GLOMERULOSCLEROSIS IN THE *COL1A2*-DEFICIENT MOUSE MODEL: HOMOTRIMER PATHOGENESIS AND MMP EXPRESSION

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ANNA M. ROBERTS-PILGRIM

Dr. Charlotte L. Phillips, Dissertation Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

GLOMERULOSCLEROSIS IN THE *COL1A2*-DEFICIENT MOUSE MODEL: HOMOTRIMER PATHOGENESIS AND MMP EXPRESSION

Presented by Anna M. Roberts-Pilgrim

A candidate for the degree of Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance

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Dr. Charlotte Phillips

_____
Dr. Craig Franklin

_____
Dr. Linda Randall

_____
Dr. Francis Schmidt

_____
Dr. Peter Tipton

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Glomerulosclerosis in the *COL1A2*-deficient mouse model: Homotrimer pathogenesis and MMP expression

Anna Roberts-Pilgrim

Dr. Charlotte L. Phillips, Dissertation Advisor

ABSTRACT

The *Col1a2*-deficient (*oim*) mouse model exclusively synthesizes homotrimeric type I collagen due to the lack of functional proα2(I) collagen chains. The mouse develops a type I collagen glomerulopathy that has previously been shown to initiate postnatally and progress in a gene dose-dependent manner, accumulating type I collagen within the renal mesangium, resulting in podocyte foot effacement and proteinuria. In this study we examine the pre- and post-translational expression of type I collagen and MMPs -2, -3, and -9 in wildtype, heterozygous and *Col1a2*-deficient glomeruli to determine whether the pathogenic collagen is homotrimeric in nature, and whether alterations in MMP expression play a role in disease progression. Analysis of whole kidney and isolated glomeruli by immunohistochemistry and CNBr peptide mapping suggest that homotrimer is the accumulating type I collagen isotype in sclerotic glomeruli of both affected and heterozygous mice. Steady state MMPs-2, and -3 mRNA levels exhibited significant increases by three months of age, with corresponding protein increases compared to age-matched wildtype mice. Steady state MMP-9 mRNA levels significantly increased by three months of age, but MMP-9 protein expression was significantly decreased. Our

findings suggest that upregulation of MMPs-2 and -3 expression is not sufficient to prevent homotrimeric type I collagen deposition and that their induction does not appear to be an initiating event, but may represent a secondary wound response.

CHAPTER I.

INTRODUCTION AND LITERATURE REVIEW

Kidney and Glomerular Function

Chronic kidney disease (CKD) is a broad term used to describe various conditions that result in damage to the filtering capacity of the kidney. According to the National Kidney Foundation approximately 26 million Americans have been diagnosed and are suffering from CKD that can lead to kidney failure, while millions of others are at an increased risk of developing the disease (Kidney Disease FAQs, 2009). In fact one in seven or eight persons will develop CKD in their lifetime.

Heart disease and hypertension have been shown to be intimately linked to the development of CKD (Taal & Brenner, 2006), which is usually identified by a persistent presence of protein in the urine (Hostetter, 2004; Anavekar, 2004; Freedman, 2007). The link between cardiovascular disease (CVD) and CKD was also studied by the Kidney Early Evaluation Program (KEEP) and the National Institutes of Health by McCullough, et al., who reports that patients with mild CKD which exhibited anemia, lowered glomerular filtration rates and increased microalbuminuria in urine also had some degree of CVD (McCullough, 2007; Go, 2004). Additionally, Elsayed, et al. reports that patients with CVD have an associated decline in kidney function, although rarely succumbing to it (Elsayed, 2007). According to the National Kidney Foundation, diabetes and hypertension are the two most common causes of CKD (Glomerular Filtration Rate, 2009). The third most common cause of CKD is sclerosis of the glomeruli, which disrupts the filtration barrier and impairs the body's ability to efficiently filter urine.

Kidneys are the major organs for waste removal from the blood. Consisting of an intertwined network of capillaries and tubules, the kidneys function by movement of

molecules and ions across several types of filters. The primary filtering unit in the kidney is glomeruli, which comprises approximately 10% of the total volume of the organ. They are made of narrow, branched capillaries held apart by mesangial cells and surrounded by a Bowman's capsule to collect ultrafiltrate and unwanted solutes. The Bowman's capsule is attached to the tubules which act to reabsorb sodium and water back into the blood stream, as well as glucose, potassium, phosphates, amino acids, and proteins, to maintain homeostasis.

The glomerulus uses an impressive combination of pressure gradients, charged barriers and pore size exclusion barriers to filter waste out of the blood as it crosses the glomerular filtration barrier. This barrier is a three layer structure of fenestrated endothelial cells, the glomerular basement membrane (GBM) and podocyte foot processes. Disruption of this delicate region leads to impaired filtration that can lead to proteinuria, sclerosis and even kidney failure (Brenner, 2000) (Figure I-1).

Each layer that make up the glomerular filtration barrier work in concert to maintain its function. The capillaries are made of endothelial cells that have thousands of tiny "pores" or fenestrae that allow filtrates up to 70KDa in size to pass through them (Bulger, 1983). These cells also have the capacity to synthesize microvasculature (Kang, 2002) and prevent coagulation of blood (Aird, 2005; Goligorsky, 2006). During pathogenic conditions, these fenestrae increase in size and shape, allowing larger molecules such as albumin to easily pass through them, occasionally leading to a reduction in angiogenesis (Yamanaka, 1999). Glomerular endothelial cells are directly in contact with the GBM and mesangial cells.

The second layer of the glomerular filtration barrier is the GBM. It has three

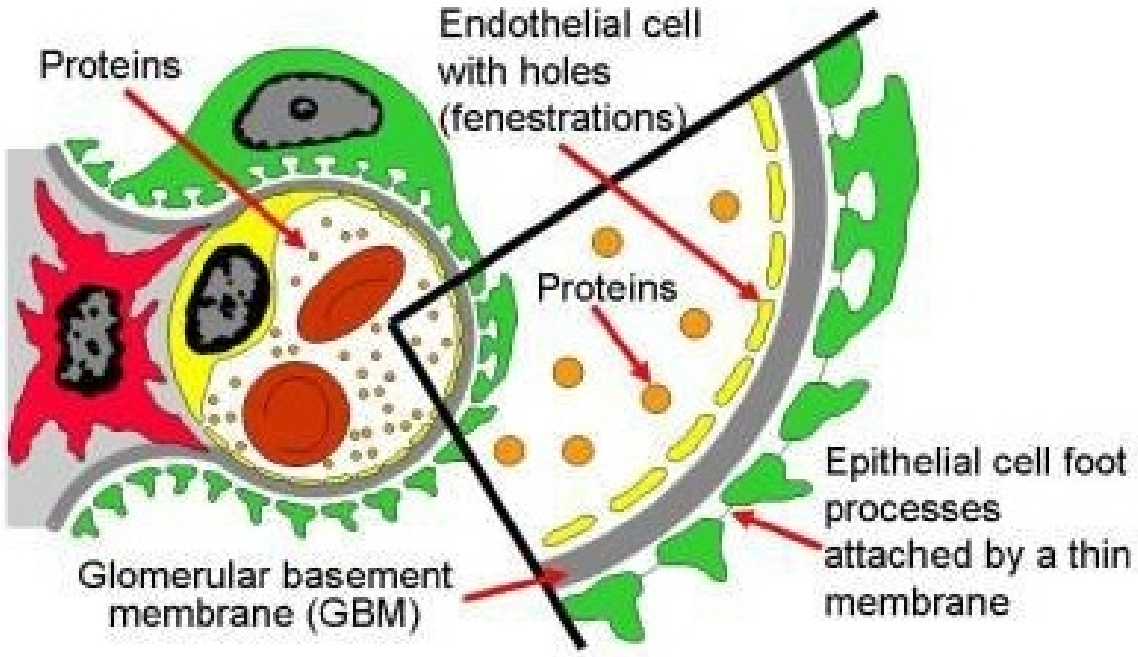


Figure I-1. The Glomerular Filtration Barrier. In the glomerulus, the filtration barrier surrounds the fenestrated capillary endothelium (yellow) which is supported by mesangial cells (red). The glomerular basement membrane (dark grey) and the podocyte cells with their interdigitating foot processes (green) completely surround the mesangial and endothelial cells. Proteins (orange) circulating in the blood are filtered across the endothelial cells, glomerular basement membrane and podocyte foot processes. Red blood cells (brown). Adapted from http://www.unckidneycenter.org/kidneyhealthlibrary/nephroticsyndrome.htm

striated layers that are a meshwork of type IV collagen, laminin, entactin, heparan sulfate, proteoglycan and fibronectin. The GBM is acellular, making its matrix a contribution from podocytes and possibly endothelial cells. The lamina rara interna, the innermost GBM layer, is directly adhered to the capillary endothelial cell. The lamina densa encompasses the middle matrix layer and the lamina rara externa is the outermost layer that is attached to the foot processes of the podocyte cell. Together these layers are between 220nm to 280nm wide and are negatively charged, using charge as its molecular selective property (Takami, 1991; Harvey, 2008; Miner, 2005).

Podocytes are the final layer of the glomerular filtration barrier. They have long finger-like extensions, called foot processes that are regularly interdigitated between foot processes on other podocyte cells as they wrap around the capillary endothelium and GBM. The spaces between the foot processes are known as glomerular slits and contain a variety of slit-associated proteins that attach the foot processes to the GBM, mediate signaling primarily to the endothelial cells and act as the final glomerular filter, although it is still unclear how (Miner, 2002; Miner, 2003; Shankland, 2006). (Figure I-1)

Giving support to the tangle of capillary endothelial cells and podocyte cells are mesangial cells, the third cell type in the glomerulus, that fill the glomerular space and excrete necessary enzymatic and extracellular matrix components to maintain the glomerulus structure (Glick, 1992; Mene, 1989; McLennan, 1999). Mesangial cells also act to stabilize the capillaries during blood pressure changes (Latta, 1992).

The landscape of the human glomerulus is a complex one and very sensitive to change. As the blood enters the glomerular capillaries, a pressure gradient is formed between the large size of the afferent arteriole, which allows blood to enter the

glomerulus, and the small size of the efferent arteriole which removes blood from the glomerulus. The change in size between the arterioles increases the pressure of the incoming blood flowing through the glomerulus and forces the ultrafiltrate and unwanted solutes through the glomerular filtration barrier and into the Bowman's capsule. In patients with hypertension, the increased pressure of the blood flowing through the glomerulus can eventually disrupt the filtration barrier.

Characteristics of Glomerulosclerosis and Wound-Healing

The scarring of glomerular capillaries that disrupts or impairs the filtration of the blood is termed as glomerulosclerosis. Glomerulosclerotic disease has many factors that make it difficult to categorize. Mouse models have been developed to mimic human renal diseases to help study the cause and effects of glomerulosclerosis in various circumstances. Glomerular pathology during disease has many different manifestations including crescent formation, basement membrane thickening, infiltration of inflammatory cells and hyalinization, mesangial hypercellularity and mesangial extracellular matrix (ECM) deposition. However, not all of these are present in every glomerulopathy.

The first method of glomerulosclerotic characterization is examining the pattern of sclerosis. There are four general categories that are consistently referred to in the literature; focal-segmental glomerulosclerosis (FSGS), diffuse mesangial glomerulosclerosis (DMS), minimal-change disease (MCD) and mesangioproliferative glomerulonephritis (MPGN). These "patterns of sclerosis" are also frequently referred to

as diseases; however, many glomerulosclerotic diseases present with these patterns of ECM accumulation. Their names give clues to the sclerotic patterns that one can expect to see during renal disease. For example, according to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) the term glomerulonephritis refers to the impairment of kidney function including the filtration barrier, whereas glomerulosclerosis refers to the specific process of glomerular capillary hardening.

Focal-segmental glomerulosclerosis (FSGS) refers to a sclerotic pattern of ECM deposition in which segmented regions of the glomerulus become affected, but other regions do not, allowing glomeruli to have partial functionality (Kwoh, 2006). This pattern shows increased production of ECM within the renal mesangium, leading to segmented mesangial space expansion. FSGS has been termed a disease of the podocyte because studies show the primary event following glomerular injury to be podocyte foot fusion, effacement and denudation of the basement membrane, resulting in adherence of the GBM to the parietal cells that form the Bowman's capsule (synechiae formation) (Asanuma, 2003; Kriz, 1994; Miner, 2003). Podocyte hypertrophy and vascularization can be especially identified surrounding the glomerular tuft region (Meyrier, 2005). It is therefore no surprise that FSGS is also accompanied by proteinuria. Patients with the inherited disease Frasier syndrome (characterized by a mutation in the Wilm's tumor suppressor gene with a phenotype of intersex, gonadoblastoma and nephropathy) display this pattern of sclerosis (Koziell, 1999). Rat models, in which one kidney is completely removed and two-thirds of the other kidney is removed (5/6 nephrectomy rats models), which are used to study hypertensive glomerulosclerosis also show this pattern of accumulation, including loss of renal blood pressure autoregulation displayed by dilation

of afferent arterioles and glomerular hyperperfusion. Glomerular lesions, proteinuria, and in many cases ischema (restriction of blood flow frequently due to blockage) follow, and finally macrophage infiltration and increased ECM synthesis are identified. Hypertensive glomerulosclerosis is the second most common cause of end stage renal disease (ESRD) (Hill, 2008).

Diffuse mesangial glomerulosclerosis (DMS) progresses by deposition of extensive fibrillary ECM within the mesangial space without mesangial cell proliferation, leading to immense distention of the mesangial area. This expansion is accompanied by podocyte hypertrophy, basement membrane thickening and proteinuria (Habib, 1993; Jeanpierre, 1998; Ito, 2001). DMS was reported in 2004 by Niaudet, et al., to be the second most common cause of infantile nephritic syndrome associated with glomerular injury and rapid progression to ESRD (Niaudet, 2004). This pattern of sclerosis can be seen in nephrotic syndromes such as the inherited Denys-Drash syndrome that results from mutations in the Wilm's tumor suppressor gene, resulting in a similar phenotype as persons with Frasier syndrome but displaying a DMS pattern of glomerulosclerotic disease progression (Gao, 2004; Koziell, 1999). The ICER Iγ (inducible cAMP early repressor in pancreatic B cells-an insulin repressor) diabetic mouse model, used to study human diabetic nephropathy resulting from hyperglycemia, also displays DMS and proteinuria precluded by increased glomerular filtration and hypertrophy by 8 weeks of age (Inada, 2008). In this model, once DMS has begun it is followed by increased expression of type IV collagen and laminin and basement membrane thickening with reduced expression of matrix metalloproteinases (MMPs), leading to a reduction in the glomerular filtration rate and increased proteinuria (Inada, 2005).

Mesangioproliferative glomerulonephritis (MPGN) is characterized by mesangial proliferation and increased matrix synthesis, resulting in mesangial expansion and a lobular glomerular tuft region. Glomerular capillaries become thickened and accumulations of immune deposits are identified in the subendothelial and intramembrane space. Newly synthesized basement membranes are also seen, leading to membrane duplication and/or splitting, and often cellular infiltration through the capillary wall (Smith, 2005; Strippoli, 2003; Rennke, 1995). MSGN has been shown to be an induced form of glomerulosclerosis by infections, notably Hepatitis-C in humans. The transgenic thymic stromal lymphopoietin (TSLP) mouse model develops cryoglobulinemic MPGN which displays all the above mentioned phenotypes of MPGN, including splitting of the glomerular capillary wall and focal intracapillary clotting (Taneda, 2001).

Minimal-change disease (MCD), the mildest pattern of glomerulosclerotic progression, shows very little or no change to glomeruli or kidney tissue when examined microscopically, with no scarring present. Miniscule lipid drops have been shown throughout the kidney, and loss of podocyte foot processes has been identified. Patients usually present with edema and proteinuria before undergoing a kidney biopsy, often idiopathically. MCD may occur at any age, but it is most common in childhood.

Fibrosis and Inflammation

Further characterization of glomerulosclerosis into inflammatory and non-inflammatory pathways has been used to study this disease. The inflammatory pathway

during sclerosis of the glomerulus is induced by circulating inflammatory cells (neutrophils, monocytes or platelets, leukocytes adhesion molecules) or local proliferating resident cells. Several glomerulosclerotic diseases that have lesions induced by the inflammatory pathway include lupus nephritis, anti-GBM glomerulonephritis, Thy-1 mesangio-proliferative glomerulonephritis, masugi nephritis and immunoglobin A (IgA) nephropathy (Schoecklmann, 1996; Striker, 1989; Couser, 1994). Non-inflammatory pathways, which do not use an inflammatory mechanism for lesion production, are usually disorders of podocyte cells or are genetically inherited diseases (Kwoh, 2006; Lenz, 2000; Couser, 1994).

A brief, general overview of what happens during wound healing, fibrosis and inflammation is necessary to orient you, the reader, to what occurs during this process and highlight events occurring during the progression of glomerulosclerosis (Figure I-2). Once injury has taken place, existing blood vessels adjacent to the wound release vascular endothelial growth factor (VEGF) and nitric oxide to vasodilate the endothelial cells, increasing the permeability of the existing vessels and allowing infiltration of cytokines and growth factors for formation of new vasculature (neovascularization). The endothelial cells eventually loose cell-to-cell contact via the secretion of plasminogen and the basement membrane is degraded by MMPs. Next, VEGF (via the VEGFR-2 endothelial cell receptor) induces endothelial cell migration toward the injury site and proliferation to form new capillaries. Pericytes and smooth muscle cells are recruited to the area by angiopoietin-1 and -2 and platelet derived growth factor (PDGF) respectively, and ECM protein deposition is induced by transforming growth factor-β1 (TGF-β1), all in an effort to stabilize neovascularization.

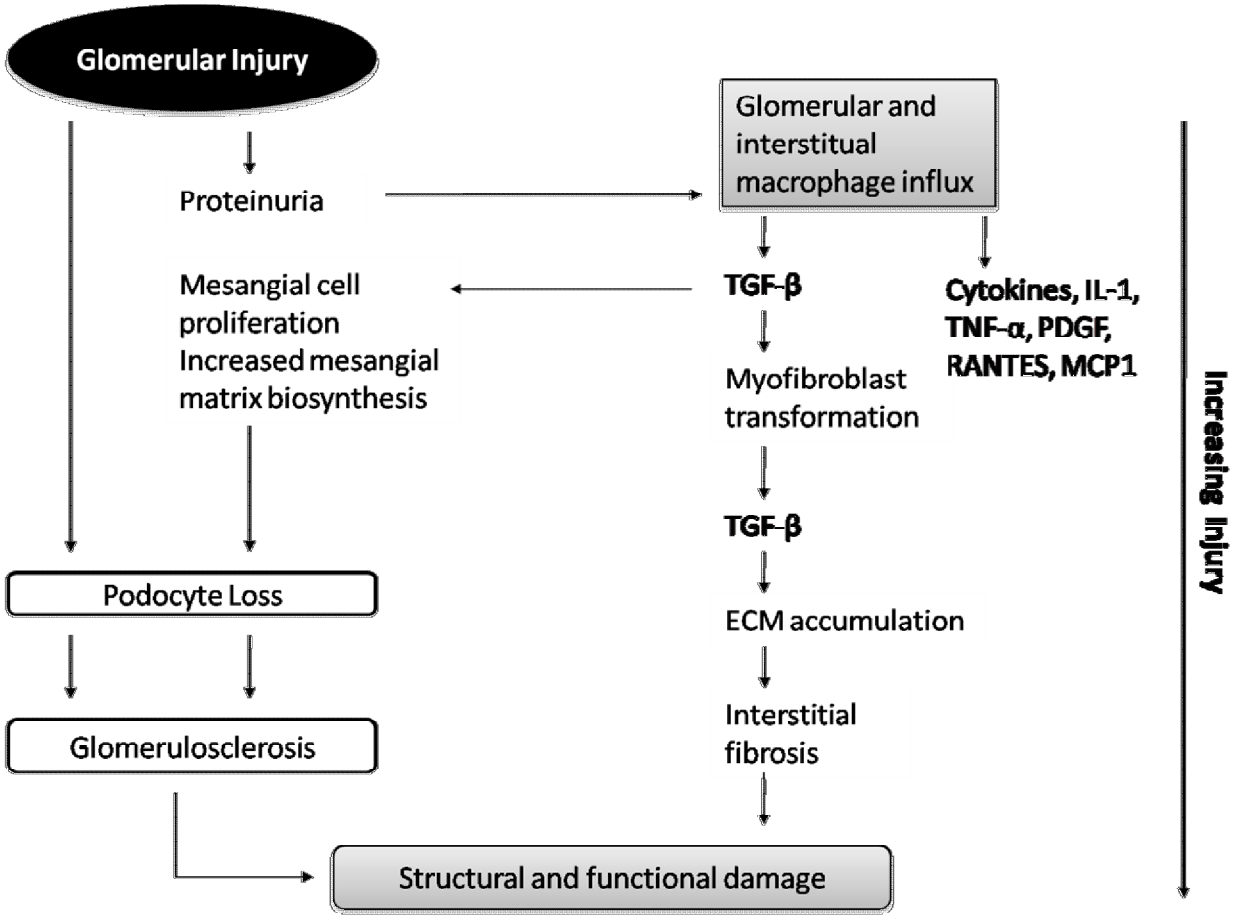


Figure I-2. The Progression of Glomerular Injury. Once glomerular injury has taken place, kidney function is impaired as exhibited by proteinuria. Glomerular injury can result in direct loss of podocytes seen in FSGS sclerotic patterns or via mesangial cell proliferation and increased mesangial matrix biosynthesis. Both pathways lead to glomerulosclerosis and glomerular damage. During inflammation, macrophages influx into the glomerulus and interstitum, releasing growth factors and cytokines that initiate myofibroblast formation and mesangial proliferation, resulting in matrix accumulation, fibrosis and damage.

The anti-inflammatory cytokine TGF-β naturally occurs in three major isoforms: β1, β2, and β3. However, TGF-β1 has been shown to be the dominant isoform present during sclerosis, although all isoforms have a role in wound healing and repair. Although the exact mechanisms are still not clear, during fibrosis TGF-β can upregulate many extracellular matrix molecules, including fibrillar and non-fibrillar collagens, fibronectin and laminin (Branton, 1999; Border, 1994). For example, podocytes increase their synthesis of collagen IV, laminin, and biglycan when TGF-β is increased. Additionally, mesangial cells in culture have increased mRNA expression of collagen I and IV and fibronectin in response to stimulation by TGF-β (MacKay, 1989; Suzuki, 1993; Nakamura, 1992). The role of TGF-β as enhancing fibrogenesis during injury, and mediating fibrotic diseases has been effectively outlined by Branton, et al., where he presents three keys points that confirm a role for TGF-β (Branton, 1999). First, TGF-β increases have been shown to be localized to sclerotic regions in various organs. Second, development of fibrosis in lab animals occurred upon administration of TGF-β, and finally, therapies that block TGF-β reduce induced fibrosis (Branton, 1999; Wells, 2000). TGF-β also aids in increasing fibroblast migration and proliferation, and decreasing degradation of ECM by MMPs. TGF-β is also a chemotactic for monocyte cell influx and can stimulate angiogenesis.

Once vasodilation is achieved, circulating plasma proteins fibrinogen and plasma fibronectin are deposited in the ECM surrounding the vessel to provide a scaffold for fibroblast and endothelial cell infiltration and growth. Platelets and activated endothelial cells release the growth factors: PDGF, epidermal growth factor (EGF) and fibroblast growth factor (FGF) and cytokines: TGFβ, interleukin-1 (IL-1) and tumor necrosis factor

(TNF), homing circulating fibroblasts (and myofibroblasts) to the site of injury. When inflammation is the process by which fibrosis occurs, inflammatory cells also release these factors. (Table I-1)

Specifically, macrophages (transient bone marrow derived cells) can synthesize TGFβ, PDGF and FGF, contributing to the process of myofibroblast migration and proliferation. During inflammatory glomerulosclerosis, monocytes/macrophages migrate into the renal mesangium via the blood and through endothelial cells in response to injury markers (chemotaxis) synthesized locally (Kwoh, 2006; Couser, 1994). One such injury marker is the cytokine intercellular adhesion molecule (ICAM) that is released by injured endothelial cells. Once at an injury site, activated macrophages start phagocytosis of injured cells and send out pro-fibrotic signals that recruit other inflammation mediators such as neutrophils, lymphocytes and leukocytes to the region which further contribute to myofibroblast migration and proliferation (Gauer, 1997; Schoecklmann, 1996), or induces expression and activation of MMPs (Shiozawa, 2000; Gomez-Guerrero, 2005).

Myofibroblast recruitment promotes wound contraction/closure by migrating along the fibrin lattice to the wound site and increasing type I collagen and ECM deposition at the injury site (Raghow, 1994). Myofibroblast ECM synthesis is regulated by growth factors (TGFβ, PDGF, FGF) and cytokines (IL-1, IL-13) released from both leukocytes and other myofibroblasts. However, in many fibrotic tissue diseases, some local cell types undergo epithelial/endothelial-to-mesenchymal cell transition (EMT) to create myofibroblast phenotypes rather than recruiting them from other areas of the body, similar to what is seen in the kidney and liver. Recent studies have described a circulating cell type called a fibrocyte that also infiltrates to an injury site and takes on a

Table I-1. Growth factors and cytokines affecting various steps in wound healing.

Wound Healing Phases	Cytokines/Growth factors
Monocyte chemotaxis	PDGF, FGF, TGFb
Fibroblast migration	PDGF, EGF, FGF, TGFb, TNF, IL-1
Fibroblast proliferation	PDGF, EGF, FGF, TNF
Angiogenesis	VEGF, Ang, FGF
Collagen synthesis	TGFb, PDGF
Collagenase secretion	PDGF, FGF, EGF, TNF, TGFb inhibits

myofibroblast phenotype (Bellini, 2007; Quan, 2006).

Myofibroblasts secrete large amounts of the cytokine marker α-smooth muscle actin (α-SMA, a marker of the cells new phenotype and function), and type I collagen (type I collagen is not normally present in the glomerulus). The final step in wound healing is the division and migration of endothelial or epithelial cells over the ECM lattice to regenerate the tissue. Fibrosis is said to occur when ECM production from myofibroblasts is not properly mediated by degradative MMPs. It is also hypothesized that constitutively expressed ECM seen in fibrosis is stimulated by various pathways such as TGFβ or SMAD; however this is still unclear.

Non-inflammatory glomerulosclerotic diseases, also termed dysregulative mechanisms, are frequently inherited, have a FSGS or DMS pattern of accumulation, and also are generally associated with down-regulation of MMPs (Lenz, 2000). Although inflammatory cells are not the cause for lesion formation in these diseases, an influx of inflammatory cells may be seen with disease progression. Here are a few examples:

The diabetic nephropathy mouse model db/db display common sclerotic patterns of FSGS, but are mediated by several cytokine factors that include VEGF, insulin-like growth factor-1 (IGF-1) and advanced glycation end products (AGEs) that stimulate ECM over-expression and lesion development by mesangial cells (Sharma, 2003; Ma, 2003).

Alport syndrome, an inherited sex-linked disease in which type IV collagen is mutated, the GBM has an irregular basketweave appearance, causing proteinuria and accompanied by podocyte damage (Kwoh, 2006). Thin basement membrane nephropathy (TBMN), also an inherited disease caused by mutations in type IV collagen,

present a GBM which is approximately 1/3 thinner than normal GBM. This phenomenon has minimal effect on renal function, exhibiting very little proteinuria (Kwoh, 2006; Lenz, 2000; van Paassen, 2004).

To study the effects of diet-induced hypercholesterolemia on glomeruli, rats fed diets high in cholesterol were observed to have mesangial matrix expansion with accumulation of type IV collagen, fibronectin and laminin, increases in glomerular size, lipid deposits and foam cell formation. Lipid deposits have been linked to mesangial cell proliferation and monocyte infiltration modification (Guijarro, 1995; Oda, 1999).

Rat models of non-inflammatory glomerulosclerosis due to hormone excess are the bGH (growth hormone excess) knockout and the (pro)-renin over-expression models. Podocytes in the bGH rats over-express growth hormone receptors that mediate growth hormone dependent pathways and stimulate podocyte actin cytoskeleton reorganization (Reddy, 2007). Hypertensive rat models display (Pro)-renin over-expression by podocytes, stimulating the mesangium to generate angiotensin II, and inducing intracellular signaling pathways leading to slow progressive nephropathy with proteinuria, breaking down the GBM (Ichihara, 2008; Huang, 2008).

Cyclosporine nephrotoxic rats develop renal vasoconstriction, but can also develop disease of the arteriole and tubulointerstitual fibrosis. Cyclosporine was originally used as an immunosuppressive drug for transplant patients, but during follow-up all patients were found to develop nephrotoxicity (Bobadilla, 2007).

During glomerular disease, sclerosis has been shown to accumulate type I, III, and IV collagens, as well as fibronectin and laminin within glomeruli, and these accumulations are believed to be caused by an imbalance between the synthesis of these

ECM molecules and their removal (Yoshioka, 1990; Alexakis, 2006; Schoecklmann, 1996; Rupprecht, 1996; Yagi, 1995). The synthesis/degradation imbalance is believed to be part of a wound healing mechanism, initiated by some form of glomerular tissue injury. This accrual of extracellular matrix results in mesangial expansion within the mesangial space. Pushing apart the mesangial matrix also force podocytes and their foot processes apart which can lead to improper removal of filtrate and hence proteinuria as determined by the creatinine/albumin ratio (McLennan, 1999; He, 2001; El-Nahas, 2003; Kriz, 2005; Bakker, 2003; Harendza, 1999; Uchio-Yamada, 2001; Glomerular Filtration Rate, 2009). As more collagen is laid down, the glomerular filtration barrier becomes disrupted by enlarged endothelial cell fenestrae and effacement of the finger-like extensions of surrounding podocyte foot processes, allowing large proteins to filter across the barrier and into the urine.

Type I collagen is present in the renal vasculature and interstitium, but not present in normal glomeruli (Alexakis, 2006; Glick, 1992; He, 2001). Several investigators (Peten, 1993; Mozes, 1999; Floege, 1992) have shown that during glomerulosclerosis, glomerular type I collagen mRNA and protein synthesis are increased and can be synthesized by parietal epithelial cells (He, 2001), podocyte cells (Osada, 1995; Shankland, 2006), and mesangial cells (Glick, 1992; Riser, 1992; Sakatsume, 1995), suggesting the possibility that the glomerulus may be reacting to a wound healing response (Lui, 2006; Eddy, 2000; Hirschberg, 2005). The postulation that kidney fibrosis is due to a wound-healing response has been further expanded with the suggestion that renal tissue is returning to an embryonic or less differentiated state to induce healing. In 1999, Safirstein laid out a few similarities between the processes seen in kidney

development and repair. Processes such as increased DNA synthesis, apoptosis, and kidney generation seen during organogenesis also occur in kidney repair (Safirstein, 1999). Vimentin expression and reduced vasoconstriction are also common to both kidney developmental and repair processes.

Glomerular Cells and Sclerosis

Renal endothelial cells are angiogenic (mediated by VEGF) and can generate new capillaries during initial disease states. The endothelial cells can become distended and lose their angiogenic capabilities, leading to loss of microvasculature (ischemia) as disease severity increases (Yamanaka, 1999; Kang, 2002; Kwoh, 2006). However, there is very little evidence to suggest endothelial cells synthesize type I collagen (Iruela-Arispe, 1991) or other ECM matrix during sclerosis. Recent data shows that along the interior of the capillary cell surface there is a thin endothelial cell surface layer (ESL) (also referred to as glycocalyx), containing a mix of membrane-associated proteoglycans, glycosaminoglycans (GAG), glycoproteins, glycolipids, and associated plasma proteins. It has been proposed that this membrane helps permselectivity and has some anticoagulant properties (Jeansson, 2006; Pries, 2000; Ballermann, 2007). During disease, this layer is thinned or removed and the cells loose their anticoagulant, anti-inflammatory phenotype and non-adhesive, protective surface. Upon activation of the endothelial cell by tumor necrosis factor receptor-2 (TNFR-2) during disease, the endothelial cell recruits neutrophils and monocytes/macrophages into the glomerular capillary tuft region by upregulating cytokines such as VCAM-1 and ICAM-1 to

Table I-2. List of commonly used abbreviations.

GBM	Glomerular Basement Membrane
ECM	Extracellular Matrix
FSGS	Focal-Segmental Glomerulosclerosis
DMS	Diffuse Mesangial Glomerulosclerosis
MPGN	Mesangioproliferative Glomerulonephritis
ESRD	End-Stage Renal Disease
MCD	Minimal Change Disease
EMT	Epithelial/Endothelial-to-Mesenchymal Cell Transition
OI	Osteogenesis Imperfecta
TIMP	Tissue Inhibitors of Matrix Metalloproteinases
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet Derived Growth Factor
TGF-β1	Transforming Growth Factor β1
IL-1β	Interleukin-1β
α-SMA	α-Smooth Muscle Actin
TNF-α	Tumor Necrosis Factor-α
RANTES	Regulated upon Activation Normal T-cell Expressed and presumably Secreted
MCP-1	Monocyte Chemotactic Protein-1

promote cellular adhesion of leukocytes before their migration into the sudendothelial and mesangial spaces (Ballermann, 2007; Haraldsson, 2008; Nagao, 2007; Segerer, 2006). There is mounting evidence that endothelial cells will undergo morphogenesis when in contact with type I collagen, but that cell morphology is maintained when in contact with laminin, the main component of the basement membrane (Davis, 2005; Liu, 2004).

In addition to increased type I collagen synthesis, podocytes contribute to glomerular disease progression and proteinuria by becoming effaced and reducing in number, allowing the exposed GBM to be adhered to by paretial cells of the Bowman's capsule, forming synechiae (also known as crescents) (Asanuma, 2003; Kriz, 1994; Kriz, 1996; Kwoh, 2006), or by uncontrolled proliferation of podocytes or parietal epithelial cells (Nagata, 1992; Nagata, 2003; Shankland, 2006). Podocytes have been shown to change their phenotype during disease states by EMT as seen by loss of adhesion to the GBM (resulting in foot process effacement), α-SMA upregulation and actin reorganization of the foot processes which lead to loss of podocyte cells, and finally fibrosis (Lui, 2004; Sam, 2006; Ng, 1999). Podocyte injury is often accompanied by proteinuria, the movement of large protein molecules through the GBM and into the urine; however, it is still unclear which comes first, podocyte injury or proteinuria (Schwartz, 1985; Schwimmer, 2003). Podocytes have also been implicated in the thickening of the glomerular basement membrane by excessive type IV collagen (Mene, 1989; Li, 2007) or laminin synthesis (Fischer, 2000; Schumacher, 2007).

The glomerular mesangium have been implicated as a major contributor to sclerosis. *In vivo*, under normal circumstances, mesangial cells synthesize very little

type III or type I collagen (Yoshioka, 1990; Herrera, 2006; Schlondorff, 1996; Glick, 1992). However, during renal fibrotic disease and in tissue culture, these mesangial cells synthesize elevated levels of either or both collagens into the mesangial space and the cell culture medium (Phillips, 2002; Glick, 1992; McLennan, 1999; Martin, 1994; Ingram, 1999). During disease, mesangial cells de-differentiate from adult, pericyte-like cells to an embryonic, myofibroblast-like cells that proliferate and contract, usually resulting in matrix accumulation within the mesangial space (Schoecklmann, 1996). It is believed that this process (EMT) is aimed to heal and restore the mesangium by reverting to an embryonic state. Mesangial cells also show altered matrix metalloproteinase expression during fibrosis (Catania, 2007; Cheng, 2006; Kuroda, 2004; Lelongt, 2002; Lemaitre, 2006).

The pending question in many sclerotic diseases is what initiates the injury that results in wound-healing? Increases in blood pressure have been linked to glomerular injury, as well as movement of large amounts of glucose across the GBM in diabetic kidneys. However, these have not been conclusively proven to be the injury insult. In the *Col1a2*-deficient mouse kidneys, collagen accumulation begins by 1 week of age and increases in severity with age. Type I collagen is believed to be synthesized by the mesangial cells, pushing them apart, although this is not confirmed. Only in the most sclerotic glomeruli is collagen deposition seen creeping between the fenestrated endothelial cell and the mesangial cells and inflammatory cells such as neutrophils are identified.

Type I Collagen Synthesis and Homotrimer

Collagen, the main structural component within the body, is the extracellular framework that participates in a variety of functions that not only gives shape to our tissues but also allows proteins and enzymes to move and communicate outside the cell. Over 27 different types of collagens have been identified to date (Kadler, 2007). Among them, collagens that align themselves to make fibrils (fibrillar collagens) are typically found to be long, uninterrupted triple-helices. The most abundant of the fibrillar collagens is type I collagen, making up approximately 30% of protein in the entire human body. It is the primary structural protein in the body and is found largely in bones, skin, teeth and connective tissue. It serves as the scaffold for many extracellular matrix components.

Type I collagen is a triple-helical, heterotrimeric protein that consists of two α1 chains [α1(1)] and one α2 chain [α2(1)] synthesized in the rough endoplasmic reticulum. Each α-chain contains 338 repeating glycine-X-Y amino acid units to facilitate the tight winding of the three individual α-chains around each other. The repeating glycine-X-Y units are coded in such a way that a proline residue has a 30% frequency of being in position X and a hydroxylated proline residue has a 30% frequency of being in position Y. This amino acid composition is no accident. The small side chains of glycine residues and the rigid, angular structures of proline residues allows the α-chain to wind tightly into a left-handed helix conformation, positioning glycine residues as the protein's backbone. To form a triple-helical molecule, these glycine backbones line up against each other as the three α-chains wind together, and form a right-handed coiled-coil motif triple-helix. This triple helical conformation situates proline and

hydroxyproline residues in an outward facing position to allow their interaction with other prolines and hydroxyprolines on adjacent type I collagen triple-helical molecules. These interactions limit the rotation of the molecule by forming hydrogen bonds and water bridges to assist in type I collagen fiber formation. The resultant molecule has a molecular weight of about 300 kilodaltons.

During the synthesis of nascent type I collagen α-chains, many residues undergo extensive covalent modifications before the collagen is able to form a triple-helical molecule. Enzymes add hydroxyl groups to selected proline and lysine residues, and galactose and glucose molecules are added to several hydroxylysine groups. Once this occurs the immature α-chains are encapsulated in secretory vesicles and begin their transport to the outside of the cell. Within these secretory vesicles, the α-chains become disulfide-bonded together by cysteine residues in the carboxyl propeptide terminus with the help of molecular chaperones which initiate the winding of the α-chains into the triple-helix, starting from the carboxyl terminus and ending at the amino terminus. This newly formed, immature protein is referred to as a procollagen, having additional amino and carboxyl termini not present on mature collagen.

Folding of the type I collagen proα1(I) and proα2(I) chains has been shown to be mediated by a molecular chaperone HSP47, that also assists in folding of type II procollagen. HSP47 has been shown to be upregulated with type I collagen synthesis and localized with type I collagen procollagen in both the endoplasmic reticulum (ER) and the *cis*-Golgi apparatus where folding occurs. HSP47 has also been shown to bind the amino terminus of both α1(I) and α2(I) chains, and aids in preventing self aggregation of the procollagen molecules chains (Kojima, 1998; Makareeva & Leikin, 2007; Tasab &

Jenkinson, 2002; Thomson & Tenni, 2003). HSP47 begins its job during the synthesis of the α-chains in the ER and continues to chaperone as the α-chains are moved from the ER to the *cis*-Golgi apperatus. Once folding is complete, HSP47 releases the collagen molecule and returns to the endoplasmic reticulum, while the folded procollagen molecule continues its transport outside the cell.

The type I collagen molecule reaches maturity when it is transported outside of the cell and the propeptide ends are cleaved. Only these mature collagen molecules can be assembled to form collagen fibrils. Once outside the cell, N-proteinases and C-proteinases cleave the amino and carboxyl terminal ends respectively, converting the procollagen into mature collagen, with approximately 1014 amino acid residues in its triple-helical region. Lysyl oxidase assisted cross-link formation between hydroxylysine residues on mature collagen molecules and both hydroxylysine and lysine residues on adjacent collagen molecules to allow fibril formation. Fibril formation occurs in a quarter-staggered fashion in which the back end of one mature triple-helical molecule overlaps the front end of another by 67nm (the common distance D-period), stabilizing the formation by lysine bonds.

Type I collagen chains are largely homologous and have been estimated to be evolutionarily conserved for approximately 500 million years. Of the total type I collagen synthesized by the body, however, it also synthesizes up to 4% of an isotype of type I collagen in the skin (Uitto, 1979) referred to as homotrimeric type I collagen (Bornstein, 1980; Keilty, 1993; Kadler, 2007; Deak, 1983). The homotrimeric isotype is comprised of three α1 chains [$\alpha1(1)_3$] and is synthesized during wound healing (Haralson, 1987; Cohen, 1992), embryogenesis (Jimenez, 1977; Scriver, 1989) and in

tumors (Moro, 1977; Uitto, 1979; Rupard, 1988). Homotrimer synthesis has raised the question as to the role and significance of the α2-chain in a heterotrimeric type I collagen molecule. Slight abnormalities in the production of collagen molecules can lead to devastating effects in the body's structural integrity (Veis, 1989; Prockop, 1995; van der Rest, 1991; Byers, 1989). It has been shown that mutations in the genes that code for either of these α chains lead to a gamut of physiological impairments, such as Ehlers-Danlos Syndrome and Osteogenesis Imperfecta (Marini, 2007; Byers, 1989). However, a clear defined role for the α2-chain has not been identified. Mesangial cells in culture also synthesize homotrimer in what is postulated to be a wound response (Haralson, 1987).

Homotrimeric type I collagen is exclusively synthesized by the *Col1a2*-deficient mouse, also referred to as the osteogenesis imperfecta mouse model or *oim* (Chipman, 1993). The *Col1a2*-deficient mouse model has a mutation that results in a frame-shift within the carboxyl terminus of the proα2(I) chain altering the last 48 amino acids and disrupting signaling peptides required for association with α1(I) procollagen to form the heterotrimeric triple helix (Keilty, 1993; Chipman, 1993). The detrimental impact of this mutation resulting in homotrimer production on the skeletal framework of the *Col1a2*-deficient mouse is clearly evident by reduced bone mineral density and increased bone fragility (Carleton, 2008; McBride, 1998; Camacho, 1999).

The development of glomerulosclerosis in *Col1a2*-deficient mice appears to result from the exclusive expression of the homotrimeric isotype of type I collagen. Heterozygous *Col1a2*-deficient mice, which synthesize both heterotrimeric and homotrimeric isotypes also develop glomerulosclerosis. Interestingly, studies by

Haralson et al demonstrated that 95% of the collagen synthesized by cultured wildtype rat mesangial cells is type I collagen, and approximately half of the type I collagen was comprised of the homotrimeric isotype (Haralson, 1987). Haralson subsequently hypothesized that by culturing mesangial cells a wound healing mechanism had been induced that led to the synthesis of homotrimer and a sclerotic-like phenotype. These factors led us to examine what type I collagen isotype will be found accumulating in the sclerotic glomeruli of heterozygous mice that synthesize both normal and homotrimeric type I collagen in their bones skin and vasculature. Additionally, the expression of homotrimer in *Col1a2*-deficient glomeruli will be examined.

Matrix Metalloproteinases and Glomerulopathy

Investigations in hepatic sclerosis brought to light the role of MMPs as an important part of the fibrotic process (Okazaki & Maruyama, 1974). Since that time MMPs have been shown to be major factors in fibrosis. MMPs are proteases that cleave extracellular matrix components such as laminin, casein, collagen and fibronectin for subsequent degradation (Stamenkovic, 2003). Similar to type I collagen, they are synthesized in the endoplasmic reticulum and transported outside the cell for activation. MMPs consist of three main segments - the prodomain, catalytic and hemopexin domains which confer localization, activation and substrate specificity respectively (Bode, 1999).

The MMP prodomain includes a hydrophobic pre-domain, which acts as an anchor to the secretory vesicle membrane for its transport outside the cell after synthesis,

and a highly conserved sequence (PR**C**GVPD) that controls its activation. This conserved sequence lies between amino acids 71 and 77, and contains a key cysteine residue that forms a bond via its sulfhydryl group with a Zn^{2+} atom on the adjacent catalytic domain to keep the enzyme in an inactive (zymogen) form. For activation of the enzyme to occur, this cysteine-Zn^{2+} bond must be cleaved.

The catalytic domain, that controls the activity of the enzyme, is coordinated with a zinc ion that binds the aforementioned conserved cysteine residue on the adjacent prodomain. The catalytic domain coordinates a highly conserved histidine-rich sequence around a Zn^{2+} atom that is integral to its degradation activity. Three histidine residues in the conserved sequence (**H**ELG**H**XXGXX**H**) make three bonds with the zinc atom, while the cysteine residue on the prodomain makes a fourth bond, stabilizing the closed confirmation of the MMP molecule. Cleavage of this zinc-sulfhydryl bond by other MMPs and cytokines basally activates the MMP enzyme, and subsequent cleavage of the prodomain fully activates the MMP. Once cleaved the free position on the Zn^{2+} atom is replaced by a water molecule, a reaction generally seen in zinc proteases. To facilitate peptide bond cleavage during auto-catalysis, a glutamic acid residue within this sequence functions as a general base that displaces the water molecule by removal of a proton. By removing a proton from the water molecule, it allows the remaining OH- group to attack the carbonyl carbon of the scissile bond between the prodomain and the catalytic domain and subsequently activating it (Springmann, 1990; Milner, 2001; Galazka, 1996). The hemopexin domain, the final domain, is connected to the catalytic domain by a flexible linker region. This domain helps to confer MMP substrate specificity by positioning the activated molecule in the proper orientation for substrate

cleavage and also assists in local unwinding of various collagen substrates (Li, 1995; Chung, 2004; Tam, 2004; Xu, 2004).

Each of the over 27 different MMPs have different substrates, although many of them overlap. Renal collagenase MMPs, such as MMP-1 and -13, cleave within the collagen triple-helix in a ¼ : ¾ ratio between Gly 775 and Ile 776. Using the hemopexin domain, collagenases determine substrate specificity and initiate triple-helicase activity to locally unwind the collagen molecule and facilitate cleavage (Li, 1995; Murphy, 1992; Hirose, 1993). The cleaved collagen molecule is thermally unstable at body temperature and unravels to form gelatin (Hasty, 1990; Pardo, 2005). It has recently been shown by Chung, et al that MMP-1, and possibly MMP-8 and -13, preferentially interact and bind the proα2(I) collagen chain within heterotrimeric type I collagen molecule before subsequently cleaving the remaining proα1(I) chains (Chung, 2004; Perumal & Orgel, 2008).

Gelatinases -2 and -9 (MMP-2 and -9) primarily degrade individual collagen chains and gelatin. They have small active sites about 5 Å in diameter that are capable of cleaving individual collagen chains. Gelatinases are also able to cleave collagen in its triple-helical form. The collagen triple-helical molecule is approximately 15 Å in diameter, but because of its dynamic nature continually opening and closing, the small active sites of gelatinases are able to bind and cleave individual α-chains while they are still a part of the triple helix (Chung, 2004). Fibronectin type II modules on gelatinases help determine substrate specificity rather than using hemopexin domains as many other MMPs do (Xu, 2004). MMP-3 is a stromelysin that also cleaves gelatins, but is also needed for activation of other MMPs, such as MMP-1 (Visse, 2003; Saus, 1988).

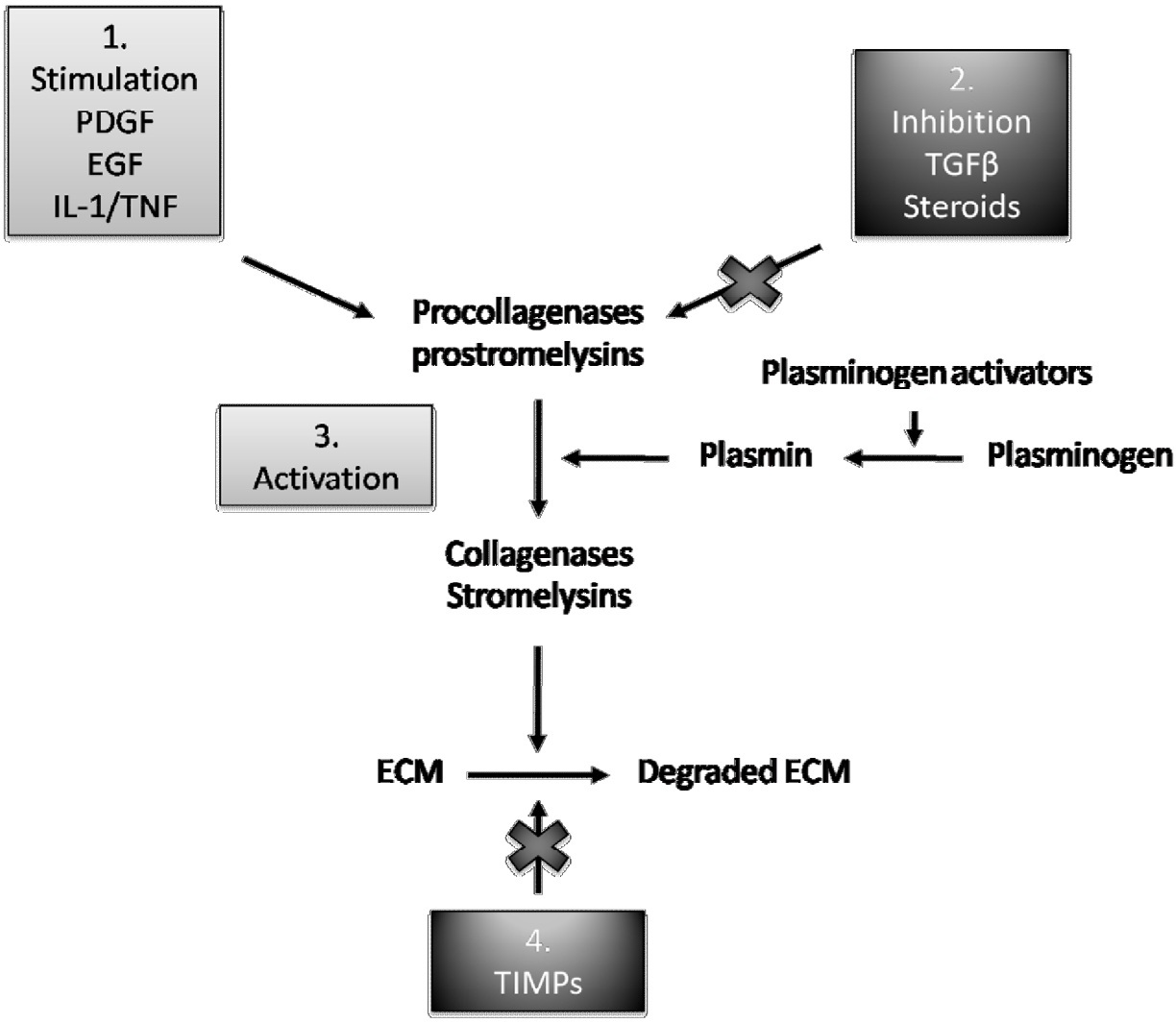


Figure I-3. Regulation of Matrix Metalloproteinases. Matrix metalloproteinases (MMPs) are regulated on many different levels. Transcription of MMPs are stimulated by the growth factors PDGF, EGF, TNF and IL-1, but can be inhibited by steroids and most notably the cytokine TGFβ. Latent forms of the proteinases (procollagenases/prostromelysins) can then be activated by other MMPs and plasminogen to their active forms (collagenases/stromelysins), which degrade ECM molecules. Active MMPs can be inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs).

MMPs are regulated by synthesis, but also by activation. The MMP genes are regulated by many different growth factors, hormones, and cytokines such as TGF-β, plasmin, urokinase-type plasminogen activator (uPA) and synthetic retinoids that induce transcription. MMP genes usually have several upstream promoters that increase their transcription rate when needed. Tissue inhibitors of matrix metalloproteinases (TIMPs) also regulate MMPs by inactivating both the zymogen and active forms of the enzyme by binding and forming complexes (Mott, 2004; Schaefer, 1997; Lechuga, 2004) (Figure I-3). There are four known TIMPs found *in vivo* (TIMPs 1-4), which each have an overlap in the inhibition of several MMPs (Woessner, 1991; Kopp, 1996; Uchio, 1999).

Matrix metalloproteinases (MMPs) are considered the ECM regulators throughout the body and are of keen interest in glomerulosclerotic disease (Norman, 1996; Shiozawa, 2000; Lelongt, 2002; Lemaitre, 2006). Human kidneys express MMPs -1, -2, -3, -9 and -13, among others, to patrol the ECM network and maintain matrix homeostasis. Mice synthesize a homolog to human MMP-1 that seems to be expressed only during embryogenesis and development, and MMP-3 and -13 are less well characterized in glomerulosclerotic disease. The most studied MMPs are the gelatinases MMP-2 and MMP-9 (Lelongt, 2001; Bolbrinker, 2006; Cheng, 2006; Rankin, 1999; Kuroda, 2004; Schaefer, 1997).

MMP-2 is constitutively expressed by both mesangial cells and glomerular epithelial cells (Turck, 1996; Davies, 1988). However, MMP-2 elevation during disease has been suggested to behave as a pro-inflammatory activator of mesangial cells causing them to proliferate and synthesize ECM components (Turck, 1997; Mertens, 1997). MMP-9 has been found to only be synthesized by glomerular epithelial cells, and unlike

MMP-2, has a restricted expression pattern during development and in adult tissues (McMillan, 1996; Yang, 2007; Ronco, 2007). Though less well studied in the kidneys, MMP-3, also known as stromelysin-1, is expressed in the kidney (Catania, 2007) and is an important activator of MMPs -2 and -9, and degrading many of the same substrates including gelatin and type I collagen (Chakraborti, 2003). Along with their common substrate preferences and glomerular localization, MMP-2, MMP-3 and MMP-9 have been shown to be strong players in fibrosis due to epithelial-mesenchymal transition (Lelongt, 2001; Turck, 1996; Orlichenko, 2008).

Several rat and mouse models that display type I collagen glomerulosclerosis show differences in MMP-expression. Bovine growth hormone (bGH) transgenic mice show an increase in MMP-2 mRNA synthesis (Doi, 1991; Carome, 1994). MMP-2 transgenic mice show over-expression of active MMP-2 in renal proximal tubular epithelial cells that lead to type I collagen deposition in tubulointerstitium and glomerular mesangium, as well as mesangial expansion and epithelial-to-mesenchymal cell transitions (Cheng, 2006). Streptozotocin-induced diabetic Sprague-Dawley rats show a decrease in MMP-2 protein levels but no change in MMP-9 protein levels as compared to non-diabetic rats after 17 weeks (Dixon, 2007). Renin-dependent hypertensive rats showed a 2-fold increase in MMP-2, but a 10% decrease in MMP-9 (Bolbrinker, 2006).

We will seek to examine the role of the expression of MMPs in *Col1a2*-deficient glomerulosclerosis and the degradative preference of renal MMPs for the heterotrimeric type I collagen isotype, or the homotrimeric type I collagen isotype.

Osteogenesis Imperfecta and the *Col1a2*-deficient Mouse Model

Osteogenesis imperfecta (OI) is a disease characterized by over 800 mutations in either COL1A1 and COL1A2 genes that encode for type I collagen α1(1) and α2(1) chains respectively. In 1979, the original classifications of the disease into 4 broad categories referred to as Types I-IV was made by Sillence, et al., based on its clinical manifestations (Sillence, 1978; Sillence, 1979). Since that time OI has been categorized into 7 general groups according to its phenotype, ranging from mild osteoporosis to perinatal lethality (Martin, 2007).

Mutations that lead to OI are frequently caused by glycine substitutions in type I collagen, but the disease severity is dependent on which α-chain the mutation occurs on, where the mutations occur, and what type of amino acid substitutions have been generated as a result of the mutation. For example, mutations that occur in regions of lethality of the triple-helical domain, where procollagen α-chains assemble with each other and between collagen molecules to form fibrils, usually exhibit a more severe phenotype compared to mutations in other regions of the molecule (Marini, 2007; Martin, 2007). OI Type III, which is of interest to this project, displays a severe phenotype and is normally a result of an inherited autosomal dominant mutation, characterized by severe, progressively deforming OI, scoliosis, and shortened stature.

In 1993, Chipman et al characterized the *Col1a2*-deficient mouse model that reflected the Type III OI genotype and phenotype similar to that found in a single patient, and characterized by phenotypic traits of reduced body size, skin laxity, long bone deformation and bone fragility (Chipman, 1993; Nicholls, 1984). The *Col1a2*-deficient mouse model (also referred to as *oim*) has a spontaneous guanidine base pair

deletion that has been mapped to position 3983 on both COL1A2 genes located on chromosome 6 of the mouse genome (Chipman, 1993). A 48 amino acid frame shift of the carboxyl terminal coding region on the COL1A2 gene results in a new stop codon and a one amino acid increase in the length of the coding region (Chipman, 1993). Once translated, the proα2(I) chain C-terminal signaling domain responsible for triple-helix assembly can no longer associate with the proα1(I) chains or incorporate themselves into the collagen triple-helix. The *Col1a2*-deficient mouse compensates by using a third proα1(I) chain in the triple-helix, creating a homotrimeric type I procollagen molecule [$\alpha1(1)_3$], and therefore can synthesize only this form of type I collagen. Proα2(I) mRNA synthesis and the complete absence of α2(I) chains in type I collagen fibrils has been shown both in patients and *Col1a2*-deficient mice (Chu, 1984; Deak, 1983; Malfait, 2006; McBride, 1997; Chipman, 1993). However, the fate of α2(I) chains is still unclear and has been hypothesized to be degraded shortly after translation.

Exclusive homotrimeric type I collagen synthesis is a rare cause of skeletal abnormalities, and can display a range of mild to severe phenotypes in both Ehlers-Danlos Syndrome (EDS) and OI patients (Nicholls, 1984; Schwarze & Byers, 2004; Hata, 1988; Sasaki, 1987). It has been hypothesized that if a mutation in the COL1A2 gene occurs closer to the amino terminus, mRNA non-sense mediated decay (NMD) occurs stopping the production of an aberrant protein and reducing the severity of the phenotype (Schwarze & Byers, 2004). However, if the aberrant proα2(I) chain is synthesized, a gain of function effect is hypothesized to disrupt the interaction with normal collagen chains that, in turn, lead to a more severe phenotype (Malfait, 2006).

The spontaneous mutation identified in the *Col1a2*-deficient mouse, occurring on exon 51 of the COL1A2 gene in the C-terminal, has been shown to be similar to that in a single human type III OI patient that has a 4 base pair mutation on exon 52, both synthesizing aberrant α2(I) chains and resulting in very similar phenotypes (Chipman, 1993). It is unlikely that NMD occurs in our mouse model because we have demonstrated the synthesis of COL1A2 steady state mRNA (Brodeur, 2007).

Heterozygous mice have one normal and one mutated copy of the COL1A2 gene. As a result, they synthesize approximately 50% normal proα2(I) chains that can successfully be incorporated into a collagen molecule and 50% aberrant proα2(1) chains that cannot incorporate themselves into the collagen triple helix (Pace, 2001; Pace, 2001), in a 1:1 ratio as seen in human patients (Gaiko-Galicka, 2002).

The *Col1a2*-deficient mouse exclusive synthesis of homotrimeric type I collagen ultimately leads to mesangial cell homotrimeric type I collagen deposition within the glomerulus and can be identified in the kidney as early as one week of age, a phenotype not studied in the human OI patient. These mice display a gene-dose dependent, non-inflammatory glomerulopathy that leads to glomerular filtration barrier disruption and podocyte foot effacement (Brodeur, 2007). Type I collagen deposition has been suggested to be the initial stage of renal fibrosis by Yoshioka, et al, however, the specific isotype, whether homotrimeric or heterotrimeric type I collagen, has not been determined (Yoshioka, 1990). Our laboratory has the unique opportunity to study the initiation of renal disease in the *Col1a2*-deficient mouse model, as well as to examine the role of proα2(I) collagen chains in the function of the kidney. The *Col1a2*-deficient mouse model provides a window to specifically study the progression of type I collagen

accumulation in sclerotic glomeruli without complications due to other confounding disease.

Hypotheses

In this body of work, our goal is to begin to elucidate the mechanism of *Col1a2*-deficient glomerulosclerosis by examining both the causes and progression of this disease. We put forth three hypotheses to be tested. First, we hypothesize that homotrimeric type I collagen is the pathogenic collagen in this mouse model, and will be reflected by the exclusive accumulation of homotrimer within heterozygous glomeruli. Next, we hypothesize that the accumulation of homotrimeric type I collagen within the *Col1a2*-deficient glomerulus is due to a combination of increased type I collagen expression and the inefficiency of renal MMPs to degrade homotrimeric type I collagen as compared to heterotrimer, resulting in an initial *in vivo* response where there is no increased expression of MMPs to clear away accumulating homotrimer. Finally, we test the hypothesis that the cytokine TGFβ has a role in the induction of homotrimeric type I collagen synthesis by mesangial cells.

CHAPTER II.

TYPE I COLLAGEN GLOMERULOPATHY: THE PATHOGENESIS OF HOMOTRIMER

Portions of this chapter have been submitted to the journal, Kidney International.

Type I Collagen Glomerulopathy: α1(I) Homotrimer and Matrix Metalloproteinase Expression

Anna M. Roberts-Pilgrim, PhD[1], Elena Makareeva, PhD[4], Juan Vera, PhD[4], Matthew H. Myles, DVM, PhD[3], Cynthia L. Besch-Williford, DVM, PhD[3], Sergey Leikin, PhD[4], Craig L. Franklin, DVM, PhD[3], Charlotte L. Phillips, PhD[1,2]

Departments of Biochemistry[1], Child Health[2], and Veterinary Pathobiology[3], University of Missouri, Columbia, Missouri 65212; and NICHD[4], National Institutes of Health, Bethesda, MD 20892.

INTRODUCTION

Historically, glomerulosclerosis has been attributed to an imbalance of collagen synthesis and its degradation (Eikmans M, 2003; Schnaper, 1995; Ahmed, 2007; Lenz, 2000; Ronco, 2007). Specifically, mesangial cells are held responsible for the excessive expression of collagen in the glomerular mesangium during disease progression (Glick, 1992; He, 2001; Sakatsume, 1995; Gibson, 1998; Striker, 1989). The matrix accumulation eventually leads to the disruption of the glomerular filtration barrier and podocyte (epithelial cells) foot effacement, leading to the development of proteinuria (Latta, 1992; Akhtar, 2004; Miner J. , 2003; Rennke, 1994; Kwoh, 2006). Often, as glomerular disease progresses, interstitial fibrosis will initiate between the tubules of the nephron. However, it remains unclear what are the initiating mechanisms involved in the glomerular extracellular matrix (ECM) deposition.

Matrix metalloproteinases (MMPs) are known regulators of the ECM and are of keen interest in glomerulosclerotic disease (Norman, 1996; Shiozawa, 2000; Lelongt, 2002; Lemaitre, 2006). Human kidneys express MMPs -1, -2, -3, -9 and -13, among others, to patrol the ECM network and maintain matrix homeostasis. Mice synthesize a homolog to human MMP-1 that seems to be only expressed during embryogenesis and development, and MMP-3 and -13 are less well characterized in glomerulosclerotic disease. The most studied MMPs in the kidney are the gelatinases MMP-2 and MMP-9 (Lelongt, 2001; Bolbrinker, 2006; Cheng, 2006; Rankin, 1999; Kuroda, 2004; Schaefer, 1997). A recent review by Catania, et al, presents the unpredictable nature of these MMPs in different models of glomerulosclerosis (Catania, 2007). MMP-2 and -9 have

been shown to be differentially regulated in several animal models; in the unilateral ureteral obstruction model MMP-2 is increased, but MMP-9 is decreased (Sharma, 1995; Iimura, 2004). Yet, the ICR-ICGN mouse model has lower activities of both MMP-2 and MMP-9 in affected glomeruli (Uchio, 2000), and MMP-2 is also down-regulated in the streptozocin (STZ)-induced diabetes rat model (Wu, 1997), but increased in Goto-Kakizaki rats, a model of type 2 diabetes, along with MMP-9 (Portik-Dobos, 2006).

The MMP-2 enzyme is constitutively expressed by both mesangial cells and glomerular epithelial cells (Turck, 1996; Davies, 1988). MMP-2 elevation during disease has been suggested to behave as a pro-inflammatory activator of mesangial cells causing them to proliferate and synthesize ECM components (Turck, 1997; Mertens, 1997), as well as possibly stimulating epithelial-mesenchymal-transition (EMT) within the glomerulus (Cheng, 2003). MMP-9, the 92kDa enzyme, has been found to only be synthesized by glomerular epithelial cells, and unlike MMP-2, has a restricted expression pattern during development and in adult tissues (McMillan, 1996; Yang, 2007; Ronco, 2007). Investigations of MMP-9 null mice suggest MMP-9 expression is not a major factor in glomerulosclerosis (Andrews, 2002), which contrasts the study by Lelongt et al that suggests a role for MMP-9 in phenotypic severity (Lelongt, 2001). Though less well studied in the renal system, MMP-3, known as stromelysin-1, is expressed in the kidney (Catania, 2007) and is an important activator of MMPs -2 and -9, and degrades many of the same substrates including gelatin and type I collagen (Chakraborti, 2003). Human renal chronic transplant nephropathy patients are reported to have higher serum levels of circulating MMP-3 (Rodrigo, 2000). Additionally, MMP-3 null mice were shown to have augmented lesions with atherosclerosis (Silence, 2001). Along with their common

substrate preferences and glomerular localization, MMP-2, MMP-3 and MMP-9 have been implicated as primary players in fibrosis due to epithelial-mesenchymal transition (Orlichenko, 2008).

Type I collagen, one of the major accumulating collagens in glomerulosclerosis, is predominantly found in tissues as a heterotrimeric isotype consisting of two α1(I) chains, and one α2(I) chain, [$\alpha1(I)_2$ α2(I)]. However the homotrimeric isotype of type I collagen consisting of three α1(I) chains, [$\alpha1(I)_3$] (Bornstein, 1980; Keilty, 1993; Kadler, 2007; Deak, 1983), is also synthesized by the body in very small amounts and is reported to compose up to 4% of normal human skin (Uitto, 1979). Homotrimer has also been shown to be present during embryogenesis (Jimenez, 1977; Scriver, 1989), in tumors (Moro, 1977; Uitto, 1979; Rupard, 1988) and at the site of healing wounds (Haralson, 1987; Cohen, 1992). Mesangial cells in culture also synthesize homotrimer in what is postulated to be a wound response (Haralson, 1987). Homotrimeric type I collagen is exclusively synthesized by the *Col1a2*-deficient mouse, also referred to as the osteogenesis imperfecta mouse model or *oim* (Chipman, 1993). The *Col1a2*-deficient mouse model has a mutation that results in a frame-shift within the carboxyl terminus of the α2(I) chain altering the last 48 amino acids and disrupting signaling peptides required for association with α1(I) procollagen to form the heterotrimeric triple helix (Keilty, 1993; Chipman, 1993). The detrimental impact of homotrimer production from this mutation on the skeletal framework of the *Col1a2*-deficient mouse is clearly evident by reduced bone mineral density and increased bone fragility (Carleton, 2008; McBride, 1998; Camacho, 1999). Less well characterized is the impact of homotrimeric expression on the kidney; the progressive glomerulosclerosis resulting from the postnatal

accumulation of homotrimeric type I collagen in the glomeruli that eventually leads to disruption of the glomerular filtration barrier, podocyte foot effacement and proteinuria (Brodeur, 2007; Phillips, 2002).

The development of glomerulosclerosis in *Col1a2*-deficient mice appears to result from the exclusive expression of the homotrimeric isotype of type I collagen. Heterozygous *Col1a2*-deficient mice, which can synthesize both heterotrimeric and homotrimeric isotypes also develop glomerulosclerosis. The mechanism responsible for initiation of the type I collagen deposition postnatally in heterozygous and homozygous *Col1a2*-deficient mice remains to be elucidated, as there is not an obvious primary insult (injury, inflammatory, etc.). Studies by Haralson et al demonstrated that cultured wildtype rat mesangial cell collagen synthesis was 95% type I collagen and that approximately half of this was comprised of the homotrimeric type I collagen isotype (Haralson, 1987). Haralson subsequently hypothesized that tissue culture conditions induce wound healing mechanisms resulting in the synthesis of homotrimeric type I collagen and a sclerotic phenotype. These findings and our own investigations in *Col1a2*-deficient mice beg the questions whether homotrimeric type I collagen is the pathogenic type I collagen present in glomerulosclerotic lesions and specifically whether homotrimeric type I collagen is the type I collagen present in heterozygous *Col1a2*-deficient mice glomeruli.

Heterozygous and homozygous *Col1a2*-deficient mice provide an unprecedented opportunity to examine the potential pathogenic role of the homotrimeric isotype of type I collagen in glomerulosclerotic disease without the confounding affects that frequently accompanies other disease states. They also provide an opportunity to explore the role of

MMP expression in glomerulosclerosis and whether alterations in MMPs have a role early or late in the disease. In the following study we investigated homo- and heterotrimeric type I collagen expression as well as MMP-2, MMP-3 and MMP-9 expression in wildtype, heterozygous and homozygous *Col1a2*-deficient mouse glomeruli and whole kidneys at both pre- and post-translational levels.

METHODS

Animals

Homozygous B6C3Fe *a/a- Col1a2*$^{oim/J}$ (*Col1a2*-deficient, -/-); heterozygous (-/+); and wildtype (+/+) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed in an AAALAC accredited animal facility, provided with water and food (Purina 5008 Formulab Diet; Purina Mills Inc., Richmond, IN) ad libitum, and cared for in accordance with an approved University of Missouri Animal Care and Use protocol. +/+, +/-, and -/- genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Phillips, 2000).

Animals were divided into two age groups, 1-month (n=85 mice) and 3-months (n=85) of age. Animals were sacrificed and either both kidneys were harvested whole or glomeruli harvested as described below, followed by snap freezing in liquid nitrogen, and stored at -80ºC. Harvested kidneys were fixed for 48 hours in either 10% phosphate buffered formalin for Picrosirius red staining or zinc chloride (0.05% calcium acetate, 0.5% zinc acetate and 0.5% zinc chloride dissolved in 0.1M Tris buffer and pH=7.0) for immunohistochemistry.

Glomerular Lesion Scoring

Longitudinal sections (5µm) of formalin-fixed kidneys embedded in paraffin and stained with Picrosirius red (PSR) stain were made into slides. Glomeruli within individual sections were evaluated blindly and a glomerular lesion score for each

(Figure II-1):

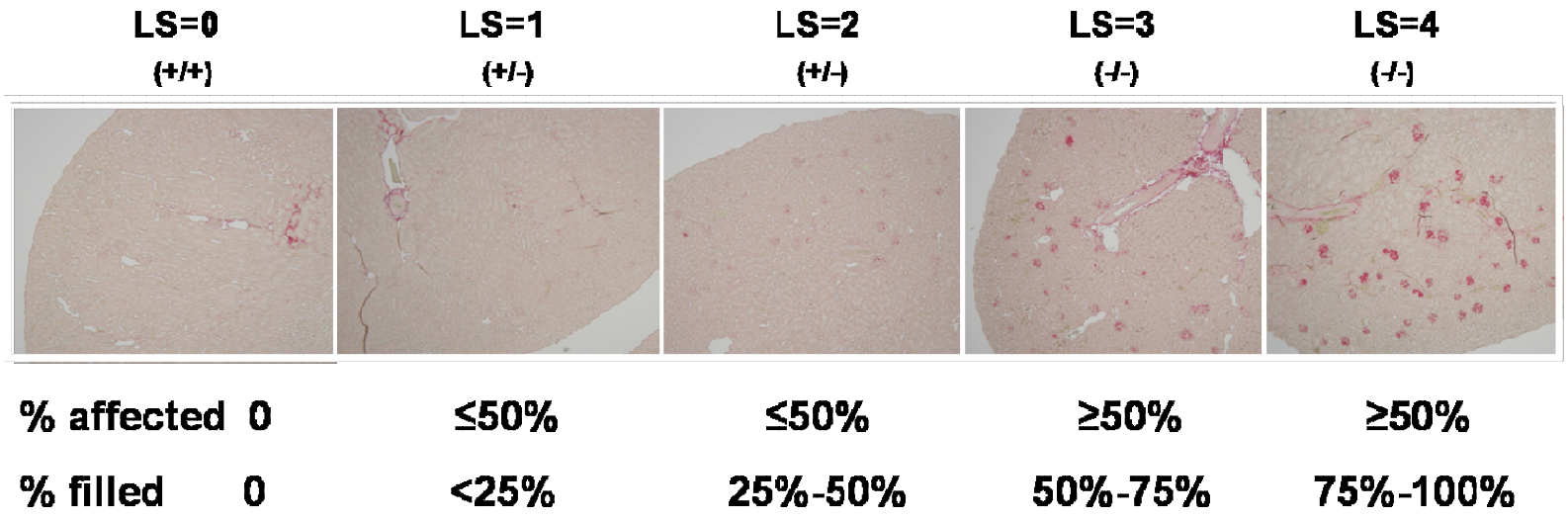


Figure II-1. Glomerular lesion scoring (LS). Picrosirius red staining of +/+ (wildtype), +/- (heterozygous), and -/- (*Col1a2*-deficient) whole kidney depicting the severity of collagen deposition. Lesion scoring (LS) refers to the severity of sclerosis seen in the kidney, 0 depicting no sclerosis, and 4 depicting the most severe sclerosis. The second line (% affected) describes the percentage of sclerotic glomeruli out of the total amount of glomeruli in a kidney section. The third line (% filled) refers to the relative amount of collagen in the average single affected glomerulus compared to an unaffected glomerulus.

LS 0 = Kidneys with no affected glomeruli.

LS 1 = Kidneys with ≤ 50% glomeruli affected, and with ≤ 25% accumulated collagen within each affected glomeruli.

LS 2 = Kidneys with ≤ 50% glomeruli affected, and with ≥ 25% but ≤ 50% accumulated collagen within each affected glomeruli.

LS 3 = Kidneys with ≥ 50% glomeruli affected, and with ≥ 50% but ≤ 75% accumulated collagen within each affected glomeruli.

LS 4 = Kidneys with ≥ 50% glomeruli affected, and with ≥ 75% or more accumulated collagen within each affected glomeruli.

Glomerular Number

PSR-stained kidney sections from 1-month and 3-month wildtype, heterozygous and *Col1a2*-deficient [-/-, n=8; +/+, n=8; and +/-, n=8] mice were also examined blindly to assess the average glomerular number within longitudinal sections. Longitudinal sections were assessed using four 200X fields that bracketed the renal cortex, from the juxtamedullary junction to the renal capsule. Each field was divided into juxtamedullary and cortical halves (zones). Within each zone, we determined the number of glomeruli and averaged the values to determine the mean glomerular number.

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. Upon confirmation of full anesthesia, the

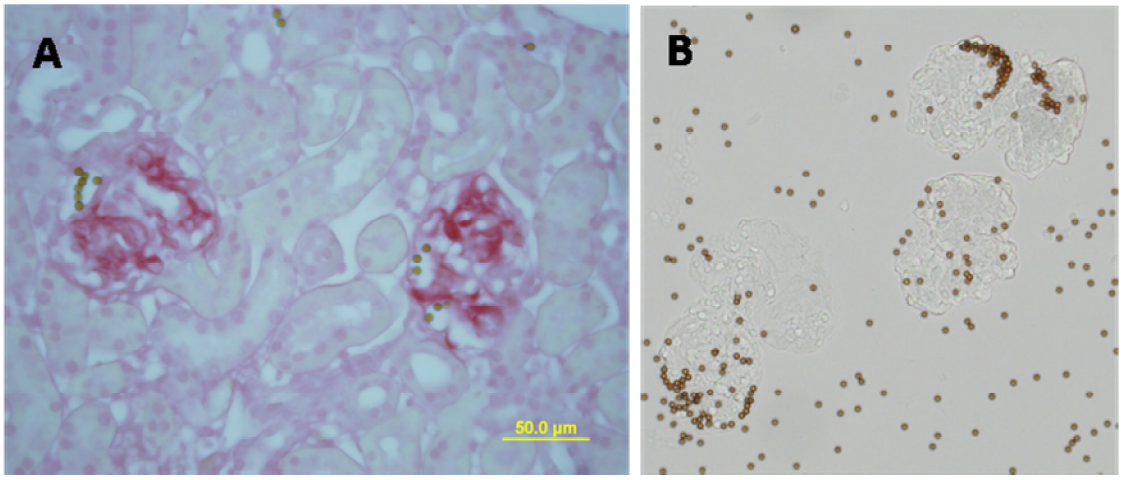


Figure II-2. Dynabead localization within glomeruli. Picrosirius red staining of whole kidney section showing perfusion of magnetic beads in sclerotic glomeruli (A), and into tubules. Aggregation of magnetic beads in the capillaries of isolated glomeruli after glomerular perfusion (B).

chest cavity was opened and vena cava cut directly above the left kidney. Perfusion of 30ml of a mixture of 1M phosphate buffered saline (PBS) and $8x10^7$ tosylactivated Dynabeads® magnetic beads (Invitrogen), deactivated prior to use according to the manufacturer's instructions, were perfused through the body via the right ventricle of the heart. Kidneys were removed and weighed, and a thin slice (about 2 mm) removed for lesion scoring.

Kidneys were minced into slurry using sterilized razor blades followed by thorough mixing with 4ml of digestion solution (1mg/ml collagenase A (Invitrogen Corporation, Carlsbad, CA), 100units/ml DNase (Invitrogen) and Hanks Balanced Salt Solution (HBSS) (GibCo-Invitrogen Corporation, Carlsbad, CA) and incubated at 37˚C for 30 minutes. The digested slurry was then sieved using two sequential disposable 100 micron cell strainers (BD Bioscience, San Jose, CA) with the addition of HBSS and a sterilized glass pestle, followed by centrifugation at 1500 rpm for 15 minutes. The kidney pellet was resuspended in 2ml of HBSS, transferred to a 1.5ml eppendorf tube, and placed onto a magnetic particle concentrator for 1 minute to allow glomeruli containing magnetic beads to be drawn to the sides of tube. Supernatant was removed and remaining tissue was resuspended in 1 ml of cold HBSS. Washing of glomeruli with HBSS was repeated 5 times to remove unwanted tubules and kidney debris. Remaining glomeruli were resuspended in 120ul of HBSS and assessed for purity and yield using a hemocytometer, followed by snap-freezing and stored at -80˚C until further use (modification of protocol by Takemoto, et al (Takemoto, 2002)) (Figure II-2).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Snap frozen glomeruli from 1-month [n=6 -/- (lesion score LS3-4), n=6 +/+ (lesion score LS0), and n=6 +/- (lesion score LS1-4)] and 3-month [n=7 -/- (lesion score LS1-4), n=6 +/+ (lesion score LS0), and n=6 +/- (lesion score LS1)] old mice were homogenized in TRIzol Reagent (Invitrogen) using a TissueLyser homogenizer (QIAGEN, Valencia, CA), or RNeasy Kit (QIAGEN, Valencia, CA) and total RNA isolated according to manufacturer's protocol. Total glomerular RNA was translated into cDNA following the manufacturer's protocol (Superscript First Strand Synthesis or VILO, Invitrogen).

PCR primer sequences were developed with the help of Matthew H. Myles, DVM, PhD, (University of Missouri-Columbia) using primer sequence program DSGene (Accelrys Inc, San Diego, CA). Each primer set chosen had to fulfill several requirements (span an intron-exon region on applicable gene, GC content less than 50%, and annealing temperatures between 50˚C-60˚C) before being used in subsequent experiments. Final primer sequences used are found in Table II-1.

Standard curve was generated by PCR amplification of the region of interest using primer sets developed above. PCR product was then cloned into TOPO vector (TOPO cloning kit, Invitrogen) following the manufacturers instructions, and transfected into E.coli cells and grown overnight. Cultured cells were then harvested and TOPO plasmid vector containing the amplified plasmid was extracted using the Fermentas Mini Wizard Plasmid Extraction Kit (Fermentas, Glen Burnie, MD). Extracted plasmids quantified using the Nanodrop Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE) and linearized by E.coR1 digestion. Linearized plasmids were re-quantified and subsequently diluted from $1x10^{8}$ copies to $1x10^{-1}$ copies by factors of ten. Standard curve

Table II-1. Primer sequences used for quantitative real time-PCR analysis

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’ – CAC CCT TGT TAC CGG ATT CTC CTT – 3’	236bp
COL1A2 reverse	5’ – TGA AGT GGG TCT TCC AGG TCT TTC – 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’	

amplification was verified by real-time PCR using relevant primers and product size was verified by SDS-PAGE electrophoresis.

Previously prepared glomerular cDNA from test subjects were diluted to 20ug/ml and mixed with SYBR®-green (Roche Diagnostics, Indianapolis, IN), nuclease free water, magnesium chloride, and the relevant primer sequences in a 20ul reaction and pipette into a 20ul glass capillaries and evaluated by Lightcycler 2.0 (Roche Diagnostics, Indianapolis, IN).

Primer sequences for hypoxanthine-guanine phosphoribosyltransferase (HPRT) have been previously reported (O'Garra, 1992). All values were normalized to HPRT levels.

SearchLight Protein Assay

Snap frozen glomeruli from 1-month [n=11 -/-, and n=9 +/+] and 3-month [n=10 -/-, and n=10 +/+] wildtype and *Col1a2*-deficient mice were thawed on ice and protease activity was inhibited by the addition of phenylmethyl sulfonyl fluoride (PMSF) to a final concentration of 2mM. Glomeruli were sonicated in an ice waterbath for 10 minutes to disrupt the glomerular unit. Disrupted glomeruli were incubated for 1 hour at room temperature with agitation (200rpm) to allow antigen-protein binding to piezoelectrically adhered MMP-2, -3, and -9 antigens in a 96-well plate format followed by washing for removal of unbound proteins. Biotinylated detection antibodies also specific for MMP-2, -3, and -9 detection were added to the 96-well plate and incubated for 30min, followed by a 30 min incubation with strepdavidin horse radish peroxidase conjugate and finally SuperSignal ELISA Femto Chemiluminescence substrate to amplify the antibody signal.

The chemiluminescent protein signal was then imaged and quantitated by Thermo Scientific SearchLight multiplex assay (Pierce Scientific, Rockford, IL) according to the manufacturer's protocols.

Immunohistochemistry for α1(I) and α2(I) collagen

To determine whether homotrimeric or heterotrimeric isotype of type I collagen is accumulating in the mesangial space, the presence of α1(I) and α2(I) were evaluated by immunohistochemistry of paraffin-embedded sections. 20 minute heat-induced epitope retrieval in 1X target retrieval solution (TRS) (Dako, Carpinterea, CA) was performed in a vegetable steamer at 95˚C. Endogenous biotin was quenched by an avidin/biotin block followed by removal of endogenous peroxidase by treating slides with 3% hydrogen peroxide, and non-specific antibody binding was blocked with a 5% bovine serum albumin (BSA) solution. Immunohistochemistry was performed using rabbit polyclonal anti-α1(I) primary antibody diluted 1:600 or rabbit polyclonal anti-α2(I) (generated in collaboration with Dr. Rolf Brekken, University of Texas-Southwestern) diluted 1:3000, a biotinylated swine anti-rabbit secondary antibody diluted 1:300 (Dako), followed by a strepavidin horseradish peroxidase conjugate (Dako), and 3,3' diaminobenzidine tetrahydrochloride (DAB) substrate followed by hematoxylin counterstain. Staining was performed on a Dako Autostainer Universal Staining System. Anti-α1(I) (data not shown) and anti-α2(I) antibody specificity was confirmed by western blot analysis of mouse tail tendon (Figure II-3).

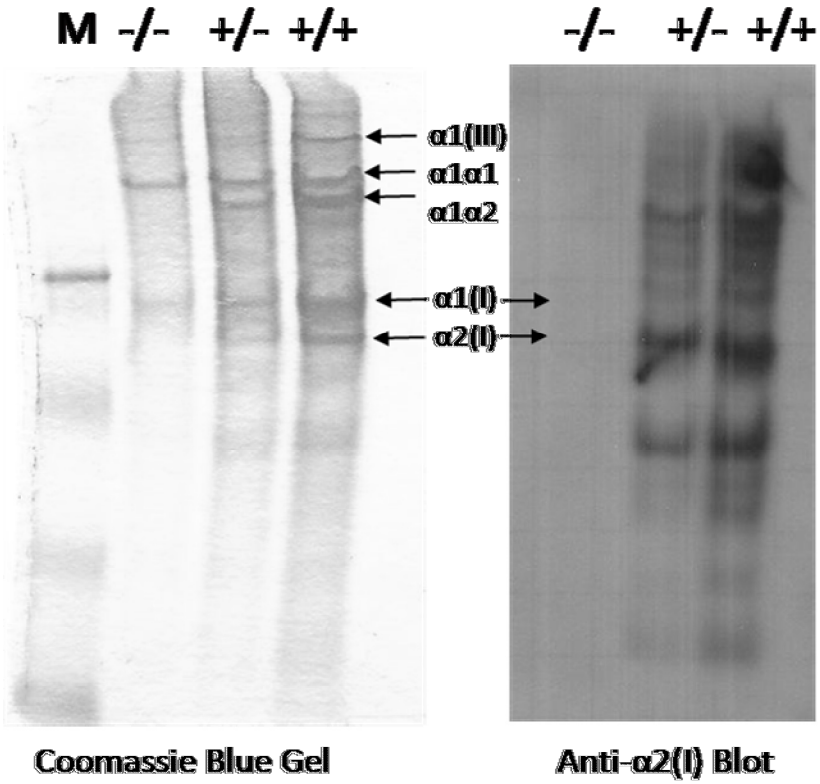


Figure II-3. Anti-α2(I) western blot analysis. Western blot analysis of type I collagen extracted from *Col1a2*-deficient (-/-), heterozygous (+/-), and wildtype (-/-) mice tails demonstrate that the anti-α2(I) collagen antibody is specific for α2(I) type I collagen chains. Bands also denote type I collagen α1α1 and α1α2 proforms and type III collagen (α1(III)) in the heterozygous and wildtype tail collagen.

Biochemical analysis of collagen composition in glomeruli and kidney

One half of a kidney from each mouse was homogenized in 40 µl of 0.5 M acetic acid, 0.5 % Brij 35 (1 min in a Branson Sonifier S-430 with a cup horn bath sonicator adapter filled with water mixed with ice). Homogenized samples were treated overnight with 0.5 mg/ml pepsin (EMD Biosciences, Darmstadt, Germany) at 4^{o}C. After removal of insoluble material by centrifugation, collagen was precipitated with 1M NaCl.

Glomerular preparations were thawed, precipitated by centrifugation, re-suspended in 0.5 M acetic acid, 0.5% Brij 35, homogenized and treated with pepsin as described above. However, virtually all collagen was found to be bound to the magnetic beads, with which the glomeruli were purified. The beads were, therefore, precipitated by centrifugation and washed with 70% ethanol, to remove residual acetic acid.

Samples of beads with bound collagen or collagen pellets from kidneys were resuspended in 10-30 µl 0.1 M Na-carbonate, 0.5 M NaCl, pH 9.3 and labeled with mono-reactive Cy5 (GE Healthcare, Piscataway, NJ) as previously described (Makareeva, 2006). Small sample aliquots were mixed with 4x LDS sample buffer (Invitrogen), denatured for 5 min at 90^{o}C and analyzed on precast 3-8% Tris-acetate mini gels (Invitrogen). Selected bands were excised from the gel, treated with CNBr (Sigma, St. Louis, MO) as described (Makareeva, 2006) and re-analyzed on precast 12% Tris-glycine gels (Invitrogen). All gels were scanned on an FLA5000 fluorescent scanner (FUJI Medical Systems, Stamford, CT). Analysis of band intensities was performed by ScienceLab software supplied with the scanner. Collagen content in glomeruli was estimated by comparing intensities of the α1(I) bands from glomerular preparations and from a standard with known collagen concentration.

Statistics

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). The RT-PCR and protein data was analyzed as a completely randomized design in which genotype and age were arranged as a 3 by 2 factorial (3 genotypes, 2 ages). Data presented are the actual mean and standard error but the differences within genotype and age were analyzed using the log or rank of the mean values.

Mean differences were ascertained using Bartlett's Homogeneity Test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Glomerular Yields in *Col1a2*-deficient Mice

To investigate whether the type I collagen glomerulosclerosis in the *Col1a2*-deficient mouse exhibits altered glomerular matrix metalloproteinase expression as seen in other glomerulosclerotic diseases (Uchio, 2000; Harendza, 1999; Carome, 1994), and to characterize the expression of both the COL1A1 and COL1A2 genes, we isolated glomeruli and whole kidneys from one and three month wildtype, heterozygous and homozygous *Col1a2*-deficient mice (Brodeur, 2007).

The one and three month old homozygous *Col1a2*-deficient mice had total body weights approximately 30% [$p \leq 0.005$] less than their wildtype littermates [12.79 ± 0.62g and 18.15 ± 0.54g (mean ± S.E.M.) respectively], and their kidneys weighed approximately 20% less than age-matched wildtype mice [*Col1a2*-deficient kidney, 0.225 ± 0.011g and wildtype kidney, 0.279 ± 0.010g, $p \leq 0.005$] (Table II-2). However, when kidney weights were normalized to the total body weight of the animal, only the *Col1a2*-deficient mice at one month of age showed a significant 13% increase in their kidney weight/ body weight ratio [*Col1a2*-deficient mice, 0.0178 ± 0.0006 and wildtype mice, 0.0155 ± 0.0004 respectively, $p \leq 0.005$].

Glomeruli were isolated from wildtype, heterozygous and homozygous *Col1a2*-deficient mice by perfusion with paramagnetic polystyrene Dynabeads® (approximately 4.5um in diameter; about half the size of a normal red blood cell), which become lodged in the glomerular capillaries. This allows for rapid purification of glomeruli that is greater than 98% glomeruli (Takemoto, 2002). Total glomerular yield from perfused kidneys were similar in mice of all genotypes at one month of age, but at three months of age

Table II-2. Wildtype (+/+), heterozygous (+/-), and *Col1a2*-deficient (-/-) perfusion data (mean ± S.E.M.)

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

* $p \leq 0.005$ compared to age-matched wildtype; ** $p \leq 0.005$ compared to same genotype at 1 month of age

glomeruli isolated from *Col1a2*-deficient mouse kidneys were significantly less [900 ± 122 total glomerular number (mean ± S.E.M.)] as compared to age matched wildtype mice and heterozygous mice [4679 ± 805 and 5398 ± 1080, respectively, $p \leq 0.005$]. To determine whether the reduction in glomerular yield in three month *Col1a2*-deficient kidneys was due to a reduction in glomerular number or represented difficulty in perfusing the sclerotic glomeruli due potentially to narrowing of the glomerular capillaries as a result of collagen deposition and mesangial expansion we quantitated the glomerular number in wildtype, heterozygous, and COL1A2 deficient mouse kidney PSR-stained histological sections. Surprisingly, one month *Col1a2*-deficient kidneys appeared to have a significant increase in their average amount of glomeruli in the combined juxtamedullary and cortical regions as compared to both the wildtype and heterozygous kidneys [5.31 ± 0.70; 3.58 ± 0.46 and 3.22 ± 0.34 and glomeruli / field (mean ± S.E.M.) respectively, $p \leq 0.05$], as well as in three month animals [4.53 ± 0.51; 3.33 ± 0.37 and 3.28 ± 0.37 glomeruli / field (mean ± S.E.M.) respectively, $p = 0.06$], though not quite significant (Table II-3).

Homotrimeric Collagen Is Accumulating in Heterozygous *Col1a2*-deficient Glomeruli

Col1a2-deficient mice are hypothesized to synthesize non-functional α2(I) chains whose altered carboxy-terminal propeptide prevents incorporation into a heterotrimeric type I collagen helix and hence *Col1a2*-deficient mice can synthesize only homotrimeric type I collagen. Alternatively, heterozygous mice can synthesize both isotypes of type I collagen (McBride, 1997). In this study we asked whether the accumulating type I

Table II-3. Average number of glomeruli per field in kidney cortex and juxtamedullary regions (mean ± S.E.M.)

Genotype (N)	Age (mo)	Avg No. Cortical Glomeruli	Avg No. Juxtamedullary Glomeruli	Avg Total Glomeruli
+ / + (8)	1	5.09 ± 0.43	2.06 ± 0.32	3.58 ± 0.46
+ / - (11)	1	4.16 ± 0.50	2.28 ± 0.21†	3.22 ± 0.34
- / - (8)	1	7.00 ± 0.92‡	3.63 ± 0.65‡	5.31 ± 0.70‡
+ / + (8)	3	4.28 ± 0.47	2.38 ± 0.31	3.33 ± 0.37
+ / - (8)	3	4.34 ± 0.43	2.22 ± 0.26	3.28 ± 0.37†
- / - (8)	3	5.34 ± 0.76	3.71 ± 0.58‡	4.53 ± 0.51

† $p \leq 0.05$ compared to age-matched *Col1a2*-deficient (-/-); ‡ $p \leq 0.05$ compared to age-matched wildtype (+/+)

collagen in the heterozygous glomeruli was either heterotrimer, homotrimer, or both. Towards this goal we evaluated wildtype, heterozygous and *Col1a2*-deficient kidneys qualitatively by immunohistochemistry and quantitated the levels of homotrimeric and heterotrimeric type I collagen from whole kidneys and isolated glomeruli.

By immunohistochemistry (IHC) using anti-α1(I) antibodies we found the pattern of α1(I) positive staining in heterozygous sclerotic glomeruli was similar to that seen in homozygous *Col1a2*-deficient mouse kidneys, although not as severe (Figure II-4). The α1(I) positive staining showed a diffuse pattern of accumulation within glomeruli in a manner similar to what is seen by picrosius red fibrillar collagen staining of heterozygous (+/-) and *Col1a2*-deficient (-/-) kidneys (Figure II-4D, E, G, H). Localization of α1(I) was also demonstrated in the vasculature of the heterozygous and *Col1a2*-deficient kidney where α1(I) positive staining is normally expressed in the wildtype kidney. Wildtype mouse kidneys show no α1(I) positive staining within glomeruli, but it is present in the renal vasculature (Figure II-4A, B). The pattern of glomerular α2(I) positive staining in wildtype and heterozygous mice are also in the renal vasculature and proximal tubule epithelium, with no anti- α2(I) staining evident in the glomeruli of wildtype kidney (Figure II-4C). However, heterozygous and homozygous *Col1a2*-deficient mice show similar patterns of positive α2(I) staining in small isolated aggregates spotted randomly within the affected *Col1a2*-deficient and heterozygous glomeruli (Figure II-4F, I). To determine whether these aggregates are artifacts of the experimental technique, or truly contain α2(I) chains, electron microscopy will have to be used.

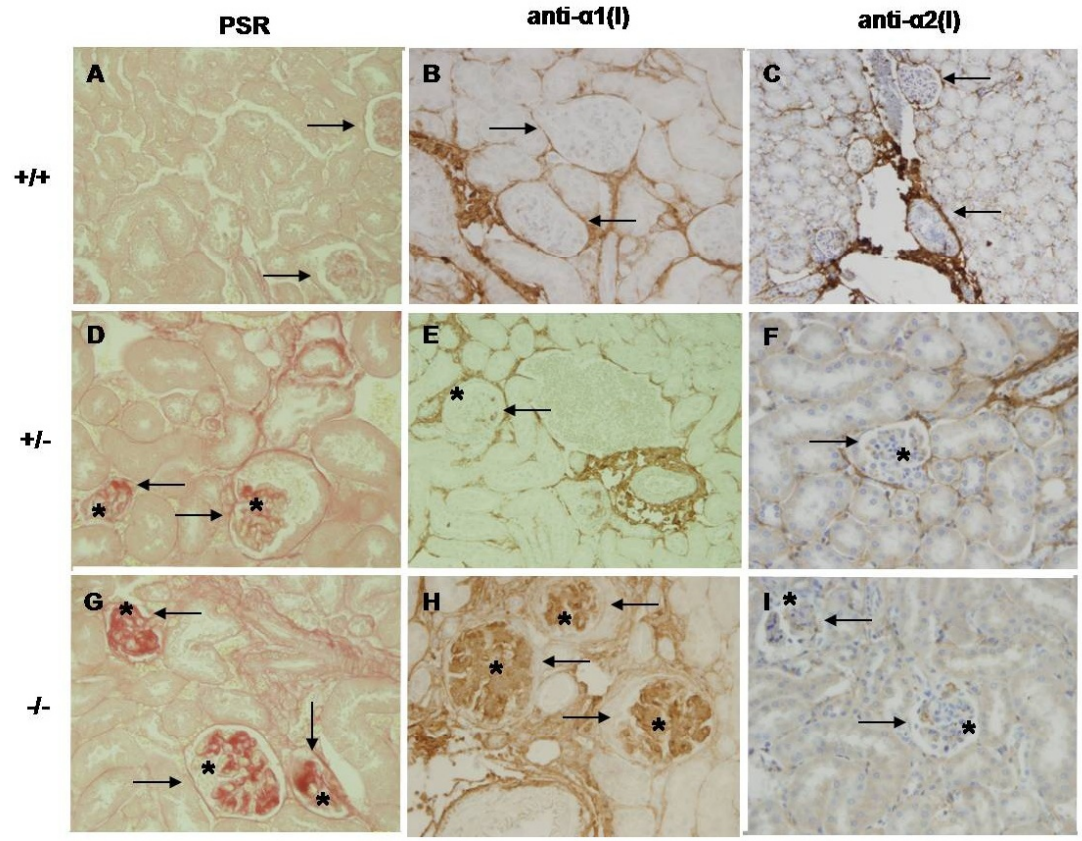
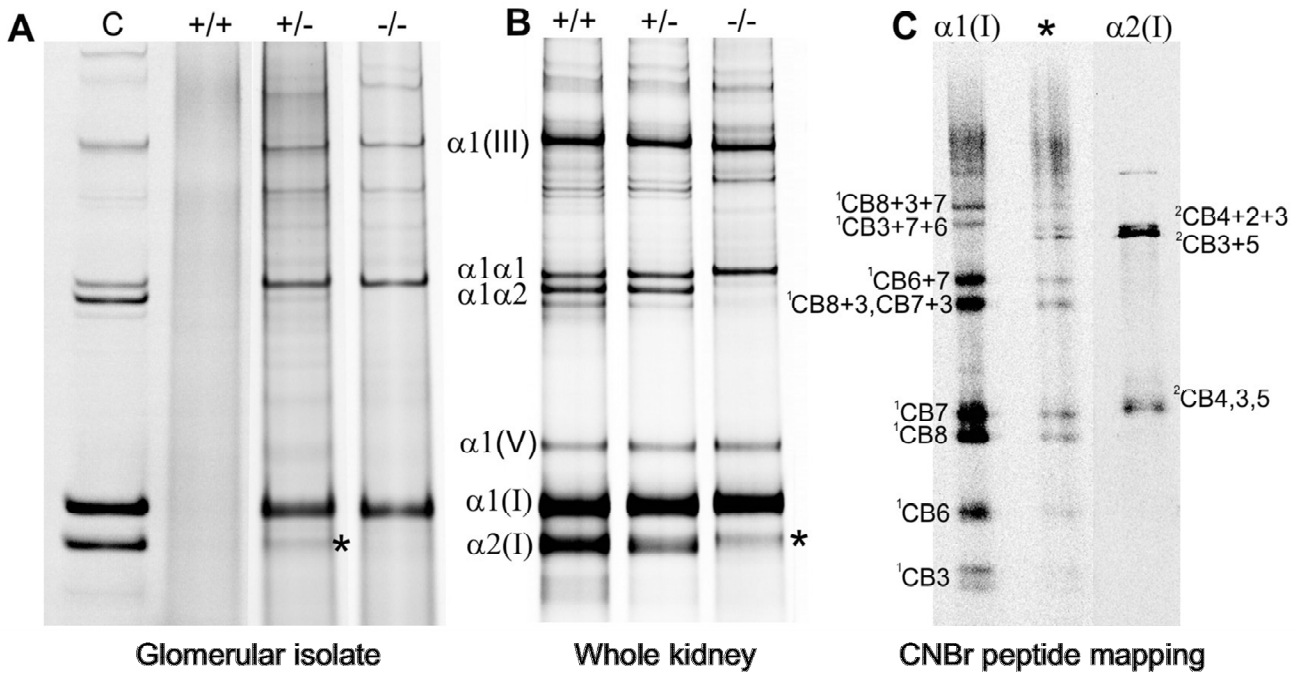


Figure II-4. Identification of type I collagen homotrimer as pathogenic collagen in heterozygous glomeruli. Picrosirius red staining of +/+ (wildtype, A) glomeruli show no deposition of fibrillar collagen, +/- (heterozygous, D) glomeruli show mild deposition of fibrillar collagen, and -/- (*Col1a2*-deficient, G) glomeruli show severe deposition of fibrillar collagen within glomeruli. By IHC, +/+ (wildtype, B-C) mice have no type I collagen α1(I) and α2(I) positive staining within glomeruli. +/- (heterozygous, E-F) mice show type I collagen α1(I) positive staining within the glomeruli and α2(I) positive staining in small aggregated spots within glomeruli. -/- (*Col1a2*-deficient, H-I) mice also show evidence of type I collagen α1(I) and α2(I) positive staining within glomeruli. α2(I)

Figure II-4 cont'd..positive staining is confined to small aggregated spots. Arrows indicate glomeruli. Asterisks denote affected (PSR+) glomeruli, 40X magnification.

The composition of the purified fibrillar collagens from whole wildtype, heterozygous and *Col1a2*-deficient kidneys was ~80 % type I, 12-16% type III and ~ 5% type V collagens (Pace, 2008). Only heterotrimeric type I collagen was present in wildtype animals, only homotrimeric type I collagen was present in *Col1a2*-deficient homozygous animals, and approximately equal amounts of both homotrimeric and heterotrimeric type I collagen were found in heterozygous animals (Figure II-5B).

Type I collagen was virtually undetectable in glomeruli from 3 month old wild type mice (0.2-0.5 pg collagen per glomerulus). In contrast, we found over 100 pg collagen per glomerulus in a homozygous *Col1a2*-deficient mouse of the same age and more careful 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 II-5A). Separation of collagen species isolated from kidneys of homozygous *Col1a2*-deficient animals and glomeruli from some heterozygous animals by SDS-PAGE demonstrated a faint band labeled with *, migrating close to the expected position of the α2(I) chain (FigureII-5A, B). CNBr peptide patterns (Figure II-5C) from the * band of the homozygous *Col1a2*-deficient 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 (Kuznetsova, 2003). 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 α2(I)-CB3, α2(I)-CB4, and α2(I)-CB5 as well as co-migrating partial CNBr cleavage products α2(I)-CB3+5 and α2(I)-CB4+2+3 (Figure II-4C). The first group



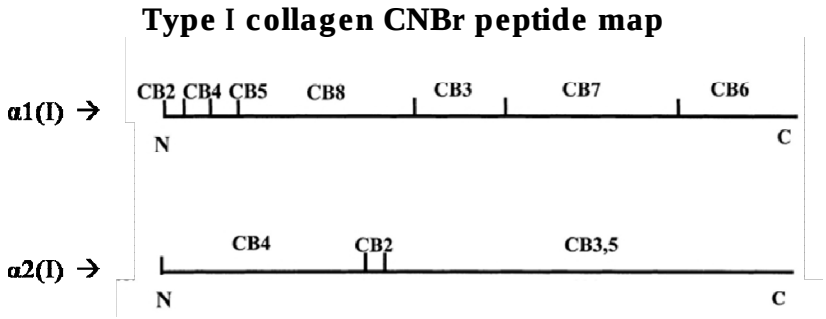


Figure II-5. Analysis of collagen composition in glomeruli (A) and kidney (B) from wild type (+/+), heterozygous (+/-) and homozygous (-/-) *Col1a2*-deficient animals. A faint band labeled with *, migrating close to the expected position of the α2(I) chain, was observed in kidneys from homozygous *Col1a2*-deficient animals and glomeruli from some heterozygous animals. 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)

Figure II-5 cont'd. homotrimers is often observed in *Col1a2*-deficient tissues (Kuznetsova, 2003). 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. CNBr type I collagen cleavage map shows the expected type I collagen cleavage products for CNBr peptide mapping.

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 α1(I)-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 (Figure II-5A, B), 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.

COL1A1 Transcript Elevation in *Col1a2*-deficient Mice

Brodeur, et al, demonstrated a 2-fold increase in steady-state COL1A1 mRNA transcripts in the whole kidney from one month old *Col1a2*-deficient mice (Brodeur, 2007). To determine whether this increase occurred specifically in the glomeruli, the focal point of this disease, we examined isolated glomeruli from one and three month wildtype heterozygous and *Col1a2*-deficient animals for steady-state expression of COL1A1 and COL1A2 mRNA transcripts and found that COL1A1 transcripts were elevated 3-fold in one month [$p \leq 0.003$] and 15-fold in three month old [$p \leq 0.0001$] *Col1a2*-deficient mice as compared to age-matched wildtype mice (Figure II-6A).

Although *Col1a2*-deficient mice do not appear to 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 (Orlichenko, 2008; McBride, 1998; Chu, 1984). In one month *Col1a2*-deficient mouse glomeruli, there is a 22% reduction, though not significant, [$p \leq 0.08$] and a significant

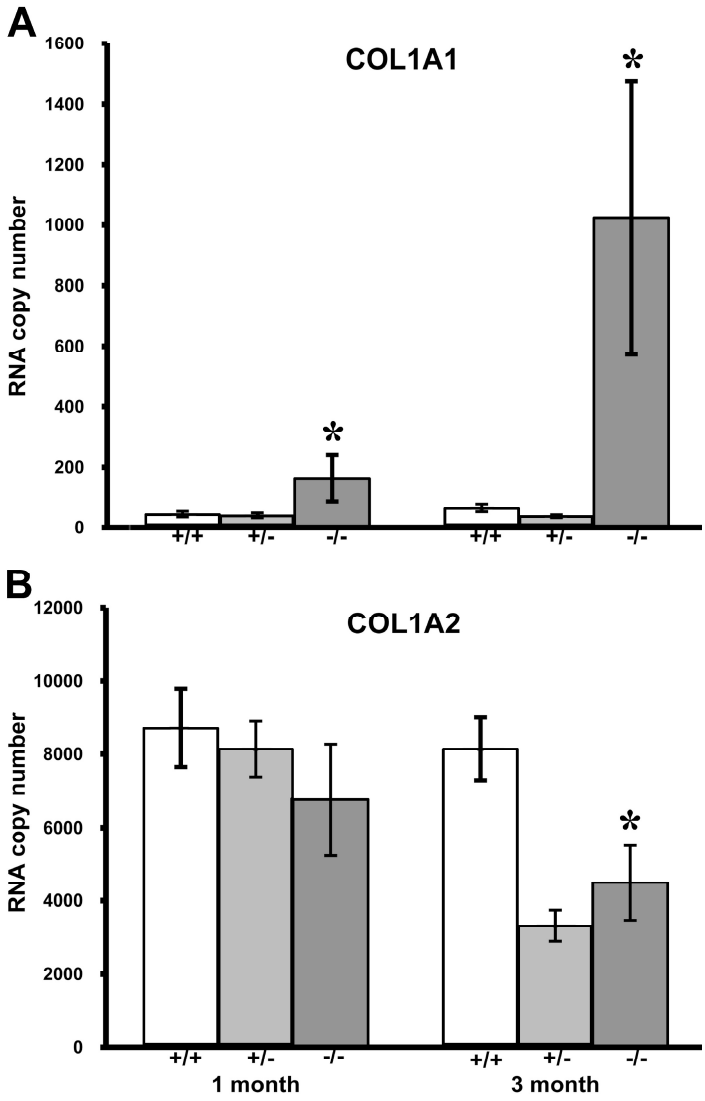


Figure II-6. Quantitative RT-PCR of steady state mRNA expression of COL1A1 (top) and COL1A2 (bottom) mRNA. Quantitative RT-PCR confirms increases in proα1(I) collagen mRNA copy number is occurring within glomeruli in 1-month and 3-month *Col1a2*-deficient (-/-) animals as compared to age-matched heterozygous (+/-) and wildtype (+/+) animals (*$p < 0.05$). 1-month and 3-month *Col1a2*-deficient (-/-) mice and heterozygous (+/-) mice demonstrate a decrease in proα2(I) collagen mRNA copy number with age.

45% reduction at three months of age [$p \leq 0.003$] in COL1A2 steady state mRNA levels as compared to age-matched wildtype mouse glomeruli (Figure II-6B).

Interestingly, heterozygous mouse glomeruli at three months of age exhibit a reduction of 42% in COL1A1 transcripts [$p \leq 0.2$], though not significant, and a significant reduction in COL1A2 transcripts by almost 60% a [$p \leq 0.0001$] as compared to age-matched wildtype mouse glomeruli (Figure II-6A, B).

Sustained Elevation of MMP-2 mRNA and Protein with Time in the *Col1a2*-deficient Mice

The gelatinase MMP-2 is one of the major degradative enzymes in the body and has been shown to have altered expression in glomerular sclerotic diseases. At one month of age glomerular MMP-2 steady-state mRNA levels were similar between *Col1a2*-deficient and wildtype glomeruli [$p \leq 0.2$]. However, by three months of age, wildtype glomerular MMP-2 mRNA levels decreased by 57% as compared to one month levels, but *Col1a2*-deficient glomerular MMP-2 mRNA levels remained elevated with values similar to one month levels [$p \leq 0.0004$]. Heterozygous mice followed a similar expression pattern displayed by wildtype mice with a 48% decrease in MMP-2 transcript synthesis by three months of age, although not significant (Figure II-7).

Examination of combined latent and active forms of the MMP-2 enzyme by protein array analysis demonstrate a significant 3-fold and 2-fold elevation of the mean MMP-2 protein concentration in *Col1a2*-deficient glomeruli at one month and three months of age [0.111 ± 0.015 and 0.110 ± 0.017 pg/glomerulus, respectively] relative to

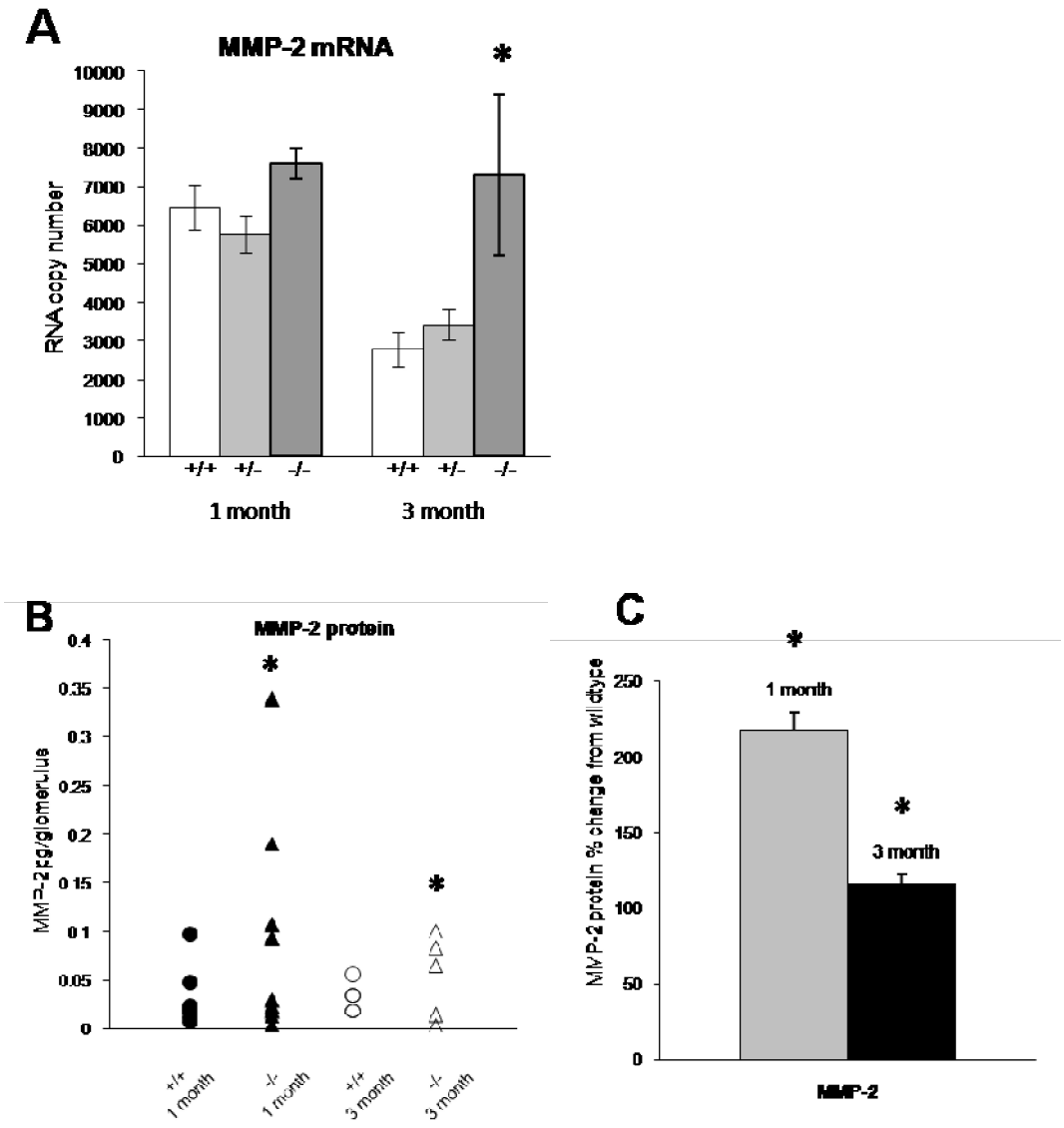


Figure II-7. MMP-2 mRNA and protein expression. Quantitative RT-PCR demonstrates a significant increase in MMP-2 (A) mRNA expression in 3-month *Col1a2*-deficient (-/-; dark grey bar) glomeruli compared to age-matched heterozygous (+/-; light grey bar) and wildtype (+/+; white bar) animals (*p <0.05). 1-month and 3-month *Col1a2*-deficient (-/-) mice show a significant increase in MMP-2 protein levels, with higher levels of MMP-2 protein in 1 month *Col1a2*-deficient glomeruli as compared to 3

Figure II-7 cont'd. month *Col1a2*-deficient glomeruli (*p ≤0.05) (B and C). (B) shows values for individual mice [circles: solid – 1month +/+, open – 3month +/+; triangles: solid-1month -/-, open-3month -/-], (C) shows the percent change of MMP-2 protein levels from wildtype glomeruli [1 month -/- light grey bar, 3 month -/- dark grey bar. Data expressed as mean ± SEM.

age-matched wildtype mouse glomeruli [0.035 ± 0.017 and 0.051 ± 0.016, pg/glomerulus, $p \leq 0.001$ and $p \leq 0.008$ respectively] (Figure II-7).

MMP-3 Expression Increases in *Col1a2*-deficient Mice

Col1a2-deficient glomerular MMP-3 steady state transcript levels showed slight elevation in one month old mice relative to age-matched wildtype glomeruli levels, although not significant [$p \leq 0.15$]. However, by three months of age steady-state MMP-3 transcripts had significantly increased by 126% in *Col1a2*-deficient glomeruli as compared to wildtype glomeruli [$p \leq 0.0018$]. Glomerular MMP-3 RNA expression in heterozygous mice followed a similar pattern as wildtype glomeruli (Figure II-8).

At the protein level glomeruli from one month old *Col1a2*-deficient mice had a 9-fold increase in glomerular MMP-3 protein content (does not distinguish between active and latent forms) as compared to age matched wildtype mice. By three months of age, both wildtype and *Col1a2*-deficient mice had a similar 2.6 fold increase in MMP-3 protein levels as compared to their one month values. This represented an increase of 9-fold in the glomerular MMP-3 levels in *Col1a2*-deficient mouse glomeruli as compared to age-matched wildtype glomeruli [0.0058 ± 0.0028, and 0.0007 ± 0.0031, pg/glomerulus (mean ± S.E.M.) ($p \leq 0.0005$) at one month; 0.015 ± 0.0031, and 0.0017 ± 0.0029, pg/glomerulus ($p \leq 0.0003$) at three months, respectively] (Figure II-8).

MMP-9 Protein Levels at Three Months of Age Does Not Correlate with an Increase in Steady-state mRNA Levels in *Col1a2*-deficient Glomeruli

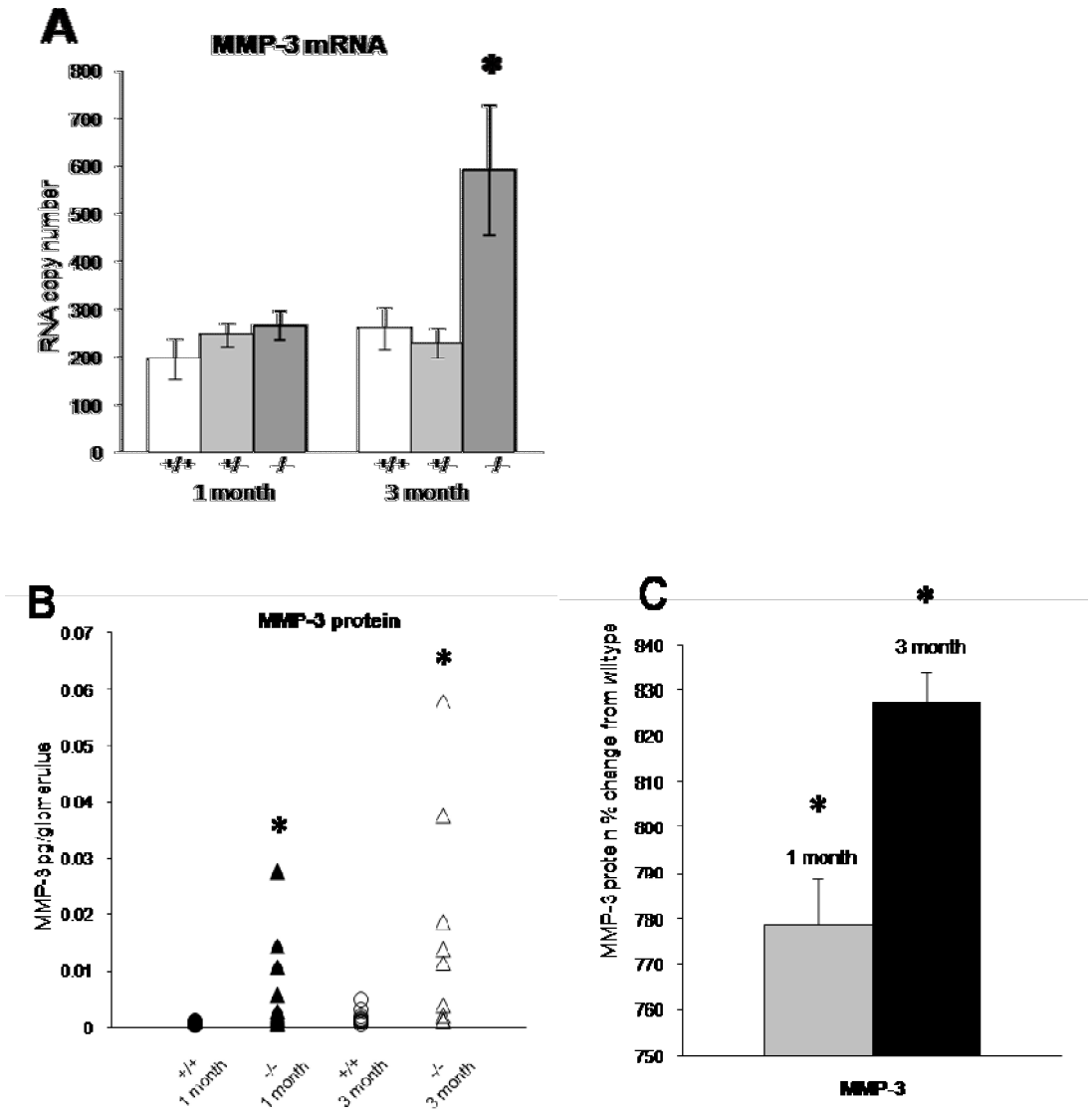


Figure II-8. MMP-3 mRNA and protein expression. Quantitative RT-PCR demonstrates a significant increase in MMP-3 (A) mRNA expression in 3-month *Col1a2*-deficient (-/-; dark grey bar) glomeruli compared to age-matched heterozygous (+/-; light grey bar) and wildtype (+/+; white bar) animals (*$p < 0.05$). 1-month and 3-month *Col1a2*-deficient (-/-) mice show a significant increase in MMP-3 protein levels, with

Figure II-8 cont'd. higher levels of MMP-3 protein in 3 month *Col1a2*-deficient glomeruli as compared to 1 month *Col1a2*-deficient glomeruli (*p ≤0.05) (B and C). (B) shows values for individual mice [circles: solid – 1month +/+, open – 3month +/+; triangles: solid-1month -/-, open-3month -/-], (C) shows the percent change of MMP-3 protein levels from wildtype glomeruli [1 month -/- light grey bar, 3 month -/- dark grey bar. Data expressed as mean ± SEM.

MMP-9 is also a gelatinase whose altered expression has been implicated in many sclerotic diseases, as well as glomerulosclerosis (Andrews, 2002; Lelongt, 2001). MMP-9 steady state mRNA levels were equivalent in wildtype and *Col1a2*-deficient glomeruli at one month of age. By three months of age both wildtype and *Col1a2*-deficient mice had elevated glomerular steady state MMP-9 mRNA levels; wildtype glomeruli showed 2-fold increase in MMP-9 mRNA and *Col1a2*-deficient glomeruli showed a 5-fold increase, although not significant. Heterozygous mouse glomeruli exhibited a 70% increase in MMP-9 mRNA at one month of age, but returned to wildtype levels by three months of age (Figure II-9).

Interestingly, the protein array analysis of relative levels of combined latent and active forms of MMP-9 protein between *Col1a2*-deficient and wildtype glomeruli (Figure II-9) demonstrated that MMP-9 was not elevated in *Col1a2*-deficient glomeruli at one month of age as compared to age matched wildtype glomeruli. However, unlike MMP-2 and -3, all forms of MMP-9 protein appeared to decrease by 20% in wildtype mice at three months of age compared to one month wildtype mouse glomeruli, and were decreased by 60% in *Col1a2*-deficient mouse glomeruli at three months of age as compared to one month of age [0.085 ± 0.014 (+/+), and 0.084 ± 0.013 (-/-), pg/glomerulus (mean ± S.E.M.) ($p \leq 0.9$) at one month respectively; 0.067 ± 0.013 (+/+), and 0.033 ± 0.014 (-/-), pg/glomerulus ($p \leq 0.05$) at three months] (Figure II-9).

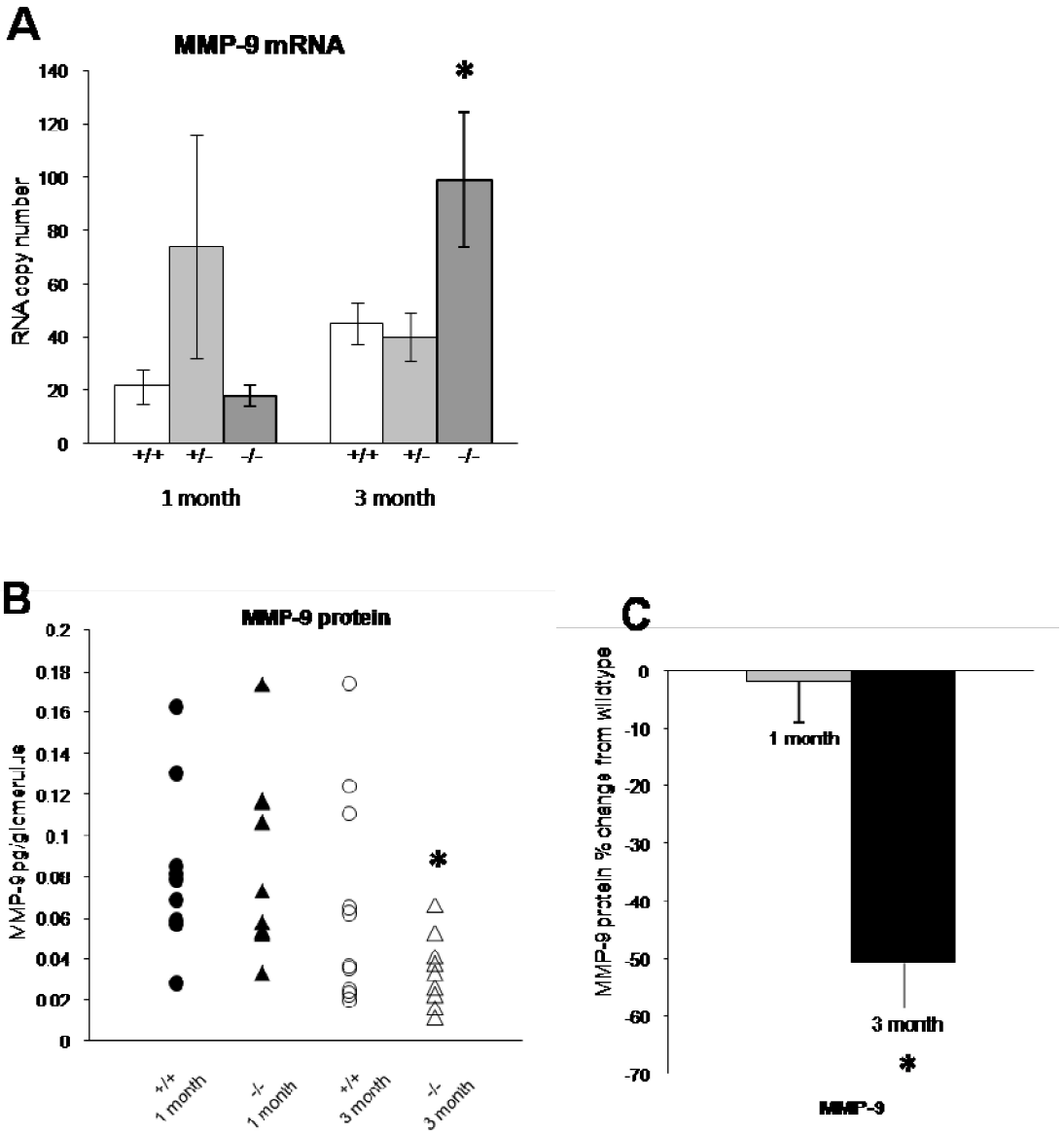


Figure II-9. MMP-9 mRNA and protein expression. Quantitative RT-PCR demonstrates a significant increase in MMP-9 (A) mRNA expression in 3-month *Col1a2*-deficient (-/-; dark grey bar) glomeruli compared to age-matched heterozygous (+/-; light grey bar) and wildtype (+/+; white bar) animals (*$p < 0.05$). 1 month *Col1a2*-deficient glomeruli have similar levels of MMP-9 protein as compared to 1 month wildtype glomeruli, but show a significant decrease in MMP-9 protein levels at 3-months of age

Figure II-9 cont'd. compared to wildtype mice (*p ≤0.05) (B and C). (B) shows values for individual mice [circles: solid – 1month +/+, open – 3month +/+; triangles: solid-1month -/-, open-3month -/-], (C) shows the percent change of MMP-9 protein levels from wildtype glomeruli [1 month -/- light grey bar, 3 month -/- dark grey bar. Data expressed as mean ± SEM.

DISCUSSION

Increased glomerular number in three month old COL1A2 deficient mouse kidneys

Examination of whole kidney and glomeruli isolated from wildtype, heterozygous and *Col1a2*-deficient mice confirmed previously published data with its significantly smaller body weights compared to age-matched wildtype littermates (Carleton, 2008; Chipman, 1993; Shapiro, 1995). However, we found that when normalized to body weight, 1 month *Col1a2*-deficient kidney weights were larger than age-matched wildtype mice, possibly suggesting an increase in size due to development, possibly even hypertrophy of the organ, or just a result of smaller body sizes.

Brodeur, et al., described initial collagen accumulation in *Col1a2*-deficient kidneys following a pattern of glomerular activation (Brodeur, 2007). When mice are born they are actively filtering waste with only the innermost glomeruli in the juxtamedullary region. As the kidney matures, glomeruli are activated in centrifugal pattern extending its use to glomeruli in the cortical regions of the kidney. This continues up to 21 days of age when all the glomeruli are activated and fully functioning (Spitzer, 1974; Kleinman, 1973). Kidneys from *Col1a2*-deficient one month old mice show a significant 33% increase in glomerular number, and three month old mice have a 26% trend in the increase in glomerular number found in the cortical and juxtamedullary regions of their kidneys as compared to age-matched wildtype mouse kidneys. This finding is consistent with studies by Bonvalet, et al, who found that normal mice have a postnatal increase in glomerular number up to three months of age, and an even greater increase of glomerular number in hypertrophied kidneys due to unilateral nephrectomy

(Bonvalet, 1977; Imbert, 1974). Bonvalet, et al, demonstrated that hypertrophied kidneys had a 49%-51% increase in glomerular number by three months of age compared to normal kidneys. It would follow that the increase in glomerular number in the *Col1a2*-deficient mice might be a reflection of the accumulation of type I collagen impacting kidney function, and the kidney subsequently responding by synthesizing more glomeruli. This increased glomerular synthesis may lead to increases in kidney weight-to-animal weight ratios in the *Col1a2*-deficient mouse.

Col1a2-deficient kidneys at three months of age also had reduced total glomerular yields by our paramagnetic extraction method. Although these mice have a significant increase in glomerular number at one month of age, the majority of the glomeruli also had significant collagen deposition. Therefore, it is likely that the reduction in glomerular yield in *Col1a2*-deficient mice is a reflection of the difficultly that the magnetic Dynabeads® may have had entering into the capillary network of the more severely affected glomeruli. This suggests a broader implication that the glomerular capillaries become restricted to the flow of blood as sclerosis increases, diminishing their filtering capacity.

***Col1a2*-deficient and heterozygous mice synthesize COL1A2 mRNA and accumulate homotrimeric type I collagen**

Type I collagen is not normally expressed in the glomerular mesangium, but is expressed in the kidney interstitium and vasculature (Alexakis, 2006; Glick, 1992). Several investigators (Peten, 1993; Mozes, 1999; Floege, 1992) have shown that during glomerulosclerosis, type I collagen mRNA and protein synthesis are increased and that

type I collagen accumulates within the mesangium, suggesting a possible wound healing response. Type I collagen accumulation was also shown to be occurring in *Col1a2*-deficient glomerulosclerosis (Phillips, 2002; Brodeur, 2007). These studies, however, did not examine whether the type I collagen isotype found in fibrotic glomeruli in heterozygote *Col1a2*-deficient kidneys is homotrimeric or heterotrimeric in nature. We have used IHC to demonstrate the absence of α2(I) positive staining within sclerotic *Col1a2*-deficient glomeruli. However, *Col1a2*-deficient glomeruli did have sporadic areas of amorphous materials, believed to be protein casts or plasma, that also showed some positive α2(I) staining (data not shown). Additionally, *Col1a2*-deficient kidneys showed isolated amorphous aggregate α2(I) positive staining within the glomerular core. Heterozygous mice, however, showed no α2(I) positive staining patterns consistent with PSR localization within the glomerular core of sclerotic glomeruli, but did show isolated α2(I) positive staining aggregates similar to that seen within *Col1a2*-deficient kidneys. We hypothesize that these aggregates within the mesangium and in the proximal tubules, which are rich in lysosomes (endocytic vacuoles) and peroxisomes (Brenner, 2000), represent α2(I) chain degradation products.

To further elucidate our findings, we isolated collagen from wildtype, heterozygous and homozygous *Col1a2*-deficient whole kidney and glomeruli and quantitated the collagen content and isotype composition by fluorescent Cy5 gels and by cyanide-bromide analyses. Collagen purified from whole kidneys contained ~80% type I, 12-16% type III and ~ 5% type V collagen. These ratios of type I, III and V collagen are consistent with the composition of collagen isolated from cultured fibroblasts (Pace, 2008). Only heterotrimeric type I collagen was present in wildtype kidneys, only

homotrimeric type I collagen was present in homozygous *Col1a2*-deficient kidneys, and as anticipated from studies of other tissues approximately equal amounts of homotrimeric and heterotrimeric type I collagen was found in whole kidney tissue extracts from heterozygous animals (Gaiko-Galicka, 2002; Han, 2008).

Evaluation of the collagen in purified glomeruli demonstrated that only negligible amounts of type I collagen were present in glomeruli from 3 month old wildtype mice. Careful analysis of heterozygous mice showed a range of accumulated type I collagen (between < 0.5 – 34 pg/glomerulus) which correlated with the degree of severity of the disease for each animal. However, in age-matched *Col1a2*-deficient glomeruli, which are the most clinically severe, we found over 100 pg of collagen per glomerulus. The estimated detection limit of this assay is 0.2-0.5 pg collagen per glomerulus.

We further analyzed whether type I collagen was homotrimeric or heterotrimeric in nature in glomeruli from wildtype, heterozygous and *Col1a2*-deficient animals and observed a faint band in several heterozygous and *Col1a2*-deficient glomerular and kidney preparations which migrated close to the expected position of the α2(I) chain. It was determined to be a proteolytic degradation product of the α1(I) chain by CNBr peptide mapping (Kuznetsova, 2003). This truncated α1(I) product has been observed in *Col1a2*-deficient tissues and in tissue culture samples from an OI patient which synthesized only homotrimeric type I collagen similar to the *Col1a2*-deficient mouse (Kuznetsova, 2003; Deak, 1985). We can not completely exclude the presence of the α2(I) chain in this extraneous band found in heterozygous glomeruli. However, based on the intensity of the observed truncated α1(I) band compared to the intensity of the α1(I) band, absence of a well defined α1(I)-α2(I) dimer band, and relative intensities of the

extraneous band compared to other bands from the CNBr cleavage pattern, we concluded that type I collagen from heterozygous glomeruli consisted of 95-98% α1(I) homotrimers,.

We also examined both COL1A1 and COL1A2 steady-state mRNA expression of glomerular type I collagen. We found that COL1A1 mRNA steady-state levels in *Col1a2*-deficient mice were elevated by three-fold at one month of age and further elevated by 15-fold at three months of age compared to age-matched wildtype mice. This finding is consistent with findings by Brodeur, et al., who previously demonstrated that *Col1a2*-deficient whole kidneys also exhibit a significant increase in COL1A1 mRNA levels at one month of age (Brodeur, 2007).

Our studies also show a 22% reduction in COL1A2 steady-state mRNA expression in *Col1a2*-deficient glomeruli at one month of age and a 45% reduction at three months of age relative to wildtype. Previous studies agree that COL1A2 mRNA continues to be synthesized by the *Col1a2*-deficient mouse, but it is uncertain whether non-functional α2(I) chains are translated. Cultured fibroblasts from the human patient which the *Col1a2*-deficient mouse was modeled suggested that there is synthesis of the aberrant proα2(I) chain protein that does not incorporate into the collagen triple helix (Nicholls, 1984; Deak, 1983). It is also still unclear why the *Col1a2*-deficient mouse continues to synthesize relatively large amounts of COL1A2 mRNA. Heterozygous mice show no significant elevation of COL1A1 steady-state mRNA levels at either one or three months of age, and no significant increase in COL1A2 steady-state mRNA levels at one month of age compared to age-matched wildtype mice. However, at three months of age, heterozygous mice show a significant 59% reduction in COL1A2 mRNA synthesis,

almost 15% less than *Col1a2*-deficient mice. Unlike *Col1a2*-deficient mice, heterozygous mice continue to be able to synthesize and use normal α2(I) chains as seen in the whole kidney tissue isolates. But it is unclear why its COL1A2 mRNA synthesis in heterozygous glomeruli is more reduced than in *Col1a2*-deficient mouse glomeruli, neither of which appear to express heterotrimeric type I collagen.

MMP upregulation is not sufficient to degrade homotrimer

Brodeur, et al, demonstrated collagen accumulation by 1 week of age in whole *Col1a2*-deficient mouse kidneys (Brodeur, 2007). Yet in *Col1a2*-deficient glomeruli steady-state mRNA levels of MMP-2 and MMP-3 were not significantly increased until three months of age, even though there was a significantly large increase in active and latent forms of MMP-2 and MMP-3 enzymes at both one and three months of age relative to wildtype. On the other hand, MMP-9 mRNA and protein levels are similar to wildtype mice at one month of age, but at three months of age MMP-9 mRNA levels *increased* with a corresponding MMP-9 protein *decrease* in *Col1a2*-deficient glomeruli.

The partial functionality of a neonatal kidney is localized to glomeruli that are within the juxtamedullary region, the innermost area of the kidney containing glomeruli. As the kidney matures with age, more glomeruli become activated in a centrifugal pattern leading outward toward the cortex of the kidney until all glomeruli are fully functional. MMP-2 and MMP-3 mRNA induction appears to occur following activation of glomeruli and the initiation of collagen deposition by demonstrating significant up-regulation by three months of age. However, the increase in MMP-2 and MMP-3 protein expression occurs at 1 month of age, prior to an increase in mRNA levels. It is possible that the

Col1a2-deficient mouse experiences increased stabilization of glomerular MMPs -2 and -3 or perhaps an increase in translational efficiencies. Additionally, the release of bound MMPs from tissue inhibitors of matrix metalloproteinases (TIMPs) may increase the availability of mesangial MMPs that are available for enzymatic activation. It is also possible that increases in MMP protein levels occurring prior to mRNA levels may be due to circulating MMPs which may be siphoned through the glomerular mesangium and contributing to early glomerular injury (Yamanaka, 1999). This effect from circulating factors can also be seen in mice with high circulating levels of TGFβ synthesized in the liver, inducing a glomerulosclerotic phenotype in a transgenic mouse mouse model (Kopp, 1996). High levels of circulating MMPs have also been associated with liver fibrosis (Hemmann, 2007) and renal disease in kidney allograft transplant patients (Rodrigo, 2000).

MMP-9 is expressed during development and in adult tissue, and has been shown not to be associated with glomerulosclerosis in a certain mouse models (Andrews, 2002). Perhaps the alterations in MMP-9 expression seen here are not directly associated with sclerosis, but have a role in EMT. MMP-9 has been shown to induce EMT in human mammary epithelial cells (Owen, 2003) and skin carcinogenesis (Coussens, 2000). MMPs have been shown to play a significant role in the induction of EMT and subsequent fibrosis (Radisky, 2007; Orlichenko, 2008; Chuang, 2008). Preliminary data from our laboratory suggests up-regulation of markers of EMT occurs later (three months of age) within the periglomerular and glomerular tuft regions of *Col1a2*-deficient glomeruli (data not shown).

Several mouse models that display type I collagen glomerulosclerosis also show differences in MMP expression. Specifically, the bovine growth hormone (bGH) transgenic mouse shows an increase in MMP-2 mRNA synthesis (Doi, 1991; Carome, 1994). MMP-2 transgenic mice show over-expression of active MMP-2 in renal proximal tubular epithelial cells that lead to type I collagen deposition in tubulointerstitium and glomerular mesangium, as well as mesangial expansion and epithelial-to-mesenchymal cell transitions (Cheng, 2006). Streptozotocin-induced diabetic Sprague-Dawley rats which exhibit type I collagen glomerulosclerosis, tubulointerstitual fibrosis, and basement membrane accumulation of collagens I and IV, fibronectin and laminin, and increased blood glucose, showed a decrease in MMP-2 protein levels but no change in MMP-9 protein levels as compared to non-diabetic and diabetic estrogen-supplemented rats after 17 weeks (Dixon, 2007). Consistent with the *Col1a2*-deficient mouse glomeruli, renin-dependent hypertensive rats which exhibit increased focal glomerulosclerosis, expansion of the mesangial matrix, and an increase in microalbuminuria, showed increases in MMP-2, with corresponding decreases in MMP-9 (Bolbrinker, 2006).

The *Col1a2*-deficient mouse model also illustrates the necessity of the α2(I) chain to normal type I collagen degradation. Although there is a 97% homology between the amino acid composition of the α1(I) and α2(I) chains that have been evolutionarily conserved for the past 500 million years (Bornstein, 1980; Deak, 1983), its absence in the *Col1a2*-deficient mouse leads to glomerular sclerosis, bone fragility, and reduced vessel integrity (Chipman, 1993; Brodeur, 2007; Carleton, 2008; Pfeiffer, 2005; Vouyouka, 2001). The α2(I) chain has been proposed to be a stabilizer of the type I collagen helix

and it plays an integral role in MMP recognition and subsequent cleavage (Miles, 2002; Kuznetsova, 2003; Nerenberg, 2008a; Chung, 2004; Nerenberg, 2008b). It would follow that in the absence of the α2(I) chain, there would be delays in cleavage of the triple-helical molecule. It is also plausible that sclerotic glomeruli do not identify homotrimer as being aberrant until collagen accumulation has reached a certain threshold or impaired glomerular function, and subsequent cell-induced recognition and wound response mechanisms are deployed. In heterozygous sclerotic glomeruli that are able to synthesis both homo- and heterotrimeric type I collagen isotypes, we can not ignore the possibility that there is no increase in homotrimer synthesis as compared to heterotrimer, but that heterotrimer is recognized and degraded more efficiently, leaving homotrimer lingering within the mesangial matrix.

The role of other renal MMPs remains to be investigated. The role of MMP-13 in the glomerulosclerotic process is unknown. Preliminary findings suggest that MMP-13 cleaves homotrimeric collagen at a slower rate than heterotrimeric collagen (data not shown). MMP-13 is a contributor to the activation of MMPs -2, -3, and -9 and is expressed during development and skeletal remodeling (Chakraborti, 2003; Stahle-Backdahl, 1997; Inada, 2004). It has also been shown to be synthesized in cultured renal mesangial cells, but its relationship to glomerulosclerosis and EMT remains to be evaluated.

Col1a2-deficient glomerulopathy and ER stress

As previously indicated, no insult or mechanism of injury has been identified to account for the induction of *Col1a2*-deficient glomerulopathy. Recently, stress of the

endoplasmic reticulum (ER) due to the unfolded protein response has been found to play a role in abnormal skeletal formation, and renal disease (Kitamura, 2008). Mutations in the human ER stress modulator PEK/EIF2AK3 gene (also known as Wolcott-Rallison syndrome), exhibit a phenotype similar to *Col1a2*-deficient osteogenesis imperfecta, including blue-gray sclera (Bonthron, 1998). Many Wolcott-Rallison syndrome patients also show signs of renal insufficiency (Bin-Abbas, 2002). Additionally, the recently discovered osteogenesis imperfecta mouse model, Aga2, has a mutated COL1A1 gene, and exhibits ER stress and abnormal fibroblast intracellular type I collagen accumulation (Lisse, 2008). ER stress and unfolded protein response (UPR) was also demonstrated in Thapsigargin-induced osteoblast-like cells with up-regulation of EIF2A and type I collagen mRNA with short term inhibitor exposure and apoptosis with long term inhibitor exposure (Hamamura, 2007). These studies suggest that a potential mechanism for the collagen deposition may be the unincorporated α2(I) chain inducing ER stress and UPR in the renal mesangium, in concert with type I collagen deposition leading to a renal pathology. Further investigations are needed to evaluate the potential role of ER stress in the *Col1a2*-deficient mouse glomerulopathy.

In summary, our findings suggest that the homotrimeric isotype of type I collagen is the type I collagen accumulating in sclerotic glomeruli of *Col1a2*-deficient and heterozygous mice. This may be due to the significant upregulation of the COL1A1 gene and down-regulation of COL1A2 gene, and suggests a pathogenic role for homotrimer synthesis in the kidney. However, homotrimer pathogenesis due to inefficient "clean-up" within the mesangium cannot be ignored. We have also shown that MMP induction is not the primary event in this disease, but appears to be a secondary response. Finally,

increases in MMP-2 and MMP-3 expression appear insufficient to prevent accumulation of homotrimeric type I collagen in the glomeruli of the *Col1a2*-deficient mouse.

CHAPTER III.

DIFFERENTIAL CLEAVAGE OF HOMOTRIMERIC AND HETEROTRIMERIC TYPE I COLLAGEN BY MATRIX METALLOPROTEINASES

INTRODUCTION

Glomerulosclerosis is believed to be a disease in which there is an imbalance in the synthesis and degradation of extracellular matrix (ECM) components. As outlined in the introduction, MMPs are the enzymes primarily responsible for degradation of ECM in the body and the kidney. During glomerulosclerosis, MMP expression has been shown to be altered. Uchio-Yamada, et al., and Tomita, et al., have both been able to demonstrate down-regulation of MMP-2 and -9 mRNA transcripts in the induced nephrotic mice models by RT-PCR (Uchio-Yamada, 2001; Tomita, 2004). Sharma, et al., and Iimora, et al., also demonstrated an increase in MMP-2 synthesis, but a decrease in MMP-9 synthesis in the unilateral ureteral obstruction rat model (Sharma, 1995; Iimura, 2004). Additionally, MMP-2 is increased in Goto-Kakizaki rats, a model of type 2 diabetes, along with MMP-9 (Portik-Dobos, 2006). However, in our mice, the *Col1a2*-deficient mouse model, quantitative real-time PCR has confirmed the upregulation of the COL1A1 gene in sclerotic glomeruli by one month of age, but upregulation of MMPs-2, -3 and -9 transcripts are not seen until three months of age. This demonstrates that the synthesis of homotrimer is increased much earlier than an increase in synthesis of MMPs, demonstrating an obvious imbalance in the synthesis/degradation mechanism, on which the mechanism of sclerosis may be based.

MMPs are regulated on many levels, both pre- and post-translationally. During sclerosis it is possible that they are down-regulated by the anti-inflammatory cytokine TGF-β or inhibited from activation by bound tissue inhibitors of matrix metalloproteinases (TIMPs) to reduce overall degradation (Wick, 2001; Esposito, 2005).

In the kidney MMPs are expressed by mesangial cells and podocyte cells, but during sclerosis myofibroblasts and macrophages may also contribute to the production of MMPs (Turck, 1996; Orlichenko, 2008; Shiozawa, 2000). Once sclerosis begins, MMP synthesis often aids in myofibroblast migration by chewing away the ECM and stopping the cell-to-cell contact which allows myofibroblasts to migrate into the region of injury. Macrophage expression of MMPs is related to an inflammatory response during glomerulosclerosis; however, the *Col1a2*-deficient mouse model show no sign of macrophage infiltration until collagen accumulation in the glomerulus is severe.

The α2(I) chain in the mature type I collagen molecule is six amino acids shorter than the α1(I) chain (Keilty, 1993) and there is 97% amino acid homology between the, α1(I) and α2(I) chain collagen chains yet the role of the α2(I) chain in type I collagen is still largely unknown. Studies of homotrimeric type I collagen have given insight into the impact of α2(I) chain on fibril formation and overall structural integrity. Researchers have shown that in the absence of the α2(I) collagen chain the homotrimer exhibits many altered physical properties as compared to heterotrimeric type I collagen, such as less organized molecular alignment in fibrils, lower melting temperatures, and higher water content (Kuznetsova, 2003; Kuznetsova, 2001). The role of the α2(I) chain has also been proposed to play a crucial part in the recognition and subsequent degradation of type I collagen by MMPs (Chung, 2004; Perumal & Orgel, 2008) .

In the following study we attempt to determine whether MMP degradation of homotrimer is equivalent to degradation of heterotrimer, as a potential explanation for the homotrimeric type I collagen accumulation seen in the glomeruli of *Col1a2*-deficient mice. Preliminary analysis in our laboratory of the proteolytic cleavage of homotrimeric

and heterotrimer type I collagen in a 24-hour period by commercially available MMPs suggest that MMPs-2 and -9 may preferentially cleave heterotrimeric type I collagen as compared to homotrimeric type I collagen

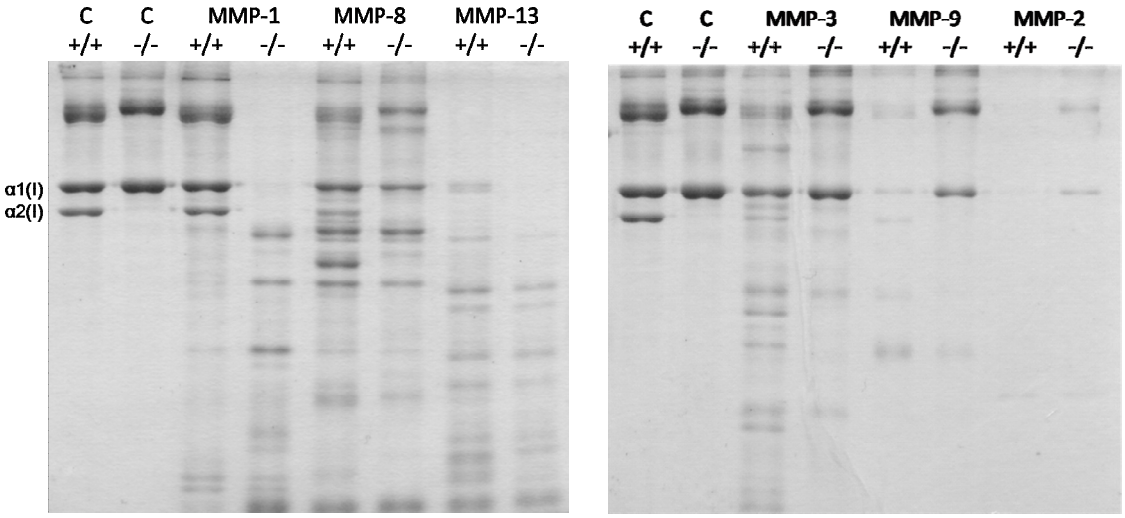


Figure III-1. Preferential proteolytic degradation of type I collagen isotypes within 24-hours using MMPs-1, -2, -3, -8, -9, and -13. Purified homotrimeric type I collagen (-/-) and heterotrimeric type I collagen (+/+) from mouse tails were incubated with specific MMPs (MMP-1, -2, -3, -8, -9, and -13) for 24 hours at 37˚C, followed by size seperation by SDS-PAGE analysis. Electrophoresis of control (C) heterotrimeric (+/+) and homotrimeric (-/-) type I collagen showing α1(I) and α2(I) bands in undigested state. When incubated with identical amounts of human recombinant MMP-1 enzyme, homotrimer was cleaved faster in a 24-hour period than heterotrimer. When incubated with MMP-3, MMP-9, and MMP-2 enzyme, heterotrimer was cleaved faster in a 24-hour period than homotrimer. When incubated with MMP-8 and MMP-13, homotrimer or heterotrimer degradative preference was not able to be determined.

(Figure 111-1). This interesting preliminary data by Amanda Brodeur and Jason Gentry gave us a look into the possible MMP degradative efficiency of MMPs-1, -2, -3, -8, -9 and -13 and lead us to examine the rates of cleavage of renal MMPs. This lead us to hypothesize that renal MMP-2, -9, and -13 expression would remain unchanged in *Col1a2*-deficient mice as compared to wildtype mice because the homotrimeric isotype of type I collagen was not being recognized by the MMPs and therefore not degraded. Alterations in the ability of the homotrimeric type I collagen to be recognized by several renal MMPs and hence their ability to efficiently degrade homotrimeric type I collagen is a potential pathogenic mechanism for glomerular homotrimeric type I collagen accumulation. In the following study we hypothesize that glomerular MMPs have altered degradative efficiency for homotrimeric type I collagen that contributes to the homotrimeric type I collagen accumulation in sclerotic *Col1a2*-deficient mouse glomeruli as compared to wildtype mice. To investigate the degradation efficiencies of MMPs-2, -9 and -13 on the homotrimeric isotype of type I collagen as compared to the heterotrimeric isotype of type I collagen, we examined the ability of MMP-2, -9 and -13 to degrade homotrimeric and heterotrimeric type I collagen by proteolysis assay and zymography.

METHODS

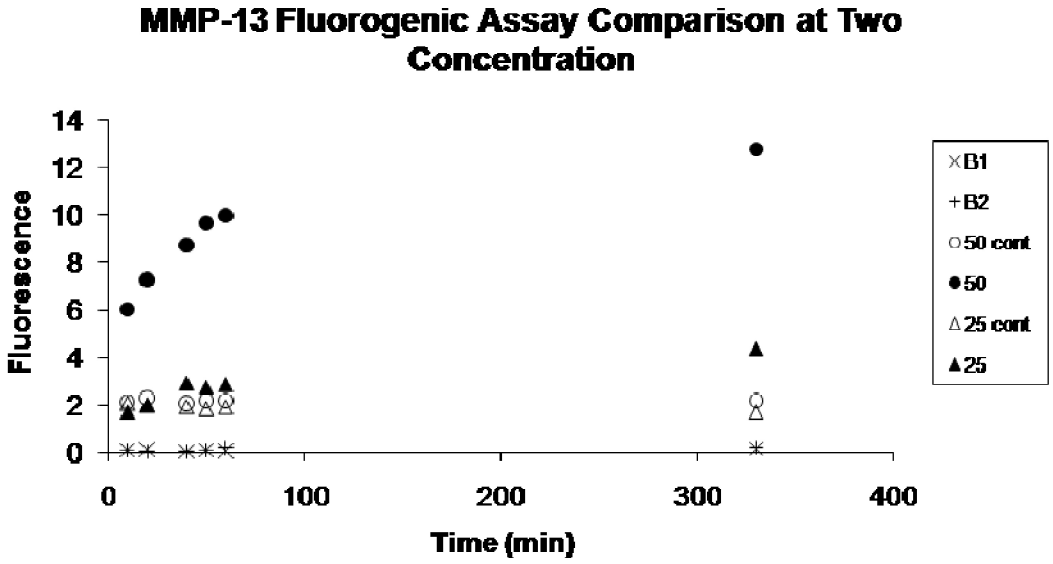
MMP and Glomerular Activity

MMPs -2, -9, and -13 (R&D Systems, Minneapolis, MN) were tested fluorogenically for manufacturers reported activity. MMPs were incubated for 2 hours in a cocktail of 10mM 4-aminophenylmercuric acetate (APMA) and tetracyanobenzene (TCNB) at 37˚C prior to use. An aliquot of each activated enzyme (MMP-2=5ng/rxn; MMP-9= 2ng/rxn; MMP-13=25ng/rxn) was then incubated with a fluorogenic substrate (Mca-PLGL-Dpa-AR-NH_2 Fluorogenic Peptide Substrate, R&D Systems, Minneapolis, MN) and fluorescence was measured at regulated time intervals (0, 10 min, 20 min, 40 min, 50 min, and 1 hour). Fluorogenic substrate emitted fluorescence at emission 320nm and excitation 405nm upon cleavage. Initial activity studies also examined activity at an arbitrary extended time (i.e. 5 hours) to verify enzyme activity past the desired time limit for proteolytic assays and to verify that optimal activity was achieved (Figure III-2).

Activation of Isolated Glomeruli: Isolated glomeruli were protected from autolytic degradation by addition of 2M phenylmethylsulphonyl fluoride (PMSF) and sonicated for 10 minutes in an ice cold waterbath. Sonicated glomeruli were activated by addition of TCNB and 10mM APMA and incubated for 2 hours at 37˚C.

Collagen Isolation and Quantitation

Col1a2-deficient and wildtype mouse tails were skinned and pulverized with mortar and pestle and washed in a series of 20% EDTA and 0.1M Phosphate buffers to remove Mg and Ca salts. Pulverized tails were agitated overnight at 4°C in 0.5N acetic



Sample	10 min	20 min	40 min	50 min	1 hr	5.5 hr
Blank 1 (TCNB+fluor substrate) (X)	0.097	0.107	0.023	0.094	0.051	0.129
Blank 2 (TCNB+fluor substrate+APMA) (+)	0.082	0.027	0.043	0.079	0.172	0.19
MMP-13 50ng (no APMA) (o)	2.106	2.281	2.063	2.157	2.151	2.147
MMP-13 50ng (●)	6.061	7.282	8.71	9.671	9.97	12.744
MMP-13 25ng (no APMA) (Δ)	2.074	1.98	1.919	1.84	1.911	1.705
MMP-13 25ng (▲)	1.671	1.976	2.887	2.711	2.843	4.367

Figure III-2. MMP-13 Fluorogenic Activity Assay. Graph shows the increased activity of two concentrations of MMP-13 (50ng and 25ng) after incubation with (closed circle and triangle) and without (open circle and triangle) APMA to verify that enzyme was activated and that MMP-13 remained activated up to 5.5 hours after activation. Controls used did not use MMP-13 (Blank 1 and Blank 2).

acid and pelleted by centrifugation at 4500 rpm for 20 minutes. Supernatant containing solublized collagen was transferred to a new tube and incubated in 30% NaCl/0.5 acetic acid (solution added periodically over a period of 45 min) overnight at 4°C, allowing collagen to precipitate out of solution. Precipitated collagen was pelleted once more at 4500 rpm for 20 minutes and resuspended in 0.1N acetic acid and stored at 4°C until use. Determination of the concentration of the extracted collagen was performed using a colormetric anionic dye Sircol™ Soluble Collagen Assay (Biocolor Ltd., Antrim, UK), and using a spectrophotometer at 540nm. Values were calculated in reference to a simultaneously generated standard curve.

Limited Proteolysis and Densitometry

5ug aliquots of isolated homotrimeric and heterotrimeric tail type I collagen suspended in 0.1N acetic acid were speed-vacuumed to allow rapid evaporation of the solvent from the collagen and resuspended for 20 min in TCNB at 37ºC to hydrate collagen. Purified MMPs purchased from R&D Systems were activated as described earlier. The activated MMPs were added to hydrolyzed collagen and incubated at 37ºC for various time intervals (0min, 5min, 10min, 15min, 20min, 30min, 45min, and 1 hour). The proteolytic reaction was stopped by addition of loading dye (glycerol, 8M urea, 0.5 tris, 10% sodium dodecyl sulphate, bromophenol blue). Samples were then examined by 8% polyacrylamide, 2M Urea SDS-PAGE electrophoresis, followed by staining with Coomassie Blue (250-R) (BioRad, Hercules, CA), and analyzed by densitometry (optical measurement of relative amount of light passing through a protein band) (Kodak 1D Image Analysis Software) compared to a no-enzyme control in arbitrary units. (Figure

III-3)

Zymography

Homotrimeric or heterotrimeric type I collagen extracted from tail tendon as previously described was co-polymerized with 33:1 Bis-polyacrylamide to make a 4% collagen/SDS-polyacrylamide gel. Activated MMPs -2, -3, -9 and -13, and glomerular isolate (as previously described) were size separated by gel electrophoresis, gels were run for 2 hours at 60mA. Glomeruli from heterozygous *Col1a2-deficient* mouse kidney (322 glomeruli/lane), glomeruli from homozygous *Col1a2*-deficient kidney (290 glomeruli/lane). Zymogen gel was then placed in zymogram renaturation buffer (BioRad) for 1 hour at room temperature to allow renaturation of MMPs and removal of SDS, followed by placement into zymogram development buffer (BioRad) overnight at 37°C to allow re-activation of enzymes and degradation of collagen. Once incubation was complete, gel was analyzed after protein staining (GelCode Blue Stain Reagent, PEIRCE, Rockford, IL) for 1 hour and destained as needed. Evaluation of MMP activity was determined by localized loss of collagen content.

Statistics

Data presented here was evaluated statistically by Student t-test for possible significance.

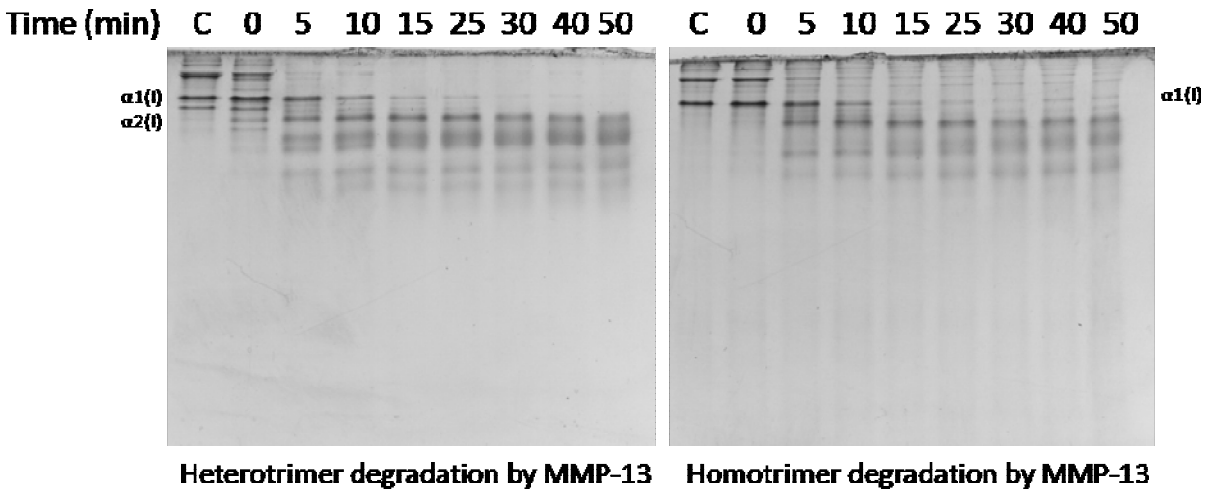


Figure III-3. SDS gel electrophoresis of heterotrimeric (left) and homotrimeric (right) degradation products with time. The degree of degradation, marked by the loss of the α1(I) band was evaluated by densitometry. Fragments running faster through the gel below the α1(I) and α2(I) chains are degradation products.

RESULTS

Limited Proteolytic Optimization of MMP Cleavage

The proteolysis assays shown here proved difficult to obtain consistent data. Among the many contributing factors, achieving reproducible MMP activity results seemed the most challenging. Although MMP activation was performed according to the manufacturer's instructions, using the manufacturer's protocol, the reported manufacturer's activity provided with each vial of purchased enzyme was not reproducible with the fluorogenic substrate. In many instances, the enzymes were unable to be activated with APMA incubation. At other times, the same nanogram amounts of enzymes had more or less activity in a vial purchased one week as compared to a vial purchased several weeks prior. Therefore, variation in the activity of the enzyme translated to large differences in rates of enzymatic degradation between purchased enzyme lots. We attempted to circumvent this problem by purchasing several vials of a specific enzyme, combining them all, aliquoting them and executing as many proteolytic experiments as possible in a span of a few days. The results reported below are the product of this effort. However, when each new batch of enzymes purchased, the problems continued.

Another problem that frequently occurred was inconsistent cleavage in one of either heterotrimeric or homotrimeric type I collagen preparations within an experimental trial. Although each experiment was executed in pairs (evaluating both heterotrimeric and homotrimeric collagen degradation simultaneously) using identical techniques and a shared master-mix, degradation of only one isotype was frequently seen, with the other

isotype showing no degradation at all even for the controls. Reflecting, this may have been due to unforeseen nuances in collagen preparation from mouse tail, the only process that was executed separately during these experiments, such as an introduction of inhibitors inherent in mouse cartilage or bone that would potentially inhibit MMP degradation. To properly evaluate results, if an experimental trial was performed and either isotype of the collagen showed incomplete or inconsistent degradation patterns, we discarded the experiment.

In light of the variability of our system and the acknowledgement of the low sensitivity, we analyzed the ability of MMP-2, -9 and -13 to cleave homotrimeric and heterotrimeric type I collagen with the realization that we would only be able to detect large differences in proteolytic efficiencies and that small changes in proteolytic cleavage would not be detected by our in vitro system. Within these limitations it is important to recognize that small changes in MMP specificity and efficiency of cleavage could have dramatic implications in vivo. To aid in our analysis, we were able to initiate collaboration with experts (S. Leiken, PhD., NIH, Bethesda, MD) that use more sensitive techniques in examining proteolytic mechanisms of MMPs, and continued my project focusing on the *in vivo* expression of MMPs.

MMP-2 Preferential Cleavage of Heterotrimer

Analysis of the degradation of homotrimeric type I collagen as compared to heterotrimeric type I collagen by MMP-2 consistently showed preferential cleavage of the native heterotrimer (data reported here was obtained from calculating the averages of six proteolytic experiments in which several vials of commercial MMP-2 were combined

and used within days). As depicted in the Figure III-4, MMP-2 cleaved heterotrimer (slope = 10555 arbitrary units/min) at twice the rate of homotrimer (slope = 5288 arbitrary units/min) in a biphasic pattern (p=0.03). To determine the initial rate of cleavage, the slope of the first four points on the graph were used to determine the rates of cleavage, before the second phase of degradation began. This data is supported by preliminary work previously performed by our lab that demonstrated greater MMP-2 degradation of heterotrimer in a 24-hour period as compared to homotrimer (Figure III-4).

MMP-9 Preference is Inconclusive

Analysis of MMP-9 using the slope of the first five points of the graph showed no significant differences in degradation rates between homotrimer (slope = 4660 arbitrary units/min) and heterotrimer (slope = 4096 arbitrary units/min; p=0.78) (Figure III-5). Whether this is true or a reflection of low sample numbers, high variability and/or low assay sensitivity is uncertain. The data reported here was obtained from calculating the averages of three successful proteolytic experiments.

MMP-13 Preference is Inconclusive

Analysis of MMP-13, again using the slope of the first four points of the graph, showed no significant difference in the proteolytic preference of homotrimer (slope = 8476 arbitrary units/min) as compared to heterotrimer (slope = 6087 arbitrary units/min; p=0.59) (Figure III-6). Again, whether this is true or a reflection of low sample numbers, high variability and/or low assay sensitivity is uncertain. MMP-13 proteolytic

experimental data reported here was obtained from calculating the averages of four successful proteolytic experiments. Whether due to the inherent reduced sensitivity of both the Sircol collagen quantitation method and the proteolytic assay, the collagen degradative preference has proved difficult to determine using our above described proteolysis method for MMP-3 and MMP-13.

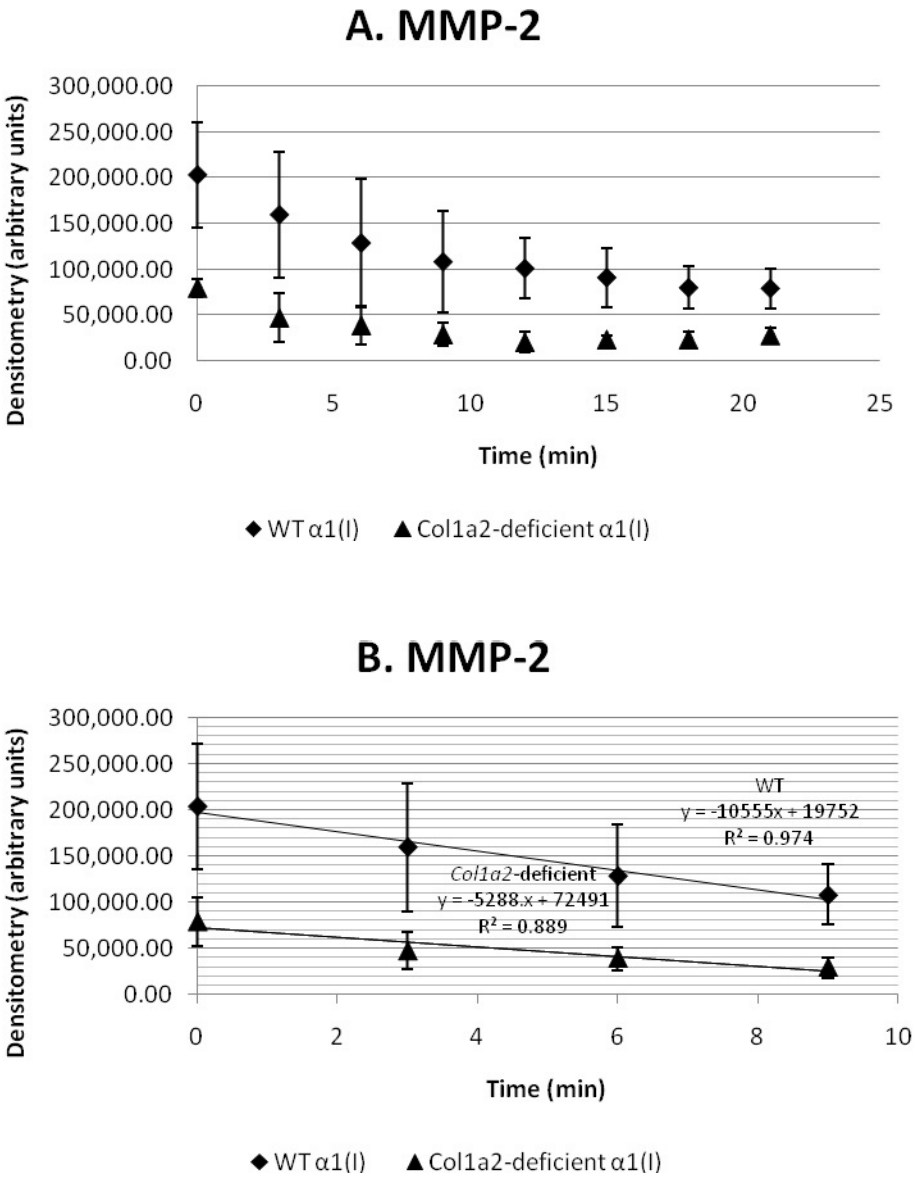


Figure III-4. Proteolytic cleavage of homotrimeric type I collagen as compared to heterotrimer by MMP-2. MMP-2 biphasic degradation of type I collagen α1(I) chains in homotrimer shows a slower rate of cleavage as compared to heterotrimeric collagen (A). Examination of the initial velocity of MMP-2 degradation of heterotrimeric α1(I) chains shows a steeper slope as compared to homotrimer (B)($p=0.03$).

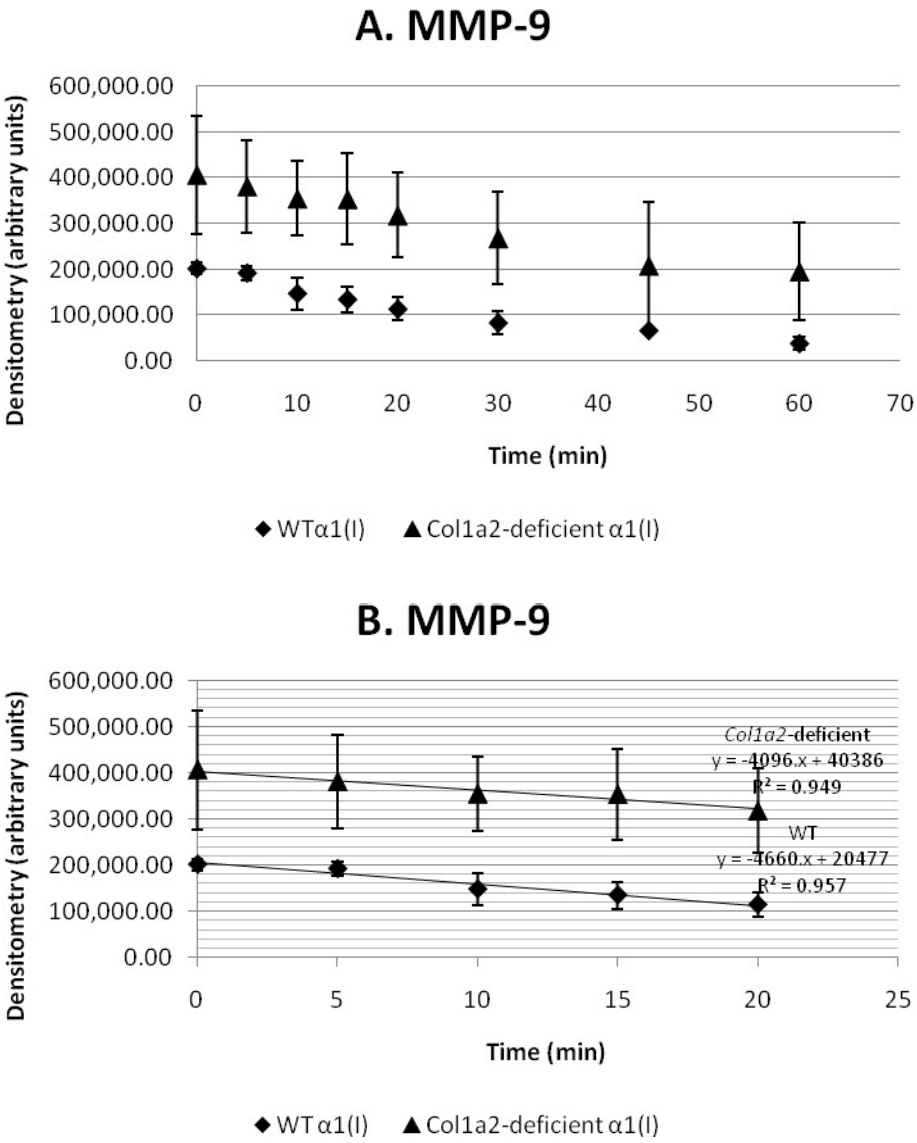


Figure III-5. Proteolytic cleavage of homotrimeric type I collagen as compared to heterotrimer by MMP-9. MMP-9 biphasic degradation of type I collagen α1(I) chains in homotrimer shows a similar rate of cleavage as compared to heterotrimeric collagen (A). Examination of the initial velocity of MMP-9 degradation of heterotrimeric α1(I) chains shows a similar slope as compared to homotrimer (B) (p=0.78).

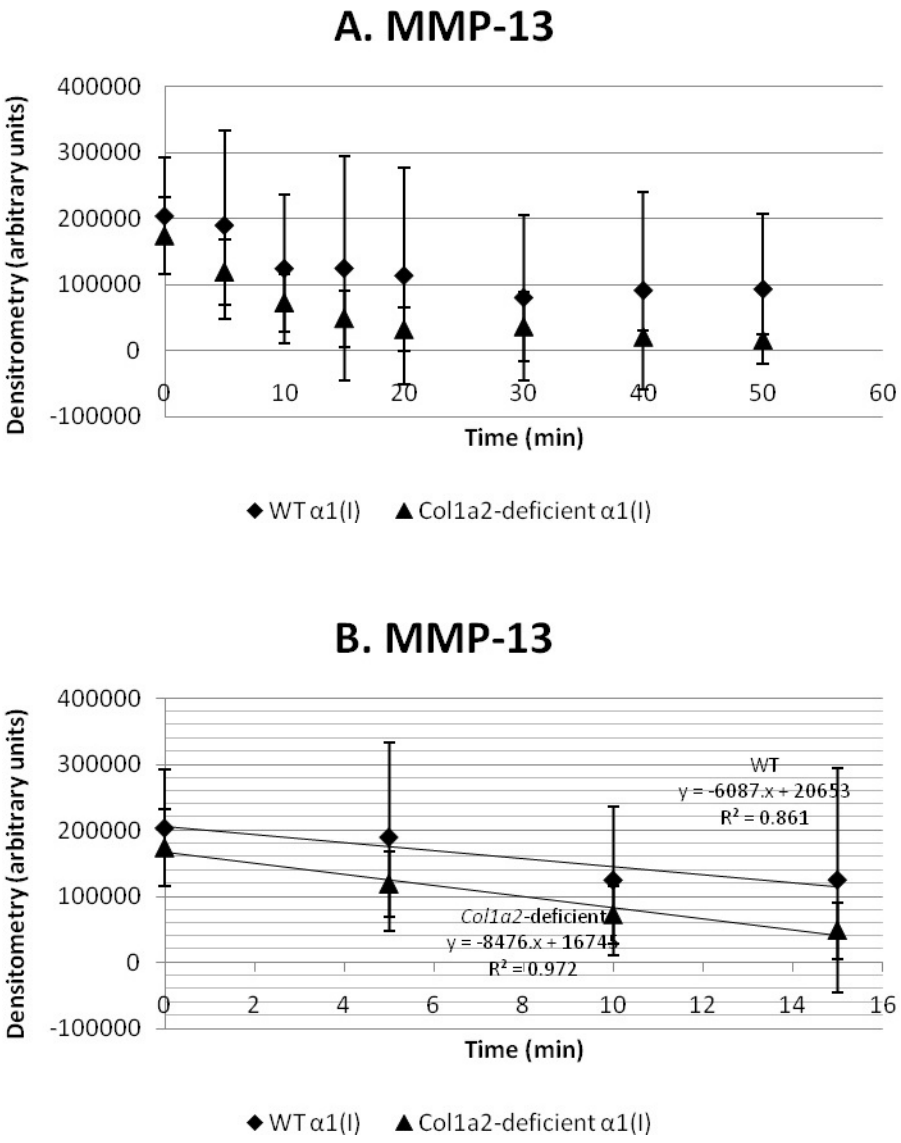


Figure III-6. Proteolytic cleavage of homotrimeric type I collagen as compared to heterotrimer by MMP-13. MMP-13 biphasic degradation of type I collagen α1(I) chains in homotrimer shows a similar rate of cleavage as compared to heterotrimeric collagen (A). Examination of the initial velocity of MMP-13 degradation of heterotrimeric α1(I) chains shows a similar slope as compared to homotrimer (B) (p=0.59).

Zymography Optimization

Qualitative analyses of MMPs can be efficiently examined using zymography. Many laboratories purchase pre-made collagen or casein zymogen gels for this use, however, because we where examining the MMP degradative efficiencies on two type I collagen substrates (homotrimer and heterotrimer) currently unavailable commercially, we needed to generate our own homo- and heterotrimeric type I collagen zymogen gels. This technique required much optimization.

Our first area of zymogen optimization was determining the appropriate collagen concentration for gels. We started with 10% collagen in our zymogen gels (an amount commonly used in commercial zymogens), but found that adding this amount of collagen prevented even mixing of the solution before polymerization and large amounts of enzyme were needed to identify degradation bands. Additionally, using higher concentrations of collagen in the zymogen allowed for more intense protein staining, masking signal intensity of MMP degradation bands. Evaluation of additional collagen concentrations (8%, 4% and 2%) was preformed and 4% collagen content in zymogen gels was determined to give the clearest results.

Next, optimization of collagen preparation to be used in zymogen gels was examined. As described in "Methods", extracted collagen is dissolved and stored in 0.1N acetic acid. We therefore needed to determine whether removal of acetic acid by dialysis or speed-vacuum methods was necessary for maximum MMP degradation. After a series of experiments using these different types of collagen preparation in our zymogen gels, we concluded that the presence of 0.1N acetic acid did not interfere with MMP activity for optimal degradative results.

Finally, we wanted to determine whether integration of collagen into the zymogen gel mix was improved by heating. Collagen triple-helices denature sharply between 41˚C and 43˚C, and once cooled the α-chains do not assemble themselves in their original triple-helical arrangement, but bind to each other randomly to form a mesh of α-chain molecules, becoming gelatin (Makareeva & Leikin, 2008). Therefore, we chose to heat the protein to 37˚C for 20 min, below the melting temperature of type I collagen to ensure that the collagen molecules remained in their triple-helical form. Indeed, we saw better integration of the collagen into the zymogen gel mix after heating. We also determined that staining of zymogen was cleaner and more even using GelCode Blue stain (Pierce Scientific) as compared to Coomassie staining. Our final conditions for zymogen gel production were adding 4% collagen heated to 37˚C for 20 minutes prior to adding to zymogen gel solution.

Further optimization of commercial MMP (MMP-1, -2, -9 and -13) was performed to ensure maximum degradative activity; however the commercial MMPs proved difficult to work with because once activated, they inconsistently cleaved the collagen zymogen, producing sporadic and unreproducible degradative results. As mentioned earlier, these troubles were not confined to this zymographic technique and therefore we decided to see whether MMPs in glomerular lysates would give us consistent degradative results. We decided to evaluate the relative degradation of wildtype, heterozygous and *Col1a2*-deficient glomerular MMPs.

Zymogen analysis of activated and unactivated MMPs in isolated glomeruli showed more intense degradation patterns on zymogens containing heterotrimer than were seen on zymogens containing homotrimer. (Figure III-7) Analysis of the

heterotrimeric zymogen shows strong proteolytic cleavage by the MMP control (a mixture of human MMP-1, -2, -3, -9 and TIMP-1, and -2; Sigma Aldrich, St Louis, MO)(A) and glomeruli isolated from heterozygous *Col1a2*-deficient mice. Although unactivated heterozygous glomeruli (Figure III-7D) did not display the same banding pattern as was seen in the activated glomeruli (Figure III-7C) (suggesting that many MMPs were not all in their active form when extracted from the kidney), there was still strong degradation by latent glomerular MMPs. This pattern of degradation was also seen in the activated (Figure III-7E) and unactivated (Figure III-7F) glomerular extracts isolated from *Col1a2*-deficient kidneys. However, equal amounts of MMP control, activated and unactivated heterozygous and *Col1a2*-deficient glomeruli cleaved zymogen containing homotrimeric type I collagen with much less intensity, suggesting that there is less degradation of the homotrimeric zymogen.

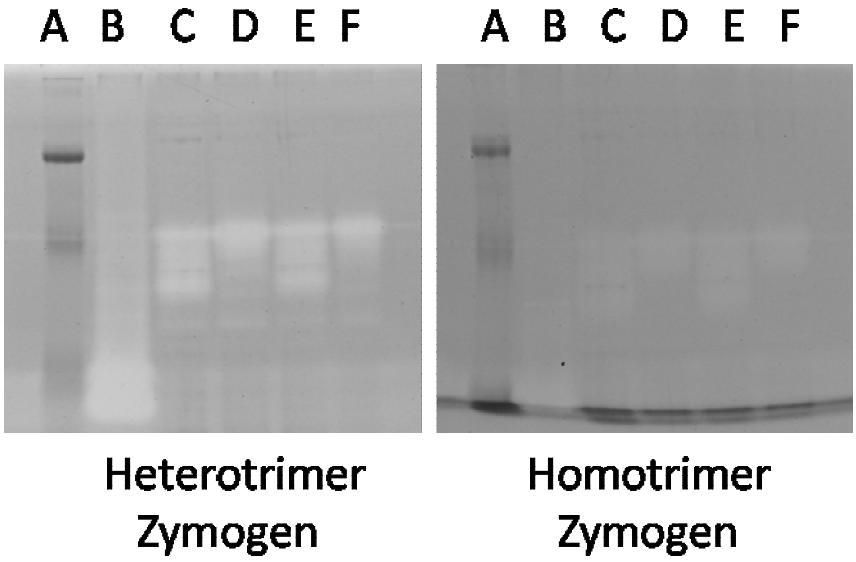


Figure III-7. Collagen zymography demonstrating the affect of glomerular proteinases on zymograms composed of heterotrimeric (left) and homotrimeric (right) type I collagen. Activated glomerular extracted proteinases cleave heterotrimeric type I collagen zymogen by showing intense degradation patterns. Molecular marker (A), MMP control (B), heterozygous mouse glomerular extract (322 glomeruli per loaded well) activated (C) and unactivated (D). *Col1a2*-deficient mouse glomerular extract (290 glomeruli loaded per well) activated (E) and unactivated (F). The degradation banding pattern is not as intense in the homotrimeric type I collagen zymogen gel, as seen on the heterotrimeric type I collagen zymogen.

DISCUSSION

Glomerulosclerosis has been described as an imbalance between collagen synthesis and degradation. As reported, the *Col1a2*-deficient mouse model has increased synthesis of COL1A1 steady state mRNA in glomeruli at one and three months of age. We further wanted to evaluate whether the degradative mechanisms of MMPs also had a role in homotrimer accumulation within affected glomeruli. Our preliminary hypothesis for the role of MMPs in the accumulation of homotrimer within sclerotic glomeruli of the *Col1a2*-deficient mouse model was that renal MMP expression was unchanged because they were unable to efficiently recognize homotrimer as its preferred substrate. By quantitative real-time PCR analysis of glomerular MMPs-2, -3 and -9, we partially confirmed this hypothesis in that the transcript expression of each MMP remained at wildtype levels at one month of age, even though type I collagen had accumulated in the renal mesangium. MMPs did show significant increases in expression at three months of age, and further we showed increases in MMP-2, and -3 protein expression at both one and three months of age. This data suggests a potential role of MMPs in *Col1a2*-deficient glomerulosclerosis as possibly not recognizing homotrimer as a preferred substrate, and therefore not increasing glomerular MMP synthesis until sclerosis is severe.

But are MMPs really not recognizing homotrimer as their preferred substrate? This question and preliminary data by Amanda Brodeur and Jason Gentry suggested that there may be a degradative preference by several renal MMPs and lead us to further examine this question. The above proteolytic data suggests that at least MMP-2 preferentially cleaves heterotrimer at a faster rate than homotrimer; further suggesting

that MMP-2 is removing homotrimer less efficiently than heterotrimer, allowing homotrimer accumulation within the glomerular space. Amanda Brodeur and Jason Gentry originally examined MMPs-1, -2, -3, -8, -9, and -13. We chose to only examine MMPs-2, -9 and -13 because the mouse ortholog of MMP-1 has been found to only be expressed embryonically and during early development in mice and MMP-8 is not expressed in the kidney. Additionally, MMP-3 proved to be very difficult to optimize using the techniques outlined in this chapter. Recent data from our collaborators (S. Leikin, PhD., E. Makareeva, PhD., NIH, Bethesda, MD) presented evidence that also suggests that MMP-1 and MMP-13 cleaves homotrimer at a slower rate than homotrimer using Cy5 fluorescent analysis (Makareeva & Leiken, 2009; Makareeva & Leikin, 2008).

Further, zymographic analyses of glomerular MMPs reinforce this hypothesis, where both activated and unactivated glomerular MMPs did not degrade homotrimer as efficiently as heterotrimer. Additionally, by zymography it can be seen that the degradative pattern of both *Col1a2*-deficient and heterozygous glomerular MMPs cleaved heterotrimeric type I collagen zymogens similarly, if not identically. This suggests that the MMP expression patterns are not grossly altered in *Col1a2*-glomerulosclerosis.

Does the loss of the α2(I) chain in homotrimer affect the mechanism of degradation? Chung, et al. and Orgel, et al., demonstrated that indeed the α2(I) chain has an important role in the initiation of type I collagen degradation by MMP-1, suggesting that in its absence degradation can be slowed down. They have hypothesized that MMP-1 binds the locally unwound α2(I) chain and subsequently cleaves the remaining α1(I) chains during the degradation process (Chung, 2004; Perumal & Orgel, 2008).

Additional studies are being done to determine whether this is the case with other collagenases.

Finally, we have shown that the absence of α2(I) chains significantly reduces the efficiency of MMP-2 cleavage, possibly leading to inefficient removal of the homotrimeric isotype in the sclerotic glomeruli of *Col1a2*-deficient mice. Subsequently, the *Col1a2*-deficient kidney does not respond to the degradative retardation until sclerosis is severe at three months of age, as reflected by the upregulation of MMPs-2, -3 and -9 transcripts. Taken together, this data suggests the mechanisms of sclerosis in this mouse model are due to both a synthesis and degradative mechanism. It would follow that increases in MMP expression in several glomerular disease, may be due to homotrimer synthesis and accumulation. However, further studies in various studies of glomerular disease will have to be performed to validate this theory before a general mechanism for glomerulosclerotic disease can be proposed.

CHAPTER IV.

ANALYSIS OF TRANSFORMIMG GROWTH FACTOR - β DURING THE PROGRESSION OF GLOMERULOSCLEROSIS IN *COL1A2*-DEFICIENT MICE

INTRODUCTION

Transforming Growth Factor-β, the wonder cytokine, has many roles. It has been shown to be involved in growth regulation and development (Goumans, 1998), cell differentiation, cellular adhesion and apoptosis (Miyazono, 2000; Roberts & Sporn, 1990). It is found as one of three isoforms, TGFβ-1, TGFβ-2 and TGFβ-3, each having overlapping functions and the ability to bind the TGFβ receptors-1 and -2 (Bottinger, 1997; Tang, 1994). TGFβ is a part of a larger family of proteins that include over 30 members, including bone morphogenic protein (Kingsley, 1994; Harland, 1997). TGFβ is synthesized as a two separate components, and dimerization allows formation of an active ligand that binds to either of the serine/threonine kinase receptors, TGFβ-receptor -1 or TGFβ-receptor-2. The TGFβ-receptor-2 is constitutively expressed and can further activate TGFβ-receptor-1. These two receptors work only in the presence of the each other (Massague, 1992; Ebner, 1993). Once activated, TGFβ-receptor-1 regulates the cell via the SMAD intercellular signaling pathway. All subsequent references to TGFβ will be referring to isoform TGFβ1.

TGFβ has been shown to be a key anti-inflammatory and profibrotic component in tissue repair and wound healing by increasing ECM deposition (Border, 1994) and down-regulating MMP expression at the site of a wound (Baricos, 1999; Uchio, 2000). TGFβ has also been implicated as an initiator and facilitator of epithelial/endothelial-to-mesenchymal cell transitions (EMT) associated with sclerosis. During repair, TGFβ is signaled locally by myofibroblasts and macrophages to increase ECM production. However, during fibrosis, TGFβ has been shown to be continually expressed, indicating a

fibrotic process that has "gone wild". Transgenic mice that over-express hepatic TGFβ display high circulating levels of TGFβ synthesized in the liver and induces severe glomerulosclerosis starting at 3 weeks of age (Sanderson, 1995; Mozes, 1999).

During glomerulopathy, many other factors are altered contributing to a sclerotic phenotype. Among them, platelet derived growth factor (PDGF) has major roles in development of the cranial and cardiac neuronal crests (Morrison-Graham, 1992; Tallquist, 2003), but its notoriety has been its roles in myofibroblast proliferation, migration and expression of MMPs (Andrae, 2008). PDGF has also been implicated to have roles outside of sclerotic diseases, such as in cancer [synthesis is stimulated by TGFβ, IL1b and TNFα]. Vascular endothelial cell growth factor (VEGF) stimulates angiogenesis under normal conditions and sclerotic conditions, and promotes endothelial cell permeability for cytokine infiltration as outlined in the introduction. Pro-inflammatory cytokines interlukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) primarily synthesized by macrophages also play a role in sclerosis. IL-1β aids leukocyte passage through blood vessel walls during infection and TNFα promotes fibroblast proliferation and migration during sclerosis. Additionally, the proinflammatory chemokines 'regulated upon activation normal T-cell expressed and presumably secreted' (RANTES) and monocyte chemotactic protein-1 (MCP-1) contribute to wound healing and sclerosis by regulating leukocyte migration and activation (Conti, 2001).

Because of their roles in wound healing and fibrosis, we sought to evaluate the role of TGFβ in *Col1a2*-deficient glomerulosclerosis, and to determine by a discovery approach whether other cytokines IL-1b and TNFα, chemokines RANTES and MCP-1, and growth factors PDGF and VEGF are involved in the *Col1a2*-deficient

glomerulosclerotic process. We hypothesize that during *Col1a2*-deficient glomerulosclerosis TGFβ is upregulated as seen in other glomerulosclerotic diseases, contributing to the accumulation of homotrimer. Examination of TGF-β cytokine expression in the glomeruli of *Col1a2*-deficient mice during the progression of glomerulopathy was performed by quantitative real-time PCR and protein array analysis.

METHODS

Animals

Animals were housed, cared for and genotyped as described in chapter II.

Animals were divided into two age groups 1-month (n=50 mice) and 3-months (n=40) of age. Animals were sacrificed and glomeruli harvested as previously described, followed by snap freezing in liquid nitrogen, and storage at -80ºC.

Glomerular Isolation

Wildtype, heterozygous and *Col1a2*-deficient mice were aged to 1-month [-/-, n=20; +/+, n=20; and +/-, n=10] and 3-months [-/-, n=15; +/+, n=15; and +/-, n=10] of age and anesthetized prior to kidney perfusion. Perfusion was performed as previously described (pg 54).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Snap frozen glomeruli from 1-month [n=10 -/- (lesion score G3-4), n=10 +/+ (lesion score G0), and n=10 +/- (lesion score G1-4)] and 3-month [n=10 -/- (lesion score G1-4), n=10 +/+ (lesion score G0), and n=10 +/- (lesion score G1)] old mice were homogenized as previously described (pg 56). Primer sequences for hypoxanthine-guanine phosphoribosyltransferase (HPRT) have been previously reported (O'Garra, 1992). All values were normalized to HPRT levels.

SearchLight Protein Assay

Snap frozen glomeruli from 1-month [n=11 -/-, and n=9 +/+] and 3-month [n=5 -/-, and n=5 +/+] wildtype and *Col1a2*-deficient mice were thawed on ice, prepared and analyzed as previously described (pg 59).

Statistics

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC). The RT-PCR and protein data was analyzed as a completely randomized design in which genotype and age were arranged as a 3 by 2 factorial (3 genotypes, 2 ages). Data presented are the actual mean and standard error but the differences within genotype and age were analyzed using the log or rank of the mean values. Mean differences were ascertained using Bartlett's Homogeneity Test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

TGFβ

Steady state mRNA expression of TGFβ shows no significant increase in *Col1a2*-deficient glomeruli at one month of age compared to age-matched wildtype mice (26083 ± 2629; 20337 ± 2291 copy number), but at three months of age *Col1a2*-deficient mouse glomeruli exhibit a significant decrease in TGFβ transcripts as compared to wildtype glomeruli (29917 ± 3470; 44411 ± 2245 copy number; p=0.0001) (Figure IV-1)

However, at both one and three months of age, *Col1a2*-deficient glomeruli do not exhibit significant differences in TGFβ protein levels as compared to age-matched wildtype mice (0.09 ± 0.03; 0.03 ± 0.01 pg protein per glomerulus; p=0.28, 1-month *Col1a2*-deficient and wildtype respectively; 0.05 ± 0.02; 0.03 ± 0.01 pg protein per glomerulus; p=0.98, 3-month *Col1a2*-deficient and wildtype respectively) (Figure IV-1). At one month of age glomeruli from two different *Col1a2*-deficient mice exhibited higher levels of TGFβ per glomeruli than any wildtype glomeruli, though the eight remaining appear to have values equivalent to wildtype glomeruli. However, when Grubb's Outlier test was performed, neither value was considered and outlier. These findings suggest that TGFβ may not have a role in ECM deposition seen in glomerulosclerotic kidneys of the *Col1a2*-deficient mouse model.

Other Cytokines and Growth Factors

IL-1β, MCP-1 and VEGF, which have been shown to be involved in glomerulosclerosis, all show trends of increased protein expression in 3 month *Col1a2*-

deficient glomeruli by SearchLight analysis, however, these findings are not significant (Figure IV-2). TNFα show a trend of decreased protein expression in 3 month *Col1a2*-deficient glomeruli, although this finding is also not significant. Finally, RANTES shows a significant decrease in *Col1a2*-deficient glomerular protein expression ($p=0.03$), while PDGF shows a significant increase in *Col1a2*-deficient glomerular protein expression ($p=0.04$) (Figure IV-2).

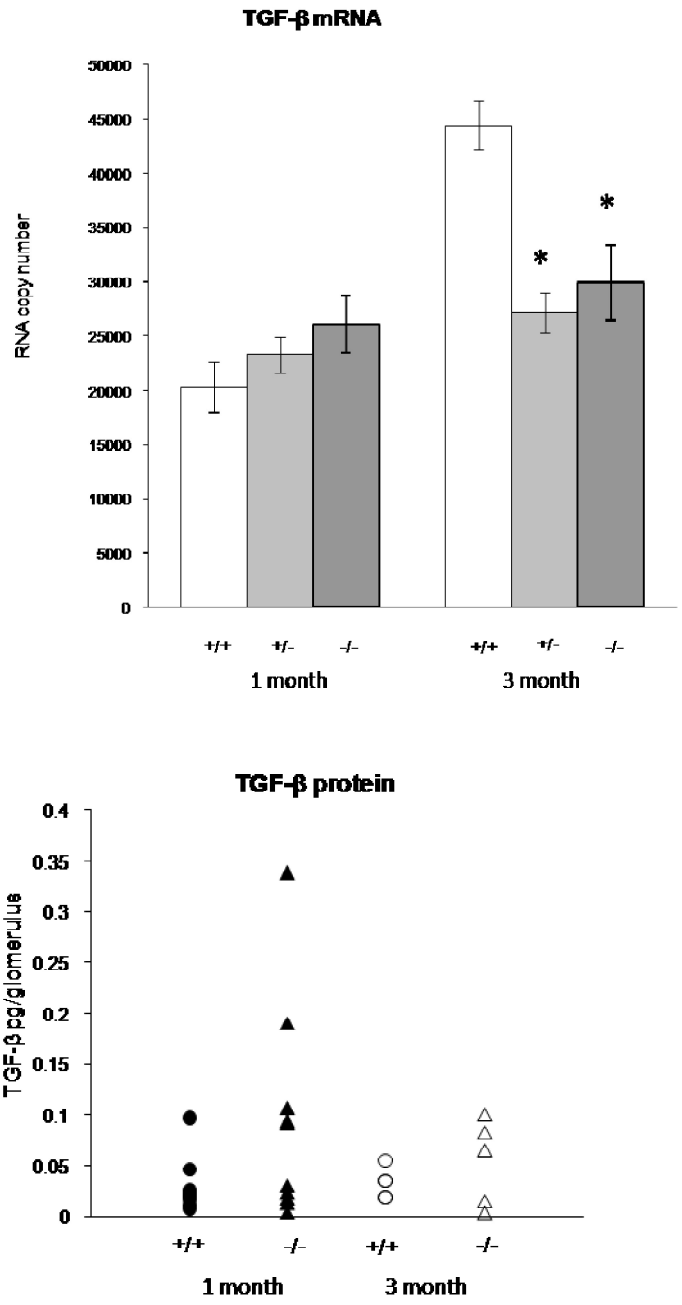


Figure IV-1. TGF-β mRNA (top) and protein expression (bottom). Heterozygous (+/-) and *Col1a2*-deficient (-/-) mice demonstrate significant reductions in TGF-β steady state mRNA expression at three months of age as compared to wildtype (+/+) mice. *Col1a2*-deficient glomeruli do not show significant differences in TGF-β protein expression at

Figure IV-1 cont'd. either one or three months of age, therefore the trends seen here are not significant. Data evaluated by Grubb's Outlier Test shows no outliers.

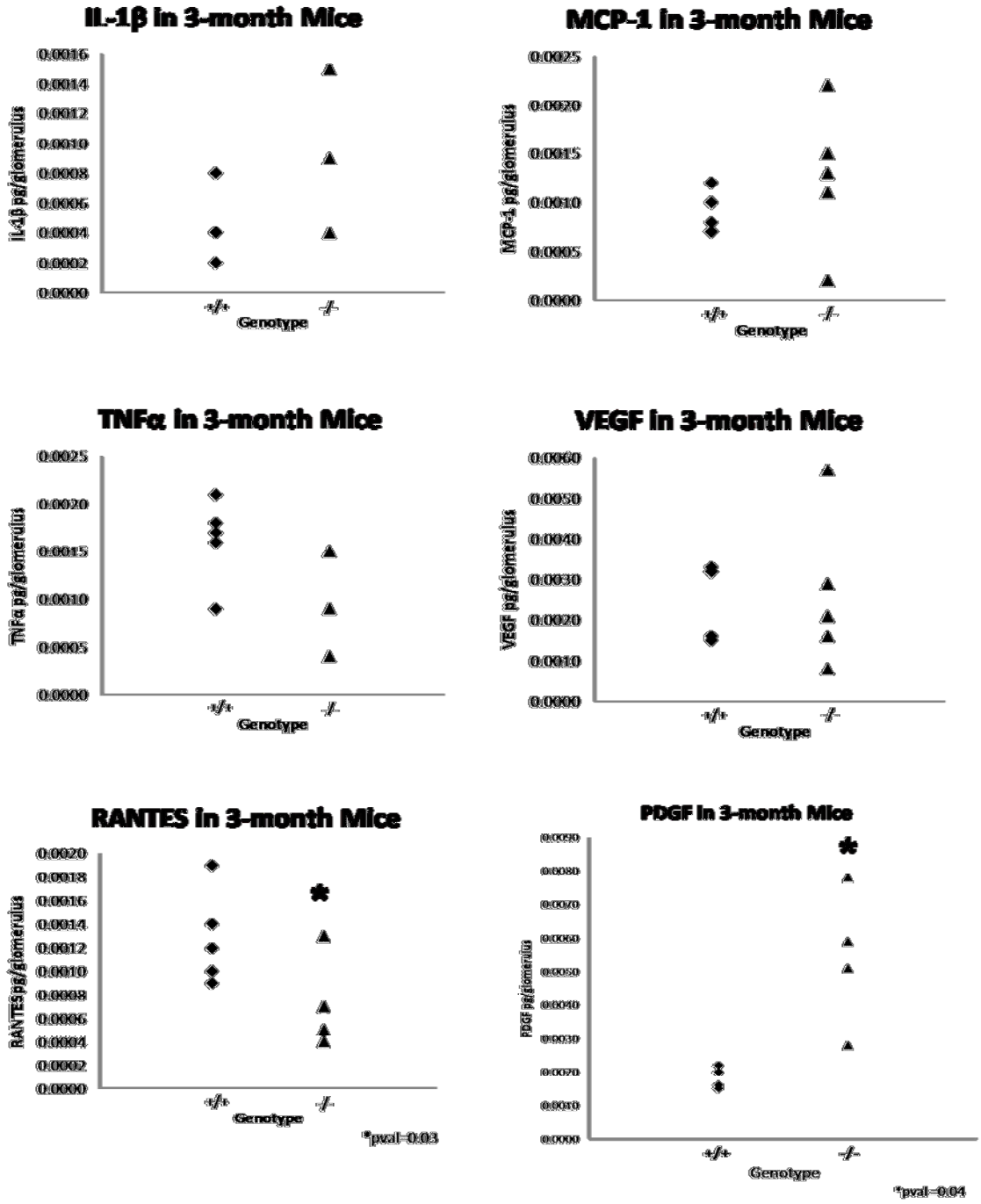


Figure IV-2. Evaluation of IL-1β, MCP-1, TNFα, VEGF, PDGF and RANTES in 3 month *Col1a2*-deficient (-/-) mouse glomeruli as compared to wildtype (+/+) mice. Cytokines IL-1β, MCP-1, TNFα and the growth factor VEGF, show no significant

differences between **Figure IV-2 cont'd.** *Col1a2*-deficient glomeruli and wildtype glomeruli. The cytokine RANTES shows a significant decrease in protein levels in *Col1a2*-deficient glomeruli as compared to wildtype glomeruli. There is a significant increase in PDGF protein levels in *Col1a2*-deficient glomeruli as compared to wildtype glomeruli. * indicates $p \leq 0.05$.

Table IV-1. Cytokine Protein Values in Glomeruli of Three Month Hybrid Mice

PDGFb (pg/glom)		MCP (pg/glom)		TNFα (pg/glom)	
Wildtype	*Col1a2*-Deficient	Wildtype	*Col1a2*-Deficient	Wildtype	*Col1a2*-Deficient
n=5	n=4	n=5	n=5	n=5	n=4
0.0022	0.0078	0.0007	0.0022	0.0016	0.0015
0.0020	0.0059	0.0008	0.0013	0.0009	0.0015
0.0015	0.0051	0.0012	0.0002	0.0021	0.0009
0.0016	0.0028	0.0010	0.0011	0.0017	0.0004
0.0020		0.0007	0.0015	0.0018	
p=0.04		p=0.31		p=0.15	

VEGF (pg/glom)		IL-1β (pg/glom)		RANTES (pg/glom)	
pg/glom		pg/glom		pg/glom	
Wildtype	*Col1a2*-Deficient	Wildtype	*Col1a2*-Deficient	Wildtype	*Col1a2*-Deficient
n=5	n=5	n=5	n=4	n=5	n=5
0.0015	0.0057	0.0004	0.0015	0.0012	0.0007
0.0016	0.0021	0.0002	0.0015	0.0014	0.0005
0.0032	0.0029	0.0008	0.0009	0.0009	0.0013
0.0032	0.0016	0.0004	0.0004	0.0010	0.0004
0.0033	0.0008	0.0004		0.0019	0.0005
p=0.95		p=0.09		p=0.04	

DISCUSSION

The pro-inflammatory effect of TGFβ aids in the wound healing response by stimulating the cells surrounding a wound to increase their type I collagen synthesis. At three months of age, the *Col1a2*-deficient mouse glomeruli show significant decreases in glomerular TGFβ mRNA synthesis, and no significant change in protein levels as compared to age-matched wildtype mouse glomeruli. This suggests that TGFβ may not play a role in the homotrimer accumulation seen in *Col1a2*-deficient glomeruli as we previously thought.

However, by our survey approach of potential candidates we did find that RANTES and PDGF levels changed significantly at three months of age in *Col1a2*-deficient mouse glomeruli compared to age matched wild-type glomeruli. RANTES has been shown to be a proinflammatory chemokine postulated to be responsible for macrophage and lymphocyte migration and activation (LLoyd, 1997; Zheng, 2004), and has also been suggested to have a role in cell proliferation (Kim, 2001). RANTES has also been shown to be expressed by lymphocytes (Schall, 1988; Lui H. , 1999). Additionally, breast cancer cells were also shown to secrete high levels of RANTES to enhance cell motility, invasion and metastasis (Karnoub, 2007), and RANTES has been implicated as an HIV suppressor (Cocchi, 1995). Tyner et al, demonstrated that RANTES aided in the impediment of macrophage apoptosis in RANTES-deficient 'murine-parainfluenza-virus effected' mice, exhibiting retarded clearance of the parainfluenza-virus and excessive airway inflammation, resulting in death (Tyner, 2005). In allograft renal transplant patients, high circulating levels of RANTES have been associated with kidney rejection, and inhibition of RANTES improved renal transplant rates (Song,

2002). In an accelerated mouse model of glomerular cresentous, blocking RANTES synthesis translated into reduced proteinuria and decreases in mononuclear cell accumulation (LLoyd, 1997), and mediating renal interstitial fibrosis (Gong, 2004).

We have also shown that PDGF significantly increases in 3 month *Col1a2*-deficient glomeruli as compared to age-matched wildtype glomeruli. And as mentioned earlier, PDGF is known to have a significant role in recruitment and proliferation of myofibroblasts and smooth muscle cells, as well as MMP expression. Although research of PDGF participation in glomerulosclerosis has been mostly associative, it has been shown that during glomerular disease PDGF and its receptors are upregulated (Fellstrom, 1989; Floege, 1999; Johnson, 1992). Interestingly, Tang et al., was able to show that systemic administration of PDGF resulted in a proliferation of mesangial cells, independent of inflammation (Tang, 1996) suggesting that the upregulation of PDGF in *Col1a2*-deficient glomeruli may not be related to an inflammation mechanism.

So how does this new information fit into the big picture? The absence of TGFβ involvement may suggest that increased PDGF expression may also be occurring independent of inflammation, although macrophages have also been shown to express PDGF (Andrae, 2008). However, because the *Col1a2*-deficient mouse model exhibts mesangial space expansion due to accumulated homotrimer, and not mesangial cell expansion due to mesangial cell proliferation, it is plausible that PDGF may be acting on locally increasing MMP-2 and -3 expression at three months of age (as outlined in Chapter II) and myofibroblast recruitment. Preliminary data in our lab suggests that there is an increase in α-smooth muscle actin, a marker of the presence of myofibroblasts and

EMT at 3-months of age, around the glomerualr tuft region in the *Col1a2*-deficient kidney (data not shown), and PDGF may be involved.

The down-regulation of RANTES has been more difficult to resolve. RANTES exclusive expression during inflammation has been shown to recruit lymophocytes and macrophages as mentioned earlier, but does not seem to be applicable in *Col1a2*-deficient glomeruli because inflammation is not seen in these mice until sclerosis is very severe. Additionally, the down-regulation of both TGFβ and RANTES compared to age-matched wildtype mice seems puzzling, rather than there being no change in expression within glomeruli between *Col1a2*-deficient and wildtype kidneys. However, we can postulate that down-regulation of TGFβ and RANTES may 'ward-off' inflammation by reducing circulating lymphocyte and macrophage signalling and infiltration during sclerosis, or they may initiate when kidney functionality is further reduced and tubulointerstital fibrosis is seen (beyond the scope of the present studies). Because inflammation is seen in *Col1a2*-deficient glomerulosclerosis when homotrimer accumulation is severe, it would follow that the decrease in the expressions of TGFβ and RANTES to prevent or reduce inflammation may be occurring as a secondary event, possibly only after podocyte effacement becomes a part of the pathology. However, prior to this, the initiating events in *Col1a2*-deficient glomerulosclerosis is most likely a combined cause of the mouse's inefficiency to degrade homotrimer at a comparable rate and the increases in COL1A1 mRNA transcript expression, both leading to homotrimer accumulation and its subsequent pathology. To further prove this theory, PDGF and RANTES protein expression and should be evaluated at one month of age and localization by IHC should be evaluated at both one and three months of age in *Col1a2*-deficient mouse glomeruli.

CHAPTER V.

PERSPECTIVES AND FUTURE DIRECTIONS

In this body of work I have been able to demonstrate that in the *Col1a2*-deficient mouse model there is an imbalance between the synthesis and degradation mechanism within the glomeruli. *Col1a2*-deficient glomeruli had increased expression levels of steady state COL1A1 mRNA for homotrimer synthesis, and MMP-2 degraded homotrimeric type I collagen at half the rate of heterotrimeric type I collagen. The increased protein synthesis and delayed removal of homotrimer leaves the protein to accumulate within the glomerular mesangial space leading to sclerosis. This imbalance may be the initiating event in *Col1a2*-deficient glomerulosclerosis. In many sclerotic kidney diseases the initiating event is elusive, and can only be correlated to other malfunctions in the body such as diabetes and hypertension associated with cardiovascular disease. This finding pinpoints the inefficiency of MMPs to degrade homotrimer as the possible catalyst causing glomerulosclerosis.

However, it is still unclear why the type I collagen accumulation phenomenon is only occurring in the glomeruli of the *Col1a2*-deficient mouse and collagen accumulation is not seen between the intracellular space throughout the entire mouse body, or in other organs sensitive to sclerosis such as the liver. More over, under normal conditions the glomeruli do not synthesize appreciable amounts of type I collagen, so why does the synthesis of homotrimer trigger upregulation of type I collagen in the glomerulus, a mechanism only seen during injury? What makes the renal environment unique and sensitive during sclerosis? Taken together, this suggests that although MMPs expressed in the kidney may not be able to efficiently degrade homotrimer, other MMPs or proteases not expressed in the kidney (whose substrate is also type I collagen) may be able to, or that other mechanisms may be in place to regulate collagen expression that

may not be present in the kidney. For this reason, future directions for this project should include examination of MMP-8, a non-renal MMP that degrades type one collagen and at present has *not* been associated with sclerosis in the liver (Hemmann, 2007) or kidney (Catania, 2007), and the only other reported MMP whose substrate is also type I collagen (Chakraborti, 2003). Additionally, and more fundamentally, future studies should include the examination of possible homotrimeric insults to the kidney that lead to glomerulosclerosis. One such insult may be ER stress within the kidney due to accumulation or impaired removal of unincorporated α2(I) chains within mesangial cells that trigger an injury response, or injury brought on by the mechanical stress of filtration shortly after birth.

Additionally, I have demonstrated that TGFβ, RANTES, and PDGF expression changes are all secondary events during *Col1a2*-deficient glomerulosclerosis. TGFβ and RANTES seem to not be involved in the progression of the disease, but PDGF may be involved in myofibroblast activation and induction of MMP synthesis. Further, increases in MMP-2 and -3 mRNA steady state expressions occurring by 3 months of age suggests that increases in MMP expression is also a secondary event. This finding allows characterization of *Col1a2*-deficient glomerulosclerosis as a non-inflammatory disease model, a finding previously unknown, and that myofibroblast or EMT involvement may be contributing to the progression of sclerosis. Further studies should include the examination and localization of several other myofibroblast or EMT markers such as α-smooth muscle actin (αSMA), vimentin and desmin and if they have a role in the progression of this disease.

I was also able to demonstrate that homotrimeric type I collagen comprises approximately 97% of the accumulating collagen in the glomeruli of heterozygous mice. It is no surprise that homozygous *Col1a2*-deficient mice accumulate homotrimeric type I collagen, because they can only synthesize homotrimer. However, the accumulation of homotrimer in heterozygous glomeruli suggests that homotrimer is the pathogenic collagen in *Col1a2*-deficient glomerulosclerosis. This notion had previously been suggested by Haralson, et al., who demonstrated that cultured mesangial cells synthesize homotrimeric type I collagen, possibly in response to injury (Haralson, 1987). More widely, it is possible that homotrimeric type I collagen accumulation and its inefficient degradation by renal MMPs may occur in several other glomerulosclerotic diseases. In type I collagen resistant mouse models of wound healing (Beare, 2003) and liver fibrosis (Hemmann, 2007), the inability to degrade the resistant collagen aggravated sclerosis. The impact of determining whether homotrimeric type I collagen is pathogenic in other glomerulosclerotic diseases provides a new target for kidney disease therapy. The evaluation of glomeruli from several other glomerulosclerotic diseases for the presence of homotrimer should be examined in future studies.

The work presented here also suggests a mechanism for glomerulosclerosis in the *Col1a2*-deficent mouse model. The type I collagen mutation in these mice force them to only synthesize homotrimer, but the glomerulus does not seem to be able to efficiently degrade the protein and this may be the reason why accumulation is seen within one week of age in these mice. Probing further, it has been well established that rats and mice are not born with fully developed kidneys as is seen in humans, guinea pigs and dogs (Dickinson, 2005; Lelievre-Pegorier, 1998; Guron, 2000; Cebrian, 2004). Brodeur,

et al., had hypothesized that collagen accumulation in our mouse model follows a centrifugal postnatal activation pattern, beginning with filtration by the innermost glomeruli shortly after birth and moving outward toward the cortical glomeruli up to one month of age, as the kidney matures (Brodeur, 2007). However, this pattern of activation is based on a guinea pig model that is born with a fully developed kidney (Spitzer, 1974). Unlike guinea pigs, rats and mice continue to develop their kidneys by moving their glomeruli toward the renal cortex up to 5-7 days postnatally, after which time we had been able to identify the beginning of collagen accumulation in our mouse model (Cebrian, 2004; Dickinson, 2005; Brodeur, 2007). Once the rat and mouse kidneys are fully developed, glomerular activation resumes according to a similar pattern of maturation as seen in guinea pigs. Additionally, Pfeiffer, et al., had been able to demonstrate vascular differences in the *Col1a2*-deficient vasculature compared to their age-matched wildtype litter mates, in which the *Col1a2*-deficient aorta was found to be much weaker due to the synthesis of homotrimer (Pfeiffer, 2005). Taken together, it is possible that in *Col1a2*-deficient glomeruli homotrimer collagen accumulation begins once kidney is fully developed at 5-7 days of age when filtering is underway, but due to the reduced vascular integrity of the glomerular afferent and efferent arterioles the mechanical stress of the blood entering the glomerulus may induce a wound healing response within glomerulus, starting at the glomerular tuft region. Because the mesangial cells (of mesenchymal origin) actively respond to changes in blood pressure by stabilizing the capillaries through contraction and swelling, it would stand to reason that injury signaled by weakened capillaries would result in a mesangial pathology (Latta, 1992; Sakatsume, 1995; Herrera, 2006; Brenner, 2000).

Whatever the mode of injury, it follows that homotrimer accumulation continues unchecked in the glomerulus until the mesangial matrix has expanded, homotrimer accumulation progresses between the fenestrated subendothelial space via the glomerular

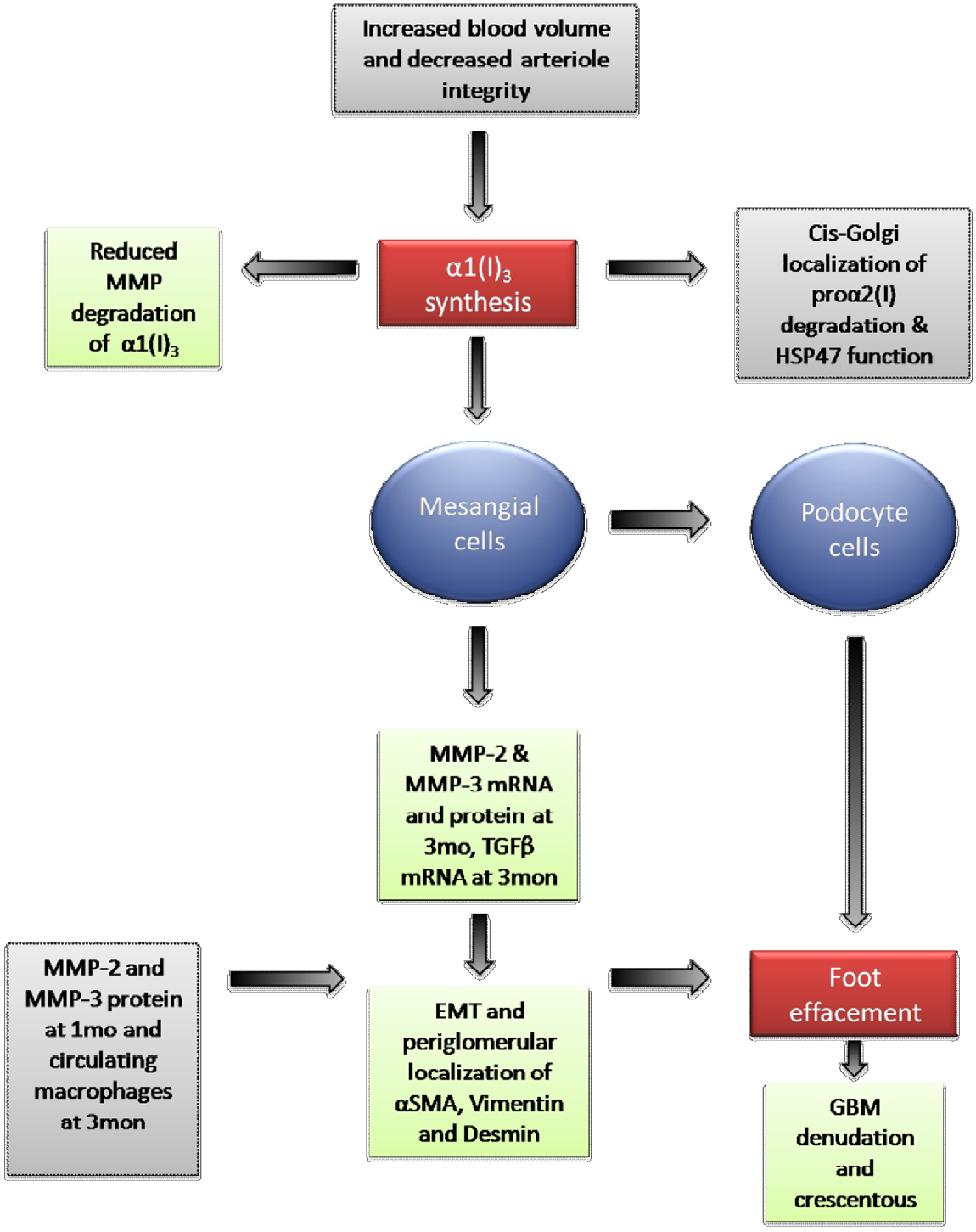


Figure V-1. Proposed Model of *Col1a2*-Deficient Glomerulosclerosis.

tuft region, and the podocytes become effaced and denuded. Consequently, as the glomeruli become more sclerotic and less useful, the mouse synthesizes more glomeruli to replace them, and as the kidney lesion scores increase, so does the presence of proteinuria in the urine. (Figure V-1)

The glomeruli then awaken and begin to recruit inflammatory cells and induce EMT to clean up the sclerosis and restore glomerular function, however it is too late. There is an increase in *Col1a2*-deficient glomerular PDGF at three months of age, suggesting myofibroblast activation and subsequent induction of MMP expression. This results in an increase in glomerular MMP-2, -3 and -9 steady state mRNA expression and an increase in MMP-2 and -3 protein expression at 3 months of age. Increases in MMP-2, and -3 protein expression at 1 month of age prior to the increases in mRNA expression may be explained by the infiltration or accumulation of circulating MMPs localizing in the sclerotic glomeruli. The decrease in MMP-9 protein expression suggests that it is not involved in the sclerotic process in this mouse model. The glomerular decrease of TGFβ steady state mRNA suggests that it is also not involved in *Col1a2*-deficient glomerulosclerosis, and may be acting to reduce homotrimeric type I collagen synthesis (Figure V-1).

Although the above theory sounds reasonable, a few more pieces will need to be added to this puzzle to lend credence to this hypothetical situation. Future directions for this project should examine the localization of HSP47 (the type I collagen chaperone) and the α2(I) chain, and the examination of the glomerular aggregates identified in the *Col1a2*-deficient kidneys by immunohistochemistry to help elucidate the fate, and possible "toxicity", of the α2(I) chain during sclerosis. The fate of the α2(I) chain has

never been conclusively determined. More over, further examination of MMP-3, -9 and -13 degradative efficiencies of homotrimer as compared to heterotrimer is needed to conclude that homotrimer accumulation is a result of reduced degradative efficiency and that a rescue effect is not occurring. Finally, macrophage infiltration should be examined to determine how large a role the wound healing process has in the progression of *Col1a2*-deficient glomerulosclerosis.

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VITA

Anna Maria Roberts-Pilgrim was born February 25th, 1977 and raised in Nassau, Bahamas. She was the sixth and final child of Thomas and Eleazer Roberts of Nassau, Bahamas. She attended and excelled in Kingsway Academy for both her elementary and high school education before beginning her college career. Her circuitous college career began at the local College of the Bahamas, Nassau, Bahamas where she completed her core courses in the area of Physics, followed by attendance at the University of North Carolina-Pembroke, Pembroke, NC where she completed her Bachelor of Science in the field of Chemistry and with a minor in Physics and got her first tastes of research under the guidance of Dr. Siva Mandjiny who encouraged her to continue her education in the field of Biochemistry. Following a successful and fruitful undergraduate career, she attended classes at the University of Miami, Miami, FL, in the field of Biomedical Engineering while working in research there for two years. During this time she met, fell in love and married Bronson A. Pilgrim. She then pursued her graduate career in the field of Biochemistry at the University of Missouri-Columbia, Columbia, MO, under the guidance of Dr. Charlotte L. Phillips PhD., where she successfully defended her doctorate on February 23, 2009. During her graduate career she gave birth to Cadence L. Pilgrim. Anna, Bronson and Cadence Pilgrim plan to stay in St. Louis, MO while she completes her post-doctoral research at Washington University, St. Louis, MO, and hope to continue her research in a warmer climate within 20 miles of the either coast.