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Modeling bladder cancer in mice: opportunities and challenges

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Abstract

The prognosis and treatment of bladder cancer have hardly improved in the last 20 years. Bladder cancer remains a debilitating and often fatal disease, and among the most costly cancers to treat. The generation of informative mouse models has the potential to improve our understanding of bladder cancer progression, as well as impact its diagnosis and treatment. However, relatively few mouse models of bladder cancer have been described and particularly few that develop invasive cancer phenotypes. This review focuses on opportunities for improving the landscape of mouse models of bladder cancer.

Introduction

Bladder cancer is one of the leading causes of cancer related deaths in Western countries. It is more common than in Western than developing countries and, for reasons that are still not well-understood, three to four times more prevalent in males than in females. Although characterized by heterogeneous subtypes that have a range of disease outcomes, the broad subgroups are non-muscle invasive bladder cancer, which is more common and usually associated with a favorable prognosis, and muscle invasive bladder cancer, which is less prevalent but typically associated with a relatively poor prognosis (for general reviews on bladder cancer see [1–3]). Notably, bladder cancer is one of the most costly cancers to treat, primarily due to the considerable costs associated with life-long clinical management of patients with non-muscle invasive disease, as well as those associated with the cost of caring for patients after surgical removal of the bladder [4].

However, despite its prevalence and adverse impact on human health, bladder cancer has been remarkably understudied relative to other cancers and remains significantly underrepresented by informative *in vivo* models, particularly genetically-engineered mouse (GEM) models. However, the tide is now changing with the recent the generation of new mouse models of bladder cancer, as well as the recent elucidation of molecular alterations

that are prevalent in bladder cancer, which provide new avenues for developing models of disease relevant genes and/or pathways. Here we introduce key concepts that are essential for the generation of informative mouse models and their effective translation to human bladder cancer. In addition, we review the status of currently available mouse models of bladder cancer, and discuss prospects for their future development.

Biology of the bladder and lineage relationships of its epithelium

The bladder is comprised of a specialized epithelium, called the urothelium, which is encapsulated by the lamina propria and surrounded by a thick layer of smooth muscle (the detrusor muscle or muscularis propria), which forms the bladder wall (Figure 1) [5, 6]. The urothelium includes three cell types: *(i)* basal cells, which are relatively small cuboidal cells that express p63 and high molecular weight cytokeratins, such as 5 and 14; *(ii)* intermediate cells, which also express p63 and high molecular weight cytokeratins, although at lower levels than the basal layer; and *(iii)* superficial cells, also called “umbrella cells”, which express uroplakin proteins and low molecular weight cytokeratins 18 and 20[7–11]. Among these, the superficial cells are the most highly specialized, relatively large, and often polynucleate. They have polarized membranes that are insoluble, and specialized structures on their apical surface, called asymmetric unit membrane (AUM), comprised of uroplakin proteins that provide a barrier against re-absorption of urine (thus the term “umbrella cells”) [12].

The bladder urothelium has among the slowest turn-over rates of any adult tissue [13, 14]. However, in response to injury, for example, as a consequence of bacterial infection or exposure to toxins, the urothelium undergoes rapid proliferation and ultimately regenerates an intact urothelium [15, 16], although the actual response may depend upon the specific inducing agent (see [17] and below). The implication of these observations is that the adult urothelium contains stem or progenitor cells that are capable of its regeneration. Such stem or progenitor cells have long been thought to reside in the basal cell layer. In particular, lineage tracing of mouse bladder following pathogen-induced regeneration demonstrated that basal cells give rise to all urothelial cell types, supporting their progenitor role [18].

However, several lines of evidence, based on analyses of both human and mouse bladder, suggest that the urothelium may have independent lineages generated by distinct progenitors [19]. Such a multiple progenitor model has been supported by an alternative lineage tracing study following chemically-induced regeneration, which concluded that umbrella cells are derived from intermediate rather than basal cells [20]. In addition, analyses of label-retaining cells in mouse bladder during development as well as following pathogen-induced regeneration also supports a multiple lineage model [11, 21]; notably, during development the progenitors are concentrated in the trigone [11], a specialized structure at the bladder. Furthermore, analyses of mice harboring a germline deletion of *p63*, which is expressed in basal but not umbrella cells, lack basal and intermediate cells but have a superficial cell layer [22, 23]. In addition to these studies of mouse bladder, analyses of clonal relationships in human bladder, inferred from analyses of mitochondrial DNA, also support the model that that the bladder has multiple progenitors [24]. Clearly, lineage relationships within the bladder urothelium are far from resolved.

Bladder cancer: a primer for the mouse modeler

The term “bladder cancer” actually refers to a heterogeneous set of diseases with a spectrum of pathologies and expected prognoses. Most (~90%) are urothelial carcinomas, which are the subject of this review and referred to simply as “bladder cancers” throughout (Figure 2); the remainder (~10%) include primary squamous cell carcinoma, adenocarcinoma, small cell carcinoma, or sarcomatoid carcinoma [1, 3, 25], which are not discussed further in this review.

As introduced above, non-muscle invasive tumors account for vast majority of bladder cancers (~75%), most of which have a relatively favorable prognosis [1, 2]. These can be further sub-grouped into low-grade, which are mainly superficial (or papillary) tumors, and high-grade, which include a subset of superficial tumors as well as carcinoma in situ (CIS) (Figure 2). As their names implies, superficial or papillary tumors grow into the bladder lumen but do not invade the muscle layer, while carcinoma in situ (CIS) is a characterized by a flattened layer of dysplastic cells that is the presumed major precursor of invasive bladder cancer [26, 27]. In contrast to the non-muscle invasive disease, muscle invasive bladder cancer accounts for ~25% of cases and has a relatively poor prognosis; in particular, muscle invasive tumors have a 5-year survival of ~50%, and for those that have metastasized, the expected 5-year survival is only ~15% [1, 3]. Below we discuss key clinical aspects of bladder cancer that impact the generation of informative mouse models.

Cells of origin of bladder cancer and their relationship to bladder cancer subtypes

Various studies in humans and mice have implicated basal cells as cells of origin of bladder cancer [19]. In the mouse, for example, analyses of lineage-tracing in a carcinogen-based model concluded that basal cells can serve as cells of origin of bladder cancer [28]. In humans, isolation of putative stem cells based on expression of cell surface markers followed by growth in xenograft models has shown that such stem cells are enriched for basal cells, particularly the most aggressive tumor subtypes [29–31]. Furthermore, gene expression profiling analyses of invasive bladder cancer have categorized a basal-like subtype, with a more aggressive phenotype, and a luminal-like subtype, with a less aggressive phenotype [32–35]. While it is not necessarily the case that the basal-subtype originates from basal cells, this observation is certainly consistent with the concept that basal cells can serve as cells of origin of bladder cancer, particularly for more aggressive subtypes.

However, studies in both humans and mice have demonstrated alternative cells of origin, which may give rise to distinct bladder cancer subtypes. In mice for example, an alternative analysis of lineage-tracing of a carcinogen-based model has shown that heterogeneous subtypes of bladder cancer can be attributed to distinct cells of origin [36]. In humans, analyses of gene signatures from sub-populations of normal urothelial cells supports the concept that distinct subtypes of bladder cancer arise from distinct cells of origin [37]. Moreover, the gene expression profiling studies discussed above, which identified molecular subtypes of bladder cancer categorized distinct basal-like and luminal-like subtypes [32–35]; although these are necessarily indicative of multiple cells of origin, this is also not inconsistent with this concept. A precise understanding of the relationships cells of origin of bladder cancer and their relationship to specific clinical subtypes is of paramount importance for generating informative mouse models of bladder cancer.

Treating bladder cancer

Treatment of bladder cancer as well as the efficacy of such treatment varies profoundly depending on the clinical stage and associated risk factors [1, 3, 38, 39] (Figure 2). The front-line treatment for non-muscle invasive bladder cancer is transurethral resection (TUR) [39], which has a high disease free survival for low-grade cases although a high rate of relapse for high-grade disease. Because of the unique biology and tissue organization of the bladder, non-muscle invasive bladder cancers can be treated locally (rather than systemically) by what is called intravesical therapy. In particular, the front line treatment for patients with non-muscle invasive bladder cancer who are at high-risk or recur following TUR is intravesical delivery of bacillus Calmette-Guerin (BCG) [40, 41], which is actually a vaccine against tuberculosis that promotes immunoreaction against cancer cells [42]. Patients that fail BCG treatment are candidates for cystectomy (surgical removal of the bladder), or alternatively for salvage intravesical therapy using chemotherapy regimens or targeted agents in an effort to preserve bladder function [43] (*e.g.*, Clinical Trial.gov; NCT02202772).

Cystectomy, with or without neoadjuvant chemotherapy [44, 45], is also the front line treatment for muscle invasive bladder cancer [39, 46]. Cystectomy has a 5-year survival ranging from 30–70% depending on the stage of the tumor, and the inevitable requirement of urinary tract diversion results in a profound impairment in quality of life [47, 48]. Notably, cystectomy is not a viable option for all patients, and is generally not recommended for patients with metastatic bladder cancer, since it has very little chance of being curative. Rather, the standard of care for metastatic bladder cancer is a multidrug chemotherapy regimen consisting of methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) or, alternatively, gemcitabine plus cisplatin (GC). Both of these regimens have a low response rate (~40–50%) and limited improvement on overall survival (~12–15 months) [39]. Moreover, cisplatin-based chemotherapy is not an option for many elderly patients, which are a large subset of those with advanced bladder cancer, due to the potential for kidney failure. Thus, treatment options for muscle invasive bladder cancer are limited and, in striking contrast with many other cancers, have not significantly improved in recent years.

What causes bladder cancer?

Although genome-wide association studies have identified various low-penetrance susceptibility loci associated with increased cancer risk [49], bladder cancer is thought to arise primarily as a consequence of environmental exposures [50–54]. Indeed, the major risk factor for bladder cancer is smoking [50, 54] and the relatively high incidence of bladder cancer in Western versus developing countries is thought to reflect the prevalence of smoking in the former. Carcinogens from tobacco smoke, as well as those associated with occupational hazards [51–53], are presumed to promote bladder cancer by virtue of their concentrated in urine, essentially bathing the urothelium with carcinogens.

Interestingly, these associated risk factors cannot fully account for the approximately three to four fold difference in the incidence of bladder cancer in men versus women, particularly with respect to smoking [50]. The implication is that there may be fundamental differences in bladder physiology and/or its development that underlie the striking prevalence of bladder cancer in men. Notably, the epithelium of the bladder and prostate, although highly

specialized and distinct, share a common embryological origin, namely the urogenital sinus [55]. Thus, it has been proposed that the increased prevalence of bladder cancer in males versus females reflects, at least in part, androgen receptor function in bladder cancer [56], as supported by recent analyses of genetically-engineered mouse models [57, 58].

Molecular subtypes and molecular alterations in bladder cancer

Non-muscle invasive versus muscle invasive bladder cancer

Several lines of evidence support the general concept that the distinct clinical outcomes of low-grade non-muscle invasive versus high-grade muscle invasive bladder tumors reflects their distinct molecular causes and, as discussed above, potentially also distinct cells of origin. Indeed, certain molecular alterations, such as gain of function mutations of *FGFR3*, are prevalent in low-grade non-muscle invasive bladder cancers whereas other alterations, such as p53 loss or mutation, are prevalent in high-grade muscle invasive bladder cancers [53, 59–62]. Although analyses of gene expression profiling [63–68] and/or genomic alterations [65, 69–73] have supported the general concept that low-grade non-invasive versus high-grade invasive bladder tumors are molecularly distinct, it is difficult to fully reconcile a mutual-exclusivity model considering that some superficial bladder tumors can progress to invasive disease. Furthermore, meta-analysis of expression profiling data from non-invasive and invasive bladder cancers failed to identify molecular subtypes that are clearly associated with pathological stage [74]. Furthermore, recent whole genome sequencing and transcriptome analyses comparing low-grade and high-grade bladder cancers supports the concept that these are evolve in parallel rather than mutually exclusively [75].

Thus, low-grade non-muscle invasive and high-grade muscle invasive bladder cancer may be more appropriately viewed as broadly distinct entities along a continuum of disease progression. In this framework, the actual phenotype and outcome may reflect the culmination of molecular alterations that tend to drive more or less aggressive phenotypes, distinct cells of origin, which may contribute to tumor aggressiveness, and potential interactions with environmental exposures, such as smoking, carcinogens or inflammation.

Molecular alterations in muscle invasive bladder cancer

The cancer genome atlas (TCGA) has recently reported a comprehensive molecular analysis of muscle invasive bladder cancer [32], which together with several additional whole exome or whole genome analyses [76–80] have both confirmed and extended the contribution of known genes/molecular pathways, as well as identified interesting new ones. In particular, as anticipated from many earlier studies, the TCGA study found that *TP53* (which encodes p53) is deleted and/or mutated in ~49% of muscle invasive bladder cancers and, more generally, that genes encoding members of the p53-RB pathway are altered in a majority of muscle-invasive bladder cancers; however, surprisingly, *FGFR3* mutations, which had long been associated almost exclusively with non-muscle invasive bladder cancer, were found to be relatively common (~12%) in muscle invasive disease [32].

Furthermore, the TCGA study, together with integrative analyses of high grade bladder tumors [81] as well as analyses of patients who are ‘exceptional responders’ to targeted therapy [82, 83], have demonstrated the relevance of the PI3K-mTOR signaling and RTK-

RAS-MAPK signaling pathways, and support the rationale for therapeutic targeting of these 'actionable' signaling pathways for treatment of advanced bladder cancer. In particular, 42% or 44% of muscle invasive tumors were found to have alterations of genes associated with PI3K-mTOR signaling or RAS-RTK-MAPK pathways, respectively, including genes such as *PIK3CA*, tuberous sclerosis 1 (*TSC1*), *TSC2*, *AKT3*, *FGFR3*, epidermal growth factor receptor (*EGFR*) and *ERBB2 (Her2)* [32].

Indeed, there has been a long standing rationale for therapeutic targeting of RTK-RAS-MAPK signaling, since *HRAS* was originally identified in bladder cancer cells [84–86], although *RAS* itself has proven difficult to target. Thus, clinical efforts have been focused on targeting relevant downstream pathways, as such *FGFR3* [87] and *EGFR* [88] (*eg.*, Clinical Trial.gov; NCT01732107; NCT01953926). Furthermore, current clinical trials targeting PI3K-mTOR pathway with various mTOR inhibitors such as temsirolimus or everolimus (also known as RAD001) are now underway (eg., Clinical Trial.gov; NCT01827943, NCT00805129 NCT02009332).

Additionally, the TCGA study identified several genes that are frequently (>10%) altered in bladder cancer but that had not been previously implicated in bladder and in some cases in any cancer, including mixed-lineage leukemia 2 *(MLL2*; also known as KMT2D), cyclin-dependent kinase inhibitor 1A (*CDKN1A*), *ERCC2*, and stromal antigen 2 (*STAG2*) [32]. Also notable is the prevalence of alterations of epigenetic regulatory genes, including *UTX* (also known as *KDM6A*), *MLL2*, CREB binding protein (*CREBBP*), and AT rich interactive domain 1A (*ARID1A*) [32, 76], thus providing new avenues for therapeutic targeting of invasive bladder cancer. Interestingly, the TCGA study, along with meta-analyses of TCGA datasets representing 12 distinct cancers [89, 90], found that bladder cancer has among the highest number of mutations per DNA megabase of any cancer. Consistent with this observation, whole genome sequencing of five muscle-invasive bladder tumors that have mutated *TP53* found a profound level of nucleotide alterations as well as chromothripsis [77], which refers to the shattering and reassembly of chromosomes as a consequence of genomic instability; potentially, this is a reflection of the unusual susceptibility of the urothelium to environmental carcinogens (discussed above).

Modeling bladder cancer in mice

Although recent studies have advanced our conceptual understanding of the biological, molecular, and environmental factors associated with bladder cancer, this knowledge has not yet advanced to the point of impacting patient care. In fact, the field is still grappling with major uncertainties regarding the nature and complexity of bladder cancer subtypes, how they are related to each other, and how they can best be treated to improve patient outcomes. Our understanding of these issues would greatly benefit from the availability of mouse models that accurately represent specific bladder cancer phenotypes or subtypes and are based on relevant genes/pathways/processes that are associated with bladder cancer (Table 1). However, in contrast to many other cancer types, which have experienced a veritable explosion of in the generation of mouse models over the last two decades, bladder cancer is relatively underrepresented by mouse models, particularly genetically-engineered mouse (GEM) models.

Presently, mouse models of bladder cancer include carcinogen-based models, in which tumors arise following treatment of mice (or rats) with carcinogens, various types of engraftment models, in which cells or tissues are grown in recipient hosts, and genetically-engineered mouse (GEM) models, based on activation or inactivation of gene function in the bladder (Table 2). An important distinction between these types of models is that carcinogen-based and GEM models are autochthonous, which means that tumors originate in the bladder, whereas graft models are non-autochthonous, since they are implanted into recipient hosts. Notably graft recipient mice are usually immunodeficient, which is of relevance given the known importance of the immune system for cancer progression and metastasis [91]. However, engraftment models have the considerable advantage of their relative ease and rapidity of generation and use for analyses of the functional relevance of candidate genes. Furthermore, although they are both autochthonous, tumors in carcinogen-based models are, by definition, induced by carcinogens, whereas those in GEM models arise following manipulation of specific genes. Thus, these different approaches to modeling bladder cancer in mice are highly complementary (Table 2). In addition to the discussion that follows, we refer the reader to recent reviews of bladder cancer cell lines and *in vivo* models of bladder cancer found in [92–94].

Carcinogen models

The classic model of bladder cancer is based on chemical carcinogenesis of the urothelium, which conceptually mimics environmental exposures that are known to be a leading cause of bladder cancer. First introduced for induction of bladder cancer in rats in the 1960's [95–97], numerous applications have used several carcinogens in various species, including mice, rats, and dogs [98]. Notably, carcinogen models were among the first preclinical models to evaluate chemotherapy for bladder cancer [99], and they continue to provide informative models for understanding processes involved in cancer progression and for elucidating cancer subtypes.

Currently, the majority of carcinogen-induced mouse models of bladder cancer utilize N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), which is delivered in the drinking water. BBN is highly relevant to human bladder cancer, since it is very similar to the major carcinogen associated with tobacco smoke [100]. Although delivered systemically (rather than directly to bladder), the urothelium is particularly susceptible to BBN treatment, as evident from analyses of the mutagenesis spectrum across various tissues [101]. BBN-treated mice develop a range of bladder cancer phenotypes, including hyperplasia, dysplasia, CIS, and muscle-invasive bladder cancer, as well as metastases in certain strain backgrounds [102]. Notably, mutation of relevant genes such as *Trp53* [102], as well as the molecular profiles of tumors from BBN-treated mice [103, 104] share similarity with those of human invasive bladder cancer. Furthermore, treatment of genetically engineered mouse models with BBN has the advantage of exacerbating the consequences of loss or gain of function phenotypes (discussed below). In particular, a recent study of bladder tumors arising following BBN-treatment of mice with or without heterozygous deletion of *Trp53* showed that the range of bladder cancer phenotypes is influenced by the status of *Trp53* [36].

Among their major advantages, carcinogen treatment simulates actual events that are known to give rise to bladder cancer in humans. In addition, these models are relatively straightforward to implement and autochthonous tumors arise in immune-competent mice. Among their disadvantages, despite the fact that the urothelium may be most susceptible, since BBN treatment is systemic, it is difficult to rule out the contribution of other tissues. Furthermore, tumor phenotypes and their temporal progression are highly heterogeneous in BBN-treated mice, and vary depending on species and strain background. Although this inherent heterogeneity may capture key elements of human bladder, it makes it difficult to implement preclinical studies or to model specific disease subtypes.

Engraftment models

Orthotopic and renal engraftment—Urothelial cancer cells can be engrafted orthotopically in mice or rats such that tumors arise within the bladder of recipient hosts. First introduced in the 1970's [105], delivery of cancer cells into the bladder lumen has been widely used for modeling bladder cancer [106–110]. The recent introduction of using ultrasound-guided implantation of cells between the urothelium and lamina propria/muscle layer [107, 108] has the benefit of being accurate in terms of cell delivery as well as minimally invasive. An alternative renal grafting approach involves recombination of urothelial cells with embryonic urogenital sinus mesenchyme (UGM) *in vitro* followed by engraftment under the kidney capsule of recipient hosts [111, 112].

The orthotopic and renal engraftment models are complementary, in that the former enables evaluation of tumor behavior in an organ-specific microenvironment, whereas the latter is particularly beneficial for investigating the role of epithelial-stromal interactions for tumor growth. Both have the advantage of their relative ease of manipulating gene expression in cell culture to introduce gain or loss of function alterations prior to engraftment, such that the consequences of such alterations for tumor growth can be evaluated *in vivo* [113]. Notably, engraftment models are not limited to tumor cells; they are adaptable to primary and/or non-transformed cells, which can be particularly beneficial for evaluating gain of function mutations, while inclusion of fluorescent or luciferase reporter genes can enable *in vivo* imaging of tumors and metastases [114, 115]. Orthotopic engraftment models have been used for preclinical evaluation of potential new treatment options, such as the targeting RTK inhibitor sunitinib and plasminogen activator inhibitor type-1 (PAI–1) [116, 117], although these agents have not yet been adapted to clinical practice.

Among their major limitations, however, engrafted tumors (as well as the patient derived xenograft tumors discussed below) are non-autochthonous, and therefore they do not model the *de novo* evolution of tumor phenotypes. Additionally, since recipient hosts are usually immunodeficient, the lack of an intact immune system may impact tumorigenesis as well as metastasis. Nonetheless, engraftment approaches are an excellent starting point to rapidly evaluate the functional significance of candidate genes and for prioritizing the generation of genetically-engineered mouse (GEM) models.

Patient derived xenografts—Engraftment of patient-derived tumor tissues into immunodeficient mouse hosts (called patient derived xenograft — PDX — models), which

have become increasingly utilized for many types of cancers [118], have been described for bladder cancer [119]. Since PDX models are derived from individual patient tumors, the expectation is that the resulting tumors capture the unique genomic and molecular properties of the individual patient from which they are derived; the further expectation is that PDX models should enable analyses of clinical responses based on the unique characteristics of a given tumor. Indeed, preclinical studies using PDX models of bladder cancer have supported the concept that co-targeting PI3K and MAP signaling may be beneficial for certain types of bladder cancers [120].

However, to date few reports have described the generation of PDX models for bladder cancer; thus, it is not clear whether such models can be generated efficiently or whether they will indeed capture all or most bladder cancer subtypes. On the other hand, whereas the generation of PDX models for certain types of cancers (such as prostate cancer for example) may be limited by tissue availability, in principal this should not be a consideration for bladder cancer because primary tissue is readily available from TUR as well as cystectomy. Thus, if indeed bladder cancer has a reasonable 'take-rate' in the recipient hosts, it should be feasible to generate a range of PDX models, ideally representative of the various subtypes of bladder cancer.

GEM models of bladder cancer

GEM models are now widely used for many applications in cancer biology, including analyses of tumor phenotypes, modeling disease subtypes, mechanistic investigations of candidate genes and signaling pathways, and preclinical evaluation of potential therapeutic agents [121–123]. Notably, GEM models complement non-autochthonous mouse models since tumors arise *de novo* in the native tissue microenvironment, and they also complement carcinogen-based models, since they are based on defined genetic alterations. However, relatively few GEM models of bladder cancer have been described, particularly those that display muscle invasive and/or metastatic phenotypes (Table 3), which we believe reflects several major challenges in their design and generation (discussed further below). In particular, relatively few promoters display bladder-specific expression and can be used to generate GEM models. Additionally, bladder tumors appear to be unusually recalcitrant to developing invasive tumors, since most single gene alterations and even many combined alterations have relatively mild phenotypes (Table 3). However, given the recent description of molecular alterations found in bladder cancer that can be modeled in mice (*e.g.*, [124]), we envision that GEM models are likely to play an increasingly prominent role in the future.

Transgenic models of bladder cancer—Similar to many of the original "oncomice" [125], the earliest GEM models of bladder cancer were transgenic mice in which SV40 large T antigen is expressed in the urothelium under the control of the *Upk2* promoter [126]. The resulting transgenic mice develop CIS and invasive bladder cancer, some progressing to metastasis [126, 127], and their molecular profiles are conserved with human bladder cancer [128]. A similar phenotype was observed when SV40 large T antigen was expressed under the control of the *Krt19* promoter [129]. Interestingly, although SV40 large T antigen inactivates *Trp53* and *Rb1*, combined loss of function of *Trp53* and *Rb1* is not sufficient for bladder tumors to arise in GEM models [112, 130]. Besides SV40 large T antigen,

other oncogenes have been expressed in the urothelium, including *Hras*, *Egfr*, and *Cyclin D1*, with the latter two resulting in urothelial hyperplasia; these have also been combined with other alleles, such as mutant *p53*, resulting in progression to dysplasia or non-invasive bladder cancer [131–135].

Conditional models of bladder cancer—The majority of recent GEM models of cancer involve tissue-specific conditional or inducible gene targeting. However, the generation of such models for bladder cancer has been challenged by the paucity of Cre alleles that restrict gene targeting specifically to the urothelium and particularly to selected cell types (discussed below) (Table 3). Moreover, most GEM models of bladder cancer described thus far display non-invasive phenotypes. Interesting, in several cases GEM models generated using "bladder-specific" Cre drivers have less aggressive phenotypes than those made using other (non-bladder specific) Cre drivers (Table 3).

In particular, conditional activation of β-catenin in the bladder using a Cre driver based on expression of the *Upk2* promoter (called UroII-Cre) results in hyperplasia, and together with activation of *Hras* or *Kras* or loss of function of *Pten*, in papillary non-invasive cancer [136–138] However, with an alternative, non-bladder-specific Cre driver, activation of β-catenin alone results in papillary non-invasive cancer [58] (Table 3). Similarly, abrogation of *Notch* function by expression of nicastrin (*Ncstn)* using a bladder specific promoter results in hyperplasia and CIS, whereas expression of *Ncstn* using a ubiquitously expressed promoter results in muscle invasive bladder cancer [139]; of course this difference may be due to the actions of *Notch* outside of the urothelium. Lastly, while loss of function of *Pten* together with *Fgfr3* activation using a UroII-Cre allele results in hyperplasia and localized dysplasia, using alternative non bladder specific Cre drivers, *Pten* loss alone or together with *Lbk1* result in papillary non-invasive tumors [140–142].

Conditional models using Adeno-Cre delivery—An alternative to using tissue-specific Cre alleles to target gene recombination in bladder, delivery of Adeno-Cre directly into the bladder lumen has been used to inactivate *Trp53* and *Pten* in the urothelium, resulting in invasive bladder cancer with prevalent metastasis [112]. This approach has also been used to delete all three members of the retinoblastoma family, resulting in papillary non-invasive cancer [85]. Interestingly, conditional activation of *Kras* and inactivation of *Trp53* via instillation of Adeno-Cre (rather than its surgical into the bladder lumen) results in sarcomas outside the bladder, while the urothelial phenotype is modest [143].

Notably, the Adeno-Cre driven *Pten; Trp53* mice display temporal progression from CIS to invasive disease and ultimately develop distant metastasis with high penetrance [112]. Thus, these mice have enabled preclinical investigations comparing intravesical therapy, evaluated at the CIS stage, with systemic therapy, evaluated at more advanced stages [112, 144, 145]. In particular, comparing intravesical versus systemic treatment of inhibition of mTOR signaling using rapamycin has demonstrated the efficacy of intravesical therapy [144]; these findings formed the basis for a clinical trial to evaluate intravesical treatment with rapamycin for high-risk early stage bladder cancer (Clinical trials.gov, NCT02009332). Similarly, other preclinical studies in this model demonstrated the efficacy of intravesical administration of multi-chemotherapy regime, which has led to new clinical trials to

evaluate intravesical delivery of this treatment for high-risk early stage bladder cancer (Clinical trials.gov, NCT02202772). These examples suggest that preclinical studies in GEM models having progressive phenotypes may be advantageous to test the effectiveness of promising drugs as well as to optimize the route of their administration.

Opportunities and challenges for GEM models of bladder cancer

Compared with other cancer types, bladder cancer is largely underrepresented by GEM models; moreover, the phenotypes of most existing GEM models are primarily non-invasive. Here we discuss major challenges that have impeded the generation of GEM models of bladder cancer and suggest various approaches to overcome these challenges.

Challenges in targeting gene expression to bladder

A key consideration for the generation of informative GEM models is the ability to restrict gene targeting to appropriate tissue layers, relevant cell(s) of origin, and at the appropriate stage of tissue development. For bladder, few promoters exist that meet these criteria. In fact, the most widely used is the *Upk2* promoter [146], which can be expressed in other tissues besides bladder and even within the bladder urothelium is not uniformly expressed but rather primarily limited to the superficial cells. Of particular concern is that this *Upk2* promoter, which used widely for the generation of transgenic mouse models as well as the development of Cre alleles, has been reported to have been cloned in the wrong orientation [94, 127], which has likely compromised its activity and specificity. Recently the *Upk3a* promoter has been used to express a tamoxifen-inducible Cre recombinase in the bladder urothelium [20]. Furthermore, since uroplakin is primarily expressed in superficial cells, ideally Cre drivers using these promoters would be complemented by promoters that direct expression to other urothelial cell layers; thus far, promoters that restrict gene targeting specifically to the bladder but preferentially to basal or intermediate cells have not been described. Other promoters that have been used to direct gene expression and/or to express Cre recombinase in the urothelium, although they are not specific for bladder, include as the fatty acid binding protein 1 (*Fabp1*), cytokeratin 19 (*Krt19*), and the msh homeobox 2 *(Msx2)* promoter [58, 129, 147]. Additionally, it has been reported that gene recombination specifically in the bladder can be achieved by delivery of tamoxifen directly into the bladder lumen of mice that have tamoxifen-inducible Cre alleles [148]; however, this approach has not been used extensively since this initial report.

In lieu of suitable Cre-drivers, an alternative approach to achieve bladder-restricted gene targeting is to introduce an adenovirus expressing Cre-recombinase (Adeno-Cre) into the bladder lumen [112, 149]. Adeno-Cre can be delivered intravesically rather than surgically [143]; however, these mice develop tumors outside the bladder and intravesical delivery is only feasible for female mice, which is a considerable limitation since bladder cancer is more prevalent in men. Although gene recombination via adeno-Cre has the benefit of being efficient and selective for the urothelium and, because it does not require the generation of mice with an additional Cre allele, can be used to ‘screen’ the consequences of gene recombination in the bladder [112], since Adeno-Cre enables recombination in all the cell layers, it is difficult to evaluate the contribution of specific urothelial cell types and thus this approach is not ideal for cell of origin analyses.

Challenges for modeling invasive bladder cancer in mice

A striking difference of modeling bladder cancer in mice compared with other cancers is that relatively few GEM models described thus far display overtly invasive or metastatic phenotypes (Table 3). More generally, with few exceptions, dysregulation in the urothelium of individual tumor suppressor genes, such as *Rb1*, *Cdkn1a* (which encodes p21), *Pten*, *Trp53*, *liver kinase B1* (*Lkb1*; also known as *Stk11*), or oncogenes, such as *Hras*, *Kras*, *Egfr*, or *Fgfr3*, have not resulted in invasive bladder cancer, irrespective of the strategy used to direct their expression or induce recombination, although in some cases these dysregulated genes collaborate with others to accelerate bladder cancer phenotypes[112, 127, 130–132, 134–137, 140–142, 150].

Although it is conceivable that the apparent difficulties in generating invasive bladder cancer phenotypes may reflect a lack of 'optimal' targeting approaches or that the models thus far have not been based on 'optimal' combinations of genes, considering the numerous examples described thus far (Table 3), it seems likely that the urothelium may be inherently refractory to developing cancer, or at least in mice. It is plausible that this reflects the characteristic slow turnover of the urothelium, such that its very limited proliferation renders it resistant to genetic assaults. Indeed, as initially demonstrated for germline loss of function of *Trp53*[151], carcinogen-treatment, even at sub-carcinogenic doses, exacerbates the bladder tumor phenotypes associated with several genes, including loss of function of patched homologue 1 (*Ptch1*), *Rb1*, secreted acidic cysteine rich glycoprotein (*Sparc*), *Cdkn1b* (which encodes p27), and gain of function of signal transducer and activator of transcription 3 (*Stat3*) and insulin growth factor1 (*IGF1*) [130, 152–158].

Why might the urothelium be inherently resistant to developing cancer? One possibility is that the current mouse models do not effectively model genomic instability, which is apparently a distinguishing feature of human bladder cancers (discussed above). Alternatively or additionally, the current models may not incorporate epigenetic modifications that are prevalent in human bladder cancer, or the "right" gene combinations to model specific cancer subtypes (discussed above). These are issues that will need to be addressed in future model development.

The future of modeling bladder cancer in mice

Historically, bladder cancer research has lagged significantly behind other cancers. This is particularly the case for the generation of mouse models and especially those that represent a spectrum of bladder cancer phenotypes and provide informative preclinical models. As discussed above, the generation of mouse models of bladder cancer has been fraught with inherent difficulties; however, we envision that these challenges are not insurmountable. Considering recent insights regarding the molecular alterations associated with bladder cancer and the description of disease subtypes associated with clinical relevance, the opportunity is now ripe for the exploration of new mouse models and particularly those that can have translatable impact to improve the therapeutic landscape for patients with bladder cancer.

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Glossary of terms

Non-muscle invasive bladder cancer	Urothelial carcinomas that invade the bladder muscle
Carcinoma in situ (CIS)	A flattened malignant transformation of the urothelium that is presumed to be a precursor of muscle invasive bladder cancer
Muscle invasive bladder cancer	Urothelial carcinomas that invade the muscle layer
Papillary (superficial) tumor	An extrusion of the urothelium into the bladder lumen without invasion of the muscle layer
Urothelium	The epithelium that lines the bladder, which is comprised of three cell types: basal, intermediate and umbrella cells
Lamina propria	The layer of connective tissue that underlies the urothelium
Detrusor muscle	The layer of smooth muscle that lines the bladder and controls the elimination of urine
Uroplakins	Transmembrane proteins that that comprise the asymmetric unit membrane (AUM) on the lumen-facing side of the umbrella cells, which provide a barrier against entry of urine
Transurethral resection (TUR)	Endoscopic surgical removal of bladder tumors or lesions
Intravesical therapy	Delivery of interventional or therapeutic agents directly to the bladder lumen
Bacillus Calmette-Guerin (BCG)	A form of immunotherapy that is a front line intravesical treatment for high-risk non-muscle invasive bladder cancer
Cystectomy	Surgical removal of the bladder, which is the front line treatment for muscle invasive bladder cancer
Autochthonous	Arising within the individual, usually refers to tumors arising *de novo* in genetically-engineered mouse models

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At a glance summary

- Bladder cancers arise in the urothelium, a specialized epithelium comprised of basal, intermediate and superficial (umbrella) cells.
- Basal cells can serve as urothelial progenitors as well as cells of origin of bladder cancer, particularly of more aggressive subtypes; however, other urothelial cell types can also serve as progenitors in normal bladder as well as cells of origin of bladder cancer, potentially of it different subtypes.
- Bladder cancer represents a heterogeneous set of tumors that vary in histopathology, molecular alterations, and potentially cells of origin; the vast majority (>90%) are urothelial carcinomas, which are the subject of this review.
- Urothelial carcinomas fall into two major categories: most (~75%) are non-muscle invasive, which include low-grade superficial (or papillary) and high-grade carcinoma *in situ*; the remainder (~25%) are muscle invasive.
- Most non-muscle invasive bladder cancers, particularly low-grade tumors, have favorable prognosis; these can be clinically-managed with bladder-sparing treatments, which are generally effective but very costly.
- Muscle invasive bladder cancers have relatively poor prognosis; those that have not metastasized are often treated by cystectomy (surgical removal of the bladder), which is reasonably effective (5-year survival of ~50%) but associated with high morbidity.
- Metastatic bladder is treated using chemotherapy, which is neither well-tolerated nor highly effective; metastatic bladder cancer has a very poor prognosis (5-year survival of ~15%).
- Unlike many other cancers, neither the prognosis nor treatment of bladder cancer has significantly improved in the past 20 years; bladder cancer remains a major cause of cancer mortality.
- The molecular pathways that give rise to low-grade non-muscle invasive versus high-grade muscle invasive bladder cancer are distinct but not mutually exclusive.
- The recent elucidation of genetic/genomic alterations prevalent in muscle invasive bladder cancer provides new avenues for understanding the underlying molecular mechanisms, as well as new targets for therapeutic intervention.
- Currently available *in vivo* models of bladder cancer include carcinogen-based and genetically-engineered mouse (GEM) models, as well as orthotopic and renal grafting, each of which has advantages and limitations.
- Bladder cancer is relatively underrepresented by GEM models, particularly those that model more aggressive phenotypes.
- The mouse bladder may be relatively recalcitrant to developing invasive tumors, which has made it challenging to develop GEM models.

- Other challenges to developing GEM models include inadequate approaches for restricting gene targeting to the urothelium and particularly to selected cells of origin.
- Improved GEM models will lead to opportunities for preclinical evaluation of new treatment options for bladder cancer.

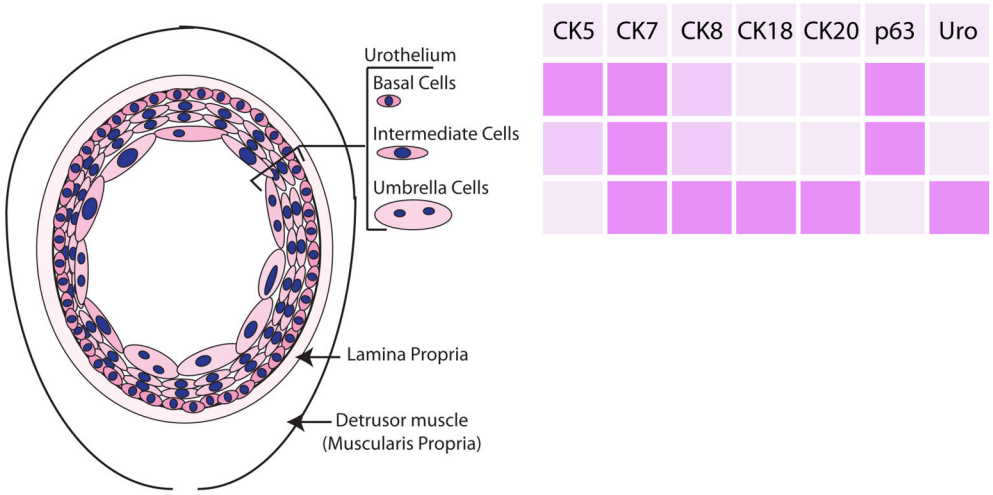

Figure 1.

A. Diagram of bladder anatomy and cell types B. Summary of expression of cytokeratins (CK), p63, and Uroplakin (Uro) in bladder urothelial cells.

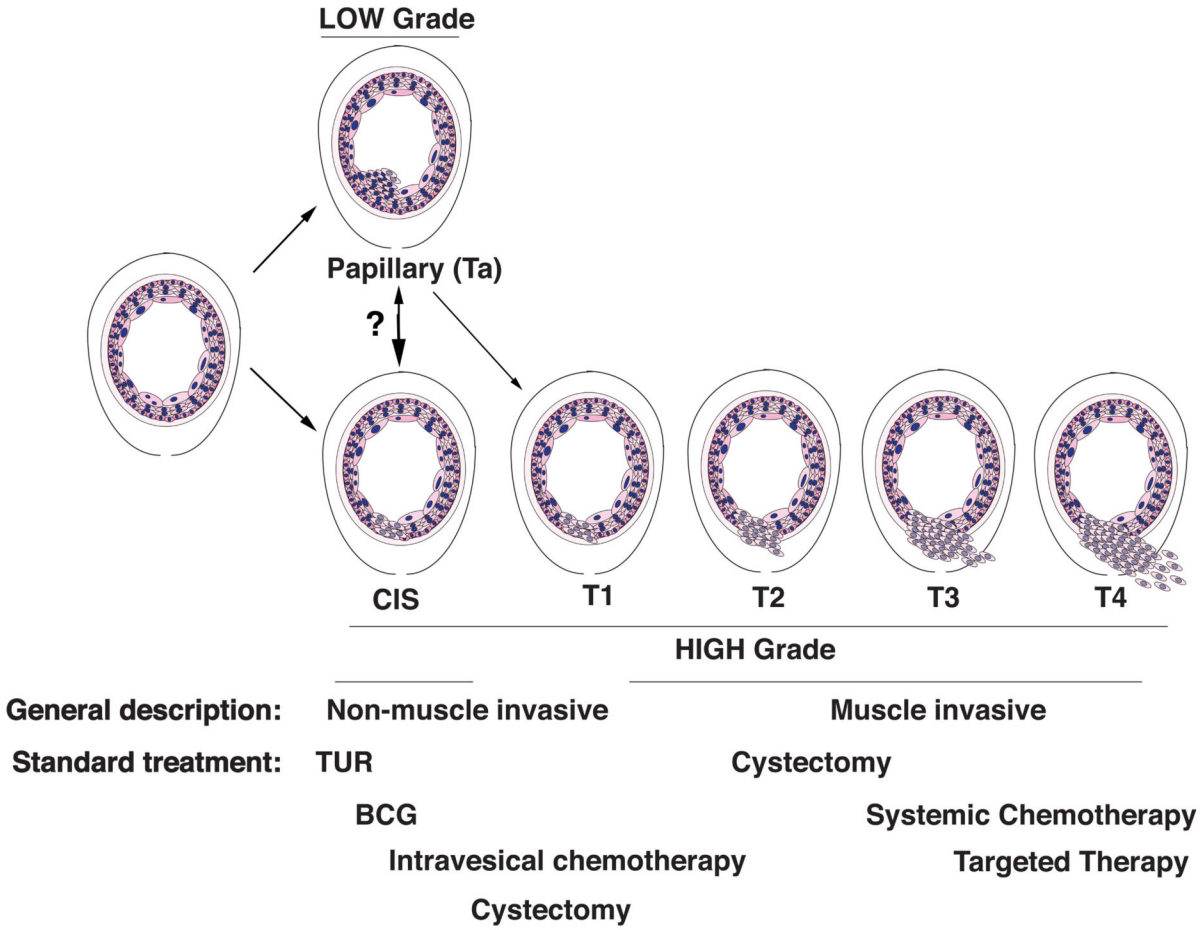

Figure 2. Clinical stages of bladder cancer

Schematic representation of the clinical stages and grades of bladder cancer and standard treatments. TUR, transurethral resection; CIS, carcinoma *in situ*.

Table 1

Major concepts in bladder cancer research that can be addressed using mouse models

Key concepts	Opportunities	Challenges
Lineage relationships and cell of origin		
Cell of origin	• Analyses of cells of origin of bladder cancer • Evaluate whether specific cell(s) of origin give rise to distinct cancer sub-types	• Inadequate approaches to restrict gene targeting to specific sub-populations of urothelial cells • Particularly challenging to restrict gene targeting to intermediate cells since these share features of both basal and superficial cells
Molecular causes of bladder cancer		
Non-muscle invasive vs muscle invasive bladder cancer	• Systematically evaluate molecular alterations that distinguish these broadly distinct bladder cancer subtypes • Elucidate the biological and molecular contexts in which low-grade non-muscle invasive bladder cancers can progress to invasive disease	• Progression of low-grade non-muscle invasive cancer may be influenced by factors such as cell of origin and/or environmental factors that may be difficult to model in mice
Muscle invasive bladder cancer	• Evaluate functional roles of specific molecular alterations • Model specific subtypes of bladder cancer	• Individual gene alterations may not lead to invasive cancer in mice • Bladder cancer subtypes may involve multiple genes and therefore may be difficult to model
Metastatic bladder cancer	• Evaluate associated molecular and biological processes	• Modeling of metastatic cancer in mice has proven challenging
Risk Factors		
Smoking	• Evaluate the molecular consequences of smoking for cancer initiation and progression • Identify molecular alterations associated with smoking-induced bladder cancer	• May be inherent challenges in translation to humans • Susceptibility loci may have very subtle phenotypes • Genetic loci may not be conserved between humans and mice
Genetic risk factors	• Model genetic susceptibility loci	
Preclinical analyses of novel therapies		
Intravesical treatment	• Evaluate new bladder-sparing treatments for high-risk bladder cancer patients	• Responses may depend on the model tested • Complexity of translating preclinical findings to human clinical trials, particularly with regard to dose and schedule • Challenges in modeling metastasis in mice • Efficacy of targeted therapies may vary depending on the specific genetic alterations of the models
Intravesical versus systemic treatment	• Compare drug responses following systemic versus local treatment • Evaluate drugs and drug combinations in distinct tumor contexts	
Systemic treatments	• Evaluate new treatments for metastatic disease	

Key concepts	Opportunities	Challenges
Targeted therapy	• Evaluate novel targeted therapies using models based on relevant molecular alterations	

Table 2

Mouse models of bladder cancer

Type of model	Description	Advantages	Disadvantages
Non-autochthonous models			
Orthotopic engraftment models	Primary urothelial or bladder cancer cells implanted into the bladder wall of recipient hosts such that tumors arise in the bladder	• Ease of functional analyses of candidate genes • Preclinical models for either intravesical or systemic therapy	• Engrafted tumors do not develop *de novo* • Tumors arise in immunodeficient mice and therefore may not inform on the role of the immune system
Renal grafting models	Urothelial or bladder cells are combined with embryonic bladder mesenchyme and grown under the kidney capsule of recipient hosts	• Ease of functional analyses of candidate genes • Investigate epithelial-mesenchymal tissue interactions • Preclinical models for systemic therapy	
Patient derived Xenografts (PDX)	Engraftment of patient tumors into recipient mice	• Potential to model individual patient tumors • Potential to model specific bladder cancer subtypes • Preclinical analysis of agents for specific patient tumors	• Tumors arise in immunodeficient mice • Take-rate may differ depending on the tumor phenotype or sub-type • No definitive evidence that responses in the PDX models will translate to patients
Autochthonous Carcinogen-based models			
Carcinogen-based	Treatment of mice with carcinogens in the drinking water, the most common is BBN	• Mimic environmental exposures associated with human bladder cancer • Not limited to mice; work in various species • Can be combined with GEM models to exacerbate their bladder cancer phenotypes	• Heterogeneity of cancer phenotypes makes it difficult to use for molecular investigations or preclinical studies • Metastases are rare
Autochthonous GEM models			
Transgenic models	Expression of oncogenes in the urothelium (such as expression of *SV40* Large T antigen)	• Original *in vivo* models of bladder cancer	• Limited options to express genes specifically in the bladder • Phenotypes of single alleles, and even most compound alleles, are relatively modest • Few models develop invasive bladder cancer • Rare occurrence of metastases
Germline models	Loss of function of tumor suppressor genes in the germline (such as *p53* null mice)	• Phenotypes can be enhanced using BBN	
Conditional alleles	Conditional or inducible gene recombination of	• Range of bladder tumor phenotypes	

Type of model	Description	Advantages	Disadvantages
	oncogenes or tumor suppressor genes in the urothelium (such as deletion of *Pten* and *p53*)		

Table 3

GEM models of bladder cancer

Allele name	Method for targeting to bladder[b]	Description	Phenotype	Ref.
Transgenic models				
UPII-SV40T[a]	Uroplakin II Promoter	Expression of SV40 large T antigen	CIS	126, 127
UPII-SV40T high			CIS with progression to invasion, in some cases metastasis	126
CK19-SV40T	Cytokeratin 19		CIS with progression to invasion, in some cases metastasis	129
UPII-*Ha-Ras*^{Q61L}	Uroplakin II	Expression of mutant (active) *H-Ras*	Papillary non-invasive cancer	131
UPII-*EGFR*		Expression of *EGFR*	Hyperplasia	132
UPII-SV40T low; *EGFR*		Expression of SV40 large T combined with *EGFR*	High-grade non-invasive cancer	132
UPII-p53DN		Expression of mutant (active) *p53*	Hyperplasia	135
UPII-*Ha-rasQ61L*; p53DN		Mutant (active) *H-Ras* combined with mutant (active) allele of *p53*	Hyperplasia with localized dysplasia	135
UPII-*Ha-rasQ61L*; p53-null		Mutant *H-Ras* combined with a germline null allele of *p53*	Papillary non-invasive cancer	135
Models using Cre Drivers expressed in the urothelium				
β-catenin$^{exon3/exon3}$	UroII-Cre allele	Mutant (active) allele of β-catenin	Hyperplasia	136
β-catenin$^{exon3/exon3}$; Pten$^{flox/flox}$		Mutant (active) allele of β-catenin combined with loss of function of Pten	Papillary non-invasive cancer	136
β-catenin$^{exon3/exon3}$; K-rasG12D		Mutant (active) allele of β-catenin combined with mutant (active) *Kras*	Papillary non-invasive cancer	137
β-catenin$^{exon3/exon3}$; H-rasQ61L		Mutant (active) allele of β-catenin combined with mutant (active) *Ha-ras*	Papillary non-invasive cancer	137
β-catenin$^{exon3/exon3}$; p21$^{-/-}$		Mutant (active) allele of β-catenin combined with germline null allele of *p21*	Papillary non-invasive cancer	137
Fgfr3$^{+/K644E}$; Pten$^{flox/flox}$		Mutant (active) allele of *Fgfr3* combined with loss of function of *Pten*	Hyperplasia with localized dysplasia	140
UPII-*Ha-rasQ61L*; CDKN2A null		Mutant *H-Ras* combined with a germline null allele of *INK4a/Arf*	Hyperplasia	134
Ncstn$^{flox/flox}$	UPII-Cre-GFP	Bladder-restricted deletion of *Nicastrin*, which abrogates *Notch* function	Hyperplasia and CIS	139
Models using Cre Drivers that are not specific for bladder				
β-catenin$^{exon3/+}$	Msx2rtTA;tetO-Cre	Mutant (active) allele of β-catenin	Papillary non-invasive cancer	58
Pten$^{flox/flox}$	Fabp-Cre	Conditional loss of function *Pten*	Papillary non-invasive cancer	150

Allele name	Method for targeting to bladder[b]	Description	Phenotype	Ref.
Lkb1$^{flox/flox}$; Pten$^{flox/flox}$	AhCreER	Conditional loss of function of *Lkb* and *Pten*	Papillary non-invasive cancer	141
Ncstn$^{flox/flox}$	Rosa26rtTA;tet O-Cre	Systemic deletion of *Nicastrin*, which abrogates *Notch* function	Muscle-invasive bladder cancer	139
Models using delivery of Adeno-Cre to the bladder				
p53$^{flox/flox}$; Pten$^{flox/flox}$	AdenoCre delivery (via surgery)	Conditional loss of function of *p53* and *Pten*	Muscle-invasive bladder cancer with frequent metastasis	112
p53$^{flox/flox}$; K-rasG12D	AdenoCre delivery (via instillation)	Mutant (active) allele of β-catenin combined with mutant (active) *Kras*	Hyperplasia	143
Rb$^{flox/flox}$; p130$^{flox/flox}$ p107$^{-/-}$	AdenoCre delivery (via surgery)	Loss of function of all retinoblastoma genes	Papillary non-invasive cancer	159

Notes:

[a] Note that distinct uroplakin 2 promoters were used to develop these SV40 large T antigen models.

[b] References for relevant Cre alleles: are as follows: UroII-Cre 160; Fabp-Cre 150; AhCreER 141; UPKII-Cre 127; Upk2-CreERT2 161; UPK3a-GFP-CreERT2 20; UPII-Cre-GFP 139.