Lgr5\(^{+}\)ve Stem/Progenitor Cells Contribute to Nephron Formation during Kidney Development

**SUMMARY**

Multipotent stem cells and their lineage-restricted progeny drive nephron formation within the developing kidney. Here, we document expression of the adult stem cell marker Lgr5 in the developing kidney and assess the stem/progenitor identity of Lgr5\(^{+}\)ve cells via in vivo lineage tracing. The appearance and localization of Lgr5\(^{+}\)ve cells coincided with that of the S-shaped body around embryonic day 14. Lgr5 expression remained restricted to cell clusters within developing nephrons in the cortex until postnatal day 7, when expression was permanently silenced. In vivo lineage tracing identified Lgr5 as a marker of a stem/progenitor population within nascent nephrons dedicated to generating the thick ascending limb of Henle’s loop and distal convoluted tubule. The Lgr5 surface marker and experimental models described here will be invaluable for deciphering the contribution of early nephron stem cells to developmental defects and for isolating human nephron progenitors as a prerequisite to evaluating their therapeutic potential.

**INTRODUCTION**

In the adult kidney, a complex epithelial network of blood filtration units termed nephrons facilitates the efficient removal of nitrogenous waste metabolites and regulates the homeostasis of water and electrolytes in the body. In contrast to intestine, stomach, and skin epithelia, which constantly self-renew throughout an individual’s lifetime (Barker et al., 2010a), mammalian nephrons are generated exclusively during late embryonic and early postnatal development, and exhibit only very limited cell turnover in adults (Guo and Cantley, 2010; Hartman et al., 2007). Despite the high regenerative potential of damaged nephrons, once damage exceeds a certain threshold, nephrons are lost without replacement.

Nephrogenesis proceeds via a series of highly coordinated reciprocal interactions between two mesoderm-derived tissues: the ureteric bud (UB) and the adjacent metanephric mesenchyme (MM) (Little et al., 2010). Early stages are characterized by invasion of the UB into the adjacent MM, when MM-derived signals induce extensive branching growth of the UB to generate the functional collecting duct system. The UB concomitantly induces the adjacent MM to convert to an epithelial structure termed the renal vesicle, driven in part by Wnt4/Wnt9b-mediated canonical Wnt signaling (Carroll et al., 2005; Karner et al., 2009; Kispert et al., 1998; Park et al., 2007; Pulkkinnen et al., 2008). The renal vesicle subsequently elongates to produce first a comma-shaped body and then an S-shaped body, which rapidly expands and patterns into a functional nephron (Carroll et al., 2005; Costantini, 2010; Georgas et al., 2009). MM-resident multipotent progenitor cells give rise to these immature nephrons, which comprise phenotypically distinct cell populations that are responsible for generating the specialized segments of the mature nephron (Georgas et al., 2009; Boyle et al., 2008; Bridgewater et al., 2008; Kobayashi et al., 2008; Marose et al., 2008; Unbekandt and Davies, 2010; Yu et al., 2002). Genetic fate mapping has revealed Six2 to be a marker of the MM multipotent progenitor population (Kobayashi et al., 2008), but the identity of the committed nephron progenitor populations is less well established. The discovery of markers that will facilitate the identification and isolation of these early stem/progenitor populations is expected to improve our understanding of nephrogenesis and should expedite a functional evaluation of their regenerative-medicine potential for treating adult kidney disease.

Wnt signaling is crucial for regulating the activity of early stem/progenitor populations that contribute to kidney development. Wnt9b and Wnt4 in particular are essential for establishing the earliest epithelial nephron structures and for the subsequent patterning into functional nephrons (Carmon et al., 2011; Carroll et al., 2012).
et al., 2005; Karner et al., 2009; Kispert et al., 1998; Kopan et al., 2007; Majumdar et al., 2003; Park et al., 2007; Yu et al., 2009). We recently identified the Wnt target gene, Lgr5 (Gpr49), as a marker of epithelial stem cells in several adult organs that display a high rate of Wnt-dependent self-renewal (Barker et al., 2007, 2008, 2010a, 2010b; Jaks et al., 2008). Lgr5, together with its close homolog, Lgr4, contributes directly to the maintenance of these stem cell compartments by binding to the R-Spondin family of secreted Wnt agonists to selectively amplify local canonical Wnt signals supplied by the niche (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011).

Preliminary quantitative PCR (qPCR) expression analyses revealed that Lgr5 is robustly expressed in early neonate kidneys but is absent from adult kidneys. Therefore, we set out to accurately document Lgr5 expression during kidney development in the mouse, and to evaluate whether Lgr5 marks kidney stem/progenitor populations using an in vivo lineage tracing approach.

RESULTS

Lgr5 Expression Is Restricted to Nascent Nephrons during Embryonic/Neonatal Kidney Development

Preliminary qPCR analyses of Lgr5 expression in the mouse kidney indicated that expression was predominantly restricted to late embryonic/early postnatal phases of development (Figure 1A). To document endogenous Lgr5 expression more accurately, we employed independent Lgr5-enhanced green fluorescent protein (EGFP)-ires-CreERT2 reporter mice (Barker et al., 2007) and highly sensitive Lgr5 florescent in situ hybridization (Lgr5-FISH) approaches (Figures 1 and S1). Lgr5-EGFP expression was first detected in discrete cell clusters within the renal cortex at embryonic day 14 (E14), coinciding with the onset of nephrogenesis (Figure 1D).

Detailed Lgr5-FISH analyses at E14 revealed Lgr5 expression to be confined to E-cadherin+ve epithelial structures resembling comma-shaped/S-shaped bodies (Figures 1E and 1F). Robust expression was maintained within the developing cortex throughout the first week of development (Figures 1G–1L) until postnatal day 6/7, when both EGFP reporter gene activity and endogenous Lgr5 transcripts became undetectable (Figures 1J–1L). Lgr5 expression was also undetectable in adult kidneys, implying that Lgr5 is permanently silenced after the first postnatal week under physiological conditions (Figures 1A–1C). Importantly, the tight correlation between Lgr5-EGFP expression and endogenous Lgr5-FISH signals validates the Lgr5-EGFP-ires-CreERT2 model as an accurate reporter of endogenous Lgr5 expression in the kidney. An identical expression pattern was observed in the developing kidneys of the independent Lgr5-lacZ reporter mouse (Figures S1M and S1N). An in situ hybridization (ISH) analysis subsequently revealed a similar expression pattern for Lgr5 within human embryonic kidney, with signals predominantly restricted to cortical structures reminiscent of S-shaped bodies (Figure S2).

Lgr5 Marks Relatively Undifferentiated Cells Residing within the Distal S-Shaped Bodies of Neonate Kidneys

Having established the general spatiotemporal organization of Lgr5-EGFP+ve cell populations within the developing mouse kidney, we set out to more accurately define their location and phenotypic identity using a panel of established lineage markers specific for the various differentiated nephron segments (Figures 2 and S3). We visualized Lgr5-EGFP+ve cells in neonate Lgr5-EGFP-ires-CreERT2 kidney sections using anti-EGFP immunohistochemistry (IHC) (Figure 2A). The specificity of the GFP antibody was confirmed by the absence of staining on neonate wild-type kidney sections (Figure 2B). Serial stains for GFP and Tamm Horsfall protein (THP; thick ascending limb of Henle’s loop; Figures 2C and 2D), calbindin (distal convoluted tubule; Figures 2E and 2F), megalin (proximal tubule; Figures 2G and 2H), and aquaporin 2 (AQP2; connecting segment/collecting duct; Figures 2I and 2J) did not reveal a phenotypic overlap between Lgr5-EGFP+ve cells and the differentiated nephron populations. Transmission electron microscopy was subsequently employed to characterize the ultrastructure of the Lgr5+ve cells in neonate (postnatal day 1 [P1]) kidneys from Lgr5-EGFP-ires-CreERT2 mice. Cryo-immunogold labeling for EGFP readily identified clusters of Lgr5-EGFP+ve cells within cortical tubular structures (Figures 3A–3D). The Lgr5-EGFP+ve cells displayed numerous mitochondria and low numbers of small apical tubules with electron-dense content. Collectively, these findings demonstrate that under physiological conditions, Lgr5 expression is exclusively restricted to clusters of polarized, relatively undifferentiated epithelial cells residing within tubular structures in the cortex of late embryonic/early postnatal kidneys.

To formally document the location of the Lgr5+ve cells within nascent nephrons, we examined P1 kidneys from Lgr5-EGFP-ires-CreERT2 mice for potential overlap of Lgr5-EGFP expression with established domain-specific markers of the S-shaped body. Substantial overlap was observed between Lgr5-EGFP and genes predominantly expressed within the distal region of the S-shaped body, including E-cadherin (Figures 3E–3G), Jagged1 (Figures 3H and 3I), and Sox9 (Figure 3J), establishing the Lgr5-expression domain within this region of the nascent nephron (Cho et al., 1998; Dahl et al., 2002; Georgas et al., 2009).

Comparative gene expression profiling of fluorescence-activated cell sorting (FACS)-sorted Lgr5-EGFPhi versus Lgr5-EGFPneg populations from embryonic Lgr5KI kidneys (E14 isolation + 10 day in vitro culture) was subsequently employed to determine the signature transcriptome of the Lgr5+ve population (see Figure 4A for a typical EGFP FACS profile). Lgr5 expression was highly enriched in the sorted Lgr5-EGFP+ve population, thus confirming its identity as the endogenous Lgr5+ve kidney population (Figure 4B). Expression of Six2 and Cited1, markers of noninduced MM (Mugford et al., 2009), was very low within the Lgr5-EGFP+ve population (Figure 4B). Similarly, expression of genes that are typically active in the proximal developing nephron, such as Wt1, was markedly lower in the Lgr5-EGFP+ve cells. In contrast, marker genes for the distal nephron (Dkk1, Papss2, UMOD, Slc12a1, and Pou3f3; Georgas et al., 2009) were highly enriched within the Lgr5-EGFP+ve population (Figure 4B). A similar expression trend was observed for selected markers when Lgr5-EGFPhi and Lgr5-EGFPneg populations isolated by FACS directly from P1 Lgr5KI kidneys were compared by qPCR (Figures 4C–4F). Of note, the highly related gene Lgr4, which is essential for kidney development (Kato et al., 2006; Mohri et al., 2011), was also robustly expressed...
Figure 1. Transient, Highly Localized Expression of Lgr5 within the Cortex of the Developing Kidney

(A) qPCR analysis of Lgr5 expression in the kidney during development and in adults.

(B) Confocal image documenting restricted Lgr5-EGFP expression in structures resembling S-shaped bodies in the cortex of neonate (P1) Lgr5-EGFP-ires-CreERT2 mice (inset: magnified Lgr5-EGFP+ve clusters).

(C) Lgr5-EGFP expression is absent from the adult kidney.

(D–L) Temporal analysis of Lgr5 expression in the developing mouse kidney reveals that Lgr5+ve cells are present at E14 and P3, but absent at P7. (D, G, and J) Lgr5-EGFP expression in the kidney cortex at E14 (D: 40x magnification), P3 (G: 40x mag) and P7 (J: 40x mag). Scale bars = 50 μm.
on the Lgr5+ve cells, although expression was not significantly enriched compared with the Lgr5-ve population (Figure 4D).

Collectively, these independent profiling approaches support the existence of a discrete population of Lgr5-expressing cells within the distal region of the S-shaped bodies of the neonatal kidney.

Lgr5 Marks a Nephron Stem/Progenitor Cell Population in the Developing Kidney

In vivo lineage tracing is a powerful technique for evaluating the stem cell identity of candidate tissue populations in their native environment. We have successfully employed this technique to establish the stem cell identity of Lgr5+ve populations in various adult tissues, including small intestine, colon, stomach, and hair follicles (Barker et al., 2007, 2010b; Jaks et al., 2008). Here, we adopted the same strategy to determine whether the Lgr5+ve populations residing within the developing kidney were functioning as renal stem cells in vivo. Neonate (P1) Lgr5-EGFP-ires-CreERT2/R26RLacZ mice were given a single 100 µg dose of 4-hydroxytamoxifen to activate the Cre enzyme and, as a consequence, permanently switch on the lacZ reporter gene within renal Lgr5+ve cells. As expected, the LacZ reporter gene was typically activated in single cells within nascent nephrons located toward the periphery of the kidney cortex (Figure 5A; 2 days postinduction). These ribbons of LacZ+ve progeny rapidly expanded to form tubular structures extending from the cortex into the medulla over the following week (Figures 5B and 5C; 7 days postinduction), consistent with an active contribution of Lgr5+ve cells to the generation of de novo structures within the developing kidney. At 13 months postinduction, multiple contiguous lacZ+ve tubules extending deep into the medulla were evident throughout the kidney, indicating a long-term contribution of Lgr5+ve cells to renal structures (Figures 5D and 5E). High-magnification whole-mount microscopy clearly identified looping tubular structures at the cortex/medulla boundary (Figure 5F, arrows) and also deep within the medulla (data not shown).

To formally identify the lacZ+ve renal structures generated by the neonate Lgr5+ve cells, we costained sections of tracing kidneys from the long-term P1 induced mice with various markers of the anatomically distinct nephron populations. LacZ+ve tubules present within the cortex, corticomedullary junction, and medulla were typically found to comprise simple, cuboidal epithelial cells lacking a brush border (not shown). Significant overlap of lacZ and THP, a marker of the thick ascending Henle’s loop, was observed in the majority of tracing units. This was particularly evident in tubules located at the cortex/medulla boundary or within the medulla proper (Figures 5G–5I, black arrows). However, lacZ+ve units lacking coexpression of THP were readily apparent throughout the kidney (red arrows). Approximately 25% of LacZ+ve tracing units located within the cortex demonstrated overlap with calbindin, a marker of the distal convoluted tubule (Figures 5J–5L). A low frequency of LacZ+ve units within the cortex overlapped with AQP2, but the AQP2+ve structures in the medulla were invariably lacZ-ve (Figures 5M and 5N). Given that the collecting ducts are non-MM-derived structures, we interpreted this as evidence of a contribution to the AQP2+ve region connecting the convoluted tubule to the collecting ducts (Roje et al., 2006). In contrast, we never observed costaining of lacZ and the proximal convoluted tubule marker megalin (Figures 5O and 5P).

Collectively, these findings demonstrate that Lgr5 marks a stem/progenitor cell population dedicated to generating the nephron segments that comprise the thick ascending Henle’s loop, the distal convoluted tubule, and the connecting segment that links the distal convoluted tubule to the collecting ducts in neonate kidneys (see cartoon summary in Figure 5Q).

To assess the contribution of individual Lgr5+ve cells to these nephron segments, we performed multicolor in vivo lineage tracing using our validated Rosa26-4color reporter mice. Analysis of kidneys from 1.5-month-old Lgr5-EGFP-ires-CreERT2/Rosa26-4color mice induced at P1 revealed the presence of 51 exclusively clonal (i.e., single-color) tracing units, highlighting the major contribution of individual Lgr5+ve stem/progenitor cells to nephron formation during development (Figures 6A and 6B). An extensive overlap between the tracing clones and THP confirmed the major contribution of the Lgr5+ve cells to formation of the thick ascending Henle’s loop (Figures 6C and 6D). Once again, THP+ve cell populations were observed within the tracing clones, indicating a contribution to nephron segments other than the thick ascending Henle’s loop (Figure 6D, white arrow). As previously documented in the ROSA26RlacZ reporter tracing experiment, overlap with the proximal tubule marker megalin was never observed (Figures 6I and 6J). However, in contrast to the lacZ tracing experiment, no overlap between the clonal tracing units and calbindin (Figures 6E and 6F) or AQP2 (Figures 6G and 6H) was found. Although this is likely a consequence of the marked lower tracing frequency achieved using the 4-color allele (typically 20-fold lower than the lacZ tracing allele), which made it technically challenging to visualize the more-limited overlap with calbindin and AQP2, we cannot exclude the possibility that distinct Lgr5+ve populations generate the thick ascending Henle’s loop and distal convoluted tubule/connecting segment.

We next set out to characterize the earliest Lgr5+ve populations identified within the embryonic kidney. We visualized Lgr5-EGFP+ve cells within E14 Lgr5-EGFP-ires-CreERT2 kidney sections using anti-GFP IHC (Figures 5A and 5B). The Lgr5-EGFP+ve cells were exclusively present within structures reminiscent of comma-shaped bodies (Figures S4A, S4D, and S4G). Serial stains for GFP and the reported distal marker genes Sox9 and Jagged1 documented extensive overlap, indicating that the Lgr5+ve population is restricted to the distal region of early nephrons (Figures S4C–S4H). We also initiated in vivo lineage tracing in E14 Lgr5-EGFP-ires-CreERT2/ROSA26RlacZ mice by administering 2 mg hydroxytamoxifen to pregnant
Figure 2. Lgr5\textsuperscript{+ve} Cells in the Developing Kidney Do Not Express Markers of Mature Nephron Lineages

(A) Neonate Lgr5-EGFP-ires-CreERT2 (P1) kidney sections stained for GFP (A: 5\times mag; inset: 40\times mag). Lgr5-EGFP\textsuperscript{+ve} cells are evident toward the periphery of the cortex.

(B) GFP staining is absent from kidneys of a P1 wild-type littermate control (5\times mag; inset: 40 times; mag). Scale bars = 500 \text{ \mu m}.

(C and D) Serial sections of P1 kidney from Lgr5-EGFP-ires-CreERT2 mice stained for GFP (C: 40\times mag) and THP (D: 40\times mag).

(E and F) Serial sections of P1 kidney from Lgr5-EGFP-ires-CreERT2 mice stained for GFP (E: 40\times mag) and calbindin (F: 40\times mag).

(G and H) Serial sections of P1 kidney from Lgr5-EGFP-ires-CreERT2 mice stained for GFP (G: 40\times mag) and megalin (H: 40\times mag).

(I and J) Serial sections of P1 kidney from Lgr5-EGFP-ires-CreERT2 mice stained for GFP (I: 40\times mag) and AQP2 (J: 40\times mag). Scale bars = 50 \text{ \mu m}.

The absence of any overlap between Lgr5-EGFP (outlined in dashed red) and the various nephron markers identifies the Lgr5\textsuperscript{+ve} cells as an undifferentiated renal population.

See also Figure S3.
females to assess the stemness of embryonic kidney Lgr5 +ve populations. Columns of tracing lacZ +ve progeny extending from the cortex to the medulla were observed at low frequency (Figures S4 I and S4J). Marker costaining experiments similar to those described above were performed on kidney sections isolated from an E14–P46 induced mouse. Overlap of lacZ and THP was again readily apparent (Figures S4 K and S4L), but no costaining of lacZ or any other nephron segment marker was observed (not shown). The lack of costaining observed with calbindin or AQP2 could indicate that Lgr5 +ve cells present at early stages of kidney development are more lineage-restricted stem/progenitor populations compared with those present at P1. However, this is more likely a consequence of the low frequency of tracing initiation achieved with these embryonic inductions, which made it technically challenging to visualize the more-limited overlap between LacZ and calbindin or AQP2.

Dynamic Expression of Lgr5 during Ex Vivo Embryonic Kidney Development

Ex vivo culture of embryonic mouse kidneys is widely recognized to be an accurate model of in vivo kidney development. We therefore employed this technique to study spatiotemporal changes in Lgr5 expression during embryonic kidney development. Embryonic kidneys were isolated from lgr5-EGFP-ires-CreERT2 mice at day 11.5 postcoitum (11.5 dpc), when the UB just invades the MM. The immature organs were subsequently cultured at the medium-air interface for 10 days as previously described (Gupta et al., 2003) and Lgr5-EGFP expression monitored via confocal microscopy. No Lgr5-EGFP signal was observed in embryonic kidneys during the first 2 days of culture (11.5±2 dpc), indicating that Lgr5 expression is absent or below detection thresholds within the UB and MM (Figures 7A–7F). In contrast, robust Lgr5-EGFP expression was initiated.
within tubular structures resembling comma-shaped/S-shaped bodies at day 3 of culture (11.5+3 dpc; Figures 7G–7I; arrows). The frequency of these Lgr5-EGFP+ve structures increased markedly within the kidney cortex during the subsequent 5 days of culture (11.5+8 dpc), consistent with de novo generation of Lgr5-expressing renal cell populations (Figures 7J–7M). High-magnification images confirmed the similarity between these embryonic Lgr5-EGFP+ve structures and comma-shaped/S-shaped bodies within the kidney cortex (not shown). Collectively, these data indicate that the stem cell marker gene Lgr5 is specifically activated within newly formed comma-shaped/S-shaped bodies during the early stages of nephrogenesis.

To more accurately determine the dynamics of Lgr5 expression during kidney development, we then captured time-lapse video footage of EGFP expression within ex vivo cultured kidneys over a 3-day period (11.5+3 dpc until 11.5+6 dpc; Movies S1 and S2). New clusters of Lgr5-EGFP+ve cells were seen to form continuously throughout the cortex during the 3-day culture. These new Lgr5+ve populations appeared to arise independently of preexisting Lgr5-EGFP+ve structures, implying that the observed increase in the frequency of Lgr5+ve structures is predominantly driven via de novo activation of Lgr5 expression within Lgr5+ve embryonic kidney populations, rather than via a simple expansion of established Lgr5+ve populations.

Strikingly, we also observed the formation of radially extending EGFP+ve trails during ex vivo kidney development. These trails consisted of EGFPhi cells at their medullary end, and cells expressing lower levels of EGFP toward the cortex. We interpreted this as evidence of rudimentary lineage tracing from Lgr5-EGFPhi cells, which generate Lgr5+ve progeny at their cortical side, with an accompanying dilution of the EGFP signal. This arrangement indicates that Lgr5+ve cells are located at the medullary end of the thick ascending limb of Henle’s loop.
DISCUSSION

Here, we identify the Wnt target gene Lgr5 (also known as GPR49) as a marker of a nephron stem/progenitor population that is responsible for generating the thick ascending limb of Henle’s loop and the distal convoluted tubule in the developing kidney. Using validated Lgr5 reporter mice and sensitive FISH techniques to document endogenous Lgr5 expression in vivo, we reveal a highly restricted expression pattern within developing kidneys during late embryogenesis and early postnatal life. Clusters of Lgr5+ve cells first appear around E14, within distal regions of tubular epithelial structures reminiscent of comma-shaped/S-shaped bodies. This expression pattern is maintained in the kidney throughout subsequent embryonic development and the first week of postnatal life. Marker analyses and an ultrastructure investigation identified these early Lgr5+ve populations as relatively immature cells distinct from the mature nephron/collecting duct lineages. Of importance, a similar Lgr5 expression pattern is evidenced by ISH within human embryonic kidneys, indicating cross-species conservation. In contrast, Lgr5 is effectively silenced toward the end of the first week of postnatal life, and expression remains undetectable in the kidney throughout adulthood under physiological conditions.

The bulk of the ~11,000 nephrons that comprise the intricate filtration network of mature murine kidneys are generated during a development window spanning 13 dpc to P5–P7. Cell turnover in the adult nephron is thought to be minimal under physiological conditions, although both the glomeruli and tubuli are able to recover from substantial damage (Benigni et al., 2010; Humphreys et al., 2008). Intriguingly, Lgr5 expression appears to be coincident with the major nephrogenesis phase of mouse development.
kidney development, originating within tubular structures resembling nascent nephrons. Indeed, expression profiling of the Lgr5-ve population revealed selective coexpression of markers of the distal S-shaped body. This observation together with the fact that Lgr5 is known to mark multiple adult stem cell populations in rapidly renewing epithelial tissues prompted us to consider the possibility that Lgr5-ve populations in the developing kidney function as nephron stem/progenitor cells.

To explore this, we employed our validated Lgr5-EGFP-ires-CreERT2 mouse model to perform in vivo lineage tracing from Lgr5-ve populations in developing kidneys. Stochastic activation of the Rosa-lacZ reporter gene in neonate (P1) mice was first evident in isolated Lgr5-ve cells scattered throughout the cortex 2 days postinduction. Tubular lacZ-ve columns extending from the cortex to the medulla appeared over the course of the next 5 days, consistent with the rapid generation of LacZ-ve progeny by actively proliferating Lgr5-ve cells. Multiple contiguous lacZ-ve tubules penetrating deep into the medulla were readily visible throughout induced kidneys 13 months later. These lacZ-ve populations frequently coexpressed THP and calbindin, identifying Lgr5-ve cells in the neonate kidney as progenitor populations dedicated to the generation of select nephron segments encompassing the thick ascending Henle’s loop and the distal convoluted tubule. A much more restricted overlap between lacZ and AQP2 was observed within the cortex of tracing kidneys, indicating a possible contribution to the AQP2-expressing region connecting the distal tubule to the collecting ducts (Rojek et al., 2006). No contribution to the proximal tubule was observed via marker analyses, confirming that Lgr5-ve cells are distinct from the truly multipotent Six2-ve population (Kobayashi et al., 2008).

Comparative gene expression profiling of different regions of nascent nephrons supports the existence of phenotypically distinct epithelial populations that are already committed to the generation of specific nephron segments (Georgas et al., 2009). However, it is currently unknown whether the subsequent expansion of nascent nephrons is driven by a limited number of segment-specific stem/progenitor cells or is simply mediated via the local expansion of committed, lineage-restricted populations within a particular segment. Our findings strongly indicate that a limited number of Lgr5-ve stem/progenitor cells within nascent nephrons are responsible for generating the functional Lgr5-ve lineages that comprise the thick ascending Henle’s loop and the distal convoluted tubule. Presumably, other Lgr5-ve stem/progenitor populations dedicated to generating the proximal nephron exist, but their definitive identification awaits the discovery of specific markers. The observed failure to maintain kidney-resident Lgr5-ve stem/progenitor populations beyond the termination of active nephrogenesis likely reflects the very limited cell turnover that occurs within adult nephrons under physiological conditions (Georgas et al., 2009; Nadasdy et al., 2009).

Figure 6. Multicolor Lineage Tracing from Neonate Lgr5-ve Populations Reveals the Activity of Individual Lgr5-ve Stem/Progenitor Cells

(A and B) Confocal images of P1–P46 induced kidneys from Lgr5-EGFP-ires-CreERT2/Rosa-4color mice. The single-color tracing shows the stem/progenitor output of a single Lgr5-ve cell.

(C and D) Extensive colocalization of THP with independent RFP tracing clones. Note the THP-ve stretch of the RFP clone in (D; white arrow), implying the presence of either less-differentiated cells or other mature cell lineages within this clone.

(E–J) No overlap is observed between the 4-color tracing clones and calbindin (E and F), AQP2 (G and H), or megalin (I and J). Scale bars = 50 μm.
This would negate a requirement for active Lgr5\(^{\text{+ve}}\) stem cell populations similar to those present in more actively renewing tissues, such as the gastrointestinal tract. We cannot exclude the possibility that Lgr5\(^{\text{-ve}}\) nephron stem/progenitor populations do exist in the adult kidney, although the observed persistence of lacZ\(^{\text{+ve}}\) tracing throughout life suggests that any such population would be derived from the neonate Lgr5\(^{\text{+ve}}\) stem/progenitor cells.

Canonical Wnt signaling is an important niche component for Lgr5\(^{\text{+ve}}\) adult stem cells in the intestine, stomach, and hair follicle (Barker et al., 2007, 2010b; Jaks et al., 2008). In turn, Lgr5 itself modulates Wnt signaling activity within the stem cells via its interaction with Wnt receptor components and the Wnt agonist R-Spondin (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). Currently, we can only speculate about the niche signals that regulate neonate Lgr5\(^{\text{+ve}}\) stem/progenitor activity in the developing kidney. However, the observed temporal overlap between Lgr5 expression and canonical Wnt signaling activity during early kidney development, together with the established role of Wnt signaling in driving early nephrogenesis (Dressler, 2009; Kopan et al., 2007), is consistent with a central role for active Wnt signaling in regulating the renal Lgr5\(^{\text{+ve}}\) stem/progenitor pool.

The robust activation of Wnt signaling in response to acute injury is known to play a major role in mediating tissue regeneration in adult tissues such as the liver and pancreas. Wnt signaling was also recently implicated in driving regeneration in the adult kidney (Lin et al., 2010). Preliminary data indicate that this local Wnt response is instrumental in driving the transient reactivation of Lgr5\(^{\text{+ve}}\) stem/progenitor populations that are capable of effecting tissue repair (H.C., unpublished data). It is tempting to speculate that...
a similar mechanism may contribute to kidney regeneration in adults following injury.

Here we demonstrate that Lgr5 marks a nephron segment-specific stem/progenitor cell population in the early patterned S-shaped body. Lgr5<sup>Cre</sup> stem/progenitor cells give rise to the thick ascending limb of Henle’s loop and distal convoluted tubule. The progressive loss of Lgr5<sup>Cre</sup> expression in the postnatal kidney suggests that under physiological conditions, postnatal tubular growth and cell turnover is independent of Lgr5<sup>Cre</sup> stem/progenitor cells. Understanding the regulatory mechanisms and progenitor cell populations involved in nephron development should support the development of therapies for congenital renal diseases and regenerative therapies.

EXPERIMENTAL PROCEDURES

Mice and Animal Care

Lgr5-EGFP-Ires-CreERT2, Lgr5-lacZ, and Rosa26-4color mice were generated as described previously (Barker et al., 2007). Rosa26RlacZ mice were obtained from Jackson Labs. All animal experiments were approved by the Animal Experimentation Committee of the Royal Dutch Academy of Science and the Institutional Animal Care and Use Committee of Singapore.

Tamoxifen Induction

Newborn (neonate) mice (P1) were injected intraperitoneally with a single 10 μl dose of 4-hydroxytamoxifen in corn oil at 10 mg/ml. For embryonic inductions, pregnant females were injected intraperitoneally with a single 200 μl dose of 4-hydroxytamoxifen in corn oil at 10 mg/ml.

Confocal Imaging Procedures

Whole-Mount Analysis

Isolated embryonic kidneys (E13 and E14) were fixed for 40 min in 4% paraformaldehyde (PFA) solution at room temperature. Fixed embryonic kidneys were mounted in Hydromount (National Diagnostics) on glass slides and covered by a coverglass. Whole-mounted organs were analyzed within 24 hr for EGFP expression by confocal microscopy (Olympus FV1000 upright).

Vibratome Sectioning

Isolated postnatal kidneys were sliced open and fixed for 2 hr in 4% PFA solution at 4°C. Fixed organs were embedded in 4% UltraPure LMP Agarose (Invitrogen) and vibratome sectioned (Leica VT 1000S) at 150 μm sections were mounted in Hydromount and analyzed within 24 hr for EGFP expression by confocal microscopy (Olympus FV1000 upright).

Immunoelectron Microscopy

Kidneys were dissected and immersion fixed in 2% PFA in 0.2 M PHEM buffer (240 mM Pipes, 40 mM EGTA, 100 mM HEPES, 8 mM MgCl<sub>2</sub>; pH 6.9), embedded in gelatine, and cryosectioned with a Leica FCS cryoultratome. Cryo-immunogold staining (Peters et al., 2006) for GFP expression was performed as previously described (Barker et al., 2007).

RNA Isolation, CDNA Synthesis, and qPCR

For gene expression analysis of whole kidneys, RNA was isolated from tissue preparations using Trizol (Invitrogen) and RNaseasy mini columns (QIAGEN), essentially according to the manufacturer’s instructions. cDNA synthesis and qPCR were performed as described in Extended Experimental Procedures.

Statistical Analysis

Gene expression data were quantified and depicted as the mean ± SEM. To determine which genes were significantly differentially expressed, the data were analyzed by one-way analysis of variance followed by Bonferroni’s post test. Significant differential gene expression is denoted as follows: ***p < 0.0001 and **p < 0.005.

In Situ Hybridization

ISH was carried out with <sup>35</sup>S antisense riboprobes essentially as described previously (Poulsom et al., 1998) using SP6 RNA polymerase and EcoRI linearized sequence-verified templates prepared in pGEM3Z by Dr. Stefanja Segditsas (human Lgr5 566 bp from 5’UTR to exon5 [UCSC Chr12 70,120,102–70,233,231, introns included]). Autoradiographic silver grains indicating the presence of Lgr5 mRNA within Giemsa-stained sections were imaged using a Nikon Eclipse ME600 epi-illumination microscope with a Qimaging MicroPublisher 5.0 camera.

Immunohistochemistry

Immunostaining was performed on formalin-fixed 4–6 μm paraffin-embedded sections. The primary antibodies were goat anti-AQP2 (1:200, C-17; Santa Cruz), goat anti-THP (1:100, G-20; Santa Cruz), goat anti-calbindin D28K (1:100, N-18; Santa Cruz), goat anti-megalin (1:100, P20; Santa Cruz) and goat anti-jagged1 (1:100, C-20; Santa Cruz). Alexa Fluor 568 and 647 anti-goat and immunoglobulin G (IgG) were used as secondary antibodies (1:500; Invitrogen). E-cadherin staining on P0 kidney vibratome sections was performed using the conjuncted Alexa Fluor 647 mouse anti-E-cadherin antibody (BD Pharmingen) at a 1:200 dilution and overnight incubation.

FISH Hybridizations and Image Analysis

FISH was performed as described previously (Itzkovitz et al., 2012). Briefly, kidneys were fixed in 4% PFA and then incubated overnight at 4°C in 30% sucrose/4% PFA. They were subsequently embedded in optimal cutting temperature compound, and 7 μm cryosections were used for hybridizations. Two Lgr5 Stellaris probe libraries totaling 96 probes, 20 bp long, complementary to the CDS of mouse Lgr5 were designed by Probe Designer at http://www.singlemoleculefish.com/ and synthesized by Biosearch Technologies. The probes were labeled using Cy3 or TAM fluorophores. An additional conjugated Alexa Fluor 488 mouse anti-E-cadherin (1:200; BD Biosciences) was added to the hybridization mix and used for protein IHC. Images were taken with a Zeiss AxioImager Z1 Upright fluorescence microscope equipped with a 63x oil-immersion objective and a CoolSnap HQ2 B/W CCD camera using MetaMorph software (images are 15–20 stacks with Z spacings of 0.2 μm, with gamma adjustment applied).

Lgr5 ISH was performed in the Histopathology Laboratory and ISH Service facility at the CRUK-London Research Institute using tissues procured and supplied specifically to facilitate localization of gene expression at the protein and mRNA level by Dr. Leslie Wong (MRC Tissue Bank, Royal Marsden Hospital, London). ISH Service work was approved by an institutional committee.
**Embryonic Kidney Culture**

Metanephric kidneys of timed pregnant mice at 11.5 days of gestation were harvested under sterile conditions using a dissecting microscope essentially as described previously (Giuliani et al., 2008). The embryos were placed in a Petri dish containing cold PBS. After removal of the head and tail, the abdominal wall was opened and intraabdominal contents were eviscerated to expose the retroperitoneum. The retroperitoneum was carefully removed and kidneys were isolated from it. The developing kidneys were placed on a 4 μm pore size transwell membrane and cultivated at the medium (Advanced Dulbecco’s modified Eagle’s medium [DMEM]/F12 supplemented with 1% penicillin/streptomycin and 10% fetal calf serum) air interface. The culture was maintained for 14 days in a fully humidified 37°C incubator with 5% CO2. Medium was replaced every second day.

**Microarray Procedure**

Metanephric kidneys of timed pregnancies of ~14 days of gestation were harvested and cultured for ~10 days as described above. Lgr5-EGFP-fires-CreERT2-expressing embryonic kidneys were identified by fluorescence microscopy and enzymatically digested to a single cell suspension using TrypLE (Sigma-Aldrich) and DNase. GFP− and GFP+ cells were sorted by microscopy and enzymatically digested to a single cell suspension using Clevers, H. (2008). Very long-term self-renewal of small intestine, colon, and hair follicles from cycling Lgr5+ve stem cells. Cold Spring Harb. Symp. Quant. Biol. 73, 351–356.


**REFERENCES**


