The transcription factor Tcf21 is required for specifying Foxd1 cells to the juxtaglomerular cell lineage

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Abstract
Renin is a crucial enzyme involved in the regulation of blood pressure and electrolyte balance. It has been shown that renin expressing cells arise from the Foxd1+ stromal progenitors, however the factors involved in guiding Foxd1+ cells towards the renin-secreting cell fate remain poorly understood. Tcf21, also known as Pod1 or Capsulin, is a bHLH transcription factor that is expressed in the metanephric mesenchyme and plays a crucial role in kidney development. We have previously shown that deletion of Tcf21 in Foxd1+ cells (*Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$) results in paucity of vascular mural cells and in disorganized renal arterial tree with fewer, shorter, and thinner arterioles. Here, we sought to examine the relationship between Tcf21 and renin cells during kidney development and test whether Tcf21 is implicated in the regulation of juxtaglomerular cell differentiation. Immunostaining for renin demonstrated that kidneys of *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ have fewer renin-positive spots at E16.5 and E18.5 compared with controls. In-situ hybridization for *renin* mRNA showed reduced expression in *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys at E14.5, E16.5, and E18.5. Together, these data suggest that stromal expression of Tcf21 is required for the emergence of renin cells. To dissect the role of Tcf21 in juxtaglomerular (JG) cells, we deleted Tcf21 upon renin promoter activation (*Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$). Interestingly, the *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ kidney showed normal arterial tree at E16.5 identical to controls. Furthermore, inactivation of Tcf21 upon renin expression did not alter kidney morphology in two- and four-month-old mice. Finally, expression *renin* mRNA was similar between *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ and controls at 2 months.
Taken together, these findings suggest that Tcf21 expression in Foxd1+ cells is essential for specifying the fate of these cells into juxtaglomerular cells. However, once renin cell identity is assumed, Tcf21 is dispensable. Uncovering the regulation of Foxd1+ cells and their derivatives, including the JG cell lineage, is crucial for understanding the mechanisms underlying renal vasculature formation.

Introduction:

Renin-producing cells, also known as juxtaglomerular cells, play a crucial role in blood pressure regulation and in fluid and electrolyte balance. Renin cells originate during kidney development from the stromal progenitors, the Forkhead box D1 positive (Foxd1+) cells, within the metanephric mesenchyme, around embryonic day 13.5 (E13.5) of the mouse (1, 2). The Foxd1+ cells also give rise to mesangial cells, vascular smooth muscle cells (VSMCs), pericytes, erythropoietin producing cells, and cells of the renal capsule (3-5) . In the mature kidney, renin cells are primarily located in the afferent arterioles of the glomeruli, whereas in the embryonic kidney, they initially appear in the smooth muscle cells of the preglomerular arterioles where they play a critical role in formation of the vasculature (6). While it has been shown that binding of the transcription factor CREB to the cAMP response element (CRE) is pivotal for transcriptional activation of the renin gene (7-9), many aspects defining the molecular identity of renin cells and their differentiation remain poorly understood. The transcription factor Tcf21, a mesoderm-specific bHLH transcription factor critical for embryogenesis, plays an important role in differentiation of proepicardial cells into fibroblasts during heart development (10, 11). In the kidney, we have previously reported that stromal expression of Tcf21 (Stromal Tcf21) is essential for the formation of the stroma and vascular mural cells (12). Here, we sought to test if Tcf21 is involved in specifying Foxd1+ cells into the JG cell lineage.

Results

Inactivation of *Tcf21* in Foxd1+ cells leads to fewer renin-positive areas in the developing kidney.

Utilizing the *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ mouse in which *Tcf21* is excised in Foxd1+ cells (12), we initially examined renin protein expression by immunostaining. At mid- and late-stages of kidney development (i.e., E16.5 and E18.5), Tcf21 stromal conditional knockout (cKO) demonstrated globally fewer renin-positive areas, representing lower renin expression in juxta glomerular apparatus and arteriolar walls, compared with controls (**Fig.1 IA-C**). Morphologic examination of the developing afferent arterioles, where renin cells normally reside, revealed thinner and shorter walls in the E16.5 Tcf21 stromal cKO compared with controls (**Fig.1 IIA-C**). To further corroborate this finding, we performed immunostaining for renin and the melanoma cell adhesion molecule (MCAM, CD146), a marker of perivascular cells (e.g. pericytes, VSMC) (13-15). This staining revealed reduced renin and MCAM expression in the walls of the afferent arterioles in Tcf21 stromal cKO compared with control (**Fig.1 IIIA-C**). This renin+MCAM+ cell population has been previously characterized as renin-positive pericytes that are believed to represent a specialized subpopulation known for their pericyte role in supporting vessel formation and for their distinct ability to synthesize renin (2, 16). Next, we examined the medullary peritubular capillaries, where we observed again a marked reduction in renin and MCAM signals in Tcf21 stromal cKO compared with control (**Fig.1 IIIE-G**).

Complementarily, we evaluated renin mRNA expression at early-, mid-, and late-stages of nephrogenesis (i.e., E14.5, E16.5, and E18.5) in control and Tcf21 mutants. Using in-situ hybridization (RNAscope), we detected a strong *Renin* signal in the arterioles leading into the developing glomeruli of control kidneys at E16.5 and E18.5 (**Fig.2 IA,B, IIA-C**). *Renin* expressing cells were primarily distributed in aggregates along the corticomedullary junction where newly formed glomeruli are known to mature (**Fig.2 IA**). In-line with the previously reported normal emergence of renin-expressing cells around E13.5 of the developing mouse kidney, we detected a scattered patten of *Renin* expression at E14.5 of control kidneys which was less robust compared with *renin* expression at E16.5 and E18.5 (**Fig.2 IIE,F**). In contrast to controls, *Renin* mRNA expression in Tcf21 stromal cKO kidneys was reduced, showing fewer *Renin*-positive areas, although cells expressing renin mRNA were still present in some pre-glomerular arterioles (**Fig.2 IA',B', IIA'-F'**).

As to *Tcf21* expression in controls, as previously described (12), we detected robust *Tcf21* signal throughout stromal regions surrounding the cortex, sub-cortex, medulla, papilla, and ureter (**Fig.2 IIA,C,E**). In addition to the stroma, a strong Tcf21 signal was detected in podocytes at E16.5 and E18.5, and in segments of developing nephron structures at E14.5 (**Fig.2 IIB,D,F**). On the other hand, *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys showed marked reduction in *Tcf21* expression in the stroma, but its expression was still detected in some regions of the medullary stroma (**Fig.2 IIC'**), which could be explained by incomplete Cre-mediated excision of Tcf21 in a population of stromal cells. As expected, we detected *Tcf21* signal in podocytes, developing nephrons and in the ureteric stromal of the mutants, as these cells are not progeny of the Foxd1+ lineage but originate from the Six2 and Tbx18 lineages (17, 18).

Taken together, these results suggest that expression of *Tcf21* in Foxd1+ cells is required for normal expression of renin mRNA and protein with ramifications to the development of the afferent arterioles.

Inactivation of Tcf21 under the *Renin* promotor does not recapitulate the vascular phenotype of *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$

The vascular defect seen in Tcf21 stromal cKO kidneys raised the possibility that continuous expression of *Tcf21* in JG cells is required for normal arterial development. To test this hypothesis, we inactivated *Tcf21* in renin expressing cells by breeding *Tcf21*$^{f/f}$ mice with *Ren1dCre*$^{+}$ mice (which express cre recombinase driven by the renin locus) to create the *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ (Tcf21 renin cKO) mouse. The *Ren1*$^{dCre+}$ model, in which Cre-recombination facilitates conversion of mTomato to GFP expression, has been previously characterized for tracing of the JG cell lineage (19-21). Following immunostaining with the pan-endothelial marker PECAM (CD31) and 3D image analysis of cleared E16.5 kidneys, we observed no difference in the vascular morphology of the Tcf21 renin cKO compared with controls (**Fig.3 I, Suppl. Fig.1**). In contrast, the arterial tree of the Tcf21 stromal cKO demonstrated fewer branches that were shorter and thinner compared with controls (**Fig.3 I** and (12)). Taken together, these findings suggest that once Foxd1+ cells assume the fate of renin cells, expression of *Tcf21* is no longer required for formation of the kidney vasculature. To ensure that inactivation of Tcf21 in renin

cells is not associated with a phenotype in the mature kidney, we evaluated the morphology of the *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ kidneys in two- and four-month-old mice. Hematoxylin-Eosin and Trichrome stainings of control and Tcf21 renin cKO kidneys demonstrated overall normal renal architecture and vessel morphology compared with controls (**Fig.3 IIA-**, **Suppl. Fig.2**). These data further supported that Tcf21 is dispensable once renin cell identity is assumed.

Inactivation of *Tcf21* in renin expressing cells does not interfere with their ability to express *renin*

Lastly, to directly test if *Tcf21* is involved in the regulation of *renin* expression, we compared renin positive spots between *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ and control kidneys using in-situ hybridization. At two-month-old mice, Tcf21 renin cKO kidneys demonstrated a similar expression pattern of *renin* mRNA spots compared with controls (**Fig.4**). This finding indicates that renin expression in the mature kidney is not regulated by *Tcf21*. Similarly, in the developing kidney as early as E14.5, we observed that *Tcf21* and *renin* mRNA signals by in-situ hybridization did not co-localize to the same cell (**Suppl. Fig.3**), further supporting that *Tcf21*'s regulatory action is completed prior to renin promoter activation.

Discussion:

The developmental pathways that guide the differentiation of Foxd1+ stromal progenitors into a myriad of cell types, such as juxtaglomerular cells, are crucial for proper kidney morphogenesis and function. Our study provides insights into the role of the transcription factor Tcf21 in this process, focusing on the specification of Foxd1+ cells towards the JG cell lineage. Firstly, our data corroborate that the mural cells of the kidney, derivatives of the Foxd1+ progenitors, are essential for renal vascular development, as previously reported (1, 22). By comparing and contrasting the *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ and *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ mouse models, the current work offers a nuanced understanding of Tcf21's involvement in determining renin cell identity that could provide important insight into renal arterial formation.

The observed reduction in renin mRNA and protein expression in *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys at crucial developmental stages (E14.5, E16.5, and E18.5) strongly suggests that Tcf21 is a pivotal factor in guiding Foxd1+ cells towards differentiating into renin-producing JG cells. In contrast, the finding that *renin* expression in *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ kidneys was comparable to controls suggests that Tcf21 is dispensable once renin cell fate has been assumed. These observations underscore that Tcf21 exerts its action in early stages of kidney development and are in agreement with our previous report (12, 23).

As to a possible explanation for the presence of residual renin-positive cells in stromal Tcf21 cKO (*Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$), these cells likely emerged from incomplete Tcf21 excision by Cre-recombinase, i.e., cell that escaped Cre-mediated recombination. Alternatively, and less likely, these could represent a sub-population of stromal cells that are not regulated by Foxd1.

This work reaffirms the essential role of Tcf21 in the development of mural cells and the organization of the renal arterial tree. By demonstrating that stromal Tcf21-deficient kidneys have disrupted (shorter and thinner) afferent arteriole morphology, with a marked decrease in renin expressing cells, we were able to further underscore the transcription factor's significance in vascular development. Interestingly, the *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ model did not recapitulate the vascular phenotype observed in the *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ model, nor did it show significant renal histologic abnormalities at two and four months. This further suggests that while Tcf21 is crucial for specifying Foxd1+ cells to the JG cell lineage, its role in the maintenance of renin cell identity and function is less critical once these cells have differentiated. This finding indicates a possible temporal and contextual specificity in Tcf21's role in renal development, where its action is indispensable during the initial differentiation phase but becomes redundant for the maintenance of the renin phenotype.

It is warranted to mention that the variability in Tcf21 deletion among cells, which may have resulted from non-absolute Cre-mediated recombination, possibly affected the uniformity of our findings. Additionally, this study did not address the complexity of the transcriptional networks governing Foxd1+ cell differentiation in terms of identifying Tcf21 binding partners, deregulators, and direct targets. These aspects should be explored in future research and could have functional implications. Finally, we did not explore potential compensatory mechanisms that might mitigate the effects of Tcf21 deficiency on JG cell differentiation.

In summary, our study highlights the complexity of the transcriptional regulation involved in the specification and maintenance of the JG cell lineage. By uncovering Tcf21's involvement in the former and its dispensability

in the latter, we provide a foundation for further research that could shed light on important signaling pathways and mechanisms involved in renal vascular development.

Methods

Mice and Genotyping

All mouse experiments were approved by the Animal Care Committee at the Center for Comparative Medicine of Northwestern University (Evanston, IL) and were performed in accordance with institutional guidelines and the NIH Guide for the Care and Use of Laboratory Animals. The *Tcf21*$^{floxf/lox}$ and *Ren1*$^{dCre/+}$mice were created as previously described Please include reference: Sequeira Lopez et al Dev Cell. 2004 May;6(5):719-28.doi: 10.1016/s1534-5807(04)00134-0 (20, 24). The *Foxd1-eGFPCre* mice were kind gifts from Dr. Andrew McMahon (University of Southern California) and are described elsewhere (25). Genotyping was performed with Quick-Load Taq 2X Master Mix (M0271L, BioLabs), on 2% agarose gel for the PCR bands. Primer information is in **Suppl. Table 1**.

Histology, Immunohistochemistry and Immunofluorescence

Mouse embryo dissections were performed at the indicated time-points. Samples were fixed with 10% Neutral buffered formalin or 4% paraformaldehyde overnight, embedded in paraffin, and sectioned into 5µm by the Mouse Histology and Phenotyping Laboratory (MHPL) of Northwestern University. Sections were deparaffinized in xylene and ethanol, and boiled in citrate buffer for antigen retrieval. Sections were then treated with 1% donkey serum with 0.3% TritonX100 in PBS for 30 minutes at room temperature before incubating with primary antibodies at 4°C overnight. Sections were washed in PBS and incubated with Alexa Fluor fluorescence-conjugated secondary antibodies for 45 minutes at room temperature. Antibody information: CD31 (R&D, AF3628, 1:200) and CD146 (abcam, ab75769, 1:200), Krt8 (TROMA-I-s, DSHB. 1:20), Renin (Alpha Diagnostic, Gomez project 1:500). All secondary antibodies were purchased from Molecular Probes ThermoFisher Scientific and used at a 1:500 dilution. For 5 channel imaging, secondary antibodies were employed as follows: AlexaFluor 488, 555, 594, 647 and DAPI.

In-situ hybridization (RNAScope)

In-situ hybridization was performed on paraffin section using RNAscope (26) and following ACD® protocol https://acdbio.com/rnascope-25-hd-duplex-assay. Probes: Tcf21 (ACD #508661), Ren1 (ACD #433461).

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Figure legends

Figure 1: **Inactivation of *Tcf21* in Foxd1+ cells results in lower renin expression at E16.5 and E18.5.** Immunostaining. I. A, A', representative E16.5 kidney of *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ shows lower renin expression level (brown staining) compare to control Scale bar 200µm.. B, B', JG area of a glomerulus of Tcf21 stromal cKO demonstrates reduced renin expression compared with control (IHC on H&E staining, Scale bar 20µm.). C, quantification of the number of renin-expressing cell aggregates (renin positive areas) in controls and Tcf21 stromal cKO kidneys at E18.5. II. A, A', B, B", and C, C', the afferent arteriole of the glomerulus, stained for CD31+ (PECAM) endothelial cells and CD146+ (MCAM) pericytes, is thinner and shorter in Tcf21 stromal cKO compared with control (Scale bar 50µm, 3D imaging in (12). III. A-C, reduced CD146+Renin+ staining along the

afferent arteriole of Tcf21 stromal cKOcompared with control. E-G, peritubular capillaries in the medulla show lower renin+ pericytes in *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys compared with control. Scale bar 50µm.

Figure 2: **Reduced *renin* mRNA expression in the developing kidney of Tcf21 stromal k/o.** In-situ hybridization (RNAscope). I. A,A',B,B', at E18.5, control kidney express *renin* in cells of the afferent arterioles and in the hilum of the glomerulus along the corticomedullary junction, while *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys demonstrate lower *renin* expression (quantification in II). II A-F, control kidneys at E14.5 through E18.5 demonstrate robust *Tcf21* expression (purple) in the stroma and glomeruli, and r*enin* expression (blue) along the afferent arterioles leading to the kidney hilum. A'-F' *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys show marked reduction in *Tcf21* expression in the majority of the stroma. *Tcf21* expression is intact in the glomeruli and stroma surrounding the ureter, cells that originate from Six2 and Tbx18 progenitors). *Renin* expression is diminished in the JG regions of Tcf21 stromal k/o. Scale bar 100µm.

Figure 3: Inactivation of *Tcf21* upon *renin* promoter expression does not recapitulate the vascular phenotype of *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys.
I. immunostaining for CD31 (PECAM, pan-endothelial) and Keratin8 (ureteric bud) demonstrates the arterial tree of E16.5 *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ (left) and E15.5 *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ (right) kidneys and controls. A,A', the *Ren1*$^{dCre/+}$*; Tcf21*$^{f/f}$ shows vascular patterning that is unchanged compared with control (3D visualization in **Suppl Fig. 1**). B,B', in contrast, the arterial tree of *Foxd1*$^{Cre/+}$*;Tcf21*$^{f/f}$ kidneys demonstrates distorted pattern with disorganized, shorter, and thinner arterioles that display abnormal centripetal pattern with abnormal vessel branching of an aberrant ramification leading into the capsule (described in detail in (12). Scale bar 100 µm. II. Histology of four month old of *Ren1*$^{dCre/+}$*; Tcf21*$^{f/f}$ kidneys do not show phenotypic changes upon H&E (left), and Trichrome (right) staining compared with controls. At 4 months, similar to the findings at E15.5, the morphology of the vascular walls and glomeruli appear intact in *Ren1*$^{dCre/+}$*; Tcf21*$^{f/f}$.

Figure 4: **Normal *renin* mRNA expression in *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ mouse kidney at 2 months.**
In-situ hybridization (RNAscope). A,A',B,B' *renin* expression (blue) is similarly detected in the arteriolar walls (yellow arrows) and in the glomerulus hilum (black arrows) of 2 month-old *Ren1*$^{dCre/+}$*; Tcf21*$^{f/f}$ and control mouse kidneys. Scale bar 75µm for A, 100µm for B. C,C', the distribution of renin-expressing cells is similar in *Ren1*$^{dCre/+}$*; Tcf21*$^{f/f}$ and controls. Scale bar 75µm.

Supplemental Table 1: Primer information (genotyping)

Supplemental Figure 1: Tissue clearing and immunostaining of *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$

Supplemental Figure 1: RNAScope of *Tcf21* and *renin* at E14.5 control kidneys

Supplemental Figure 2: Histology of two-month old mice of control and *Ren1*$^{dCre/+}$*;Tcf21*$^{f/f}$ kidneys

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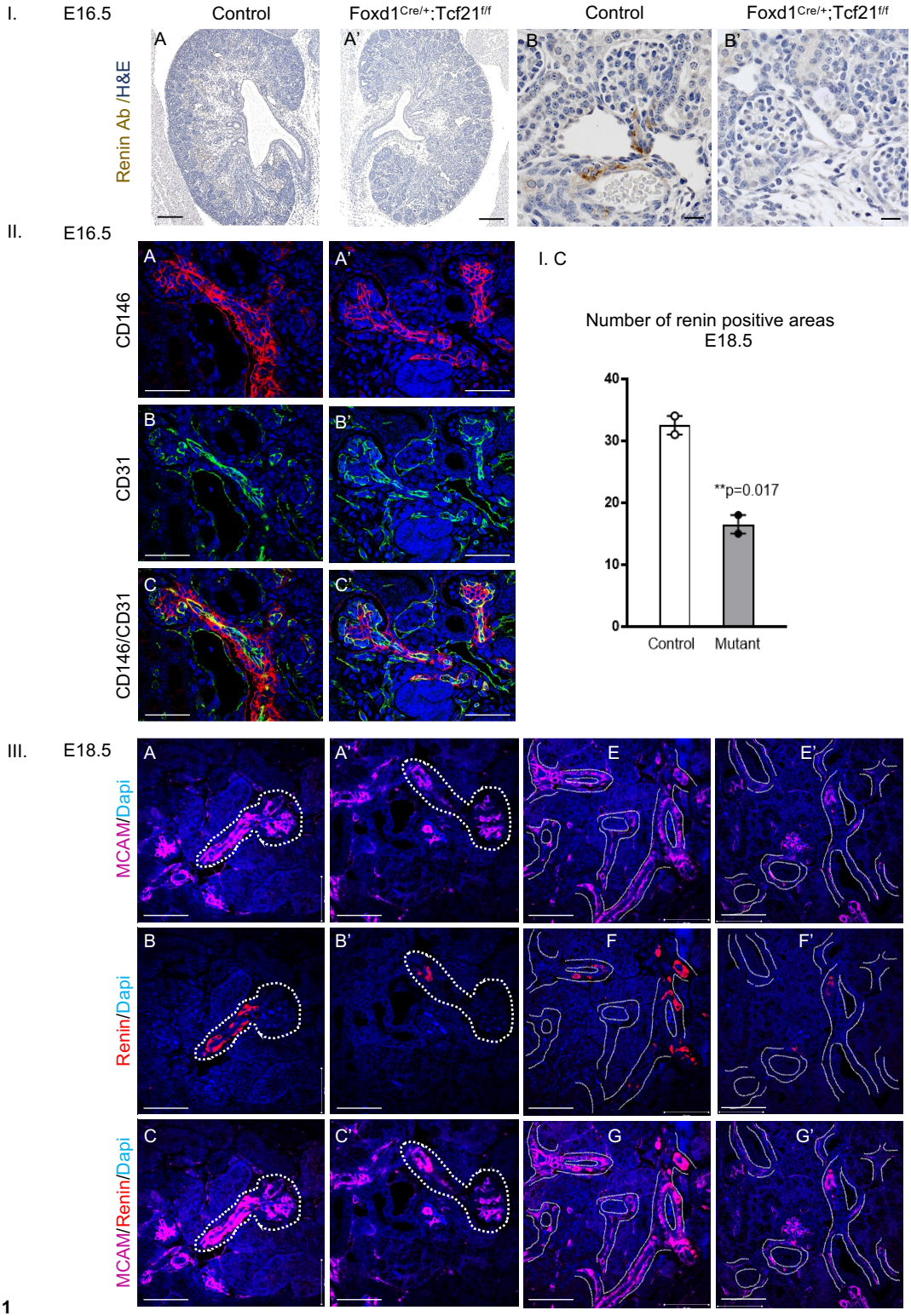
I.
E16.5
Control
Foxd1Cre/+;Tcf21f/f
Control
Foxd1Cre/+;Tcf21f/f
Renin Ab /H&E
A
A'
B
B'
II.
E16.5
CD146
CD31
CD146/CD31
C
C'
I. C
Number of renin positive areas
E18.5
40
30
20
10
0
**p=0.017
Control
Mutant
III.
E18.5
MCAM/Dapi
Renin/Dapi
MCAM/Renin/Dapi
E
E'
F
F'
G
G'

Figure 1

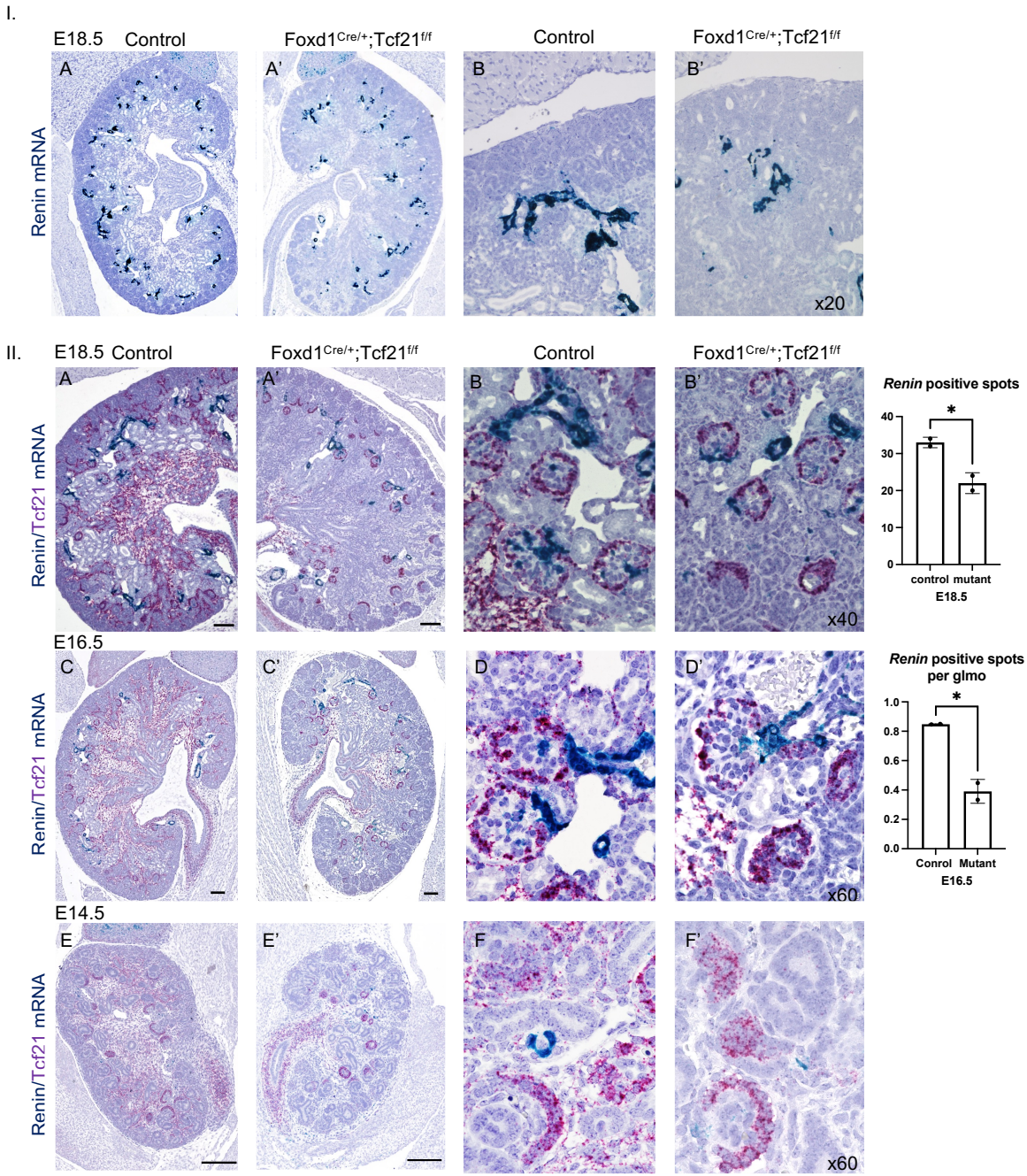
I.
E18.5
Control
Foxd1Cre/+;Tcf21f/f
Control
Foxd1Cre/+;Tcf21f/f
Renin mRNA
A
A'
B
B'
x20
II.
E18.5
Control
Foxd1Cre/+;Tcf21f/f
Control
Foxd1Cre/+;Tcf21f/f
Renin/Tcf21 mRNA
A
A'
B
B'
x40
Renin positive spots
40
30
20
10
0
*
control
mutant
E18.5
E16.5
Renin/Tcf21 mRNA
C
C'
D
D'
x60
Renin positive spots per glmo
1.0
0.8
0.6
0.4
0.2
0.0
*
Conrol
Mutant
E16.5
E14.5
Renin/Tcf21 mRNA
E
E'
F
F'
x60

Figure 2

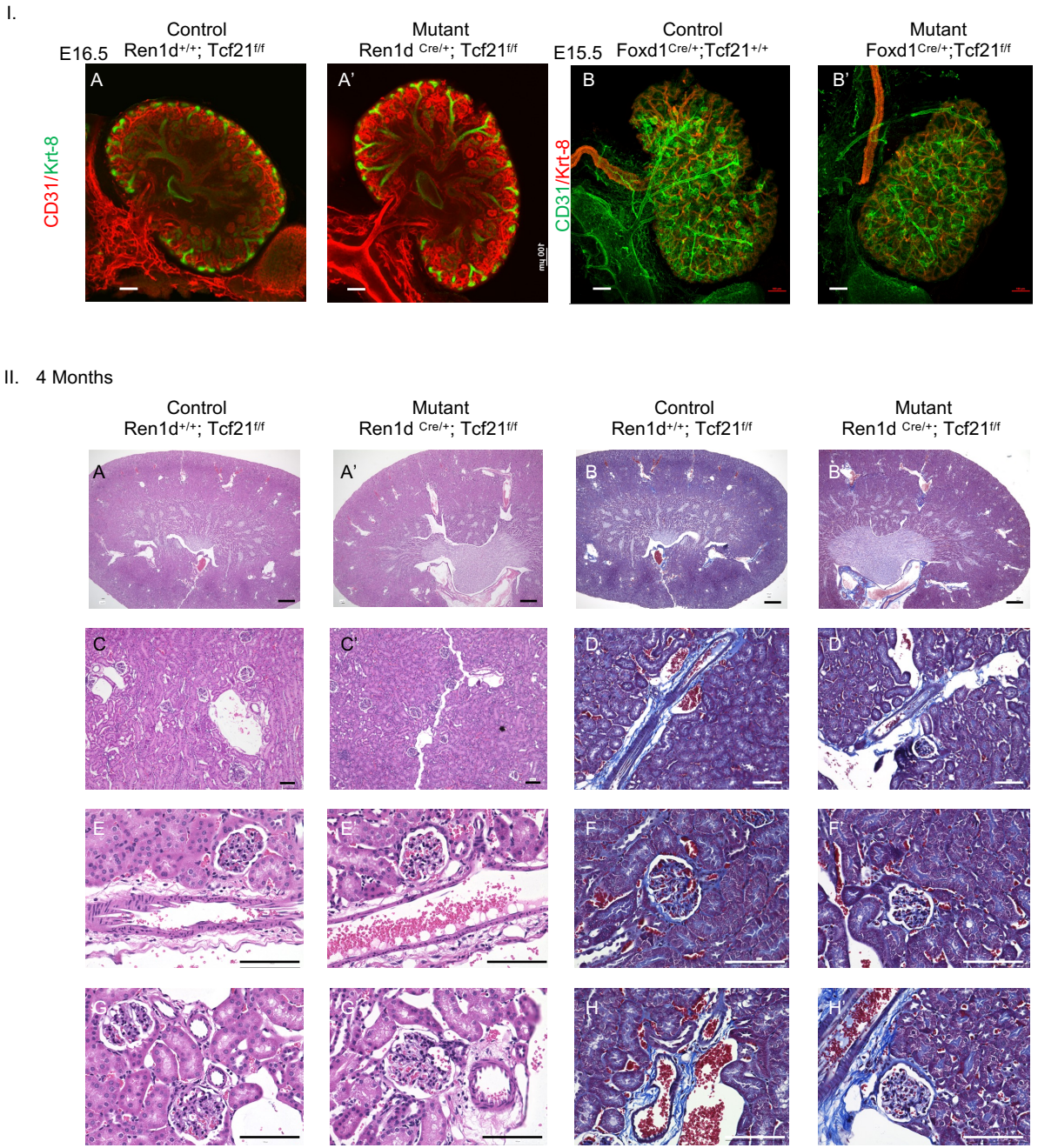
I.
Control
Ren1d+/+; Tcf21f/f
Mutant
Ren1d Cre/+; Tcf21f/f
Control
Foxd1Cre/+;Tcf21+/+
Mutant
Foxd1Cre/+;Tcf21f/f
E16.5
E15.5
CD31/Krt-8
CD31/Krt-8
A
A'
B
B'
II. 4 Months
Control
Ren1d+/+; Tcf21f/f
Mutant
Ren1d Cre/+; Tcf21f/f
Control
Ren1d+/+; Tcf21f/f
Mutant
Ren1d Cre/+; Tcf21f/f
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'

Figure 3

2 Months

Control
Ren1$^{d+/+}$; Tcf21$^{f/f}$

Mutant
Ren1$^{d\ Cre/+}$; Tcf21$^{f/f}$

Renin/Tcf21 mRNA

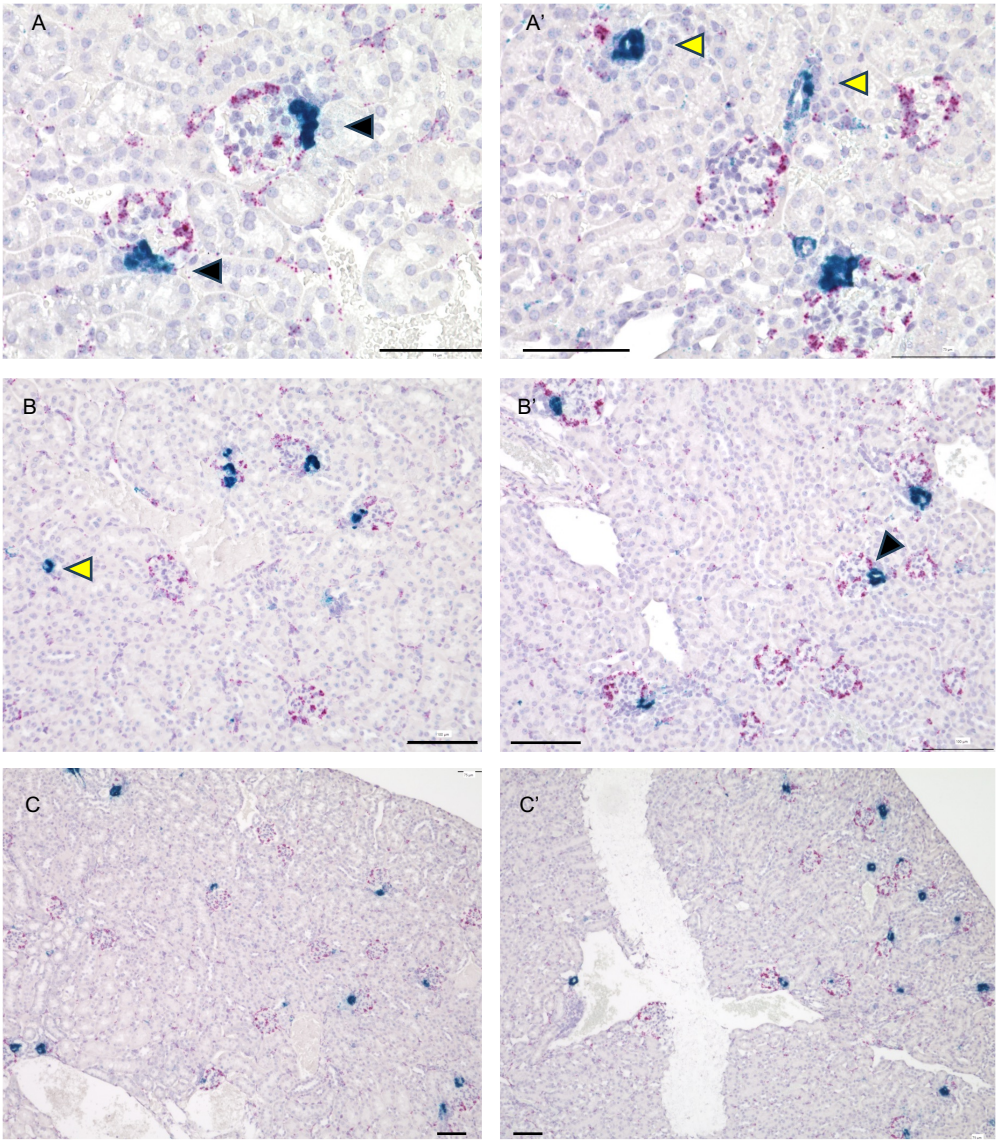

Figure 4