Genetic tracing of the epithelial lineage during mammalian kidney repair

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Developing new therapeutic approaches to treat acute kidney injury requires a detailed understanding of endogenous cellular repair. Genetic fate mapping defines cellular hierarchies *in vivo* and we used this technique to assess a possible contribution of non-epithelial stem cells to renal repair after ischemic injury. Mice with efficient labeling of renal epithelial cells, but not non-epithelial interstitial cells, were subjected to a single cycle or sequential cycles of kidney injury and repair. No dilution of the epithelial cell fate marker was observed despite robust epithelial cell proliferation. Thus, non-tubular cells do not have the ability to migrate across the basement membrane and differentiate into epithelial cells in this model. Instead, surviving tubular epithelial cells are responsible for repair of the damaged nephron. Future studies will need to distinguish between uniform dedifferentiation and proliferation of all epithelial cells after injury versus selective expansion of an intratubular epithelial stem cell.

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KIDNEY REPAIR AND INTERSTITIAL STEM CELLS

Acute kidney injury (AKI) is common, costly, and independently associated with increased risk of death.[1] Patient outcomes are directly related to AKI severity, including what are considered minor changes in serum creatinine.[2] More severe AKI is an independent risk factor for death, and the modest improvement in mortality observed in recent years is offset by the increasing prevalence of AKI in our aging population.[3] Novel directed therapies are needed for the treatment of AKI, and stem cells have generated a tremendous amount of excitement because of their potential to lead to new treatment approaches. Tissue-specific adult stem cells have important roles in early development, as well as in tissue homeostasis and regeneration in the hematopoietic system, skin, intestine, and muscle. However, the existence of epithelial stem cells in the kidney is still controversial.[4–6]

Adult renal stem cells could have important therapeutic uses.[7–11] For example, manipulating the signaling pathways that regulate growth and differentiation of renal progenitors might enhance repair after AKI. Renal stem cells might serve as a cellular source for preclinical toxicity testing or to seed the bioartificial kidney, a hemofiltration system containing live human tubular epithelial cells.[12,13] Mesenchymal stem cells (MSCs) ameliorate AKI by paracrine mechanisms[14] and they are currently being tested in a Phase 1 trial.[15] As accumulating evidence implicates an episode of AKI in the development of chronic kidney disease, stem cell therapies for AKI may provide long-term benefits as well.[16] Finally, the isolation of renal stem cells from human kidney biopsy specimens could enable the creation of disease-specific models for research. A necessary first step for many of these applications, however, is definitive proof that such cells exist in the adult kidney to allow isolation, as well as phenotypic and functional characterization of such cells. Progress toward achieving these goals has been slow in the kidney because of the complexity of the tissue architecture and a lack of stem cell markers.

Several lines of evidence have supported the existence of a non-tubular, interstitial stem cell population in kidney. During nephrogenesis, the cells that make up nephron epithelia, including podocytes and tubule segment epithelia, are derived from a common progenitor that also gives rise to

the interstitial cell types, such as pericytes or perivascular fibroblasts and mesangium. These progenitors are present in metanephric mesenchyme, a prespecified group of cells that is induced toward kidney fates by inductive signals secreted by the ureteric bud. As interstitial and tubular cells share a common mesenchymal progenitor, persistence of a small number of these mesenchymal cells in the adult interstitium could serve as a stem cell niche for tubule regeneration after injury. Indeed, in lower vertebrates such as the adult skate (*Leucoraja erinacea*), the zebrafish (*Danio rerio*), and the goldfish (*Carassius auratus*), for example, partial nephrectomy or nephrotoxic damage induces *de novo* nephrogenesis in a process recapitulating embryonic kidney development, including condensation of interstitial mesenchyme-like cells.[17–19] In mammals, certain interstitial populations express stem cell markers such as Sca-1,[20,21] CD34 and CD44,[22] CD133,[23] or long-term label retention,[24] and in some cases these cells appear to engraft into endogenous tubular epithelium when injected during nephrogenesis or after kidney injury.

LINEAGE ANALYSIS TO DEFINE EPITHELIAL CELL ORIGINS

To definitively test whether an endogenous interstitial stem cell population might contribute to the epithelial lineage during mammalian renal repair, all mesenchyme-derived renal epithelial cells were genetically labeled using a Cre/Lox strategy. The *Six2* gene is expressed exclusively in metanephric mesenchyme cells fated to become renal epithelia,[25] and we crossed a transgenic mouse in which the Six2 promoter drives expression of a green fluorescence protein (GFP)–Cre recombinase fusion protein (the Six2-GC mouse) against two different reporter mice, the Rosa26-LacZ (R26R) reporter and the ACTB-Bgeo,-DsRed.MST reporter (Z/Red). Because the *Six2* gene is active very early in nephrogenesis, recombination efficiency is high and we achieved 95% labeling of all tubular epithelial cells derived from the metanephric mesenchyme (collecting duct epithelia are derived from the ureteric bud, and therefore not labeled in this model).[26] The rationale is outlined in Figure 1. As interstitial cells are not labeled, after a cycle of injury and repair, dilution of the fate marker would indicate contribution to the epithelial lineage by unlabeled interstitial cells. If repaired tubules still express the fate marker, by contrast, then repairing epithelial cells originated from within the tubule.

Close inspection of kidney sections confirmed that no interstitial cells were labeled, either with β-galactosidase (LacZ) or with red fluorescent protein (RFP). GFPCre fusion protein expression was undetectable after the completion of nephrogenesis (P3-5), whether assessed by epifluorescence or antibody-based detection of GFP (the GFPCre fusion protein retains GFP fluorescence), Six2 mRNA, or Six2 protein using a specific antisera (as GFPCre expression is regulated by the Six2 promoter, the GFP expression pattern mirrors expression of endogenous Six2 mRNA and protein). Mice were subjected to renal ischemia/reperfusion injury to trigger the proliferative response. Two days after injury, 47.4% of cells in the outer medulla expressed the proliferation marker Ki67, and these cells coexpressed RFP. Many tubules had flattened epithelial cells characteristic of the dedifferentiated state, and these cells also expressed either RFP or LacZ. When mice were injured and given an injection of 5-bromo-2′-deoxyuridine once daily for 7 days, 66.9% of outer medulla epithelial cells had incorporated 5-bromo-2′-deoxyuridine, compared with 3.5% in uninjured, control kidneys. Despite this robust proliferation, there was no dilution of the fate marker in kidneys that had been allowed to repair for 15 days. A total of $94.3 \pm 3.7\%$ of outer medullary epithelia were RFP positive before injury, and $94.4 \pm 2.4\%$ expressed RFP after repair with similar results calculated for LacZ as the fate marker.[26]

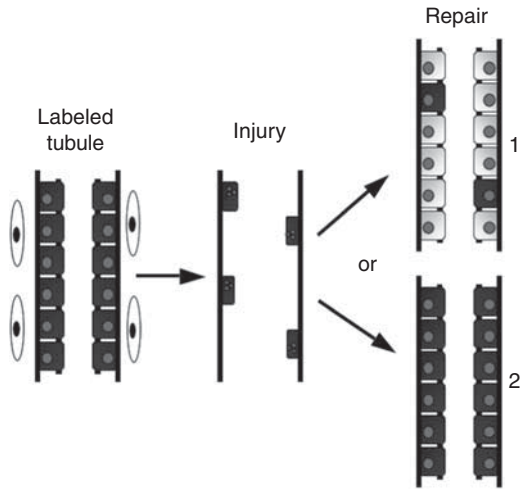

Figure 1 | Lineage tracing to determine origin of epithelial cells after kidney repair. Genetically labeled tubule cells are dark gray, and interstitial cells are unlabeled. After injury, there is necrosis and apoptosis of labeled epithelial cells. If tubule repair involves an interstitial stem cell, these unlabeled cells will dilute the genetic marker and tubules will lose the label after completion of repair (model 1). If surviving epithelial cells are responsible for tubule repair, then the genetic marker will not be diluted by unlabeled cells after completion of repair (model 2).

If the GFPCre fusion protein were re-expressed in epithelial cells after injury, then a non-labeled interstitial cell that had migrated into the damaged tubule might also be induced to express GFPCre, and thus activate expression of either RFP or LacZ, compromising our ability to detect a dilution of the fate marker. However, we did not detect re-expression of the transgene, as assessed by sensitive GFP immunofluorescence despite very robust GFP fluorescence in the cap mesenchyme of P1 kidneys from Six2-GC mice, a stage at which Six2 is still expressed. We were also unable to detect Cre mRNA after injury by qualitative reverse transcriptase-polymerase chain reaction, and we did not detect endogenous Six2 protein in adult kidney after injury using a specific anti-Six2 antibody.

To verify these results, we also performed the converse experiment by labeling interstitial but not epithelial cells, using a FoxD1-Cre driver. In this case, the interstitial

compartment, including pericytes, fibroblasts, and mesangium, was efficiently labeled. After a cycle of injury and repair, there was no increase in labeled epithelial cells in the tubule, providing further strong evidence against the possibility that an interstitial cell population is capable of contributing to the epithelial lineage during endogenous repair.

UNRESOLVED QUESTIONS

If surviving epithelial cells are responsible for tubule repair, how can this be reconciled with the notion that renal interstitium contains a stem cell population with multilineage differentiation capability—cells that also ameliorate ischemic renal injury when administered exogenously?[20,21,23,24,27,28] An intriguing hypothesis has been proposed that these interstitial cell populations are all MSCs derived from the perivascular niche.[20,29,30] Indeed, pericytes themselves—mesenchymal-like cells associated with the walls of small blood vessels directly adjacent to endothelial cells—may directly give rise to MSCs.[31] These cells are poised to respond to vascular injury and integrate injury signals from the vasculature to regulate epithelial repair and modulate the immune response. Through secretion of bioactive molecules that exert antiapoptotic and proproliferative effects on tubular epithelial cells, as well as immunomodulatory actions on migrating inflammatory cells, these adult kidney-specific MSCs could function in a paracrine manner to promote tissue repair.[14] The ability of exogenous, bone marrow-derived MSCs to protect against kidney injury through secretion of soluble mediators suggests that endogenous, organ-specific MSCs in renal perivasculature may also function on tubular cells in a similar paracrine manner.[32,33] Whether nestin-positive, interstitial slow cycling cells in renal papilla might represent such a population of kidney-specific MSCs requires investigation.[24,26]

Another unresolved question is whether an intratubular epithelial stem cell might exist, or whether every epithelial cell has the equivalent capacity to dedifferentiate and proliferate after injury. In support of an intratubular stem cell, stem cell markers including Oct4 and label retention have been used to derive cell lines with stem cell properties.[28,34] In *Drosophila*, an intratubular pluripotent has been definitively identified, bolstering the contention that a mammalian equivalent might exist.[35] Arguing against an intratubular stem cell is the rapidity of the epithelial proliferative response to injury and the failure to detect phenotypic differences between quiescent and dividing epithelial cells during kidney homeostasis.[36,37] An intriguing recent study by Vogetseder and colleagues[38] in rat, using a lead acetate mitotic stimulus, suggested that $\sim$40% of proximal tubules express cyclin D1, indicating that they are in G_1 rather than G_0, and this proportion falls after the proliferative stimulus suggesting progression through the cell cycle. As cyclin D1 helps regulate cell cycle re-entry, one interpretation of this observation is that the capacity to divide is not restricted to a small subset of proximal tubule epithelia, as would be expected for an intratubular stem cell model. It will be important to re-address this conclusion using a model that induces tubule injury including epithelial dedifferentiation, such as renal ischemia.

DISCLOSURE

The author declared no competing interests.

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