Stem cells in the biology of normal urothelium and urothelial carcinoma

Minireview**

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Urothelium is a special type of stratified epithelium that lines the distal portion of the urinary tract. For a long time, basal urothelial cells have been suspected to include a population of urothelial stem cells. Recent experiments identifying label-retaining cells as well as lineage tracing analyses corroborate this notion. There are striking morphological and antigenic similarities between basal or differentiated urothelial cells and the corresponding cells in some urothelial carcinomas. In this respect, basal cell-specific markers provide good candidates to identify urothelial carcinoma stem cells, e.g. specific cytokeratins (CK5, CK14, CK17) or adhesion molecules (specific integrin subspecies, CD44). Common properties of the stem cells of normal urothelium and urothelial cancer have thus emerged. Both are characterized by a remarkable plasticity and both rely on reciprocal interactions with stromal fibroblasts. However, the stem cells of individual urothelial carcinomas appear to differ considerably and may contribute to the heterogeneity of this disease. The presence, quantity, and particular biological nature of urothelial carcinoma stem cells in each case may thus carry important clinical information that might allow a rationale stratification of urothelial cancer patients for treatment in the near future.

Key words: urothelium, urothelial cancer, stem cells, epithelial-stromal interactions, cell culture models, clinical risk assessment

Urothelium, a specialized epithelium of the distal urinary tract, is essential for the safe excretion of urine. It is also medically highly important, with two major diseases dominating. First, uropathogenic infections belong to the most common and costly infectious diseases worldwide, and, second, urothelial carcinoma is associated with considerable morbidity and is one of the most expensive cancer diagnoses, by virtue of its high prevalence and high recurrence rate. Both normal and transformed urothelium rely on similar biological principles. Evidence is mounting that a urothelial stem cell occupies a central position in normal urothelial homeostasis and that its transformed counterpart, a urothelial cancer stem cell, is a driving force of urothelial carcinogenesis. Research into both the normal and transformed urothelial stem cells could thus be mutually inspiring, accelerating our knowledge in both fields. As targeting cancer stem cells is counted among the most promising therapeutic options for the future, it is crucial to explore the properties of normal and cancerous stem cell populations from this point of view as well. Evidently, only the specific targeting of cancer stem cells, sparing the normal tissue stem cells, could provide a major therapeutic benefit to cancer patients, by eliminating tumors but not severely damaging normal tissues in the process. The purpose of this review article is to summarize current knowledge on normal urothelial and urothelial cancer stem cells.

Urothelial stem cells. Urothelium is a specialized epithelium lining the distal portion of the urinary tract which comprises the renal pelvis, ureters, urinary bladder, upper

urethra and glandular ducts of the prostate. The main function of the urothelium is to provide a barrier against the free exchange of substances between urine and blood while being able to accommodate a highly variable urine volume. The major contribution to this specific barrier function is provided by the uppermost cellular layer of the urothelium, which is composed of highly specialized and fully differentiated umbrella cells. These are giant (80-120 μm), frequently multinucleated cells with a specific composition of apical membranes, high-resistance tight junctions and an apical glycan layer. In other respects, the urothelium resembles common stratified epithelia. Beneath the umbrella cells, a variable number of intermediate cell layers are found, and a single layer of basal cells is in full adhesive contact with the basal lamina. Until relatively recently, the term "transitional epithelium" was in use for the urothelium, implying that all urothelial cell layers are in contact with the basal lamina. Recent electron microscopic analyses have essentially disproved this classical model of urothelial structural organization, by showing that interactions with the basal lamina are reserved to basal and occasional intermediate cells, but never involve umbrella cells. Urothelium is a slowly cycling tissue, with the life-time of umbrella cells being estimated as up to 200 days and the turnover interval of the urothelial epithelium to 6 – 12 month. On the other hand, urothelium has a remarkable regenerative capacity, with tissue damage resulting in a very rapid proliferation coupled to differentiation until full urothelial integrity is restored [1].

By analogy to other stratified epithelia, it has been assumed for a long time that both regenerative and homeostatic urothelial proliferation originate from cells of the basal cell layer, where the urothelial stem cells were suspected to reside. In this model, the intermediate cells would represent the population of transit amplifying (TA) cells that terminally differentiate into the umbrella cells. Several recent studies have indeed borne out this model, at least in its basic outlines [2]. Kurzrock et al. localized label retaining cells to the basal layer of rat bladder urothelium [3]. Pulse labelling with bromo-deoxyuridine (BrdU) followed by long term observation allows to distinguish mitotically active TA cells, which gradually dilute the label at each round of DNA replication, from stem cells, which because of slow cycling and, possibly, retention of the same DNA strand (the hypothetical immortal DNA strand) throughout many replications retain the label [4, 5]. One year after the BrdU pulse, ~ 10% of bladder urothelium basal cells were still BrdU-positive, strongly implicating this minor fraction as urothelial stem cells [3]. It should be noted, nevertheless, that identification of BrdU – label retaining cells is technically demanding and thus prone to error. Indeed, a recent attempt to reproduce these results was unsuccessful [6]. On the other hand, the slow cycling of urothelium and especially the urothelial stem cells has made it possible to map the unit of proliferative succession by mutational analysis as well. Both X-inactivation based [7] and mitochondrial mutation-based [8] studies gave, principally, identical results – monoclonal proliferative units (i.e. subpopulations of cells sharing the same

copy of inactivated X chromosome or the same mutation in the mitochondrial DNA) could be identified in urothelium. In each experiment they originated in basal cells and extended across a section of the epithelium towards umbrella cells. Importantly, not all monoclonal proliferative units reached the urothelial surface, corroborating the basal cells as the cells of origin (i.e. stem cells) of the monoclonal units.

The basal urothelial cells are small (5-10 μm), display low granularity and a high nucleo-cytoplasmic ratio, express β_1 and β_4 integrins, laminin receptor, CD44, and specific "basal" cytokeratins (CK-5/14, CK-17) [1, 2, 3, 9]. Importantly, all these markers are common to the entire basal cell layer. The label retaining study cited above revealed, however, that only about 10% of basal cells might correspond to true stem cells. There is, to our knowledge, currently no marker available to distinguish the subpopulation of urothelial stem cells from the remaining basal cells, which probably correspond to TA cells.

Another difficulty about urothelial stem cells is that the relationship between *in vivo* stem cell properties and *in vitro* clonogenic properties is by far not as unequivocal as, for example, in the epidermis. Classical clonogenic analysis of epidermal cells yields three types of colonies – holoclones, i.e. colonies of small compactly arranged cells with an extensive proliferative capacity, meroclones, i.e. colonies of more diffuse and flatter cells of limited proliferative capacity, and paraclones – colonies of terminally differentiated keratinocytes that could not be sub-cultured at all [10, 11]. A highly plausible interpretation is that holoclones are founded by a self-renewing stem cell and can therefore be serially propagated, with essentially the same full spectrum of secondary colonies as total epidermal cells, whereas meroclones originate from a TA-cell, yielding a limited number of secondary colonies without holoclones, and paraclones are derived from fully differentiated cells. While essentially the same basic pattern of colony morphologies could also be evidenced by *in vitro* culture of normal porcine bladder urothelium, all colony types gave a similar distribution of secondary colony types. In other words, secondary holoclones were generated not only from primary holoclones, but also from primary meroclones and paraclones [12]. Whether this obvious dedifferentiation capacity originates from *in vitro* culture conditions or whether it reflects a true biological capacity of urothelium remains to be analyzed.

Urothelial cancer stem cells. Urothelial carcinoma is a frequent cancer diagnosis, representing the fifth most common malignancy worldwide with an annual incidence of ~386 000 cases and an annual mortality approaching 150 000 cases [13]. Urothelial cancer is distinguished by the existence of two different carcinogenic pathways (so called dual track carcinogenesis). The majority of newly diagnosed cases are superficial papillary tumors that develop from low grade hyperplastic precursor lesions. These tumors frequently recur and their long-term management is among the most expensive of all malignancies. Non-papillary invasive carcinomas develop from high grade dysplastic precursor lesions

known as carcinoma in situ (CIS) and have a high propensity of metastatic progression, with quite a poor prognosis. The majority of invasive carcinomas develop without a preceding history of papillary disease, but 10 – 15% of papillary tumors eventually progress towards invasive stage; there are at present no prognostic markers available to forecast this progression of papillary tumors. Each carcinogenesis pathway features typical molecular changes. With a certain level of simplification, one could summarize the primary defects in papillary tumors as overactivation of growth factor signalling pathways, with activating mutations in the fibroblast growth factor receptor 3 or *Ras* oncogenes as two common alternatives. Along the same lines of simplification, invasive tumors could be characterized as a disease of disturbed cell cycle regulation, with loss-of-function mutations in *TP53* and *pRB1* tumor suppressor genes as typical changes [14]. Urothelial (especially bladder) cancer is presumed to be initiated by mutagenic insults incurred by urothelium as a consequence of mutagen concentration in urine – a series of initially mutated forerunner genes has been mapped recently [15]. Beyond their typical molecular changes, an important difference between papillary and invasive tumors has been hypothesized to consist in the particular initiated cell. Thus, papillary tumors may derive from a TA cell, while invasive tumors might be derived from an initiated urothelial stem cell [16]. However, the exact identity of the urothelial cancer cell of origin – in either subtype – is still open.

In urothelial carcinomas, especially in low grade papillary tumors, areas strikingly reminiscent of normal urothelial differentiation patterns can be readily identified. This similarity extends beyond the mere morphological level – experiments performed in the late 1980's demonstrated a great antigenic similarity between normal urothelial basal cells and the basal cell layer of papillary carcinomas, and the same was true of the respective superficial cell populations [17]. By analogy to the hierarchical organization of normal urothelium discussed above, a similar cellular hierarchy can be invoked in urothelial carcinoma. The "classical" stem cell model (see accompanying paper of Hatina, this issue of Neoplasma) is probably more appropriate for the papillary carcinomas and the "dynamic" stem cell model might be more suitable for many invasive carcinomas, where the stem cell compartment might be continuously replenished by ongoing epithelial-mesenchymal transition. Irrespective of this possible intrinsic difference, many reseachers believe that normal urothelial stem cell markers could be good candidates to approach the urothelial cancer stem cell population. Indeed, basal-type cytokeratins CK5, CK14 and CK17 are common to normal and urothelial carcinoma basal cell layers, and CK20 might similarly mark normal as well as cancerous differentiated cells [9]. In some urothelial carcinoma cell lines, morphological variability is evident, and, interestingly, small compactly arranged cells frequently express basal type cytokeratins as well. In addition, in the HT-1197 cell line, these small compactly arranged cells express CD44v6 [18]. CD44 is another general marker of basal urothelial cells that has attracted much attention as a putative bladder cancer stem cell marker. In a fraction of tumor samples, FACS-purified CD44+ cells were of small size and low granularity and, notably, were serially tumorigenic, meeting probably the most compelling criterion for stem cells. CD44 co-localizes with basal cells in normal urothelium and with CK5 in both normal urothelium and tumors. On the other hand, CD44 behaved in this manner in only two out of 14 tumors analyzed and there was no correlation between the proportion of CD44+ cells and tumorigenicity [19]. In keeping with this finding, immunohistochemical examination of a large series of bladder tumors revealed that CD44 indeed marked basal cells in normal urothelium, its expression became ubiquitous in superficial papillary tumors and was progressively lost in invasive tumors, with over 60% of bladder tumors being negative [20]. Collectively, these results show that CD44 might be a useful stem cell marker for some tumors, but it cannot be regarded as a universal bladder cancer stem cell marker.

One of the basic biologic characteristics of stem cells is an enhanced ability to protect themselves against genotoxic insults. This general mechanism has come to be employed to purify putative cancer stem cells as well. Su et al. used the Aldefluor assay to purify cancer stem cells from three low-grade carcinoma cell lines HBT-2, -4, and -9 [21]. In all the three cell lines, ALDH1A1+ cells were highly enriched for clonogenic (both in classical two-dimensional cell culture and in anchorage-independent growth conditions) and tumorigenic cells. Interestingly, the aldehyde dehydrogenase A1 seemed to be actively involved in maintaining the stem cell phenotype, as its knockdown via specific siRNA significantly diminished both clonogenicity and tumorigenicity. Notably, CD44 could be used as a second independent stem cell marker to enrich for tumorigenic cells, albeit to a distinctly lesser extent than ALDH1A1. When both markers were used in combination, there was no difference in tumorigenicity between ALDH1A1+ CD44- and ALDH1A1+ CD44+ cells (as well as single positive ALDH1A1+ cells). CD44 was widely expressed in the three cell lines (52-72% positivity) and ALDH1A1+ cells (6.4 -8.2% of the bulk population) were significantly enriched in the CD44+ fraction (12.8 – 16.5%) [21]. Collectively, these results corroborate the above conclusion that CD44 can be used as a stem cell marker only in a fraction of bladder tumors.

The side population (SP) assay, not surprisingly, could not go unnoticed in attempts to isolate bladder cancer stem cells. Both Hoechst 33342 and Dye Cycle Violet have been used and both staining procedures were able to enrich for clonogenic and tumorigenic cells [22-24]. Surprisingly, a side-by-side comparison of both stains revealed that in some urothelial cancer cell lines, the proportion of SP cells detected by the Dye Cycle Violet staining could be far higher (up to 20 times) than for the Hoechst 33342 (e.g. UM-UC-6, 253-B-V, HT-1197) [23] – an unexplained phenomenon deserving further attention. There was no correlation between the proportion of SP-cells and the transformation status of the respective cell line. Strikingly, Dye cycle Violet purified non-SP cells could, upon

further sub-culturing, generate a small proportion of SP cells and the authors exclude the possibility of contamination [24]. Moreover, in the bladder carcinoma cell line J82, cell cultures founded by purified SP cells displayed a sharp drop in the SP fraction immediately after seeding (i.e. seeded SP cells rapidly differentiated into non-SP cells) followed by dedifferentiation into SP cells as the cultures approached confluence [23]. Collectively, these observations are remarkably reminiscent of the plasticity of paraclones in primary cell culture of normal urothelial cells mentioned above.

Epithelial-stroma interactions in normal urothelium and urothelial carcinogenesis. Because of the overall low level of homeostatic proliferation, the most convenient experimental approach to study the dynamics of urothelial regeneration is to introduce an acute urothelial injury, e.g. by urothelial instillation of uropathogenic Escherichia coli strains. Obviously, this experimental model goes far beyond the theoretical study of stem cell biology, as urinary tract infections are among the most frequent infectious diseases in humans. Uropathogenic E. coli infections cause acute severe damage to the urothelium, with exfoliation of the umbrella cell layer, which is followed by an intense proliferation in the regeneration phase [25]. Importantly, the regenerative proliferation involves both basal urothelial cells and underlying stromal fibroblasts located just beneath the basic lamina in the lamina propria [26, 27]. Two signalling systems mediating this complex regenerative cellular response have recently been characterized in murine bladder. Bladder fibroblasts specifically express the TGF-β family member bone morphogenic protein 4 (Bmp-4), whereas the urothelial cells express the cognate receptor Bmpr1a, predominantly at the basolateral surface of the basal cell layer. Following E coli infection, Bmp-4 levels in the stroma cells became reduced, and the nuclear transcription factor activated by the Bmp-4 signal, phosphorylated Smad-1, declined in basal and suprabasal urothelial cells. Given that TGF-β – family factors frequently suppress proliferation of normal epithelial cells [28] and in light of the observed proliferative response of regenerating urothelium, a picture emerged in which the stromal derived Bmp-4 could be responsible for the relative quiescence of urothelial cells, thus contributing to the normal stem cell niche. The issue is certainly more complicated, as urothelium – specific *Bmpr1a* knockout led, contrary to expectations, to diminished basal cell proliferation in response to uropathogenic E. coli infection, accompanied by a differentiation dysbalance of regenerating urothelium [26].

Another signalling system activated in regenerating murine urothelium is Sonic hedgehog (Shh). The response to this growth factor proceeds via the specific cell surface receptor Patched to transmit the signal further to the nucleus via the Gli-1 transcription factor [29]. In this case, however, cards are dealt in the opposite way as in the Bmp-4 – Bmpr1a signalling system. The regenerating urothelial cells express the growth factor, i.e. Shh coincides with CK-5 and is largely confined to basal cells, whereas activated Gli-1 is found exclusively in stro-

mal fibroblasts. This positive paracrine interaction activated proliferation and specific gene expression of the fibroblasts. Among the genes activated by the Shh signal, several crucial growth factors have been identified, prominently two Wnt factors Wnt-2 and Wnt-4. The activated stroma thus signals back to the urothelium, with the canonical Wnt-β-catenin pathway being, probably, mainly responsible for the proliferation activation in basal urothelial cells [27]. Collectively, these results indicate that underlying stromal cells make crucial contributions to the biology of normal urothelial stem cells and that one of their specific functions might be to create an appropriate microenvironment for urothelial stem cells, i.e. the stem cell niche.

Could any analogy be drawn for urothelial carcinoma? Several independent experiments clearly showed that bladder cancer xenografts, both freshly transplanted tumor fragments and xenotransplanted bladder cancer cell lines, are organized in the manner that basal-like cells (identified by basal-type cytokeratins CK5 or CK-17, or, in some tumors, CD44) are in direct contact with mouse-provided tumor stroma, reproducing the distribution of basal-like and superficial-like cells in clinical bladder cancer specimens [19, 30]. This suggests a unique requirement of urothelial cancer stem cells for supportive stromal elements. The intuitive explanation is that stromal cells provide a sort of cancer stem cell niche resulting in preservation of the stem cell phenotype in cancer cells that directly interact with the stroma, in a quite similar manner as in normal urothelium. Obviously, cancer cells possess some capacity to maintain a stable stem cell fraction autonomously – illustrated by the experiments described above with purified ALDH1A1+ or SP cells performed with cancer cell lines in pure culture. Nonetheless, stromal cells appear to provide this function much more efficiently. In an attempt to define this exquisite stromal influence, the group of Bergman et al. searched for suitable cell surface molecules, which would be strongly co-expressed with CK-17 in SW780 bladder cancer cell line xenografts, and discovered the 67 KDa laminin receptor (67LR) as a possible bladder stem cell marker. FACS-sorting of 67LR[high] and 67LR^{low} cells validated this strategy. The 67LR^{high} cells were greatly enriched for tumorigenic cells and their gene expression profile involved many genes with known or supposed function in stem cell biology, including typical signalling molecules or genes coupled to various self protection mechanisms. A key observation was that the 67LR acted as a stem cell marker only in xenograft tumors, but not in pure SW780 cell cultures [30]. Apparently, upon withdrawal of supportive stromal elements, different mechanisms of stem cell phenotype preservation, carried out by cancer cells themselves, are invoked, and, accordingly, the 67LR expression becomes entirely uncoupled from the stem cell phenotype. These findings reveal a remarkable flexibility of the stem cell phenotype in bladder cancer, a recurring theme discussed in several place above.

The necessity to rely on xenografted tumors in order to spot the stroma-responsive bladder cancer stem cells is a major
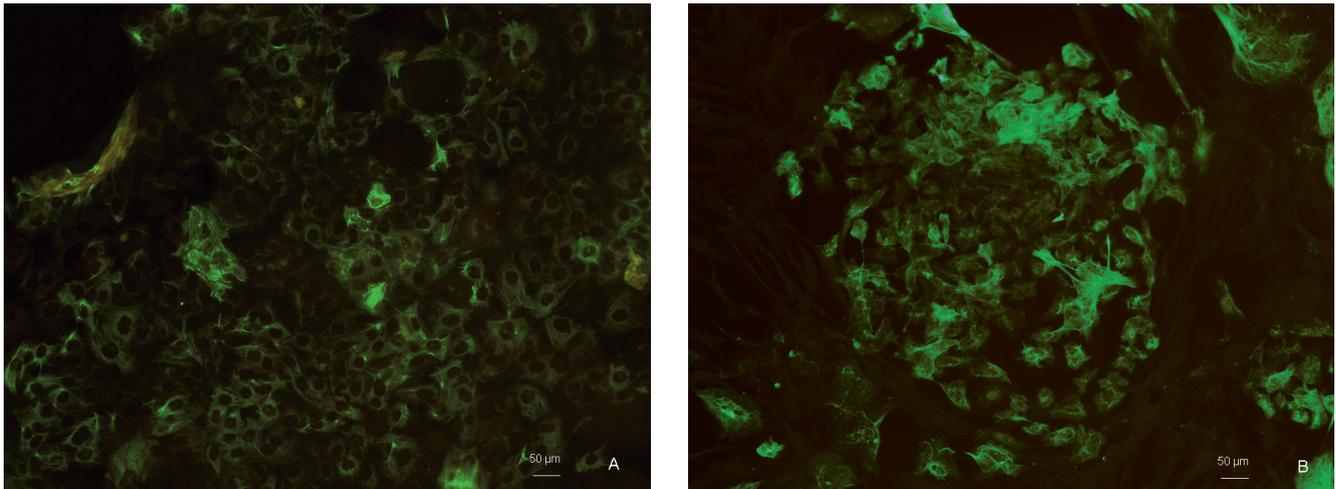
Fig. 1. Carcinoma-associated fibroblasts promote bladder carcinoma stem cells. Cells were cultured in 1:1 mixture of supplemented Epilife medium (Gibco) [43] and high glucose DMEM (Sigma) supplemented with 10% foetal bovine serum (PPA) and antibiotics in a humidified atmosphere containing 5 % CO_2. 20 000 BC44 cells were seeded either alone or together with 50 000 BC44Fibr cells per well on coverslips in 6-well plates and incubated until the culture approached confluence. Fixation was performed by ice-cold methanol for 30 min. Permeabilization was carried out by incubation with 0.5 % Saponin (Sigma) solution in PBS for 5 min. Blocking with 2 % normal goat serum (Milipore) followed for 1 h at room temperature. Indirect immunofluorescence staining was done with the α-CK17 monoclonal antibody clone CK-E3 (1 : 100) and goat α–mouse Atto488 conjugated secondary antibody (1 : 100), both purchased from Sigma. Antibodies were diluted in PBS, and slides incubated for 90 min at room temperature. Coverslips were mounted in Vectashield mounting medium (Vector Laboratories) and analyzed using the Olympus AX70 fluorescent microscope equipped with the Olympus DP71 camera system at 200x magnification. A. BC44 in pure cell culture. B. Cocultute of BC44 and BC44Fibr.

drawback of this approach. This method is slow, cumbersome and laborious, even when disregarding the implicit suffering of experimental animals. We have recently derived, from a single bladder cancer specimen, a pair of carcinoma and stromal cell lines, BC44 and BC44Fibr, respectively [31]. Availability of a genuine CAF (carcinoma-associated fibroblasts) cell line could greatly facilitate the search for these niche-providing signals from tumor stroma (see accompanying paper of Weiland et al.). In an initial attempt to evidence the niche-providing activity of the carcinoma fibroblasts, we stained the BC44 carcinoma cells for the basal type CK-17 either in pure culture, or in co-culture with the BC44Fibr cognate carcinoma fibroblasts (Fig.1). The increase in frequency of CK-17 highly positive cells as well as their preferential localization in the close proximity to the co-cultured fibroblasts is evident. We hope that the use of these and more complex co-culture systems might greatly expedite the molecular analysis of bladder cancer stem cells and their regulation by tumor stromal components.

Beyond the fundamental biology of urothelium and urothelial cancer stem cells, another pertinent avenue of research has recently received attention. In addition to the local fibroblasts in lamina propria, recruited mesenchymal stem cells (MSC) might constitute a source of stromal cells. This recruitment may lead to various effects according to the nature of the recruiting stimulus. Bone marrow derived MSC were shown to be specifically recruited to rat bladders following experimental partial bladder outlet obstruction, and in this specific situation, they contributed to tissue normalization by limiting the extent of both fibrosis and smooth muscle hypertrophy [32]. Likewise, bone marrow-derived MSC were recruited to several cellular matrix bladder grafts, where they both differentiated into and promoted the recruitment of smooth muscle cells as well as the ingrowth of the surrounding urothelium [33]. In the future, both approaches might bring important therapeutic benefits in treating obstructed bladders or for the purpose of bladder augmentation. From the point of view of cancer biology, MSC can be used as specific therapeutic vehicles (see accompanying paper of Altaner). For example, amniotic fluid derived MSC engineered to overexpress interferon-β significantly reduced the growth of transplanted experimental urothelial carcinoma xenografts [34]. On the other hand, MSC may constitute a source of carcinoma-associated fibroblasts, essential for promoting tumor growth and especially supporting CSC, as discussed above [35]. The relationship between MSC and cancer is thus a sort of double-edged sword, with many variables that ought to be carefully weighted before considering a direct therapeutic application.

Molecular characterization of urothelial cancer stem cells. Bladder cancer is a highly heterogeneous disease, far beyond the dual pathway carcinogenesis discussed above. Pronounced heterogeneity seems to exist at the stem cell level as well, even though only a fraction of this heterogeneity could be analyzed to date. It was reported that among carcinomas in which CD44 might mark cancer stem cells, 5% displayed nuclear (i.e. active) β-catenin, 20% Bmi-1, 40% STAT-3 and 80% Gli-1 [19]. Does this tell us something about the cell of origin

of urothelial cancer? As discussed above, CD44 is one of the markers of basal urothelial cells. One can thus speculate that tumors in which CD44 is specifically expressed in the stem cell compartment originate from urothelial basal cells (possibly, but not necessarily from urothelial stem cells), whereas CD44 negative tumors (totalling 60% of cases) may develop from more differentiated, possibly intermediate cells [19]. Taking lessons from regenerative biology of normal urothelium, it is quite surprising that only 5% of CD44+ tumor cases show the activated form of β-catenin; as described above, proliferation of basal urothelial cells depends on Wnt-signals produced by stromal fibroblasts. A possible explanation could be that homeostatic regulation might not be identical to regulation during regeneration in that only a small fraction of basal urothelial cells might be dependent on the Wnt-β-catenin signalling pathway under homeostatic conditions. For tumors relying on Gli-1, we can envision that the originally paracrine action of the Sonic hedgehog signalling pathway via stromal fibroblasts is switched over to the autocrine mode. Chronic exposure to arsenic, a well established bladder carcinogen, was recently associated with activation of Hedgehog signaling and pronounced Gli-1 positivity in a series of clinical bladder carcinoma samples [36]. Bladder cancer stem cells expressing Bmi-1 could derive from epithelial-mesenchymal transition (EMT) instead, since Bmi-1 has been reported to represent a downstream gene of Twist-1 [37], a crucial EMT trigger, whose role in bladder cancer progression is documented [38]. As for STAT-3, this transcription factor is activated, among other signals, by inflammatory cytokines [39]. Whether this could reflect a possible role of inflammation in bladder cancer initiation, however, remains to be seen. We would like to stress, though, that all these deductions are highly speculative. In addition, a lot of bladder tumors co-expressed several of these putative stem cell markers [19], further obscuring the possibility to infer their mechanism of initiation.

Interestingly, Oct-4, another general stem cell marker, could not be evidenced in any of the bladder tumors in the above cited study [19]. Two independent studies, nonetheless, reported that Oct-4 could be readily detected in bladder tumors [40, 41]. In this context, however, the Oct-4 effect is probably largely independent from its putative role in stem cells. In fact, the reported expression levels were distinctly lower than in embryonic stem cells analyzed in parallel. In this setting, Oct-4 seems to act mainly by activation of an invasive phenotype (motility, secretion of proteases etc.) [40]. Not surprisingly, high Oct-4 expression represented a significant negative prognostic factor [40, 41].

The prognostic value of urothelial cancer stem cells represents another important issue. Among the proposed stem cell markers, ALDH1A1 expression seems to have an important prognostic value for both cancer-specific and overall survival, with high expression marking a significantly worse prognosis [21]. Likewise, a specific expression profile was generated for 67LR+ cancer stem cells from the SW780 bladder cancer cell line and compared to several publically available clinical bladder cancer expression profiles. Significant overlaps with signatures distinguishing normal urothelium from bladder cancer, unifocal from multifocal bladder cancer and superficial from invasive bladder cancer, respectively, were reported [30]. Importantly, 67LR- non-stem cells were essentially indistinguishable from unfractionated SW780 cells. This shows another important aspect. As stem cells frequently represent only a minor fraction of the total cell population, their expression profile remains, frequently, hidden when profiling the total cancer cells. The results above clearly illustrate that it is especially this specific expression profile of cancer stem cells that might be clinically particularly important [30].

Regarding CD44, an expression profile of CD44+ bladder cancer stem cells, too, was generated, with high expression of this specific signature being significantly negatively correlated with the time to recurrence and/or progression of superficial bladder cancer [19]. In a recent extension of this study [42], the authors were able, with the aid of newly developed algorithms, to distinguish three successive differentiation steps of bladder carcinoma, on the basis of specific cytokeratin expression (basal: CK14+ CK5+ CK20-, intermediate: CK14- CK5+ CK20-, differentiated: CK14- CK5- CK20+) and of corresponding cell surface marker expression profiles (basal: CD90+ CD44+ CD49f+, early intermediate: CD90- CD44+ CD49f+, late intermediate: CD90- CD44- CD49f+, differentiated: CD90- CD44- CD49f-). There are two crucial conclusions from this analysis. First, bladder tumors are very heterogenous as to their differentiation status – only a fraction of tumors involved the whole differentiation sequence from basal through intermediate to differentiated cells, whereas a significant proportion of tumor samples involved only intermediate and differentiated cell populations or even only phenotypically apparently differentiated cells. Importantly, irrespective of the tumor differentiation status, it was always the least differentiated subpopulation that comprised the putative cancer stem cells, defined as tumor initiating cells upon xenotransplantation into immunodeficient mice. Second, the tumor differentiation status carried important clinical information. The presence of basal bladder carcinoma cells (either CK14+ CK5+ CK20- or CD90+ CD44+ CD49f+) was associated with a significantly worse clinical course compared to all other tumor groups. From a practical point of view, the presence of basal bladder cancer cells could be readily evidenced by a simple immunohistochemical staining for CK-14, which could be realistically accomplished as a part of the standard pathologic examination. If validated, this approach would open the way to the clinical stratification of bladder cancer patients by virtue of biological properties of the stem cells of their respective tumors.

At the same time, this point illustrates the greatest challenge facing the current urothelial cancer stem cell field – to translate the wealth of knowledge gained into improvements in the care of cancer patients. In this context, it should be repeated once more that any effort to treat urothelial carcinoma by targeting

Table 1. Comparison of biological and molecular characteristics between normal urothelial stem cells and urothelial cancer stem cells.

Characteristic	Urothelial stem cell	Urothelial cancer stem cell	Reference
Morphology	small size, display low granularity and a high nucleo-cytoplasmic ratio	small size, display low granularity and a high nucleo-cytoplasmic ratio in a fraction of CSCs	12, 19
Clonogenicity	high, holoclones	high, holoclones, ancorage-independent growth	12, 22, 24
Tumorigenicity	not applicable	high	19, 21, 24, 42
Plasticity (dedifferentiation capacity)	yes, paraclones can dedifferentiate into holoclones	yes, non-SP cell can dedifferentiate into SP cells	12, 23, 24
Position in the epithelium	basal	basal, if applicable	3, 7, 8, 19, 42
Stromal dependence	yes, at least during tissue regeneration	yes, interaction with stromal cells imparts stem cell character	19, 26, 27, 30
CD44 positivity	yes	yes, in ~40 % of tumor samples	19, 20, 21
Positivity for basal-type cytokeratins (CK5, CK14, CK17)	yes	yes	19, 26, 27, 42
Aldehyde dehydrogenase activity	not reported	yes	21
Activity of ABC-efflux pumps	not reported	yes (side population)	22,23,24

urothelial cancer stem cells can only be successful if normal urothelial stem cells are not fatally damaged. From this point of view, simultaneous investigation and careful comparison (Tab 1) of both stem cell populations is crucial.

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