Conserved and divergent features of human and mouse kidney organogenesis

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Complete Methods

Dissemination of data

The histological and video image data are visible online at www.gudmap.org. High-resolution tile-scan images for SISH, IF, and H&E can be viewed through standard web-browsers. Specific DOIs for the histological and 3D data can be viewed at: https://doi.org/10.25548/BURB-6P44 and https://doi.org/10.25548/S5RE-NVCE : McMahon, A. GUDMAP Consortium.

Sample preparation for sectioning and immunofluorescent staining

For immunoanalysis, human kidney samples were fixed in 4% formaldehyde overnight at 4°C with mixed-motion provide by a Nutator (Thomas Scientific). Samples were subsequently washed twice with PBS then placed in 30% sucrose, 24 hours for week 8 and 48 hours for week 16 samples, prior to embedding and freezing in Optimal Cutting Temperature compound (Tissue-Tek, 4583). Mouse kidney samples were fixed in in 4% formaldehyde at 4°C for 20 min and washed twice with PBS and then placed in 30% sucrose for 24 hours prior to embedding and sectioning. All samples were sectioned at 10 µm intervals, placed on slides, and stored at -80°C before use. Immunofluorescent detection of target antigens was largely performed as previously described [1] with some modifications. Slides were washed in PBS for 5 min to remove OCT and subsequently blocked for 30 min in PBS with 1.5% SEA block (ThermoFisher Scientific, 37527) and 0.25% TritonX100. Primary antibodies were diluted in the blocking solution and applied to the samples overnight at 4 °C: SIX1 (Cell Signaling, 12891, 1:1000), JAG1 (R&D, AF599, 1:300) CUBN (Santa Cruz, sc-20607, 1:500), AQP2 (Santa Cruz, sc-9882, 1:300), CALB1 (Sigma, C9848, 1:300), VEGFR2 (Cell Signaling, 2479, 1:150), MAFB (Santa Cruz, sc-10022, 1:300), NPHS2 (Abcam, ab50339, 1:10,000), LRP2 (My Bio Source, MBS690201, 1:1000), SLC12A1 (Sigma, HPA018107, 1:1000), UMOD (R&D, AF5144 and AF5175, 1:500), SLC12A3 (Sigma, HPA028748, 1:300, SIX2 (Sigma Aldrich, SAB1401533; 1:500), SIX2 (MyBioSource, MBS610128; 1:1000), CITED1 (Abcam, ab55467; 1:300), FOXD1 (Santa Cruz, sc-47585; 1:750), KRT8 (DSHB, troma-1; 1:50), β-laminin (Santa Cruz, sc-33709; 1:300), PHH3 (Cell Signaling; 9706, 1:500), CDH1 (BD Transduction Laboratories, 610182; 1:300). After incubation in primary antibodies the samples were washed 3 times in PBS with 0.25% TritonX100 (PBT). Secondary antibodies were diluted in the blocking solution and applied to the sample for 1 hour at room temperature. All secondary antibodies were purchased from Molecular Probes ThermoFisher Scientific and used at a 1:1000 dilution. For 5 channel imaging, secondary antibodies were employed as follows: AlexaFluor 488, 555, 594, 647. After incubating with secondary antibodies, samples were washed 3 times in PBT and once in PBS. Nuclei were stained with 1 µg/ml Hoechst 33342 (Invitrogen) in PBS for 5 min before a final PBS wash. Sections were mounted in ProLong Gold Antifade Reagent (Life technologies) and imaged on either a Leica SP8 (confocal microscope) or a Zeiss Axio Scan.Z1 Slide Scanner.

Paraffin sectioning and H&E staining

To preserve optimum histology, samples used for H&E staining were fixed in Bouin's solution (Sigma) at 4°C overnight. Samples were subsequently rinsed 4 times in 70% ethanol with 0.85% NaCl. Samples were dehydrated through an ethanol series, washed in xylene then placed in paraffin (Shandon/Thermo Scientific) at 56°C for embedding. Sections were collected from wax blocks at 5µm intervals, dried, and stored at room temperature for long-term use. Prior to hematoxylin and eosin (H&E) staining, sections were dewaxed and rehydrated through a reverse xylene and ethanol series to PBS. Samples were stained for 30-45 sec each in hematoxylin then for 15-30 sec in eosin and finally counterstained with fast red for 1 minute. Samples were dehydrated and scanned at high resolution (10X) on a Zeiss Axio Scan.Z1 Slide Scanner to generate high-resolution tiled image files of the entire tissue section. The number and age-distribution of samples can be found in Table 1.

In situ hybridization

In situ hybridization stains were performed on frozen sectioned samples as previously described (https://www.gudmap.org/Research/Protocols/McMahon.html). The number and age-distribution of samples can be found in Table 1.

3D sample preparation and whole mount staining

As the renal capsule interferes with whole-mount immunofluorescence procedures, isolated kidneys were carefully decapsulated in PBS to not disturb the underlying tissue organization. To obtain kidney samples for whole-mount study, kidneys were placed on surgical gauze soaked in PBS with a petri dish and an approximately 3mm slice removed from each kidney lobe, parallel to the kidney surface. Each slice was fixed in 4% formaldehyde in PBS for 45 minutes at 4˚C without shaking, then washed in PBS. Whole mouse kidneys were isolated and similarly decapsulated but processed whole during the whole-mount staining procedure. Slices or whole kidneys were incubated for 1hr in PBS with 2% SEA Block and 0.1% TritonX100 at 4°C while shaking on a Nutator platform. Primary antibodies were resuspended in blocking solution, this mix was added to each tissue sample, then incubated for 48 hours with Nutator-directed sample agitation. Then, samples were washed for up to 8 hours in 5 washes of PBT. Secondary antibodies were diluted in blocking solution, and samples were incubated in secondary antibodies similarly to primary antibody incubation. The samples were washed 3 times in PBT before being incubated in 1 µg/ml Hoechst 33342 for 2 hours. The slices or kidneys were then washed in PBS, and dehydrated through a methanol series (50%, 75%, 100%), through a 50% Benzyl alcohol, Benzyl benzoate (BABB) / 50% methanol solution (1 hr) and finally into

100% BABB. Specimens were stored in BABB at 4°C until imaging. The number and age-distribution of samples can be found in Table 1.

Confocal imaging

Imaging of cortical slices (3D) were performed on a Leica SP8 using a 10x objective and a 40x objective (40x/1.30 Oil HC PL APO CS2). 1024x1024 images were captured at 5 µm and 0.35 µm optical resolution, respectively. Images for quantitative comparison of protein levels in the nephron progenitor compartment were performed on images captured using a 63x objective.

Image preparation for publishing declaration

Three dimensional (3D) images were opened and processed in LAS X (Leica), Imaris (Bitplane) and Photoshop (Adobe) while 2D sectional images were opened and processed in LAS X, Imaris, Photoshop, and Fiji. For both sets of images brightness, contrast, and transparency were altered for optimal rendering of fluorescent signals and tissue structures.

Image and sample quantification

2D immunofluorescent analyses

Frozen and sectioned samples were stained as described above to detect SIX2, SIX2, LEF1, FOXD1, and CITED1. KRT8 and β-laminin were used as structural markers to determine the location of the ureteric epithelium and nephrons. Images were captured with a 63x objective on a Leica SP8. Data were captured as 8-bit images. IMARIS 8.2 (Bitplane) was used for quantification of nuclear antibody signals. The Spot function was used to manually add circular spots to mark all nuclei on the image frame using DAPI-highlighted nuclei as a reference. Due to the convoluted shape of nuclei in 2D sections and the circular shape of the spot function, we used multiple smaller spots to represent single nuclei to ensure accurate quantitation and coverage across the nuclei. Spots were grouped into three cell populations: (1) cap mesenchyme (SIX2^{+} cells), (2) cortical interstitium (FOXD1^{+} cells), (3) all other cells. To compare the mean intensity of spots we first normalized the mean intensity values taking into consideration the background signal and the maximum signal for each channel. To do this we measured the intensity for each channel throughout all spots and identified the lower 5th percentile intensity mean (background), as well as the maximum value. Each spot's intensity mean was thereafter normalized as follows:

$$\left(\frac{Intensity\ mean\ of\ spot - 5th\ percentile\ intensity}{Maximum\ intensity - 5th\ percentile\ intensity}\right)x100$$

This transforms the intensity mean of each spot onto a 0-100 scale with the 5th percentile equaling 0 and the maximum being 100, respectively. To plot the normalized intensity of spots against their position within the cap mesenchyme population we marked the most cortical point of the cap mesenchyme and utilized this as point 0. A line was extended in a medullary direction parallel to the ureteric epithelium around which the cap mesenchyme was located.

3D immunofluorescent analyses

Three-dimensional image analyses were performed using Imaris 8.2 (Bitplane) and Amira (FEI Thermo Fisher Scientific). To count nephron progenitor cells around nephron progenitor tips, progenitor populations (SIX1+ human SIX2+ mouse) were segmented and the spot function was used to detect individual nuclei. These analyses were performed on data collected from the whole-mounts cortical slices (human) or whole kidneys (mouse). The human cortical slices were imaged at three different positions across the cortical slice surface. The mouse kidneys were image twice per kidney in triplicate kidneys. For the analyses the following number of (individual niches) were examined per stage: wk11 (9); wk13 (13); wk15 (18); wk16 (16); wk17 (17); wk18 (18); wk23 (20); ms E15.5 (13); ms P2 (20). To analyze ureteric branch tip spacing, tips were identified tips and manually marked using the spot function. The XYZ coordinate of all tips within an imaged area was compared to each other to identify the nearest neighboring tip. Distance intervals were determined using Matlab (MathWorks). The following (number of tips) were analyzed for each stage from 1 kidney per age: wk11 (217); wk13 (233); wk15 (273); wk16 (297); wk17 (266); wk18 (161); wk23 (310); ms E15.5 (264); ms P2 (281). To quantify the number of ureteric epithelial tips per niche, niches were individually selected and tips counted. The following (number of niches) were analyzed: wk11 (60); wk13 (29); wk15 (25); wk16 (46); wk17 (27); wk18 (20); wk23 (69); ms E15.5 (18); ms P2 (31). The number and age-distribution of separate samples can be found in Table 1.

RNA expression analyses

RNA isolation for whole kidneys

Week 9 kidneys were treated whole and larger kidneys were cut to generate a wedge from the cortex to the medulla. The ureter was removed prior to processing. The tissue was manually cut into small pieces using a scalpel and placed in an Eppendorf tube with 500 µl Trizol LS (Thermo Fisher Scientific) and subsequently homogenized. 100 µl Chloroform (Thermo Fisher Scientific) was added per sample and the samples vigorously mixed. The samples

were incubated for 5-10min at room temperature followed by centrifugation at 14,000 RPM for 15 min at 4°C. After centrifugation, the aqueous phase was moved to a new tube, 500 µl Isopropanol (Thermo Fisher Scientific) was added and the samples separately mixed. After 5-10 min at room temperature the samples were again centrifuged at 14,000 RPM for 15 min at 4°C. The resultant RNA pellet was washed with 80% Ethanol and centrifuged at 14,000 RPM for 5 min at 4°C. Finally, the RNA pellet was air dried and then resuspend in RNAse free water. DNA was at this point removed by treating the samples with Dnase I, and collection using spin columns RNeasy Micro Kit (QIAGEN). The number and age-distribution of samples can be found in Table 1.

RNA-seq data generation and analysis

All RNA samples were polyA selected, synthesized into Illumina NGS libraries with Kapa stranded mRNA-Seq kit, and were sequenced on Illumina NextSeq500 platform by the pair-end 75 bp option. Mouse and human mRNA-Seq reads were aligned to human reference genome (hg38) using TopHat2 [2]. To quantify gene expression, we calculated Reads Per Kilobase Million (RPKM) values with aligned mRNA-Seq reads using Partek Genomics Suite (version 6.6, St. Louis, MO, USA. To remove bias created by the polyA selection procedure, Transcripts Per Million (TPM) was calculated by normalizing the RPKM to the proportion of exon reads within individual libraries. Gene ontology enrichment analyses were performed using DAVID (Huang et al., 2009).

For results shown in Figure 8 due to insufficient number of samples for t-tests, we identified differentially expressed genes between week 9 and week 21 human kidney samples by the following threshold: 1) mean TPM > 5 in at least one of the groups; 2) mean fold difference > 2 between groups. For all the rest of the comparison between stages, due to overall similarity between samples, we used following threshold: 1) mean TPM > 5 in at least one of the groups; 2) mean fold difference > 1.5 between groups.

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