Supplemental Material

LRG1 promotes DKD progression by enhancing TGF-β-induced angiogenesis

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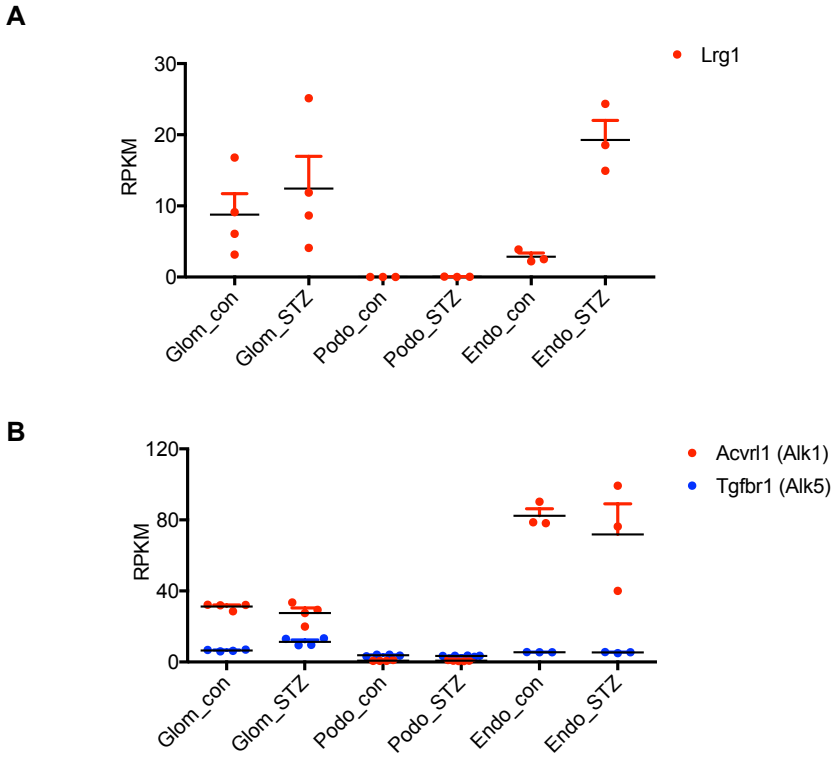
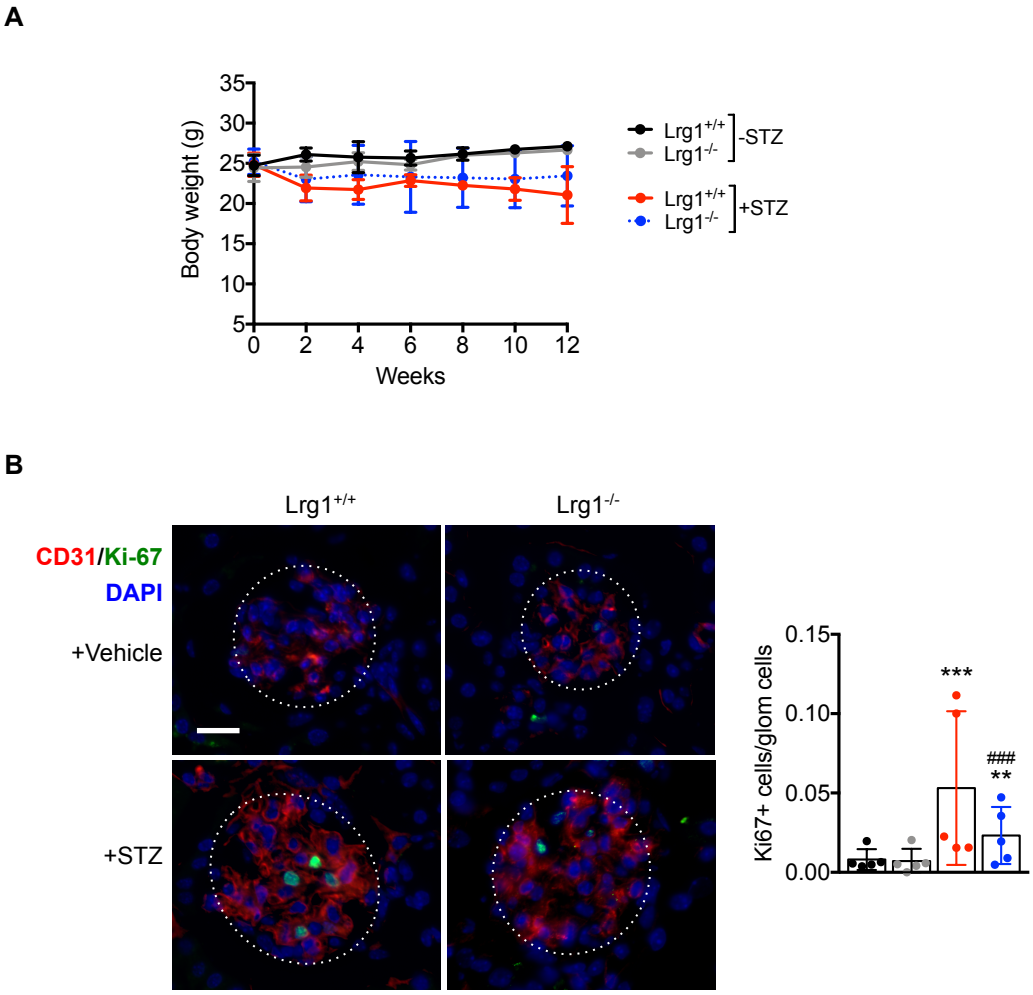
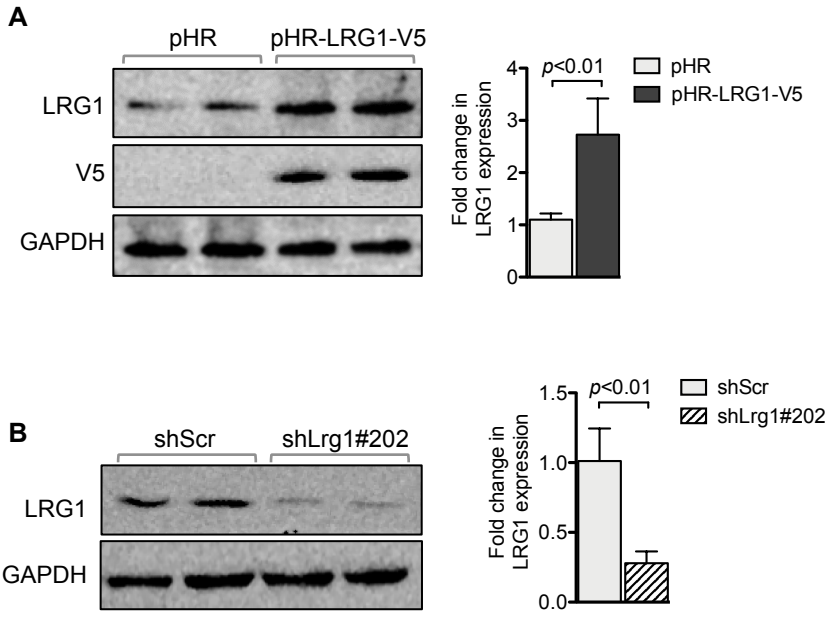


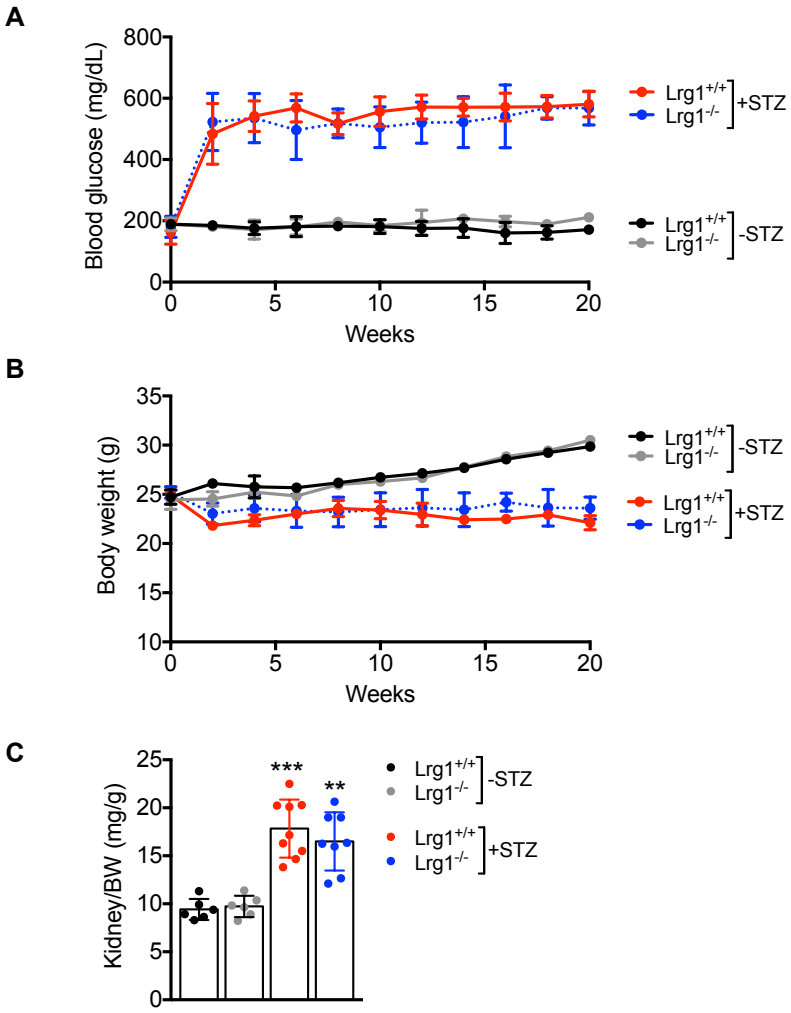
Suppl. Fig. 1: RNA-seq expression of mouse *Lrg1* mRNA in isolated glomeruli, podocytes, or GECs. mRNA sequencing data (combined data from Fu *et al.* 2016 and Fu *et al.* 2018) shows the normalized expression of (A) *Lrg1* and (B) *Alk1* and *Alk5* mRNA in isolated glomeruli (Glom), sorted podocytes, and sorted glomerular endothelial cells (GECs) from STZ-induced diabetic eNOS$^{-/-}$ (STZ) and nondiabetic eNOS$^{-/-}$ (con) mice. n=3-4 samples per group, where each sample is pooled from 3-4 mice. RPKM, reads per kilobase million.



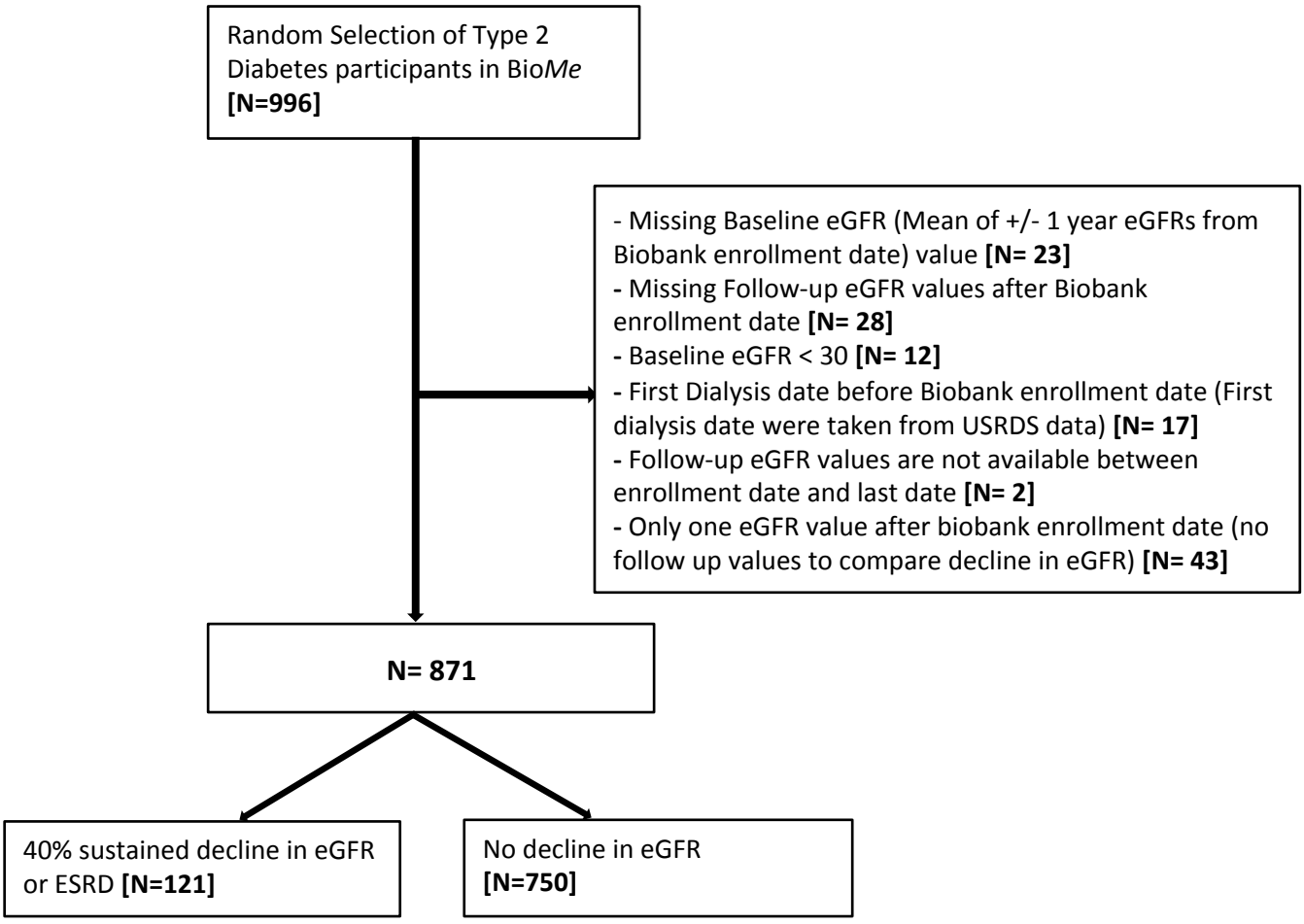
Suppl. Fig. 2: Additional characterization of diabetic and control Lrg1$^{+/+}$ and Lrg1$^{-/-}$ mice at 12 weeks post-DM. (A) Body weight measurements in control and diabetic mice. (B) Ki-67/CD31 immunofluorescence on kidney sections of control and diabetic mice. Scale bar, 20µm. Quantification of Ki-67^{+} cell per total glomerular cells per mouse is shown on the right (n=5 mice per group, 20-30 glomeruli per mouse). $^{**}P<0.01$ and $^{***}P<0.001$ vs. nondiabetic controls; $^{\#\#\#}P<0.001$ vs. diabetic *Lrg1*$^{+/+}$ mice.



Suppl. Fig. 3: Overexpression and knockdown of LRG1 in mGECs. (A) Immortalized mouse GECs (mGECs) was transduced with either empty pHR lentivector or with pHR vector expressing V5 tagged LRG1 (pHR-LRG1-V5). Lysates from stably tranduced cells were subjected to western blot analysis using LRG1 and V5 antibodies. GAPDH was used as loading control. Representative blot is shown on the left and quantification of the western blots are shown on the right. (B) mGECs were transduced with pLKO lentivector expressing scrambled shRNA (shScr) or shRNA against LRG1 (shLrg1#202). Representative blot is shown on the left and quantification of the western blots are shown on the right.



Suppl. Fig. 4: Additional characterization of diabetic and control *Lrg1*$^{+/+}$ and *Lrg1*$^{-/-}$ mice at 20 weeks post-DM. (A-C) Blood glucose (A), body weight (B), and kidney-to-body weight (C) measurements in control and diabetic mice. ***P*<0.01 and ****P*<0.001 when compared with respective nondiabetic control mice.



Suppl. Fig. 5: Flow diagram of patient selection into study cohort.

Supplemental Tables

	12 weeks post-injection				20 weeks post-injection	
	Vehicle-Lrg1$^{+/+}$	**Vehicle-Lrg1$^{-/-}$**	**STZ-Lrg1$^{+/+}$**	**STZ-Lrg1$^{-/-}$**	**STZ-Lrg1$^{+/+}$**	**STZ-Lrg1$^{-/-}$**
SBP (mean±SEM)	103.3±14.6	95.73±6.4	117.1±15.4	107.5±12.3	100.0±7.4	98.4±4.9
DBP (mean±SEM)	56.6±8.9	72.87±5.5	62.93±12.2	83.8±12.7	70.5±6.5	77.6±4.2

Suppl. Table 1: Blood pressure measurements of diabetic and control mice. Blood pressure was measured by tail-cuff blood pressure monitor at 12 or 20 weeks post vehicle or STZ injections.

	Overall (n= 871)	Without Renal Endpoint (n= 750)	With Renal Endpoint (n= 121)	p value
Clinical Characteristics				
Age in years, Median (IQR)	60 [53 - 66]	60 [53 - 66]	61 [51 - 66]	0.64
Female, n (%)	507 (58.2)	434 (57.9)	73 (60.3)	0.62
Body Mass Index in kg/m^2, Median [IQR]	30.9 [26.6 - 36.1]	31.0 [26.9 - 36.2]	29.7 [24.7 - 35.5]	0.28
Race, n (%)				0.09
White	62 (71.2)	59 (7.9)	3 (2.5)	
African-American	346 (39.7)	290 (38.7)	56 (46.3)	
Hispanic/Latino	412 (47.3)	355 (47.3)	57 (47.1)	
Other	51 (58.6)	46 (6.1)	5 (4.1)	
Hypertension, n (%)	813 (93.3)	694 (92.5)	119 (98.4)	0.02
Coronary Artery Disease, n (%)	432 (49.6)	392 (52.3)	40 (33.1)	<0.001
Heart Failure, n (%)	192 (22.0)	144 (19.2)	48 (39.7)	<0.001
Systolic Blood Pressure in mm Hg, Median [IQR]	131.9 [123.7 - 143.4]	131.2 [123.5 - 141.5]	140.3 [125.1 - 150.1]	0.01
Diastolic Blood Pressure in mm Hg, Median [IQR]	73.4 [68.7 - 79.3]	73.2 [68.5 - 78.9]	75.2 [70.6 - 81.0]	0.04
Follow up Time in years, Median [IQR]	4.5 [3.3 - 6.1]	4.4 [3.2 - 5.9]	5.2 [3.8 - 6.8]	0.01
Laboratory Characteristics				
Baseline eGFR, Median [IQR}	68.4 [55.3 - 80.0]	69.0 [56.3 - 80.3]	62.6 [50.5 - 78.0]	0.02
Baseline Hemoglobin A1C, Median [IQR]	7.0 [6.2 - 8.7]	6.9 [6.2 - 8.5]	7.6 [6.3 - 9.7]	0.01
Baseline urine albumin/creatinine, Median [IQR]	13.0 [4.0 - 66.3]	11.0 [4.0 - 38.0]	204.0 [25.0 - 1240.0]	<0.001
Medications				
ACE/ARB, n (%)	675 (77.5)	575 (76.7)	100 (82.6)	0.15
Plasma LRG1 Concentrations				
LRG1, in ng/ml, Median [IQR]	57.0 [33.8 – 105.5]	53.8 [32.8 – 96.9]	80.7 [53.2 – 172.1]	<0.0001

Suppl. Table 2: Baseline characteristics. Values are presented as mean (SD) for normally distributed continuous values, median (interquartile range) for skewed continuous values, and *N* (%) for categorical values. Renal Endpoint is defined as ESRD (defined by the initiation of maintenance dialysis or receipt of kidney transplant) or a sustained (on two or more time intervals ≥3 months apart) decline in eGFR of ≥40% from baseline eGFR.

Supplemental Methods

Human archival kidney samples

Human kidney biopsy samples were obtained from archival biopsy specimens collected at Icahn School of Medicine at Mount Sinai (ISMMS) under a protocol approved by the Institutional Review Board at ISMMS.

RNA in situ hybridization

Mouse kidneys (dual ISH): Lrg1 mRNA expression was assessed by RNAscope 2.5 HD Duplex Reagent Kit (Advanced Cell Diagnostics, Hayward, CA; #322430) according to the manufacturer's instructions. Briefly, deparaffinized 4-μm FFPE kidney sections were pre-treated with RNAscope 2.5 Universal Pretreatment reagent at 100°C for 15 minutes, rinsed in deionized water, and treated with Protease Plus reagent at 40°C for 30 minutes in a HybEZ Hybridization System. *mLrg1* (#423381) and *mPecam1*-C2 (#316721-C2) probes were then hybridized for 2 hours at 40 °C in a hybridization oven. After amplification, chromogenic substrate was added for the visualization of the target mRNA. Freshly cut sections of same batch of FFPE specimens were used in parallel with positive (*mPolr2a* probe, #321651) and negative (bacterial *DapB* probe, #320751) controls. The specimens which exhibited negative staining with a negative control probe (*DapB*) and grade 4 + or higher staining with the positive control (*mPolr2a*) probe, were considered adequate for *mLrg1* analysis. Two independent observers blinded to mouse genotypes evaluated the FFPE slides for *mLrg1* expression using five graded categories (0-4), according to the manufacturer's guidelines.

Human kidneys (dual ISH-IF): Deparaffinized FFPE kidney sections were processed similarly as above using *hLRG1* probe (#439311) with RNAscope 2.5 HD Assay-RED (#439311). Sections were then subjected to immunofluorescence with CD31 antibody (Abbiotec, 250590). *hLRG1* probe was detected with a filter set at Ex554nm/Em576nm, and CD31 was detected using the Alexa Fluor 488-conjugated secondary antibody.

Measurements of urinary albumin and BUN

Urine albumin was quantified using the ELISA kit from Bethyl Laboratories, Inc. (Houston, TX). Urine creatinine levels were measured in the same samples using the Creatinine Colorimetric Assay Kit (Cayman, MI, USA) according to the manufacturer's instructions. 24-hour urine collections in the metabolic cages were also used for determination of urinary albumin excretion. Blood urea nitrogen (BUN) levels were measured using a commercially available kit according to manufacturer's protocol (Bioassay Systems, Hayward, CA).

Kidney histologic and morphometric analyses

Kidney samples were fixed in 10% formalin, embedded in paraffin and sectioned to 4-μm thickness. Sections were stained with periodic acid–Schiff (PAS) for analysis of glomerular area and mesangial matrix expansion. The mean glomerular tuft volume was determined from the mean glomerular cross-sectional area by light microscopy. The glomerular tuft volume was calculated using the following equation: $GV=(\beta/\kappa) \times GA3/2$, where $\beta=1.38$, the shape coefficient of spheres (the idealized shape of glomeruli), and $\kappa=1.1$, the size distribution coefficient. Mesangial expansion was defined as a periodic acid–Schiff-positive and nuclei-free area in the mesangium. Quantification of mesangial expansion was based on a minimum of 10 glomeruli per section in a blinded fashion, under 400x magnification (Zeiss AX10 microscope, Carl Zeiss Canada Ltd, Toronto, ON,

Canada). For immunofluorescence staining, kidney samples were frozen in OCT embedding compound. For transmission electron microscopy, kidney cortex samples fixed in 2.5% glutaraldehyde were sectioned and mounted on a copper grid; then images were photographed using a Hitachi H7650 microscope (Tokyo, Japan). Briefly, digitized images were scanned and profile areas were traced using ImageJ. The quantification of podocyte foot process width was determined as previously described [1].

Immunofluorescence

Frozen kidney sections were used for immunofluorescence staining as previously described [2] [3] using CD31 (BD Biosciences Inc., #BDB550274) and phospho-Smad1/5/8 (Cell Signaling Technologies, #9516) primary antibodies. Fluorescent images were taken using the Zeiss Axioplan 2 IE microscope.

Directed in vivo angiogenesis angioreactor (DIVAA)

In vivo angiogenesis was assessed using the Directed In Vivo Angiogenesis Assay (DIVAA) kit from Trevigen (Gaithersburg, MD; #3450-048-K) according to manufacturer's protocol. Briefly, at 7 weeks post diabetes induction mice were anesthetized with isoflurane, and the angioreactors were subcutaneously implanted into the dorsal flank. Angiogenesis was measured as the amount of FITC-Lectin detected 14 days post-implantation. Amount of fluorescence in each sample was collected using the SpectraMax M3 plate reader (Molecular Devices, Sunnyvale CA).

Quantification of EFYP+ cells with two-photon microscopy

4% formaldehyde-perfused kidneys were cut in 500μm sections with the Vibratome (Leica VT1200, Germany), and processed as described in Puelles et al.[4] Images from each sections were obtained with Leica TCP-SP5 Two-Photon Microscope (Leica Microsystems) equipped with a 20x and 0.95 numerical aperture oil–immersion objective and Chameleon Laser (Coherent, Santa Clara, CA). Fluorescence emission was guided directly to supersensitive external hybrid photodetectors (Leica/Hammamatsu). Images were reconstructed to 3D view, and EYFP+ cells were quantified using the Imaris software (Bitplane AG, CH-8048 Zurich, Switzerland).

Overexexpression and knockdown of LRG1 in mGECs

Based on mouse *Lrg1* NCBI reference sequence (NM_029796.2), full length *Lrg1* cDNA tagged with V5 tag was cloned into pGEM-Teasy vector with the following primers: 5'-TTA AGA TCT GGA TCC GCC ACC ATG GTC TCT TGG CAG CAT CAA GGA AG-3', 5'-AAC CGG TGG ATC CTT ATG TAG AAT CAA GTC CCA GAA GAG GAT TTG GAA TAG GTT TTC CCA GGG ACC CCA GCT CTG CCA C-3', then subcloned into BamH I and Sma I restriction enzyme site of pHR-CMV-MCS-IRES-GFP-delta B lentivirus vector [5], named pHR-LRG1-V5. Together with psPAX2 packaging and VSVG envelope plasmids, pHR-LRG1-V5 plasmid or empty pHR-V5 control plasmid was transfected in human embryonic kidney (HEK)-293T cells using PolyJet transfection reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer's manual. Collection of supernatant 48 hours post-transfection was harvested and concentrated. Lentivectors expressing short hairpin RNA (shRNA) for mouse *Lrg1* (pLKO.1-shLRG1; 4 independent clones) and scrambled shRNA (pLKO.1-shScr) were obtained from Sigma-Aldrich (St. Louis, MO), as described previously [6].

Western Blot

Cells were homogenized in NP-40 lysis buffer containing protease and phosphatase inhibitor cocktail. Equal amounts of protein samples were separated on SDS polyacrylamide gel, transferred to PVDF membranes (Millipore) and probed with primary antibodies (~1:1000). Membranes were then washed with PBST and incubated with a secondary antibody (horseradish peroxidase conjugated antibodies to mouse IgG or to rabbit IgG, ~1;10,000). Blots were developed with the enhanced chemi-luminescence system and imaged with Odyssey FC Imager (LI-COR Biosciences, NE).

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