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Deficient degradation of homotrimeric type I collagen,α1(I)$_3$ glomerulopathy in *oim* mice

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Abstract

Col1a2-deficient (*oim*) mice synthesize homotrimeric type I collagen due to nonfunctional proα2(I) collagen chains. Our previous studies revealed a postnatal, progressive type I collagen glomerulopathy in this mouse model, but the mechanism of the sclerotic collagen accumulation within the renal mesangium remains unclear. The recent demonstration of the resistance of homotrimeric type I collagen to cleavage by matrix metalloproteinases (MMPs), led us to investigate the role of MMP-resistance in the glomerulosclerosis of *Col1a2*-deficient mice. We measured the pre- and post-translational expression of type I collagen and MMPs in glomeruli from heterozygous and homozygous animals. Both the heterotrimeric and homotrimeric isotypes of type I collagen were equally present in whole kidneys of heterozygous mice by immunohistochemistry and biochemical analysis, but the sclerotic glomerular collagen was at least 95–98% homotrimeric, suggesting homotrimeric type I collagen is the pathogenic isotype of type I collagen in glomerular disease. Although steady-state MMP and *Col1a1* mRNA levels increased with the disease progression, we found these changes to be a secondary response to the deficient clearance of MMP-resistant homotrimers. Increased renal MMP expression was not sufficient to prevent homotrimeric type I collagen accumulation.

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Keywords

collagen; extracellular matrix; glomerular sclerosis; fibrosis; matrix metalloproteinase

1. Introduction

Type I collagen is predominantly found in tissues as a heterotrimeric isotype consisting of two α1(I) chains and one α2(I) chain, [$\alpha1(I)_2\alpha2(I)$]. However the homotrimers consisting of three α1(I) chains, [$\alpha1(I)_3$] [1–4] have been shown to be present during embryogenesis [5], in tumors [6–8], fibrotic tissues [9–12] and in stressed mesangial cells [13].

Haralson et al demonstrated that cultured wildtype rat mesangial cells synthesized the homotrimeric type I collagen in what was postulated to be a wound response [13]. They hypothesized that the synthesis of the homotrimeric isotype may contribute to sclerotic accumulation of type I collagen within the renal mesangium. Historically, glomerulosclerosis has been attributed to an imbalance of collagen synthesis and degradation [14–17]. Mesangial cells are thought to be responsible for the excessive expression of collagen in the glomerular mesangium during disease progression [18–21]. Although the resulting matrix accumulation leads to glomerulosclerosis [22–25], the initiating mechanisms of the pathology and the specific role of homotrimeric type I collagen still remain unclear.

A clue to answering the latter question may be contained in the recent finding that type I collagen homotrimers are much more resistant to degradation by matrix metalloproteinases (MMPs) than the heterotrimers [8, 26]. MMPs are known regulators of the extracellular matrix (ECM) and are of keen interest in glomerulosclerotic disease [27–30]. To maintain ECM homeostasis, human kidneys express collagenases (MMP-1, -13 and -14), gelatinases (MMP-2 and -9) and stromelysin-1 (MMP-3) [31]. MMP-2 has been most widely studied across species and shown to be constitutively expressed by both mesangial and glomerular epithelial cells [31–33] and is postulated to act as a pro-inflammatory activator of mesangial cells [34, 35] while possibly contributing to epithelial-to-mesenchymal-transition (EMT) [36]. MMP-9 [31, 37, 38] is also synthesized by glomerular epithelial cells, but unlike MMP-2, has been shown to have restricted temporal expression during development and in adult rodent tissues [39, 40] and is differentially regulated in several animal models [41–44]. Though less well studied in kidneys, MMP-3 is an important activator of MMP-2, -9 and -13, degrading many of the same substrates including gelatin [45]. It has also been shown to be elevated in the serum of human renal transplant patients with chronic nephropathy [46]. MMP-13 is synthesized by mesangial cells, activated by MMP-2, -3 and -9, and plays an important role in cancer progression [8, 31]. The membrane collagenase MMP-14 (also known as MT1-MMP) has multiple functions, including but not limited to pericellular collagen cleavage and activation of MMP-2 [47]. It is known to be crucial for general collagen turnover [48] as well as wound healing and tumor invasion [49]. MMP-3, -13 and -14 have also been identified as primary players in fibrosis due to EMT [50].

Mice constitutively expressing the MMP-resistant homotrimers provide an unprecedented opportunity to examine the potential pathogenic role of this collagen isotype in glomerulosclerotic disease without confounding factors of artificially induced renal injury. The *Col1a2*-deficient mouse has a single nucleotide deletion that causes a frame-shift and disrupts the carboxyl terminus (C-propeptide) of the α2(I) chain, required for the association with α1(I) procollagen chains to form the heterotrimeric triple helix [51]. The resulting exclusion of the α2(I) chain from the type I collagen triple helix leads to synthesis and secretion of only the homotrimeric isotype in homozygous *Col1a2*-deficient (−/−) mice. The

genetic defect itself, reduced bone mineral density and increased bone fragility in the *Col1a2*-deficient mouse [52–54] resemble a patient with an autosomal recessive form of osteogenesis imperfecta [55–57], which is why this mouse is also known as the osteogenesis imperfecta murine (*oim*) model. In addition to the bone phenotype, our previous studies of the *Col1a2*-deficient mouse revealed postnatal accumulation of homotrimeric type I collagen in the renal mesangium, resulting in progressive glomerulosclerosis, podocyte foot process effacement and proteinuria [12, 58]. These findings and the synthesis of the homotrimers by mesangial cells that have no genetic *Col1a2* deficiency [13] beg the question whether this isotype is the primary pathogenic collagen in glomerulosclerotic lesions.

In the present study, we addressed the potential role of delayed degradation and clearance of this MMP-resistant collagen isotype from renal mesangium. We compared pre- and post-translational expression and distribution of type I collagen isotypes and MMPs in whole kidneys and isolated glomeruli from 1 month old and 3 month old heterozygous and *Col1a2*-deficient mice. Since mouse MMP-1 is only synthesized during embryogenesis [31, 59, 60], we limited the study to MMP-2, -3, -9, -13 and -14.

2. Materials and methods

2.1. Animals

Heterozygous B6C3Fe *a/a*- *Col1a2*$^{oim/J}$ (−/+) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred to produce wildtype (+/+), heterozygous (+/−) and homozygous (−/−) animals. Animals were housed and fed (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN) *ad libitum* in an AAALAC accredited animal facility in accordance with an approved University of Missouri Animal Care and Use protocol. Animal genotypes were determined as previously described [61] and aged to 1 (n=168 mice) or 3 months (n=151) of age. Animals were sacrificed and kidneys or glomeruli harvested as described below.

2.2. Glomerular isolation

Wildtype, heterozygous and *Col1a2*-deficient mice were aged to 1-month [−/−, n=20; +/+, n=21; and +/−, n=38] and 3-months [−/−, n=13; +/+, n=22; and +/−, n=23] of age and anesthetized prior to kidney perfusion [62]. Perfusion of 8×10^7 tosylactivated Dynabeads® magnetic beads (deactivated according to the manufacturers instructions) in 1M PBS were perfused through the body via the heart. Perfused kidneys were removed, weighed and minced followed by a 30 minute digestion in 1mg/ml collagenase A (Invitrogen Corporation, Carlsbad, CA), 100units/ml DNase (Invitrogen Corp., Carlsbad, CA) and Hanks Balanced Salt Solution (HBSS) (GibCo-Invitrogen Corporation, Carlsbad, CA) at 37°C. The digested slurry was sieved twice through 100μm cell strainers (BD Bioscience, San Jose, CA) with the addition of HBSS, followed by centrifugation at 1500rpm for 15 minutes. The pellet was resuspended in HBSS and placed onto a magnetic particle concentrator for 1 minute to separate glomeruli from extraneous tissue, and repeated 5 times. Remaining glomeruli were resuspended in HBSS and assessed for purity (greater than 98% purification) and yield using a hemocytometer, followed by snap-freezing and storage at −80°C.

2.3. Microscopy and glomerular counting

Longitudinal sections (5μm) of formalin-fixed kidneys from 1 and 3 month wildtype, heterozygous and *Col1a2*-deficient [n=8 of each genotype] were embedded in paraffin and stained with picrosirius red (PSR) fibrillar collagen stain. The PSR-stained sections were examined by conventional light microscopy and with polarized light. Glomeruli within

individual sections were evaluated blindly to obtain a glomerular lesion score for each kidney as previously described [58], and to assess the average glomerular number within longitudinal sections. Mean glomerular number was calculated as the average of the number of glomeruli within 4 sections.

2.4. Immunohistochemistry (IHC) for α1(I) and α2(I) collagen

Longitudinal sections (10μm) of zinc fixed kidneys were embedded in paraffin and placed on slides. Heat-induced epitope retrieval in target retrieval solution (1X TRS) (Dako, Carpinterea, CA) was followed by quenching of endogenous biotin using a avidin/biotin block. Endogenous peroxidase was removed by treating slides with 3% hydrogen peroxide and non-specific antibody binding was blocked with a 5% bovine serum albumin (BSA) solution. Next, kidneys were incubated in either rabbit polyclonal anti-α1(I) collagen primary antibody (MD Biosciences, St Paul, MN) diluted 1:600 or rabbit polyclonal anti-α2(I) collagen primary antibody diluted 1:3000. The anti-α2(I) collagen primary antibody was produced by the antibody production core at UT-Southwestern Medical Center. In brief, two rabbits (U6410 and U6425) were immunized genetically with 1 mg each of cDNA encoding murine α2(I) collagen chain three times over 42 days. The rabbits were boosted every two weeks (five times total) with collagen purified from homozygous *Col1a2*-deficient mice. Pre-immune serum and sera collected after the final boost were evaluated by ELISA for reactivity with collagen isolated from wildtype and *Col1a2*-deficient mice animals. Following the addition of the primary antibodies, biotinylated swine anti-rabbit secondary antibody diluted 1:300 was added, followed by a strepavidin horseradish peroxidase conjugate, and 3,3’ diaminobenzidine tetrahydrochloride (DAB) substrate with hematoxylin counterstaining. Staining was performed on a Dako Autostainer Universal Staining System. Anti-α1(I) and anti-α2(I) collagen antibody specificity was confirmed by western blot analysis of wildtype and *Col1a2*-deficient mouse tail tendon collagen. The anti-α2(I) collagen also specifically binds acid-solublized and pepsin treated rat and human α2(I) collagen chains by western blot analyses.

2.5. Biochemical analysis of collagen composition in glomeruli and kidney

One half of harvested whole kidney was homogenized by ice-bath sonication in 0.5M acetic acid and 0.5 % Brij 35 for 1 minute and treated overnight with 0.5 mg/ml pepsin (EMD Biosciences, Darmstadt, Germany) at 4°C. Solublized collagen was precipitated with 1M NaCl. Glomerular preparations were thawed, precipitated by centrifugation, homogenized, pepsin treated, and precipitated with NaCl as described above. The beads with precipitated collagen were separated from pepsin solution by centrifugation and washed with 70% ethanol, to remove residual acetic acid.

For analysis, kidney or glomerular collagen were resuspended in 0.1M Na_2CO_3, 0.5M NaCl, pH 9.3 and labeled with mono-reactive Cy5 (GE Healthcare, Piscataway, NJ) as previously described [63]. Aliquots of 3–4μg of protein were denatured for 5 min at 90°C in 4X LDS sample buffer (Invitrogen) and analyzed on precast 3–8% Tris-acetate mini gels (Invitrogen) with α1(I) molecular standards. Selected bands were excised from the gel, treated with CNBr (Sigma, St. Louis, MO), and re-analyzed on precast 12% Tris-glycine gels (Invitrogen). All gels were scanned on a FLA5000 fluorescent scanner (FUJI Medical Systems, Stamford, CT) and analysized by ScienceLab software. The estimated detection limit of this assay is 0.2–0.5 pg collagen/glomerulus.

2.6. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Snap frozen glomeruli from 1 month and 3 month old +/+, +/− and −/− mice were homogenized in TRIzol Reagent (Invitrogen Corporation) using a TissueLyser homogenizer (QIAGEN, Valencia, CA), or RNeasy Kit (QIAGEN) and total RNA was isolated according

to manufacturer's protocol. Total glomerular RNA was transcribed following the manufacturer's protocol (Superscript First Strand Synthesis or VILO, Invitrogen Corporation). Real-time RT-PCR amplification was performed on individual mouse samples and standard curves generated as previously described [58]. One month [+/+ (lesion score G0), n=10; +/− (lesion score G1–4), n=16; −/− (lesion score G3–4), n=16] and 3 month [+/+ (lesion score G0), n=16; +/− (lesion score G1), n=16; −/− (lesion score G1–4), n=17] old mice were individually evaluated for *COL1A1, COL1A2* MMP-2, -3, and -9 transcript levels, and 1 month [+/+ (lesion score G0), n=8; +/− (lesion score G1–4), n=9; −/− (lesion score G3–4), n=8] and 3 month [+/+ (lesion score G0), n=8; +/− (lesion score G1), n=9; −/− (lesion score G1–4), n=8] old mice were evaluated for MMP-13 and -14. PCR primer sequences for *COL1A1, COL1A2* MMP-2, -3, and -9 are found in Table 3. MMP-13 and -14 transcripts were evaluated with purchased primer/probe sets (Applied Biosystems, Foster City, CA) and individually evaluated using TaqMan® gene expression assay. RNA copy number values were evaluated and normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) [64].

2.7. Quantitation of MMPs

Protein levels of MMP-2, MMP-3, and MMP-9 in 1 month [n=9 +/+; n=11 −/−] and 3 month [n=10 +/+; n=10 −/−] wildtype and *Col1a2*-deficient mouse glomeruli were measured commercially using the Thermo Scientific SearchLight multiplex assay (Pierce Scientific, Rockford, IL) [65, 66]. Briefly, snap frozen glomeruli were thawed on ice and protease activity inhibited by the addition of phenylmethyl sulfonyl fluoride (PMSF) to a final concentration of 2mM. Glomeruli were disrupted by a 10 minute ice-bath followed by a 1 hour incubation in 96-well format at room temperature with agitation (200rpm) for antigen-protein binding to MMP-2, -3, and -9 antigens. Wells were washed and incubated with biotinylated detection antibodies for 30min, followed by a 30 min incubation with strepdavidin horseradish peroxidase conjugate and imaged by SuperSignal ELISA Femto Chemiluminescence substrate.

2.8. Statistics

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC) by complete randomization design in which genotype and age were arranged as a 3 × 2 factorial (3 genotypes, 2 ages). Stabilization of heterogeneous variations were made when necessary by using the log transformation of the mean values. Mean differences were determined using Fisher's protected least significant differences (LSD). Means and standard errors presented are untransformed values, although all p values, with the exception of MMP-14 mRNA data, were determined from transformed data. All results are presented as mean ± standard error with statistical significance as noted.

3. Results

3.1. Kidney size and glomerular content

Col1a2-deficient mice had smaller total body weights [51, 53, 67] and kidney weights than their wildtype and heterozygous littermates, but larger kidney/body weight ratios (Table 1); although the increased kidney/body weight ratio was significant ($p \leq 0.005$) only at one month of age. The yield of glomeruli isolated by perfusion from *Col1a2*-deficient kidneys (6510 ± 1350 glomeruli) was similar to the wildtype and heterozygous kidneys at 1 month of age, and much lower at 3 month of age (900 ±122 glomeruli, $p \leq 0.005$) (Table 1). To investigate this further, 1 and 3 month wildtype, heterozygous and *Col1a2*-deficient deficient kidneys were evaluated histologically and the glomerular number determined (Table 2). This confirmed that there was not a decrease in the number of glomeruli (per field of view) in the 3 month old *Col1a2*-deficient animals, but rather suggested that the

decreased glomerular yield was a result of inefficient perfusion of sclerotic glomeruli in the older *Col1a2*-deficient animals. The increase in glomerular number in *Col1a2*-deficient kidneys (Table 2) can likely be attributed to the reduction in size and area of *Col1a2*-deficient kidneys (Table 1) as compared to their littermates, and rather than a true increase in the total number of glomeruli.

3.2. Glomerular accumulation of homotrimeric collagen

Immunohistological examination of kidney sections demonstrated a divergence between the α1(I) and α2(I) chain localizations, suggesting that only homotrimeric type I collagen is depositing in the glomeruli (Figure 1). The adventitia of the renal vasculature of all genotypes, where type I collagen is normally expressed, showed picrosirius red (PSR) collagen staining and anti-α1(I) antibody staining. As expected, the same region also showed anti-α2(I) staining in wildtype and heterozygous kidneys, but not in *Col1a2*-deficient kidneys. Sclerotic glomeruli in heterozygous and *Col1a2*-deficient kidneys were positive for both anti-α1(I) and PSR, but negative for anti-α2(I).

Further biochemical examination of extracted collagen showed that type I collagen comprised ~80% of total fibrillar collagen in whole kidneys, while type III and V collagens comprised ~12–16% and ~5%, respectively (Figure 2A). These ratios are consistent with the composition of collagen isolated from cultured fibroblasts. Additionally, type I collagen was present in wildtype kidneys only as the heterotrimeric isotype, in *Col1a2*-deficient kidneys only as the homotrimeric isotype, and approximately equal amounts of both homotrimeric and heterotrimeric isotypes were present in heterozygous kidneys (Figure 2A).

Of key interest, no glomerular type I collagen was detected in wildtype mouse glomeruli at 0.2–0.5 pg/glomerulus detection limit, but over 100 pg of homotrimeric type I collagen per glomerulus (evidenced by the lack of α2(I) chains) was found in an age-matched *Col1a2*-deficient mouse. Analysis of heterozygous mice revealed <0.5 pg/glomerulus in one of six animals, 0.8–7 pg/glomerulus in four animals and 34 pg/glomerulus in a single animal with the most severe lesions of the six heterozygous mice evaluated (Figure 2B). In Figures 2A & B, a faint pepsin-resistant protein species (indicated by the asterick) was seen migrating slightly slower than the pepsin-treated α2(I) chains in the extracts from kidneys from homozygous mice and glomeruli from some heterozygous mice. To determine if this protein was of α1(I) or α2(I) chain origin CNBr pepetide mapping was done (Figure 2C). The CNBr peptide patterns from of this protein species (*) was similar and consistent with the α1(I) chain cleaved within CB6 or CB5. Such proteolytic degradation of α1(I) homotrimers is often observed in *Col1a2*-deficient tissues [68]. Based on the observed protein species (*)/α1(I) intensity ratio, absence of the well defined α1(I)-α2(I) dimer band, and the relative intensities of different fragments in the CNBr cleavage pattern of the protein species (*), we estimated that at least 95–98% of type I collagen molecules in heterozygous glomeruli were α1(I) homotrimers, confirming selective accumulation of the homotrimeric isotype.

3.3. Col1a1 and col1a2 transcription

Analysis of steady-state mRNA levels by quantitative RT-PCR (Figure 3A) revealed similar *col1a1* expression in wildtype and heterozygous glomeruli (both at one and at three month of age). *Col1a1* expression in *Col1a2*-deficient glomeruli was elevated 3-fold at one month ($p \leq 0.003$) and 15-fold at three month of age ($p \leq 0.0001$), consistent with previously observed 2-fold increase in steady-state *col1a1* mRNA transcripts in the whole kidney at one month [58].

Although *Col1a2*-deficient mice do not incorporate α2(I) chains in their type I collagen triple helix, they continue to synthesize *col1a2* transcripts which are hypothesized to be

translated and the aberrant protein product degraded shortly thereafter [4, 69]. Both in heterozygous and *Col1a2*-deficient glomeruli, we observed progressive reduction in *col1a2* steady-state mRNA with age, compared to matched wildtype glomeruli (Figure 3B).

3.4. MMP transcription and translation

Analysis of mRNA showed similar MMP-2 expression in all genotypes at one month of age (Figure 4A). MMP-2 mRNA levels decreased 2-fold in wildtype and heterozygous glomeruli at three months of age, but remained elevated in *Col1a2*-deficient glomeruli ($p \leq 0.0004$). Analysis of the protein showed a 3-fold and 2-fold elevation in MMP-2 in *Col1a2*-deficient glomeruli at one month ($p \leq 0.001$) and three months ($p \leq 0.008$), respectively (Figure 4D).

MMP-3 steady-state transcript levels (Figure 4B) appeared similar in all genotypes at one month of age. At three months of age, they increased more than 2-fold in *Col1a2*-deficient ($p \leq 0.002$) and remained unchanged in wildtype and heterozygous glomeruli. At the protein level, however, *Col1a2*-deficient mice had 9-fold higher MMP-3 content compared to age-matched wildtype mice ($p \leq 0.0005$), both at one and at three months (Figure 4E). Furthermore, the MMP-3 protein content increased 2.6 fold at three months compared to one month in both genotypes.

MMP-9 steady-state mRNA levels (Figure 4C) were similar in one month old wildtype and *Col1a2*-deficient glomeruli and 70% greater, though not significantly, in heterozygous glomeruli. At three months of age, MMP-9 mRNA levels were similar in wildtype and heterozygous glomeruli, and 2-fold higher in *Col1a2*-deficient glomeruli, although not significant. MMP-9 protein was similar in one month old *Col1a2*-deficient and wildtype glomeruli, but MMP-9 protein level was 2-fold lower in *Col1a2*-deficient glomeruli than in the wildtype ($p \leq 0.05$) at three months of age, opposite to the change in mRNA (Figure 4F).

MMP-13 steady-state mRNA levels (Figure 5A) were higher in *Col1a2*-deficient than wildtype glomeruli ($p \leq 0.02$) and increased with age ($p \leq 0.02$). MMP-14 steady state mRNA levels (Figure 5B) were also higher in *Col1a2*-deficient glomeruli than in wildtype glomeruli ($p \leq 0.02$), but decreased in all genotypes with age ($p \leq 0.0001$). Due to very small amounts of tissue and very low expression levels, protein data was not analyzed for MMP-13 and MT1-MMP.

4. Discussion

4.1. Collagen accumulation results in increased number and diminished filtration capacity of glomeruli in Col1a2-deficient mice

In utero, fetal glomeruli function to generate the placental fluid, however glomerular functionality and filtration is dramatically changed postnatally. In the postnatal mouse kidney, functionality of the glomeruli appears to start with the innermost glomeruli, increasing circumferentially outward with age until all glomeruli are functioning [58, 70]. *Col1a2* deficiency in *oim* mice results in a progressive glomerulopathy caused by accumulation of type I collagen [58], and the reduced glomerular yields seen in three month *Col1a2*-deficient kidneys are potentially due to narrowing of glomerular capillaries as a result of collagen deposition and mesangial expansion suggesting that the glomerular capillaries may become restricted to the flow of blood as sclerosis increases, thus diminishing their filtering capacity as well as potentially altering their mechanical properties similar to that observed in glomeruli of the Col4a3$^{-/-}$ and HIV-associated nephropathy mice [71].

4.2. Mice that synthesize heterotrimeric and homotrimeric type I collagen accumulate the homotrimeric isotype in glomeruli

Type I collagen is normally found in the kidney vasculature, to a lesser extent interstitium, but not within the glomerular mesangium [19, 72]. However, during glomerulosclerosis, increases in type I collagen mRNA and accumulation of the protein has been shown within the mesangium [73–75], yet it is not known which isotype of type I collagen is present, or why it accumulates. Although we have previously shown homotrimeric type I collagen accumulation in sclerotic glomeruli of *Col1a2*-deficient kidneys [12, 58], we did not examine sclerotic glomeruli in heterozygous mice, which synthesize both collagen isotypes, to determine whether homotrimer was indeed the pathogenic accumulating collagen in the glomeruli. As shown in Figures 1&2, homotrimer is the predominant type I collagen isotype (95–98%) accumulating in sclerotic heterozygous *Col1a2*-deficient glomeruli, while both heterotrimeric and homotrimeric type I collagen appear equally present in tissues outside the glomeruli, suggesting a major role of homotrimeric type I collagen in the pathogenesis of the disease.

4.3. The homotrimer accumulation in heterozygous glomeruli is caused by deficient degradation of the homotrimers rather than increased synthesis of α1(I) chains

Stressed mesangial cells were shown to produce the homotrimeric collagen isotype [13]. However, our examination of steady-state mRNA revealed no increases in *COL1A1* expression in heterozygous glomeruli at 1 and 3 months (Figure 3). *COL1A1* expression was increased only in homozygous animals as a response to more severe overall collagen deficiency, consistent with previous observations in whole kidneys [58]. Apparently, increased *COL1A1* expression was not the primary cause but a contributing factor to glomerulosclerosis severity in these animals.

Our data suggest that type I collagen is weakly expressed in wildtype, heterozygous, and *Col1a2*-deficient glomeruli. The protein seems to be effectively degraded in wildtype glomeruli, so that it is usually not detected and it does not accumulate. Collagen accumulation in heterozygous glomeruli with normal or even reduced expression of *COL1A1* and *COL1A2* mRNA (Figure 3) indicates that normal heterotrimers might be recognized and degraded more efficiently, leaving homotrimers lingering within the mesangial matrix.

The inefficient degradation appears to be associated with the absence of the α2(I) chain in homotrimer molecules. Although the 97% homology between amino acid compositions of the α1(I) and α2(I) chains has been evolutionarily conserved for the past 500 million years [1, 2], absence of the α2(I) chain leads to significant changes in type I collagen properties. The α2(I) chain alters crosslinking and tensile strength of collagen fibers [76] as well as interactions between the triple helices in fibers [77]. It reduces the overall triple helix stability [78, 79] and changes the local stability of differing regions along the helix [79]. The α2(I) chain has been proposed to play an important role in collagen recognition and subsequent cleavage by MMPs [80–82]. Our recent study has revealed that $\alpha1(I)_3$ homotrimers are 5–10 times more resistant to cleavage by all collagenolytic MMPs than normal type I heterotrimers [8] due to increased triple helix stability at the cleavage site [26].

4.4. MMP upregulation follows the homotrimer build up but is not sufficient to degrade the accumulating collagen

Collagenases (MMP-13, -14), gelatinases (MMP-2, -9), and stromelysin (MMP-3) are all crucial enzymes for type I collagen degradation in soft mouse tissues [83]. Although MMP-3 does not cleave collagen by itself, it is essential for activating collagenolytic MMPs.

In addition to collagen degradation, the same MMPs also have other functions and substrates that may be important in EMT induction and fibrosis [84]. MMPs not only activate themselves as previously mentioned, but have been shown to be transcriptionally regulated by TGF-α and other growth factors that take part in the process of fibrosis[42, 85, 86]. Further, MMP collagenolytic activity has also been shown to be influenced by the presence of TIMPs." Nonetheless, we have observed significant changes in MMP mRNA expression only in homozygous animals and only at three month of age (Figures 4 and 5). In these mice, homotrimer accumulation begins as early as 1 week after birth, suggesting that the MMP upregulation is not a primary pathogenic event [58].

The simplest interpretation of our observations is that the upregulation of the collagen degradation pathway occurs in response to collagen accumulation, although it is not sufficient for degradation of the MMP-resistant homotrimers. On a cautionary note, however, increased levels of MMP-2 and MMP-3 proteins seem to occur before an increase in their mRNA. These discrepancies suggest potential changes in protein translation, circulation, or life span of the MMPs in the tissue. Alterations in circulating levels of MMPs have been associated with inflammation and tumor progression [46, 50, 87–89]. However, decreases in MMP-9 protein seem to suggest that it has little or no role in this type of glomerulopathy. Additional investigation is necessary to elucidate the regulation and functional mechanisms of MMPs in *Col1a2*-deficient glomeruli.

4.5. ER stress may exacerbate Col1a2-deficient glomerulopathy

The aberrant proα2(I) chains, which are not incorporated into the collagen triple helix, appear to be translated and retained in the endoplasmic reticulum (ER), triggering an unfolded protein response (UPR) and degradation [2, 55, 90]. The resulting ER stress may explain why syntheses on nonfunctional proα2(I) chains leads to severe skeletal deformities [55] while complete lack of proα2(I) chain translation does not [91]. ER stress due to UPR has recently been found to play a role in abnormal skeletal formation and renal disease [92–96]. ER stress and UPR may result not only in cell malfunction but also in increased synthesis of type I collagen homotrimers, which is a known response of mesangial cells to stress [13]. ER stress and UPR may underlie the increased *COL1A1* expression and decreased *COL1A2* expression in homozygous animals at three month of age. The increased homotrimer synthesis may worsen the disease. Further studies are needed to define the potential role of ER stress in the glomerulopathy in *Col1a2*-deficient mice.

In summary, our findings demonstrate that homotrimeric type I collagen is the pathogenic type I collagen isotype accumulating in sclerotic glomeruli of heterozygous and homozygous mice with *Col1a2* deficiency. This accumulation appears to be associated with the type I collagen homotrimer resistance to MMPs and inefficient homotrimer “clean-up” within the mesangium. It may be exacerbated by UPR to nonfunctional proα2(I) chains, resulting in ER stress, and accompanying upregulation of the *COL1A1* gene and down-regulation of the *COL1A2* gene. Alterations in MMP-2, -3, -9, -13 and -14 expression appear to be a secondary response rather than primary events in the disease process. Increased expression of collagenolytic MMPs is insufficient to prevent accumulation of homotrimeric type I collagen in the glomeruli of the *Col1a2*-deficient mouse.

Abbreviations

+/+	wildtype mouse
+/−	heterozygous *oim* mouse
−/−	homozygous *oim* mouse

BSA	bovine serum albumin
DAB	3,3’ diaminobenzidine tetrahydrochloride
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal-transition
ER	endoplasmic reticulum
HBSS	Hanks Balanced Salt Solution
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IHC	immunohistochemistry
MMP	matrix metalloproteinase
OI	osteogenesis imperfecta
oim	osteogenesis imperfecta murine
PBS	phosphate buffered saline
PMSF	phenylmethyl sulfonyl fluoride
PSR	picrosirius red
TRS	target retrieval solution
UPR	unfolded protein response

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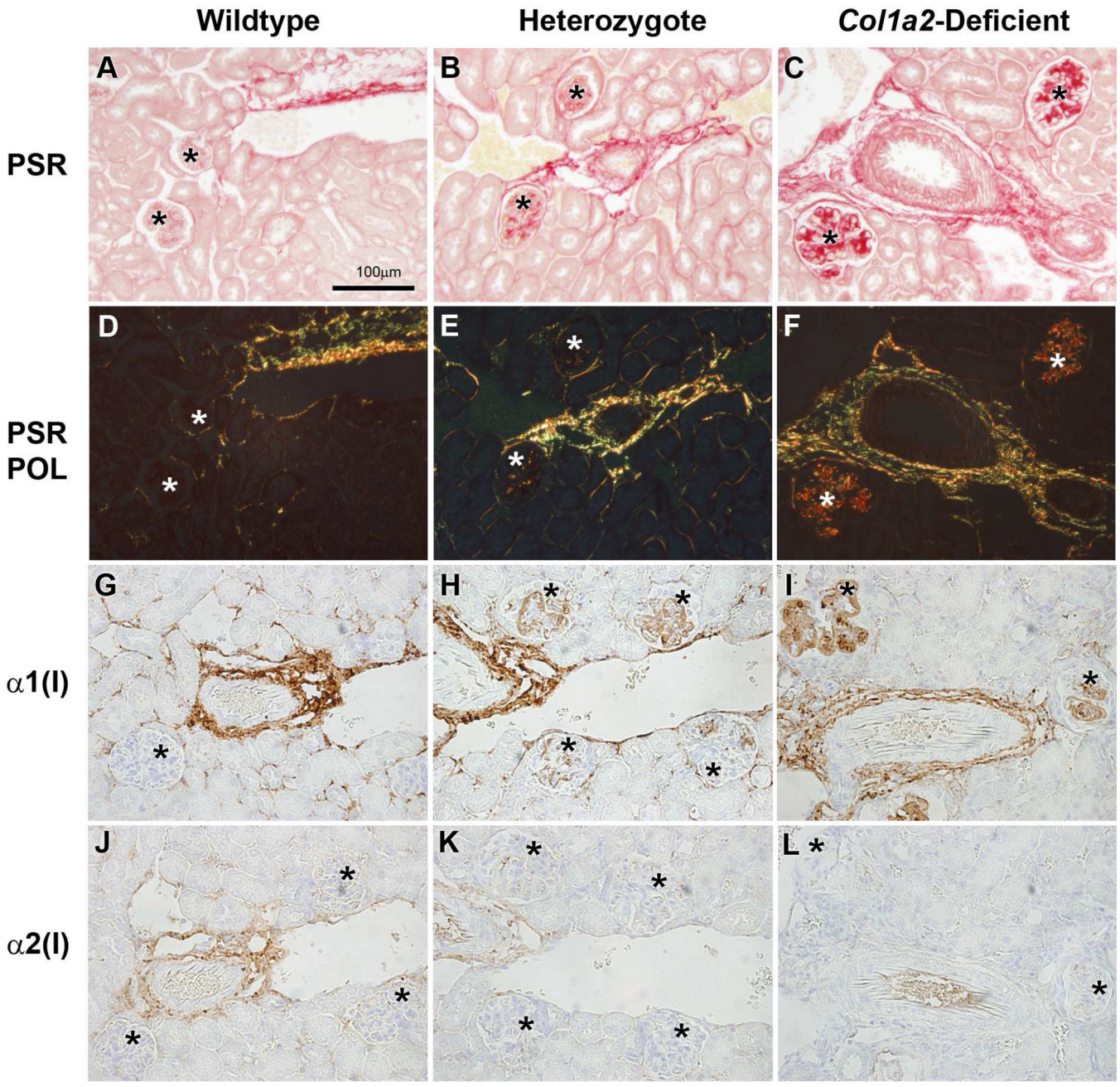

Figure 1.

Identification of type I collagen homotrimer in wildtype (+/+) (A,D,G,J), heterozygous (+/−) (B,E,H,K) and homozygous (−/−)(C,F,I,L) glomeruli. Picrosirius red (PSR) staining under normal (A,B,C)and polarized light (D,E,F) of wildtype glomeruli (A,D) show no deposition of type I collagen, heterozygous glomeruli (B,E) show mild deposition of type I collagen, and homozygous glomeruli (C,F) show severe deposition of collagen within glomeruli. Anti-α1(I) collagen immunohistochemistry (IHC)(G–I) of wildtype kidneys (G) demonstrate no localization of α1(I) chains within glomeruli, only in the vasculature. Heterozygous kidneys (H) show localization of type I collagen α1(I) chains within the glomeruli and vasculature. Homozygous kidneys (I) mice also show evidence of type I collagen α1(I) in the glomeruli and vasculature. Anti-α2(I) collagen IHC (J–L) of wildtype (J), heterozygous (K), and

homozygous (L) kidneys demonstrate the presence of α2(I) chains in the vasculature of wildtype and heterozygous kidneys, and the absence of anti-α2(I) positive staining within glomeruli of wildtype, heterozygous, and homozygous kidneys. Asterisks indicate glomeruli.

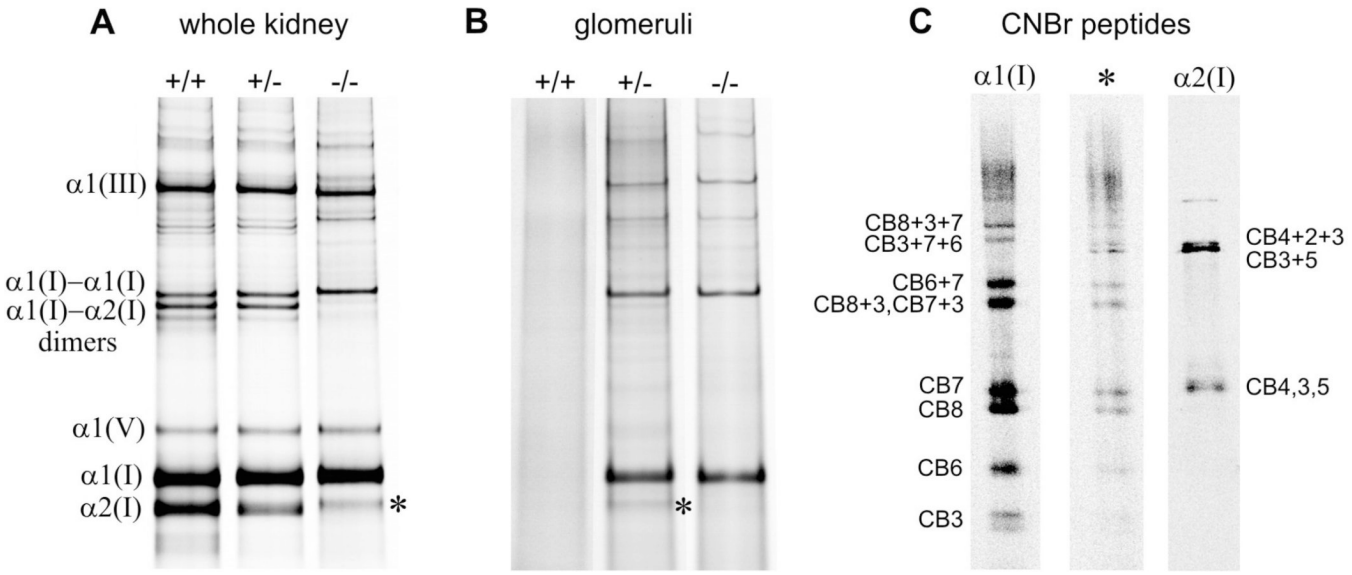

Figure 2.

Analysis of collagen composition in whole kidney (A) and glomeruli (B) from wildtype (+/+), heterozygous (+/−) and homozygous *Col1a2*-deficient (−/−) mice. The fraction of homotrimeric type I collagen was determined from the intensity ratios of α1(I) : α2(I) and α1(I)-α1(I) : α1(I)-α2(I) as previously described [90]. A faint band labeled with *, migrating close to the expected position of the α2(I) chain, was observed in kidneys from homozygous mice and glomeruli from some heterozygous mice. CNBr peptide patterns (C) from the * band of the homozygous kidney and heterozygous glomeruli were similar and consistent with the α1(I) chain cleaved within CB6 or CB5. Such proteolytic degradation of α1(I) homotrimers is often observed in *Col1a2*-deficient tissues [68]. However, we could not completely exclude the presence of the α2(I) chain in the * band from heterozygous glomeruli. Indeed, the most intense bands of α2(I) CNBr peptides are co-migrating CB3, CB4, and CB5 as well as co-migrating partial CNBr cleavage products CB3+5 and CB4+2+3 (C). The first group migrates close to and may overlap with α1(I)-CB7. The second group migrates close to and may overlap with α1(I)-CB3+7+6, in which CB6 truncated by the proteolytic cleavage discussed above. Based on the observed */α1(I) intensity ratio, absence of the well defined α1(I)-α2(I) dimer band, and relative intensities of different bands in the CNBr cleavage pattern of the * band, we estimated that type I collagen from heterozygous glomeruli consisted of at least 95–98 % α1(I) homotrimers. Note that fluorescent labeling with Cy5 allows evaluation of the relative content of α1(I) and α2(I) chains with approximately 5% accuracy.

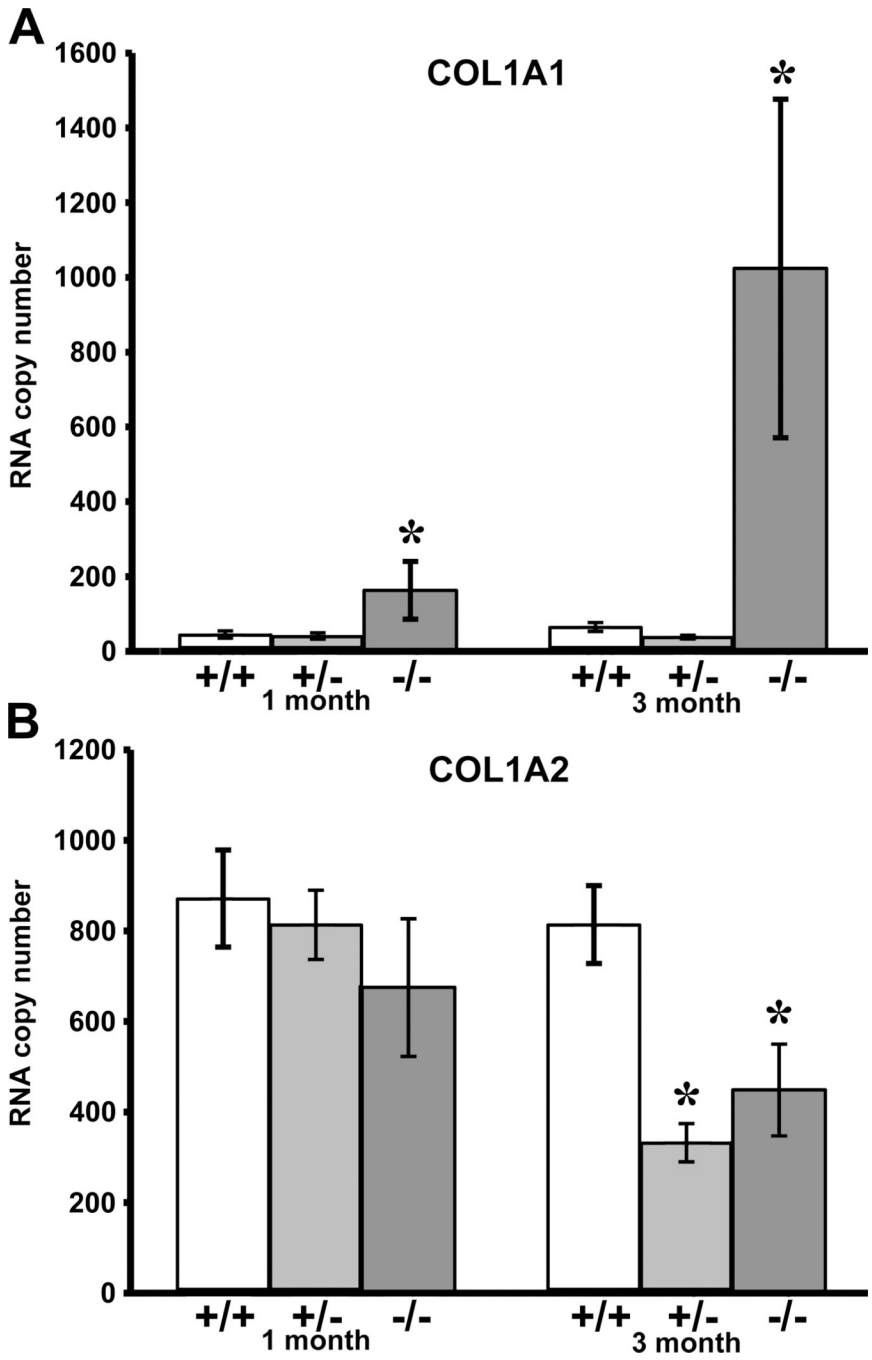

Figure 3.

Quantitative RT-PCR steady-state mRNA expression of COL1A1 (top) and COL1A2 (bottom) transcripts in wildtype (+/+), heterozygous (+/−) and homozygous (−/−) *Col1a2*-deficient glomeruli. 1-month and 3-month old homozygous glomeruli demonstrate increases in proα1(I) collagen mRNA copy as compared to age-matched wildtype and heterozygous glomeruli (*p <0.003 and *p <0.0001 respectively). Proα2(I)collagen mRNA copy number decreases in 3-month old heterozygous (*p <0.0001) and homozygous (*p <0.003) glomeruli as compared to age-matched wildtype glomeruli.

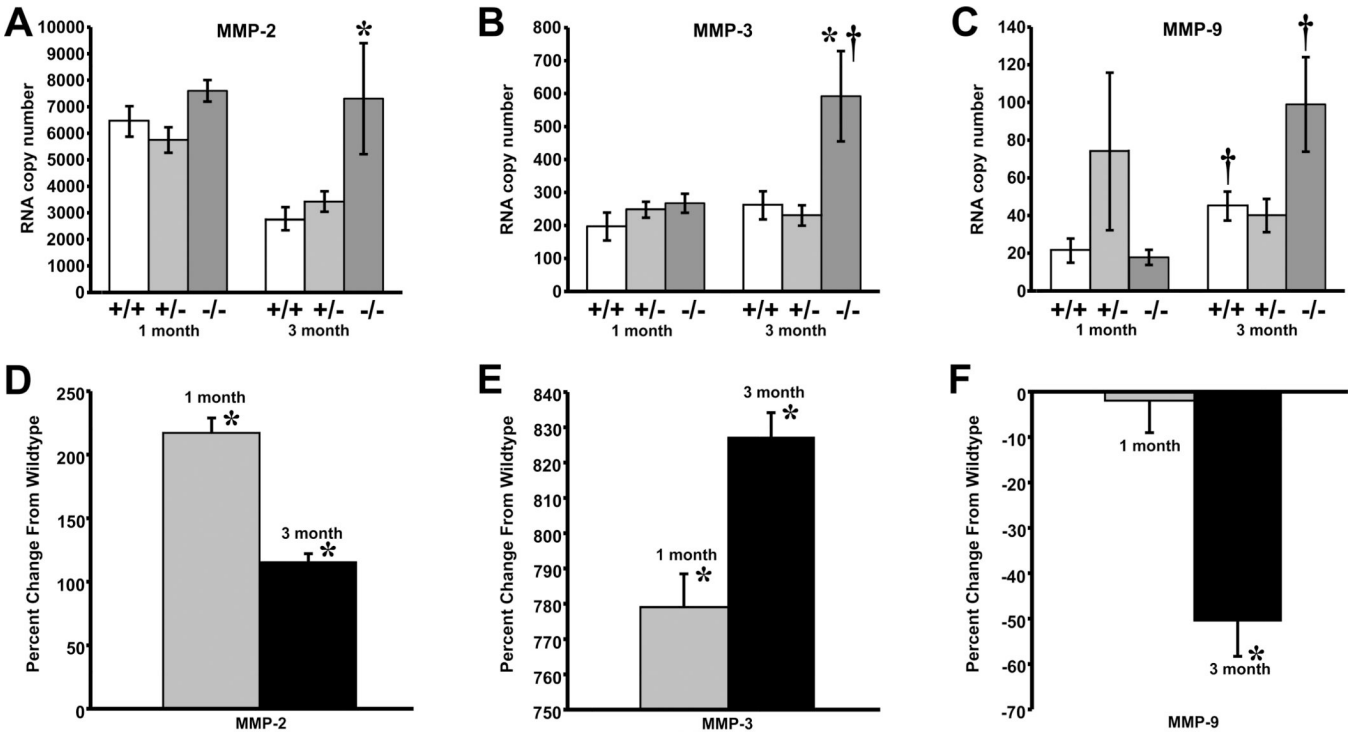

Figure 4.

Quantitative RT-PCR demonstrates an increase in MMP-2 (A), and MMP-3 (B) mRNA expression in 3-month *Col1a2*-deficient (−/−) glomeruli compared to age-matched wildtype (+/+) [MMP-2, *p <0.0004; MMP-3,*p <0.04]. MMP-3 (B) and MMP-9 (C) demonstrate significant increases between 1-month and 3-month wildtype and *Col1a2*-deficient glomeruli [MMP-3, †p <0.04 *Col1a2*-deficient glomeruli; MMP-9, †p <0.01 wildtype and MMP-9, †p <0.0007 *Col1a2*-deficient glomeruli]. 1-month and 3-month *Col1a2*-deficient glomeruli show an increase in MMP-2 (D) (*p ≤0.001 and *p ≤0.008 respectively), and MMP-3 (E) (*p ≤0.0005 and *p ≤0.0003 respectively) protein expression as compared to age-matched wildtype mice, while MMP-9 (F) shows a decrease in protein expression at three months of age (*p ≤0.05). Data expressed as mean ±SEM.

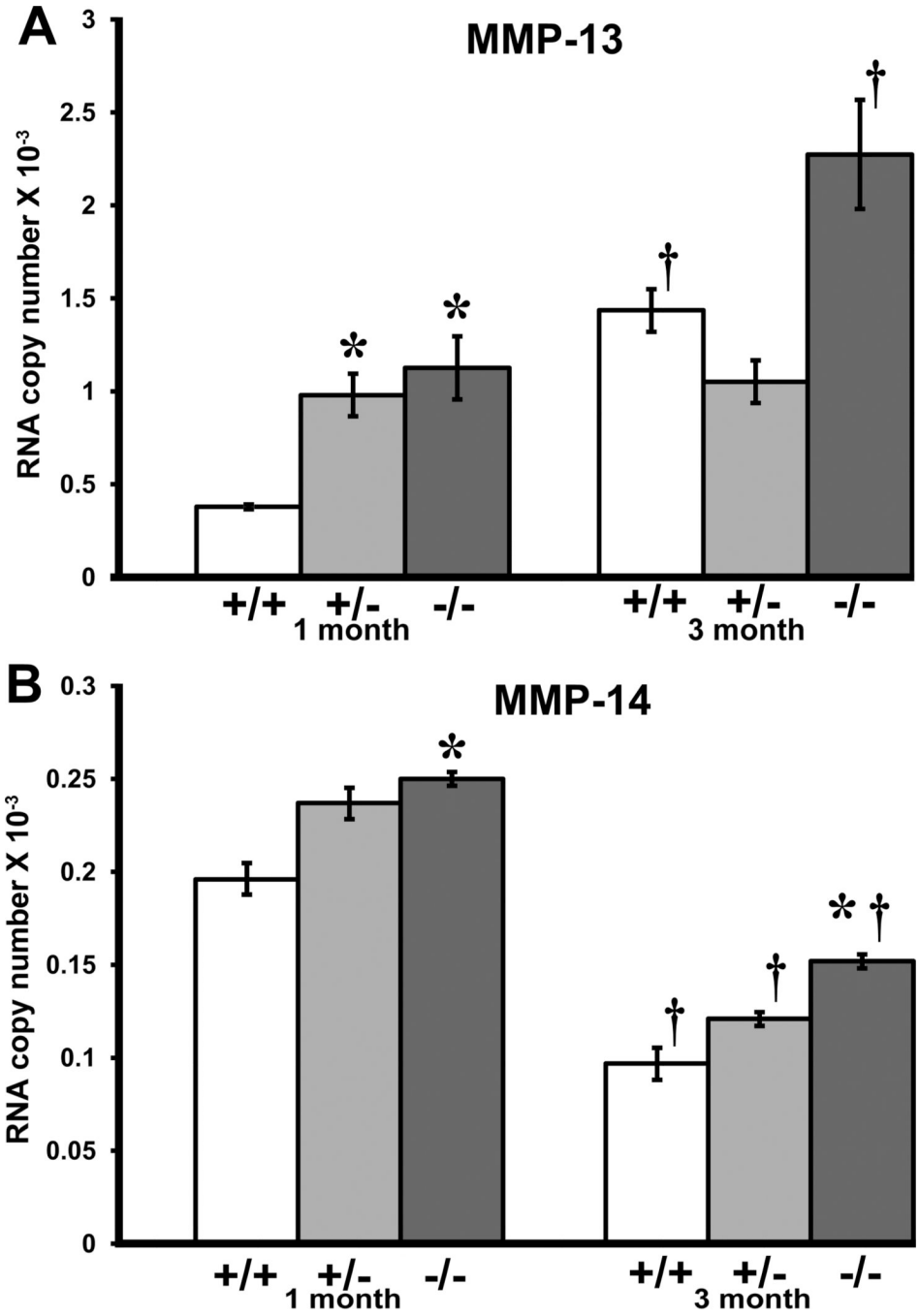

Figure 5.

Quantitative RT-PCR steady-state expression of MMP-13 and MMP-14 transcripts in wildtype (+/+), heterozygous (+/−) and homozygous (−/−) *Col1a2*-deficient glomeruli. 1-month homozygous (*p <0.02) and heterozygous *Col1a2*-deficient glomeruli (*p <0.03) show an increase in MMP-13 (A) mRNA expression compared to age-matched wildtype glomeruli and increases in wildtype (†p<0.02) and *Col1a2*-deficient glomeruli (†p<0.02) between 1 and 3-months of age. MMP-14 (B) mRNA transcript levels are elevated in 1-month homozygous glomeruli compared to age-matched wildtype glomeruli (*p<0.02), and in 3-month homozygous glomeruli (*p ≤0.02). Wildtype glomeruli show a decrease in

MMP-14 mRNA transcripts at 3 months of age compared to 1-month wildtype glomeruli and this trend is seen across all genotypes ($^{\dagger}p \leq 0.0001$). Data expressed as mean ±SEM.

Table 1

Wildtype (+/+), heterozygous (+/−), and *Col1a2*-deficient (−/−) perfusion data (mean ± S.E.M.)

Genotype (n)	Age (mo)	Animal Wt (g)	Kidney Wt (g)	Kidney/ Animal Wt	Total Glomerular Yield	Glomerular Yield/Kidney Wt
Wildtype (21)	1	18.15 ± 0.54	0.279 ± 0.010	0.0155 ± 0.0004	6942 ± 818	25826 ± 3204
Heterozygous (38)	1	17.38 ± 0.45	0.281 ± 0.008	0.0163 ± 0.0004	6750 ± 659	25160 ± 2797
Col1a2-deficient (20)	1	12.79 ± 0.62[a]	0.225 ± 0.011[a]	0.0178 ± 0.0006[a]	6510 ± 1350	30568 ± 6905
Wildtype (22)	3	29.40 ± 0.66	0.437 ± 0.013	0.0149 ± 0.0004	4679 ± 805	10880 ± 2041[b]
Heterozygous (23)	3	27.91 ± 0.95	0.441 ± 0.022	0.0157 ± 0.0004	5398 ± 1080	12011 ± 2322[b]
Col1a2-deficient (13)	3	21.05 ± 1.13[a]	0.343 ± 0.032[a]	0.0162 ± 0.0012	900 ± 122[a,b]	3087 ± 584[a,b]

[a] $p \leq 0.005$ compared to age-matched wildtype;

[b] $p \leq 0.005$ compared to same genotype at 1 month of age

Table 2

Average number of glomeruli per field in kidney cortex and juxtamedullary regions (mean ± S.E.M.)

Genotype (n)	Age (mo)	Cortical Glomeruli	Juxtamedullary Glomeruli	Total Glomeruli
Wildtype (8)	1	5.09 ± 0.43	2.06 ± 0.32	3.58 ± 0.46
Heterozygous (11)	1	4.16 ± 0.50	2.28 ± 0.21	3.22 ± 0.34
Col1a2-deficient (8)	1	7.00 ± 0.92[b]	3.63 ± 0.65[a,b]	5.31 ± 0.70[b]
Wildtype (8)	3	4.28 ± 0.47	2.38 ± 0.31	3.33 ± 0.37
Heterozygous (8)	3	4.34 ± 0.43	2.22 ± 0.26	3.28 ± 0.37
Col1a2-deficient (8)	3	5.34 ± 0.76	3.71 ± 0.58[b]	4.53 ± 0.51[a]

[a] $p \leq 0.05$ compared to age-matched heterozygous;

[b] $p \leq 0.05$ compared to age-matched wildtype

Table 3

Quantitative real time PCR primers

Primer	Sequence	Amplicon size
COL1A1 forward	5' - TGG ATT CCC GTT CGA GTA CG - 3'	202bp
COL1A1 reverse	5’ – ATT AGG CGC AGG AAG GTC AG - 3’	
COL1A2 forward	5’ – TGA AGT GGG TCT TCC AGG TCT TTC – 3’	236bp
COL1A2 reverse	5’ – CAC CCT TGT TAC CGG ATT CTC CTT – 3’	
MMP-2 forward	5’ – AAA GGA CTC GGG TTG TCT GA – 3’	150bp
MMP-2 reverse	5’ – CAA GAA GGC TGA GCA GGA AG – 3’	
MMP-3 forward	5’ – TAA AGA CAG GCA CTT TTG GC – 3’	114bp
MMP-3 reverse	5’ – GTA ACC TCA TAT GCA GCA TCC – 3’	
MMP-9 forward	5’ – TCC AGT ACC AAG ACA AAG CC – 3’	169bp
MMP-9 reverse	5’ – TGA AGC AAA GAA GGA GCC C – 3’	
MMP-13	Mm00439491_m1 TaqMan® assay (Applied Biosciences)	65 bp
MMP-14	Mm01318966_m1 TaqMan® assay (Applied Biosciences)	82 bp