Renin Lineage Cells Repopulate the Glomerular Mesangium after Injury

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ABSTRACT

Mesangial cell injury has a major role in many CKDs. Because renin-positive precursor cells give rise to mesangial cells during nephrogenesis, this study tested the hypothesis that the same phenomenon contributes to glomerular regeneration after murine experimental mesangial injury. Mesangiolysis was induced by administration of an anti-mesangial cell serum in combination with LPS. In enhanced green fluorescent protein–reporter mice with constitutively labeled renin lineage cells, the size of the enhanced green fluorescent protein–positive area in the glomerular tufts increased after mesangial injury. Furthermore, we generated a novel Tet-on inducible triple-transgenic LacZ reporter line that allowed selective labeling of renin cells along renal afferent arterioles of adult mice. Although no intraglomerular LacZ expression was detected in healthy mice, about two-thirds of the glomerular tufts became LacZ positive during the regenerative phase after severe mesangial injury. Intraglomerular renin descendant LacZ-expressing cells colocalized with mesangial cell markers α8-integrin and PDGF receptor-β but not with endothelial, podocyte, or parietal epithelial cell markers. In contrast with LacZ-positive cells in the afferent arterioles, LacZ-positive cells in the glomerular tuft did not express renin. These data demonstrate that extraglomerular renin lineage cells represent a major source of repopulating cells for reconstitution of the intraglomerular mesangium after injury.


Mesangial cells are considered to play a central role in maintaining the structure and function of the glomerulus.1 Unbalanced mesangial cell injury/lysis and response to injury reactions are characteristic for various CKDs such as IgA nephropathy, hemolytic uremic syndrome, lupus nephritis, diabetic nephropathy, and thrombotic microangiopathy leading to renal dysfunction.2–4 By contrast, animal models such as the rat anti-Thy1 nephritis model demonstrate that even a complete lysis of all intraglomerular mesangial cells can be successfully repaired within several days without giving rise to chronic progressive kidney failure.5 In this reversible anti-Thy1 model, our studies previously suggested that cells residing in the juxtaglomerular apparatus (JGA) migrate into the glomerular tuft after induction of severe mesangiolysis.5,6 Therefore, the juxtaglomerular region is considered to be a source of progenitor cells with regenerative capacity. However, the precise origin of the repopulating mesangial cells after injury remains elusive.

Among the different cellular populations of the JGA, the renin-producing cells in the afferent arterioles are the best-characterized cell type.7 Renin is the limiting enzyme of the renin-angiotensin-aldosterone system, which in turn plays a central role in the regulation of arterial BP and electrolyte homeostasis. Previous studies have shown that the renin-expressing cells can be precursors for a variety of cell types, including the mesangial cells, during kidney development.8 Moreover, a recent study suggests a podocyte and glomerular parietal epithelial cell renewal from renin lineage cells during experimental FSGS.9

Using cell-tracing experiments, we examined whether cells of renin lineage are precursor cells that contribute to the repopulation of the intraglomerular mesangium after injury. For this purpose, we used the mouse model of mesangial...
proliferative GN, in which injury is induced by anti-mesangial cell serum as previously reported.\textsuperscript{10} Although this model is similar to the anti-Thy1 nephritis model in rats, the degree of mesangiolysis is not as profound (data not shown; see Yo et al.\textsuperscript{10}) and requires additional application of LPS to aggravate mesangial injury.\textsuperscript{11,12} In several pilot experiments combining anti-mesangial cell serum with varying doses of either Habu snake venom (data not shown) or LPS, we determined the best model system with maximal mesangial cell injury without an increase in mortality, which was used for all further experiments. On days 2–3, the optimized LPS/anti-mesangial cell serum–induced experimental disease model revealed overall pronounced mesangiolysis at varying degrees in individual glomeruli, which was followed by cellular repopulation up to days 10–13 (Figure 1) as indicated by several lines of evidence. Using the well established mesangiolysis periodic acid–Schiff reagent (PAS) scoring system,\textsuperscript{13} disruption of the mesangial area was markedly upregulated on days 2–3 and was already back to normal levels on days 10–13 (Figure 1, A and C). These data are consistent with a decrease of glomerular cells per cross-section on days 2–3 and a significant increase on days 10–13, considering

Figure 1. Severe mesangial cell injury induced by administration of LPS and anti-mesangial cell serum. (A) Histologic evaluation of PAS staining shows mesangiolysis and decreased glomerular cell number (D) on day 3 after model induction compared with the healthy control. (C) The PAS-mesangiolysis score further indicates mesangial cell loss on days 2–3 followed by hypercellularity on days 10–13 (D). (B and E) Expression of the mesangial cell marker α8-integrin is detected in all glomeruli of healthy control mice. Staining of α8-integrin is significantly reduced on days 2–3 after disease induction but mesangiolysis is not homogeneously distributed in all glomeruli on days 2–3. On day 10 after injury, α8-integrin–positive mesangial cells repopulate the glomerular tuft. Box plots show the median with 25th and 75th percentiles and error bars show 10th and 90th percentiles (n=5–8 per group). *P<0.05; **P<0.01; ***P<0.001.
that the mesangium represents only about one-third of all glomerular cells (Figure 1D). In addition, the mesangial cell marker α8-integrin14 was markedly reduced on days 2–3 compared with healthy controls and was almost completely back to normal levels on days 10–13 as evaluated by immunohistochemistry (Figure 1, B and E). Similar results were obtained when immunostaining of PDGF receptor-β (PDGFR-β) was used as a marker of mesangial cells (data not shown).15 These data demonstrate that our model represents a valuable and reliable tool to study the mechanism of mesangial cell repopulation after injury.

To fate-map cells of renin lineage during glomerular injury, we first generated double transgenic mRenCre/tdTomato-enhanced green fluorescent protein (EGFP) reporter mice. These animals constitutively express EGFP in all renin lineage cells. Intraglomerular EGFP expression with typical mesangial cell localization occurred in approximately 5% of the glomerular area per cross-section in healthy mRenCre/tdTomato-EGFP mice immediately before disease induction (Figure 2, A and B). Strikingly, on day 10 after disease onset, the intraglomerular EGFP-positive area increased by 2-fold up to 10% (Figure 2, A and B). Nevertheless, because of the constitutive nature of EGFP expression by renin descendants, these data do not allow us to distinguish whether the increased intraglomerular expression of the renin lineage marker was due to migration of extraglomerular cells or proliferation of the resident labeled intraglomerular cells.

To answer the latter question, we generated a new triple-transgenic mouse line (termed mRen-rtTAm2/LC1/R26R-LacZ) (Supplemental Figure 1), in which irreversible LacZ labeling of renin-expressing cells is induced only upon tetracycline application. Sections were costained for renin and α-smooth muscle actin (α-SMA) to validate that the β-galactosidase (β-gal) expression was limited to renin lineage cells (Supplemental Figure 2, A and B). β-gal and renin colocalization was restricted to the extraglomerular cells of the JGA (Supplemental Figure 2A). For increased renin and LacZ expression, transgenic mice were treated with the angiotensin I–converting enzyme (ACE) inhibitor enalapril, which led to recruitment of double-positive cells along the arterioles.

Figure 2. Fate-mapped cells of renin lineage are located in the glomerular tuft after experimental mesangial injury. (A) Mesangial injury is induced in mRenCre/tdTomato-EGFP mice to trace renin lineage cells using EGFP as the reporter. No antibody is required to visualize EGFP reporter cells, which localize to the afferent arterioles or parietal cells of Bowman’s capsule. Representative images demonstrate that only a small glomerular area shows EGFP-positive reporter cells within the glomerular tuft in healthy control mice, as indicated by arrowheads (left). On day 10 after disease induction, an increased intraglomerular area displays EGFP-positive reporter cells (right, arrowheads). A higher magnification view of the boxed region shows EGFP-tagged cells with typical localization for mesangial cells on day 10. (B) Statistical analysis of EGFP-positive staining area per glomerulus shows a significantly increased percentage on day 10 after mesangial cell injury compared with healthy controls (n=6–10 per group). (C) To determine whether renin lineage cells are recruited from an extraglomerular origin, experimental mesangial damage was induced in mRen-rtTAm2/LC1/R26R-LacZ mice. Genetically tagged cells of renin lineage are visualized using β-gal staining. A representative glomerulus on day 10 after disease induction shows a considerable fraction of LacZ-expressing cells in the glomerular tuft (left, arrowhead). In contrast with the juxtaglomerular apparatus, intraglomerular β-gal–stained cells do not overlap with renin staining (right). (D) The percentage of glomeruli with intraglomerular LacZ-expressing cells per cross-section is markedly increased in mice on day 10 after injury (n=7) compared with healthy controls (n=5). Data represent the mean±SD. *P<0.05. n.d., not detectable.
walls of the afferent arterioles (Supple-
mental Figure 2B). We found that β-gal
staining overlapped in 79% of all glomer-
uli with renin expression at the JGA in
untreated mice versus 89% in enalapril-
treated transgenic mice (Supplemental
Figure 2C). Because of this higher ef-
ciency of β-gal labeling of renin descen-
dants, the ACE inhibitor was adminis-
tered for all subsequent experiments.

In healthy labeled mRen-rTA2/LC1/
R26R-LacZ mice, none of the LacZ-
positive cells were localized within the
glomerular tuft (Supplemental Figure 2,
A and B). By contrast, when pulse-
labeled mice were subjected to mesangial
injury, about 25% of all glomerular tufts
per cross-section expressed LacZ during
the regenerative phase of the disease on
day 10 (Figure 2, C and D). This result
conforms to the phenomenon of a
glomerular replenishment by EGFP-
positive renin descendant cells in the
mRenCre/tdTomato-EGFP mice under
comparable experimental conditions.
This finding further demonstrates that
after mesangial injury, all repopulating
renin descendants are exclusively of ex-
traglomerular origin. These consistent
data from two mouse lines also demon-
strate that the increase of intraglomeru-
lar renin lineage cells is unlikely a side
effect of genomic manipulation in the
transgenic animals. Comparison of
three-dimensional reconstructions of
LacZ-stained cross-sections of animals
before and after model induction con-
fi rmed that strictly extraglomerular-
derived renin progenitors move to
intraglomerular sites of the glomerular
tuft upon injury, although not all replen-
ished mesangial cells are of renin lineage
origin (Figure 3, Supplemental Figure 3).
Intriguingly, the intraglomerular LacZ-
expressing renin descendants did not ex-
press renin any longer (Figure 2C). They
also did not express the podocyte marker
WT-1, the parietal epithelial protein
Claudin-1, or the endothelial cell marker
CD31 (Figure 4). However, the geneti-
cally labeled renin lineage cells in the
glomerular tuft were positive for α8-
integrin and PDGFR-β, both characteristic
for mesangial cells (Figure 4, Supplemental
Figure 4).

Altogether, our results demonstrate
that extraglomerular renin-positive cells
give rise to intraglomerular mesangial
cells after injury. This process recapitu-
lates the development of mesangial cells
from renin-expressing precursors during
nephrogenesis. Therefore, it appears
that the renin-producing cells represent
a specific progenitor niche because they
retain this developmental differentiation
program throughout life.

Next, we tried to better estimate the
quantitative importance of this finding.
Considering that all glomeruli were only
segmentally repopulated by LacZ-positive
renin descendant cells, two-dimensional
evaluation of cross-sections underestimates
the real frequency of this phenomenon.
Therefore, accurate three-dimensional asses-
m ent of the intraglomerular appearance
of pulse-labeled renin-derived cells was
performed via examination of serial sec-
tions comprising 90-μm stack portions
of mRen-rTA2/LC1/R26R-LacZ mouse
kidneys 10 days after mesangial injury.
We evaluated >300 glomeruli in three
separate mice with >100 glomeruli per
individual mouse within a 90-μm stack
portion, and our results showed that
87% of all fully captured glomeruli ex-
pressed LacZ in their JGA and afferent
arterioles. Furthermore, 69% of these
glomeruli also expressed LacZ in their
glomerular tufts during the repopulation
phase after mesangial damage, thereby
supporting the concept that this phe-
nomenon represents a central repair
mechanism.

Our findings also show that not all
intraglomerular mesangial cells were
replaced by pulse-labeled renin cells. Therefore, we must consider that despite aggravated mesangiolysis in our LPS/anti-mesangial cell serum model, mesangial cell loss is incomplete and variable in individual glomeruli, as discussed above (see also Figure 1B). In this context, we found in at least 50 randomly selected glomeruli of six mice, that only about 21% of glomeruli show an almost complete mesangiolysis on day 2 or 3, whereas about 13% of glomeruli reveal almost no mesangiolysis, therefore requiring different degrees and potentially distinct mechanisms of repair. In addition, our study could not exclude the possibility that proliferation of remnant resident mesangial cells or recruitment of renin lineage unrelated cells also contributes to glomerular repopulation after injury. In this context, a previous report shows that bone marrow–derived cells also participate in the repopulation of the mesangium after Thy1-induced mesangiolysis in rats.16

In conclusion, our studies prove the novel concept that renin-positive cells represent a major precursor cell source in the adult organism that leads to the repair of injured mesangium. Upon injury, renin-positive precursor cells move into intraglomerular sites and differentiate into (renin-negative) mesangial cells, a process that recapitulates the cell-differentiation program of renin cells during glomerulogenesis. Future studies are needed to examine why this fascinating repair program fails in the case of chronic progressive glomerular disease and how it can be successfully reactivated.

CONCISE METHODS

Transgenic Mice
First, we generated transgenic mRenCre/tdTomato-EGFP mice by crossing the previously described mRenCre (Ren14-cre)8 mice and ROSA mT/mG mice purchased from the Jackson Laboratories with a C57BL/6J background. Only adult mice that were heterozygous for both Cre and mT/mG were used for our experiments. In these mice, red fluorescence is expressed in all cells except for the renin lineage cells, in which the floxed membrane-targeted tdTomato cassette is deleted in order to allow constitutive expression of the membrane-targeted EGFP cassette instead.

Furthermore, we created novel transgenic mice expressing a modified reverse tetracycline transactivator (rTAm2) from the mouse renin gene locus (mRen-rTAm2
mice). To this end, the coding region of the mouse renin gene (all exons and the interposed introns) was replaced by the rtTam2 sequence in BAC clone RP23-240p23 (BacPac Resources; Supplemental Figure 1A). RP23-240p23 contains the complete mouse renin gene. The rtTam2 encodes a highly specific reverse tetracycline transactivator. Modified RP23-240p23 was purified, linearized, and purified again to obtain the mRen-rtTam2 transgene. This construct was microinjected into one-cell mouse embryos that were then transferred to C57/BL6 foster mothers following standard protocols. The progeny was screened for the full-length transgenic construct. After the identification of the positive founder, it was crossed to the LC1 strain. The LC1 mice express cre-recombinase and firefly luciferase under the control of the tetracycline response element TRE from a bidirectional transcription unit (Supplemental Figure 1B). To localize the expression of the mRen-rtTam2 construct more precisely, we further crossed the mRen-rtTA2/LC1 line to the Rosa26-lacZ reporter strain to generate mRen-rtTA2/LC1/R26R-LacZ triple-transgenic mice. Animals were housed under standard specific pathogen-free conditions including a 12-hour light cycle. All experimental groups contained a similar distribution of male and female mice. All animal studies were approved by the local authorities (Landesdirektion Sachsen Az 24-9168.11-1/2012-29).

**Doxycycline and Enalapril Treatment**

The mRen-rtTA2/LC1/LacZ mice received doxycycline hydrochloride at an age of 8 weeks *via* drinking water *ad libitum* for 16 days (2 mg doxycycline/ml, 5% sucrose, protected from light), which was exchanged every 2 days. In addition to doxycycline, another group of animals also received the ACE inhibitor enalapril (10 mg/kg body wt per day) for 14 days *via* drinking water. To ensure that the mice were free from doxycycline and enalapril during the experimental procedures, they received normal drinking water for at least 7 days.

**Mesangial Cell Injury Model**

To induce experimental mesangiolysis, we used a modified protocol of acute GN in mice as previously described. For our study, mRen-rtTA2/LC1/R26R-LacZ mice and mRenCre/tdTomato-EGFP mice received a single intraperitoneal injection of 1 mg/kg body weight LPS. Two hours later, heat-inactivated sheep anti-mouse mesangial cell serum was administered at a dose of 5 μl/g body weight by intravenous tail injection twice in a 24-hour interval. To examine the efficiency of model induction, a kidney survival biopsy was taken on day 2 or 3 after the last serum administration. At the end of the experiment on day 10, one kidney was removed and fixed in zinc fixative solution before the mice were perfusion-fixed with 3% paraformaldehyde (PFA) and the second kidney was removed.

**β-Gal and Immunofluorescence Staining**

Enzymatic X-gal staining was used to detect β-gal activity. For this purpose, PFA-fixed kidneys were embedded in Tissue-Tek (Thermo Fisher Scientific), frozen on dry ice, and sectioned at 7 μm and slides were washed in LacZ wash buffer (PBS containing 0.01% sodium deoxycholate, 0.2% nonidet-P40). Cryosections were incubated overnight at 37°C in staining solution (1 mg/ml X-gal in dimethylformamide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS, all in LacZ wash buffer), subsequently washed, and blocked before immunostaining. Tissue sections were incubated with specific primary antibodies for renin (1:200, MAB42771; R&D Systems), α-SMA (1:500, ab5694; Abcam, Inc.), WT1 (1:200, sc-15421; Santa Cruz Biotechnology), CD31 (1:100, 550274; BD Biosciences), and Claudin-1 (1:200, RB-9209-P1; Thermo Lab Vision). Sections were heated in citrate buffer for staining of α8-integrin (1:200, BAF4076; R&D Systems) and PDGFR-α (1:200, ab32570; Abcam, Inc.). Visualization was performed by incubation with secondary antibodies, including donkey anti-rabbit antibody (1:500, A31572; Life Technologies), donkey anti-rat antibody (1:500, A21208; Life Technologies), or a fluorescent streptavidin conjugate (1:2000, NL997; R&D Systems). Cellular nuclei were stained with 4′,6-diamidino-2-phenylindole.

Positivity of intraglomerular EGFP staining area in PFA-fixed cryosections of transgenic mRenCre/tdTomato-EGFP mice was quantified in at least 50 randomly selected glomeruli by using computer-assisted image analysis software.

**Three-Dimensional Reconstruction**

For three-dimensional reconstructions, serial slices of X-gal–stained renal cryosections were immunofluorescently stained against α-SMA and α8-integrin as described. After digitalization, glomeruli were reconstructed by using AMIRA software (FEI) as described.

**Statistical Analyses**

In a first step, Kolmogorov–Smirnov and D’Agostino and Pearson omnibus normality tests were performed. Depending on data distribution, statistical significance was
calculated by using one-way ANOVA, followed by Bonferroni’s multiple-comparison test or the Mann–Whitney U test. Analysis was performed using GraphPad Prism 4.0 software (GraphPad Software Inc.). Data are presented as the mean±SD as specified.

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DISCLOSURES

None.

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