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RESEARCH ARTICLE

Intact prostaglandin signaling through EP2 and EP4 receptors in stromal progenitor cells is required for normal development of the renal cortex in mice

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Abstract

Cyclooxygenase (Cox) inhibitors are known to have severe side effects during renal development. These consist of reduced renal function, underdeveloped subcapsular glomeruli, interstitial fibrosis, and thinner cortical tissue. Global genetic deletion of Cox-2 mimics the phenotype observed after application of Cox inhibitors. This study aimed to investigate which cell types express Cox-2 and prostaglandin E_2 receptors and what functions are mediated through this pathway during renal development. Expression of EP2 and EP4 mRNA was detected by RNAscope mainly in descendants of FoxD1$^+$ stromal progenitors; EP1 and EP3, on the other hand, were expressed in tubules. Cox-2 mRNA was detected in medullary interstitial cells and macula densa cells. Functional investigations were performed with a cell-specific approach to delete Cox-2, EP2, and EP4 in FoxD1$^+$ stromal progenitor cells. Our data show that Cox-2 expression in macula densa cells is sufficient to drive renal development. Deletion of EP2 or EP4 in FoxD1$^+$ cells had no functional effect on renal development. Codeletion of EP2 and EP4 in FoxD1$^+$ stromal cells, however, led to severe glomerular defects and a strong decline of glomerular filtration rate (1.316 ± 69.7 µL/min/100 g body wt in controls vs. 644.1 ± 64.58 µL/min/100 g body wt in FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice), similar to global deletion of Cox-2. Furthermore, EP2/EP4-deficient mice showed a significant increase in collagen production with a strong downregulation of renal renin expression. This study shows the distinct localization of EP receptors in mice. Functionally, we could identify EP2 and EP4 receptors in stromal FoxD1$^+$ progenitor cells as essential receptor subtypes for normal renal development.

NEW & NOTEWORTHY Cyclooxygenase-2 (Cox-2) produces prostaglandins that are essential for normal renal development. It is unclear in which cells Cox-2 and the receptors for prostaglandin E_2 (EP receptors) are expressed during late nephrogenesis. This study identified the expression sites for EP subtypes and Cox-2 in neonatal mouse kidneys. Furthermore, it shows that stromal progenitor cells may require intact prostaglandin E_2 signaling through EP2 and EP4 receptors for normal renal development.

cyclooxygenase-2; interstitial cells; prostaglandin receptors; renal development; renal function

INTRODUCTION

Nephrogenesis is a complex process involving the timed expression of various transcription factors to develop glomeruli, their attached nephron structure, and the stromal cells responsible for the kidney's structural integrity (1, 2). Reduced cyclooxygenase-2 (Cox-2) activity in a critical timeframe near the end of renal development is associated with defective kidneys, leading to cortical thinning, subcapsular glomerulosclerosis, and a decline of renal function (3–6).

Experimental and epidemiological evidence suggests that the critical timeframe for Cox-2 expression is the final stage of renal development in the last trimester for humans and correspondently in the first 10–14 days postpartum in mice (2, 7–9). The enzyme Cox-2 catalyzes the reaction of arachidonic acid into prostaglandin (PG)H_2. PGH_2 is a versatile precursor for different biologically active products, such as PGE_2, PGD_2, PGI_2, and thromboxane A_2 (10). The different PGs derived from PGH_2 are generated by specialized synthases differentially expressed in the tissues. Three isoforms (microsomal PGE synthase-1, microsomal PGE synthase-2, and cytosolic PGE_2 synthase) have been described for the enzyme that produces PGE_2, the major PG in the kidneys (7, 11–13). Like the synthases that process PGH_2, the various prostanoids derived from it have specific receptors (11, 14, 15). Four different receptors, designated as EP1–EP4, have been identified for PGE_2. Although these receptors share only a low degree of homology, all are G protein-coupled transmembranous receptors with a high affinity for PGE_2 (11, 16). EP1 and EP3 have been localized mainly in the tubular system of different species, with EP3 also present in the vasculature (11, 16–18). Functionally, EP1/IP_3 and EP3/G_i receptors are important regulators of aquaporin (AQP) function and urine concentration in the kidney. Although EP2 and EP4 are both G_s/cAMP-coupled, different localizations, functions, and selective inhibitors for these receptors have been described in the literature (11, 16). EP2 and EP4 have been reported in tubular segments of the kidneys, in

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Table 1. *Primers used for genotyping of mice and size of the resulting PCR products*

Construct	Sequence (5′–3′)	Product Size, bp
Cox-2^{del}	Sense: AATTACTGCTGAAGCCCACC Antisense: GAATCTCCTAGAACTGACTGG	1,054 = del
Cox-2^{flox}	Sense: AATTACTGCTGAAGCCCACC Antisense: AGAAGGCTTCCCAGCTTTTGTAACC	1,058 = flox 823 = wt
EP2$^{-/-}$	Sense: 1: TAATGGCCAGGAGAATGAGG Antisense: 1: GCTATCAGGACATAGCGTTGG Sense: 2: TAATGGCCAGGAGAATGAGG Antisense: 2: CTGGTAACGGAATTGGTGCT	1,100 = flox575 = wt
EP4^{flox}	Sense: GTTAGATGGGGGGAGGGGACAACT Antisense: CGCACTCTCTCTCTCCCAAGGAA	127 = wt231 = flox
FoxD1$^{+/Cre}$	Sense 1: CTCCTCCGTGTCCTCGTC Sense: 2: TCTGGTCCAAGAATCCGAAG Antisense: GGGAGGATTGGGAAGACAAT	450 = Cre237 = wt
SMCX-1 and SMC4-1 (for sex genotyping)	X: CCGCTGCCAAATTCTTTGG Y: TGAAGCTTTTGGCTTTGAG	350 = female350 + 300 = male

Cox-2, cyclooxygenase-2. EP2/4, prostaglandin E_2 receptor 2 or 4; FoxD1, forkhead box protein D1.

glomeruli, and in the vascular system. Functionally, they are involved in salt handling, blood pressure homeostasis, renin regulation, and progression of renal damage (19–25).

However, little is known about the expression of these receptors and their role in renal development. A number of drugs that have a good side effect profile in adults may cause kidney damage when they are applied during nephrogenesis (26–32). New selective antagonists for EP2 and EP4 are currently under development for a large variety of different indications (11, 33–35). Due to their role in inflammation, selective inhibitors for EP4 are being investigated as an alternative to classical Cox inhibitors (11, 33, 34, 36). However, the potential side effects of these new drugs on nephrogenesis are not yet clear (11).

Murine kidneys develop from three major cell populations characterized by the expression of distinct transcription factors (37). HoxB7$^+$ cells give rise to collecting ducts, whereas the tubular system derives from Six2-expressing cells. Stromal FoxD1$^+$ cells differentiate into glomerular mesangial cells, vascular smooth muscle cells, renin-producing cells, and interstitial fibroblast-like cells of the kidneys (37, 38). All cells derived from FoxD1$^+$ progenitor cells are affected by the renal phenotype observed in mice with global deletion of Cox-2 or in mice treated with Cox-2 inhibitors during the final phase of renal development. The phenotype resulting from disrupted PG synthesis at this stage is highly comparable in rodents and humans, making rodents a suitable model organism for studying the mechanisms leading to the renal abnormalities.

Therefore, this study investigated which PGE_2 receptors are expressed in stromal progenitor cells during renal development and whether they are necessary for normal renal development.

To this aim, we first performed high-resolution in situ hybridization on neonatal mouse kidneys with strong mRNA expression and well-distinguishable structures to localize cells expressing the different EP receptor isoforms and Cox-2. We used a cell-specific approach in mouse models to target the genes encoding Cox-2, EP2, and EP4 and to study their function during development. First, we disrupted PG production through Cox-2 deletion in FoxD1$^+$ stromal progenitor cells. Second, we investigated the effects of deficient receptor signaling by deleting either EP2 globally or EP4 only in FoxD1$^+$ cells. To investigate a possible compensation between these two receptors, we also generated a mouse strain with cell-specific deletion of EP4 in the FoxD1 lineage with simultaneous systemic deletion of EP2. In adult animals of the different mouse models, we analyzed renal morphology, renal function, and expression of fibrotic factors as well as renin expression.

MATERIALS AND METHODS

Mouse Models

All animal experiments were conducted according to the National Institutes of Health *Guide for the Care and Use of Animals in Research* and were approved by the local ethics committee. All mice were kept under optimal conditions, consisting of constant 23°C room temperature, relative humidity of 55 ± 5%, and a constant 12:12-h dark-light cycle. Standard rodent chow (0.6% NaCl, Ssniff) and tab water were provided ad libitum. All mice were bred in a C57/Bl6/J background. To study the function of Cox-2 during nephrogenesis, mice with Cre recombinase under control of the FoxD1 promotor, FoxD$^{1tm1(GFP/cre)Amc}$ (FoxD1$^{+/Cre}$, stock no. 012463, Jackson Laboratories, RRID:MGI:4437922) (39, 40) were crossed with mice bearing the floxed alleles for Cox-2

Table 2. *Primers used for real-time PCR*

Gene	Sequence (5′-3′)
Col1a1	Sense: CTGACGCATGGCCAAGAAGA Antisense: ATACCTCGGGTTTCCACGTC
EP1	Sense: GACGATTCCGAAAGACCGCAG Antisense: CAACACCACCAACACCAGCAG
EP2	Sense: TGCTGGCTTCATATTCAAGAAA Antisense:TGGCCAGACTAAAGAAGGTCA
EP3	Sense: TGCTGGCTCTGGTGGTGAC Antisense: ACTCCTTCTCCTTTCCCATCTGTG
EP4	Sense: TCATCTGCTCCATTCCGCTC Antisense: GTCCAGGATGGGGTTCACAG
Renin	Sense: ATGAAGGGGGTGTCTGTGGGGTC Antisense: ATGTCGGGGAGGGTGGGCACCTG
GAPDH	Sense: CACCAGGGCTGCCATTTGCA Antisense: GCTCCACCCTTCAAGTGG

Col1a1, collagen type I-α_1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. *Probes used for RNAscope in situ hybridization*

Probe	Cat. No.
RNAscope negative control probe DapB	320751 bacterial
RNAscope positive control probe PPIB	321651 mouse
RNAscope probe Mm-Aqp-2 C1 & C3	452411 and 452411-C3
RNAscope probe Mm-Col1a1	319371
RNAscope probe Mm-Myh11	316101
RNAscope probe Mm-Nphs1-C2	433571-C2
RNAscope probe Mm-PDGFRb C1 & C2	411381 and 411381-C2
RNAscope Probe Mm-Pecam1	316721
RNAscope Probe Mm-Ptger1 C2	501821-C2
RNAscope probe Mm-Ptger2	456481
RNAscope probe Mm-Ptger3	504481
RNAscope probe Mm-Ptger4-C3	441461-C3
RNAscope probe Mm-Ptgs2-O1 C1 & C2	518051 and 518051-C2
RNAscope probe Mm-Ren1-C2	433461-C2

(Cox-2$^{fl/fl}$, stock no. 030785, Jackson Laboratories, RRID: IMSR_JAX:030785) (41, 42). To study the relevance of different PG receptors, mice with general deletion of the *Ptger2* gene (EP2$^{-/-}$ mice, Stock No. 004376, Jackson Laboratories, RRID:IMSR_JAX:004376) (43) were crossbred with FoxD1$^{+/Cre}$ mice and mice bearing floxed alleles for the *Ptger4* gene (EP4^{flox} mice, Stock No. 028102, Jackson Laboratories, RRID: IMSR_JAX:028102) (44). The sex of embryonic and neonatal mice was determined by PCR (45). Genotyping was performed with the primers shown in Table 1.

Male mice at an average age of 15 wk were used for the experiments unless stated otherwise. For linearity, only male embryos or neonatal mice of wild-type C57/Bl6/J breeding pairs were used. Adult animals were euthanized by dislocation of the neck after sedation [ketamine (80 mg/kg body wt) and xylazine (10 mg/kg body wt), ip]. Neonatal mice were euthanized by decapitation after anesthesia overdose.

Successful excision of exons 4 and 5 of the Cox-2 gene was verified by genotype PCR as previously described (42). Deletion of the receptors was verified by measurement of mRNA content in the respective animals. Archival tissue of male mice with constitutive global deletion of Cox-2 in all cells (Cox-2$^{-/-}$, Stock No. 002476, Jackson Laboratories, RRID:IMSR_JAX:002476) was used to compare the morphological changes. No live Cox-2$^{-/-}$ mice were used for this study. The experimental structure of this study is provided in Supplemental Fig. S1 (all Supplemental Material is available at https://doi.org/10.6084/m9.figshare.17313557).

Fixation of Renal Tissue

Adult mice were anesthetized and euthanized as described in *Mouse Models*. To fixate the renal tissue for RNAscope or

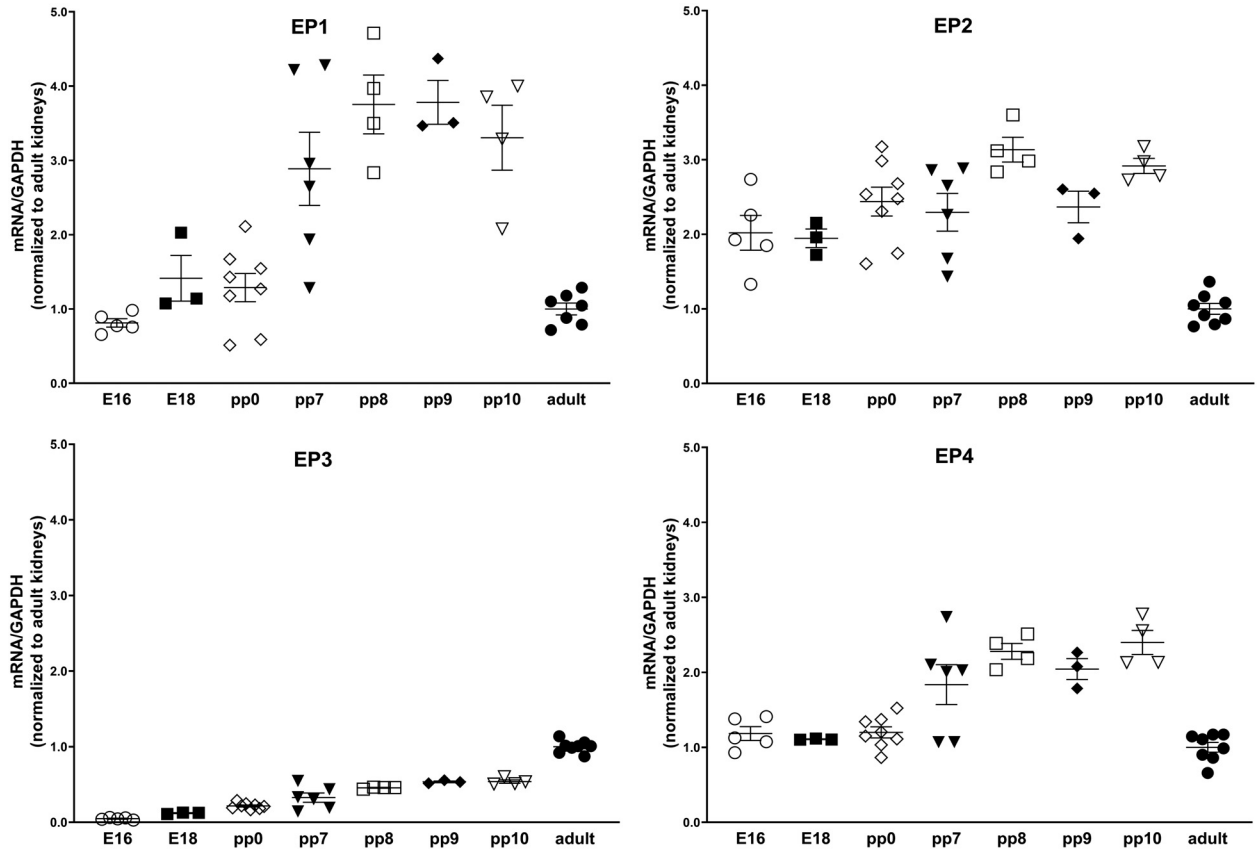

Figure 1. mRNA expression of the four prostaglandin E_2 receptor subtypes during renal development of wild-type mice. mRNA was measured at embryonic stages and during postpartum (pp) kidney development of mice up to *day 10*. Kidneys of adult wild-type mice were used for comparison. With the exception of EP3 (*bottom left*), the receptors for prostaglandin E_2 were all strongly upregulated during the final stages of renal development, followed by lower expression in adult mice. mRNA was measured for $n \geq 3$ animals per time point. Values are means ± SE. mRNA abundance of adult mice was set to 1 for better comparison. E, embryonic day.

histology, the abdominal cavity was opened. To reduce the number of mice needed for this study, the right kidney was ligated to preserve it for mRNA measurements and the left kidney was fixed by retrograde perfusion through the abdominal artery. All mice were first perfused with 40 mL of sterile PBS to clear erythrocytes and then with 40 mL of 10% neutral buffered formalin solution (for RNAscope) or 40 mL of 3% buffered paraformaldehyde (PFA) solution (for histology). After perfusion, the ligated (nonperfused) right kidneys were removed, frozen in liquid nitrogen, and stored at −80°C for mRNA measurements. For embryonic and neonatal tissues, one kidney was frozen for mRNA measurements and the other kidney was fixed by immersion in 15 mL of 10% neutral buffered formalin solution under slow agitation.

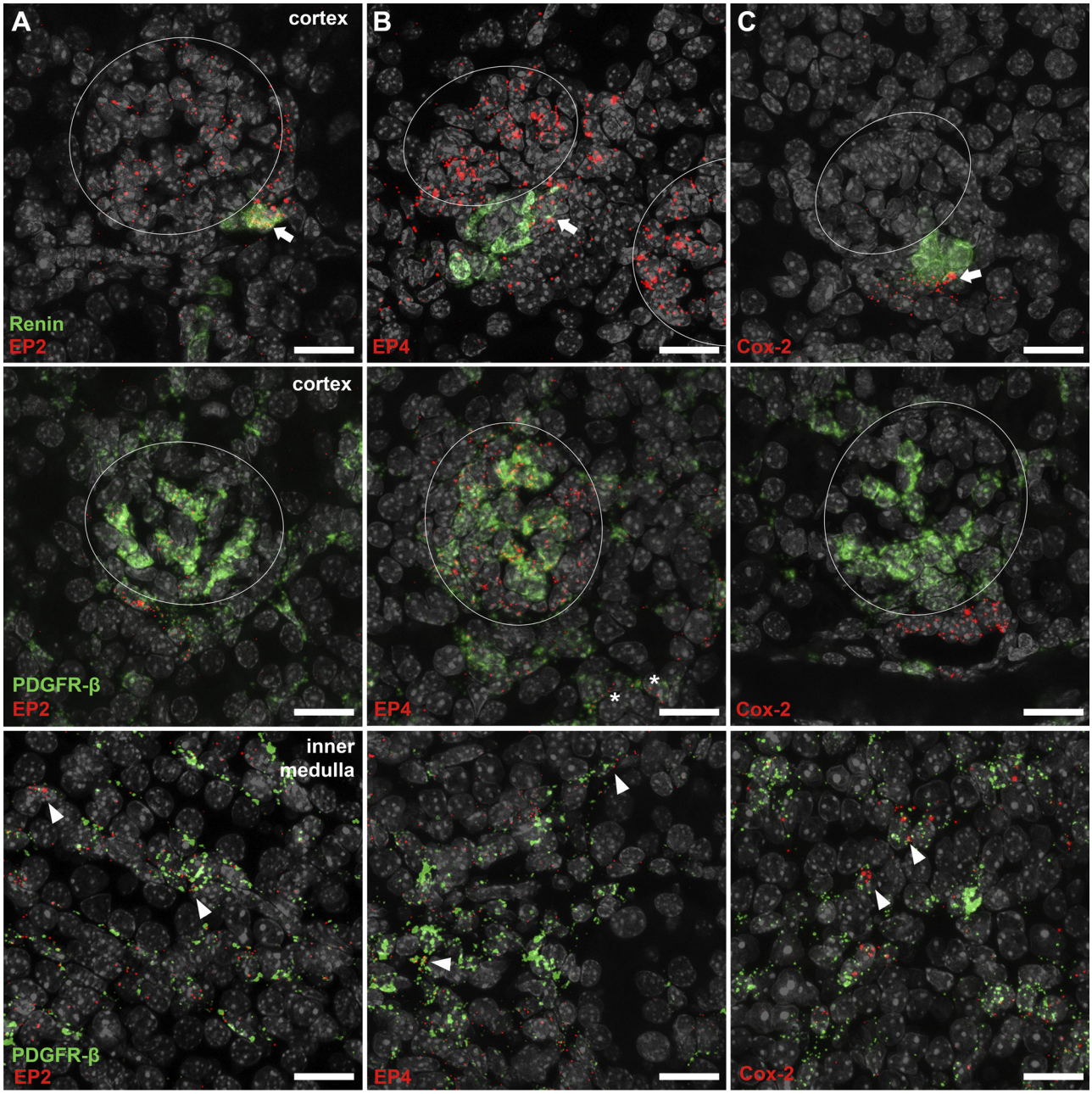

Figure 2. RNAscope on kidney sections of 9-day-old wild-type mice for different components of the prostaglandin signaling pathway and marker mRNAs. *Top*: cohybridization with renin (green) mRNA showed a strong expression for EP2 (red; *A*) and EP4 (red; *B*) in renin-expressing cells (arrows) at the glomeruli (circles). Expression of cortical cyclooxygenase-2 (Cox-2) mRNA (red; *C*) was restricted to tubular macula densa cells (arrow) in close proximity to renin-producing cells. *Middle*: platelet-derived growth factor receptor-β (PDGFR-β) mRNA (green) as a marker for intraglomerular mesangial cells, extraglomerular mesangial cells, and interstitial cells colocalized well with EP2 in intraglomerular and extraglomerular mesangial cells (red; *A*). EP4 expression (red; *B*) was more pronounced in mesangial cells than EP2. EP4 was also expressed in interstitial cells (*). There was no overlap of Cox-2 and PDGFR-β mRNA in the renal cortex (red; *C*). *Bottom*: in the inner and outer medulla, both EP2 (red; *A*) and EP4 (red; *B*) as well as Cox-2 (red; *C*) were expressed by interstitial PDGFR-β$^+$ cells (green, arrowheads). Nuclei were counterstained with DAPI (gray). Circles indicate glomeruli. Scale bars = 20 μm.

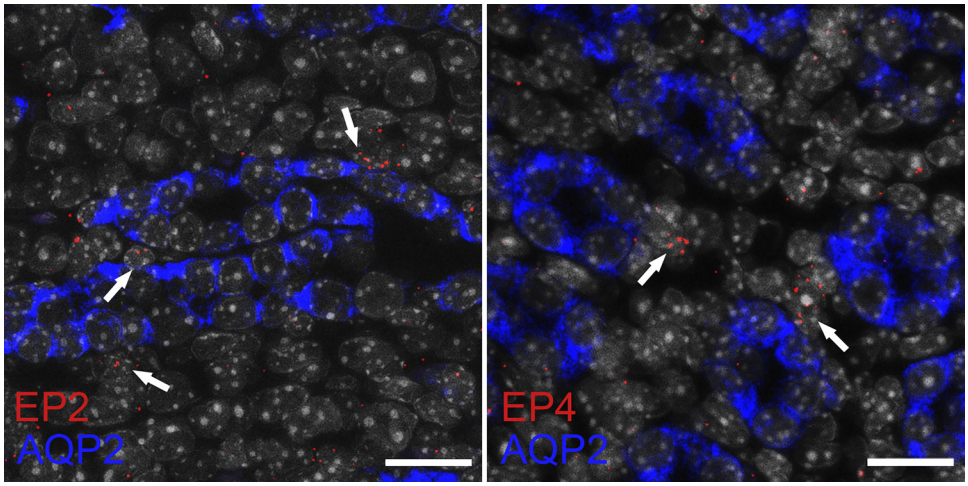

Figure 3. RNAscope for aquaporin-2 (AQP2; blue) mRNA and EP2 or EP4 mRNA (red) in the inner medulla in kidney sections of 9-day-old wild-type mice. Expression of EP2 mRNA (*left*; red) was detected in interstitial cells (arrows) associated with AQP2-positive (blue) tubules. The expression pattern was similar for EP4 mRNA (*right*; red). Nuclei were counterstained with DAPI (gray). Scale bars = 20 μm.

Determination of mRNA Expression by Real-Time PCR

Total RNA content was isolated from frozen kidneys as previously described by Chomczynski and Sacchi (46) and quantified by photometer. Of the resulting RNA, 1 μg was used for reverse transcription. cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Waltham, MA). For quantification of mRNA expression, real-time PCR was performed using a Light Cycler Instrument and the LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics, Mannheim, Germany). mRNA expression data were normalized to

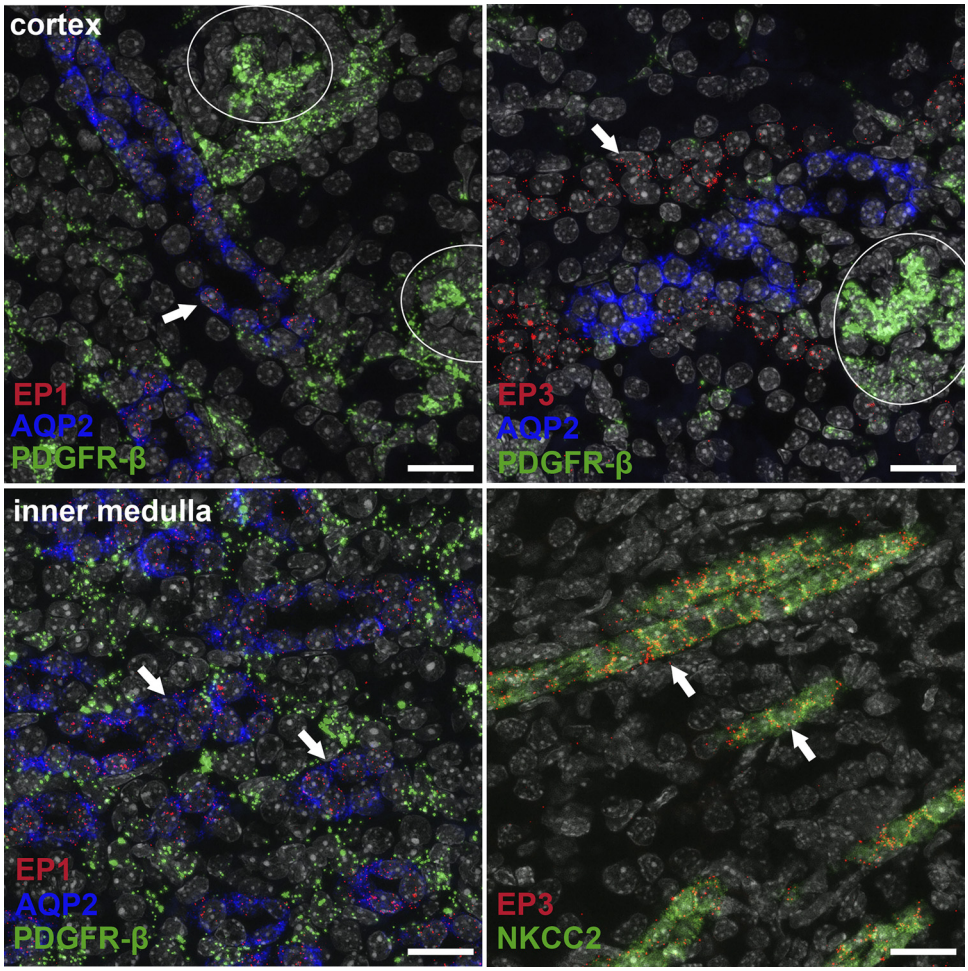

Figure 4. Tissue of 9-day-old mice with triple RNAscope for EP1 (red; *left*) or EP3 mRNA (red; *right*) with platelet-derived growth factor receptor-β (PDGFR-β; green) and aquaporin-2 (AQP2; blue) or with Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) mRNA as cell type markers. *Left*: the highest density of EP1 mRNA (red) could be detected in AQP2$^+$ collecting ducts (blue, arrows) of the developing cortex. There was no colocalization with the interstitial cell marker PDGFR-β. In the inner medulla, EP1 was only expressed in AQP2$^+$ tubules, with sporadic signals in PDGFR-β$^+$ interstitial cells. *Right*: expression of EP3 was restricted to distal tubules in the cortex (arrows) but not in AQP2$^+$ collecting ducts. There was no colocalization for EP3 and PDGFR-β. In the medullary region, expression of EP3 followed the same pattern as in the cortex, with the highest signal density in NKCC2 (green)-expressing distal tubules. Nuclei were counterstained with DAPI (gray). Circles indicate glomeruli. Scale bars = 20 μm.

GAPDH. Table 2 shows the primer sequences for real-time PCR.

RNAscope In Situ Hybridization

Localization of mRNA expression was studied with the RNAscope Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics, Hayward, CA) according to the manufacturer's instructions (47, 48). The kidneys were perfusion fixed with 10% neutral buffered formalin solution, dehydrated in an ethanol series, and embedded in paraffin. Hybridization signals were detected on 5-μm tissue sections using Opal fluorophores 480, 570, and 690 (Akoya Biosciences, Marlborough, MA). Slices were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Positive and negative controls were routinely enclosed. The RNAscope probes are shown in Table 3.

Microscopy

All micrographs were captured with an Axio Observer.Z1 Microscope (Zeiss, Jena, Germany) and a Plan-Apochromat ×20/0.8 objective, a ×1 tube lens, and the Apotome.2 system. The Colibri7 (Zeiss) was used as light source. Chromogenic images were taken with the Axiocam 305color; fluorescent images were taken with an Axiocam 506mono. The excitation wavelengths used were 630, 567, 475, and 385 nm, and emissions were detected at 673, 561, 500, and 465 nm, respectively. For detail images, 5–15 *z*-stacked images were combined for maximum projection. Overviews of whole kidney sections were generated by combination of tiles taken at ×20 magnification. Images represented in the same figure were captured with the same light intensities and exposure times.

Glomerular Filtration Rate Measurement

To measure the glomerular filtration rate (GFR) in conscious mice, a slightly modified protocol using the clearance of FITC-sinistrin by the kidney was used (49). GFR/body weight was calculated and used for analysis.

Sirius Red Staining

Kidney tissue fixed for histology was cut in 5-μm sections. Tissue sections were deparaffinized in Xylol and a

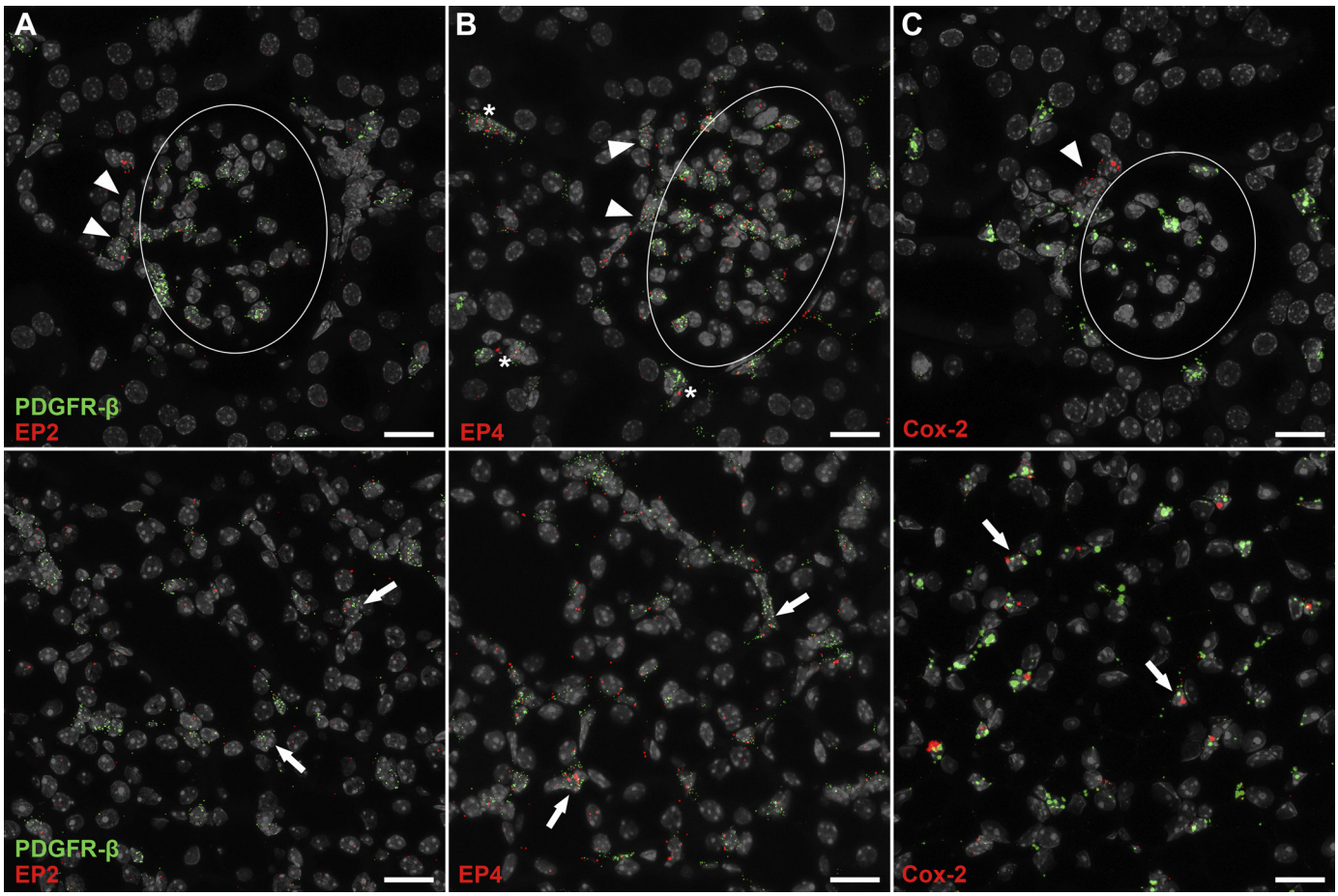

Figure 5. Expression of EP2, EP4, and cyclooxygenase-2 (Cox-2) mRNA in kidney sections of 15-wk-old adult mice. In adult mice, expression of EP2 (red; *A*), EP4 (red; *B*), and Cox-2 mRNA (red; *C*) was detected in the same cell types as in 9-day-old mice, but the density and number of mRNA signals detected were clearly lower for all three targets. EP2 expression was detected in extraglomerular mesangial cells (arrowheads) and medullary interstitial cells coexpressing platelet-derived growth factor receptor-β (PDGFR-β; green, arrows). Signals for EP4 mRNA were detected in intraglomerular and extraglomerular mesangial cells (arrowheads), some interstitial cells of the cortex (*B*, *), and interstitial cells of the medulla (arrows). Cox-2 was expressed in the cortex only by cells of the macula densa (arrowhead) that were negative for PDGFR-β mRNA, whereas medullary Cox-2 expression could only be detected in PDGFR-β$^+$ interstitial cells (arrows). Nuclei were counterstained with DAPI (gray). Circles indicate glomeruli. Scale bars = 20 μm.

descending isopropanol series, followed by a wash step in Millipore-H_2O. Sections were incubated for 45 min with picrosirius red solution (Cat. No. ab256832, Abcam, Cambridge, UK) and 0.1% fast green (Cat. No. F7252, Merck). Sections were repeatedly washed with Millipore-H_2O and then incubated shortly with 0.5% acidic acid. Dehydration was performed with washing in 100% isopropanol twice, followed by two wash steps in xylene for 5 min each. Sections were mounted with dibutylphthalate polystyrene xylene (DPX) mounting medium (Cat. No. 06522, Merck).

Statistical Analyses

All data are presented as means ± SE. To test for normal distribution of values, a Shapiro-Wilk test was performed and the corresponding Q-Q plots were created for graphical verification. Statistical significance between groups was determined by ANOVA with Bonferroni correction. $P < 0.05$ was considered statistically significant. The data were analyzed using Graph Pad Prism9 (Graphpad Software, San Diego, CA, RRID:SCR_002798).

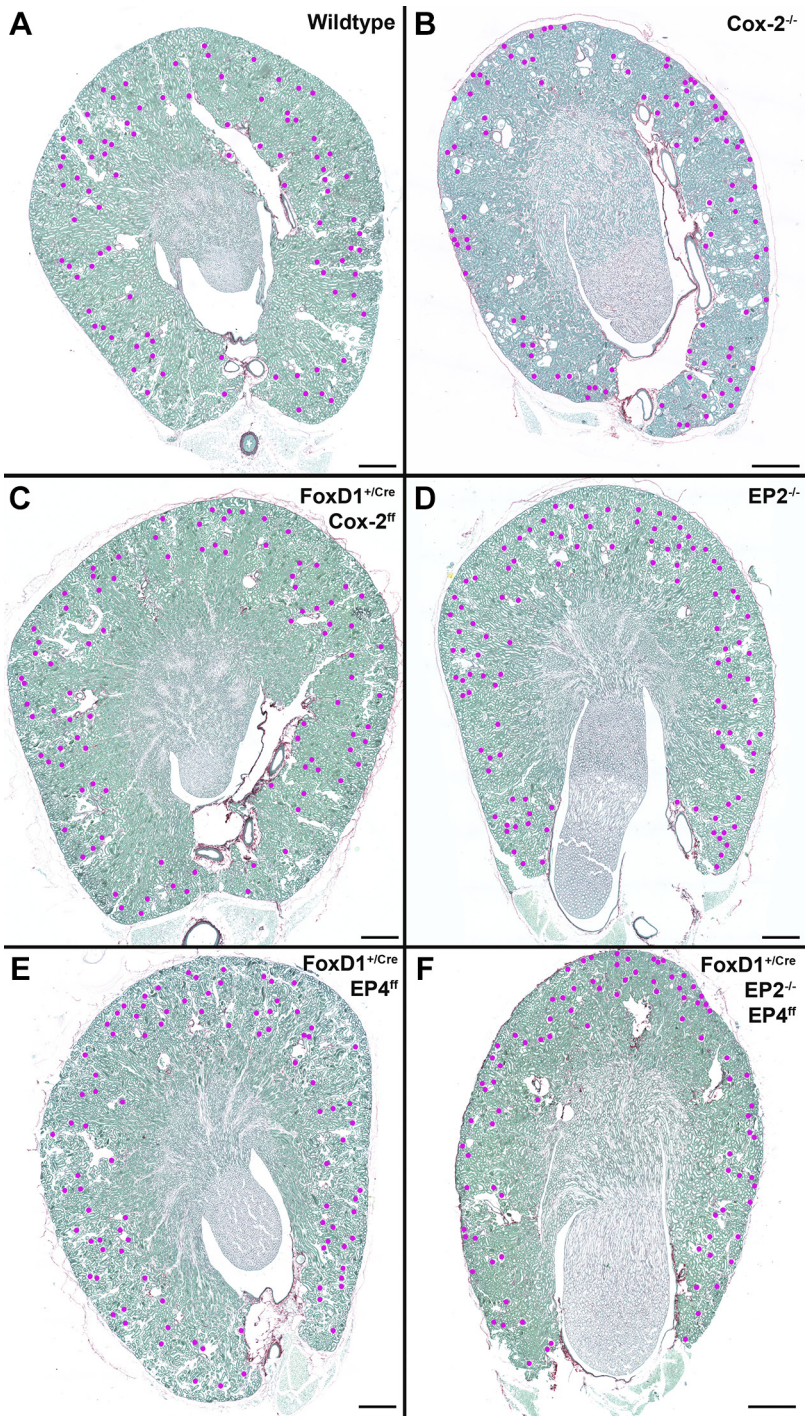

Figure 6. Whole kidney sections of wild-type mice and mice with different disruptions of the prostaglandin pathway. To illustrate the structural changes of the kidneys, sirius red staining was performed on renal tissue of adult mice and glomeruli were highlighted with pink dots. Compared with wild-type mice (*A*), mice with a constitutive deletion of cyclooxygenase-2 (Cox-2) in all cells (*B*) had a thinner cortex with many underdeveloped subcapsular glomeruli (pink dots), and the renal medulla appeared bigger in contrast to the thin cortical tissue. Cell-specific deletion of Cox-2 only in stromal FoxD1$^+$ progenitor cells (*C*) had no effect on cortical development or the placement and formation of glomeruli. Animals bearing either deletion of EP2 in all cells (*D*) or cell-specific deletion of EP4 in renal stromal progenitors (*E*) showed no major defects in the development of the cortex. Codeletion of both EP2 and EP4 in cells of the FoxD1 compartment (*F*) led to a thin cortex with underdeveloped subcapsular glomeruli. Scale bars = 500 μm.

RESULTS

Receptor Subtypes for PGE_2 Are Highly Expressed During the Late Stages of Renal Development and Expressed by Distinct Cell Populations

The strong upregulation of Cox-2 expression during postpartum nephrogenesis in mice, with a peak expression between 6 and 8 days postpartum, has been previously reported (7). We measured mRNA levels of the four receptors for PGE_2 at *embryonic days 16* and *18*, at the day of birth, and during the late stages of kidney development (Fig. 1). Except for EP3, we detected increased expression of the EP receptor subtypes during the postnatal period compared with adult kidneys.

We used highly specific RNAscope in situ hybridization to localize cells expressing EP receptors and Cox-2 mRNA in kidneys of 9-day-old wild-type mice (Figs. 2, 3, and 4). Cohybridization with cellular markers allowed the identification of different cell types expressing mRNA for Cox-2 or one of the EP receptors.

In the kidney cortex, glomerular mesangial cells most strongly expressed EP2 and EP4 in neonatal tissues. Renin-expressing cells at the glomerular pole were also positive for both receptor mRNAs (Fig. 2, *A* and *B*). Moreover, mRNA signals for EP4 were detectable in interstitial cells and in podocytes of the developing cortex (Supplemental Fig. S2). Overall, EP4 mRNA signals were more numerous than EP2 mRNA signals, indicating a higher number of EP4 mRNA molecules. Cortical expression of Cox-2 was restricted to tubular cells forming the macula densa in direct contact with renin-expressing cells (Fig. 2*C*). Cohybridization with the interstitial cell marker platelet-derived growth factor receptor (PDGFR)-β showed that in the inner and outer medulla EP2, EP4, and Cox-2 were expressed by interstitial cells (Fig. 2, *bottom*). In AQP2-positive tubules, only very sporadic mRNA signals for EP2 or EP4 could be detected. Cox-2 mRNA was not detected in any tubules outside macula densa cells. Expression of both receptors was stronger in interstitial cells than in the tubules (Fig. 3).

Mesangial cells, renin-expressing cells, and PDGFR-β$^+$ interstitial cells, which all derive from FoxD1$^+$ progenitors, could be identified as the main expression sites for EP2 and EP4 in developing kidneys. These cells are in direct contact to PG-producing Cox-2$^+$ cells of the macula densa in the renal cortex.

The receptor subtypes EP1 and EP3 were mainly expressed in the tubular compartment (Fig. 4). EP1 was detected in AQP2-expressing collecting ducts (Fig. 4, *left*). In the inner medulla, few PDGFR-β$^+$ cells showed weak EP1 signals. EP3 was mainly expressed by distal Na^+-K^+-$2Cl^-$ cotransporter (NKCC2)-expressing tubules in the cortex and inner medulla (Fig. 4, *right*). There was no colocalization of EP3 and

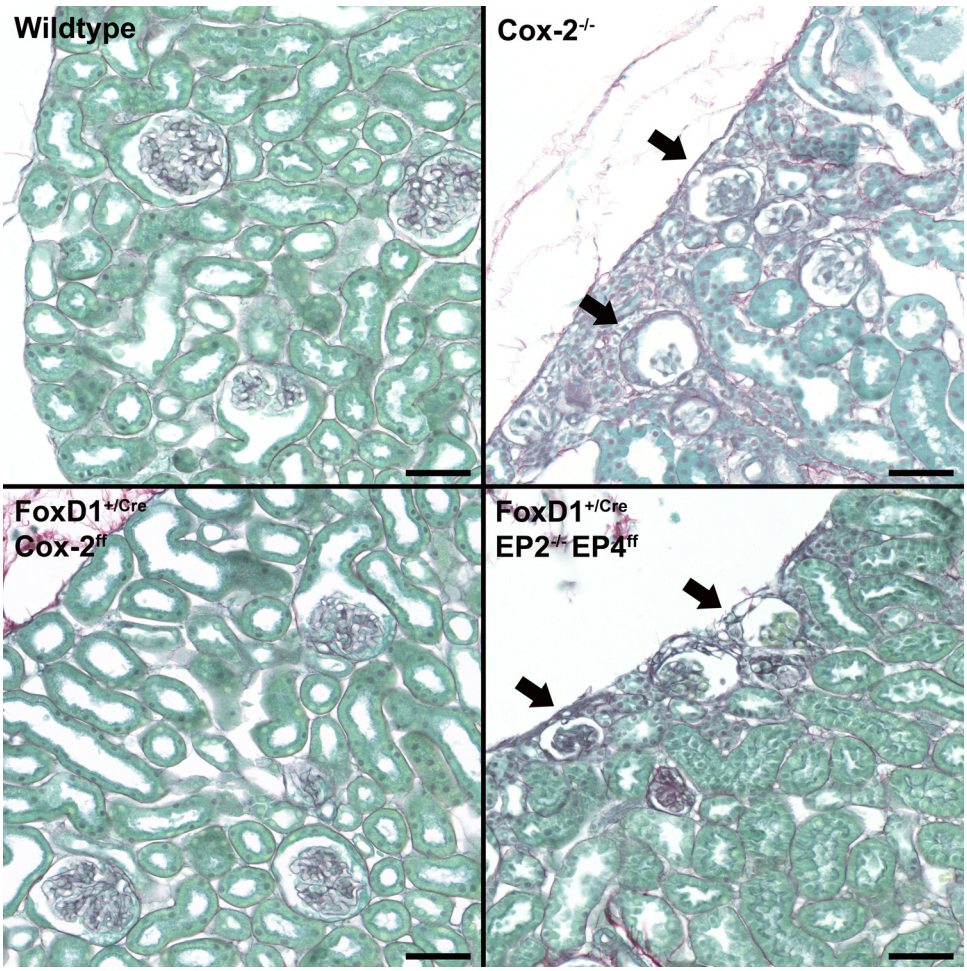

Figure 7. Details of cortical tissue after sirius red staining, taken from adult mice with different disruptions of the prostaglandin pathway. There was no apparent difference in the development of the glomeruli or the surrounding tissue of wild-type mice and mice with targeted deletion of cyclooxygenase-2 (Cox-2) in FoxD1$^+$ cells during development. Mice with the global deletion of Cox-2 showed numerous small subcapsular glomeruli surrounded by collagenous tissue (purple, arrows). A similar phenotype could be observed in FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} animals, which also showed small subcapsular, underdeveloped glomeruli and increased collagenic fibers sourrounding the damaged glomeruli. Scale bars = 50 μm.

PDGFR-β in interstitial cells of any kidney zone, but few signals for EP3 mRNA could be detected in larger vessels.

In line with real-time PCR measurements (Fig. 1), adult kidneys showed fewer mRNA signals for EP2, EP4, and Cox-2 than tissues of 9-day-old mice (Fig. 5). However, the expression pattern in regard to the cell types expressing Cox-2 and the different EP receptors remained the same. We did not detect mRNA signals for EP2 or EP4 in vascular smooth muscle cells or endothelial cells (Supplemental Fig. S3).

EP2 and EP4 in FoxD1$^+$ Stromal Progenitor Cells Are Essential for Normal Renal Development

In mice with global deletion of Cox-2 or mice treated with Cox-2 inhibitors in the first 10 days after birth, cells differentiated from FoxD1$^+$ progenitor cells are affected by the resulting renal phenotype. The localization experiments showed that only EP2 and EP4 are strongly expressed in descendants of FoxD1$^+$ progenitors (Fig. 2). Therefore, we focused on these two receptor subtypes and Cox-2 as the relevant PG-producing enzyme.

We analyzed different mouse models with cell-specific deletion of Cox-2 and EP4 under control of the FoxD1 promotor (FoxD1$^{+/Cre}$ Cox-2^{ff} and FoxD1$^{+/Cre}$ EP4^{ff} mice). To exclude a possible compensation between receptor subtypes, we crossed FoxD1$^{+/Cre}$ EP4^{ff} mice with mice that had global deletion of EP2 (EP2$^{-/-}$ mice) to create mice deficient for both receptors in all cells derived from FoxD1 progenitors (FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice). Tissue from mice with constitutive deletion of Cox-2 in all cells (Cox-2$^{-/-}$) was used for comparison of the resulting phenotype. All mouse strains were viable, survived to adulthood, and were fertile. Heterozygous breeding pairs produced offspring in the expected Mendelian ratios.

Morphological analysis of the kidney structure showed that mice with deletion of Cox-2 in the FoxD1$^+$ stromal progenitors developed no abnormalities (Figs. 6, 7, and 8). Deletion of EP2 or EP4 alone seemed to reduce the distance of glomeruli to the renal capsule but had no other effects on renal morphology (Fig. 6). Renal function, as measured by GFR, was not affected by these changes in EP2$^{-/-}$ and FoxD1$^{+/Cre}$ EP4^{ff} mice (Fig. 8).

Codeletion of EP2 and EP4 in FoxD1$^+$ cells, however, led to severe defects, similar to the global deletion of Cox-2. As in Cox-2$^{-/-}$ mice, the kidneys of FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice appeared to have fewer and smaller glomeruli per section than control animals, and the glomeruli were located closer to the renal capsule (Fig. 6, *B* and *F*, and Fig. 7).

The cortical tissue of FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice appeared disproportionally thinner, and the kidneys were smaller overall. Therefore, the medullary tissue of these mice seemed larger in comparison (Fig. 6*F*). The body weight of FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice was not significantly lower than that of control animals. The subcapsular glomeruli of Cox-2$^{-/-}$ and FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice were small and surrounded by collagenous deposits (Figs. 7 and 9).

In line with the structural changes in FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} animals, the kidney function of these mice, as measured by GFR, was severely compromised (Fig. 8).

Interestingly, in situ hybridization and whole kidney mRNA measurements for collagen type I-α_1 (Col1a1) in adult mice showed that interstitial myofibroblasts surrounded the subcapsular glomeruli in FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice. These cells showed strong Col1a1 mRNA expression compared with interstitial cells of adult control animals and the other genotypes analyzed for this study (Fig. 9). Furthermore, kidneys of FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice expressed significantly less renin mRNA than the other genotypes (Fig. 9).

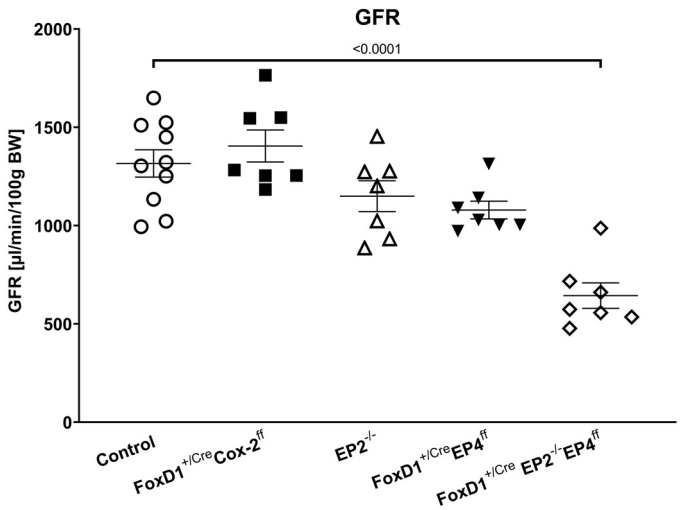

Figure 8. Measurement of glomerular filtration rate (GFR) for mice with different deletions in the prostaglandin pathway. Deletion of cyclooxygenase-2 (Cox-2) in FoxD1$^+$ cells had no effect on renal development or function. FoxD1$^{+/Cre}$ EP4^{ff} and EP2$^{-/-}$ mice showed no changes in GFR. FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice had a significantly reduced GFR in line with morphological changes. At least five animals were analyzed per genotype. Significant *P* values are indicated in the graph between relevant pairs. BW, body weight.

DISCUSSION

This study aimed to investigate the cell-specific expression and relevance of the PG-producing enzyme Cox-2 and the effect of PG signaling through cAMP-coupled EP2 and EP4 receptors during renal development. Due to the lack of reliable antibodies for G protein-coupled receptors (50) and difficulties with microdissected developing mouse kidneys, the localization of EP receptors and Cox-2 in the postnatal period of mice has not been reported to date. Previous studies in adult mice and rats have reported expression of EP receptors in different tubular segments in addition to glomeruli and renal vessels (16, 23–25, 51–53). In human kidneys, EP1 and EP3 receptors were detected in tubular segments and vessels and EP2 and EP4 were located in glomeruli, renal vessels, and some interstitial cells (54–56).

This study showed distinct cellular expression of EP2 and EP4 during the neonatal period of mice that was mirrored in adult animals. The two G_s/cAMP-coupled receptors for PGE_2 were expressed mainly in cells derived from FoxD1$^+$ renal stromal progenitor cells (Fig. 2). This is in good accordance with a previous report on the expression of EP2 and EP4 in rats (51). We detected EP4 mRNA in pericyte-like cells associated with the renal vasculature; these cells might influence the vascular tone of the vessels (24). No mRNA signals for EP2 and EP4 could be detected in tubules or vascular smooth muscle cells, as previously reported (Fig. 3 and Supplemental Fig. S3) (23, 25, 53). It is possible that mRNA expression in these cells was below the detection limit of the RNAscope technique but still sufficient for functional protein expression.

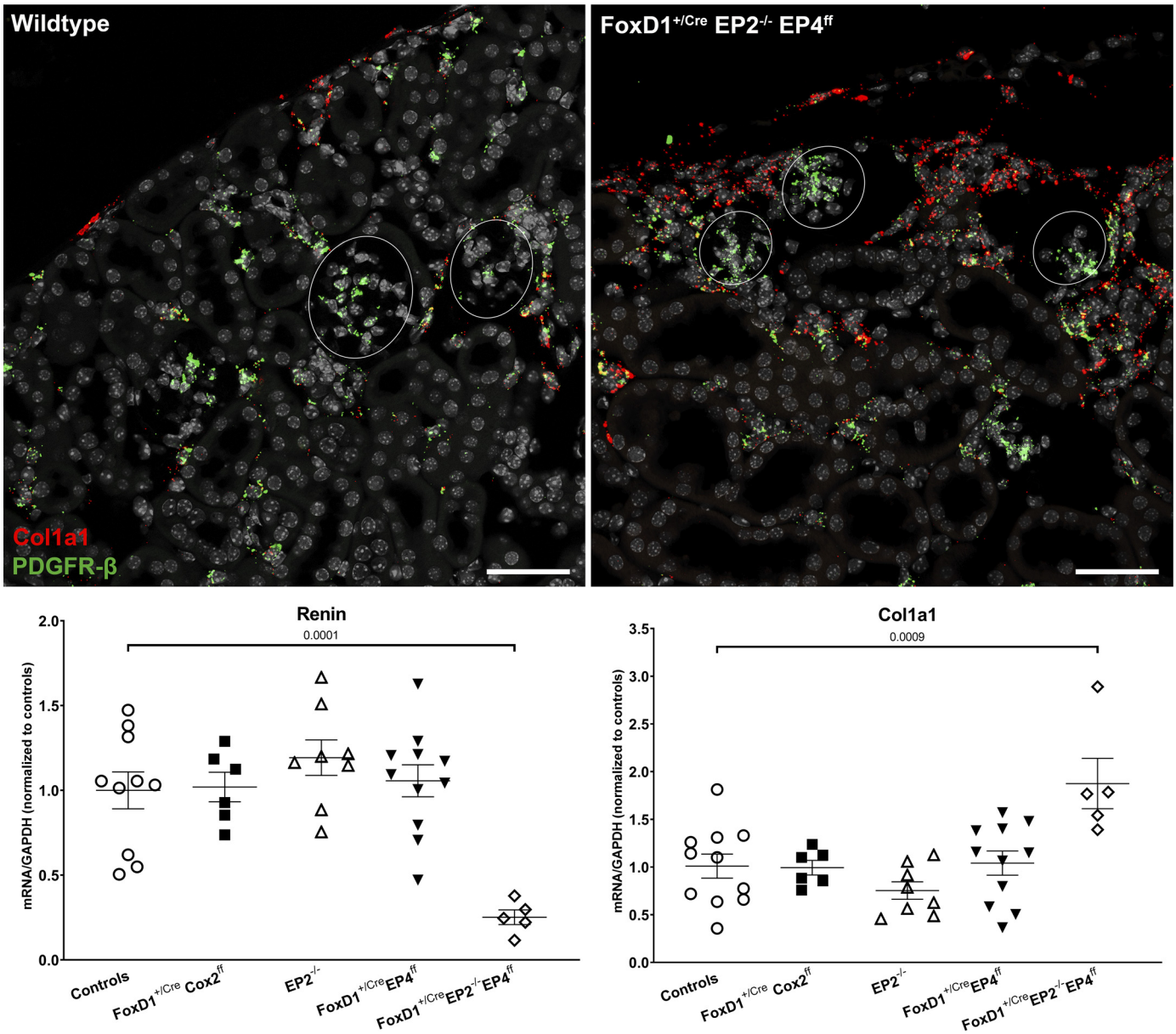

Figure 9. Cortical details of in situ hybridization for collagen type I-α_1 (Col1a1; red) and platelet-derived growth factor receptor-β (PDGFR-β; green) mRNA in tissue of adult control mice (*left*) and FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice (*right*) and mRNA abundance of Col1a1 and renin in adult kidneys of the different genotypes analyzed in this study. *Top*: tissue of adult FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice showed substantial Col1a1 mRNA expression around underdeveloped subcapsular glomeruli. The majority of Col1a1 mRNA was expressed by PDGFR-β$^+$ interstitial cells surrounding the glomeruli and located in the tubulointerstitial space, but not by intraglomerular mesangial cells. In control mice, Col1a1 mRNA was also expressed exclusively in interstitial PDGFR-β$^+$ cells, but to a much lower degree than in FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice. Nuclei were counterstained with DAPI (gray). Scale bars = 50 μm. *Bottom*: mRNA abundance of renin and Col1a1 in adult kidneys of mice with different cell-specific deletions in the prostaglandin pathway. FoxD1$^{+/Cre}$ EP2$^{-/-}$ EP4^{ff} mice showed significantly lower renin expression and higher Col1a1 expression compared with the other genotypes. EP2$^{-/-}$ and FoxD1$^{+/Cre}$ EP4^{ff} mice did not show any elevated Col1a1 expression and maintained normal renin expression. At least five animals per genotype were analyzed. Significant *P* values are indicated in the graph between relevant pairs.

EP1 was strongly expressed in AQP2$^+$ collecting ducts, and EP3 mRNA was detected in NKCC2-expressing distal tubules. This is in accordance with previous findings (16–18, 25). However, neither EP1 nor EP3 mRNA was detected in glomeruli, renin cells, or interstitial cells (Fig. 4).

Expression of Cox-2, the relevant Cox isoform during renal development (3, 4, 7), was detected in two developmentally different cell populations. In addition to the classical expression in tubular cells of the macula densa at the glomerular pole, Cox-2 was localized in medullary PDGFR-β$^+$ interstitial cells. Interestingly, interstitial PDGFR-β$^+$ cells, derived from FoxD1$^+$ progenitors, showed a zonally different expression pattern for the investigated targets. Medullary interstitial cells expressed Cox-2, EP2, and EP4 mRNA (Fig. 2) in neonates and later in adult animals (Fig. 5), whereas cortical interstitial cells expressed only EP4. This zonally differential expression is in good accordance with recent findings on different subpopulations of interstitial cells (41, 47).

To investigate possible functions between EP2, EP4, and Cox-2 expressed by stromal cells, we generated specific mouse models. Mice with a deletion of Cox-2 in FoxD1$^+$ cells showed no apparent morphological or functional alterations in this study. Another study (57) reported a very mild phenotype for FoxD1$^{+/Cre}$ Cox-2^{ff} mice with reduced glomerular size and thinner cortical tissue. A difference in the genetic background of the mice used to generate this genotype (4) and the age of the mice might account for the divergent findings. We examined kidney morphology at an average age of 15 wk, whereas Nelson et al. used 21-day-old mice. The mild phenotype might no longer be detectable in FoxD1$^{+/Cre}$ Cox-2^{ff} mice at this later point. In line with our results for the present study, the authors did not report functional changes in these mice.

The strong and overlapping expression of EP2 and EP4 receptors in FoxD1-derived cells surrounding Cox-2$^+$ macula densa cells (Fig. 2) indicates an important role for PG-based cross-talk between these cells. EP2 and EP4 are the only two G_s/cAMP-coupled receptors for PGE_2 (11) and are important for the regulation of renal renin expression (22, 24).

Deletion of only one receptor subtype appeared to have only minor morphological effects that did not result in functional impairment of GFR or renal renin expression (Figs. 7, 8, and 9). Although renin expression could be maintained by the remaining G_s/cAMP-coupled receptor (Fig. 9), slight morphological changes could be detected. We hypothesize that, in addition to the already well-known necessity of timed activation of the renin-angiotensin system (26, 27, 32, 58, 59), PGs confer a direct effect on cortical development through EP2 and EP4. This is supported by a study using salt supplementation and aldosterone agonists (8) in neonate Cox-2$^{-/-}$ mice. These experiments compensated the lower renin expression but thinning of the cortical tissue persisted. The data of animals with deletion of only one EP isoform of this study are in line with these findings. However, deletion of both receptors in FoxD1$^+$ cells resulted in a severe phenotype (Figs. 6–9) that is comparable to global deletion of Cox-2. On the one hand, this indicates an "evolutionary insurance policy" for maintaining renin expression in the kidneys; on the other hand, it suggests unique functions for receptors that are only 31% homologous (11). It is conceivable that a reduction of intracellular cAMP during nephrogenesis in cells affected by deletion of either EP2 or EP4 leads to the morphological changes observed in this study. Thus, our data concerning cell-specific expression of EP2 and EP4 and those of a previous study (8) might contribute to a better understanding of the role that Cox-2 and PGE_2 play during renal development.

Taken together, we were able to localize, for the first time, the distinct expression sites of EP receptor mRNA in neonatal and adult mouse kidneys. Furthermore, we could show that PG signaling through EP2 and EP4 receptors in stromal cells is essential for normal kidney development. The similarities between global Cox-2$^{-/-}$ mice and EP2/4-deficient mice suggest that PGE_2 is the essential Cox-2-derived product for renal development. However, other products, such as prostacyclin or thromboxane A_2, should not be entirely discounted.

This study demonstrates the need for further research into the distinct functions of the different PGE_2 receptors beyond their involvement in pain, inflammation, and cancer. This could help to ensure the safety of newly developed drugs in this field.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S3: https://doi.org/10.6084/m9.figshare.17313557.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.A.A.F., A.K., and K.A.-E.B. conceived and designed research; M.A.A.F., J.S., C.L., and K.A.-E.B. performed experiments; M.A.A.F., C.L., and K.A.-E.B. analyzed data; M.A.A.F., J.S., C.L., and K.A.-E.B. interpreted results of experiments; M.A.A.F. prepared figures; M.A.A.F., J.S., and K.A.-E.B. drafted manuscript; M.A.A.F., J.S., C.W., A.K., and K.A.-E.B. edited and revised manuscript; M.A.A.F., J.S., C.L., C.W., A.K., and K.A.-E.B. approved final version of manuscript.

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