

Label-retaining cells of the bladder: candidate urothelial stem cells

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Kurzrock EA, Lieu DK, deGraffenried LA, Chan CW, Isseroff RR. Label-retaining cells of the bladder: candidate urothelial stem cells. *Am J Physiol Renal Physiol* 294: F1415–F1421, 2008. First published March 26, 2008; doi:10.1152/ajprenal.00533.2007.—Adult tissue stem cells replicate infrequently, retaining DNA nucleotide label (BrdU) for much longer periods than mature, dividing cells in which the label is diluted during a chase period. Those “label-retaining cells” (LRCs) have been identified as the tissue stem cells in skin, cornea, intestine, and prostate. However, in the urinary tract uroepithelial stem cells have not yet been identified. In this study, BrdU administration identified urothelial LRCs in the rat bladder with 9% of the epithelial basal cells retaining BrdU label 1 yr after its administration. Markers for stem cells in other tissues, Bcl, p63, cytokeratin 14, and $\beta 1$ integrin, were immunolocalized in the basal bladder epithelium in or near urothelial LRCs, but not uniquely limited to these cells. Flow cytometry demonstrated that urothelial LRCs were small, had low granularity, and were uniquely $\beta 4$ integrin bright. Urothelium from long-term labeled bladders was cultured and LRCs were found to be significantly more clonogenic and proliferative, characteristics of stem cells, than unlabeled urothelial cells. Thus, this work demonstrates that LRCs in the bladder localize to the basal layer, are small, low granularity, uniquely $\beta 4$ integrin rich, slowly cycling and demonstrate superior clonogenic and proliferative ability compared with unlabeled epithelial cells. We propose that LRCs represent putative urothelial stem cells.

adult stem cells; epithelium; rats

SELF-RENEWING EPITHELIAL TISSUES have been shown to contain stem cells to provide the tissue’s regenerative potential. The stem cell theory holds that a proliferative hierarchy exists with distinct subpopulations: slowly-cycling, undifferentiated stem cells with unlimited regenerative potential; transit-amplifying cells that have limited proliferative potential; and differentiated nonproliferative cells. Lineage-specific stem cells have been identified in epithelia such as skin, intestine, and cornea (8, 16). Although urothelial stem cells have not been identified, we demonstrated a progenitor population rich in $\beta 1$ integrin (21).

Although the definition of a stem cell is somewhat contentious, the prevailing view is that a stem cell is able to reproduce itself, has a high capacity for cell division, and can produce at least one type of highly differentiated descendant (36, 43). Other attributes given to epithelial stem cells include slowly-cycling in vivo, clonogenic, high proliferative potential, primitive differentiation, reside in protected niche, and target of tumor initiation. Recent work has stressed the difference between lineage-specific stem cells and transit amplifying (TA) cells which carry the major replicative duties of epidermis. Whether such a cell hierarchy exists in urothelium is unknown.

In contrast to the hematopoietic system, specific biochemical markers for identifying epithelial stem cells have not been defined. A particularly powerful method for localizing epithelial stem cells takes advantage of their slowly-cycling nature (40). Once an epithelial stem cell incorporates labeled nucleic acids, ^3H -thymidine or BrdU, into its DNA, it retains that label for longer periods of time. More rapidly-cycling cells incorporate the label faster, mature, and die. Thus, the slower-cycling stem cells remain as the only label-retaining cells (LRCs). This approach has been used to identify stem cells in the bulge region of the hair follicle, the limbus of the cornea, endometrium of the uterus, crypts of intestine, and proximal region of prostatic ducts and by some is considered the gold standard for epithelial stem cell identification (2, 6, 8, 16, 19, 27, 40).

The bladder lacks crypts and ducts, thus the presumed protected “niche” for urothelial stem cells is along the basement membrane. In skin, a “columnar unit” of histogenesis has been suggested by the spatial orientation of mitotic figures, pulse-labeling with tritiated thymidine and transduction of cells in vitro with replication-deficient retroviral vectors (23, 35). A similar vertical unit of urothelial cells possibly founded by one stem cell is supported by evidence of macroscopic areas of monoclonality, “monoclonal patches,” covering the bladder (39).

Higher levels of expression of p63, cytokeratins 15 and 19, and $\beta 1$ and $\beta 4$ integrins have been noted in epidermal stem cells and have been utilized for stem cell isolation via adhesion and FACS (3, 5, 7, 9, 10, 20, 22, 25, 33). We employed similar strategies to enrich for urothelial progenitor cells (21). Some investigators believe that, unlike the hematopoietic system, the proteins proposed to identify epithelial stem cells exhibit too broad a specificity to be considered as specific markers (12, 41). Many of these markers are also expressed in TA cells (10, 20).

The regulatory mechanisms of stem cells, i.e., induction of proliferation or self renewal, are of increasing interest and intense investigation (28). Although slowly cycling in vivo, stem cells demonstrate greater clonogenic and proliferative capacity in vitro (1, 10, 14, 20, 27, 31, 32, 34, 40). Similarly, stem cells can be induced to proliferate in vivo using models of repair or regeneration (5, 8, 19). Particular interest in identifying the urothelial stem cells is prompted by intense efforts at bioengineering bladder and other urothelial tissues (30). In addition, identification, characterization, and isolation of these cells will increase our understanding and further investigation of the processes of urothelial differentiation and carcinogenesis (26).

Our goal was to identify LRCs in the bladder using the strategies that have been successful in identifying LRCs in epidermis, cornea, and prostate (2, 8, 19, 27, 40). In previous studies of epidermis and cornea, a cell was considered to be

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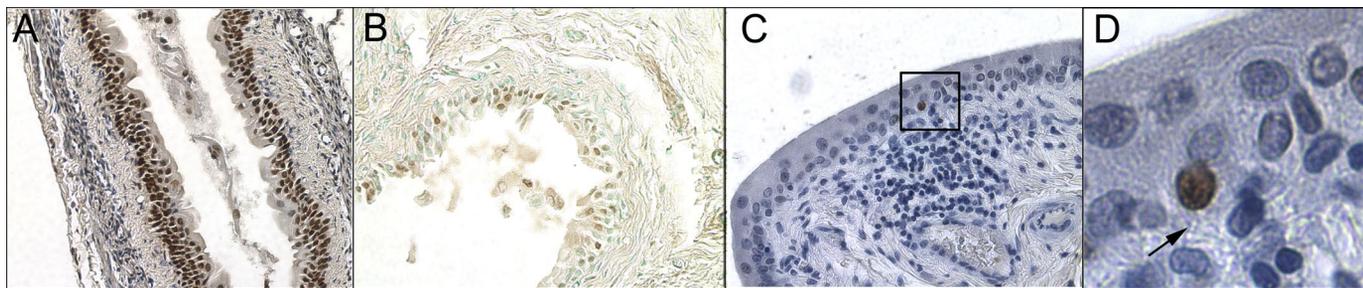


Fig. 1. Rats were administered BrdU for 4 consecutive days at 6 wk of age. Animals were euthanized at different time points postinjection. Anti-BrdU staining (DAB-brown) demonstrates label-retaining cells (LRCs) at 1 mo ($\times 20$; A), 6 mo ($\times 40$; B), and 12 mo ($\times 60$; C) after BrdU administration. C, inset: magnified ($\times 5$) in D demonstrating the LRC atop the basement membrane (arrow). These findings are representative of 45 animals evaluated. Counter stains: hematoxylin (A, C) and methyl green (B).

label-retaining 30 days after labeling with BrdU or tritiated thymidine. Due to the slower cell cycle time of homeostatic urothelium (11), we evaluated animals periodically up to 1 yr after labeling. To characterize potential urothelial stem cell markers, protein expression of LRCs was investigated with immunohistochemistry and flow cytometry. Labeled cells were evaluated in culture to determine whether they carried the clonogenic attributes of stem cells.

MATERIALS AND METHODS

Animals and generation of LRCs. Sprague-Dawley and Long-Evans rats were purchased from Charles River Laboratories (Wilmington, MA). The animals and procedures used in this study were in accordance with the guidelines and approval of the University of California–Animal Care and Use Committee. BrdU ($50 \mu\text{g}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$) was administered intraperitoneally to juvenile female rats (6 wk, 130–180 g) daily for 4 consecutive days. Bladders were harvested at selected time points from 1-wk to 1-yr post-BrdU administration.

Immunohistochemical analysis of LRCs. Bladders were removed, fixed in Streck tissue fixative (Streck Laboratories, LaVista, NE), dehydrated, and embedded in paraffin. Tissue sections ($6 \mu\text{m}$) were deparaffinized and endogenous peroxidase was blocked by immersing sections in $3\% \text{H}_2\text{O}_2$ in methanol for 5 min. Nonspecific binding sites were blocked by incubation in PBS containing 10% normal horse serum and the slides were then incubated with mouse anti-BrdU antibody (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. A secondary biotinylated donkey anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was then used, followed by incubation in an avidin-biotin complex (Vector Laboratories, Burlingame, CA). The antibody binding sites were visualized using the substrate DAB (Vector Laboratories). Slides were lightly counterstained with hematoxylin (Vector Laboratories) or methyl green (Dako, Carpinteria, CA). A negative control, mouse IgG (Vector Laboratories), was utilized for each staining session.

Each time point was represented by 4 to 10 animals. Each bladder was evaluated with a minimum of 2 staining sessions and 3 to 10 sections were evaluated at $\times 20$, $\times 40$, and $\times 60$ magnification for each animal. Serial sections were not utilized to avoid duplicate counting of the same cells. The number of basal cells labeled divided by total basal cells (range 75 to 500) was determined for each section [labeling index (LI)]. The evaluator was blinded to animal age and the highest LI was recorded for each animal. The LIs were compared between time points with unpaired Student's *t*-test (significance defined as $P \leq 0.05$). We previously demonstrated in another study sequestration of clonogenic cells in the rat bladder trigone (29); we therefore compared the LIs of the bladder neck, midbladder, and dome with paired Student's *t*-test.

Stem cell markers and proteins of interest. Although none are considered highly specific, p63, integrins, Bcl-2 (low Bax), and CK19

are proposed epithelial stem cell markers. Using antibodies against these proteins, we sought to determine whether LRCs had a qualitatively different immunostaining pattern compared with other basal cells. Due to its unique basal localization in rat bladder, CK14 was studied, rather than CK19, which is a proposed stem cell marker in skin.

Sections of bladder with LRCs (1 yr post-BrdU labeling) were doublestained with anti-BrdU and antibodies against p63 (sc-8431, goat, Santa Cruz Biotechnology), β_4 integrin (rabbit, Santa Cruz Biotechnology), CK14 (mouse, clone LL002, Labvision, Fremont, CA), and Bcl-2 (mouse, Santa Cruz Biotechnology). Where antigen retrieval was required, sections were stained with anti-BrdU as described above before antigen retrieval. Following BrdU/DAB staining, sections were blocked and stained with a second primary antibody followed by a biotinylated secondary, avidin-biotin complex and Vector VIP chromogen (Vector Laboratories). Slides were lightly counterstained with hematoxylin (Vector Laboratories) or methyl green (Dako).

Each bladder was evaluated with a minimum of 2 staining sessions and 3 to 10 sections were evaluated for each animal. The staining pattern of LRCs was analyzed compared with other basal cells. Mouse, rabbit, or goat IgG was utilized as a negative control.

Flow cytometry analysis of proteins of interest. To determine expression of proteins in LRCs, urothelial cells were harvested from 10 rats that were labeled with BrdU 1 yr previously and 2 unlabeled animals as controls. Cell harvest was performed as described (15). Briefly, bladders were inverted and incubated with trypsin/EDTA at 37°C for 1 h. After separating the epithelial cells from the connective tissue, cells were resuspended in DMEM medium containing 10% fetal bovine serum to neutralize the trypsin, and then centrifuged at 1,000 rpm for 5 min. Cells were then resuspended in KGM medium (Cambrex, East Rutherford, NJ). Cell counts and viability were determined. Each bladder yielded 5×10^5 to 1.5×10^6 cells with greater than 80% viability. Each bladder was evaluated individually.

Cells were stained with PE-anti-mouse β_1 integrin (CD29, HM β_1 -1, BioLegend, San Diego, CA), PE-Cy 5 anti-mouse α_6 integrin

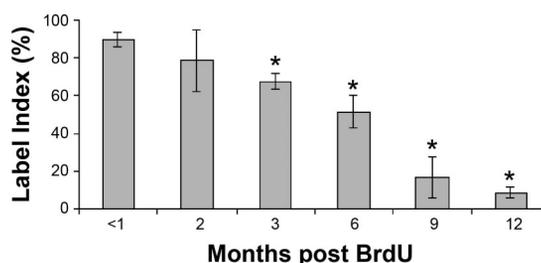


Fig. 2. Mean BrdU label index of urothelial basal cells after BrdU administration to juvenile rats. Each time point post-BrdU administration represented by 4 to 10 animals. Error bars represent \pm SE. * $P \leq 0.05$.

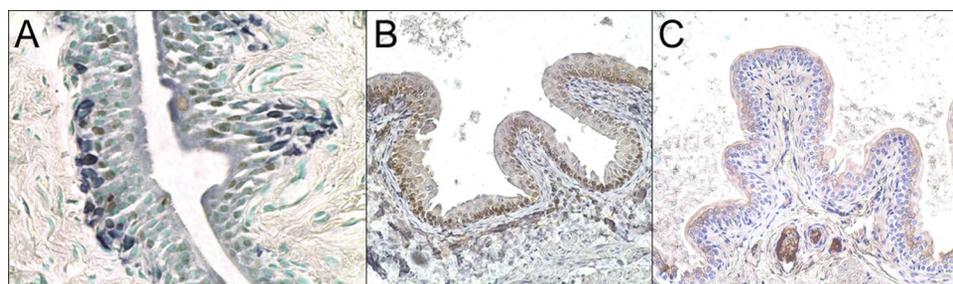


Fig. 3. Immunohistochemical staining of rat bladder (1 yr post-BrdU). A: CK14 (purple) colocalizes with LRCs (brown nuclei, BrdU) and other unlabeled basal cells ($\times 60$). B: ubiquitous staining of basal nuclei for p63 (brown; $\times 20$). C: Bcl-2 (brown) appears along basement membrane without variation between basal cells ($\times 40$). Counter stains: methyl green (A) and hematoxylin (B, C).

(CD49f, mAB-5A, ABED Serotec, Kidlington, UK), and biotinylated anti-mouse CK14 (LL002, Labvision) followed by streptavidin-APC. Cells were stained with polyclonal rabbit anti-human/rat/mouse β_4 integrin IgG (H-101, Santa Cruz Biotechnology) or normal rabbit IgG isotype control (Imgenex) followed by secondary goat anti-rabbit IgG pre-conjugated with Alexa Fluor 610. Before staining with labeled primary antibodies, cells were preincubated with 2% rat serum to block Fc γ II/III receptors. Cells were stained with the appropriate isotype controls in each experiment. BrdU incorporation in cells was analyzed using the FITC BrdU Flow Kit (BD Biosciences, San Jose, CA). All cells were analyzed using a Dako Cyan Cytomation instrument (Dako, Carpinteria, CA) and the results were analyzed using FlowJo software (TreeStar, Ashland, OR).

Cell culture and analysis of LRC clonogenicity and proliferation. To determine the clonogenic potential of LRCs, urothelial cells were harvested from 10 rats that were labeled with BrdU, 4 mo (4), 6 mo (4), 9 mo (2), and 12 mo (2), previously. Before cell harvest, the bladder neck was excised and later analyzed by immunohistochemistry (IHC) to determine the LI.

Cells were plated in modified KGM medium (15) with mitomycin C-treated 3T3 feeder cells (Swiss albino, graciously supplied by R. H. Rice, PhD., U.C. Davis). Urothelial cells were seeded at 500 cells/cm²

on two-chambered Permanox slides (Nalgene/Nunc, Rochester, NY) and incubated in a 37°C/5% CO₂ cell culture incubator. Medium was changed every 2 to 3 days.

Cultures were fixed at 2- to 3-day intervals between 3 and 12 days in 1:1 methanol/acetone and stained against BrdU. With this protocol, immunostaining with antibodies to cytokeratins demonstrated that all cultured cells were keratin positive (data not shown) indicating a pure epithelial culture. Positive controls consisting of urothelial cells exposed to BrdU in culture were utilized. Negative controls consisted of cells not exposed to BrdU in vitro or in vivo. A negative control for the anti-BrdU antibody consisted of mouse IgG.

The number of BrdU-labeled and -unlabeled colonies was determined in each well. Colonies were scored by the number of cells: 20–50, 50–100, 100–500, and greater than 500 cells. A colony was described as “large” if the cell count exceeded 50 (culture day 6), 100 (culture day 9), and 500 (culture day 12).

Large-colony and small-colony formation efficiencies (CFE) were determined by the following equations: CFE-labeled cells (%) = [# colonies with label/(# cells plated \times LI)] \times 100 and CFE-unlabeled cells (%) = [# colonies without label/# cells plated \times (1 – LI)] \times 100. LI was determined from the respective bladder neck. CFEs of

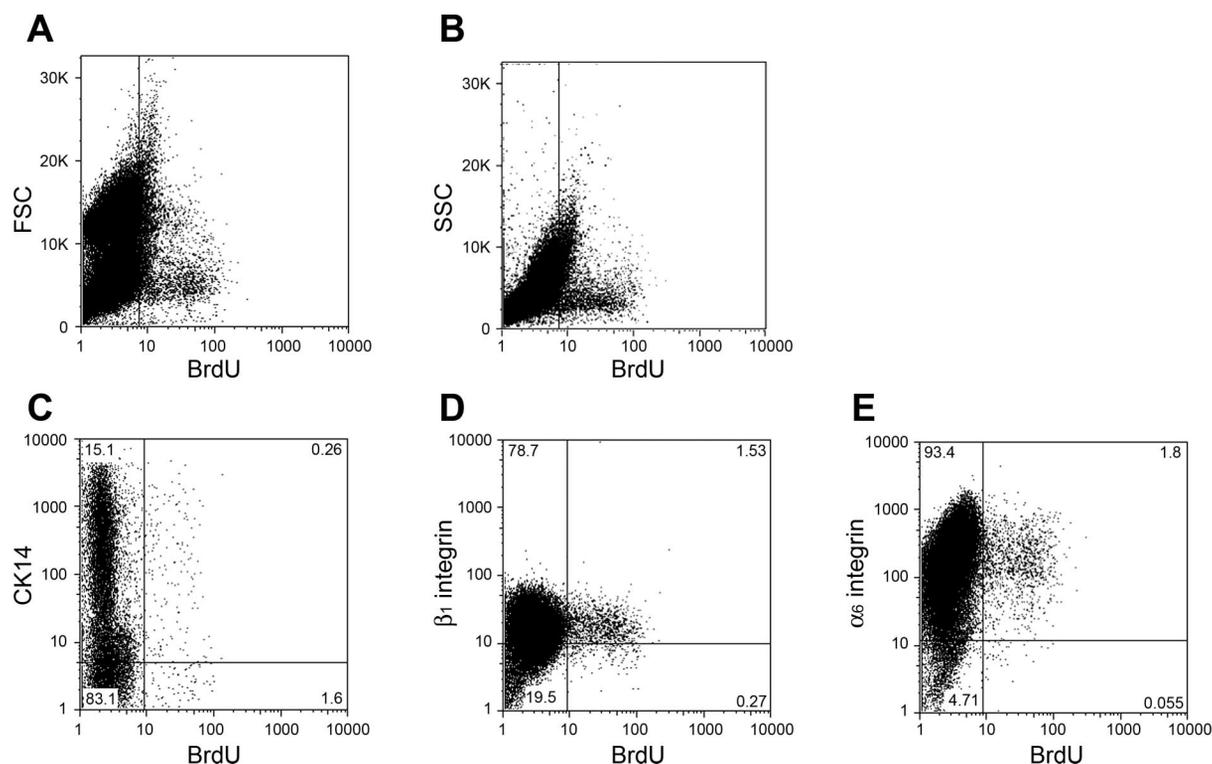


Fig. 4. Representative flow cytometry plots of urothelial cells 1 yr post-BrdU labeling. A, B: forward- and side-scatter demonstrate LRCs to be small with low granularity. C, D: CK14 and β_1 integrin expression were similar for LRCs and unlabeled cells. E: unlabeled cells were α_6 integrin positive and negative, whereas LRCs were only α_6 integrin positive.

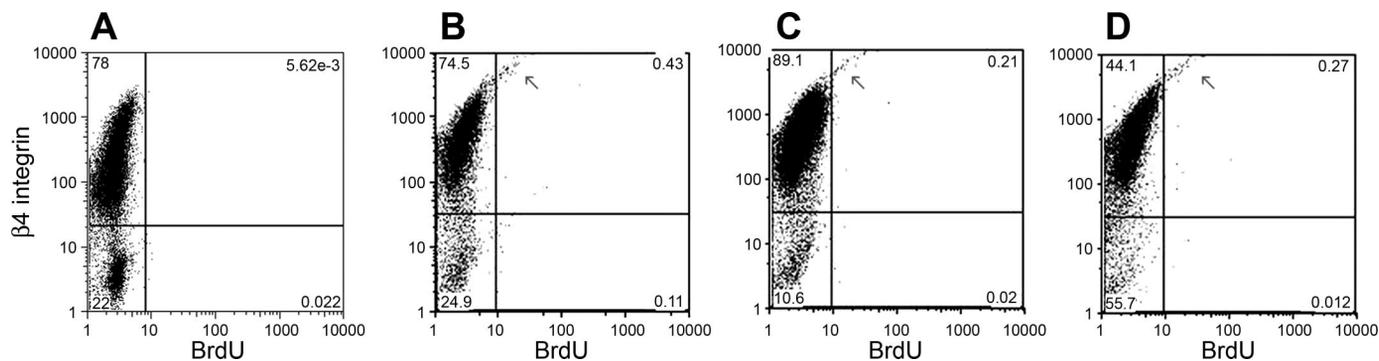


Fig. 5. Representative flow cytometry plots of urothelial cells stained against β_4 integrin and BrdU. *A*: negative control animal without exposure to BrdU. *B–D*: 3 animals 1 yr post-BrdU labeling. Within the LRC population, over 90% of the cells were β_4 integrin bright (arrow).

labeled cells were compared with CFEs of unlabeled cells after 6, 9, and 12 days in culture with paired Student's *t*-test.

RESULTS

LRCs in the rat bladder. There was variability between animals, staining sessions, and sections, therefore the following approaches were used to obtain representative data. Each time point was represented by 4 to 10 animals. Each bladder was evaluated with a minimum of 2 staining sessions and 3 to 10 sections were evaluated for each animal.

IHC demonstrated incorporation of BrdU into most epithelial basal nuclei less than 1 mo post-BrdU administration (Figs. 1 and 2). While not the focus of this study, we noted that only a few mesenchymal cells incorporated BrdU label. This may be due to the slower turnover of stromal cells compared with the epithelial compartment. Epithelial BrdU labeling did not significantly vary between animals within a month of BrdU administration. We found no significant difference in LI between the bladder neck, midsection, or dome. There was substantial variability between animals after 2 mo. After 1 yr, the mean LI was 9% of basal cells. The intranuclear label intensity decreased over time. The mean LI of all time points, except at 2 mo, was significantly different than the mean LI at 1 mo.

Stem cell markers and proteins of interest. CK14 (Fig. 3A) and β_4 integrin expression is evident in BrdU-positive LRCs. However, IHC did not demonstrate a higher intensity of staining for these protein markers in LRCs compared with other basal cells. IHC demonstrated ubiquitous staining of basal nuclei for p63 (Fig. 3B). Bcl-2 appeared in the cells located

along the basement membrane without significant variation between basal cells (Fig. 3C).

Flow cytometry analysis of proteins of interest. The 10 animals evaluated 1 yr after BrdU labeling showed an LRC population of 0.2 to 2%. The two (unlabeled) control animals were BrdU negative. The LRC population in the labeled animals was primarily represented by small, low granularity cells (Fig. 4, *A* and *B*). Further analysis of protein expression only included small, low granularity cells. Figure 4, *A* and *B*, shows the representative flow cytometry plots of all the acquired cells (>200,000 events/sample) without any gating strategy. The subsequent analyses were gated on the FSC low and SSC low (without the dead cell population). This would include both the BrdU-positive and -negative cells and represent 30–45% of the total live population.

CK14 expression was similar for LRCs and unlabeled cells ($n = 10$; Fig. 4C). LRCs expressed β_1 integrin at similar levels to unlabeled cells ($n = 10$; Fig. 4D). Unlabeled cells were represented by α_6 -positive and -negative cells, whereas there were no α_6 -negative LRCs ($n = 4$; Fig. 4E). Within the LRC population over 90% of the cells were β_4 bright ($n = 6$; Fig. 5).

Clonogenic and proliferative capacity of LRCs. In the vast majority (>95%) of colonies, the intranuclear staining for BrdU was clear and unequivocal (compare Fig. 6A unlabeled with Fig. 6B labeled). In a minority of colonies, the labeling was less clear, and these colonies were counted as unlabeled. In colonies established from cells isolated from animals that were 12 mo post-BrdU administration, only a few cells in any colony exhibited BrdU staining, and thus, these colonies were excluded from analysis.

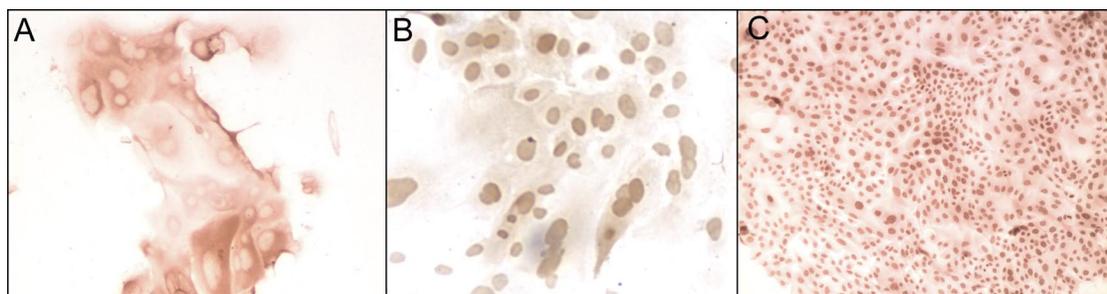


Fig. 6. Representative urothelial colonies from a rat labeled with BrdU 6 mo before cell harvest. Slides were stained against BrdU (brown-DAB), no counterstain. *A*: unlabeled small colony at day 12 of culture demonstrates background without nuclear staining ($\times 20$), labeled small colony at day 12 of culture with nuclear staining of BrdU ($\times 20$; *B*), and labeled large colony at day 12 of culture with nuclear staining of BrdU ($\times 10$; *C*).

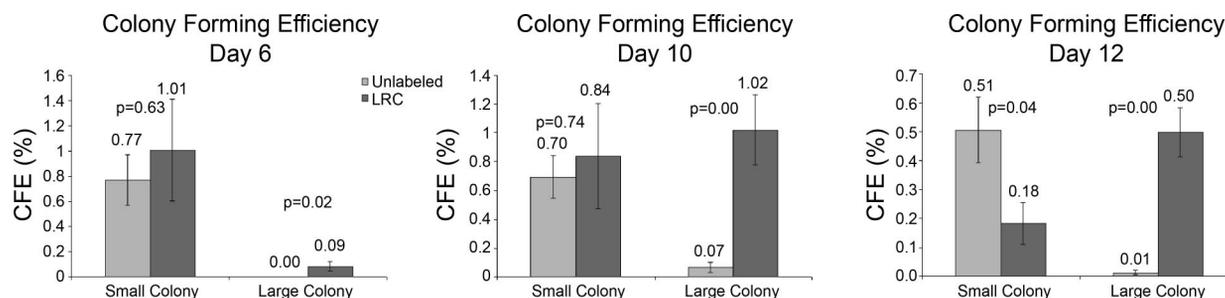


Fig. 7. Colony-forming efficiency (CFE) of urothelial cells derived from bladders taken from animals 4, 6, and 9 mo after in vivo labeling with BrdU. Urothelial cells were cultured as outlined in MATERIALS AND METHODS and the resultant colonies were scored at 6, 10, and 12 days after plating. Means are of data collected from 10 different animals. Essentially, all large colonies were founded from LRCs. Error bars represent \pm SE.

Significant differences of large and small CFEs were found between LRCs and unlabeled cells for all culture time points (Fig. 7). The vast majority of large colonies were comprised of labeled cells. Analysis of individual animal groups, 4, 6, and 9 mo after BrdU, demonstrated similar results with one exception. Due to the smaller sample size and paucity of large colonies early in culture, the difference between labeled and unlabeled large-colony CFE at culture *day 6* did not reach statistical significance, *P* value 0.08, 0.9, and 0.12, respectively. However, at culture *day 12*, there was a significant difference between labeled and unlabeled large-colony CFE for all individual groups, *P* value 0.01, 0.00, and 0.01, respectively (Fig. 8).

DISCUSSION

Epithelial stem cells have been identified in skin, intestine, and cornea. The slowly-cycling nature of stem cells in vivo and high proliferative potential in vitro have been used to localize stem cells to possible geographic tissue niches: the bulge region of the hair follicle, the limbus of the cornea, and proximal region of prostatic ducts (2, 8, 16, 19, 27, 40). We modified the methodologies of other studies to identify and characterize urothelial stem cells. Since urothelium has a very slow cell cycle time in vivo (11), we studied rats up to 1 yr after BrdU administration, compared with similar studies in skin that utilized a chase period of 1 mo (2, 24, 27).

At early time points after BrdU administration, there was almost complete saturation of urothelial basal nuclei with the labeled nucleotide. This intense saturation decreased the variability of the LI between rats. Two months after labeling, the mean LI decreased but the variability between animals in-

creased likely due to differences in epithelial turnover. The intensity of staining also decreased over time. Ultimately, \sim 9% of urothelial basal cells retained label at 1 yr. This is notable, since the life expectancy of a rat is 2 to 3 yr. Some portion of the LRC population may represent TA cells.

The LI difference between IHC and flow analysis (9 vs. 2%) may be secondary to the selection (gating) of labeled cells with flow being set at a higher BrdU signal level than IHC. In other words, cells with minimal BrdU expression on IHC were not counted by flow. The difference in LRC density between IHC and flow may also be due to cell sampling. Although we monitored the stroma for persistence of basal cells during our isolation procedure, nevertheless, the harvest of urothelial cells for flow may have been incomplete and some labeled cells were left attached to the basement membrane. On the other hand, IHC of the intact bladder would demonstrate and count every basal cell, yielding a higher LI. Our previous work demonstrated a very thorough harvest technique (15), yet the most likely cell to be left adherent to the basement membrane would be a stem cell (21).

Stem cells appear to localize in well-protected areas of epithelium. Corneal stem cells localize in the basal layer of the limbus in the peripheral cornea, not the central cornea (8, 34). In the epidermis and intestine, stem cells are located at the bottom of deep rete ridges (17) and intestinal crypts (18), respectively. Urothelial stem cells have not been localized, but are presumed to reside in the basal layer. Our data support this contention.

IHC and flow cytometry demonstrated LRCs to be basal and small with low granularity, an attribute of stem cells (13, 44). Our previous work demonstrated more clonogenic cells in the

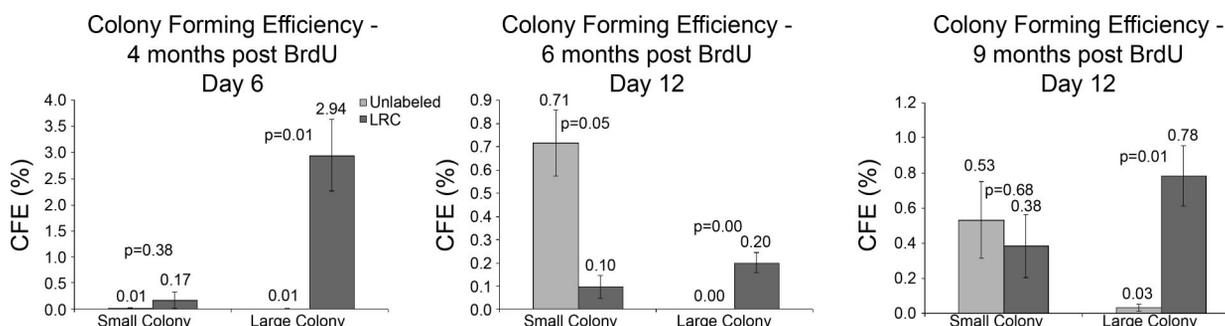


Fig. 8. CFE of urothelial cells derived from bladders taken from animals 4, 6, and 9 mo after in vivo labeling with BrdU. Urothelial cells were cultured as outlined in MATERIALS AND METHODS and the resultant colonies were scored at 12 days after plating. Essentially, all large colonies were founded from LRCs. Error bars represent \pm SE.

bladder trigone (29), yet we found no difference in the LRC density between bladder regions in the current study. With only 1 to 2% of basal cells being clonogenic in vitro, statistical correlation of the regional CFE in culture with the density of LRCs in vivo, at 9%, is not possible. On the other hand, LRCs contributed to over 90% of the clonogenic capacity in vitro, which we believe reflects their role as progenitors in vivo.

The search for a specific epithelial stem cell marker has been difficult. Numerous studies evaluated potential markers, i.e., p63, CK15, CK19, and various integrins (5, 7, 20, 22, 33); however, these do not appear to be specific to stem cells (12, 41). Without using a labeled nucleotide, Tumber et al. (41) elegantly characterized epidermal stem cells by marking slowly-cycling cells with GFP in engineered transgenic mice. Transcriptional profiling of GFP-labeled LRCs demonstrated over-expression of numerous mRNAs relative to their progeny. These included proteins regulating cell growth, transcription factors, and receptors to hormones and extracellular matrix.

Integrins are involved in cell-cell and cell-substrate adhesion, as well as transduction of extracellular signals that regulate apoptosis, proliferation, and differentiation (4, 42). Kaur et al. (20, 38) demonstrated that keratinocyte LRCs are α_6 bright and CD71 dim, which is a marker of proliferative activity. They also demonstrated that this population had the greatest regenerative and long-term growth capacity. Similar to skin, urothelial stem cells may express higher levels of integrins. β_1 Integrin staining was ubiquitous in all layers of the rat bladder (data not shown), whereas β_4 integrin is predominantly expressed in the basal cells. This is consistent with the expression of integrins in the human bladder (37).

Although flow cytometry demonstrated that LRCs were α_6 positive, and not α_6 negative, it also demonstrated α_6 staining in unlabeled cells. The strong α_6 expression of unlabeled cells is likely a reflection of the $\alpha_6 \beta_1$ heterodimer. This reinforces previous findings that separation of urothelial cells by β_1 expression does not allow for efficient stem cell enrichment (21) and α_6 integrin is also a nonspecific marker. We were encouraged to find that the LRC population was uniquely and extremely β_4 integrin bright, whereas the unlabeled population had no corresponding cells with such a high level of β_4 expression. This is consistent with epidermal LRCs (5).

An important attribute of epithelial stem cells is their high clonogenic and proliferative capacity in vitro (1, 10, 20, 27, 31, 32, 34, 40). Bickenbach et al. (2, 24) demonstrated the proliferative capacity of epithelial LRCs in vitro. Keratinocyte cultures from TdR-labeled mice (30 days postlabel) demonstrated an increasing percentage of labeled cells between 3 and 7 days in culture. On the other hand, work by Morris and Potten (27) at a later time point (8–10 wk postlabel) showed that labeled cells did not increase significantly in culture but LRCs appeared to found colonies without labeled progeny.

One possible explanation for the finding of unlabeled progeny is that each stem cell produced one or more TA cells in vitro. Then, the TA cells multiplied and diluted their labeled DNA. Progeny have minimal to no appreciable labeled nucleotide. The only cell in the colony with any appreciable BrdU is the stem cell. This is consistent with the “slowly-cycling” theory of stem cell label retention in vivo. Whether this phenomenon is preserved in vitro is theoretical and would only become evident when intranuclear BrdU is scarce. If this occurred in our experiment, colonies founded by LRCs would

have been undercounted. Yet, there were few unlabeled large colonies until 12 mo post-BrdU.

Despite the possible undercounting of LRC clones, LRCs appear to carry the role of forming large colonies. Labeled and unlabeled cells demonstrated equivalent small CFE at earlier culture points. Unlabeled small colonies did not progress. On the other hand, LRC small colonies continued to grow into large colonies. Essentially, all the proliferation at 12 days was represented by LRCs.

The identification, characterization, and enrichment of the previously elusive adult urothelial stem cell will have significant impact on laboratory and clinical investigation of normal and neoplastic urothelial differentiation, bioengineering, gene therapy, and cancer treatment. Successful bioengineering of durable urologic organs, needed in patients with congenital abnormalities or cancer of the bladder, may depend on the harvest and transplantation of urothelial stem cells. Likewise, successful and efficient gene therapy, as proposed for bladder cancer, will depend on the transduction of urothelial stem cells that will propagate the intended gene and/or gene product. A better understanding and availability of urothelial stem cells will allow further investigation of normal and abnormal differentiation pathways. This will lead to further study of cancer stem cells and better treatments for transitional cell carcinoma.

In conclusion, LRCs in the bladder localize to the basal layer, are small, low granularity, slowly cycling, and demonstrate superior clonogenic and proliferative ability compared with more differentiated cells. LRCs had a uniquely high expression of β_4 integrin, which was not seen in unlabeled cells. We propose that these LRCs represent a population of lineage-specific urothelial stem cells.

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GRANTS

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