Published in final edited form as:

Cancer Metastasis Rev. 2009 December ; 28(3-4): 291–304. doi:10.1007/s10555-009-9187-6.

Urothelial carcinoma: Stem cells on the edge

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Abstract

Tumors are heterogeneous collections of cells with highly variable abilities to survive, grow, and metastasize. This variability likely stems from epigenetic and genetic influences, either stochastic or hardwired by cell type-specific lineage programs. That differentiation underlies tumor cell heterogeneity was elegantly demonstrated in hematopoietic tumors, in which rare primitive cells (cancer stem cells (CSCs)) resembling normal hematopoietic stem cells are ultimately responsible for tumor growth and viability. Because of the compelling clinical implications CSCs pose—across the entire spectrum of cancers—investigators applied the CSC model to cancers arising in tissues with crudely understood differentiation programs. Instead of relying on differentiation, these studies used empirically selected markers and statistical arguments to identify CSCs. The empirical approach has stimulated important questions about “stemness” in cancer cells as well as the validity and stoichiometry of CSC assays. The recent identification of urothelial differentiation programs in urothelial carcinomas (UroCas) supports the idea that solid epithelial cancers (carcinomas) develop and differentiate analogously to normal epithelia and provides new insights about the spatial localization and molecular makeup of carcinoma CSCs. Importantly, CSCs from invasive UroCas (UroCSCs) appear well situated to exchange important signals with adjacent stroma, to escape immune surveillance, and to survive cytotoxic therapy. These signals have potential roles in treatment

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Disclosure UroCSC research in DMB's laboratory was partially supported by Stemline Therapeutics which licenses associated inventions from the Johns Hopkins University. DMB and the University are entitled to royalty payments from this arrangement. DMB is also a paid consultant to Stemline Therapeutics and owns Stemline Therapeutics stock options. The terms of this arrangement are managed by the Johns Hopkins University in accordance with its conflict of interest policies.

resistance and many participate in druggable cellular pathways. In this review, we discuss the implications of these findings in understanding CSCs and in better understanding how UroCas form, progress, and should be treated.

Keywords

Differentiation; Cancer stem cell; Stroma; Bladder; Wnt; Carcinoma *in situ*

1 Introduction

Most cancers are collections of phenotypically mixed cell populations with variable proliferative potential, a feature shared with normally developing organs. Two primary models have gained support to account for this variability. The clonal evolution model proposes that multiple genetic mutations confer upon a cell the ability to proliferate indefinitely, and that a subsequent mutation will confer a growth advantage upon one of this cell's progeny. The new, more rapidly growing clone will outcompete other clones and dominate the tumor. Repetition of this process brings about worse iterations of the disease over time [1,2]. The cancer stem cell (CSC) model provides an alternative explanation, assigning cellular heterogeneity to differing degrees of differentiation. According to this model, heterogeneity is predictable rather than random, recapitulating aspects of the developmental and homeostatic mechanisms of normal tissues [3]. Understanding the operation of normal developmental and homeostatic mechanisms, according to this theory, becomes useful for designing therapeutic strategies that target the heterogeneous subpopulations that constitute a cancer.

Here, we review the concept that CSCs are the basis for cancer cell heterogeneity and mediate resistance to common cancer therapies. We will apply lessons from landmark studies in hematologic CSCs to the controversial topic of solid epithelial tumors (carcinomas) in general and to urothelial carcinomas (UroCa) stem cells in particular. UroCa stem cells underlie a distinctly urothelial differentiation program originating at the tumor–stroma interface [3–5]. The spatial and molecular organization of this program offers special opportunities for insights into the CSC niche, UroCa pathophysiology, and treatment.

1.1 Differentiation in cancer

Cancer cells at the origins of differentiation hierarchies can resemble benign tissue stem/progenitor cells, both in terms of the proteins they express and in their unlimited replicative potential, which sustains long-term homeostasis in tissues, and long-term growth and metastasis in cancer. An important principle that appears conserved in normal and malignant cells is the inverse correlation between the capacity for long-term growth and differentiation. Although both the starting point from which differentiation proceeds, and the degree to which tumors differentiate are highly variable (see below), a typical cancer can be pictured as having cancer cells with low (primitive), intermediate, and high degrees of differentiation.

CSCs are the most primitive, can proliferate indefinitely, and have genoprotective mechanisms, similar to benign stem cells (see below). Thus, they are uniquely suited to colonize new sites (metastasize) and to mediate recurrence after chemo- or radiation therapy. More differentiated cells have a limited capacity to replicate. However, depending on the balance between proliferation and cell death, the proportion of proliferating cells in the differentiated compartment can be high, and presumably a significant source of cancer morbidity and mortality. Given their differing characteristics, elucidating the growth regulatory signals and dynamics of these different cellular compartments should prove to be critical in understanding and interfering with tumor growth.

The first evidence that some human cancer cells are more tumorigenic than others was gathered in the 1950s through ethically controversial autotransplantation experiments which showed that large numbers of cancer cells from one site were required to initiate a tumor when injected into a new site in the same patient [6]. This result indicated that most cancer cells were incapable of colonizing a new site. The ability to identify and characterize the rare, more capable cells came some years later from studies of hematologic malignancy.

1.2 Hematologic cancer stem cells

Highly tumorigenic subpopulations from hematologic malignancies were shown to resemble hematopoietic stem cells, both functionally and phenotypically. Early functional studies suggested that normal hematopoiesis was governed by a strict cellular hierarchy and maintained by self-renewing stem cells with the capacity to give rise, through a multistep differentiation process, to distinct myeloid, lymphoid, and erythroid populations (reviewed in [7]). Later studies demonstrated that specific cellular properties, including the expression of certain cell surface antigens, could identify specific cellular subsets within this hierarchy. These studies have produced a map that can be used to match most hematologic malignancies to a specific step in normal hematopoiesis, with each developmentally defined cancer expressing its own distinctive biologic and clinical properties.

The assignment of specific functional attributes to defined human cell populations was initially demonstrated in myeloid leukemias. Bedi et al. [8] characterized human chronic myelogenous leukemia (CML) stem cells carrying the iconic Philadelphia chromosome translocation. Like normal hematopoietic stem cells, CML CSCs were small, expressed the primitive hematopoietic marker CD34, and lacked markers of myeloid and lymphoid lineage commitment. *In vitro*, these cells self-renewed and produced highly proliferative committed progenitor cells as well as more mature differentiated progeny. The following year, Dick and colleagues demonstrated that cells with a similarly primitive phenotype were tumorigenic using transplantation of acute myeloid leukemia (AML) cells into immunodeficient mice [9]. Regardless of the subtype of AML (based upon the standard French-American-British classification), CD34+CD38- cells gave rise to leukemias that were phenotypically indistinguishable from the original clinical specimens. In addition, the ability of these cells to self-renew was demonstrated through their ability to give rise to leukemic growth during serial rounds of transplantation. These studies suggested human leukemias arise from the transformation of normal hematopoietic cells at the CD34+CD38- stem cell/progenitor stage [10] and challenged a popular notion that leukemias displaying varying degrees of myeloid differentiation represented transformation of different cell types along the differentiation spectrum.

These studies suggested that normal stem cells represent the cell of origin for human cancers. Indeed, it was assumed that stem cells should be efficiently transformed into cancers since they possess unlimited capacities to self-renew [11]. However, later studies suggested that more committed and normally replication-limited progenitors could acquire the ability to self renew through malignant transformation. Utilizing a model system of leukemia, Krivtsov et al. [12] found that myeloid progenitors with limited replicative potential could be transformed into self-renewing leukemia stem cells upon viral transduction with the MLL:AF9 fusion gene. Similarly, during blast crisis in human CML, myeloid progenitors that normally have limited replicative potential were found to serve as the clonogenic and self-renewing stem cell. Notably, MLL CSCs differentiate into hierarchically arranged tumors. Thus, it appears that transformative genetic or epigenetic events in progenitors may result in the development of cancer, but a cellular hierarchy is still retained, and originates with the least phenotypically differentiated cell [13].

Having concluded that CSCs function like stem cells, but do not always arise from or resemble them phenotypically, an American Association for Cancer Research meeting in early 2006 operationally defined a CSC as a malignant cell that has the ability to both self-renew and differentiate to recapitulate all of the cell types in a given tumor [14]. Thus defined, a CSC is essentially equivalent to a “highly tumorigenic cancer cell” or “tumor-initiating cell” [3,4, 15]. Since CSC is the most broadly used of these terms, we will use it in this review.

A final important lesson for CSCs from hematologic malignancies is their emerging role in resistance to therapy and recurrence. One of the most dramatic examples is the profound clinical response to imatinib (Gleevec) seen in CML patients. These responses are invariably followed by recurrent disease upon discontinuation of the drug because CML stem cells are relatively imatinib resistant compared to their differentiated progeny. The precise mechanism of resistance is unknown, but possibilities include low expression of the characteristic fusion protein BCR–ABL targeted by imatinib [8], mutations in the ABL kinase-binding domain preventing imatinib binding [16], BCR–ABL gene amplification [17], or cellular quiescence exhibited by CML stem cells [18].

Multiple myeloma represents an analogous case history. Several novel drugs effectively target the malignant plasma cells that define both the diagnosis and the clinical manifestations of this disease. These drugs include the immunomodulatory agent lenalomide (Revlimid) and the proteasome inhibitor bortezomib (Velcade), both of which debuted in cancer therapy as treatments for myeloma. Recent studies, however, have shown that myeloma is propagated not by malignant plasma cells, but by CSCs with a less mature postgerminal B cell phenotype that constitute less than 5% of the tumor cell population [19]. These myeloma CSCs are insensitive to lenalomide and bortezomib, providing an intellectually satisfying explanation to the unfortunate fact that responses to these drugs are almost invariably only temporary [20]. The relative insensitivity of CSCs to therapy—even the latest and most specifically targeted therapies—highlights the need to establish and attack the specific therapeutic vulnerabilities of these cells as a means of obtaining durably effective cancer treatments.

1.3 Carcinoma stem cells

Unlike hematopoiesis, epithelial cell lineage programs are poorly understood. Accordingly, investigations into carcinoma stem cells have chosen empirically based strategies to isolate CSCs. Studies relying on differential expression of surface markers such as CD24, CD44, and CD133 have identified highly tumorigenic carcinoma subpopulations that self-renew and produce phenotypically distinct non-tumorigenic carcinoma cells in tumors from the breast [21,22], lung [23], colon [24–26], prostate [27], ovary [28], and oropharyngeal mucosa (head and neck) [29]. The roles of these markers in epithelial morphogenesis and homeostasis have not been established. Thus, the relationship between cancer stem cells and native tissue stem cells has yet to be elucidated.

Recent studies have indicated the need for caution when interpreting the relationship between surface marker expression and the ability to form tumors in xenografting assays. In particular, factors other than self renewal can affect xenografting efficiency. CSCs were originally believed to be a rare population of cells, as many human tumors are quite inefficient at forming tumors in T and B cell-deficient mice typically used as recipients, requiring thousands of cells to form a single tumor [10,11]. Recent studies have shown that the host immune response likely accounts for some of this inefficiency. Compared to several thousand human cancer cells required in mouse xenografting experiments by other investigators, Strasser and colleagues found that as few as ten unselected Burkitt's lymphoma cells can form a tumor when the tumor originates in and is transplanted into syngeneic mouse hosts [15]. Likewise, as few as four unselected human melanoma cells formed tumors when injected into immunocompromised mice that lack not only T and B cells, but also natural killer cells [30]. Side-by-side experiments

injecting the same tumor populations into different hosts would be required to understand the true significance of these findings, and other investigators have reported much lower tumor-forming efficiency from leukemias inoculated into optimally immunocompromised or syngeneic hosts [31]. These inconsistent results from different laboratories indicate that host factors likely affect the results of tumor transplantation experiments.

Tumor-forming potency can also vary intrinsically [31], with variation within and between different types of cancer. For example, of the six mammary carcinoma cases analyzed by Al Hajj and colleagues [21], five showed minority populations of highly tumorigenic CD44⁺CD24⁻ lineage cells, whereas this phenotype dominated the sixth patient's sample, constituting 62% of the tumor. Similarly, our laboratory characterized human pancreatic xenograft lines that showed varying degrees of differentiation that correlated with the number of cells required to form a tumor in an athymic mouse, a "standard" immunodeficient strain (XH, WM, and DMB unpublished observations). Tumor formation from the least differentiated line required only 20 unselected cells. Furthermore, it would also be reasonable to expect highly variable fractions of CSCs between different cancer types. For example, Burkitt's lymphoma and melanoma are highly proliferative, widely metastatic tumors with little phenotypic heterogeneity. As such, they might, on average, have particularly high fractions of CSCs [11], as reported by Kelly [15] and Quintana [30]. In contrast, urothelial cancers show a wide degree of heterogeneity within and between tumors and recapitulate aspects of a relatively well-characterized differentiation program established in normal urothelium. Just as stromal cells provide polarity and a base for differentiation of normal epithelium [32], tumor stroma appears to play a similar role.

2 The tumor–stroma interface

In addition to intrinsic properties of CSCs, their local microenvironment (analogous to the "niche" that supports benign stem cells) also plays an important role in regulating the balance between quiescence, active growth, and invasion [33,34]. Removing stem cells from their native environment causes them to undergo physiological changes [35], some of which are irreversible [36]. The significance of these changes is not yet fully understood, but they highlight tumor–stroma interactions as a potential route to manipulating the behavior of CSCs. For example, acute myeloid leukemia stem cells require the adhesion molecule CD44 to bind to their niche. Abrogation of this binding leads to disruption of homing to the niche and increased differentiation of leukemic stem cells, leading to decreased tumor burden in NOD-SCID mice [37].

Another intriguing role for the stroma in carcinomas was recently reported wherein mesenchymal stem cells (MSCs) localizing to tumor-associated stroma were shown to enhance breast CSC motility, invasion, and metastasis by elaborating the chemokine CCL5 [38]. If suitable CSC-suppressing signals are identified, it is possible that MSCs engineered to provide these signals could seek out and home to tumors, regardless of their location, providing a unique and useful tool for combating metastatic disease.

Inflammatory cells are typically transient, variable constituents of tumor stroma, but also have well-established roles in cancer biology that may impact CSCs. Innate immune cells contribute to cancer development through the release of potent soluble mediators that regulate cell angiogenesis, genomic integrity, metabolism, migration, proliferation, and tissue remodeling [39,40]. Chronic inflammation is associated with increased cancer risk, particularly for carcinomas, through cytokine-mediated growth. The use of anti-inflammatory compounds has been shown to reduce cancer risk in various solid organs by 20–50% [39]. Elucidation of the mechanisms by which inflammatory cells promote carcinogenesis could lead to novel therapeutic targets.

Numerous other studies have demonstrated that the microenvironment is capable of initiating tumorigenesis in normal stem cells, augmenting tumor progression in premalignant CSCs, and at least temporally, preventing tumor progression depending on the overall genetic state of each cell type [41–46]. Our group has recently demonstrated a niche-like function for tumor stromal cells in directing the differentiation of UroCa xenografts [3]. Based on these studies, we propose a model whereby invasive properties of UroCa are determined by the ability of malignant CSCs to interact with the stromal compartment. This ability, in turn is a function of the differentiation state of the cancer cell.

Urothelial CSCs have recently been characterized in invasive bladder cancer and display many properties similar to urothelial stem cells [3,4], including expression of known and novel biomarkers and a distinctive anatomic position at the stromal interface. With this in mind, we review normal urothelial stem cells and the lineages they generate and explore the advent, progression and recurrence of UroCa. Finally, we discuss molecular therapeutic targets for CSCs from invasive UroCas (UroCSC) and mechanisms by which these cells might preferentially survive therapies currently in use. Insights into these areas should provide better strategies for altering the course of this relentless disease.

3 Urothelial biology

The bladder is responsible for receiving urine from the kidney and storing it prior to excretion. Three cell types in distinct layers constitute urothelium, which is a specialized epithelial lining of the bladder utilized for urine storage. The hierarchical organization of these cells can be deduced from the observation that differentiation is lowest and proliferative capacity greatest at the edge of the basement membrane where basal stem cells reside. Proliferative capacity diminishes, and cells become larger and more differentiated toward the lumen, where large fully differentiated superficial/umbrella cells form the urinary barrier (Fig. 1a) [47,48].

3.1 Basal cells

The single layer of basal stem cells interacts with the basement membrane, a thin band of extracellular matrix material that serves as the nidus for stromal epithelial interactions [49]. This interaction is likely facilitated by basal cell expression of receptors for laminin (the 67 kDa laminin receptor, or 67LR; [3,50]), integrins ($\beta 4$ integrin, [5,51]), and hyaluronic acid (CD44; [4,52]). Like basal cells in other epithelia, urothelial basal cells are also distinguished by high levels of keratins 5, 14, and 17, and low/absent levels of the carcinoembryonic antigen-related cell adhesion molecule CEACAM6 [3] and keratins 8 and 18. Kurzrock and colleagues demonstrated the stem cell properties of basal cells in adult rats [5] by labeling newly synthesized DNA with bromo-deoxyuridine (BrdU) over a 4-day period and visualizing cells that retained the label. At 1 month after labeling, BrdU was detectable in all three urothelial layers (basal, intermediate, and superficial), including almost 90% of basal cells. A year after labeling, BrdU was found exclusively in basal cells, and the proportion of labeled basal cells dropped by tenfold. These results indicate that urothelial stem cells reside exclusively in the basal layer where they comprise approximately 10% of the population. Furthermore, as the only long-lived and proliferative cells in the urothelium, basal stem cells serve as the sole source of all urothelial cells present during the life of the individual.

3.2 Intermediate and superficial urothelial cells

Basal cells generate an adjacent multicellular layer of intermediate cells [49]. Their kinetics follow those of classic “transient amplifying cells,” [47] a high fraction can proliferate, but only for a few cycles. Intermediate cells show variable expression of CD44 [52], low/absent expression of keratins 5 and 17 [53], and high levels of CEACAM6 [3] and cytokeratin18 (CK18) [53,54]. These cells give rise to a single overlying layer of fully differentiated

superficial or umbrella cells, which have an even more limited ability to proliferate and form the elastic protective barrier that protects underlying tissue from constituents of the urine [47,48]. Terminal urothelial cell differentiation into umbrella cells is marked by production of uroplakin proteins [55] and keratin 20 (CK20) [56].

4 Urothelial carcinoma: two pathways

The two most distinctive aspects of urothelial carcinoma biology are its dual pathways of carcinogenesis (papillary/noninvasive versus flat/invasive) and its notorious propensity to recur in every clinical stage and setting. UroCSCs have the potential to play important roles in both of these phenomena.

Carcinogenesis is a multistep process. Urothelial cells are thought to accumulate genetic abnormalities through chronic exposure to toxins that are concentrated in the urine. Certain cells may accumulate mutations that favor their expansion over the growth of neighboring cells—increasing the prevalence of this clone and its susceptibility to further oncogenic “hits” [2]. Depending on the gene affected, the initial mutation appears to send urothelial cells down one of two pathways, toward invasive or noninvasive cancer. Early mutations insufficient to cause cancer underlie a “field effect” by which local recurrence is thought to form. Since stem cells should be the only cells long lived enough to accumulate all of the mutations involved in multistep carcinogenesis [11], they are likely to mediate the field effect [57–59]. The final oncogenic event that leads to unrestrained growth and a histologically recognized cancer could occur in a CSC or in its progeny.

4.1 Low-grade noninvasive papillary urothelial carcinoma

Activating mutations in the fibroblast growth receptor FGFR3 gene constitute the most prominent “hit” in noninvasive/papillary UroCas [60,61], which represent ~80% of all bladder cancers. These mutations occur in a background of intact Retinoblastoma (Rb) and p53 tumor suppressor function, and combined with other crudely understood events lead to tumors that grow toward the urinary lumen from the surface urothelium in a distinctive arborizing growth pattern. As evidence for a field effect, these tumors are often multifocal and have a 70% rate of recurrence after local excision (reviewed in [62]). Frequent recurrence and a ~15% lifetime risk of a subsequent high grade or invasive lesion necessitates frequent monitoring by cystoscopy—an invasive and costly procedure that makes bladder cancer one of the most expensive of all cancer types to manage. The differentiation capacity of low-grade noninvasive UroCas has not been well studied, but one gene-profiling study comparing these tumors to invasive cancers found that noninvasive UroCas preferentially express messenger RNAs (mRNAs) encoding markers of differentiated urothelial cells, including the cell adhesion proteins LAMB3 and ITGB4 and the superficial/umbrella cell marker uroplakin 2 [63]. If confirmed by protein expression, these results would indicate a more advanced state of differentiation than that seen in invasive tumors.

Given their limited capacity to grow and spread, it is our hypothesis that papillary carcinomas typically arise from or predominantly differentiate into an intermediate cell phenotype. This phenotype resembles these tumors, which have limited replicative potential and do not normally participate directly in stromal–epithelial interactions that might facilitate invasion. Indeed, benign self-limited urothelial neoplasms called “papillomas,” like most papillary urothelial carcinomas, harbor activating mutations in FGFR3 [61,64], supporting the notion that this pathway initiates in a separate indolent form compared with invasive tumors that lack FGFR3 mutations. Further genetic and/or epigenetic changes that accrue in these indolent lesions might expand their growth potential to make low-grade, noninvasive papillary tumors (Fig. 1b). Investigators have found evidence for basal, intermediate, and superficial differentiation in invasive UroCas but the expression of these differentiation programs in

noninvasive/papillary tumors remains incompletely investigated. Sorting out the cell type of origin for UroCas will therefore require a variety of additional studies.

4.2 High-grade flat-invasive urothelial carcinoma

The small proportion of cases with noninvasive papillary tumors that progress to muscle-invasive disease may represent either *de novo* appearance of invasive tumors or true progression of noninvasive lesions. In contrast, the flat/invasive pathway involves a separate 15–20% of patients whose lesions arise *de novo* or as flat, high-grade carcinoma *in situ* (CIS). The oncogenic mutations seen in these tumors typically involve loss of p53 and/or retinoblastoma (Rb) protein tumor-suppressor activity, whereas FGFR3 mutations are absent. These tumors are highly proliferative and invasive, with ~50% of patients progressing from CIS to invasive disease, and 50% of invasive cases progressing to lethal metastasis [65–67]. The capacity to sustain long-term growth and invade is complemented by enhanced expression of matrix-remodeling proteins and other genes and pathways supporting angiogenesis and the immune response [63]. These abilities parallel the enhanced capacities for growth and stromal interactions possessed by benign urothelial basal cells, and suggest an appealing hypothesis: that invasive carcinomas arise from or are at least phenotypically similar to basal cells (Fig. 1c).

5 Mouse models of urothelial carcinoma

Interestingly, mouse models of UroCa have recapitulated aspects of the two tracks of UroCa pathogenesis. Mutations in the tumor suppressor p53 are important in modeling the flat/invasive carcinogenesis pathway. While not sufficient to cause UroCa alone, p53 inactivation cooperates with inactivation of either Rb [68] or the phosphatase and tensin homolog (PTEN) [69] to cause invasive UroCa in mice.

Noninvasive cancers, on the other hand, arise in mice engineered to express activating mutations in H-ras in the bladder. Activating H-ras mutations are not frequently found in human bladder cancers [70,71], but H-ras activation may mimic the Ras-stimulating effects of activating mutations in FGFR3, a common finding in human papillary/noninvasive cases [60,61]. As is the case in humans, these lesions do not require p53 mutations to form, and the addition of p53 mutations does not induce invasion, further reinforcing the notion that invasive and papillary cancers form by distinct mechanisms [72]. The differentiation programs in these cancer models have not been delineated, but doing so could lead to improved abilities to interrogate the role of CSCs in urothelial carcinogenesis and the molecular pathways that underlie their biology.

6 Urothelial differentiation in urothelial carcinoma

Based on the operation of stem cell programs previously reported in hematopoietic and central nervous system tumors, several groups have hypothesized that urothelial CSCs also share properties with bladder urothelial stem cells (i.e., basal cells).

Yang and Chang found benign basal and intermediate urothelial cells that express the hyaluronic acid receptor CD44 but not epithelial membrane antigen (EMA) [52]. Their studies in five primary papillary/noninvasive UroCas demonstrated that these CD44+EMA– basal/intermediate-like cancer cells constituted approximately one third of the total cell population. As demonstrated by *in vitro* single-cell cloning assays, these CD44+EMA–cells also contained all of the clonogenic capacity of the parental tumor [52]. Our subsequent study focused more narrowly on basal cells, and we found that two thirds of invasive UroCas expressed the basal cell marker keratin 17 (KER17) [3]. The dominant pattern of expression indicated a distinct basal cell compartment at the edge of tumor nodules where they abut the stroma, mirroring the

arrangement of normal basal cells at the urothelial–stromal interface [3]. Working in human SW780 UroCa xenografts, we showed that the 67-kDa laminin receptor (67LR), which preferentially marks invasive UroCas [50], colocalized with KER17 in basal tumor cells [3]. In single-cell xenograft suspensions, 67LR bright basal UroCa cells constituted ~13% of the parental tumor but possessed essentially all of its tumor forming ability when assayed by the ability to form a new tumor xenografts *in vivo* [3]. Our group independently validated this finding using a second tumor model and an independent sorting strategy that isolated basal cells by virtue of their lack of CEACAM6 (CD66) expression. Chan et al. subsequently validated enhanced tumorigenicity of basal-like UroCa cells, with CD44+CK5+CK20–CSCs from five newly established human xenograft lines showing enrichment in their ability to form tumors upon initial and serial transplantation when compared to CD44–CK5–CK20+ tumor cells [4].

7 Urothelial carcinoma recurrence

UroCa recurs in three distinct settings: after resection of intravesical tumors for noninvasive disease, after bladder removal (cystectomy) for invasive disease, and after chemotherapy for metastatic disease. After local resection, recurrence likely results from the appearance of new local lesions that result from a field effect (see above), or from metastasis that occurred prior to surgery. In contrast, the ability to survive chemotherapy (chemoresistance) has been linked to the operation of enhanced detoxification and genoprotective mechanisms in normal stem cells and in their malignant counterparts.

UroCa provides an excellent subject for the study of chemoresistance in cancer because most patients respond to chemotherapy with dramatic tumor shrinkage, only to have their cancers recur later, and in a manner that is resistant to these drugs. Patients undergoing bladder removal (cystectomy in women or cystoprostatectomy in men) for muscle-invasive bladder cancer experience metastatic relapses about 50% of the time due to occult micrometastases [73]. Neoadjuvant chemotherapy prior to surgery modestly improves this cure rate to 55–60%, suggesting that these occult cells are somewhat chemosensitive [73,74]. Interestingly, adjuvant chemotherapy following cystectomy has never been proven to provide a survival benefit [75]. Whether this lack of benefit is due to poor trial design or an inherent flaw in the treatment strategy is unclear [76]. However, since chemotherapy is typically delayed for several months during recovery from surgery, this delay may allow CSCs adequate time to establish themselves at distant sites, making them more difficult to eradicate with systemic chemotherapy. In addition, disruption of normal blood flow and lymphatic drainage in the pelvis following surgery might reduce chemotherapeutic drug penetration in the tissue, resulting in insufficient drug concentrations for cytotoxicity in occult pelvic sites.

In addition to its use as an adjuvant to surgery, chemotherapy is also used in the setting of metastatic disease, and we suspect that urothelial CSCs are likely the reason for treatment resistance in this cohort of patients. Metastatic bladder cancer is also highly responsive to chemotherapy as measured by tumor shrinkage. Between 40% and 60% of patients with metastatic disease experience responses to cisplatin-based combination chemotherapy. However, complete responses, with total eradication of visible tumor, occur in only 10–20% of patients with metastatic disease [77,78]. In total, only 10–15% of patients with advanced disease will be long-term survivors, suggesting that, in a small cohort of patients, standard chemotherapy can target and eradicate UroCa stem cells even in metastases [77,79]. These results suggest that the nontumorigenic cancer cell burden may be reduced by cytotoxic chemotherapy, but that the underlying CSCs remain viable in the vast majority of patients with metastatic disease. This further underscores the need to identify and specifically target UroCa stem cells to provide more complete and durable responses for UroCa patients.

8 Targeting cancer stem cells

Although none are in current clinical use, evidence is mounting that CSC-directed therapies may yield more durable responses than standard chemotherapeutic regimens. A recent study in breast cancer has demonstrated the feasibility of targeting CSCs in an *in vitro* system. A screen of ~16,000 compounds identified a drug called salinomycin that reduced the proportion of CSCs by greater than 100-fold when compared to the currently utilized breast cancer chemotherapeutic drug paclitaxel [80]. The target of this drug is not yet known, but its characterization should be of biologic as well as clinical interest.

Another set of recent studies has shown that leukemic CSCs and their normal stem cell counterparts have mechanistic differences in their self-renewal in response to conditional PTEN deletion; leukemic CSCs were expanded while normal stem cells were depleted. However, upon mTOR inhibition by the drug rapamycin, the leukemic cells were reduced while the number of normal stem cells was restored, possibly through feedback inhibition of the AKT pathway [81–83]. This result implies that a single targeted therapy may be able to both deplete CSCs and restore their normal stem cell counterpart, indicating that targeted CSC treatments may not only be feasible but also tolerated by normal stem cells.

8.1 Genomic profiles of cancer stem cells identify potential therapeutic targets

“Invasiveness” gene signatures have been developed in an attempt to identify patients who merit particularly aggressive treatment. These profiles have been adopted for guiding treatment in mammary cancers [84,85], but are not in general use for UroCa despite promise shown in initial studies. Highlighted by distinct markers for papillary (e.g., *FGFR3*) and invasive (e.g., *p53*, *Rb*) UroCa [62], more comprehensive gene expression profiles have been established to differentiate between these two pathways [86]. Furthermore, the use of a 16-gene classifier on normal urothelium can help detect whether or not adjacent flat CIS is present [63]. Both fatty acid binding protein 4 (FABP4) and cathepsin E (CTSE) can identify papillary tumors that are more likely to progress to invasive disease [87]. These studies, along with others, have implicated specific genomic changes that occur in distinct cohorts of UroCa patients [64,88]. Integration and elaboration of these studies could allow for more accurate prognosis and better tailored therapy for this disease.

Our work [3], and that of Chan et al. [4], linked invasiveness profiles in UroCa to CSCs, indicating that basal-like UroCSCs promote invasive bladder cancer growth, invasion, and spread in human patients. Accordingly, we discovered an UroCa CSC gene signature that is enriched in more aggressive tumors and negatively associated with survival in UC patients [3]. Genes enriched in basal-like urothelial CSCs participate in a variety of cellular pathways that are known to promote cell survival and growth, and resist toxic and DNA damaging agents as well as oxidative stress. By arranging these genes according to biochemical pathway, several pharmaceutically vulnerable targets emerge, including Notch, ErbB, Focal Adhesion, mTor, and Wnt (Table 1). Upon appropriate validation, these observations may lead to more durable treatments for UroCa by eliminating CSC-mediated recurrence.

8.2 Wnt signaling in urothelial carcinoma stem cells

For the purposes of brevity and to reflect the pathway with the most historical interest in stem cells, cancer, and UroCa, we will briefly review Wnt signaling. Wnt maintains stem cells in an undifferentiated state [89]. Several Wnt signaling components are differentially expressed between UroCSCs and nontumorigenic cancer cells, including Wnt10a ligand and the MYC oncogene [3], which is a known target of WNT signaling in UroCa cells [90]. In addition, elevated protein levels of the Wnt pathway effector and oncogene β -catenin (CTTNB) in UroCSCs indicate that the pathway is more active in these cells than in nontumorigenic cells

[3]. The functions of these components in urothelial differentiation and UroCSCs are essentially unexplored, but should be fruitful areas for future work.

There are multiple Wnt ligands, with many cell types expressing a variety of these secreted proteins [89]. Receptor binding by Wnt ligands rescues CTTNB from a destruction complex that includes the adenomatous polyposis coli (APC) tumor suppressor gene. Remarkably, the Wnt 7b ligand is upregulated in papillary noninvasive carcinomas, but is not in invasive UroCas [91], suggesting that this Wnt ligand may distinguish between the two pathways of urothelial carcinogenesis. Further evidence that activation of Wnt signaling confers a selective advantage on UroCa cells comes from studies that identified frequent gene silencing of endogenous Wnt inhibitors [92] and less frequently, APC mutations in UroCa [93,94]. Gene silencing by DNA methylation downregulates expression of several secreted Wnt ligand antagonists, including the Wnt inhibitory factor WIF1, and dickkopf homolog 1 (DKK1) [90,92,95,96]. WIF1 silencing was found in 67% of cases, and associated with increased nuclear translocation of CTTNB, indicating that it enhanced Wnt signaling [90]. Small interfering RNA [90] and overexpression [97] studies in human UroCa lines indicated that inhibiting Wnt signaling through WIF1 restrains UroCa cell growth by suppressing the expression of cyclin D1 and the c-Myc oncogene. Thus, Wnt signaling appears to promote UroCa growth potentially in both noninvasive and invasive UroCa. Delineating the Wnt antagonist compounds that are currently available for research purposes and focusing on responses defined in UroCa stem cells may enhance the transition to clinical applications.

9 Mechanisms of urothelial carcinoma stem cell chemoresistance

In addition to novel targets for mechanism-based CSC therapy, we found evidence for pathways mediating chemoresistance in UroCSCs. Interfering with chemoresistance mechanisms may allow established therapies to work more effectively. Urothelial CSCs likely use a variety of mechanisms to evade chemotherapy-mediated death. Evidence from the literature includes enhanced abilities to: (1) dispose of chemotherapeutic drugs by pumping them out of the cell, (2) enzymatically neutralize toxic drugs, their metabolites, and byproducts such as oxidants and free radicals, (3) suppress programmed cell death pathways, and (4) modulate cytokines and other immune mediators to dampen anti-tumor immune responses by the host.

9.1 ABC transporters

ABC transporters belong to an ancient family of ATP-dependent transmembrane proteins that transport substrates across plasma membranes. In stem cells, ABC transporters, particularly the verapamil-sensitive ABCG2 family member, expel a variety of substrates, including metabolic products, drugs, and toxins [98]. ABCG2 activity can be measured by the verapamil-sensitive ability to exclude Hoechst 33342 fluorescent dye. Cells with this ability are called “side population” (SP) cells and coincide with the stem cell fraction of a number of cell types [99]. More recent studies have shown that the SP assay identifies CSCs in leukemia, glioma, medulloblastoma, hepatocarcinoma, breast, prostate, thyroid, colorectal, and ovarian carcinoma [100–106]. SP populations have been identified in single-cell suspensions from UroCa tumors [107] and from the T24 human UroCa cell line [108]. In culture, these SP cells showed enhanced clonogenic capacity and were resistant to chemotherapy and radiation [107,108] indicating that this mechanism of drug efflux chemoresistance also operates in UroCa, is associated with aspects of CSC phenotype, and may be expected to play a role in UroCa chemoresistance (Fig. 2).

9.2 Aldehyde dehydrogenase

We found that aldehyde dehydrogenase 1 (ALDH1) is significantly upregulated in UroCa CSCs compared to nontumorigenic UroCa cells [3], indicating a potential mode of chemoresistance

in urothelial CSCs (Fig. 2). Cytoplasmic ALDH1 contributes to chemoresistance, and like Hoechst dye exclusion is the basis for assays used to identify stem cells and CSCs [20]. Its role in CSC chemoresistance was recently highlighted in serially transplanted colorectal carcinoma xenografts treated with the apoptosis-inducing alkylating agent cyclophosphamide [109]. During therapy, tumors became enriched in CSCs, which survived through oxidation and inactivation of bioactive cyclophosphamide metabolites, a known function of ALDH enzymatic activity [110]. These results suggest that cyclophosphamide might have reduced activity in UroCa and indicate the potential utility of testing sensitivity and resistance of UroCSCs to other cytotoxic chemotherapy agents in current use.

9.3 Neutralizing reactive oxygen species

Reactive oxygen species (ROS) accumulate in cells during cytotoxic chemotherapy and in response to radiation [111]. ROS species damage DNA leading to decreased cellular replication and survival. Similar to normal stem cells, epithelial CSCs contain lower levels of reactive oxygen species and increased expression of free radical scavenging systems, which protect their genetic DNA from damage due to endogenous and exogenous ROS [112]. In UroCSCs, we found high levels of the antioxidant enzyme superoxide dismutase SOD2 and heme oxygenase [3], which also mediate resistance to DNA damage through ionizing radiation or cytotoxic chemotherapy [113–115]. These enzymes may contribute to the enhanced survival of CSCs in the setting of chemotherapy, and as such, represent possible therapeutic targets (Fig. 2).

9.4 Apoptosis inhibition

When DNA damage reaches a certain threshold, apoptotic pathways are activated. However, inhibitor of apoptosis proteins (IAPs) can block apoptosis. IAPs prevent cell death by blocking caspase activation pathways, more specifically caspases 3, 7, and 9 [116]. UroCa stem cells upregulate the IAP protein BIRC3 compared to nontumorigenic UroCa cells (Table 1) [3]. This class of proteins has a wide variety of activities in cancer cells, modulating cell division, cell cycle progression, and signal transduction pathways [116].

UroCa stem cells show enhanced expression of mRNAs encoding a number of interleukins, including IL-11, 18, and 23 [3]. Interleukins have been shown to promote cancer cell survival and growth by stimulating the expression of antiapoptotic genes, including cFLIP/FLAME-1 and Bcl-x_L *in vitro*; high expression levels of these proteins, including IL-4, have been found in prostate, breast, bladder, and colorectal cancer *in vivo* [117,118]. IL-23 has been implicated in the inflammatory response by preferentially activating STAT-3 [40]. STAT3 has a large number of reported protumorigenic activities, including promotion of survival, proliferation, invasion, and angiogenesis [119]. Further investigation into the interleukin–STAT3 axis may yield novel therapeutic strategies aimed at UroCa stem cells.

Additionally, CD47 expression in hematopoietic and leukemia stem cells can bind an inhibitory receptor on macrophages, preventing them from being phagocytosed [120,121]. This protein is also highly expressed in UroCa CSCs compared to non-CSCs, and was shown to prevent macrophage engulfment of bladder cancer cells *in vitro* [4]. These studies provide a potential mechanism of CSC escape from immune surveillance, making this a potentially valuable therapeutic target for UroCa (Fig. 2).

In sum, UroCa stem cells are equipped with a variety of intrinsic factors that enable them to survive chemotherapy and mediate cancer recurrence. The ability to identify these factors in patient samples should provide tools for identifying therapy-resistant tumors and designing novel, more effective therapeutic approaches (Fig. 2).

10 Future directions

Muscle-invasive UroCa is an aggressive and often terminal disease. Emerging data suggest that much of their aggressiveness is driven by UroCa stem cells. Thus, we propose that future research more intensively investigates this population. In particular, it is likely that the UroCSC phenotype will need to be further refined before UroCSCs can be reliably identified and isolated in the majority of individuals, including animal models. In parallel with these efforts, reliable and validated UroCSC culture systems could be used in high-throughput drug screens of novel and existing drugs to identify agents with specific activity against these cells. Promising candidates can then be tested in animal models that cover the spectrum of disease, from early low-grade lesions to metastases. The availability of SW780 human xenograft models with a spatially demarcated UroCSC compartment should markedly facilitate the analysis of drug studies. Results from this system can subsequently be validated in primary human tissue collections as a guide to selecting promising therapies for clinical trials.

Acknowledgments

Our work is supported by NIH Grant Numbers R01DK072000, P01CA077664; The Bladder Cancer Research Center at Johns Hopkins University, and Stemline Therapeutics.

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Fig. 1.

Development of low-grade papillary versus high-grade muscle-invasive UroCa. The bladder consists of a stratified polarized epithelium. **a** The urothelial lining of the bladder originates with basal cells (*B*) located next to the stroma (*S*). The basal cells give rise to transient amplifying intermediate cells (*I*), which in turn differentiate into superficial umbrella cells (*U*). Umbrella cells form the barrier that prevents urinary solutes from re-entering the bloodstream. **b** Genetic and/or epigenetic changes leading to malignant transformation of intermediate cells by FGFR3 lead to hyperplasia and low-grade papillary tumors. **c** Malignant transformation of basal stem cells through modification of p53, retinoblastoma, and Pten genes

lead to CIS and high-grade muscle (*M*) invasive UC. High-grade UC can also be initiated, or propagated, by inflammation or other stromal factors

Fig. 2.

CSCs drug resistance methodology. Cancer stem cells have developed multiple mechanisms to resist chemo- and radiotherapy. The nuclei of CSCs retain lower levels of reactive oxygen species, resulting in less DNA damage. The DNA damage response pathway is preferentially activated allowing for increased DNA repair capacity. A modified epigenetic signature can prevent CSCs from undergoing apoptosis through repression of retinoblastoma (*Rb*) protein activity. ATP-binding cassette (*ABC*) transporters efficiently pump chemotherapeutic drugs out of the cell. The *ALDH1* enzyme can effectively oxidize and inactivate certain bioactive metabolic drugs. IAP proteins block the function of death caspases preempting cell death. UroCa stem cells upregulate the expression of CD47 to prevent phagocytosis by macrophages

Table 1

The table lists selected functional pathways that were significantly enriched (adjusted $p < 0.05$) among differentially expressed genes between UroCa CSCs (67LR bright) and UroCa Non-CSCs (67LR dim), along with selected genes in each pathway showing statistically significant (adjusted $p < 0.001$) differential expression

Gene expression profile of UroCa CSC vs UroCa non-CSC Cells		
Hallmarks of cancer pathways:	Genes up in UroCa CSC:	Genes down in UroCa CSC:
Response to oxidative stress	APOE, SOD2, HMOX1, PRNP, KRT1	IDH1
Anti-apoptosis	APOE, NOL3, NRG2, BNIP3, SOCS3, HMOX1, PRNP, CRYAB, IGF1R	BIRC3
DNA repair	SOD2, FANCE	
Cell growth	IGFBP2, DLC	PRSS2, PLCE1, ING3
Potential drug target pathways:		
Focal adhesion	ITGA6, ITGAV, CAV1, LAMB3, LAMC2, PGF, SPP1, CAV3, IGFR, LAMA3	BIRC3
ErbB signaling pathway	NRG2, HBEGF, MYC	AREG
Notch signaling pathway	DLL1, MFNG	
mTOR signaling pathway	HIF1A, PGF, DDIT4	
Wnt signaling pathway	MYC, NFATC1, WNT10A	PRKACB

Genes are listed according to official symbols in descending order of statistical significance. Kremen2 was assigned to the Wnt pathway category, but was not considered in statistical analysis. Table modified from [3]