Inventory of Supplemental Information

I Supplemental Figures and Legends

Figure S1

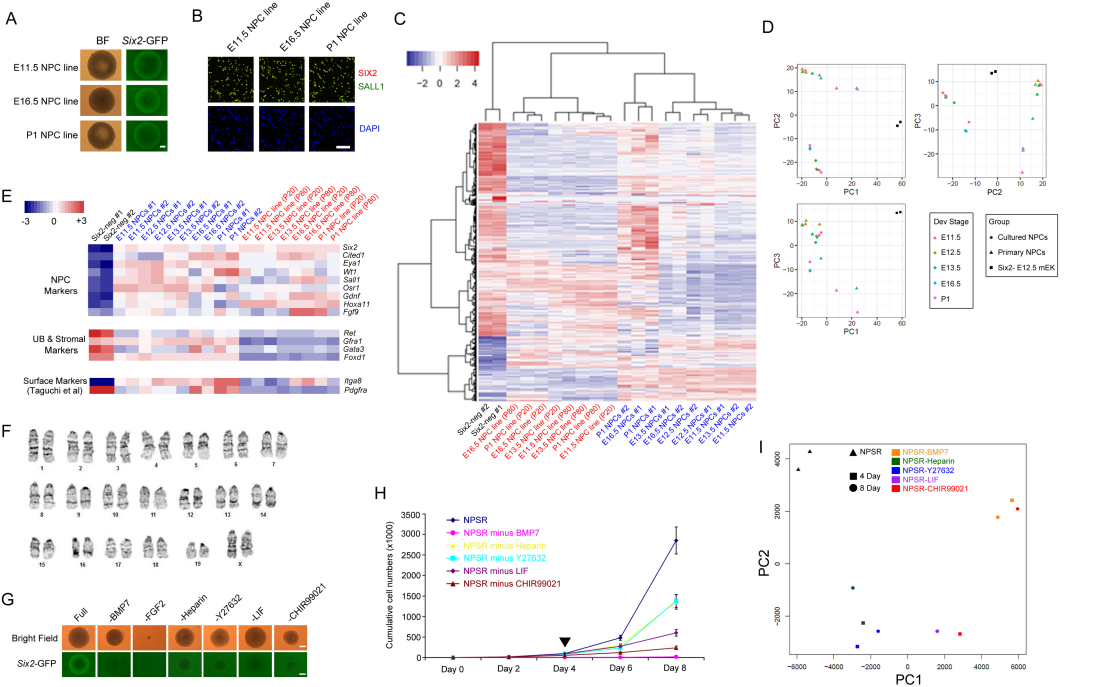

A
BF
Six2-GFP
E11.5 NPC line
E16.5 NPC line
P1 NPC line
B
E11.5 NPC line
E16.5 NPC line
P1 NPC line
SIX2
SALL1
DAPI
C
−2 0 2 4
D
PC1
PC2
PC3
Dev Stage
E11.5
E12.5
E13.5
E16.5
P1
Group
Cultured NPCs
Primary NPCs
Six2- E12.5 mEK
E
-3 0 +3
NPC Markers
Six2
Cited1
Eya1
Wt1
Sall1
Osr1
Gdnf
Hoxa11
Fgf9
UB & Stromal Markers
Ret
Gfra1
Gata3
Foxd1
Surface Markers (Taguchi et al)
Itga8
Pdgfra
F
G
Full
-BMP7
-FGF2
-Heparin
-Y27632
-LIF
-CHIR99021
Bright Field
Six2-GFP
H
Cumulative cell numbers (x1000)
NPSR
NPSR minus BMP7
NPSR minus Heparin
NPSR minus Y27632
NPSR minus LIF
NPSR minus CHIR99021
Day 0
Day 2
Day 4
Day 6
Day 8
I
NPSR
4 Day
8 Day
NPSR-BMP7
NPSR-Heparin
NPSR-Y27632
NPSR-LIF
NPSR-CHIR99021
PC1
PC2

Figure S1. Related to Figure 1. Characterization of murine NPC lines derived from different developmental stages

(A) Bright field and fluorescence images of NPC lines derived from E11.5, E16.5 and P1 mouse kidneys (all the NPC lines were cultured for more than 60 passages and more than 8 months). Scale bar, 200 μm.

(B) Immunofluorescence analyses of NPC lines derived from E11.5, E16.5 and P1 mouse kidney (all the NPC lines were cultured for more than 60 passages and more than 8 months) for NPC markers SIX2 and SALL1. Scale bar, 100 μm.

(C) Hierarchical clustering analysis of NPC signature gene expressions among cultured NPC lines and primary NPC samples from the RNA-seq dataset. *Six2*-GFP- E12.5 mouse embryonic kidney cells were used as control.

(D) Principal component analysis (PCA) of the NPC signature gene expression in 2D views. Colors indicate the different developmental stages and shapes represent different sources of cells.

(E) Heat-map representation of RNA-seq data of the expression profiles of NPC and UB/stromal markers, and surface markers Itga8 and Pdgfra, in cultured NPC lines and primary NPCs. *Six2*-GFP- E12.5 mouse embryonic kidney cells (Six2-neg) were used as controls.

(F) Karyotyping of E13.5-NPC line (P60, more than 8 month culture).

(G) Bright field images (Top) and GFP signals (Bottom) of NPC aggregates cultured in NPSR medium without indicated components for 4 days. NPCs cultured in complete NPSR medium (Full) is used as a control. Scale bar, 200μm.

(H) Cumulative growth curve of NPCs in NPSR medium or in NPSR medium without each component up to 8 days in culture. Black arrowhead indicates the time when cells were passaged.

(I) Principal component analysis (PCA) of global gene expression upon removal of indicated NPSR components. Colors indicate different factor deprivations and shapes represent different time points after factor removal. Black triangles indicate NPCs cultured in complete NPSR medium.

Figure S2

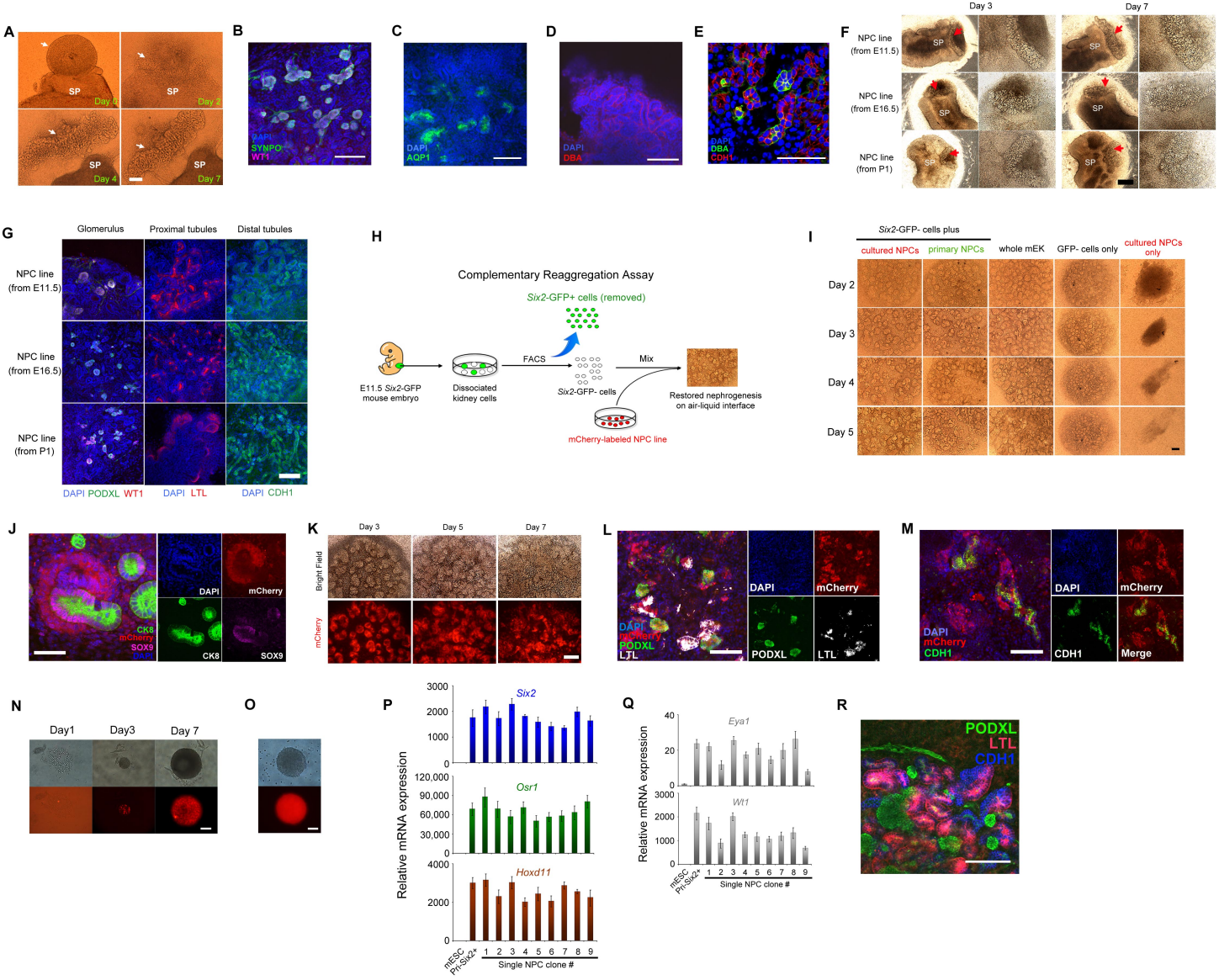

A
SP
Day 2
Day 4
Day 7
B
DAPI
SYNPO
WT1
C
DAPI
AQP1
D
DAPI
DBA
E
DAPI
DBA
CDH1
F
Day 3
Day 7
NPC line (from E11.5)
NPC line (from E16.5)
NPC line (from P1)
G
Glomerulus
Proximal tubules
Distal tubules
NPC line (from E11.5)
NPC line (from E16.5)
NPC line (from P1)
DAPI PODXL WT1
DAPI LTL
DAPI CDH1
H
Complementary Reaggregation Assay
Six2-GFP+ cells (removed)
FACS
Mix
E11.5 Six2-GFP mouse embryo
Dissociated kidney cells
Six2-GFP- cells
Restored nephrogenesis on air-liquid interface
mCherry-labeled NPC line
I
Six2-GFP- cells plus
cultured NPCs
primary NPCs
whole mEK
GFP- cells only
cultured NPCs only
Day 2
Day 3
Day 4
Day 5
J
CK8
mCherry
SOX9
DAPI
DAPI
mCherry
CK8
SOX9
K
Day 3
Day 5
Day 7
Bright Field
mCherry
L
DAPI
mCherry
PODXL
LTL
DAPI
mCherry
PODXL
LTL
M
DAPI
mCherry
CDH1
DAPI
mCherry
CDH1
Merge
N
Day1
Day3
Day 7
O
P
Six2
Osr1
Hoxd11
Relative mRNA expression
3000
2000
1000
0
120,000
90,000
60,000
30,000
4000
mESC
Pri-Six2+
1 2 3 4 5 6 7 8 9
Single NPC clone #
Q
Eya1
Wt1
Relative mRNA expression
40
20
3000
2000
1000
mESC
Pri-Six2+
1 2 3 4 5 6 7 8 9
Single NPC clone #
R
PODXL
LTL
CDH1

Figure S2. Related to Figure 2. Long-term cultured NPC lines interact with UB and are functionally homogeneous.

(A) Time-lapse bright field images of spinal cord induction assay with E13.5-derived NPCs (P60, more than 8 month culture). The white arrows mark NPCs and their derivatives. SP, spinal cord. Scale bar, 200 μm.

(B-E) Whole-mount immunofluorescence (B-D) and immunofluorescence (E) analyses of E13.5-derived NPC line (P60, more than 8 months culture) induced by dorsal spinal cord for 7 days. Podocyte markers, Wilms tumor 1 (WT1) and Synaptopodin (SYNPO), proximal tubule marker, Aquaporin 1 (AQP1), Henle's loop/distal tubule marker, CHD1, and distal tubule marker, DBA. Scale bars, 100μm.

(F) Bright field images of NPCs (P20, ~3 months culture) derived from E11.5, E16.5 embryonic kidney, and P1 neonatal kidney co-cultured with the spinal cord for 3 days and 7 days (higher magnification images on the right side). The red arrows indicate NPC-derived structures. SP, spinal cord. Scale bar, 1mm.

(G) Whole-mount immunostaining for podocyte markers, PODXL and WT1, proximal tubule marker LTL, and Henle's loop/distal tubule marker CDH1 in NPCs (P20, ~3 months culture) derived from E11.5, E16.5, and P1 mouse kidneys after 7 days of spinal cord induction. Scale bars, 100 μm.

(H) Schematic of complementary reaggregation assay. E11.5 mouse embryonic kidneys from $Six2^{GCE}$ mouse were dissociated into single cells followed by FACS sorting. *Six2*-GFP- populations were collected and mixed with mCherry-labeled NPCs. Formed reaggregates were transferred to air-liquid interface for further culture.

(I) Bright field images showing morphologies of indicated aggregates cultured in air-liquid interface at different time points. P60 (more than 8 month culture) mCherry-labeled E13.5-NPC line was used. Scale bar, 200μm.

(J) Whole-mount immunofluorescence images showing ureteric epithelia tip cell marker SOX9 and UB marker CK8 in reaggregates formed by mCherry-labeled E13.5-NPC line (P60, more than 8 month culture) plus *Six2*-GFP- cells in air-liquid interface cultured for 2 days. Scale bar, 100μm.

(K) Time-lapse bright field and fluorescence images showing the reaggregates in air-liquid interface culture after complementary reaggregation assay using mCherry-labeled NPCs (P60, more than 8 month culture) and *Six2*-GFP- cells. Scale bar, 200 μm.

(L and M) Whole-mount immunofluorescence images showing glomerulus marker PODXL, proximal tubule marker LTL expression (L) and Henle's loop/distal tubule marker CDH1 expression (M) in reaggregates formed by mCherry-labeled NPCs (P60, more than 8 month culture) plus *Six2*-GFP- cells cultured for 7 days in air-liquid interface. Scale bars, 100μm.

(N) Time-lapse bright field and fluorescence images showing the NPC aggregate with a single mCherry/Puro-NPC and ~300 wild-type helper NPCs. Scale bar, 200 μm.

(O) Bright field and fluorescence images show that after puromycin selection for 2 passages, single clone mCherry/Puro-NPCs was generated by using helper NPCs. Scale bar, 200 μm.

(P and Q) qRT-PCR analyses of NPC markers *Six2, Osr1, Hoxd1, Eya1*, and *Wt1* in mESCs (control), primary NPCs, and single NPC clones. Data are presented as mean ±SD.

(R) Representative whole-mount immunostaining for podocyte marker PODXL, proximal tubule marker LTL, and Henle's loop/distal tubule marker CDH1 in single NPC clones derived from E13.5-NPC line after 7 days of spinal cord induction. Scale bars, 100 μm.

Figure S3

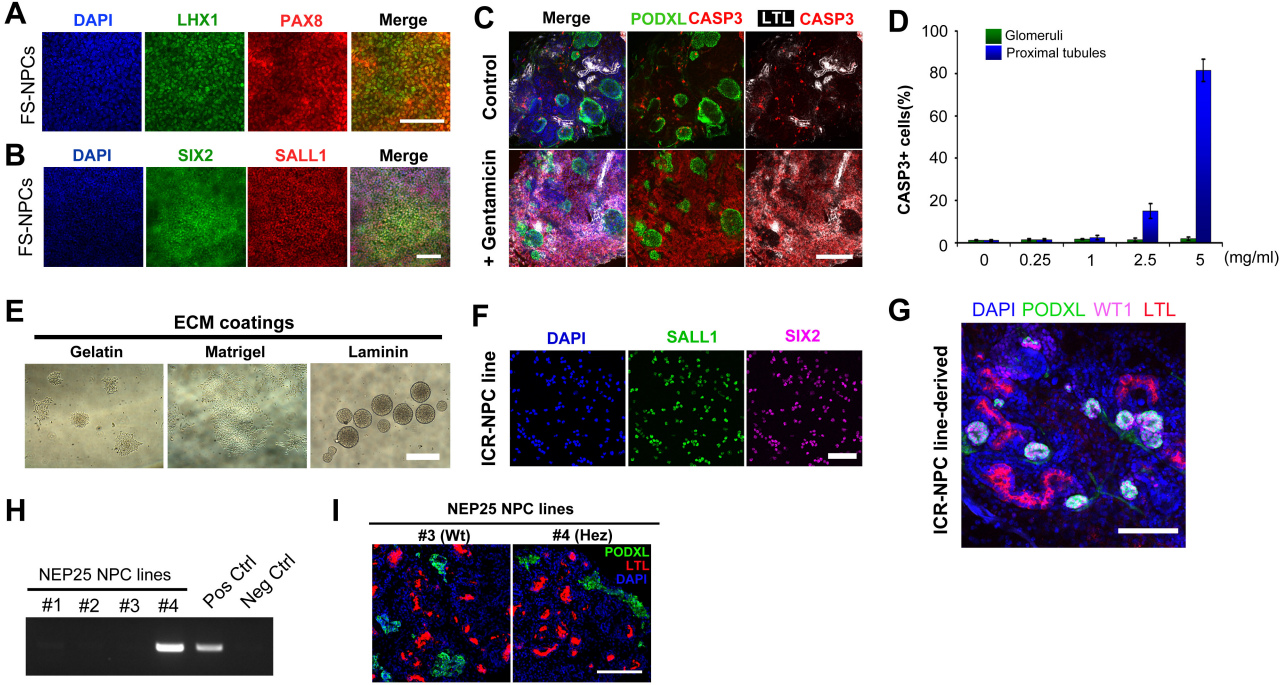

A
DAPI
LHX1
PAX8
Merge
FS-NPCs
B
DAPI
SIX2
SALL1
Merge
FS-NPCs
C
Merge
PODXL CASP3
LTL CASP3
Control
+ Gentamicin
D
Glomeruli
Proximal tubules
CASP3+ cells(%)
100
80
60
40
20
0
0
0.25
1
2.5
5
(mg/ml)
E
ECM coatings
Gelatin
Matrigel
Laminin
F
DAPI
SALL1
SIX2
ICR-NPC line
G
DAPI PODXL WT1 LTL
ICR-NPC line-derived
H
NEP25 NPC lines
#1
#2
#3
#4
Pos Ctrl
Neg Ctrl
I
NEP25 NPC lines
#3 (Wt)
#4 (Hez)
PODXL
LTL
DAPI

Figure S3. Related to Figure 3. Fast and efficient nephron organoid generation from cultured NPCs via FS-NPCs

(A and B) Immunofluorescence analyses for common PTA and RV markers LHX1 and PAX8 (A), and NPC markers, SIX2 and SALL1 (B) of FS-NPCs. Scale bar, 100 μm.

(C) Whole-mount immunofluorescence analyses for PODXL, LTL, and Caspase 3 (CASP3) of FS-NPC derived nephron organoids receiving gentamicin (5 mg/ml) for 24 hrs. Scale bar, 100 μm.

(D) Percentage of CASP3+ cells co-stained with PODXL or LTL of FS-NPC derived nephron organoids receiving different doses of gentamicin for 24 hrs.

(E) Representative bright-field images of mouse embryonic kidney (mEK) culture in dishes coated with 3 different types of extracellular matrixes (ECMs). Single cells of freshly dissociated E12.5 mouse embryonic kidney (mEK) from $Six2^{GCE}$ mouse were seeded into dishes coated with Gelatin, Matrigel and Laminin. The images were taken after 4 days. Scale bar, 500 μm.

(F) Immunofluorescence analyses for SIX2 and SALL1 of NPC line derived from outbred ICR mouse strain (P20, 3 month culture) using CDP method. Scale bar, 100 μm.

(G) Whole-mount immunofluorescence analyses for PODXL, WT1, and LTL of nephron organoids derived from ICR NPCs. Scale bar, 100 μm.

(H) Genotyping result of four NPC lines derived from NEP25 transgenic mice.

(I) Immunofluorescence analyses for PODXL and LTL of NEP25 NPC line-derived nephron organoids treated with LMB2 (20nM) for 4 days. Wt, wild-type; Hez, heterozygous. Scale bars, 100 μm.

Figure S4

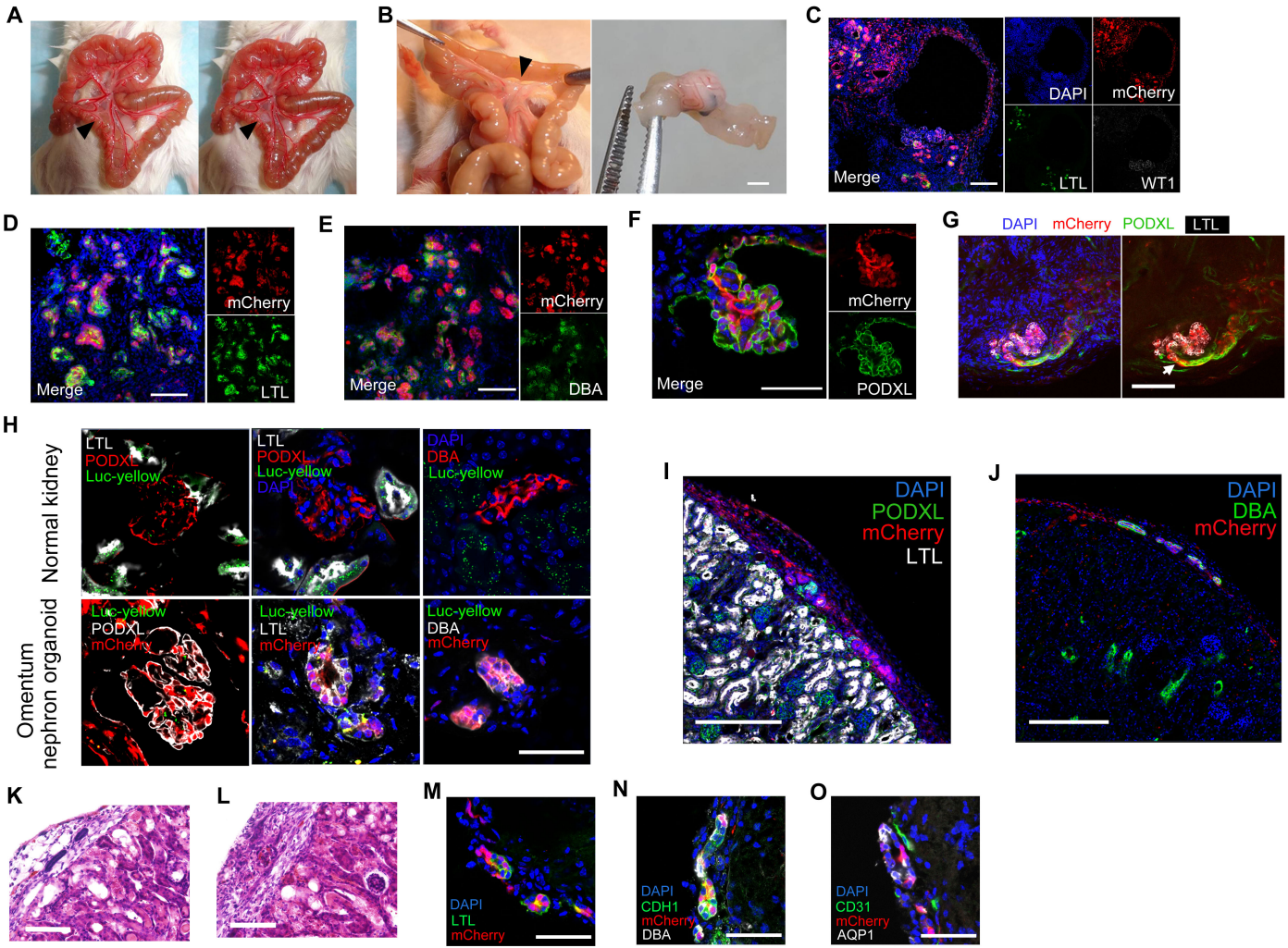

A
B
C
DAPI
mCherry
Merge
LTL
WT1
D
Merge
mCherry
LTL
E
Merge
mCherry
DBA
F
Merge
mCherry
PODXL
G
DAPI
mCherry
PODXL
LTL
H
Normal kidney
Omentum nephron organoid
LTL
PODXL
Luc-yellow
DAPI
DBA
mCherry
I
DAPI
PODXL
mCherry
LTL
J
DAPI
DBA
mCherry
K
L
M
DAPI
LTL
mCherry
N
DAPI
CDH1
mCherry
DBA
O
DAPI
CD31
mCherry
AQP1

Figure S4. Related to Figure 5. Cultured NPCs generate ectopic functional nephron-like structures in vivo.
(A) Images showing transplantation of cultured mouse NPCs together with spinal cord to omentum. Left image shows the omentum before transplantation. Right image shows omentum after transplantation. Black arrowheads indicate the location where NPCs and spinal cord (white color) are transplanted.
(B) Images showing the structures generated by NPCs and spinal cord 2 weeks after omentum transplantation. Left image shows the transplant inside omentum. Right image shows the dissected out transplant from the omentum. Black arrowhead shows the location of transplant. Scale bar, 2 mm.
(C) Immunofluorescence analyses for LTL and WT1 from sections of cystic structures derived from transplanted mCherry-labeled NPCs. Scale bar, 100 μm.
(D and E) Immunofluorescence analyses for LTL (D) and DBA (E) of cystic structures derived from mCherry-labeled NPCs after 2-week omentum transplantation. Scale bar, 100 μm.
(F) Immunofluorescence analysis for PODXL in cystic structures derived from mCherry-labeled NPCs after 2-week omentum transplantation. Scale bar, 50 μm.
(G) Whole-mount immunofluorescence analyses of the cystic structures derived from mCherry-labeled NPCs after 2 weeks of omentum transplantation for PODXL and LTL. White arrow indicates the connecting site of glomerulus and proximal tubule. Scale bar, 100 μm.
(H) Representative immunofluorescence images show the Lucifer-yellow dextran accumulation in PODXL+ glomeruli, LTL+ proximal tubule, and DBA+ distal tubule from normal kidney (top) and cultured NPC-derived nephron organoid (bottom) 2 hrs after tail vein injection. Scale bar, 50 μm.
(I and J) Low-power field immunofluorescence analyses for PODXL, LTL and DBA of cisplatin-treated NSG mouse kidney receiving subcapsule transplantation of FS-NPCs. Scale bar, 100 μm.
(K and L) Representative H&E staining shows tubular structure (K) and glomerular structure (L) of histological sections of cisplatin-treated NSG mouse kidney receiving subcapsule transplantation of FS-NPCs. Scale bars, 50 μm
(M-O) Immunofluorescence analyses for LTL, AQP1, CDH1, DBA, and CD31 of cisplatin-treated NSG mouse kidney receiving subcapsule transplantation of mCherry-labeled FS-NPCs. Scale bars, 50 μm.

Figure S5

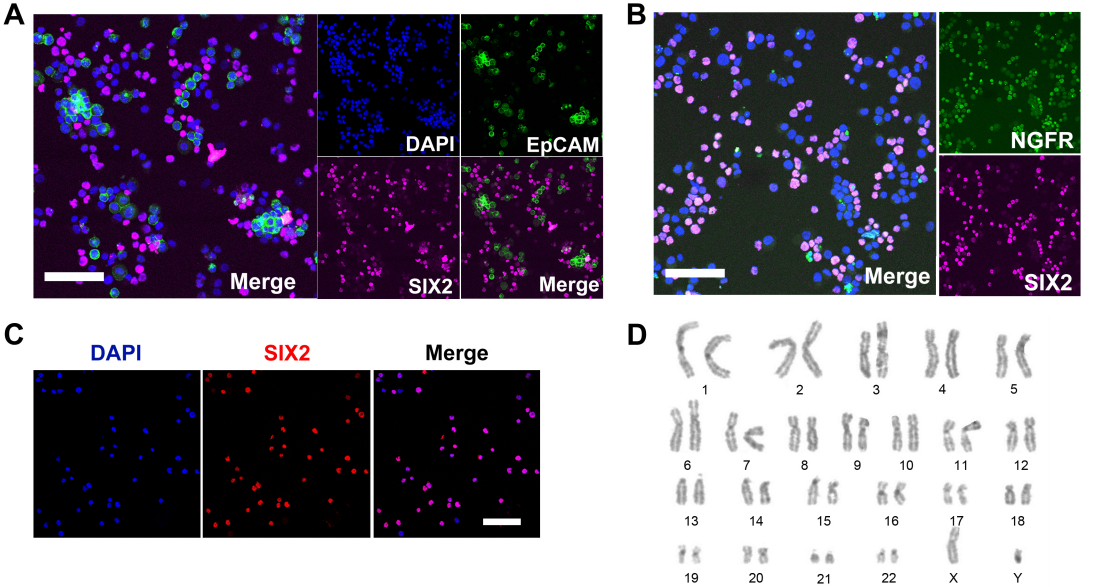
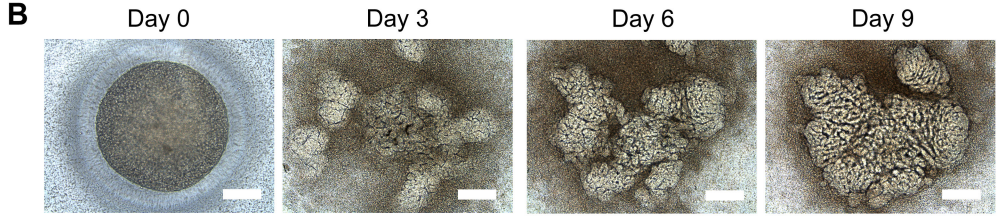
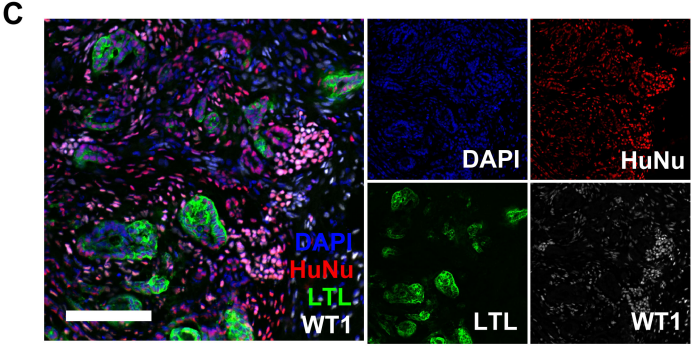
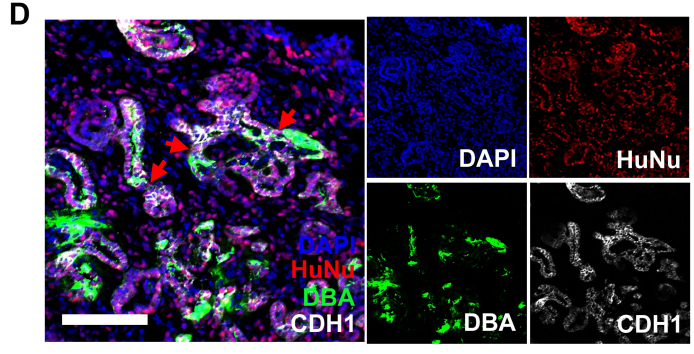

A
DAPI
EpCAM
Merge
SIX2
Merge
B
NGFR
Merge
SIX2
C
DAPI
SIX2
Merge
D
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

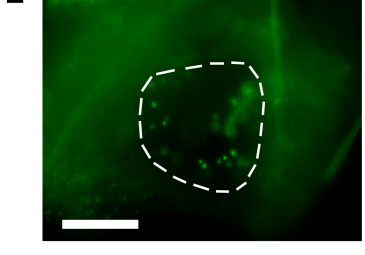
Figure S5. Related to Figure 6. Isolation of EpCAM$^-$/NGFR$^+$ cells from fetal human kidney enriched SIX2+ cells.
(A) Immunofluorescence analyses of primary human fetal kidney cells (around 11 weeks of gestation) for EpCAM and SIX2. Scale bar, 100 μm.
(B) Immunofluorescence analyses of primary human fetal kidney cells (around 11 weeks of gestation) for NGFR and SIX2. Scale bar, 100 μm.
(C) Immunostaining for SIX2 in EpCAM-/NGFR+ fraction from primary fetal kidney cells (around 11 weeks of gestation). Scale bar, 100 μm.
(D) Normal Karyotype of human NPC line cultured in hNPSR/3D condition for 5 months.

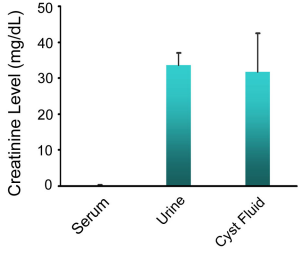
Figure S6











G

1st Round (Knock-in)	Colonies Picked	Heterogeneous Clones	Heterogeneous Percentage	Homogeneous Clones	Homogeneous Percentage	Targeting Efficiency
	56	29	51.8%	4	7.1%	58.9%

H

2nd Round (Neo excision)	Colonies Picked	Neo Cassette Removed	Efficiency
	31	25	80.6%

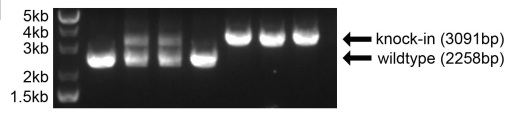


Figure S6. Related to Figure 7. *SIX2*-GFP knock-in reporter hiPSC-derived NPCs are expanded in hNPSR/3D condition.
(A) Schematic representation of the nephron organoid formation protocol from human NPC lines. The 3D aggregate of cultured human NPCs were transferred to air-liquid interface with basal medium supplemented with CHIR99021 (CHIR) and FGF2 for 1 to 2 days before switching to basal medium without CHIR and FGF2 for additional 5~7 days. Dramatic morphological change is typically observed after 5~7 days in total with the formation of numerous tubular structures.
(B) Time-lapse bright field images of nephron organoid formation from 11~12wk-derived hNPC line (P43, more than 6 months culture). Scale bar, 200 μm.
(C) Immunofluorescence analyses for WT1, LTL and human nuclei (HuNu) of renal structures derived from human NPCs after 3 weeks of omentum transplantation. Scale bar, 100 μm.
(D) Immunofluorescence analyses for CDH1, DBA and HuNu of renal structures derived from human NPCs after 3 weeks of omentum transplantation. Scale bar, 100 μm. The red arrows indicate the connecting sites between CDH1+/DBA- Henle's loop and CDH1+/DBA+ distal tubules.
(E) A representative fluorescence image shows the Lucifer-yellow dextran accumulation in the cyst after 2 hr of tail vein injection. Dashed circle indicates the boundaries of cyst. Scale bar, 500 μm.
(F) Creatinine levels in serum, urine, and cyst fluid. Serum and urine samples were obtained from wild-type mice (n=3) and cyst fluids were collected from mice receiving hNPCs plus spinal cord transplantation (n=3). Data are presented as mean ±SD.
(G and H) Summary of *SIX2*-EGFP knock-in reporter iPSC line generation efficiency in the 1st round of knock-in process (G) and second round of excision process (H).
(I) Representative PCR-based genotyping to discriminate between wild-type and knock-in alleles (after excision of PGK-Neo cassette).

II Supplemental Tables

Table S1: Summary of available mouse NPC culture methods, related to Figures 1-5.

		Our culture method (NPSR)		Brown et al, 2015 (NPEM)		Tanigawa et al, 2015		Tanigawa et al, 2016	
Culture period		Until now, more than 17 months and more than 110 passages		Up to 10 passages were shown.		"No more than P5, or 1 month."		Up to around 19 days	
Culture condition	**Format**	3D		2D (Matrigel coating)		2D (Fibronectin coating)		2D (iMatrix coating)	
	Basal medium	DMEM/F12		APEL		Not shown in paper		DMEM/F12	
	Specific factors	Factor	Final Concentration	Factor	Final Concentration	Factor	Final Concentration	Factor	Final Concentration
		BMP7	50ng/ml	BMP7	30ng/ml	TGF-α	10ng/ml	TGF-α	10ng/ml
		FGF2	200ng/ml	FGF9	200ng/ml	FGF2	50ng/ml	FGF2/9	50ng/ml
		Heparin	1ug/ml	Heparin	1ug/ml	LIF	1ng/ml	LIF	5ng/ml
		Y27632	10uM	Y27632	10uM	Y27632	10uM	Y27632	10uM
		CHIR99021	1uM	CHIR99021	1.25uM			CHIR99021	1uM
		LIF	10ng/ml	LDN193189	125nM			BMP7	5ng/ml
				BMP4	30ng/ml			DAPT	2.5uM
				IGF1	20ng/ml				
				IGF2	2ng/ml				
Nephrogenic potential	**Glomeruli**	Yes. Markers: PODXL, SYNPO, WT1, Nephrin		Not shown		Yes. Markers: WT1, Nephrin		Yes. Markers: WT1, Nephrin	
	Proximal tubules	Yes. Markers: LTL, AQP1,		Yes. Markers: LTL		Yes. Markers: CDH6		Yes. Markers: CDH6	
	Distal tubules	Yes. Markers: BRN1+/DBA+, DBA, CDH1, PAX2		Yes. Markers: DBA, CDH1, NCC		Yes. Markers: CDH1		Yes. Markers: CDH1	
	Loop of Henle	Yes. Marker: BRN1+/DBA-, DBA-/CDH1+		Not shown.		Not shown.		Not shown.	
	Potential from single cell clones	Homogeneous, single-cell derived clones display nephrogenic potential		Heterogeneous and the percentage of competent cells decrease gradually since P5		Heterogeneous and the percentage of competent cells decrease with each passage		Heterogeneous and the percentage of competent cells decrease with each passage	
	In vivo potential	1. Form chimeric nephron structures with host kidney upon injection to neonatal mouse kidney (chimerism observed in major nephron segments). 2. Form continuous glomerulus-PT-DT structure upon transplanted to early chicken embryos 3. Form vascularized organoid producing urine-like fluid upon transplanted to mouse omentum		Not shown		Not shown		Not shown	
NPC marker gene expression		Homogeneous expression of NPC markers after 17 months culture with more than 110 passages		Heterogeneous and Cited1+ cell percentages decrease with each passage after P5		Heterogeneous and Six2+ cell percentages decrease dramatically with each passage after P2		Heterogeneous and most of NPC marker genes decrease gradually with each passage	
Culture difficulties	**NPC line derivation**	1. 100% successful derivation efficiency of NPC lines from FACS sorted Six2-GFP+ cells from E11.5 to P1 2. Simple CDP-dependent derivation of NPC lines from E12.5 kidneys of mouse strain from all genetic backgrounds examined. Only 4 days is needed for the process.		Specific dissection procedure is needed to obtain nephrogenic zone (NGZ) cells, followed by MACS sorting.		Enzymatic separation of MM from UB is needed.		Enzymatic separation of MM and FACS-based sorting of purified Six2-GFP+ cell were both described.	
	NPC line passaging	Highly repeatable 4 day passaging cycle from initial ~3000 cells per aggregate to ~100,000 cells per aggregate. Passage at the ratio of 1: 30 every 4 days.		Spontaneous differentiation takes place when cells reach more than 70% to 80% confluence.		No special instructions mentioned in the paper.		Cells were dissociated to clumps, but not single cells, and divided into plates at the ratio of 1:3.	
Applications	**Gene editing**	Very efficient as shown by Cas9-mediated gene editing		Not shown.		Not shown.		Not shown.	
	Toxicity testing	Yes. As shown by Gentamycin induced tubule injury.		Not shown		Not shown		Not shown	
	Disease modeling	Yes. As shown by NEP25 and Nphs1 KO models		Not shown		Not shown		Not shown	

Table S2: Summary of available human NPC culture methods, related to Figures 6 and 7.

		Our culture method (human NPSR)		Brown et al, 2015 (NPEM)		Tanigawa et al, 2016	
Culture period		Human NPC lines were derived from different gestational ages and until now, more than 7 months stable expansion with more than 50 passages.		2 passages		P0 (8 days without passage)	
Human NPC sources		Primary human fetal kidneys (9 week to 17week old). Human NPCs were first enriched by FACS.		From hESC differentiation (unpurified) (Takasato et al 2014)		From hiPSC differentiation (unpurified) (Taguchi et al 2014)	
Culture condition	**Format**	3D		2D (Matrigel coating)		2D (iMatrix coating)	
	Basal medium	DMEM/F12		APEL		DMEM/F12	
	Specific factors	Factor	Final Concentration	Factor	Final Concentration	Factor	Final Concentration
		BMP7	50ng/ml	BMP7	30ng/ml	TGF-α	10ng/ml
		FGF2	200ng/ml	FGF9	200ng/ml	FGF2/9	50ng/ml
		Heparin	1ug/ml	Heparin	1ug/ml	LIF	5ng/ml
		Y27632	10uM	Y27632	10uM	Y27632	10uM
		CHIR99021	1uM	CHIR99021	1.25uM	CHIR99021	1uM
		LIF	10ng/ml	LDN193189	125nM	BMP7	5ng/ml
		LDN193189	10-100nM	BMP4	30ng/ml	DAPT	2.5uM
		A83-01	0.05-0.5uM	IGF1	20ng/ml		
				IGF2	2ng/ml		
Nephrogenic potential	**Glomeruli**	Yes. Markers: PODXL, WT1		Not shown		Yes. Markers: WT1, Nephrin	
	Proximal tubules	Yes. Markers: LTL		Yes. Markers: LTL		Yes. Markers: CDH6	
	Distal tubules	Yes. Markers: DBA+/CDH1+		Yes. Markers: CDH1		Yes. Markers: CDH1	
	Loop of Henle	Yes. Marker: DBA-/CDH1+		Not shown		Not shown	
	In vivo potential	Differentiation to all nephron structures upon transplantation to mouse omentum		Not shown		Not shown	
NPC marker gene expression		Homogeneous NPC marker gene (SIX2, SALL1, CITED1, PAX2) expression after 7 months culture (more than 50 passages)		A few cells expressing Cited1 or Pax2 were observed. No data available after 2 passages.		Immediate decrease of some NPC marker gene (PAX2, WT1) expression during the first 8 day culture. No data available after 8 days.	
Culture difficulties	**NPC line derivation**	Derivation of hNPC lines from all gestational ages tested (9 week to 17 week old).		No hNPC lines were derived.		No hNPC lines were derived.	
	NPC line passaging	Highly repeatable 4 day passaging cycle from initial ~10000 cells per aggregate to ~100,000 cells per aggregate. Passage at the ratio of 1: 10 every 4~5 days.		No serial cell passaging showed after P2.		No serial cell passaging showed.	

Table S3: Growth factors and small molecules tested, related to Figure 1.

Growth factor/chemical	Concentrations tested	Company	Cat. No.
Activin A	5ng/ml, 20ng/ml, 100ng/ml	Stemgent	03-0001
TGF-β1	10pg/ml, 50pg/ml	Peprotech	100-21C
SB431542	2uM, 10uM	Reagents Direct	21-A94
A83-01	0.2uM, 1uM	STEMGENT	04-0014
BMP2	20ng/ml, 100ng/ml	R&D	355-BM-010
BMP4	5ng/ml, 20ng/ml, 100ng/ml	Stemgent	03-0007
LDN-193189	50nM, 100nM, 200nM	Reagents Direct	36-F52
DMH1	1uM, 10uM	Millipore	203646-5MG
Wnt3a	10ng/ml, 50ng/ml	R&D	5036-WN-010
CHIR99021	0.5uM, 1uM, 2uM, 3uM, 10uM	Reagents Direct	27-H76
IWR-1	2.5uM, 5uM, 50uM	Sigma	10161-5MG
IWP-2	5uM, 20uM, 50uM	Sigma	10536-5MG
PD0325901	1uM	Reagents Direct	39-C68
FGF9	200ng/ml	Peprotech	100-23
FGF20	100ng/ml	Peprotech	100-41
FGF8	100ng/ml	Peprotech	AF-100-25
FGF10	100ng/ml	Peprotech	AF-100-26
EGF	100ng/ml	R&D	236-EG-200
BMP8a	100ng/ml	R&D	1073-BPC-025
SCF	100ng/ml	R&D	255-SC-010
IGF-1	100ng/ml	Sigma	I1271-.1MG
IGF-2	10ng/ml	Peprotech	AF-100-12
LIF	1ng/ml, 5ng/ml, 10ng/ml	Millipore	ESG1107
Retinoic Acid, all trans	0.1uM, 1uM	Santa Cruz	sc-200898
TTNPB	0.1uM, 1uM	TOCRIS	0761
BMS 195614	1uM	TOCRIS	3660
MM 11253	1uM	TOCRIS	3822
LE 135	1uM	TOCRIS	2021
Trichostatin A	200nM	Sigma	T1952-200UL
5-Azacytidine	1uM	Sigma	A2385-100MG
SP600125	10uM	TOCRIS	1496
SB202190	5uM	Axon Medchem	1364
Go6983	5uM	TOCRIS	2285
DAPT	5uM, 10uM	Sigma	D5942-5MG
Purmorphamine	1uM	STEMGENT	04-0009
KAAD-Cyclopamine	20nM	STEMGENT	04-0028

Table S4. RPKM values of RNA-seq data and list of NPC signature genes, related to Figure 1.
See the attached Excel file.

Table S5. qRT-PCR primer sequences, related to Figures 3 and 7.

qRT-PCR Primers (Mouse)		
Gene Name	**Forward Primer**	**Reverse Primer**
Gapdh	CATGGCCTTCCGTGTTCCTA	CCTGCTTCACCACCTTCTTGAT
Lhx1	CTTCTTCCGATGTTTCGGTA	TCATGCAGGTGAAGCAGTTG
Pax8	GGCTCTACCTACTCTATCAA	CTGCTGCTGCTCTGTGAGTC
Six2	AGGAAAGGGAGAACAGCGAGAA	GGACTGGACGACGAGTGGT
Wt1	CCACACCCCTACTGACAGTT	TCACTCTCATACCCTGTGCC
Osr1	CTGCCCAACCTGTATGGTTT	TGGCACTTTAGAAAAAGAGG
Eya1	GGACAGGCACCGTACAGCTACC	GTGTGCTGGATACGGCGAGCTG
Hoxd11	TGGAACGCGAGTTTTTCTTT	TTGCAGACGGTCCCTGTTCA
qRT-PCR Primers (Human)		
Gene Name	**Forward Primer**	**Reverse Primer**
ACTB	CAATGTGGCCGAGGACTTTG	CATTCTCCTTAGAGAGAAGTGG
SIX2	AGGAAAGGGAGAACAACGAGAA	GGGCTGGATGATGAGTGGT
EYA1	GGACAGGCACCATACAGCTACC	ATGTGCTGGATACGGTGAGCTG
HOXD11	TGGAACGCGAGTTTTTCTTT	CTGCAGACGGTCTCTGTTCA
PAX2	CCCAAAGTGGTGGACAAGAT	GAAAGGCTGCTGAACTTTGG
OSR1	CTGCCCAACCTGTATGGTTT	CGGCACTTTGGAGAAAGAAG

Table S6. Antibody information, related to Figures 1-7.

Antibodies	Company	Cat. No.	Dilution
SIX2	Proteintech	11562-1-AP	(1:200)
SALL1	R&D	PP-K9814-00	(1:200)
CITED1	Life Technologies	PA1-24469	(1:200)
WT1	Abcam	ab89901	(1:200)
PAX2	Covance	PRB-276P	(1:200)
LHX1	DSHB	4F2	(1:100)
PAX8	Proteintech	10336-1-AP	(1:100)
PODXL (mouse)	R&D	MAB1556	(1:300)
PODXL (human)	R&D	AF1658	(1:200)
LTL	Vector laboratories	B-1325	(1:200)
DBA	Vector laboratories	B-1035	(1:200)
CDH1	Cell Signling	3195	(1:200)
SYNPO	PROGEN Biotechnik	65294	(1:100)
NPHS1	PROGEN Biotechnik	GP-N2	(1:100)
AQP1	Santa Cruz	sc-25287	(1:200)
CD31	BD Biosciences	553370	(1:100)
HuNu	Millipore	MAB1281	(1:500)
HuNu	StemCells	AB-101-U-050	(1:500)
EpCAM	R&D	FAB9601G	(1:100)
NGFR	BioLegend	345106	(1:50)
CK8	Covance	MMS-162P-250	(1:200)
BRN1	Santa Cruz	sc-6028-R	(1:100)
SOX9	Millipore	AB5535	(1:100)
Cleaved CASP3	Cell Signaling	9661	(1:200)

III Supplemental Experimental Procedures

Complementary Reaggregation Assay
E11.5 mouse embryonic kidneys from F1 embryos crossed between heterozygotes $Six2^{GCE}$ mouse strain and wildtype B6 mice were dissected and $Six2^{GCE}$ heterozygous kidneys were picked up under epifluorescence microscope based on GFP expression in the metanephric mesenchyme region. The $Six2^{GCE}$ heterozygous kidneys were dissociated to single cells and sorted by FACS to collect

Six2-GFP- populations. mCherry-labeled mouse NPCs were dissociated to single cells and reaggregated with *Six2*-GFP- cells at the ratio of 1:3 to 1:4, mimicking the ratio of Six2+:Six2- ratio in vivo. The reaggregates were formed overnight in 96-well U-bottom low attachment plate in culture medium containing 10% FBS and 10μM Y27632. The next day reaggregates were transferred to air-liquid interface with culture medium containing 10% FBS for ex vivo nephrogenesis.

Chemical Treatment on mouse nephron organoids

NPC aggregates were placed onto transwell inserts at the air-liquid interface with culture medium at the bottom containing CHIR99021 and FGF2 for the first 2 days to generate fate-specified NPCs (FS-NPCs). The culture medium was then switched to basal medium containing 5% knockout serum replacement (KSR) for another 5 to 10 days to generate nephron organoids. DAPT at final concentration of 5μM was used to specifically block glomerulus and proximal tubule differentiation from day 2 to day7 (FS-NPCs to nephron organoids period). Gentamicin (IBI Scientific, IB02030) was used at different doses (0, 0.25, 1, 2.5, 5 mg/ml) to treat nephron organoids for 24 hrs to induce proximal tubular damage. Nephron organoids derived from NEP25 mice were treated with 20nM of immunotoxin anti-Tac (Fv)-PE38 (LMB2) (Matsusaka et al., 2005) for 4 days.

Lentiviral Labeling of mCherry and CRISPR-Cas9 Mediated *Nphs1* Gene Knockout in cultured NPCs

CMV-driven mCherry expressing lentivirus was generated as described previously (Liao et al., 2015). 0.5 μg/ml of puromycin was used to select for mCherry-expressing NPCs after 3 days of infection. NPCs with homogeneous expression of mCherry were obtained after 2 passages. For the lentiviral based CRISPR vector, the Cas9 vector used was modified from the lenti-CRISPR vector (Addgene, plasmid #49535) with the replacement of original EFS to CMV promoter in order to enhance the expression of Cas9 in target cells, and cloning individual gRNAs into the vector. The gRNA sequence targeting mouse *Nphs1* is "*gctgctggtgatgggagcta*". The production, purification and titration of lenti-CRISPR virus were described previously (Liao et al., 2015). 0.5 μg/ml of puromycin was used to select for CRISPR-Cas9 integrated NPCs. To select for single clones of Nphs1 KO NPCs, puromycin selected mixed population of CRISPR-Cas9 integrated NPCs was dissociated into single cells and one cell was mixed with 300~500 helper NPCs without expression of puromycin resistant gene to form aggregate in NPSR medium. Another 7 to 10 days was allowed for the aggregate to grow and puromycin was then used to selectively kill the helper NPCs. Genomic DNA of established single cell clones in this way were extracted and the sequences surrounding the gRNA targeted site was amplified by PCR. DNA sequencing was used to verify if mutations were generated. To further discriminate between biallelic or monoallelic mutations, TA-cloning with pGEM-T vector was carried out using PCR product flanking the targeted site. Plasmids from Single *E. Coli* clones each carrying one copy of the PCR product were sent for sequencing. Analysis was then carried out by comparing the sequencing results with PubMed reference sequences of Nphs1.

Chick Embryo Grafting

Small sized mouse mCherry-labeled NPC aggregates (diameter around 300μm) were carefully placed into the lateral plate mesoderm of stage HH18 chick embryos. 7 days after grafting, the chick embryos were analyzed and mCherry+ tubular structures were identified and dissected under an epifluorescence microscope. Whole-mount immunostaining was performed to characterize the identity of the tubular structures formed *in vivo*.

Omentum Transplantation

mCherry-labeled mouse or unlabeled human NPCs were co-cultured with spinal cord for 2 days before transplanted to the omentum of NSG mice (male and female, 8 to 12 weeks old) anesthetized by isoflurane as reported before (Yokoo et al., 2006). After 2-3 weeks mice were sacrificed and transplants were isolated and analyzed. 200 uL of 5 mg/ml Lucifer Yellow-labeled dextran (10,000 MW) (Thermo Fisher Scientific, D-1825) was injected as an intravenous bolus 2 hrs before the mice were sacrificed and examined for presence of Lucifer Yellow-labeled dextran in the cyst fluid (Hackl et al., 2013). Cyst fluid, urine, and serum were collected and assayed for creatinine levels in the same way as serum.

Neonatal Renal Cortex Transplantation

For neonatal mice, newborn C57BL/6 mice (P0.5) were placed on an ice pack for up to 15 minutes to induce hypothermic anesthesia and received a 5-10 mm paramedian incision for exposure of the kidney. 3 aggregates (approximately 1.0 x 10^5 cells/aggregate) of mCherry-labeled NPCs or FS-NPCs were injected into the renal cortex area of neonates using a 29-gauge Hamilton syringe. 7 days later, the mice were sacrificed and the kidney tissues were collected for analysis.

Cisplatin-Induced Acute Kidney Injury Mouse Model

NOD/SCID IL-2R^{null} (NSG) mice (male and female, 8 to 12 weeks old) received a subcutaneous injection of 20 mg/Kg cisplatin (Tocris Bioscience, Ellisville, Missouri). After 24 h, mice were transplanted with mCherry-labeled FS-NPCs (20 aggregates, about 2 million cells), or mouse embryonic stem cells (mESCs, 2 million cells), or PBS (Control) into left side renal subcapsule. Kidney tissues and blood serum samples were collected 4 days after cisplatin administration. Mice receiving mouse FS-NPCs transplantation survived longer and the kidney tissue and blood serum samples were collected 10 days after transplantation. Blood serum was assayed for blood urea nitrogen (BUN) and serum creatinine (S-Cre) levels using commercially available assay kits (QuantiChrom Urea Assay Kit and QuaintChrom Creatinine Assay Kit; BioAssay Systems, Hayward, CA) as renal function parameters. For renal subcapsule transplantation, the left kidney of NSG mice (male and female, 8 to 12 weeks old) anesthetized by isoflurane was lifted and a pocket was created under the subcapsule using a 24-gauge catheter after puncture of needle. Mouse FS-NPC aggregates, mESCs or PBS were then delivered into the pocket of renal subcapsule through the 24-gauge catheter. Collected kidney samples were fixed in 4%

Paraformaldehyde (PFA) and embedded in OCT compound after PBS wash and quickly frozen in ethanol. Cryostat sections (10μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff's reagent (PAS). Tubular necrosis, urinary casts, tubular dilation, and tubular borders were assessed in non-overlapping fields (high power field, HPF) as described before (Imberti et al., 2015). For collection of conditioned medium, 100 aggregates of cultured NPCs with 1mm in diameter were placed on air-liquid interface of transwell insert (6-well plate format) and induced to FS-NPCs by using KR5 medium (DMEM/F12 with 5% KSR, see detailed protocol section below for detailed recipe) containing CHIR99021 and FGF2. Medium was changed to KR5 only after 2 days, and from this point conditioned medium was collected everyday from day 3 to day 7 and pooled together for use in subsequent intraperitoneal injection to NSG mice receiving cisplatin treatment. 24 hours post-cisplatin injection, conditioned medium (CM) was injected every 12 hours (two times per day) for 3 consecutive days. None conditioned KR5 medium was used as control.

SIX2-EGFP knock-in reporter hiPSC line generation and directed differentiation

The hiPSCs were generated using previously reported integration-free episomal vectors (Okita et al., 2011). TALEN-based homologous recombination was used to insert 2A-EGFP-PGK-Neo cassette downstream of the stop codon (removed) of endogenous *SIX2* gene. DNA sequences ~1Kb upstream and ~1K downstream of endogenous SIX2 stop codon were cloned upstream and downstream of 2A-EGFP-PGK-Neo cassette respectively to facilitate homologous recombination. 2A-EGFP fragment was cloned from pCAS9_GFP (Addgene #44719) and the FRT-PGK-Neo-FRT cassette was cloned from pZero-FRT-Neo3R (kindly provided by Dr. Keiichiro Suzuki). The different fragments were then cloned to pUC19 plasmid to make the complete donor plasmid. TALEN and donor plasmids were tranfected to hiPSCs via electroporation. Neomycin-resistant single cell colonies were picked up manually and genotyping was performed based on PCR. Clones with biallelic knock-in were chosen for second round screen where plasmid encoding Flippase was delivered to allow the transient expression Flippase, whose activities excise the FRT-flanked PGK-Neo cassette from the knock-in alleles. PCR was performed to identify the single cell clones in which PGK-Neo cassettes were excised from both alleles. PCR products were sequenced to further verify the correct knock-in. SIX2-EGFP reporter hiPSCs were then subjected to NPC differentiation following previously established protocol (Taguchi et al., 2014). EGFP+ cells were enriched using FACS and then cultured in hNPSR/3D condition similar to the culture of primary human NPCs.

Immunostaining, RNA Purification, qRT-PCR and Immunoblotting

Whole-mount immunostaining was performed as described previously (Wu et al., 2015; Xia et al., 2013). RNA purification, qRT-PCR and immunoblotting were performed as described previously (Li et al., 2012). qRT-PCR primers are listed in Table S5 and antibody information can be found in Table S6. For cytospin immunostaining quantification, 6 different fields of view were randomly selected to count the number of positively stained cell numbers and total cell numbers. At least 300 cells in total were counted. Error bars represent standard derivation between different field views.

RNA-seq and Principal Component Analysis

Primary *Six2*-GFP- cells from E12.5, *Six2*-GFP+ cells from E11.5, E12.5, E13.5, E16.5 and P1 were isolated from $Six2^{GCE}$ mouse kidneys and NPC lines from E11.5, E13.5, E16.5 and P1 (P20, ~3 months culture; P80, ~11 months culture) were used for RNA-seq. To generate rpkm values from raw data, single-end 50bp reads were mapped to the UCSC mouse transcriptome (mm9) by STAR (Dobin et al., 2013), allowing for up to 10 mismatches (which is the default by STAR). Only the reads aligned uniquely to one genomic location were retained for subsequent analysis. And expression levels of all genes were estimated by Cufflink (Trapnell et al., 2012) using only the reads with exact matches. The gene expression levels of the NPC-signature genes (Table S4) were firstly transformed as logarithm scales. And then the program "prcomp", a built-in program for principal component analysis in R packages, was employed with default parameters. We evaluated the variance percentage of each principal component, and found the top 3 components accounted for 84.1% of the total variance, where PC1 accounted for 46.42%, PC2 23.87% and PC3 13.81%. Those three PCs are therefore selected as candidate principal components in the further analysis. Another program "scatterplot3d" in the R packages was used to plot the 3D view of PCA, and "ggplot2" was used in 2D view of PCA. RPKM values of all RNA-seq data are also included in Table S4.

Protocols for Derivation and Culture of Mouse and Human NPC lines in NPSR/3D Culture Condition and Subsequent Differentiation to Nephron Organoids

INDEX

- **Protocol #7 Derivation of human NPC lines from human fetal kidneys.**
- **Protocol #8 Chemically defined nephron organoid formation from human NPC lines.**

SECTION 1: MOUSE STRAIN AND REAGENTS

Six2$^{tm3(EGFP/cre/ERT2)Amc}$, JAX Stock No. 009600
ICR (CD01), Charles river, Strain code 022
C57BL/6J, JAX Stock No. 000664
TrypLE (Invitrogen, Cat. No. 12563-011)
40um cell strainer (Greiner bio-one, Cat. No. 542040)
U-bottom low-attachment 96-well plates (Thermo, Cat. No. 174929)
Accumax (Innovative Cell Technologies, Cat. No. AM-105)
Laminin (BD, Cat. No. 354232)
Transwell inserts (6-well format, #07200170; 12-well format, #07200161; 24-well format, #07200154; all from Fisher)
Human EpCAM/TROP1 Alexa Fluor® 488 conjugated Antibody (R&D, #FAB9601G)
FITC anti-human CD271 (NGFR) Antibody (Biolegend, #345104)

SECTION 2: CULTURE MEDIUM RECIPES

1. NPSR medium

Basal medium: DMEM/F12 (1:1) (1X), Invitrogen, Cat. No. 11330-032.

Supplements:

Reagent Name	**Company**	**Cat. No.**	**Final Concentration**
GlutaMAX-I (100X)	Invitrogen	35050-079	1X
MEM NEAA (100X)	Invitrogen	11140-050	1X
2-Mercaptoethanol (55mM)	Invitrogen	21985-023	0.1μM
Pen Strep (100X)	Invitrogen	15140-122	1X
B-27 Supplement (50X), minus vitamin A	Invitrogen	12587-010	1X
ITS Liquid Media Supplement (100×)	Sigma	I3146-5ML	1X
BMP-7	R&D	354-BP-010	50ng/ml
FGF-2	Peprotech	100-18B	200ng/ml
Heparin	Sigma	H3149-100KU	1ug/ml
Y-27632	Enzo	ALX-270-333-M025	10 uM
Mouse LIF	Millipore	ESG1107	1000 units/ml
CHIR99021	Reagents Direct	27-H76	1 μM

2. hNPSR medium

Basal medium: DMEM/F12 (1:1) (1X), Invitrogen, Cat. No. 11330-032.

Supplements:

Reagent Name	**Company**	**Cat. No.**	**Final Concentration**
GlutaMAX-I (100X)	Invitrogen	35050-079	1X
MEM NEAA (100X)	Invitrogen	11140-050	1X
2-Mercaptoethanol (55mM)	Invitrogen	21985-023	0.1μM
Pen Strep (100X)	Invitrogen	15140-122	1X
B-27 Supplement (50X), minus vitamin A	Invitrogen	12587-010	1X

ITS Liquid Media Supplement (100×)	Sigma	I3146-5ML	1X
BMP-7	R&D	354-BP-010	50ng/ml
FGF-2	Peprotech	100-18B	200ng/ml
Heparin	Sigma	H3149-100KU	1ug/ml
Y-27632	Enzo	ALX-270-333-M025	10uM
Human LIF	Millipore	LIF1050	10ng/ml
CHIR99021	Reagents Direct	27-H76	1uM
LDN193189	Reagents Direct	36-F52	10 to 100 nM
A83-01	STEMGENT	04-0014	0.05 to 0.5 uM

3. KR5 medium

Basal medium: DMEM/F12 (1:1) (1X), Invitrogen, Cat. No. 11330-032.

Supplements:

Reagent Name	**Company**	**Cat. No.**	**Final Concentration**
GlutaMAX-I (100X)	Invitrogen	35050-079	1X
MEM NEAA (100X)	Invitrogen	11140-050	1X
2-Mercaptoethanol (55mM)	Invitrogen	21985-023	0.1μM
Pen Strep (100X)	Invitrogen	15140-122	1X
KSR	Invitrogen	10828-028	5%

4. Dissection medium

Basal medium: DMEM (1X), Invitrogen, Cat. No. 11995-040.

Supplements:

Reagent Name	**Company**	**Cat. No.**	**Final Concentration**
GlutaMAX-I (100X)	Invitrogen	35050-079	1X
MEM NEAA (100X)	Invitrogen	11140-050	1X
2-Mercaptoethanol (55mM)	Invitrogen	21985-023	0.1μM
Pen Strep (100X)	Invitrogen	15140-122	1X
FBS	GEMINI	100-106	10%

SECTION 3: PROTOCOLS

Protocol #1 Derivation of mouse NPC lines from Six2^{GCE} mice.

1. Heterozygous Six2^{GCE} mice were purchased from The Jackson Laboratory (*Six2$^{tm3(EGFP/cre/ERT2)Amc}$*, JAX Stock No. 009600). Wild-type C57BL/6J mice were used to mate with Six2^{GCE} mice to expand heterozygous Six2^{GCE} mouse colony. Genotyping was performed routinely following the protocols from the JAX website (Note that the genotyping primers for this strain was somehow listed wrong on the JAX website, the primers from the original Kobayashi et al.'s paper (Kobayashi et al., 2008) were used: Primer sets: Cre-Fw1, Cre-Rv2 (Transgenic amplicon, expected size: 219bp) Primer sets: mSix2-Fw35, mSix2-Rv36 (Wild-type amplicon, expected size: 286bp)

Cre-Fw1-Six2	GGACATGTTCAGGGATCGCCAGGC
Cre-Rv2-Six2	CGACGATGAAGCATGTTTAGCTG
mSix2-Fw35	CCACCTTCGGCTTCACGCAGGAGCAAGT

mSix2-Rv36	CCGCGCAGCTTCTCCGCCTCGATGTAGT

2. To get timed embryonic kidneys with $Six2^{GCE}$ reporter, heterozygous $Six2^{GCE}$ mice were mated with wild-type C57BL/6J mice and plugs were checked next morning, midday of plug positive was designated as day 0.5. Pregnant mice were sacrificed at designed embryonic days from E11.5 to P1, kidneys were dissected out from mouse embryos/P1 pups with standard dissection technique. The embryonic kidneys were placed in dissection medium on ice before dissociation.

   For dissociation of E11.5 to E13.5 embryonic kidneys:
   a. collect kidneys into 1.5ml tubes with dissection medium
   b. carefully aspirate dissection medium and wash the kidneys once with sterile PBS
   c. add 300ul of TrypLE (Invitrogen, Cat. No. 12563-011) and incubate in 37 degree incubator for 15~20 minutes to dissociate the embryonic kidneys.
   d. add 500ul dissection medium to the tube, pipette up and down GENTALLY 20 to 30 times to further dissociate the embryonic kidneys.
   e. spin down the cells at 300g for 5min and resuspend the cell pellet with FACS medium (cold PBS with 2%FBS).
   f. filter the cell suspension through 40um cell strainer (Greiner bio-one, Cat. No. 542040) to remove the remaining cell clumps. The cells that go through the strainer are then placed on ice before FACS.
   g. For dissociation of kidneys from E16.5 to P1, first manually cut isolated kidneys into small pieces (to the sizes comparable to E12.5 embryonic kidneys, the smaller the better) under the dissection microscope in cold dissection medium. After that, follow the same steps from a to f as listed above for E11.5 to E13.5 embryonic kidneys. From our experience some kidney tubules are difficult to dissociate using this method, but very few *Six2*-GFP+ cells were in those fractions and the strainer step can eliminate those clumps of tubules. After preparing the dissociated embryonic kidney cells, FACS is then carried out to isolate *Six2*-GFP+ cells.

3. FACS isolated *Six2*-GFP+ cells are spun down and resuspended in NPSR medium and transferred into U-bottom low-attachment 96-well plates (Thermo, Cat. No. 174929) at ~3000 cells/100ul per well. After cell transfer, spin down the 96-well plate at 300g for 3min to make sure the cells form a monolayer at the bottom of the plate. Place the plate into 37-degree incubator and 3D aggregate will be formed following overnight incubation. Please note that the endogenous *Six2*-GFP reporter expression is very dim and it is very difficult to see clear GFP signal in single cells. However, after overnight formation of aggregate, the GFP signal becomes very clear. Change the medium every 2 days (each well with 100ul fresh NPSR medium) and after 4~5 days' growth, the aggregate reached around 1mm in diameter and around 100,000 cells in cell number. At this time point, they are ready for passage.

Passage cultured mouse NPCs:
   a. collect 3D aggregates to 1.5ml tubes. Note: the aggregates are very soft in nature (mesenchymal origin), do not use 200ul or 20ul pipette tips to transfer the aggregates because the aggregates will be disrupted. 1ml pipette tips are routinely used in our lab for this purpose.
   b. aspirate the culture medium and wash once with 1ml sterile PBS.
   c. aspirate PBS and add room temperature Accumax (Innovative Cell Technologies, Cat. No. AM-105) to the NPC aggregates (the volume of Accumax depends on number of NPC aggregates to be dissociated. Our experience are: 1~2 aggregates, 50ul; 3~5 aggregates, 100ul; 10~20 aggregates, 200~300ul.
   d. incubate NPCs with Accumax in 37 degree incubator for 7~10 minutes.
   e. add 500ul dissection medium, pipette up and down VERY GENTALLY for 5 to 10 times. The clumps of cells should be invisible after 2~3 times pipetting. NEVER use force for pipetting the NPCs to obtain single cells, which will negatively affect their self-renewal thereafter.
   f. spin down the cells at 300g for 5min, resuspend the cell pellet with NPSR medium
   g. split the NPCs at the ratio around 1:30 to U-bottom low-attachment 96-well plates
   h. spin down the 96-well plates at 300g for 3min and then place the plates back to 37 degree incubator
   i. change medium with 100ul fresh NPSR medium every 2 days and passage the NPC aggregates every 4~5 days after the aggregate reached the optimal size for passaging (~1mm in diameter). In our hand, 1:30 every 4 days is routinely used and the proliferation rate of cultured NPCs are very stable using this method. GFP signal from the *Six2*-GFP knock-in reporter is checked regularly to make sure that the NPCs are kept at the self-renewing state. The NPSR/3D culture is very robust and we never saw loss of GFP signal once the protocol is followed correctly.

4. Repeat the passage cycles for maintenance and expansion of mouse NPC lines.
   Note: Most of the NPSR medium components are relatively stable, but special attention should be paid to two components: B27 supplements and FGF2. We noticed B27 varies from batch to batch in supporting NPC growth, similar to what was observed in neural stem cell culture. We usually test a few different lots and stock those batches with good activities to support NPC growth. FGF2 is unstable in 37 degree and thus if the medium is warmed up using 37 degree water bath every time before use, the activity of FGF2 will be lost fairly quickly. Loss of FGF2 activity will lead to dramatic cell death of NPCs in culture, similar to what was observed in removal of FGF2 from the culture medium. We do not recommend pre-warm-up for the NPSR medium before use. Keep the medium in the hood at room temperature for 5-10 minutes is enough. Apart from the potential variations of these two components, the other components are very stable even after one-month daily use in our hand. After we established the hNPSR

medium recipe, we also tested the effects of A83-01 and LDN193189 on mouse NPC culture. Our results indicated that both components are dispensable for mouse NPC culture. However, the addition of these two components to mouse NPSR culture medium appears at least not harmful for mouse NPC self-renewal. We did observe that the addition of these two components allow mouse NPCs to grow at seeding numbers as low as 50 to 100 cells, which is not allowed in NPSR medium without the two components (minimal 300 cells in our hand).

Protocol #2 Derivation of mouse NPC lines with CDP method.

We developed this protocol to derive NPC lines from mouse strains other than Six2-GFP reporter or any surface marker purification. It depends on the robust culture condition (culture-dependent purification, CDP) and adherence properties of NPCs and NPC lines can be derived with CDP method within as short as 4 days.

a. Pre-treat flat bottom 12-well plate (regular plate for tissue culture, not low-attachment) with laminin (BD, Cat. No. 354232). 10ug of laminin is mixed with 1ml DMEM/F12 basal medium to coat one well in 12-well plate. The 12-well plate with laminin/DMEM/F12 coating is transferred to 37 degree incubator for at least one hour and is washed once with PBS before seeding mouse embryonic kidney cells.
b. 1 pair of E12.5 embryonic kidneys of a given mouse strain are dissected out and put into cold dissection medium on ice.
c. carefully aspirate dissection medium and wash the kidneys once with sterile PBS
d. add 100ul of TrypLE and incubate the pair of kidneys in 37 degree incubator for 15~20 minutes to dissociate the embryonic kidneys.
e. add 500ul dissection medium to the tube, pipette up and down GENTALLY 20 to 30 times to further dissociate the kidneys (use the minimal pipetting times that can dissociate more than 95% of cells to single cells).
f. spin down the cells at 300g for 5min and resuspend the cell pellet in NPSR medium (from our experience, total cell number of 1 pair E12.5 kidneys is around 100,000. We usually add 100ul NPSR medium to make the cell density of around 1 million/ml)
g. cell number is counted and 10,000 unfractioned mouse embryonic kidney cells are seeded in one well of 12-well plate with 500ul NPSR medium. The 12-well plate is placed into 37 degree incubator.
h. 2 days later, add another 500ul NPSR medium to the well (do not change medium, just add 500ul more). At this time point, small round colonies can be observed.
i. on day 4, lots of aggregates with various sizes have detached from the plate and are clustered at the center of the plate and while some still loosely attached NPC aggregates are observed at the periphery of the plate. Collect the floating aggregates using 1ml tips to 1.5ml tube. Note: sometimes due to the variations of cell counting, the actual initial seeding number could be less than 10,000. In this case, after 4 days the aggregates are very small and additional 1~2 days is needed for the NPC aggregates to grow before collection. If this is the case, add 1ml NPSR on day 4 (still do not change medium) and collect the floating NPC aggregates on day 5 or day 6.
j. spin down the aggregates at 300g for 5min in 1.5ml tube. CAREFULLY aspirate the medium to avoid any loss of these small aggregates and then directly add 200ul room temperature Accumax without wash.
k. incubate in 37 degree incubator for 5~10 minutes and add 500ul dissection medium, carefully pipetting up and down for 5 to 10 times.
l. count the cell density and seed 3,000 cells in one well of U-bottom low attachment plate.
m. spin down the plate at 300g for 3min and transfer the plate to 37 degree incubator.
n. from now on, follow the same procedure to culture and passage the CDP-derived NPCs, as to culture NPCs from FACS sorted *Six2*-GFP+ NPCs.

Protocol #3 Clonal expansion of mouse NPCs with helper NPCs.

NPCs cultured in NPSR/3D condition cannot be cultured as single cells with currently unknown reasons. To circumvent this problem to enable its applications such as gene-editing, we developed this method. The following protocol details an example to generate mChery+/puromycin-resistant (mCherry/Puro for short) single clones. First, a mixture of mCherry/Puro NPCs is generated using lentivirus infection:

a. Lentivirus that express both mCherry and puromycin resistant gene was generated via a previously published protocol (Liao et al., 2015). 1 aggregate of NPCs at around 100,000 cells were dissociated and passaged according to above protocols, at the split ratio of 1:5 into 5 wells of low-attachment 96-well plate. Only 50ul NPSR medium, but not 100ul was used for each well. 1ul of concentrated mCherry/Puro lentivirus particles were added to each well, together with polybrene at final concentration of 10ug/ml. After mixing, the plate was spun down at 300g for 3min and then placed into 37-degree incubator. After 10 to 12 hours, the aggregates were formed. Aggregates were transferred into a 1.5ml eppendorf tube and washed with 1ml PBS for 3 times. After wash aggregates were placed into new low attachment wells with fresh NPSR medium (100ul per well). Note: the volume of lentivirus particles here is just an example. Different virus particle generated with different method has quite different titers and thus the infection volume should be empirically tested.
b. 2 days later, change the medium with 100ul fresh NPSR medium; 3 days later, change the medium to NPSR supplemented with 0.5ug/ml puromycin. Culture the aggregate with NPSR+puromycin for 2 passages to eliminate the cells without lentivirus integration. After 2 passages, the aggregate has bright and homogeneous mCherry expression under the fluorescence microscope (more than 99% mCherry+).
c. Next, single clones were generated with helper NPCs:

d. dissociate mCherry/puro mixture NPCs into single cells, calculate the cell densities and dilute it to 0.5 cell/10ul in NPSR medium.
e. add 10ul dissociated mCherry/puro cells at 0.5 cell/10ul to each well of low attachment plate. Spin down the plate at 300g for 5min and then label the wells that have only one cell in each well. According to our experience, serial dilution might be the best choice since FACS based single cell sorting causes a lot of damage to NPCs. On average, 30% to 70% of the wells should be single cells as long as the cell densities were calculated accurately.
f. dissociate wild-type NPCs and dilute it to 300 cells/10ul. Add 10ul of wild-type NPCs to the wells with single mCherry/puro cell.
g. spin down at 300g for 5min and place the plate back to 37 degree incubator.
h. add 30 ul NPSR medium to each well after two days; add another 50 ul NPSR medium to each well after 4 days.
i. change the medium with 100ul NPSR medium after 6 days and change medium every 2 days for another 4 to 6 days until the aggregate reached the size suitable for passage. At this time point, change to NPSR medium containing 0.5ug/ml puromycin to kill the helper wild-type NPCs.
j. keep changing medium with NPSR+puromycin until the aggregate grows big enough for passaging. After puromycin selection the whole aggregate represents a clonally expanded mCherry/puro NPC line. Note: 300 helper NPCs is the minimal cell number to form a healthy NPC aggregate from our experience. Less than 300 is not recommended and in case of variations in counting cell density, 300 to 500 cells is recommended to be used to form the aggregate.

Protocol #4 Cryopreservation and reviving of NPC lines.

A general protocol used for commonly used cell lines can be used for the cryopreservation and thawing of cultured NPCs. Detailed protocols are as follows:

To cryopreserve cultured NPCs:

a. dissociate NPC aggregates following the same procedure described above for passaging NPCs.
b. spin down and resuspend the cell pellet in cryo-medium (90% NPSR or hNPSR plus 10% DMSO) at the cell density of no less than 1million/ml. For mouse NPCs, we put around 100,000 cells to one cryotube and for human NPCs, we put 200,000 cells to one cryotube.
c. the cryotubes were transfered into Mr. Frosty and put into -80 degree freezer immediately.
d. the next morning, the cryotubes were transferred to liquid nitrogen tanks for long term storage.

To thaw the cryopreserved NPC lines:

a. immediately place the cryotube into 37 degree water bath after taking it out from the liquid nitrogen tank.
b. swirl the tubes in the water bath until half of the cells are thawed and the other half frozen. Use 70% ethanol to spray the tubes before bringing into cell culture hood. Transfer cells to a 15-ml falcon tube filled with 10ml NPSR medium, close the cap and invert the tubes several times before spinning down at 300g for 5min
c. resuspend the cell pellets in culture medium (for mouse NPCs, one vial of 100,000 cells resuspended in 500ul NPSR and split into 5 wells; for human NPCs, one vial of 200,000 cells resuspended in 500ul hNPSR medium and split into 5 wells)
d. spin down the plate at 300g for 3min and place the plate into 37 degree incubator.

Protocol #5 Spinal cord induced nephron organoid formation from NPC lines.

The spinal cord induction assay is well established and widely used. Since a lot of results in this study were generated from this assay. We list our detailed protocol here.

a. E11.5 to E13.5 mouse embryonic spinal cords were dissected and placed into cold dissection medium on ice.
b. The spinal cords were peeled into two halves from the dorsal cleave and put onto transwell membrane in an air-liquid interface (6-well, 12-well and 24-well transwell membranes were chosen according to different purposes. The Cat. No. for these plates: 6-well format, #07200170; 12-well format, #07200161; 24-well format, #07200154. All of them are from Fisher). 0.8ml dissection medium was added to the lower chamber in 6-well format; 0.35ml for the 12-well format and 180ul for the 24-well format.
c. NPC aggregates are placed in close contact with the dorsal side of the spinal cord (the ventral side has minimal inductive effect). Excessive medium on the transwell membrane during the NPC transfer process was removed using 20ul tip pipette.
d. the medium is changed every 2 days until the samples are collected at designed time points for analysis.

Protocol #6 Chemically defined nephron organoid formation from mouse NPC lines.

The following differentiation protocol efficiently generates nephron organoid from culture NPCs under chemically defined culture conditions in as short as 7 days.

a. 3D NPC aggregates were transferred into 1.5ml tube with KR5 medium to dilute the carry-over NPSR medium from transfer.
b. KR5 medium supplemented with 4.5uM CHIR99021 and 200ng/ml FGF2 (KR5-CF) was added to the lower chamber of transwell membrane (0.8ml for 6-well format; 0.35ml for the 12-well format and 180ul for the 24-well format). Note: CHIR99021 concentration has dramatic effects on the differentiation to nephron segments. Our experience is that with low CHIR99021 concentration at around 3uM, more glomeruli are generated, but less distal tubules are generated; higher concentration of CHIR99021 at around 6uM, less glomeruli but more distal tubules are generated. 4.5uM is a balanced concentration we used in our hand. This should be empirically optimized.

c. transfer NPC aggregates from KR5 medium to the transwell membrane and remove the excessive KR5 medium during the transfer using 20ul tip pipette.
d. 2 days after KR5-CF treatment, change medium to KR5 medium in the lower chamber (0.8ml for 6-well format; 0.35ml for the 12-well format and 180ul for the 24-well format). After that, change to fresh KR5 medium every 2 days until the samples are collected at designed time points for analysis.

Protocol #7 Derivation of human NPC lines from human fetal kidneys.

The following protocol generates long-term proliferative human SIX2+ NPC lines from human fetal kidneys with nephrogenic potential.

a. fresh human fetal kidneys (between the gestation ages of 9 weeks to 17 weeks) were collected and placed into cold dissection medium on ice.
b. dissect human fetal kidneys into small pieces with comparable sizes to E12.5 mouse embryonic kidneys in cold dissection medium.
c. collect the small kidney tissue pieces into a 1.5ml tube, wash 3 times with PBS and then use TrypLE to dissociate the tissues (37 degree, 20~30 min).
d. add 500ul of dissection medium and pipette up and down for 30 to 50 times GENTALLY. Renal tubules are difficult to dissociate, but most of SIX2+ NPCs are liberated from the kidney tissue using this method. 40um cell strainer was used to remove those undigested tubules and also big cell clumps.
e. spin down the dissociated cells and resuspend the cell pellet with FACS medium (cold PBS with 2%FBS). Count the cell number and dilute the cells to the density of 2 million/100ul in a 1.5ml eppendorf tube.
f. incubate with AF488-conjugated EpCAM antibody (1:100 use, R&D, # FAB9601G) and PE-conjugated NGFR antibody (1:50 use, BioLegend, #345104) on ice for 20min.
g. add 1ml cold FACS medium to the 1.5ml eppendoff tube, spin down at 300g for 5min, remove the FACS medium and resuspend the cell pellet with fresh FACS medium (2million/300ul).
h. FACS was used to sort EpCAM-/NGFR+ cell population, which enriched human SIX2+ cells. Note: EpCAM antibody has very high specificity and clear boundaries were observed between EpCAM- and EpCAM+ populations; NGFR antibodies have background staining from batch to batch and we always selected $NGFR^{Hi}$ population for FACS gating. We also tested the recently reported surface marker ITGA8 (O'Brien et al., 2016) and it also works well in our hand in enriching SIX2+ cells. After enrichment with either EpCAM-/NGFR+ or EpCAM-/ITGA8+, SIX2+ cells could be 50% to 80% depending on the gestational stages.
i. FACS enriched SIX2+ cells were spun down and resuspended in hNPSR medium. 5,000~10,000 cells in 100ul hNPSR were seeded into one well of low-attachment 96-well plate.
j. spin down the plate at 300g for 3min and transfer the plate to 37 degree incubator.
k. Aggregate is formed within 2 days and we change medium every 2 days with fresh hNPSR. SIX2+ cells grow quickly and form morphologically mesenchymal-type 3D aggregates while the other SIX2- cells grow slowly and form tubular structures. The mesenchymal-type aggregate, when reaching the diameter of ~0.5mm, was dissected out under the microscope and cultured separately. These aggregates are almost 100% SIX2+ cells. Note: the growth kinetics of mesenchymal-type aggregates vary with gestational stages of the human fetal kidneys (the earlier the quicker) as well as the viability of cells after dissociation. Allow the growth of the mesenchymal-type aggregate to ~0.5mm in diameter before separated for culture. After this stage, the dissected aggregates could grow stably.
l. After the dissected mesenchymal-type human NPC aggregates reach diameter of ~1mm, we passage the aggregates at the ratio of 1:10 every 4~5 days.

The passage of human NPC aggregates is very similar to that of mouse NPCs:

a. collect 3D aggregates to 1.5ml eppendoff tubes.
b. aspirate the culture medium and wash once with 1ml sterile PBS.
c. aspirate PBS and add room temperature Accumax to the NPC aggregates (the volume of Accumax depends on number of NPC aggregates to be dissociated. Our experience are shared here as a guideline: 1~2 aggregates, 50ul; 3~5 aggregates, 100ul; 10~20 aggregates, 200~300ul)
d. incubate NPCs with Accumax in 37 degree incubator for 7~10 minutes.
e. add 500ul dissection medium, pipette up and down VERY GENTALLY for 5 to 10 times. The clumps of cells should be invisible after 2~3 pipettes. Never ever strongly pipette the NPCs, which would be harmful for their self-renewal condition.
f. spin down the cells at 300g for 5min, resuspend the cell pellet with hNPSR medium
g. split the NPCs at the ratio around 1:10 to U-bottom low-attachment 96-well plates
h. spin down the 96-well plates at 300g for 3min and then put the plates back to 37 degree incubator
i. change medium with 100ul fresh hNPSR medium every 2 days and passage the NPC aggregates every 4~5 days

Note: we noticed different optimal concentration of LDN193189 and A83-01 in derivation of human NPC lines from different gestational ages. The ranges are 10 to 100 nM for LDN193189 and 0.05 to 0.5uM for A83-01. We recommend the following starting concentrations for testing: 50nM LDN193189 and 0.2 uM A83-01.

Protocol #8 Chemically defined nephron organoid formation from human NPC lines

The following differentiation protocol efficiently generates nephron organoids from human NPC lines under chemically defined culture conditions within 7~9 days in total.

a. 3D NPC aggregates were transferred into 1.5ml tube with KR5 medium to dilute the carry-over NPSR medium from transfer.
b. KR5 medium supplemented with 4.5uM CHIR99021 and 200ng/ml FGF2 (KR5-CF) was added to the lower chamber of transwell membrane (0.8ml for 6-well format; 0.35ml for the 12-well format and 180ul for the 24-well format). Note: CHIR99021 concentration has dramatic effects on the differentiation to nephron segments. Our experience is that with low CHIR99021 concentration at around 3uM, more glomeruli are generated, but less distal tubules are generated; higher concentration of CHIR99021 at around 6uM, less glomeruli but more distal tubules are generated. 4.5uM is a balanced concentration we used in our hand. This should be empirically optimized.
c. transfer NPC aggregates from KR5 medium to the transwell membrane and remove the excessive KR5 medium during the transfer using 20ul tip pipette.
d. 1~2 days after KR5-CF treatment (this timing should be optimized for each hNPC line), change medium to KR5 medium in the lower chamber (0.8ml for 6-well format; 0.35ml for the 12-well format and 180ul for the 24-well format). After that, change to fresh KR5 medium every 2 days until the samples are collected at designed time points for analysis.

IV Supplemental References

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