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ORIGINAL ARTICLE

Glomerular basement membrane deposition of collagen α1(III) in Alport glomeruli by mesangial filopodia injures podocytes via aberrant signaling through DDR1 and integrin α2β1

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Abstract

In Alport mice, activation of the endothelin A receptor (ET_AR) in mesangial cells results in sub-endothelial invasion of glomerular capillaries by mesangial filopodia. Filopodia deposit mesangial matrix in the glomerular basement membrane (GBM), including laminin 211 which activates NF-κB, resulting in induction of inflammatory cytokines. Herein we show that collagen α1(III) is also deposited in the GBM. Collagen α1(III) localized to the mesangium in wild-type mice and was found in both the mesangium and the GBM in Alport mice. We show that collagen α1(III) activates discoidin domain receptor family, member 1 (DDR1) receptors both *in vitro* and *in vivo*. To elucidate whether collagen α1(III) might cause podocyte injury, cultured murine Alport podocytes were overlaid with recombinant collagen α1(III), or not, for 24 h and RNA was analyzed by RNA sequencing (RNA-seq). These same cells were subjected to siRNA knockdown for integrin α2 or DDR1 and the RNA was analyzed by RNA-seq. Results were validated *in vivo* using RNA-seq from RNA isolated from wild-type and Alport mouse glomeruli. Numerous genes associated with podocyte injury were up- or down-regulated in both Alport glomeruli and cultured podocytes treated with collagen α1(III), 18 of which have been associated previously with podocyte injury or glomerulonephritis. The data indicate α2β1 integrin/DDR1 co-receptor signaling as the dominant regulatory mechanism. This may explain earlier studies where deletion of either DDR1 or α2β1 integrin in Alport mice ameliorates renal pathology.

Keywords: Alport syndrome; podocyte injury; collagen α1(III); integrin α2β1; discoidin domain receptor 1

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Introduction

Alport syndrome, characterized by congenital onset and variably progressive (based on the specific mutation) glomerular disease associated with hearing loss, results from mutations in type IV collagen genes, *COL4A3*, *COL4A4*, and *COL4A5*. There are two inherited forms of the disease: autosomal recessive (involving *COL4A3* or *COL4A4*) [1,2], accounting for about 20% of cases, and X-linked (involving *COL4A5*) [3], accounting for about 80%. Mutations in any of these three genes usually results in the absence of all three collagen chains in the glomerular basement membrane (GBM), due to an obligatory association of the three chains to form protomers. This results in a GBM that contains only a type IV collagen α1/α2 network with two distinct consequences that likely contribute to glomerular disease initiation. The first is that the type IV collagen network is thinner and contains fewer interchain crosslinks than the type IV collagen α3/α4/α5 network [4]. This imparts greater elasticity to the GBM, resulting in biomechanical stress on the cells comprising the glomerular capillary tufts. This biomechanical stress directly drives the expression of genes contributing to glomerular pathology and accelerates the progression of glomerular disease in Alport mice [5]. The second consequence, which is more theoretical in nature, is based on super-resolution microscopic analysis of the GBM in wild-type and Alport mice that placed the GBM collagen network in Alport mice, but not wild-type mice, within range to interact with collagen receptors on podocytes [6].

The mechanism of biomechanical strain-mediated glomerular pathology is more complex. Abnormal stresses on the glomerular capillary tuft result in up-regulation of endothelin-1 in glomerular endothelial cells that binds to endothelin A receptors (ET_ARs) on

the glomerular mesangial cells. ET_AR signaling results in activation of CDC42, which induces the migration of mesangial filopodia into the sub-endothelial space of the glomerular capillaries [7,8]. One consequence of the mesangial filopodial invasion is the deposition of mesangial proteins in the GBM. It was shown by our lab and others two decades ago that laminin α2 progressively accumulates in the GBM of Alport mice, dogs, and humans [9,10]. We later showed that the presence of this laminin directly injures podocytes, activating focal adhesion kinase (FAK) with downstream activation of the transcription factor NF-κB, which activates the expression of pro-inflammatory mediators [11]; however, the specific receptor(s) mediating these effects was not identified in this study.

In the present study, we identified for the first time a second mesangial extracellular matrix (ECM) molecule accumulating in the GBM, collagen α1(III). The pattern of progressive accumulation of collagen α1(III) mirrored that for laminin 211, suggesting that they are both secreted by mesangial filopodia. Cultured wild-type and Alport mouse podocytes do not have detectable mRNA for *Lama2*, suggesting that ectopic activation in podocytes is not a source of laminin α2 protein. Cultured Alport mouse podocytes overlaid with collagen α1(III) demonstrated a myriad of effects on gene expression that were consistent with podocyte injury. There are two known collagen receptors expressed by podocytes: integrin α2β1 and discoidin domain receptor 1 (DDR1). Alport mice lacking either receptor show attenuated glomerular disease progression, indicating a functional importance for these receptors in driving podocyte dysfunction, likely via aberrant collagen-mediated signaling [12,13]. Knockdown of DDR1 or integrin α2β1 in Alport mouse podocytes prior to overlay with collagen α1(III) produced transcriptome profiles with suppression of receptor knockdown-specific pathologic genes as well as most genes for which knockdown of either receptor suppresses expression. This indicates that these receptors can signal in both distinct and overlapping ways. We validated cell culture data using RNA-seq data produced from isolated glomeruli from autosomal Alport mice at 5 and 7 weeks of age. The results revealed several genes that have been previously shown to be associated with podocyte injury, glomerulonephritis, or chronic kidney disease (CKD); two of these genes encode proteins previously implicated in Alport renal disease progression (CTGF and ITGA2).

Materials and methods

Animals

129 Sv autosomal Alport mice (*Col4a3*$^{-/-}$) were developed in the Cosgrove lab [14]. All mice were on a pure 129 Sv genetic background and maintained in house. All procedures involving animals were conducted in accordance with an approved IACUC protocol at Boys Town National Research Hospital and were consistent with the NIH Guide for the Care and Use of Laboratory Animals. Dogs used by Dr Nabity (College of Veterinary Medicine and Biological Sciences, Texas A&M University, College Station, TX, USA) [15] were maintained under strict USDA-approved conditions. Every effort was made to minimize usage as well as minimize any pain or distress. Both males and females were utilized. Animals were housed in groups in rooms with a 14/10-h light/dark cycle, except for dogs, an inbred stain of Beagle, where only males were used, given they have X-linked Alport syndrome.

Podocyte culture

Conditionally immortalized podocytes were derived from 6-week-old 129 Sv wild-type or Alport mice carrying the immortomouse transgene as previously described [16]. The clonal cell line was characterized by verifying induced expression of podocin, α-actinin-4, nephrin, and von Willibrand factor (absence of expression) in differentiated cells compared with non-differentiated cells prior to use. Cells were grown under permissive conditions (10% FCS, 10 U/ml γ-interferon at 33 °C) and then plated on human fibronectin (356008; Corning, Bedford, MA, USA) at a density to achieve a 50% final confluency and allowed to differentiate (no γ-interferon at 37 °C) for 10 days. We chose to culture cells on fibronectin rather than collagen I, which is typically used, because collagen I activates DDR1, which would confound our results [17]. During differentiation, podocytes were cultured in 5% fetal calf serum (FCS), reduced to 2% FCS on day 3 and 0.5% FCS on day 7. After 10 days of differentiation, siRNA transfections were performed by complexing 5 µl of *Transit-X2* transfection system (MIR6004; Mirus, Madison, WI, USA) and 1 µg of Silencer® Select Pre-designed siRNAs (Ambion, Carlsbarg, CA, USA) in 500 µl of DMEM/F-12 medium, –FCS, –antibiotics for 20 min. Complexes were applied dropwise following the manufacturer's guidelines. Thirty-six hours post-transfection, soluble collagen α1(III) (NBP1-97267; Novus, Centennial, CO, USA) was added to the culture media at a concentration of 5 µg/ml. After 24 h, cells were lysed in Trizol® Reagent (Ambion) and RNA was isolated from the aqueous phase utilizing PureLink™ RNA Micro Kits (12183; Invitrogen, Carlsbad, CA, USA). An RNA Quality Number (RQN) was determined for each sample using a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc, Ames, IA, USA). Samples with RQNs of ≥8 were processed using a TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) and cDNA synthesis and libraries were generated utilizing a Nextera™ XT DNA Library Preparation Kit (Illumina). RNA-seq analysis was performed using the Illumina® NextSeq™ 550 system.

Immunohistochemistry

An agrin antibody (AF550; R&D Systems, Minneapolis, MN, USA) was used at 1:200. A laminin α2 antibody (L-0663; Sigma, St Louis, MO, USA) was used at

1:200. A collagen α1(III) antibody (NB600-594; Novus Biologicals, Littleton, CO, USA) was used at 1:500. This antibody had been extensively qualified by the manufacturer and showed minimal cross-reactivity with collagen types I, II, IV, V, and VI in ELISA assays. A DDR1 antibody (AF2396, R&D Systems) was used at 1:75. For dog kidney immunostaining, an anti-collagen α1(III) antibody raised in goat (NBP1-26547; Novus Biologicals) was used at 1:50. The anti-laminin β2 antibody was from the Developmental Studies Hybridoma Bank and was a mouse monoclonal used at 1:25 dilution. An anti-collagen I antibody (CL50151AP; Cedar Lane, Burlington, ON, Canada) was used at 1:200 dilution. Anti-phospho-DDR1 (Tyr513) (PA5-37501; Thermo Fisher, Waltham, MA, USA) was used at 1:25. Anti-integrin α2 (PA5-47193; Thermo Fisher) was used at 1:25. Anti-laminin α5 (EWL004; Kerafast, Boston, MA, USA) was used at 1:800. Nidogen 1 antibody (MAB1946; EMD Millipore, Burlington, MA, USA) was used at 1:200. Anti-podocin (a gift from Dr Corinne Antignac, Institut National de la Santé et de la Recherche Médicale, Inserm UMR 1163, Laboratory of Inherited Kidney Diseases, Imagine Institute, Paris, France) was used at 1:100. Anti-WT1 (SC-15421; Santa Cruz Biotechnology, Dallas, TX, USA) was used at 1:50 dilution. Anti-collagen V (1350-01; Southern Biotech, Birmingham, AL, USA) was used at 1:100. All immunostaining was done on 4-μm-thick sections of frozen mouse tissue fixed with cold acetone. Sections were incubated with primary antibodies in 1% BSA in PBST (PBS plus 0.05% Tween-20) overnight at 4 °C. All fluorescent secondary antibodies (Alexa Fluor, Thermo Fisher) were used at 1:500 dilution and applied in 1% BSA/PBST for 1 h at room temperature. Three washes in PBS, each 5 min, were performed after primary and secondary antibody incubations. Vectashield with DAPI was used as the mounting medium (Vector Labs, Burlingame, CA, USA) before coverslips were applied. Confocal microscopy was performed using a Zeiss (White Plains, NY, USA) LSM 710 and a Nikon (Melville, NY, USA) Eclipse Ti, and the images were processed using the software program Fiji (https://imagej.net/software/fiji/; Accessed 19 May 2022).

Structured illumination microscopy (SIM)

SIM images were captured using a Zeiss ELYRA PS.1 super-resolution microscope.

Western blotting

Glomeruli were isolated, as previously described [8], from 7-week-old wild-type and collagen α3(IV)-null mice. Glomerular ECM proteins were enriched as previously described [18,19]. Protein from a single mouse was fractionated in one lane of a 4–20% TGX™ acrylamide gel (Cat# 4561093; Bio-Rad, Hercules, CA, USA). Proteins were electrotransferred onto PVDF membranes (Cat# 88518, Thermo Scientific), 30 V overnight at 4 °C. Membranes were blocked with 5% Blotting-Grade Blocker (Cat# 1706404, Bio-Rad) in Tris-buffered saline containing 0.05% Tween-20 for 1 h. Membranes were incubated with a 1:5,000 dilution of collagen α1(III) antibody (Cat# NB600-594, Novus Biologicals) overnight at 4 °C. Blots were washed and incubated with a 1:10,000 dilution of anti-rabbit HRP-conjugated secondary antibody (Cat# 12-348, Sigma). Blots were then developed with Radiance Chemiluminescent HRP Substrate (Cat# AC2100; Azure Biosystems, Dublin, CA, USA) according to the manufacturer's recommendation. Exposures were generated using the Azure Biosystems 600 Imaging System. Blots were stained with TotalStain Q (Cat# AC2225, Azure Biosystems) following the manufacturer's protocols. Quantification of bands was performed using the Fiji software suite. Normalization to total protein and removal of background were performed using the mean gray value for all exposures. Statistical analyses used Student's *t*-test.

Collagen α1(III) mediated activation of DDR1 in cultured podocytes

Podocytes were differentiated on human fibronectin (356008, Corning)-coated cytology slides (VWR, Batavia, IL, USA) for 10 days and treated with collagen α1(III) (NBP1-97267, Novus) for 24 h. Slides were rinsed 5 times with PBS, fixed with −20 °C acetone for 5 min, and dried for 2 h at 25 °C. Slides were incubated with antibodies to DDR1 and pDDR1 as described above. Images were captured on a Zeiss AX10 imager, A1.

RNA-seq analysis for podocytes and glomeruli

All experiments were performed in triplicate. Samples were processed by the Illumina NextSeq 550 platform at the University of Nebraska Medical Center's Sequencing Core Facility. The resulting Fastq files were sent through a pipeline utilizing the Trimmomatic suite [20] and a 2-Pass STAR protocol [21] equipping the GRCm38 genome (mm10) from the National Center for Biotechnological Information (NCBI) and a curated genomic regions file. The genomic regions file was trimmed of pseudogenes, RIKEN cDNA clones, and other novel regions to clarify the resulting output.

The aligned binary files were processed using the default settings of the featureCounts program of the Rsubread package from Bioconductor, allowing multi-overlap [22]. The resulting gene feature count values were derived using the list of curated genomic regions that are synonymous with the Mouse Genome Index (MGI) and the NCBI, filtering the same genomic regions file used for alignment. These genes had descriptions within the MGI and were also present in the NCBI database. The resulting 19,743 genomic features were used to score the alignments in featureCounts. Samples were normalized across the experiment to genes *Gapdh*, *Atp5pb*, and *Pgk1* [23]. Normalized read counts were then analyzed with the Gene Set Enrichment Analysis (GSEA) Software Package v4.1.0 from the Broad Institute [24] using the Signal2Noise metric to identify

differentially expressed genes and gene sets from those which are provided in the c5.all.v7.0 subset in the GSEA Molecular Signatures Database. We chose the Signal2Noise metric to discover genes with no expression to be included in the analysis that may have been excluded via the classic fold-change metric. Genes

Table 1. Collagen III responsive genes in cell cultures validated by glomerular RNA-seq from 7-week-old WT and Alport mice

Gene	Role in podocyte injury or protection	Reference	FC	DDR1 KD	ITGA2 KD
Tnfsf15	Associated with nephrotic syndrome	[25]	3.8	**2.2**	**2.2**
Egfl7	Reduces vascular elasticity increasing biomechanical strain	[26]	3.7	**1.3**	**1.6**
Fgf1	Suppresses oxidative stress and inflammation	[27]	3.1	**2.0**	**−1.2**
Itga2	Promotes progression of CKD in Alport syndrome	[13]	3.0	**−4.4**	**−7.5**
Ccn2	Encodes CTGF, associated with glomerulonephritis/inflammation	[28]	2.7	**2.0**	**1.9**
Mcub	Mitochondrial calcium uptake mediator	[29]	2.7	**1.7**	2.8
Nyap	PI3K Akt Rac1 cell signaling regulator/acting remodeling	[30]	2.7	**1.1**	**1.3**
Tlr5	Toll-like receptor shown to induce podocyte apoptosis	[31]	2.7	**2.1**	**1.7**
Itgb3	αvβ3 heterodimer has been shown to promote podocyte injury	[32]	2.4	**1.4**	2.1
Tle6	Regulates podocyte proliferation	[33]	2.4	**1.8**	**1.9**
Ntrk2	Susceptibility gene for childhood IgA nephropathy	[34]	−2.3	−2.0	**−4.2**
Cacna1b	Its deletion ameliorates diabetic nephropathy	[35]	−2.5	**−4.5**	**−4.8**
Apela	Regulates renal fluid homeostasis	[36]	−2.7	**−1.0**	**−1.2**
Iqgap2	Required for the glomerular filtration barrier	[37]	−2.8	−2.4	**−3.7**
Cyp2s1	Associated with CKD in mice and humans	[38]	−3.1	**−4.2**	−2.8
Kcnma1	Associated with TRPC6-mediated podocyte injury	[39]	−3.9	−3.7	**−6.6**
Lrp3	Associated with the formation of inflammasomes in podocytes	[40]	−6.4	**−1.1**	**−2.0**
Steap4	Associated with the progression of diabetic nephropathy	[41]	−16.1	**−10.3**	**−7.9**

FC, fold-change; KD, knockdown.
KD values in bold trend towards baseline.

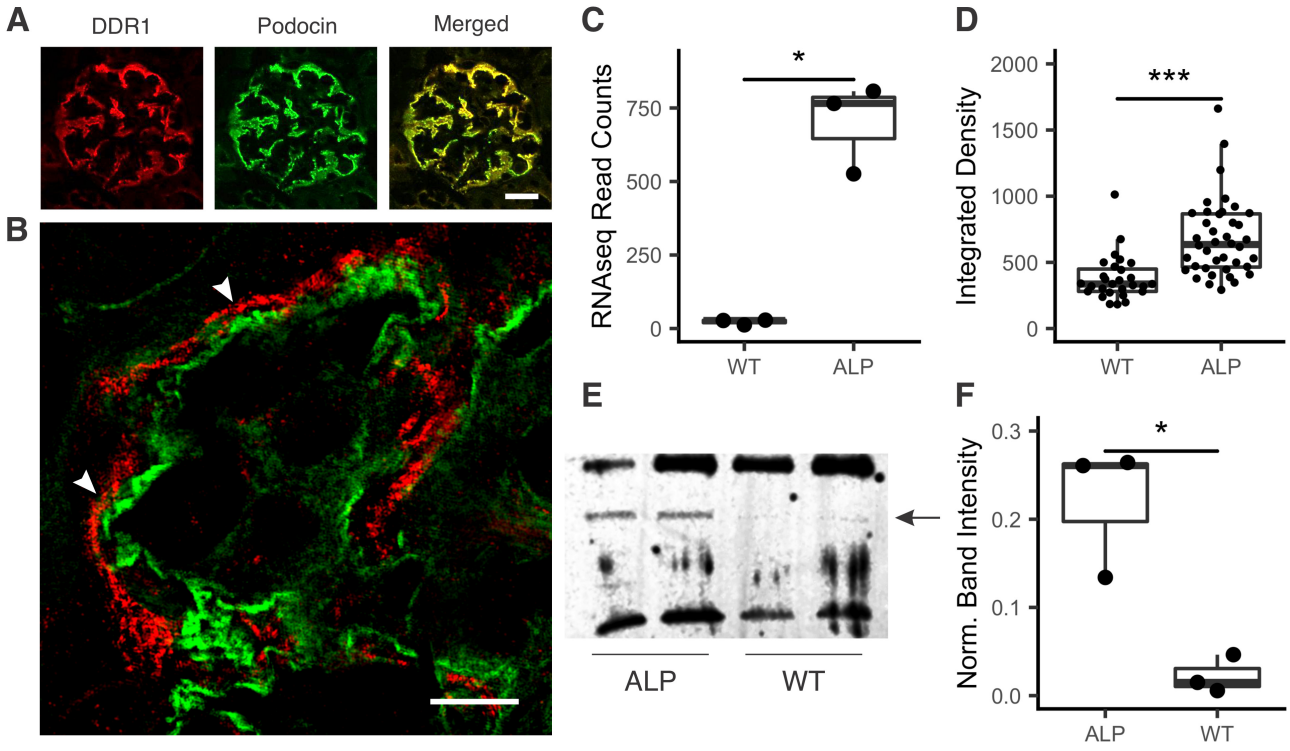

Figure 1. Collagen α1(III) is expressed in the glomerular basement membrane in Alport mice. (A) Dual immunofluorescence analysis was performed on kidney cryosections from 7-week-old wild-type and Alport mice using antibodies for podocin (a slit diaphragm protein) and DDR1 (a collagen receptor). Clear co-localization is apparent, placing DDR1 at the foot processes (bar = 15 μm). (B) Super-resolution structured illumination microscopy (SR-SIM) of dual immunofluorescence staining of a capillary loop from a 7-week-old Alport mouse stained with anti-DDR1 antibodies (in red) and anti-collagen α1(III) antibodies (in green). The adjacent localization (arrowheads) indicates basement membrane localization of collagen α1(III) (bar = 5 μm). (C) RNA-seq results from wild-type and Alport glomeruli show a marked (>20-fold) increase in the expression of *Col3a1* mRNA relative to wild-type. These results were confirmed using real-time RT-PCR (data not shown) and microarray analysis [8]. (D) ImageJ analysis of the relative fluorescence for immunostains of wild-type and Alport glomeruli (six independent glomeruli each) shows significant increases of fluorescence intensity in Alport mice. (E) Western blotting shows clear increases in the 139 kDa band corresponding to collagen α1(III). (F) Quantification of the relative band intensity for triplicate blots of wild-type and Alport mouse glomeruli indicates significantly elevated abundance of collagen α1(III) in Alport glomeruli relative to wild-type, consistent with the RNA-seq findings. *$p < 0.05$, ***$p < 0.001$.

were ranked from most induced (relative to untreated cells) to most suppressed. Those genes whose ranking metric score modulated from ±2 to 5 were cross-referenced with data from the DDR1 and integrin α2 knockdown cells to look for return of wild-type gene expression. These data were then validated for relevance using RNA-seq data from 2- and 5-week Alport glomeruli (three individual samples run in triplicate and analyzed as per above) to confirm induction or suppression of gene expression *in vivo*. It is these data that are presented in Table 1. GSEA pathway analysis was also performed for collagen α1(III)-treated cells versus untreated control, and 7-week-old *Col4a3*$^{-/-}$ mice versus wild-type control. All pathways listed in supplementary material, Table S2, showed statistics that had nominal P value < 0.05, false discovery rate (FDR) < 0.25, and family-wise error rate (FWER) < 0.25. Using the top 100 pathways from collagen α1(III)-treated cells versus non-treated, 5-week Alport versus WT glomeruli, and 7-week Alport versus WT glomeruli, we found the pathways in these lists that are duplicated in either the 5-week or the 7-week data set, listed in supplementary material, Table S3.

Glomerular isolation was performed as described previously [8]. The complete data set for RNA-seq results for both cell cultures and glomeruli are provided in the NCBI database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA690102; Accessed 3 November 2021).

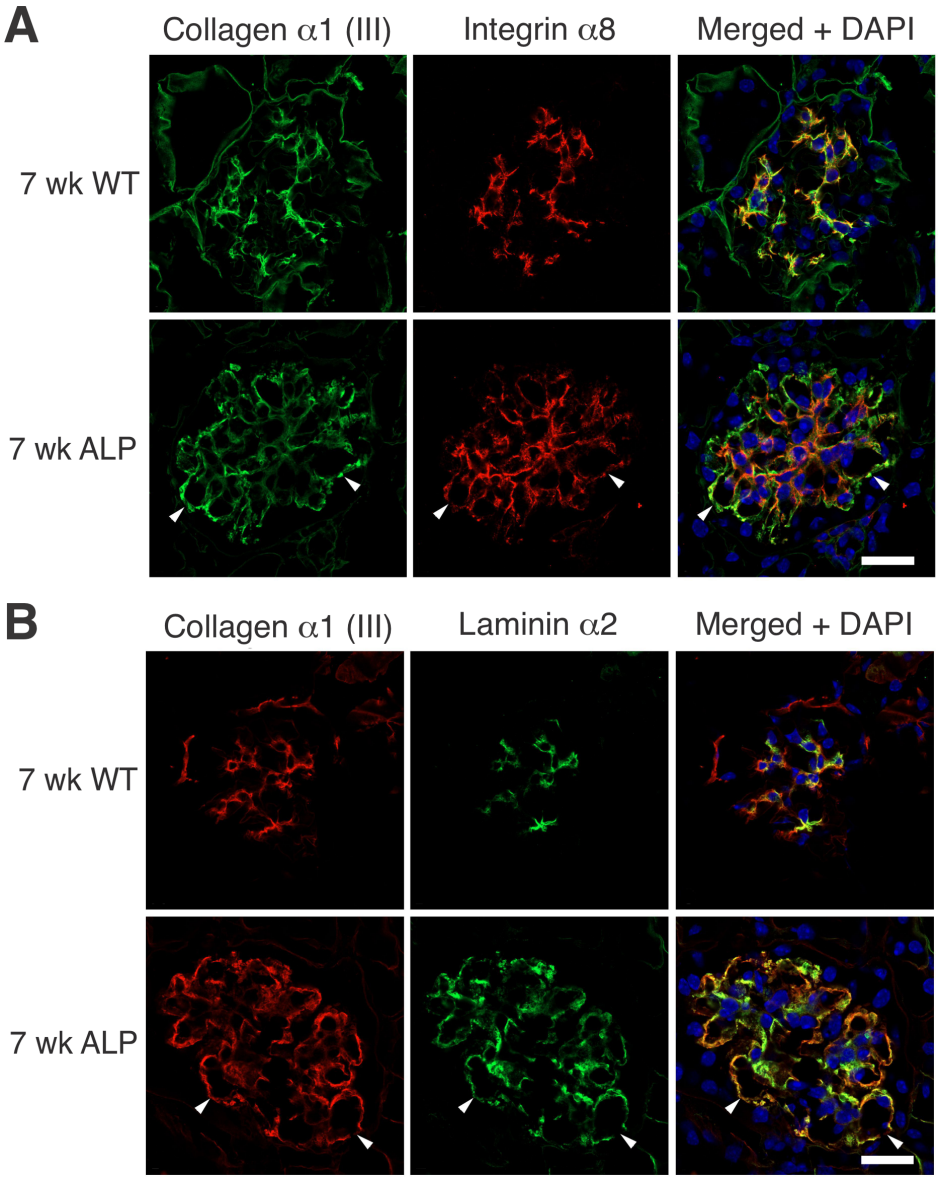

Figure 2. Collagen α1(III) co-localization with glomerular mesangial matrix proteins. (A) Mesangial filopodia are evident in the areas of the GBM where collagen III is observed. Dual immunofluorescence analysis was performed on kidney cryosections from 7-week-old wild-type and Alport mice using antibodies for integrin α8 (a mesangial integrin) and collagen III. Integrin α8 localizes to capillary loops where collagen α1(III) is present, consistent with a mesangial ECM protein. The dual fluorescence localizes collagen α1(III) to the GBM. Arrowheads denote areas in the GBM with clear co-localization. Bar = 15 μm. (B) Laminin α2 and collagen α1(III) co-localize in the GBM, consistent with their being secreted by mesangial filopodia. Dual immunofluorescence analysis was performed on kidney cryosections from 7-week-old wild-type and Alport mice using antibodies for laminin α2 and collagen α1(III). Arrowheads denote areas in the GBM with clear co-localization. Bar = 15 μm.

Statistical analysis

Quantitative PCR (qPCR) and RNA-seq results were statistically analyzed using two-tailed Student's *t*-tests with Bonferroni correction.

Results

Previous work showed that DDR1 co-localized with nephrin, positioning it at the interface between the podocyte pedicles and the GBM [42]. In Figure 1A, we show that the nephrin binding protein podocin also co-localizes with DDR1 (as expected). Using super-resolution structured illumination microscopy (SR-SIM), we showed that collagen α1(III) is localized immediately adjacent to DDR1, positioning collagen α1(III) in the GBM of Alport mice. Collagen α1(III) is restricted to the mesangium in wild-type mice (supplementary material, Figure S1) and co-localizes with the GBM in 7-week-old Alport mice (supplementary material, Figure S2). It was noted from RNA-seq data that *Col3a1* mRNA was markedly induced (>10-fold) in Alport mouse glomeruli relative to wild-type (Figure 1C). Immunostaining intensity for collagen α1(III) in Alport glomeruli was also significantly elevated relative to wild-type, as quantified using ImageJ software (Figure 1D; six independent glomerular images measured for each, shown in supplementary material, Figure S1). The induction of collagen α1(III) at the protein level was further confirmed using western blot analysis of extracts from isolated glomeruli from wild-type and Alport mice (duplicate blots from two groups of wild-type and Alport mice shown in Figure 1E; quantification and statistical analysis using Student's *t*-test from three independent animals per group shown in Figure 1F). We also observed GBM staining for

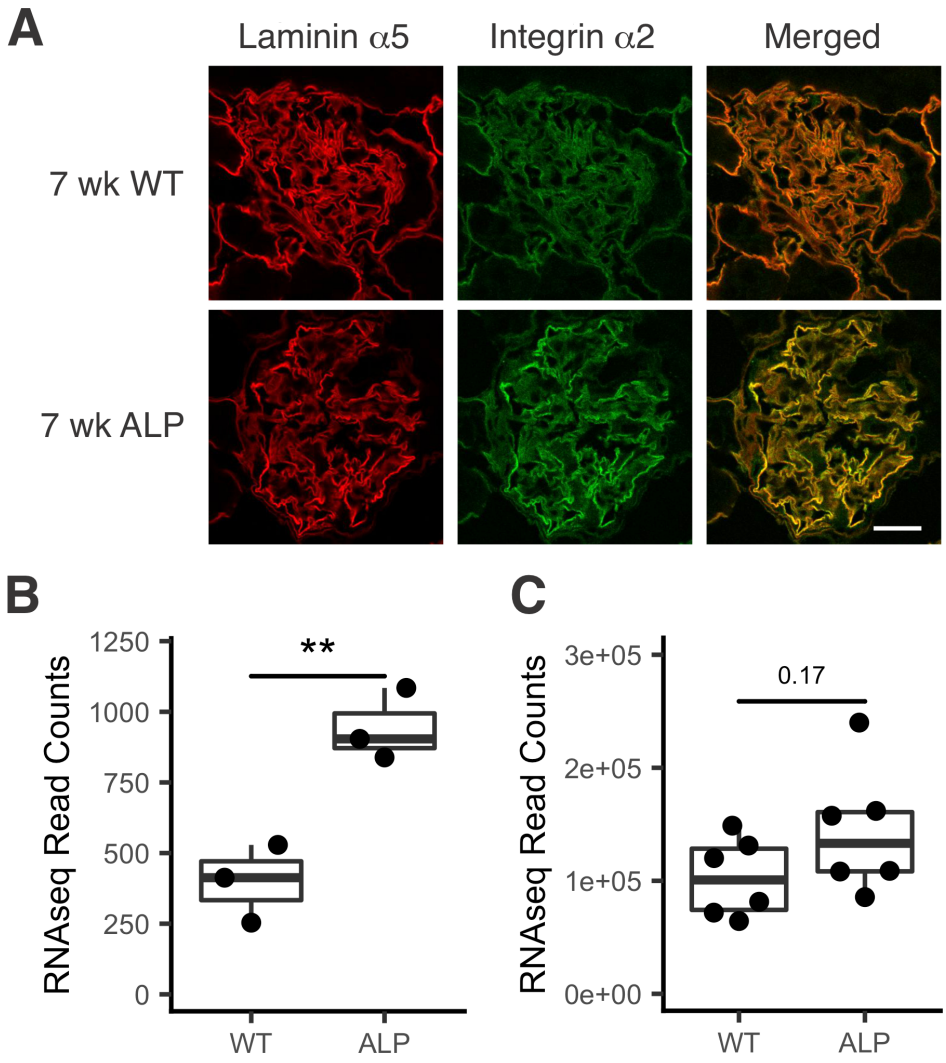

Figure 3. Integrin α2 co-localizes with laminin α5 and is induced in Alport glomeruli relative to wild-type. (A) Kidney cryosections from wild-type and Alport mice were dual immunostained with antibodies specific for integrin α2 and laminin α5. Immunostaining indicates co-localization with elevated levels of integrin α2 in Alport mice relative to wild-type. Bar = 15 μm. (B) RNA-seq of triplicate RNA samples from wild-type and Alport glomeruli shows a significant increase of *Itga2* in Alport glomeruli relative to wild-type $^{**}p < 0.01$. (C) ImageJ analysis of the relative fluorescence for immunostains of wild-type and Alport glomeruli (six independent glomeruli each) shows an increase in Alport fluorescence intensity that trends towards significance.

collagen α1(III) in Alport dog glomeruli, where it co-localizes with laminin β2 (supplementary material, Figure S3).

Dual immunofluorescence analysis for collagen α1 (III) and integrin α8, a marker for mesangial cells, shows extensive invasion of mesangial filopodia into the sub-

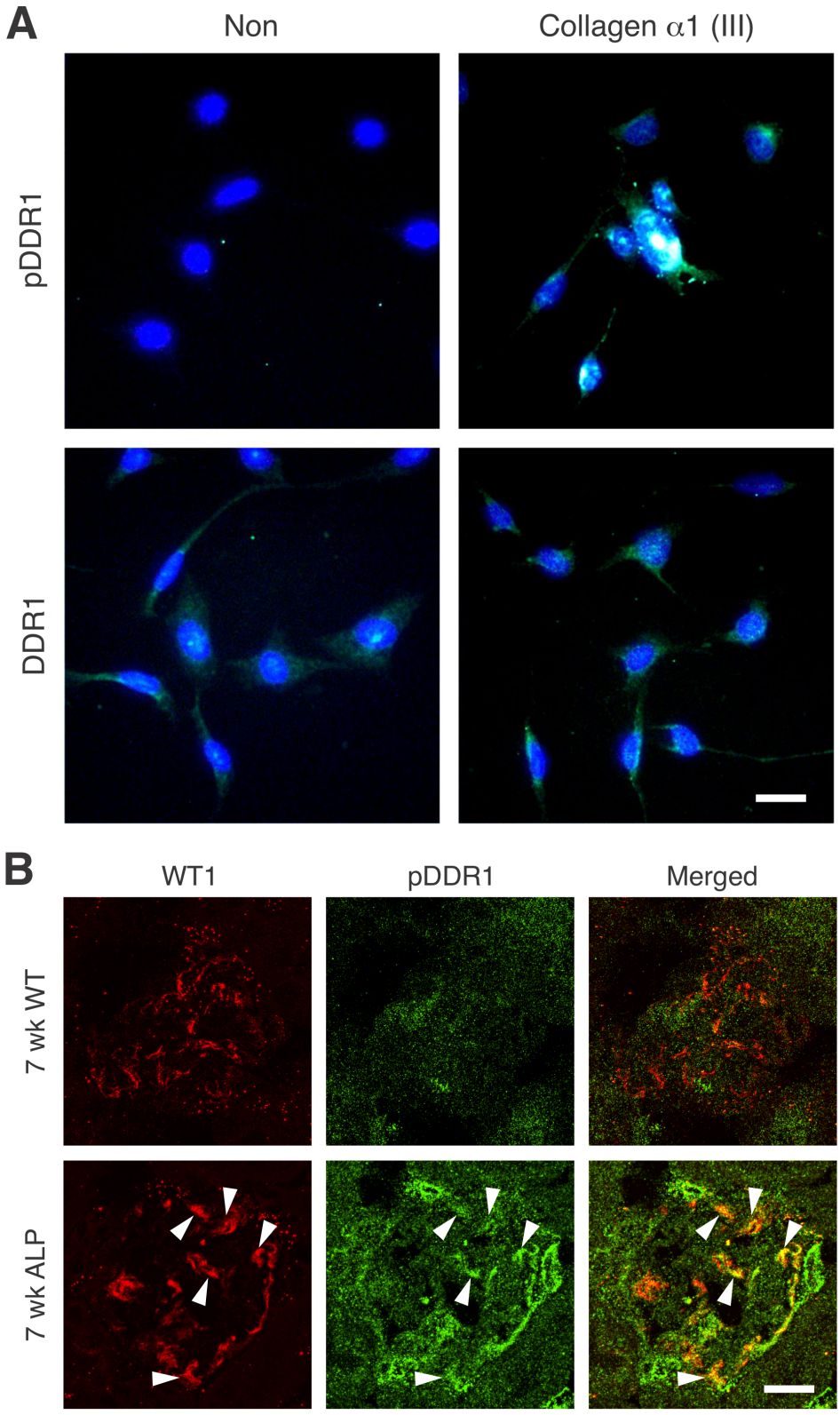

Figure 4. Collagen α1(III) activates DDR1 receptors both *in vitro* and *in vivo*. (A) Cells were treated or not with collagen III and after 12 h, stained with antibodies against either total DDR1 or phospho-DDR1 (pDDR1) (bar = 5 μm). (B) Cryosections from 7-week-old wild-type and Alport mice were dual immunostained with antibodies specific for pDDR1 or WT1 (a podocyte nuclear marker) (bar = 15 μm). Results indicate that collagen III activates DDR1 receptors both *in vitro* and *in vivo* in glomerular podocytes. Arrowheads denote areas of WT1 and pDDR1 co-localization.

endothelial spaces of mesangial capillaries in regions where GBM deposition of collagen α1(III) is observed (Figure 2A). Dual staining for collagen α1(III) and laminin α2 (which have previously been shown to be deposited in the GBM) [9,11] showed that the mesangial-derived proteins demonstrate overlapping immunostaining in the GBM, as would be expected if they were both deposited by mesangial filopodia (Figure 2B).

There are two collagen receptors expressed on glomerular podocytes: integrin α2β1 and DDR1. Deletion of either of these two receptors in Alport mice results in attenuated progression of renal disease and extended lifespan. This clearly implicates collagen-mediated signaling via these receptors in the pathobiologic mechanism of Alport glomerular disease in the model [12,13]. If DDR1 and integrin α2β1 receptors are to engage collagen ligands in the GBM, they must localize to the interface between the GBM and the podocyte foot processes. To determine if this is the case, we performed dual immunofluorescence analysis with antibodies to podocin and DDR1 (Figure 1A) or antibodies to integrin α2 and laminin α5 (Figure 3). Different marker antibodies were used due to compatibility with test antibodies. The results confirm proper localization of both DDR1 and integrin α2β1 for interaction with collagen ligand in the GBM. Further, the results show elevated expression of α2β1 integrin in Alport glomeruli relative to wild-type glomeruli (Figure 3A), which was confirmed at the mRNA level (Figure 3B) and at the protein level by ImageJ analysis of replicate images (Figure 3C, six each for wild-type and Alport).

For collagen α1(III) to modulate gene expression in the nucleus, it must activate receptors on podocytes. To determine whether this is the case, we overlaid cultured differentiated podocytes with collagen α1(III). DDR1 activation is easy to visualize, as it results in its translocation to the nucleus of podocytes [32]. After 24 h treatment, DDR1 was clearly localized to the nucleus in the collagen α1(III)-treated cells, but not untreated cells (Figure 4A). As shown in Figure 1B, the GBM of 7-week-old Alport mice contained a significant amount of collagen α1(III), while that of wild-type mice did not (supplementary material, Figure S1). We expected to see activated pDDR1 in Alport podocytes but not in wild-type podocytes, and Figure 4B shows that this is indeed what we observed, with some, but indeed not all, of the pDDR1 staining localizing to the podocyte nucleus, where it co-localizes with WT1. Super-resolution microscopy studies suggested that the type IV collagen α3/α4/α5 network is too distant from the podocyte pedicles to interact with collagen receptors [6]. There is no direct evidence that this in fact occurs, or that the collagen IV network activates collagen receptors on podocytes. Figure 5 shows that DDR1 is not activated in glomeruli from 5-week-old integrin α1-null Alport mice [a stage where collagen α1(III) is not yet significantly observed in the GBM]; thus, the type IV collagen network is likely too distant from the receptors to bind and activate them.

To determine whether collagen α1(III) directly injures podocytes, we utilized cultures of Alport mouse podocytes. Alport podocytes were developed because we assumed that they would be under endoplasmic reticulum (ER) stress due to the inability to form collagen α3/4/5 (IV) heterotrimers, resulting in the accumulation of type IV collagen α4 and α5 chains in the ER. In Figure 6, we show that these cells are indeed under ER

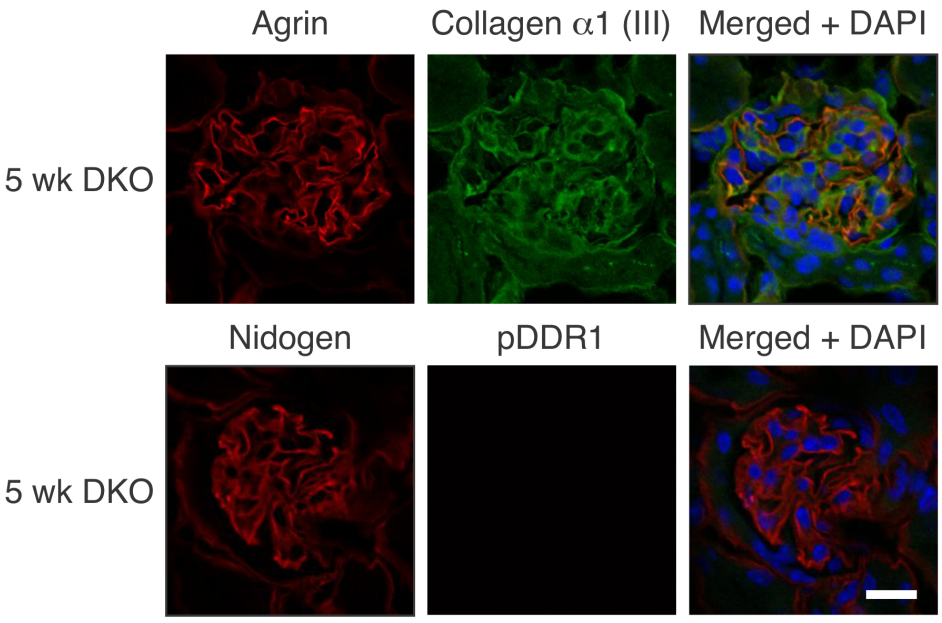

Figure 5. The collagen IV α1/α2 network in Alport GBM does not activate DDR1. Cryosections from 5-week-old integrin α1-null Alport mice were stained with antibodies for the indicated proteins. Note the absence of collagen α1(III) in the GBM and the absence of pDDR1 nuclear immunostaining in the podocytes. This indicates that the collagen IV α1/α2 network does not activate DDR1. Bar = 15 μm.

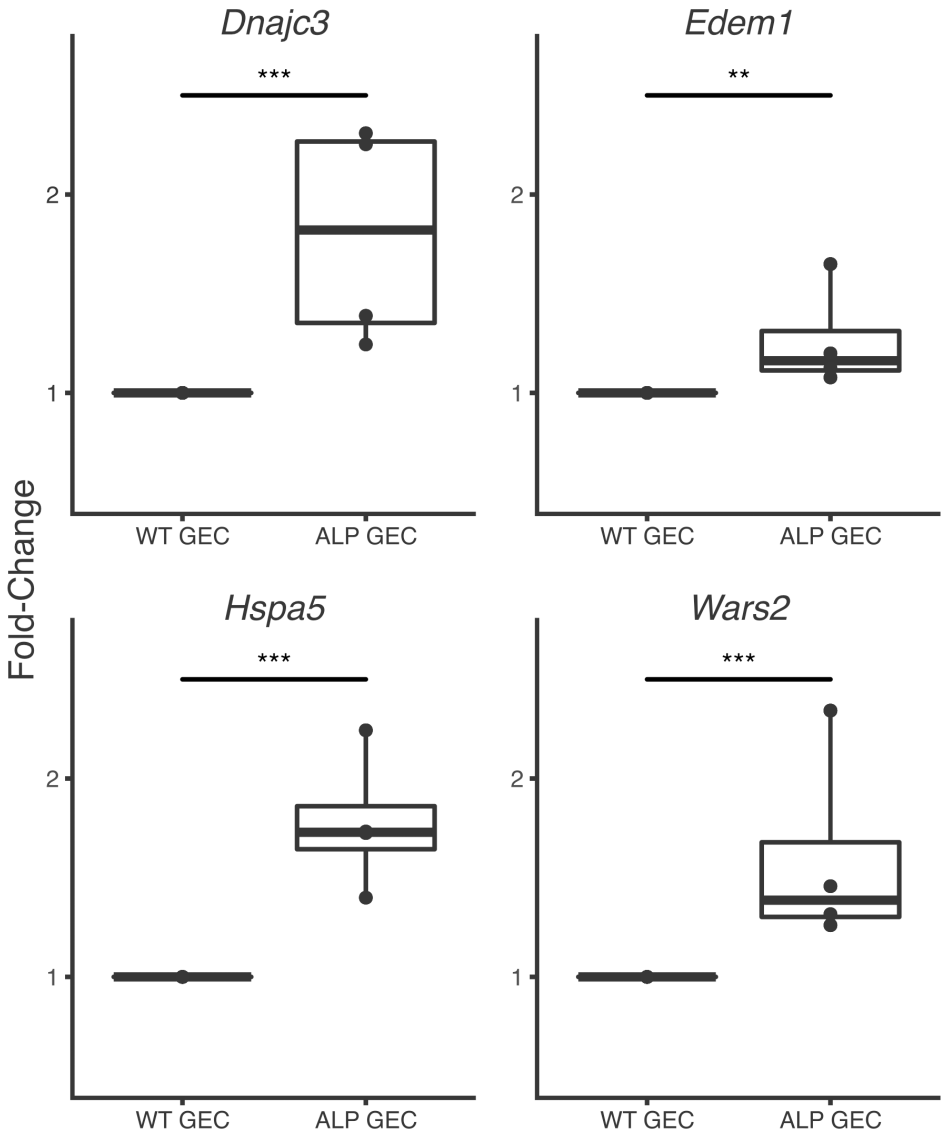

Figure 6. Genes associated with ER stress are induced in Alport podocytes relative to wild-type podocytes. Cultured podocytes were differentiated for 14 days and the RNA was isolated and analyzed for the indicated transcripts by RT-qPCR. The experiment was run in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$.

stress, based on significantly elevated expression of four ER stress-associated transcripts relative to wild-type podocytes [43–46]. It is likely that because of ER stress, Alport podocytes may respond differently to mesangial ECM proteins than wild-type podocytes. Podocytes were transfected with scrambled siRNA or siRNA knockdown for either integrin α2 or DDR1. In all cases, more than 80% knockdown of transcripts was achieved based on real-time RT-PCR analysis (supplementary material, Figure S4). After differentiating for 8 days, transfections were performed and the podocytes were overlaid with recombinant collagen α1(III) from Novus [or not overlaid with collagen α1(III), as a control] and cultured for 24 h. RNA was isolated and analyzed by RNA-seq. The experiment was performed three independent times and each sample analyzed independently to allow statistical analysis.

There were about 150 transcripts significantly increased in relative abundance (based on Student's *t*-tests) and about 150 that were significantly reduced in relative abundance in collagen α1(III)-treated cells versus untreated cells. The complete list is provided in supplementary material, Table S1. We validated whether these transcripts are induced or suppressed *in vivo* using RNA-seq data from glomerular RNA derived from 5- and 7-week-old 129 Sv autosomal Alport mice. Literature searches were performed for each gene to identify those previously implicated in glomerular disease or CKD. The genes that met these two criteria are listed in Table 1. Of the 18 genes identified that are induced or suppressed by collagen α1 (III), only *CTGF* and *ITGA2* have been previously implicated in Alport syndrome [13]. Thus, the remaining genes in Table 1 will be of interest for further studies. The data indicate not only genes that are implicated in podocyte injury but also some that have been shown to be protective, which may reflect a compensatory mechanism.

For most (12) of these genes, knockdown of either DDR1 or laminin α2 restored expression towards baseline levels, indicating that collagen α1(III) is inducing co-receptor signaling via the two collagen receptors to regulate most of the genes. For three genes (Table 1), expression was restored towards baseline levels in DDR1-knockdown cells but was unaffected in integrin α2-knockdown cells, and for three genes (Table 1), expression was restored towards baseline levels in integrin α2-knockdown cells but was unaffected in DDR1-knockdown cells. These data clearly demonstrate that the two collagen receptors can either signal independently or jointly, with co-receptor signaling being the dominant mechanism in podocytes.

Regarding the genes that are regulated by both DDR1 and α2β1 integrin, crosstalk between these two collagen receptors has been previously described. It is established that DDR1 activation can promote adhesion of α2β1 integrin to collagen [47], and to prevent α2β1-dependent cell spreading on collagen via inhibition of CDC42 activity [48].

Discussion

The mesangial matrix protein collagen α1(III) progressively accumulates in the GBM of Alport mice and causes podocyte injury via aberrant cell signaling through either DDR1 or α2β1 integrin (rarely) or through DDR1/α2β1 co-receptor signaling (mostly). Our transcriptome analysis approach identified many genes that are involved in the podocyte injury mechanism through their collagen α1(III)-mediated dysregulation. This phenomenon likely accounts for why deletion of either DDR1 or integrin α2 ameliorates the renal phenotype in Alport mice [12,13].

Super-resolution microscopy studies suggested that the type IV collagen α3/α4/α5 network is too distant from the podocyte pedicles to interact with collagen receptors [6]. There is no direct evidence that this in fact occurs or that the collagen IV network activates collagen receptors on podocytes. The results in Figure 4B showed that DDR1 is not activated in podocytes of wild-type glomeruli but is activated in podocytes of Alport glomeruli. Figure 4A showed that collagen α1(III) directly activates DDR1 in cultured podocytes. We have previously shown that Alport mice on the integrin α1-null background show markedly delayed deposition of laminin α2 in the GBM [9]. In 5-week-old integrin α1-null Alport mice, GBM deposition of collagen α1(III) is not yet apparent. Since these are Alport mice, the collagen network is entirely composed of type IV collagen α1/α2 protomers (supplementary material, Figure S5). DDR1 activation is not observed, demonstrating that the collagen α1/α2 network is not activating DDR1 receptors (Figure 5). Thus, collagen α1(III) in the GBM results in DDR1-mediated podocyte injury. The fact that many of the same podocyte injury genes are activated in both collagen α1(III)-treated podocytes and Alport glomeruli as a function of collagen α1(III) deposition in the GBM (Table 1) supports this notion.

One interesting and unexpected aspect of the data was that for 12 of these up- or down-regulated genes validated *in vivo* and implicated in renal disease that are regulated by collagen α1(III), knockdown of *either* DDR1 or integrin α2 restored expression towards baseline levels. This clearly indicates that collagen α1(III) is inducing co-receptor signaling in podocytes via the two collagen receptors to regulate most of the genes. For three genes, expression was restored towards baseline levels in DDR1-knockdown cells but was unaffected in integrin α2-knockdown cells. For three genes, expression was restored towards baseline levels in integrin α2-knockdown cells but was unaffected in DDR1-knockdown cells. None of the genes examined were unaffected in the knockdown studies, suggesting that we have identified all the collagen receptors involved. Remarkably, these data clearly demonstrate that the two collagen receptors can signal either independently or jointly.

It should be noted that early work in humans showed the presence of type V and type VI collagen in the GBM of Alport glomeruli [49]. The collagen antibodies in this study appeared to be properly validated by the manufacturer. Given concern that our results might reflect activation by either collagen V or collagen VI, we performed immunostaining of wild-type and Alport glomeruli in 7-week-old mice using antibodies specific for these two collagens. Anti-collagen V staining was not observed in either the mesangium or the GBM (supplementary material, Figure S6). Recently, however, punctate deposition of collagen VI in Alport GBM was reported in the 129 Sv ARAS Alport mouse GBM [50]. Thus, our *in vivo* results might reflect activation of DDR1 by collagen α1(III) and collagen VI. Collagen I has been shown to accumulate in the mesangium upon glomerular injury and to contribute to the pathobiology in diabetic nephropathy [51]. We did not see any significant difference in immunostaining for collagen I when comparing wild-type and Alport glomeruli (supplementary material, Figure S7).

Our approach of exploring the role of collagen α1(III) in modulating gene expression first *in vitro* using conditionally immortalized podocytes from Alport mice and then validating that these genes are similarly and progressively modulated *in vivo* [given that collagen α1(III) progressively accumulated in the GBM] provides confidence that these genes likely underlie podocyte injury in Alport syndrome. One way to definitively test this would be to evaluate whether the genes return to baseline levels in glomeruli from integrin α2-null Alport mice and/or DDR1-null Alport mice. This approach would also allow one to explore whether co-receptor signaling and DDR1-specific or α2β1 integrin-specific signaling are happening *in vivo*. It would then be possible to go back to the cell line approach to map the signaling cascades involved in mono- and co-receptor signaling using small molecule inhibitors and/or siRNA knockdown approaches.

As shown in Table 1, many of the genes identified to be regulated by collagen α1(III) *in vitro* and *in vivo* have been previously associated with renal disease. Only CTGF (encoded by *CCN2*) and *ITGA2* have been previously associated with Alport renal disease progression [13,28]. Thus, this work reveals new and unexplored pathways that likely contribute to podocyte injury in Alport syndrome. In future work, these genes should have priority over ones that have not yet been associated with the pathobiology of the glomerulus.

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Author contributions statement

JM performed bioinformatics analysis, data analysis, microscopy, and immunohistochemistry, and edited the manuscript. KW and DM performed cell culture work, immunohistochemistry, and western blotting. DD and GS maintained mouse colonies for analysis. DC conceived the project and wrote the manuscript.

Data availability statement

The complete data set for RNA-seq results for both cell cultures and glomeruli are provided in the NCBI database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA690102).

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Immunofluorescence for collagen α1(III) in glomeruli from wild-type and Alport mice for semi-quantification

Figure S2. Collagen α1(III) is expressed in the glomerular basement membrane of Alport mice

Figure S3. Alport dog glomeruli also have collagen α1(III) in the GBM

Figure S4. siRNA knockdown of integrin α2 (*Itga2*) or DDR1 (*Ddr1*) mRNAs treated with collagen α1(III) (C3a1)

Figure S5. Collagen α1(IV) is restricted to the mesangium of glomeruli from wild-type mice and localizes to both the mesangium and the GBM of glomeruli from Alport mice

Figure S6. Collagen V is not expressed in the GBM of either wild-type or Alport mice

Figure S7. Collagen I is weakly expressed in the mesangium of both wild-type and Alport glomeruli and there is no difference in staining intensity when comparing the two

Table S1. Genes up-regulated by collagen α1(III) in podocytes

Table S2. GSEA pathway analysis results from collagen α1(III)-treated cells versus non-treated, and 7-week Alport versus WT glomerular data

Table S3. Gene Ontology Consortium pathways enriched in collagen α1(III)-treated cells, synonymous with the top 100 pathways found in either the 5-week Alport versus wild-type, or the 7-week Alport versus wild-type glomerular RNA-seq data processed using GSEA