Nephrons require Rho-kinase for proximal-distal polarity development

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Epithelial tubules must have the right length and pattern for proper function. In the nephron, planar cell polarity controls elongation along the proximal-distal axis. As the tubule lengthens, specialized segments (proximal, distal etc.) begin to differentiate along it. Other epithelia need Rho-kinase for planar cell polarity but it is not known whether Rho-kinase is involved in this way in the nephron. We show that Rho-kinase is essential for the morphogenesis of nephrons, specifically for correct cell orientation and volume. We use fluorescent reporter-models and progenitor-specific markers to demonstrate that inhibition of Rho-kinase prevents proper proximal-distal axis formation, causes segments to develop abnormally, and progenitor-cell segregation to fail. Our data demonstrate the importance of Rho-kinase in normal nephron tubulogenesis and patterning.

Results

Rho-Kinase is required for normal nephron formation. We blocked ROCK function during nephron formation by applying ROCK-inhibitors glycyl-H1152 (hereafter H1152)22,23 and Y2763224, separately, to various embryonic kidney culture systems3,25,26. To monitor effects on nephron development we used kidneys from E12.5 Wt11/GFP knock-in reporter mice27 and wild-type embryos to track the early nephron and podocyte progenitors28, and antibodies against tubule basement membrane (β-laminin) and the ureteric bud (Calbindin-D28K)29. ROCK-inhibited kidneys contained morphologically abnormal nephrons (Fig. 1b and Fig. S1b–d) compared to controls (Fig. 1a and Fig. S1a,c). We counted the number of morphologically normal and abnormal nephrons in treated and untreated wild-type kidneys (Fig. 1c; see Materials and Methods for scoring criteria). 61% of nephrons forming in ROCK inhibiting conditions (n = 76; from 3 kidneys) displayed morphological abnormalities whilst only 5% of control nephrons (n = 58; from 3 kidneys) did so (p = 0.00145 by Student’s t-test).

We and others have previously shown that the ureteric bud is itself affected by ROCK inhibitors22,25 (Fig. 1b and Fig. S1b). We tested whether the there was also a reduction in the number of nephrons, which
there was (Student’s t-test p = 0.003 for control vs. 2.5 μM H1152), and whether this correlated with a decrease in the number of ureteric bud tips and nephrons plotted against specified concentrations after 96 hrs of culture. Error bars indicates SEM in both graphs. P-values related to differences in categories. (c) Mean percentage of abnormal, normal, and ambiguous nephrons in controls and experimental wild-type kidneys after 120 hrs of culture. P-values related to differences in categories. (d) Mean number of ureteric bud tips and nephrons plotted against specified concentrations after 96 hrs of culture. Error bars indicates SEM in both graphs. P-values related to reduction in tip-numbers. (e) Induced isolated mesenchyme in control conditions and (f) ROCK conditions. Abnormal epithelialisation (arrows), normal nephrons (arrowheads). Antibody stains and culture times as specified on images.

Figure 1 | Inhibition of ROCK reduces nephron formation and disturbs normal morphology. (a) Control culture of Wt1-GFP kidney. (b) Culture of Wt1-GFP kidney grown in ROCK inhibiting conditions. Nephrons indicated with arrowheads. (c) Mean percentage of abnormal, normal, and ambiguous nephrons in controls and experimental wild-type kidneys after 120 hrs of culture. P-values related to differences in categories. (d) Mean number of ureteric bud tips and nephrons plotted against specified concentrations after 96 hrs of culture. Error bars indicates SEM in both graphs. P-values related to reduction in tip-numbers. (e) Induced isolated mesenchyme in control conditions and (f) ROCK conditions. Abnormal epithelialisation (arrows), normal nephrons (arrowheads). Antibody stains and culture times as specified on images.

of ROCK still caused the formation of aberrant and tangled epithelial structures (Fig. 1f and Fig. S1f). As suggested by previous studies, the nephron mesenchymal-to-epithelial transition itself is not blocked by ROCK inhibition. To test whether the timing of ROCK inhibition influenced the degree to which nephron formation was disturbed, we added the inhibitor at different time-points or withdrew it once added (Fig. S2). E12.5 kidneys were used. Withdrawing the inhibitor after 24 hrs or 48 hrs and culturing in normal medium to a total of 72 hrs allowed nephrogenesis to recover to produce 90% and 60%, respectively, of the total nephrons found in controls. Cultures continuously maintained
Rho-Kinase is required for tubule morphogenesis. Nephron development is highly dynamic so, to visualise and characterise the nephron defects, we set up time-lapse cultures of kidney from Pax8\(^{−/−}\);YFP\(_{pax8}\)-reporter mice\(^{22,25}\). Pax8 is expressed in the ureteric bud, in induced nephrogen progenitor cells, and in the nephron itself\(^{3}\). Pax8\(^{−/−}\);YFP\(_{pax8}\)-reporter kidneys stably express YFP in all nephron lineages (Movie 1: the Pax8\(^{−/−}\);YFP\(_{pax8}\)-reporter mouse cross and lineage tracing experiments are to be fully described in Berry et al. in preparation). We added the ROCK inhibitor either at the start of the experiment, before nephrons formed (Fig. 2b), or only after 48 hrs of culture (Fig. S3a and Movie 2) when nephrons had epithelialised and started segmentation. Both cases resulted in a variety of defects. Nephrons that formed in ROCK-inhibitor conditions epithelialised (i.e. they expressed Cdh1) and initiated segmentation (i.e. they expressed Jag1-in their medial domain) (Fig. 2b and Fig. S3a) in a similar manner to controls (Fig. 2a). Consistently, however, they failed to elongate normally and instead rounded up into cyst-like structures (Movie 2).

Kidney tubules elongate along their proximal-distal axis by a mechanism of convergent extension, resulting in cells that have completed the process being positioned at an angle perpendicular to that of the direction of the tubule\(^{4}\). We investigated whether the morphological defects could be caused by the cells being abnormally polarised. To do this we generated cell 3D reconstructions and measured the orientation of these nephron cells within the tubules, as described elsewhere\(^{5}\) and in Materials and Methods. We measured each cell's orientation compared to the direction of the tubule and calculated the deviation of this value from the 90° angle expected by a perfectly perpendicularly aligned cell. The cell angles were measured as positive numbers, regardless of the direction of the deviation, in order to gain an absolute measure of error that would not be cancelled by equal errors in opposite directions by different cells. In controls, the mean deviation was 17° (Fig. 2c, f; n = 83 cells from 7 nephrons). ROCK inhibitor treated nephrons displayed two distinct sets of cell populations, those which were located within morphologically more normal epithelial tubules (Fig. 2d, g; n = 85 cells; from 6 nephrons) and those within bloated portions of tubules (Fig. 2e, h; n = 61 cells from 5 nephrons). The cells in the more normal tubules averaged a higher mean deviation 25° compared to controls (Fig. 2g; 25°, p = 0.006). Those cells located within bloated tubules displayed a much greater degree of deviation 33° compared to controls (p = 4 × 10\(^{−4}\)).

In other tissues, ROCK activity is important for the regulation of cell size\(^{5,5}\). We tested whether the cell size was also altered by ROCK inhibition by analysing the volume of the cells (measured by 3D reconstruction from optical sections). ROCK-inhibited cells from both ‘normal’ and bloated nephron segments showed a large increase (+78%) in cell volume compared to controls (p = 6 × 10\(^{−4}\); n = 94 cells from control conditions vs. 159 cells from H1152 conditions) (Fig. S3b).

Rho-Kinase is required for progenitor segregation but not differentiation. The proximal-distal axis is populated by domains containing different progenitor cells that will give rise to segment-specific lineages. To test whether the cell-polarisation defects affected the specification or location of these progenitor cell populations we performed antibody stains against segment-specific proteins and examined kidneys for regions of abnormal expression overlap. Wt1 and Podxl were used to detect glomerular/podocyte progenitors. Lotus tetragonolobus lectin (LTL) for proximal tubule cells and Jag1 for Loop of Henle and proximal tubule progenitors\(^{28,57−59}\). The distal tubule displays strong Cdh1 and is negative for the other markers used\(^{6}\). Remarkably, in controls only 4/891 (0.4%) of nephrons examined displayed spatial overlap of segment markers in any area of the nephron (Fig. S4a). In ROCK-inhibitor treated samples, this overlap was still uncommon but increased 10-fold (25/549; 4.5%; Fig. S4a). A Chi Square test was significant to p = 0.001, d.f. = 1 x\(^{2}\) = 29.0. Overlap between Wt1 and Jag1 domains (i.e. glomerular podocyte and proximal/medial tubule) accounted for 80% of these cases. Wt1 and Jag1 are expressed during the earliest stages of nephron development, whilst LTL binding and Podxl expression is initiated at later time-points. We carried out confocal scans at higher magnification (60×) and these showed a range of segmentation abnormalities: engulfed glomeruli (Fig. 3a and Fig. S4b), overlap of segment-specific expression (Fig. 3b and Fig. S4c), and what appeared as loosely structured glomeruli (Fig. 3c and Fig. S4d).

As ROCK activity can also affect differentiation\(^{46}\) we used qRT-PCR to analyse the expression of 11 genes exclusively expressed in specific nephron-segments (Fig. 3d). It would be expected from data presented above that by applying the ROCK inhibitor we would reduce nephron numbers by ~70% (Fig. 1d), thus, any significant reduction would have to be greater than this. Expression levels were significantly decreased for 6/11 genes analysed; however, they were decreased by an average of 64% (Fig. 3d), not far from the expected 70% decrease. This suggests that the reduction in gene expression is caused by the decrease in nephron formation rather than by loss of differentiation of any particular nephron segment.

We focused on the nephron defects that displayed abnormal segregation of segment-progenitor cells (Fig. 3b). To determine whether the Wt1\(^{+}\) podocyte progenitor cells (Fig. 3b) also extended into the tubular portion of nephrons we again used the Pax8\(^{−/−}\);YFP\(_{pax8}\)-reporter to mark all nephron cells (Fig. 4a). Nephrons displayed Wt1\(^{+}\) cells within tubular regions that were also Cdh1\(^{+}\) (Fig. 4a). We confirmed this by 3D visualisation (Fig. 4b, Fig. S5a). Another defect perhaps related to tubule identities was the occurrence of nephrons with two connections to the ureteric bud. These were fused at their Jag1 proximal domain (Fig. 4c and Fig. S5c). To confirm that such nephrons were indeed caused by nephrons abnormally fusing to other nephrons we set up time-lapse experiments with Wt1\(^{−/−}\)GFP knock-in reporter mice. In ROCK-inhibitor treated Wt1\(^{−/−}\)GFP kidneys, nephrons occasionally came head-to-head and fused with each other (Fig. 4d and Movie 4). In experimental conditions, 6/6 kidneys displayed such nephron-to-nephron fusions. The number of fusions per kidney ranged from 5 sets to only 1 (average was 2.7). In control conditions (Fig. 4d and Movie 3) such nephron-to-nephron fusions were less frequent (p = 0.049). 4/6 control kidneys displayed fusions at an average of 0.83 fusions per kidney.
Discussion

As the nephron forms, it develops multiple segments that express genes and display properties distinct to each segment. During the process of segmentation it also undergoes a series of complex morphogenetic changes; for instance it connects with the ureteric bud, it elongates, and it develops the Bowman’s capsule. This division of the nephron into domains is evident from the stage directly after epithelialisation.37,42,43 Because the segmentation, patterning, elongation, and morphogenesis occur simultaneously, they are likely to at some level be co-dependent. Here, we have demonstrated that Rho-kinase (ROCK) activity is required for both morphogenesis and the patterning of the nephron. With reduced ROCK activity, serious failures of morphogenesis occur and we show that these are associated with the disorientation of cell axes within the nephron tubule.

Figure 2 | Inhibition of ROCK perturb normal planar cell polarity and elongation. Time-lapse of Pax8Cre-YFPlox-stop kidneys in (a) control conditions, or in (b) ROCK inhibitor conditions. Boxed area - confocal scans performed on same kidneys. Nephrons indicated with arrowheads. 3D reconstructions of tubular cells in (c) control conditions (d, e) ROCK inhibiting conditions. Total cell numbers shown on cell-orientation graphs. Graphs display the percentage frequency of cells exhibiting a particular angle of deviation from the perpendicular. Antibody stains and culture times as specified on images. p-values were calculated using Student’s t-tests.
In Xenopus nephrons, the elongation of the nephron tubules is regulated via a process of convergent extension. This elongation is dependent on Wnt-signalling and a mechanism which controls the precise alignment of cell axes. Mouse nephrons rely on a similar alignment of cells as the nephron elongates, and this requires Wnt9b-PCP signalling, which is associated with JNK activation and therefore probably with the PCP pathway. Because ROCK is known to be activated in the PCP pathway, our work therefore brings these

Figure 3 | ROCK is required for tubule morphogenesis. Nephrons in ROCK inhibitor conditions display a range of defects as characterised using segment-specific markers. (a) WT1+ glomeruli forming within LTL+ proximal tubule. (b) WT1+ and JAG1+ cells overlap in their expression domains. (c) PODXL+ podocytes within glomeruli are poorly packed and fragment from glomeruli. Dashed lines outline nephrons. Arrowheads indicate cells with overlapping expression of WT1 and JAG1. (d) qRT-PCR analysis on segment-specific genes. RNA was extracted from whole treated kidneys and the qRT-PCR analysis was performed against genes expressed in specific cell-types within particular nephron segments. Error bars indicate SEM, p = values stated in Methods section. Antibody stains as specified on images.
observations together to suggest that the PCP pathway acts through ROCK to control cell orientation in the nephron tubule.

ROCK is also required for normal cell migration of pre-epithelial nephron cells. Although, it is not known at what precise stage the nephron is patterned into separate segments, it is known that, the renal vesicle already displays polarised gene expression directly after epithelialisation. One possibility is that the specification of nephron segment identities begins prior to epithelialisation when mesenchymal nephron progenitor-cells aggregate and prepare for a mesenchymal-to-epithelial transition. If so, this could indicate that some of the defects observed here result from abnormal migration of cells during pre-epithelial stages of nephron development.

**Figure 4** | ROCK is necessary for progenitor cell segregation and segment identity. Confocal projections of Pax8\(^{cre}\)/YFP\(^{lox-stop}\) kidneys cultured in control or in ROCK inhibiting conditions (a). Arrowheads indicate nephrons. (b) High magnification of WT1\(^{+}\) tubular region. Arrowheads indicate WT1\(^{+}\) and CDH1\(^{+}\) cells. (c) Nephrons in control conditions and in ROCK inhibiting conditions, the latter fused to another nephron. (d) Time-lapse of Wt1\(^{+}\)/GFP kidney in control and ROCK inhibiting conditions, the latter displaying fusion of three GFP\(^{+}\) glomeruli. Antibody stains and culture times as specified on images.

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however unlikely to be the sole mechanism as post-epithelial nephrons are also affected by inhibition of ROCK but it raises questions regarding the importance of cell-migration for early nephron patterning.

Other consequences of losing normal ROCK activity included the failure of some nephrons to connect with the ureteric bud (Figure 2b), the abnormal positioning of the glomerulus within the tubule (Figure 3a), and the disorderly segregation of different nephron segments along the tubules (Figure 3b). ROCK serves an important function for the remodelling of epithelia in other tissues via myosin-dependent mechanisms45,46 and in Drosophila, actin-myosin tension is known to be required for the maintenance of compartment boundaries47. Since ROCK is an activator of myosin, it is possible that in kidneys too, proper epithelial remodelling and compartment boundary formation require adequate actin-myosin tension, and this is required for segment segregation. Our data highlight the importance of integrating the mechanisms driving the morphogenesis of the nephron with those that regulate the differentiation programmes that are occurring as the nephron segments are formed.

**Methods**

**Animal experiments.** All animal experiments were approved by the Edinburgh University Animal Welfare and Ethical Review Body (AWERB). All animals were kept at the MRC Human Genetics Unit and University of Edinburgh animal facilities. All mice were kept according to regulations specified by the Home Office. Animals were kept and bred under P.H. Project Licence 60/3788 as approved by the Home Office.

**Organ cultures.** Kidneys were isolated from E11.5–E12.5 mice. Wt1+EYFP (Wt1+/EYFP) mice were crossed with CD1. Pax8+EYFP (Pax8+/EYFP) mice were crossed to Rosa26YFP+YFP reporter kidneys were used. 14 in control conditions, 9 with H1152 added at 0 hrs, 7 with H1152 added at 48 hrs, and 5 with H1152 added at 72 hrs. 8 kidneys were used for time-lapse. Kidneys were cultured as previously described by Michael et al.48. Isolated mesenchyme was induced using E11.5 dorsal spinal cord in transfilter cultures with the mesenchyme being placed on the top and the spinal cord at the bottom. Controls were carried out using cultures of nephrons formed in transfilter spinal cord-induce mesenchyme where the spinal cord was removed after 24 hrs of induction in order to test whether the effects of inhibitors, on nephrons, were direct (and not secondary effects on the spinal cord). Control conditions contained an equal volume of inhibitor vehicle. Inhibitors were added at the beginning of culture (t = 0) unless otherwise specified. Culture medium was changed every three days unless otherwise specified.

**Quantification of kidney morphology and nephron formation.** To quantify the kidney response to ROCK inhibitor, E11.5 kidneys were cultured and treated with a range of ROCK inhibitor concentrations (0, 0.08, 0.16, 0.63, 1.25, and 2.5 μM) for 96 hrs in organ culture conditions. Ureteric bud marker Cadherin D-28K and ureteric bud and nephron marker laminin. Ureteric bud tips and nephrons were counted for each condition starting (t = 0) unless otherwise specified. Culture medium was changed every three days unless otherwise specified.

**3D reconstructions, volumetric analyses, and cell orientation.** Confocal stacks (1 μm steps, captured at 60 ×, 1024 × 1024 pixels at 0.2071602 μm/pixel) of whole nephrons were loaded into TrakEM2 - Fiji (http://fiji.sc/). The automatic segmentation tools were found to be insufficient in separating individual cells whereas manual marking was found to be more robust and was therefore utilised. Cells were thus manually marked throughout each cell’s z-plane. The annotated cells were subsequently clustered using confocal microscopy. Zeiss Axio Imager.A1 or Nikon A1R. Time-lapse imaging was carried out using a Nikon T2E microscope and nuclei were stained with DAPI. Images were used at the following concentrations: anti-Laminin (L9393/ Sigma), anti-Cd1 (M0733/DAKO), anti-CdH1 (610181/BD), anti-RS-pan-cytokeratin (C2562/Sigma), anti-WT1 (sc-192/SANTA CRUZ), anti-JAG1 (R&D Systems). Nuclei were stained with TO-PRO-3 iodide (T3605/MOLECULAR PROBES), 1: 250. Secondary antibodies were purchased from Sigma and Molecular Probes. Rho-kinase inhibitor Y27632 (Y0503/Sigma) was reconstituted in dH2O, stored at −20°C and used at 10 μM–20 μM; 20 μM was used. Glycyl-H1152 dihydrochloride (2485/Tocris) was reconstituted in dH2O, stored at −20°C and used at a range of 1.25 μM–2.5 μM; 1.25 μM was used in all experiments except for experiment shown in (Fig. 1d) looking at dosage response.

**Segment-specific gene expression.** To isolate RNA, cultured control and experimental kidneys were placed in RNAlater (Ambion) for stabilisation. A minimum of 9 kidneys were used per condition, with 3 kidneys per replicate. The RNA isolated and used for quantification was the same in Figure 3 and Figure S1. RNA isolation was performed using RNeasy Micro kits (Qiagen) from the whole kidney. RNA was used for microarray analysis and RT-qPCR. Primers were designed using SuperScript II Reverse Transcriptase (Invitrogen) with Primers (Promega). qRT-PCR assays were performed using the Universal Probe Library (Roche) and all primers/probes were designed using the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?uid=U093000). The primers and probes used were: Slc3a1 (F: cattttgaattggttggtcctac ggtatatttgcttgaactggag R: cgccttggggcttctctcttg probe:4), Slc12a1 (F: gactgttcctgggctctccctac ggtactgctggctgctct gggtgctctctcgctgac R: ggccttggggcttctctcttg probe:9). TaqMan assays were run on a Light Cycler 480 (ROCHE) using 384-well plates, 60 cycles of melting of 95°C 10 s, 60°C 30 s, 72°C 1 s.

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**Author contributions**

N.O.L. and J.A.D. designed the experiments and N.O.L. performed experiments. P.H. provided Pax8<sup>+/+<sup>,<sup>YFP<sup>+/+<sup> and Wt1<sup>−/−<sup> models and assisted with manuscript preparations. N.O.L. and J.A.D. analysed experiments and wrote the manuscript.

**Additional information**

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