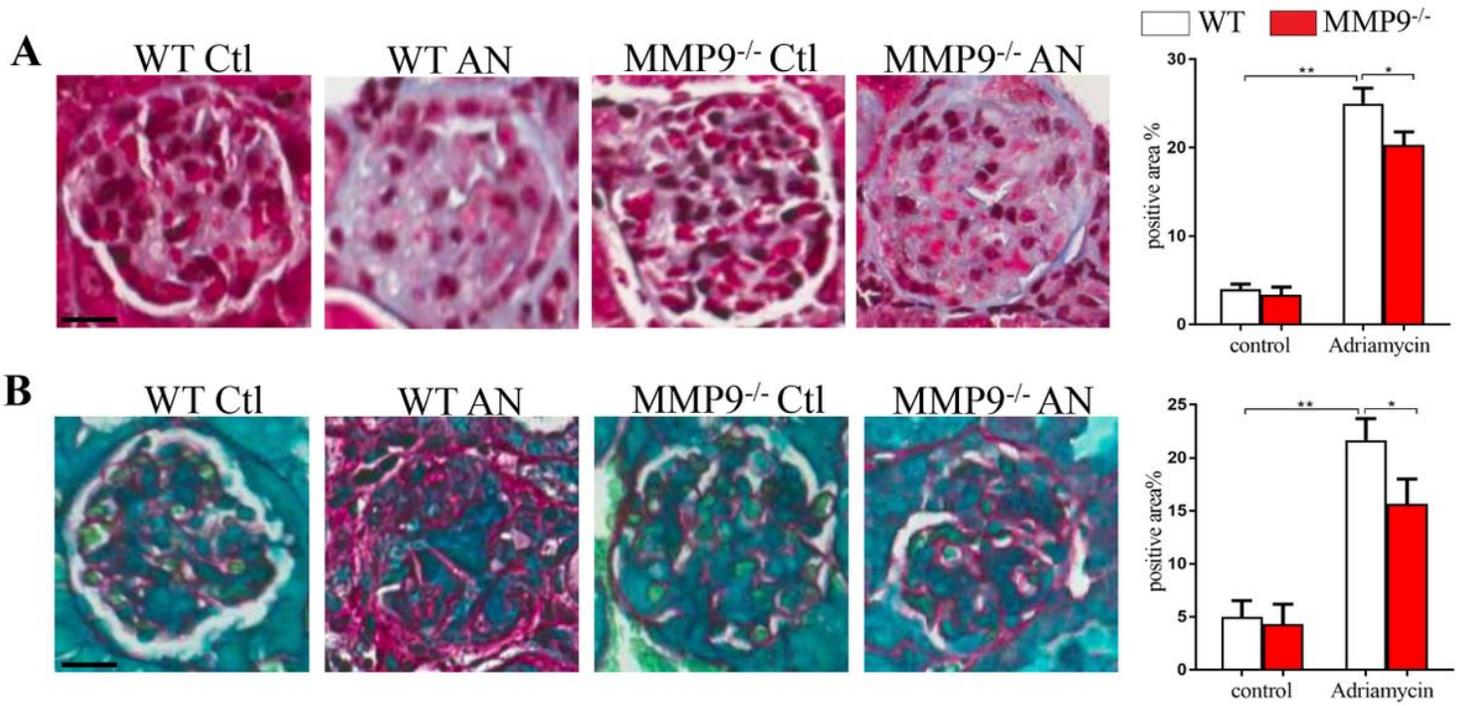


Figure 3

Effects of MMP-9 on the progression of glomerulosclerosis in adriamycin nephropathy. Representative Gomori trichrome (A) and Sirius red (B) stained images are shown. Original magnification, X60. Scale bar, 40 μ m. Quantitation of the glomerular area, using unpaired two-tailed t test. Results are shown as \pm SEM (n=5 for each group). *P<0.05, **P<0.01.

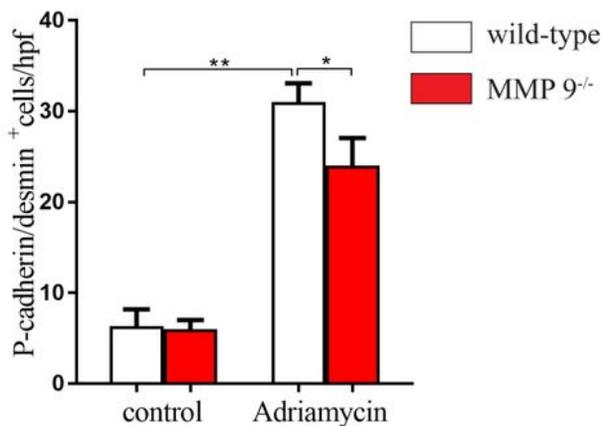
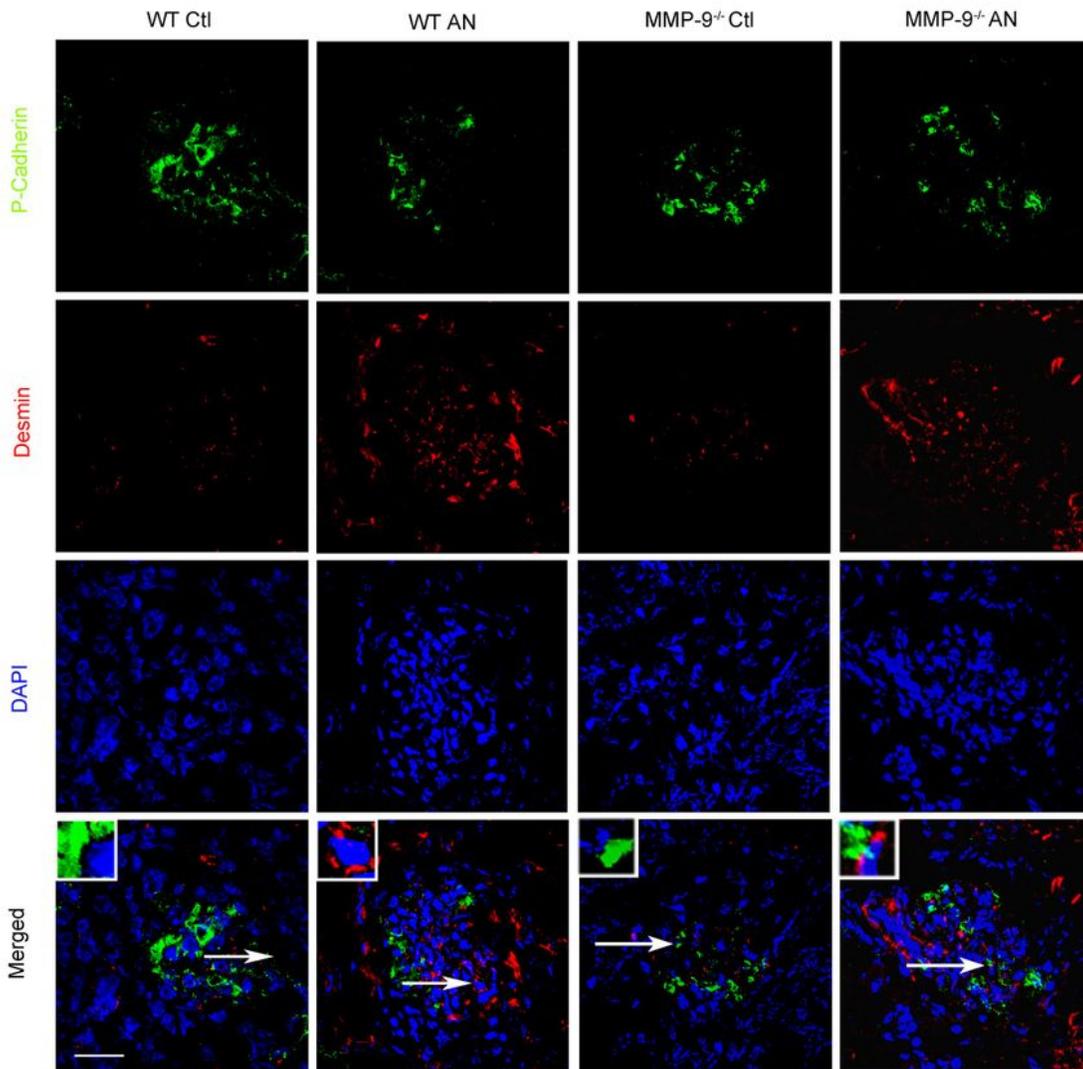


Figure 4

Effects of MMP-9 on podocyte P-cadherin and desmin distribution in adriamycin nephropathy. Representative immunofluorescence images of P-cadherin (green color) and desmin (red color) and their glomerular co-localization with DAPI (blue). Original magnification, X40. Scale bar, 40 μ m. Quantitation of the area per HPF with cells double-positive cells P-cadherin and desmin was compared using unpaired two-tailed t test. Results are shown as \pm SEM (n=5 for each group). *P<0.05, **P<0.01.

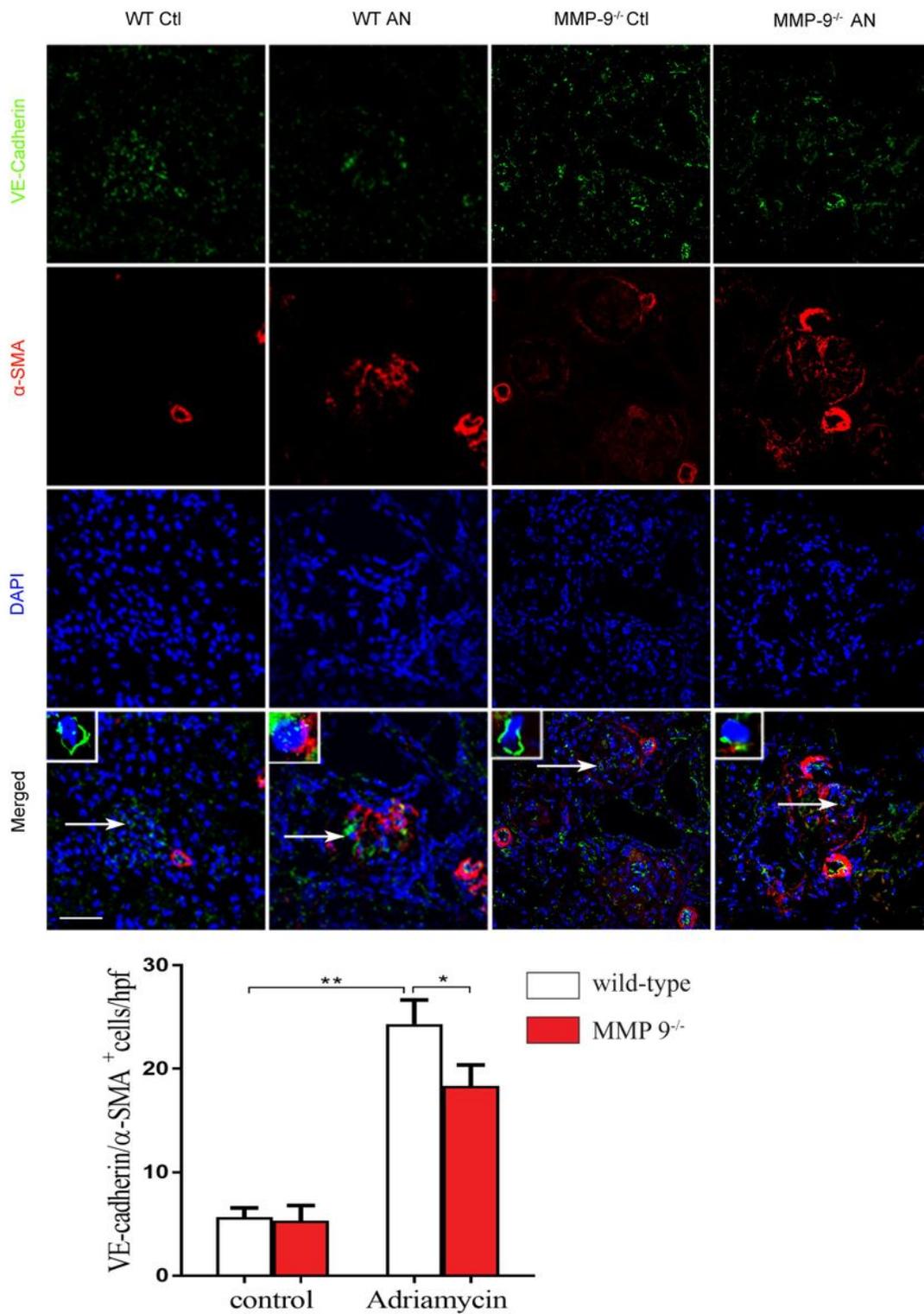


Figure 5

Effects of MMP-9 on endothelial cell VE-cadherin and α-SMA distribution in adriamycin nephropathy. Representative immunofluorescence images of VE-cadherin (green color) and α-SMA (red color) and their glomerular co-localization with DAPI (blue). Original magnification, X40. Scale bar, 40μm. Quantitation of the area per HPF with cells double-positive for VE-cadherin and α-SMA was compared using unpaired two-tailed t test. Results are shown as ± SEM (n=5 for each group). *P<0.05, **P<0.01.