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REVIEW

Development, regeneration and tumorigenesis of the urothelium

Gregory B. Wiessner[1,2], Sakina A. Plumber[1], Tina Xiang[1] and Cathy L. Mendelsohn[1,2,*]

ABSTRACT

The urothelium of the bladder functions as a waterproof barrier between tissue and outflowing urine. Largely quiescent during homeostasis, this unique epithelium rapidly regenerates in response to bacterial or chemical injury. The specification of the proper cell types during development and injury repair is crucial for tissue function. This Review surveys the current understanding of urothelial progenitor populations in the contexts of organogenesis, regeneration and tumorigenesis. Furthermore, we discuss pathways and signaling mechanisms involved in urothelial differentiation, and consider the relevance of this knowledge to stem cell biology and tissue regeneration.

KEY WORDS: Basal cells, Progenitors, Retinoids, Urothelial development, Urothelium

Introduction

The bladder mediates crucial functions required for the storage and excretion of urine. It is composed of a thick layer of muscle (the detrusor) and a submucosa consisting of interstitial cells, fibroblasts and myofibroblasts, and is lined by a specialized epithelium called the urothelium. The urothelium is a unique stratified epithelium that lines most of the urinary outflow tract. This includes the bladder and the ureters, as well as parts of the renal pelvis and the urethra. The urothelium is structurally and sensorily specialized to create a waterproof barrier that is protective against pathogens, ions, water and chemicals (reviewed by Hicks, 1975). Furthermore, the urothelium of the bladder is uniquely responsive to stretch, allowing this organ to expand and contract as it fills and empties (Berry et al., 2011; Dalghi et al., 2020, 2021; Marshall et al., 2020). Defects in the urothelial lining due to persistent injury from chemical exposure, radiation or infection may lead to benign urological conditions, including bladder pain syndrome and voiding dysfunction (Berry et al., 2011; Birder, 2005; Birder et al., 2012; Parsons, 2011). The urothelium is also the source of cells that undergo tumorigenesis and contribute to bladder cancer. In view of this, identifying the urothelial cell populations and signaling pathways involved in the development, homeostasis and injury of the urothelium is important for understanding the pathologies of both benign and malignant urological conditions. Moreover, an important goal in the field is to generate urothelial cell types that can be used to augment or repair the injured the urothelium. Here, we review the current understanding of bladder urothelial progenitor populations during embryogenesis and adulthood.

[1]Departments of Urology, Genetics and Development, Pathology and Cell Biology, Columbia Stem Cell Initiative and Institute of Human Nutrition, Columbia University, New York, NY 10032, USA. [2]Institute of Human Nutrition, Columbia University, New York, NY 10032, USA.

*Author for correspondence (clm20@cumc.columbia.edu)

C.L.M., 0000-0002-4026-4073

Cell types in the bladder urothelium

In the mouse, the bladder urothelium can be categorized into three major cell types based on their expression of molecular markers, morphology and location (Fig. 1): superficial cells (S cells), which line the apical (luminal) surface; intermediate cells (I cells); and basal cells, which populate the basal and suprabasal layers. S cells are large, terminally differentiated, often binucleated, polyhedral-shaped cells that are also referred to as 'umbrella cells' due to their expansive morphology (Fig. 1). They range in diameter from 50-150 µm, depending on the volume of urine in the bladder, and may cover as many as 50 underlying cells (Walker, 1957). S cells are chiefly responsible for creating the waterproof barrier of the urothelium due to their repertoire of expressed surface and intercellular proteins (Hu et al., 2002). Between adjacent S cells are tight junctions formed by claudins and zona occludens 1 (Zo1), which limit the paracellular exchange of solutes and ions (Acharya et al., 2004; Negrete et al., 1996). Additionally, S cells produce crystalline plaques that further contribute to barrier function (Hu et al., 2002). These plaques consist of transmembrane uroplakin proteins (Upk1a, Upk1b, Upk2 and Upk3), which assemble into asymmetric unit membranes (AUMs) consisting of thousands of plaque particles (Sun et al., 1996; Wu et al., 1990, 1995). Uroplakins are shuttled from the endoplasmic reticulum to the apical surface of S cells by discoidal and/or fusiform-shaped vesicles (DFVs) (Tu et al., 2002; Yu et al., 2009a). Interestingly, this shuttling increases in response to bladder filling, in order to accommodate volumetric expansion of the urothelium, and uroplakin proteins are then returned from the membrane to endosomes for degradation to allow bladder contraction after voiding (Khandelwal et al., 2010; Kreft et al., 2009). Genetic experiments on uroplakins have invariably demonstrated that loss of these proteins results in failed plaque formation and compromised barrier function (Aboushwareb et al., 2009; Carpenter et al., 2016; Hu et al., 2000).

Directly beneath the S cells are the relatively small population of intermediate (I) cells (Fig. 1) that also express uroplakin proteins but can be distinguished by their smaller size (~20 µm in diameter) (Keshtkar et al., 2007). Accounting for fewer than 10% of urothelial cells, I cells retain connections to the basement membrane through long thin cytoplasmic extensions (Jost et al., 1989; Wang et al., 2018). Pyriform in shape, the majority of I cells are mononucleate, although they may undergo failed cytokinesis to generate new binucleated (2n+2n) precursors that replace S cells when they die off during homeostasis or after acute injury (Wang et al., 2018). Basal cells, which line the bottom-most layers along the basement membrane, account for the majority (>80%) of cells in the bladder urothelium. Basal cells are the smallest in size (10 µm in diameter) and retain connections to the basement membrane via hemidesmosomes (Alroy and Gould, 1980; Jones, 2001; Owaribe et al., 1990).

As mentioned above, urothelial cell types may be distinguished by their combinatorial expression of protein markers (Fig. 1). There are two populations of basal cells (BCs): K5-BCs express the heavy-chain cytokeratin 5 (Krt5) and Trp63 (p63), but have minimal or

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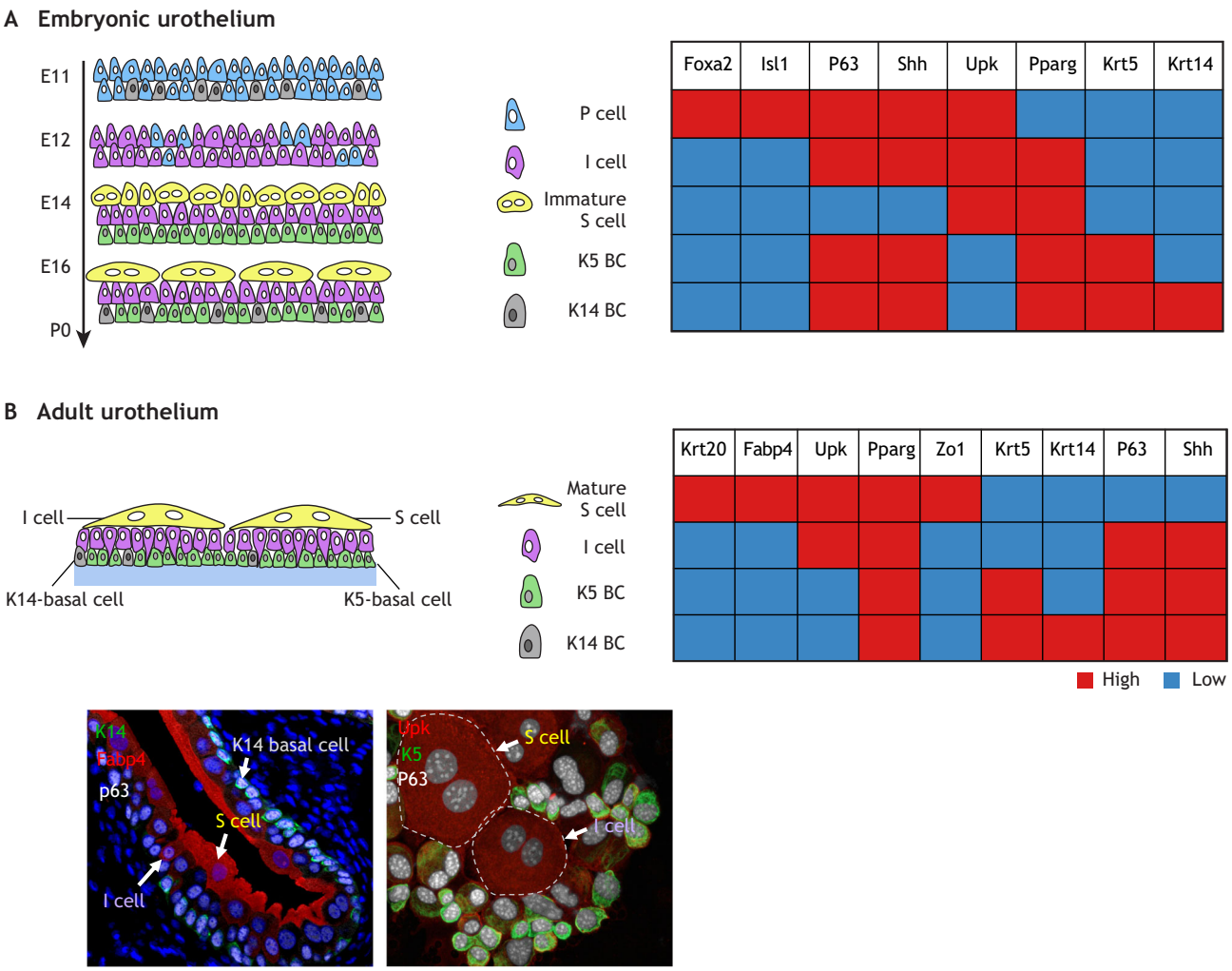

Fig. 1. Schematic of urothelial development and homeostasis. (A) Formation of the mouse embryonic urothelium. The image (left) depicts changes in urothelial cell types as the tissue develops (from E11 to P0). The plot (right) highlights some of the key genes expressed in each cell type; red indicates high expression, blue indicates low expression. (B) Composition of the adult mouse urothelium. The image (left) depicts the cell types present within the adult urothelium. The plot (right) highlights some of the key genes expressed in each cell type. Sections through the adult urothelium (bottom panels) showing expression of combinatorial markers in urothelial populations. Isl1, islet 1; K5-basal cell, Krt5-expressing basal cells; K14-basal cells, Krt14-expressing basal cells; P cell, progenitor cell; P63, tumor protein P63 (also known as TRP63); Pparg, peroxisome proliferator-activated receptor γ; S cell, superficial cell; Shh, sonic hedgehog; Upk, uroplakin; Zo1, zona occludens 1.

undetectable expression of uroplakin proteins. A second population of basal cells (K14-BCs) expresses Krt14, Krt15 and p63. These cells are progenitor cell types, similar to those present in the skin and airways, and they drive tumor invasion and dissemination in a number of epithelial cancers (Cheung and Ewald, 2016; Cheung et al., 2013; Chu et al., 2001; Sánchez-Danés and Blanpain, 2018). The K14-BC population dramatically expands in response to urothelial injury and has been of increasing interest in recent years due to its reported role as a progenitor cell population during regeneration (Papafotiou et al., 2016). K14-BCs can also, based on lineage studies in mouse models, give rise to bladder tumors after BBN exposure (Papafotiou et al., 2016), although several lines of evidence suggest that I cells are also able to produce tumors (Van Batavia et al., 2014; Wu, 2009; Zhang et al., 2001). It remains unclear whether K14-BCs are a distinct cell population or, alternatively, whether K14 expression may represent a state that is induced in response to injury.

I cells express all major uroplakins, sonic hedgehog (Shh) and p63, but Krt5 and Krt14 proteins are low or undetectable (Fig. 1). Recent single cell RNA-seq analysis of the human and mouse bladder reveals that Sprr2f is perhaps the first specific marker for I cells (Cheng et al., 2021). Uroplakin-expressing S cells are easily distinguishable from other urothelial cell types by the presence of Uchl1 (Liu et al., 2019) and Krt20 (Romih et al., 1999), which mark mature S cells together with the absence of p63 and basal cell markers. Interestingly, although there is considerable specificity between urothelial cell types at the level of protein expression, there is significant overlap at the mRNA level. For example, Krt5 mRNA is observed at particularly high levels in K5-BCs but is also expressed at moderately high levels in I cells. Likewise, Upk mRNAs are expressed at high levels in S cells and I cells, but are also detectable in K5-BCs, while protein expression of Upk family members is much more tightly regulated (www.gudmap.org). Going forward, a deeper analysis of urothelial populations at the single cell level, as well as at the bulk level, may prove useful in assessing the state of K14 basal cells and for identifying additional markers that define functional urothelial cell types (Cheng et al., 2021; Santos et al., 2019; Yu et al., 2019).

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Urothelial development

The urothelium is derived from an endodermal sac known as the cloaca (Georgas et al., 2015; Seifert et al., 2008). At embryonic day (E) 10.5 in mice, the dorsal region of the cloaca begins to differentiate into the hindgut (which will ultimately become the large intestine), whereas the ventral cloaca partitions into what will become the urogenital sinus, which in turn gives rise to the bladder and urethra. At E11.5, the primitive bladder begins to emerge at the anterior aspect of the urogenital sinus (UGS), while the urethra begins to develop from the posterior aspect of the UGS. The stratification of the developing urothelium follows a unique course (Fig. 1A) that differs from the bottom-to-top (basal-to-suprabasal) formation of cell types observed in other epithelia, such as the epidermis. Our lab previously identified and characterized a population of endoderm-derived urothelial progenitor cells in the developing bladder, referred to as progenitor cells (P cells; Gandhi et al., 2013). Transiently detectable from E11.5 to E14.5, P cells are identifiable by their expression of endodermal markers, including Isl1 and Foxa2 (Fig. 1A). Lineage-tracing experiments using *Foxa2*^{CreERT}*;mTmG* to indelibly label P cells demonstrated that this population gives rise to the uroplakin-expressing I and S cells of the urothelium. I cells (Shh^{+}, p63^{+}, Upk^{+}) are first detectable between E11 and E12 (Fig. 2A; Gandhi et al., 2013; Georgas et al., 2015). I cells produce S cells (Upk^{+}, Shh^{-}, p63^{-}), which are initially similar in size to I cells. Binucleated S cells then form by a process of endoreplication: they enter mitosis and undergo nuclear division, but they fail to complete cytokinesis. This process generates binucleated S-cell precursors that subsequently enlarge and double their DNA content (from 2n to 4n) via endoreplication; newly formed S cells leave the cell cycle without entering mitosis (Wang et al., 2018). K5-BCs (K5^{+}, Shh^{+}, p63^{+}, Upk^{-}) have a later ontogeny, first appearing at stage E15.5. The K14-BC subpopulation is detectable roughly 1 day later, around E16.5 (Georgas et al., 2015). The origin of basal cell populations is unclear at present; however, their appearance coincides with the time when urinary outflow increases in the embryo (Mendelsohn, 2009), raising the question of whether stress stimuli might regulate the differentiation or expansion of this unique cell population.

A similar urothelium consisting of basal, intermediate and superficial cells lines the ureter and a region of the renal pelvis, although the ureteral urothelium is derived from intermediate mesoderm (Bohnenpoll and Kispert, 2014; Jackson et al., 2021), whereas the bladder urothelium is derived from endoderm (Georgas et al., 2015; Seifert et al., 2008). In both cases, reciprocal signaling between the urothelium and sub-urothelial stroma are important drivers of specification and differentiation, of both epithelial and non-urothelial cell types, including stroma and muscle. This raises intriguing questions surrounding the commonalities and distinctions of the progenitor populations, developmental ontogenies, signaling pathways and composition of cell types of each region. Several studies using mouse models have demonstrated that the bladder and ureters have relatively similar mature cell types and ontogenies during development (Bohnenpoll et al., 2017a; Bohnenpoll and Kispert, 2014), suggesting the ureter and bladder urothelium form via conserved specification programs. Consistent with this, a number of signaling pathways, including those involving Shh, Rars, Pparg and Grhl3, are important for differentiation of the urothelium of both the bladder and ureter (Bell et al., 2011; Bohnenpoll et al., 2017b; Liu et al., 2019; Tate et al., 2021; Varley et al., 2009, 2004b; Weiss et al., 2013; Yu et al., 2009b).

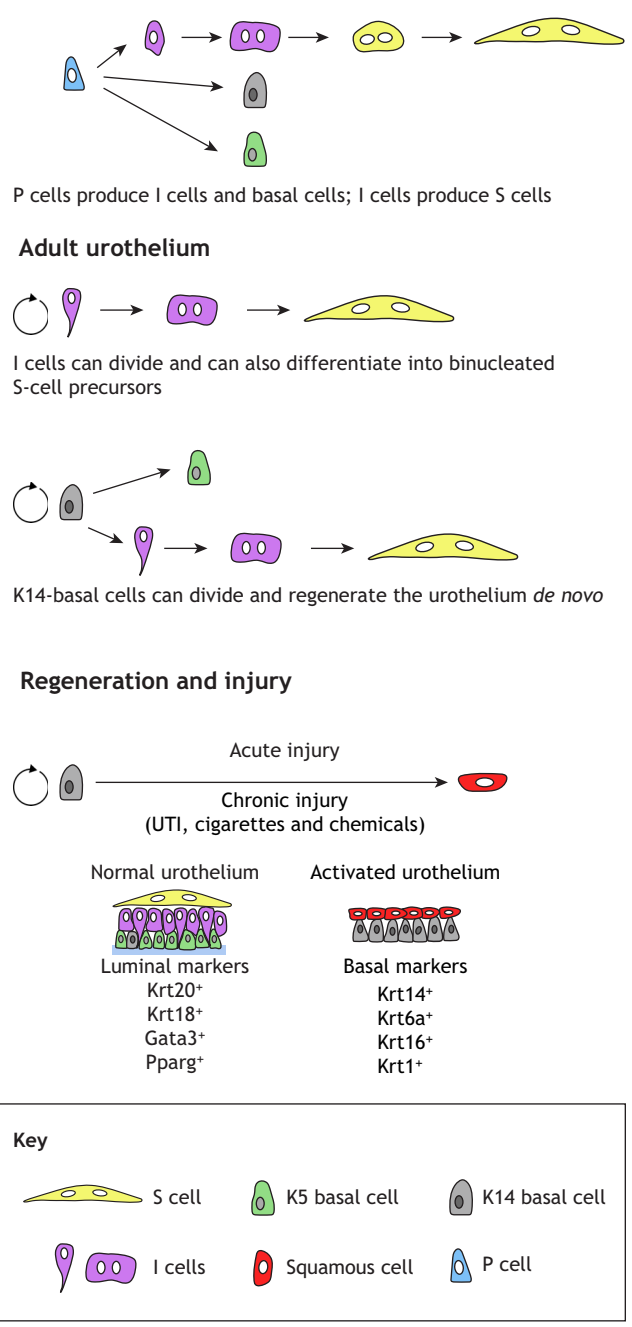

Fig. 2. Urothelial progenitors during differentiation, homeostasis and regeneration. (A) During development, progenitor (P) cells give rise to intermediate (I) cells, which in turn undergo failed cytokinesis to produce immature superficial (S) cells. Immature S cells enlarge and replicate their DNA via endoreplication to form mature S cells. Basal cells (BCs) appear later in development and exist in two forms: K5-BCs populate the basal and suprabasal layers, while K14-BCs are rare and are found only in the basal layer. (B) Repair during urothelial homeostasis and after acute injury. I cells can replace S cells when they die off during homeostasis or after acute injury. K14-BCs can regenerate the urothelium *de novo* after injury. (C) Regeneration and injury. K14-BCs undergo a transient activation state induced by injury, expanding and upregulating Krt6a and Krt16. These changes can persist after chronic injury from recurrent UTI, cigarette smoking and exposure to chemicals.

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Urothelial homeostasis and regeneration

The urothelium, compared with other epithelia, such as the intestinal epithelium or epidermis, is remarkably quiescent. Various pulse-labeling experiments using both rodent bladders and human biopsies have demonstrated extremely low mitotic and cellular turnover rates in this tissue (Martin, 1972). Indeed, cells of the rodent urothelium are estimated to turn over every 200 days, putting it among the slowest cycling epithelia in the body (Hicks, 1975). However, after chemical, bacterial or spinal cord injury, the urothelium undergoes a swift process of exfoliation of its luminal S cells, followed by regenerative cell divisions that restore the barrier lining (Fig. 2B; Balsara and Li, 2017; Lavelle et al., 2002; Mysorekar et al., 2002). For example, in the mouse, the urothelium has the capacity to respond to injury and undergo a repair process that occurs rapidly over a period of 72 h (Fig. 2B). Chronic or repetitive injury and inflammation, however, can permanently disrupt urothelial differentiation, resulting in squamous differentiation (Fig. 2C), a state that may make urothelium cells more susceptible to tumor formation.

The cellular events involved in urothelial regeneration following injury have been explored using a variety of injury models. For example, urothelial regeneration is often evaluated in the context of urinary tract infection (UTI), a common bacterial infection caused by the uropathogenic *Escherichia coli* (UPEC), which most women experience at least once in their lifetime (Silverman et al., 2013). UPEC are specialized at infecting urothelial cells through their expression of FimH adhesions on their type I pili that enable them to attach to the Upk1a protein expressed on S cells (Min et al., 2002; Mulvey et al., 1998; Mysorekar et al., 2002). This attachment leads to their uptake by S cells through endocytic mechanisms, triggering a robust immune response that provokes exfoliation and regeneration of the urothelial lining (Khandelwal et al., 2009; Thumbikat et al., 2009; Wang et al., 2017, 2018).

Chemicals, including cyclophosphamide (CPP) and protamine sulfate, have also been used to study urothelial regeneration in animal models (Lavelle et al., 2002; Mysorekar and Hultgren, 2006). CPP, which in humans is administered as a chemotherapeutic compound, is converted into acrolein in the urine, through which it exerts toxicity in the urothelium to provoke desquamation (Cox, 1979; Farsund, 1976; Golubeva et al., 2014). Initial lineage-tracing studies using the *Shh^{CreERT2};mTmG* mouse line demonstrated that Shh-expressing cells, which include basal cells and I cells, regenerate all layers of the urothelium following injury by CPP or UTI (Shin et al., 2011). The basal population in the urothelium contains mostly K5-BCs (K5^{+}/K14^{-}) and a small proportion of K14-BCs (K14^{+}/K5^{+}). Using lineage studies in mice, it was shown that K14-BCs can regenerate the urothelium in response to injury, as well as produce tumors in a mouse model of carcinogenesis, indicating that these cells are likely to be the major progenitor population (Papafotiou et al., 2016). However, studies from our lab suggest that I cells are also capable of urothelial repair, producing S cells during homeostasis and after acute injury. Indeed, lineage studies using *K5^{CreERT2};mTmG* and *Upk3GCE;mCherry* to indelibly label K5-BCs and I cells during CPP- or UTI-induced injury revealed that I cells are the major source of S cells during homeostasis and after acute injury, whereas basal cells have the capacity to repair the urothelium after repeated injury, leading to depletion of the I-cell population (Gandhi et al., 2013; Wang et al., 2018). Furthermore, we have demonstrated that mononuclear I cells undergo failed cytokinesis in order to generate binucleated cells, which subsequently endoreplicate to double their DNA content as they differentiate into polyploid terminally differentiated S cells (Wang et al., 2018). Consistent with these observations, studies using *K5^{CreERT2}* and *Upk2^{CreERT}* lines to lineage trace basal and I cells, respectively, in a surgical model of augmentation cystoplasty, indicate that I cells have the capacity to replenish the S-cell layer after focal damage, but only basal cells are able to repopulate the denuded urothelium *de novo* (Schäfer et al., 2017). In view of these observations, I cells demonstrate progenitor potential in the context of acute urothelial injury, thereby contradicting the linear model of bottom-to-top stratification observed in other epithelia, such as the epidermis.

Damage to the urothelial barrier from infection or injury also triggers an inflammatory response, which includes activation of the Nf-κb pathway in urothelial cells, and secretion of cytokines and chemokines that mediate innate immunity (Eckmann and Neish, 2011; Schilling et al., 2003). Recent studies suggest that the basal progenitor population in the mouse urothelium (K14-BCs) becomes activated in response acute and chronic injury (Fig. 2C; Tate et al., 2021), a response observed in the skin and other epithelial barriers (Freedberg et al., 2001). Massive expansion of the K14-BC population also occurs, along with upregulation of keratins expressed in squamous cells, including Krt6a and Krt16, that are not detected in the healthy urothelium. These studies further suggest that a persistent activation state and associated inflammation are likely to promote squamous metaplasia and may also confer increased susceptibility to bladder cancer (urothelial carcinoma).

Pathways and transcription factors that control urothelial development, specification and regeneration

Development, homeostasis and regeneration of the bladder urothelium are regulated by a multitude of signaling pathways (summarized in Table 1), many of which involve communication between the epithelium and the underlying mesenchyme or stroma via the diffusion of secreted signals. Below, we highlight some of the key factors that control urothelial development, specification and regeneration.

Bmp4

As is the case for epithelia throughout the body, signaling pathways operating between the urothelium and the sub-urothelial stroma are important for regulating specification and cell behavior in response to injury. Work from the Hultgen lab using mouse models showed several years ago that Bmp4 secreted from stromal cells immediately beneath the urothelium activates Bmp signaling in urothelial cells via Bmpr1a, evidenced by increased expression of p-Smad1 (Mysorekar et al., 2009, 2002). Inactivation of Bmp signaling by deletion of Bmpr1a results in aberrant urothelial regeneration, indicating that this pathway is likely to be important for regulating the urothelial response to injury.

Shh

Shh is a secreted signaling molecule that binds to the patched (Ptch) receptor to regulate a variety of cellular behaviors mediated by the downstream Gli transcription factors. In the bladder, *Shh* is constitutively expressed in the urothelium from the onset of development and into adulthood, whereas Ptch and Gli1 are expressed in the underlying mesenchyme (Shin et al., 2011). The urothelium responds to UTI by upregulating Shh and Wnt signaling, suggesting that these pathways interact to control proliferation during injury-induced regeneration (Shin et al., 2011). On the other hand, decreased Shh expression is observed in the urothelium during BBN-induced carcinogenesis. The role of the Shh pathway in counteracting bladder cancer progression was further

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Table 1. Signaling pathways and transcription regulators involved in urothelial differentiation in bladder and ureter

Signaling pathway/transcription factor	References
Bone morphogenic protein (Bmp)	Mamo et al. (2017); Mysorekar et al. (2009)
Fibroblast growth factor (Fgf)	Narla et al. (2020); Narla et al. (2021); Tash et al. (2001)
Hedgehog	Haraguchi et al. (2012); Haraguchi et al. (2007); Shin et al. (2011)
Notch	Santos et al. (2019)
Wnt/β-catenin	Miyagawa et al. (2014); Santos et al. (2019); Shin et al. (2011)
Brahma-related gene 1 (Brg1)	Weiss et al. (2013)
Forkhead box A1 (Foxa1)	Reddy et al. (2015)
Grainyhead-like transcription factor 3	Yu et al. (2009b)
Kruppel-like factor 5 (Klf5)	Bell et al. (2011)
Peroxisome proliferator-activated receptor gamma (Pparg)	Liu et al. (2019); Tate et al. (2021); Varley et al. (2004a,b)
Polycomb repressive complex II (Prc2)	Guo et al. (2017)
Retinoic acid receptors (Rars)	Gandhi et al. (2013); Goldblatt and Benischek (1927); Molloy and Laskin (1988); Wolbach and Howe (1925)
Tumor protein p63 (p63)	Karni-Schmidt et al. (2011)

demonstrated in experiments using mice lacking smoothened (Smo) in the mesenchyme, which exhibit accelerated tumor progression in response to BBN-induced carcinogenesis (Kim et al., 2019; Shin et al., 2014b).

Notch

The Notch pathway, which is a crucial regulator of differentiation across numerous epithelia and other tissues (Siebel and Lendahl, 2017), is active at baseline levels during urothelial homeostasis throughout all cell layers, as indicated by the detectability of Notch intracellular domain 1 (NICD1) and downstream gene targets, including *Hes1* and *Hey1* (Paraskevopoulou et al., 2020). Notch pathway activation is primarily mediated by the jagged 1 (Jag1) Notch ligand (Rampias et al., 2014). During adult homeostasis, inhibition of Notch signaling in basal cells through $K5^{CreERT2}$-mediated deletion of either *Nicastrin* ($Ncstn^{fl}$) or *RBPJ* ($RBPJ^{fl}$) was observed to result in urothelial hypoplasia, inflammation and downregulation of Krt20 and zona occludens 1 (Zo1), indicative of loss of S-cell integrity (Paraskevopoulou et al., 2020). Somewhat relatedly, pharmacological inhibition of Notch signaling in a urothelial organoid system (see Box 1) impaired differentiation of luminal I- and S-cell types, indicating a role for this pathway in terminal differentiation in the urothelium (Santos et al., 2019). Interestingly, inactivating mutations in the *NOTCH1* and *NOTCH2* genes are frequently found in urothelial carcinoma, suggesting that this pathway may be protective against cancer in the human urothelium (Greife et al., 2014; Maraver et al., 2015; Rampias et al., 2014).

Transcription factors

The basal cell activation response, which is likely to induce squamous differentiation, also occurs mutants lacking *Pparg*, a member of the nuclear receptor superfamily. Pparg forms heterodimers with Rxr, and these bind to Pparg-response elements in regulatory regions of target genes. Transcriptional activity is induced by the binding of ligands, which include fatty acids and synthetic agonists (Davies et al., 2001; Kliewer et al., 1995; Lehmann et al., 1995). A number of years ago, in studies that were remarkably prescient, it was shown that cultures of K14-expressing normal human urothelial cells can be converted to a luminal differentiation program by addition of an Egfr antagonist together with a Pparg agonist, inducing the expression of Upk and Krt20, which are I- and S-cell markers (Varley et al., 2004a,b). Interestingly, loss of Pparg expression results in persistent inflammation and mis-programming of basal cell progenitors, which become activated and produce squamous cells instead of endogenous urothelial cells (Fig. 2C). Squamous differentiation in Pparg knockout mice worsens after UTI, most likely as a result of persistent upregulation of Nf-κb, a transcriptional target of Pparg, which leads to chronic inflammation (Liu et al., 2019).

Retinoic acid signaling

Lack of vitamin A, the inactive precursor of retinoic acid (RA), results in squamous metaplasia in the urothelium, as well as in other organs (Wolbach and Howe, 1925). Recent work suggests that retinoid signaling is required both for suppressing squamous differentiation and promoting urothelial differentiation (Gandhi et al., 2013). Retinoids activate retinoic acid receptors (Rars), transcription factors belonging to the nuclear receptor superfamily. Rar genes expressed in urothelial cells are activated by RA-produced by Aldh1a2 and Aldh1a3, which are expressed in the sub-urothelial stroma and urothelium, respectively. Inhibition of RA signaling using the retinoic acid receptor dominant negative (*RaraDN*) allele, driven by Shh^{Cre}, to selectively inhibit RA signaling in the urothelium from the onset of bladder development, demonstrated the importance of this transcriptional regulator in normal urothelial differentiation (Gandhi et al., 2013). In adults, RA signaling in the bladder remains at baseline levels during homeostasis. However, injury provokes a transient upregulation of *Aldh1*a2 in the stroma and activation of RA signaling in all cell layers of the urothelium. Studies have also demonstrated that RA signaling in I cells is required for their proper regeneration, as *Up3GCE*-driven expression of *RaraDN* results in failed specification of I cells into terminally differentiated S cells (Gandhi et al., 2013).

Epigenetic pathways

Epigenetic pathways are also important for urothelial specification. *Ezh2* and *Eed1* are subunits of the polycomb repressive complex 2 (Prc2), which catalyzes di-methylation or tri-methylation of histone H3 lysine 27 (H3K27me3; Barski et al., 2007; Cao et al., 2002; Margueron and Reinberg, 2011). Studies have shown that PRC2 is important for controlling both cell fate and the timing of urothelial progenitor cell differentiation during development and regeneration (Guo et al., 2017). Indeed, an *Eed1* mutant epithelium undergoes squamous differentiation during development and exhibits downregulation of major signaling pathways, including those involving Shh, retinoids, Foxa1 and Pparg, all of which are important drivers of urothelial differentiation and regeneration.

Recent studies have also shed light on the role of epigenetic regulators in bladder cancer, which affects males three to four times

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Box 1. Mimicking urothelial development *in vitro*

The Southgate lab pioneered the use of 2D cultures of human urothelial cells derived from the ureters or bladder as a means of studying urothelial biology (Southgate et al., 1994). More recently, spheroids and/or organoids have been used as a model system for studying both the benign urothelium and urothelial tumors from bladder cancer patients (Lee et al., 2018; Mullenders et al., 2019). Organoids derived from wild-type mouse urothelium have also been generated (Mullenders et al., 2019; Santos et al., 2019). For example, a recent study used FACS-sorted CD49f^{+} basal cells, which include K14 progenitors, to produce organoids (Santos et al., 2019). Using this approach, it was shown that K14 progenitors take on a basal differentiation program when cultured in medium containing Egf, Wnt3a and R-spondin, while the addition of a Pparg agonist, γ-secretase (a Notch inhibitor), and inhibitors of canonical Wnt signaling induces urothelial differentiation. In this case, basal marker expression was decreased, and expression of luminal markers (Upk, Krt18, Krt20, Pparg and Foxa1) was increased (Santos et al., 2019). These findings suggest that Wnt, Notch and Egf drive the progenitor population to take on a basal-like differentiation program, while Pparg induces progenitors to take on a luminal differentiation program. Interestingly, umbrella cells (S cells) were not detected in either of the mouse studies, suggesting that additional factors are needed to support this polyploid terminally differentiated population.

more than females. These studies used mouse models to investigate the role of X-linked lysine demethylase 6A (KDM6A), which functions to remove the methyl groups of H3K27me3 and is often mutated in bladder cancer, revealing that *KDM6A* is a likely tumor suppressor and contributes to the protective effect of the X-chromosome observed in females (Kaneko and Li, 2018).

Urothelial carcinoma

Urothelial carcinoma (commonly referred to as bladder cancer) is the fourth most frequent malignancy among males in the USA (Siegel et al., 2019). For unidentified reasons, this disease is roughly four times more common in males than in females (Bray et al., 2018; Richters et al., 2020). To date, smoking is the greatest identified risk factor for bladder cancer (Zeegers et al., 2000), particularly sidestream smoke (passive smoking) (Lee et al., 2015), with arsenic, contamination and exposure to chemicals containing aromatic amines also being risk factors (Kiriluk et al., 2012; Saginala et al., 2020).

Broadly speaking, urothelial carcinoma can be graded into non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). Low-grade NMIBC has a low incidence of invasion, a high frequency of recurrence and is often papillary in morphology (Babjuk et al., 2022; American Cancer Society, 2020; Viale, 2020). Several genes are frequently mutated in NMIBC, including *FGFR3*, *PPARG*, *STAG2* and *PIK3CA* (Choi et al., 2017; Hurst and Knowles, 2018; Platt et al., 2009; Solomon et al., 2013; Rochel et al., 2019). Such tumors are typically surgically removed through transurethral resection (TURBT). MIBC, on the other hand, is characterized as being 'genomically unstable' and is associated with mutations in the tumor suppressor genes *PTEN*, *RBP1* and *TP53* (Kamoun et al., 2020; Robertson et al., 2017). The frontline treatment for MIBC is radical cystectomy (removal of the bladder and parts of adjacent organs) in combination with adjuvant or neoadjuvant chemotherapy (Lobo et al., 2017). More recently, immune checkpoint inhibitors and several small molecules or antibody-drug conjugates have been approved as second- or third-line treatments (Meeks et al., 2012; Patel et al., 2020; Pietzak et al., 2019; Sonpavde and Lerner, 2007).

Throughout the past decade, a handful of independent research teams have performed extensive expression profiling that has led to the broad subtyping of 'basal' and 'luminal' subtypes that are somewhat analogous to those established for breast tumors (Choi et al., 2014; Damrauer et al., 2014; Sjodahl et al., 2012; Volkmer et al., 2012). Based on an amalgamation of the most recent sequencing experiments, the 2020 consensus publication categorized MIBC into six subtypes: luminal papillary (LumP), luminal unstable (LumU), luminal non-specified (LumNS), stroma-rich, basal/squamous (Ba/Sq) and neuroendocrine like (NE like) (Kamoun et al., 2020). Basal/squamous bladder tumors express basal cell markers (e.g. KRT5, KRT6A and KRT14) and exhibit activated EGFR signaling. These tumors frequently have *TP53* and *RB1* mutations, and are immune 'hot' (i.e. enriched for NK cells and cytotoxic lymphocytes) (Lerner et al., 2016). Luminal bladder tumors, on the other hand, express luminal urothelial markers (uroplakins and KRT20), have activated PPARG and GATA3 signaling, and exhibit mutations in *FGFR3*, RXR genes, *ELF3* and *ERBB2*, depending on subtype (Kamoun et al., 2020). In view of the heterogeneity of MIBC, further research is needed to analyze the immune characteristics, signaling activities and mutational loads of tumor subtypes in order to generate improved patient-specific therapeutic strategies.

In the field of preclinical bladder cancer research, the predominant animal model – spontaneous urothelial carcinoma – is generated via continuous exposure to the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). BBN is an alkylating metabolite of an N-nitroso compound found in tobacco smoke (Suzuki and Okada, 1980). In rodents, specific BBN metabolites are excreted in the urine and, during storage in the bladder, these agents covalently bind to the DNA within urothelial cells to create adducts that ultimately generate mutations (Fantini and Meeks, 2018; Oliveira et al., 2006; Weng et al., 2018). When introduced at low concentrations in drinking water, consistent BBN exposure leads to invasive bladder cancer in mice within 5 months (Vasconcelos-Nobrega et al., 2012). In wild-type mice, BBN-induced tumors resemble the basal/squamous subtype of bladder tumors (Fantini and Meeks, 2018).

To identify cell types that give rise to tumors, the Beachy lab performed lineage-tracing experiments using the *Shh^{CreERT2};mTmG* line to label the basal population in conjunction with a BBN-induced model of carcinogenesis (Shin et al., 2014a). They observed lineage-marked cells in MIBC lesions suggesting that basal cells, which are by far the most abundant population labeled by Shh, are progenitors that drive tumor formation (Shin et al., 2014a). More-recent lineage studies identified K14-BCs as the likely progenitor population. In this case, the Klinakis lab generated a *K14^{CreERT}* mouse in which K14-BCs are selectively labeled and used this line in lineage studies with the BBN model of carcinogenesis. They found that K14-BCs but not K5-BCs contribute to basal/squamous tumors (Papafotiou et al., 2016).

Several studies suggest that I cells can also contribute to bladder cancer. Our lab performed lineage tracing using both *K5^{CreERT2};mTmG* and *Up2^{CreERT2};mTmG* to trace the fate of basal and I cells during BBN-induced carcinogenesis. In wild-type mice, we found that basal cells contributed to muscle-invasive squamous/basal tumors, as well as to non-invasive carcinoma *in situ* (CIS), whereas I cells made an undetectable contribution to tumorigenesis (Van Batavia et al., 2014). However, in mice heterozygous for *Trp53*, I cells labeled via *Up2^{CreERT2};mTmG* contributed to papillary-like tumors (Van Batavia et al., 2014). Consistent with this, studies from the Wu lab found that *Upk2;HRAS*** mice expressing two copies of

a mutated form of *HRAS* develop papillary and/or non-muscle invasive lesions, while *Upk2;HRas;WT/*;p53fl/fl* mice that harbor a single copy of mutated HRAS in a *Trp53* mutant background develop basal/squamous MIBC (Wu, 2009; Zhang et al., 2001). Similarly, the Kim lab generated a luminal-like mouse model of bladder cancer by expressing mutant forms of *Trp53* and *Pten* under control of the *Upk3a* promoter (Saito et al., 2018). Studies performed in the Abate-Shen lab using a combination of Adeno-cre and transgenic Cre lines to drive expression of mutated *Trp53* and *Pten* in basal versus I and/or S cells observed lineage-labeled basal tumors derived from basal cells, but not from I and/or S cells (Park et al., 2021). On the other hand, our recent studies suggest that basal cells can produce luminal tumors (Tate et al., 2021). Specifically, we showed that expression of a constitutively activated form of *Pparg* (*VP16;Pparg*) in basal cells that have been activated by injury induces the formation of luminal papillary tumors, whereas expression of the *VP16;Pparg* mutant in basal cells during homeostasis induces a luminal differentiation program and exit from the cell cycle (Tate et al., 2021). Overall, these results suggest that tumors can form from both from basal and I-cell populations, depending on which mutations are present and the activation state of the population.

Conclusions

The urothelium of the bladder serves as a protective epithelial barrier. Damage to the urothelium thus compromises organ function and is a potential cause of benign urological conditions. In view of this, robust methods for restoring the urothelium are needed and constitute a major unmet need in urological care. Knowledge of the progenitor cell populations and mechanisms of specification of this tissue may ultimately guide strategies for augmenting repair in individuals prone to bladder damage.

Our understanding of the gene signatures and signaling pathways involved in urothelial development and regeneration has recently shaped methods for differentiating pluripotent stem cells into urothelium-resembling cells *in vitro*. Existing protocols for both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) rely on RA in the culture media to induce differentiation of definitive endoderm towards uroplakin-expressing urothelium (Mauney et al., 2010; Osborn et al., 2014). Inhibition of β-catenin and activation of *Pparg* signaling have been found to further augment terminal differentiation of $Upk^{+}/K20^{+}$ superficial cell types *in vitro* (Suzuki et al., 2019). A potential question for consideration, therefore, is whether such small molecules could similarly function through administration *in vivo* to augment urothelial repair.

Competing interests

The authors declare no competing or financial interests.

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