Tmem100-GCE Allele Characterization

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Findings: VALIDATED

Our analysis confirms the expression of eGFPCreER^{T2} at 15.5dpc under the regulation of Tmem100 in developing nephrons in the kidney, the Wolfian and Mullerian ducts and vasculature of the UGS. At 15.5dpc eGFP localizes to the proximal region of the early renal vesicle, at later stages eGFP is present in the parietal epithelium adjacent to Wilms Tumor (WT1) positive cells demarcating the future podocytes of Bowman's capsule. Cre dependent R26R-LacZ expression is observed in the renal vesicle derivatives and male and female reproductive systems following Tamoxifen treatment.

Data:

Crosses

The Tmem100-GCE strain is a BAC transgenic line with eGFPCreER^{T2} (GCE) expressed in the Tmem100 domain. Pronuclear injection of the BAC construct DNA into C57Bl6/DBA F1 embryos resulted in the birth of 97 pups of which 9 male and 10 females carried the transgene. Five male founders were crossed to Rosa26R$^{lacZ/+}$ (R26R) females and the urogenital system (UGS) was collected from 15.5dpc embryos. Four founder males transmitted the transgene: M30, M40, M51 and M72; M51 subsequently died without fathering progeny. Tmem100$^{GCE/+;}$ R26R$^{lacZ/+}$ embryos collected for each founder exhibited similar expression patterns as determined by native GFP fluorescence or by lacZ staining 15.5-16.5dpc UGS. In some of the founders the GFP fluorescence was either not strong enough to view in whole mount (M51 and M72) or the transgene was not expressed in all Tmem100$^{GCE/+;}$ R26R$^{lacZ/+}$ embryos (M40). Therefore, subsequent analysis was carried out on founder M30.

We crossed Tmem100$^{GCE/+;}$ males from founder line 30 with Rosa26R$^{lacZ/+}$ (R26R) female mice to obtain Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$embryos. In order to activate ß-galactosidase (β-gal) reporter expression from the R26R$^{lacZ/+}$allele, an intraperitoneal injection of tamoxifen in corn oil (1mg to 40g body weight) was injected into pregnant 13.5dpc mice. A control group was injected with the same volume of corn oil. Three litters of embryos from tamoxifen induced females and two litters from corn oil injected females were dissected at 15.5-16.5dpc to collect the urogenital system (UGS). GFP expression and tamoxifen dependant β-gal activity was observed in the kidney and male and female reproductive tracts.

Genotyping

Tail samples of the embryos were collected and incubated in tail digestion buffer overnight at 55ºC. PCR was performed as per the protocol below and the PCR products were run on a 1.5% agarose gel (Fig1).

Oligonucleotides: for targeted/transgenic allele Size: 359bp
DNA sequence (forward): 5'- GACCCCACAAATGGAGAATG -3'
DNA sequence (reverse 2) 5'- GAACTTCAGGGTCAGCTTGC -3'
Amplifies 5' arm into GFP sequence within GFP-Cre region.

Rxn Buffer and Conditions: (25µl reaction)

10X PCR Buffer	2.5ul			
1.25mM dNTP	4ul	94ºC	3min	1 cycle
10uM primer F	1ul	94ºC	30sec	
10uM primer R1	1ul	58.5ºC	30sec	35cycles
5x cresol red dye	5ul	72ºC	45sec	
Amplify Taq	0.2ul (5u/ul)	72ºC	10min	1 cycle
Genomic DNA	1ul			
Total volume	**25 ul**			

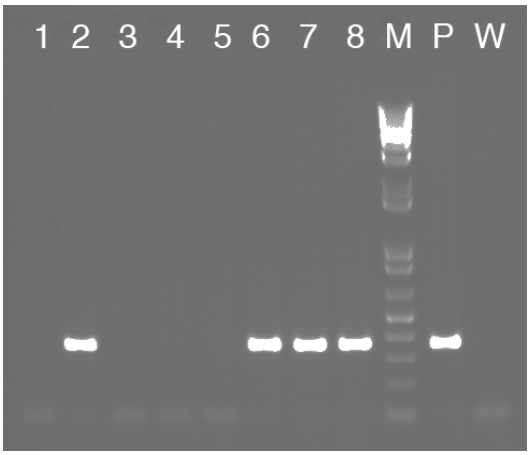

Fig1: Numbers 2, 6, 7 and 8 Tmem100$^{GCE/+}$, numbers 1, 3, 4 and 5 wildtype **P:** Tmem100$^{GCE/+}$ positive control, **W**: Wildtype control**.**

Native Fluorescence

15.5dpc UGSs where examined with a fluorescent microscope to view GFP expression. GFP can be seen in whole mount in the developing nephrons in the kidney and in the male and female reproductive track (Fig 2).

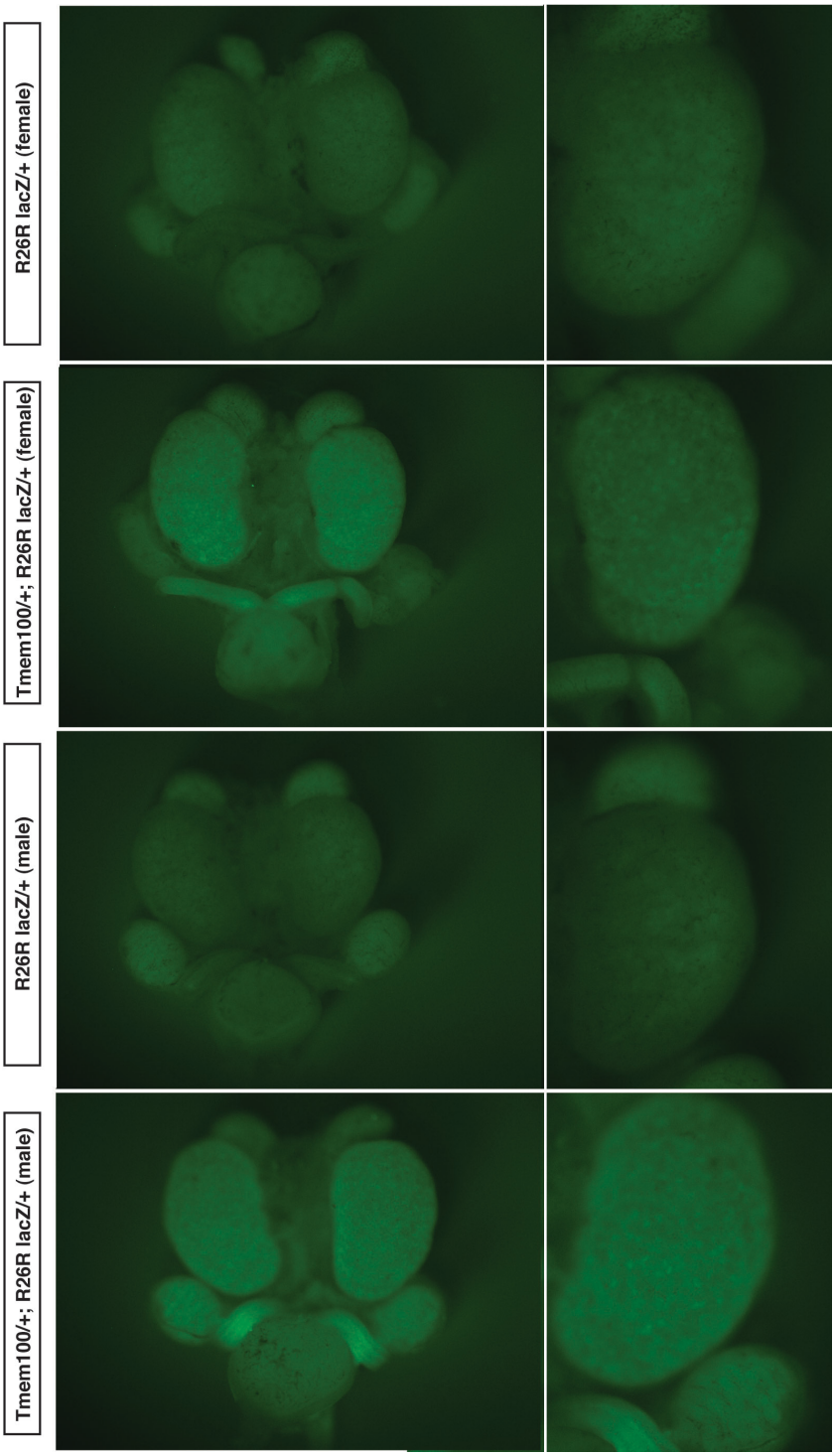

Fig 2. Wholemount GFP detection in 15.5dpc Tmem100$^{GCE/+}$ UGS.

GFP fluorescence was visible in nephrogenic zone in 15.5dpc kidneys and the Mullerian and Wolfian ducts in male and female embryos. GFP expression was limited to the Tmem100 domain.

Cre-recombinase Activity

Dissected 15.5dpc UGS samples were stained with X-gal to assay for β-gal activity. Tamoxifen dependent Cre activity was detected in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ samples but not in corn oil injected Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ controls nor in the UGS of male or female wildtype litter mates (Fig 3, 4 and 5).

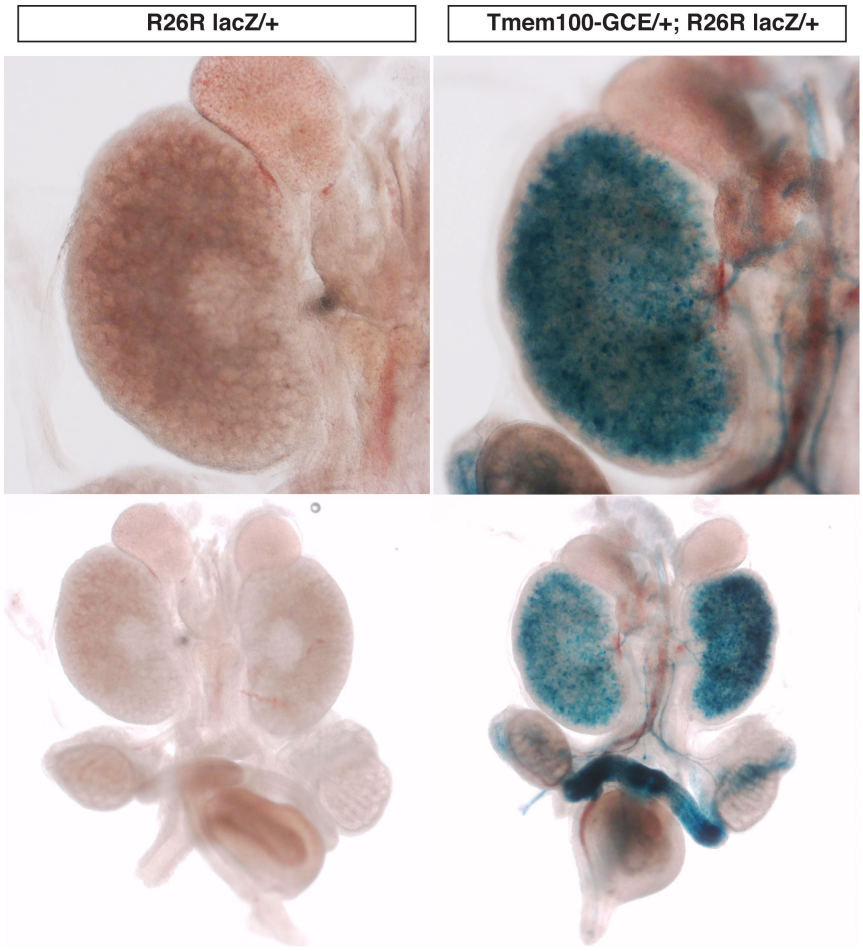

Fig 3. Cre-dependent β-gal activity in male Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ UGSs.
A single dose of 2mg/40g tamoxifen, injected at 13.5dpc resulted in ß-gal activity in the nephrogenic zone, vasculature and Wolfian duct of male Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ but not in R26R$^{lacZ/+}$ 15.5dpc UGS.

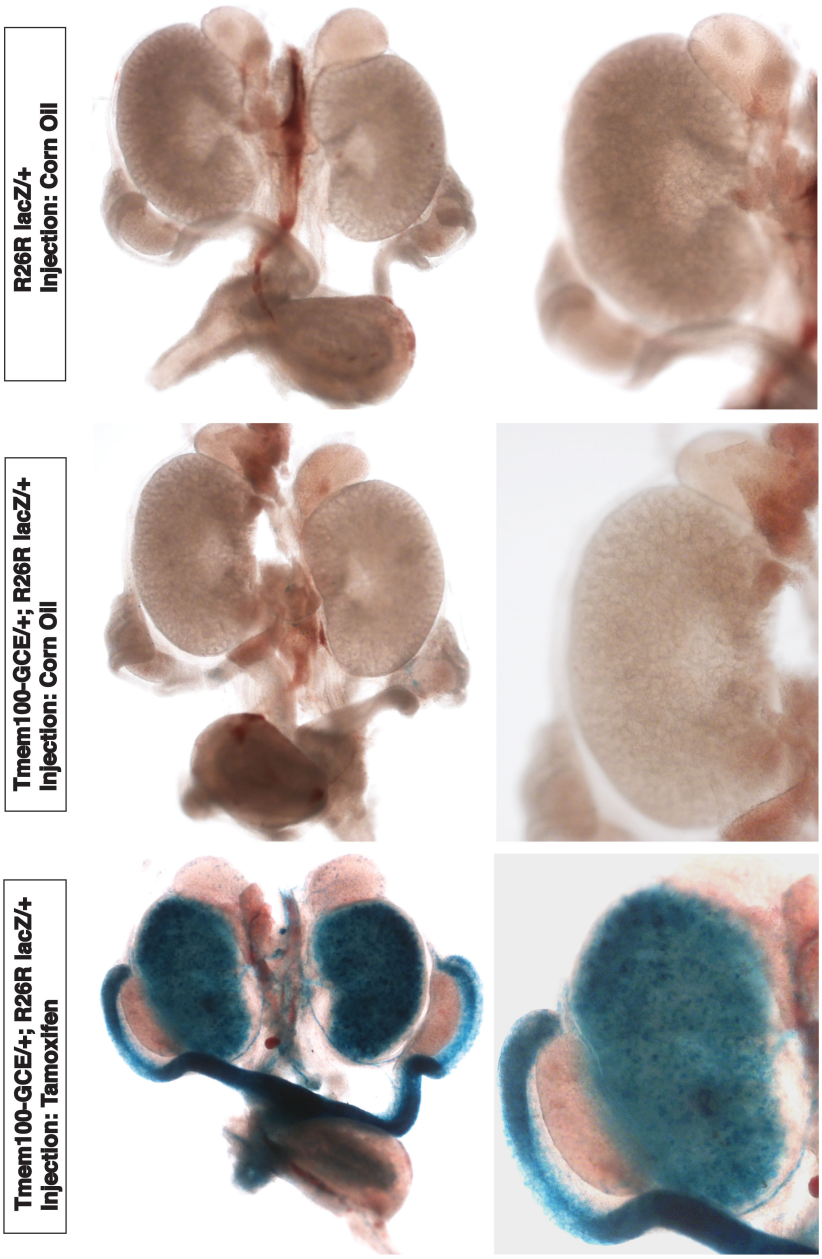

Fig 4. Cre-dependent β-gal activity in female Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ UGS. A single dose of 2mg/40g tamoxifen, injected at 13.5dpc resulted in ß-gal activity in the nephrogenic zone, vasculature and Mullerian duct of female Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ embryos but not in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ corn oil controls or R26R$^{lacZ/+}$ 15.5dpc UGS.

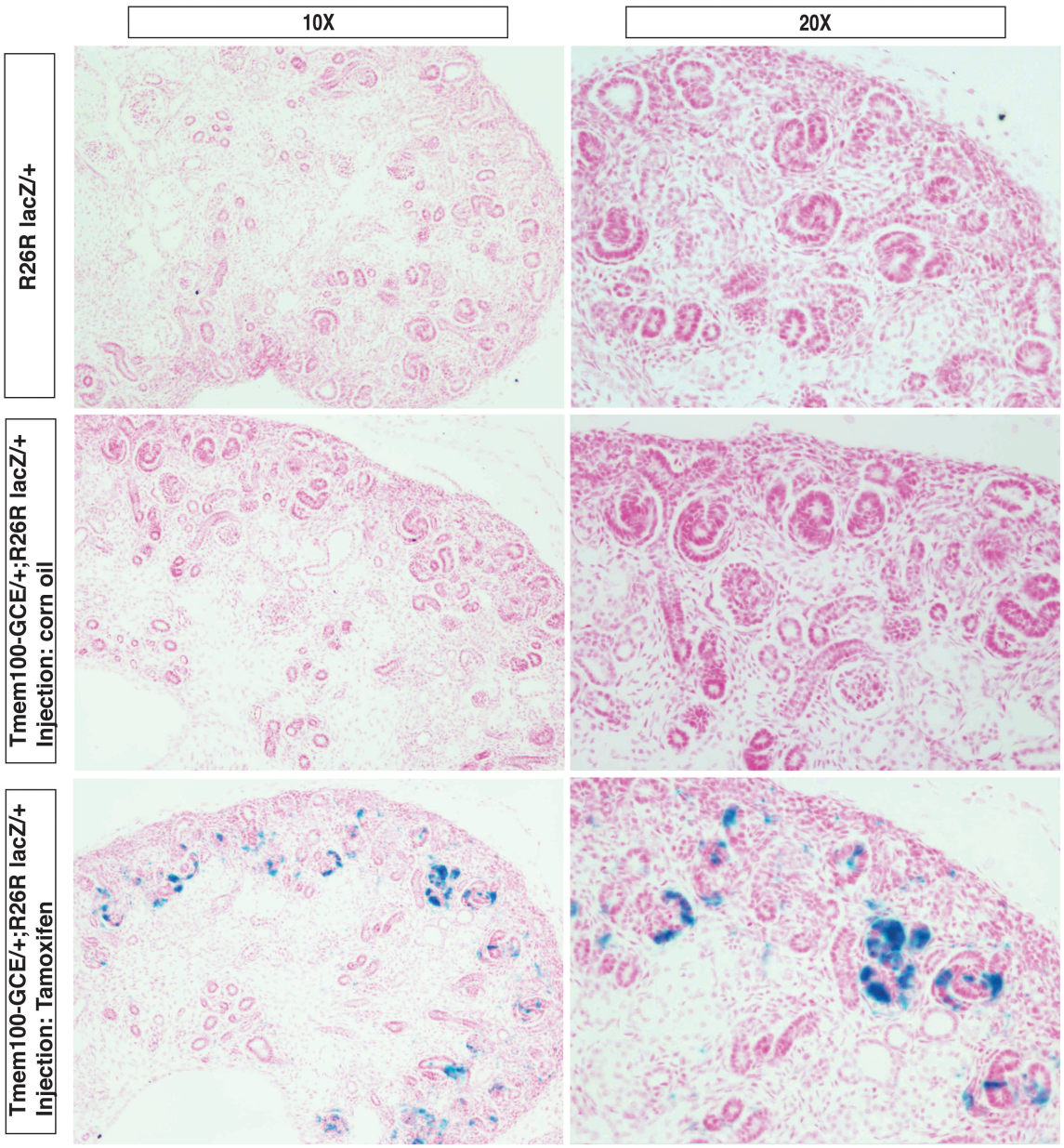

Fig 5. Cre-dependent β-gal activity in the cortical region of the kidney of Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ UGS. A single dose of 2mg/40g tamoxifen, injected at 13.5dpc resulted in ß-gal activity at multiple stages of nephron development and potentially small arteries in the kidneys of Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ embryos but not in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ corn oil controls or R26R$^{lacZ/+}$ 15.5dpc UGS.

Immunohistochemistry

Immunohistochemistry was performed to examine if the eGFPCreER^{T2} allele was expressed in the expected Tmem100 domain. To test for Cre function, Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ UGSs from Tamoxifen injected mice, Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ corn oil control and R26R$^{lacZ/+}$ were assayed. β-gal and GFP expression were examined by probing with rabbit anti-β-gal, chicken-anti-GFP and anti-cytokeratin 5 antibody. WT1 and GFP expression were examined by probing with rabbit anti-WT1, chicken-anti-GFP and anti-cytokeratin 5 antibody.

Whole UGSs were fixed in 4% paraformaldehyde at 4ºC 2 hours, washed 3 times in PBS, equilibrated in 30% sucrose overnight and then embedded in OCT and flash frozen on dry-ice. The UGSs were sectioned at 16um and stained with rabbit-anti-WT1/chicken-anti-GFP/mouse-anti-Cytokeratin, rabbit-anti-ß-gal/Chicken-anti-GFP/mouse-anti-Cytokeratin respectively, GFP (Chicken, Aves Labs, Inc, GFP-1020, 1:500); beta-gal (Rabbit, MP Biomedicals, LLC, 55976, 1: 20000), WT1 (Rabbit, Santa Cruz Sc-192, 1:50), Cytokeratin (Mouse IgG1, Sigma, C 2562, 1:500) were incubated overnight at 4ºC and detected with secondary antibodies Alexafluor 488, 555, 633, and 647 (Molecular probes).

GFP was observed in the proximal region of the early renal vesicle and at later stages was present in the parietal epithelium adjacent to WT1 positive cells marking the future podicytes of Bowman's capsule (Figs 8, 9 and 10). ß-gal expressing cells co-localized with a subset of the GFP positive cells in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ Tamoxifen injected UGS, but not in either Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ corn oil control or R26R$^{lacZ/+}$ samples (Fig. 6 & 7)

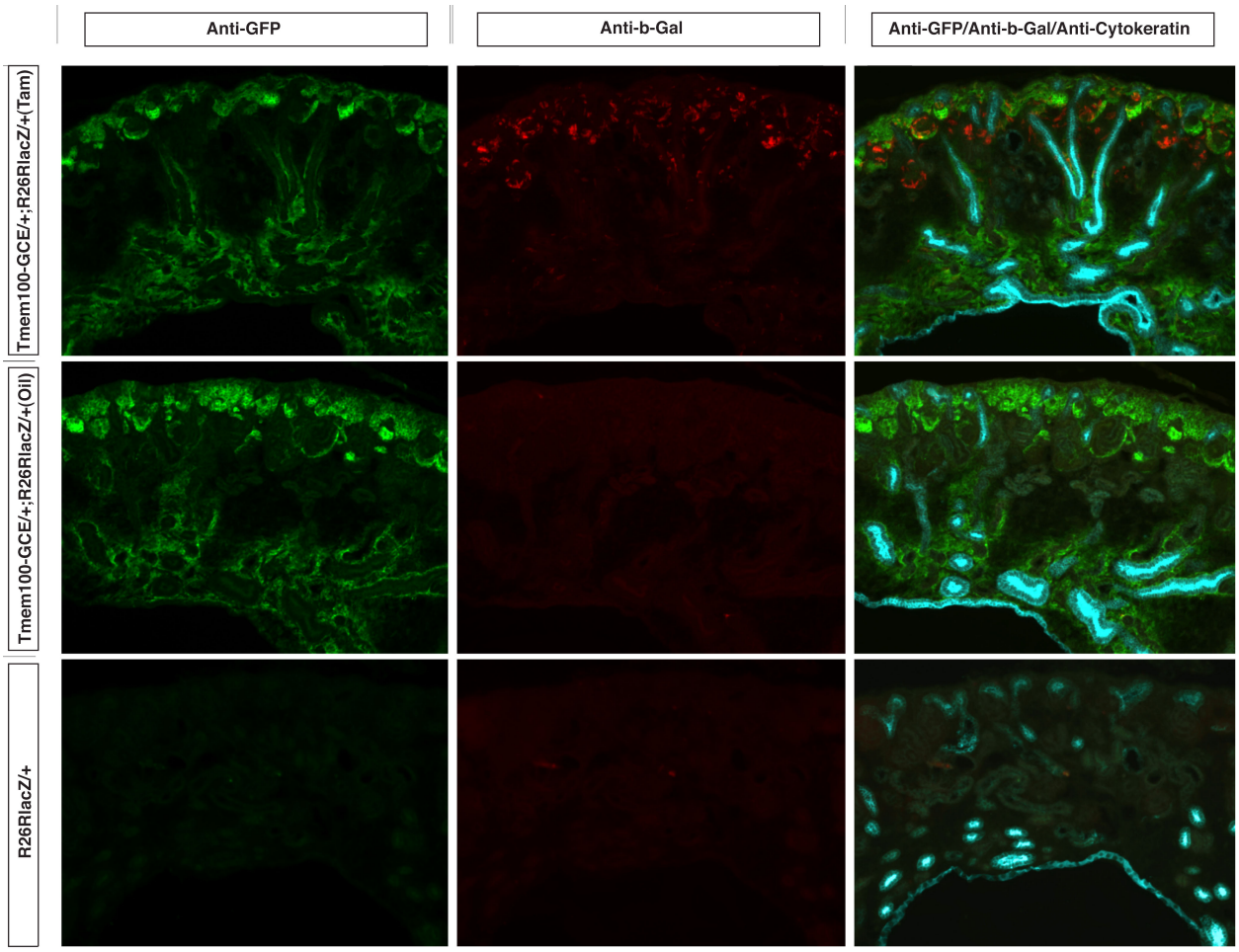

Fig 6. ß-galactosidase detected in the cortex of the kidney in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ tamoxifen injected kidneys. Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ and R26R$^{lacZ/+}$kidneys were probed with anti-ß-gal, anti-GFP and anti-cytokeratin 5 antibodies. Co-localization of GFP and Tamoxifen-dependent ß-gal expression is detected in in multiple stages of nephrogenesis and in kidney vasculature.

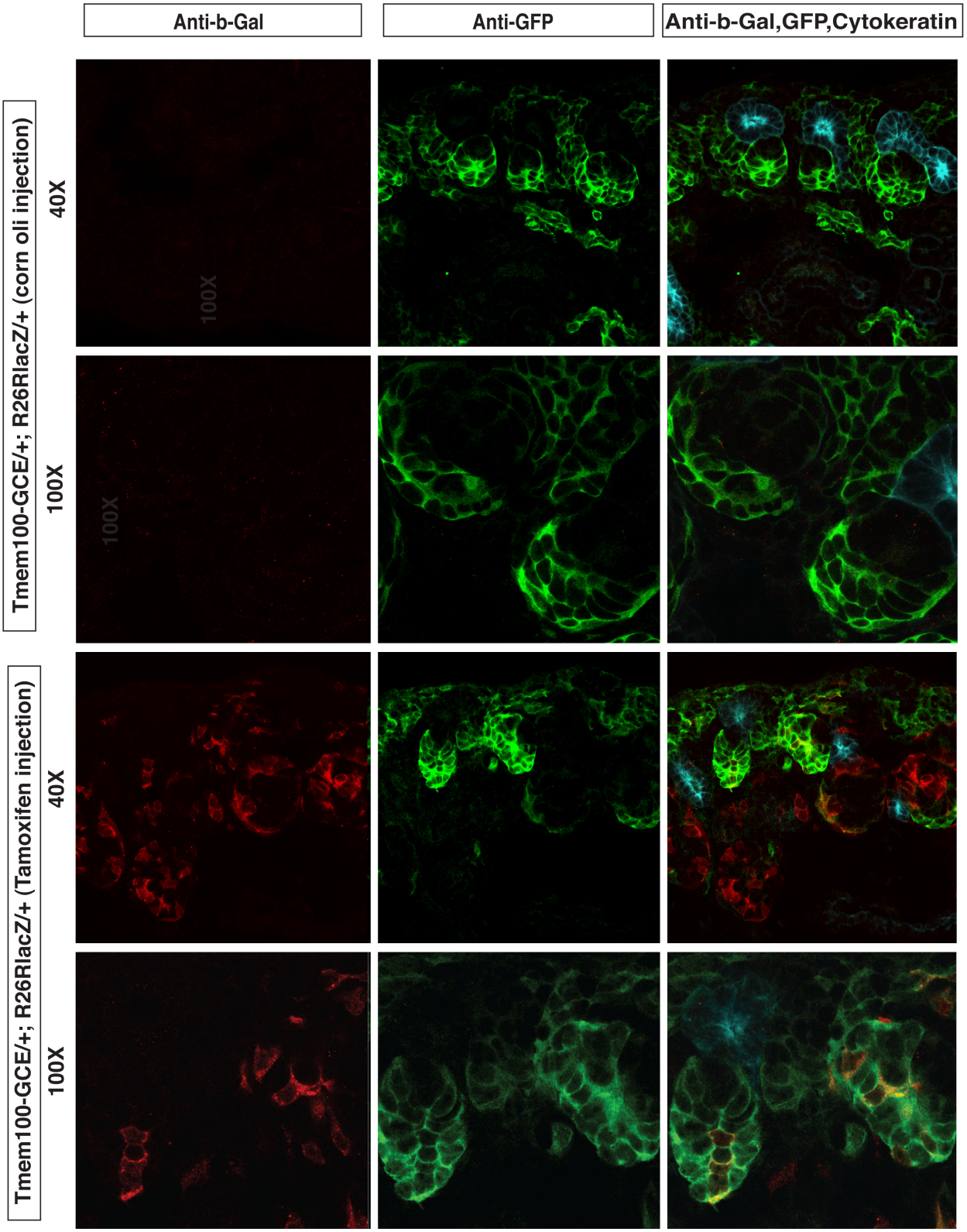

Fig 7. ß-galactosidase detected in renal vesicle derivatives in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ Tamoxifen injected kidneys. Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ and R26R$^{lacZ/+}$kidneys were probed with anti-ß-gal, anti-GFP, and anti-cytokeratin 5

antibodies. Co-localization of GFP and Tamoxifen-dependent ß-gal expression is detected in renal vesicle derivatives in the nephrogenic zone.

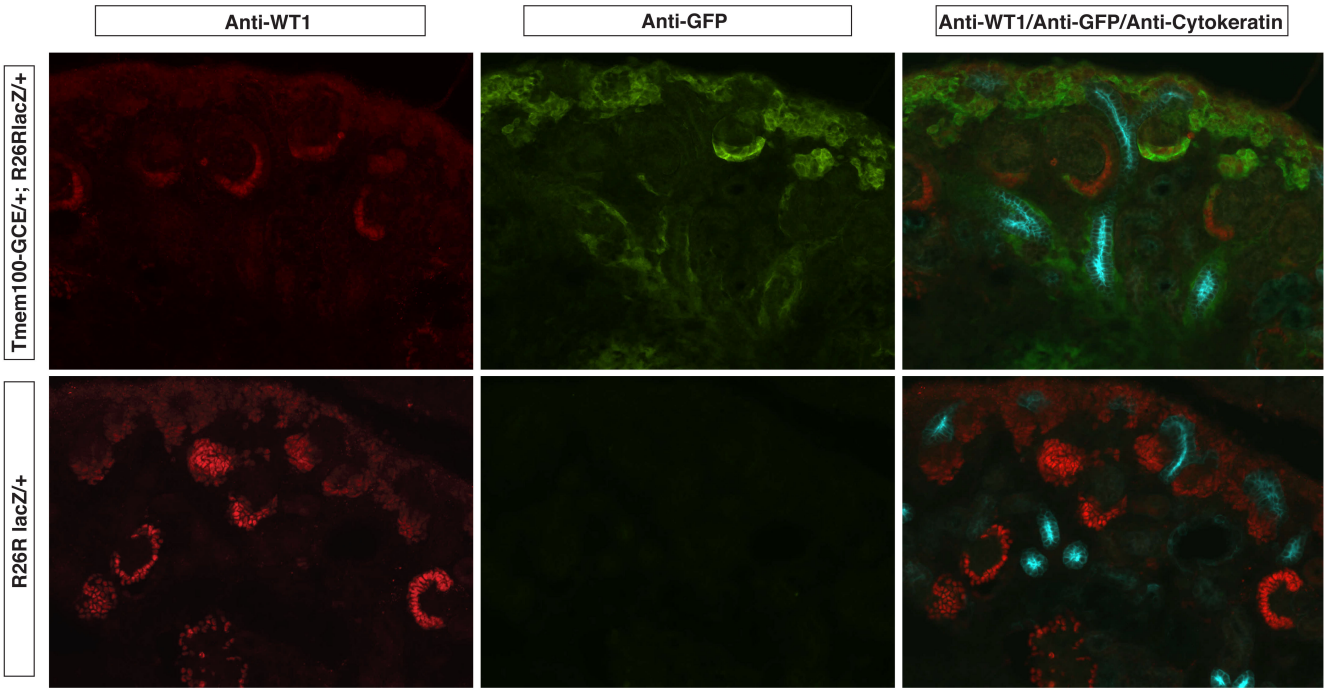

Fig 8. WT1 and GFP positive cells in developing renal vesicles of Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ 15.5dpc kidneys. Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ and R26R$^{lacZ/+}$kidneys were probed with anti-WT1, anti-GFP and anti-cytokeratin 5 antibodies.

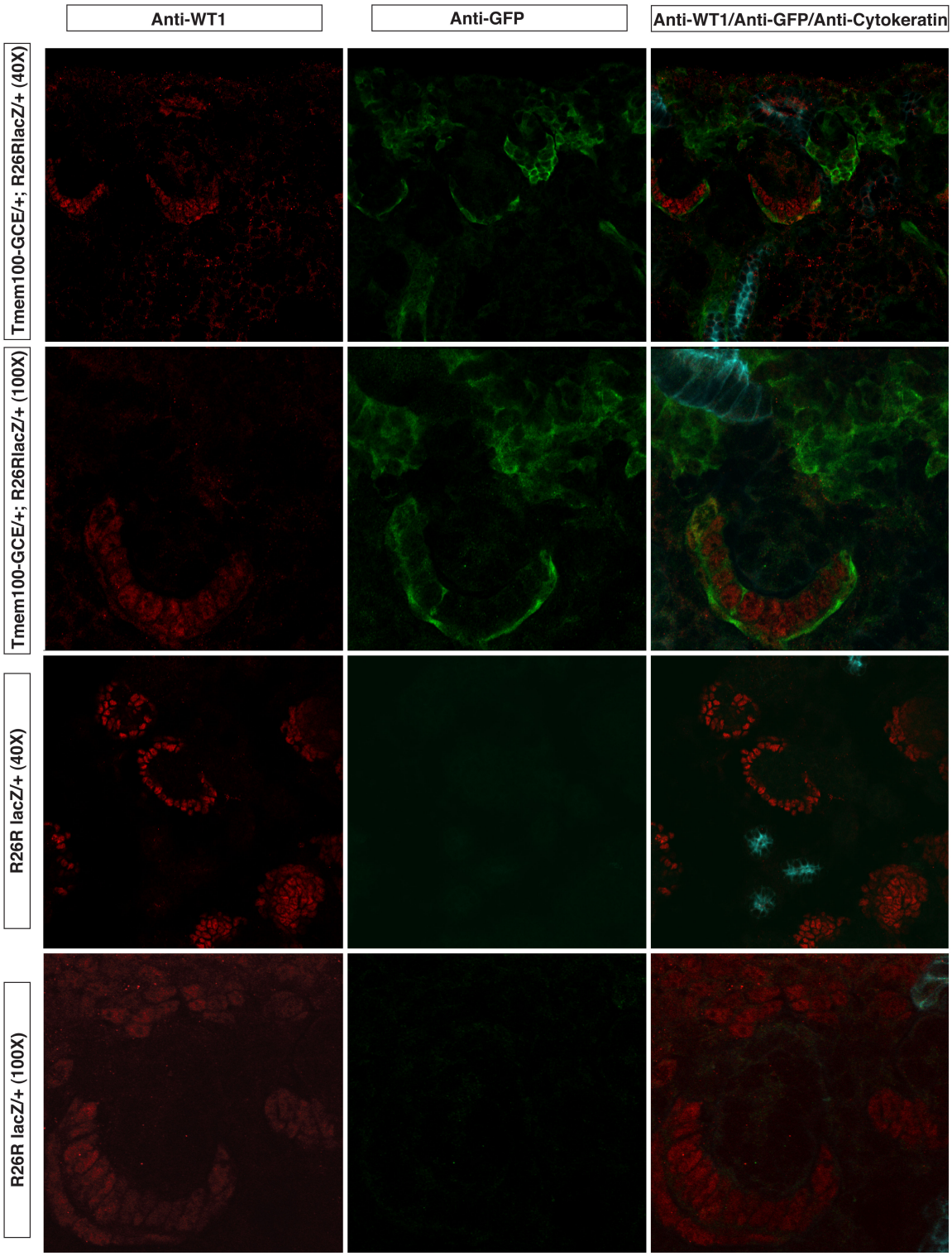

Fig 9. WT1 and GFP positive cells in late renal vesicles Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ 15.5dpc kidneys. Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ and R26R$^{lacZ/+}$kidneys were probed with anti-WT1, anti-GFP and anti-cytokeratin 5 antibodies. GFP detected in parietal epithelium layer adjacent to WT1 positive cells.

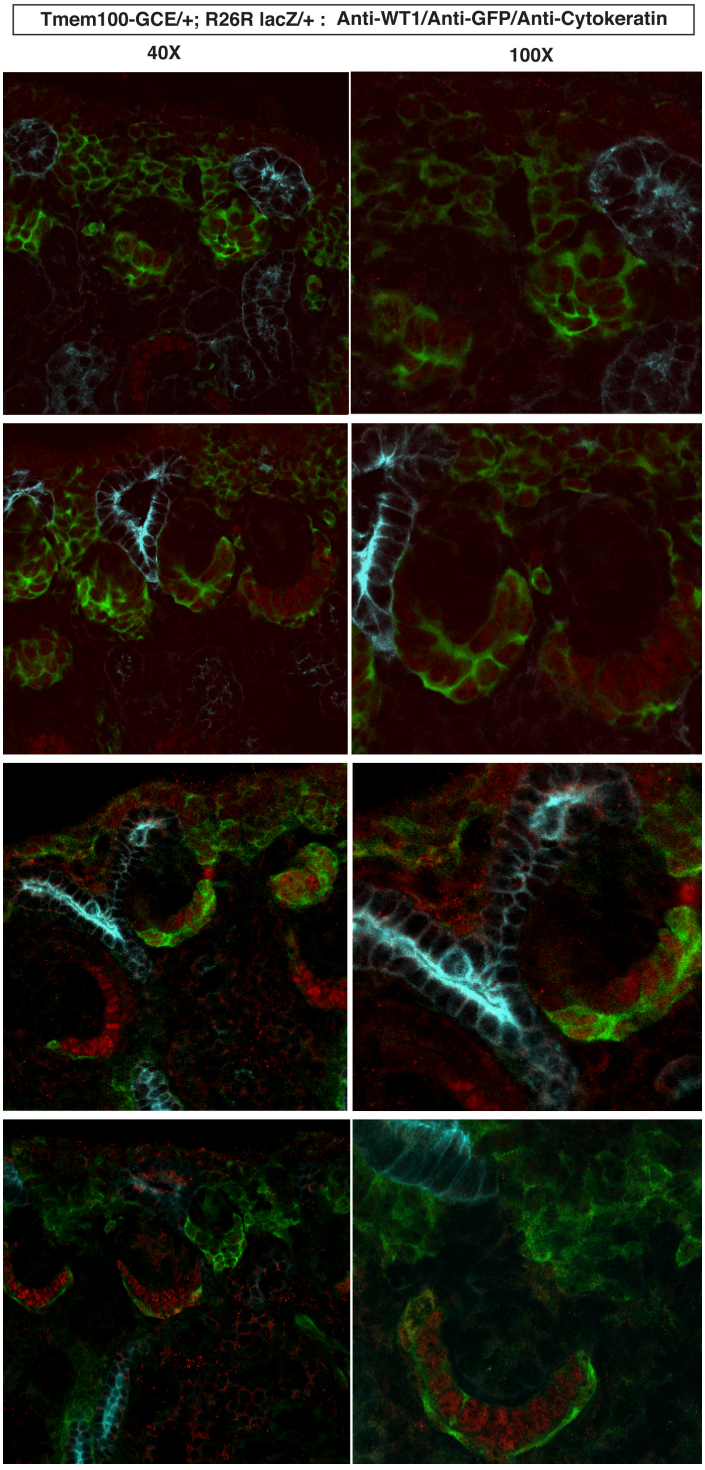

Fig 10. WT1 and GFP positive cells in developing renal vesicles in Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ 15.5dpc kidneys. Tmem100$^{GCE/+}$; R26R$^{lacZ/+}$ and R26R$^{lacZ/+}$kidneys were probed with anti-WT1 (red), anti-GFP (green) and anti-cytokeratin 5 (cyano) antibodies. GFP was detected in early through late stages of renal vesicle derivatives adjacent to WT1 positive cells.