Immunohistochemistry as a paramount tool in research of normal urothelium, bladder cancer and bladder pain syndrome

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ABSTRACT

The urothelium, an epithelium of the urinary bladder, primarily functions as blood-urine permeability barrier. The urothelium has a very slow turnover under normal conditions but is capable of extremely fast response to injury. During regeneration urothelium either restores normal function or undergoes altered differentiation pathways, the latter being the cause of several bladder diseases. In this review, we describe the structure of the apical plasma membrane that enables barrier function, the role of urothelium specific proteins uroplakins and the machinery for polarized membrane transports in terminally differentiated superficial umbrella cells. We address key markers, such as keratins, cancer stem cell markers, retinoic acid signalling pathway proteins and transient receptor potential channels and purinergic receptors that drive normal and altered differentiation in bladder cancer and bladder pain syndrome. Finally, we discuss uncertainties regarding research, diagnosis and treatment of bladder pain syndrome. Throughout the review, we emphasise the contribution of immunohistochemistry in advancing our understanding of processes in normal and diseased bladder as well as the most promising possibilities for improved bladder cancer and bladder pain syndrome management.

Key words: Urothelium; bladder cancer; bladder pain syndrome; uroplakins; keratins; retinoic acid; transient receptor potential channels; purinergic receptors.

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Introduction

The urothelium is a stratified epithelium of the lower urinary tract covering the renal pelvis, ureters, urinary bladder and proximal urethra.[1] Bladder urothelium is composed of three distinctive cell layers, *i.e.* poorly differentiated basal cells, partially differentiated intermediate cells, and terminally differentiated superficial umbrella cells. The urothelium plus basal lamina and lamina propria form the mucosal layer of the bladder wall.[2,3] The other two layers are the muscularis propria (detrusor) and the adventitia/serosa.

Functionally, normal urothelium forms a blood-urine permeability barrier against toxic metabolites and pathogenic bacteria in the urine. Transepithelial resistance of the urothelium is 10,000 – 75,000 Ωcm^2, which is extremely high and puts urothelium among the tightest barriers in mammalian body.[4] The barrier must be maintained during micturition cycles, particularly when bladder is stretched by filling with urine.[2] This requirement is accomplished mainly by terminal differentiation of umbrella cells.

The ability of the bladder to perform its function of urine storage and voiding depends on the central nervous system receiving accurate information on the state of the bladder fullness, which was first described in 1933 by Denny-Brown and Robertson.[5] Recent evidence suggests that sensory proteins like transient receptor potential (TRP) channels and purinergic P2X receptors in the urothelium are activated by binding of ATP or upon distension, respectively.[6,7] This leads to release of signaling molecules such as ATP, NO and acetylcholine from urothelium and their action on interstitial cells, afferent fibres and smooth muscle cells in the bladder wall.[8,9] Therefore urothelium acts as a part of bladder sensory web, which coordinates the micturition cycle.[10]

The permeability barrier and sensory function of the urothelium are often compromised in bladder diseases like bladder cancer (BC) and bladder pain syndrome (BPS). Immunohistochemistry (IHC) provided crucial contribution to our comprehension of urothelial structure and function in health and disease. Data were obtained by IHC based on peroxidase reaction (IHC-Px), but the most informative were immunofluorescence (IF) and immunoelectron microscopy (IEM).

Immunohistochemistry reveals the mysteries of normal urothelial differentiation

Seminal transmission electron microscopy analyses done by Marian R. Hicks in the sixties revealed thickened plasma membrane of the bladder's luminal surface and of angular cytoplasmic vesicles, which suggested that these membrane are unusually rigid.[11] Leopold G. Koss named this membrane asymmetric unit membrane (AUM).[3] It was confirmed that AUM forms apical plasma membrane and fusiform vesicles (FVs) of the umbrella cells.[2,12] The designation 'urothelial plaques' was coined to describe rigid AUM structure.[13] Urothelial plaques cover up to 90 % of the apical surface and they are separated by narrow hinge regions of unthickened symmetric membrane.[14] Another evidence for this organisation came from scanning electron microscopy and atomic force microscopy, which showed that plaques are surrounded by microridges, which correspond to hinge regions.[15-18] Isolation, freeze fracturing and quick-freeze/deep-etch demonstrated that urothelial plaques (diameter 0.2-0.5 µm) contain hexagonally arranged 16-nm intramembrane particles.[19-21] Cryo-electron microscopy provided evidence that these particles create structural basis for urothelial permeability barrier.[22]

Ground-breaking understanding of molecular structure of intramembrane particles came in the beginning of nineties with the discovery of 4 transmembrane proteins, uroplakins (UPs) Ia, Ib, II and IIIa by the group of Tung-Tien Sun.[20,23-25] IHC-Px and IF confirmed their expression in umbrella cells and IEM showed their association with urothelial plaques[17-19] (Figure 1). A model of UPs assembly into 16-nm particles[26] suggests that UPs acquire high mannose glycans and form two heterodimers (UPIa/UPII and UPIb/UPIII) in the endoplasmic reticulum.[27,28] These heterodimers exit from the endoplasmic reticulum and they are transported to the Golgi apparatus (GA).[29,30] In the GA, specific glycosylation occurs, which causes conformational changes, thus allowing heterotetramer (UPIa/UPII + UPIb/UPIII) formation.[31] In the trans-Golgi network (TGN), the prosequence of UPII is removed, triggering the oligomerization of 6 heterotetramers into one urothelial particle.[26,32] IEM revealed polarised transport of UPs from the TGN to the apical plasma membrane of the umbrella cell. A crucial necessity for such polarised transport are tight junctions of the umbrella cells.[33] Combination of transmission electron microscopy, IEM, IF, freez-fracture and electron tomography

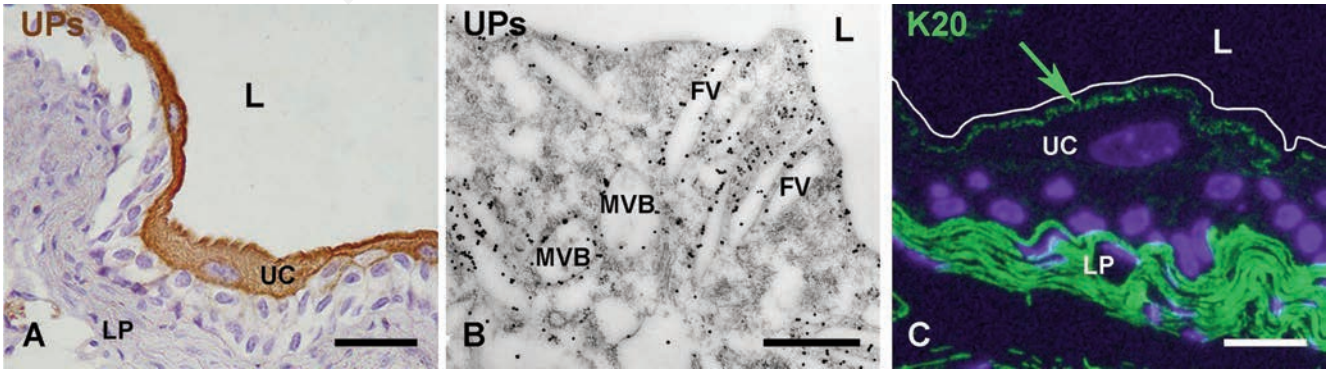

Figure 1. Normal urothelium of the mouse urinary bladder. A) IHC-Px of uroplakins (UPs) is positive (brown) in the superficial umbrella cells; B) IEM of UPs is positive in the membranes of fusiform vesicle, multivesicular bodies and in the apical plasma membrane of umbrella cell; C) IF of keratin K20 is positive (green arrow) in the subapical region of umbrella cell. Labelling of the lamina propria is unspecific. L, lumen; UC, umbrella cell; FV, fusiform vesicle; MVB, multivesicular body; LP, lamina propria; white line, location of the apical plasma membrane. Scale bars: A) 50 µm; B) 500 nm; C) 20 µm.

demonstrated the sequential steps of urothelial plaque formation in post-Golgi compartments. First, small rounded uroplakin-positive transporting vesicles detach from the TGN and subsequently fuse into immature FVs with slightly dilated profiles. With additional fusions and buddings, they mature into flattened FVs, which transport urothelial plaques toward the apical surface of the umbrella cells.[34,35] According to IF and IEM, FVs contain Rab8/11 and Rab27b/Slac2-a, which mediate apical transport along actin filaments by the action of myosin Va. FVs pass trajectorial network of K20 to reach the subapical region of the umbrella cells (Figure 1).[36] This is followed by membrane tethering and a final step of SNARE mediated and MAL (myelin-and-lymphocyte protein) facilitated membrane fusion with the apical plasma membrane.[37,38]

To sum up, uroplakins and K20 are two major markers, which have to be analyzed by IHC, IF and IEM to assess differentiation stage of urothelial cells. Therefore, the detection of their expression and localisation is crucial to discriminate between normal and pathologically altered urothelium.

How immunohistochemistry contributes to understanding the bladder cancer

The majority of BC are urothelial carcinomas. More than a half of them are papillomas or the papillary carcinomas (pTa, pT1), which have a relatively good prognosis.[39] Squamous cell carcinomas have variable survival outcomes, while carcinoma *in situ* (CIS) can progress to muscle-invasive urothelial carcinoma (pT2) with poor prognosis.[40] The most challenging feature of BC is its high recurrence rate, which is between 50% and 90% of cases.[41]

Somehow surprising is the finding that immunohistochemical labelling of UPs does not correlate with BC stage and grade.[42,43] However, IEM showed that despite the expression of UPs is preserved their subcellular localization is changed in urothelial cancer cells. In contrast to normal superficial cells, they appear also in the basolateral plasma membrane, which points to altered regulation of membrane transports (Figure 2).[44,45]

Accepted diagnostic and prognostic markers for BC are changes of keratin expression profiles. For comparison, IF demonstrated the expression of K7, K8, K18 and K19 in all cell layers, small quantity of K5 in basal cells, K13 in basal and some intermediate cells and K20 only in umbrella cells in the normal urothelium.[46-48] Normal K20 expression pattern is correlated with non-recurrent tumours and it can therefore be used for objective differential diagnosis between papillomas and carcinomas.[49] Increased expression of K20 in all urothelial cell layers is distinctive feature of CIS,[50] while intense IHC-Px staining of K8 and K18 might point to invasive cancer.[49] Furthermore, the loss of K13 is associated with high cancer grade and stage, while *de novo* expression of K14 may indicate squamous differentiation of urothelial cells and an unfavourable outcome for patients.[49]

It turned out that keratin immunolabelling is important for the studies of BC progression and recurrence, which both rely on cancer stem cells (CSCs). Their identification and immunolocalization remain elusive, mainly because it is not clear weather cancer originate form one or more subpopulations of CSCs. A subset of K5, K14 and sonic hedgehog positive basal cells were proposed to be the urothelial CSCs.[51] Furthermore, Lin *et al.* suggested that K5-positive, K7-negative basal cells with constitutive expression of β-catenin were the possible CSCs.[52] In accordance with these results, Shin et al. discovered that K5-positive basal cells, which also express sonic hedgehog, were necessary for CIS and invasive carcinoma development.[53] The origin of urothelial carcinomas was challenged by lineage tracing studies, which showed that papillary carcinoma arises from intermediate cells, while CIS and muscle-invasive carcinoma arise through the transformation of K5-negative basal cells.[51]

Several pieces of evidence show that the retinoic acid (RA) signalling pathway is often compromised in carcinomas.[54,55] For example, due to low intake of vitamin A (a generic term referring to a group of retinoids, such as retinol, retinal and RA), normal urothelium is replaced by urothelial squamous metaplasia, a precancerous stage, which may progress to squamous carcinoma.[5] Additionally, several epidemiological studies and meta-analyses show that high vitamin A intake and high vitamin A serum levels are associated with lower risk of BC in humans.[57-59] In an animal model of early bladder carcinogenesis, we demonstrated that vitamin A-rich diet altered RA signalling and decreased atypia and apoptosis of urothelial cells.[60] Moreover, IF revealed that during early bladder carcinogenesis, lecithin retinol acyltransferase (LRAT), which transforms retinol to inactive retinyl esters, is

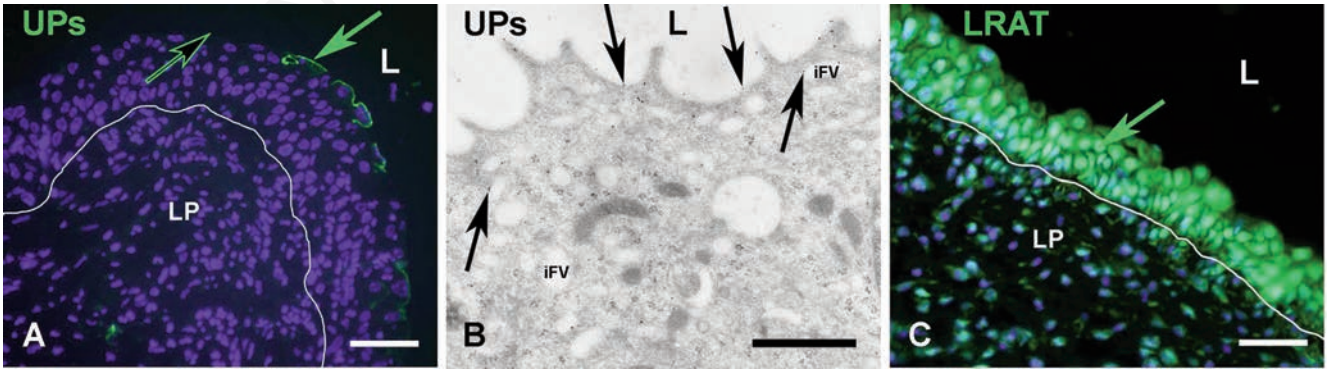

Figure 2. Animal model of bladder carcinogenesis induced by N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN). A) IF of uroplakins (UPs) is positive (green arrow) or negative (black arrow) in the apical plasma membrane of superficial urothelial cells after 10 weeks of BBN treatment; B) IEM of UPs is positive (arrows) in the membranes of immature fusiform vesicle (iFV) and in the apical plasma membrane of superficial urothelial cell after 5 weeks of BBN treatment; C) IF of lecithin retinol acyltransferase (LRAT) is positive (green) in the urothelium after 2 weeks of BBN treatment; LRAT is present in the cytoplasm of the urothelial cells and also in their nuclei (green arrow). L, lumen; UPs, uroplakins; iFV, immature fusiform vesicle; LP, lamina propria; white line, approximate location of basal lamina. Scale bars: A,C) 50 μm; B) 1 μm.

translocated from cytoplasm of urothelial cells into their nuclei (Figure 2).[60] We assume that LRAT exerts its tumour-suppressing role in the nuclei of urothelial cells.[61] This notion is supported by the study of Boorjian *et al*., which showed by IHC-Px that LRAT is inversely correlated to tumour stage in BC.[62] Although various findings from *in vivo* and *in vitro* models of BC demonstrate a potential for the use of synthetic and natural retinoids for BC prevention and treatment, the successful clinical trials are needed before they can be used in clinical settings.

Bladder pain syndrome remains a major challenge for immunohistochemistry

The urothelial-associated sensory web is affected in BPS. BPS belong to a group of bladder diseases under the term interstitial cystitis/bladder pain syndrome (IC/BPS).[63,64] Main presenting symptoms of BPS are frequency, urgency, nocturia, and bladder pain, which often increases as the bladder fills. Despite unknown etiology, difficult diagnosis and lack of effective treatment (https://uroweb.org/, https://www.auanet.org/), aberrant urothelial cell differentiation, together with accompanying changes in sensory protein expressions are unifying hallmarks of BPS that are studied by IHC. BPS is associated with incomplete urothelial differentiation as demonstrated by IEM that showed lower expression of UPs and absence of urothelial plaques in the apical plasma membrane.[65-67] This may lead to a leaky barrier followed by enhanced signalling from the urothelium.[65] TRP channels and P2X receptors are among the candidates to play a role in BPS since they are dysregulated during various micturition-related disorders.[68] TRP channels are nonspecific cation channels that are permeable to Ca^{2+} and might act as sensors of stretch and/or chemical irritation. The TRP channel superfamily consists of subfamilies TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPN (no mechanopotential), TRPP (polycystin) and TRPV (vanilloid).[69] P2X receptors are membrane ion channels preferably permeable to sodium, potassium and calcium. Various homo- and hetero-trimers of P2X1-P2X7 subunits associate to form a functional ion channels that open upon binding of extracellular ATP. Almost all subtypes of TRPV channels and P2X receptors were shown in the bladder wall by various methods, but we consider here only those that were immunohistochemically demonstrated in the urothelium.

Several members of TRP superfamily (*e.g.* TRPV1, TRPV4, TRPV2, TRPM4, TRPM7, TRPM8, TRPA1, TRPC1 and TRPC4) were shown in normal urothelium by IHC.[70] The majority of stud-

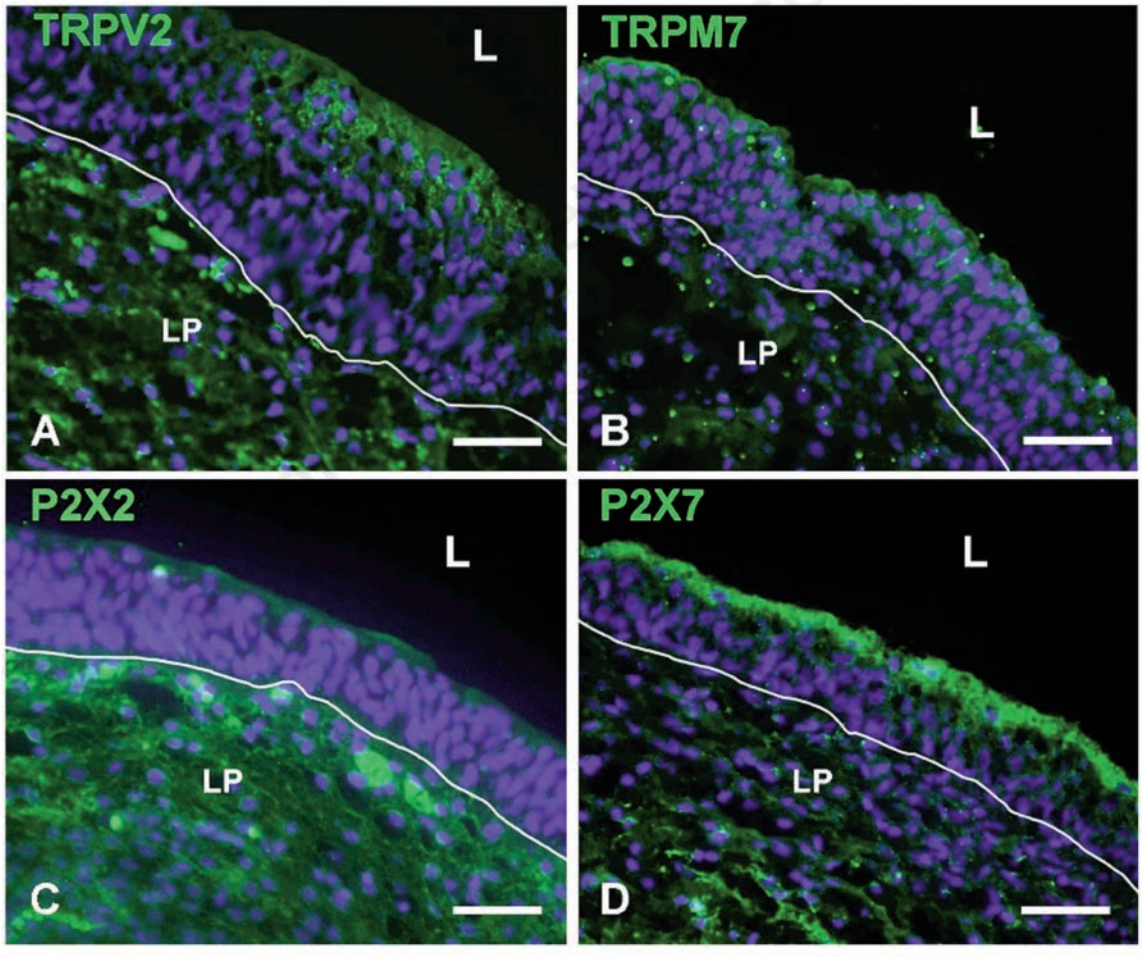

Figure 3. Our preliminary IF results on normal human urothelium of the urinary bladder. A) IF of TRPV2 is positive (green) in the superficial layers of the urothelium and in some areas of the lamina propria; B) IF of TRPM7 is positive (green) in the superficial layers of the urothelium; C) IF of P2X2 is weakly positive (green) in the superficial urothelial cells; D) IF of P2X7 is positive (green) in the superficial layers of the urothelium. L, lumen; LP, lamina propria; white line, approximate location of basal lamina. Scale bars: 100 µm.

ies focus on TRPV1 and TRPV4. TRPV1 is essential for pain sensation associated with BPS, while TRPV4 is the main urothelial mechanosensor, which is activated by bladder distension.[71] There is still a controversy about TRPV1 expression in urothelium, since variable labelling results were reported by different groups.[72-76] For example, a weak anti-TRPV1 labelling in superficial urothelial layer by IF.[74] However, a similar staining pattern was observed in the urothelium of wild-type and TRPV1 knockout mice, which shows that currently used anti-TRPV1 antibodies can cause an unspecific labelling.[77] Several studies showed altered expression of TRPV1 in patients with BPS as well as in animal models, but until now only one study demonstrated this by IF.[74,75,78,79] On the other hand, there is more consistency about TRPV4 localization in the normal urothelium, particularly in the basal cell layer.[76,80,81] IEM further showed that TRPV4 is localized near the basal plasma membranes adjacent to the basal lamina.[73] Although some studies reported on important role of TRPV4 in BPS,[82,83] no IHC confirmation is available, yet. Regarding other members of TRP family, IF studies showed that TRPV2 and TRPM4 were prominently localized to the umbrella cell apical plasma membrane, TRPC4 and TRPV4 on their abluminal surfaces, while TRPC1, TRPM7, and TRPML1 in their cytoplasm (Figure 3).[73,76,80,84] To the best of our knowledge, the studies of their possible contributions to BPS were not performed.

All of seven P2X receptors subtypes were expressed throughout the urothelium as suggested by an IF study on feline bladders.[85] In comparison, clear P2X7, less distinct P2X2 and weaker P2X3 labelling was observed in the normal human urothelium (Figure 3).[86] In the mouse urothelium, IF revealed that P2X7 receptor is confined to the superficial urothelial cells.[87] Additionally, in the rat urothelium, P2X7 and P2X4 are expressed both intracellularly and on the apical surface.[88] The most studied member of P2X family is P2X3, yet again, the IF results are inconsistent about its urothelial expression, since studies report positive, weak or negative labelling.[85-87,89-92] While there was a marked reduction in P2X1 receptor staining observed in feline interstitial cystitis,[85] studies on human biopsy samples from patients with BPS revealed that the expression of P2X2 and P2X3 is increased in the urothelium.[93,94] Moreover, P2X3 expression is abnormally upregulated in response to stretch in urothelial cells isolated from patients with BPS.[95,96]

Since TRPV1 and P2X3 represent the most important therapeutic targets for BPS, their adequate immunolocalization is of huge importance. Unfortunately, as mentioned above, IHC is still generating confusing results about the presence and distribution of TRP channels and P2X receptors in the urothelium. The reasons for that are diverse, but they probably include low expression levels of these proteins, poor specificity of antibodies, lack of adequate controls and the fact that the urothelium is particularly susceptible to nonspecific adsorption of antibodies.[97] Moreover, other experimental approaches (*e.g.*, immunoblotting, RT-PCR, *in situ* hybridization, fluorescently activated cell sorting, functional and pharmacological assays) introduced additional controversies regarding the expression of these and other sensory and signalling proteins in the urothelium.[97]

What more can immunohistochemistry do in the field of urothelial biology?

Despite a vast amount of knowledge on urothelial biology gained in the recent years, there are still many open questions. Some of them, which are closely related to the discussions above, emerged also from our work and they are briefly described here.

In contrast to well established pathway of apical plasma membrane biosynthesis in umbrella cells, less understood remain the function, mechanism and regulation of the endocytosis of UPs. It is possible that in umbrella cells a small fraction of fusiform vesicles is formed by urothelial plaque internalization upon voiding. Yet, we have shown that apical plasma membrane is internalized mainly from hinge regions by small endocytic vesicles.[98,99] Internalized UPs become integrated into the membranes of multivesicular bodies (Figure 1B) to be later degraded in lysosomes. Recently it was shown that sorting nexin Snx31 plays an active role in the regulation of UPs degradation.[100] It seems that increased UPs synthesis, together with the rearrangement of actin filaments and microtubules, hinder apical endocytosis.[98,101] Moreover, Khandelwal *et al.* showed that apical endocytosis occurs *via* non-clathrin pathway that requires RhoA and dynamin 2.[102]

We have gained some insights into altered RA signalling during early bladder carcinogenesis (Figure 2C), but there are no data about RA signalling in developed urothelial carcinomas.[60] We have discussed the role of P2Xs and TRPs, particularly in BPS patients, where we have seen that the IHC results are inconclusive or preliminary (Figure 3). Other receptors and channels involved in urothelial signalling have been reported,[9] including adrenoreceptors, muscarinic and nicotinic receptors.[103] However, with all of them there is the same problem of conflicting IHC results. The problem of inconclusive localization results can be resolved in two ways. First, by the production of more specific antibodies against these proteins, which will be suitable also for IEM. Second, by applying new or improved techniques, such as cryosections according to the Tokuyasu, freeze-fracture replica immunolabelling, combined lectin- and immuno-histochemistry and correlative light and electron microscopy.[104-108]

We believe that IHC will remain indispensable for bladder research in the future, not only for resolving basic biological questions, but also for helping find and implement new diagnostic and treatment strategies for BC, BPS and other bladder disorders.

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