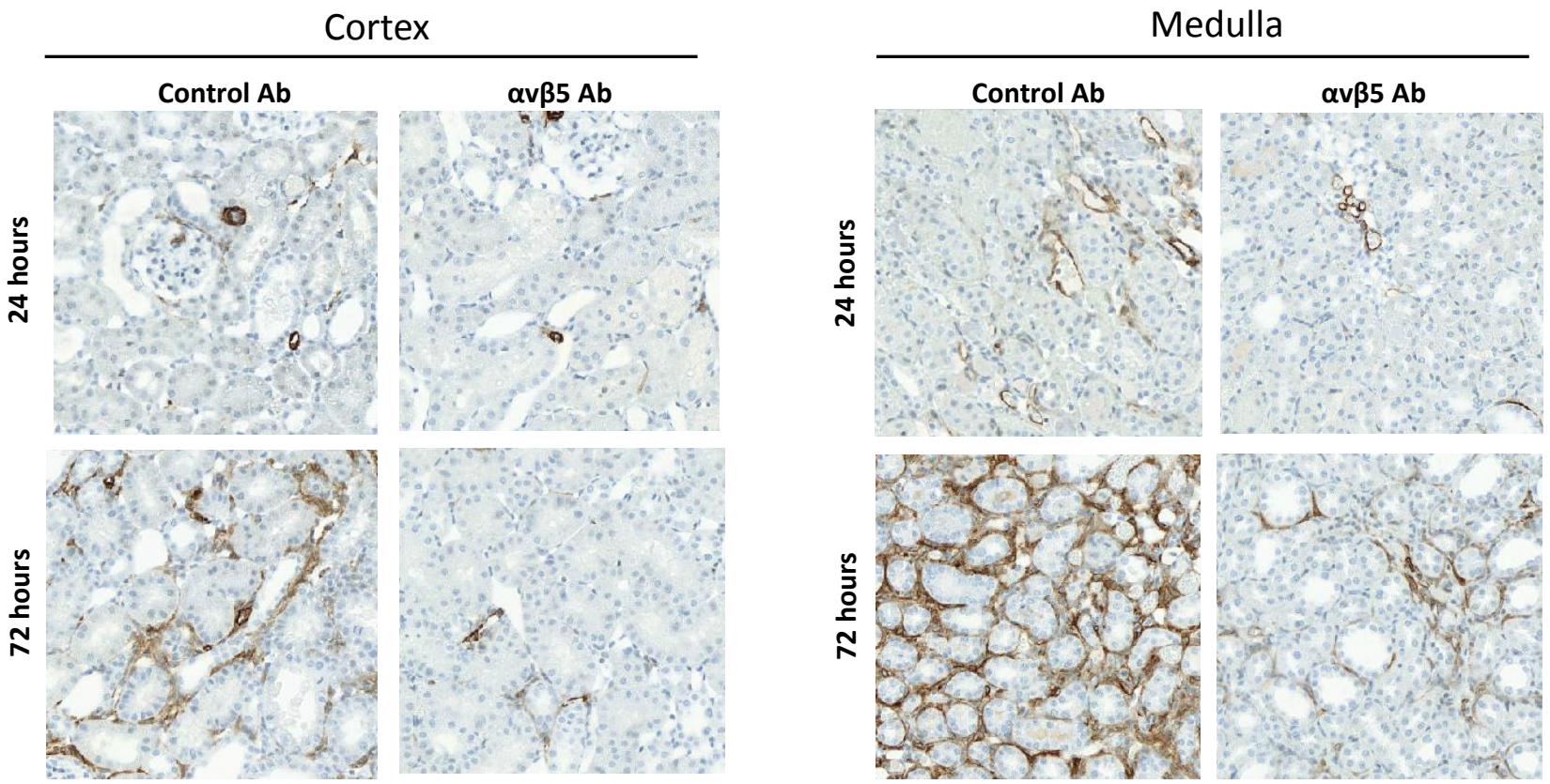
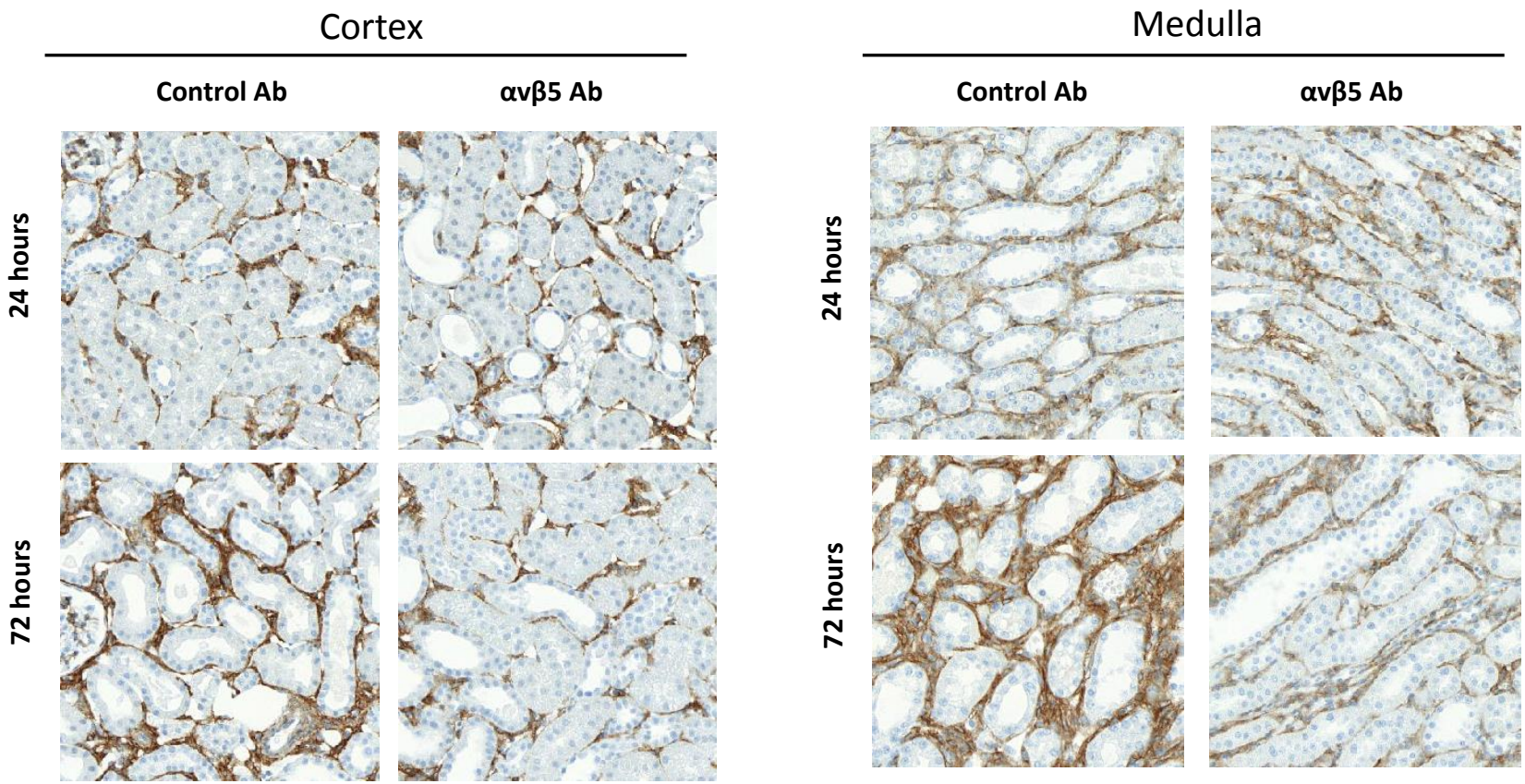
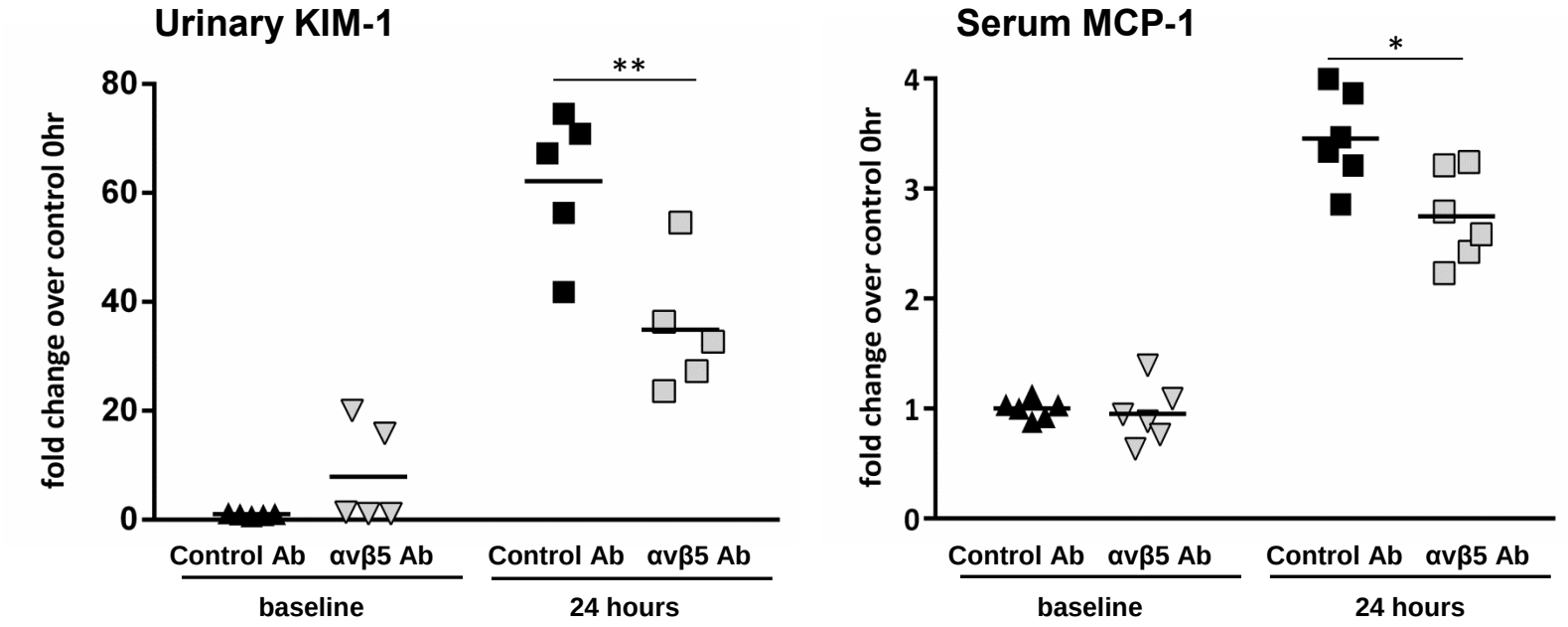
Supplemental material and online methods



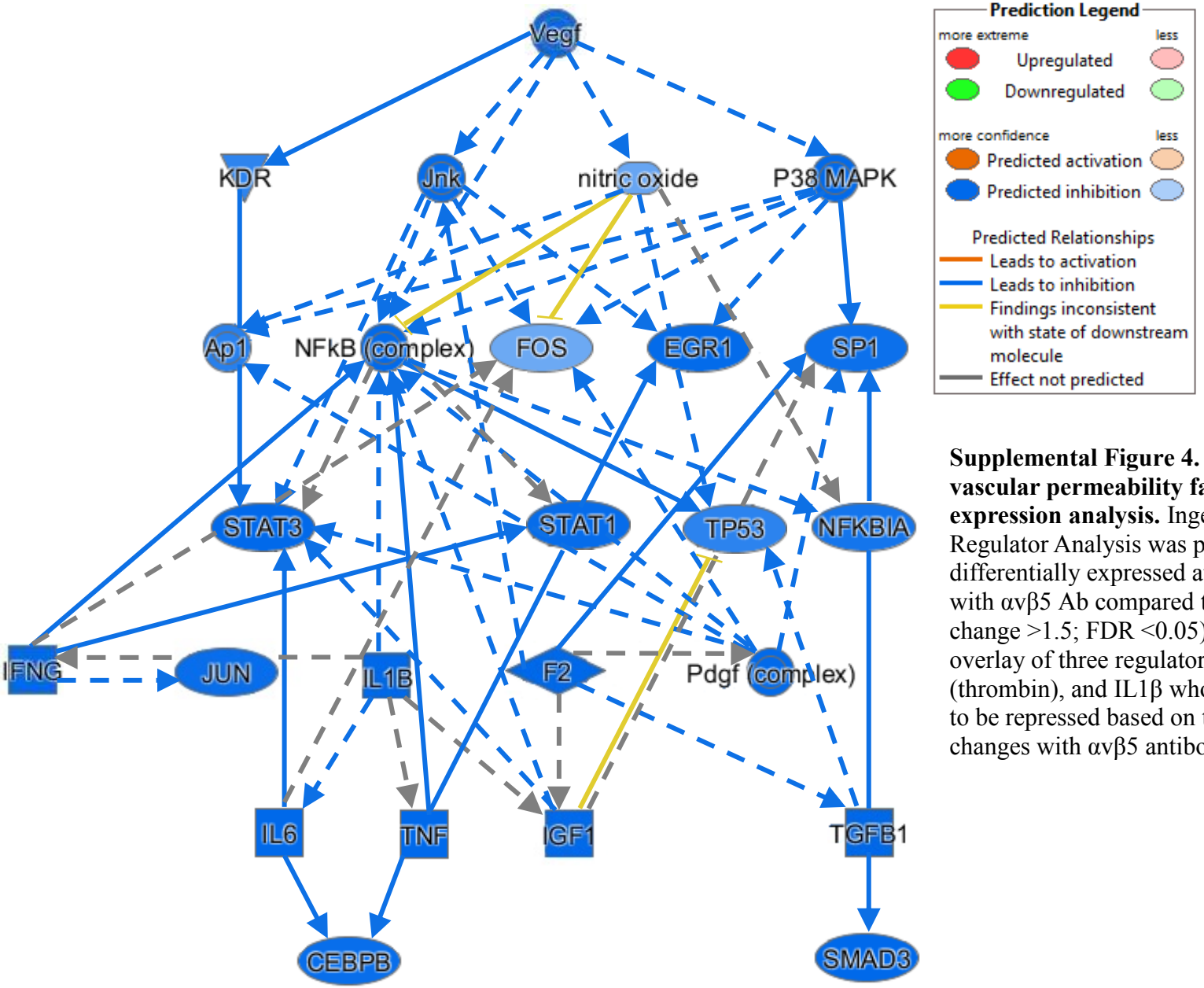
Supplemental Figure 1. αvβ5 inhibition decreases ischemia-induced α smooth muscle actin (αSMA) expression. Alpha smooth muscle actin staining was used as a measure of myofibroblast differentiation in post-IRI kidney sections. The degree of αSMA staining increased with injury in the control antibody-treated rats at 72 hours post-IRI. Rats treated with αvβ5 Ab had significantly reduced myofibroblast activation/expansion in both the renal cortex and medulla at 72 hours post-injury.



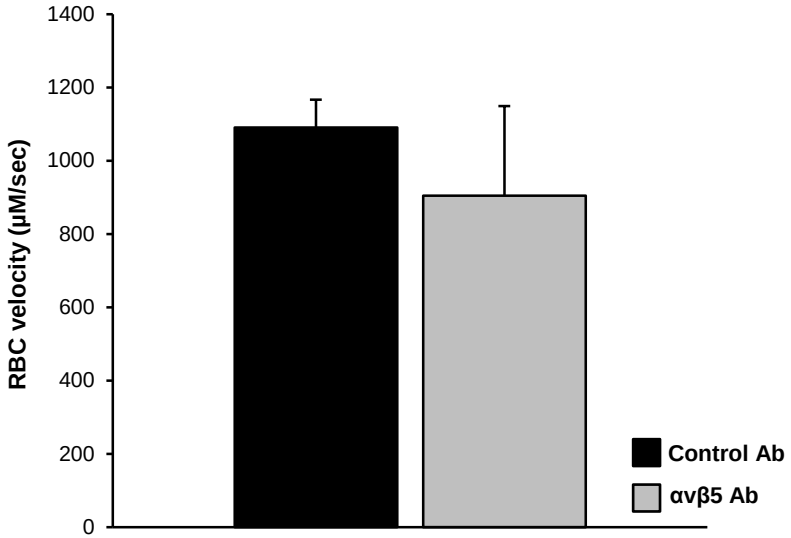
Supplemental Figure 2. αvβ5 inhibition decreases ischemia-induced PDGFRβ expression. PDGFRβ staining was used as a measure of myofibroblast differentiation in post-IRI kidney sections. The degree of PDGFRβ staining increased with injury in the control antibody-treated rats at 72 hours post-IRI. Rats treated with αvβ5 Ab had significantly reduced myofibroblast activation/expansion in both the renal cortex and medulla at 72 hours post-injury.



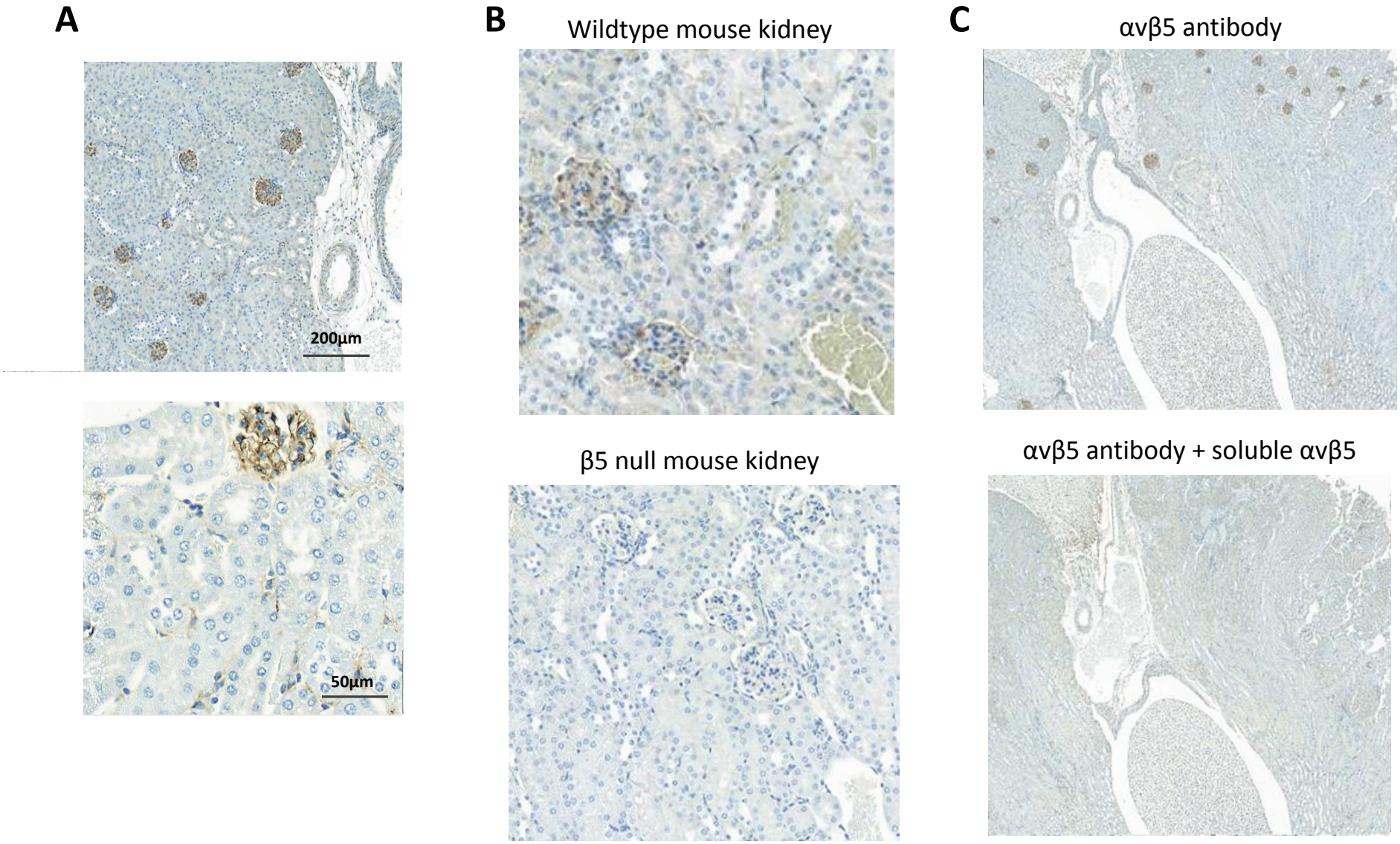
Supplemental Figure 3. αvβ5 inhibition reduces levels of injury markers KIM-1 and MCP-1. A rat kidney multianalyte panel was used to determine plasma and urinary levels of proteins associated with renal injury. Two proteins, urinary KIM-1 and serum MCP-1 exhibited patterns consistent with the biochemical and histological indicators showing decreased injury with αvβ5 Ab treatment at 24 hours post-IRI (*P<0.05, **P<0.01 vs. control Ab).



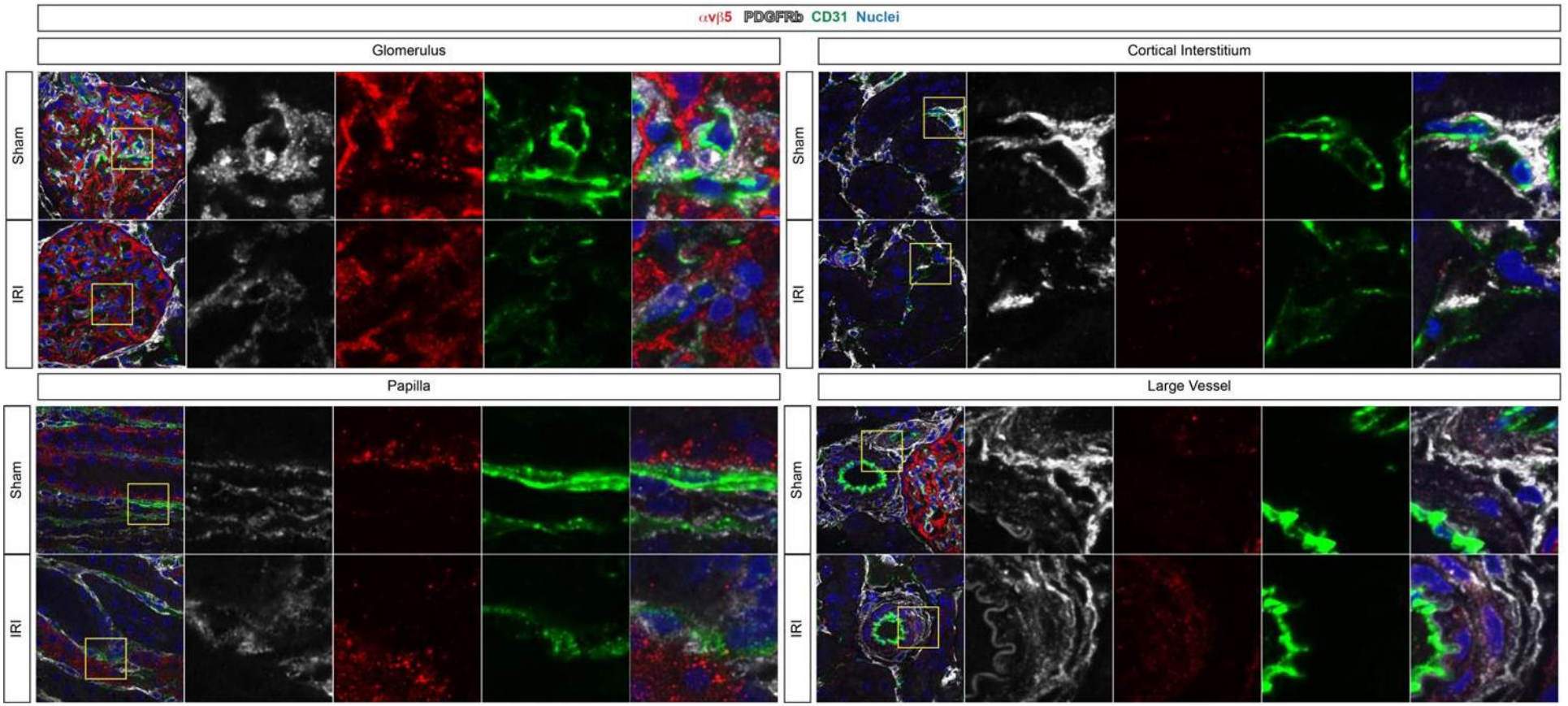
Supplemental Figure 4. Predicted repression of vascular permeability factors in global gene expression analysis. Ingenuity Upstream Regulator Analysis was performed on transcripts differentially expressed at 24 and 72 hrs post-IRI with αvβ5 Ab compared to control Ab (fold change >1.5; FDR <0.05). Figure depicts the overlay of three regulator networks: Vegf, F2 (thrombin), and IL1β whose signaling is predicted to be repressed based on the gene expression changes with αvβ5 antibody treatment..



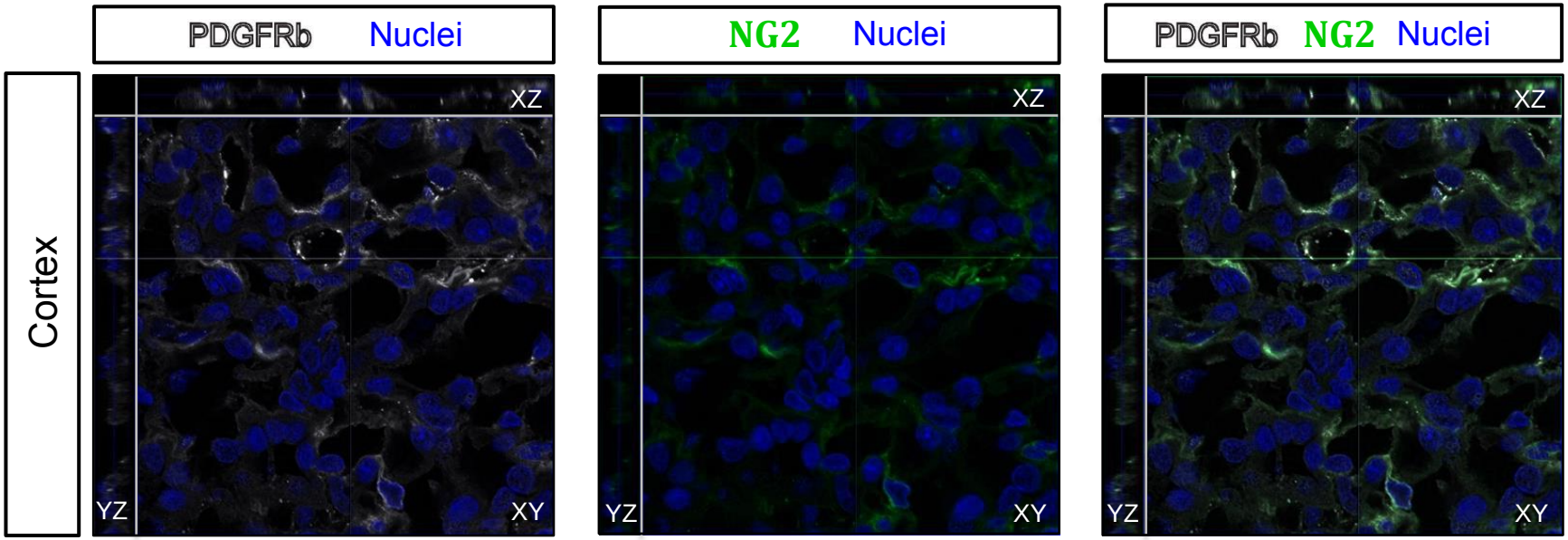
Supplemental Figure 5. Red blood cell (RBC) flow rate is not altered by αvβ5 antibody treatment. 150kD fluorescein-dextran was intravenously injected into rats at 24 hrs post-ischemia to evaluate RBC velocity following injury. Intravital imaging was utilized to determine RBC velocity in vivo by calculating the slope induced during image acquisition of the RBCs. No significant difference in RBC velocity between control Ab or αvβ5-Ab treatment was observed. Error bars =standard error



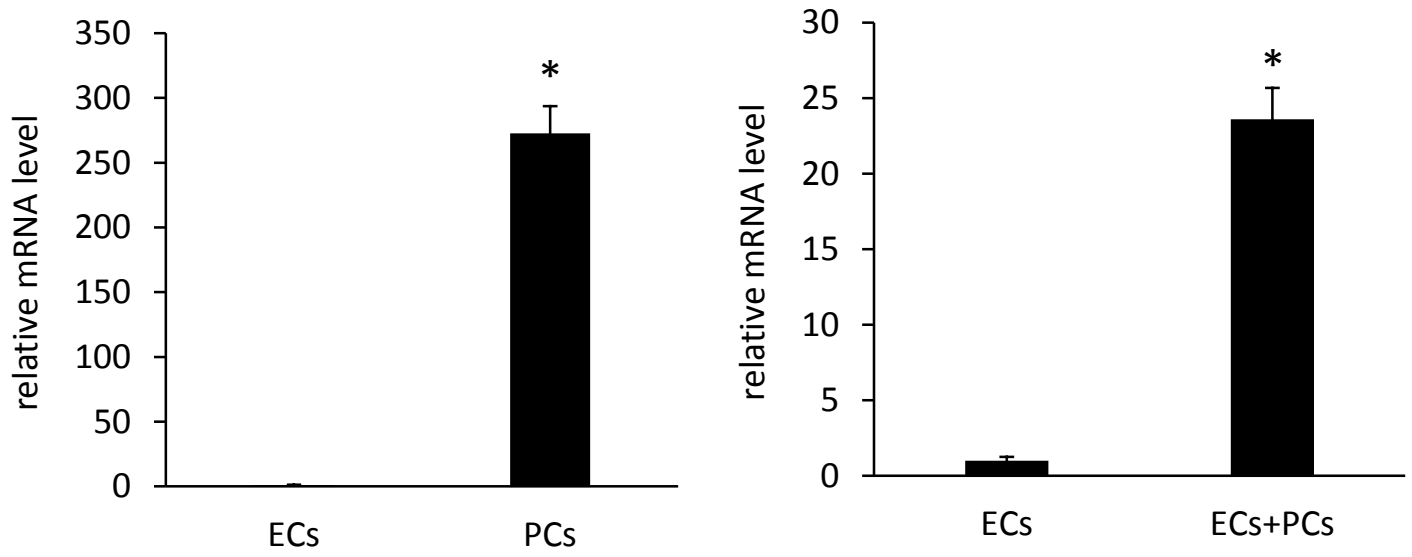
Supplemental Figure 6. αvβ5 expression in the mouse kidney. (A) The αvβ5 integrin is expressed in the glomerulus, tubular interstitial cells and proximal tubules as seen in low power (top) and a higher power image (bottom) of the kidney. **(B)** Additional staining performed in the β5-/- mouse and its corresponding wildtype 129 strain demonstrating the specificity of the antibody (10x images). **(C)** Competition with soluble αvβ5 was performed to further confirm the specific staining observed with the αvβ5 antibody (2x images).



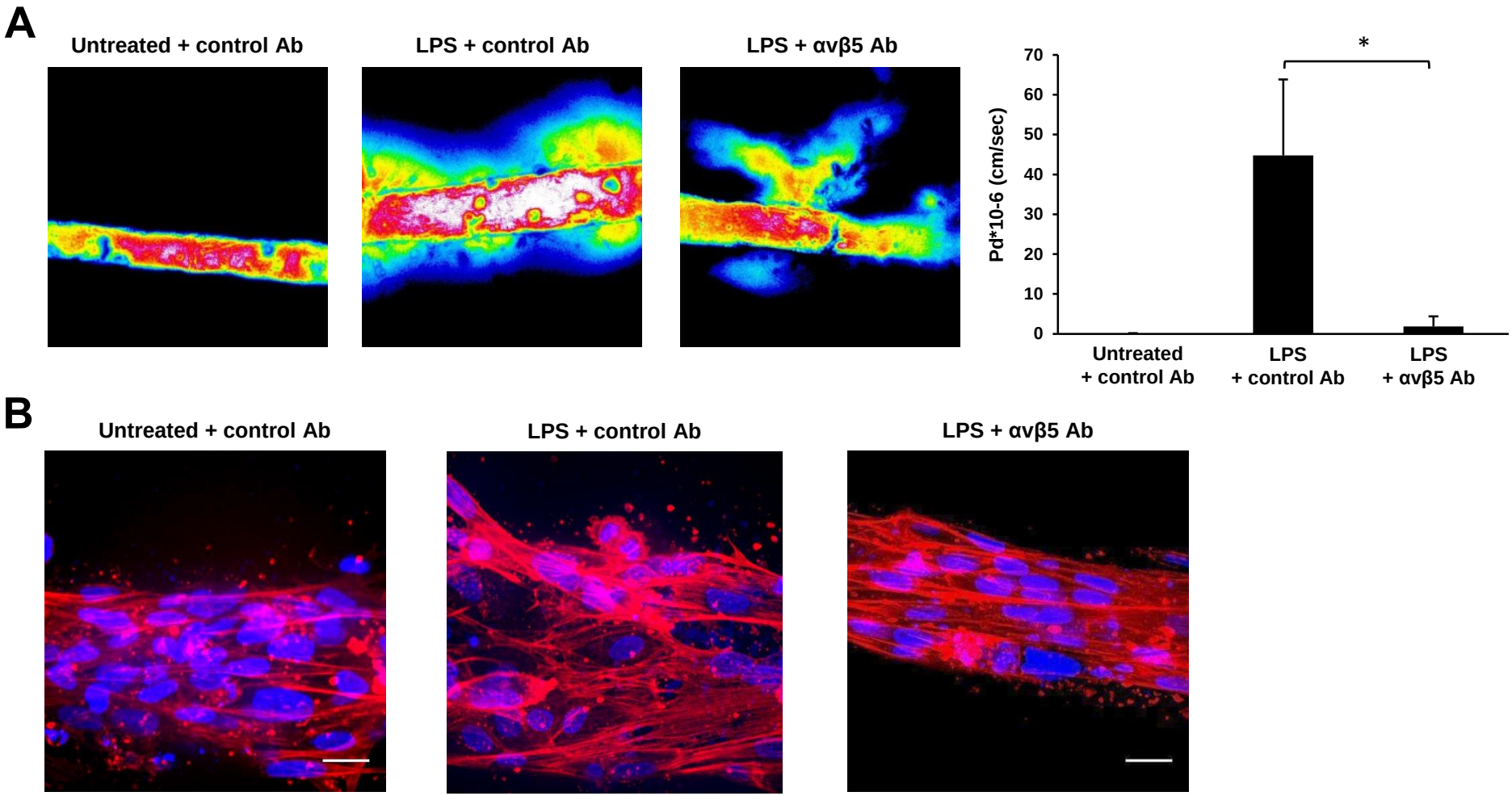
Supplemental Figure 7. αvβ5 expression in the normal and injured rat kidney. Frozen sections of rat kidney were stained for αvβ5 (red), CD31 (green) and PDGFRβ (white) with nuclei in blue. Representative images from the glomerulus, cortical interstitium, and papilla as well as an artery are shown. αvβ5 expression in the sham rat kidney is very similar to what is seen in the mouse kidney, with predominant expression in the glomerulus and less pronounced staining in the cortical interstitium. Again, αvβ5 appears to co-localize with PDGFRβ positive cells and to be excluded from CD31 positive endothelial cells. Expression of αvβ5 24 hours following ischemia reperfusion injury (IRI) did not substantially alter the amount or localization of αvβ5 in the kidney.



Supplemental Figure 8. Co-localization of PDGFRβ and NG2 proteoglycan in rat kidney. Frozen sections of rat kidney were stained for PDGFRβ (white) and NG2 proteoglycan (green) with nuclei in blue. Representative images from the renal cortex are shown. There is substantial co-localization of PDGFRβ and NG2 expression indicating the presence of perivascular cells (pericytes and vascular smooth muscle cells). These data help support the conclusion that αvβ5 immunostaining in PDGFRβ positive cells signifies αvβ5 expression in perivascular cells of the kidney.



Supplemental Figure 9. β5 mRNA expression levels in cells isolated from microfluidic devices. RNA was isolated from renal endothelial cells (ECs) and renal pericytes (PCs) cultured in 3D microfluidic devices under flow conditions. Levels of β5 (*itgb5*) mRNA were measured by quantitative PCR**.** Significantly higher levels of β5 were present in pericytes or the co-culture compared to ECs alone. (*P<0.05 compared to ECs alone; error bars =standard error).



Supplemental Figure 10. αvβ5 antibody treatment inhibits lipopolysaccharide (LPS)-induced vascular leak. (A) Human kidney endothelial cells and pericytes were seeded into a 3D microfluidic device and allowed to form a tube in the presence of flow. Cells were treated with control or αvβ5 antibody 1 hr prior to challenge with LPS (100ng/ml). 70 kDa Texas Red albumin was perfused into the vessel and the diffusion was measured by confocal microscopy to calculate the diffusive permeability coefficient (P_d). αvβ5 antibody significantly inhibited the LPS-induced vascular permeability (N=6) *P<0.05 vs. LPS + control Ab; error bars=standard error. **(B)** Phalloidin staining was performed to visualize F-actin filaments in cells within the microfluidics device ±LPS, ±αvβ5 antibody treatment.

Supplemental Table 1. Confirmation of microarray differential gene expression by quantitative PCR. Selected genes that had a fold change greater than 1.5 and a p-value of less than 0.05 by microarray analysis at one or both times post-ischemia were confirmed by quantitative PCR**.** Fold change represents the fold change of the αvβ5 antibody treated versus the control antibody treated at the respective time points.

	24 hours post-ischemia				72 hours post-ischemia			
	Microarray		qPCR		Microarray		qPCR	
	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
rgn	2.152	0.0463	2.259	0.0524	4.052	0.0008	4.706	0.0068
klk1	5.408	0.0002	15.986	1.02E-05	1.836	0.0003	2.741	0.0010
egf	4.714	0.0001	15.233	0.0043	3.304	0.0004	4.922	4.44E-06
fgb	-2.132	0.0092	-2.132	0.0036	-2.676	0.0003	-2.637	0.0040
hmox1	-1.68	0.1075	-1.597	0.1329	-1.783	0.0006	-2.260	0.0018
atf3	-2.088	0.0016	-2.261	0.0019	-1.794	0.0008	-2.013	2.05E-05
gadd45b	-1.547	0.0068	-1.949	0.0164	-1.148	0.0151	-1.015	0.8294
lcn2	-1.324	0.3540	-1.375	0.5293	-2.236	0.0191	-2.190	0.0066

Online Methods

Unilateral Clamp Ischemia Model

Animal experiments were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Male Sprague Dawley rats (Harlan Laboratories) weighing 250-320g were anesthetized with an Isofluorane / O2 mixture, 5% for induction and 1-2% for maintenance of anesthesia. An induction chamber is used for induction and an anesthesia circuit is used during surgery. The abdomen is shaved with clippers and washed with germicidal soap and water, towel dried and swabbed with Betadine. The animal is placed on a sterile disposable absorbent towel over a warming pad thermostatically controlled by a rectal thermometer. The animal is monitored continuously for pulse, oximetry, respiratory rate and blood pressure. The abdomen is opened using a 3 cm midline incision. Each kidney is isolated and the fat and connective tissue surrounding the renal artery and vein are dissected away using sterile cotton swabs. The right kidney is removed and the renal artery and vein sutured off. Ischemia of the left kidney is initiated by clamping the renal artery and vein for 30 minutes using non-traumatic clamps (Fine Scientific Tools) on each renal pedicle. For sham surgery, the kidneys are isolated as above but not clamped. The incision is covered with sterile saline saturated gauze sponge during the ischemic period. At the conclusion of the ischemic period, the clamps are removed and the kidneys are observed to insure rapid re-establishment of blood flow. The animal is rehydrated with 2cc of sterile saline introduced into the abdominal cavity. The muscle layer is closed with 3-0 silk; the skin is closed with surgical 3-0 silk. The αvβ5 blocking antibody (ALULA) used in these studies was previously described[1]. The 2H6 mouse monoclonal IgG2b antibody was used as a control. Antibodies were administered at a volume of 300uL by subcutaneous injection at defined times before or after clamping. Serum creatinine was

measured using the Creatinine Analyzer 2 (Beckman) at 0, 24, 48, and 72 hrs post-surgery and reported as mg per deciliter.

Immunostaining of fixed kidneys

To detect αvβ5 in formalin-fixed, paraffin-embedded tissues, a human β5 integrin specific polyclonal antiserum was prepared by immunizing a rabbit with purified soluble human αvβ5 ectodomain protein generated with recombinant constructs in CHO cells. The total IgG fraction was purified from the antiserum using protein-A affinity chromatography, and subjected to affinity depletion using Sepharose beads conjugated to soluble human αvβ6 protein, in order to remove human αv-reactive components. The human β5-integrin specific rabbit antibodies were then affinity purified using Sepharose beads conjugated to soluble human αvβ5 protein. The final isolated rabbit IgG fraction was only reactive to human β5-containing integrin but not any other integrin subunits as tested by ELISA (data not shown). Formalin fixed tissues were embedded in paraffin, sectioned at 5μM and processed on the Ventana Discovery XT platform (Roche) using the purified anti-human β5 polyclonal antibody. Immunohistochemistry of additional markers was performed as above using the following antibodies and dilutions: PDGFRβ (1:50; Thermo Fisher), α-smooth muscle actin (1:8000; αSMA; Abcam), and activated (cleaved) caspase-3 (0.24 μg/ml; Cell Signaling). Quantification of caspase-3 immunoreactive area was accomplished by custom-designing algorithms in Visiopharm (Denmark) software.

Additional immunostaining for αvβ5 was performed on frozen tissue from rat (Sprague-Dawley strain; Harlan/ENVIGO) and mouse (Itgb5tm1Des β5 null strain and wildtype 129 SvJ strain; Jackson Laboratories). Tissues were bisected and fixed in cold 4% PFA (Electron Microscopy Sciences) for 1.5 hours, washed with cold PBS, incubated in 30% sucrose/PBS

overnight at 4˚C and then embedded in OTC (Electron Microscopy Sciences). Frozen blocks were sectioned on a cryostat at 10-20µm thickness and slides were stored at -80˚C. For immunofluorescence OTC was dissolved in PBS, slides were blocked (in PBS + 0.1% TritonX100 + 1% normal donkey serum + 2% BSA) for 1hr and incubated overnight in primary antibody at 4˚C. Primary and secondary antibodies were as follows: αvβ5 antibody ALULA1 (humanized variant), mouse anti-rat CD31 or rat anti-mouse CD31 (eBioscience), rabbit anti-PDGFRb (clone 28E1, Cell Signaling), mouse anti-rat NG2 (clone 50009, Abcam), anti-human Cy3 (Jackson ImmunoResearch), anti-rabbit AF647, anti-rat AF488, and anti-mouse AF488 (Life Technologies). Images were acquired on a Marianas confocal system (3i) at sub-micron z-axis resolution (0.8um per image). Three-dimensional image stacks were pseudocolored and reconstructed through 5 slices with Volocity (Perkin-Elmer). Imaging was performed on the same day with the same imaging settings for negative controls.

Histological evaluation of injured kidneys

H&E stained sections from formalin-fixed, paraffin-embedded kidneys were examined by a blinded, board certified veterinary pathologist for injury and regeneration using a scoring method modified from[2]. The following criteria were scored: (1) Injury score: tubular necrosis; calcific debris in tubluar lumina; interstitial inflammation; casts; juxta-glomerular apparatus prominence; interstitial edema; brush border loss; tubular proteinosis; pelvic congestion. (2) Regeneration score: neutrophils in vasa recti; tubular regeneration; tubular cell mitoses. Two lesions, tubular proteinosis and pelvic congestion, were observed in the present study and added as scored criteria. Lesions were scored on a scale of 0-2 with 0, none; 0.5, minimal; 1, mild; 1.5

moderate; and 2, marked. Composite lesion scores presented are a combination of the injury and regeneration scores.

Serum and urine biomarker measurements

Serum creatinine of all rats was measured at baseline and at days 1, 2, and 3 post-clamp (animals sacrificed at 24hr post-clamp have only baseline and day 1 serum creatinine measurements). A 0.15 ml blood sample was drawn from the tail vein and 24hr urine was collected in a metabolic cage. Serum creatinine levels were measured using an AU480 Chemistry Analyzer (Beckman) and reported as mg per deciliter. Profiling of serum and urine biomarkers of renal injury was conducted using the Luminex Bead assay platform with the multianalyte panel Rat KidneyMAP v1.0 (Myriad RBM).

RNA isolation, microarray analysis and quantitative PCR

Total RNA from the kidney was isolated in QIAzol (Qiagen) using mechanical disruption on the FastPrep system (MP Biomedical) and purified using the RNeasy Mini Kit (Qiagen). RNA was quantitated (NanoDrop; Thermo Fisher) and was evaluated for integrity using the RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent).

Gene expression microarray experiments were performed at Expression Analysis (a Quintiles company) as follows: Total RNA was labeled and hybridized using the Ambion WT Expression kit according to the manufactur's instructions. Briefly, 200ng input of total RNA was reverse transcribed using engineered primers that are designed to prime non-ribosomal RNA. The cDNA was then converted to dsDNA and RNA was degraded using DNA polymerase and RNase H, respectively. cRNA synthesis occurred using T7 RNA polymerase. The resulting

cRNA was purified using the supplied nucleic acid binding beads and quantified using spectrophotometry. A second reverse transcription occurred using random primers and dUTP was incorporated into the cDNA synthesis, after which RNase H was again used to degrade the RNA, leaving ssDNA with dUTP incorporated. Another purification step was performed using nucleic acid binding beads and ssDNA quantified using spectrophotometry. This ssDNA was then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that breaks the DNA at dUTP sites. DNA was subsequently labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix® proprietary DNA Labeling Reagent that is covalently linked to biotin. Labeled, fragmented ssDNA was then hybridized to arrays at 45°C for 16 hrs. Following hybridization, arrays were washed and stained using standard Affymetrix procedures before scanning on the Affymetrix GeneChip Scanner.

For qPCR confirmation of microarray results and post-treatment biomarker transcripts, cDNA was synthesized from 1µg of RNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher) and qPCR was performed using Taqman Gene Expression assays run on the QuantStudio 7 (Thermo Fisher). Ct values of target genes were normalized to the *eif1a* control gene to generate ΔCt values.

Intravital microscopy to evaluate vascular permeability in vivo

Rat serum albumin was conjugated to Texas Red (TR-RSA) as previously described[3]. Briefly, Rat serum albumin (Sigma Aldrich, cat# A-6272) is dissolved in 100mM bicarbonate buffer, pH 9.0 at a final concentration of 15mg/mL. Texas Red sulfonly chloride (Life Technologies) is dissolved in high quality anhydrous N,N-Dimethylformamide. Unconjugated hydrolyzed fluorophore was removed by dialysis against a 0.9% saline solution using a 50kDa

MWCO Spectra-Por Float-A-Lyzer membrane (Pierce).

A 150kDa fluorescein-conjugated dextran (TdB Consultancy, Uppsala, Sweden) was dissolved at 40mg/mL in 0.9% normal saline. To remove any small molecular weight components, the stock solution was dialyzed against normal saline using a 100kDa MWCO Spectra-Por Float-A-Lyzer membrane (Pierce).

Imaging: Images were acquired using an Olympus FV-1000 microscope with a 60x 1.2NA water immersion objectives and highly sensitive GaAsP non-descanned (external) detectors. Ten random fields were marked using a prior mechanized stage and imaged at 5, 15 and 30 minutes post infusion of the dual TR-RSA/150kDa fluorescein dextran bolus. All of the images were taken approximately 5 to 10 um below the kidney capsule where interstitial space (the space between basolateral side of the tubules and the renal vasculature) is prevalent.

Extravasation and Stratification: The degree of extravasation of the two different molecular weight markers (~66kDa for the TR-RSA and 150kDa for the fluorescein-dextran) was used examine the severity of damage to vascular integrity. Texas Red RSA was used to rank mild to moderate vascular damage as this compound, over time, is transcytosed from the apical membrane to the basolateral membranes and excocytosed into the interstitial space. This process does take time. Therefore having an abundance of this compound in the interstitial space over a short time period can only come from compromised renal microvasculaure.

The 150 kDa fluorescein-dextran was used to rank more severe damage. This compound typically does not cross over into the interstitial space from the vascular side. This compound has a glomerular sieving coefficient of ~0.005, so there is a very small amount that does cross the glomerular filtration barrier. However unlike TR-RSA, once it is internalized by the

proximal tubule cells it is trafficked to the lysosomes where it is sequestered and does not undergo transcytosis to the basolateral membrane for exocytosis into the interstitial space.

By working in consort, stratifying the amount seen for each of the compounds can be used to determine a gradation of injury that either compound alone could not present. A score of "0" denotes no amount of the compound is present in the interstitial space. Here the vasculature is clearly seen in contrast against the empty interstitial space (asterisks). A score of "1" is given to an image where some amount of the compound is seen in the interstitial space of a small region of the total image. A score of "2" is given to an image where the compound leaks into the interstitial space of the majority of the image. Note that the amount or concentration of the compound in the interstitial space for scores "1" and "2" are well below that seen in the vasculature. A score of "3" is given to an image where the concentration of the compound in the interstitial space starts to match the intensities seen in the circulating microvasculature. This is indicative of severe damage particularly for TR-RSA at 5 and 15 minute time points or the 150kda fluorescein-dextran at any time point.

Red blood cell (RBC) velocity was calculated as previously described[4]. Briefly, utilizing the 150kDa dextran retained in the vasculature, RBC velocity was determined from single plane images or line scans as the dextran labels only the plasma and is excluded from RBC and white blood cells causing them to appear as dark streaks. This was accomplished by determination of the slope induced during image acquisition of the RBCs; faster moving RBC produce a more shallow slope while slower moving RBCs produce a steeper slope. Speed was calculated by using the dimensions of time (the y-axis) and distance (the x-axis).

Microfluidic platform for vascular permeability

Microfluidic devices were fabricated using soft lithography as described previously[5]. Polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) devices were bonded to glass and treated with 0.01% poly-L-lysine and 1% gluteraldehyde to promote collagen adhesion with PDMS. After washing overnight in water, steel acupuncture needles (160μm diameter, Seirin) were introduced into the device. Collagen type I (Thermo Fisher Scientific) solution was buffered with 10x DMEM, titrated to a pH of 8.0 with NaOH, and brought to a final concentration of 2.5 mg/mL collagen I in total solution. Collagen solution was injected into microfluidic devices and allowed to polymerize for 20 min at 37°C. Growth medium was then added to the devices and needles were removed to create 160μm diameter channels in the collagen gel. Human renal glomerular endothelial cells (ScienCell) were harvested with 0.05% Trypsin/EDTA and centrifuged at 200 x *g* for 5 min. The cells were resupsended at 0.5×10^6 cells/mL of endothelial cell medium (ScienCell), and 70μL of cell suspension was introduced into devices to allow cells to adhere to collagen gel for 15 min before washing with growth medium. In pericyte-endothelial cell co-culture experiments, devices were first seeded with isolated kidney pericytes at 0.5×10^6 cells/mL, allowed to adhere for 15 min, washed with growth medium, and incubated for 4 hrs before adding renal glomerular endothelial cells.

Cells were allowed to recover and spread overnight, and the following day the resulting microvessels were treated with 10μg/mL control Ab antibody; or 0.3U/mL thrombin+ control Ab antibody; or 10μg/mL αvβ5 blocking antibody+ 0.3U/mL thrombin for 1 hr. For LPS experiments, microvessels were treated with 100ng/ml LPS (sigma) for 1hr. To measure the permeability of the endothelium in the microfluidic platform, fluorescent dextran (70 kDa Texas Red, Thermo Fisher) was introduced into perfusion media at a concentration of 12.5 μg/mL, and diffusion of the dextran was imaged in real time with a confocal microscope (LSM 710, Carl

Zeiss) at 10x magnification. The diffusive permeability coefficient was calculated by measuring the flux of dextran into the collagen gel and fitting the resulting diffusion profiles to a dynamic mass conservation equation as described previously[6].

In vitro pericyte adhesion assay

96-well high binding plates (Costar) were coated overnight at 4^{o}C with 0.1% collagen (Sigma) or 10ug/mL vitronectin in PBS (R&D Systems). Plates were washed twice with assay buffer (TBS + 1mM CaCl + 1mM MgCl), filled with blocking buffer (1% BSA in PBS), blocked for 1hr at 37^{o} then plates were washed again with assay buffer. Antibodies (10µg/ml) 2H6 (mouse monoclonal IgG2b Ab used as control), anti-αVβ5 (ALULA), and anti- αVβ3 (Millipore MAB1976) were diluted in assay buffer and added prior to cell seeding. Human fetal kidney pericytes (gift from Jeremy Duffield) were cultured in Pericyte Medium (ScienCell) and fluorescently labelled with 2uM Calcein-AM (Molecular Probes). 150,000 cells were seeded into antibody-containing wells and plates were incubated at 37^{o}C for 1.5 hrs. After incubation, plates were washed with assay buffer and read (Ex: 485nm, Em: 515nm) on a Synergy H1 microplate reader (BioTek) to quantify adherent cells via fluorescent intensity. Replicate wells were averaged and standard error was calculated.

Scratch wound migration assay

96-well ImageLock plates (Essen Bioscience) were coated overnight at 4^{o}C with 0.1% collagen (Sigma) or 10ug/mL vitronectin in PBS (R&D) then washed with 1x PBS. Human fetal kidney pericytes were cultured in Pericyte Medium (ScienCell), re-suspended in Opti-MEM (Thermo Fisher), seeded at 50,000 cells per well and incubated at 37^{o}C for 6 hrs to allow cells to

adhere. Scratches were made using the Woundmaker (Essen Biosceince) and plates were washed with Optimem. Antibodies 2H6, anti-αvβ5, anti- αvβ3 (Millipore MAB1976) and a JNK inhibitor (Selleckchem SP600125) were diluted in Optimem and added to the wells 1 hr prior to scratch. Plates were set in the Incucyte ZOOM system (Essen Bioscience) and imaged every two hours for 48 hours. Incucyte ZOOM software was used to generate a processing definition specific to human kidney pericytes. An analysis was run on each plate and wound confluence was calculated at each individual time point for each treatment condition.

References

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