

Uroplakins in urothelial biology, function, and disease

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Urothelium covers the inner surfaces of the renal pelvis, ureter, bladder, and prostatic urethra. Although morphologically similar, the urothelia in these anatomic locations differ in their embryonic origin and lineages of cellular differentiation, as reflected in their different uroplakin content, expandability during micturition, and susceptibility to chemical carcinogens. Previously thought to be an inert tissue forming a passive barrier between the urine and blood, urothelia have recently been shown to have a secretory activity that actively modifies urine composition. Urothelial cells express a number of ion channels, receptors, and ligands, enabling them to receive and send signals and communicate with adjoining cells and their broader environment. The urothelial surface bears specific receptors that not only allow uropathogenic *E. coli* to attach to and invade the bladder mucosa, but also provide a route by which the bacteria ascend through the ureters to the kidney to cause pyelonephritis. Genetic ablation of one or more uroplakin genes in mice causes severe retrograde vesicoureteral reflux, hydronephrosis, and renal failure, conditions that mirror certain human congenital diseases. Clearly, abnormalities of the lower urinary tract can impact the upper tract, and vice versa, through the urothelial connection. In this review, we highlight recent advances in the field of urothelial biology by focusing on the uroplakins, a group of urothelium-specific and differentiation-dependent integral membrane proteins. We discuss these proteins' biochemistry, structure, assembly, intracellular trafficking, and their emerging roles in urothelial biology, function, and pathological processes. We also call attention to

important areas where greater investigative efforts are warranted.

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Urothelium is also known as 'transitional epithelium' in histology textbooks and scientific literature, largely based on the erroneous assumption that although it appears as a *stratified* epithelium, all cell layers are in direct contact with the basement membrane, a characteristic of *simple* epithelia. This notion has been contradicted, however, by electron microscopic and immunohistochemical studies, which have failed to show any upper urothelial layer/basement-membrane connection but instead delineate distinct urothelial layers. In rodents, for example, there are at least three discernable cell layers (basal, intermediate, and superficial/umbrella), whereas in higher mammals such as cattle and humans more than one intermediate layer is present. Thus, the term 'transitional epithelium' is evidently a misnomer and should be replaced by 'urothelium', as has been proposed earlier.¹ Similarly, the term 'transitional cell carcinoma' should be replaced by 'urothelial carcinoma'.²

Urothelium is one of the slowest cycling epithelia in the body with a turnover rate of ~200 days and a tritium-thymidine labeling index of ~0.01%.^{3,4} Such a remarkable durability is functionally desirable as the urothelium needs to act as a constant permeability barrier to protect the blood from toxic urinary substances. In fact, the urothelium is among the most effective barriers of any biomembrane, with a transepithelial electric resistance of up to 75,000 Ω/cm^2 , making it a far more effective barrier than the epidermis.^{5,6} Urothelium also needs to remain highly flexible throughout the micturition cycle so that it can accommodate significant changes in surface area.^{4,7,8} How does urothelium achieve a high degree of impermeability and flexibility all at once? The answer seems to rest with a membrane specialization called

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the *urothelial plaque* elaborated by the urothelium during an advanced stage of differentiation.

Urothelial plaques are rigid-looking, concave-shaped biomembrane structures that are visible only by electron microscopy. On cross-sections, the plaques (0.3–1 μm in diameter) exhibit outer membrane leaflets twice as thick as the inner ones, hence the name asymmetric unit membrane (see Porter and Bonneville⁹; Hicks¹⁰; Koss¹¹; Figure 1a, inset). These plaques occupy ~90% of the urothelial apical surface, interrupted by narrow ‘hinge’ regions (see Porter *et al.*¹⁵; Figure 1a and b). Negative staining and quick-freeze/deep-etch of the urothelial plaques revealed hexagonally arranged, 16-nm protein particles.^{12,16–20} Not only are the plaques found on the luminal surface, they are also present in great

abundance in fusiform vesicles in the cytoplasm of the superficial umbrella cells (Figure 1a and c). These cytoplasmic vesicles were thought to travel reversibly between the luminal surface and cytoplasm to adjust the apical surface area during the urothelial contraction/extension cycle,^{7,8,10,15} although whether the movement is uni- or bi-directional remains an open question.^{21,22} Despite the abundance and the striking structural features of these urothelial plaques, relatively little was known about their protein composition and their exact role(s) in urothelial physiology and diseases. In the following sections, we will review the identification and characterization of the protein components of the urothelial plaques and their biological functions and disease implications. The recently discovered secretory²³

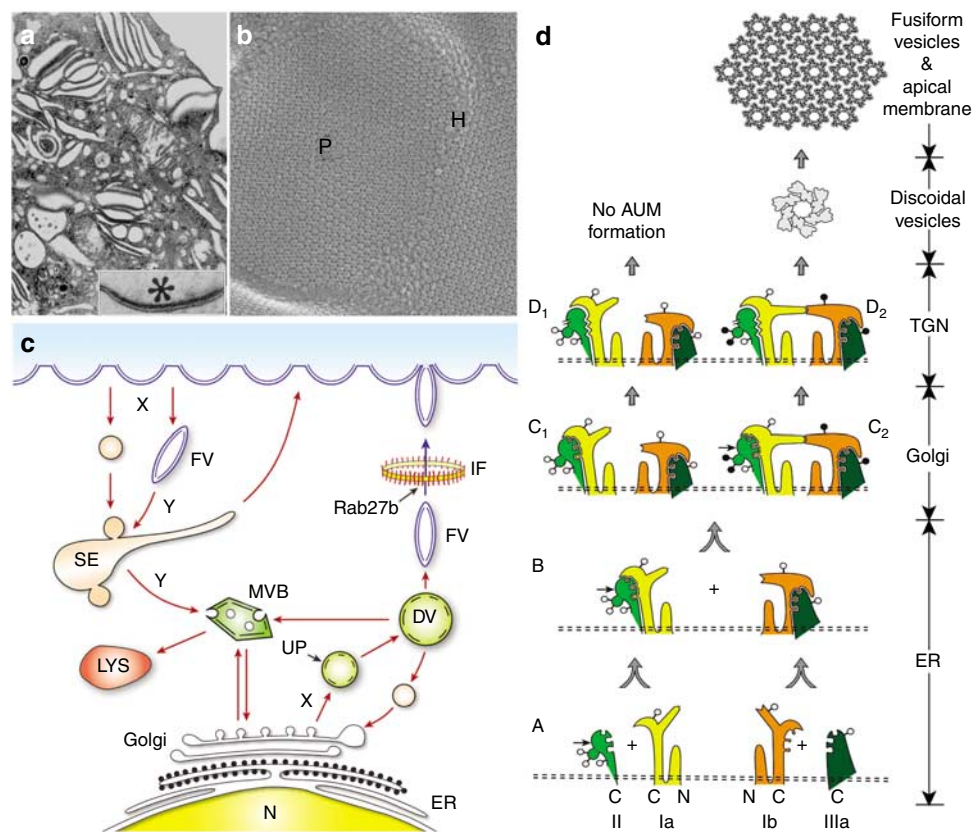


Figure 1 | Structure, assembly, and trafficking of uroplakins. (a) Transmission electron micrograph of a mouse urothelial superficial umbrella cell. Note dense clusters of fusiform vesicles (flattened disks), which most likely mature from the spherical discoidal vesicles deep in the cytoplasm. Inset shows a higher magnification view of a rigid-looking plaque showing asymmetric unit membrane. (b) Quick-freeze deep-etch image of the apical surface of a mouse umbrella cell showing urothelial plaques (P) containing hexagonal arrays of 16-nm particles interconnected by particle-free hinge (H) areas.¹² (c) Schematic diagram illustrating the vesicular traffic in umbrella cells. Uroplakin heterodimers assembled in the ER and modified in the Golgi apparatus accumulate in small vesicles budding off the *trans*-Golgi network (TGN). They form discoidal vesicles (DVs) where an assembly of crystalline arrays continues. The mature fusiform vesicles (FVs) traverse a meshwork of intermediate filaments (IF; see Veranic and Jezernik¹³) and fuse with the apical plasma membrane in a regulated fashion mediated by Rab27b. Degradation of UPs requires the formation of endocytic vesicles and/or modified FVs that form sorting endosomes (SE) and multivesicular bodies (MVB), which fuse with mature lysosomes (LYS). (d) A model depicting the assembly of the four major uroplakins (UPIa, Ib, II, and IIIa) into 2D crystals of urothelial particles. Stages A and B: The four uroplakins acquire high-mannose and complex glycans in the ER and form two heterodimers (UPIa/II and UPIb/IIIa), which undergo major conformational changes. Symbols: the small, horizontal arrows on UPII mark the furin cleavage site at the end of the prosequence; the open and closed circles represent high-mannose and complex glycans, respectively. In normal urothelium (pathway on the right), the glycans on two of the three N-glycosylation sites on the prosequence of UPII become complex glycans in the Golgi apparatus (stage C2), and the furin-mediated cleavage/removal of the prosequence in the TGN (stage D2) then triggers oligomerization to form a 16-nm particle. In cultured urothelial cells (pathway on the left), the differentiation-dependent glycosylation of pro-UPII does not occur, thus hampering the formation of the uroplakin heterotetramer and the 16-nm particle, thus no asymmetric unit membrane assembly (Adapted from Hu *et al.*¹⁴ with permission).

and neuronal properties of the urothelium and their possible roles in bladder functions have been detailed in excellent reviews^{24,25} and will not be discussed here.

UROPLAKINS ARE THE PROTEIN BUILDING BLOCKS OF UROTHELIAL PLAQUES

Although urothelial plaques are a hallmark of urothelial differentiation, earlier attempts to define their protein composition were hampered by difficulties in their purification and inability to generate antibodies to any plaque-associated proteins.^{26–28} Using sucrose gradient centrifugation coupled with detergent wash, we succeeded in isolating milligram quantities of highly purified bovine urothelial plaques.²⁹ These plaques exhibited typical 2D crystals of hexagonally packed 16-nm particles and contained four major proteins of 15, 27, 28, and 47 kDa. By generating monospecific antibodies and EM-localizing each of these proteins to the urothelial plaques *in situ*, we named these novel integral membrane proteins uroplakins Ia (27 kDa), Ib (28 kDa), II (15 kDa), and IIIa (47 kDa).^{30–32} Subsequent studies demonstrated that the urothelial plaques isolated from a wide range of species including mouse and human were ultrastructurally identical, and all contained the four uroplakins, demonstrating their high degree of conservation during mammalian evolution.^{33,34} The onset of uroplakin expression during urothelial differentiation varies, however, from species to species. In higher mammals such as cattle and human that possess a thick urothelium, uroplakins are detected primarily in the superficial umbrella cells, whereas in rodents that possess a thin 3–4 cell-layered urothelium they are found in all urothelial layers including the basal cells (see Wu *et al.*; Yu *et al.*; Mo *et al.*; Zhang *et al.*^{29,35–37}; also see the section on UPII promoter below). Such a species and/or urothelial thickness-dependent expression of uroplakins may be related to the expression patterns of certain transcription factors in different urothelial cell layers.³⁸

Among the uroplakins (UPs), UPIa, and UPIb share ~40% of their amino acid sequence, and both have four transmembrane domains (TMD) with a minor (first) and a major (second) hydrophilic domain extending extracellularly.³² These two UPs belong to the tetraspanin family comprised of many leukocyte differentiation antigens such as CD9, CD27, CD63, CD81, and CD83.^{39,40} UPII possesses an N-terminal signal peptide followed by a heavily glycosylated pro-peptide of 59 amino acids ending in an RGRR consensus substrate for furin, a *trans*-Golgi network-associated processing enzyme. The mature UPII is not glycosylated and has only a single TMD located close to its C terminus, with very little cytoplasmic domain.³¹ Similar to UPII, UPIIIa has a single TMD, which divides the protein into a long (189 residue) N-terminal luminal domain and a C-terminal (52 residue) cytoplasmic domain.³⁰ The N-terminal luminal domain is heavily glycosylated with 20-kDa equivalents of complex-type carbohydrates, whereas the cytoplasmic domain harbors several potential serine/threonine and tyrosine phosphorylation sites. Interestingly, UPIIIa shares a stretch of

about 12 amino acid residues, located on the N-terminal side of the single TMD, with a similarly located domain in UPII suggesting that these two UPs are related.^{30,31} Because UPIIIa is the only UP that possesses a significant cytoplasmic domain, this raises the interesting possibility that UPIIIa may be involved in anchoring the urothelial plaques to the underlying cytoskeleton⁴¹ and that its C-terminal phosphorylation may be involved in regulating such an association.³⁰ It is notable that, overall, the mass of extracellular domains of UPs greatly exceeds that of their cytoplasmic domains (Figure 1d). Such an asymmetric mass distribution of UPs across the lipid bilayer explains the TEM observation that the outer leaflets of urothelial plaques are almost twice as thick as the inner ones.^{30–32}

Three lines of evidence suggest that the four major UPs form two specific pairs. First, treatment of purified bovine urothelial plaques with bifunctional crosslinking reagents led to the crosslinking of UPIa and UPIb, the two tetraspanin-related uroplakins, to UPII and UPIIIa, respectively, suggesting the existence of UPIa/II and UPIb/IIIa heterodimers.⁴² Second, ion exchange chromatography co-purified UPIa with II and UPIb with IIIa.⁴³ Third, transfection of 293T cells with single uroplakin cDNAs resulted in UPs being trapped in the ER (except UPIb which can exit by itself). In contrast, double transfection of UPIa/II or UPIb/IIIa enabled the heterodimers to reach the cell surface.⁴⁴ These results clearly indicate that the formation of UPIa/II or UPIb/IIIa heterodimers is the first step of UP assembly that is required for ER-exit (see Hu *et al.*¹⁴; Tu *et al.*⁴⁴; Figure 1d).

FORMATION OF 2D CRYSTALS BY UPs PROVIDES STRUCTURAL INSIGHTS INTO THESE PROTEINS

The existence of UPs in naturally occurring 2D crystals of the urothelial plaques has offered unique opportunities for in-depth structural studies. Quick-freeze deep-etch,¹² negative staining coupled with image processing,^{17–20} cryo-EM,^{45–47} and atomic force microscopy⁴⁸ resolved each 16-nm particle into six inner and six outer subdomains forming two concentric rings. One inner and one outer subdomain interconnect to form a subunit – one sixth of the hexagonal particle. Cryo-EM 3D reconstructions revealed that each subdomain contains a total of 5 transmembrane helices, corresponding to one tetraspanin and a single-pass uroplakin (see Min *et al.*^{46,47}; Figure 2). EM localization using the *E. coli* FimH adhesin, which specifically binds to UPIa, indicates that the UPIa/II pair occupies the inner subdomain, whereas the UP Ib/III pair occupies the outer subdomain (see Min *et al.*⁴⁸; Figures 2 and 3a and b). The four transmembrane helices of UPIa and UPIb are tightly packed into bundles aligned with the extracellular domains, giving these two UPs an overall cylindrical shape. The single-pass UPII and UPIIIa adopt an inverted ‘L’-shape, anchored by its transmembrane helix packed against the four transmembrane helices of their partner tetraspanin UPs, thus forming a five-helix bundle within each subdomain. The long arm of the inverted ‘L’ continues up against the cylindrical UP tetraspanins.

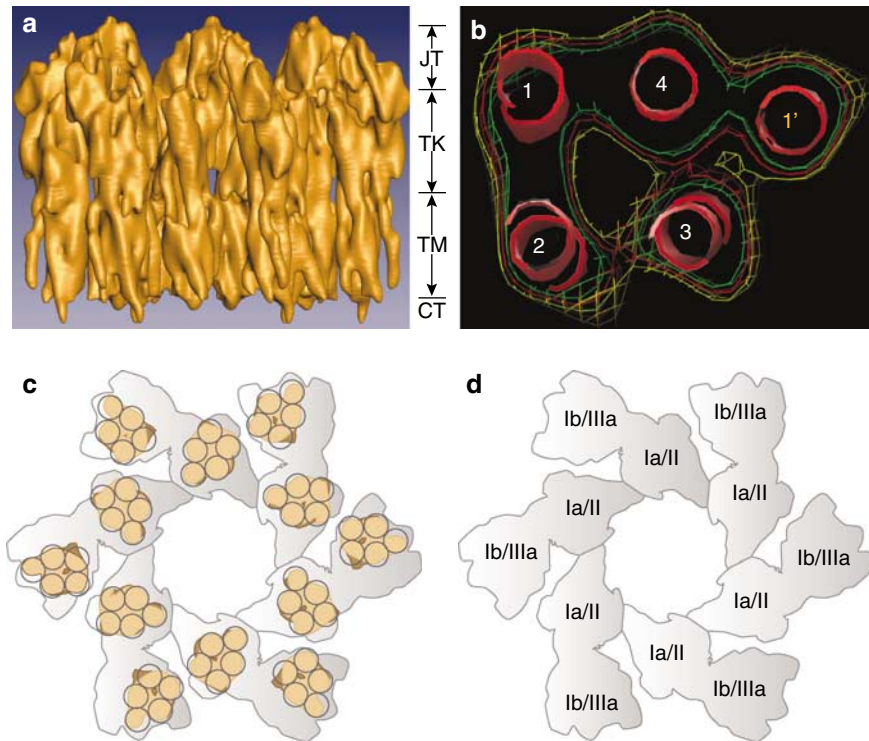


Figure 2 | Structure of the 16-nm uroplakin particle. (a) The side view of the cryo-EM structure of the 16-nm mouse uroplakin particle at 6 Å resolution. The vertical dimension of the particle can be divided into four regions: the joint (JT), the trunk (TK), the transmembrane region (TM), and the cytoplasmic region (CT). (b) Electron densities of the transmembrane region of an inner subdomain of the particle, showing a five-helix bundle formed by the transmembrane helices from the tetraspanin UPIa (helices 1–4) and the single transmembrane domain from UPII (helix 1'). (c) Positions of the transmembrane helices in the 16-nm particle (yellow). (d) A model showing that the inner and outer subdomains of the particle are formed by the UPIa/II and UPIb/IIIa pair, respectively (see text). Panels a and b are reprinted with permission from Min *et al.*⁴⁶.

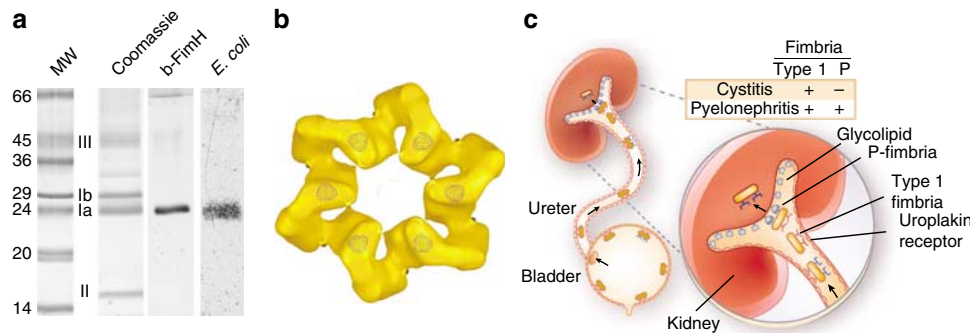


Figure 3 | Identification of uroplakin Ia as bacterial receptor and its role in urinary tract infection. (a) The four major mouse uroplakins (Ia, Ib, II, and IIIa) were resolved by SDS-PAGE, electroblotted (lane 2) and incubated with biotinylated, bacterial FimH/C complex (lane 3) or with type 1-fimbriated *E. coli* that were metabolically labeled with ³⁵S-methionine (lane 4). Note the specific binding of FimH and the uropathogenic *E. coli* to UPIa. MW: molecular weight standards. (b) Localization of the FimH binding site to the six inner subdomains of the mouse 16-nm uroplakin particle, based on the difference map of the mouse urothelial plaques that have been negatively stained with and without a saturating amount of FimH. (c) Schematic model of ascending UTI in mice by uropathogenic *E. coli*. *E. coli* expressing the type 1 fimbriae can attach to the uroplakin receptor and cause bladder infection (cystitis). Although these type-1 positive bacteria can bind to the uroplakin receptor on the ureteral urothelial surface and ascend to the kidneys, they usually do not cause kidney infection (pyelonephritis) because they lack P fimbria, which is required for binding to the kidney's glycolipids. In contrast, *E. coli* expressing both type 1 and P fimbriae can not only ascend to the renal pelvis (RP), but also bind to and invade the renal parenchyma, causing pyelonephritis. Once there, the expression of type 1 fimbria can sometimes be turned off by phase variation. Panels a and b are reprinted with permission from Min *et al.* and Zhou *et al.*,^{48,49} respectively.

The short arm of the 'L' extends to join the short arm of the other inverted 'L' from the paired subdomain within the same subunit, thus forming a 'joint'.⁴⁶ Interestingly, the joint

provides the only contact between the two subdomains within a subunit, while the two tetraspanins, UPIa and UPIb, do not appear to have any direct contact.⁴⁶ This type of loose

connection between the two subdomains within a subunit suggests a flexible interaction between the inner and outer subdomains, thus providing a basis for possible structural changes of UPs upon binding to the *E. coli* FimH adhesin.

ASSEMBLY OF UPs INTO 2D CRYSTALS IS A DYNAMIC AND HIGHLY REGULATED PROCESS

A crucial step in elucidating the biological functions of urothelial plaques is to understand how UPs are assembled into higher order protein complexes and how the resultant urothelial plaques are delivered to the urothelial apical surface. Transfection studies showed that, immediately after their synthesis in the ER, the four uroplakins acquire high-mannose glycans and form two heterodimers (UPIa/II and UPIb/IIIa).⁴⁴ Recent data suggest a model in which major conformational changes occur during UP dimerization leading to a more stable protein complex that can exit from the ER.¹⁴ The glycans on two of the three N-glycosylation sites of the pro-peptide of UPII become complex glycans in the Golgi apparatus, causing conformational changes in pro-UPII and its partner UPIa and thereby allowing heterotetramer formation (see Hu *et al.*^{14,50}; Figure 1d). Furin-mediated removal of the pro-peptide in the TGN then triggers oligomerization to form a 16-nm particle in which UPIa/II and UPIb/IIIa are associated with the inner and outer subdomains, respectively,⁴⁸ and to later form 2D crystals (Figure 1d). Interestingly, in cultured bovine urothelial cells, the differentiation-dependent glycosylation of pro-UPII does not occur, thus hampering the formation of the UP heterotetramer and the 16-nm particle.^{44,51}

While UPII and UPIIIa are transported to the plasma membrane only after heterodimerization with UPIa and UPIb, respectively, UPIb when expressed alone can exit the ER and move to the plasma membrane. In contrast, the structurally closely related UPIa by itself remains trapped in this compartment.^{44,52} This may explain why UPIb is the only UP that is expressed by itself in tissues other than the urothelium, namely the cornea, conjunctiva, and possibly the lung.^{53,54} The finding that UPIb can exit from the ER without forming a heterodimeric complex with UPIIIa is also consistent with the results from UPIIIa-knockout mice where UPIb can be detected at the urothelial cell surface.⁵⁵

The identification of specific UPIb domains that are required for this tetraspanin to exit the ER was greatly facilitated by swapping UPIb domains with its closely related tetraspanin UPIa. As it turns out, the four TMDs of UPIb do not simply provide hydrophobic anchors; they contain sequence and structural information that affects intra-TMD interactions, which are critical for the transport of UPIb from the ER to the Golgi apparatus.⁵² Mutations in such TMDs cause UPIb to aggregate, perhaps due to interactions with chaperone-like proteins that recognize improperly assembled TMDs resulting in their retention in the ER.^{56,57} However, deletion of the N-linked oligosaccharide from UPIb or elimination of the disulfide bridges does not result in the ER-retention of UPIb. Therefore, these two post-translational

modifications are apparently not essential for the proper folding of the large loop of UPIb.⁵² This is in striking contrast to studies on other tetraspanins such as CD81 and CD82, which after reduction of their disulfide bridges, can no longer exit from the ER.⁵⁸

How are pre-assembled crystalline arrays of UPs delivered to the urothelial apical surface? We have attempted to address this question by investigating the possible role of Rab proteins. Using RT-PCR, we identified in urothelial cells 12 Rab or Rab-related proteins: Rab4, Rab5, Rab8, Rab11, Rab13, Rab15, Rab27b, Rab28, Rab32, RhoA, RhoC, and Ras1.⁵⁹ Although most Rabs were broadly distributed, Rab27b was exceptionally enriched in urothelium, representing as much as 0.1% of the total urothelial proteins. Furthermore, on Western blots, only Rab27b, but not its isoform Rb27a, was detected in urothelium.⁵⁹ The abundance of Rab27b in urothelium and its differentiation-dependent co-expression with the UPs raise the interesting possibility that the expression of Rab27b and the formation of UP-containing fusiform vesicles are highly coordinated, and that Rab27b may play a role in the delivery of urothelial plaques to the plasma membrane (see for example, Tiwari *et al.*⁶⁰). Consistent with this idea, we showed through immuno-EM that Rab27b was associated with the UP-containing fusiform vesicles.⁵⁹ These results indicate that Rab27b is involved in targeting UP-containing fusiform vesicles to the plasma membrane of urothelial umbrella cells (Figure 1c). Recent results indicate the involvement of an additional Rab protein, Rab11a, in the exocytosis of urothelial plaques.⁶¹

UROTHELIA OF DIFFERENT ANATOMIC LOCATIONS REPRESENT DIFFERENT CELL LINEAGES

Although the term 'urothelium' is ordinarily used to describe the epithelial lining of both upper and lower urinary tracts, recent data indicate that urothelia at different anatomical sites are ultrastructurally and biochemically different due to intrinsic divergence acquired during development.⁶² For instance, ureteral urothelium contains fewer cytoplasmic fusiform vesicles and less uroplakin proteins per total cellular proteins than does the bladder urothelium.⁶²⁻⁶⁴ Importantly, this difference in uroplakin content persists even after the bovine bladder and ureteral urothelial cells have been serially subcultured under identical cell culture conditions using lethally irradiated 3T3 cells as a feeder layer. Moreover, such cultured bovine bladder urothelial cells exhibit a higher growth rate than the ureteral urothelial cells. These results indicate that the observed phenotypic differences cannot be due to extrinsic regulation.⁶⁵⁻⁶⁸ Feeding vitamin A-deficient diet to mice induces bladder urothelial keratinization (bladder metaplasia) that originates not from the bladder dome but from the bladder neck/proximal urethra (see Liang *et al.*⁶²; Figure 4a). Combined with the fact that renal pelvis/ureteral urothelium is mesoderm-derived, whereas the bladder/urethral urothelium is endoderm-derived,^{69,70} these observations suggest that urothelium consists of at least three cell lineages, that is, (i) renal pelvis/ureter, (ii) bladder and

(iii) bladder neck/proximal urethra.⁶² Therefore, to use the general term ‘urothelium’ without specifying its anatomic location can be confusing and misleading. The heterogeneity of urothelium has implications about the cellular basis of urothelial metaplasia (Figure 4b–f), and raises questions about the common practice of using human ureteral urothelium, which is readily available from surgical specimens, as a surrogate for adult human bladder urothelium, which is more difficult to obtain and to grow in culture. Specifically, autologous bladder urothelial cells provide an ideal source for bladder reconstruction.⁷¹ Ureteral urothelial cells may be used as a substitute for bladder urothelial cells, although these two cell types may have different functional and biomechanical properties.^{72,73} Finally, urothelia at different locations appear to respond differently to various

carcinogens. Although experimental carcinogens like *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine or *N*-nitrosomethylurea cause primarily urothelial carcinomas of the bladder,⁷⁴ aristolochic acid, a mycotoxin contaminant in wheat and a component in certain Chinese herbal products, induces urothelial carcinomas of only the renal pelvis and the upper ureter.^{75,76} It will be very interesting to determine whether intrinsic properties underlying the urothelial heterogeneity are responsible for the varied susceptibilities of the urothelia of different anatomic sites to different carcinogens.

GENE INACTIVATION REVEALS IMPORTANT PHYSIOLOGICAL FUNCTIONS OF UPs

A strategy was taken to ablate mouse genes encoding UPII or UPIIIa because inactivating these two UPs should abolish the formation of UPIa/II and UPIb/III pairs, respectively.^{55,77} Knockout of the UPIIIa gene led to the loss of about 70–80% of the urothelial plaques of the urothelial apical surface.⁵⁵ The retention of some plaques in the UPIIIa knockouts may be due to the presence of a minor UPIII isoform, UPIIIb, which was identified in a bovine urothelial subtraction cDNA library.⁷⁸ However, ablation of the UPII gene, which does not appear to have an isoform, led to the complete loss of urothelial plaques.⁷⁷ These results establish UPs as the integral protein subunits of the urothelial plaques.

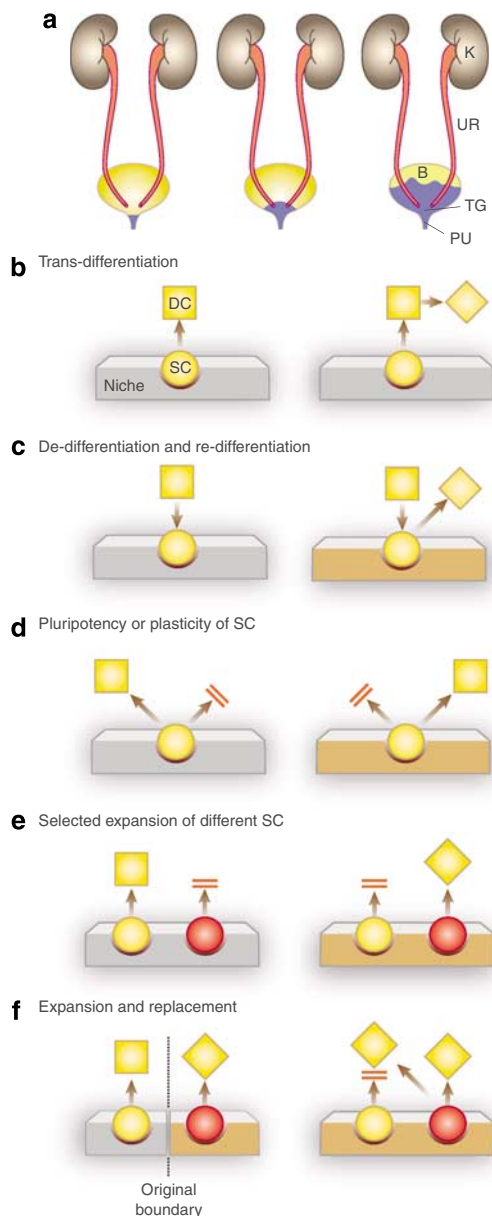


Figure 4 | Models of squamous metaplasia of the mouse urothelium induced by vitamin A deficiency. (a) Schematic diagram showing gradual expansion of the vitamin A deficiency-induced squamous metaplasia from the proximal urethra (PU) and the trigone (TG) region to the rest of the bladder (B). UR, ureter; K, kidney. (b–f) Models for various cellular mechanisms of bladder metaplasia. (b) The transdifferentiation model: A terminally differentiated cell (DC; square) can directly transform into a different kind of terminally differentiated cell (diamond). (c) The dedifferentiation-and-redifferentiation model: A terminally differentiated cell can revert back to an undifferentiated or stem cell (SC), which can then differentiate along a different pathway yielding a distinct phenotype in response to environmental and/or mesenchymal changes (beige). (d) The pluripotent stem cell model: Under normal conditions, the pluripotent stem cells give rise to terminal differentiated cells of a particular phenotype; mesenchymal changes may induce such a stem cell to undergo an alternative pathway of differentiation. (e) The selective expansion model. The tissue contains two separate populations of stem cells: one of them (yellow) normally gives rise to the main phenotype, whereas the other lies dormant. Mesenchymal and/or environmental changes, including alterations of the stem cell niche, suppress the growth and differentiation of the originally dominant stem cell while activating the originally dormant stem cell (red) that now gives rise to a different phenotype. (f) The expansion and replacement model. The tissue contains two separate cell lineages that occupy different domains separated by well-defined boundaries. Mesenchymal and/or environmental changes such as vitamin A deficiency favor the expansion of one cell lineage over another, thus allowing one cell type to expand and invade another cell lineage’s domain. This last model can best explain the existing data on urothelial keratinizing squamous metaplasia that is induced by vitamin A deficiency. (d–f) The parallel red bars denote that the process/pathway is blocked. SC, stem cell (adapted from Liang *et al.*⁶²).

Physiological measurements of bladder mucosa using modified Ussing chambers showed that the permeability of the UPII- or UPIIIa-deficient mouse urothelium to ^{14}C -urea and ^3H -water was significantly higher than that of the wild-type mice, suggesting that uroplakin plaques contribute to the urothelial permeability barrier function.^{55,77,79} It is likely that under normal conditions the crystalline network of UPs imposes structural constraints on the lipid molecules, limiting their ability to undergo lateral diffusion and thus enhancing the barrier function.^{6,47,79} Together, these results indicate that the UP-containing urothelial plaques, in conjunction with the lipids and the tight junctions, play an important part in the urothelial permeability barrier function.^{5,51,80} It is worth noting that increased urothelial permeability is a common manifestation in interstitial cystitis, an excruciatingly painful bladder syndrome.^{81–83} Whether defects of UPs and their associated proteins, possibly including the ‘glycocalyx’ components,^{82,84} are involved in the pathogenesis of this important disease remains to be seen.

Other prominent features in mice lacking UPII, UPIIIa or both are severe vesicoureteral reflux (VUR), hydronephrosis and impaired renal function.^{55,77} The retrograde overflow of urine into the upper tract appeared to result from enlarged ureteral orifices in the knockout mice.⁵⁵ In an attempt to determine whether UP defects might be involved in human VUR, we examined a panel of 76 well-documented VUR patients and 90 race-matched controls for single nucleotide polymorphisms of all four major UPs. Most single nucleotide polymorphisms found were not significantly associated with VUR, although several single nucleotide polymorphisms were marginally associated.⁸⁵ The fact that no truncation or frame shift mutations were found in any of the VUR patients coupled with our recent finding that some breeding pairs of UPIIIa and UPII knockout mice yielded litters that die neonatally,^{55,77} raises the intriguing possibility that major UP mutations are not tolerated in humans. By analyzing a few single nucleotide polymorphisms of only the UPIIIa gene, others have come to a similar conclusion.^{86,87} More recent data indicate that certain UPIIIa mutations can be correlated with human renal hypodysplasia and adysplasia, which can lead to renal failure.^{88,89}

UPs PLAY A ROLE IN *E. coli* ADHESION, INVASION, AND UPPER TRACT DISSEMINATION

The first and essential step for uropathogenic *E. coli* (UPEC), which causes ~85% of all urinary tract infections, to colonize the bladder is for the bacteria to adhere to the urothelial surface by specific interactions between UPEC’s FimH adhesin and the urothelial surface’s mannosylated glycoprotein(s).^{90–95} However, despite much conjecture, the identity of such urothelial receptors had long been elusive. We tested whether some of the glycosylated UPs, as the principal protein components that account for over 90% of the urothelial surface, can serve as the urothelial receptor(s) for type 1-fimbriated *E. coli*. We discovered that UPEC strains

expressing the type 1-fimbriae, but not those expressing P-fimbriae or no fimbriae, bound to purified urothelial plaques in large numbers, in a saturable and species-conserved manner.⁹⁶ The binding was completely abolished by pre-incubating type 1-fimbriated *E. coli* with D-mannose. Gel-overlay assay revealed that the type 1-fimbriated *E. coli* bound specifically to protein bands in the UPIa/Ib region, without binding to UPII and III. The fact that the highly glycosylated UPIIIa did not interact with type 1-fimbriae suggested that only UPIa/Ib contained unmodified, terminal mannose moieties that represent the structural fit for the FimH-binding pocket. Indeed, endo-H treatment, which removes the high mannose residues, or the addition of D-mannose, completely abolished the *E. coli*-UPIa/Ib binding. These results provided the first experimental evidence indicating that the urothelial surface contains a principal mannosylated glycoprotein (UPIa/Ib) that is capable of interacting with type 1-fimbriated *E. coli*.⁹⁶

This conclusion was further refined using mouse UPs and recombinant FimH/FimC as a probe. Unlike bovine UPIa and UPIb, which were not well resolved on SDS-PAGE because of their close molecular masses, the mouse counterparts were well separated, allowing the conclusive identification of mouse UPIa as the sole binder for FimH (see Zhou *et al.*⁴⁹; Figure 3a). Additional studies of the glycomoiety of UPIa and UPIb established that only UPIa contains high mannose glycans that are capable of interacting with FimH, making it the sole urothelial receptor for FimH *in vitro*.^{49,97} Glycopeptide analysis also established Asn169 of UPIa as the site that bears the Man(6)GlcNAc(2). Importantly, this glycomoiety is conserved in UPIa isolated from mouse, bovine, and human bladder urothelia.⁹⁷

Mounting evidence suggests that the interaction of UPECs with the urothelial surface is critical not only for adhesion, but also for triggering a cascade of events that lead to bacterial invasion into the urothelial cells.^{98,99} UPECs appear to exploit the membrane trafficking machinery that normally exists in the urothelial cells. Abraham and co-workers recently showed that UPECs initially reside in the Rab27b-positive, UP-containing, fusiform vesicles.¹⁰⁰ Once inside the urothelial cells, UPECs are insulated from the attack of host immunity and are resistant to antibiotic treatment. Hultgren and co-workers showed that the intracellular UPECs can not only survive, but also proliferate, forming so-called intracellular bacterial communities.^{101,102} Some of these intracellular bacterial communities can either stay dormant forming ‘quiescent intracellular reservoirs’ or break out of the urothelial cells to seed a new round of infection. Recent evidence indicates that intracellular bacterial community formation can occur in many mouse strains and is reproducible with different clinical *E. coli* isolates from human UTIs.¹⁰³ More importantly, desquamated urothelial cells from UTI patients harbor intracellular bacterial communities.¹⁰⁴ The discovery of UPECs as intracellular pathogens is highly significant because it reveals a potentially important mechanism for recurrent UTIs. Thus, instead of

re-inoculation of the urinary system with *E. coli* from the peri-urethral region, UPECs causing recurrent UTIs could be derived from those inhabiting the urothelial cells between active infections. Clearly, this concept, if further explored, can have a major impact on devising novel treatment strategies for recurrent UTIs.

In addition to its role in mediating urothelial adhesion and invasion within the confines of the bladder, the binding of UPECs to UPIa may also facilitate the ascent of the bacteria into the kidney to cause upper tract infections (Figure 3c). This scenario is based on the fact that UPIa is also present on the surfaces of the ureteral and renal pelvic urothelia.^{62,64,85} The interactions of type 1-fimbriae with UPIa in these anatomic locations may help the bacteria resist the urine flow and enable them to migrate upwards (see Wu *et al.*⁹⁶; Figure 3c), with the help of bacterial motility apparatus such as flagella.¹⁰⁵ Most UPECs that cause pyelonephritis express both type 1 and P fimbriae.¹⁰⁶ Although type 1 fimbriae are crucial for cystitis, P fimbriae seem dispensable in the lower urinary tract, perhaps due to the lack of specific receptors for P fimbriae on the urothelial cells.^{107–109} Once attaching to the renal papillary epithelium and invading into the kidney parenchyma where nutrients are more abundant, the expression of type 1 fimbriae ceases due to a transcriptionally controlled process called ‘phase variation’.¹¹⁰ There, P fimbriae can bind to the globo-series of the glycolipids expressed by the renal tubular cells.^{111–113} Epigenetic control of P fimbrial expression can also suppress type 1 fimbrial expression through cross-talk that is not yet completely understood.¹¹⁴ Taken together, the discrete uroplakin/glycolipid receptor expression at different anatomic locations within the urinary tract, as depicted in Figure 3c, can explain the ability of UPECs to spread from lower to upper tract as well as the highly characteristic fimbria expression patterns of the UPEC strain isolates from cystitis and pyelonephritis patients (Figure 3c).

UPs ARE USEFUL MARKERS FOR DIAGNOSIS, DETECTION, AND PROGNOSTIC PREDICTION OF UROTHELIAL CARCINOMAS

As discussed in the preceding sections, UPs are expressed abundantly by the normal urothelium, but are undetectable in non-urothelial tissues in humans. This urothelium-specificity is by and large maintained after urothelial transformation, as UPs are readily detected by immunohistochemistry in urothelial carcinomas, but not in non-urothelial tumors.^{115–117} The detection rate in urothelial carcinomas has been fairly consistent among different cohorts, ranging from 50 to 60%. Somewhat surprisingly, UP expression is not strictly correlative with low pathological grade, despite their being the terminal differentiation products of normal urothelium.^{118,119} About half of the muscle-invasive urothelial carcinomas and lymph node metastases retain UPs. The clinico-pathological implications of UP detection are manifold. *First*, although a lack of UP expression does not rule out a urothelial carcinoma, positive identification of UPs is strongly indicative of a carcinoma of

urothelial origin. UPs can therefore be of significant value, particularly when combined with other urothelium-restricted markers, such as CK20, in the differential diagnosis of poorly differentiated pelvic carcinomas whose sites of origin are in question.^{115,118,120–124} *Second*, UP detection by RT-PCR is more sensitive than histopathology for detecting local tissue and lymph node spread of urothelial carcinoma cells. Copp *et al.*¹²⁵ observed that, among the pathologically node-negative patients, 33% turned out to be node-positive upon UPII-based RT-PCR and in 91% of these PCR-positive cases the urothelial carcinomas recurred. Thus, UP identification by RT-PCR appears to be an excellent tool for aiding pathologists to detect lymph node metastasis. *Third*, detection of UP mRNAs in the circulation by RT-PCR could be an indication of micro-metastatic spread of urothelial carcinomas into the bloodstream.^{126–130} In extending this concept, the UP status in the peripheral blood could be used to monitor the efficacy of chemotherapeutic regimens, although prospective studies establishing this utility have yet to be carried out. Overall, challenges remain with RT-PCR regarding its sensitivity and specificity. Nested PCR and increased cycles both improved the sensitivity, whereas inevitably compromising the specificity. Genomic DNA contamination of the RNA samples, a universal problem associated with the use of commercial kits for RNA isolation, can lead to false positive results, although digestion of DNA in the RNA samples and using PCR primers on separate exons can alleviate some of these problems. Like other tissue-specific markers, it requires refinement and standardization before the RT-PCR detection of UPs can be employed reliably in a clinical setting. *Finally*, we have recently shown by using immunohistochemistry of arrayed human urothelial carcinomas that the absence of UPs expression was significantly associated with advanced pathologic stage, lymph node metastases, disease recurrence and bladder cancer-specific mortality in univariate analyses.¹¹⁹ The UP status is independent of cell cycle regulators p53, pRb, p27, and cyclin D1, and can therefore be a useful adjunct marker to predict the prognosis of patients with advanced urothelial carcinomas.

MOUSE UPII PROMOTER PROVIDES AN *IN VIVO* PLATFORM FOR STUDYING BLADDER TUMORIGENESIS

A major by-product from characterizing UPs was the successful isolation of the mouse UPII promoter.¹³¹ Although this promoter functions both in bladder and ureteral urothelia, it apparently is much more active in the former so that it has become an excellent tool for driving bladder-specific gene expression and interrogating the divergent molecular pathways of bladder tumorigenesis. This transgenic approach would not have been feasible, had the UPII promoter been active only in terminally differentiated urothelial umbrella cells, which would be highly refractory to oncogenic transformation. Fortunately, unlike the *human* urothelium, UP expression in *mouse* urothelium has a much earlier onset, beginning in the basal layer.³⁶ The ability to

drive gene expression even in the urothelial basal layer is an important requirement for tumorigenesis, because this layer is where the stem cells most likely reside and oncogenic transformation originates.

Using the mouse UPII promoter to express activated oncogenes and/or inactivate specific tumor suppressor genes in the urothelium, we have systematically evaluated whether specific molecular defects are capable of driving bladder tumorigenesis along divergent phenotypic pathways (Figure 5a and b). We discovered that activation of the ras pathway, given sufficient gene dosage, induces exclusively low-grade,

non-invasive superficial papillary bladder tumors (see Mo *et al.*¹³²; Figure 5b). Molecularly, the over-activation of Akt and Stat3/5 and the functional inactivation of PTEN by C-terminal hyper-phosphorylation seem crucial for triggering the nodular urothelial hyperplasia and low-grade papillary bladder tumors (see Mo *et al.*¹³²; Figure 5c). The onset and time course of these lesions are, however, not affected by the concurrent deficiency of p16Ink4a and p19Arf – an event found to synergize with ras activation in a wide range of tissues.^{133–135} These results provide compelling experimental evidence indicating that deficiency of the

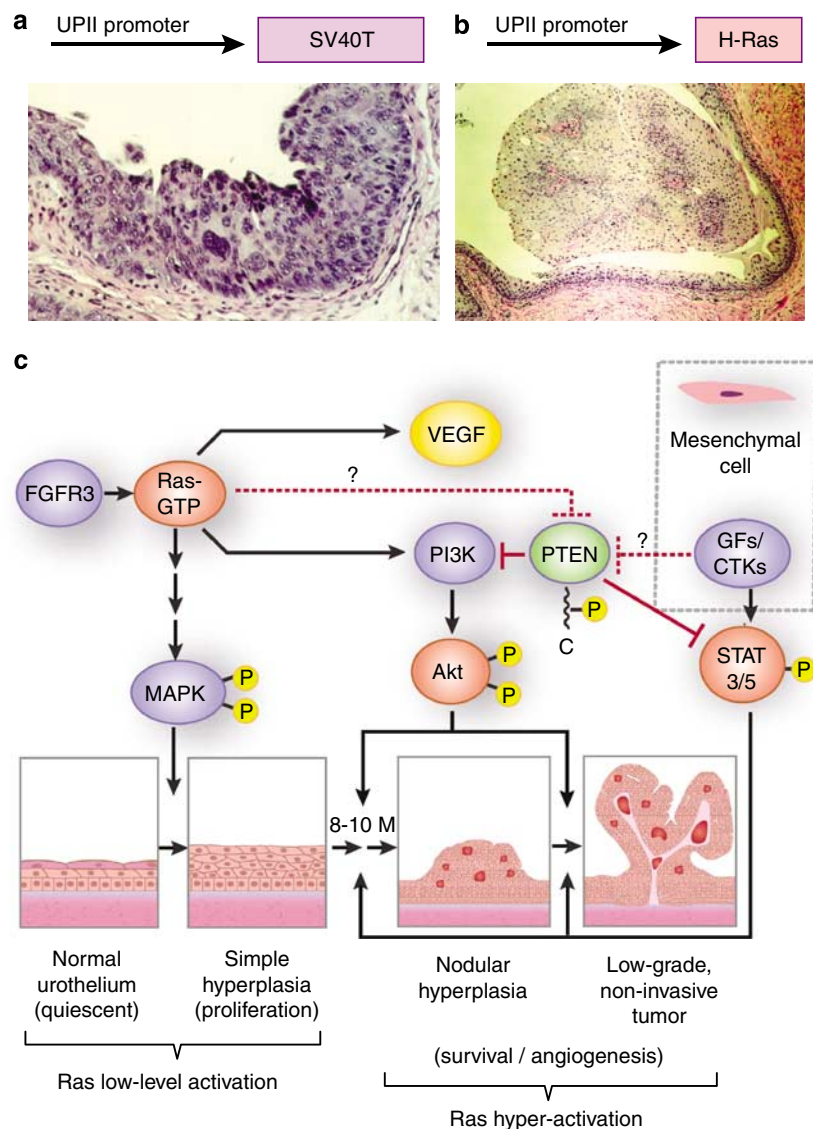


Figure 5 | Divergent molecular pathways of urothelial tumorigenesis. Transgenic mouse models recapitulate the two main phenotypes of urothelial carcinomas. Whereas urothelial expression of SV40 large T antigen elicits exclusively high-grade, flat urothelial lesions (a) bearing strong resemblance to carcinoma *in situ* of the human counterparts, urothelial expression of activated Ha-ras induces exclusively low-grade, non-invasive superficial papillary urothelial tumors (b). (c) Dosage-dependence of ras activation in triggering urothelial tumorigenesis. Although low-level ras activation activates the MAPK pathway resulting in urothelial hyperplasia, it is insufficient to induce tumorigenesis. Hyperactivation of ras can, however, activate the PI3K/AKT and STAT3/5 pathways, leading to nodular urothelial hyperplasia and low-grade, superficial papillary tumors. The activation of these two pathways may also be facilitated by the functional inactivation of PTEN through its C-terminal hyper-phosphorylation. Fibroblast growth factor receptor 3, whose mutations occur in up to 80% of the low-grade, superficial papillary tumors in humans, may trigger this urothelial tumor variant through similar signal mechanisms (adapted from Mo *et al.*¹³²).

INK4a gene, long thought to be a critical event in urothelial tumor initiation,¹³⁶ is unnecessary for urothelial tumor initiation; and that hyper-activation of *ras per se* is sufficient to trigger urothelial tumors. The results obtained in mice are highly relevant to humans because *ras* activation through point mutations and overexpression occurs in 30 and 50%, respectively, of the human low-grade, superficial papillary bladder tumors.^{137,138} Interestingly, mutations of fibroblast growth factor receptor 3, which can activate the *ras* signaling pathways, are found in up to 70% of these papillary tumors.^{139–141} Given the fact that *ras* and fibroblast growth factor receptor 3 mutations are almost always mutually exclusive,¹⁴² perhaps reflecting the fact that they function in the same signaling pathway, there is strong reason to believe that *ras* pathway activation, through *ras* mutation/overexpression or fibroblast growth factor receptor 3 mutation/overexpression, occurs in an overwhelming majority of the low-grade, superficial papillary bladder tumors in humans (see Mo *et al.*¹³²; Wu¹³⁸; Figure 5c). Finally, patients with Costello syndrome, a genetic disease caused by germline mutations in the *Ha-ras* gene, frequently develop low-grade papillary bladder tumors,¹⁴³ further supporting the relevance of *ras* pathway activation in this type of bladder tumors.

Contrary to the *Ha-ras* mutation, which consistently induces low-grade papillary bladder tumors, urothelial expression of a Simian virus 40 large T antigen (SV40T) elicited exclusively high-grade, flat urothelial carcinomas that strongly resembled carcinoma *in situ* in humans (see Zhang *et al.*³⁷; Figure 5a). Over time, these carcinoma *in situ*-like lesions evolve to high-grade papillary tumors that are occasionally muscle-invasive and metastatic to lymph node, liver, and lungs.¹⁴⁴ In support of our findings, Sandgren and co-workers showed by using a keratin 5 promoter to drive the SV40T expression in urothelium and other epithelial tissues, that bladder-invasive tumors were a consistent finding.¹⁴⁵ Together, these results suggest that SV40T-mediated functional inactivation of the p53 and Rb tumor suppressors play a critical role in bladder tumorigenesis along the high-grade, invasive pathway. Interestingly, in humans, defects affecting both p53 and Rb are rare in low-grade, superficial papillary bladder tumors but occur in over half of the high-grade, invasive bladder carcinomas.^{146–148} Although the SV40T data from the transgenic mice are strongly supportive of the human data on p53 and Rb, one needs to be cautious because SV40T exerts wider oncogenic effects than simply inactivating p53 and Rb.^{149,150} For this reason, specific inactivation of both p53 and Rb genes in urothelium should provide more insightful information regarding the role of these two genes in triggering muscle-invasive bladder tumors. Recently, we have developed a Cre-recombinase based system that allows urothelium-specific ablation of genes flanked by the loxP sequences.³⁶ With this system, we have been able to inactivate p53 or Rb or both in the urothelium, thus avoiding the embryonic lethality and premature death associated with the global knockout of these two genes. Experiments are underway to determine whether p53 and/or Rb deficiency

is sufficient to trigger invasive bladder tumors. Overall, the UPII promoter-based transgenic and knockout systems have been instrumental in defining the *in vivo* roles of genetic alterations in bladder tumor initiation and progression. Given their resemblance to the human counterparts and their highly predictable time course and biological behavior, the transgenic models of bladder tumors will also be useful for evaluating novel preventive and therapeutic strategies.¹⁵¹

SUMMARY AND PERSPECTIVES

Over the last two decades, the field of urothelial biology has seen considerable advances in understanding how the urothelial cells differentiate to perform their unique biological functions. We now know that uroplakins are the essential structural components of the urothelial apical surface whose deficiency compromises the urothelial permeability barrier and leads to global urinary tract anomalies. The assembly of UPs and subsequent translocation of the urothelial plaques from the cytoplasm to the apical surface are a highly coordinated and regulated process. As one of the few known urothelium-specific markers that are retained by most of its malignancies, UPs can serve as excellent adjunct biomarkers for differential diagnosis and early identification of urothelial cancer cell spread to local tissues, lymph nodes, and blood stream. UPs are major mediators in the adhesion, host cell invasion, and upward urinary tract spread of type 1-fimbriated *E. coli*. Finally, the uroplakin promoter-based transgenic and knockout models have yielded new insights into the molecular pathways of urothelial tumorigenesis and progression and will provide a platform for evaluating novel preventive and therapeutic approaches. Notwithstanding the progress, many important questions remain. For instance, how is uroplakin-trafficking between the cytoplasm and the apical surface of the urothelial umbrella cells regulated in the non-stretched vis-à-vis the stretched state of the urothelium? What are the relative contributions of different Rab family proteins in tethering the uroplakin-containing fusiform vesicles to the urothelial apical surface, and precisely what cellular machineries control the degradative pathway of uroplakins? Are these membrane trafficking processes involved in bacterial invasion into and exit from the urothelial cells? Through what specific mechanisms do urothelial plaques interact with a meshwork of underlying cytoskeletons allowing dynamic membrane movement during the normal micturition cycle as well as the pathophysiological processes such as bacterial invasion? Exactly how are the signals from FimH/UPIa interaction at the extracellular surface propagated across the membrane to the cytoplasm? Is it possible to resolve the uroplakin complex at atomic resolution so that the structure/function relationship of these membrane proteins can be eventually elucidated? Finally, will gene expression and/or knockout in urothelium in a temporally controlled manner yield more realistic models of urothelial diseases including carcinomas over the current, constitutive approaches? Answers to some of these questions will undoubtedly further advance the field of urothelial

biology and, more importantly, they should shed light on the molecular mechanisms whereby urothelium-related diseases arise and evolve.

DISCLOSURE

All the authors declared no competing interests.

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