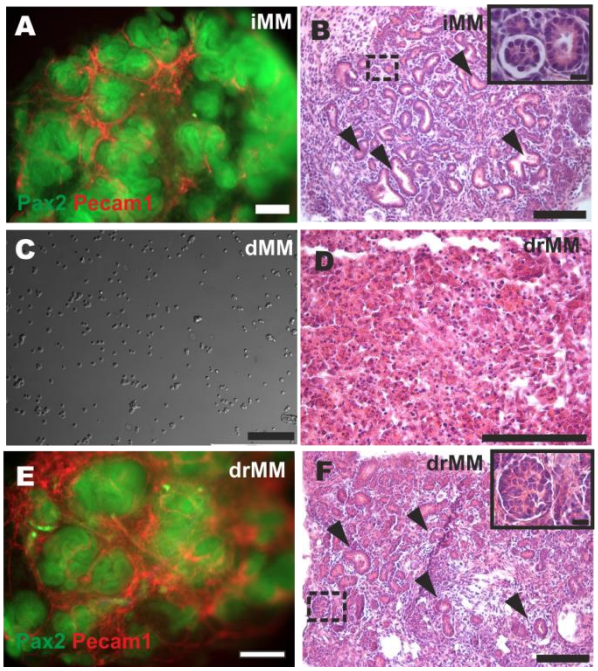
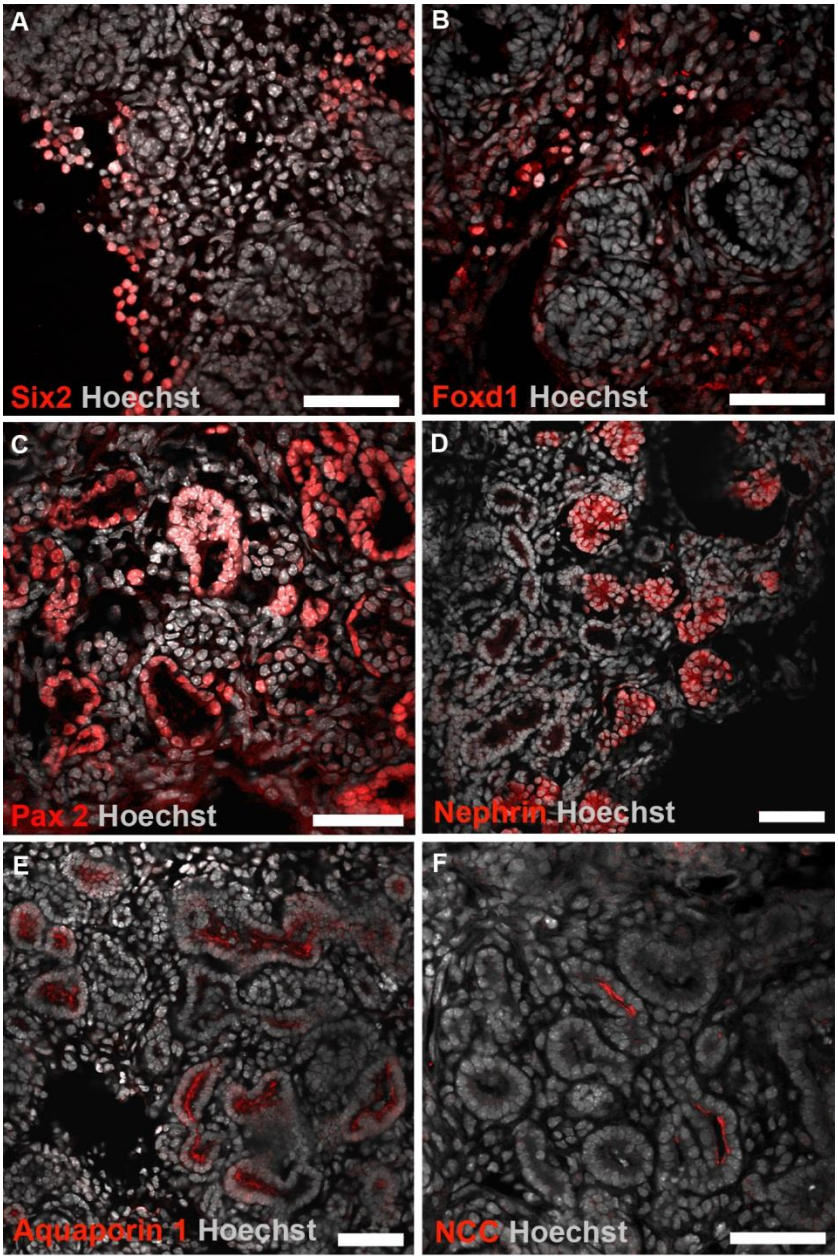
Supplemental Figures (SI-1-3) and Legends:



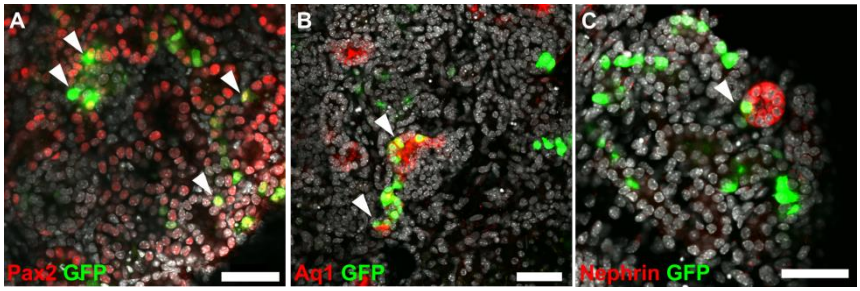
SI-1. Nephrogenesis potential of the embryonic kidney mesenchymal progenitor cells can be maintained with hrBMP7 and hrFGF2 even after their dissociation and reaggregation. The embryonic kidney was prepared from mouse embryos at E11.5 and the metanephric mesenchyme (MM) was separated and processed as illustrated in Figure 1. **A)** Classical intact, non-dissociated, induced MM (iMM) cultured for nine days. Pax2 expression (in green) and CD31/Pecam1 expression (in red) depict the induced tubules and endothelial cells differentiation, respectively indicating induction of nephrogenesis. **B)** Hematoxylin/eosin stained sections of the induced and subcultured iMM. Proximal and distal tubule-like structures (arrowheads) and renal corpuscle-like cellular assemblies (the boxed area, the insert) have developed. **C) A** differential interference contrast image of the dissociated MM cells reveals that for the most part the adopted protocol dissociates the MM (dMM) well to a single cell suspension. **D)** Depiction of a section of the MM that was dissociated and reaggregated (drMM) in the presence of hrBMP7 and hrFGF2. The drMM was cultured for 24 hours without the tubule inducing tissue. Note that the drMM cells that normally undergo apoptosis survive but remain uninduced since no epithelial

tubules develop. **E)** An example of an induced drMM resembling iMM control with respect to Pax2 and Pecam1 expression. **F)** Histology of the drMM resembles that of the iMM. Proximal and distal tubule-like structures (arrowheads) and renal corpuscle-like cellular assemblies (the boxed area, the insert) have developed. Scale bars: 100µm except (C) 1mm, inserts 10µm.



SI-2. Evidence that the nephrogenesis potential is restored in the embryonic kidney mesenchymal progenitor cells even after their dissociation and reaggregation. The embryonic kidney mesenchyme progenitor cells (MM) were separated, dissociated,

reaggregated (drMM), and induced as illustrated in Figure 1 (Steps 1-B6). They were then stained with a panel of nephrogenesis markers. After induction and a nine day culture of the drMM, the nephron progenitor cell marker Six2 (**A**) and the stromal progenitor marker Foxd1 (**B**) are expressed in the drMM. Similarly, tubular marker Pax2 **(C)**, podocyte marker Nephrin (**D**), and nephron segment markers Aquaporin1 (proximal tubules) (**E**) and NCC (distal tubules) (**F**) have become induced in the drMM. The red color depicts antibody staining. Scale bar: 50µm.



SI-3. Evidence that the roles of *Lhx1* function within the embryonic kidney mesenchyme progenitor cells can be targeted by viral *shRNA* mediated knock down *ex vivo*. The embryonic kidneys were prepared and the respective mesenchymes (MM) was separated, processed, and transduced as depicted in Figure 1 (Steps 1-B6). **(A-C)** When the *GFP-Lhx1-shRNA* virus is introduced directly into dissociated MM cells prior to their reaggregation, the cells transduced by the *Lhx1* knock down (KD) express the GFP (in green). The GFP expression makes it possible to identify the cells that express the Lhx1 *shRNA* sequences and to follow their fate. Note that the *Lhx1* KD in the MM has for the most part inhibited the capacity of the infected MM cells to start the tubulogenesis (A-C, arrowheads) process, since most of the GFP^{+} cells are not on the tubules as indicated by Pax2 expression (A), Aquaporin 1 (Aq1) (B) and Nephrin (C) markers. Scale bar: 50µm.

Legends to Supplemental Movies (Sl4 and Sl5)

SI-4. Behavior of the embryonic kidney mesenchymal progenitor cells in the dissociation/reaggregation culture set up as illustrated by time-lapse image capture.

The kidney mesenchyme progenitor cell reaggregates (drMM) were done as shown in Figure 1. The drMM was placed onto a Transwell permeable membrane and set into direct contact with a fragment of embryonic spinal cord (eSC, E11.5) to induce tubulogenesis. The eSC is present in the upper left corner and later at the center of the field of view. Induction of the drMM leads to the formation of drMM cell clusters, which can be seen as several translucent areas that become visible after around 1330min in culture. The translucent foci represent the drMM regions that develop the pretubular cell aggregates. The cells assemble into clearly distinguishable morphological cell clusters and resemble comma-shaped bodies at 3000min and S-shaped bodies structures at around the 3800 min culture time point. The counter time in minutes.

SI-5. Time-lapse illustration of the microscopic images captured from cultured chimeric embryonic kidney mesenchymal progenitor cells composed of GFP+ and wild-type cells. The kidney mesenchyme progenitor cell reaggregates (drMM) were done as indicated in Figure 1. The movie on the left is a bright field, the one in the middle an epifluorescence (GFP^+), and the one on the right a combination of the other two to illustrate the behavior of the induced drMM cells. The start of the *ex vivo* culture (0 hours) depicts the assembled drMM that is composed of the GFP^+ and GFP^-/wild-type MM cells. A fragment of embryonic spinal cord (eSC) serves as the tubule inducer. The drMM and its induction lead to the formation of several translucent foci in the MM cells, which appear around 1200min of culture time. Note that there are no nephrons that are exclusively

composed of GFP^+ cells. This illustrates the cellular foundation of the nephrogenesis. The chimeric drMMs reveal the highly motile and migratory nature of the drMM cells induced to undergo nephrogenesis in response to the eSC signaling in the repatterning drMM.

Material and Methods

Mouse lines, generation of embryos, dissection and culture of embryonic kidneys

The animal care and experimental procedures in this study were done in accordance with Finnish national legislation on the use of laboratory animals, the European Convention for the protection of vertebrate animals used for experimental and other scientific purpose (ETS 123), and EU Directive 86/609/EEC or were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

The embryos were obtained from pregnant wild-type CD-1, the CD-$1^{GFP\ 1}$ female mice, or mice that were generated by crossing the *Foxd1Cre*+ with the *GTRosaCAG* reporter mice that had inherited the floxed tdTomato gene encoding the Red Fluorescent Protein (RFP). The embryos that were positive for the *Foxd1Cre*+ and the *GTRosaCAG* were used. This permanently labels the daughters of the stromal cells that express the *Foxd1* gene[2]**.**The age of the embryos was considered to be E0.5 at noon the next day from the appearance of the vaginal plug. The metanephric kidneys were dissected from E11.25–11.5 mouse embryos in chilled Dulbecco´s PBS buffer **(**0.9mM $CaCl_2$, 2.7mM KCl, 1.5mM KH_2PO_4, 0.5mM $MgCl_2$, 137mM NaCl, and 8.1mM Na_2HPO_4**).**

For the experiments the whole embryonic kidney was prepared for culture. The dissected organ rudiments were placed on a Trowel-type culture system in Dulbecco's Modified Eagle's Medium (Gibco) with 10% fetal bovine serum (FBS, Sigma), 1%

penicillin/streptomycin (Sigma), and 10mM HEPES (Sigma).This medium is subsequently referred to as the normal medium. The explants were cultured at 37°C in a 5% CO2 environment typically for five days**.**

Dissociation of embryonic metanephric mesenchyme progenitor cells into a single cell suspension, their reaggregation and culture

To make the embryonic kidney mesenchyme progenitor dissociation and reaggregation assays (drMM, see Figure 1), the MMs were loosened from the ureteric bud (UB) with the aid of 2.25% trypsin (Sigma) and 0.125% pancreatin (Sigma) incubation for 30 to 40 seconds at room temperature (RT). The kidneys were given time to recover in the normal medium for 10–20 min at RT to inhibit the enzyme activity. Next, the UB was separated mechanically from the MM with 27G needles in normal medium.

In making the drMM explants, eight prepared MM tissues were pooled in a 1.5-ml LoBind Eppendorf tube (Eppendorf) in 240 µl of physiological buffer (137mM NaCl, 5.6mM KCl, 2.2mM $CaCl_2$, 1.2mM $MgCl_2$ x $6H_2O$, 2.5mM glucose, 10mM HEPES). The dissociation of the MM was achieved by adding 9400U/ml of collagenase III (Worthington) for 40 min to the solution at 37°C. The dissociation process of the MM was enhanced by constant stirring and gentle pipetting of the MMs. The stirring and pipetting was continued until a single-cell suspension was obtained. We used stereomicroscopic inspection to monitor the progress. Once dissociation of the MM was reached, fresh media was added to inhibit the enzyme activity. The cell suspension was washed twice with fresh medium coupled with centrifugation at 1380g for four minutes each time (See Figure 1).

It is critical that when assembling the drMM, the culture medium is supplemented with 50ng/ml of human recombinant BMP7 (Insight Biotechnology) and 100ng/ml of human

recombinant FGF2 (PeproTech). These growth factors are present in the culture medium throughout the drMM process but are removed prior to nephrogenesis induction.

During the final centrifugation step, the dMM cells are reaggregated (drMM) by pelleting the cells at 1380g for 20 min at RT. The cell pellet was then incubated at 37°C in a 5% CO_2 for two hours to promote the recovery of cell-cell adhesion between the MM cells[3]. After this, the MM tissue pellet was transferred to a Nuclepore Polycarbonate Track-etched membrane with 1.0-µm pore-size (Whatman).

The assembled drMM was transferred to the Trowel-type organ culture system at 37°C in a 5% CO_2 for 24 hours whilestill in the culture medium that contained the hrBMP7 and hrFGF2 growth factors so the tissue recovers well from the drMM step. Tubulogenesis was then induced by washing the hrBMP7/hrFGF2 away and by introducing the tubule inducing tissue, in this case, eSC. The tissue conjugate was cultured up to nine days in the absence of hrBMP7 and hrFGF2.

To assemble the chimeric MMs composed of the GFP^+ and GFP^- wildtype MM progenitor cells, one freshly dissected GFP^+ dMM was mixed with the cells derived from six wildtype/GFP^- dMMs.

Reconstitution of kidney development between the dissociated and reaggregated embryonic kidney mesenchyme and the ureteric bud

For the ureteric bud (UB) recombination experiments with the drMM, the micro surgically separated UBs were incubated for 30 min in 100ng/ml of human recombinant glial-cell-line-derived neurotrophic factor (GDNF) (PeproTech, USA) supplemented media. The GDNF was then washed away with fresh culture medium. Using a small glass capillary,

the UBs were transferred to the drMM pellet, which was left to reconstitute for one to two hours in medium supplemented with hrBMP7 and hrFGF2. Two to four UBs per eight MMs were used to recover the epithelial and mesenchymal interactions to advance kidney organogenesis *ex vivo*. It should be highlighted that hrBMP7 and hrFGF2 factors were removed when the UBs were added and the explants were cultured four to eight days. Ten pooled samples were generated and studied.

Extraction of the *Foxd1cre+* marked stromal progenitor cell lineages from embryonic kidney mesenchymal cells and their reaggregation assays

We applied the fluorescent activated cell sorting (FACS) technology for the mouse embryonic kidney to assess whether specificcell types could be extracted in this setting as reported earlier[2]. The *Foxd1cre+/floxed Rosa26Tdtomato* (RFP)-marked embryonic kidneys were prepared from the double positive embryos and a single cell suspension was made as described above. A UB marker FITC-conjugated Dolichos Biflorus Agglutinin (DBA) enabled FACS extraction of the cells from a cell suspension of the whole embryonic kidney rudiment. The *Foxd1cre+/floxed Rosa26Tdtomato*-marker served in turn to remove the stromal MM cells from the rest of the MM cells. Embryos that did not express the RFP were subjected to sorting with the DBA to extract the UB cells and to set up the drMM cultures (Figure 1, steps 1-B6).

The FACS extracted MM cells, which represent 1) the *Foxd1cre+*-labeled homotypic cells, 2) the rest of the MM cells, and 3) their reaggregates (drMM), were all pelleted as described above. The cell pellets were maintained for a maximum of 24 hours in a medium supplemented with hrBMP7/hrFGF2 growth factors to promote recovery. The growth factors were washed away and the eSC inducer tissue was placed onto the Nuclepore

filter on a metal grid. The eSC was covered with a second filter and the drMM placed directly on top of it. The conjugate was then cultured for five days, stained with Pax2 and Pecam1 (CD31) antibodies as whole mount, and photographed.

Monitoring the behavior of the dissociated and reaggregated embryonic kidney mesenchymal progenitor cell cultures via time-lapse image capture in culture

Embryonic kidney mesenchyme's (MM) were prepared at E11.5, dissociated, reaggregated (drMM, see Figure 1), and cultured in the presence of hrBMP7 and hrFGF2 for 24 hours as described above. The drMM explants were then positioned together with the eSC tissue on the Transwell permeable polyester membranes (Costar 3450, Corning, pore diameter 0.4 µm) and placed into a microscope stage incubator (OkoLab). DMEM without phenol red (Gibco) supplemented with 10 % FBS and 1 % penicillin/streptomycin was used as the culture medium. The Control Basic 2.3 software was used to keep the humidified air inside the incubator at a temperature of 37°C and the CO_2 concentration at 5%. The Olympus IX81 inverted microscope with a XM-10 digital camera and Cell^P software were used to record images at 20-minute intervals.

Cloning of the *shRNA* sequences to a retroviral viral vector

Short hairpin-expressing retrovirus vectors were created as described previously[4]. Oligos used in this study were: *Lhx1*_sense; 5'-GAT CTC CGG AGA TTA CCA GAG TGA GTA TTT CAA GAG AAT ACT CAC TCT GGT AAT CTC CTT TTT GGA AC-3'; *Lhx1*_antisense; 5'-TCG AGT TCC AAA AAG GAG ATT ACC AGA GTG AGT ATT CTC TTG AAA TAC TCA CTC TGG TAA TCT CCG GA-3'; The oligo pairs were annealed and

cloned into a BglII/XhoI-digested pRVH-GFP retroviral vector as described previously[4]. To determine the knockdown efficiencies of each shRNA-constructs, the oligos were cloned into a BglII/XhoI-digested pRVH-puro retroviral vector as previously described[4]. The KD efficiencies were determined in puromycine-selected mouse-kidney-derived MK4 cell line5 by quantitative PCR using the following primers: Lhx1Left: 5'-ATC CTG GAC CGT TTC CTC TT-3' and Lhx1Right: 5'-TCG GTC AGG TTG CAT TTA CA-3'.

Production an enrichment of the recombinant retroviruses

A human Phoenix gag-pol packaging cell line (http://www.stanford.edu/ group/nolan/retroviral_systems/phx.html; obtained from the American Type Culture Collection with authorization by Garry Nolan, School of Medicine, Stanford University, Stanford, CA) was kept in high-glucose DMEM (4.5g/L) containing 10% FCS, 2mM of glutamine, and 100units/ml of penicillin and streptomycin. Nearly confluent cells in 6-wells were transfected with 4µg of RVH1-puro and 0.4µg of pMD.G using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours post transfection, the medium was changed to low-glucose DMEM (1g/L) with the same supplements as above (1ml per 6-well), and kept at 37°C. Forty-eight hours post transfection the medium was collected, centrifuged for 5min at 200g to remove cell debris, and used for infection.

To produce concentrated virus stocks, Phoenix cells in 10-cm dishes were transfected with 24µg of retroviral vector and 2.4µg of pMD.G. The medium was changed to low-glucose DMEM (4ml per dish) 24 hours post transfection. Batches of virus-containing supernatant were then collected every 24 hours for up to four days. The supernatant was concentrated with Spin-X® UF concentrator columns (Corning) (used in experiments described in Figure 7 and 8) or by ultracentrifugation (used in experiments described in Figures 9 and SI-3).

The collected virus supernatant was filtered through a 0.45-µm filter. HEPES was added to 10mM and the supernatant was concentrated by Spin-X® UF concentrator (Corning) at 2000rpm at +4°C.

The approximately twelve times concentrated virus was aliquoted and kept at -70°C. When ultracentrifugation was used as the concentration method, the virus medium was centrifuged 16 800 rpm for two hours at +4°C. The supernatant was removed once approximately twenty times concentration had been achieved. The functional titers for SpinX- concentrated virus suspensions (assessed using Madin Darby Canine Kidney cells) ranged between 0.5–1x106 PFU/ml (for unconcentrated virus) and 0.5–1x107 PFU/ml (12x concentrated virus).

Transduction of the embryonic kidney mesenchymal progenitor cells with the recombinant retroviruses

To obtain protocols by which to transduce the embryonic kidney mesenchymal progenitor cells (See Figure 1, steps 1-6), the dissociated cells were washed as described above, with the exception that 2µg/ml of polybrene was included in the final wash with the culture medium. The enzymatically dissociated mesenchymal cells were spun down and the polybrene-supplemented medium was replaced by a solution composed of 50–100µl of the generated virus suspension (~106 PFU), 50ng/ml of hrBMP7 (Insight Biotechnology), 100ng/ml of hrFGF2 (PeproTech), 10mM HEPES (Sigma), and 2µg/ml of polybrene. The drMM pellet was dispersed by pipetting. The dMM with the viruses were brought into solution, incubated for an hour, and reaggregated by the centrifugation step. The drMM viral complex was transferred to the Trowel-type culture as described above. For

quantification of the Lhx1KD effect, three control and three knock down specimen were used.

Immunohistochemistry of the embryonic kidney mesenchymal progenitor cell reaggregates

The cultured induced or uninduced drMMs were fixed in 4% formaldehyde for 20 min at RT, washed once with PBS, and subjected either to immunochemical staining as whole mount specimen or embedded directly in O.C.T™ Compound (Tissue-Tek) for making cryostat sections. For O.C.T processing the explants were transferred via a sucrose gradient, first in 15% sucrose in PBS, then 30% sucrose, 30% sucrose/O.C.T. (1:1), and finally O.C.T only. The specimens were frozen in liquid nitrogen and stored at -75°C until sectioned with a Leica CM3050S cryotome into 5-µm-thick sections.

Both the whole mount specimens and the section specimens were permeabilized with 0.5% Triton-100X in 1 x PBS for 20 minutes at room temperature, and then blocked with 10% goat or donkey serum and 1% BSA in 0.02M glycine-PBS for 1–2 hours at room temperature. The diluted primary antibodies were incubated overnight at either room temperature (anti-Nephrin), +4°C (anti-AQ1, anti-Pax2, Troma-1, E-Cad, anti-CD-31, anti-Foxd1) +12°C (anti-NCC) or +37°C (anti-GFP). The specimens were washed several times with 0.02M glycine-PBS and blocked again as described above. The diluted secondary antibodies were incubated for 1–2 hours at room temperature.

The specimens were washed several times with 0.02M glycine-PBS and incubated in nuclear stain Hoechst (1:2000 in PBS) for 10 minutes at room temperature. After washing with PBS and sterile water, both the whole mount specimens and section specimens were

mounted in ImmuMount (Thermo Shadon). The whole mount specimens were gently pressed between an objective glass and a cover slip.

The antibodies used were polyclonal rabbit anti-Pax2 (Covance), mouse anti-CD-31 (BD Pharmigen), mouse anti-Aquaporin1 (CELL Applications), anti-Nephrin (kind gift from Prof. Karl Tryggvason), anti-NCC (Chemicon), mouse anti-Troma-1 (DSHB), anti-mouse E-cadherin (Invitrogen), goat anti-Foxd1 (Santa Cruz), rabbit anti-Six2 (Proteintech Europe), rabbit anti-Slc12a1 (Abcam), Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-rat IgG, Alexa Fluor 546 donkey anti-rabbit IgG (Molecular Probes) and donkey anti-goat Cy3 (Jackson ImmunoResearch). Immunohistochemical-stained specimens were examined under a confocal Olympus IX81 microscope with Olympus Fluoview Ver. 3.0a software or with an epifluorescent Olympus BX51WI microscope and Cell^M software.

Section *in situ* hybridization and histological staining of embryonic kidney mesenchymal progenitor cell reaggregates

The cultured MM and the induced drMM tissues were fixed as described above with the exception that the formaldehyde used was depc-treated and embedded in paraffin with Tissue processor Vip5 Junior (Tissue-Tek) and sectioned with a Microm microtome into 5-µm-thick sections. The plasmids for the digoxigening-labeled RNA *in situ* probes, which were used to identify a panel of markers of the segments of the nephron, were *Slc34a1, Clcn-Kb, Podxl* (kind gifts from Dr. Ritsuko Takada), *Slc12a1, and Slc12a3* (kind gifts from Prof. Andre W. Brändli). The *in situ* hybridization of mRNA in the paraffin-embedded sections was performed essentially as described by Breitschopf *et al.* (1992)[5]. The commercial reagents used for RNA probe labeling and detection were Digoxigenin RNA Labeling mix, Blocking Reagent, Anti-Digoxigenin-AP, and NBT/BCIP Stock Solution and

were all from Roche. For histological analysis the sections were stained with Hematoxylin and Eosin according routine protocols. Both the RNA *in situ* and histological specimens were photographed with a DM LB2 Research Microscope (Leica) combined with a Leica DCF320 Twain camera (Leica).

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