Formation and regeneration of the urothelium

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Formation and regeneration of the urothelium

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Purpose of review
This review addresses significant changes in our understanding of urothelial development and regeneration. Understanding urothelial differentiation will be important in the push to find new methods of bladder reconstruction and augmentation, as well as identification of bladder cancer stem cells.

Recent findings
This review will cover recent findings including the identification of novel progenitor cells in the embryo and adult urothelium, function of the urothelium, and regeneration of the urothelium. Using Cre-lox recombination with cell-type-specific Cre lines, lineage studies from our laboratory have revealed novel urothelial cell types and progenitors that are critical for formation and regeneration of the urothelium. Interestingly, our studies indicate that Keratin-5-expressing basal cells, which have previously been proposed to be urothelial stem cells, are a self-renewing unipotent population, whereas P-cells, a novel urothelial cell type, are progenitors in the embryo, and intermediate cells serve as a progenitor pool in the adult.

Summary
These findings could have important implications for our understanding of cancer tumorigenesis and could move the fields of regeneration and reconstruction forward.

Keywords
progenitors, regeneration, urothelium

INTRODUCTION

The urothelium is a stratified epithelium that extends from the renal pelvis to the proximal urethra and serves as a crucial barrier between the blood and urine. Mature urothelium consists of a layer of Keratin-5-expressing basal cells (K5-BCs), several layers of intermediate cells, and a luminal layer of superficial cells. The adult urothelium is quiescent but can rapidly regenerate in response to acute damage such as urinary tract infection or exposure to drugs and toxins. Chronic damage, however, can compromise bladder function, leading to inflammation, and exposure of sub-urothelial nerve fiber receptors to urinary toxins, a possible mechanism behind chronic bladder pain or interstitial cystitis. In this review, we will discuss function of the urothelium and uroplakins, distinguishing aspects of the urothelial cell types, formation and differentiation of the urothelium, and regeneration of the urothelium.

UROTHELIUM FUNCTION

The urothelium serves many functions within the bladder. Like the esophagus, the primary function is to provide a protective barrier. Unlike the urethra, the bladder epithelium must withstand dramatic increases in pressure during the micturition process; hence, the urothelial barrier has specialized features that prevent exchange of water, metabolites, pathogens, and toxic substances between the urine and blood [1]. Historically, the urothelium was thought to function solely as a passive barrier, impermeable to all water, ions, solutes, and large macromolecules. More recent research, however, has shown that in addition to its barrier function, urothelium plays an active role in altering ion and protein composition of the urine as well as responding to a multitude of external stimuli [1].

One recent study suggests that urothelial plaques, which are composed of uroplakins and play an important role in forming the permeability barrier of the urothelium, are unlikely to be involved in

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KEY POINTS

- The urothelium is a stratified epithelium with three cell types (basal, intermediate, and superficial) with a multitude of functions that includes forming a permeability barrier, acting as a sensory organ, and accommodating large volumes of urine.
- The P-cell, a recently identified cell type present only in the embryonic urothelium, serves as the progenitor for intermediate and superficial cells in embryos, and intermediate cells serve as superficial progenitors in adults.
- Basal cells are not the progenitors of intermediate or superficial cells in the developing urothelium and are likely to arise from a distinct progenitor population.
- Retinoids regulate a stromal-epithelial signaling pathway that is important for development and regeneration of the urothelium.

the prevention of CO_2 flux across the urothelium. When the kidneys secrete acidic urine, the partial pressure of CO_2 in the urine can reach as high as three to four times that found in the blood. Zocher *et al.* [2▪] using a mouse model found that this low permeability to CO_2 was not related to uroplakin expression but rather to the lack of carbonic anhydrase in the urothelium. Thus, the urothelial barrier function is a complex phenomenon with much still to be elucidated.

The urothelium also has an important role in the bladders ability to accommodate a wide range of volume. The bladder is unique in this ability to rapidly change capacity in a cyclic fashion. The mucosal surface, which is wrinkled in the decompressed state of the empty bladder, unfolds as the bladder fills, while the urothelium itself thins, likely as a result of the intermediate cells sliding horizontally [3]. The umbrella cells can increase their surface area as much as 50% via exocytosis of uroplakins in response to hydrostatic pressure [4]. Myelin-and-lymphocyte protein (MAL) is involved in the incorporation of discoidal or fusiform-shaped vesicle (DFV) with the apical membrane, while Rab11a, Rab8a and Myo5B function together to promote stretch-regulated exocytosis [5▪,6▪].

In addition to its functions as a barrier and in increasing bladder capacity, the urothelium also has various sensory functions [7▪▪]. Urothelial cells act like neural cells in three ways: expression of ion channels and receptors; responsive to external molecular/chemical, mechanical, or thermal stimuli; and release of chemicals and neurotransmitters in response to stimuli. For instance, exposure to bradykinin, purines, noradrenaline, and acetylcholine can lead to stimulation of urothelial cells with the release of ATP, prostaglandins, acetylcholine, and nitric oxide. All of these second messengers can have excitatory or inhibitory effects on nerve fibers both in and adjacent to the urothelium [8▪]. One recent in-vitro study of mouse bladders noted that bladder distension evoked reproducible pressure-dependent increases in afferent nerve firing. Interestingly, after exposure to onabotulinumtoxin A (a potent neurotoxin that inhibits release of neurotransmitters at cholinergic neuromuscular junctions, commonly used to treat various lower urinary tract conditions), the urothelium was noted to release significantly less ATP and more nitric oxide [9▪]. ATP release is also inhibited by adenosine via a negative feedback mechanism and stimulated by changes in the transepithelial potential [10▪]. Abnormal urothelial-afferent nerve interactions have been suggested to contribute to bladder dysfunction and pose a viable option for targeted therapy [11▪▪].

FORMATION AND DIFFERENTIATION OF THE UROTHELIM

Discussion of the formation of the urothelium requires knowledge of the different cell types within the urothelium. Adult bladder urothelium is composed of three cell types: basal cells, intermediate cells, and superficial cells. The cells can be distinguished based on combinatorial markers (Fig. 1).

KERATIN-5 BASAL CELLS

K5-BCs comprise the layer of cells in close contact with the basement membrane. These cells are mononuclear and range from 10 to 20 μm in size [12]. They were originally assumed to be a homogenous population; however, our recent studies indicate that there are two distinct K5-BC populations: K5-BCa (Krt5+ Krt14+ Upk− p63+) and K5-BCb (Krt5+ Krt14− Upk− p63+). K5-BCs are stem cells in the skin and have been considered to be a stem cell population in the adult urothelium [13,14▪▪]. Cells expressing similar marker profiles to K5-BCs are present in the prostate and airways where they have also been proposed to be progenitor populations [15▪▪,16,17].

INTERMEDIATE CELLS

The intermediate cell layer ranges from 1 to 4 cells thick depending on the species and the degree of distension in the bladder. Intermediate cells (Upk+ P63+ Krt5−) differ from basal cells based on their expression of uroplakins and their lack of expression of K5 [18,19]. The cells range from 10 to 15 μm in

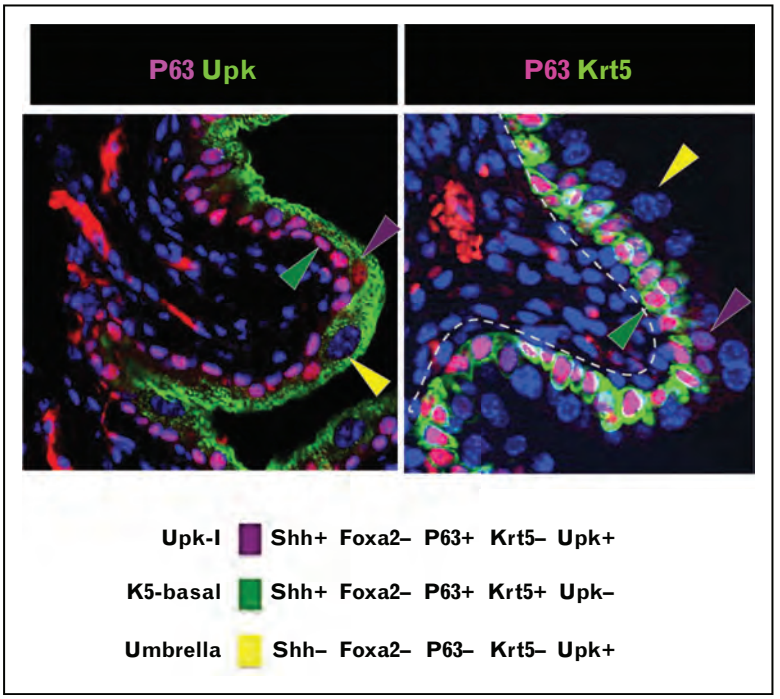

FIGURE 1. Cell types of the urothelium. This original figure illustrates the three cell types of the urothelium, distinguished in this case by their expression of keratin-5, p63 and uroplakin. Basal cells (green arrow) express keratin-5 and p63, but do not express uroplakin. Intermediate cells (purple arrow) express p63 and uroplakin, but do not express keratin-5. Superficial cells (yellow arrow) express uroplakin, but do not express p63 or keratin-5.

width and have a number of interesting characteristics [12]. Our studies suggest that they are progenitors of superficial cells during development and in the adult urothelium. Interestingly, others and we have observed cytoplasmic structures extending from intermediate cells to the basement membrane, suggesting that they may obtain signals or growth factors from the stroma.

SUPERFICIAL CELLS

Superficial cells, or umbrella cells, form a single layer of terminally differentiated cells on the luminal surface of the urothelium that are specialized for the synthesis and transport of uroplakins. These cells can range in size from 25 to 250 μm depending on the degree of distension of the bladder, and they can cover as many as 50 underlying intermediate cells, hence the name umbrella cell [12,20]. Superficial cells are typically multinucleated in contrast with both basal cells and intermediate cells.

Superficial cells are highly polarized. Desmosomes allow for attachment to sub-superficial cell layers and tight junctions between superficial cells aid in forming the high-resistance barrier of the urothelium [1,20,21]. Urothelial tight junctions are comprised of Tight junction protein 1 (ZO-1), occludin, Claudin-4, 8, and 12 [21]. Many physiologic changes occur with tight junctions during filling of the bladder [22▪]. Carratino *et al.* describe how stretching the bladder causes a reversible expansion of tight junction rings and a decrease in paracellular resistance. Therefore, tight junctions accommodate stretching of the bladder, while increasing ion permeability that may alter the function of nearby sensory neurons or muscle cells.

A distinguishing characteristic of superficial cells is the presence of apically located DFVs [12]. In response to bladder filling, DFVs fuse with the apical membrane of superficial cells and release uroplakins and other proteins to the surface, which adjusts the permeability barrier and increases the surface area of the urothelium [4,5▪,6▪]. The reverse process occurs during emptying of the bladder, which causes the urothelial surface area to decrease.

Superficial cells express high levels of two low-molecular-weight cytokeratins, keratin-18 and keratin-20 [23,24]. Keratin-20 contributes to a dense cytokeratin network found below the apical surface of superficial cells that guides DFVs to the surface [25].

UROPLAKINS

Uroplakins are a group of tetraspanin membrane proteins, produced by superficial and intermediate

cells that contribute to the formation of urothelial plaques [18]. Uroplakins UPIa, UPIb, UPII, UPIIIa, and UPIIIb are the major constituents of urothelial plaques and asymmetric unit membranes (AUMs), which is the characteristic apical membrane of superficial cells [18,19]. They are believed to assemble within the endoplasmic reticulum and the golgi apparatus before they are packaged into DFVs [26]. When DFVs fuse with the apical membrane, uroplakins form AUM particles, which are 16-nm crystalline particles with six subunits arranged in inner and outer rings [27]. Uroplakins have various functions within the urothelial barrier best demonstrated by the fact that UPIIIa knockout mice have decreased urothelial barrier function, including increased permeability to water and urea [28]. Loss of UPIIIa can also lead to urothelial hyperplasia, vesicoureteral reflux, and enlarged ureters for currently unknown reasons [28]. Altered uroplakin expression is found in urothelial papillomas and papillary carcinoma [29].

THE EMBRYONIC BLADDER

The bladder is an anterior extension of the urogenital sinus, which develops from cloacal endoderm [30]. The junction of the bladder and urogenital sinus will become the trigone, and the posterior portion of the urogenital sinus will extend and differentiate into the urethra. In males, the urethra has a prostatic portion, a spongy portion, and phallic portion that differentiate late in development in response to sex hormones. In females, the urogenital sinus forms the vagina and the urethra, which is much shorter than the male counterpart. Based on uroplakin expression, the urothelium extends to the bladder neck, a transitional zone that contains a mixture of urethral and urothelial cell types. Until recently, the adult and embryonic urothelium were considered to contain three cell types: K5-BCs, intermediate cells, and superficial cells. These cell populations can be readily distinguished using combinatorial markers including Krt5, Uroplakins and p63, a transcription factor widely expressed in endoderm and its derivatives (Fig. 1). K5-BCs (Krt5+ Upk− p63+) have been considered to be a stem cell population that generates intermediate and superficial cells [14▪▪,31]. Intermediate cells (Krt5− Upk+ p63+) reside in the middle layers of the urothelium, and superficial cells (Krt5− Upk+ p63−) line the upper layer.

IDENTIFICATION OF EMBRYONIC AND ADULT UROTHELIAL PROGENITOR POPULATIONS

Owing to its stratified appearance, urothelium was traditionally believed to assemble and differentiate in a linear sequence similar to the differentiation of skin epithelium, where basal cells generate the suprabasal keratinocytes [32]. A number of studies suggest that K5-BCs in the urothelium are also stem cells that are self-renewing and are able to generate intermediate and superficial cell daughters [14▪▪,31,33▪,34–39]. However, immunophenotypic characterization of the urothelial cell layers led to the proposal by Castillo-Martin *et al.* in 2010 that two distinct progenitor cells are responsible for basal/intermediate cells and superficial cells, respectively. Their conclusion is based on differential expression of cytokeratins, blood group antigens, uroplakins, and p63 among the different cell layers [35]. The same group published in 2011 that p63 mutants contain only a single layer of cells with a superficial cell phenotype as evidence that there are two distinct progenitor cells [40].

In the last year, molecular analysis of urothelial development has indicated the importance of p63 signaling for urothelium formation [14▪▪]. Lineage studies using p63Cre mice in combination with R26R lines to permanently label p63+ cells indicate that all cell types in the urothelium are daughters of p63-expressing cells [14▪▪]. P63 is expressed throughout the undifferentiated endoderm prior to the stage when the bladder forms and in intermediate and K5-BCs in the mature urothelium [14▪▪,40,41]. Hence, it is not possible from these studies to determine whether p63+ progenitors are an endodermal cell type or an urothelial cell type. The observation that epithelia lining the prostate and hindgut, which are endoderm derivatives, are also composed of p63 daughters suggests that there is a common p63+ endodermal stem cell that gives rise to more specialized progenitor cells that populate the urothelium [41]. Recent studies in our laboratory support this hypothesis and shed doubt on previous models of urothelial formation.

We identified a novel cell population, P-cells (Foxa2+ Upk+ P63+ Krt5−), which are a transient cell type that makes up most of the urothelium at E11.5. P-cells persist only as far as E13.5 when the bladder is still in a primitive form. Using the Foxa2CreERT2;mTmG line as an indelible label in fate mapping experiments, we showed that P-cells generate intermediate and superficial cell daughters during development (Fig. 2a) [42▪].

Similarly, we performed fate mapping with a Upk3aGCE;mTmG line to permanently label intermediate cells and their daughters (Fig. 2b). These experiments revealed that intermediate cells, rather than K5-BCs, generate superficial cells, both in the embryonic and adult urothelium [42▪]. In the embryo, intermediate cells appear on E12.5, which is prior to the stage when superficial cells appear on E14. This suggests that intermediate cells may also

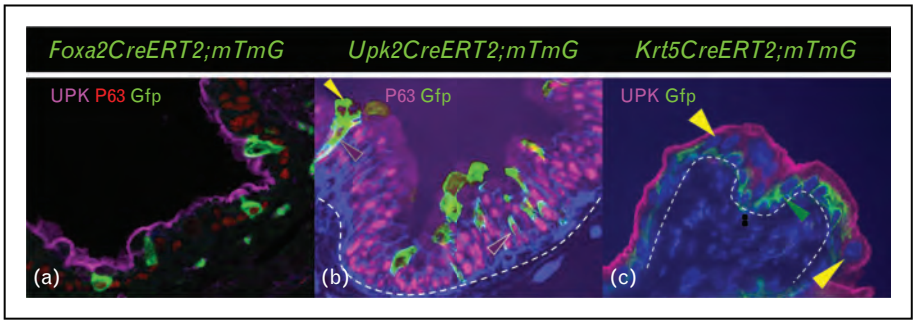

FIGURE 2. P-cells are progenitor cells of intermediate cells and superficial cells in the embryonic urothelium. This original figure illustrates a lineage-tagging analysis in which P-cells were labeled using the Foxa2CreERT2;mTmG line (a), intermediate cells are labeled using the Upk2CreERT2;mtmg line (b), and basal cells are labeled using the Krt5CreERT2;mtmg line (c). (a) Shows that intermediate cells and superficial cells are derived from P-cells in the embryonic urothelium. We see green fluorescent protein (GFP)-labeled cells in the intermediate and superficial cell layers at E18, indicating the progenitor ability of the FoxA2-expressing P cell. (b) Shows that intermediate cells are progenitors for superficial cells in the embryonic urothelium, and they can self-renew. GFP-labeled cells are found in the intermediate and superficial cell layers, but they are not found in the basal cell layer. (c) Shows that basal cells can self-renew, but they are not progenitors for intermediate or superficial cells in the developing urothelium. GFP is only found in the basal cell layer.

be superficial progenitors during development (Fig. 2b). However, this has not been confirmed, as we at present have no marker expressed that is selective for intermediate cells over P-cells. Superficial cells are initially mononuclear, but increase in size and are multinucleated by E17, a stage when urine production begins to increase in the embryo.

Interestingly, K5-BCs, which were thought to be stem cells, form after P-cells, intermediate cells, and superficial cells. It is unclear whether these cells originate from an unknown urothelial progenitor or migrate into the urothelium from adjacent tissues. Studies with the p63Cre mice indicate that K5-BCs are derived from endoderm, but our fate mapping studies indicate that they are not derived from either P-cells or intermediate cells [14▪▪]. Lineage analysis using the Krt5CreERT2 line in conjunction with R26R reporters indicates that K5-BCs are a self-renewing, unipotent population (Figs 2c and 3) [42▪]. Whether K5-Bcs arise from an unknown progenitor population or migrate into the urothelium from adjacent tissues is an interesting question that will be pursued in the future.

THE UROTHELIUM IS REGULATED BY STROMAL-DERIVED SIGNALS, INCLUDING RETINOIC ACID

Landmark studies from the Cunha laboratory indicate that mesenchyme in the embryonic

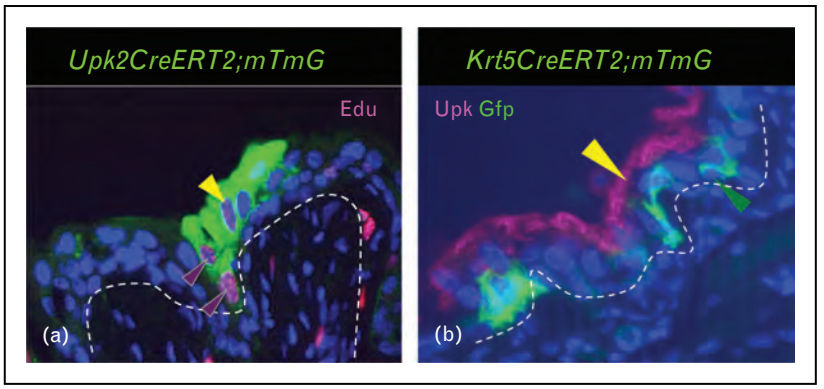

FIGURE 3. Intermediate cells are the progenitors for superficial cells in the adult urothelium. This is an original figure. Uroplakin-expressing cells are labeled using the Upk2CreERT2;mtmg line (a) and basal cells are labeled using the Krt5CreERT2;mtmg line (b). Following cyclophosphamide administration, urothelial regeneration occurs. (a) Shows that intermediate cells are the progenitors for superficial cells. Edu labeling shows proliferation in the intermediate and superficial cell layers. (b) Shows that basal cells proliferate as well, but they do not differentiate into intermediate or superficial cells based on lineage-tracing studies.

urogenital tract can reprogram overlying epithelia to form different cell types [43,44]. These observations have been confirmed in gene inactivation studies in mice. Mysorekar *et al.* [45] showed that Bmp4, secreted from sub-urothelial stroma, and the Bmpr1 receptor, expressed in urothelial cells, are critical for development and regeneration of the urothelium. Likewise, Shh and Wnt-signaling pathways are important for this bi-directional signaling between the urothelium and underlying stroma [31,46]. Our studies indicate that retinoid signaling is also critical for regulating the stromal-epithelial patterning. Retinoic acid, the active form of vitamin A, is synthesized in the stroma and is required for urothelial differentiation and maintenance in adults [42▪]. Additional signaling pathways that direct urothelial development include ELF3, Brg1, Oct4, and Fibroblast growth factor 10 (FGF-10) [47▪,48▪,49,50▪▪]. With continued improvement in techniques for genetic analysis, we are sure many more genes will emerge that are crucial for urothelial and bladder development.

REGENERATION OF THE UROTHELIUM

Many studies have been performed looking at bladder regeneration given its importance to bladder augmentation procedures in patients with limited bladder capacity. The current method for performing bladder augmentation involves the use of gastrointestinal mucosa to increase the volume of the bladder. A multitude of complications occur with intestinal augmentation cystoplasty, including electrolyte imbalances, stone formation, infections, and malignancy [51]. Therefore, the need to find an alternative tissue engineering method is evident. In recent years, there has been a move toward bladder reconstruction techniques that utilize urothelium [44]. An understanding of urothelial regeneration can further aid our ability to develop well tolerated and functional bladder reconstruction techniques.

Studies published recently indicate that K5-BCs are likely progenitors in the adult urothelium following urinary tract infection-induced damage and regeneration [31]. However, these experiments were performed with ShhCreERT2 as a lineage tag, which labels both K5-BCs and intermediate cells [42▪]. To further evaluate which cell types are important for regeneration in adults, we used K5CreERT2;mTmG and UpkGCE;mCherry lines to selectively label K5-BCs and intermediate cells, respectively, then we followed their fates during cyclophosphamide (CPP)-induced damage and regeneration. Treatment of mice with CPP results in rapid desquamation, proliferation, and regeneration of the urothelium, with a cycle that lasts about 3 days. We found that intermediate cells can self-renew and generate superficial daughters, indicating that these are a pool of adult urothelial progenitors (Fig. 3a). However, K5-BCs can self-renew in adults and, as in the embryonic urothelium, are unipotent (Fig. 3b). Interestingly, we found that, as during development, retinoid signaling is critical for the differentiation process [42▪].

Other studies from Erman *et al.* [52▪▪] used chitosan-induced injury to study functional and structural regeneration of the urothelium *ex vivo*. They found that excised bladder tissue maintains the ability to functionally and structurally regenerate urothelium. Other groups have explored the potential use of keratinocyte growth factor (KGF) to protect the urothelium from potentially toxic substances and to support urothelial regeneration following damage [53]. KGF can ameliorate cyclophosphamide-induced ulcerative hemorrhagic cystitis in rats by causing rapid proliferation of urothelial cells.

Additional research on urothelial regeneration in the last year has focused on tissue-engineered urothelium development. Tu *et al.* [54▪] used an acellular silk scaffold to perform augmentation cystoplasty in pigs. They were able to achieve formation of a structurally accurate urothelium, in addition to formation of innervated and vascularized smooth muscle. Similarly, Jerman *et al.* [55▪] used an amniotic membrane scaffold seeded with normal urothelial cells to develop a highly differentiated urothelium. This group found that the most robust growth of urothelial cells occurred on stromal amniotic membrane. Mechanisms for urothelial regeneration were not discussed in either article and are worthy of investigation given the potential for stromal-urothelial interactions as the driver of robust urothelial regeneration.

A final interesting topic that was well researched in the last year was generation of urothelial cells from nonurothelial cells. The idea of using stem cells in urothelial regeneration has become increasingly popular given the potential for many therapeutic applications. A 2010 study by Mauney *et al.* showed that retinoids could induce embryonic stem cells to differentiate into urothelial cells *in vitro* [56]. In the last year, other studies have used amniotic fluid stem cells, adipose-derived stromal cells, adipose-derived stem cells, endometrial stem cells, and umbilical cord-derived stromal cells to generate urothelial cells [57▪▪,58▪,59▪▪,60▪,61,62▪]. One study from Moad *et al.* [63▪▪] used bladder stromal cells to form urothelial tract induced pluripotent stem cells (UT-iPSC) that could then be directed to undergo bladder differentiation. This is an exciting and new area for urothelium research with many potential applications.

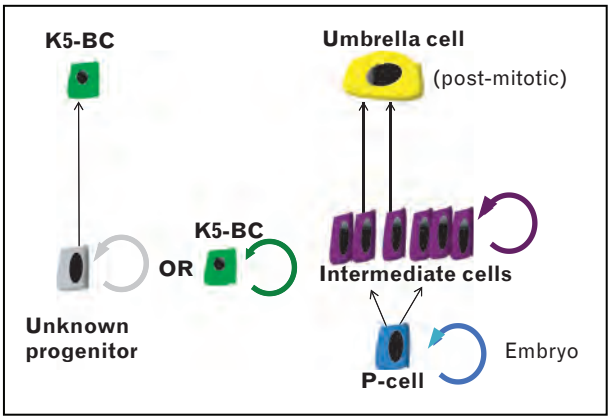

FIGURE 4. Schematic of urothelial formation and regeneration. This is an original figure that demonstrates our proposed model of urothelial formation during embryogenesis and urothelial regeneration in the adult bladder. In the developing bladder, P-cells are progenitors for intermediate and superficial cells. Basal cells have a currently unknown progenitor, or they are a self-renewing cell population. In the adult bladder, following acute injury, intermediate cells function as the progenitors for superficial cells, and they are self-renewing. Basal cells are not progenitors for intermediate or superficial cells.

CONCLUSION

The urothelium is much more than a simple barrier between urine and blood. It can respond to external stimuli and alter the composition of urine. The urothelium also contains distinct cell types, with unique cell progenitors. The identification of unique cell progenitors in the intermediate and basal cells, respectively, can have important implications for our understanding of both bladder regeneration and cancer tumorigenesis. Recent advances in our understanding of urothelial formation and regeneration necessitate a new model of urothelial differentiation to be proposed (Fig. 4).

Acknowledgements

Disclosures: Nothing to disclose.

Conflicts of interest

There are no conflicts of interest.

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