RAAS-Deficient Organoids Reveal that Delayed Angiogenesis Is The Pathomechanism Underlying Autosomal Recessive Renal Tubular Dysplasia

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Supplemental information – Table of content:

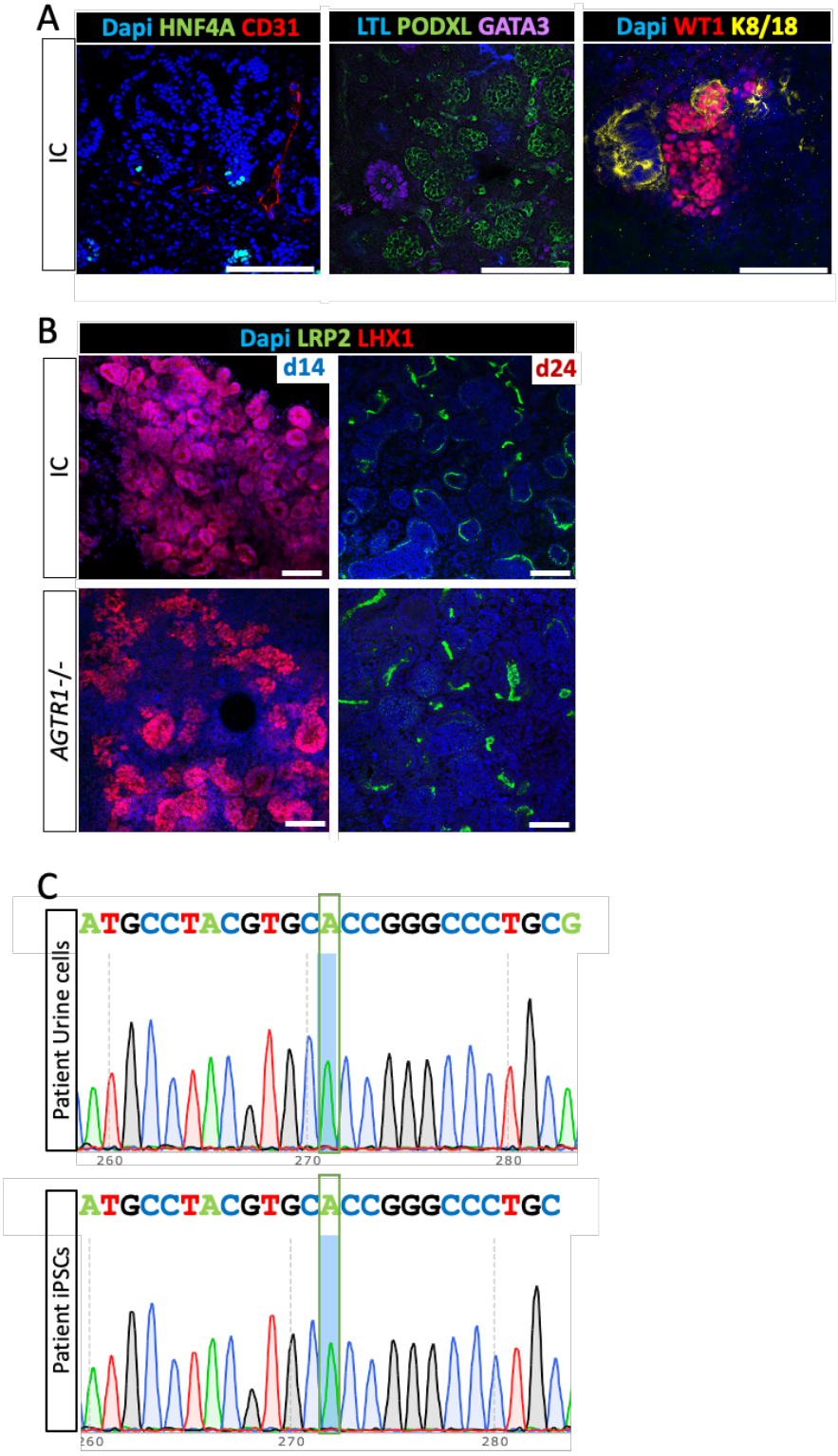

Fig. S1, supporting Figures 1 and 2. Immunofluorescent (IF) characterization of fully differentiated organoids. (A) Representative IF staining for nephron and endothelial markers in wild type iPSC-derived kidney organoids. (B) IF staining for LHX1 and LRP2 in both isogenic control (IC) and mutated (AGTR1-/-) d14 and d24 organoids. Scale bars=100μm are indicated in each image. (C) Sequencing of reprogrammed urine cells illustrating biallelic c.2570G>A missense mutation in the *ACE* gene was retained in the iPSCs. Scale bars=100μm are indicated in each image.

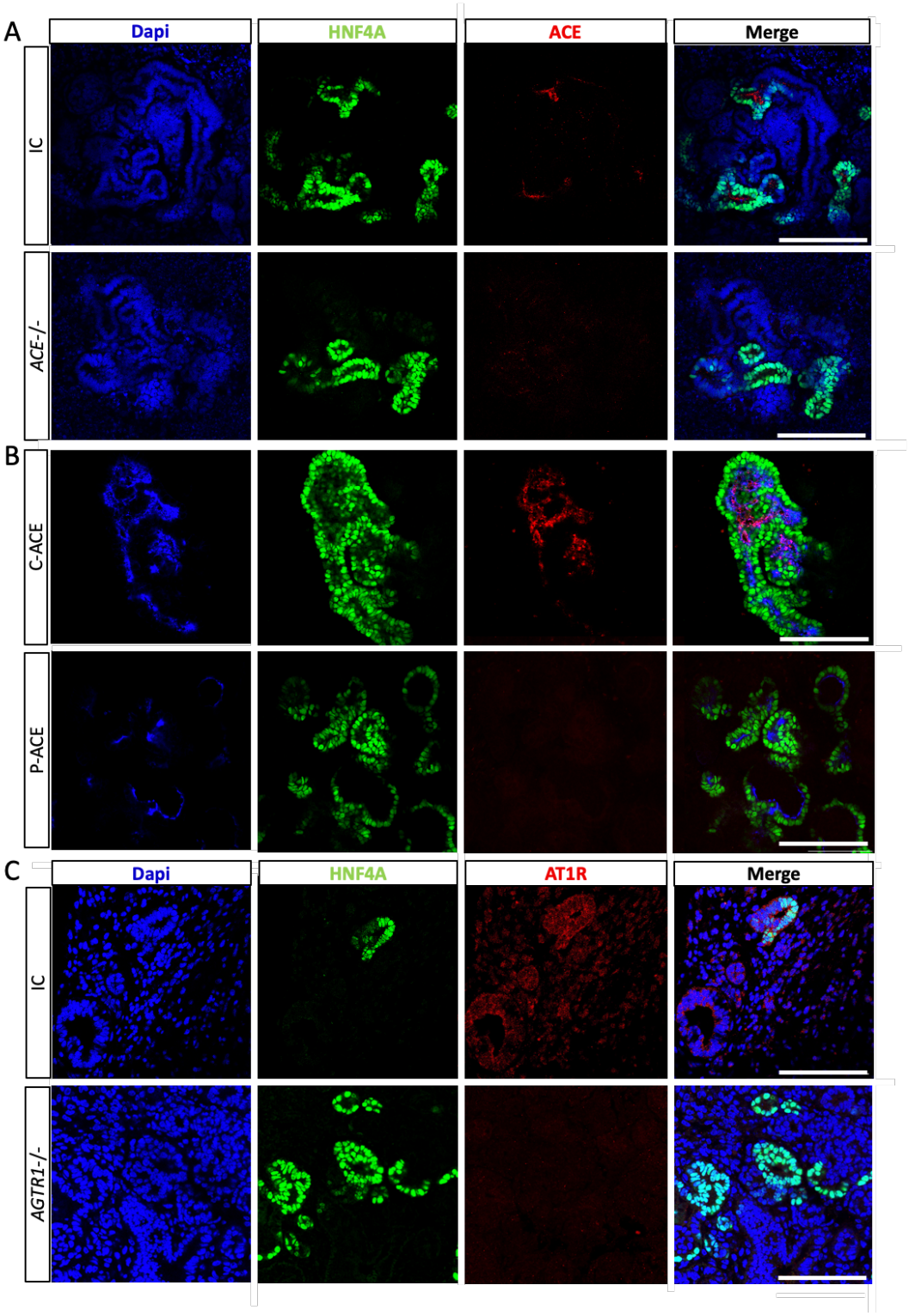

Fig. S2, supporting Figure 2. Validation of ACE and AT1R in iPSC lines. Immunofluorescent (IF) staining of *ACE*-/- and patient (P-ACE) iPSC-derived organoids compared with their corresponding ICs (IC, C-ACE) for ACE and of *AGTR1*-/- and IC for AT1R. IC=Isogenic control. P-ACE=AR-RTD patient derived iPSC. C-ACE=CRISPR-corrected iPSCs. Scale bars=100μm are indicated in each image.

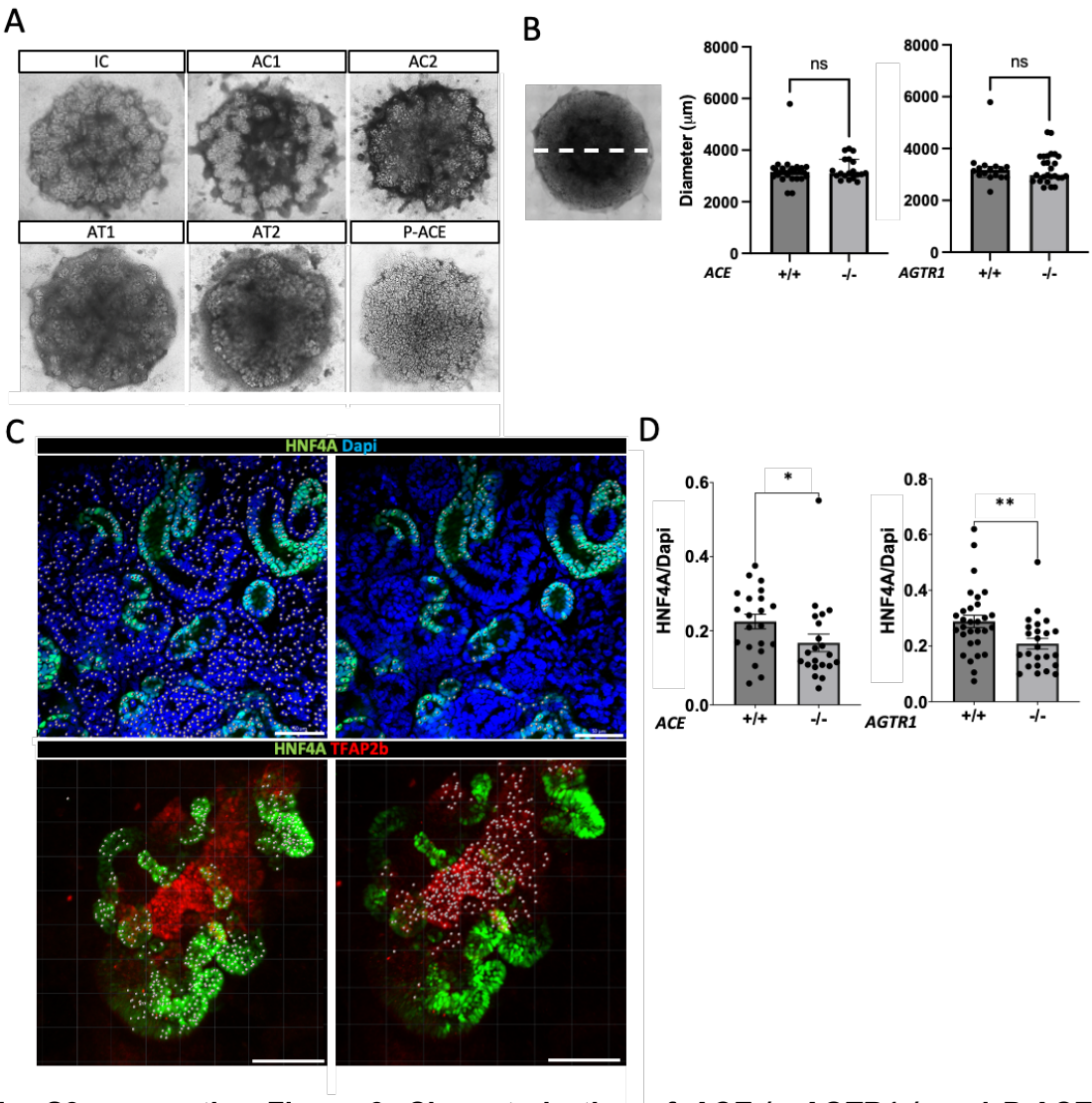

Fig. S3, supporting Figure 3. Characterization of *ACE-/-*, *AGTR1-/-* and P-ACE iPSC derived organoids. (A) Representative bright field images of IC, *ACE -/-*, *AGTR -/-,* and P-ACE iPSC-derived organoids. (B) Diameter of *ACE-/-* and *AGTR1-/-* and their respective ICs. Graphs present the mean±S.E.M of diameters calculated for n≥20 organoids per clone. Each dot represents the mean cell number of x4 z-sections per organoid. ns=not significant. (C) Representative images from z-sections of IF staining for HNF4A, TFAP2b and Dapi in IC kidney organoids with IMARIS marking individual cells for quantification of either HNFA+ and Dapi+ or HNF4A+ and TFAP2b+ to calculate ratios. (D) Ratios of HNF4A/Dapi in ACE-/- or AGTR1-/- organoids and their respective ICs. Graphs present the mean ratio of HNF4a (PT cells) to Dapi positive cells (all cells) in mutant organoids and ICs. Each dot represents the mean cell number of x4 z-sections per organoid. Quantification was performed on n≥20 organoids per iPSC line. Data is presented as mean±S.E.M. *p=0.013, **p=0.004. Scale bars=100µm are indicated in each image

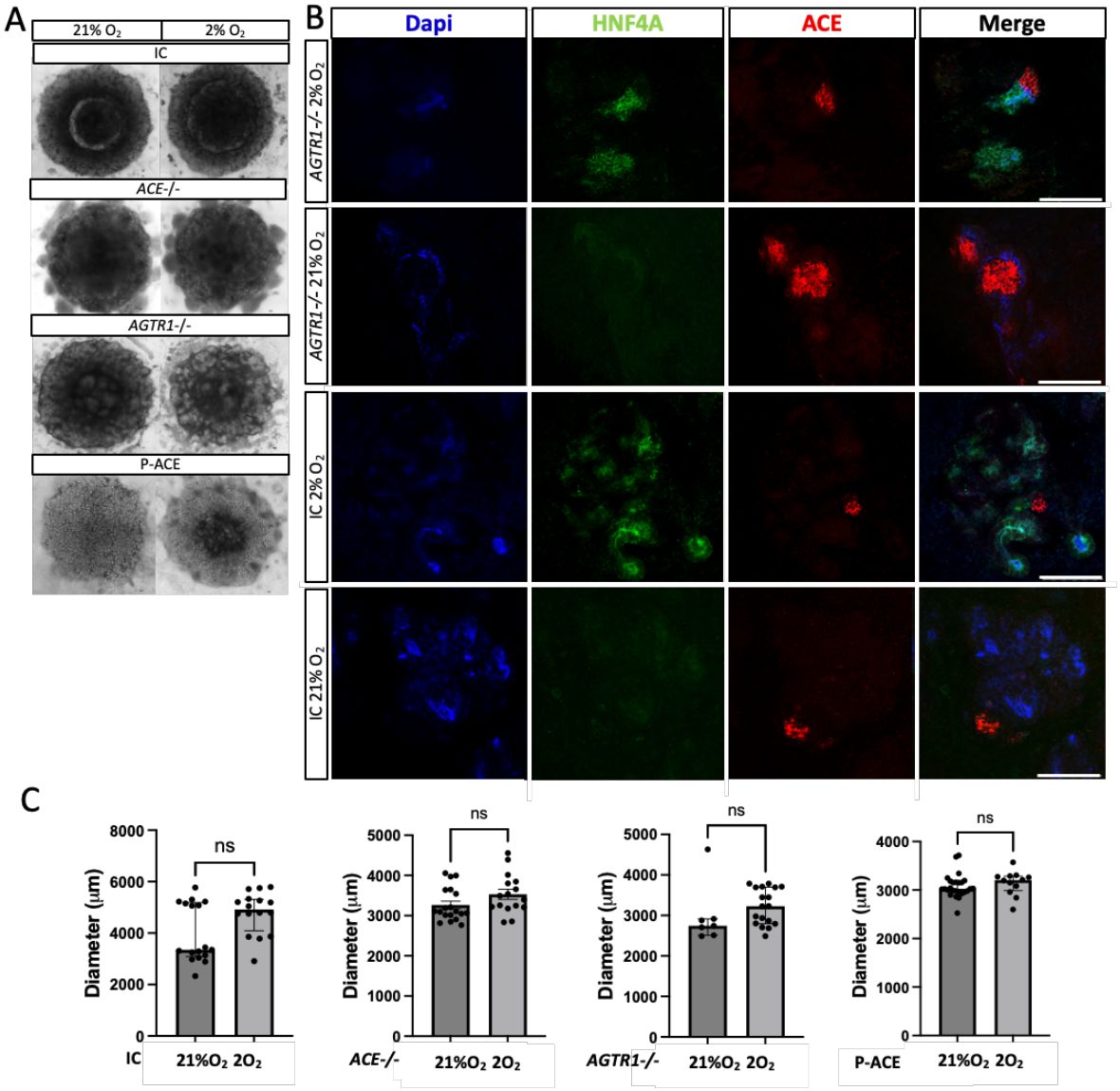

Fig. S4, supporting Figure 4. Characterization of *ACE-/-*, *AGTR1-/-,* and P-ACE iPSC derived organoids grown in standard versus hypoxic conditions. (A) Representative bright field images of IC, *ACE -/-*, *AGTR -/-,* and P-ACE iPSC-derived organoids grown in 21%O_2 versus hypoxic (2%O_2) conditions. (B) Immunofluorescent staining for pimonidazole (hypoxia marker, green) in *AGTR1-/-* and IC organoids grown in standard (21%O_2) versus hypoxic (2%O_2) conditions. Scale bars=100µm are indicated in each image. (C) Diameter of IC, *ACE-/-*, *AGTR1-/-,* and P-ACE iPSC-derived organoids grown in standard compared to hypoxic conditions. Graphs present mean±S.E.M of diameter calculated for 7-20 organoids per clone. ns=not significant. Scale bars=100µm are indicated in each image.

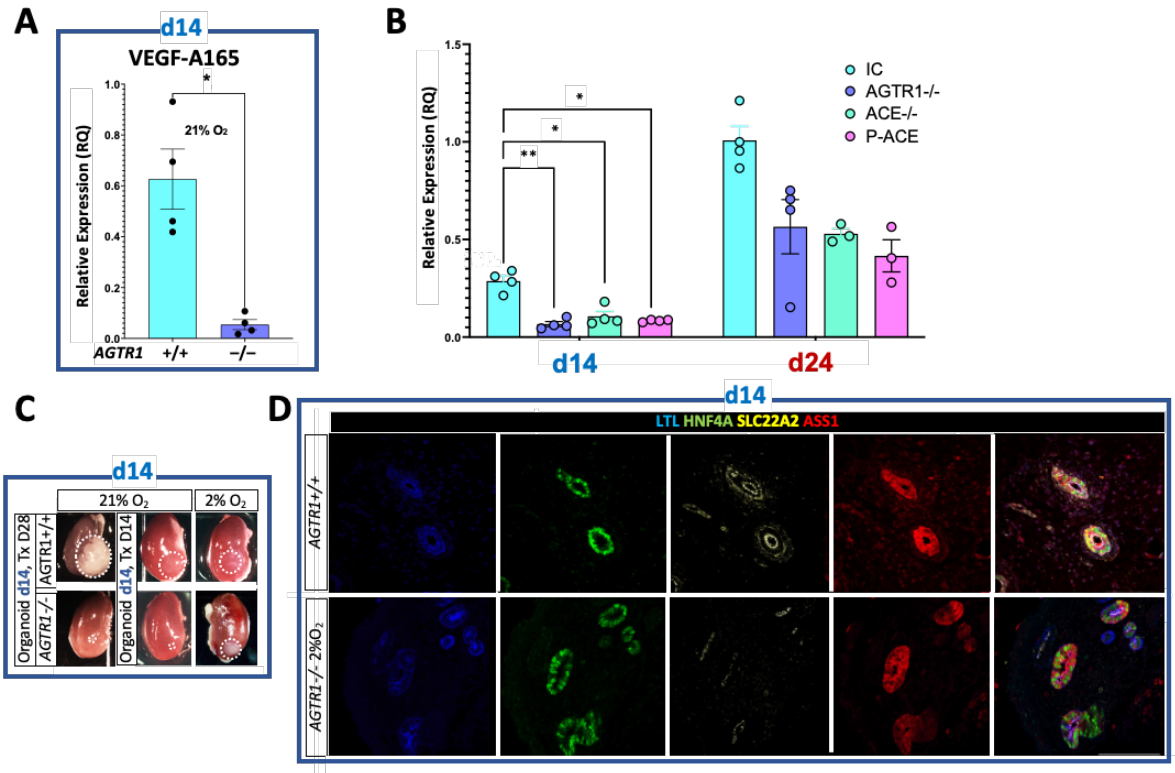

Fig. S5, supporting Figures 5 and 6. Q-PCR for VEGF-A165 gene expression in d14 *AGTR1*-/- and IC organoids grown in standard (21%O_2) conditions; Results are presented as mean±S.E.M of n=4 independent experiments. **p*=0.03. (B) Q-PCR for VEGF-A gene expression in *AGTR1*-/-, *ACE*-/-, P-ACE and IC iPSC-derived organoids at d14 and d24; Results are presented as mean±S.E.M of n=4 independent experiments. **p*=0.02, ***p*=0.006. (C) Images of explanted IC and *AGTR1*-/- iPSC derived organoids transplanted at d14 and extracted either 28 days or 14 days after transplantation. (D) IF staining for PT maturation markers (LTL, HNF4A, SLC22A2 and ASS1) in explanted *AGTR1*-/- and IC-derived organoids, grown in hypoxia (2%O_2) prior to transplantation at d14. Scale bars=100µm are indicated in each image.

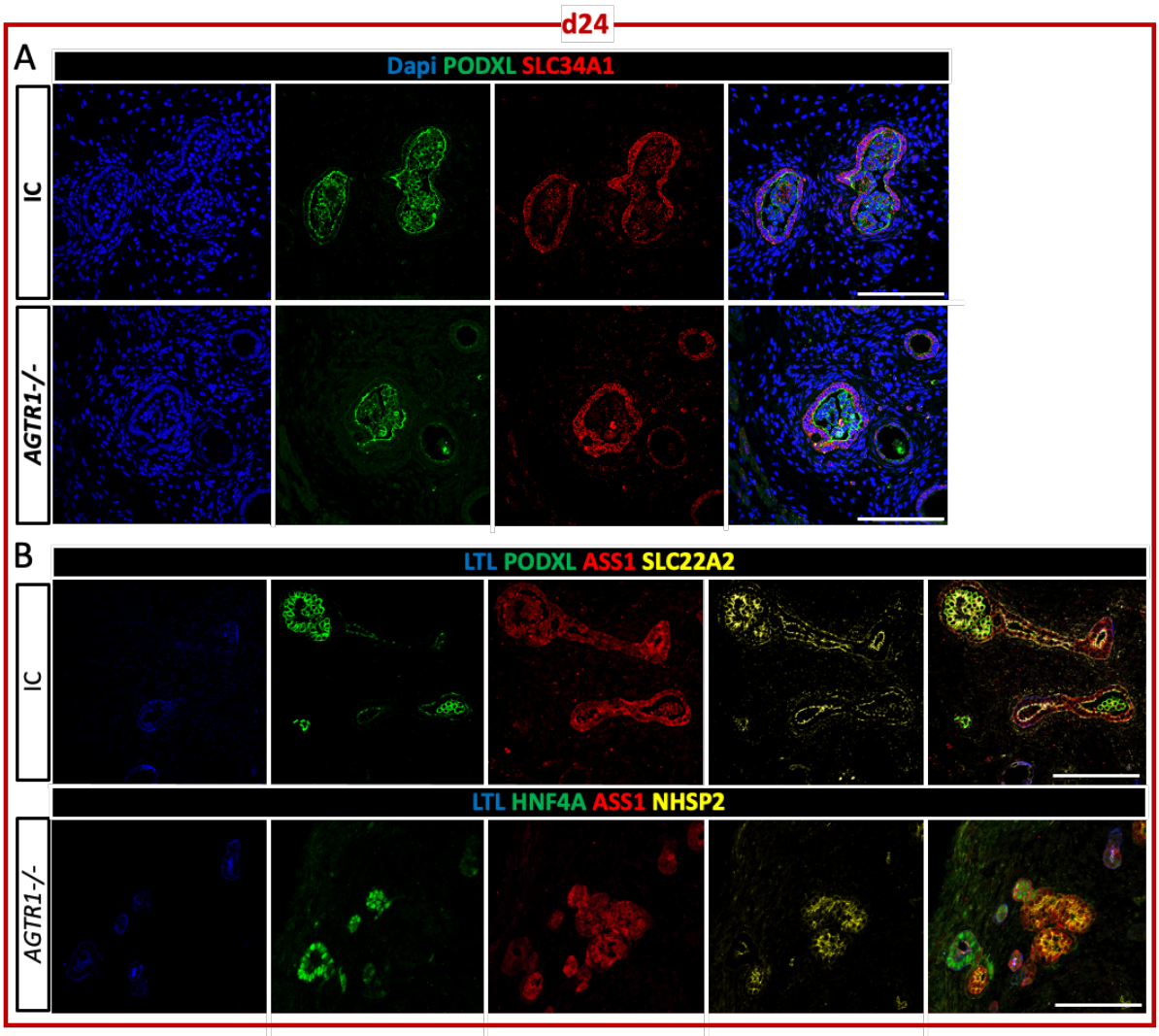

Fig. S6, supporting Figure 5. Characterization of *AGTR1*-/- and IC iPSC-derived organoids transplanted at d24 under the kidney capsule of immunodeficient mice. (A) IF staining of *AGTR1*-/- and IC extracted organoids for Dapi, PODXL and SLC34A1. (B) IF staining of *AGTR1*-/- and IC extracted organoids for LTL, PODXL, ASS1, SLC22A2, HNF4a and NPHS2. Scale bars=100µm are indicated in the images.

Table S10| Antibodies used in this manuscript.

Antibody	Source	Catalog/clone number	Dilution factor
ACE	Sigma-Aldrich	HPA029298	1:200
AGTR1	Bioss Antibodies	BS-2097R	1:200
ASS1	Abcam	ab77590	1:100
CD31	Novus Biologicals	NBP2-80640	1:200
CUBN	Sigma-Aldrich	SAB4301904	1:100
E-Cadherin	Bio-techne Novus Biologicals	NBP1-43347-0.025	1:350
GATA3	BioTechne R&D	AF2605	1:500
HNF4A	Abcam	ab41898	1:200
Kim1	R&D Systems	AF1750	1:200
KRT8/18	Abcam	ab194130	1:1000
LIM1/LHX1	Abcam	ab229474	1:250
LRP2/Megalin	Bio-techne R&D	MAB9578-100	1:100
LTL	Vector Laboratories	B-1325-2	1:500
Nephrin/NPHS1	R&D Systems	AF4269	1:300
Podocalyxin	Bio-techne R&D	MAB1658	1:50
Podocin/NPHS2	Proteintech	20384-1-AP	1:100
SLC22A2	Abcam	ab170871	1:100
SLC34A1	ThermoFisher	PA5-62358	1:100
TFAP2B	Cell Signaling Technology	2509S	1:200
TROMA-1	Sigma-Aldrich	MABT329	1:50
VE-Cadherin	Novus Biologicals	NBP1-43347	1:200
VEGF-A	Abcam	ab52917	1:250
WT1	Santa Cruz	sc-393498	1:20

Table S11| Primers and guide RNAs (gRNA) used in this manuscript

Gene	Forward	Reverse
VEGF-A	AGGGCAGAATCATCACGAAG	AGGGTCTCGATTGGATGGCA
VEGF-A165	GAGCAAGACAAGAAAATCCC	CCTCGGCTTGTCACATCTG
VEGF-A121	AGGCCAGCACATAGGAGAGA	GCCTCGGCTTGTCACATTTTT
VEGF-A189	TAAGTCCTGGAGCGTTCCCT	ACGCGAGTCTGTGTTTTTGC
ACE flanking the deletion (Exon 10 to intron 15)	GCCTGAAACTCCCTCTTCCAG	CCATTTGGAGTCCAAGCCCATG
ACE within the deletion (intron 13)	CTTGAAGCCAGGAGTTGGAG	CCTTAGGAAGGAGCCAGCTT
AGTR1 flanking the deletion	AGGCTTTATCAGTTCACAGTGT	TGTGGCTTTGCTTTGTCTTGT
AGTR1 within the deletion	ACTCACGTGTCTCAGCATTG	TCACGTATGATGCCTAGTTGAA
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
hSLC22A2	AGGGTCTCGATTGGATGGCA	GTTAAACTCGGTGACGATGGAC
hSLC34A1	CCCTCAGGTCCTACACAGGAT	GGAGCAGACGAAGAGGTAGAG
hSLC22A5	TCCACCATTGTGACCGAGTG	ACCCACGAAGAACAAGGAGATT
hSLC5A12	AGGCAACTTCCCGAGAGTTC	CCCCAAAGCGGTAGACTTCAG
hSLC7A13	TTGGCTCCACGGTTGCTTTT	CCAATGCCAGACATTTCTTAGGC
ACE Exon 17 for c.2570 A>G sequencing	GCAGGTAATGTGGTGTTGGG	GGTGACTTGCCCGACATAGA
gRNA sg320	GTTTCGTTTCGGGTAACAGG AGG	
gRNA sg321	GCATGGTATGAAGTAGGTGC CGG	
gRNA Sg431	GAGAATCATTTTGATCACCTGGG	
gRNA Sg433	TTGGTAGTGAAGTGCTGCAGAGG	

Table S12| Number of mice used for organoids transplantations

	No. of mice per in vitro growth condition (engrafted organoids/total no. implanted)		
	21% O_2		**2% O_2**
iPSC line	**Transplantation of D24 organoids**	**Transplantation of D14 organoids**	**Transplantation of D14 organoids**
IC	4 (4/4)	22 (22/22)	4 (4/4)
AGTR1-/-	4 (4/4)	22 (0/22)	6 (6/6)

Supplemental data:

AR-RTD patient's phenotype. The patient is a female from an Arab-Muslim decent who was born preterm following oligohydramnios and development of IUGR. Following birth, she was anuric and developed hyperkalemia for which peritoneal dialysis was initiated. She required vasopressors for profound hypotension. ACE serum levels were undetectable, Renin serum levels were elevated >5000uIU/ml, Aldosterone was at the normal range. Following treatment with Fludrocortisone and blood pressure stabilization kidney function was partially recovered. She was discharged from the NICU and continues close follow-up by the pediatric nephrology unit at the Soroka Medical Center. She is currently 12 years old with CKD stage 3. ACE level remains undetectable and Renin remains >5000uIU/ml. Kidney ultrasound remains unremarkable apart for simple cysts (x1 in each kidney). IUGR- Intra-uterine growth restriction.