Stem Cell Reports, Volume *3*

Supplemental Information

Identification of a Multipotent Self-Renewing Stromal Progenitor Population during Mammalian Kidney Organogenesis

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

Foxd1-Cre knock-in alleles were generated as follows. *Foxd1* targeting vectors were generated with sequence-confirmed homologous arms subcloned by PCR from a BAC clone RPCI23-164L13. The *eGFPCre (GC)*, *CreER^{T2} (CE)*, or *eGFPCreER^{T2} (GCE)* constructs followed by an *FRT-PGKneobpA-FRT* selection cassette (Kobayashi et al., 2008) was introduced into the 5' UTR of the *Foxd1* locus. Mice were generated as previously described (Behringer et al., 2013; Kobayashi et al., 2008). *Foxd1$^{GC/+}$*, *Foxd1$^{CE/+}$*, and *Foxd1$^{GCE/+}$* mouse lines were maintained on 129/Sv x C57BL/6J mixed backgrounds.

Foxd1-Cre alleles were genotyped using the following primers (*Cre*-Fw14; TTGCCTGCATTACCGGTCGATGCAACGAGT, *Cre*-Rv15; CCTGGTCGAAATCAGTGCGTTCGAACGCTA, *mFoxd1*-Fw30; GATCTGCGAGTTCATCAGCAGCCGCTTCCCT, *mFoxd1*-Rv31; GGAAGCTGCCGTTGTCGAACATATCT), which give a 403-bp band for the *Foxd1-Cre* alleles (*Cre*-Fw14 and *Cre*-Rv15) and a 194-bp band for the *Foxd1* wild-type allele (*mFoxd1*-Fw30 and *mFoxd1*-Rv31).

Six2$^{TGC/+}$ mice (Kobayashi et al., 2008) were maintained on 129/Sv x C57BL/6J mixed backgrounds. *R26R-lacZ* Cre reporter (Kobayashi et al., 2008) mice were purchased from Jackson Laboratory and maintained on C57BL/6J x Swiss Webster mixed backgrounds. The mice were genotyped s described previously (Kobayashi et al., 2008).

FACS analysis

FACS analysis of kidney cells was performed as described previously (Kobayashi et al., 2008). Briefly, kidneys from *Foxd1$^{GC/+}$* mice were dissected and treated in 300 µl Trypsin (Invitrogen, 25200-072) at 37 ºC for 3-5 min. After adding 600 µl DMEM media (Invitrogen, 11965) containing 10% sheep serum (Sigma, S2263), a single cell suspension was prepared by pipetting. Cells were collected by centrifugation and resuspended in 100 µl of PBS (Mediatech, 21-031-CV) containing 2% sheep serum. FACS analysis was performed using DAKO Cytomation MoFlo.

Histology

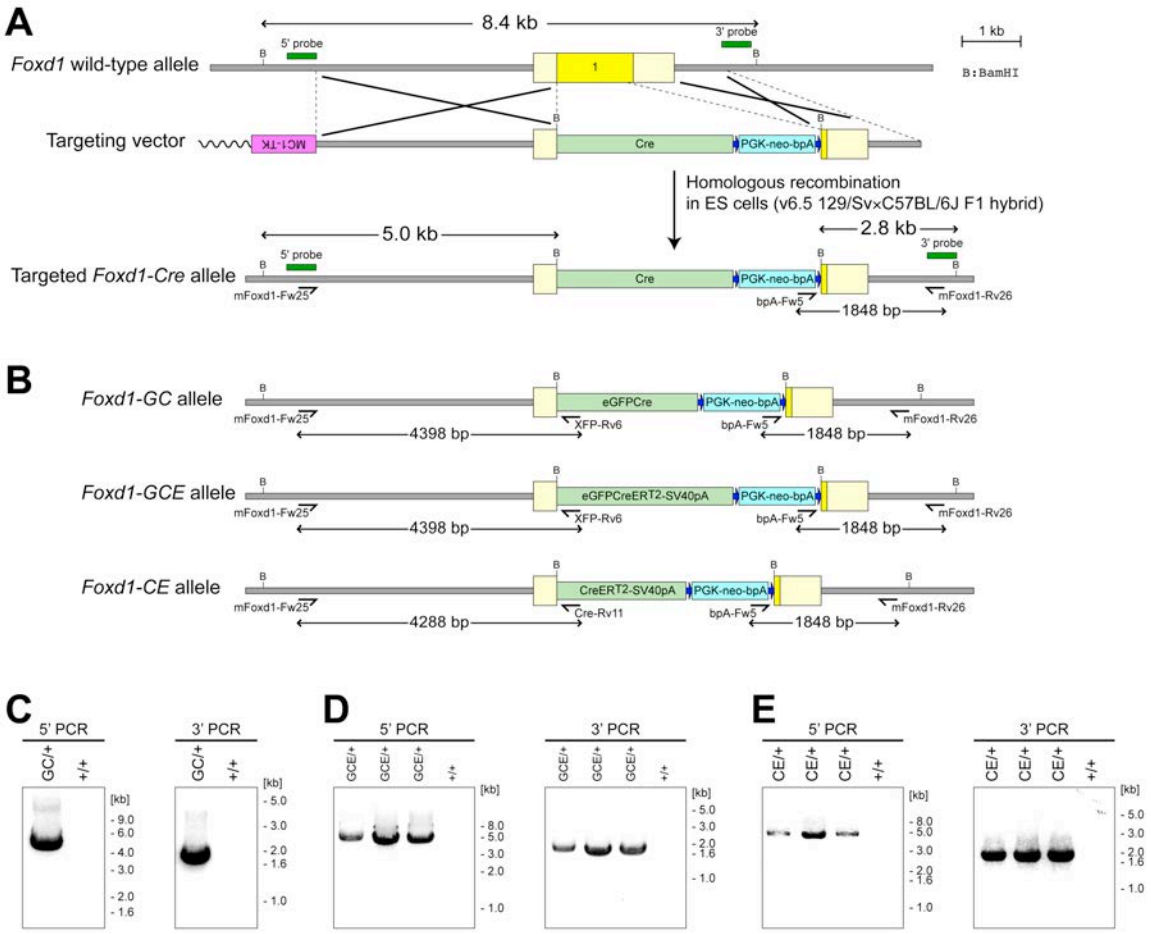
Histology on kidneys was performed as described previously (Kobayashi et al., 2008). Briefly, dissected kidneys were fixed in 4 % paraformaldehyde for 1 hr at 4 ºC and soaked in 30 % sucrose overnight at 4 ºC. After embedding in OCT (Sakura, 4583), cryosections were generated at 16 µm using a Microm HM 550 cryostat.

X-gal staining

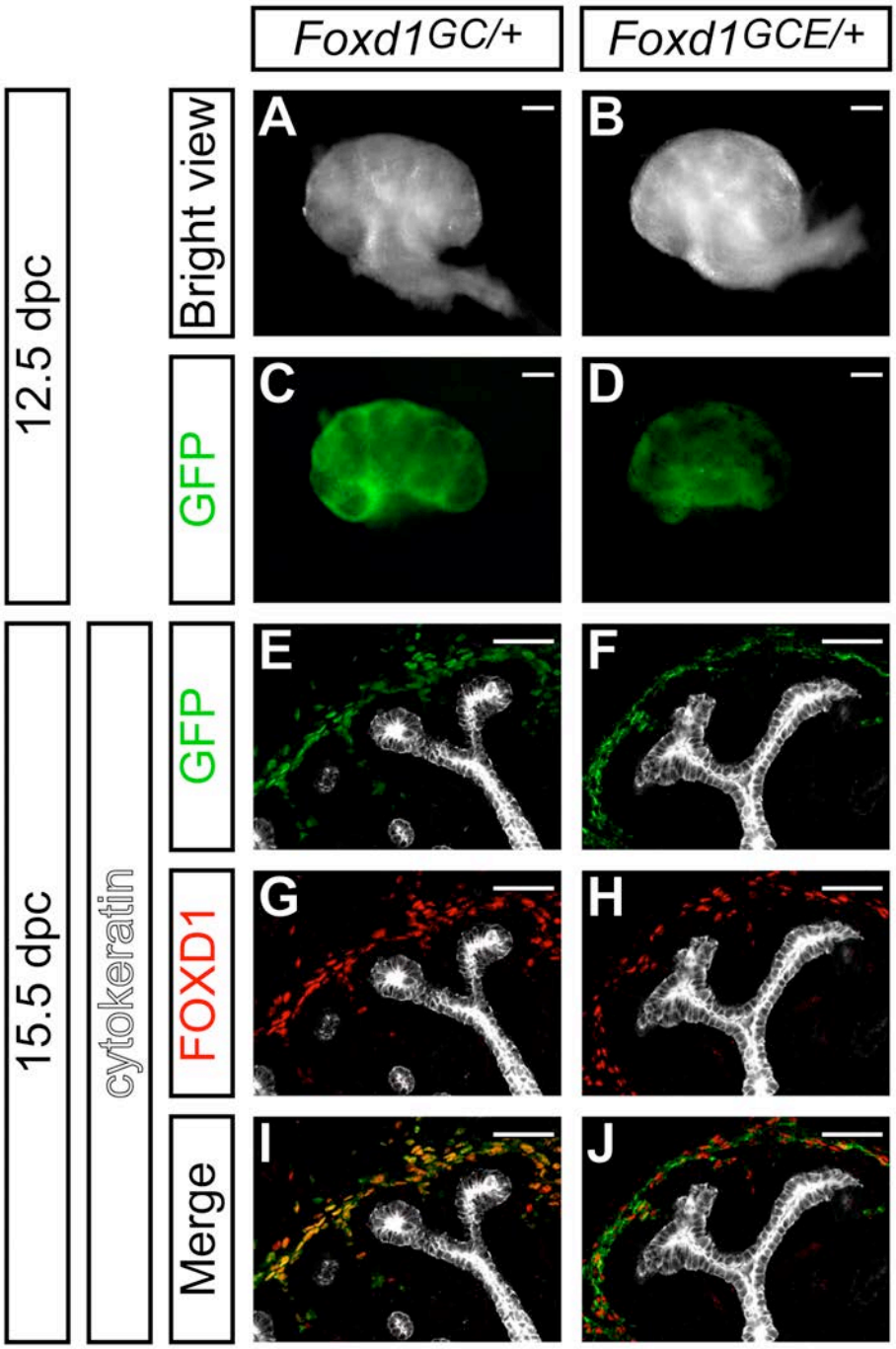
X-gal staining was performed as described previously (Kobayashi et al., 2005; Kobayashi et al., 2004). Cryosections were stained with X-gal at 37 ºC overnight and counter-stained with 0.2 % Eosin-Y (Polysciences Inc.). Whole-mount kidneys were fixed in 4 % paraformaldehyde for 1 hr at 4 ºC and stained at 37 ºC overnight for embryonic samples, or at 4 ºC for 2-3 days for neonate samples.

Immunofluorescence

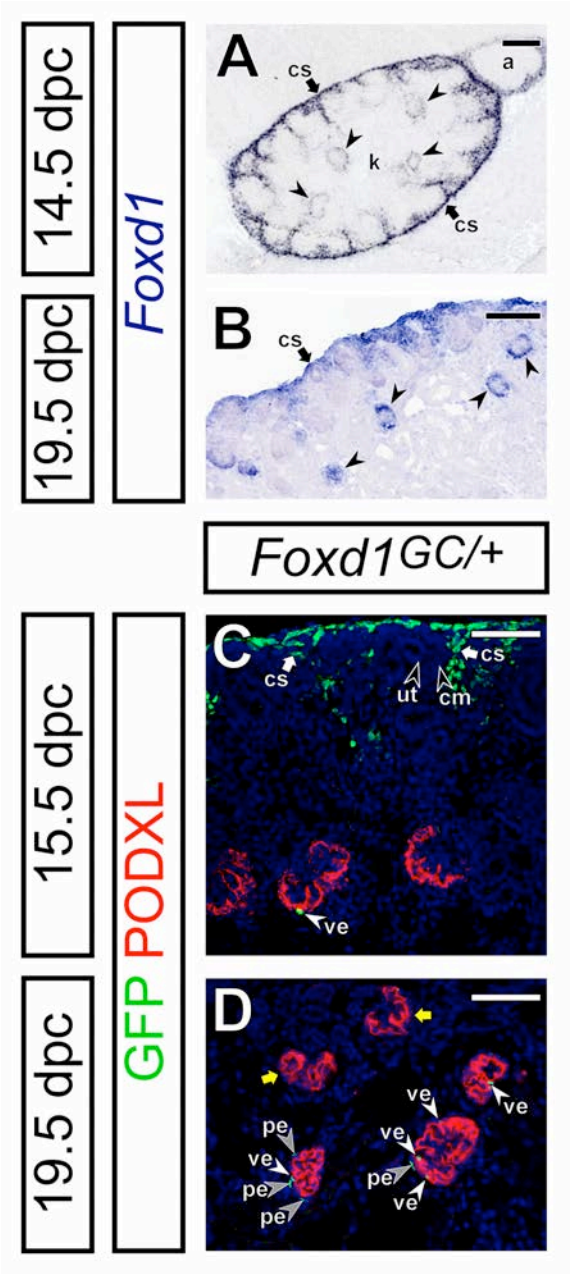
Sections were incubated with primary antibodies against SIX2 (Kobayashi et al., 2008), FOXD1 (Mugford et al., 2008), WT1 (Santa Cruz, sc-192), NPHS1 (Abcam, ab58968), PODXL (R&D Systems, MAB1556), FLK1 (Pharmingen, 555307), PDGFRB (eBiosciences, 14-1402-82), F4/80 (eBioscience, 14-4801), CDKN1C (Abcam, ab4058), LIV2 (MBL International, D118-3), SMA (Sigma, A5228), GFP (Aves labs, GFP-1020; Rockland, 200341-215), β-gal (Cappel, #55976; Abcam, ab9361; Promega, Z3781) and pan-cytokeratin (Sigma, C2562) and detected by the secondary antibodies with DyLight 488, 549 and 649 (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488, 568, 633 and 647 (Invitrogen). Sections were stained with Hoechst (Invitrogen, H3570) prior to mounting with Immu-Mount Medium (Fisher, 9990412). Fluorescent images were photographed on Zeiss LSM510, LSM710 and LSM780 confocal microscopes, and a Nikon Eclipse C1si confocal microscope.



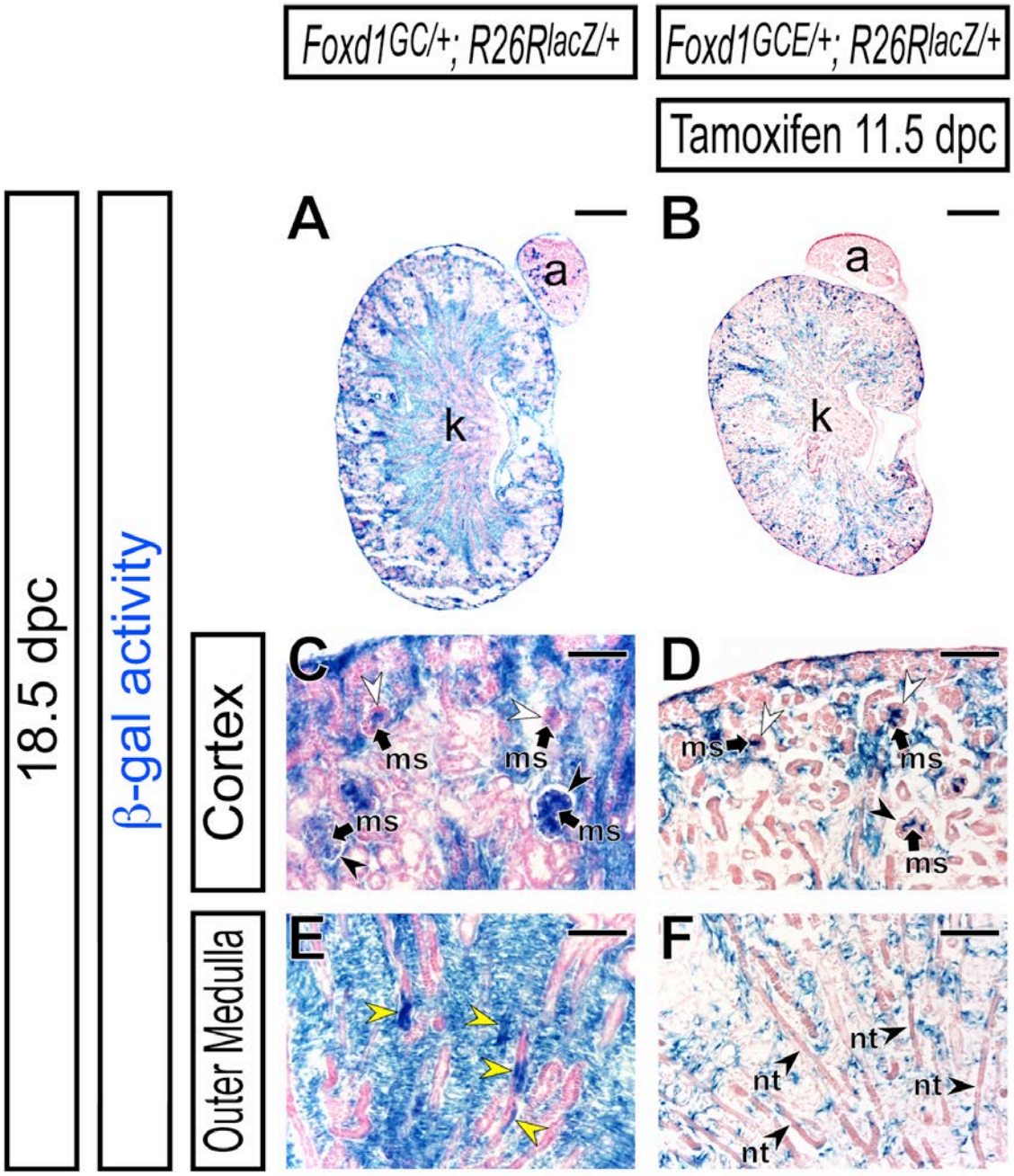
Supplemental Figure 1. Generation of *Foxd1-Cre* knock-in alleles. (A) Schematic illustration of targeting strategy for *Foxd1-Cre* knock-in alleles. **(B)** Targeted *Foxd1-Cre* loci. **(C-E)** Confirmation of targeted *Foxd1-Cre* loci by long-range PCR. (C) *Foxd1-GC*. (D) *Foxd1-GCE*. (E) *Foxd1-CE*. *Foxd1-Cre* alleles were screened using the following primers (bpA-Fw5; GCTGGGGATGCGGTGGGCTCTATGGCTTCT, Cre-Rv11; GGTTCTTGCGAACCTCATCACTCGTTGCAT, mFoxd1-Rv26; CCATCTGCTATAAGCAGGGCTCGCAGTA, mFoxd1-Fw25; CTGTGGGTTGGAATTAGCTCAGCGTTAGGTA, XFP-Rv6; GCACGGGCAGCTTGCCGGTGGTGCAGAT and Cre-Rv11; GGTTCTTGCGAACCTCATCACTCGTTGCAT). Detailed description of the targeting strategy is available upon a request.



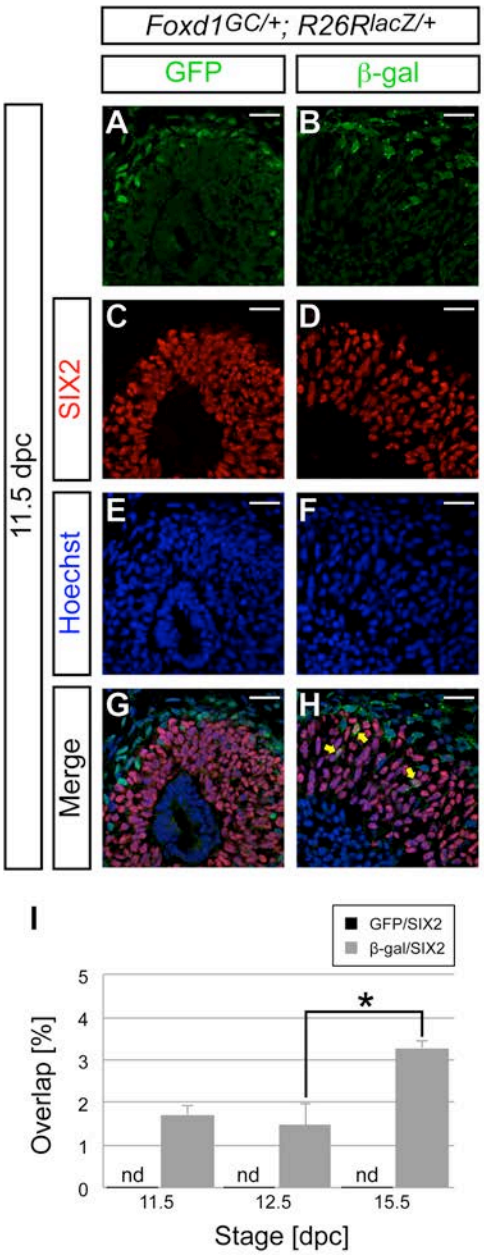
Supplemental Figure 2. *Foxd1-eGFPCre* transgenes are expressed in the FOXD1+ cortical stroma. Kidneys from *Foxd1*$^{GC/+}$ (A,C,E,G,I) and *Foxd1*$^{GCE/+}$ (B,D,F,H,J) knock-in alleles at 12.5 dpc (A-D) and 15.5 dpc (E-J). **(A-D)** Whole-mount kidneys. (A,B) Bright view. (C,D) GFP expression. **(E-J)** Confocal immunofluorescence images of GFP (green), FOXD1 (red) and cytokeratin (KRT, white). At this stage, high levels of cytokeratin are evident in the ureteric tip and collecting duct. Note, the FOXD1 transcriptional regulator localizes in the nucleus, while eGFPCre and eGFPCreER^{T2} proteins show nuclear and cytoplasmic localization, respectively. Scale bars, 100 μm in A-D, 50 μm in E-J.



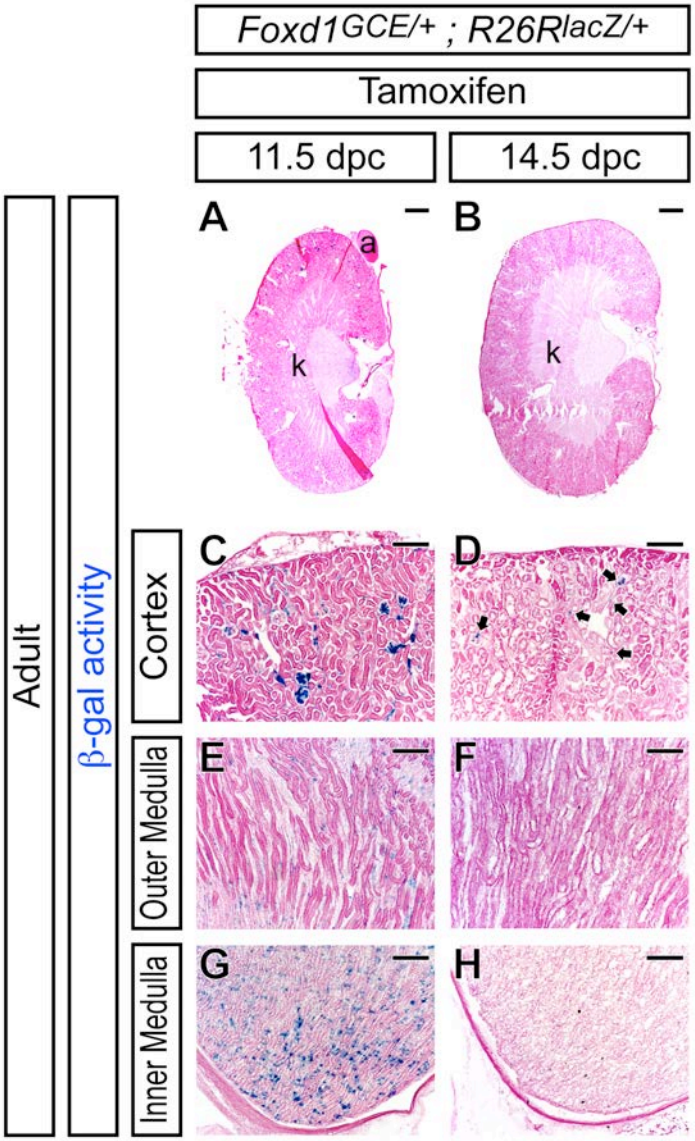
Supplemental Figure 3. The *Foxd1-GFP* is expressed in the visceral and parietal epithelium of the glomerulus. (A,B) *Foxd1* expression in the wild-type kidney by section *in situ* hybridization at 14.5 dpc adapted from the GenePaint database (Visel et al., 2004) and 19.5 dpc adapted from the GUDMAP database (Harding et al., 2011; McMahon et al., 2008). *Foxd1* is expressed in the cortical stroma (cs) and glomerulus (arrowheads). **(C,D)** Confocal immunofluorescence images of *Foxd1*$^{GC/+}$ kidneys at 15.5 dpc (C) and 19.5 dpc (D) with anti-GFP (green), anti-PODXL (Podocalyxin, red) and Hoechst (blue) staining. *Foxd1-GFP* expression was observed in some cells in the both visceral (podocytes) and parietal epithelium of maturing glomeruli, but not in less-differentiated glomeruli (yellow arrows in D). a, adrenal gland; cm, cap mesenchyme; cs, cortical stroma; k, kidney; pe, parietal epithelium; ut, ureteric tip; ve, visceral epithelium (podocytes). Scale bars, 200 μm in A, 100 μm in B, 50 μm in C and D.



Supplemental Figure 4. A few tubular epithelial cells in some nephrons are derived from the *Foxd1*+ population. X-gal stained (blue) kidneys at 18.5 dpc with eosin counter-staining (pink) from *Foxd1*$^{GC/+}$*; R26R*$^{lacZ/+}$ embryos (A,C,E) and *Foxd1*$^{GCE/+}$*; R26R*$^{lacZ/+}$ embryos with 6 mg tamoxifen injection at 11.5 dpc (B,D,F). **(A,B)** Whole view. **(C-F)** Higher magnification of the renal cortex with glomeruli (C,D) and the outer medulla (E,F). White and black arrowheads in C and D indicate podocytes of glomeruli at less differentiated capillary loop stage stages and maturing stages, respectively. Yellow arrowheads in E indicate β-gal+ cell in the nephron tubule in *Foxd1*$^{GC/+}$*; R26R*$^{lacZ/+}$ kidneys. a, adrenal gland; k, kidney; ms, mesangium; nt, nephron tubule. Scale bars, 500 μm in A and B, 100 μm in C-F.



Supplemental Figure 5. *Foxd1*+ cells contribute to a small portion of SIX2+ cap mesenchyme cells. (**A-H**) Confocal immunofluorescence images of the nephrogenic zone of the kidney at 11.5 dpc with anti-GFP (A, green), anti-β-gal (B, green), anti-SIX2 (C,D, red), and Hoechst (E,F, blue) staining and merged images (G,H). Yellow arrows in H indicate β-gal+ SIX2+ cells. Scale bars, 25 μm. (**I**) While no overlap was detected between GFP and SIX2 at 11.5, 12.5, and 15.5 dpc, the frequency of β-gal+ SIX2+ cells in the SIX2+ population significantly increases during development (1.74 ± 0.20, 1.49 ± 0.48, 3.31 ± 0.16 at 11.5, 12.5 and 15.5 dpc, respectively; * $p<0.05$, unpaired t-test) from three biological replicates (n=3) analyzed for each stage. Total numbers of SIX2+ cells counted were 2,389, 1,433 and 1,156 at 11.5, 12.5 and 15.5 dpc, respectively. Data are presented as mean ± SEM (standard error of the mean). nd, not detected. Scale bars, 25 μm.



Supplemental Figure 6. The stage-dependent contribution of *Foxd1*+ cortical stromal cells to renal stromal tissues along the cortico-medullary axis is maintained in adult kidneys. X-gal stained (blue) sections counter-stained with eosin (pink) of 6-week old kidneys from *Foxd1*$^{GCE/+}$*; R26R*$^{lacZ/+}$ adult males after injections of 2 mg tamoxifen at 11.5 dpc (A,C,E,G) and 14.5 dpc (B,D,F,H). **(A,B)** Whole kidney. **(C,D)** Renal cortex. **(E,F)** Outer renal medulla. **(G,H)** Inner renal medulla (papilla). Arrows in D indicate β-gal+ cells in the renal cortex. While *Foxd1*+ cortical stromal cells at 11.5 dpc contributed to renal stromal cells in the inner medulla, medulla and cortex (C,E,G), contribution of *Foxd1*+ cortical stromal cells at 14.5 dpc is restricted to the cortex only. Although we observed very rare β-gal+ cells in the inner medulla in kidneys with tamoxifen injection at 14.5 dpc (H and data not shown), our confocal immunofluorescence showed that these rare β-gal+ cells do not express stromal markers including PDGFRB (data not shown). Therefore, it is unlikely that these β-gal+ cells are renal stromal cells, although their identities and origins are currently unclear. a, adrenal gland; k, kidney. Scale bars, 1 mm in A and B, 200 μm in C-H.

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