Inventory of Supplementary Materials

Supplementary data

Supplementary Experimental Procedures

Supplementary data

Figure S1

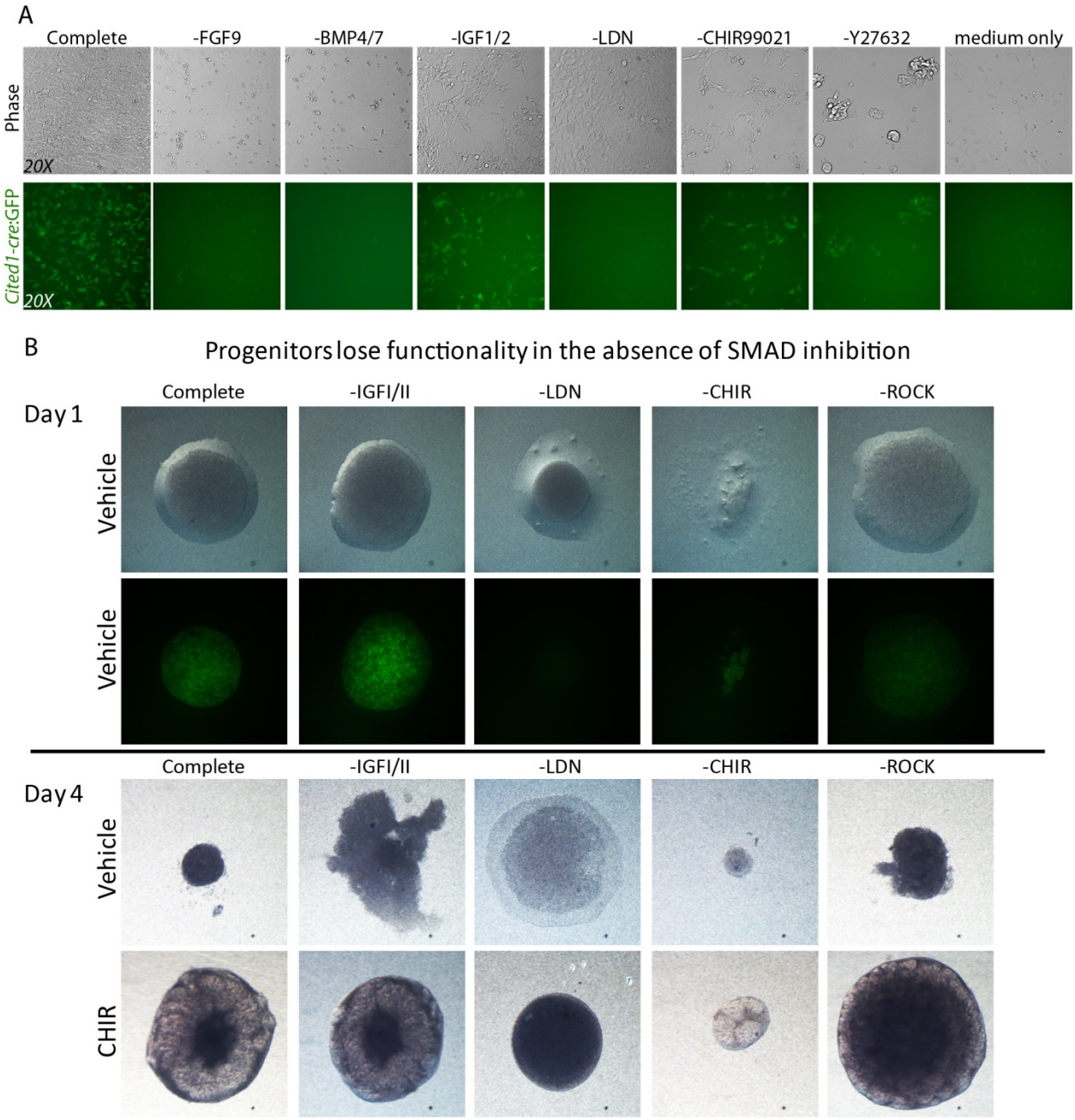


Figure S1| Nephron progenitor cultures growth in the absence of individual factors, Related to Figure 3.

(A) Individual factors were removed from NPEM and purified CITED1 progenitors from *Cited1creERT2-EGFP* x ICR mice were cultured in monolayer for 3 days. Cultures contain a 50/50 starting mix of GFP+ and GFP- cells. Top panels: Phase microscopy of cell morphology after 3 days in the absence of individual factors. Bottom panels: Corresponding immunofluorescence of GFP signal after 3 days in culture.

(B) GFP immunofluorescence and corresponding light microscopy of 3D aggregates grown for 1 and 4 days respectively. Individual factors were removed from NPEM and purified CITED1 progenitors from *Cited1creERT2-EGFP* x ICR mice were cultured for 3 days in monolayer culture. Cells were dissociated

and spotted in 3D aggregate on floating filters for the indicated times. Top panels: Vehicle only control shown after 1 day in culture. Bottom panels: Tubulogenesis can be seen when cultures are treated with CHIR, except when monolayer cultures were grown in the absence LDN. 3 monolayer replicates were pooled per 3D aggregate. 1 of 2 experimental replicates shown. Note: FGF9 and BMP4/7 minus cultures died during the monolayer phase.

Figure S2

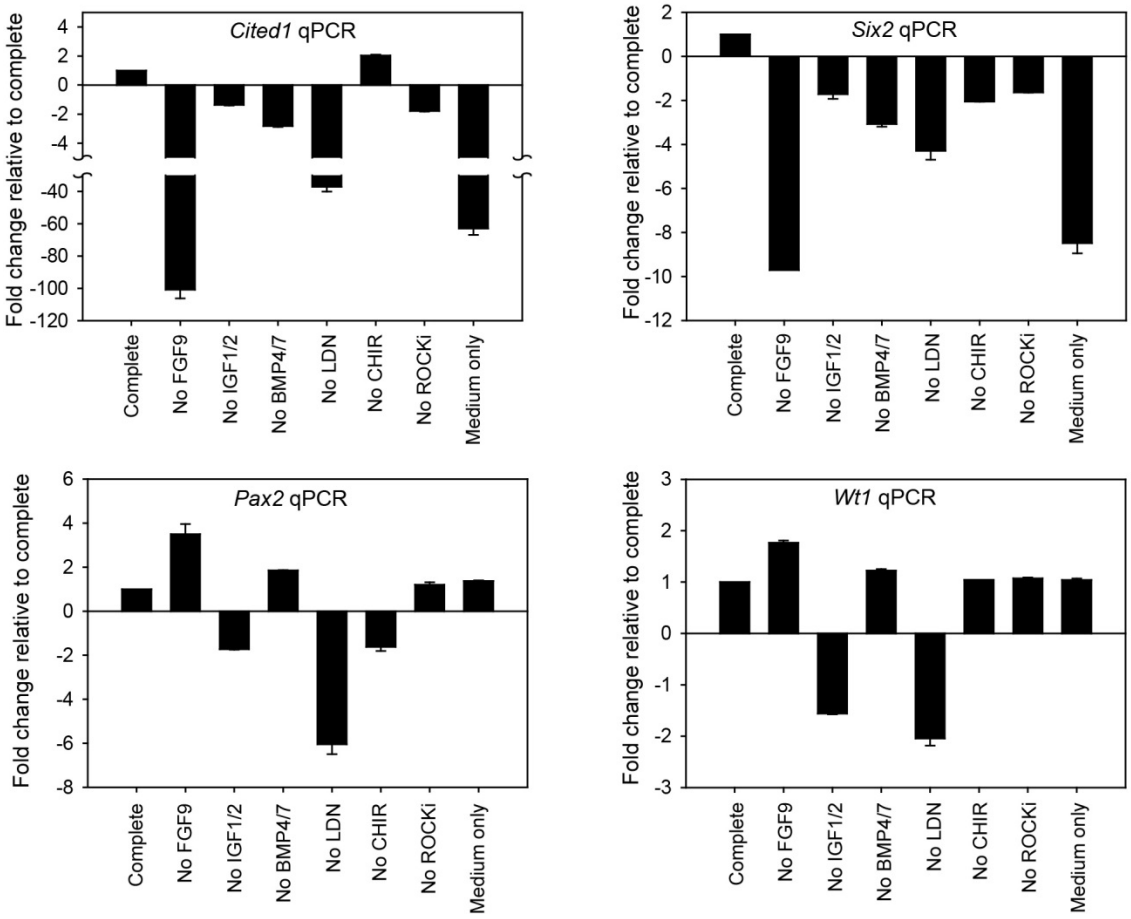
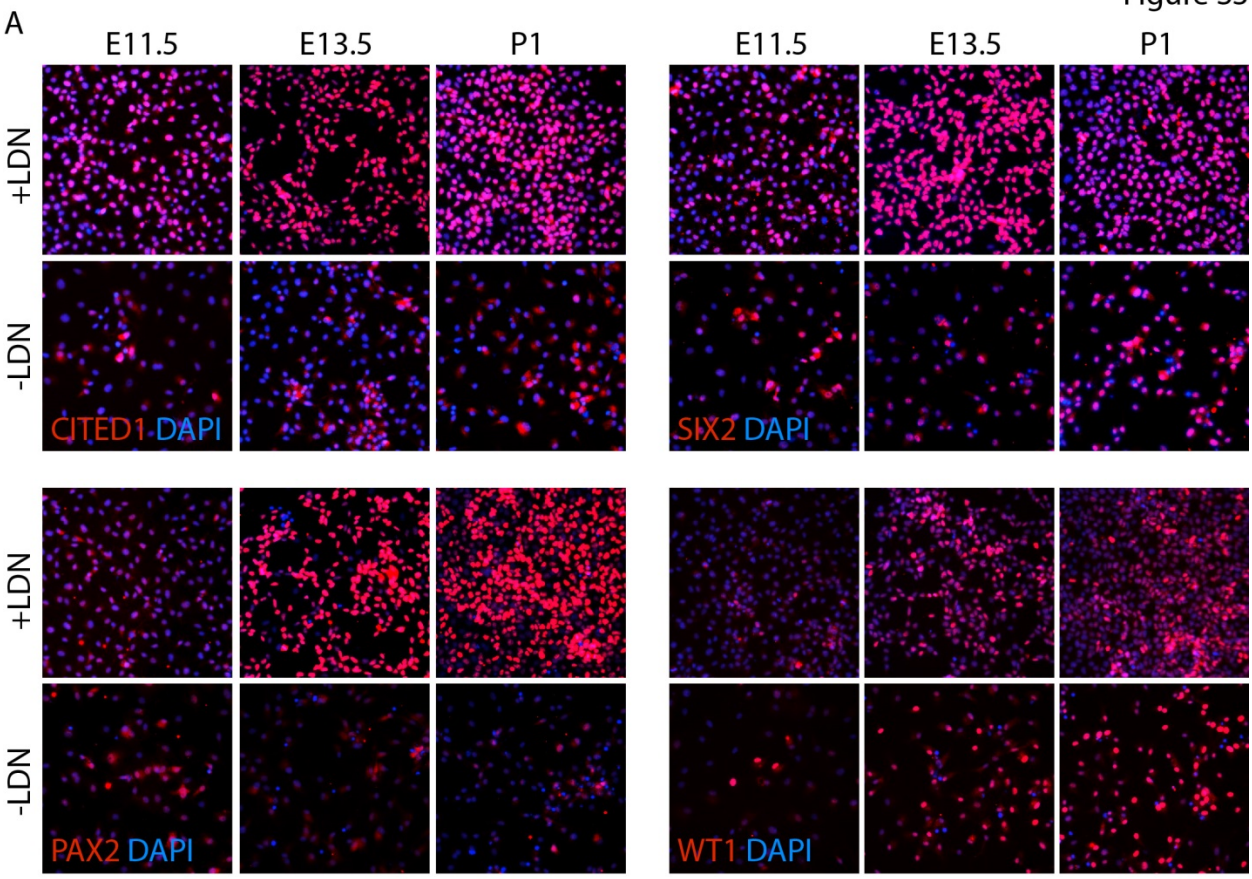


Figure S2| Nephron progenitor marker expression in cultures grown in the absence of individual factors, Related to Figure 3. Purified CITED1 progenitors were grown in NPEM in the absence of individual factors for 3 days in monolayer culture. Cells were lysed and gene expression was measured by quantitative PCR. Fold changes are relative to cells grown in complete NPEM for each primer set. Results shown represent 3 pooled culture replicates derived from 20-24 pooled embryonic kidneys.

Figure S3


A
E11.5
E13.5
P1
E11.5
E13.5
P1
+LDN
-LDN
CITED1 DAPI
SIX2 DAPI
+LDN
-LDN
PAX2 DAPI
WT1 DAPI

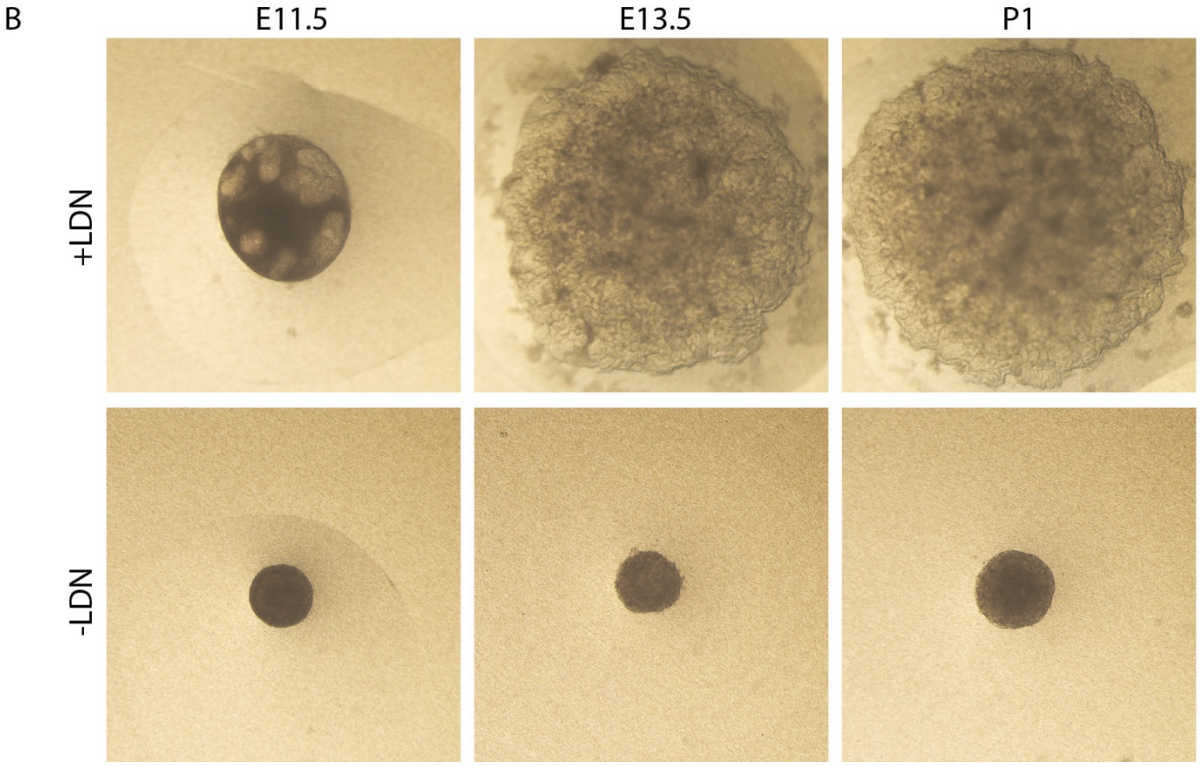

B
E11.5
E13.5
P1
+LDN
-LDN

Figure S3| NPCs isolated from E11.5 kidneys require LDN to remain competent to undergo differentiation, Related to Figure 4.
(A) LDN is necessary to maintain maximal expression of cap mesenchyme markers compared to NPCs cultured for 24 hours in the absence of LDN.
(B) Only NPCs propagated in NPEM containing LDN retain the capacity to undergo tubulogenesis when subsequently differentiated in 3D aggregate culture with CHIR.

Figure S4

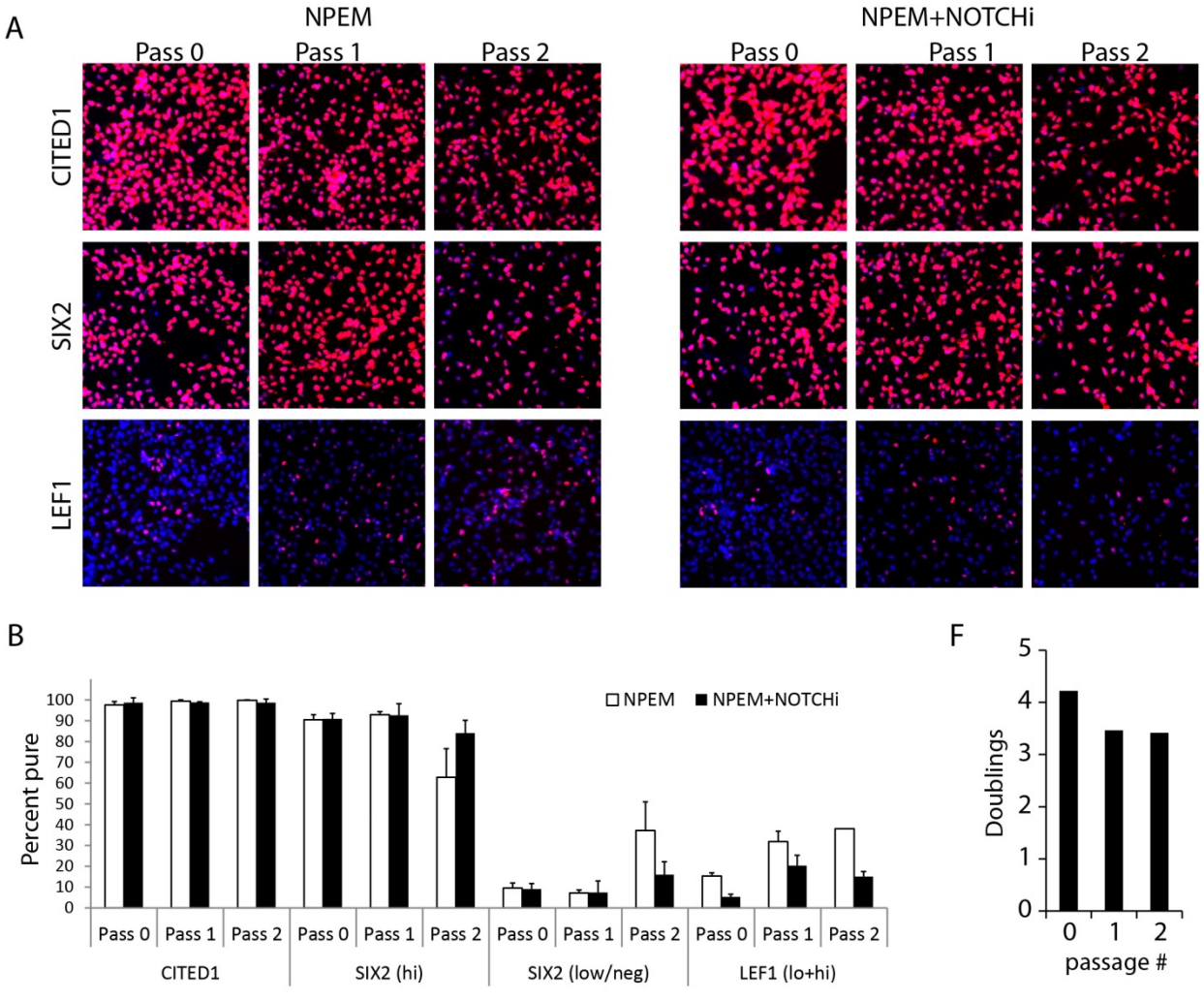

A
NPEM
NPEM+NOTCHi
Pass 0
Pass 1
Pass 2
CITED1
SIX2
LEF1
B
Percent pure
NPEM
NPEM+NOTCHi
CITED1
SIX2 (hi)
SIX2 (low/neg)
LEF1 (lo+hi)
F
Doublings
passage #
C
Pass 0
SIX2
LEF1
D
NPEM
Pass 1
Pass 2
LTL ECAD
E
NPEM+Notchi
Pass 1
Pass 2
LTL ECAD

Figure S4| Analysis and expansion of progenitors isolated from E11.5 mesenchymes, Related to Figure 4.

(A, B) Unlike cells isolated from later developmental stages, E11.5 cultures consisted of approximately 10% $SIX2^{low}$ and 15% LEF1+ cells at the end passage 0, and the proportion of cells with a differentiated profile continued to increase through 2 passages.

(C) Examples of SIX2 and LEF1 high and low positive cells at the end of passage 0.

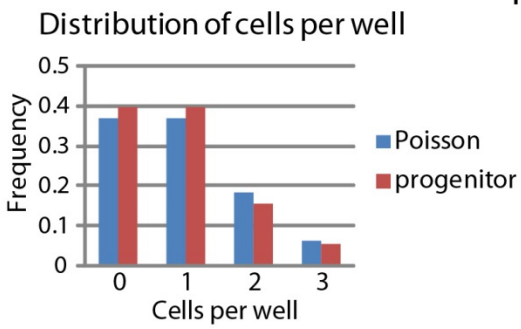
(D) Aggregates of expanded E11.5 progenitors treated with 3μM CHIR in aggregate culture formed LTL+ and ECAD+ tubules through passage 1. By passage 2, progenitors lost the potential for differentiation into tubules and the percentage of $SIX2^{low}$ and LEF1+ cells had increased further (B). More than 99% of cells remained CITED1+ through passage 2, indicating a significant difference in marker gene expression between E11.5 progenitors and cells from later stages cultured in NPEM (B).

(E) To overcome the premature differentiation of cells and further increase expansion potential, we attempted to block the spontaneous differentiation of progenitors by adding a Notch inhibitor (Compound E, Calbiochem, 125nM) to the expansion cocktail. Addition of this inhibitor decreased the number of $SIX2^{low}$ and LEF1+ cells through passage 2 (A and B) and yielded more robust differentiation compared to progenitors expanded without inhibitor.

(F) Notch inhibitor supplemented NPEM is sufficient to expand differentiation competent E11.5 nephron progenitors through 11 doublings (approximately 2000 fold, passages 0-2).

Figure S5

A



B Representative CITED1 immunostain for clone #s 1-24

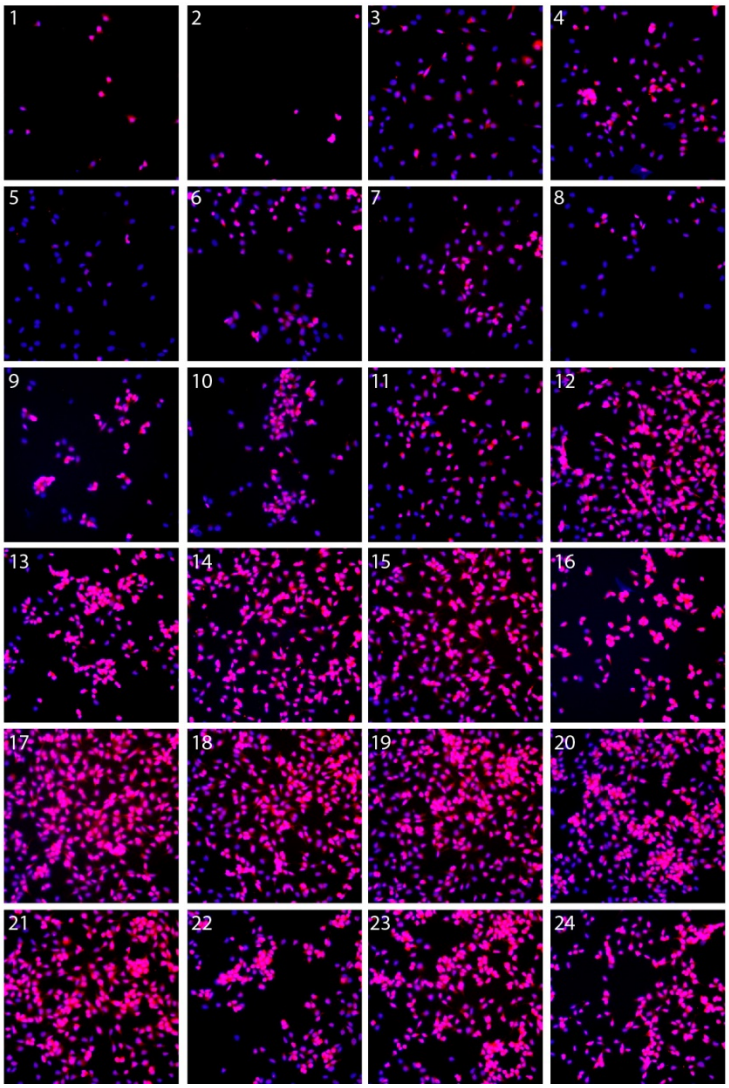


Figure S5| Initial cell seeding distribution and final expanded CITED1+ purity, Related to Figure 6. (A) Expected and observed Poisson distribution of limiting dilution assay. A random sampling of 192 wells was chosen for the analysis and screened by light microscopy.

(B) Representative CITED1 immunostaining of expanded clones. DAPI shown in blue.

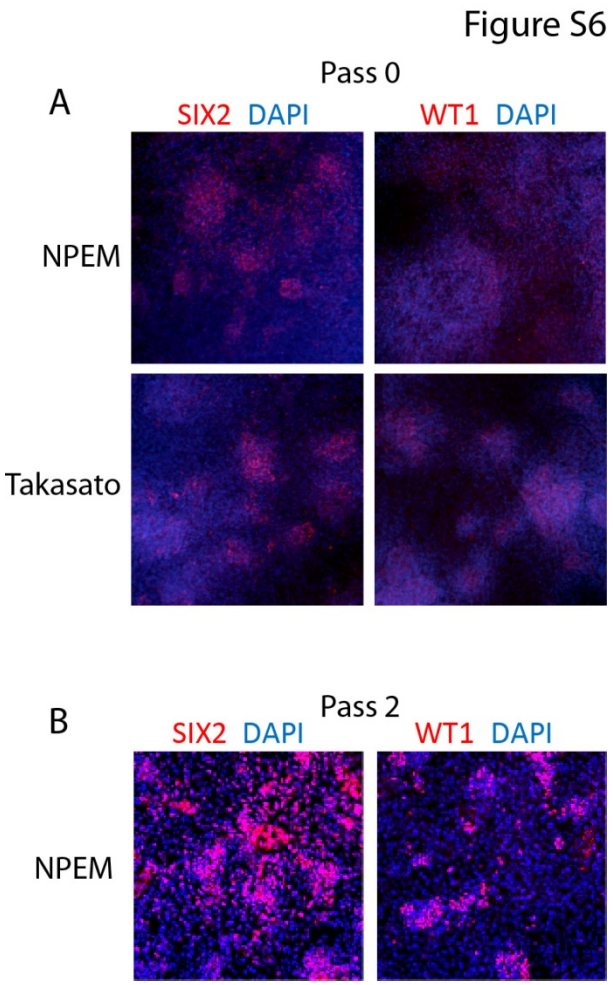


Figure S6| SIX2 and WT1 expression in human nephron progenitors derived from human ES cells after pass 0 and 2, Related to Figure 7.
(A) Nephron progenitor cells immunostained for SIX2 and WT1 were differentiated for 5 days from H9 hESCs using the conditions reported by Takasato et al and switched to NPEM for an additional 5 days (top panels). Cells differentiated using the Takasato procedure for 10 days (bottom panels).
(B) Cells differentiated using the Takasato procedure were switched to NPEM after 5 days and expanded for 2 passages (1:8 split) with retained expression of WT1 and SIX2.

Figure S7

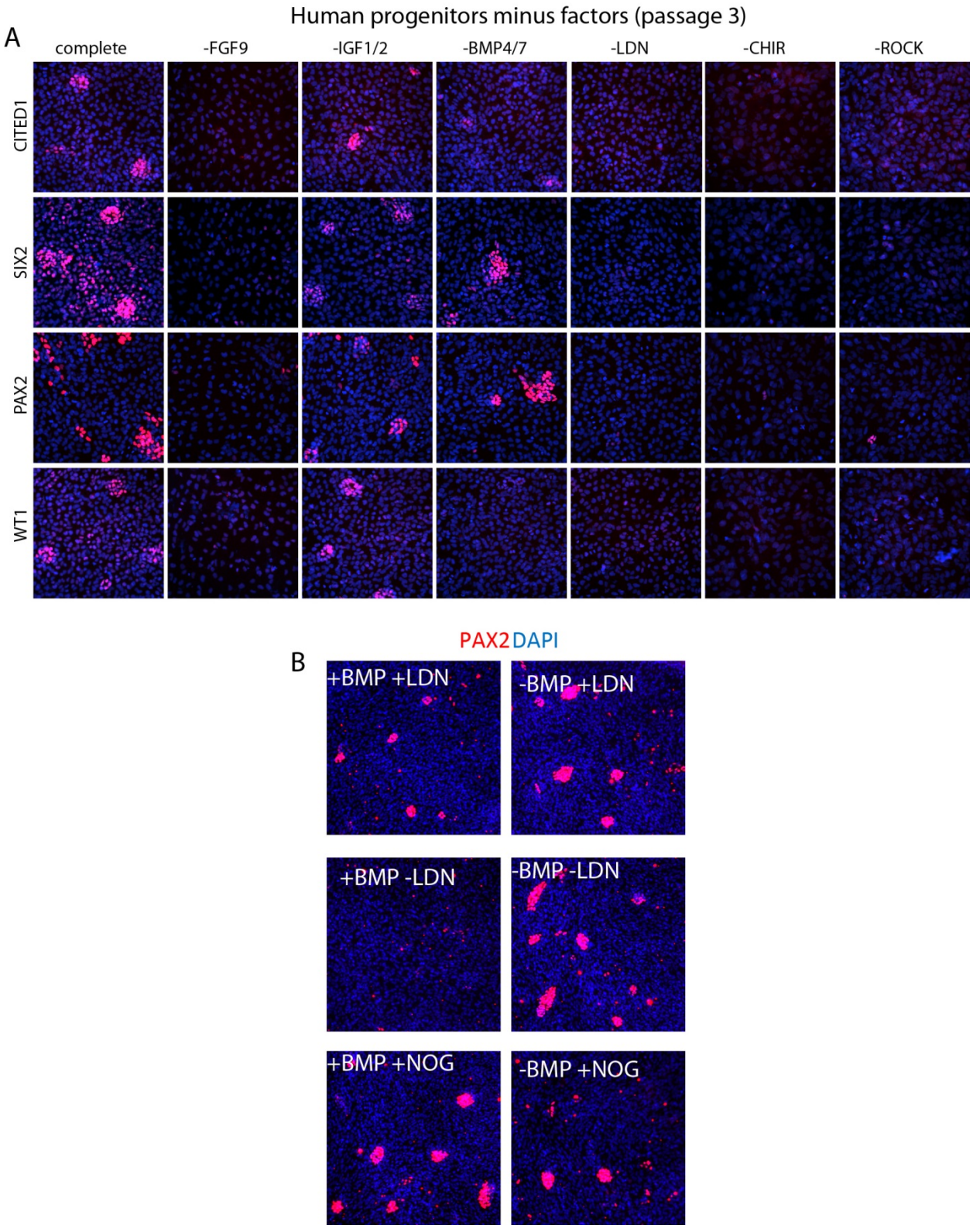


Figure S7| Nephron progenitor marker expression in human ES cell derived cultures grown in the absence of individual factors, Related to Figure 7.

(A) Passage 3 progenitors were grown in NPEM in the absence of individual factors for 3 days in monolayer culture and immunostained for CITED1, SIX2, PAX2 and WT1.
(B) Passage 3 progenitors were grown in NPEM in the absence of the indicated factors for 3 days in monolayer culture and immunostained for PAX2.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Primer sequences used in this study

Gene	Forward	Reverse
Cited1	CCACTAGCTCCTCTGGATCG	AGCCCCTTGGTACTGGCTAT
Cv2	CCTGCTGTGAACGATGCAAAGG	ACCTCAGACTCTGTCACCACAC
Dpf3	CCTCTCAGGAAGACCACGACAA	CCAGGTGAGTATGAGCGTAGTG
Hoxb7	GCCGCAAGTTCGGTTTTCG	GCAAAGGCGCAAGAAGTTTGT
Id1	TTGGTCTGTCGGAGCAAAGCGT	CGTGAGTAGCAGCCGTTCATGT
Id3	GCGTGTCATAGACTACATCCTCG	GTCCTTGGAGATCACAAGTTCCG
Id4	AGTGCGATATGAACGACTGCTAC	AGCAAAGCAGGGTGAGTCTCCA
Jag1	TCGCACCGATACCAGTTGTCTC	TGCGTGGTCAATGGAGACTCCT
Lef1	ACTGTCAGGCGACACTTCCATG	GTGCTCCTGTTTGACCTGAGGT
Meox1	GGAGGATTGCATGGTACTTGGG	CTTTGCTGCTGCCTTCTGGCTT
Pax2	ATTCCTCGCTCCAACGGTGAGA	CAGACCAGATGTAAACCTCCACC
Six2	CACCTCCACAAGAATGAAAGCG	CTCCGCCTCGATGTAGTGC
Sp5	TCGCACCGATACCAGTTGTCTC	AGGTGATCGCTTCGCATGAAGC
Wnt4	GAGAACTGGAGAAGTGTGGCTG	CTGTGAGAAGGCTACGCCATAG
Wt1	GGTTTTCTCGCTCAGACCAGCT	ATGAGTCCTGGTGTGGGTCTTC

Protocol for the isolation and culture of CITED1+ nephron progenitor cells

Sections:

Abbreviations

FBS – Fetal Bovine Serum
NPC – Nephron Progenitor Cell
NPEM – Nephron Progenitor Expansion Medium
NZC – Nephrogenic Zone Cell
RT – Room Temperature

Section 1.1: Equipment

Nutator placed at Room Temperature (RT)
Nutator placed at 37°C
AutoMACS Pro Separator (Miltenyi, 130-092-545)
96, 24 or 6 well culture plates (Corning, 3595, 3524, 3516)
Transfer Pipette (Corning, 357575)

Section 1.2: Reagents

Collagenase A (Roche, 11 088 793 001)
Pancreatin (porcine) (Sigma, P1625)
Matrigel (Corning, 354277)
HBSS without calcium or magnesium (Life Technologies, 14175-095)
PBS without calcium or magnesium (Lonza, 17-513F)
FBS (Biowest, S1620)
AutoMACS running buffer (Miltenyi, 130-091-221)
Pre-Separation Filters (30 μm) (Miltenyi, 130-041-407)
anti-CD105-PE (Miltenyi, 130-102-548)
anti-CD140-PE (Miltenyi, 130-102-502)
anti-Ter119-PE (Miltenyi, 130-102-893)
anti-CD326-PE (Miltenyi, 130-102-265)
anti-PE microbeads (Miltenyi, 130-048-801)
APEL Medium (Stemcell Technologies, 05210)
Human recombinant FGF9 (R&D, 273-F9-025)
Human recombinant BMP7 (R&D, 354-BP-010)
Human recombinant BMP4 (R&D, 314-BP-010)

Human recombinant IGF1 (R&D, 791-MG-050)
Mouse recombinant IGF2 (R&D, 792-MG-050)
CHIR99021 (Stemgent, 04-0004)
LDN-193189 (Stemgent, 04-0074-02)
Y27632 (Millipore, 688001)
Heparin (Sigma, H3393)
TrypLE™ dissociation solution (Life Technologies, 12563029)

Section 1.3: Preparation of reagents prior to isolation

1. Prepare collagenase A/pancreatin enzyme digest solution 2 hours before kidney dissection by adding 25 mg collagenase A to 10 ml PBS without calcium or magnesium. Place on a nutator at RT for 15 minutes or until collagenase A is dissolved. Add 100 mg pancreatin and place on nutator until kidney harvest is complete. Filter sterilize (0.2 µm filter) before use to remove any undissolved particles. Alternatively, large batches of the enzyme digest solution can be made, filter sterilized, and frozen at -20°C for up to 6 months.

2. Dilute Matrigel 1:25 in cold DMEM/F12 and coat plates. Volumes of Matrigel for coating are 50 µl/well for 96 well, 300 µl/well for 24 well and 1 ml/well for 6 well. Make sure Matrigel is distributed over the entire surface of the well and allow to sit undisturbed in a laminar flow cabinet for cell culture for at least 1 hour.

3. Prepare round bottomed 5 ml tubes to receive dissected kidneys by filling them with 2 ml HBSS without magnesium or calcium.

4. Transfer 50 ml of autoMACS running buffer to a 50 ml conical tube and warm to RT.

5. Start autoMACS instrument: check solutions, install autoMACs running buffer bottle, run clean program.

6. Make appropriate volume of NPEM in a 50 ml conical tube.

Section 1.4: Nephron progenitor expansion medium (NPEM)

Factor	stock concentration	working concentration	for 10 mL of medium
APEL medium	1X	1X	10 ml
Penstrep	100X	1X	100 µl
FGF9	100 µg/ml	200 ng/ml	20 µl
Y27632	5 mM	10 µM	20 µl
IGF1	50 µg/ml	20 ng/ml	20 µl of 1:100 dilution (in APEL)
IGF2	1 µg/ml	2 ng/ml	20 µl of 1:100 dilution (in APEL)
BMP4	100 µg/ml	30 ng/ml	3 µl
BMP7	100 µg/ml	30 ng/ml	3 µl
LDN-193189	10 mM	125 nM	12.5 ul of 1:100 dilution (in APEL)
CHIR99021	10 mM	1.25 uM	1.25 µl
Heparin	10 mg/ml	1 µg/ml	1 µl

Make NPEM in a laminar flow cabinet for cell culture and thoroughly mix by inverting several times.

Section 1.5: Isolation of nephrogenic zone cells (NZCs) from developing kidneys

1. Dissect the kidneys from E13 to P1 mice in a 10 cm dish containing a sufficient volume of PBS without calcium or magnesium to cover the embryos. Completely remove the ureter and kidney capsule to expose the nephrogenic zone and transfer kidneys to the HBSS-containing tube using a transfer pipette with an orifice cut to at least twice the diameter of the kidneys to be transferred. Discard ruptured or broken kidneys as they will reduce the purity of the final NPC preparation. Up to 24 kidneys can be placed in one tube.

2. Incubate the tube with kidneys in HBSS on a nutator for 2 minutes to dislodge debris that may still be attached to the kidneys (e.g. red blood cells and remaining capsule fragments).

3. Remove the HBSS and add another 2 ml of HBSS to wash. Be careful not to damage the kidneys with pipette tip.

4. Carefully remove as much of the HBSS as possible and add 2 ml of RT collagenase A/pancreatin enzyme digest solution. Place tube on the nutator at 37 °C for 12-15 minutes. Digestion times can vary greatly depending on the enzyme activities of different lots of collagenase A and pancreatin. Adjust digestion times to obtain 3-7 million NZCs from 20 E17 kidneys.

5. After digestion, remove tube containing kidneys and immediately add 125 µl of FBS to stop enzyme reaction. Invert to mix. Perform steps from here on in a laminar flow cabinet for cell culture.

6. Allow kidneys to sink to the bottom of tube and remove any floating particles with a 1 ml micropipette while removing as little of the cell suspension as possible. Floating particles at this

stage in the purification are usually capsule fragments that were not removed prior to the digestion.

7. Remove remaining cell suspension with a 1 ml micropipette. There is no need to take all of the solution and leaving 200 µl behind is recommended to avoid unwanted particles at the bottom of the tube.

8. Transfer the cell suspension evenly to two 1.5 ml microfuge tubes and spin in a microfuge at 2,000 rpm (300 g) for 5minutes.

9. Discard supernatant, gently resuspend each cell pellet in 500 µl autoMACS running buffer, then combine cell suspensions into one 1.5 ml microfuge tube.

10. Place a 30 µm pre-separation filter on a 15 ml conical tube and wash with 4 ml of autoMACS running buffer. Discard the flow through.

11. Add cell suspensions to the washed 30 µm pre-separation filter and wash with 500 µl of autoMACS running buffer. Transfer the 1.5 ml of filtered cell suspension to a 1.5 ml microfuge tube.

12. Transfer 10 µl of the cell suspension into a microfuge tube containing 90 µl autoMACS running buffer and determine cell count using a hemocytometer.

13a. For a mixed population of NZCs: Spin cells at 300g for 5 minutes to pellet and resuspend in medium for plating.
13b. For NPC purification proceed to Step 1 in Section 1.6.

Section 1.6: NPC purification from a mixed population of NZCs
This section describes a magnetic depletion protocol based on antibody labeling and cell capture using a Miltenyi Biotec autoMACS Pro Separator. For separations using manual columns it is recommended to consult Miltenyi Biotec for protocol modifications.

1. For each 10 million NZCs, gently resuspend cell pellet to a total volume of 76 ul (taking into consideration the volume of pellet) with autoMACS running buffer in a 1.5 ml microfuge tube and add the following antibodies for a total volume of 110 µl:

a. 9 µl of anti-CD105-PE
b. 9 µl of anti-CD140-PE
c. 8 µl of anti-Ter119-PE
d. 8 µl of anti-CD326-PE

2. Triturate cell/antibody suspension gently with a 200 µl pipettor and incubate at 4°C for 13 minutes without agitation.

3. Add 1 ml autoMACS running buffer and triturate gently 3 times. Centrifuge sample at 300g for 5 minutes.

4. Discard supernatant and gently resuspend pellet in 1 ml autoMACS running buffer. Centrifuge sample at 300 g for 5 minutes.

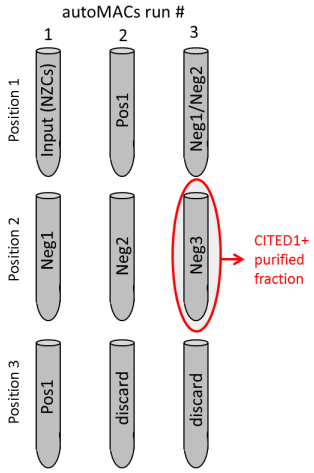
5. Discard supernatant and gently resuspend the pellet in a total volume of 80 µl autoMACS running buffer (taking into consideration the volume of pellet).

6. Add 20 µl of anti-PE microbeads to cell suspension. Gently mix by triturating several times and incubate at 4°C for 18 minutes without agitation.

7. Add 1 ml autoMACS running buffer and triturate gently 3 times. Centrifuge sample at 300 g for 5 minutes.

8. Discard supernatant and resuspend cell pellet in 1 ml autoMACS running buffer. Centrifuge again at 300 g for 5 minutes.

9. During centrifugation, label five 15 ml conical tubes: Input, Pos1, Neg1, Neg2, and Neg3. Three sequential autoMACs Pro Separator runs must be performed in order to obtain the purified CITED1+ cell population (see illustration to right).



AutoMACs run 1:

10. Discard supernatant and resuspend the pellet in 500 µl autoMACS buffer and transfer to the 15 ml conical tube labeled "input".

11. Place "input" tube into position 1 of the autoMACS Pro Separator, and Neg1 and Pos1 in positions 2 and 3.

12. Start separation using the Deplete(s) program and run a Qrinse (quick rinse) at the end of each separation. The NPCs will be collected as the negative fractions.

AutoMACs run 2:

13. After the first separation place the Pos1 tube with positive fraction in the inlet position with Neg2 in position 2 and an empty tube in position 3 and rerun the Deplete(s) program. In this step the positive fraction from the first separation will be run again to collect many non-labeled NPCs that are still present in the positive fraction. This step can increase the yield by up to 50%. Any remaining positive fractions can be discarded.

AutoMACs run 3:

14. Combine the Neg1 and Neg2 fractions that contain the NPCs into the Neg1 labeled conical tube and place this tube as the input (position 1). Place the Neg3 tube in position 2 and an empty tube in position 3. Rerun the Deplete(s) program to further enrich the NPC fraction to near 100 % CITED1-expressing cells, which will be deposited in the Neg3 tube.

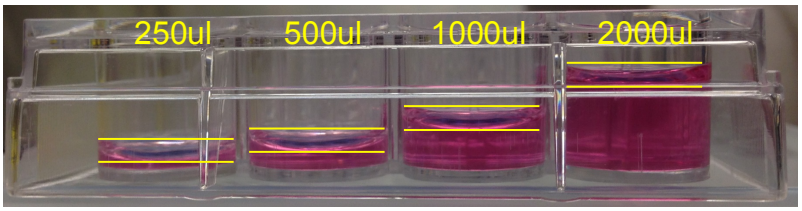
15. Spin the Neg3 fraction at 300 g for 5 minutes to pellet the purified NPCs.

16. Remove the supernatant and resuspend the NPCs in 1 ml of NPEM. Use 10 µl of the suspension for a cell count. The average yield of NPCs recovered from 5 million NZCs is about 1.2 million in our hands.

1.7: Cell plating and culture

1. Suggested cell seeding density is between 5,000 and 25,000 cells per cm^2. Increase the volume of NPC suspension with NPEM such that the desired cell density per ml is achieved for plating.

Note: To prevent clustering of cells near the edge of the well, which will result in earlier cellular overgrowth and the need for more frequent passaging, it is highly recommended to initially plate the cells in a high volume of NPEM. This will reduce the meniscus effect, which results in a higher cell density at the edge of the well due to the greater vertical column height of cells in suspension.



The meniscus effect at different culture medium volumes in a 24 well plate. The ratio of the meniscus height (yellow) to total vertical height (red) at well edge decreases at higher volumes.

The following volumes can be used as a guide:

Medium volume for various well sizes:
96 well plate – 200 µl
24 well plate – 2 ml
6 well plate – 4 ml

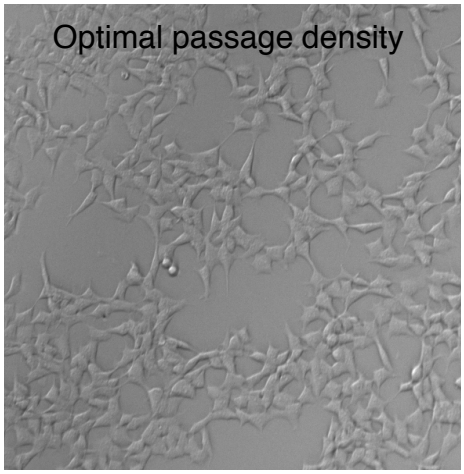
2. Immediately prior to plating, remove Matrigel from the culture plate wells using a vacuum aspirator connected to a sterile pipette tip and immediately add the desired volume of cell suspension in NPEM to each well. Agitate the plate to spread cells evenly.

3. Several hours after plating when cells have adhered to the culture plate, excess NPEM can be removed and stored at 4 °C for several days to be used for the next passage. Prior to NPEM storage, pellet any dead cells by centrifugation at 300 g and transfer the supernatant to a new tube while leaving pelleted cells or cellular debris behind. For example, cells are plated a 2 ml volume in a 24 well culture plate and 1.5 ml of NPEM is removed after 2 hours.

4. Change 100% of the medium every 48 hours.

1.8: Cell passaging

1. When the cells have reached 70-80% confluence at the areas of the well with the highest cell density they must be passaged for further expansion. The highest cell densities usually occur at the edge of the well, but sometimes can occur in the middle of the well. If cells are allowed to become too dense, they will substantially lose the potential for multiple passages. The image to the right can be used as a guide to show the optimal density that cells should be allowed to grow to before passage.



2. To passage, warm autoMACS running buffer to RT and TrypLE dissociation solution to 37 °C.

3. Aspirate NPEM and add pre-warmed TrypLE solution in the following amounts based upon well size.

96 well – 50 µl
24 well – 250 µl
6 well – 500 µl

4. Place culture plate in a 37 °C incubator for 2 minutes and then check that the cells have started to dislodge from the plate surface under a light microscope. If not, extend the treatment in 1 minute increments until cell detachment becomes noticeable.

5. Add the following volumes of autoMACS running buffer to each well and wash cells from plate by triturating gently.

96 well – 200 µl
24 well – 1000 µl
6 well – 2000 µl

6. Transfer the cells to an appropriate tube and spin at 300 g for 5 minutes to pellet the cells.

7. Remove the supernatant and resuspend the CITED1+ progenitors in 1 ml of NPEM. Perform a cell count on a 1:10 dilution of cells.

8. Increase the volume of NPEM and plate cells at the desired density on Matrigel coated culture plates (see **1.7: Cell plating and culture** above).

1.9: Cryopreserving CITED1+ progenitors

1. NPCs can be frozen in cryopreservation medium consisting of 10% DMSO, 40% FBS and 50% DMEM either immediately after isolation (uncultured) or following propagation and passaging in NPEM. 100,000 to 1 million cells per vial is recommended.

2. Pellet uncultured cells or TrypLE dissociated cells that have been diluted in autoMACS running buffer by centrifugation at 300g and remove supernatant. If cryopreserving cultured cells, wash one additional time in autoMACS running buffer prior to adding freeze medium to ensure that all TrypLE has been removed. Cells have been successfully cryopreserved from 80% confluent cultures in 24 well and 6 well plates up to passage 5, with subsequent expansion in NPEM.

3. Resuspend cell pellet in an appropriate volume of chilled cryopreservation medium depending on the number of cells per vial needed and transfer cell suspension to screw cap cryovials.

4. Place cryovial containing cells in a Styrofoam tube holder with cover at -70 °C overnight to slowly freeze the cells. The next day transfer frozen cells to liquid nitrogen for long term storage.

1.10: Thawing CITED1 progenitors

1. Thaw cells rapidly in a 37 °C water bath by swirling until just a small sliver of ice is left in the cryovial. This takes approximately 90-120 seconds.

2. Triturate once with 1 ml micropipette and transfer to a 15 ml conical tube. Slowly drip 2 ml RT autoMACS running buffer into the thawed cell suspension. After 2 ml has been added by dripping, add additional autoMACS running buffer to 10 ml.

3. Spin cell suspension at 300 g for 5 minutes to pellet cells.

4. Remove the majority of autoMACS running buffer (leave 50 µl behind to avoid disrupting the pellet) and wash pellet with 1 ml of NPEM by triturating gently several times.

5. Transfer cells to a 1.5 ml microfuge tube and spin in a microfuge for 5 minutes at 300 g.

6. Discard NPEM and resuspend cell pellet in the appropriate volume of fresh NPEM for plating (see **1.7: Cell plating and culture** above). It is important that cells do not clump as they will then cluster, causing focal overgrowth.

7. Plate cells on Matrigel-coated culture plates as described in section 1.7.

8. We typically observe greater than 90 % recovery of viable cells after thawing.