Collecting Duct-Derived Cells Display Mesenchymal Stem Cell Properties and Retain Selective In Vitro and In Vivo Epithelial Capacity

Joan Li,* Usukhbayar Ariunbold,* Norseha Suhaimi,* Nana Sunn,† Jinjin Guo,‡ Jill A. McMahon,‡ Andrew P. McMahon,‡ and Melissa Little*

*Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia; †Diamantina Institute, University of Queensland, Woolloongabba, Queensland, Australia; and ‡Department of Stem Cell Biology and Regenerative Medicine, Broad-CIRM Center for Regenerative Medicine and Stem Cell Research, University of Southern California Keck School of Medicine, Los Angeles, California

ABSTRACT
We previously described a mesenchymal stem cell (MSC)-like population within the adult mouse kidney that displays long-term colony-forming efficiency, clonogenicity, immunosuppression, and panmesodermal potential. Although phenotypically similar to bone marrow (BM)-MSCs, kidney MSC–like cells display a distinct expression profile. FACS sorting from Hoxb7/enhanced green fluorescent protein (GFP) mice identified the collecting duct as a source of kidney MSC–like cells, with these cells undergoing an epithelial-to-mesenchymal transition to form clonogenic, long-term, self-renewing MSC-like cells. Notably, after extensive passage, kidney MSC–like cells selectively integrated into the aquaporin 2–positive medullary collecting duct when microinjected into the kidneys of neonatal mice. No epithelial integration was observed after injection of BM-MSCs. Indeed, kidney MSC–like cells retained a capacity to form epithelial structures in vitro and in vivo, and conditioned media from these cells supported epithelial repair in vitro. To investigate the origin of kidney MSC–like cells, we further examined Hoxb7+ fractions within the kidney across postnatal development, identifying a neonatal interstitial GFPlo (Hoxb7lo) population displaying an expression profile intermediate between epithelium and interstitium. Temporal analyses with Wnt4GCE/+;R26tdTomato/+ mice revealed evidence for the intercalation of a Wnt4-expressing interstitial population into the neonatal collecting duct, suggesting that such intercalation may represent a normal developmental mechanism giving rise to a distinct collecting duct subpopulation. These results extend previous observations of papillary stem cell activity and collecting duct plasticity and imply a role for such cells in collecting duct formation and, possibly, repair.


Mesenchymal stromal cells (MSCs; also known as mesenchymal stem cells)1 were first isolated from the bone marrow (BM) on the basis of their ability to adhere to plastic and display a fibroblastic phenotype.2 Although initially proposed to play a critical homeostatic function within the BM, these cells show panmesodermal potential, including bone, fat, and cartilage,3–5 suggesting a role as stem cells for mesenchymal tissues.6 BM-MSCs are immunomodulatory and can also home to damaged tissues,6,7 with their participation in tissue repair at distant sites of considerable interest. The systemic delivery of BM-MSCs after a variety of acute renal injuries (glycerol, mercury chloride, cisplatin, and ischemic injury) elicits a reparative effect in animal models.8–13 Although it was initially thought to occur by the transdifferentiation of MSCs into renal
epithelium,\(^8\) the observed improvements in histology and function are now considered to result from the secretion of proreparative factors.\(^9,12,14,15\)

Cells with MSC-like properties have now been isolated from many solid organs.\(^16\) These tissue-derived MSC-like cells apparently represent perivascular cells on the basis of marker expression (CD146\(^+\)NG2\(^+\)CD140b\(^+\))\(^16–18\) and have been proposed to support local tissue turnover and/or repair. We have previously reported the isolation of an MSC-like population from the adult mouse kidney that displayed long-term colony-forming capacity and clonogenicity.\(^19\) These kidney MSC-like cells displayed an immunophenotypic profile and functional properties extremely similar to mouse BM-MSCs. However, this population also displayed a kidney-specific gene expression signature,\(^19\) including the differential expression of the collecting duct markers natriuretic peptide precursor type B,\(^20\) Uroplakin I\(b,\)\(^21\) and Hoxb7.\(^22\) The expression of such markers after extensive culture suggested epithelial origin and/or epithelial potential.

In this study, we show that kidney MSC-like cells are enriched in the renal papilla, can be derived from the mature collecting duct epithelium, and undergo an epithelial-mesenchymal transition (EMT), giving rise to a robust, long-term, self-renewing, and clonogenic MSC-like population. When microinjected into the kidney of a neonatal mouse, kidney MSC-like cells selectively home and integrate into collecting duct epithelium. Together with in vitro and in vivo epithelial potential, conditioned media from kidney MSC-like cells display proreparative activity.

**RESULTS**

**Specific In Vivo Epithelial Potential of Kidney MSC-Like Cells**

On the basis of the differential expression of renal epithelial markers by kidney MSC-like cells, we investigated the fate of these cells after microinjection into the neonatal kidney at postnatal day 1 (PND1), a time when nephron formation and papillary maturation were still active (Figure 1, A and B). MSCs isolated and cultured from total kidney or BM of adult ubiquitous green fluorescent protein-positive (GFP\(^+\)) mice (Bulk cultures) were used for microinjection at late passage (approximately passage 10 [P10]). After microinjection, GFP\(^+\) BM-MSCs were occasionally detected as rare scattered single cells within the medullary interstitium but never detected within tubular epithelia (Figure 1C, Supplemental Figure 1A). GFP\(^+\) kidney MSC-like cells, although not detected in the cortex, were detected in the medulla/papilla region (Figure 1D, Supplemental Figure 1A). These GFP\(^+\) cells were predominantly within tubular structures surrounded by a Collagen IV\(^+\) basement membrane (Figure 1E), although occasional interstitial GFP\(^+\) cells were also seen, often closely associated with aquaporin 2–positive (Aqp2\(^+\)) collecting ducts (Figure 1E). Colocalization with Aqp2, but not Umod, indicated a selective homing to and integration into collecting duct epithelium (Figure 1E), with 12±0.8% of collecting duct cells being GFP\(^+\) (Supplemental Figure 1B). Coimmunofluorescence with the mitotic marker, pH3, indicated proliferation of these integrated GFP\(^+\) cells (Figure 1E, Supplemental Figure 2). Nuclei double-positive for GFP and pH3 represented 1.2±0.2% of all GFP\(^+\) cells. By way of comparison, 0.5±0.2% collecting duct cells were pH3\(^+\) in a normal PND6 Hoxb7/EGFP\(^+\) kidney (comparable age) (Supplemental Figure 2B); the incorporating kidney MSC-like cells showed a statistically higher mitotic rate than normal collecting duct epithelium (P=0.04). Two weeks after injection, GFP\(^+\) cells could still be detected within Aqp2\(^+\) tubules, including some pH3\(^+\) cells (Figure 1F), eliminating the possibility that these cells were being phagocytosed by the collecting duct epithelium\(^23\) and indicating a long-term contribution to epithelial structures. No GFP signals were ever detected within F4/80\(^+\) macrophages located in the same region (Supplemental Figure 3).

**Enrichment of the Kidney MSC-Like Cells in the Collecting Duct Epithelium**

To identify the origin of MSC-forming cells within the kidney, three isolation approaches were used and compared with MSC-like populations derived from Bulk kidney digests. (1) On the basis of the previously described stem cell markers, CD24 and Sca-1,\(^24–26\) only the Lin\(^–\)CD31\(^–\)CD24\(^–\)Sca-1\(^+\) fractions could form MSC colonies in vitro (Supplemental Figure 4A) (termed the Sorted fraction). (2) Regional isolation was achieved by enzymatic digestion of manually dissected adult kidney cortex, medulla, and papilla. (3) Isolation of cells specifically from the collecting duct was performed using Hoxb7/EGFP mice,\(^27\) with both GFP\(^+\) and GFP\(^–\) fractions being isolated. Freshly isolated GFP\(^+(\text{Hoxb7}^+)\) cells have low levels of CD24 expression (CD24\(^–\)) (Supplemental Figure 4B), similar to the Sorted population.

After establishment and extended culture as MSCs, colony-forming efficiency, clonogenicity from a single colony, and population doubling time were assessed for these different isolated populations (Figure 2, Supplemental Figure 5).\(^19\) For colony-forming efficiency, colonies were classified into small (<25 cells), medium (25–100 cells), or large (>100 cells) (Figure 2A) as an indicator of stem versus progenitor status.\(^28,29\) Cells isolated from papilla showed the highest capability to form colonies of all sizes at 14 days postplating (Figure 2A), suggesting the strongest enrichment for stem cell properties. GFP\(^+(\text{Hoxb7}^+)\) populations displayed a colony-forming efficiency similar to cells from the papilla, whereas the GFP\(^–\) fraction only produced small and medium-sized colonies at lower frequencies (Figure 2A). Clonogenicity of the Hoxb7\(^+\) fraction was >15-fold higher than Bulk and >3-fold higher than Sorted populations (Figure 2B). Finally, population doubling times indicate that Hoxb7-derived MSC-like cells proliferate slightly faster than Bulk-cultured cells (Supplemental Figure 5). Together, these results suggest a more stem-like
phenotype for populations derived from medulla/papilla or directly isolated from collecting ducts.

**Kidney MSC–Like Populations Arise by an EMT**

Because the GFP+ fraction isolated from adult Hoxb7/EGFP mice is expected to represent mature collecting duct epithelium, we investigated whether the formation of kidney MSC-like cells represented an EMT. After isolation, cells remained GFP+ for up to 3 weeks while displaying an epithelial morphology. By passage 4 (approximately 2 months), these cells changed morphology and downregulated Hoxb7 (loss of GFP) (Figure 2C). Initially, Hoxb7-derived cultures were immunopositive for collecting duct (Aqp2 and Pax2) and epithelial (ZO-1) markers (Figure 2D). With passage, these cells lost Aqp2 and ZO-1 and acquired the pericyte/mesenchymal marker NG2 (Figure 2D). At passage 2, Hoxb7-derived cultures were uniformly positive for the principal cell marker Aqp2+ and showed no staining for intercalated cell markers Pendrin (∝-intercalated) or AE1 (∝-intercalated) (Figure 2E), suggesting that the MSC-like cultures arise from mature principal cells or previously described bipotential Aqp2+ collecting duct progenitors.30

**Kidney MSC–Like Cells Show Panmesodermal Potential**

MSCs derived from Bulk, Sorted (CD24lo Sca1+), and GFP+(Hoxb7+) fractions all displayed a characteristic murine MSC immunophenotypic profile (CD44, CD49e, and Sca-1)18 (Figure 3A). Uniquely, by passage 6, 100% of Hoxb7-derived cells were

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**Figure 1.** Specific epithelial integration of the kidney MSC–like cells. (A) Ultrasound image of PND1 mouse kidney. Dashed line outlines the kidney as viewed under an Ultrasound Biomicroscope. (B) Fluorescence microscopy showing the detection of Fluoresbrite Yellow Green microspheres (green), which were mixed with cells and used to localize the injection site within the nephrogenic zone 24 hours postinjection. (C and D) Characterization of the location of GFP+ cells in the neonatal kidney 4 days after injection with (C) BM-MSCs or (D) kidney MSC–like cells isolated from a ubiquitously GFP-expressing mouse. (C) Arrowhead indicates the presence of a single interstitial GFP+ BM-MSC. (D) GFP+ Aqp2+ tubular structures in the medullary region after injection of kidney MSC–like cells. (E) Coimmunostaining for GFP (green), Collagen IV (red; basement membrane), Aqp2 (red; collecting duct), Umod (red; loops of Henle), and pH3 (red; mitotic cells) in kidney sections injected with GFP+ kidney MSC–like cells at 4 days postdelivery. Arrowheads indicate occasional interstitial GFP+ cells in all panels but pH3 immunofluorescence, where they indicate pH3+ cells. Arrows indicate GFP+ cells within the tubular structures. Scale bar, 20 μm. (F) GFP+ tubular cells at 14 days postdelivery. They can be colocalized with Aqp2+ collecting duct, with some positive for the mitosis marker pH3. Arrowheads indicate a pH3+ cell within a tubule. Scale bar, 20 μm. All images were captured using an upright laser-scanning confocal microscope for bright field and epifluorescence (Carl Zeiss LSM 710).
positive for CD24, a marker previously associated with tubular progenitor/stem cells, including renal progenitors.\textsuperscript{31,32} Hoxb7\textsuperscript{+} -derived cultures were also negative for CD140a and CD140b (PDGFR\textsubscript{a} and PDGFR\textsubscript{b}), known markers of pericytes previously associated with other tissue-derived MSC populations.\textsuperscript{19,33} This finding reflects the active selection against the pericytic compartment when isolating this population from the Hoxb7/EGFP mouse. Despite this finding, all three populations displayed panmesodermal potential (Figure 3B), which was previously reported for BM-MSCs, reinforcing the MSC-like properties of the Hoxb7-derived cells, despite the absence of classic pericytic marker expression.

**Epithelial Potential of Kidney MSC–Like Cells Derived from Collecting Duct**

Although our data identify a kidney MSC–like population within the mature collecting duct, it may have represented a distinct MSC-like population to that isolated from initial Bulk cultures. We evaluated the epithelial potential of Hoxb7-derived MSC–like cells in vitro using three-dimensional culture in Collagen I.\textsuperscript{34} These cells formed E-cadherin\textsuperscript{+} PECAM\textsuperscript{–} branching tubular structures after 9 days, suggesting tubulogenesis but not vasculogenesis (Figure 4A). No similar structures were observed using BM-MSCs. To test their in vivo epithelial integration capacity, Hoxb7-derived kidney MSC–like cells were cultured for 12–15 passages, labeled with PKH26, and injected into PND1 neonatal kidney. PKH26-labeled cells were only detected in Aqp2\textsuperscript{+} tubular structures within the medulla/papilla and displayed a slightly higher level of integration into the collecting duct than observed for Bulk GFP\textsuperscript{+} kidney MSC–like cells (19±3.2% versus 12±0.8%; \(P=0.02\))

**Figure 2.** Papillary enrichment and phenotypic changes of kidney MSC–like cells. (A) Colony-forming efficiency of kidney MSC–like populations isolated by either regional dissection (cortex, medulla, or papilla) or FACS sorting as a CD24\textsuperscript{lo}Sca-1\textsuperscript{+} fraction (Sorted) from wild-type mice or GFP\textsuperscript{+} (Hoxb7\textsuperscript{+}) and GFP\textsuperscript{–} (Hoxb7\textsuperscript{–}) fractions from Hoxb7/EGFP mice (\(n\geq3\)). *\(P<0.01\) compared with the Sorted group. (B) Clonogenicity of kidney MSC–like cultures isolated as Bulk, Sorted, and Hoxb7/GFP\textsuperscript{+} fractions (\(n=3\)). Passage number is indicated in parentheses. *\(P<0.01\) compared with Bulk culture. (C) Bright-field (BF) and fluorescence images of a GFP\textsuperscript{+} colony derived from the Hoxb7\textsuperscript{+} fraction at passages 0 and 4. Note the loss of GFP on passage suggesting the downregulation of Hoxb7 in culture. Scale bar, 100 \(\mu\text{m}\). (D) Characterization of cells derived from Hoxb7\textsuperscript{+} fractions at passages 2, 6, and 21. Immunofluorescence was performed for markers of the collecting duct (Aqp2 and Pax2) as well as characteristic markers of mesenchyme/pericyte (NG2) and epithelium (ZO-1). Scale bar, 50 \(\mu\text{m}\). (E) Expression of the principal cell marker (Aqp2) and absence of intercalated cell markers (Pendrin and AE1) on cells derived from Hoxb7\textsuperscript{+} fraction at passage 2. Scale bar, 50 \(\mu\text{m}\).
After incorporated into the collecting duct, these PKH26-labeled cells re-expressed GFP, suggesting the readoption of an Hoxb7+ collecting duct phenotype (Figure 4B).

**Repair Activity of Kidney-Derived Stromal Cells in Scrape Injury Assay**

Tissue-derived MSCs have been proposed to play a role in tissue turnover, homeostasis, and repair through humoral mechanisms. Indeed, BM-MSC conditioned medium can promote and accelerate wound healing in vitro. To test whether kidney MSC–like cells can facilitate repair, a scratch wound repair assay was performed. Madin–Darby Canine Kidney (MDCK) cells were cultured to confluence, subjected to scratch injury, and continuously cultured in the presence of normal culture medium or conditioned medium from Bulk or Hoxb7-derived kidney MSC–like cells. Accelerated scratch closure ensued in the presence of conditioned media from both kidney MSC–like populations (Figure 5), suggesting the production of paracrine reparative factors by these cells.

**Detection of Hoxb7lo Interstitial Cells within the Postnatal Kidney**

Although present in adult kidney, we were unable to isolate kidney MSC–like populations from embryonic kidney (data not shown), despite the presence of an extensive Hoxb7+ ureteric epithelium throughout development. Although the expression of Hoxb7 in the embryonic collecting duct is well characterized, the location and identity of Hoxb7+ cells in the postnatal kidney are less clear. Analyses of Hoxb7/EGFP transgenic mice between birth and adulthood confirmed that most GFP+ cells were tubular epithelial cells largely colocalized with Aqp2+ (Figure 6A). However, rare medullary Hoxb7GFPlo cells were also detected between PND0 and PND14. They were interstitial as assessed by Collagen IV staining (Figure 6B). FACS analysis confirmed the presence of a GFPloEpCAM2 population within the neonatal kidney (Figure 6C). Although barely detectable at PND0, this nonepithelial GFPloEpCAM2 population reached 4.8% of total GFP+ cells in the PND5 kidney before disappearing from the adult kidney (Figure 6D). Both GFPlo EpCAM− (interstitial) and GFP+EpCAM+ (epithelial) fractions showed the capacity to form small, medium, and large MSC-like colonies with equivalent efficiency (Figure 6E). Immunofluorescence and FACS for F4/80 confirmed that this interstitial GFPlo population did not represent granulocyte macrophages (Supplemental Figure 6), despite previous reports of Hoxb7 expression in that population. Cells isolated from PND5 kidneys were subsequently sorted into GFPloEpCAM+, GFPloEpCAM−, GFP+EpCAM+, and GFP−EpCAM− fractions for quantitative PCR (qPCR). GFPloEpCAM− cells displayed differential Wnt4 expression similar to neonatal medullary interstitial colonies (Supplemental Figure 1B).
cells [GenitoUrinary Development molecular Anatomy Project (GUDMAP), www.gudmap.org], whereas E-cadherin expression was comparable with other epithelial fractions (Figure 6F). Hence, the GFPloEpCAM2 population displays a metastable phenotype intermediate between interstitium and collecting duct epithelium.

Possible Origin of Hoxb7-Derived Kidney Stromal Cells
Strong Wnt4 expression has previously been observed in the renal interstitium as early as embryo day 15.5 (E15.5) and within occasional collecting duct cells within the papilla at PND2 and PND6 (GUDMAP accession ID’s: 7156, 14182, 14082, and 14084; www.gudmap.org) (Supplemental Figure 7). We investigated the expression and distribution of Wnt4+ cells using tissue sections from Wnt4 GCE/+ :R26 TdTomato/+ mice. Tamoxifen (25 mg/kg) was injected at E17.5 and kidneys were collected at either E19.5 (immediately before birth) or PND49 (adult). A contribution of Wnt4-expressing cells to the developing nephrons was evident at both E19.5 and PND49, but we will focus on the medulla/papilla. At E19.5, no Wnt4+ (EGFP+) or TdTomato+ cells were located within Aqp2+ collecting ducts; however, both EGFP+ and EGFP+TdTomato+ cells (representing 8.35% of all GFP+ cells) were present within the interstitium of the medulla/papilla (Figure 7A, Supplemental Figure 8B). All TdTomato+ cells also expressed EGFP. In the PND49 kidney, as previously reported, active Wnt4 expression (EGFP+) was detected in all Aqp2+ collecting ducts39 (Figure 7, Band C, Supplemental Figure 8A). In addition, approximately 8% of Aqp2+ medullary/papillary collecting duct cells were double-positive for TdTomato and EGFP (Figure 7, B and C, Supplemental Figure 8B), indicating a population of collecting duct–located cells derived from the interstitial Wnt4-expressing cells present at the time of Tamoxifen injection. This finding would suggest a process of interstitial intercalation during the immediate postnatal kidney maturation, potentially occurring by a similar mechanism to that observed after the neonatal injection of kidney MSC–like cells.

DISCUSSION
In this study, we describe the isolation of MSC-like cells from the adult mouse kidney collecting duct. These cells are able to transition between epithelial and mesenchymal states and display robust MSC characteristics, including long-term expansion (>20 passages), clonogenicity, panmesodermal potential, and a characteristic epitope profile. Most remarkably, even after extensive culture, these cells retain a capacity to re-epithelialize both in vitro and in vivo. Indeed, if reintroduced into the neonatal kidney by microinjection, this phenotypically mesenchymal population specifically homed to and integrated back into the collecting duct, thereafter proliferating as an epithelial component of that compartment.

Although a variety of renal epithelia have been reported to undergo EMT, including the proximal tubular epithelium, mesenchymal-epithelial transition is an unusual response for a cultured MSC-like population. Indeed, there has been no evidence of an in vitro or in vivo epithelial potential for BM-MSCs.11,40,41 Collecting duct cells can undergo EMT in response to urinary obstruction, resulting in the formation of...
interstitial fibrosis. However, it is thought to predominantly involve the myofibroblastic transformation of intercalated cells, a cell type not evident in the Hoxb7/GFP\(^+\) fraction at isolation. The selective integration of injected kidney MSC–like cells into the collecting duct epithelium suggests both an active homing process and a memory of tissue origin. Although we cannot rule out fusion, the fact that we can show both EMT and mesenchymal-epithelial transition in these cells and the re-expression of Aqp2 and Hoxb7 after integration support genuine epithelial transdifferentiation. This result poses the question of whether the integration of kidney MSC–like cells into the neonatal collecting duct represents a normal process during papillary collecting duct elongation and maturation. The renal papilla undergo dramatic elongation within a short period of time immediately after birth. Although the underlying mechanism is poorly understood, collecting duct elongation does involve convergent extension (Supplemental Figure 9), a process that can also incorporate surrounding cells. Curiously, a kidney MSC–like population could not be derived from embryonic kidney (J. Li, U. Ariunbold, and M. Little, unpublished data), despite the presence of abundant Hoxb7\(^+\) collecting duct epithelium. This finding implies that MSC-forming cells within the collecting duct represent a subpopulation rather than it being a phenotypic option available to all Aqp2\(^+\) collecting duct cells. Indeed, we provide evidence that this subpopulation possibly arises from the medullary interstitium during the immediate postnatal period by intercalation of rare interstitial cells. Several previous reports indicate the presence of stem cells in the renal papilla. Bromodeoxyuridine, 5-bromo-2\(^9\)-deoxyuridine pulse chase experiments identified enrichment of long-term label-retaining cells within the papilla. However, such experiments performed after the immediate neonatal period no longer label a papillary population, instead identifying label-retaining cells within the tubules. This result suggests the presence of a dividing neonatal papillary population, which then becomes relatively quiescent, an observation not inconsistent with our lineage analysis or the appearance and disappearance of the Hoxb7\(^-\) population within the papilla. The human renal papilla has also been reported to contain epithelial Oct4-expressing prominin/CD133\(^+\) cells in loops of Henle that form epithelial structures \textit{in vitro}. Our kidney MSC–like cells are derived from collecting duct and home specifically back to that tubular segment, suggesting no overlap with these studies.

Lineage-tracing analyses have shown that tubular repair in the adult kidney after acute injury only involves epithelial cells within the nephrons. The integration of a nontubular cell type into the collecting duct would not have been identified in these previous lineage-tracing studies, because the lineage marker used, Six2, only identifies cells derived from the capsule mesenchyme, a compartment that does not give rise to the collecting duct epithelium. To date, we have no evidence suggesting that continued contribution of interstitial cells into the collecting duct compartment occurs in the adult mouse.

**Figure 5.** Kidney MSC–like cultures produce factors able to enhance wound repair in a scratch assay. (A) Bright-field images showing repair of a scratch wound within a lawn of MDCK cells across a 12-hour period. MDCK cells were cultured with control medium or conditioned medium from Bulk cultured kidney MSC–like cells or Hoxb7-derived kidney MSC–like cells. Scale bar, 100 \(\mu\)m. (B) Quantification of the rate of scratch wound closure in all three conditions measured as wound width at 4- and 12-hour time points normalized for the wound width at 0 hours. The wound closed significantly faster in the presence of conditioned medium, especially Hoxb7-derived culture, compared with control culture and Hoxb7 culture. cond, conditioned.
Figure 6. Characterization of the location and properties of endogenous Hoxb7+ populations within the postnatal kidney. (A) Detection of endogenous GFP+ (Hoxb7+) and GFPlo populations across kidney development. Immunofluorescence shows the presence of Hoxb7 expression (GFP; green) and staining for Aqp2 (red) on kidney sections at PND0, PND9, and PND14 and adult Hoxb7/EGFP transgenic mouse kidneys. Arrowheads indicate GFPlo interstitial cells. Scale bar, 20 μm. (B) Immunofluorescence staining for Collagen IV (red) plus endogenous GFP on sections of PND9 Hoxb7/EGFP transgenic mouse kidney. Arrowheads indicate interstitial GFPlo cells outside of Collagen IV+ tubule basement membranes. Scale bar, 10 μm. (C) FACS analysis for GFP and EpCAM (marker of epithelium) from
However, the capacity for kidney MSC-like cells to proliferate long-term and switch phenotype does raise the prospect that these cells respond to injury in a reparative fashion, even within the confines of the collecting duct epithelium. Although more analyses are required, these cells are able to produce growth factors that promote epithelial wound repair in vitro, which would support a role for such cells in response to damage in vivo. Indeed, the capacity to switch phenotype may also endow this population with more substantial roles in collecting duct development, homeostasis, and response to injury, which remain to be investigated.

**CONCISE METHODS**

**Animals**

Animal experiments were approved by the University of Queensland Animal Ethics Committee (Institute for Molecular Bioscience) and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice expressing ubiquitous EGFP\textsuperscript{22} or Hoxb7\textsuperscript{7}EGFP\textsuperscript{22} were used for cell isolation and FACS sorting. Wnt\textsubscript{4}^{GCE\textsuperscript{-}}:R26\textsuperscript{TdTomato\textsuperscript{+}} mice were used for lineage-tracing experiments. Neonates of outbred CD1 mice were used for neonatal injections. Wild-type, EGFP, and Hoxb7/EGFP transgenic mice used for experimentation were housed within the University of Queensland Biologic Resources in clean, temperature-controlled mouse facilities. Wild-type, EGFP, and Hoxb7/EGFP transgenic mice used for lineage-tracing experiments were housed within the University of Queensland Biologic Resources in clean, temperature-controlled mouse facilities. Animals for scientific purposes were approved by the University of Queensland Animal Ethics Committee (Institute for Molecular Bioscience) and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animals for scientific purposes were approved by the University of Queensland Animal Ethics Committee (Institute for Molecular Bioscience) and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

**Isolation of Kidney MSC-Like Cells**

Kidney stromal cells were isolated from adult mice (male, 6–8 weeks old) with or without FACS sorting followed by standard culture as previously described.\textsuperscript{28} In brief, kidneys were dissected and subjected to enzymatic digestion (Collagenase B, 1 mg/ml; Dispase II, 1.2 unit/ml) followed by two rounds of filtration with a cell strainer (70 and 40 μm; BD Falcon) to produce single-cells yield. Isolated cells were then either cultured directly as Bulk or Sorted to collect different fractions. For Bulk culture, cells were preplated for 24 hours in 20% FCS/α-MEM (Gibco, Basel, Switzerland). Nonadherent cells were then washed off, and adherent cells were continuously cultured and expanded over time. Sorted populations were established through FACs fractionation on the basis of previously described stem cell markers CD24 and Sca-1.\textsuperscript{24–26} Total kidney isolates, excluding Lin\textsuperscript{–}CD31\textsuperscript{+} cells, were sorted into CD24\textsuperscript{+}Sca1\textsuperscript{+}, CD24\textsuperscript{+} Sca-1\textsuperscript{−}, and CD24\textsuperscript{+}Sca-1\textsuperscript{−} fractions. Only the CD24\textsuperscript{+}Sca-1\textsuperscript{−} fractions could form MSC colonies in vitro (Supplemental Figure 4A). For regional isolation, kidneys were mechanically dissected into cortex, medulla, and papilla regions and subjected to enzymatic digestion; then, they were plated as total isolates from that region. Hoxb7-derived cultures were obtained by FACs sorting of GFP\textsuperscript{+}EpCAM\textsuperscript{+} fractions after single-cell isolation from Hoxb7/EGFP transgenic mice.

**Neonatal Injection Model**

Cells isolated from ubiquitous EGFP mice or cells derived from Hoxb7/GFP\textsuperscript{+} fraction and labeled with PKH26 (see PKH26 Labeling and Detection) were resuspended at 0.5–1×10\textsuperscript{7}/ml in PBS and injected into the neonatal kidney at PND1 using a microinjection pipette under the guidance of Ultrasound Biomicroscope (Vevo770; VisualSonics). Neonates were anesthetized and mounted on the stage of a rail injection platform with gauze cushion tapes. The pups were then moved into the scan plane using the XYZ controls on the platform stage. The kidney was visualized with Ultrasound Biomicroscope fitted with a 55-MHz probe (RMV711; VisualSonics) (Figure 1A). The microinjection needle tip was aligned in the scan plane using the XYZ controls so that the tip is centered within the focal zone of the transducer (area of image with the greatest resolution). Using the Nanojet II, cells were delivered at a regulated pulse (69 nl/pulse) with three to four pulses per injection, and the final delivery volume was around 300 nl. Coinjection of Fluoresbrite Yellow Green microspheres (2.0 μm; Polysciences, Inc.) was used to confirm the injection site within the nephrogenic zone of the neonatal kidney (Figure 1B).

Before injection, cell suspensions were mixed with Fluoresbrite Yellow Green microspheres (2.0 μm; Polysciences, Inc.) at a ratio of 20:1. Microspheres alone, GFP\textsuperscript{+} kidney–derived stromal cells, GFP\textsuperscript{+} BM

PND0, PND9, PND14, and adult Hoxb7/GFP transgenic mouse kidneys highlighting the percentage of the Hoxb7\textsuperscript{lo} population (boxed) at each time point. (D) Quantitation of GFP\textsuperscript{lo}EpCAM\textsuperscript{−} and GFP\textsuperscript{−}EpCAM\textsuperscript{−} populations in the postnatal Hoxb7/GFP kidney (PND0, n=3; PND4, n=5; PND6, n=9; PND14, n=2; adult, n=4). Data show the changes in relative percentage of Hoxb7\textsuperscript{lo} versus Hoxb7\textsuperscript{hi} populations, illustrating the appearance and decline of an interstitial Hoxb7\textsuperscript{lo} population in the postnatal kidney. (E) Comparison of the colony-forming efficiency between GFP\textsuperscript{lo}EpCAM\textsuperscript{−} and GFP\textsuperscript{−}EpCAM\textsuperscript{−} populations isolated from PND5 kidney. There is no significant difference in capacity to form small, medium, or large colonies between these two populations (n=3). (F) Gene expression analysis showing expression of interstitial (Wnt4) and epithelial (E-cadherin) markers in all four fractions isolated from PND5 Hoxb7/GFP mice kidneys (n=3). *P<0.05 compared with whole kidney.
stromal cells, or PKH26-labeled Hoxb7-derived kidney stromal cells, all mixed with microspheres, were microinjected into the nephrogenic zone (3000–5000 cells in 300 nl), and kidneys were harvested at 4 and 14 days postinjection. Except for the beads themselves, no GFP signals were detected in the beads only control. For the neonatal injection, six independent experiments have been conducted with varying numbers of samples collected from each time point: P4, n = 8; P5, n = 1; P7, n = 6; P9, n = 1; and P14, n = 5. Three independent experiments have been conducted using PKH26-labeled cells derived from Hoxb7 culture, and on average, three samples were collected from P3, P5, and P8. For presentation purposes, we only included data from days 4 and 14 postinjection.

In Vitro Scratch Wound Repair Assay
For the in vitro wound-healing assay, MDCK cells (1 × 10^6 cells/well; 10% FCS/DMEM) were plated in a six-well plate and cultured until confluent. A straight scratch wound was then created in MDCK cultures using a 200-μl pipette tip held perpendicular to the plate. After the scratch, cells were washed two times with PBS and then provided fresh culture media (20% FCS/a-MEM) plus 10% FCS/DMEM (1:1 ratio) or conditional media (1:1 ratio). Cells were imaged at 0, 4, and 12 hours at the same position using an inverted bright-field/fluorescence microscope (Nikon ECLIPSE Ti-U). Scratch area at each time point was measured using NIS-Elements BR 4.10 software and used for final quantitation.

FACS Analyses and Immunophenotype Analyses
Freshly isolated cells were sorted into different fractions on the basis of either specific antibody staining or GFP (Hoxb7) expression using FACSAria Cell Sorter (BD Falcon) and then cultured for additional analysis. For FACS sorting, bioconjugated Lin cocktail plus Streptavidin-Allophycocyanin and directly conjugated APC-CD31, FITC-Sca-1, PE-CD24, and APC-EpCAM (BD Falcon) were used. Epitope controls conjugated to different fluorochromes were used as nonspecific staining controls for flow cytometry sorting. For immunophenotyping, cultured cells were stained with directly conjugated antibodies (PE-Cd24, APC-Cd44, PE-Cd49e, PE-Cd51, PE-Cd81, PE-Cd140a, PE-Cd140b, and PE-Sca-1; BD Falcon) and analyzed on FACS Canto II (BD Falcon). Dead cells were excluded on the basis of 7AAD staining. Data were collected and analyzed using FACSDiva software (BD Falcon) and FlowJo (version 7.6.5; Tree Star).

Figure 7. Expression and distribution of Wnt4+ cells in full-term embryo (E19.5) and adult (PND49) kidney. (A) Analysis of Wnt4 (EGFP; green), TdTomato (red), and Aqp2 (white) in the cortex, medulla, and papilla of an E19.5 kidney (immediately before anticipated birth) post-Tamoxifen injection (25 mg/kg body wt) at E17.5. As expected, Wnt4 (EGFP) expression can be detected in renal vesicles within the cortex, and some of the renal vesicles cells are positive for TdTomato. In the medulla, no collecting ducts were EGFP+ or TdTomato+. All medullary EGFP+ cells appeared to be interstitially located between Collagen IV+ basement membranes. A small number of these cells was also positive for TdTomato (arrows indicate a single TdTomato+ cell within the interstitium). Scale bar, 20 μm. (B) Analysis of Wnt4 (EGFP; green), TdTomato (red), and Aqp2 (white) in the cortex, medulla, and papilla of the PND49 kidney post-Tamoxifen injection (25 mg/kg body wt at E17.5). As expected, given the expression of Wnt4 in the renal vesicle before nephron maturation, extensive numbers of TdTomato+ cells are seen along the length of the renal vesicle-derived nephron, especially in the renal cortex. However, ongoing Wnt4 expression (green) was only detected in Aqp2+ collecting duct cells, with it being strongest in the papillary collecting duct as previously reported. Of note, approximately 8% of Aqp2+ medullary/papillary collecting duct cells were double-positive for TdTomato+ and EGFP. Scale bar, 20 μm. (C) High-resolution images of PND49 kidney showing Wnt4 expression (EGFP) and the presence of TdTomato+ cells within Aqp2+ papilla collecting duct epithelium at PND49. Arrows indicate TdTomato+ cells within the epithelium. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars, 20 μm.
Colony-Forming Efficiency, Clonogenicity, and Proliferation
Fractionated or total cell isolates were plated at an initial density of $5 \times 10^3$ cells/well on a six-well plate in α-MEM (Gibco) with 20% FCS, 100 units/ml Penicillin, and 100 μg/ml Streptomycin and cultured in 5% CO$_2$ in a 37°C incubator. After being cultured for 14 days, cells were washed with PBS, fixed with ethanol for 5 minutes at room temperature, and stained with 0.5% Crystal Violet (Sigma-Aldrich) in methanol for 8 minutes at room temperature. The number of colonies was counted under light microscope. Colonies consisting of >25 cells were scored and classified into three sizes: large is >100 cells, medium is 50–100 cells, and small is 25–50 cells. For colony-forming efficiency, the numbers of colonies with different sizes are counted and normalized to the numbers of cells plated. To evaluate clonogenicity, single cells were sorted directly into a 96-well plate with one cell per well, and the numbers of colonies were recorded after 14 days and analyzed. Population-doubling and population-doubling time were calculated to show the proliferation property of isolated cells in culture using the following equations:

$$PDs = \log_2 \left( \frac{N_t}{N_0} \right)$$

and

$$PDT = T_i / PDs.$$ 

PDs is population doubling number, $N_t$ is the accumulated cell number at the end of incubation time, $N_0$ is the accumulated cell number at the beginning of the incubation time, $PDT$ is population doubling time, and $T_i$ is the incubation time in any unit.

Mesodermal Differentiation Assays
Mesodermal differentiation was carried out as per the work by Barlow et al.$^{53}$

Adipogenic Differentiation
Cells were seeded into six-well plates at a density of $8 \times 10^4$ cells/well. When cells reached 80% confluence, they were washed in PBS and cultured for 7–21 days in adipogenic differentiation medium consisting of α-MEM, 10% FCS, 0.5 mM 3-isobutyl-1-methylxanthine, 60 μM indomethacin, 10 μg/ml insulin, 1 μM dexamethasone, and 40 μg/ml gentamicin.$^{54,55}$ Medium was replaced two times per week, and after the formation of intracellular lipid droplets was observed, cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature and stained with oil red-O stain (Sigma-Aldrich).

Osteogenic Differentiation
Cells were seeded onto coverslips as above, grown to 100% confluence, and cultured for 3 weeks in osteogenic differentiation media consisting of α-MEM, 10% FCS, 0.1 μM dexamethasone, 100 mM β-glycerol phosphate, 10 mM l-ascorbate-2-phosphate, and 40 μg/ml gentamicin. Medium was replaced two times per week for 3 weeks, and cells were fixed as above and stained with Alizarin red-S solution.$^{18,56}$

Chondrogenic Differentiation
Cells were centrifuged at $150 \times g$ for 5 minutes to pellet the cells. Cells were then cultured as a pellet in chondrogenic differentiation medium consisting of α-MEM, 0.1 μM dexamethasone, 2 mM l-ascorbate-2-phosphate, 1 mM sodium pyruvate, proline, TGF-β1, 50 μg/ml insulin/transferin/selenium, and 40 μg/ml gentamicin. Medium was replaced two times per week for 3 weeks. Pellets were fixed in 4% PFA and embedded as paraffin block and sectioned using microtome. Sections were stained with Alcian blue stain, and nuclei were counterstained with nuclear fast red.$^{55}$

PKH26 Labeling and Detection
Cells were labeled with PKH-26 red fluorescence cell linker (Sigma-Aldrich) following the manufacturer’s instruction and used for neonatal injection or coculture in a three-dimensional system. In brief, cells were harvested, counted, and resuspended at desired density, and then, they were washed one time with PBS. The cell pellet was then resuspended in Solution C provided in the kit, and PKH26 dye was added to achieve the desired final concentration. Cells were incubated at room temperature for 5 minutes. The dye was then washed off, and the cells were washed one more time with PBS to get rid of any residue; then, it is ready to be used. Labeling efficacy was >98%. Cell viability was evaluated by Trypan blue exclusion and was >96%. PKH26 signal was directly detected using either an upright fluorescence microscope (Olympus BX-51) or a confocal microscope (Carl Zeiss LSM 510 Meta UV or Carl Zeiss LSM 710 upright).

Immunofluorescence of Kidney Sections
For immunofluorescence staining, kidney samples were fixed in 4% PFA, infiltrated with 30% sucrose/PBS overnight, then embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Ten-micrometer-thick sections were cut and postfixed in 4% PFA for 10 minutes at room temperature and then incubated with various primary antibodies, including anti-GFP (Sapphire Bioscience), Aqp2 (EMD Millipore, Australia), Collagen IV (Chemicon International), Umod (Chemicon International), pH3 (Upstate Cell Signaling Solutions), and F4/80 (Serotec). For immunocytochemistry, cultured cells were fixed with ice-cold methanol at 4°C for 10 minutes and stained with GFP (Sapphire Bioscience), Pax2 (Zymed Laboratories Inc.), NG2 (Chemicon International), ZO-1 (Invitrogen), Pendrin (Santa Cruz Biotechnology), AE-1 (Alpha Diagnostic), and E-cadherin (BD Biosciences) followed by secondary antibody. All images were captured on a fluorescence microscope (Olympus BX-51) or a confocal microscope (Carl Zeiss LSM 710 upright).

Kidneys collected from Wnt3a-Cre;R26TdTmato/+ time-mated males, which were injected with Tamoxifen at E17.5, were fixed for 1 hour at 4°C in 4% PFA, infiltrated with 30% sucrose/PBS overnight, then embedded in Tissue-Tek OCT Compound (Sakura Finetek), and frozen in an ethanol/dry ice bath. Samples were shipped as cryopreserved blocks in dry ice. Ten-micrometer-thick sections were cut and postfixed in 4% PFA for 10 minutes at room temperature and then stained with various primary antibodies, including anti-GFP (Sapphire Bioscience), Aqp2 (EMD Millipore), and Collagen IV (Chemicon International). TdTmato was detected directly using a confocal microscope (Carl Zeiss LSM 710 upright).

Three-Dimensional Collagen Gel Culture
MDCK cells (strain II) were harvested from confluent culture using trypsin-EDTA and resuspended at a concentration of $4 \times 10^6$ cells/ml
in ice-cold Collagen I solution prepared as described previously. Aliquots of the cell suspension were dispensed into 24-well plates (250 μl/well; Nunc; Kampstrup, Roskilide, Denmark) and allowed to gel for 30 minutes at 37°C before adding 500 μl culture medium (either 10% FCS/MEM or 20% FCS/α-MEM; Gibco). Culture medium was changed every 2–3 days. The cultures were monitored and photographed using an inverted fluorescence microscope (Nikon Ti-U) every 2–3 days, fixed on day 14 with 4% PFA, and subjected to immunofluorescence for additional analysis.

Quantification
For quantification of the proportion of medullary collecting duct cells derived from injected kidney MSC-like cells, kidney samples were examined from three independent neonatal injection experiments, in which cells of interest were delivered into CD1 neonates at PND1 and then collected at PND6 for analysis. Two to three sections were chosen from each sample and stained with antibody to detect injected cells (anti-GFP) and collecting duct epithelial cells (anti-Aqp2). Staining with 4′,6-diamidino-2-phenylindole was also used to detect individual nuclei. When injecting PKH26-labeled cells, they were by direct fluorescence without any staining. Under the microscope, the field with the most significant incorporation events within the medullary/papillary region was chosen to be imaged. All images (300 dpi at x20) were captured with a fluorescence microscope (Olympus BX-51) and uploaded into Imaris software (version 7.2; BitPlane AG) for quantification. Imaris software was then used to create masks on the basis of specific staining (Aqp2, GFP, or PKH26), and spot counting was performed. The number of nuclei (identified using 4′,6-diamidino-2-phenylindole) within each specific mask were counted separately and used to calculate integration efficiency (GFP*Aqp2+/Aqp2+ or PKH26*Aqp2+/Aqp2+). For the quantification of the relative proliferation rate of incorporated kidney MSC-like cells, kidney samples were again examined from three independent neonatal injection experiments, in which GFP+ kidney MSC-like cells were delivered into CD1 neonates at PND1 and then collected at PND6. In this instance, immunofluorescence was performed for anti-GFP and anti-pH3, a marker of mitosis. Imaris software was again used to spot count nuclei that were GFP+ or pH3+, such that the ratio of GFP+/pH3+ to GFP− could be determined. To investigate the relative proliferation within normal collecting duct, samples were collected from PND6 Hoxb7/EGFP mice, immunofluorescence was performed for anti-pH3, and the ratio of EGFP+/pH3+/EGFP− cells was quantified.

Gene Expression Analyses
Total RNA was extracted from cells using TRIzol (Life Technologies), and cDNA was synthesized from >100 ng RNA using Super Script III reverse transcription (Life Technologies). qPCR analyses were performed with Syber Green (Applied Biosystems) by an ABI PRISM 7500 Real-Time PCR Machine. The sequences of primers used for qPCR are as listed (Supplemental Table 1).

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DISCLOSURES
None.

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