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Systematic Review

Modeling Tumor Heterogeneity in Bladder Cancer: The Current State of the Field and Future Needs

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Abstract.
BACKGROUND: Tumor heterogeneity has been recognized in many cancer types for decades. However, the significance of tumor heterogeneity on disease course and clinical outcome in bladder cancer is of more recent interest to researchers and clinicians. This is especially true as morphologic and molecular heterogeneity has the potential to confound accurate diagnosis, efficient prognostication, and subsequent clinical management. While this is true, it is not always clear what laboratory models are available or suitable for the study of these important clinical phenomena.
OBJECTIVE: To review *in vitro* and *in vivo* laboratory models for the study of morphologic and molecular tumor heterogeneity in bladder cancer.
METHODS: We undertook a review of PubMed with a focus on identifying suitable models for the study of tumor heterogeneity in bladder cancer.
RESULTS: We provide a review of common *in vivo* (genetically engineered mice and patient-derived xenografts) and *in vitro* (established cell lines and organoid systems) models and discuss their utility in the study of morphologic and molecular tumor heterogeneity in bladder cancer.
CONCLUSION: Genetically engineered mouse models and patient-derived xenografts provide complementary approaches for the study of tumor heterogeneity in bladder cancer. In addition, cell culture-based systems provide a system amenable to genetic manipulation and mechanistic studies, while organoid systems bridge the gap between *in vivo* and *in vitro* systems. However, the availability of models to study molecular heterogeneity is limited, partly because of a relative lack of molecular characterization of available models. In summary, while models for the study of specific subsets of morphologic heterogeneity are available, more models are required for studies of molecular heterogeneity. This shortcoming could be partially addressed by more comprehensively characterizing currently available model systems. In addition, each system/approach has advantages and disadvantages, and care should be taken when selecting a given model.

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INTRODUCTION TO TUMOR HETEROGENEITY

The recognition of tumor heterogeneity and its clinical implications is not new. In fact, as early as the 1950 s, Foulds et al. [1] suggested that cancer

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development was not a simple progression as in the single-cell theory; rather, cancers develop via complex, nonlinear advancements of genetic mutations that could vary over time, within single tumors, and between individuals [1]. Indeed, the presence of phenotypically (clinically) evident tumor heterogeneity is common following disease progression. For example, numerous researchers provided evidence for the existence of multiple subpopulations of neoplastic cells within single tumors, as some of the first acknowledgements of tumor heterogeneity (reviewed in G. H. Heppner's "Tumor Heterogeneity" essay, 1984 [2]). Although this morphologic tumor heterogeneity has been recognized for some time, relatively recent technological advancements in the areas of genomics and computational biology have enhanced our understanding of the complexities of tumor heterogeneity, specifically at the molecular level. Indeed, these technologic advancements have helped to shed light on the impact of tumor heterogeneity on diagnostic intricacies, mechanisms of drug resistance, and clinical management strategies. For many of these reasons, interest in tumor heterogeneity has gained recent momentum.

In general, there are four types of tumor heterogeneity (reviewed in [3]). These include (1) molecular or cellular differences between tumors of a similar type in different patients (i.e., interpatient or intertumoral heterogeneity); (2) differences in cancer cell types or molecular attributes within a single lesion within one patient (intratumoral heterogeneity); (3) differences in cancer cell types or molecular attributes between primary and metastatic lesions, or between two different metastatic lesions within one patient (intermetastatic heterogeneity); and (4) differences in cancer cell types or molecular attributes within a single metastatic lesion (intrametastatic heterogeneity). Inherent in these definitions, tumor heterogeneity can be conceptualized at many levels, including the tissue, cellular and molecular (i.e., genetic, epigenetic) levels.

MORPHOLOGY: THE FIRST LEVEL OF TUMOR HETEROGENEITY IN BLADDER CANCER

Morphologically, while urothelial cell carcinoma (UCC) accounts for 90% of all muscle invasive bladder cancers in the United States and Europe, a striking percentage of muscle invasive UCC cases display histomorphometric variation and associated molecular alterations. These morphologic and molecular variants of UCC are clinically significant, as they are associated with poor clinical outcomes [4], and may be associated with treatment resistance. In addition, as we move towards molecular diagnostics in bladder cancer, presence of morphologic and molecular variants can complicate accurate diagnosis. The most common morphologic variant of UCC is squamous differentiation, which is characterized by the presence of intercellular bridging and the formation of keratin "pearls" [5]. It has been estimated that up to 40% of muscle invasive UCC cases present with patterns of squamous differentiation. While the influence of squamous differentiation on prognosis and disease course is unclear, this morphology is enriched in patients with basal (now basal-squamous) bladder cancer, which confers a worse prognosis [6–10]. Similar to squamous differentiation, glandular differentiation is present in an estimated 10% of muscle invasive UCC cases [11], and is associated with a higher incidence of extravesical tumors and metastasis to lymph nodes, as well as a higher risk of recurrence after treatment [8, 9]. In addition to these morphologic variants, others including neuroendocrine/small cell, plasmacytoid, micropapillary, etc (reviewed here [5, 12]) are frequently present in bladder cancer. As discussed below, morphologic and molecular heterogeneity are often linked, which adds to the complex nature of this disease state.

CONNECTIONS BETWEEN MORPHOLOGIC AND MOLECULAR HETEROGENEITY IN BLADDER CANCER

As is the case in other malignancies such as prostate cancer ([13–16]), molecular heterogeneity is often tied to morphologic heterogeneity in bladder cancer. For example, the original identification of a subset of bladder cancers that exhibit an expression pattern similar to basal urothelial cells (i.e., high molecular weight cytokeratins) revealed these tumors are often (but not always) enriched for squamous differentiation [17]. Indeed, recent studies have confirmed connections between the basal gene expression pattern and squamous differentiation in the setting of intratumoral heterogeneity as well [18]. Another example regarding connections between morphologic variation and specific genetic alterations is provided by studies of the aggressive

plasmacytoid morphologic variant of bladder cancer. Interestingly, loss of function mutations in *CDH1* (encoding E-cadherin) is detected in over 80% of plasmacytoid bladder cancers [19]. In a more recent study [20], we showed that among patients with more than one tumor morphology (intratumoral heterogeneity), 39% demonstrated associated molecular heterogeneity across the different morphologies, further suggesting a link between heterogeneity at the morphologic and molecular levels. Unfortunately however, as we discuss below, there is a relative lack of models that "connect" the common morphologic alterations in human bladder cancer with specific molecular alterations.

Therefore, it is clear that morphologic and molecular tumor heterogeneity in bladder cancer is of significant clinical significance and further research is required. For those interested in the relative strengths and weaknesses of specific models, as well as a discussion of their utility for addressing specific questions, we refer to previous reviews by us and others [21–24]. However, the types of models available for the study of tumor heterogeneity in bladder cancer may be unclear to investigators. To address this potential knowledge gap, as well as to highlight areas of potential focus for further model development and characterization, we provide an overview of models that depict elements of tumor heterogeneity in bladder cancer and how the studies utilizing these models are advancing our insights into this common disease.

IN VIVO MODELS OF TUMOR HETEROGENEITY

Genetically engineered mice

By enabling investigators to determine the impact of individual and combined genetic alterations on bladder cancer pathophysiology in an *in vivo* setting with an intact immune system, genetically engineered mice (GEM) provide an invaluable research tool. For example, GEM models have been created to examine the role of several genes, including (but not limited to) *HRAS*, *TP53*, *RB1*, *CDKN2A, FOXA1, ATDC9* and *PTEN* in bladder cancer development and progression. With some notable exceptions (see below), the targeted manipulation (i.e., knockout of tumor suppressor or overexpression of mutant transgene) is often insufficient to drive the development of frank tumor heterogeneity in the form of variant morphology. This may be related to the inability of a subset of genetic alterations to induce genomic instability, which can be overcome by the addition of chemical carcinogens (see section on carcinogen models below). As such, many of these studies describe the development of morphologic heterogeneity following use of a multi-gene approach in order to more closely mimic the molecular characteristics of the bladder cancer. Furthermore, several of these studies have identified a potential role for the aforementioned genes and others in the development of heterogeneous bladder cancer.

Upk2-HRAS$^{*/WT}$*/Upk2-cre/p53*$^{LOX/LOX}$

Using a transgenic mouse model, He et al. [25] (2016) investigated the effect of a constitutively activating HRAS mutation (HRAS*) and conditional inactivation of *Tp53* in urothelial cells on the development of muscle-invasive bladder cancer. As described in a prior publication [26], these transgenic mice harbor a fusion of the murine uroplakin-2 promoter and a 3.0 kb activated rabbit c-Ha-Ras harboring a point mutation (*Upk2-HRAS**). Specifically, the point mutation is at codon 61 of the second exon, resulting in a CAG (glutamine) to CTG (leucine) change. Importantly, this mutation results in the activation of kinase activity, and has been shown to transform immortalized cells *in vitro.* Constitutively expressed low levels of *Upk2-HRAS** results in simple urothelial hyperplasia that fails to progress to frank invasion. However, expression of two copies of *Upk2-HRAS** results in early onset, papillary urothelial carcinoma. Importantly, expression of one or two copies of mutant HRAS alone does not result in any detectible morphologic heterogeneity. While individual *Tp53* knockout results in a normal urothelial phenotype, *Tp53* KO (which almost certainly results in underlying genomic instability) combined with *Upk*-HRAS* expression results in the development of carcinoma-*in-situ* (CIS) and progression to muscle-invasive bladder cancers. Intriguingly, muscle-invasive tumors exhibit focal squamous differentiation as well as high molecular weight keratins (Krt5 and Krt14), typically associated with squamous differentiation and the basal-squamous subtype [25]. Therefore, the *Upk2*-HRAS$^{*/WT}$/*Upk2*-cre/p53$^{LOX/LOX}$ represents one model suitable for the study of basal-squamous bladder cancer.

Adeno-Cre/Tp53$^{loxp/loxp}$*/Pten*$^{loxp/loxp}$

Inactivation of *Tp53* was also investigated in a transgenic model that showcased intratumoral heterogeneity, developed by Puzio-Kuter et al. [27] (2009).

This novel mouse model used to investigate invasive bladder cancer uses combination *Tp53 loxp/loxp; Pten loxp/loxp* conditional KO mice with urothelial tumorigenesis induced following injection of Adeno-Cre directly into the bladder. Investigation of the effects of *Tp53* or *Pten* single mutants demonstrated normal bladder epithelium and no apparent tumors, even after a year post-injection with Adeno-Cre. However, bladders of the aforementioned combination KO mice with both *Tp53* and *Pten* deletion showed CIS, as well as muscle-invasive UCC with areas of squamous and sarcomatoid carcinoma that were positive for cytokeratins on IHC staining [27].

AhCreER + Lkb1$^{fl/fl}$*Pten* $^{fl/fl}$

Yet another notable use of a transgenic mouse model, conducted by Shorning et al. [28], involved the combined deletion of *Lkb1* and *Pten*. While *Lkb1* is a tumor suppressor previously shown to be dysregulated in bladder cancer, the deletion of *Lkb1* alone did not result in any morphologic changes in the bladder epithelium of mice. This was also the case for single deletion of *Pten* (which reportedly results in a range of phenotypes including hyperplasia and non-invasive cancer) [29–31]. The combined deletion model was developed by crossing mice with an inducible *AhCreER* transgene (induced with beta-napthoflavone and tamoxifen) with mice bearing a LoxP flanked *Pten* locus. Mice with deletion of both *Lkb1* and *Pten* developed large papillary tumors with tissue heterogeneity. Tumors had areas with vacuoles and apoptotic cells, as well as more proliferative sections; the tumors also showed some spindle-shaped cells, squamous metaplasia, and focal microvesicular change [28].

UBC-Cre/ERT2/Foxa1$^{loxp/loxp}$

Forkhead Box A1 (FOXA1) is a master regulator of urothelial differentiation, and decreased FOXA1 expression is a hallmark of basal-squamous bladder cancer [32]. To investigate the direct contribution of FOXA1 loss to the development of basal-squamous bladder cancer, Reddy et al. [33] investigated the effects of *Foxa1* knockout on urothelial differentiation in a GEM model. Using this model, tissue from female mice showed keratinizing squamous metaplasia with high levels of cytokeratin 14 expression, whereas tumors from male nice showed urothelial hyperplasia without squamous differentiation. While this study identified the sexually dimorphic effects resulting from *Foxa1* knockout, and provided direct evidence regarding a role for this developmental transcription factor in urothelial differentiation [33], it also suggested that *Foxa1* deletion alone was insufficient to drive tumorigenesis. Ongoing studies by our group [34] have now shown *FOXA1* silencing and *PTEN* copy number loss frequently co-occur in bladder cancer, and that Upk2-Cre mediated deletion of *Foxa1* and *Pten* in luminal cells (and a subset of intermediate basal urothelial cells) results in the development of frank bladder cancer with significant squamous differentiation.

CAG-ATDC transgenic mice

The tripartite motif protein family member and transcriptional regulator ataxia-telangiectasia group D complementing (ATDC/TRIM29) is overexpressed in bladder cancer, and recent studies suggest a role for ATDC in basal-squamous bladder cancers. Palmbos et al. [35] used a CAG-promoter transgenic model to constitutively overexpress ATDC in every tissue, including the bladder. In addition to the development of bladder outlet obstruction, mutant mice developed a spectrum of phenotypes in an ATDC copy number-dependent manner. Phenotypes included hyperplasia, dysplasia, low-grade non-invasive bladder cancer, and high-grade muscle-invasive bladder cancers. In a follow-up study, Palmbos et al. [36] identified ATDC as a marker of basal-squamous bladder cancer, and showed ATDC expression was significantly correlated with expression of all three isoforms of delta-N-TP63 (dNP63α, -β and -γ), as well as total TP63. Furthermore, this work showed that TP63 positively and directly regulates ATDC expression, increasing the phenotypic aggressiveness of bladder cancer cells. Although basal-squamous disease is enriched with tumors that exhibit squamous differentiation, not all tumors present with this morphologic attribute. Taken together, these results suggest that high levels of ATDC expression are associated with a basal-squamous subtype of bladder cancer, and indicate the ATDC transgenic mouse developed by this group is suitable for the study of basal-squamous disease.

Carcinogen-induced mouse models

Wide ranges of carcinogens have been utilized to induce bladder cancer in mice. The relevance of chemical carcinogens to urothelial tumor development and progression is significant, given that exposure to cigarette smoke, environmental pollutants and industrial chemicals represent major risk factors for the development of bladder cancer.

Additionally, carcinogen-induced bladder cancer models provide *in vivo* systems that are immune competent, a necessary component for immunotherapeutic studies. Perhaps the most commonly used chemical carcinogen for bladder cancer studies is *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). Recently, Fantini et al. [37] investigated the effects of BBN on the molecular and mutational profiles of carcinogen-induced mouse bladder tumors and provided evidence that BBN produces a basal molecular subtype of bladder cancer. Tumors specifically demonstrated frequent mutations in *Kmt2c, Trp53,* and *Kmt2d*, levels of which closely correlated with human muscle-invasive bladder tumors when compared to data from The Cancer Genome Atlas study [17]. Genetic similarities between carcinogen-induced mouse models of muscle invasive bladder cancer and human bladder tumors reaffirms the utility of BBN carcinogen-based models to study the basal molecular subtype of bladder cancer in hosts with intact immune systems. However, it should be noted that phenotypes which arise following BBN exposure (i.e., rapidity of disease onset and progression, as well as presence and type of tumor morphology) often vary according to genetic background [38], and are almost certainly related to strain-specific molecular differences/alterations.

Patient-derived xenografts (PDX) provide an additional in vivo approach

Relative to human disease, patient-derived xenograft (PDX) models are arguably the most faithful *in vivo* models available for the study of tumor-associated epithelial heterogeneity. This is true because PDX models appear to largely maintain tumor histology, including morphologic heterogeneity, as well as genetic alterations and molecular heterogeneity, seen in parent tumors [39]. However, (like any model system) there are issues associated with the use of PDX models for bladder cancer studies. For example, the existence of significant patient-to-patient variability requires the use of a number of models for a given study, which can drive the relatively high cost associated with incorporating PDX models into a study. In addition, PDX models require specific expertise, and (like all xenograft systems) the need for an immune-deficient host (unless creating a syngeneic line from a strain-matched transgenic, knockout or carcinogen-induced bladder cancer from mice). As demonstrated following the molecular analysis of 22 PDX lines by Pan et al. [40], PDX models faithfully represent the original morphologic and molecular characteristics associated with the clinical specimens from which these models are derived. While there is significant interest in the use of PDX models for select therapeutics studies, this approach is not new. For instance, in 1986 Russel et al. [41] utilized tumor biopsies from twenty-two patients to establish and analyze PDX lines. Of eleven biopsies that successfully implanted, tumor specimens maintained the same histological grade and features as the original patient tumors following xenografting. In three PDX lines that were established and serially transplantable, focal areas of squamous and glandular differentiation were present in subsequently analyzed samples. Indeed, a number of PDX lines with variant morphologic patterns have been established, including those exhibiting small cell, inverted papillary, micropapillary, and neuroendocrine histology types [42–44]. In one study of note, Hofner et al. [44] developed an accurate preclinical PDX model of aggressive neuroendocrine bladder cancer, which was used to identify diagnostic markers and potential therapeutic targets. This study is noteworthy, as there currently are no transgenic or cell line models of neuroendocrine/small cell bladder cancer. While PDX models have many advantages in preclinical bladder cancer research, their lengthy development time and variable take rates (in addition to other limitations described above) preclude many researchers from using them. To address this limitation, Gheibi et al. [45] successfully established cultures of PDX-derived ellipsoids in microchambers, which could help to maintain these patient-derived cells for extended periods of time. Ellipsoids demonstrated considerable heterogeneity in drug susceptibility, reflective of heterogeneity that may be seen *in vivo* [45].

IN VITRO MODELS OF TUMOR HETEROGENEITY

Cell lines as models of tumor heterogeneity

Perhaps the most important advantage of cell line-based approaches is the fact that they present a system suitable for mechanistic studies. For example, our group used publicly available data from the Cancer Cell Line Encyclopedia (CCLE) for 27 bladder cancer cell lines, as well as bladder cancer data available through the TCGA bladder cancer study, to identify cell lines suitable for mechanistic studies related to

molecular subtype [46]. Specifically, we identified 7 and 10 cell lines representative of luminal and basal molecular subtypes of bladder cancer, respectively. In keeping with previous molecular subtyping studies, luminal cell lines were characterized by high levels of FOXA1, GATA Binding Protein 3 (GATA3) and Peroxisome Proliferator Activated Receptor Gamma (PPARγ) expression, while basal cell lines exhibited reduced expression of these transcriptional regulators. Although not all tested cell lines were capable of forming tumors *in vivo*, xenografting studies showed that SCaBER bladder cancer cells exhibited extensive levels of squamous differentiation, while two luminal bladder cancer cell lines exhibited papillary (RT4) and urothelial cell carcinoma histology with elements of clear cell differentiation (UMUC1). These molecular and morphologic attributes are consistent with clinical history provided when these lines were established [47–49]. Recent studies by our group and others suggest molecular subtype is plastic and can change during tumor progression [18, 20] with the basal molecular subtype expanding over time. While such progression-associated tumor plasticity is potentially associated with intratumoral heterogeneity and therapeutic resistance, the molecular mechanism(s) related to this plasticity are unknown. As FOXA1, GATA3 and PPARγ are involved in urothelial differentiation and expressed in a highly subtype-specific manner, we leveraged our cell line analysis to test the hypothesis that these factors cooperate to control subtype-specific gene expression events in bladder cancer. Interestingly, while no single factor was capable of "reprogramming" this basal cell line to a luminal gene expression pattern, combinations of FOXA1 or GATA3 overexpression in conjunction with PPARγ activation was sufficient to classify the basal 5637 bladder cancer cell line as luminal. In addition to characterizing available cell lines in regard to their molecular subtype and providing a cancer-specific context for the long-described phenomenon of urothelial plasticity, these results additionally suggest human bladder cancer cell lines are useful for studies designed to identify the mechanistic drivers of molecular heterogeneity.

De-novo and acquired resistance to systemic chemotherapy is a significant clinical issue in the management of patients with advanced bladder cancer. For this reason, bladder cancer cell lines have been extensively used to determine the impact of specific genetic alterations on chemotherapeutic sensitivity and predict patient response to cisplatin [50], as well as a model for resistance to systemic chemotherapy. However, recent clinical studies have shown that systemic chemotherapy treatment is also a significant contributor to intratumoral and intermetastatic tumor heterogeneity in bladder cancer patients. For example, one recent study reported that only $\sim$28% of mutations are shared in patient-matched, pre and post chemotherapy treated samples [51]. Although cell lines cannot recapitulate the complete physiologic complexity of a living organism, they do provide one system for analyzing the contribution of chemotherapeutic treatment to tumor heterogeneity.

In addition to commonly used human cell lines, Saito et al. [52] have developed two unique mouse cell lines to specifically model luminal-like and basal-like bladder cancer (UPPL1541 and BBN963, respectively). Utilizing an inducible *Upk3* promoter system for directed knockout of *Trp53* and *Pten*, they produced *Upk3a-Cre*^{ERT2}*; Trp53*$^{L/L}$*; Pten*$^{L/L}$*; Rosa26*$^{LSL-Luc}$ (UPPL) mice with high-grade, muscle-invasive bladder cancer. These mice develop bladder cancer with papillary histology and a luminal molecular subtype. Their cell line derived from UPPL tumors maintains luminal subtype, demonstrated by expression of PPARγ and GATA3. For their BBN model, C57BL/6 mice were exposed to BBN, resulting in the development of bladder tumors with basal phenotype. These lines have been utilized for immune checkpoint studies [52, 53], and provide an important new research tool.

Organoids: A conceptual middle ground between established cell lines and PDX models

While useful for biochemical and mechanistic studies, traditional monolayer cell cultures are relatively artificial. In addition to being cultured *in vitro* for decades, monolayer cell culture does not support growth patterns that faithfully recreate all attributes of an *in vivo* tissue microenvironment. Organoid culture systems overcome some of the artificial qualities of monolayer cultures. By definition, organoids are "organ-like" models that recapitulate the *in vivo* physiology of their "parent" tissue of origin *in vitro* in three-dimensional culture [54]. Organoid culture systems have been described for a number of organ systems and cancers, including prostate [55], gastric [56], intestinal [57], pancreatic [58],

Table 1
Models for the Study of Tumor Heterogeneity in Bladder Cancer

Model type	Model examples	Benefits	Limitations
Transgenic or genetically engineered models (GEM)	1. *Upk2*-HRAS$^{*/WT}$/*Upk2*-cre/p53$^{LOX/LOX}$ [25] 2. Adeno-Cre/Tp53$^{loxp/loxp}$/Pten$^{loxp/loxp}$ [27] 3. AhCreER^{+}Lkb1$^{fl/fl}$Pten $^{fl/fl}$ [28] 4. UBC-Cre/ERT2/Foxa1$^{loxp/loxp}$ [33] 5. CAG-ATDC [35]	–Investigators can target individual and combined genetic alterations to determine impact on tumor development and progression *in vivo.* –Living organism with organ system of focus and functional immune system –Can be combined with carcinogen-based studies	–High cost often related to long generation time –Specialize expertise is required –Potentially simple repertoire of genetic alterations relative to human disease
Carcinogen-induced models	BBN-induced MIBC: [37]	–Rapid induction of tumorigenesis and progression *in vivo*	–Identity and sequence of genetic alterations responsible for tumorigenesis is unknown –Rapid onset of tumor development and disease progression can make intervention-associated differences difficult to detect/observe Phenotype is often strain-dependent [38]
Patient-derived xenografts	Analysis of PDX lines: [40, 41] Variant morphologic patterns: 1. Small cell [42] 2. Inverted papillary [42] 3. Micropapillary [43] 4. Neuroendocrine [44]	–PDX models largely maintain tumor histology and molecular heterogeneity from parent tumors –Significant utility in preclinical therapeutics studies	–PDX use limited by the requirement of specific expertise for use, lengthy development time, and variable take rates –Need for immune deficient hosts –Associated with relatively high cost and need for special expertise relative to other models
Cell lines	1. SCaBER [47] 2. RT4 [48] 3. UMUC1 [49] 4. 5637 [18] 5. UPPL1541 [52] 6. BBN963 [52] 7. See Table 1 [22] 8. For molecular characterization see [46]	–Simple, reductionist system ideal for mechanistic studies –Relative ease of genetic manipulation –Well characterized –Often can be grown *in vivo* in xenograft studies –Rapid generation time and with low cost –In some respects ideal for preclinical therapeutics studies	–Often Passaged for extended periods of time *in vitro*, which can lead to phenotypic drift/changes –Required immunodeficent hosts for *in vivo* studies, and not all lines are tumorigenic –Not all cell lines are representative of human disease
Organoids	Originating from: 1. Patient-derived tissue [60, 62] 2. Carcinogen-induced rodent bladder tumors [61] 3. Cell lines [60]	–Organoid models recreate a more accurate, three-dimensional *in vivo* tissue microenvironment in an *in vitro* setting. –Largely retain heterogeneity consistent with parent tumors. –Can be established form a number of sources, including patient-derived tissue, carcinogen-induced tumors from animal bladders, and cell lines Ideal for preclinical therapeutics studies	–Use limited by the need for favorable *in vitro* growth conditions –Reduced availability relative to cell lines

liver [59], among others. Similar to PDX models, innovative organoid lines developed from patient-derived tissue can be utilized to study bladder tumor progression and drug susceptibility. Bladder cancer organoids have been developed from patient samples [60], carcinogen-induced tumors from rodent bladders [61], and well as cell lines. In addition, organoids can be developed from transgenic animal systems, and be used for "syngeneic" (i.e., immune-competent) *in vivo* studies. Because organoids can provide some authentic aspects of an *in vivo* environment in an *in vitro* setting, these may be more suitable than established cell lines grown in a monolayer format. Following the establishment of 22 bladder cancer organoid cultures, Lee et al. [62] showed that organoid lines retain elements of tumor heterogeneity identified in the parental tumor and effectively model human tumor evolution and treatment response. More specifically, organoids originating from both high-grade and low-grade urothelial cell carcinoma samples, as well as one sample of squamous cell carcinoma of the bladder, were established. On genomic analysis, organoids were found to retain significant heterogeneity, consistent with "parent" tumors; in fact, there was over 80% concordance between the majority of organoid lines and corresponding parental tumors [63]. Lee et al. also made the observation that some organoid lines exhibit phenotypic changes *in vitro*. Specifically, some organoid lines (while exhibiting mixed or luminal molecular subtype in the parental tumor) exhibited a shift to the basal subtype. The development of cellular plasticity may reflect the stages that occur during tumor progression [62, 63], and potentially arise (as the authors suggest) through epigenetic changes. In summary, organoid cultures provide an innovative model resource for tumor heterogeneity studies.

DISCUSSION AND FUTURE DIRECTIONS

Instead of reviewing the relative strengths and weaknesses of a given set of approaches, we have endeavored to review the most commonly utilized preclinical models for tumor heterogeneity studies in bladder cancer. For a more detailed discussion of strengths and weaknesses of model systems, readers are referred elsewhere [22].

As we describe in our introduction, bladder cancer is extremely heterogeneous at the morphologic and molecular levels, and tumor heterogeneity can both impact and result from clinical management [51]. However, while there exists a subset of models suitable for the study of specific elements of tumor heterogeneity (i.e., squamous differentiation at the morphology level) within the primary tumor, and there is a paucity of models for the study of other morphologic variants. Because of lack of thorough molecular characterization, it is difficult to know what extent these models faithfully and/or completely model human disease. We now have an increased understanding of the molecular biology associated with tumor heterogeneity. However, the availability of models to study molecular intratumoral heterogeneity is especially limited. Moreover, *in vivo* models suitable for the study of tumor heterogeneity (broadly defined) resulting in response to clinical management and regarding morphologic and molecular heterogeneity between primary tumors and metastatic lesions are seemingly nonexistent.

In the opinion of the authors, these represent significant impediments to translational bladder cancer research and discovery. One easy "fix" would be to place increased focused on fully characterizing available *in vivo* models, especially at the genetic level. An important aspect of this process is to compare these models to human disease whenever possible. In addition, increased efforts are required to develop additional *in vivo* and *in vitro* models to address these gaps.

Morphologic assessment provides architectural and contextual information, and will unlikely ever be replaced completely by molecular techniques. However, alterations in gene expression and genomics are an essential component of our understanding of tumor heterogeneity. Although morphologic and molecular variation and differences are often linked, it is true that molecular heterogeneity in the form of gene expression differences can exist in a manner independent of morphologic variation. For this reason, there are clear limitations with the use of morphologic heterogeneity as a surrogate to identify elements of molecular heterogeneity. Therefore, resources for the credentialing of currently available model systems in regard to their relationship to molecular subtypes present in human bladder cancer should also be a high priority for the research community. Indeed, lack of thorough characterization (and funding available to support these efforts) of available models is perhaps the most significant roadblock to the identification of additional models, which represent a more complete representation of the spectrum of molecular heterogeneity in this common disease.

AUTHOR CONTRIBUTIONS

All authors contributed equally to review concept and design, drafting of the manuscript, and critical revision of the manuscript.

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ETHICAL CONSIDERATIONS

This study, as a literature review is exempt from any requirement for Institutional Review Board approval.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

REFERENCES

[1] Foulds L. The natural history of cancer. J Chronic Dis. 1958;8(1):2-37.
[2] Heppner GH. Tumor heterogeneity. Cancer Res. 1984; 44(6):2259-65.
[3] Jamal-Hanjani M, Quezada SA, Larkin J, Swanton C. Translational implications of tumor heterogeneity. Clin Cancer Res. 2015;21(6):1258-66.
[4] Pons F, Orsola A, Morote J, Bellmunt J. Variant forms of bladder cancer: Basic considerations on treatment approaches. Curr Oncol Rep. 2011;13(3):216-21.
[5] Lopez-Beltran A, Henriques V, Montironi R, Cimadamore A, Raspollini MR, Cheng L. Variants and new entities of bladder cancer. Histopathology. 2019;74(1):77-96.
[6] Li G, Yu J, Song H, Zhu S, Sun L, Shang Z, et al. Squamous differentiation in patients with superficial bladder urothelial carcinoma is associated with high risk of recurrence and poor survival. BMC Cancer. 2017;17(1):530.
[7] Antunes AA, Nesrallah LJ, Dall'Oglio MF, Maluf CE, Camara C, Leite KR, et al. The role of squamous differentiation in patients with transitional cell carcinoma of the bladder treated with radical cystectomy. Int Braz J Urol. 2007;33(3):339-45; discussion 46.
[8] Kim SP, Frank I, Cheville JC, Thompson RH, Weight CJ, Thapa P, et al. The impact of squamous and glandular differentiation on survival after radical cystectomy for urothelial carcinoma. J Urol. 2012;188(2):405-9.
[9] Lee YJ, Moon KC, Jeong CW, Kwak C, Kim HH, Ku JH. Impact of squamous and glandular differentiation on oncologic outcomes in upper and lower tract urothelial carcinoma. PLoS One. 2014;9(9):e107027.
[10] Clark PE. Urothelial carcinoma with squamous differentiation: Response to chemotherapy and radiation. Urol Oncol. 2015;33(10):434-6.
[11] Amin MB. Histological variants of urothelial carcinoma: Diagnostic, therapeutic and prognostic implications. Mod Pathol. 2009;22(Suppl 2):S96-S118.
[12] Al-Ahmadie H, Iyer G. Updates on the Genetics and Molecular Subtypes of Urothelial Carcinoma and Select Variants. Surg Pathol Clin. 2018;11(4):713-23.
[13] Mu P, Zhang Z, Benelli M, Karthaus WR, Hoover E, Chen CC, et al. SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer. Science. 2017;355(6320):84-8.
[14] Guo H, Ci X, Ahmed M, Hua JT, Soares F, Lin D, et al. ONECUT2 is a driver of neuroendocrine prostate cancer. Nat Commun. 2019;10(1):278.
[15] Ku SY, Rosario S, Wang Y, Mu P, Seshadri M, Goodrich ZW, et al. Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and antiandrogen resistance. Science. 2017;355(6320):78-83.
[16] Park JW, Lee JK, Sheu KM, Wang L, Balanis NG, Nguyen K, et al. Reprogramming normal human epithelial tissues to a common, lethal neuroendocrine cancer lineage. Science. 2018;362(6410):91-5.
[17] Network CGAR. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014;507(7492):315-22.
[18] Hovelson DH, Udager AM, McDaniel AS, Grivas P, Palmbos P, Tamura S, et al. Targeted DNA and RNA Sequencing of Paired Urothelial and Squamous Bladder Cancers Reveals Discordant Genomic and Transcriptomic Events and Unique Therapeutic Implications. Eur Urol. 2018;74(6):741-53.
[19] Al-Ahmadie HA, Iyer G, Lee BH, Scott SN, Mehra R, Bagrodia A, et al. Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer. Nat Genet. 2016;48(4):356-8.
[20] Warrick JI, Sjödahl G, Kaag M, Raman JD, Merrill S, Shuman L, et al. Intratumoral Heterogeneity of Bladder Cancer by Molecular Subtypes and Histologic Variants. Eur Urol. 2019;75(1):18-22.
[21] Wu XR. Biology of urothelial tumorigenesis: Insights from genetically engineered mice. Cancer Metastasis Rev. 2009;28(3-4):281-90.
[22] DeGraff DJ, Robinson VL, Shah JB, Brandt WD, Sonpavde G, Kang Y, et al. Current preclinical models for the advancement of translational bladder cancer research. Mol Cancer Ther. 2013;12(2):121-30.
[23] Lin-Tsai O, Taylor JA, Clark PE, Adam RM, Wu XR, DeGraff DJ. Progress made in the use of animal models for the study of high-risk, nonmuscle invasive bladder cancer. Curr Opin Urol. 2014;24(5):512-6.
[24] Kobayashi T, Owczarek TB, McKiernan JM, Abate-Shen C. Modelling bladder cancer in mice: Opportunities and challenges. Nat Rev Cancer. 2015;15(1): 42-54.
[25] He F, Melamed J, Tang MS, Huang C, Wu XR. Oncogenic HRAS Activates Epithelial-to-Mesenchymal Transition and Confers Stemness to p53-Deficient Urothelial Cells to Drive Muscle Invasion of Basal Subtype Carcinomas. Cancer Res. 2015;75(10):2017-28.
[26] Zhang ZT, Pak J, Huang HY, Shapiro E, Sun TT, Pellicer A, et al. Role of Ha-ras activation in superficial papillary pathway of urothelial tumor formation. Oncogene. 2001;20(16):1973-80.
[27] Puzio-Kuter AM, Castillo-Martin M, Kinkade CW, Wang X, Shen TH, Matos T, et al. Inactivation of p53 and Pten promotes invasive bladder cancer. Genes Dev. 2009;23(6):675-80.
[28] Shorning BY, Griffiths D, Clarke AR. Lkb1 and Pten synergise to suppress mTOR-mediated tumorigenesis and

epithelial-mesenchymal transition in the mouse bladder. PLoS One. 2011;6(1):e16209.

[29] Tsuruta H, Kishimoto H, Sasaki T, Horie Y, Natsui M, Shibata Y, et al. Hyperplasia and carcinomas in Pten-deficient mice and reduced PTEN protein in human bladder cancer patients. Cancer Res. 2006;66(17):8389-96.

[30] Yoo LI, Liu DW, Le Vu S, Bronson RT, Wu H, Yuan J. Pten deficiency activates distinct downstream signaling pathways in a tissue-specific manner. Cancer Res. 2006;66(4):1929-39.

[31] Foth M, Ahmad I, van Rhijn BW, van der Kwast T, Bergman AM, King L, et al. Fibroblast growth factor receptor 3 activation plays a causative role in urothelial cancer pathogenesis in cooperation with Pten loss in mice. J Pathol. 2014;233(2):148-58.

[32] DeGraff DJ, Clark PE, Cates JM, Yamashita H, Robinson VL, Yu X, et al. Loss of the urothelial differentiation marker FOXA1 is associated with high grade, late stage bladder cancer and increased tumor proliferation. PLoS One. 2012;7(5):e36669.

[33] Reddy OL, Cates JM, Gellert LL, Crist HS, Yang Z, Yamashita H, et al. Loss of FOXA1 Drives Sexually Dimorphic Changes in Urothelial Differentiation and Is an Independent Predictor of Poor Prognosis in Bladder Cancer. Am J Pathol. 2015;185(5):1385-95.

[34] Osei-Amponsa V, Buckwalter J, Shuman L, Zheng Z, Hironobu Y, Walter V, et al. Hypermethylation of FOXA1 and Allelic Loss of PTEN Drive Squamous Differentiation and Promote Heterogeneity in Bladder Cancer. Under revision.

[35] Palmbos PL, Wang L, Yang H, Wang Y, Leflein J, Ahmet ML, et al. ATDC/TRIM29 Drives Invasive Bladder Cancer Formation through miRNA-Mediated and Epigenetic Mechanisms. Cancer Res. 2015;75(23):5155-66.

[36] Palmbos PL, Wang Y, Bankhead Iii A, Kelleher AJ, Wang L, Yang H, et al. ATDC mediates a TP63-regulated basal cancer invasive program. Oncogene. 2019;38(18):3340-54.

[37] Fantini D, Glaser AP, Rimar KJ, Wang Y, Schipma M, Varghese N, et al. A Carcinogen-induced mouse model recapitulates the molecular alterations of human muscle invasive bladder cancer. Oncogene. 2018;37(14):1911-25.

[38] Amponsa VO, Shuman L, Ellis J, Wang E, Walter V, Owens RG, et al. Carcinogen-induced bladder cancer in the FVB mouse strain is associated with glandular differentiation and increased Cd274/Pdl-1 expression. Am J Clin Exp Urol. 2019;7(3):139-52.

[39] Jäger W, Xue H, Hayashi T, Janssen C, Awrey S, Wyatt AW, et al. Patient-derived bladder cancer xenografts in the preclinical development of novel targeted therapies. Oncotarget. 2015;6(25):21522-32.

[40] Pan CX, Zhang H, Tepper CG, Lin TY, Davis RR, Keck J, et al. Development and Characterization of Bladder Cancer Patient-Derived Xenografts for Molecularly Guided Targeted Therapy. PLoS One. 2015;10(8):e0134346.

[41] Russell PJ, Raghavan D, Gregory P, Philips J, Wills EJ, Jelbart M, et al. Bladder cancer xenografts: A model of tumor cell heterogeneity. Cancer Res. 1986;46(4 Pt 2):2035-40.

[42] Abe T, Tada M, Shinohara N, Okada F, Itoh T, Hamada J, et al. Establishment and characterization of human urothelial cancer xenografts in severe combined immunodeficient mice. Int J Urol. 2006;13(1):47-57.

[43] Park B, Jeong BC, Choi YL, Kwon GY, Lim JE, Seo SI, et al. Development and characterization of a bladder cancer xenograft model using patient-derived tumor tissue. Cancer Sci. 2013;104(5):631-8.

[44] Hofner T, Macher-Goeppinger S, Klein C, Rigo-Watermeier T, Eisen C, Pahernik S, et al. Development and characteristics of preclinical experimental models for the research of rare neuroendocrine bladder cancer. J Urol. 2013;190(6):2263-70.

[45] Gheibi P, Zeng S, Son KJ, Vu T, Ma AH, Dall'Era MA, et al. Microchamber Cultures of Bladder Cancer: A Platform for Characterizing Drug Responsiveness and Resistance in PDX and Primary Cancer Cells. Sci Rep. 2017;7(1):12277.

[46] Warrick JI, Walter V, Yamashita H, Chung E, Shuman L, Amponsa VO, et al. FOXA1, GATA3 and PPARγ Cooperate to Drive Luminal Subtype in Bladder Cancer: A Molecular Analysis of Established Human Cell Lines. Sci Rep. 2016;6:38531.

[47] O'Toole C, Nayak S, Price Z, Gilbert WH, Waisman J. A cell line (SCABER) derived from squamous cell carcinoma of the human urinary bladder. Int J Cancer. 1976;17(6):707-14.

[48] Rigby CC, Franks LM. A human tissue culture cell line from a transitional cell tumour of the urinary bladder: Growth, chromosone pattern and ultrastructure. Br J Cancer. 1970;24(4):746-54.

[49] Grossman HB, Wedemeyer G, Ren L. UM-UC-1 and UM-UC-2: Characterization of two new human transitional cell carcinoma lines. J Urol. 1984;132(4):834-7.

[50] Nickerson ML, Witte N, Im KM, Turan S, Owens C, Misner K, et al. Molecular analysis of urothelial cancer cell lines for modeling tumor biology and drug response. Oncogene. 2017;36(1):35-46.

[51] Faltas BM, Prandi D, Tagawa ST, Molina AM, Nanus DM, Sternberg C, et al. Clonal evolution of chemotherapy-resistant urothelial carcinoma. Nat Genet. 2016;48(12):1490-9.

[52] Saito R, Smith CC, Utsumi T, Bixby LM, Kardos J, Wobker SE, et al. Molecular Subtype-Specific Immunocompetent Models of High-Grade Urothelial Carcinoma Reveal Differential Neoantigen Expression and Response to Immunotherapy. Cancer Res. 2018;78(14):3954-68.

[53] Kardos J, Chai S, Mose LE, Selitsky SR, Krishnan B, Saito R, et al. Claudin-low bladder tumors are immune infiltrated and actively immune suppressed. JCI Insight. 2016;1(3):e85902.

[54] Yoshida T, Singh AK, Bishai WR, McConkey DJ, Bivalacqua TJ. Organoid culture of bladder cancer cells. Investig Clin Urol. 2018;59(3):149-51.

[55] Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, et al. Organoid cultures derived from patients with advanced prostate cancer. Cell. 2014;159(1):176-87.

[56] Seidlitz T, Merker SR, Rothe A, Zakrzewski F, von Neubeck C, Grützmann K, et al. Human gastric cancer modelling using organoids. Gut. 2019;68(2):207-17.

[57] Vlachogiannis G, Hedayat S, Vatsiou A, Jamin Y, Fernández-Mateos J, Khan K, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science. 2018;359(6378):920-6.

[58] Boj SF, Hwang CI, Baker LA, Chio II, Engle DD, Corbo V, et al. Organoid models of human and mouse ductal pancreatic cancer. Cell. 2015;160(1-2):324-38.

[59] Nuciforo S, Fofana I, Matter MS, Blumer T, Calabrese D, Boldanova T, et al. Organoid Models of Human Liver Cancers Derived from Tumor Needle Biopsies. Cell Rep. 2018;24(5):1363-76.

[60] Yoshida T, Sopko NA, Kates M, Liu X, Joice G, McConkey DJ, et al. Three-dimensional organoid culture reveals involvement of Wnt/β-catenin pathway in proliferation of bladder cancer cells. Oncotarget. 2018;9(13):11060-70.

[61] Yoshida T, Kates M, Sopko NA, Liu X, Singh AK, Bishai WR, et al. Ex vivo culture of tumor cells from N-methyl-N-nitrosourea-induced bladder cancer in rats: Development of organoids and an immortalized cell line. Urol Oncol. 2018;36(4):160.e23-.e32.

[62] Lee SH, Hu W, Matulay JT, Silva MV, Owczarek TB, Kim K, et al. Tumor Evolution and Drug Response in Patient-Derived Organoid Models of Bladder Cancer. Cell. 2018;173(2):515-28.e17.

[63] Adam RM, DeGraff DJ. Molecular mechanisms of squamous differentiation in urothelial cell carcinoma: A paradigm for molecular subtyping of urothelial cell carcinoma of the bladder. Urol Oncol. 2015;33(10):444-50.