In vivo clonal analysis reveals lineage-restricted progenitor characteristics in mammalian kidney development, maintenance, and regeneration


Published in:
Cell Reports

DOI:
10.1016/j.celrep.2014.04.018

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
In Vivo Clonal Analysis Reveals Lineage-Restricted Progenitor Characteristics in Mammalian Kidney Development, Maintenance, and Regeneration

Yuval Rinkevich,1,* Daniel T. Montoro,2 Humberto Contreras-Trujillo,1 Orit Harari-Steinberg,3 Aaron M. Newman,1 Jonathan M. Tsai,1 Xinhong Lim,1 Renee Van-Amerongen,1 Angela Bowman,1 Michael Januszyk,2 Oren Pleniceanu,3 Roel Nusse,1,4 Michael T. Longaker,1,2 Irving L. Weissman,1,5,6 and Benjamin Dekel3,6,*

1Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA
2Hagey Laboratory for Pediatric Regenerative Medicine, Department of Surgery, Plastic and Reconstructive Surgery, Stanford University School of Medicine, Stanford, CA 94305, USA
3Pediatric Stem Cell Research Institute, Edmond and Lily Safra Children’s Hospital, Sheba Medical Center, Tel Hashomer, Sackler School of Medicine, Tel Aviv University, Tel Aviv 52621, Israel
4Department of Developmental Biology, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA
5Ludwig Center for Cancer Stem Cell Research, Stanford University, Stanford, CA 94305, USA
6Co-senior author
*Correspondence: ryuval@stanford.edu (Y.R.), binyamin.dekel@sheba.health.gov.il (B.D.)
http://dx.doi.org/10.1016/j.celrep.2014.04.018
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

The mechanism and magnitude by which the mammalian kidney generates and maintains its proximal tubules, distal tubules, and collecting ducts remain controversial. Here, we use long-term in vivo genetic lineage tracing and clonal analysis of individual cells from kidneys undergoing development, maintenance, and regeneration. We show that the adult mammalian kidney undergoes continuous tubulogenesis via expansions of fate-restricted clones. Kidneys recovering from damage undergo tubulogenesis through expansions of clones with segment-specific borders, and renal spheres developing in vitro from individual cells maintain distinct, segment-specific fates. Analysis of mice derived by transfer of color-marked embryonic stem cells (ESCs) into uncolored blastocysts demonstrates that nephrons are polyclonal, developing from expansions of singly fated clones. Finally, we show that adult renal clones are derived from Wnt-responsive precursors, and their tracing in vivo generates tubules that are segment specific. Collectively, these analyses demonstrate that fate-restricted precursors functioning as unipotent progenitors continuously maintain and self-preserve the mouse kidney throughout life.

INTRODUCTION

The adult mammalian kidney has been classically regarded as a static organ with limited cellular turnover and regenerative capacity (Little, 2006). Nephrogenesis, the process by which new nephrons are generated, initiates in a unique anatomical site of the developing kidney cortex: the cap mesenchyme (CM). In the CM, multipotent stem/progenitor cells are believed to form whole nephrons until 34 weeks of human gestation and for 1–2 weeks in the postnatal mouse, after which the nephrogenic zone exhausts its stem/progenitors (Dressler, 2009; Rosenblum, 2008; Hartman et al., 2007). Humans are estimated to be born with 300,000 to 1 million nephrons and are unable to generate additional whole nephrons under physiological or pathological conditions (Rosenblum, 2008). This is in contrast to invertebrates (Singh et al., 2007) (fly) and lower vertebrates (Diep et al., 2011) (fish), where multipotent renal stem/progenitors are thought to exist in adulthood. In the developing mouse embryo, lineage-tracing studies demonstrate that undifferentiated CM gives rise to all epithelial cell types of the adult neprphon (Herzlinger et al., 1992; Kobayashi et al., 2008; Boyle et al., 2008) and that this population self-renews, fulfilling the requirements for stem cells similar to the reports from the adult fly and fish (Singh et al., 2007; Diep et al., 2011).

Although the regenerative capacity of the adult mammalian kidney remains largely unexplored at the single cell level, the appearance of epithelial cells in the urine due to normal shedding (68,000–72,000 cells/hr; Prescott, 1966) and the documented renal repair that follows damage suggest that the mammalian kidney undergoes constant cellular renewal (Little, 2006). Various cellular models for the maintenance and repair of the adult mammalian kidney have been proposed, including (1) circulating extrarenal cells (Pleniceanu et al., 2010), (2) renal epithelial cells undergoing limited and terminal cell divisions (Bonventre, 2003; Humphreys et al., 2008, 2011), and (3) multipotent adult renal stem cells (Oliver et al., 2004; Bussolati et al., 2005; Sagrinati et al., 2006; Dekel et al., 2006; Maeshima et al., 2006; Appel et al., 2009).

As such, a fundamental question in renal biology still exists as to how and to what extent the kidney physiologically maintains and regenerates its entire compartments in vivo. We utilized
long-term genetic lineage tracing and clonal analysis of individual cells to analyze the magnitude of tissue renewal within the adult mammalian kidney and to discriminate between the proposed cellular modes. Our results reveal a mechanism of continuous regeneration and cellular renewal of kidney epithelia by fate-restricted and segment-specific clones, beginning from fetal stage and persisting throughout adult life.

RESULTS AND DISCUSSION

Clonal Analysis in the Adult Kidney

Mouse kidneys continue to grow in mass for the first 12 weeks postparturition (from 8 to 12 weeks; $p = 0.001046$), a time point from which they maintain a steady-state mass (Figure 1A; from 12 to 40 weeks; $p = 0.208876$). These values were used to establish periods in which renal growth was absent and in which the magnitude and cellular mechanisms underlying renal maintenance could be assessed.

We genetically lineage traced individual cells within the adult kidney (>12 weeks) using “Rainbow” mice (Rinkevich et al., 2011), which harbor a multicolor (red, yellow, green, and blue) Cre-dependent reporter construct within the ROSA locus ($R_{26}^{VT2/GK3}$). To analyze all renal epithelial cell types, including rare and presumed multipotent stem cells, we employed a lineage-tracing strategy that was independent of candidate markers by crossing Rainbow mice with mice harboring an inducible Cre-ER fusion protein under the ubiquitous Actin promoter ($Actin^{CreER}$, $Actin^{CreER};R_{26}^{VT2/GK3}$). Tamoxifen at 12 weeks of age to induce cytoplasmic fusion protein to enter the nucleus and randomly recombine permanently a single color-encoding gene. Mice were sacrificed following 1, 2, 4, and 7 months, at which times the fluorescent patterns were analyzed. Over a 1-month period, small two to three epithelial cell clones were scattered throughout the cortex, medulla, and papillae (Figure S1A, 1 month, $n = 400$). Over extended periods, a subset of epithelial cells increased in size over time, giving rise to large (more than eight cells) clones that have contributed substantially to existing tubules within the cortex (Figure 1B, dotted white line), medulla (Figure 1C, dotted white line), and papillae (Figure 1D).

Each single color segment was uninterrupted by any single cell of another color, ruling out migration of other cells into the clonal region. In each time point (1, 2, 4, and 7 months), only epithelial cell types were in the clone, indicating that physiological epithelial-mesenchymal transformation (EMT) is not apparent within the kidney (Duffield and Humphreys, 2011; Duffield, 2010). These results are not consistent with interstitial mesenchymal cells contributing to renal epithelial repair (Humphreys et al., 2008, 2010) but are consistent with intrinsic renal epithelial cells that mediate tubulogenesis. To rule out the possibility of cellular contributions from adjacent and similarly colored cells to these clones, we performed genetic lineage tracing by administering single and low doses of tamoxifen (see the Experimental Procedures). In these experiments, the frequency of recombination was low (<1%) such that renal epithelial cells were sparsely labeled within large and noncolored kidney domains. Despite the scarcity of labeled cells, kidneys subjected to lineage tracing over 7 months showed single-colored and large clones of 10–12 cells in all kidney regions (Figures 1E–1G), highlighting genuine clonal outputs by individual cells.

To visualize the full sizes and distributions of clones, we isolated intact nephron segments (Schafer et al., 1997) from $Actin^{CreER},R_{26}^{VT2/GK3}$ mice that were chased for 7 months. Intact tubules either showed no significant tubulogenesis in some instances (Figure 1H) or contained large epithelial clones (Figures 1I and 1J) within individual segments ($n = 75$), consistent with epifluorescence analysis of serial sections. Large clones of more than ten cells expanded along the longitudinal and perpendicular axes of tubules (Figures 1I and 1J), contributing circumferentially as well as longitudinally to tubule segments. These results indicate that substantial tubulogenesis has occurred within the adult renal epithelium. Despite the lack of increase in renal mass from 12 weeks postparturition onward (Figure 1A), cumulative counting of cell divisions (see the Experimental Procedures) in the adult kidney over 7 months indicated that the magnitude of tubulogenesis within the adult kidney is equivalent to 4.6–6 times complete renewal of the renal epithelium ($\times 4.6$, cortex; $\times 6$, medulla; $\times 5$, papilla). Tissue renewal occurred despite 20%–25% of renal epithelial cells failing to divide, indicating that only a subset of adult epithelial cells generates new tubule segments postnatally.

The composition of new tubule segments generated postnatally by renal clones was determined by using distinguishing markers for the various tubule types (Laitinen et al., 1987) (Figure 2A; see the Experimental Procedures). A total of 31 clones were examined within convoluted distal tubules (DTs) and stained entirely with DT-specific marker peanut agglutinin (PNA) in the mouse cortex (Figures 2B–2B’). A total of 54 clones were examined within convoluted proximal tubules (PTs) and stained entirely with PT-specific marker lotus tetragonolobus lectin (LTA) (Figures 2F–2F’), whereas 115 clones examined within the collecting ducts (CDs) stained entirely for CD-specific markers aquaporin 2 (AQP2) and aquaporin 3 (AQP3) in the mouse papilla (Figures 2C–2C’ and 2D–2D’). Indeed, combinations of single and double staining using LTA/Mucin 1 (MUC1) (Figures 2E–2E’), LTA/PNA (Figures 2F–2F’), and LTA/Calbindin (Figures 2G–2G’) indicated that clones maintained composition of a single renal lineage and tubule type, including clones ($n = 200$) at the interface between tubule segments (Figures S1B–S1B’, S1C–S1C’, and S1D–S1D’). Given the complete absence of clonal expansion into different segments on the scale of these experiments, it is likely that the clone-initiating cells maintain constant tubulogenesis within the adult kidney are intrinsically limited in their capacity for differentiation and that a multipotent stem cell, if at all present, is physiologically negligible to adult tissue renewal. We did not co-stain MUC1 and PNA because MUC1 also expressed in interstitial mesenchyme (EMT) is not apparent within the kidney (Wagers et al., 2002). Wild-type mice were

Negligible Contributions from Circulating Cells to Kidney Maintenance

To address the possibility of a circulating population of cells that contribute to adult tubulogenesis, we generated pairs of genetically marked parabiotic mice that have a shared anastomosed circulatory system (Wagers et al., 2002). Wild-type mice were
Figure 1. Clonal Analysis during Adult Kidney Growth

(A) Line chart depicting the mean change in kidney mass over time. x axis represents age in days, y axis represents kidney mass in grams.

(B–D) Composite (Rainbow and DAPI) images from Actin^{CreER}; R26^{YFP-DRK1} mice that were chased for 7 months. Singly colored clones contribute to distinct segments in the renal cortex (B, single red clone is outlined), medulla (C, single yellow clone is outlined), and papilla (D, blue clone is outlined).

(legend continued on next page)
surgically conjoined to mice expressing GFP under a ubiquitous (chicken β-actin) promoter. These mice develop full hematopoietic chimerism within a month (Wagers et al., 2003). Kidneys from wild-type mice that were removed after 12 months of parabiosis showed donor-derived GFP⁺ cells throughout interstitial spaces of the kidney, in the renal pelvis, and in cell foci surrounding some glomeruli (Figure S2) that did not incorporate into any glomerular, tubular, vascular, or epithelial structures, or express endothelial or renal epithelial markers (Figure S2). All GFP⁺ cells expressed the pan hematopoietic marker CD45 (Figures S2 and S2D–S2D⁺) as well as CD11c and F4/80 (Figures S2, S2E–S2E⁺, and S2F–S2F⁺), indicating that they belong to the monocyte/macrophage lineage. We found no evidence of contributions from circulating cells, of any type, to cell turnover within the kidney, including glomerular mesangium, which was previously suggested to repopulate from bone marrow cells (Cornachia et al., 2001).

These results of renal tubule-restricted clonal segment-specific epithelial cells are complemented by our previous studies that demonstrated generation of nonepithelial fibroblasts and smooth muscle cells from a mesothelial precursor lineage (Rinkevich et al., 2012) and collectively demonstrate a mechanism of organ renewal by tissue- and lineage-restricted precursors for both renal tubule and nontubule (endothelial, smooth muscle, mesothelium, and fibroblast) components. Like our studies on digit-tip regeneration in mice, wherein lineage-restricted local tissue-type-specific stem/progenitor cells (Rinkevich et al., 2012) rather than dedifferentiated pluripotent blastema cells are responsible for regeneration, and our previous studies that blood-forming stem cells can only make blood (Wagers et al., 2002), and not other tissues such as heart cells (Balsam et al., 2004), brain cells (Massengale et al., 2005), or any endoderm-derived epithelial cells (Wagers and Weissman, 2004) by transdifferentiation, it appears that the mouse and human body plans for tissue maintenance occurs via tissue-restricted and tissue subregion-specific cells with stem/progenitor characteristics.

Clonal Analysis of the Developing Kidney
During kidney development, early mesenchyme of the nephrogenic cortex is induced to undergo mesenchymal-epithelial transition (MET) and differentiates into kidney epithelium (Dressler, 2009; Rosenblum, 2008; Harari-Steinberg et al., 2013). In the pre-MET developmental stage, Six2 specifies mesenchymal progenitors that can give rise to multiple cell types of the nephron tubule (Kobayashi et al., 2008). Post-MET developmental stages represent induced epithelia at varying stages of differentiation and mature nephron tubules. We performed clonal analysis of renal tubules at post-MET stages to identify cellular mechanisms of tubule growth.

Using the ActinCreER ; R26VT2/GK3 system, we traced embryonic renal progenitors from gestational stage of embryonic day 13.5 (E13.5) up to postnatal day 1 (P1) (see Experimental Procedures).

In these post-MET stages, tubules were polyclonal, derived from multiple mixed progenitors (Figures 3B–3D, dotted white lines) similar to lineage-tracing observations of adult kidneys. We separately generated tetrachimeric mice by injecting mouse embryonic stem cells (mESCs) that stably express separate fluorescent proteins (GFP-mESCs), red fluorescent protein (RFP-mESCs), and cyan fluorescent protein (CFP-mESCs) into wild-type blastocysts (Jeno and Weissman, 2006). Within tetrachimera kidneys, mature nephrons were polyclonal, revealing mixed contributions of clones to individual tubule segments (Figures 3E–3H). A similar polyclonal pattern was observed under confocal fluorescent microscopy in both renal tubules (Figure 3I) and glomeruli (Figures 3J–3J⁺). Within the renal papilla, we found a pattern of clonal foci (Y.R., unpublished data), indicating that their progenitors directly seed the renal papilla without prior cellular intermixing. This mosaic polyclonal composition of kidney epithelia (both in CM-derived nephron epithelia and CDs derived from ureteric buds) suggests that several types of stem/progenitor cells are present during kidney organogenesis. Immunostaining for renal epithelial subtypes in tetrachimeras illustrated that clones were separately composed of PT, DT, or CD fates (Figures 3K–3P). Thus, whereas the in vivo clonal analysis cannot definitely assess the pre-MET stage, it indicates that similar to adulthood, at least during the post-MET developmental stages, the immediate contributing precursors to the kidney tubules are locally restricted to a single lineage and tubule type.

Clonal Analysis of the Kidney during Repair
We investigated the response of the kidney to acute injury by performing unilateral ischemia/reperfusion (I/R) to the left kidneys of adult mice (Bonventre and Yang, 2011). Kidneys subjected to I/R showed extensive tubular damage characterized by loss of the epithelial brush border (Figure S3B, black asterisk), flattening of epithelial cells, necrotic tubular cells, and tubular casts at 72 hr postinjury (Figure S3B, black arrowheads). At 4 days postinjury, trichrome-stained kidney sections revealed ectopic collagen deposition and interstitial fibrosis (Figure S3D), especially in those kidneys subjected to prolonged occlusion times (60 and 75 min). To investigate the clonal responses of the kidney to renal injury, tamoxifen was injected into ActinCreER ; R26VT2/GK3 mice, followed by clamping of the left renal arteries. Kidneys were harvested and sectioned after 2 months to allow the cumulative representations of all potential clones (early and late) triggered during the injury response, including cell activities involved in physiologic renal maintenance during this period. Single colored clones appeared in the damaged kidney cortex (Figures 4A–4A⁺, dotted white lines), medulla (Figures 4B–4B⁺, dotted white lines), and papilla (Figures 4C–4C⁺, dotted white lines) and were restricted to their nascent tubule segments of PTs, DTs, and CDs. In areas of damage, we found significant tubulogenesis (Figures 4B and 4C), with clones that have
contributed circumferentially to an entire tubule. Confocal and epifluorescence analyses of serial sections indicated that clones expanded in parallel to the longitudinal and perpendicular axes of a tubule (Figures 4D and 4D') but that did not cross to adjacent segments within a nephron (on the transverse plane) nor to neighboring nephrons (on the sagittal plane). Quantitative counting of clones indicated that significantly more epithelial cells entered cell cycle following injury than did during maintenance, with the highest surge of cell division occurring in the renal cortex (60% versus 41%, Figure S4A).

The possibility of a multipotent cell phenotype emerging in response to damage was determined by analyzing the long-term fates of renal clones. Clones within I/R-subjected kidneys were entirely retained within the domain of a single tubule marker (or completely absent; Figures 4E–4E", 4F–4F", and 4G–4G"). Double immunostaining of dolichos biflorus agglutinin (DBA)/LTA (Figures 4H–4H") and AQ3/LTA (Figures 4I–4I") in areas of renal damage confirmed that clones maintained the fate of one single renal lineage and tubule type, similar to adult maintenance and fetal development.

We explored a second model of acute renal failure using intramuscular injection of glycerol (Figures S3 and S3E–S3H), which results in rhabdomyolysis and myoglobinuria leading to toxic tubular damage (Buzhor et al., 2013). Significant tubulogenesis was independently observed in kidneys that were acutely subjected to glycerol-induced injury (Figure S5) and by expansions of clonal precursors that are fate restricted (Figures S5F and S5G).

Establishment of Renal Organoids In Vitro
To investigate the in vitro fates from individual renal precursors, we established a culture system of growing renal epithelial organoids (Ootani et al., 2009; Buzhor et al., 2011) (see the Experimental Procedures). Kidneys were harvested from Actin\textsuperscript{CreER\textsuperscript{}}; R26\textsuperscript{VTZ2/GRK3} mice following tamoxifen injection, dissociated into single cells, and plated with Matrigel on 24- and 48-well plates. Within several days of culturing, multiple renal organoids developed, gradually enlarged, and then opened into hollow spheres resembling renal tubules in vivo. We found that monoclonal, biclonal, and polyclonal spheres emerged in our culture systems (Figures 5A–5A") with similar propensity, appearance, and size.

To test the in vitro clonal efficiency of renal progenitors, we plated Actin\textsuperscript{CreER\textsuperscript{}}; R26\textsuperscript{VTZ2/GRK3} renal cells in limiting dilution. Approximately 30, 6, and 1 spheres emerged per well following the culture of 1 \times 10\textsuperscript{5}, 1 \times 10\textsuperscript{4}, and 1 \times 10\textsuperscript{3} cells, respectively (average formation of spheres/well was 1.4 spheres/10,000 cells or 0.014%, Figure 5C). Within all dilutions, a similar propensity for monoclonal/polyclonal spheres emerged (43%/57% in 1 \times 10\textsuperscript{6} cells, 50%/50% in 1 \times 10\textsuperscript{5} cells, and 52%/48% in 1 \times 10\textsuperscript{4} cells), indicating that progenitor frequency, not aggregations between renal cells, most likely underlies the frequency of renal spheres expanded in our culture system. Serial passaging of renal spheres over three sequential passages (Figure 5D) revealed that monoclonal spheres are constantly formed, and with increased propensity over time, which is consistent with a subset of cells with self-renewal potential. We then analyzed the fate of monoclonal renal spheres derived from individual cells by staining with antibodies to distinct renal tubule segments. For each segment-specific marker we tested, spheres either stained entirely within all epithelial cells (Figures 5E–5E") or were entirely absent of label (n = 48). Double and triple staining using combinations of segment-specific markers revealed that each renal sphere is fate restricted to one tubule segment only (Figures 5G–5G", 5H–5H", and 5I–5I"), indicating that renal precursors give rise in vitro to epithelial descendants of the same tubule type (PTs, DTs, and CDs). Although our culture conditions support all developmental fates, and spheres in serial passages, we cannot exclude the possibility that the culture conditions biased against a multipotent fate, an increasingly unlikely possibility given the concordance of our in vivo and in vitro data presented here.

Clonal Analysis of Wnt-Responsive Cells
To examine renal precursors’ response to Wnt signaling, we analyzed the expression of Axin2 (Conductin) protein, a gene whose transcription is induced by \&-catenin that has translocated into the nucleus and that provides negative feedback in the Wnt-\&-catenin signaling pathway (Lustig et al., 2002). Kidneys from Axin2\textsuperscript{lacz} transgenic mice, which express LacZ protein under the control of the endogenous Axin2 promoter/enhancer region, showed expression in single cells within the collecting system and the PTs (Figures 6A and 6A'). We then lineage traced the fate of single Wnt-responding cells (WRCs) using mice harboring an inducible Cre-ER under the promoter of the Axin2 gene (van Amerongen et al., 2012) (Axin2\textsuperscript{CreER\textsuperscript{}}). Axin2\textsuperscript{CreER\textsuperscript{}} transgenic mice were crossed with R26\textsuperscript{mTmG}, a double-fluorescent reporter mouse that replaces the expression of tomato red with GFP after Cre-mediated excision (Muzumdar et al., 2007). Kidneys from Axin2\textsuperscript{CreER\textsuperscript{}}; R26\textsuperscript{mTmG} (double-heterozygote) offspring showed GFP expression within single cells of the collecting system, and PTs 4 days after tamoxifen injection, similar to the pattern observed in Axin2\textsuperscript{lacz} transgenic mice (Figure 6A''). Single WRCs that were traced from gestational stage E17.5 up to the
third postnatal month showed large GFP+ clones within the cortex that were developmentally restricted to a PT fate (Figures 6B and 6B'). Separate GFP+ clones appeared within the medullary papilla that were developmentally restricted to a CD fate (Figures 6B" and 6B'"'). Isolation of intact nephron segments from $Axin^{CreER} \times R26^{VT2/GK3}$ mice indicated that single WRCs regenerated new tubule segments (Figures 6C–6C", 6D–6D", and 6E–6E") with more than one tubule segment renewing per PT (Figures 6C", 6D", and 6E"). A similar fate restriction of clones was documented in kidneys from $Axin^{CreER}$ mice crossed to "Rainbow" reporter mice (Figures 6F–6F"). We then counted the clone sizes that emerged in $Axin^{CreER} \times R26^{VT2/GK3}$ kidneys ($n = 1,580$ and found that 97% of all WRCs at the time of tamoxifen injection formed large clones of up to 11 cells (Figure 6G). This represents a substantial increase in the proliferative capacity of WRCs compared to non-Wnt-distinguished cells, with the distribution of the former resembling the upper 5% of the latter.

Immunostaining of single WRC-derived clones for segment-specific markers showed that clones were restricted to either a

Figure 3. Clonal Analysis of the Developing Kidney

(A–D) Composite images (Rainbow and DAPI) from $Actin^{CreER} \times R26^{VT2/GK3}$ mice that were traced from gestational stage of E13.5 up to P1. (A) Lineage-traced cells in the CM are intermixed at the cortex prior to differentiation (white line). (B and C) At post-MET stages, tubules are expanding from mixed progenitors (dotted white line) creating the future polyclonal nephrons. (D) A later stage in a developing tubule shows separate red and green clones contributing to a single tubule segment. (E–H) Composite (Rainbow and DAPI) images of renal tubules from a tetrachimeric mouse. (E) An image of the medulla showing polyclonal nephron and CD tubules. White arrows indicate boarders between clones within individual nephrons. (F) High-power image showing nondividing cells (white arrows) and cell processes interspersed within a green clone. Dotted white line indicates the boarder of the green clone within adjacent and separately colored tubule. (G and H) Separately colored clones are retained within tubules of the CD. (I and J") Confoilucent fluorescent microscopy image of the medulla and a glomerular mesangium showing separate red and green clones of mesangial cells. (K–P) Singly colored clones from tetrachimeric mice are fate restricted to PTs (K–M, LTA+ PNA"), DT (N and O, PNA"), or CD fates (P, AQP3"). Scale bars represent 50 µm (I–P) and 25 µm (A–H).
PT or CD fate. Within the renal cortex, clones were LTA⁺ (Figures 6H–6H‴) and PNA⁻ (Figures 6I–6I‴), consistent with a PT fate. Within the renal papilla, clones were DBA⁺ (Figures 6J–6J‴), AQP2⁺ (Figures 6K–6K‴), and AQP3⁺ (Figures 6L–6L‴), consistent with a CD fate.

We then analyzed the contributions of WRCs to tubulogenesis that follows acute renal failure, using intramuscular injection of glycerol (Figure S6). After 2 months, Axin2CreER; R26mTmG mice (n = 3) that were subjected to glycerol-induced damage revealed predominantly (95%) large clones that contributed new tubule segments within PTs (Figures S6A–S6A‴) and CDs (Figures S6B–S6B‴ and S6C–S6C‴). Lineage tracing of individual Axin2⁺ cells in a similar manner using the Axin2CreER; R26Y2ZGK3 system (n = 3) revealed single-colored and large clones within the renal epithelium and that significant tubulogenesis has occurred via single Axin2⁺ precursors within PTs (Figures S6D–S6D‴) and CDs (Figures S6E–S6E‴). These observations are consistent with the subsets of cells with highest proliferative capacity observed using the ActinCreER system. The fact that Axin2 is expressed (at the time of recombination) within a subset of renal epithelial cells, that then trace long-lived and large clones of up to 11 cells, suggests that the molecular phenotype of the clone-forming cells is distinct from those of their daughter cells and is consistent with a stem cell and/or progenitor characteristic of proximal and collecting system tubules. Going beyond the scope of this manuscript, it would be interesting

Figure 4. Clonal Analysis of the Mammalian Kidney following I/R

(A–C) Composite Rainbow images from ActinCreER; R26Y2ZGK3 mice following 2 months of tracing.

(A–A‴) Singly colored red and green clones contributing to the damaged renal cortex.

(B–B‴) Singly colored yellow and red clones contributing to the damaged renal medulla.

(C–C‴) High-power image showing red and green clones contributing to the damaged CDs.

(D and D‴) Confocal Rainbow images of the renal medulla showing that clones are retained within segments following renal damage.

(E–I) Singly colored clones that emerge following renal damage are fate restricted. Clones are either completely absent (PNA⁻ for non-DT fate, E–E‴) or entirely express (DBA⁺ for CD fate, F–F‴ and G–G‴) the segment-specific markers. Double immunostaining of segment-specific markers shows a green clone that is LTA⁺ DBA⁺ (H) and LTA⁺ AQP3⁻ (I) both representing a PT fate.

Scale bars represent 50 μm (A and C–I) and 25 μm (B).
to assess whether WRCs are a predetermined subset or represent a transient step of cell differentiation induced by local Wnt stimuli.

A recent report by Barker et al. (2012) has identified LGR5+ cells as the immediate progenitors that generate the thick ascending limb of Henle’s loop and distal convoluted tubule during kidney development. Although LGR5, itself a Wnt-responsive gene, is silenced at later postnatal stages of development and fails to trace clone-forming cells in the adult, our analysis demonstrates that constant tubulogenesis is occurring within the mammalian kidney via a similar mechanism involving fate-restricted precursors throughout physiologic renal maintenance and following regeneration-induced damage. During the revision stages of this manuscript, two publications described fate mapping of PT epithelia during renal injury: Kusaba et al. (2014) and Berger et al. (2014). Different from our long-term and unbiased clonal analysis regimen, these groups use marker genes to follow the fates of PT epithelia and independently demonstrate that expanding PT epithelia are fate restricted in their development during renal injury.

Thus, the daily shedding of epithelial cells from all compartments into the urine (Prescott, 1966) can be replenished by local cell production from Wnt-responsive, fate-restricted, and clone-forming cells that may function as unipotent stem/progenitor cells. It is possible that the scattered distribution of single WRCs indicates that they are self-renewed and, thus, are unipotent stem cells, but a more formal analysis of this possibility requires further study.

This mechanism could equally explain the compensatory renal growth that has been documented following nephrectomy (Kaufman et al., 1975) and the idiopathic renal growth documented in pediatric patients with either solitary or single-functioning kidneys (Spira et al., 2009). It also serves to explain the restricted fates and subtypes that have been observed within renal cell carcinomas (Valladares Ayerbe et al., 2009) and inherited kidney disorders (Klootwijk et al., 2014; Bockenhauer et al., 2009) arising from specific kidney segments.

These experiments emphasize the importance of using genetic labeling of individual cells. Histological/immunohistochemical data (Witzgall et al., 1994), staining patterns of bromodeoxyuridine label retention by cells (Oliver et al., 2004), or experiments where multiple thymidine analogs have been pulsed then chased (Humphreys et al., 2008) would greatly depend on previous knowledge of the cell-cycle kinetics of resident cells. Without that knowledge, the distinction between a slow-cycling progenitor and a differentiated cell undergoing its last cell division could not be made.

A similar cellular framework may also take place in liver and pancreas, where self-duplications of adult pancreatic islet cells (Dor et al., 2004) and liver hepatocytes have been reported. In those organs, as in the kidney, a morphologically homogeneous population can nevertheless contain clonogenic subsets, here shown to be the Wnt-responsive cells, that produce the fate-restricted kidney epithelial cells, display enhanced proliferative capacity, as well as retain the low frequency of WRCs, therefore offering a therapeutic target to increase or restore the regenerative capacity of the mammalian kidney.

**EXPERIMENTAL PROCEDURES**

**Mice**

Mice were derived and maintained at the Stanford University Research Animal Facility in accordance with Stanford University guidelines. All the animals were housed in sterile microisolators and given water and rodent chow ad libitum. ActcrtmER transgenic mice were a gift from Dr. Liqun Luo (Stanford University). Axin2tm1(cre/ERT2)Rnu and Axin2tmER (The Jackson Laboratory; strain name B6.129(Cg)-Axin2tm1(cre/ERT2);J, stock number 018867) were a gift from Dr. Roel Nusse (Stanford University). R26rtmG transgenic mice were obtained from The Jackson Laboratory (strain name B6.129(Cg)-R26RosA26Sortm4(ACTB-tdTomato,EGFP); strain number 007676). Male mice were used in all experiments, unless stated otherwise. Parabiosed wild-type mice were all females.

**Mice Genotyping**

The following primers and PCR conditions were used for genotyping:

- Cre: 5’-CGGTGATGCAACGAGTGATGAGG-3’ and 5’-CCAGAGACGGAAATCCATGGCTCG-3’; 94°C for 10 min, 94°C for 30 s, 56°C for 1 min, and 72°C for 1:30 min; and repeat 35 cycles, 72°C for 8 min.
- mTmG: 5’-CTCTTGCTGCCCTGCTTCTC-3’ and 5’-CGAGGGGATCACACCAAGCAATAA-3’ and 5’-TCAATGGGGGGGGGCGCTTT-3’; 94°C for 3 min, 94°C for 30 s, 61°C for 1 min, and 72°C for 1 min; and repeat 35 cycles, 72°C for 2 min.

**Tamoxifen Injections**

Male mice were used in all experiments, unless stated otherwise. All strains used for genetic lineage tracing (including ActcrtmER, R26rtmG and Axin2tmER, R26rtmG) were assessed for leakiness in adult kidneys and show negative Rainbow fluorescence (using R26rtmG reporter) or GFP (using R26rtmG reporter) in the absence of tamoxifen administration.

Tamoxifen (Sigma-Aldrich) was prepared by dissolving in corn oil (Sigma-Aldrich) to a concentration of 20 mg/ml. A total of 4–6 mg tamoxifen was injected intraperitoneally (i.p.) every other day for 1 week, using a tuberculin syringe and 25G needle.

To find tamoxifen concentrations where sparse labeling would take place within the renal epithelium, we injected male mice at >2.5 months of age with a single dose of tamoxifen/corn oil at various concentrations. At these postnatal stages, we found that injecting tamoxifen i.p. at concentrations below 1 mg (n = 5 mice) does not lead to recombination within the renal cortex and medulla, but only in papillary epithelium. A dose of 1–2 mg

---

**Figure 5. Renal Spheres that Develop from Individual Cells Are Lineage Restricted In Vitro**

(A–A’) Composite fluorescent images of spheres from single-cell suspensions of ActcrtmER, R26rtmG kidneys. (B–B’) Representative sections of renal spheres stained with hematoxylin and eosin. (C) Histogram that graphically represents the frequency of spheres formed from cells cultured in limited dilution. (D) Graphical representation of the frequency of monoclonal/polyclonal spheres emerging after serial passaging. Following three passages, most emerging spheres are monoclonal (red line), and not polyclonal (blue line). (E and F) Images of sections from renal spheres immunostained with antibodies to distinct renal segments reveal that each segment-specific marker stains some but not all spheres. (G–I) Immunostaining of three representative spheres with antibodies for segment-specific markers. Each renal sphere is fate restricted to DTs (PNA+), PTs (LTA+), or Cds (AQ3+). Scale bars represent 50 μm (A–A’, B–B’, and E–I).
tamoxifen/corn oil was found to be the minimal concentration where sparse labeling of around 1% would take place within all renal epithelium (n = 5). Lineage Tracing of Embryonic Kidney Adult female \textit{R26\textsuperscript{Rainbow}} mice older than 2 months of age were mated with adult \textit{Axin\textsuperscript{CreER}} or \textit{Actin\textsuperscript{CreER}} males. Plugs were checked every morning, and 0.25 mg/25 g of tamoxifen diluted in corn oil was injected i.p. at days E10.5–E13.5 with a Becton Dickinson 1 ml insulin syringe with a 27G needle. Eight neonatal pups were sacked at day 1, fixed in 2% paraformaldehyde overnight, and set in optimal cutting temperature compound (OCT). Kidneys (n = 16) were subjected to 5 \textmu m sections and analyzed for multicolor fluorescence. Generation of Tetrachimera Mice C57BL/6J female mice (Jackson Laboratory; strain/000664) were superovulated with 5 IU of pregnant mare serum gonadotropin (Sigma-Aldrich; G4877) and 5 IU of human chorionic gonadotropin (Sigma-Aldrich; CG 10) and mated by a standard protocol. Morulas were collected at E2.5 and cultured with EmbryoMax KSOM (Millipore; MR-106-D) overnight to blastocysts. For each blastocyst, a mixture of 15 ESCs was injected. For single-cell injection, Rosa-EGFP, Rosa-ECFP, and Rosa-mRFP1 ESC clones were put in a separate place on an injection chamber, and each one of the three clones was picked up and injected into a blastocyst. Injected blastocysts were then transferred into the uterus of day 2.5 pseudopregnant CD1 mice (Charles River Laboratories; #022) and allowed to reach postnatal stages of development, at which time five tetrachimera mice (male and female) were sacrificed at 30 days postparturition, and their kidneys (n = 10) were analyzed. Renal I/R Injury A left peritoneal incision was performed on five male mice 6–8 weeks of age to expose the kidney, followed by clamping of the left renal pedicle for 30–75 min.
Glycerol-Induced Acute Kidney Injury

Following 22 hr of water deprivation, five male mice 6–10 weeks of age received an intramuscular injection of 6–8 mg/kg hypertonic glycerol (50% v/v; Sigma-Aldrich) as divided injections in both the hindlimbs. Blood samples were collected from the retro-orbital sinus. Mice serum was extracted using 0.8 ml MiniCollect Tube (Greiner Bio-One) according to manufacturer’s instructions, and blood levels of creatinine and urea were measured using the Olympus AU2700 analyzer. Blood urea nitrogen (BUN) equals urea (mg/dl) divided by 2.14.

Mice exposed to a single intramuscular injection of glycerol showed a significant rise in BUN and creatinine following 3 days (Figures S3E and S3F), which was associated with tubular epithelial injury and generalized disorganization of the normal kidney structures, including necrosis, cast formation (hyaline and cellular), and desegregation of tubular membranes (Figure S7). Clonal analysis was associated with tubular epithelial injury and generalized disorganization of renal epithelial cells entered cell cycle within the cortex, medulla, and papilla, respectively (Figure S5E). The IR-induced clonal response is significantly different from that of the glycerol-induced response and may be an outcome of regional susceptibility to hypoxia that exists between the compartments of the kidney.

Histology and Tissue Analysis

For fixation, kidney samples were placed in 2% paraformaldehyde for 12–16 hr at 4 °C and then prepared for embedding by soaking in 30% sucrose in PBS at 4 °C for 24 hr. Kidney samples were removed from the sucrose solution, and tissue blocks were prepared by embedding in Tissue-Tek O.C.T. (Sakura Fine-tek) under dry ice to freeze the samples within the compound. Frozen blocks were mounted on a Microm HM550 cryostat (MICROM International), and 5- to 8- or 12-μm-thick sections were transferred to Superfrost Plus Adhesive Slides (Fisherbrand).

Hematoxylin and Eosin Staining

All histological stains were carried out on 8 μm formalin-fixed paraffin-embedded sections. Nuclei were stained with Gill’s hematoxylin, blue with diluted ammonium hydroxide, and the cytoplasm was counterstained with acidified eosin.

Trichrome Staining

Trichrome stains of kidney samples 4 days after IR injury were carried out according to Masson’s method. Samples were deparaffinized and rehydrated, refixed in Bouin’s solution, and stained with Weigert’s iron hematoxylin to stain nuclei, Biebrich scarlet-acid fuchsin to stain cytoplasm and keratin, and aniline blue to stain collagen fibers.

Periodic Acid-Schiff Staining

Periodic Acid-Schiff (PAS) stains of kidney samples 3 days after glycerol administration were carried out using PAS kit (Sigma-Aldrich) according to the instructions of the manufacturer.

Confocal Microscopy

Confocal microscopy was carried out on 8–12 μM cryosections counterstained with Hoechst 33424. A Leica SP2 AOBS (Acousto-Optical Beam Splitters) confocal laser-scanning microscope was used to capture images allowing spectral detection and unmixing of overlapping spectrum with 20×, 40×, and 63× oil objectives. For resolution of nuclei-dense regions, z stacked confocal images were rendered into three dimensions using Velocity 6.0.1 software for clonal analysis.

Immunohistochemistry

Immunostaining was performed using the following primary antibodies: CD31 (Abcam), CD90 (Bioscience), PEA (BD PharMingen), Cytokeratin 5 (Abcam), Cytokeratin 14 (Covance), CD45 (BioLegend), LTA (Covance), DBA (Covance), PNA (Covance), AQP3 (Abcam), and MUC1 (Abcam). LTA immunostains PTs, PNA, and Calbindin, immunostain D Ts, AQP2, AQP3, DBA, and MUC1 immunostain CDs.

Briefly, slides were blocked for 30 min in 10% BSA with 2% goat serum followed by incubation with primary antibody for 12–16 hr. For immunoassaying on sections from ActinCreER, R26T2/GK3 mice, Alexa Fluor 647-conjugated antibody was used as secondary 1:1,000 for 1 hr (Invitrogen) and was visualized in the far-red channel (Cy5). Fluorescent and bright-field images were taken with a Leica DM4000B microscope (Leica Microsystems) and RETIGA 2000R camera (QImaging Scientific Cameras).

Cell Counting

Clones were visualized using a Leica DM4000B microscope. For each experiment, 400 clones from the renal cortex, medulla, and papilla were counted separately and then averaged. Clone size was established by incorporating the number of nuclei in each clone per section and by incorporating the total clone sizes from serial sections.

Physiological tissue renewal over 1 year was approximated by counting clones in the renal epithelium, only, and in areas with recombination frequency >95%. Overall cell division within a field was calculated by estimating the percentage of cells in each clone size times the number of cell divisions required to reach each clone size. For example, 20% of renal epithelial cells exhibiting an average clone size of ten cells/clone would indicate renewal of 20% x 9 = 180%. These values do not take into account renal epithelial cell death and shedding and, therefore, represent a lower estimation.

In Vitro Culture of Renal Cells

To isolate renal cells, adult ActinCreER, R26T2/GK3 (Rainbow) mice were injected i.p. with 4–6 mg of tamoxifen (Sigma-Aldrich). Kidneys were then collected and mechanically and enzymatically dissociated in Medium 199 containing Liberase TM, TH enzymes (Roche), and D Nase ( Worthington) treated at 37 °C until a single cell suspension was achieved. Dissociated cells were washed with PBS and serially filtered through 70 and 40 μm cell strainers.

Red blood cells were lysed using ACK Lysing Buffer (Invitrogen). Single-cell suspensions were mixed with 25 μl of ice-cold Matrigel (reduced growth factors; BD Biosciences), plated in 48-well plates, and allowed to solidify at 37 °C. After polymerization of Matrigel, 250 μl of culture medium was added: advanced Dulbecco’s modified Eagle’s medium/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, 1× N2, 1× B27-A (Invitrogen), and N-acetylcysteine (Sigma-Aldrich) containing growth factors (50 ng/ml epidermal growth factor [PeproTech], 500 ng/ml R-spondin-1 [R&D Systems], and 100 ng/ml Noggin [PeproTech]). Growth factors were added every fourth day, and the entire medium was changed every 8 days.

Kidney Weight

Various aged CD-1 wild-type mice (sharing background with ActinCreER, R26T2/GK3 experimental animals) were purchased from Jackson Laboratory as individuals from separate litters to control for variable husbandry and litter conditions. Left and right kidneys were collected, and mesothelium was removed for accurate weighing in triplicate on a Mettler-Toledo AB54-S/FACT Electronic Analytical Balance to the nearest 10−4 g. Control kidneys perfused with saline prior to weighing tended to be heavier than raw processed kidneys but did not show a significant difference between kidneys of the same weight, nor did it change the trend of kidney mass over time. Statistics between individual age groups was determined with two-tailed t test; each experiment, 400 clones from the renal cortex, medulla, and papilla were counted separately and then averaged. Clone size was established by incorporating the number of nuclei in each clone per section and by incorporating the total clone sizes from serial sections.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.016.

AUTHOR CONTRIBUTIONS

Y.R. and B.D. designed and performed the kidney experiments with suggestions from I.L.W. and R.N. Y.R. imaged and analyzed the data from all kidney experiments. D.T.M. performed the confocal imaging and assisted with experiments and collection and analysis of data. H.C.-T. developed, performed, and analyzed the in vitro assays. O.H.-S. analyzed outcomes of renal damage. R.W.-A. and X.L. performed the lineage tracing in ActinCreER,R26T2/GK3 mice.
ACKNOWLEDGMENTS

We thank Adriane Mosley for assistance with animal care and parabiosis experiments and Charlene Wang for making the tetrachimeric mice. We thank Kitty Lee and the Cell Sciences Imaging Facility Fluorescent Microscopy Core at Stanford University for technical assistance with confocal microscopy. This work was supported in part by a grant from the California Institute of Regenerative Medicine (RC1 00354) and the Smith Family Trust (to I.L.W.), the Oak Foundation and the Hagey Laboratory for Pediatric Regenerative Medicine (to M.T.L.), the Israel Scientific Foundation (910-11), Israel Cancer Research Fund (PO-27013), and the Feldman Family Visiting Professorship, Stanford School of Medicine (to B.D.). Y.R. was supported by the Human Frontier Science Program Long-Term Fellowship, the Machiaj Foundation Fellowship, and the Siebel foundation (1119368-104-GHBJ).

Received: July 7, 2013
Revised: March 2, 2014
Accepted: April 9, 2014
Published: May 15, 2014

REFERENCES


Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pletsch, T., Karsten, U., van de Watering, M., Clevers, H., Schlag, P.M., Birchmeier, W., and Behrens, J.

