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A Population of Progenitor Cells in the Basal and Intermediate Layers of the Murine Bladder Urothelium Contributes to Urothelial Development and Regeneration

Sara A. Colopy, DVM, PhD, DACVS, **Dale E. Bjorling, DVM, MS, DACVS**, **William A. Mulligan**, and **Wade Bushman, MD, PhD**
Department of Surgical Sciences, School of Veterinary Medicine (SAC, DEB) and Department of Urology, School of Medicine (WB, WAM), University of Wisconsin, Madison, Wisconsin

Abstract

Background—Homeostatic maintenance and repair of the bladder urothelium has been attributed to proliferation of keratin 5-expressing basal cells (K5-BC) with subsequent differentiation into superficial cells. Recent evidence, however, suggests that the intermediate cell layer harbors a population of progenitor cells. We use label-retaining cell (LRC) methodology in conjunction with a clinically relevant model of uropathogenic *Escherichia coli* (UPEC)-induced injury to characterize urothelial ontogeny during development and in response to diffuse urothelial injury.

Results—In the developing urothelium, proliferating cells were dispersed throughout the K5-BC and intermediate cells layers, becoming progressively concentrated in the K5-BC layer with age. When 5-bromo-2-deoxyuridine (BrdU) was administered during urothelial development, LRCs in the adult were found within the K5-BC, intermediate, and superficial cell layers, the location dependent upon time of labeling. UPEC inoculation resulted in loss of the superficial cell layer followed by robust proliferation of K5-BCs and intermediate cells. LRCs within the K5-BC and intermediate cell layers proliferated in response to injury.

Conclusions—Urothelial development and regeneration following injury relies on proliferation of K5-BC and intermediate cells. The existence and proliferation of LRCs within both the K5-BC and intermediate cell layers suggests the presence of two populations of urothelial progenitor cells.

Keywords

bacteria; cystitis; progenitor; urinary injury

INTRODUCTION

The adult bladder epithelium (urothelium) is a specialized epithelium, comprised of three layers: the keratin 5-expressing basal cell (K5-BC) layer, the intermediate cell layer, and the terminally differentiated superficial (“umbrella”) cell layer. Together, these layers provide a

Correspondence: Dr. Sara A. Colopy, Department of Surgical Sciences, School of Veterinary Medicine, 2015 Linden Drive, Madison, WI 53706 (telephone: 608-262-4580, FAX: 608-263-7930; colopys@svm.vetmed.wisc.edu).

protective barrier to hypertonic urine and toxic metabolites within the urine (Hicks, 1975). Damage to the urothelium results in compromised urothelial barrier function and leakage of urine into the underlying subcutaneous tissues. Chronic bladder inflammation, such as experienced in interstitial cystitis/painful bladder syndrome (IC/PBS) is thought to result from a dysfunctional response to urothelial injury, inadequate repair of urothelial integrity, and chronic pain due to exposure of afferent nerve fibers to urine in the suburothelial space (Dasgupta and Tincello, 2009; Keay et al., 2003; Slobodov et al., 2004; Zhang et al., 2005; Keay et al., 2012). The identification of urothelial progenitor cells and the mechanisms that allow these cells to proliferate and differentiate could therefore shed light on the mechanisms that underlie chronic urothelial disease and ultimately lead to new treatment strategies for tissue regeneration.

The urothelium exhibits one of the slowest turnover rates among mammalian epithelia under homeostatic conditions (Hicks, 1965; Jost, 1989); however, urothelial proliferation is dramatically upregulated in response to injury, resulting in complete restoration of differentiated superficial cells within seven days (Koss and Lavin, 1970; Romih et al., 2001; Mulvey et al., 1998). Homeostatic maintenance and repair of the urothelium have been attributed to proliferation of K5-BCs with subsequent differentiation into intermediate and superficial cells, but there is very little direct evidence to support this concept. The preponderance of evidence supporting this hypothesis comes from observational studies utilizing injury-repair models in which the majority of proliferating urothelial cells have been observed within the basal layer of the urothelium subsequent to injury (Romih et al., 2001; Messier and Leblond, 1960; Mysorekar et al., 2009). Critical assessment of urothelial ontogeny during development and repair using lineage-tracing studies has only recently become the subject of study (Shin et al., 2011; Gandhi et al., 2013).

Two recent studies provide conflicting evidence as to the location of progenitor cells within the bladder urothelium. Shin et al. (2011) utilized a tamoxifen (TM)-inducible *ShhCreERT2;mTmG* transgenic mouse to label sonic hedgehog expressing (Shh^{+}) cells in adult urothelium. Results from this study support existence of a population of Shh-expressing progenitors with long-term regenerative potential, and co-localization of Shh with the basal cell marker keratin 5 (Krt5), led the authors to conclude that the urothelial progenitor is a K5-BC (Shin et al., 2011).

Recognizing that Shh^{+} cells are found both within the K5-BC and intermediate cell layer, Gandhi et al. (2013), performed fate-mapping analysis of K5-BCs and intermediate cells separately in urothelial development and in a cyclophosphamide-induced urothelial injury model to determine which cell population is responsible for replenishing the superficial cell layer. Interestingly, results from this study suggest that the urothelial progenitor cell is *not* a K5-BC, neither in development nor in the adult regenerating epithelium. In development, the authors identified a transient population of $Foxa2^{+}/P63^{+}/Shh^{+}/Upk^{+}/Krt5^{-}$ progenitor cells (P cells) that generate intermediate and superficial cells in development, but not in the adult. In the adult, superficial cells were found to be derived from proliferation of intermediate cells after injury (Gandhi et al., 2013). This concept is supported by recent findings that all layers of the urothelium develop from p63-expressing cells (present in K5-BCs and intermediate cells), rather than the K5-BCs (Pignon et al., 2013). Clearly, further

investigation is needed to understand location and behavior of progenitor cells within the bladder urothelium.

The label-retaining cell (LRC) strategy is a popular method of localizing potential epithelial progenitor cells because of the lack of specific markers for these cells. This technique entails pulse-labeling mitotic nuclei by intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU) and subsequently examining tissues for the presence of BrdU-positive cells. It has been speculated that asymmetric cell division and/or a slow-cycling phenotype leads to retention of BrdU by a small subset of potential progenitor cells (Potten *et al*., 2002; Teng *et al*., 2007; Kimoto *et al*., 2008). This strategy has been used successfully in a variety of organs, including hair follicles, cornea, mammary glands, and prostate, to enrich for a subset of potential progenitor cells (Maeshima, 2007; Teng et al., 2007).

The purpose of this study was to characterize urothelial ontogeny during development and in a clinically relevant murine model of UPEC-induced urothelial injury. We employed LRC methodology in conjunction with UPEC infection to increase understanding as to the location and behavior of urothelial progenitor cells. By tracing BrdU-labeled urothelial cells from embryological development into adulthood, we found that LRCs were located within the K5-BC, intermediate, and superficial cell layers, the location dependent upon the time of labeling during development. Intravesicular instillation of UPEC 1677 resulted in loss of the superficial cell layer followed by robust proliferation of both K5-BCs and intermediate cells. The regenerative response to injury included proliferation of LRCs in both the K5-BC and intermediate cell layers. Our data suggest that both the K5-BC and intermediate cell layers contain a population of progenitor cells that contribute to urothelial development and regeneration after injury.

RESULTS

Urothelial proliferation during development

Pregnant females or neonates were treated with BrdU at various time points and sacrificed one hour later to determine the population of current dividing cells. Urothelial cells from different layers of the urothelium express different types of biomarkers, among them distinct keratins (Krt). Krt5 is a high molecular weight Krt found within the basal cell layer. In contrast, Krt20 is a low molecular weight Krt found only within superficial cells. Thus, Krt5 and Krt20 can be used as biomarkers for immunophenotypic characterization of basal and superficial cells, respectively (Castillo-Martin et al., 2010).

At E15, the developing urothelium was ~3–5 cells layers thick, and proliferating cells were found throughout all layers of the urothelium. Proliferating cells were phenotypically consistent with basal and intermediate cells (either positive or negative Krt5 staining and negative Krt20 staining). Krt20 immunostaining was absent at E15, indicating the lack of differentiated superficial cells at this time (Figures 1a and 1b). At P1, the developing urothelium had reduced to 2-cell layers in thickness, and proliferating cells were predominantly K5-BCs (Figures 1c and 1d). At P5, P10, and in the adult, the developing urothelium had returned to a multilayer state, and actively proliferating cells were predominantly K5-BCs (Figures 1e–1j). Proliferating cell counts are summarized in Table 1.

At no time point analyzed, during development or adulthood, were differentiated ($Krt20^+$) superficial cells found to be actively proliferating.

Urothelial Response to UPEC 1677 Inoculation

We instilled UPEC 1677 transurethrally into the bladders of 8-week-old female mice to examine the urothelial response to injury. The presence or absence of infection was confirmed by culture of bladder tissue from bacteria- and PBS-instilled groups, respectively (data not shown). Histological examination of infected bladders revealed extensive infiltration by inflammatory cells, expansion of the vasculature, and edema of the lamina propria. These changes were present within one day of bacterial inoculation and progressively decreased over time (Figure 2a). Urothelial thickness was significantly increased one day post-inoculation (Figure 2b), correlating with an increased number of cell layers and edema, and gradually returned to baseline. The thickness of the muscularis propria did not change at any time point following bacterial inoculation (data not shown).

It has previously been reported in other bladder infection models that superficial cells slough in response to UPEC infection as a mechanism to rapidly clear infection (Mulvey et al., 1998; Anderson et al., 2003). Using Krt20 staining as a specific marker of fully differentiated superficial cells, we confirmed that the superficial cell layer was sloughed in response to inoculation with UPEC 1677. Rare $Krt20^+$ cells were identified in the urothelium one day post-inoculation. Two days post-inoculation, Krt20 staining increased. Krt20 staining was present within the majority of the luminal cells by three days post-inoculation, indicating replenishment of differentiated superficial cells. Panuroplakin antibody has been shown to stain all urothelial cell layers of the mouse bladder (Wu et al., 2009). In the present study, the intensity of the staining was reduced one day after bacterial inoculation, consistent with loss of the superficial cell layer and the associated thick uroplakin plaques on the surface of these cells. The remaining urothelial cells expressed uroplakin (previously described to be within fusiform vesicles (Wu et al., 2009)) and continued to stain positive. Panuroplakin staining gradually increased as the superficial cell layer was restored (Figure 3).

A robust proliferative response ensued within the remaining K5-BC and intermediate cell layers after loss of the superficial cell layer

To characterize urothelial proliferation following bacterial inoculation, BrdU was injected one hour prior to sacrifice, and co-staining for BrdU and urothelial differentiation markers was performed. We found very little proliferation (0–1 cell/hpf) in the urothelium of naïve mice (no intravesical instillation) or in PBS-treated controls (Figure 4a and 4b). The superficial cell layer sloughed, and robust proliferation ensued in the remaining urothelial cell layers within 24 hours after bacterial inoculation. Co-staining with BrdU and Krt5 or Krt20 revealed that proliferating cells were distributed throughout the K5-BC and intermediate cell layers (Figure 4c and 4d). Between 2–7 days after bacterial inoculation, the superficial cell layer reformed, urothelial proliferation significantly declined, and proliferating cells were found to become more concentrated in the K5-BC layer (Figures 4e–4l). Quantification of proliferating cells confirmed that proliferation was highest during the earliest time point observed (one day post-inoculation), declined by two days, and returned

to baseline three days post-inoculation (Figure 4m). Quantitative analysis of the contribution of K5-BCs and intermediate cells to the regenerative response revealed the surprising observation that infection induced proliferation in a much larger percentage of intermediate cells than K5-BCs (31% versus 10.5%, respectively) (Figure 4n).

The regenerative response of the adult recapitulated the distribution of proliferating cells during urothelial development

A comparison of urothelial proliferation during development and following bacterial-induced injury in the adult was made. As noted previously, urothelial proliferation was quantified by BrdU labeling at various time points during development (E15, P1, P5, P10, and P14), in 8-week-old naïve mice, and in 8-week-old mice one day after transurethral inoculation with UPEC 1677. BrdU-labeled cells were quantified in the K5-BC and intermediate cell layers and expressed as a percentage of total proliferating urothelial cells. Urothelial proliferation was abundant in both K5-BCs and intermediate cells early in development and became progressively more concentrated in K5-BCs with age. In the adult mouse, homeostatic urothelial proliferation occurred predominantly in K5-BCs. One day after bacterial inoculation, proliferation was increased in both K5-BCs and intermediate cells. The relative increase in proliferation of intermediate cells was striking. This distribution of proliferative activity resembles the distribution of proliferative activity during prenatal development and was remarkably different from any time point postnatal (Table 1).

Urothelial label-retaining cells

To provide a unique perspective on the location of urothelial progenitor cells and augment our findings in the injury model, we employed the LRC methodology. Animals in this study were pulse-labeled with BrdU at various time points during development and then sacrificed at one month of age to characterize the label-retaining population of cells. When animals were given BrdU prior to E13, no LRCs were identified within the urothelium at one month of age (not shown). When animals given BrdU at E15 were evaluated at one month of age, LRCs were found almost exclusively within the superficial cell layer as indicated by a lack of Krt5 staining and positive Krt20 staining (Figures 5a and 5b). When animals were given BrdU at P1 and evaluated at one month of age, LRCs were found within the K5-BC, intermediate, and superficial cell layers (Figures 5c and 5d). In animals given BrdU at P7, LRCs were found within K5-BCs and intermediate cells at one month of age, as evidenced by both positive and negative Krt5 co-staining and a lack of co-staining with Krt20 (Figures 5e – 5f). When animals were given BrdU at P14, LRCs were found predominantly in K5-BCs (Figures 5g and 5h).

UPEC-induced urothelial injury elicited proliferation of LRCs within the urothelium

One limitation of the LRC methodology is that LRCs residing in the adult urothelium could be cells that ceased dividing and underwent terminal differentiation while still bearing the BrdU label, or alternatively, the LRCs could be slow-cycling cells that retain the ability to proliferate. If the LRCs were terminally differentiated, one would expect that these cells to be replaced secondary to normal turnover of the urothelium. To examine this possibility, we administered BrdU to neonatal animals on P7 and sacrificed them at two (Figures 6a and 6b) and four (not shown) months of age and found that LRCs persisted in the K5-BC and

intermediate cell layers at both time points. We then inoculated previously BrdU-labeled two-month-old mice with UPEC 1677. Examination of bladders one day post-inoculation revealed persistence of LRCs within both the K5-BCs and intermediate cell layers (Figures 6c and 6d). Co-staining for Ki67 and BrdU one day post-inoculation revealed that a small subpopulation of LRCs in the basal cell layer and intermediate cell layer proliferated in response to bacterial inoculation (Figures 6e–6g). Some LRCs were found to be incorporated in the newly developed superficial cell layer 14 days post-inoculation, (Figure 6h).

DISCUSSION

The multi-layered urothelial lining of the urinary bladder is a highly specialized epithelium that provides a waterproof barrier to toxic metabolites in the urine. Differentiated superficial cells line the surface of the urothelium and are covered with thick uroplakin plaques that interact to form the “asymmetric unit membrane” (AUM), an effective barrier to transcellular permeability (Hicks et al., 1975; Hu et al., 2002; Min et al., 2003). Unique junctional complexes, located at the intersection of the apical and lateral membranes, form a zone of attachment between adjacent cells and restrict paracellular transport (Acharya et al., 2004; Kreft et al., 2005; Khandelwal et al., 2009; Bryan and Tselepsis, 2010). After UPEC-induced urothelial injury, barrier function is temporarily disrupted until a new, fully differentiated superficial cell layer is formed (Wood et al., 2012; Shin et al., 2011). Urothelial cell proliferation must be dramatically upregulated from its normally quiescent state to rapidly accomplish this important task.

Conflicting evidence exists as to the location of progenitor cells responsible for urothelial restoration after injury. Lack of specific epithelial stem cell markers makes identification of progenitor cells in epithelial tissue difficult. Until recently, it was generally accepted that homeostatic maintenance and repair of the urothelium results from proliferation of K5-BCs with subsequent differentiation into superficial cells. Evidence supporting this hypothesis comes from observational studies utilizing injury-repair models in which proliferating urothelial cells have been observed predominantly within the basal layer of the urothelium after injury (Romih et al., 2001; Messier and Leblond, 1960; Mysorekar et al., 2009). Recent fate-mapping studies, however, suggest that the intermediate cell layer harbors a population of progenitor cells that is responsible for repopulating superficial cells following injury (Gandhi et al., 2013).

The initial goal of the studies described here was to compare and contrast the urothelial proliferative pattern in bladder development, during homeostasis in the adult, and following urothelial injury in the adult to determine if urothelial injury evokes an upregulation of K5-BC proliferation seen during homeostasis or a qualitatively different response. Our analysis of proliferation during homeostatic conditions in the normal adult bladder concurs with previous evidence that the mitotic index is extremely low and the majority of proliferation occurs within K5-BCs (Romih et al., 2001; Messier and Leblond, 1960; Martin, 1972). Following injury, however, we found that both K5-BCs and intermediate cells contribute to urothelial regeneration. This distribution of proliferative activity is quite different from the

restricted K5-BC proliferation observed in the normal adult bladder, and mimics the proliferative pattern observed during early prenatal urothelial development.

The remarkable proliferative response of the intermediate cell layer led us to the hypothesis that the intermediate cell layer harbors a population of progenitor cells that is recruited in response to injury. Using LRC methodology in conjunction with a clinically relevant model of UPEC-induce urothelial injury provided a unique perspective that compliments the previously existing fate-mapping studies of Shin et al. (2011) and Gandhi et al. (2013). We found that both the K5-BC layer and the intermediate cell layer harbor a population of slow-cycling LRCs that have the capacity to proliferate in response to diffuse urothelial cell injury. We speculate that having a population of progenitor cells in the intermediate cell layer allows the urothelium to rapidly upregulate proliferation to rapidly restore the barrier-forming superficial cell layer.

Interestingly, our results reconcile previous conflicting evidence as to the location of LRCs in the bladder urothelium. Kurzrock et al. (2008) initially found that when proliferating urothelial cells were BrdU-labeled at 6 week of age, LRCs were found exclusively in the basal cell layer. We made a similar observation when two-week-old mice received BrdU. However, the prevailing LRC protocol uses neonatal animals to label progenitor cells that are dividing during tissue development (Bickenbach and Chism, 1998; Duvillié et al., 2003; Chan and Gargett, 2006). Thus, another group (Zhang et al., 2012) repeated these studies utilizing 5-ethynyl-2-deoxyuridine (EdU) in neonatal (P1) rats. Though the investigators did not specify the urothelial layer within which LRCs were located, they reported that LRCs were “randomly” distributed in the adult rat urothelium and stated that their results differed from the previous study, in which “preferential labeling of urothelial basal cells was noted.” These findings are similar to our results when mice received BrdU at P1. Comprehensive analysis of urothelial proliferation during different time points of development followed by characterization of LRCs generated at the these different time points in our study led to the discovery that the location of LRCs depends upon the time of labeling during development.

During early prenatal development, when the urothelium is reported to be three cell layers in thickness (Erman et al., 2006), cells are rapidly dividing in all cell layers of the urothelium. This has been found in previous studies (Castillo-Martin et al., 2010; Erman et al., 2001) and confirmed in our study. Proliferating cells exhibit either a basal or intermediate cell phenotype, as evidence by positive or negative Krt5 staining and lack of Krt20 staining. Based on recent work by Gandhi et al. (2013), these proliferating cells may also contain a population of transient $Foxa2^{+}/P63^{+}/Shh^{+}/Upk^{+}/Krt5^{-}$ progenitor cells (P cells), present only during prenatal urothelial development. By pulse-labeling proliferating cells with BrdU and tracking them into adulthood, we found that many of these cells are incorporated into, and retained within, the superficial cell layer into adulthood. We suspect that these superficial LRCs are terminally differentiated, as the superficial cell layer was sloughed following inoculation of the bladder with bacteria.

As reported previously, we found that proliferation of intermediate cells is dramatically reduced postnatally, with proliferation being confined almost exclusively to the K5-BC layer after P7 (Castillo-Martin, et al., 2010; Erman et al., 2001). Accordingly, we found that

generation of LRCs during post-natal urothelial development modeled this pattern. LRCs generated at P1 were found in all urothelial cell layers of the adult, LRCs generated at P7 were found distributed within the K5-BC and intermediate cell layers of the adult, and LRCs generated at P14 were found almost exclusively in the K5-BC layer of the adult. The implication of this finding is that urothelial ontogeny is a complex, time dependent process with different cell populations proliferating at different time points during development. Identification of progenitor cell populations by LRC methodology depends on what time during development the labeling is performed. Our data reveal a unique temporal ontogeny of urothelial development in which the progenitor cell population changes from prenatal to postnatal and from development into adulthood.

BrdU labeling at P7 demonstrated that LRCs generated at this time point persist in K5-BCs and intermediate cell layers until at least 4 months of age. To determine whether these cells were terminally differentiated versus mitotically quiescent cells with proliferative potential, we tracked their fate following bacteria-induced urothelial injury. We found that a subpopulation of LRCs within both K5-BCs and intermediate cells proliferates in response to injury, and that some of these cells are incorporated into the newly developed superficial cell layer. Future fate-mapping studies are required to determine: 1) whether LRCs in K5-BC layer, intermediate cell layer, or both are incorporated into the superficial cell layer; and 2) whether urothelial LRCs represent a subpopulation of slow-cycling progenitor cells with the ability to self-renew.

Understanding the location and function of progenitor cells within the bladder urothelium has important implications for understanding the pathogenesis of chronic urothelial disease in people and for developing novel regenerative treatments in this patient population. The results of this study lays the groundwork for future studies designed to identify biomarkers of this progenitor cell population and factors that allow this subpopulation of cells to retain the capacity to proliferate and differentiate. Success in this effort would help elucidate mechanisms underlying urothelial dysregulation in chronic disease and/or allow manipulation of this cellular population for tissue regeneration.

In summary, we demonstrated that urothelial regeneration following diffuse injury recapitulates the pattern witnessed in urothelial development, relying on robust proliferation of K5-BCs and intermediate cells. Label-retaining cells present within the K5-BC and intermediate cell layers proliferate in response to bacteria-induced injury and are incorporated into the newly developed superficial cell layer. Our data suggest that both the K5-BC and intermediate cell layers contain a population of progenitor cells that contribute to urothelial regeneration after injury.

EXPERIMENTAL PROCEDURES

Animals

All experimental protocols were reviewed and approved by the Animal Care and Use Committee of the University of Wisconsin. C57Bl/6J wild type female mice (Jackson Laboratories) were used in these experiments. Ages ranged from embryonic day 15 (E15) to 4 months of age.

In-vivo BrdU labeling to identify urothelial LRCs

Adult pregnant C57Bl/6J female mice or neonatal C57Bl/6J mice received intraperitoneal (IP) injection of sterile BrdU (10mM, Roche), 1–2 ml/100g body weight at various time points during development (E6–10, E10–12, E13, E15, P1, P7, or P14). They were injected with BrdU once daily during the designated labeling period. Half of the animals were sacrificed one hour after the last injection (to determine location/quantity of currently proliferating cells), and the other half were sacrificed at one month of age (to characterize the label-retaining population of cells).

Bacteria

The UPEC 1677 bacteria were isolated previously from a patient with a severe urinary tract infection (Hopkins et al., 1986) and stored in liquid nitrogen. Virulence characteristics of this strain include type 1 and P fimbriae, hemolysin, aerobactin, and the O6 serotype (Hopkins et al., 1998). The bacteria were grown overnight in lysogeny broth medium, and concentrations of bacteria were determined by spectrophotometry.

Transurethral Intravesical Instillation

Mice were anesthetized with isoflurane, and a lubricated sterile 24 G x 0.75 inch BD Angiocath™ peripheral venous catheter was inserted via the urethra into the bladder. The bladder was emptied by application of digital pressure to the lower abdomen. UPEC 1677, 10^8 colony-forming units (CFUs) in 50 μl sterile phosphate buffered saline (PBS), or 50 μl sterile PBS was instilled into the bladder slowly over 10 seconds. Age-equivalent mice in which a urethral catheter was not passed (naïve group) were also included as a negative control to account for mechanical injury from the instillation process. Animals were sacrificed 1, 2, 3, 5, 7, or 14 days after instillation of PBS or bacteria, one hour after IP injection with BrdU (10mM, Roche) 1–2 mL/100g bodyweight.

In vivo assessment of LRC response to injury

Neonatal C57Bl/6J female mice were injected with sterile BrdU (10mM, Roche) IP at age P7 as previously described. When the mice reached two or four months of age, they were anesthetized, and intravesicular instillation with UPEC was performed as previously described. PBS-instilled animals and naïve animals were used as controls (both control groups were previously labeled with BrdU at P7). Mice were sacrificed one day or 14 days after PBS or bacterial instillation, and their urinary bladders were removed. Immunohistochemical staining for Ki67, BrdU, Krt5, and Krt20 was performed to determine the location and proliferative response of LRCs in the bladder urothelium after bacterial inoculation.

Sacrifice and Tissue Collection

Adult animals were sacrificed by cervical dislocation after sedation with isoflurane. Neonates (six days and younger) were sacrificed by inducing a state of hypothermia (placing the animals near ice for 30 minutes) followed by decapitation. The bladders were collected in sterile fashion, weighed, and divided in half. A sample of the bladder was excised for microbial culture, and the remaining bladder tissue was processed for histology

Bacterial Culture

A sample of bladder was excised in sterile fashion, weighed, and placed in a 1.5 mL microtube. Tissues were homogenized using a high-speed pestle grinder system (Fisher Scientific) in PBS, and serially diluted to 1:100, 1:500, and 1:1000. Standard plate counts were calculated by plating onto Levine's eosin methylene blue agar (Becton, Dickinson and Company) at 37°C. Bacterial titers were calculated as the number of CFUs per mg of bladder tissue.

Histology

Bladders were fixed in 4% paraformaldehyde at 4°C overnight, rinsed in PBS, and then positioned sagittally in 1% agarose under a dissecting microscope to ensure that each bladder was positioned and sectioned in the same orientation. Agarose blocks were processed, embedded in paraffin, and cut into 5-μm sections with a microtome.

Quantification of epithelial and muscularis propria thickness

After paraffin-embedded tissue sections were stained with hematoxylin and eosin, slides were imaged using a Nikon microscope and camera attached to a dedicated computer compatible with Image-Pro Plus software (Media Cybernetics). Approximately 10 random high-power images were obtained per bladder section. Within each image, 3–5 random epithelial measurements were made, generating a total of approximately 40 random epithelial measurements per bladder. Measurements were averaged to obtain the average epithelial thickness for that animal. The same methodology was used to determine the average thickness of the muscularis propria.

Protein localization by immunohistochemistry

Slides were deparaffinized and hydrated through a xylene and methanol gradient and subjected to heat-induced antigen retrieval by boiling in either 10 mM citrate buffer solution (pH 6.0) or in Tris-EDTA buffer (pH 9.0) for 15 minutes. Blocking for non-specific background was performed using a 1% bovine serum albumin (BSA)/10% donkey serum/ PBS-Tween solution for one hour. Sections were incubated with primary antibody overnight at 4°C. Primary antibodies and dilutions were as follows: BrdU labeling Detection Kit II (1:20, Roche); rabbit anti-Ki67 (1:100, Abcam), mouse anti-Krt20 (1:200, Dako); mouse anti-Krt 5/6 (1:100, Dako); rabbit anti-Krt5 (1:100, Abcam); and rabbit anti-panuroplakin (generously provided by X.R. Wu, New York University Langone Medical Center). Sections were washed and incubated with secondary antibodies for one hour at room temperature. Secondary antibodies and dilutions were as follows: donkey anti-mouse IgG-Alexa 488 (1:100, Invitrogen); donkey anti-rabbit IgG-Alexa 594 (1:100, Invitrogen); donkey anti-rabbit-Alexa 488 (1:200, Invitrogen); donkey anti-mouse-Alexa 594 (1:200, Invitrogen); donkey anti-rat-Alexa 594 (1:50, Invitrogen); donkey anti-goat Alexa 488 (1:100, Invitrogen). Sections were incubated with Hoechst counterstain (4 μg/ml, Invitrogen) for 10 minutes, washed, and cover-slipped using an aqueous mounting medium. Slides were imaged using an Olympus model BX51 fluorescent microscope and Spot Advanced software v. 3.5.2.

Quantification of proliferating urothelial cells

We determined the percentage of proliferating cells using the following protocol. Briefly, the total number of urothelial cells per high power field was determined and subdivided into $Krt5^+$ and $Krt5^-$ cell populations. Proliferating cells were quantified by counting the number of $BrdU^+$ nuclei within the urothelium. We then expressed the number of proliferating cells as a percentage of total urothelial cells, total $Krt5^+$ cells, and total $Krt5^-$ cells to quantify total proliferating urothelial cells, proliferating basal cells, and proliferating intermediate cells, respectively. Analysis was performed using Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/).

Statistical Analysis

Data were collected chronologically and group sizes were no less than 6 mice per interval (e.g. at least 6 mice per treatment were sacrificed at each time point). Data are presented as arithmetic means ± SEM, and two group comparisons were done with a two-tailed Student's *t*-test. A p-value < 0.05 was considered statistically significant.

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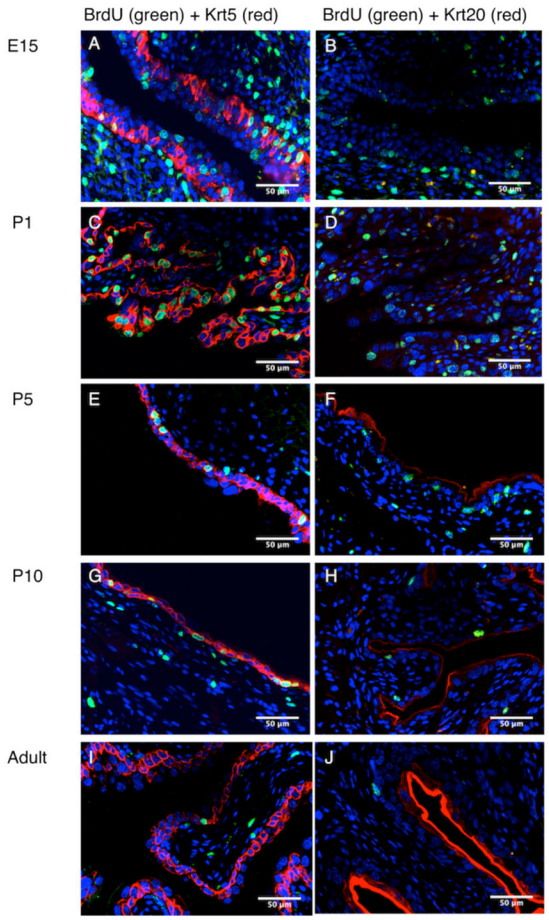


Figure 1.

Immunostaining for BrdU (green) and Krt5 (red, basal cells) or Krt20 (red, superficial cells) to identify proliferating urothelial cells at various time points during development. The time points during which the animals were labeled are noted to the left of the images. At E15, the developing urothelium was ~3–5 cells layers thick, and proliferating cells were found within the K5-BC layer and Krt5^{-} intermediate cell layer (Figure 1a). Differentiated superficial cells are absent during this time (Figure 1b). At P1, the developing urothelium had reduced to 2-cell layers, and proliferating cells were seen predominantly in the K5-BC cell layer and occasionally in the intermediate cell layer (Figures 1c and 1d). At P5, P10, and in the adult, the developing urothelium had returned to a multilayer state, and actively proliferating cells were found almost exclusively in the K5-BC layer (Figures 1e–1j).

FIGURE 2A:

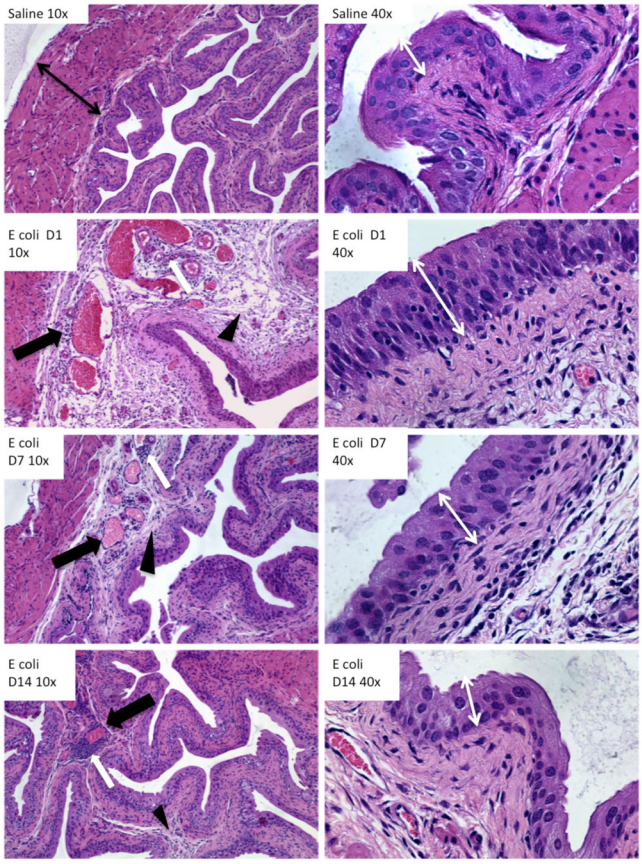


FIGURE 2B:

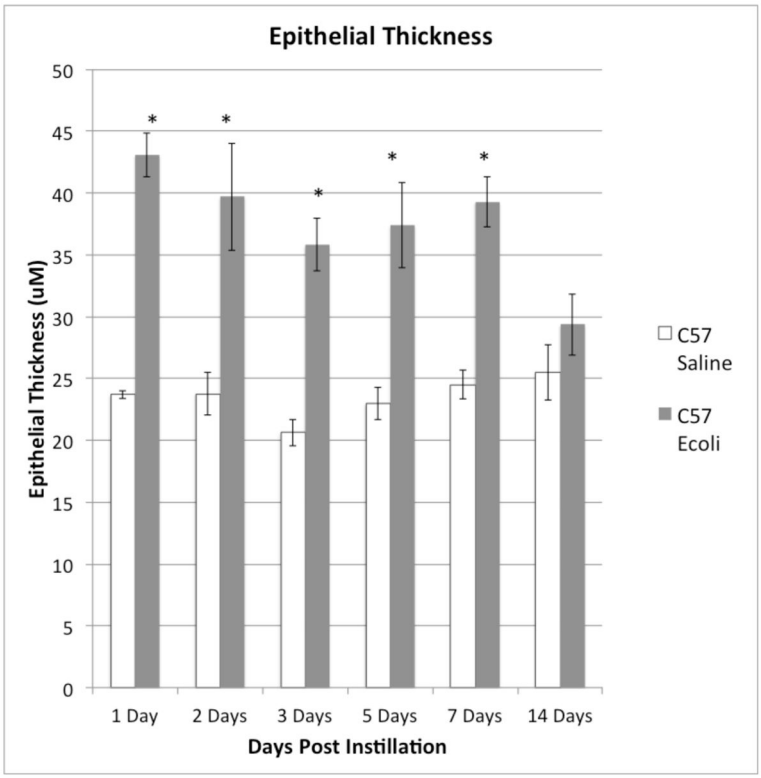


Figure 2.

Characterization of the bladder response to UPEC 1677 inoculation. **(2a)** As compared to PBS-instilled controls, infected bladders exhibited increased vascularity (black arrow), edema (black arrowhead), and inflammatory infiltrate (white arrow) at all time points examined. Thickness of the muscularis propria (black double-headed arrow) was unchanged. The white double-headed arrow indicates thickness of the urothelium. **(2b)** Epithelial thickness was significantly increased one day after bacterial inoculation and persisted for at least 7 days. At 14 days post-inoculation, epithelial thickness was not significantly different from PBS-instilled controls. Data are expressed as the mean ± SEM; * $p < 0.05$ compared to PBS-instilled controls.

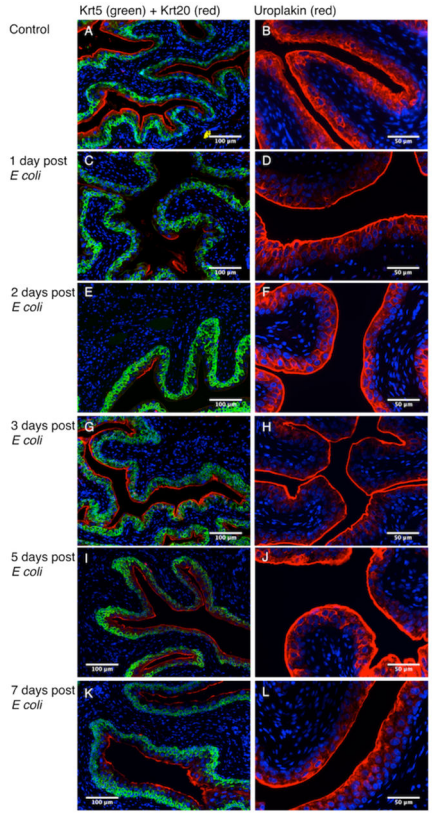
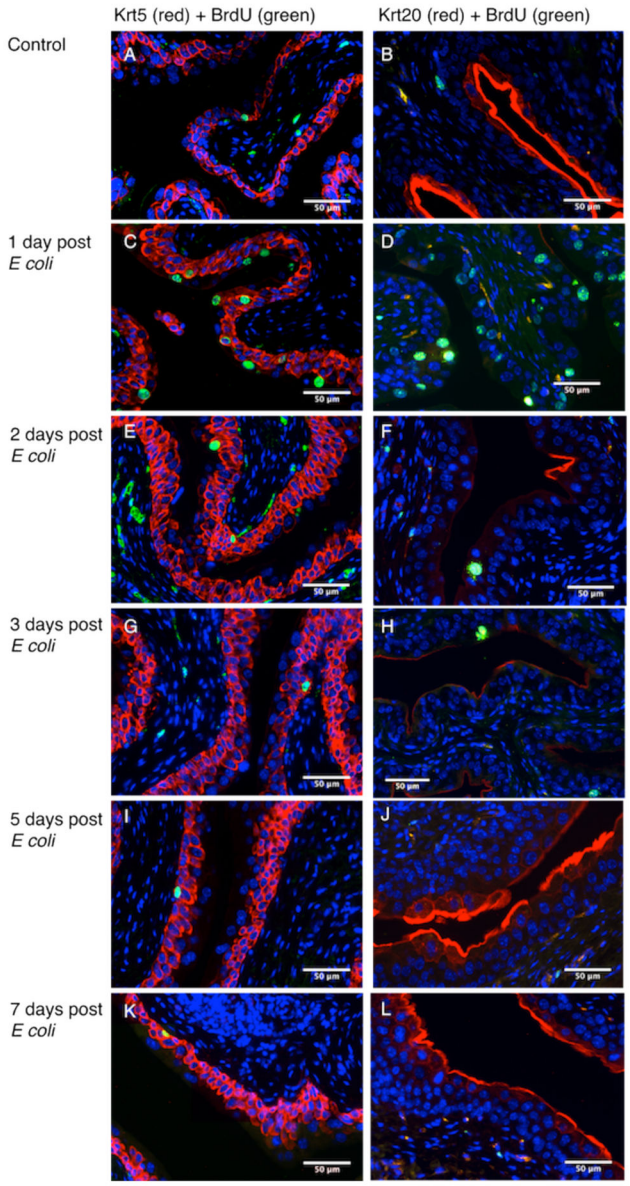


Figure 3.

Co-staining with Krt5 (green) and Krt20 (red) in the left-hand panels demonstrated loss of the superficial cell layer following bacterial inoculation. Rare Krt20^{+} superficial cells were identified one day post-inoculation. By three days post-inoculation, Krt20 staining was present within the majority of the luminal cell layer, indicating renewal of differentiated superficial cells. The right hand panels demonstrate staining of the urothelium with panuroplakin (red). Typically present within all urothelial cell layers, uroplakin staining was less intense from 1–3 days post-inoculation, coinciding with loss of the superficial cell layer.

FIGURE 4A-L:



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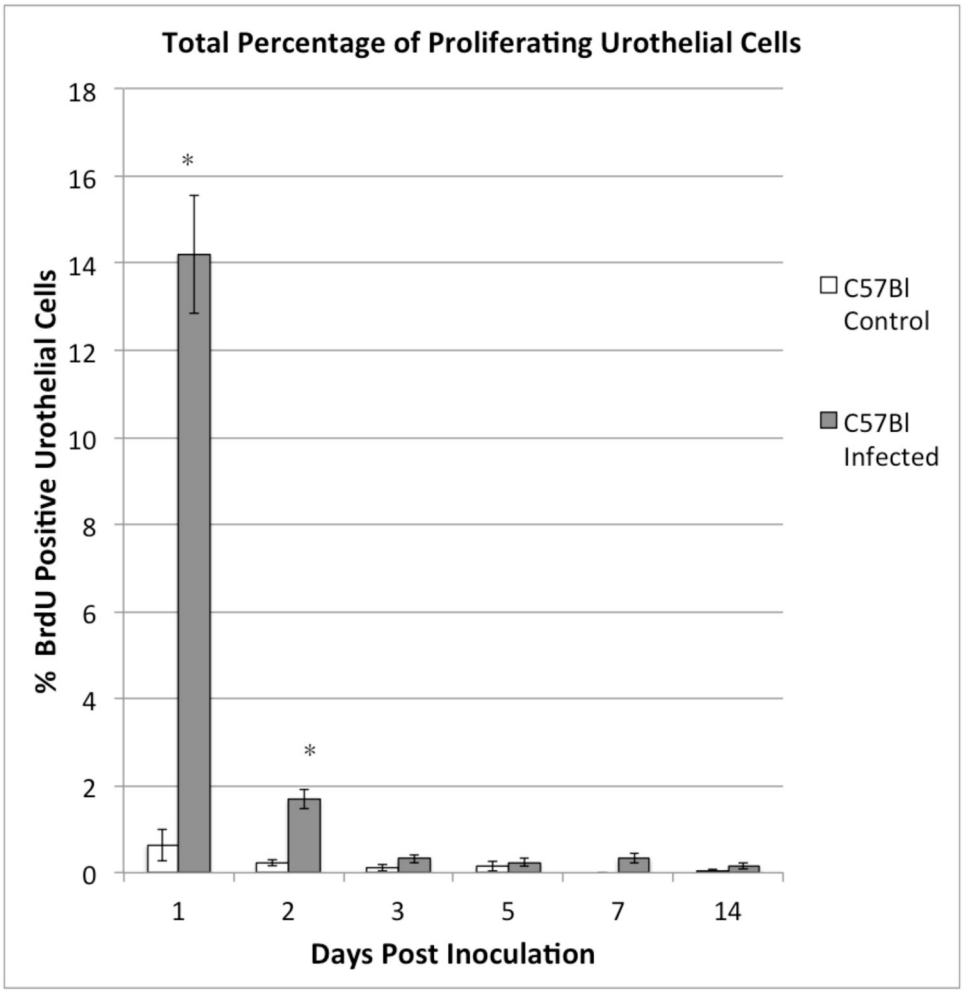

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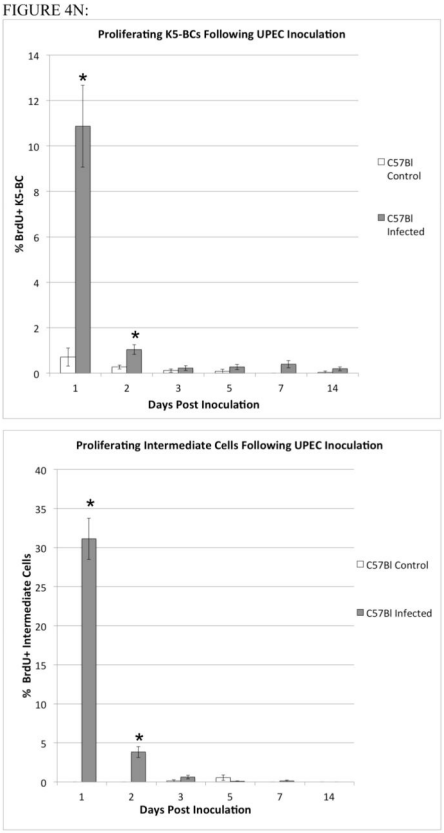

Figure 4.

(4a–l) Immunostaining for BrdU (green, proliferating cells), Krt5 (left, red, urothelial basal cells), and Krt20 (right, red, urothelial superficial cells). Occasional proliferating cells (0–1 cell/hpf) are seen in the K5-BC layer of the naïve urothelium. 24 hours after bacterial inoculation, urothelial proliferation increased within the K5-BC and intermediate cell layers **(4c and 4d)** and declines rapidly to baseline within 3 days **(4e–4l).** Quantitative analysis revealed dramatically increased urothelial proliferation one day post-inoculation, with ~14% of all urothelial cells positive for BrdU. Data are expressed as the mean ± SEM; * $p < 0.05$ compared to PBS-instilled controls **(4m).** Proliferating cells were significantly increased in both the K5-BC layer (~11%) and the $Krt5^{-}$ intermediate cell layer (~32%) one day post-inoculation and returned to baseline within three days. Data are expressed as the mean ± SEM; * $p < 0.05$ compared to PBS-instilled controls **(4n).**

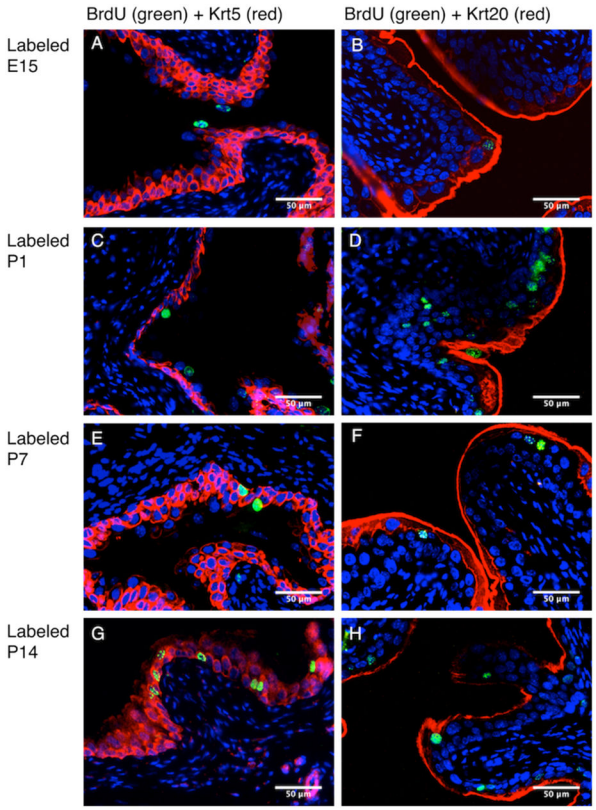

Figure 5.

Identification of label-retaining cells in the bladder urothelium. Immunostaining for BrdU (green), Krt5 (left, red, urothelial basal cells), and Krt20 (right, red, urothelial superficial cells). When animals given BrdU at E15 were evaluated at one month of age, LRCs were found almost exclusively within the superficial cell layer as indicated by a lack of Krt5 staining and positive Krt20 staining (Figures 5a and 5b). When animals were given BrdU at P1 and evaluated at one month of age, LRCs were found within the K5-BC, intermediate, and superficial cell layers (Figures 5c and 5d). In animals given BrdU at P7, LRCs were found within the K5-BC and intermediate cell layer at one month of age, as evidenced by both positive and negative Krt5 co-staining and a lack of co-staining with Krt20 (Figures 5e – 5f). When animals were given BrdU at P14, LRCs are predominantly K5-BCs (Figures 5g and 5h).

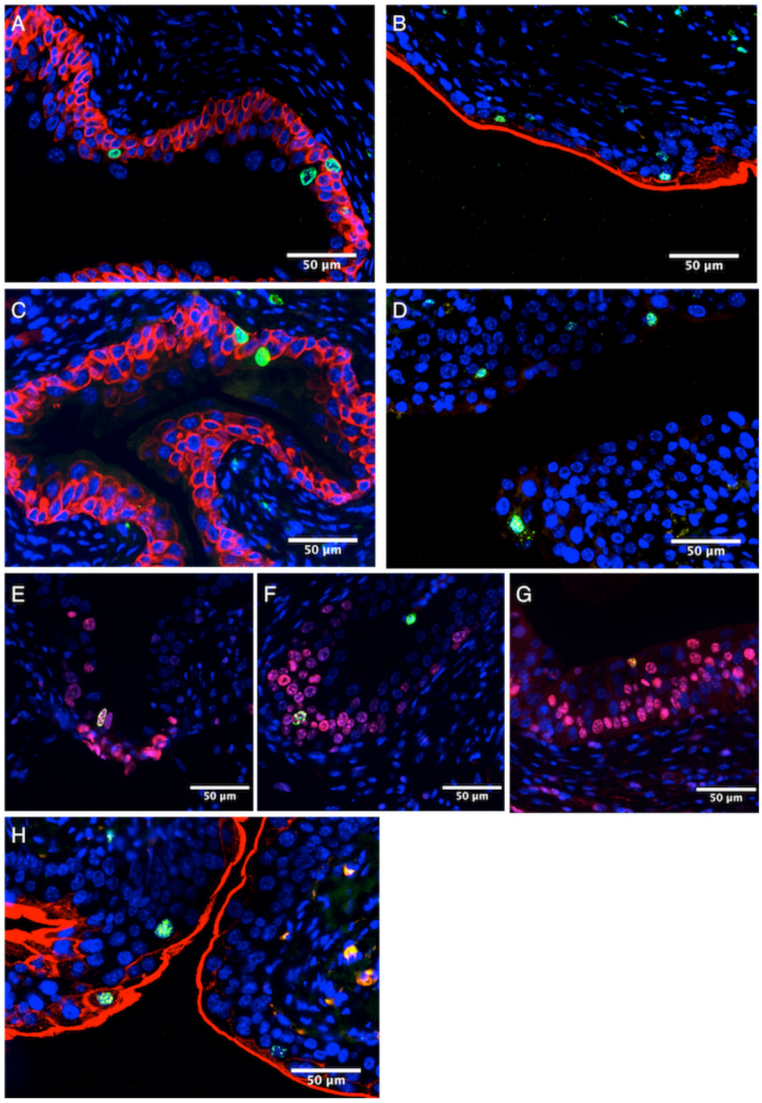

Figure 6.

6a and 6b: 2-month-old mice, previously labeled with BrdU at age P7. Immunostaining for BrdU was performed to identify LRCs (green) and for Krt5 **(6a)** or Krt20 **(6b)** to identify the basal or superficial urothelium (red), respectively. $BrdU^{+}$ LRCs are present in the $Krt5^{-}$ intermediate cell population and within the K5-BC layer, but not within the superficial cell layer. **6c and 6d:** LRCs persisted in the K5-BC and intermediate cell layer one day after inoculation with UPEC 1677 and superficial cell exfoliation. Immunostaining for BrdU was performed to identify LRCs (green) and for Krt5 **(6c)** or Krt20 **(6d)** to identify the basal or superficial urothelium (red), respectively. The superficial cell layer is absent 24 hours after bacterial inoculation **(6d). 6e–6g:** Immunostaining for Ki67 (red) and BrdU (green) in the urothelium. 2-month-old mice, previously labeled with BrdU at age P7, were instilled with UPEC 1677 and sacrificed one day later. Cells that were double-labeled for Ki67 and BrdU represent LRCs that proliferated in response to bacterial inoculation. Proliferating LRCs were located both with the basal cell layer and intermediate cell layer. **6h**: Immunostaining

for BrdU to identify LRCs (green) and for Krt20 to identify the superficial urothelium (red). 14 days post-inoculation, some of the LRCs were found to be incorporated in the newly developed superficial cell layer.

Table 1

Proliferation of urothelial cells in the K5-BC and intermediate cell layers during development, adult homeostasis, and after transurethral inoculation with UPEC 1677.

Group	Condition	% BrdU Positive	Percent of Proliferating Cells that are:	
			Basal Cells	Intermediate Cells
E15	Not infected	17.9	53.1	46.9
P1	Not infected	30.4	95.9	4.1
P5	Not infected	5.8	99.4	0.6
P10	Not infected	4.3	100	0
P14	Not infected	1.1	100	0
8 weeks	Not infected	0.6	91.4	8.6
8 weeks	24 hours post infection	14.2	65.6	34.4