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Metallothionein over-expression in podocytes reduces adriamycin nephrotoxicity

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Abstract
Adriamycin (ADR) is nephrotoxic. One component of ADR-induced nephropathy may be oxidative stress. This study used a recently developed line of transgenic mice (Nmt) on the FVB background strain, which over-express the antioxidant protein metallothionein (MT) in podocytes. Cultured podocytes from Nmt mice were resistant to H_2O_2 injury, as judged by disruption of F-actin filaments. FVB control and transgenic mice received 11 mg/kg body weight ADR by tail vein injection and 24-h urine samples were then collected for albumin analysis. Also renal morphology was investigated by light and electron microscopy. Urine albumin analysis showed that ADR treatment significantly increased albuminuria in control mice, indicating that the FVB strain is sensitive to ADR nephropathy and Nmt mice were significantly protected from elevated albuminuria. Glomerular histopathology revealed that ADR reduced podocyte number and produced foot process effacement in FVB mice. The Nmt transgene protected podocyte numbers and podocyte foot processes from the effects of ADR. These results show that metallothionein can protect podocytes from ADR toxicity.

Keywords: *Adriamycin, nephrotoxicity, metallothionein, oxidative stress, albuminuria*

Abbreviations: *ADR, Adriamycin; MT, Metallothionein; Nmt, Transgenic mice line that over-expresses MT in podocytes; UAE, Urine albumin excretion*

Introduction

Adriamycin (ADR) is commonly used to treat leukaemia, lymphoma and other cancers. However, clinical use of ADR is limited due to its toxicity to kidney, heart and other organs. Its complex cytotoxic mechanisms [1] are known to include enzyme inhibition, DNA intercalation, reactive oxygen species generation [2] and inductions of apoptosis. ADR-induced nephropathy has been well characterized in rodents and demonstrated to include albuminuria, impaired glomerular function, glomerulosclerosis, morphological changes and other features [3,4]. In mice ADR nephropathy varies among strains, with BALB/C mice being highly susceptible while C57BL/6J mice and most other strains are resistant [5].

Reactive oxygen species (ROS) are thought to be involved in the mechanism of ADR-induced kidney damage. ADR undergoes one-electron reduction catalysed by flavin-containing enzymes [6] such as NADPH-cytochrome-P-450 reductase [7]. This reduction generates a semiquinone free-radical. In the presence of molecular oxygen, the semiquinone reduces oxygen to superoxide and regenerates intact ADR. The ADR semiquinone can also react with hydrogen peroxide to yield hydroxyl radical [8]. These toxic ROS react with cellular molecules, including nucleic acids, protein and lipids, causing cell damage. Most evidence for this free radical hypothesis comes from *in vitro* studies on cell lines, including reports that: (1) ADR increases ROS levels and lipid peroxidation [9] and (2) the finding that

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free radical scavengers such as N-acetylcysteine [10], vitamin E [11] and superoxide dismutase [11] decrease the severity of ADR-induced damage. The free radical mechanism of ADR toxicity has been shown to apply to kidney cells. In cultured glomerular epithelial cells, ADR increased ROS production and produced cytoxicity [12]. ADR damage to cultured glomerular epithelial cells could be reduced by prior incubation with an ROS scavenger [12].

Podocytes are one of the major cell types of the renal glomerulus. They surround the glomerular capillaries and are a component of the glomerular filtration barrier which prevents leakage of protein in the urine. Podocytes are needed to maintain a functional glomerular basement membrane [13] and glomerular capillaries [14]. Our lab recently developed a transgenic mouse line that over-expresses MT specifically in podocytes [15]. We demonstrated that this line was significantly protected from diabetic nephropathy. In severely diabetic mice MT over-expression reduced both albuminuria and podocyte damage. Since MT possesses potent antioxidant action we elected to test whether MT could also reduce ADR nephrotoxicity. MT protects from ROS due to its very high thiol content. Our prior work in pancreatic beta cells [16] and cardiomyocytes [17] demonstrated that transgenic over-expression of MT scavenges a broad range of ROS. Importantly, we previously demonstrated that cardiac over-expression of MT protected the heart against ADR-induced cardiotoxicity [18]. These results indicated that MT would be a good antioxidant to protect against ADR nephropathy. Podocytes are a glomerular cell-type sensitive to ADR cytotoxicity [19] and podocytes are critical to maintaining normal glomerular structure and function. Therefore, in this study we tested whether targeted over-expression of MT protein in podocytes could both reduce podocyte damage and decrease ADR nephropathy.

Material and methods

Chemicals

Adriamycin (Doxorubicin hydrochloride, 2 mg/ml) was purchased from Sigma-Aldrich (St. Louis, MO). Oregon Green 488 phalloidin was purchased from Invitrogen (Carlsbad, CA). All the other chemicals and solvents were of analytical grade.

Transgenic mice

Our lab produced transgenic mice with podocyte-specific over-expression of MT on the FVB background. The transgene designated Nmt contained 8300 bp of the podocyte-specific mouse nephrin promoter (Dr Moeller, University of Michigan) ligated to a 2400 bp fragment containing the human MTII gene [20]. Podocye-specific over-expression of MT was evaluated by immunohistochemistry and western blotting of glomerular protein with an MT-specific antibody. All mice were housed in ventilated cages at the University of Louisville Research Resource Center with free access to water and standard mice diet. All animal procedures adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee.

Adriamycin treatment

To induce adriamycin (ADR) nephropathy in male mice of FVB background, Nmt or FVB mice at 3 months of age were injected via the tail vein at a dose 11 mg/kg ADR. The original solution of ADR (2 mg/ml) was used directly for injection.

F-actin immunofluorescence assay of hydrogen peroxide injury to podocytes

Cultured glomeruli were used in order to directly expose podocytes to oxidative injury and to be able to identify podocytes. In brief, cultured glomeruli were obtained by the following procedure: Glomeruli were purified from normal or transgenic mice by the Dynal beads perfusion procedure [21]. Glomeruli were then plated on glass coverslips in DMEM/F12 media for 4–5 days at 37°C in 95% air, 5% CO_2. During this period podocytes extended out as monolayers from the centre of the glomerular tuft. After treatment with hydrogen peroxide cells were fixed in 3.7% formaldehyde in sodium phosphate buffer for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 in sodium phosphate buffer for 3–5 min. Podocytes were stained with antibody to the podocytes-specific marker WT1 (1:50; rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA). F-actin was detected using Oregon Green 488 phalloidin (Invitrogen, Carlsbad, CA) diluted 1:40 in sodium phosphate buffer. The mounted cells were then photographed on a Nikon E600 fluorescent microscope. Images were rated by a blind observer for location and sharpness of F-actin filaments.

Histopathological studies

Kidneys removed from anaesthetized mice were immediately cut in half and fixed in 10% formaldehyde in 0.1 M PBS (pH 7.2) and then transferred to 70% ethanol after 24 h. The kidney tissue was then embedded in paraffin and sectioned at 5 μm. Sections stained with haemotoxylin and eosin (H&E) were used to evaluate general structural changes in glomeruli and tubules and for calculation of glomerular volume. The cross-sectional area of the glomerular tuft (AG) was determined from outlines of the tuft

using the program Adobe Photoshop 7.0. Glomerular volume (VG) was calculated from the cross-sectional area using the formula $VG = \beta/k\ (AG)3/2$, where $\beta = 1.38$ is the shape coefficient for a sphere and $k = 1.1$ is the size distribution coefficient [22,23].

Immunohistochemistry analysis

Formalin-fixed-paraffin-embedded sections were stained with antibody to the podocyte-specific marker WT1 (1:50; rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) and for MT (1:40, mouse, monoclonal, DAKO). To quantify podocyte numbers, on each section we randomly picked 20 glomeruli and counted the WT1 positive cells. The counting was performed by an observer blind to the identity of the sections.

Measurement of urinary albumin excretion

For determination of urinary albumin excretion, mice were placed individually in metabolic cages with free access to chow and 10% liquid diet (Glucerna, Abbot Laboratories). Urine was collected for 24 h and albuminuria was measured using a mouse albumin ELISA kit (Bethyl Laboratories). The dilution of sample urine was adjusted to place the albumin concentrations within the linear range of the ELISA assay.

Electron microscopy

Tissue from ADR treated mice and control FVB mice were used, mice were deeply anaesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (32 mg/kg) then perfused through the heart with Tyrode solution, followed by a fixative of 1% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB). The kidneys were removed, weighed and decapsulated, they were then sliced longitudinally and the medulla from each slice was removed and cortical strips were cut into 1 mm^3 tissue blocks. The blocks from each kidney were selected by unbiased technical personnel and placed into cold fixative overnight and then the selected blocks were post-fixed in 2% osmium tetroxide, dehydrated with an ethanol series, embedded in Durcupan resin (Ted Pella Co.). Thick sections (250 nm) were cut and stained by toluidine blue for light microscope observation, ultra-thin sections (70–80 nm, silver-gray interference colour) were cut serially using a diamond knife. To avoid examining the same cells on multiple sections, every 10^{th} section was collected on Formvar-coated copper slot grids. Sections were then stained with 10% uranyl acetate in methanol for 30 min before examination with the transmission electron microscope.

Statistical analysis

Values are expressed as mean ± SE. Statistical analysis was performed using Microsoft-Excel and Sigma-Stat software. Significance of differences was determined by the appropriate two-tail *t*-test for single comparisons. Two-way analysis of variance (ANOVA) with Holm-Sidak post-hoc test was used for multiple comparisons. *P*-values less than 0.05 were considered statistically significant.

Results

We checked 24-h urine excretion of albumin in FVB mice 5 days after ADR injection. This time point was chosen based on the results of Wang et al. [3], who reported that albuminuria increases 5 days after ADR injection. There was a 2–3- fold, significant elevation of urine albumin 5 days after ADR treatment (shown in Figure 1). This showed that ADR treatment injured kidneys of FVB mice.

Oxidative stress has been reported to play an important role in Adriamycin (ADR)-induced nephropathy; and some antioxidant treatments have shown efficacy against ADR nephrotoxicity. Metallothionein is a low molecular weight, cysteine-rich, inducible protein, which is capable of scavenging many different types of ROS. Our lab developed a transgenic mouse line in FVB mice designated Nmt, which we have recently described [15]. In Nmt mice MT is over-expressed in podocytes using the podocyte-specific nephrin promoter (a more complete description of the Nmt transgene is contained in Zheng et al. [15]). The morphology of Nmt glomeruli appears normal and MT staining is clearly increased in Nmt glomeruli compared to FVB glomeruli, as shown in Figure 2. Also, elevated MT staining appears to be in the same cells that stain for WT1, illustrating that the increase in MT content is in podocytes. Using Nmt mice we demonstrated that over-expression of MT in podocytes protects the

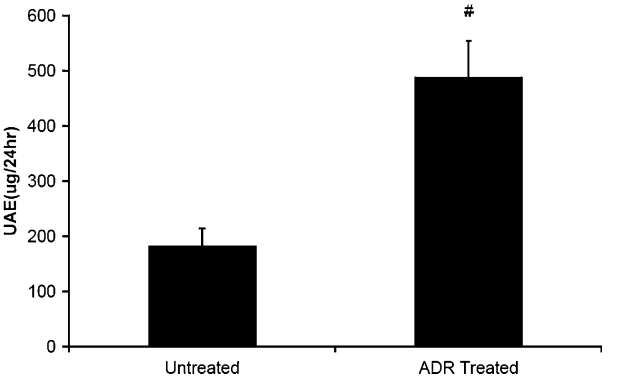

Figure 1. ADR increases urine albumin (UAE) and total protein excretion in FVB mice; 24-h urine albumin excretion, respectively, from FVB mice before and 5 days after ADR treatment (values are the means ± SE of 15 mice, # indicates $p < 0.01$).

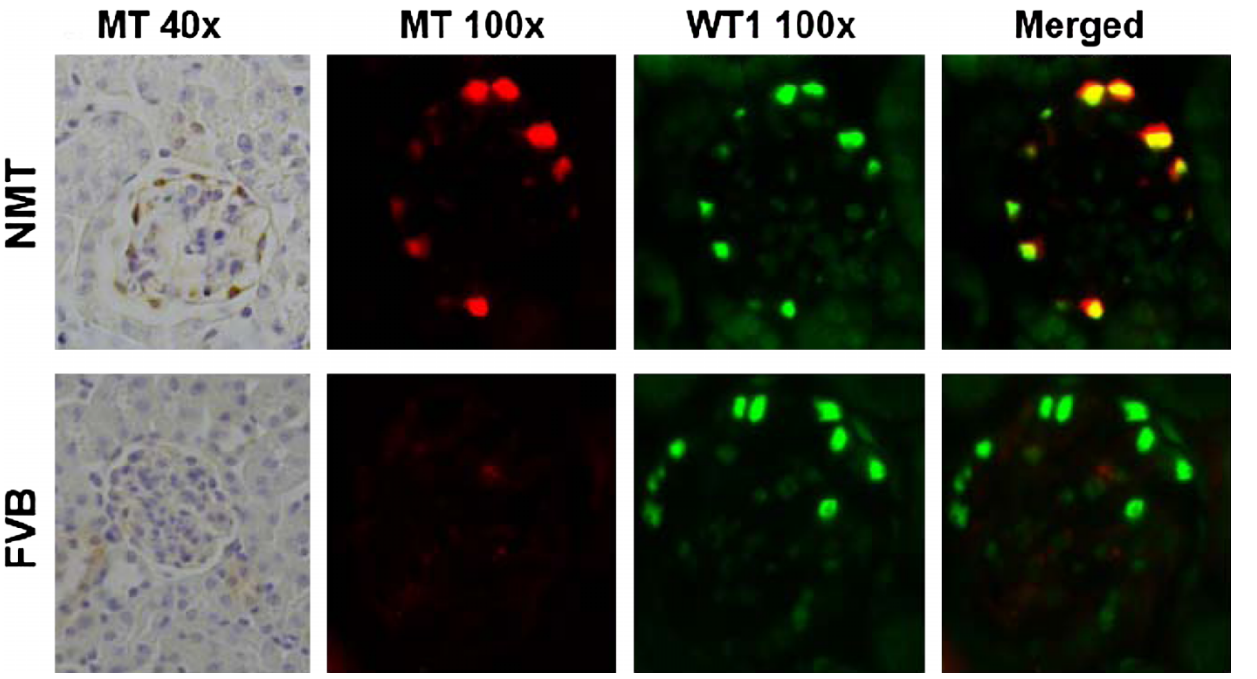

Figure 2. Expression of MT in Nmt transgenic and FVB control mice. Light microscopy images show normal morphology in Nmt glomeruli. MT staining with DAB chromogen is indicated by brown colour in the left panels. The fluorescent images show double-staining for MT (red) and WT1 (green). Original light micrographs were taken at 40× and fluorescent images were taken at 100× magnification. This figure is reproduced in colour in *Free Radical Research* online.

diabetic mouse model OVE26 from albuminuria and reduces injury to podocytes and glomeruli [15].

We next determined if MT over-expression protected transgenic podocytes from oxidative damage. Because podocytes are less than 20% of glomerular cells we needed a histological procedure that would allow us to identify both injury to cells and which cells were podocytes. To achieve this we examined the effect of H_2O_2 on cultured glomeruli from transgenic and control mice. Cultured glomeruli were exposed to 880 μm H_2O_2 for 16 h. They were then stained for F-actin using Oregon Green 488 phalloidin to examine cell injury. The morphology and fragmentation state of F-actin filaments provides a marker for the health of many cultured cell-types [24,25], including podocytes [26]. The podocytes in the cell population were identified by staining for the podocyte marker WT1. Both F-actin filaments and WT1 were then examined by fluorescence microscopy. In podocytes, we observed that F-actin filaments were less sharply defined after H_2O_2 treatment (Figure 3). Also the filaments were localized less along the cell boundary and more towards the nucleus, which is similar to the findings of Huot et al. [24]. Figure 3 also shows that most Nmt podocytes were less susceptible to the disruption by hydrogen peroxide than control podocytes. More Nmt podocytes retained well-organized longitudinal stress fibre structure. Semi-quantitive studies of F-actin morphology after H_2O_2 treatment confirmed our observations that podocytes over-expressing MT were protected against H_2O_2-induced oxidative injury at the F-actin level.

Based on our hypothesis that ADR nephrotoxicity would be attenuated by the MT-transgene, we measured 24-h urine albumin excretion following ADR injection in a group of non-transgenic FVB mice and a group of transgenic Nmt mice (Figure 4). In FVB mice, urinary albumin excretion (UAE) was elevated by the 5th day after ADR injection and tended to increase out to 21 days post-injection. Nmt mice also had elevated UAE by 5 days post-injection, but they tended towards recovery much more rapidly than FVB mice. UAE in Nmt mice was significantly lower than in the FVB mice at 5, 21 and 28 days after ADR injection.

To see if MT over-expression helped maintain podocyte numbers, we counted the number of podocytes per glomerular cross-section in untreated and ADR-treated FVB and Nmt mice (Figure 5). Podocytes were identified by staining with an antibody against the podocyte marker WT1. Mice were sacrificed 5 days after ADR injection for podocyte counting. As shown in Figure 5, ADR treatment produced a significant ($p < 0.05$) reduction in podocyte number in FVB mice of ~20%. However, in Nmt mice ADR treatment did not significantly reduce podocyte number and the Nmt-ADR mice had significantly more podocytes per glomerulus than FVB-ADR mice. Podocytes ultrastructure was further examined using electronic microscopy. Compared to the distinct brush-like structure of podocytes foot processes shown in control mice (Figures 6A and D), fusion of

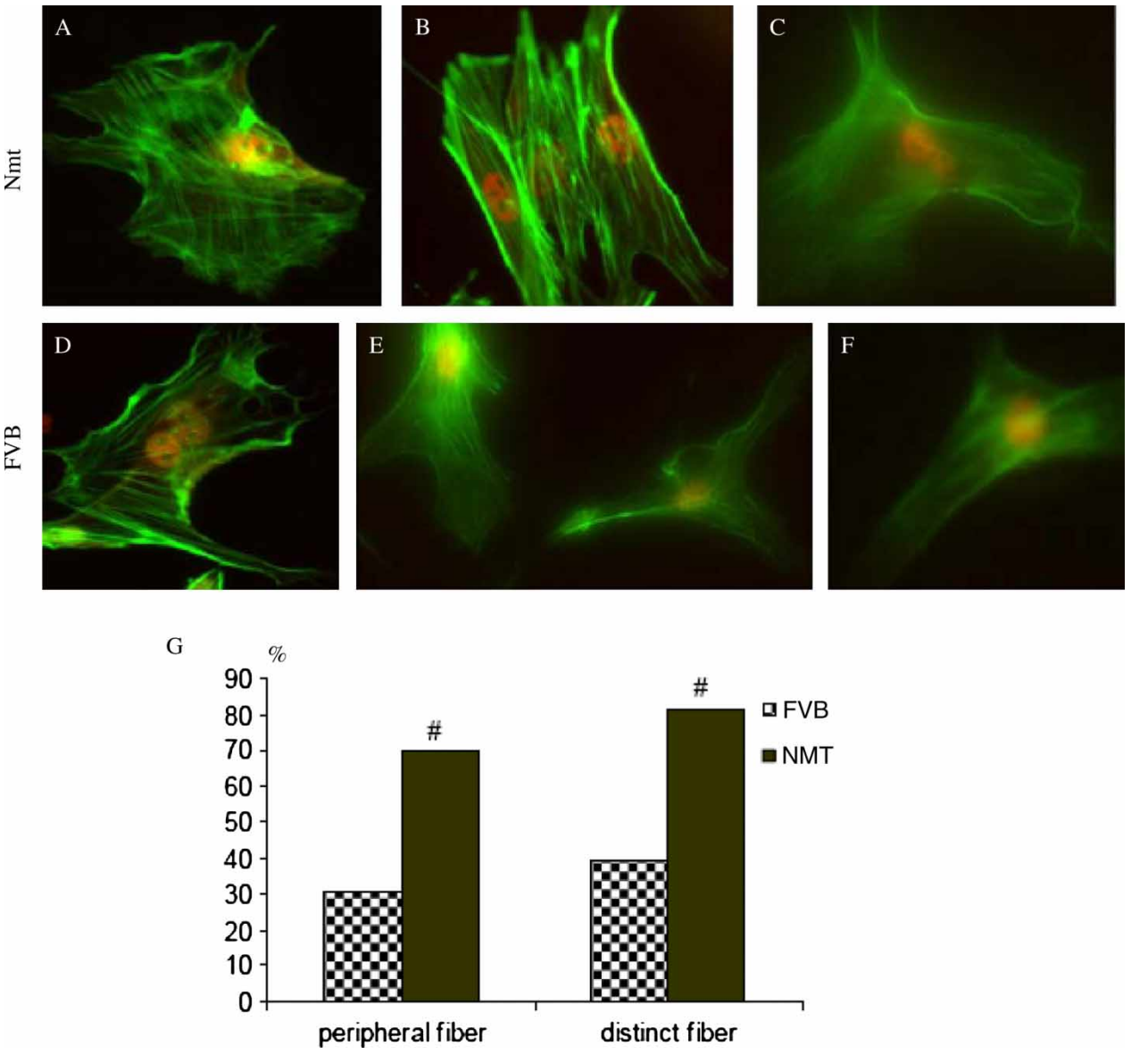

Figure 3. F-actin filaments are resistant to H_2O_2 in Nmt podocytes. Green is Oregon green phalloidin staining for F-actin. Red is WT1 staining for identification of podocyte nuclei. (A and D) Untreated Nmt and FVB podocytes. (B and C) Two examples of Nmt podocytes after 16 h of 880 μM H_2O_2 exposure. (E and F) Two examples of FVB podocytes after H_2O_2 exposure. In many Nmt podocytes (B) the F-actin filaments retained their distinct structure and peripheral location after H_2O_2 exposure. (G) Analysis of F-actin morphology after H_2O_2 exposure. F-actin fibres were rated by a blind observer whether they did or did not retain peripheral localization and whether or not the fibres were clearly defined (distinct). Scoring is of 20 podocytes from three separate glomerular preparations per group. # indicates that Nmt is higher that FVB ($p < 0.02$) by Chi-square test. This figure is reproduced in colour in *Free Radical Research* online.

podocytes foot processes developed in FVB mice at 5 days after ADR injection (Figures 6B and E). Nmt mice podocytes tended to retain relatively normal ultrastructures (Figures 6C and F). These results were representative of two Nmt and two FVB mice treated with ADR.

Glomerular volume was estimated by light microscopy on H&E stained kidney sections according to the mathematical model of Weibel and Gomez [22,23]. FVB mice receiving ADR showed a trend towards reduced glomerular volume compared to control FVB mice (Figure 7), though the difference did not reach significance ($p = 0.117$). In Nmt mice glomerular volume was not reduced at all by ADR injection and the glomerular volume of NMT-ADR mice was significantly greater than that of FVB-ADR mice ($p < 0.05$) (Figure 7).

Discussion

In this study, we demonstrated that inbred FVB mice are susceptible to ADR-induced renal damage. This was evident as decreased podocyte number, reduced glomerular volume and significantly increased albuminuria. Furthermore, when we tested ADR toxicity in Nmt transgenic mice [15] that over-express MT specifically in podocytes, all parameters tested for

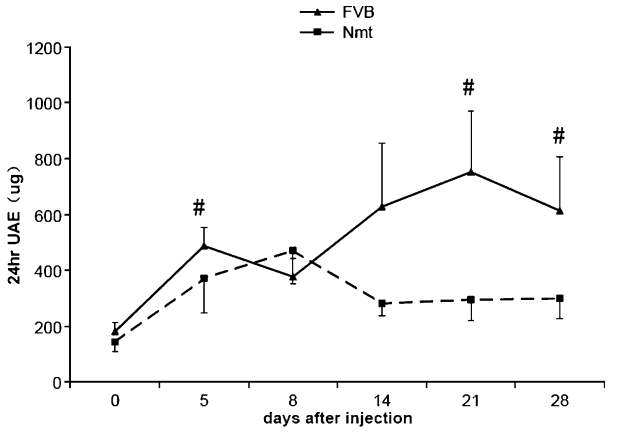

Figure 4. MT over-expression reduces ADR-induced albuminuria. Twenty-four hour urine albumin excretion plotted against time after ADR injection. Values are the mean ± SE. # indicates $p < 0.05$ for the difference between Nmt and FVB at that time point by two-way ANOVA ($n \geq 6$ mice for FVB group, $n > 8$ mice for Nmt group).

ADR-induced nephropathy were significantly reduced. These findings show that podocytes are a direct target of ADR damage and that protection of podocytes by increasing expression of MT reduces ADR nephropathy.

The FVB strain of mice was selected for this study because it is the strain that Nmt transgenic mice was developed on. However, Zheng et al. [15] reported that among many strains tested, including FVB, only BALB/C mice were susceptible to ADR nephropathy. In contrast to their findings we observed that FVB mice were sensitive to ADR: ADR treatment increased albuminuria more than 2-fold, to ~ 0.75 mg/24 h. ADR also affected glomerular structure: Glomeruli tended to be smaller, as has been reported in ADR treated rats [27], and podocyte number per glomerular section was reduced. We believe that the reason our conclusion about ADR toxicity to FVB mice was different from that of Zheng et al. is that BALB/C mice are much more sensitive: ADR

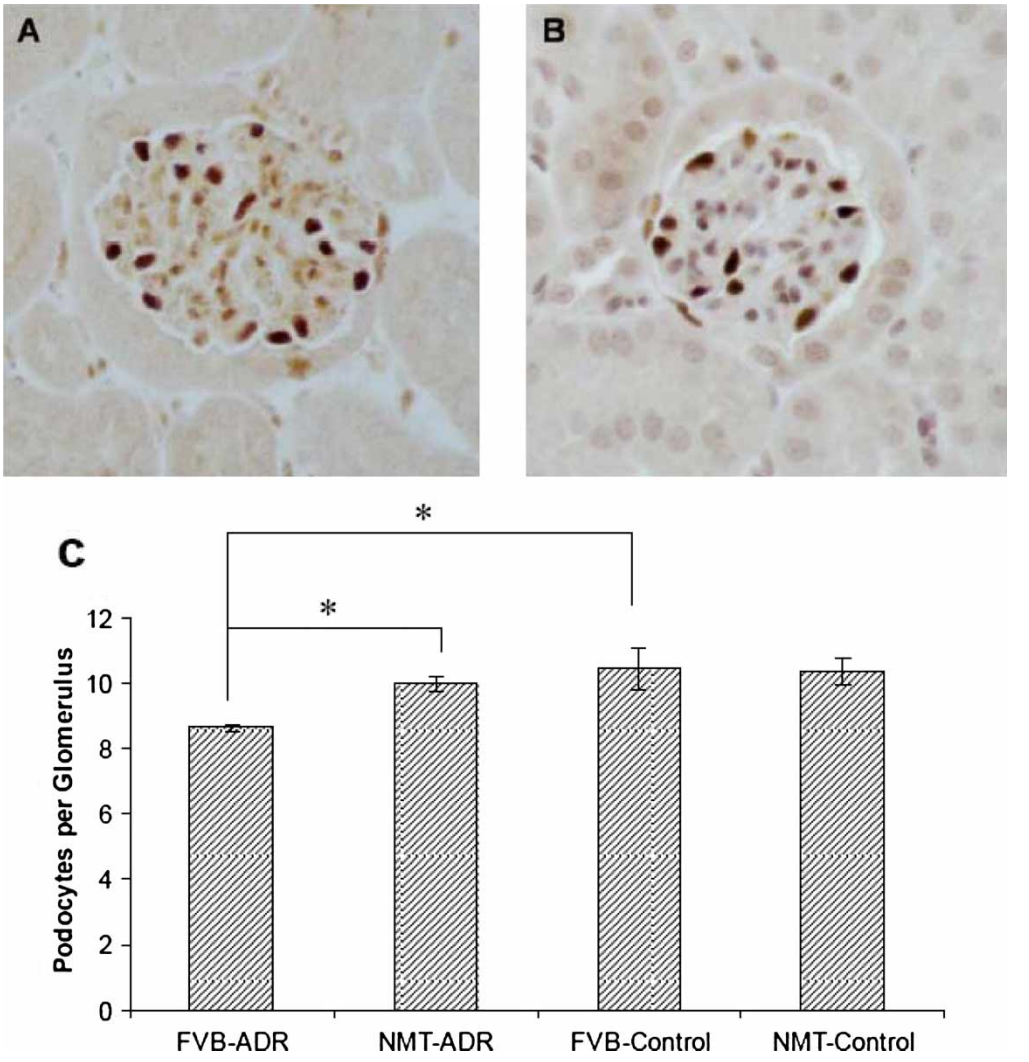

Figure 5. The NMT transgene preserves podocyte number. (A) FVB-control and (B) FVB-ADR show typical WT1 staining of control and ADR-treated FVB glomeruli. Brown staining indicates podocyte nuclei. (C) Quantitative comparison of podocyte number among different groups. Kidney samples were taken 5 days after ADR injection. The asterisks indicate that podocyte number in ADR-treated FVB mice is lower than in either ADR-treated Nmt or non-treated FVB glomeruli ($p < 0.05$ by two-way ANOVA, $n \geq 4$ mice per group). Vertical bars are the SEM.

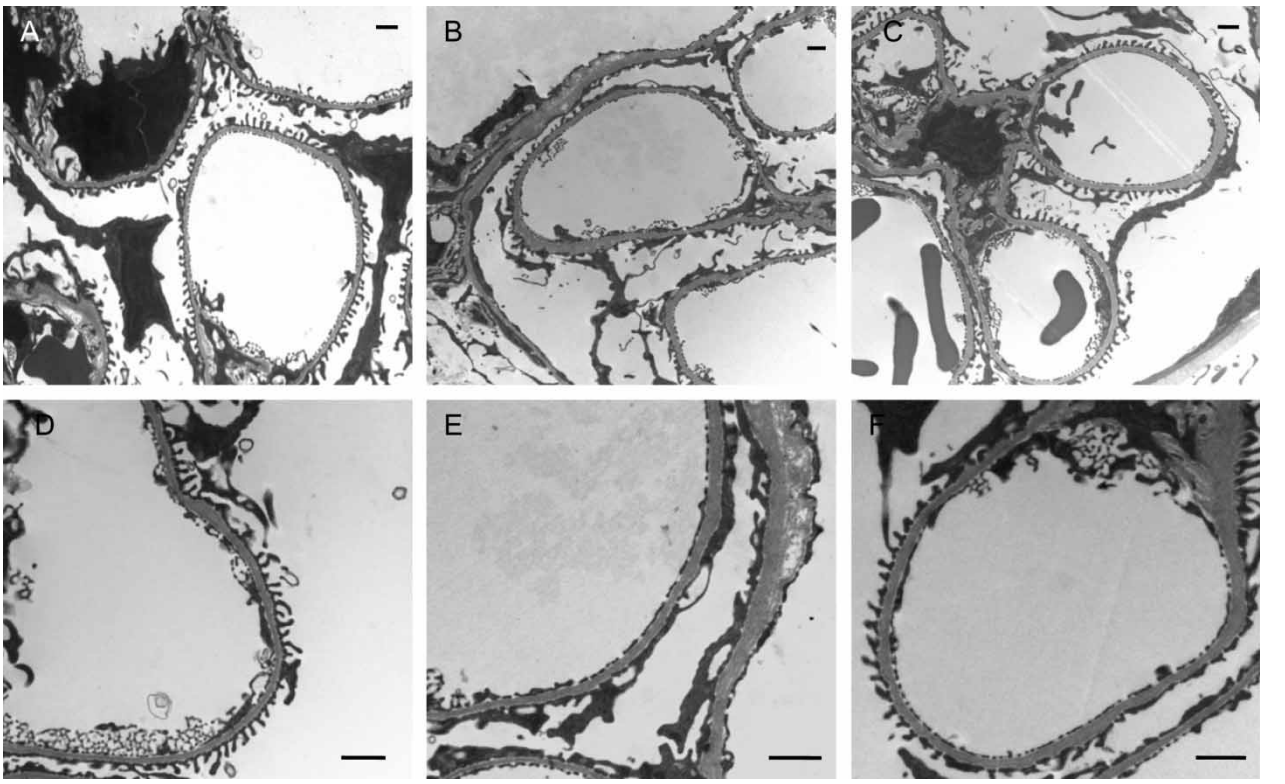

Figure 6. The Nmt transgene improves podocyte ultrastructure of ADR-injected mice. (A and D) Normal structure of podocytes foot processes in a FVB mouse without ADR injection at low (5600×) and high (9800×) magnification. (B) 4900× and (E) 8800× show effacement of podocytes foot processes in FVB mice 5 days after ADR injection. (C) 4900× and (F) 8800× show relatively normal foot processes in Nmt mice 5 days after ADR injection. (The scale bars on each panel represent 1 μM.) ADR results are representative of two mice per group.

treatment increases albuminuria more than 20-times in BALB/C mice [28] but only two times in FVB mice. Also, ADR-induced morphological changes in FVB mice are less obvious and more difficult to measure than morphological changes in BALB/C mice. Both our morphological and albuminuria studies show that FVB mice are damaged by ADR, albeit to a lesser extent than in BALB/C mice. The methods we used to detect ADR damage were more sensitive than the methods used by Zheng et al. and we believe that is why we observed ADR-induced injury to FVB mice when Zheng et al. did not. Zheng et al. identified the DOXNPH haplotype of BALB/C mice as critical to this strain's sensitivity to ADR. FVB mice do not carry this haplotype. It is possible that other strains of mice, in addition to FVB, that do not have the BALB/C haplotype also exhibit modest sensitivity to ADR.

The renal cell-type(s) sensitive to ADR are uncertain. A number of renal cells are damaged by ADR treatment, including tubular epithelial cells [27] and glomerular cells [29]. Since different renal cell-types interact *in vivo* it is difficult to determine which cells are damaged directly and which are damaged indirectly. Glomerular podocytes are an important determinant of proteinuria. They are an essential component of the normal glomerular filtration barrier and injury to podocytes produces albuminuria. If ADR damages podocytes *in vivo* then this could cause the prominent proteinuria of ADR nephropathy. *In vitro* experiments with cultured podocytes indicate that they are sensitive to ADR toxicity [30]. To assess whether podocytes are a direct site of ADR injury *in vivo*, we protected them by targeted over-expression of MT using the transgene Nmt, which uses the podocyte-specific nephrin promoter. Our results show that podocytes are a direct target of ADR toxicity, since the transgene was not expressed in any other cell type.

The mechanism of toxicity by ADR has not been clearly established in the kidney. It is widely supposed that a major cytotoxic mechanism of ADR is to increase ROS generation. MT is a potent antioxidant and our prior work demonstrated that transgenic over-expression of MT in pancreatic beta cells and cardiomyocytes [16,17] increases scavenging of a broad range of ROS including peroxynitrite, superoxide and hydrogen peroxide. The *in vitro* experiments needed in this study to specifically examine podocyte damage showed that MT over-expression protected podocytes from oxidative injury. Therefore, MT protection against nephrotoxicity induced by ADR provides stronger evidence that ROS are a cause of ADR toxicity. However, because MT has

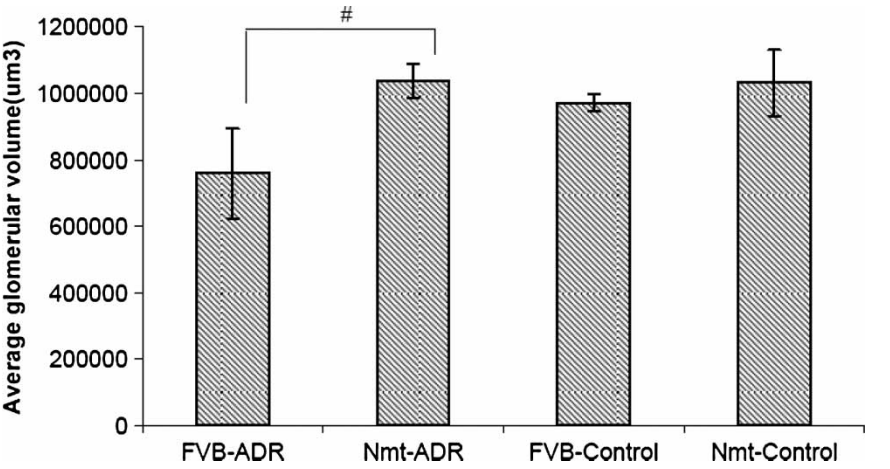

Figure 7. Comparison of glomerular volume in ADR-treated FVB mice (FVB-ADR), ADR-treated Nmt mice (Nmt-ADR), control FVB mice (FVB-Control) and control Nmt mice (Nmt-Control). The average volume of ADR treated FVB glomeruli was significantly less than the volume of ADR-treated Nmt glomeruli (* indicates $p < 0.05$ by two way ANOVA). Kidney samples were fixed 5 days after ADR injection. Average glomerular volumes for each group were determined from measurement of 80 glomerular cross-sections measured in four different mice of that group. Values are the mean ± SEM.

actions in additions to scavenging ROS [31] and because we did not demonstrate changes in oxidative damage in podocytes, it is possible that MT reduced ADR toxicity by mechanisms other than reduced oxidative stress.

Protective effects of MT over-expression were evident by morphological analysis. Five days after ADR treatment, Nmt glomeruli were larger and had more podocytes than FVB glomeruli. The ADR-induced decline in podocyte number was completely eliminated by the Nmt transgene. This action of MT may have been due to preventing ADR-induced podocyte death or it may have been due to reducing podocyte detachment. Electronic microscopy studies also showed that adriamycin caused podocyte foot process effacement in FVB mice and this damage appeared to be reduced by the NMT transgene. The fact that MT over-expression in podocytes provided protection indicates that the injury was a direct action of ADR on the podocyte and that the damage was mediated through ROS toxicity. The mechanism behind the trend to reduced glomerular volume after ADR treatment is less clear. It is an abnormality that has also been observed in glomeruli of ADR-treated rats [27]. The reduction in podocyte number is not sufficient to explain the reduction in total glomerular volume, because podocytes represent too small a fraction of the glomerulus. Since MT podocyte protection eliminated the reduction in volume, it is probable that the decrease in glomerular volume developed secondary to podocyte injury. Podocyte damage has been shown to have significant effects on other glomerular cells *in vivo*, such as mesanagial cells [32,33].

The clearest effect of MT over-expression was that Nmt mice had less albuminuria after ADR injection. The initial rise in albuminuria at 5 days post-treatment was significantly less in Nmt mice and albuminuria rapidly declined in Nmt mice, whereas it continued to rise in FVB mice. This result is consistent with the protection observed for podocyte number and demonstrates that the cause of ADR-induced albuminuria in FVB mice is injury to the podocyte.

In summary, our results show that ADR produce nephropathy in FVB mice and that MT protection of one glomerular cell type, the podocyte, is sufficient to protect from all components of ADR nephrotoxicity.

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