Integrin $\alpha 1\beta 1$ and Transforming Growth Factor- $\beta 1$ Play Distinct Roles in Alport Glomerular Pathogenesis and Serve as Dual Targets for Metabolic Therapy

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Alport syndrome is a genetic disorder resulting from mutations in type IV collagen genes. The defect results in pathological changes in kidney glomerular and inner-ear basement membranes. In the kidney, progressive glomerulonephritis culminates in tubulointerstitial fibrosis and death. Using gene knockout-mouse models, we demonstrate that two different pathways, one mediated by transforming growth factor (TGF)- $\beta 1$ and the other by integrin $\alpha 1\beta 1$, affect Alport glomerular pathogenesis in distinct ways. In Alport mice that are also null for integrin $\alpha 1$ expression, expansion of the mesangial matrix and podocyte foot process effacement are attenuated. The novel observation of nonnative laminin isoforms (laminin-2 and/or laminin-4) accumulating in the glomerular basement membrane of Alport mice is markedly reduced in the double knockouts. The second pathway, mediated by TGF- $\beta 1$, was blocked using a soluble fusion protein comprising the extracellular domain of the TGF- $\beta 1$ type II receptor. This inhibitor prevents focal thickening of the glomerular basement membrane, but does not prevent effacement of the podocyte foot processes. If both integrin $\alpha 1\beta 1$ and TGF- $\beta 1$ pathways are functionally inhibited, glomerular foot process and glomerular basement membrane morphology are primarily restored and renal function is markedly improved. These data suggest that integrin $\alpha 1\beta 1$ and TGF- $\beta 1$ may provide useful targets for a dual therapy aimed at slowing disease progression in Alport glomerulonephritis. (*Am J Pathol* 2000, 157:1649–1659)

Alport syndrome is a hereditary basement membrane disease affecting approximately one in 5,000 people.¹ The disease is manifest by juvenile to adult onset progressive glomerulonephritis usually associated with a high-frequency-specific sensorineural hearing loss, dot and fleck retinopathy, and lens abnormalities. No effective drug therapy exists for this disease, which is currently treated by dialysis and renal transplant.^{1,2} The most common form of the disease is X-linked, and caused primarily by mutations in the collagen $\alpha 5(\text{IV})$ gene,³ accounting for ~80% of the cases. Mutations in the collagen $\alpha 3(\text{IV})$ or $\alpha 4(\text{IV})$ genes lead to the recessive forms of the disease.^{4,5} The absence of any one of these type IV collagen chains can result in the absence of all three chains in the glomerular basement membrane (GBM), presumably due to an obligatory association of the three chains in forming the type IV collagen superstructure.^{6,7} Normal distribution of the three α chains is observed in approximately one third of patients.⁸

The adult GBM contains a thin subendothelial network of collagen $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains, and a thick subepithelial network of collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains.⁹ These networks are thought to be physically separate from one another.^{10,11} In Alport syndrome the entire width of the GBM is comprised of collagen $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains, which is the normal collagen composition of the embryonic GBM.^{12,13} These changes result in progressive loss of glomerular function because of alterations in the GBM, podocyte effacement, and mesangial matrix expansion.

Type IV collagen networks comprised of only $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains are more susceptible to endoproteolysis than GBM containing all five type IV collagen chains,¹³ which is likely because of the greater number of

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crosslinks formed in a network of collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains.¹¹ Based on these observations, it has been proposed that the irregular ultrastructure of Alport GBM might be attributed to focal endoproteolysis of the GBM.

Two independently produced gene knockout murine models for Alport syndrome have been described,^{14,15} as well as one resulting from a random transgene insertion event.¹⁶ These models have proven to have progressive renal disease that is remarkably similar to that in humans.

Expansion of the mesangial matrix occurs early in Alport renal pathogenesis. The most abundant integrin on mesangial cells is the $\alpha 1\beta 1$ heterodimer.^{17,18} An $\alpha 1$ integrin knockout has been produced that shows no renal abnormalities and no phenotype detrimental to the survival of the animal.¹⁹ Considering the recently described roles for $\alpha 1\beta 1$ integrin in collagen-dependent cell proliferation, cell adhesion, mesangial matrix remodeling, and mesangial cell migration,^{19–21} we suspected that integrin $\alpha 1\beta 1$ might play a specific role in Alport renal disease progression. To test this notion, we produced a mouse null at both the collagen $\alpha 3(\text{IV})$ gene (Alport mouse) and the $\alpha 1$ integrin gene. These double-knockout mice have delayed onset and slowed progression of glomerular disease, attenuated expansion of the mesangial matrix, and markedly improved foot process architecture, illustrating a major role for $\alpha 1\beta 1$ integrin in Alport glomerular disease progression.

Transforming growth factor (TGF)- β has been shown to promote accumulation of extracellular matrix in both wound repair and fibrotic diseases, including glomerulonephritis.²² In recent studies, we demonstrated a likely role for TGF- $\beta 1$ in Alport glomerular and tubulointerstitial disease.²³ Herein, we extend these earlier studies by illustrating that inhibition of TGF- $\beta 1$, by injecting a type II TGF- β soluble receptor as a competitive inhibitor, prevents the irregular thickening of the GBM.

Treating the double knockouts with the TGF- $\beta 1$ soluble receptor provides synergistic benefits, restoring podocyte foot process architecture, inhibiting matrix deposition in the GBM, and slowing mesangial matrix expansion. Based on this new evidence, we conclude that renal pathogenesis in Alport syndrome involves biochemical pathways modulated by TGF- $\beta 1$ and integrin $\alpha 1\beta 1$, and that the two pathways affect distinct aspects of glomerular pathology.

Materials and Methods

Mice

The collagen $\alpha 3(\text{IV})$ knockout mice were described previously.¹⁴ These mice, which were originally in the 129 Sv/J background, were successively back-crossed 10 times with 129 Sv mice. Heterozygotes were then crossed with homozygote integrin $\alpha 1$ knockouts, also described previously,¹⁹ which were on a pure 129 Sv background. A breeding stock of mice heterozygous for the collagen $\alpha 3(\text{IV})$ mutation and homozygous for the

integrin $\alpha 1$ mutation was established. No difference in the kinetics of renal pathogenesis was observed for Alport mice on the 129 Sv/J *versus* 129 Sv backgrounds.

Urinary Protein Analysis

Semiquantitative measurements of urinary protein were performed using Albustix reagent strips (Bayer Corporation, Elkhart, IN), and reading the relative amounts from the color chart provided with the kit. Successive readings were performed weekly on fresh urine from the same experimental animals. Microhematuria was assessed using Hemastix reagent strips (Bayer Corporation).

Fractions of the above urine samples were subjected to gel analysis. Samples (the equivalent of 0.5 μl of undiluted urine) were fractionated by electrophoresis through 10% denaturing acrylamide gels. Urine creatinine levels were measured when sufficient urine was collected for meaningful readings ($>100 \mu\text{l}$). We observed no significant effect of the TGF- $\beta 1$ inhibitor on urine creatinine levels, and these levels fluctuated by no more than 20% throughout the time course. The protein in the gels was stained with Coomassie blue and photographed. Bovine serum albumin was used as a molecular weight standard.

Determination of End-Stage Renal Disease

End-stage renal disease was defined as the point in the disease progression where a weight loss of $>15\%$ of the body mass was observed. Animals were euthanized at this point and end stage was confirmed by visual examination. Blood urea nitrogen levels at this stage were consistently $>200 \text{ mg/dl}$. The kidneys are visibly smaller with a granular surface and are pale in color. When such visual characteristics were present, histological examination invariably confirmed advanced fibrosis.

Transmission Electron Microscopy

Fresh external renal cortex was minced in 4% paraformaldehyde, allowed to fix for 2 hours, and stored at 5°C in phosphate-buffered saline (PBS). The tissue was washed extensively (5 times for 10 minutes each at 4°C) with 0.1 mol/L Sorenson's buffer (Sorenson's buffer was made by combining 100 ml of a 200 mmol/L monobasic sodium phosphate with 400 ml of 200 mmol/L dibasic sodium phosphate, with 500 ml of water, and adjusted to pH 7.4), and postfixed in 1% osmium tetroxide in Sorenson's buffer for 1 hour. The tissue was then dehydrated in graded ethanol (70%, then 80%, then 90%, then 100% for 10 minutes each), and finally in propylene oxide and embedded in Poly/Bed 812 epoxy resin (Polysciences, Inc., Warrington, PA) following the procedures described by the manufacturer. Glomeruli were identified in 1- μm sections stained with toluidine blue, and thin sections were cut at 70-nm thickness using a Reichert Jung Ultracut E ultramicrotome (Cambridge Instrument Co., Vienna, Austria). Sections were mounted onto grids and stained with uranyl acetate and lead citrate. Grid-

mounted sections were examined and photographed using a Phillips CM10 electron microscope.

Scanning Electron Microscopy

Small pieces (approximately 2-mm cubes) of kidney cortex were fixed in 3% phosphate-buffered glutaraldehyde, then postfixed in 1% phosphate-buffered osmium tetroxide. Samples were then dehydrated in graded ethanols, and critical point-dried in carbon dioxide. The cubes were then cracked in pieces by stressing them with the edge of a razor blade, and mounted with glue onto stubs with the cracked surface facing upward. The surface was sputter-coated using gold/palladium and visualized with a scanning electron microscope.

Immunofluorescence Analysis

Fresh kidneys were removed and embedded in Tissue Tek OCT aqueous compound. They were frozen at -150°C and sectioned at $4\text{ }\mu\text{m}$ on a Microm cryostat. Slides were fixed in cold 100% acetone for 10 minutes then air-dried overnight. Slides were washed three times in cold $1\times$ PBS. Primary antibodies were diluted together in 7% nonfat dry milk (1:200 for entactin, 1:10 for laminin $\alpha 2$), applied to the sections, and incubated overnight at 4°C in a humidified dish. Slides were then washed successively for 5, 7, and 10 minutes with cold $1\times$ PBS. Secondary antibodies were diluted together at 1:100 (Texas red α -rabbit for lam- $\alpha 2$ and fluorescein isothiocyanate α -rat for entactin) in the blocking agent and applied for 4 hours at 4°C . After PBS washes, Vectashield (Vector Laboratories, App Imaging, Santa Clara, CA) anti-fade mounting media was applied and sections were coverslipped, sealed, and imaged. Images were collected using a Cytovision Ultra image analysis system (Applied Imaging, Inc.) interfaced with an Olympus BH-2 fluorescence microscope.

For the remaining laminin chain-specific antibodies, slides were brought to room temperature, then postfixed in cold (-20°C) acetone for 15 minutes, and air-dried overnight at 5°C . The specimens were rehydrated by three successive washes in PBS for 10 minutes each at room temperature, then denatured by immersing them in 0.1% sodium dodecyl sulfate at 37°C for 45 minutes. This step greatly enhanced immunoreactivity, presumably by exposing the masked laminin epitopes (B. Patton, personal communication). The specificity of the laminin chain-specific antibodies have been established in previous publications.^{24,25} The anti-laminin $\alpha 1$ (8B3) and $\beta 1$ (5A2) antibodies were mouse monoclonals, and a gift from D. Abrahamson (Department of Anatomy and Cell Biology, University of Kansas Medicine Center, Kansas City, KS).²⁶ Anti-laminin $\alpha 2$ chain-specific rabbit antibodies were a gift from Dr. Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, New Jersey).²⁷ The anti-laminin $\alpha 3$ chain-specific antibodies were a gift from Dr. Bob Burgeson (anti laminin-5 rabbit antiserum No. 4101) and described previously.^{28,29} The anti-laminin $\alpha 4$ (C0877) and $\alpha 5$ (8948) rabbit antisera were provided

by Jeff Miner (Washington University School of Medicine Department of Nephrology, St. Louis, MO).²⁴ The anti-laminin $\beta 2$ (Gpb1) chain-specific guinea pig antisera were a gift from Joshua Sanes (Washington University School of Medicine Department of Anatomy and Neurobiology, St. Louis, MO).³¹ Anti-laminin $\beta 3$ antibodies were provided by Yoshi Yamada (National Institute of Dental and Craniofacial Research, Bethesda, MD).³¹ A rat monoclonal antibody for the laminin $\gamma 1$ chain was purchased from Chemicon (Temecula, CA). All secondary reagents were purchased from Vector Laboratories.

For fibronectin immunostaining, the antibody was purchased from Sigma (St. Louis, MO; catalogue no. F3648). The same procedure was used as used for the laminin $\alpha 2$ chain.

Construction, Purification, and Activity of the Murine TGF β R:Fc

The extracellular domain of the murine TGF- β type II receptor was amplified from a murine lung cDNA library (Clontech, Palo Alto, CA) by PCR, and engineered to contain a 5' *NotI*, and a 3' *SalI* restriction site. The Fc region of murine IgG2a was amplified by PCR from a murine hybridoma, and engineered to contain a 5' *SalI* restriction site and a 3' *NotI* restriction site. The receptor and Fc fragments were purified, digested with the appropriate restriction enzymes, and ligated into the expression vector pEAG347, which contains tandem SV40, and adenovirus major late promoters; the SV40 late polyA termination signal; an ampicillin resistance gene; and a pSV2 dihydrofolate reductase-derived selection marker. The resulting construct (pAA002) was transformed into competent JM109, and plasmids were selected for the correct orientation of the receptor-Fc fusion gene. Proper sequence and alignment was confirmed by DNA sequencing.

pAA002 was transfected in Chinese hamster ovary cells (CHO DUKX-B1) by electroporation. After selection in 200 nmol/L methotrexate, single clones were selected and screened for the expression of mTGF β R:Fc. The clone with the highest titer was picked for expansion to 20 L fermentors, and the expressed protein was purified over protein A-Sepharose (Pharmacia, Piscataway, NJ), under sterile, endotoxin-free conditions. The protein is $>95\%$ pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and contains <1 U endotoxin per mg protein.

Activity of the mTGF β R:Fc was assessed in the mink lung epithelial cell assay as described.³²⁻³⁴ Briefly, Mv1Lu cells (ATCC CCL-64) were maintained in minimal essential medium supplemented with 100 U/ml penicillin, 100 Tg/ml streptomycin, 10% fetal bovine serum, and 4 mmol/L L-glutamine. To test, serial dilutions of TGF β R:Fc were incubated with 0.1 ng/ml TGF- $\beta 1$, 0.5 ng/ml TGF- $\beta 2$, and 0.05 ng/ml TGF- $\beta 3$ (R&D Systems, Minneapolis, MN) for 1 hour in assay medium (minimal essential medium supplemented with 100 U/ml of penicillin, 100 Tg/ml of streptomycin, and 10% fetal bovine serum) in a 96-well microtiter tissue culture plate. Mv1Lu cells were resus-

Figure 1. Proteinuria in experimental mice. Proteinuria was measured by gel electrophoresis of urine samples taken at weekly intervals from an individual set of experimental animals. The age of the mice (in weeks) at the time of sample collection is indicated above each lane. Group A(1) is the profile for an untreated 129 Sv/J Alport mouse; Group A(2) is that for a double-knockout mouse; Group B(1) is the extended profile for a double-knockout mouse; Group B(2) is a littermate double knockout treated with mTGF β R:Fc. Mr, molecular weight standards.

pended in assay medium and added to the assay plate at a concentration of 6,000 cells per well. The cells were incubated at 37°C for 48 hours and pulsed with [3 H]thymidine (70 to 86 Ci/mmol; Amersham) for an additional 6 hours. DNA synthesis, which reflects cell proliferation, was determined by measuring incorporation of [3 H]thymidine. As reported for similar TGF- β receptor antagonists,^{32,33} mTGF β R:Fc blocks TGF- β 1 (IC₅₀ = 1 nmol/L), and TGF- β 3 (IC₅₀ = 1 nmol/L), but not TGF- β 2-mediated inhibition of Mv1Lu cell proliferation.

Results

Loss of α 1 β 1 Integrin Slows Renal Disease Progression, Attenuates Expansion of the Mesangial Matrix, and Rescues Podocyte Foot Process Architecture in Alport Mice

The collagen α 3(IV)-deficient mouse developed in this laboratory¹⁴ was crossed with the integrin α 1-deficient mouse¹⁹ to produce an animal null at both alleles. Comparative analysis of renal disease pathogenesis of Alport mice *versus* these double-knockout mice revealed a markedly slower rate of renal disease in the double knockouts. Both the time of onset and the rate of progression of proteinuria was delayed relative to the Alport mice (Figure 1A and Table 1), suggesting improved function of the glomerular filter. The data in Table 1 represent successive analysis of urinary albumin in three sets of experimental animals, and illustrate that differences in onset and progression of proteinuria in the Alport mouse *versus* the double knockout are highly reproducible. Microhematuria was also assessed, and did not vary significantly among the experimental groups. The mean age of end-stage renal failure (as defined in the Methods section), based on analysis of 10 experimental sets (10 controls and 10 Alport, 10 integrin α 1 null mice, and 10 double-knockout animals) was extended from 8.5 $\pm$ 0.5 weeks in the Alport mice to 14.5 $\pm$ 0.9 weeks in the double knockouts.

Transmission electron microscopic analysis (TEM) of the glomerular capillary loops revealed attenuated GBM damage in 7-week-old double-knockout *versus* age-matched Alport mice (compare focal thickening of the GBM in Figure 2G with D), thus the absence of α 1 integrin appears to have provided a protective effect on GBM damage in the Alport mouse model. The morphology of

Table 1. Inactivating α 1 β 1 Integrin and TGF- β 1 Results in Additive Improvement in Slowing Both the Rate of Onset and Progression of Albuminuria in the Alport Mouse System

	Age (Wks)									
	1	2	3	4	5	6	7	8	9	10
Control	—	—	—	—	—	—	—	—	—	—
Control Sol Rec	—	—	—	—	—	—	—	—	—	—
Alport	—	—	±	+	+	++	+++	+++	Renal failure	—
DKO	—	—	±	+	+	++	+++	+++	++	++
DKO Sol Rec	—	—	—	—	—	+	+	+	++	++
	—	—	—	—	—	—	±	±	±	+
	—	—	—	—	—	—	—	±	±	±

Semiquantitative assessment of proteinuria was performed using Albustix (Bayer Corporation, Elkhart, IN) at the indicated times. Each symbol represents an individual urine analysis. The data represents successive analysis of three individual animals for each of the indicated experimental groups. Approximate albumin concentrations: (—) = negative; (±) = trace; (+) = about 0.3 mg/ml; (++) = about 1 mg/ml; (+++) = greater than 3 mg/ml. DKO = double knockout; SolRec = animals treated with mTGF β R:Fc.

Figure 2. Ultrastructural damage to the glomerular capillary loop and glomerular distribution of the laminin $\alpha2$ chain in control and double-mutant mice. Renal cortex from normal (A–C), Alport (D–F), and double-knockout (G–I) mice was harvested at 7 weeks. Tissue was analyzed by transmission electron microscopy (A, D, and G), scanning electron microscopy (B, E, and H), or dual immunofluorescence using antibodies specific for entactin (green), and the laminin $\alpha2$ chain (red) (C, F, and I). Arrows denote foot processes. C, capillary lumen; U, urinary space. Scale bars, 1.5 μ m for transmission electron microscopic photographs and 2 μ m for scanning electron microscopy photographs. Immunofluorescence panels illustrate sections of whole glomeruli at $\times 400$ magnification.

the podocyte foot processes was normal in the double knockout as compared with the Alport mouse both by transmission electron microscopy (Figure 2; A, D, and G) and scanning electron microscopy (Figure 2; B, E, and H). Effacement of the podocyte foot processes, as observed in the Alport mouse (Figure 2, D and E), obliterates the slit diaphragms, potentially blocking the glomerular filter.

One underlying mechanism driving effacement of the foot processes is thought to be the loss of focal contact adhesion between the podocytes and the GBM.³⁵ Thus, we performed a comparative examination of the molecular composition of the GBM in normal mice and Alport mice. Earlier studies have shown that type IV collagen and fibronectin, which localize primarily to the mesangial matrix in control mice, are abundant in the GBM of Alport mice.^{14,15} A comprehensive analysis of the known laminin chains in normal *versus* Alport mice has not yet been performed. We used antibodies specific for the different laminin chains to examine the GBM composition of normal *versus* Alport mice. The results in Figure 3 illustrate a

dramatic shift in laminin $\alpha2$ (Figure 3, C *versus* D) and laminin $\beta1$ (Figure 3, G *versus* H) chain expression from the mesangial matrix in control mice to the GBM in Alport mice. No changes in the glomerular localization of the $\alpha5$, $\beta2$, or $\gamma1$ chains were observed in normal *versus* Alport mice. Consistent with earlier reports,³⁶ the $\alpha3$, $\alpha4$, and $\beta3$ chains are not expressed in the renal glomerulus. By co-localization inference, these data suggest that either laminin-2 (a heterotrimer comprised of the $\alpha2$, $\beta1$, and $\gamma1$ chains) or a combination of laminin-2 and laminin-4 (comprised of the $\alpha2$, $\beta2$, and $\gamma1$ chains), whereas they are completely absent from the GBM of normal mice, are abundant in the GBM of Alport mice.

In Figure 2, C, F, and I, the GBM is stained in green, and laminin $\alpha2$ chain is stained in red to illustrate that the laminin $\alpha2$ chain, which normally localizes specifically to the glomerular mesangium (Figure 2C) localized heavily in the GBM of Alport mice (Figure 2F). In double-knockout mice, the laminin $\alpha2$ -chain localization was similar to that in normal mice, however some laminin $\alpha2$ staining is observed in the GBM (Figure 2I). Similar results were

Figure 3. Analysis of the laminin chain composition of the GBM in normal *versus* Alport mice. Antibodies specific for the indicated laminin chains were used to immunostain glomeruli from 7-week-old normal mice and Alport littermates. The laminin chain specificity of the antibody used is indicated to the left of each set of panels. C, control; A, Alport. Based on co-localization, either laminin-2 ($\alpha1\beta1\gamma1$) or both laminin-2 and laminin-4 ($\alpha1\beta2\gamma1$) are present in the GBM of the Alport mouse, but not in the GBM of the control.

obtained using antisera specific for the $\beta1$ -laminin chain (data not shown). The changes in laminin composition of the GBM in normal *versus* Alport mice may be causally linked to effacement of the podocyte foot processes.

As $\alpha1\beta1$ integrin plays a key role in cell adhesion, spreading, and migration in other systems,^{19,37,38} it seemed plausible that expansion of the mesangial matrix, an early marker of Alport glomerular pathogenesis, might be attenuated in the double-knockout mice. In Figure 4 a fibronectin immunostain was used to illustrate that this is indeed the case. This abundant mesangial matrix marker reveals extensive expansion of the mesangial matrix in Alport *versus* normal mice at 7 weeks of age (Figure 4, A *versus* B). Age matched double-knockout mice, however, do not exhibit noticeable expansion of the glomerular mesangium (Figure 4C). By 12 weeks of age, however, the mesangial matrix in double-knockout mice is expanded (data not shown), illustrating that the absence of $\alpha1$ integrin attenuates, but does not inhibit expansion of the mesangial matrix.

Blocking TGF- $\beta1$ Inhibits Focal GBM Thickening, but Not Podocyte Foot Process Effacement in Alport Mice

Recently we described induction of TGF- $\beta1$ mRNA as well as fibronectin, laminin $\beta1$, and collagen $\alpha1(IV)$ mRNAs in the podocytes of the Alport mouse.²³ We further evaluated the role of TGF- $\beta1$ on renal disease progression in the Alport mouse by using a newly designed soluble receptor inhibitor. A number of groups, including ours, have reported the development of soluble TGF- β type II receptors as *in vitro* and *in vivo* antagonists of TGF- β .^{32,33,39} As reported for these similar TGF- β receptor antagonists, mouse TGF β R:Fc blocks TGF- $\beta1$ (IC_{50} = 1 nmol/L), and TGF- $\beta3$ (IC_{50} = 1 nmol/L), but not TGF- $\beta2$ -mediated inhibition of mink lung tumor (Mv1Lu) cell proliferation (Figure 5). We noted no inflammation or immune infiltration in normal mice chronically administered effective doses of this inhibitor (data not shown).

Alport mice were injected twice weekly through the tail vein with 25 μ g of mTGF β R:Fc starting at 3 weeks of age and kidneys were harvested at 7 weeks of age. Transmission electron microscopy illustrates that treatment with the inhibitor prevents irregular thickening of the GBM, but does not prevent podocyte foot process effacement (Figure 6, A–C). Scanning electron microscopy reveals the podocyte foot processes are effaced in Alport animals treated with mTGF β R:Fc (Figure 6F). Laminin $\alpha2$ is present in the GBM of Alport mice treated with mTGF β R:Fc (Figure 6G). Alport mice treated with mTGF β R:Fc die of renal failure at approximately the same time as untreated Alport mice, presumably because of effacement of the podocyte foot processes which results in massive proteinuria (data not shown).

Blocking TGF- $\beta1$ and Integrin $\alpha1\beta1$ in Alport Mice Restores Both GBM and Podocyte Architecture

The results shown in Figures 2 and 6 suggest $\alpha1\beta1$ integrin and TGF- $\beta1$ play distinct roles in Alport glomerular pathogenesis. If so, blocking TGF- $\beta1$ in Alport mice

Figure 4. Absence of integrin $\alpha1\beta1$ is associated with attenuated expansion of the mesangial matrix. The glomerular mesangium from 7-week-old control (A), Alport (B), and double-knockout (C) mice was stained using antibodies specific for fibronectin.

null for $\alpha1\beta1$ integrin should prevent both podocyte foot process effacement and GBM thickening. To test this hypothesis, mTGF β R:Fc was administered to double-knockout mice starting at 4 weeks of age, and the kidneys were analyzed at 10 weeks of age. The treated animals were compared directly with untreated double-knockout littermates. Data shown in Table 1 and Figure 1B illustrates that both the time of onset and progression of proteinuria are markedly attenuated in double knockouts treated with mTGF β R:Fc relative to untreated double-knockout mice. Double-knockout animals treated with mTGF β R:Fc die at 18 to 24 weeks of age. As shown in Figure 7, the GBM of the double-knockout mice at 10 weeks of age contains many areas of focal thickening, indicating progression of the glomerular pathology relative to 7-week-old double knockouts (compare Figures 7B and 2G). The podocyte foot processes have normal morphology, except for areas where the GBM is focally thickened. In these regions, the foot processes appear

fused. In the double knockouts treated with the soluble receptor, both basement membrane thickening and effacement of the podocyte foot processes are virtually absent. Most of the glomeruli in the treated double knockouts reflect that illustrated in Figure 7C, where some irregularities in the glomerular basement membrane are visible. Approximately 20% of the glomeruli, however, are morphologically indistinguishable from those of control animals (Figure 7D). By comparison, out of >50 glomeruli examined by transmission electron microscopy, none of the glomeruli in 3-week-old Alport mice are morphologically normal. Laminin $\alpha2$ in the untreated 10-week-old double knockouts shows considerable staining in the GBM (Figure 7E), however the staining is focally deposited rather than distributed throughout the full length of the GBM, as observed in the 7-week-old Alport mice (compare Figure 7E with 2F). This is consistent with the focal thickening observed on transmission electron microscopy micrographs (Figure 7B). In 10-week-old double knockouts treated with mTGF β R:Fc, laminin $\alpha2$ staining is restricted to the glomerular mesangium (Figure 7F), identical to that observed in normal animals (Figure 2C). Thus, blocking $\alpha1\beta1$ integrin and TGF- $\beta1$ is synergistic in slowing the rate of both onset and progression of glomerular pathology in the Alport mouse.

Figure 5. The soluble TGF- β type II receptor (mTGF β R:Fc) inhibits TGF- $\beta1$ and TGF- $\beta3$ activities, but not TGF- $\beta2$ activity. The biological activity of mTGF β R:Fc was tested in the mink lung cell (Mv1Lu) proliferation assay. Mv1Lu cells were pre-incubated with either 0.1 ng/ml TGF- $\beta1$, 0.5 ng/ml TGF- $\beta2$, or 0.05 ng/ml TGF- $\beta3$ for 1 hour, then transferred to wells containing the indicated concentrations of the mTGF β R:Fc. Cells were incubated for 48 hours, pulsed with [3 H]thymidine for 6 hours, and [3 H]thymidine incorporation into the DNA measured by liquid scintillation spectrometry (y axis).

Discussion

The delayed onset and progressive nature of Alport renal pathogenesis suggests the existence of a molecular triggering mechanism which initiates an imbalance in GBM homeostasis. The age of onset for Alport renal disease, although usually in the juvenile, can be quite variable, suggesting that the metabolic pathways affecting onset and progression are modulated by differences in the genetic background between individuals. Thus, identification of the specific metabolic imbalances might provide targets for therapies that delay onset, progression, or both.

Herein we describe that at least two pathways play distinct contributory roles in the glomerular pathology of Alport syndrome. The first involves integrin $\alpha1\beta1$, which is the most prevalent integrin found on the surface of mesangial cells.^{17,18} Our data illustrates this integrin is in-

Figure 6. TGF- β 1 inhibition prevents GBM thickening, but not podocyte foot process effacement in the Alport mouse. Renal cortex from 7-week-old normal mice (**A** and **D**), Alport mice (**B** and **D**), and Alport mice treated with mTGF β R:Fc (**C** and **E**) was analyzed by both transmission electron microscopy (**A**, **B**, and **C**) and scanning electron microscopy (**D**, **E**, and **F**). Glomerular capillary loops and podocyte surface architecture are shown. **F**: Dual immunofluorescence immunostain (laminin α 2 in red and entactin, a marker for GBM, in green) of a glomerulus from a 7-week-old Alport mouse treated with mTGF β R:Fc.

volved in the mechanisms of both podocyte foot process effacement and mesangial matrix expansion. Its effect on podocyte effacement is likely mediated via GBM deposition of laminin isoforms not native to the GBM. The second pathway involves TGF- β 1, which we show is driving focal thickening of the GBM, a hallmark for diagnosis of Alport GBM pathogenesis. When both TGF- β 1 and α 1 β 1 integrin are removed from Alport mice, synergistic improvement in renal morphology and function are observed, indicating that these two pathways work in distinct ways to drive renal disease progression in Alport syndrome.

Integrin α 1 β 1 and Alport Renal Disease

A major indicator of Alport glomerular pathogenesis is expansion of the mesangial matrix.² How or whether changes in the glomerular mesangium have anything to do with observed GBM damage in Alport syndrome, however, is not clear. Integrin α 1 β 1 has been shown to be important for gel contraction of mesangial cells *in vitro*.²¹ It is well documented that mesangial cells depend on β 1 integrins for adhesion and migration,⁴⁰ and α 1 β 1 integrin has been directly implicated in both adhesion and spreading of chondrocytes.³⁸ Thus, the absence of α 1 β 1

Figure 7. TGF- $\beta 1$ inhibition primarily restores both GBM and podocyte architecture in double-knockout mice. Renal cortex from normal mice (**A**), double-knockout mice (**B**), and double-knockout mice treated with mTGF β R:Fc (**C** and **D**) was harvested at 10 weeks of age and analyzed by transmission electron microscopy. Fields represent the GBM architecture of typical glomerular capillary loops. **E** and **F**: Dual immunofluorescence staining using antibodies specific for entactin (green) and the laminin $\alpha 2$ chain (red). **E**: Glomerulus from a 10-week-old untreated double-knockout mouse. **F**: Glomerulus from a 10-week-old double-knockout mouse treated with mTGF β R:Fc. Scale bars, 3 μ m.

in the double knockouts might affect the migration of cytoplasmic processes that interface the mesangium with the glomerular capillary loops.^{41,42} These contact points provide direct access for mesangial enzymes and proteins to be deposited into the GBM. Focal degradation by matrix metalloproteinase derived from the glomerular mesangium with concomitant deposition of mesangial matrix proteins might explain why focal thickening and splitting are observed in Alport GBM disease. Indeed, the proteins that accumulate in the GBM as a function of Alport renal disease pathogenesis include many matrix proteins normally synthesized by mesangial cells,^{14,23}

and mesangial cells are known to produce both matrix metalloproteinase (MMP)-2, MMP-9 and the tissue inhibitors of metalloproteinases-I, -II, and -III.^{43,44} The mRNAs encoding both the integrin $\alpha 1$ and $\beta 1$ subunits are induced by TGF- $\beta 1$,⁴⁵ as are mesangial expression of laminins, fibronectin, and collagen $\alpha 1(IV)$ and $\alpha 2(IV)$. An alternative explanation for the appearance of typically mesangial proteins in the GBM is gene activation in the podocytes. Activation in podocytes of the genes encoding collagen $\alpha 1(IV)$, fibronectin, and the laminin $\beta 1$ chain, all typically mesangial matrix proteins, has been demonstrated in Alport mice.²³ Thus, an integrin $\alpha 1\beta 1$ regulated

paracrine factor may exist that can activate these genes in podocytes.

Absence of $\alpha 1\beta 1$ integrin results in an inhibition of laminin $\alpha 2$ and $\beta 1$ chain deposition in the GBM. This report is the first to illustrate GBM deposition of these laminin chains in GBM pathogenesis. The importance of this observation lies with its likely association with effacement of the podocyte foot processes. Known laminin heterotrimers that contain the $\alpha 2$ chain include laminin-2 ($\alpha 2\beta 1\gamma 1$) and laminin-4 ($\alpha 2\beta 2\gamma 1$).⁴⁶ Normal mature GBM contains only laminin-11 ($\alpha 5\beta 2\gamma 1$).²⁴ Recent studies used a recombinant soluble $\alpha 3\beta 1$ integrin receptor to illustrate that the integrin heterodimer binds specifically to laminin heterotrimers that contain the $\alpha 5$ chain, but not those that contain the $\alpha 1$ or $\alpha 2$ chains.⁴⁷ It is widely believed that adhesion of the podocyte foot processes to the GBM requires $\alpha 3\beta 1$ integrin.³⁵ Immunogold localization studies place $\alpha 3\beta 1$ integrin at the interface between the podocytes and the basement membrane.⁴⁸ An $\alpha 3$ integrin knockout has been produced, and dies at birth from acute renal failure, with complete effacement of the podocyte foot processes.⁴⁹ Integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind to the amino terminus of the laminin $\alpha 1$ chain and the laminin $\alpha 2$ chain,^{50,51} creating precedent for laminin α chains as specific ligands for integrins. Thus, deposition of the laminin heterotrimers containing the $\alpha 2$ chain might mask binding of $\alpha 3\beta 1$ integrin to its preferred ligand, laminin-11. Such masking might lead to progressive loss of focal contact adhesion, resulting in foot process effacement. Because laminin $\alpha 2$ is found only in the mesangial matrix of normal mice and humans, how it gets into the GBM in Alport syndrome is an important unanswered question. Clearly $\alpha 1\beta 1$ integrin plays an key role in this process.

Role of TGF- $\beta 1$ in Alport Glomerular Pathogenesis

TGF- $\beta 1$ is strongly implicated as a molecular driver in the rat model for acute mesangial proliferative glomerulonephritis.⁵² The induced disease can be suppressed by injecting the animals with neutralizing antibodies against TGF- $\beta 1$ or antisense oligonucleotides complimentary to the TGF- $\beta 1$ mRNA.^{53,54} More recently, this work has been extended to include gene therapy by transduction with an expression vector expressing a soluble TGF- β type II receptor/Fc fusion peptide, much like the soluble receptor used in our studies.³⁹ TGF- $\beta 1$ has been implicated in a number of glomerular diseases,^{54,55} including Alport syndrome.²³

In this report we show that by inhibiting TGF- $\beta 1$ we can inhibit thickening of the GBM. We do not, however, inhibit effacement of the podocyte foot processes. Laminin $\alpha 2$ is present in the GBM of 7-week-old Alport mice treated with mTGF β R:Fc, suggesting that the events resulting in deposition of laminin $\alpha 2$ containing isoforms in the GBM are not mediated by TGF- $\beta 1$. The animals die at about the same time as untreated animals, however why they die is not clear. They do develop acute massive proteinuria, so the cause of death may be hypoalbuminemia and hypovolemia. This is very different from the results obtained

with experimental glomerulonephritis, where inhibition of TGF- $\beta 1$ was sufficient to restore normal glomerular function.^{22,53} Thus, although TGF- $\beta 1$ might be induced in a variety of glomerular disease, it is likely that its contributory role may be unique for each.

Combined, the observations reported herein support a potential therapeutic paradigm for Alport renal disease by treatment with inhibitors for both integrin $\alpha 1\beta 1$ and TGF- $\beta 1$.

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References

1. Flinter F: Alport syndrome. *J Med Genet* 1992, 34:326–330
2. Gregory MC, Terreros DA, Barker DF, Fain PN, Denison JC, Atkin CL: Alport syndrome—clinical phenotypes, incidence, and pathology. *Contrib Nephrol* 1996, 117:1–28
3. Barker DE, Hostikka SL, Zhou J, Show LT, Oliphant AR, Gerken SC, Gregory MC, Skolnick MH, Atkin CL, Tryggvason K: Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science* 1990, 248:1224–1227
4. Lemmink HH, Mochizuki T, van den Heuvel LPWJ, Schroder CH, Barrientos A, Monnens LAH, van Oost BA, Brunner HG, Reeders ST, Smeets HJM: Mutations in the type IV collagen $\alpha 3$ (COL4A3) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* 1994, 3:1269–1273
5. Mochizuki T, Lemmink HH, Mariyama M, Antignac C, Gubler MC, Pirson Y, Verellen-Dumoulin C, Chan B, Schroder CH, Smeets HJ, Reeders ST: Identification of mutations in the $\alpha 3$ (IV) and $\alpha 4$ (IV) collagen genes in autosomal recessive Alport syndrome. *Nat Genet* 1994, 8:77–82
6. Kashtan CE, Kim Y: Distribution of the $\alpha 1$ and $\alpha 2$ chains of collagen IV and of collagens V and VI in Alport syndrome. *Kidney Int* 1992, 42:115–126
7. Gubler M-C, Knebelmann B, Beziau A, Broyer M, Pirson Y, Haddoum F, Kleppel MM, Antignac C: Autosomal recessive Alport syndrome: immunohistochemical study of type IV collagen chain distribution. *Kidney Int* 1995, 47:1142–1147
8. Mazzucco G, Barsotti P, Muda AO, Fortunato M, Mihatsch M, Torri-Tarelli L, Renieri A, Faraggiana T, De Marchi M, Monga G: Ultrastructural and immunohistochemical findings in Alport's syndrome: a study of 108 patients from 97 Italian families with particular emphasis on COL4A5 gene mutation correlations. *J Am Soc Nephrol* 1998, 9:1023–1031
9. Desjardins M, Bendayan M: Ontogenesis of glomerular basement membrane: structural and functional properties. *J Cell Biol* 1991, 113:689–700
10. Kleppel MM, Fan WW, Cheong HI, Michael AF: Evidence for separate networks of classical and novel basement membrane collagen. *J Biol Chem* 1992, 267:4137–4142
11. Gunwar S, Ballester F, Noelken ME, Ninomiya Y, Hudson B: Glomerular basement membrane. Identification of a novel disulfide-cross-linked network of α , $\alpha 4$, and $\alpha 5$ chains of type IV collagen and its implications for the pathogenesis of Alport syndrome. *J Biol Chem* 1998, 273:8767–8775
12. Miner JH, Sanes JR: Collagen IV $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains in rodent basal lamina: sequence, distribution, association with laminins, and developmental switches. *J Cell Biol* 1994, 127:879–891
13. Kalluri R, Shield III CF, Todd P, Hudson BG, Nielson EG: Isoform switching of type IV collagen is developmentally arrested in X-linked Alport syndrome leading to increased susceptibility of renal basement membranes to endoproteolysis. *J Clin Invest* 1997, 99:2470–2478
14. Cosgrove D, Meehan DT, Grunkemeyer JA, Kornak JM, Sayers R, Hunter WJ, Samuelson GC: Collagen COL4A3 knockout: a mouse

- model for autosomal Alport syndrome. *Genes Dev* 1996, 10:2981–2992
15. Miner JH, Sanes JR: Molecular and functional defects in kidneys of mice lacking collagen $\alpha 3(IV)$: implications for Alport syndrome. *J Cell Biol* 1996, 135:1403–1413
16. Lu W, Phillips CL, Killen PD, Hlaing WR, Elder FF, Miner JH, Overbeek PA, Meisler MH: Insertional mutation of the collagen genes Col4a3 and Col4a4 in a mouse model for Alport syndrome. *Genomics* 1999, 15:113–124
17. Patey N, Halbwachs-Mecarelli D, Droz D, LeSavre P, Noel LH: Distribution of integrin subunits in normal human kidney. *Cell Adhes Commun* 1994, 2:159–167
18. Sterk LM, deMelker AA, Kramer D, Kuikman I, Chand A, Claessen N, Weening JJ, Sonnenberg A: Glomerular extracellular matrix components and integrins. *Cell Adhes Commun* 1998, 5:177–192
19. Gardner H, Kreidberg J, Koteliensky V, Jaenisch R: Deletion of integrin $\alpha 1$ by homologous recombination permits normal murine development, but gives rise to a specific deficit in cell adhesion. *Dev Biol* 1996, 175:301–313
20. Pozzi A, Wary KK, Giancotti FG, Gardner HA: Integrin $\alpha 1\beta 1$ mediates a unique collagen-dependent proliferation pathway in vivo. *J Cell Biol* 1999, 142:587–594
21. Kagami S, Kondo S, Loster K, Reutter W, Kuhara T, Yatsumoto K, Kuroda Y: $\alpha 1\beta 1$ integrin-mediated collagen matrix remodeling by rat mesangial cells is differentially regulated by transforming growth factor- β and platelet-derived growth factor-BB. *J Am Soc Nephrol* 1999, 10:779–789
22. Border WA, Ruoslahti E: Transforming growth factor- β in disease: the dark side of tissue repair. *J Clin Invest* 1992, 90:1–7
23. Sayers R, Kalluri R, Rodgers KD, Shield III CF, Meehan DT, Cosgrove D: Role for TGF- β in Alport renal disease progression. *Kidney Int* 1999, 56:1662–1673
24. Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG, Sanes JR: The laminin α chains: expression, developmental transitions, and chromosomal locations of $\alpha 1$ – $\alpha 5$, identification of heterotrimeric laminins 8–11, and cloning of a novel $\alpha 3$ isoform. *J Cell Biol* 1997, 137:685–701
25. Patton BL, Miner JH, Chiu AY, Sanes JH: Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. *J Cell Biol* 1997, 139:1507–1521
26. Abrahamson DR, Irwin MH, St John PL, Perry EW, Accavitti MA, Heck LW, Couchman JR: Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete microdomains. *Cell Biol* 1989, 109:2477–2491
27. Vachon PH, Loechel F, Xu H, Wewer UM, Engvall E: Merosin and laminin in myogenesis: specific requirement for merosin in myotube stability and survival. *J Cell Biol* 1996, 134:1483–1497
28. Rousselle P, Lundstrum GP, Keene DR, Burgeson RE: Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J Cell Biol* 1991, 114:567–576
29. Marinkovich MP, Lunstrum GP, Keene DR, Burgeson RE: The dermal-epidermal junction of human skin contains a novel laminin variant. *J Cell Biol* 1992, 119:695–703
30. Sanes JR, Engvall E, Butkowski R, Hunter DD: Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. *J Cell Biol* 1990, 111:1685–1699
31. Utani A, Kopp JB, Kozak CA, Matsuki Y, Amizuka N, Sugiyama S, Yamada Y: Mouse kalinin B1 (laminin $\beta 3$ chain): cloning and tissue distribution. *Lab Invest* 1995, 72:300–310
32. Tsang ML-S, Zhou L, Zheng BL, Wenker J, Fransen G, Humphrey J, Smith JM, O'Connor-McCourt M, Lucas R, Weatherbee JA: Characterization of recombinant soluble human transforming growth factor- β Type II (rhTGF- β RII). *Cytokine* 1995, 7:389–397
33. O'Connor-McCourt M, Segarini P, Grothe S, Tsang ML-S, Weatherbee JA: Analysis of the interaction between two TGF- β binding proteins and three TGF- β isoforms using surface plasmon resonance. *Ann NY Acad Sci* 1995, 766:300–302
34. Smith JD, Bryant SR, Couper LL, Vary CPH, Gotwals PJ, Koteliensky VE, Lindner V: Soluble transforming growth factor- β type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial regrowth. *Circ Res* 1999, 84:1212–1222
35. Smoyer WE, Mundel P: Regulation of podocyte structure during the development of nephrotic syndrome. *J Mol Med* 1998, 76:172–183
36. Miner JH: Renal basement membrane components. *Kidney Int* 1999, 56:2016–2024
37. Carver W, Molano I, Reaves TA, Borg TK, Terracio L: Role of the $\alpha 1\beta 1$ integrin complex in collagen gel contraction in vitro by fibroblasts. *J Cell Physiol* 1995, 165:425–437
38. Makihiro S, Yan W, Ohno S, Kawamoto T, Fujimoto K, Okimura A, Yoshida E, Noshiro M, Hamada T, Kato Y: Enhancement of cell adhesion and spreading by a cartilage-specific noncollagenous protein, cartilage matrix protein (CMP/Matrilin-1), via integrin $\alpha 1\beta 1$. *J Biol Chem* 1999, 274:11417–11423
39. Isaka Y, Akagi Y, Ando Y, Tsujie M, Sudo T, Ohno N, Border W, Noble NA, Kaneda Y, Hori M, Imai E: Gene therapy by transforming growth factor- β receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 1999, 55:465–475
40. Prols F, Hartner A, Schlockman HO, Stersel RB: Mesangial cells and their adhesive properties. *Exp Nephrol* 1999, 7:137–146
41. Sakai T, Kriz W: The structural relationship between mesangial cells and basement membrane of the renal glomerulus. *Anat Embryol* 1987, 176:373–386
42. Kriz W, Elger M, Lemley KV, Sakai T: Mesangial cell-glomerular basement membrane connections counteract glomerular capillary and mesangium expansion. *Am J Nephrol* 1990, 10(Suppl 1):4–13
43. Anderson SS, Wu K, Nagase H, Stettler-Stevenson WG, Kim Y, Tsilibary EC: Effect of matrix glycation on expression of type IV collagen, MMP-2, MMP-9, and TIMP-1 by human mesangial cells. *Cell Adhes Commun* 1996, 2:89–101
44. Mozes MM, Bottinger EP, Jacot TA, Kopp JB: Renal expression of fibrotic matrix proteins and of transforming growth factor- β (TGF- β) isoforms in TGF- β transgenic mice. *J Am Soc Nephrol* 1999, 10:271–280
45. Kagami S, Kuhara T, Yatsumoto K, Okada K, Loster K, Reutter W, Kuroda Y: Transforming growth factor β (TGF- β) stimulates the expression of $\beta 1$ integrins and adhesion by rat mesangial cells. *Exp Cell Res* 1996, 229:1–6
46. Engvall E, Earwicker D, Haaparanta T, Ruoslahti E, Sanes JR: Distribution and isolation of four laminin variants: tissue restricted distribution of heterotrimers assembled from five different subunits. *Cell Regul* 1990, 1:731–740
47. Eble JA, Wucherpfennig KW, Gauthier L, Dersch P, Krukonis E, Isberg RR, Hemler ME: Recombinant soluble human $\alpha 3\beta 1$ integrin: purification, processing, regulation, and specific binding to laminin-5 and invasion in a mutually exclusive manner. *Biochemistry* 1998, 37:10945–10955
48. Regoli M, Bendayan M: Alterations in the expression of the $\alpha 3\beta 1$ integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes mellitus. *Diabetologia* 1997, 40:15–22
49. Kreidberg JA, Donovan MJ, Goldstein SL, Rennke H, Shepherd K, Jones RC, Jaenisch R: $\alpha 3\beta 1$ integrin has a crucial role in kidney and lung organogenesis. *Development* 1996, 122:3537–3547
50. Colognato H, MacCarrick M, O'Rear JJ, Yurchenco PD: The laminin $\alpha 2$ -chain short arm mediates cell adhesion through both the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *J Biol Chem* 1997, 272:29330–29336
51. Ettner N, Gohring W, Sasaki T, Mann K, Timmler R: The N-terminal globular domains of the laminin $\alpha 1$ chain binds to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins and to the heparin sulfate-containing domains of perlecan. *FEBS Lett* 1998, 430:217–222
52. W.A., Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor $\beta 1$ *Nature (London)* 1990, 346:371–374
53. Agaki Y, Isaka Y, Arai M, Kaneko T, Takenaka M, Moriyama T, Kaneda Y, Ando A, Orita Y, Kamada T, Ueda N, Imai E: Inhibition of TGF- $\beta 1$ expression by antisense oligonucleotides: suppressed extracellular matrix accumulation in experimental border glomerulonephritis. *Kidney Int* 1996, 50:148–155
54. Yamamoto T, Noble NA, Miller DE, Border WA: Sustained expression of TGF- $\beta 1$ underlies development of progressive kidney fibrosis. *Kidney Int* 1994, 45:916–927
55. Yang C-W, Vlassara H, Striker GE, Striker LJ: Administration of AGEs in vivo induces genes implicated in diabetic glomerulosclerosis. *Kidney Int* 1995, 47:S53–S58