Three clonal types of urothelium with different capacities for replication

R. Thangappan and E. A. Kurzrock

Urology and Pediatrics, University of California, Davis, Sacramento, CA, USA

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Abstract

Objectives: Similar to other epithelia, urothelium *in vivo* has a hierarchal organization of cells each with specific gradients of differentiation. While distinct cell types have been described as important in bladder cancer *in vitro*, clonal and proliferative capacities of normal urothelial cells have not been characterized.

Materials and methods: Three cell types and colony types were identified from primary porcine urothelial culture. Proliferative activity, patterns of apoptosis and differentiation, colony forming efficiency and ability to change phenotype with passage were determined and compared.

Results: Small, T-I colonies with large flattened (type-1) cells had low levels of proliferation and high levels of apoptosis. Large T-III colonies had a central area of small (type-3) cells surrounded by type-1 and type-2 cells. Proliferation and apoptosis were asymmetrically distributed in the periphery of T-II and T-III colonies. T-III colonies proved to be significantly more clonogenic and proliferative. With appropriate induction, type-1 cells were able to proliferate upon passage and form type-3 cells, yet long-term culture demonstrated that progeny of type-1 cells appeared to have inherited a clonogenic handicap.

Conclusions: Type-3 cells in the centre of T-III colonies appear to harbour stem-like qualities with a relatively low proliferative and apoptotic index at homeostasis and the ability to become highly proliferative upon passage. This study demonstrates that distinct urothelial cell types with differing clonal capacities can be isolated from the bladder and these

cells may have implications for tissue engineering and carcinogenesis.

Introduction

Three different clonal types of human keratinocytes in culture, with different replicative capacities, were demonstrated by Barrandon and Green in 1986 (1). Distinction between clonal types was assigned based on frequency of terminal colonies produced when a clone was passaged. These colony types were termed paraclones, meroclones and holoclones. When no colonies formed or when all colonies formed were terminal, the clone was classified as a paraclone. Holoclones, which by definition produce less than 5% terminal colonies, were defined as clones with the greatest replicative and lifespan potential and it was suggested that they might harbour stem cells.

In a further study using murine keratinocytes, Tudor *et al.* described analogous colony and cell types, which correlated with para-, mero- and holoclones. Using different terminology, they found that type-III colonies (analogous to holoclones) contained a central zone of type-3 cells that behaved like the stem-cell component of the cultures (2). These cells were not present in type-I or type-II colonies. In addition to dissimilarity in morphology, colony types could be differentiated based on clonogenic capacity, apoptotic rate and protein expression. Tudor *et al.* found that only type-III colonies contained cells that were capable of expansive growth and reformation of new type-III colonies.

In the urogenital system, similar clonal analysis has only been performed with prostate epithelial cells. Hudson *et al.* described two colony types and considerable proliferative heterogeneity, with type-II colonies being presumptive progeny of stem cells (3). Similar study characterizing the clonal proliferative capabilities of uro-epithelium is, to date, lacking. Two phenotypically different cell types have been identified in primary culture. Relatively smaller, homogeneous round cells with a high nuclear-to-cytoplasmic ratio are believed to be responsible for proliferation, whereas a population of larger cells

Correspondence: E. A. Kurzrock, MD, Assoc. Professor of Urology and Pediatrics, University of California, Davis, 4860 Y Street, Suite 3500, Sacramento, CA 95817, USA. Tel.: 916-734-5154; Fax: 916-734-8094; E-mail: eric.kurzrock@ucdmc.ucdavis.edu

appears to have declining proliferative capacity upon culture (4,5). Although Mackillop *et al.* studied clonal heterogeneity in normal and neoplastic urothelium, they did so in semisolid culture conditions. Under these circumstances, none of the normal bladder specimens showed any evidence of proliferation and thus, their observations on clonal capacity of urothelial cells were limited to the neoplastic specimens (6).

Current models of urothelial homeostasis and regeneration are based on a belief that a proliferative basal cell layer contains a pool of stem cells responsible for replenishing the more differentiated, uroplakin (UP)-expressing umbrella cells, which form the superficial layer. In the skin, an epithelial proliferative or 'columnar' unit with a stem cell at its base is evident, so the possibility of a similar vertical unit of urothelial cells founded by one basal stem cell is supported by evidence of macroscopic areas of monoclonality, 'monoclonal patches', which cover the bladder (7). Columnar histogenic units are also suggested by the spatial distribution of urothelial nuclei (8). Recent data from our laboratory suggest that urothelial stem cells are localized in the basal layer of bladder epithelium (9).

A number of questions regarding bladder urothelial cells remain unanswered. Does urothelium have a proliferative 'stem-like' cell in culture? Are differentiated cells truly terminal or can intermediate or terminally differentiated cells proliferate and replicate *in vitro*? After *in vitro* investigations of skin and prostate, our goals were to determine whether urothelium demonstrates distinguishable colony types in culture and if these colony types have heterogeneous functional and phenotypic qualities. Furthermore, we sought to determine whether these different clones could replicate long-term and whether their proliferation was limited to self-replication of their respective clonal phenotype, as has been observed for skin-derived keratinocytes, or whether de-differentiation of mature cells to a more proliferative state was possible.

Materials and methods

Animals

Bladders from pigs 4–5 months of age were acquired from the slaughterhouse at University of California, Davis.

Urothelial cell harvest

The bladders were bisected lengthwise and incubated in 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA, USA), with the urothelial-side submerged, overnight at 4 °C. Next day the porcine urothelial cells (PUC) were gently scraped off bladder extracellular matrix using a sterile glass slide. The basement membrane was treated

with collagenase IV (1%; Worthington Biochemicals, Lakewood, NJ, USA) to remove remaining adherent cells. Red blood cells present in the cell suspension were lysed using NH_4Cl solution. The cell suspension was filtered through 40 μm cell strainer to eliminate remnants of the basement membrane (10).

Primary cell culture (P0)

Cells were plated on to ten 48-well (78.5 mm^2/well) cell-culture plates (Corning, Corning, NY, USA) pre-seeded with mitomycin C-treated murine 3T3 (Swiss albino) cells (11) at a density of five urothelial cells per well. This density was chosen because of the low colony forming efficiency of urothelium. By evaluating the wells every 2 days during primary culture, preliminary observation demonstrated that 26% of wells had more than one colony. These wells were not used for colony typing or secondary culture.

Cells were cultivated in Keratinocyte Basal Medium (calcium < 0.2 mM) (Lonza, Walkersville, MD, USA) supplemented with (Clonetics Single Quot CC-4131) (KGM+) bovine pituitary extract (BPE) (60 μg protein/ml), hydrocortisone (0.5 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), epidermal growth factor (EGF) (0.1 ng/ml), gentamicin (30 $\mu\text{g}/\text{ml}$; BW), cholera toxin (10^{-10} M), amphotericin (15 ng/ml; BW) and 2% FBS (Omega Scientific Inc., Tarzana, CA, USA), and medium was changed every 2–4 days. Plates were incubated in a 37 °C humidified atmosphere with 5% carbon dioxide and the medium was changed every 2 days. At 14 days, colonies were counted. The urothelial colonies were easily distinguished from the loosely associated or non-associated 3T3 fibroblasts.

Colony typing

Preliminary investigation by phase contrast microscopy (Nikon Eclipse TS100, Nikon Instruments Inc., Melville, NY, USA) demonstrated three cell types (types -1, -2 and -3) and colony types (T-I, T-II and T-III) using a low power objective (10 $\times$); these are demonstrated in Fig. 1 and defined in Table 1. After urothelium from one bladder had been cultured for 14 days at ten 48-well plates per bladder (as described above), primary colonies (passage zero – P0) were typed as either T-I, T-II or T-III by two independent investigators, based on criteria described in Table 1. Correlation of investigators' typing was analysed using the Pearson Correlation Coefficient statistical test. In addition, epithelium was harvested from four further bladders and primary cell cultures were established on ten 48-well plates per bladder as described earlier. P0 colonies from 1920 wells were typed and counted. Prevalence of each colony type was compared using Student's paired *t*-test.

Figure 1. (a) Representative examples of T-I, T-II and T-III urothelial colony types on a 48-well plate. (b, d and f) illustrate 4× magnification of colonies, T-I, T-II and T-III respectively. (c, e and g) illustrate 20× magnification of inset boxes to demonstrate cellular morphology.

Immunohistochemistry

Primary urothelial colonies (P0, 14 days) in 48-well plates and P-60 wells (19.63 cm²), were treated with EDTA (1 mM) to remove adherent 3T3 cells, and then were washed with PBS three to four times. In brief, colonies were treated with cold methanol (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min, washed with PBS twice and treated with 0.2% Triton X 100 for 10 min at room temperature, to permeabilize cell membranes. Colonies were treated with 10% horse serum and were incubated with the requisite primary antibodies at 4 °C overnight. After primary antibody treatment, cells were washed in

PBS three times for 5 min each, incubated with appropriate secondary antibody conjugated with Alexa fluorochrome, and again washed three times with PBS for at least 5 min each, then counterstained with DAPI (1:1000 dilution) for 20 min. Samples were viewed using a fluorescence microscope (Nikon Eclipse E400).

Determining proliferation at P0 (Ki-67)

Rabbit monoclonal antibody against Ki-67 (Abcam, Cambridge, MA, USA) and anti-rabbit secondary antibody pre-conjugated with Alexa 594 fluorochrome were used to identify actively proliferating cells at P0. All three

Table 1. Histological parameters used to categorize porcine urothelial cells and colonies after primary culture (P0) under the phase contrast microscope with low power objective (10×)

Description of cells	
Type-1	Large flattened cells
Type-2	Smaller, relatively uniform cells
Type-3	Even smaller, uniform cells
Description of colonies	
T-I	Consist only of type-1 cells, (0.5–2 mm width), cells relatively dispersed
T-II	Consist of type-2 cells; +/- type-1 cells at periphery, (2–4 mm width)
T-III	Central zone of type-3 cells surrounded by type-2 cells; may have swirling or ridge pattern; +/- type-1 cells at periphery, (4–6 mm width)

colony types were stained to determine Ki-67 labelling. A minimum of six colonies of each type were photographed at the fluorescence microscope (Nikon Eclipse E400) using the 10× objective. T-I colonies were accommodated within a single frame of 10× objective (0.7 mm²). Red fluorescent nuclei were counted as positive for Ki-67 expression and nuclei with only DAPI blue counterstain (Invitrogen, Minneapolis, MN, USA) were considered negative. Preliminary observation demonstrated more Ki-67⁺ cells in peripheral areas than in the central areas of T-II and T-III colonies. As T-II and T-III colonies were larger than T-I colonies, six to eight pictures were taken of the periphery and two to three pictures of the middle of each T-II and T-III colony. Care was taken to avoid overlapping images and SPOT Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) was used to count positive and negative cells for calculation of labelling index.

Determining apoptosis at P0

Rabbit polyclonal antibody against caspase-3 (Lab Vision, Fremont, CA, USA) and anti-rabbit secondary antibody pre-conjugated with Alexa 594 fluorochrome were used to identify cells undergoing apoptosis. Labelling difference was also noted between peripheral and central zones of T-II and T-III colonies. Stained cells/total cells were counted and labelling indices were calculated and analysed in a method similar to that used for the Ki-67 protocol described above.

Evaluation of colony differentiation and proliferation at P0

Epithelial differentiation was analysed using mouse monoclonal antibodies against cytokeratin (CK) 8 (Abcam) and CK14 (BD Biosciences, San Jose, CA, USA). For CK staining, a biotinylated anti-mouse secondary antibody

was utilized followed by routine DAB chromogen protocol. Terminal differentiation was analysed using a goat, monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) against uroplakin (UP) III, a marker of terminal differentiation specific for urothelium. P0 colonies were also double-stained for Ki-67 and UPIII. After primary antibody incubation, slides were incubated with appropriate pre-conjugated secondary antibodies, anti-rabbit-Alexa 594 (Ki-67) and anti-goat-Alexa 633 (UPIII). Two frames (10× objective) in the peripheral zone and one frame in the central zone were photographed and analysed per colony. Labelled cells were counted, labelling indices were calculated and analysed similar to Ki-67 protocol described earlier.

Secondary cell culture of P0 colonies

After typing, colonies from wells with only one colony type were trypsinized (5 min/0.25% trypsin) and pooled in separate 15 ml tubes (Fig. 2). P0 cells were termed 'P0 T-I, T-II or T-III groups' and fresh medium was added to terminate trypsin activity. Cells were centrifuged, and live and dead cells were identified and counted (Gibco, Invitrogen Corporation, Invitrogen, Minneapolis, MN, USA) using a haemocytometer after trypan blue staining. Cells were plated onto P-60 (19.6 cm²) cell-culture plates seeded with mitomycin C-treated murine 3T3 (Swiss albino) feeder layer at a density of 100 live urothelial cells/cm² for colony formation efficiency (CFE) plates and separate proliferation plates. Cells were cultivated in KGM+ and medium was changed every 2 days. Each sample was plated in triplicate. Plates were incubated at 37 °C in a humidified atmosphere with 5% carbon dioxide. At 14 days, CFE plates were fixed in Streck tissue fixative (Streck Labs, La Vista, NE, USA) and stained with rhodamine blue (Sigma-Aldrich) for counting and typing colonies at P1.

At the same time point as CFE plates, proliferation plates were treated with 0.25% trypsin – and 1 mM EDTA to detach the cells for counting. Cell generation was calculated using the formula $\log_2 (N/N_0)/(t - t_0)$ where t , t_0 indicate time points at counting and at initial plating, respectively; N , N_0 indicate number of cells at respective time points. N_0 was determined by multiplying total cells plated by the respective colony forming efficiency.

The CFE, cell yield and generations at P1 that were derived from P0 T-I, T-II and T-III groups were compared using Student's paired t -test and results are depicted as average of triplicate samples for four independent cell culture experiments (four separate bladders and times). As described, the colonies were typed at P1 after fixation. The 'rendering' of each P0 group was analysed by comparing distribution of P1 colonies derived from each P0

Figure 2. Diagrammatic representation of clonal analysis of porcine urothelial cells. At P0, colonies were typed 'I, II or III' and pooled into P0 groups I, II and III, green, blue and red respectively. P1 plates from each group were analysed for distribution of colony types.

group using Student's paired *t*-test. Thus, we sought to determine whether the P0 T-I group only rendered T-I colonies at P1 and so forth.

Long-term culture of P0 colonies

To calculate total cell yield in long-term culture, 10×10^3 cells from each P0 group were seeded separately on to P-60 plates with no feeder layer. Each experiment was replicated on five plates for each group (15 plates), carried in parallel and passaged at the same time. Total cell yield per plate was determined at each passage. At each passage, cells were re-plated at 10×10^3 cells per plate (five plates) at 14 days, irrespective of cell yield. After five passages, the experiment was terminated. To compare cell yields, cell outputs were extrapolated by assuming that all cells from the previous passage had been re-plated.

Results

Inter-observer colony type correlation

After primary culture of urothelium on ten 48-well plates, 81 colonies on one 48-well plate were typed by two investigators based on criteria described in Table 1. Correlation of colony type assignment was 70% for T-I, 85% for T-II and 81% for T-III. Most differences in assignment of colony type were between T-II and T-III colonies or between T-I and T-II colonies. There was only one occurrence of difference in T-I/T-III assignment where a cluster of assigned T-I colonies was interpreted as T-III by the second observer.

P0 colony type distribution

P0 colonies were counted and typed from four bladders (1920 wells). Mean distribution of colony types at P0 was essentially equal at 33% for each colony type (Fig. 2). There was substantial variation in P0 colony distribution

between experiments with standard errors of 10%, 6% and 7% for T-I, T-II and T-III colony types respectively. There was no statistically significant difference in colony type prevalence (*P*-value > 0.9).

Proliferation (Ki-67 index) of colony types

T-I colonies had significantly lower Ki-67 labelling index than T-II and T-III colonies (Fig. 3). Ki-67⁺ cell distribution within T-II and T-III colonies demonstrated distinct difference between labelling of peripheral and central areas with more Ki-67⁺ cells, indicative of proliferation, in periphery of colonies (*P*-value ≤ 0.05). T-I colonies did not have distinct central and peripheral zones.

Pattern of apoptosis in colony types

Caspase-3 staining of these porcine urothelial cell colony types at P0 indicated that a majority of cells in T-I colonies were undergoing apoptosis (Fig. 4). Cells in T-II and T-III colonies appeared to have lower prevalence of apoptosis with the exception of T-II peripheral cells (*P*-value ≤ 0.05). Peripheral zones of T-II and T-III colonies demonstrated higher apoptotic indices than respective central regions (*P*-value ≤ 0.05).

Colony differentiation – cytokeratins, uroplakin and Ki-67 double staining

All colony types showed near ubiquitous staining for cytokeratins without any particular pattern within a colony. On the other hand, uroplakin (UP) expression was different across colony types. UPIII and Ki-67 positive cells were identified by double immunofluorescence staining (Fig. 5). T-I colonies had higher prevalence of terminally differentiated (UPIII⁺) cells than T-II and T-III colonies (32%, 24% and 16%, respectively), and only the difference between T-I and T-III reached significance (*P*-value ≤ 0.05). Unlike proliferative activity, there was

Figure 3. Representative Ki-67 immunostaining of peripheral zone of T-III colony (20 $\times$). Pink nuclei indicate active proliferating cells (counterstain DAPI). T-II and T-III colonies had distinct central and peripheral areas, whereas smaller T-I colonies did not. Bar diagram illustrates low Ki-67 labelling index of T-I colonies (values are mean $\pm$ SD). The periphery of T-II and T-III colonies harbours more proliferative cells than their respective central zones (P -value ≤ 0.05).

no identified pattern of UPIII staining within each colony, peripheral *versus* central zones. Less than 6% of the cells in all colony types demonstrated simultaneous Ki-67 and UPIII staining, indicating that terminally differentiated cells are an infrequent participant in the overall proliferative pool.

P0 to P1 colony type rendering

After separation of P0 colonies into T-I, T-II and T-III cell suspension groups, cells were cultured for 14 days and each group rendered a very similar distribution of T-I, T-II and T-III colonies at P1, approximately 20%, 40% and 40% respectively (Fig. 2). Thus, moderate decrease in T-I colonies and moderate increases in T-II and T-III colonies with passage were observed. This change was irrespective of P0 cell group of origin. Thus, type-1 cells were not restricted to production of type-1 cells and T-I colonies.

Figure 4. Caspase-3 immunostaining. Pink nuclei indicate caspase-3 staining (counterstain DAPI) (20 $\times$). Bar diagram illustrates very high apoptotic index of cells within T-I colony (values are means $\pm$ SD). Peripheral cells of T-II and T-III colonies demonstrate higher apoptotic index than cells in their respective central regions (P -value ≤ 0.05).

CFE, cell yield and generation of P0 colony groups

After separation of P0 colonies into T-I, T-II and T-III cell suspension groups, the cells were cultured for 14 days and CFE, cell yield and generations were determined and compared using Student's *t*-test. T-III cell group produced almost twice as many colonies as T-I group ($P < 0.05$), and there was approximately 4-fold increase in cell number over that from the T-I group (P -value ≤ 0.05) (Fig. 6).

Figure 5. (a–c) Ki-67 and uroplakin III double immunofluorescent staining of porcine urothelial colonies, T-I, T-II and T-III, (10 $\times$, 20 $\times$ and 20 $\times$, respectively). UPIII expression (green cytoplasm) indicates terminal differentiation of the cell. Ki-67⁺ staining (pink nucleus) indicates active proliferation, and DAPI counterstain (blue nucleus). Bar diagram illustrates that T-I colonies had a higher prevalence of terminally differentiated (UPIII⁺) cells than T-II and T-III colonies (values are means $\pm$ SD) (P -value $\leq$ 0.05). Less than 6% of cells in all colony types demonstrated simultaneous Ki-67 and UPIII staining (hatched bar).

Difference between T-II and T-III cell groups was not statistically significant (P -value = 0.2). All three cell groups had similar cell generations and doubling times.

Long-term culture of P0 colonies

Similar to results of experiments mentioned earlier (at P1), long-term culture demonstrated that the T-III cell group was significantly more proliferative than T-I and T-II groups, and T-II was more proliferative than T-I (Fig. 7). There was stable replication with an approximate 20-, 40- and 80-fold increase in cell number at each passage for groups I, II and III respectively. The difference in proliferation became more dramatic with each passage.

Discussion

Similar to other epithelia, urothelium displays distinct colony types when dissociated epithelial cells are grown in primary culture. Barrandon *et al.* suggested that heterogeneity of epidermal colony types is because of differences in colony founding cells so that holoclones, or T-III colonies, are founded by stem cells (1). Although, by definition, stem cells have high self-renewal capacity, they may actually divide relatively infrequently when tissue is physiologically homeostatic (12). This quality has allowed identification of stem cells in epithelia based on long-term retention of labelled nucleotide in nuclei of these quiescent cells (13–15). Less than 10% of bladder basal cells have been found to be slowly cycling *in vivo* and demonstrate superior clonogenic capacity, consistent with stem cell qualities (9).

Between a stem cell and its terminally differentiated progeny, there is an intermediate population of committed

progenitors with limited proliferative capacity that are responsible for amplification of each stem-cell division. In the skin, these relatively rapidly cycling cells are termed 'transit amplifying (TA)' cells. TA cells of the bladder may reside in the basal and intermediate compartments of bladder epithelium where there is relatively higher proliferative activity. Categorizing *in vivo* and *in vitro* cells into three types may be oversimplification and most likely there is a spectrum of differentiation and function between basal and superficial cells and between type-1 and type-3 cells.

Here, we have hypothesized that *in vitro* colony organization may reflect *in vivo* relationships. Near confluence at 14 days in culture P0 T-III urothelial colonies might model homeostatic bladder epithelium. Central type-3 cells with lower cytoplasm-to-nuclear ratio and relatively lower proliferative and apoptotic indices manifest basal, stem-like qualities found in other epithelial stem cells. The peripheral zone with more active type-2 cells would simulate TA cells. These patterns are not as neatly organized as a prototypical, layered epithelium *in vivo*. The size of T-III colonies far surpasses the usual cell layering of bladder epithelium. Non-organized pattern of terminal differentiation (UPIII) may be a result of this robust growth.

Prior to confluence and at a plateau in their growth phase, double staining of primary colonies for Ki-67 and UPIII demonstrated that very few terminally differentiated cells were participating in proliferation. This is consistent between *in vivo* and *in vitro* studies of the bladder (6,10,16,17). T-III colonies demonstrate a lower degree of terminal differentiation (16% UPIII⁺), higher peripheral proliferative population and lower, central proliferative and apoptotic indices. T-I colonies have more 'terminally'

Figure 6. Bar diagrams demonstrating colony forming efficiency, cell generation and cell yield for T-I, T-II and T-III urothelial P0 colony groups after one passage (P1) (values are mean $\pm$ SD).

differentiated cells (32% UPIII⁺) and have a significant population of apoptotic cells, which might explain the lower CFE in comparison with T-II and T-III colonies. We raise the caveat that in contrast to the tissue, UPIII may not be the best marker of *in vitro* terminal differentiation.

T-I colonies were strictly defined as a small colony consisting of large flattened type-1 cells only. We hypothesized that type-1 cells pooled into the T-I cell group were responsible for the colony distribution after passage. This

implies that type-1 cells are able to proliferate under culture conditions utilized and create a very similar distribution of colony types as type-2 and type-3 cells. We do not discount that T-I colonies may harbour unrecognized type-3 cells, yet, the amount of missed type-3 cells within a type I colony would be miniscule. We do not believe that this small, theoretic population of cells could explain production of an equivalent amount of T-III colonies from the T-I and T-III cell groups upon one passage.

Apparently, type-1 urothelial cells are not locked into a non-proliferative state and with appropriate growth factors, they can be induced to replicate. Results of this proliferation are surprising in that our colony type distribution was very similar for all cell groups. This implies that colony distribution may be under greater influence of growth conditions (5), such as plate size, seeding density and growth media, than the cell-type of origin. The *in vivo* correlate of this phenomenon is that the daughter cell's phenotype is not determined by the parent cell's phenotype or classification, stem cell or de-differentiated cell, but rather by the needs and architecture of the epithelial/tissue environment (18).

Our results with urothelium are different from the findings of Tudor *et al.* with keratinocytes (2). They found that cells from T-I colonies generated few colonies consisting of flattened cells showing T-I colony morphology. Tudor *et al.* suggested that keratinocyte type-2 cells have characteristics of TA cells and reported that re-plating of T-II colonies produced only T-I and T-II colonies (2). Our results are quite different and may reflect different regulatory mechanisms within the tissues and/or medium conditions of the experiments. Both studies utilized a relatively low calcium medium (<0.2 and <0.06 mM, respectively) with EGF supplementation, yet urothelium was grown on a feeder layer with FBS supplementation. This difference in culture environment might be a factor in differentiation of cultured urothelium. In a recent study, Signoretti *et al.* using a p63^{+/−} chimaeric mouse model suggest that terminally differentiated umbrella cells of the bladder epithelium can develop independently from basal or intermediate cells (19). This somewhat supports the plasticity of urothelium found with our *in vitro* study. Ability to de-differentiate with appropriate growth factors is not unique to urothelium and has been demonstrated using keratinocytes (20). Indeed, Southgate *et al.* (5) cited that "on occasion, cultures [of urothelium] that were thought to have become senescent and were populated by the large 'non-proliferative' cell type would recover after becoming repopulated by the small 'proliferative' cell type."

Despite the fact that primary (P0) T-I, T-II and T-III colonies produce an equal distribution of P1 T-I, T-II and T-III colonies upon passage (Fig. 2), the P0 T-III cell group produced twice as many colonies as T-I cell group

Figure 7. Porcine urothelial colony groups were grown to P5 to assess long-term growth. For reasons of the dramatic difference in cell yield between the groups (1000-fold), a logarithmic scale was necessary to display the growth curve. Bar diagram demonstrates cumulative cell yield after five passages.

(Fig. 6). This finding implies that type-3 cells are more clonogenic than type-1 cells. All three primary cell groups had similar doubling times. Although primary type-1 cells are able to produce type-2 and type-3 cells and T-II and T-III colonies upon passage, the overall number of colonies is less.

Lower clonogenic capacity of primary T-I colonies continued on long-term culture. Of note, colony types were not separated after P0 so that all progeny were passaged. Cells from T-III cell group increased around 80-fold with each passage, whereas cells from T-I cell group increased 20-fold with each passage. This raises many questions. If the T-I group produces type-2 and type-3 cells and T-II and T-III colonies at equal distribution but lower colony count than T-II and T-III cell groups, why is the replication rate of the progeny not catching up? Although the type-1 cell can produce a type-3 cell and T-III colony phenotypically, type-1-derived, type-3 cells are possibly limited by their parental origin and have inherited a clonogenic handicap.

Here, the study was limited by lack of separation of cell types in T-II and T-III colonies for individual analysis. Similar to Tudor's study with keratinocytes, the fact that T-I colonies, by definition, only contain type-1 cells somewhat mitigates this problem, but does not eliminate the potential for contamination and improvement. Tudor *et al.* were able to obtain cleaner subpopulations of keratinocyte cell-types using laser capture for evaluation of RNA expression, but live cells were not separated for culture (2). Having defined urothelial heterogeneity in culture, our next goal is to further analyse protein expression of cell types, beyond uroplakins, to distinguish cell-surface patterns of each cell type. This would allow flow-assisted sorting of live cells for analysis of both RNA expression and growth capacity.

Although distinct cell types and clones were presumed to exist in normal urothelium, this is the first study to describe and characterize them explicitly in non-neoplastic urothelium. Similar to epidermal keratinocytes, three cell types and colony types were identified with distinct phenotypic and functional qualities. Similar to epidermis, type-3 cells proved to be significantly more clonogenic and proliferative than type-1 cells. Yet, unlike epidermis, type-1 urothelial cells were able to proliferate on passage

and form type-3 cells and T-III colonies. Progeny of type-1 cells were not able to catch up to the proliferative capacity of T-III progeny after multiple passages. This suggests that the stem-like type-3 cells may have a unique clonogenic ability that cannot be replicated without neoplastic transformation (21). There are two distinct forms of urothelial carcinoma, one being recurrent and non-malignant and the other being invasive and malignant. We hypothesize that there may be a relationship between the described cell types in normal urothelium and forms of clinical cancer. In tissue engineering and regenerative medicine, a stem cell-like population may allow construction of more durable bioengineered bladder tissue. For our laboratory, the next goal is to isolate the three urothelial subpopulations analyze differential gene expression and susceptibility to carcinogenesis.

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