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Normal and neoplastic urothelial stem cells: getting to the root of the problem

Philip Levy Ho, Antonina Kurtova, and Keith Syson Chan

Scott Department of Urology, Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

Abstract

Most epithelial tissues contain self-renewing stem cells that mature into downstream progenies with increasingly limited differentiation potential. It is not surprising that cancers arising from such hierarchically organized epithelial tissues retain features of cellular differentiation. Accumulating evidence suggests that the urothelium of the urinary bladder is a hierarchically organized tissue, containing tissue-specific stem cells that are important for both normal homeostasis and injury response. The phenotypic and functional properties of cancer stem cells (CSCs; also known as tumour-initiating cells) from bladder cancer tissue have been studied in detail. Urothelial CSCs are not isolated by a ‘one-marker-fits-all’ approach; instead, various cell surface marker combinations (possibly reflecting the cell-of-origin) are used to isolate CSCs from distinct differentiation subtypes of urothelial carcinomas. Additional CSC markers, including cytokeratin 14 (CK14), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), and tumour protein 63 (p63), have revealed prognostic value for urothelial carcinomas. Signalling pathways involved in normal stem cell self-renewal and differentiation are implicated in the malignant transformation of different subsets of urothelial carcinomas. Early expansion of primitive CK14⁺ cells—driven by genetic pathways such as STAT3—can lead to the development of carcinoma *in situ*, and CSC-enriched urothelial carcinomas are associated with poor clinical outcomes. Given that bladder CSCs are the proposed root of malignancy and drivers of cancer initiation and progression for urothelial carcinomas, these cells are ideal targets for anticancer therapies.

Introduction

Stem cells are unspecialized cells that undergo unlimited self-renewal and multilineage differentiation to become specialized cells. Stem cells are categorized according to their differentiation potential as totipotent, pluripotent, multipotent, oligopotent, or unipotent.¹ Totipotent stem cells can develop into any cell type present in an organism. In terms of human development, the zygote is the earliest possible totipotent stem cell that can fully develop into all three germ cell layers (ectoderm, mesoderm, and endoderm) and extraembryonic tissues.¹ Typical embryonic stem cells are pluripotent and derived from the inner cell mass (about 200–300 cells) of blastocysts.² Unlike totipotent stem cells, pluripotent embryonic stem cells lack the ability to form extraembryonic tissues, but can fully develop into all three germ layers. Stem cell biology was revolutionized when it was demonstrated that four transcription factors can induce the generation of pluripotent stem

Correspondence to: K. S. Chan, kc1@bcm.edu.

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cells from terminally differentiated or adult somatic cells.³ These cells are known as induced pluripotent stem (iPS) cells.

Multipotent stem cells give rise to specific cell types in multilineages, such as haematopoietic stem cells, bulge stem cells (present in the skin), and intestinal stem cells. Haematopoietic stem cells can give rise to multiple downstream lineages—such as the myeloid lineage (including monocytes, macrophages, neutrophils, and dendritic cells) and the lymphoid lineage (including T, B, and NK cells)—and are responsible for the development of all mature blood cells in the system.⁴ Bulge stem cells in skin are multipotent and capable of forming multiple lineages, including the epidermis, hair follicle, and sebaceous gland.⁵

Oligopotent stem cells can give rise to only a few different cell types within a certain lineage. Examples include the common lymphoid progenitors that give rise to NK, T, and B lymphocytes in the haematopoietic system,⁴ as well as the oligopotent stem cells of the cornea that produce epithelial and goblet cells.⁶ Unipotent cells can only give rise to a single lineage. For example, basal stem cells in the epidermis only give rise to mature cells within the epidermal compartment.⁵

It is unsurprising that cancers arising from adult tissues with a hierarchical organization retain some of these biological features. Cancer stem cells (CSCs) behave similarly to normal stem cells in that they maintain the same functional ability to limitlessly self-renew and differentiate into heterogeneous cell populations. In addition, CSCs have the unique potential to initiate tumours. However, it should be noted that ‘cancer stem cell’ is a functional term; its use does not necessarily mean that CSCs only arise from normal stem cells. CSCs have been isolated from leukaemias,⁷ breast,⁸ brain,⁹ and colon¹⁰ cancers, as well as many other epithelial tumour types. It is also worth noting that the concept of CSCs is not mutually exclusive to the clonal evolution model.

Emerging evidence supports the existence of normal urothelial stem cells and CSCs in the bladder. Early characterization of these cells revealed that similar signalling pathways are activated during developmental lineage specification and bladder cancer pathogenesis. In this Review, we highlight historical perspectives and recent progress in the study of normal urothelial and neoplastic bladder stem cells. We focus on developments in stem cell isolation, molecular characterization (in terms of the signalling pathways involved in cellular differentiation), and global gene expression profiling, with particular emphasis on the clinical implications of these developments.

Isolating stem cells

Two key developments—fluorescence-activated cell sorting (FACS) and monoclonal antibody production—enabled isolation of the first mouse and human haematopoietic stem cells.^{11–14} These techniques have since become fundamental tools for the prospective isolation of viable stem cells, including CSCs, from multiple tissue types.

In vivo xenotransplantation and reconstitution

The most stringent test of a functional stem cell is the demonstration of its unique abilities to self-renew and maintain multilineage differentiation at a single-cell level in a reconstitution assay *in vivo*. For example, a single fully functioning mammary stem cell should be able to develop into a whole functional mammary duct in a cleared fat pad. The self-renewal property of stem cells is determined by their ability to serially form the entire hierarchical organization of tissue in a secondary transplantation.¹⁵

The current gold standard for assaying CSCs is the xenotransplantation assay (typically in immunocompromised mice). Primary tumours, xenografts, or immortalized cell lines are usually subfractionated by FACS or magnetic bead separation and the relative tumorigenic potential of cell subpopulations is determined by their latency and xenograft-take rate.¹⁶ The ability of CSCs to self-renew is examined by serial transplantation into a second recipient. Using flow cytometry and immunohistochemistry, differentiation ability is determined by the extent to which a pure CSC population can regenerate the heterogeneity of the original tumour.¹⁶

***In vitro* stem cell assays**

Several *in vitro* stem cell assays have been adapted from neural and skin stem cell systems. These assays include the sphere-forming assay and the fibroblast-supported clonogenic assay.¹⁷ For the sphere-forming assay, non-adherent plates and serum-free media are generally used to culture stem cells at low or clonal density. The fibroblast-supported clonogenic assay requires an irradiated or mitomycin-C-treated feeder cell layer, usually composed of 3T3 fibroblasts. Stem cells are then plated at low density onto the irradiated feeders. The study endpoint is generally the size and number of spheres or colonies that form. Differentiation is expressed through the heterogeneity of spheres or clones, whereas self-renewal is evident by the ability of sphere cells or clones to undergo serial passage.¹⁷

Normal bladder urothelial stem cells

The adult urinary bladder is composed of the urothelium (mucosa), lamina propria (submucosa), muscularis propria, and perivesical soft tissues. The adult urothelium is histologically classified as transitional epithelium, which comprises at least 3–6 layers of basal, intermediate, and superficial (umbrella) cells (Figure 1a).^{18,19} Basal cells are small and polygonal (~10 µm), usually forming a single layer in direct contact with the basement membrane. Intermediate cells are pyriform (~10–40 µm) and can form several cell layers. Umbrella cells are large bi nucleated or multinucleated cuboidal cells (~70–100 µm) that form a single layer in direct contact with the urinary space.¹⁸ A unique feature of transitional urothelium is that it can accommodate bladder filling and emptying through distension and subsequent retraction.

The adult human urothelium is estimated to renew itself every 3–6 months during normal homeostasis.¹⁹ However, upon pathological damage (for example, bacteria-induced) or chemically induced injury (for example, following exposure to cyclophosphamide),²⁰ basal cells of the murine urothelium rapidly proliferate and the urothelium is completely regenerated within 72 h.²¹ Similarly, when rat urothelium is exposed to protamine sulphate (which induces restricted abrasion of umbrella cells but spares the intermediate cell layer), intermediate cells possess the regenerative capacity to form a functionally intact urothelium by day 10.²²

The urothelium also displays properties of plasticity and can undergo metaplastic transdifferentiation. For example, squamous metaplasia of the trigonal region of the bladder²³ and cystitis glandularis (metaplastic transformation of the mucosal bladder lining)²⁴ are both commonly reported. Taken together, these characteristics support the existence of normal adult stem cells within the urothelium.

Localizing normal bladder stem cells

Epithelial stem cells have been shown to possess a unique label-retention property, resulting from either the asymmetric segregation of DNA strands or the slow-cycling characteristics of these stem cells.²⁵ In label-retaining assays, cells are exposed to labelled or synthetic nucleosides, such as ³H-thymidine and 5-bromo-2'-deoxyuridine, respectively. Replicating

cells incorporate these nucleosides into nascent strands during S phase. Repeated incorporation of these nucleosides ensures the labelling of all cell populations, including stem cells. With each round of cell division, presence of the labelled nucleoside diminishes. Rapidly proliferating cells lose their nucleoside label more rapidly than stem cells, which are slow-cycling and can asymmetrically retain the labelled DNA strand. Thus, stem cells are commonly referred to as label-retaining cells (LRCs).

Using this approach, multipotent adult stem cells have been localized in the crypts of intestine,²⁶ bulge region of the hair follicle,² corneal limbus,¹¹ endometrium,⁵ prostatic ducts,¹² and bladder.^{27,28} In one study of rat bladders, it was estimated that LRCs account for about 9% of all basal cells.²⁸ These basal LRCs expressed $\beta 4$ integrin and were found to be highly clonogenic in an *in vitro* stem cell assay (Figure 1b).²⁸ Lineage tracing experiments in mice also revealed that basal cells can give rise to all layers of mouse urothelium.²⁹ A separate study utilized naturally occurring mitochondrial DNA mutations as markers of clonal expansion in human urothelium.³⁰ The study authors reported that patches of clonally related urothelial cells from the basal layer were consistently connected to patches of intermediate and umbrella cells. These data from rodents and humans indicate a basal cell origin for urothelial stem cells (Figure 1b).

However, contradictory findings have been reported in another study, which investigated the localization of LRC in rat bladders using a different synthetic nucleoside—5-ethynyl-2-deoxyuridine—and demonstrated that LRC distribution is random, with no clear evidence of preferential basal cell labelling.³¹ These contrasting findings could result from differences in labelling efficiency and chase period, warranting further investigation. Alternatively, these disparities could be explained by findings from other studies,^{32,33} which suggest that, although some bladder stem cells originate from basal cells, others develop from an alternative pool of urothelial stem cells that can give rise to umbrella cells.

One such study utilized a mouse model deficient in tumour protein 63 (p63)—a p53 family member that is normally localized to the basal cell layer of bladder urothelium—to study urothelial development.³² The investigators demonstrated that the urothelia of p63-deficient embryonic mouse bladders were defective and only contained umbrella cells, suggesting that functional p63 is not required for the generation of umbrella cells. By complementing p63-deficient mouse blastocysts with wild-type embryonic stem cells to create chimeric mouse bladders, the study authors revealed that, although both p63⁻ and p63⁺ cells developed into umbrella cells, p63⁺ cells only accounted for about 0–15% of umbrella cells.³² These results suggest that umbrella cells do not necessarily arise from p63⁺ basal cells, supporting the existence of an alternative hypothesis to that of an exclusive basal origin for urothelial stem cells (Figure 1c).³²

These findings are supported by a second study, which demonstrated that, although basal and intermediate urothelial cells were lacking from the adult bladders of p63-null mice, a single layer of umbrella cells still formed.³³ Furthermore, these umbrella cells stained positive for uroplakin 2—a protein that is crucial for regulating permeability and providing tensile strength to urothelial umbrella cells. Collectively, these results suggest that there are at least two independent pools of urothelial stem cells in the bladder that can give rise to mature urothelial cells (Figures 1b and 1c).

Bladder urothelial CSCs

The urothelium is an organized tissue in which functional stem cells can give rise to downstream differentiated cells (Figure 2a). It is perhaps unsurprising, therefore, that cancers arising from this structured tissue architecture retain some level of hierarchical organization. Indeed, pathological analyses have revealed that all undifferentiated or poorly

differentiated (high-grade) bladder urothelial carcinomas are either invasive or have the potential to become invasive.³⁴ By comparison, well or moderately differentiated (low-grade) bladder urothelial carcinomas are generally associated with better clinical outcomes (Figures 2a and 2b).³⁴ Pathological grading—classified by histological analysis of bladder cancer differentiation—supports the existence of a modified tissue hierarchy and remains an important independent marker for clinical prognosis.³⁴

The CSC hypothesis, which builds upon pathological observations and our understanding of normal developmental biology, provides a biological and molecular explanation for the intratumoural heterogeneity of bladder urothelial carcinomas. This heterogeneity has been well established in humans, both histologically and functionally.³⁵ Evidence suggests that CSCs in bladder urothelial carcinomas can give rise to biological heterogeneity within a tumour by differentiating into downstream differentiated tumour cells. ‘Cancer stem cell’ is a functional term, which defines a tumour subpopulation with tumour-initiating potential, self-renewal properties, and the ability to generate cellular tumour heterogeneity via differentiation.¹⁶ These CSCs do not necessarily arise from normal stem cells; they are also derived from differentiated progenies that have acquired tumorigenic properties via genetic or epigenetic alteration.

Isolating bladder CSCs

Cell surface markers—Following the development of monoclonal antibodies that preferentially bind to the basal layer of normal urothelium and superficial cells (MoAb 21.48 and MoAb 5.48, respectively), researchers have analysed the staining patterns of cell surface proteins in urothelial carcinomas.³⁶ A high degree of MoAb 21.48 (basal) staining has been reported in high-grade urothelial carcinoma specimens, whereas MoAb 5.48 has demonstrated higher specificity for superficial cells, with more diffuse staining in well-differentiated tumours.³⁶ These early studies were probably the first to examine urothelial cancer development in parallel to normal urothelial developmental biology. The study results suggest that the cellular heterogeneity and hierarchical tissue organization observed in normal urothelium are conserved in tumours.

Since these initial studies were published, cell surface markers have remained a routine method for the isolation of viable CSCs. Given that cancer cells in these studies have undergone both *in vivo* immortalization and subsequent long-term *in vitro* culture, it is likely that alterations to their behaviour and antigenic expression have taken place—potentially resulting in characteristics that deviate from the source tumour. Primary or early *in vivo* passage tumour cells from patients are, therefore, the preferred source for CSC isolation and characterization.^{35,37}

Our laboratory has screened a panel of cell surface markers to analyse cell suspensions created from primary human bladder cancer specimens.^{35,37} Of the markers studied, CD44 was the most consistently expressed.^{35,37} Using FACS, infiltrating haematopoietic and endothelial cells were excluded and tumour cells were subfractionated into CD44⁺ and CD44[−] cells. The relative tumorigenic potential of these two cell subpopulations was then determined, based on their ability to form tumours in immunocompromised mice devoid of T, B, and NK cells—created by crossing mice deficient in recombination activating gene 2 (RAG2) and common cytokine receptor gamma chain (γc). The tumorigenic potential of CD44⁺ tumour cells was at least 10–200 times greater than that of CD44[−] tumour cells. CD44⁺ tumour cells underwent serial passage and were able to recapitulate the heterogeneity of the original tumour (Table 1).³⁷ Thus, these cells fulfilled all of the functional criteria of a CSC.

Using an *in vitro* colony-forming assay to assess tumour cell ‘stemness’, one research group utilized a CD44 spliced variant (CD44v6) to isolate CD44v6⁺ epithelial membrane antigen negative (EMA⁻) bladder CSC subpopulations (Table 1).³⁸ In another study, xenografts from the established bladder cancer cell line SW780 and from the once-passaged xenograft line XBL8 (derived from an invasive urothelial carcinoma) were used to identify tumorigenic cells. The 67L-kDa laminin receptor (67LR; a marker expressed at the tumour–stroma interface and upregulated in 80% of high-grade invasive urothelial carcinomas)³⁹ and carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6; a nonspecific crossreactive antigen) were used to isolate tumorigenic cells (Table 1). CSCs were identified as 67LR⁺ and CEACAM6⁻ cells; both of these markers are normally expressed in basal cells (Figure 1b).⁴⁰ Collectively, these studies highlight a distinct characteristic of tumour cell subpopulations—namely, that they express cellular markers that are also present in normal urothelial basal cells.

Cytokeratins—Cytokeratins are intermediate filaments that are expressed in the cytoskeleton of epithelial tissues (including the urothelium) and are differentially expressed during cellular differentiation and in different cell types (for example, basal, intermediate, and umbrella cells). Although cytokeratins cannot be used as markers for the isolation of viable tumour cells by FACS (as they are intra-cellular proteins that require cell permeabilization for detection), this protein family can provide information regarding the differentiation status of tumour cells.

It has been shown that intracellular cytokeratin 5 (CK5), which is expressed in normal basal cells, can differentiate into CK20-expressing superficial umbrella cells (Figure 1b).³⁷ In this study, immunofluorescence analysis of xenografts from CD44⁺ tumour cells revealed colocalization of CD44⁺ and CK5⁺ cells. CD44⁻ cells, on the other hand, frequently colocalized with CK20⁺ cells. In approximately half of the patients analysed, CD44⁺ tumour cells were relatively small and homogeneous in size, with a high nuclear:cytoplasmic ratio (characteristic of basal cells).³⁷ By comparison, CD44⁻ tumour cells were heterogeneously moderate or large in size; a characteristic associated with well differentiated cells.³⁷ In a separate study, CK17—another urothelial basal cell marker—was shown to colocalize with 67LR in SW780 xenograft tumour cells (Table 1).⁴⁰

There is evidence to suggest that the expression of cytokeratins can be altered during the transformation process. Observations from our laboratory suggest that the cytokeratin staining patterns of early versus late differentiation markers are usually mutually exclusive in bladder cancer. However, some cytokeratins that are expressed in the intermediate stages of normal cellular differentiation are sometimes aberrantly expressed in cancer cells.³⁷ As the cytokeratin family of intermediate filaments contains at least 20 proteins, it is possible that this bladder cancer subgroup aberrantly expresses cytokeratins that are not routinely expressed or analysed in the urothelium.

Side-populations and aldehyde dehydrogenase—In addition to cell surface and intracellular markers, the distinct biological properties of CSCs have been exploited for their prospective isolation. Stem cells are known to express high levels of ATP-binding cassette (ABC) transporters, enabling them to effectively efflux drugs.⁴¹ A subpopulation of stem cells with the ability to efflux the vital dye Hoechst 33342 is commonly referred to as a ‘side-population’. This unique property of stem cells has been used to isolate CSCs. Two independent research groups used established bladder cancer cell lines SW780 and T24, respectively, to isolate side-population cells by flow cytometry. These cells demonstrated colony-forming, self-renewal, and differentiation characteristics that are typical of CSCs and absent in non-side-population cells (Table 1).^{42,43}

Another promising marker for bladder CSCs is aldehyde dehydrogenase 1 A1 (ALDH1A1), which demonstrates increased expression in bladder CSCs.⁴⁴ Using an *in vitro* sphere-forming assay and *in vivo* xenograft assays, one study demonstrated greater clonogenicity and tumorigenicity for ALDH1A1⁺ bladder cancer cells compared to ALDH1A1⁻ cells. Xenografts from ALDH1A1⁺ cells retained the phenotypic heterogeneity of the source tumour with every serial passage (Table 1).⁴⁴ Functional disruption of the ALDH1A1 gene with a small hairpin RNA construct resulted in decreased CSC clonogenic and tumorigenic potential. Interestingly, these data suggest that ALDH1A1⁺ cells constitute a subpopulation of CD44⁺ cells and might represent more primitive CSCs.

Collectively, these findings support the existence of functional CSC subpopulations within primary bladder cancer specimens, xenografts, and immortalized cancer cell lines. Isolation techniques for each subpopulation have generally utilized different antigenic markers and functional assays, although some degree of overlap has been reported (for example, between CD44 and ALDH1A1 markers). As novel markers and approaches to CSC isolation continue to emerge, elucidation of the relationships between different CSC subpopulations becomes increasingly important.³⁵

Bladder CSC signalling

Peroxisome proliferator-activated receptor signalling

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors and transcription factors that are known to mediate cellular differentiation in a variety of tissue types, including the urothelium. All PPARs heterodimerize with the retinoid X receptor (RXR) and bind to the peroxisome proliferator hormone response elements (PPREs) to mediate transcriptional activation. There are three major members of the PPAR family—PPAR α , PPAR β/δ , and PPAR γ . PPAR γ agonists, such as troglitazone, have been shown to repress urothelial basal cell proliferation by inducing cell cycle arrest at G0/G1 (via upregulation of the cyclin-dependent kinase inhibitor p21).⁴⁵ Furthermore, activation of PPAR γ by troglitazone has been shown to promote urothelial differentiation in normal human urothelial cells *in vitro*, as demonstrated by upregulation of late or terminal differentiation markers such as CK13, UPII, UPIb, and CK20 (Figure 1b).^{46–48} Pretreatment of human urothelial cell culture with the PPAR γ antagonist GW9662 attenuated the troglitazone-induced differentiation response (Figure 1c).⁴⁶ Interestingly, tissue expression of PPAR γ in human bladder urothelial carcinoma is inversely associated with stage, tumour grade, and the expression of proliferation marker Ki67.⁴⁷ These results are consistent with the hypothesis that PPAR γ signalling is involved in both normal urothelial and neoplastic differentiation.

Epidermal growth factor receptor signalling

Early *in vitro* data revealed that epidermal growth factor (EGF) ligands and EGF receptors (EGFRs) are activated transiently in the early stages of wound response in both rodent^{49,50} and human^{51,52} urothelial cells, suggesting an early involvement in urothelial cell renewal. The EGFR ligands implicated in wound response include transforming growth factor alpha, EGF, keratinocyte growth factor, heparin-binding EGF, and amphiregulin. In one study, a neutralizing anti-amphiregulin antibody was shown to attenuate wound repair *in vitro*,⁵² implying a functional involvement of EGFR signalling in wound-induced urothelial cell renewal.

In another study, nuclear active transcription factor SOX-9 (SOX9) was implicated as a downstream mediator of EGFR signalling. SOX9 expression was found to be transiently induced following urothelial repair, in conjunction with increased EGFR, receptor tyrosine-protein kinase ERBB2 (also known as HER2), and receptor tyrosine-protein kinase ERBB3

(also known as HER3) mRNA expression.⁵³ SOX9 expression was also induced by exogenous treatment with EGF and heparin-binding EGF-like growth factor in immortalized human urothelial cells.⁵³ Induction of SOX9 expression by EGF ligands was diminished by MAPK kinase inhibitors, suggesting a role for EGFR-MAPK-SOX9 signalling during wound-induced urothelial cell renewal (Figure 1c).⁵³ In bladder urothelial carcinomas, EGFR overexpression correlates with poor prognosis parameters, including histological grade, tumour stage, and recurrence.⁵⁴ These results indicate that functional EGFR signalling is active during urothelial regeneration and neoplastic transformation.

Self-renewal and differentiation signalling

Signalling pathways that regulate cellular renewal and differentiation are not restricted to the urothelial cell compartment. An interplay between the epithelial and stromal compartments is essential in the regeneration of injured urothelium from basal stem cells.²⁹ Using genetically modified mice marked by fluorescent analogues of proteins such as enhanced green fluorescent protein, studies have demonstrated that the sonic hedgehog protein (SHH) is expressed in urothelial basal cells during steady-state conditions. Interestingly, genetic lineage tracing has revealed that SHH-expressing urothelial basal cells can give rise to all layers of the urothelium (including uroplakin-3a-expressing terminally differentiated urothelial cells) following bacteria-induced injury of the urothelium.²⁹

However, regeneration of urothelium from SHH⁺/CK5⁺ urothelial basal cells is dependent on feedback paracrine signals from neighboring stromal cells (Figure 1c).²⁹ SHH secretion from epithelial basal cells signals the activation of zinc finger protein GLI1—a transcription factor that is primarily localized and expressed in neighbouring stromal cells in the lamina propria. GLI1 activation in stromal cells triggers a Wnt paracrine signal that induces the expansion of urothelial basal cells and replenishes the injured urothelium. It has been suggested that this regenerative process mirrors the process of carcinogenic transformation in epithelial tumour cells.

Several of the signalling pathways that are involved in the self-renewal of adult and embryonic stem cells are also often activated during tumorigenesis.³⁷ β -Catenin is a downstream activator of the Wnt ligand, which has a role in both self-renewal and oncogenesis in haematopoietic and chronic myelogenous leukaemic stem cells.⁵⁵ Another protein—polycomb complex protein BMI-1—is essential for the self-renewal of adult haematopoietic stem cells and neural stem cells,^{56,57} and has also been implicated in the tumorigenesis of many malignancies. Signal transducer and activator of transcription 3 (STAT3) is an essential protein for the self-renewal of human embryonic stem cells⁵⁸ that is constitutively active in a number of epithelial cancers.^{59,60} POU domain, class 5, transcription factor 1 (also known as Oct-4) and homeobox protein NANOG—key transcription factors for maintaining the self-renewal properties of embryonic stem cells⁶¹—are also expressed in some somatic and germ cell tumours. In epithelial tissues, Oct-4 expression leads to proliferation of progenitor cells, dysplastic growth, and block of differentiation.^{61,62}

Comprehensive analysis of the expression of these oncoproteins in human bladder urothelial carcinomas has revealed a tremendous heterogeneity in their activation status among patients. Nuclear active β -catenin was observed in only 5% of bladder urothelial carcinoma samples, whereas nuclear localization of other self-renewal signal transducers, such as BMI-1 and STAT3, was more pronounced (20% and 40% localization, respectively) and 80% of bladder urothelial carcinomas expressed GLI1 mRNA.³⁷ There was no evidence of expression of NANOG or Oct-4 in any of the urothelial carcinoma samples. Expression levels of STAT3 and BMI-1 strongly correlated with expression of CD44. This molecular heterogeneity in the signalling pathways within CSCs probably reflects the diverse aetiology

of bladder urothelial carcinomas, which has been strongly linked to tobacco smoking and exposure to industrial chemicals.³⁴

Prognostic role of bladder CSCs

CSC markers

The basal characteristics of bladder CSCs have been well documented; however, 58% of tumours do not express the basal cell marker CD44.³⁷ This suggests that either CD44[−] tumours do not arise from the basal cell compartment or that they exhibit aberrant protein expression during the transformation process. We have developed a novel biologically supervised computational approach to predicting markers with roles in bladder cancer differentiation. Using this approach to extrapolate information from existing gene expression databases, we have shown that basal cells preferentially express CK5 (which colocalizes with cell surface marker CD44), whereas CK20 expression is restricted to terminally differentiated cells (Figure 1b). Cytokeratin 14 (CK14) was identified as a more primitive upstream keratin marker to CK5 and CK20.⁶³ Further analysis revealed CD90 as a corresponding primitive stem cell marker and loss of CD49 as another terminal differentiation marker (Figure 2a).⁶³

Using these marker combinations, we have been able to risk-stratify urothelial carcinomas into three subtypes based on their differentiation status—basal, intermediate, and differentiated (Figure 2a).⁶³ The most primitive basal subtype correlates with the poorest clinical outcomes and, importantly, the most primitive cell subpopulation within each tumour subtype exhibits CSC-like characteristics.⁶³ These results suggest, but do not directly demonstrate, that distinct subtypes of bladder urothelial carcinoma probably arise from normal urothelial cells of distinct cellular differentiation status (Figure 2a). Nevertheless, cancer derived from more differentiated urothelial cells can acquire the characteristics of CSCs via genetic and epigenetic alterations.

CK14—Our research team evaluated the prognostic significance of the primitive stem cell marker CK14 using formalin-fixed paraffin embedded (FFPE) tissue samples from patients with bladder urothelial carcinoma.⁶³ Kaplan-Meier analyses of FFPE samples from two independent cohorts of patients revealed that patients with CK14⁺ bladder urothelial carcinomas demonstrated significantly reduced overall survival compared to patients with CK14[−] tumours. *CK14* gene expression was a strong predictor of poor survival— independent of tumour stage and grade.⁶³ Furthermore, noninvasive Ta tumours with upregulated CK14 expression were positively associated with tumour recurrence and progression, and negatively associated with overall survival.⁶³

ALDH1A1—In one study of bladder urothelial carcinomas, patients with high expression of the CSC marker ALDH1A1 were over four times more likely to experience tumour progression and decreased cancer-specific and overall survival than patients with low expression of ALDH1A1. *ALDH1A1* gene expression was also shown to be an independent predictor of survival (Table 1).⁴⁴

p63—p63 is a protein that is primarily expressed in the basal cells, as well as some intermediate cells, of the bladder urothelium. Different research groups have evaluated the clinical significance of p63 expression and reported conflicting results. Earlier studies established that loss of p63 was associated with muscle-invasive bladder urothelial carcinomas and tumour progression.^{64,65} However, a later study demonstrated an association between high p63 expression and decreased survival in patients with muscle-invasive bladder urothelial carcinomas.⁶⁶ These conflicting results could be explained by changes in the methodology used to analyse patient populations. In the more recent study, patients with

non-muscle-invasive cancers (who generally exhibit high p63 expression but good long-term survival) were excluded from analysis. Additionally, this discrepancy could also result from the limited ability of antibodies to distinguish between the distinct isoforms of p63 (TAp63 and Δ Np63). To address this issue, another research group utilized a Δ Np63-specific antibody and demonstrated that Δ Np63 expression correlated negatively with clinical outcomes in patients with invasive urothelial carcinomas.³³ Collectively, these findings highlight the prognostic significance of primitive CSC markers and basal cell markers in patients with urothelial carcinomas.

Interestingly, some CSC markers have also been investigated in the context of prognosing histological urothelial carcinoma variants, such as squamous cell carcinomas (SCCs) and micropapillary urothelial carcinomas. These variants, which comprise less than 5–6% of all bladder cancer specimens in the USA, are associated with poor prognosis and aggressive phenotype.³⁴ In a study that analyzed 48 pure bladder SCCs and 56 mixed (SCC and urothelial carcinoma) tumours, 96% of pure SCCs stained strongly positively for CK5 and CK14 markers and negative for CK20.⁶⁷ By comparison, 86–93% of mixed tumours stained strongly positive for CK5 and CK14, negative for uroplakin, and rarely positive for CK20.⁶⁷ Another study analyzed 20 micropapillary variants of urothelial carcinomas and demonstrated that these cancers stained positive for both 343E12 (basal cell marker) and CK20.⁶⁸ However, they did not report a significant difference between the staining patterns of micropapillary variants and pure urothelial carcinomas.⁶⁸ In fact, the most aggressive pure urothelial carcinomas and histological variants all stained positive for primitive CSC markers, suggesting that aggressive forms of bladder cancer might arise from a common primitive stem cell origin (Figure 2b).

Gene expression signatures

As CSCs represent the most tumorigenic subpopulation of urothelial carcinomas, global molecular profiling of these cells might reveal additional biological insights into tumour development. Next-generation sequencing of human urothelial carcinomas has revealed mutations in several chromatin-remodelling genes.⁷⁵ Mutation frequency of one of the chromatin-remodelling genes, UTX, is significantly associated with early-stage urothelial carcinomas.⁷⁵ UTX expression levels were 35% for grade 1, 15% for grade 2, and 4% for grade 3 urothelial carcinomas. Although the role of chromatin-remodelling proteins in urothelial carcinoma development is still unclear, this study highlighted the importance of epigenetic programming in the pathogenesis of bladder urothelial carcinomas. Another group used whole-organ genetic mapping to identify genetic changes associated with clonal expansion and step-wise progression of bladder cancer, and generate a genome-wide map of bladder cancer progression.⁷⁶ Furthermore, global gene expression profiling of bulk cancer masses has led to initial success in the molecular subclassification of bladder cancer, with meaningful clinical parameters.^{55,69–74,77}

Using a global gene expression platform, we identified a panel of 477 genes upregulated in CD44⁺ CSCs (referred to as a bladder CSC gene signature).³⁷ Gene expression data from two independent published data-sets were used to evaluate the prognostic value of this CSC gene signature. We established that unsupervised hierarchical clustering of the data can be used to categorize patients according to whether they have an activated or repressed bladder CSC gene signature; the latter being highly reliable for identifying noninvasive tumours and predicting better clinical outcomes.³⁷ Additionally, CSC gene signatures can be used to identify subgroups of patients with noninvasive bladder cancers at risk of shorter time to progression and poorer survival.³⁷

Another research group independently derived a gene signature by examining the global gene expression of 67LR⁺ and 67LR[−] cells from xenografts generated from the SW780

cancer cell line. The investigators reported similarities between the gene expression profiles of the 67LR⁺ highly tumorigenic cells (HTCs) and other aggressive forms of urothelial carcinoma.⁴⁰ Publicly available gene expression datasets were used to show that genes with altered expression status in HTCs have also been used to differentiate between normal and neoplastic urothelium, solitary and multifocal cancers, and non-muscle-invasive and muscle-invasive tumours. Further data mining for this gene signature revealed HTCs enriched for stem cell self-renewal pathways involving Janus kinase-signal transducers and activators of transcription, such as Wnt, mTOR, Notch, focal adhesion kinase, and EGFR.

Another group of investigators utilized a different analytical approach to assess whether an embryonic stem cell gene signature can be used to subclassify bladder urothelial carcinomas. Their analysis revealed that high-grade urothelial carcinomas (poorly differentiated) have an enriched embryonic stem cell gene signature, although this signature does not effectively segregate noninvasive from invasive bladder urothelial carcinomas.⁷⁸ Taken together, these findings suggest that genes that are upregulated in CSCs could have a key role in bladder cancer cell invasion, whereas genes enriched in embryonic stem cells are associated with poorly differentiated tumours.

Collectively, these findings could help to identify candidate genes associated with the clinical behaviour of aggressive bladder cancers. This could potentially lead to the establishment of standardized microarray-based tests—as pioneered for breast cancer (for example, MammaPrint^{®79} [Agendia, Inc., CA, USA] and Oncotype DX^{®80} [Genomic Health, Inc., CA, USA])—and the development of a personalized approach to the clinical management of bladder cancer.

Two-pathway progression

Previous research in the field has suggested that the development of noninvasive and invasive bladder urothelial carcinomas can occur via two separate pathways with distinct pathobiologies (Figure 2b).^{81–83} The first pathway is associated with early-stage urothelial carcinomas (pTa or pT1), which account for 70–80% of all urothelial carcinomas. These urothelial carcinomas commonly contain activating mutations of the fibroblast growth factor receptor 3 (FGFR3), Harvey Ras (HRAS) or PI3-kinase (PIK3CA).^{84–86} They develop through a step-wise progression from hyperplastic lesions to papillary non-invasive urothelial carcinomas that have a high propensity to recur; only a small proportion (about 15%) of the high-risk subgroup will progress to invasive urothelial carcinomas. The second pathway has been associated with advanced-stage urothelial carcinomas (>pT2), which account for 20–30% of all urothelial carcinomas. These urothelial carcinomas are commonly linked to mutations in the tumour suppressor genes *P53*, *RB*, and *PTEN*.^{87–89} These advanced-stage urothelial carcinomas commonly arise from high-grade dysplastic lesions or carcinoma *in situ* (CIS), usually without an intermediary stage of noninvasive papillary disease.

It is important to understand how urothelial CSCs might fit into this two-pathway model of bladder carcinogenesis. The majority of studies have reported that urothelial CSCs display basal cell characteristics. Normal urothelial basal cells express CK5, CK17, CD44, and 67LR, and are in direct contact with the basement membrane. We have demonstrated that CK14⁺ cells constitute a subpopulation of CK5⁺ basal cells and could represent a primitive stem cell population in both human⁶³ and murine⁹⁰ urothelial tissues. Human bladder urothelial carcinomas with a higher frequency of CK14⁺ CSCs have been associated with poorer survival in three independent gene expression datasets and two independent FFPE tissue sample sets (at the protein level).⁶³ CK14 expression is not restricted to noninvasive (pTa or pT1) or invasive (>pT2) urothelial carcinomas. Independent of pathology, urothelial carcinomas with high levels of CK14 expression have been associated with reduced

progression-free and overall survival.⁶³ These results suggest that differentiation status (basal subtype) can reflect the aggressive behaviour of bladder cancers.

Further investigation revealed that activation of the STAT3 signalling pathway directed urothelial cells towards the CIS-invasive urothelial carcinoma pathway (Figure 2b).⁹⁰ Using a mouse model, it has been demonstrated that treatment with a carcinogen leads to direct progression to CIS and subsequent invasive urothelial carcinoma development, bypassing a noninvasive tumour stage.⁹⁰ Interestingly, STAT3-driven invasive urothelial carcinomas are heavily populated with primitive CK14⁺ stem cells.⁹⁰ Taken together, these data suggest that primitive cellular differentiation status and genetic alteration (for example, STAT3 activation) both contribute towards driving CIS-invasive urothelial carcinoma development. It seems probable that low-grade noninvasive urothelial carcinomas arise from a more differentiated cell-of-origin, whereas invasive urothelial carcinomas arise from a more primitive cell-of-origin (Figure 2a); however, definitive lineage-tracing experiments are required to support this hypothesis. Nevertheless, these initial studies of urothelial CSCs provide important biological insights into the two-pathway model of bladder carcinogenesis and could, in the future, be useful for predicting and monitoring disease progression.

Therapeutic role of bladder CSCs

Conventional cytotoxic therapy

CSCs possess intrinsic unique biological properties that enable them to survive and repopulate residual tumours following cytotoxic treatment.⁹¹ Studies in other organ systems have shown that CSCs are more resistant than non-CSCs to conventional cytotoxic treatment approaches, such as radiation and chemotherapy.⁹¹ In one study, CSCs were isolated in the 5637 bladder cancer cell line on the basis of CD44 expression.⁹² Following exposure to cisplatin, CD44⁺ cells demonstrated improved survival relative to CD44⁻ cells with half-maximal inhibitory concentrations of 0.43 µg/ml and 0.25 µg/ml, respectively. CD44⁺ cells also demonstrated a higher transforming ability than CD44⁻ cells when exposed to cisplatin in a clonogenic assay.⁹²

In another study, the ALDEFLUOR™ (STEMCELL Technologies, Inc., Grenoble, France) assay was used to isolate populations of cells with high ALDH activity—also known as ALDH(High) tumour cells—from T24 and 5637 bladder cancer cell lines. ALDH(High) tumour cells demonstrated resistance to cisplatin *in vitro*, colony-forming properties, and other aggressive characteristics of CSCs.⁹³ Using an alternative approach, a recent study established derivatives of the T24 bladder cancer cell line (DR-T24) via continuous exposure to cisplatin *in vitro*. These DR-T24 derivatives contained a greater subpopulation of side population cells and increased *in vitro* clonogenic capacity compared to the T24 parental line.⁹⁴ Collectively, these data support the existence of a bladder CSC population that differentially responds to cisplatin-based chemotherapy.

Interestingly, normal stem cells also show evidence of resistance to chemotherapy and radiation. For example, nonmyeloablative chemotherapy results in pancytopenia. This associated adverse effect is not permanent—numbers of mature white and red blood cells are restored after treatment. It seems that normal haematopoietic stem cells are also immune to these treatment modalities. One possible explanation is that both normal stem cells and CSCs contain lower levels of reactive oxygen species as a result of upregulated free radical scavenging systems.⁹⁵

Further studies of the molecular mechanisms responsible for bladder CSC response to chemotherapy could be clinically significant. Blockade of these signalling pathways—in combination with chemotherapy, BCG immunotherapy, or both modalities—might improve

therapeutic efficacy. Pending further validation, CSC markers could be used to predict response to conventional therapies for high-risk noninvasive bladder cancers, including BCG immunotherapy and neoadjuvant chemotherapy. These markers could provide molecular information to further risk stratify patients with bladder cancer.

Targeted therapy

A widely used and effective approach for anticancer therapy is targeting monoclonal antibodies to cancer-specific cell antigens. CD47 is one of the proteins that is significantly downregulated during bladder cancer cell differentiation.^{37,67} Immunofluorescence and FACS analysis have confirmed this finding, showing widespread expression of CD47 in bulk bladder urothelial carcinoma cells. CD47 levels are preferentially higher in CD44⁺ CSCs in comparison to CD44⁻ tumour cell subpopulations. CD47 acts as a 'don't-eat-me' signal, interacting with signal regulatory protein α (SIRP α)—a plasma membrane protein expressed mainly in myeloid cells (macrophages, neutrophils, basophils, and dendritic cells). Ligation of CD47 to SIRP α leads to the suppression of immune cell activity and, ultimately, to the blockade of phagocytosis by macrophages.⁶¹

We have previously shown that the *in vitro* incubation of human bladder urothelial carcinoma cells with macrophages and a blocking anti-CD47 antibody leads to phagocytosis of tumour cells, whereas incubation with a negative control (IgG1 isotype) does not lead to tumour cell engulfment.³⁷ Human bladder urothelial carcinoma xenografts treated *in vivo* with the same blocking anti-CD47 antibody exhibited a significant reduction in metastatic lymph node size and micrometastasis to the lung (compared with an IgG-isotype-treated control).⁹⁶ These data support the hypothesis that CD47, and potentially other proteins overexpressed by CSCs, are promising targets for cancer therapy. Unlike the conventional chemotherapy-based and radiation-based approaches to cancer treatment, targeted therapy with CSCs—whether it involves antibodies, CSC-targeted radiosensitizers, CSC-targeted chemosensitizers, or small molecule inhibitors to stem cell pathways—can, hopefully, prove to be of curative benefit.

Conclusions

Functionally distinct cancer stem cells exist within human urothelial carcinomas. These cancer stem cells can arise from different cells-of-origin and can not be identified by a 'one-marker-fits-all' approach; instead, distinct marker combinations are used to isolate these CSCs from various differentiation subtypes of urothelial carcinomas. Advances relating to the molecular and functional characterization of CSCs in human urothelial carcinomas have revealed insights into our current understanding of the two-pathway model of bladder carcinogenesis. Early expansion of primitive CK14⁺ cells, driven by genetic pathways such as STAT3, leads to progression towards the CIS-invasive pathway. Additionally, retrospective studies have demonstrated the potential of several CSC markers—particularly CK14, ALDH1A1, and p63—as prognostic markers for stratifying high-risk bladder urothelial carcinomas. To evaluate and validate the clinical utility of these markers (for example, in predicting therapeutic response), large-scale prospective clinical trials are required. Further biological subtyping (by differentiation status) and molecular subtyping (by gene expression) of urothelial carcinomas will be important to relate cancer stem cell biology to cancer phenotype. Although many signalling pathways that are intrinsic to urothelial CSCs are implicated in tumorigenesis, an improved understanding of the genetic and epigenetic changes that occur in CSCs—and how the tumour microenvironment contributes extrinsically to regulate these CSCs—could provide novel targets for therapeutic action against bladder urothelial carcinomas.

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Key points

- Normal slow-cycling urothelium demonstrates rapid regenerative potential and the ability to transdifferentiate into multiple cell types; characteristics that support the existence of normal urothelial stem cells
- Evidence suggests that urothelial stem cells primarily originate from basal cells, whereas an alternative pool of stem cells might exist that could give rise to umbrella cells within the urothelium
- Tumorigenic subpopulations of cancer stem cells (CSCs) with basal cell characteristics and phenotypic markers are evident in primary bladder cancers, xenografts, and immortalized cell lines
- Signalling pathways implicated in normal stem cell self-renewal and lineage differentiation have major roles in bladder cancer development; heterogeneity in their activation status is evident among patients
- Bladder cancers can be categorized into subtypes on the basis of differentiation status; the most primitive basal subtypes and cell markers correlate with poor clinical outcomes
- Novel targeted approaches for treating bladder CSCs might improve the efficacy of current standard-of-care treatment regimens when administered as combination therapy

Figure 1.

Markers and signalling pathways associated with urothelial cellular differentiation. **a** | Composition of the normal adult bladder urothelium. Spiky bright-green symbols represent infiltrating immune cells, stromal cells are shown in grey, basal cells are shown in light brown, a single stem cell is shown in dark brown, intermediate cells are shown in blue, and umbrella cells are shown in purple. **b** | The association of markers with normal urothelial cellular differentiation. Thin black arrows depict transitions from one cell type to another. Dotted thin black lines show an alternative (hypothetical) pathway for cellular differentiation. Thick black arrows depict increases in levels of expression. **c** | The role of signalling proteins and pathways in injury-induced cell regeneration. Thin black arrows depict transitions from one cell type to another. Dotted thin black lines show alternative (hypothetical) signalling pathways for cellular regeneration. Abbreviations: 67LR, 67L-kDa laminin receptor; CD, cluster of differentiation; CK, cytokeratin; EGF, epidermal growth

factor; EGFR, epidermal growth factor receptor; GLI1, zinc finger protein GLI1; LRC, label-retaining cell; MAPK, mitogen-activated protein kinase; PPAR γ , peroxisome proliferator-activated receptor γ ; SHH, sonic hedgehog protein; SOX9, transcription factor SOX-9; Wnt, protein Wnt.

Figure 2.

Clinical relevance of urothelial CSCs. **a** | Process of cellular differentiation in the normal urothelium. During the process of differentiation, loss of expression marker CD90 occurs early on, followed by changes to expression status of CD44, and, in later stages, CD49. At least three subtypes of UC exist on the basis of differentiation status. Differentiated and intermediate subtypes are associated with better outcomes, whereas basal subtypes are associated with poor clinical outcomes. **b** | Early-stage UCs (pTa or pT1) commonly contain activating mutations in *FGFR3*, *HRAS*, or *PIK3CA*. About 15% of the high-risk subgroup will progress to invasive UCs. Advanced-stage UCs (>pT2) are commonly linked to mutations in the tumour suppressor genes *P53*, *RB*, and *PTEN*. These advanced-stage UCs commonly arise from high-grade dysplastic lesions or CIS, usually without an intermediary stage of noninvasive papillary disease. STAT3-driven expansion of CK14 primitive stem cells directs urothelial cells towards the CIS-invasive pathway. Some CSC markers (CK5, CK14, and 34BE12) have been linked to histological UC variants, such as squamous cell carcinomas and micropapillary UCs, which are associated with poor prognosis and aggressive phenotype. Abbreviations: CIS, carcinoma *in situ*; CK, cytokeratin; CSCs, cancer stem cells; FGFR3, fibroblast growth factor receptor 3; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; PIK3CA, phosphoinositide-3-kinase, catalytic, alpha polypeptide; PTEN, phosphatase and tensin homolog; p53, tumour protein 53; RB, retinoblastoma protein; SCC, squamous cell carcinoma; STAT3, signal transducer and activator of transcription 3 (acute-phase response factor).

Table 1

Selected studies of urothelial CSC markers

Study	Study design	CSC markers	Tumour cell type	Tumorigenic potential
Yang & Chang ³⁸	<i>In vitro</i> colony-forming assay	EMA ⁺ ; CD44v6 ⁺	Primary patient	None reported
She <i>et al.</i> ⁴²	<i>In vitro</i> colony-forming assay	SP (DyeCycle violet staining)	SW780	>10-fold
Ning <i>et al.</i> ⁴³	<i>In vitro</i> colony-forming assay	SP (Hoechst 33342)	T24	6-fold
He <i>et al.</i> ⁴⁰	<i>In vivo</i> colony-forming assay and athymic nude mouse model assay	67LR ⁺ ; CEACAM6 ⁺ ; CK17 ⁺	SW780 and primary patient (once-passaged)	5–10-fold
Chan <i>et al.</i> ³⁷	<i>In vivo</i> colony-forming assay and <i>in vitro</i> RAG2 ⁻ /γ ⁻ mouse model assay	CD44 ⁺ ; CK5 ⁺ ; CK20 ⁻	Primary patient and patient-derived xenografts	10–200-fold
Su <i>et al.</i> ⁴⁴	<i>In vivo</i> colony-forming assay and athymic Swiss nude mouse model assay	ALDH1A1	HTB-2; HTB-9; HTB-4	100-fold
Volkmer <i>et al.</i> ⁶³	<i>In vivo</i> RAG2 ⁻ /γ ⁻ mouse model assay	CD90 ⁺ /CK14 ⁺	Primary patient and patient-derived xenografts	17-fold

Abbreviations: 67LR, 67 kDa laminin receptor; ALDH1A1, aldehyde dehydrogenase 1 family, member A1 (retinal dehydrogenase 1); CD, cluster of differentiation; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen); CK, cytokeratin; CSC, cancer stem cell; EMA, epithelial membrane antigen; HTB, human tumour cell bank; γc, common cytokine receptor gamma chain; RAG2, recombination activating gene 2; SP, side-population.