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Preclinical models for bladder cancer research

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SYNOPSIS

At diagnosis, over 70% of bladder cancers (BC) are at the non-muscle-invasive bladder cancer (NMIBC) stages which are usually treated with transurethral resection followed by intravesical instillation. For the remaining advanced cancers, systemic therapy is the standard of care with addition of radical cystectomy in case of locally advanced cancers. Because of the difference in treatment modalities, different models are needed to advance the care of NMIBC and advanced BC. This article gives a comprehensive review of both *in vitro* and *in vivo* BC models and compares the advantages and drawbacks of these preclinical systems in BC research.

Keywords

Bladder cancer; organoid; conditionally reprogrammed cell culture; genetically engineered mouse model; patient-derived xenograft; humanized mouse

Bladder cancer (BC) is the most common tumor in the urinary system, ranking at the 11th among all human malignancies worldwide. It is more common in males than females, with a ratio of 3:1 to 5:1 [1]. In 2017, approximately 430,000 new cases were diagnosed with BC

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(330,000 males and 99,000 females) worldwide with 51,000 deaths in the European Union and 18,000 deaths in North America [2]. Some of the risk factors include smoking, occupational chemical exposure, male gender and social economic status [2–4]. Depending on whether cancer cells invade into the muscle layer of the bladder wall, BC is divided into non-muscle-invasive bladder cancer (NMIBC) and advanced BC which includes locally advanced and metastatic cancer. NMIBC includes Ta, T1 and CIS (cancer in situ), and accounts for approximately 75% of newly diagnosed BC cases [2]. The standard of care for NMIBC is transurethral resection followed by intravesical therapy for high risk patients. With this treatment, over 70% patients develop cancer recurrence and one third of patients have cancer progression to advanced stages [5]. For advanced BC, the standard of care for locally advanced muscle-invasive BC (MIBC) is neoadjuvant chemotherapy followed by radical cystectomy, and approximately 55% of MIBC patients survive at 5 years. The standard treatment for metastatic BC is systemic therapy and the median survival is less than two years [6].

There has been no significant improvement in BC treatment until recently with the approval of immunotherapy for both advanced and NMIBC, as well as one targeted therapy with a fibroblast growth factor receptor inhibitor erdafitinib and one antibody-drug conjugate enfortumab vedotin for advanced BC. The development for therapeutic interventions is still disappointing and advanced BC is generally incurable. For example, recurrent genomic alterations have been identified in BC[7]. But, so far, only erdafitinib has been approved and yields a modest incremental benefit and no cures. Immunotherapy using PD1/L1 inhibitors has shown promising activity, but the response rate is only around 20% in advanced BC [8]. The purpose of this review article is to review various *in vitro* and *in vivo* models that can be used to advance BC research and care for both NMIBC and advanced BC.

IN VITRO TWO-DIMENSIONAL (2D) MODELS

1. Cancer cell lines

Compared to animal models, cancer cell lines are faster, cheaper, easier to manipulate, more widely available and have been extensively used in BC research. They are the most used models for cancer research.

BC is classified to low and high grade based on differentiation. Accordingly, human BC cell lines can also be classified into low-grade cancer cell lines, such as RT4 and RT112, and high-grade ones, such as T24, J82, 5637, UM-UC1, UM-UC3, HB-CLS-2, TCCSUP and EJ-1 [9]. Furthermore, the genetic characteristics and drug sensitivity information of cell lines can be found at some of the commonly used databases, such as Catalog of Somatic Mutations In Cancer (COSMIC) [10], Cancer Cell Line Encyclopedia (CCLE) [11], and the Genomics of Drug Sensitivity in Cancer [12]. Based on gene expression profiling, BC cell lines can recapitulate the expression profiles of different molecular subtypes of human BC [13].

Besides human BC, cell lines from dog, rat and mouse BC have also been developed [13–15]. The advantage of rat and mouse BC cell lines is that they can be implanted into immunocompetent syngeneic hosts to allow studies in immunotherapy. When human and

canine cell lines are implanted *in vivo,* immunodeficient animals are usually used that do not permit studies in immunotherapy.

There are several major drawbacks associated cell lines. First, cell lines and human BCs may have different genetic and epigenetic alterations. Cancer cell lines have been cultured *in vitro* for a long time, leading to the acquisition and accumulation of additional genetic and epigenetic aberrations that can be dramatically different from that of the original cancers. Even after a few generations, there was a great irreversible genetic divergence between a primary tumor and a cell line derived from that tumor [16]. Hence it is not surprising that prediction models based on cell lines frequently fail to predict drug efficacy in the clinic [17]. Second, cell lines use synthetic artificial culture medium. Different culture environment, nutrition availability and different endo/para/autocrine growth factor support during cell culture apply selection pressure to cells, skew cancer cell composition and alter genetic and epigenetic alterations of the survived cancer cells. Furthermore, long-term culture *in vitro* enables tumor cells adapted to the culture environment, selects the fittest cells and loses the heterogeneity as seen in primary tumor tissues. Third, lack of 3D structure and supporting environment can alter cell behavior. *In vivo* cancer cells grow in a three-dimensional (3D) environment and communicate with other components, such as epithelial cells, stromal cells, immune cells, matrix, etc. The communication plays important roles in tumorigenesis and progression. Fourth, for *in vivo* studies, human and dog BC cell lines can only be implanted into mice or rats that are immunocompromised and, hence, are not suitable for research in immunotherapy.

2. Conditionally reprogrammed cell culture (CRC)

CRC has recently emerged as a promising primary culture of both normal and cancer cells [18]. To establish CRC, after specimens are reviewed by pathology, they are digested with enzymes to generate single cell suspension and co-cultured with irradiated 3T3-J2 mouse fibroblasts (serve as feeder cells) in a medium containing a Rho-associated kinase inhibitor Y-27632. Under this culture condition, cells rapidly convert to a stem-like state, are highly proliferative, but retain the original karyotype [19]. After its development, this method has been used to establish CRC cultures of many normal epithelial tissues, including skin, prostate, lung, breast, kidney, salivary gland and liver cells, and cells across many species, such as mouse, rat, dog, ferret, horse, and cow. CRC can be established for all cancer types that have been tested so far.

CRC has been extensively studied in BC. CRC can be easily established using urine specimens with the overall success rate of 83.3% (50/60), including 85.4% for high grade BC (41⁄48) and 75.0% (9/12) for low grade BC [20]. This suggests that patient-derived CRC models can be established in most patients without requiring biopsy. CRC retains the genetic alterations and shares similar drug sensitivities of their corresponding *in vivo* parental cancer models [21].

There are several advantages associated with CRC. First, CRC cells can be propagated in long-term culture, yet retain cell lineage commitment with the capacity to fully differentiate into the original tissue types they are derived from [18]. Second, CRC is highly efficient in establishing cultures. Epithelial colonies are readily observed at two days and proliferate

rapidly. Third, CRC maintains the heterogeneity of cells present in a biopsy. The drawbacks of CRC are similar to those of cell lines except that CRCs are directly derived from cancer tissues, retain genetic alterations and high concordance of drug sensitivity as the *in vivo* counterparts, and can differentiate into the original tissues that CRC was developed.

IN VITRO 3D MODELS

1. Organoid

An organoid is a miniaturized and simplified version of an organ produced *in vitro* from differentiated cells, embryonic stem cells or induced pluripotent stem cells that self-organize in a 3D structure and can self-renew and replicate. Compared to traditional 2D cell line culture models, organoids better retain the intrinsic characteristics of tumor and its microenvironment, including cell-cell interaction, cell-stroma interaction, tissue polarity and nutrition gradients, etc. With the development of culture matrix, a variety of organoid culture models are increasingly applied to BC research, such as BC stem cells and co-culture system. However, there is still a lack of unified quantitative standards in correlating primary BCs and cultured organoids. Genomic and transcriptomic sequencing have been widely used in analyzing the consistency between organoids and primary BCs while sensitivity to treatments have been used to correlate with and predict the response of primary BCs to the same treatments.

The most common sources of organoids include cell lines (murine and human BC cell lines), patient-derived organoids (PDOs) and cancer-tissue originated spheroid (CTOS) [22]. To grow BC organoids, cell suspensions from cell lines or cancer specimens are implanted into a medium containing growth factors and cell matrix to grow into 3-D structures (Figure 1) [23]. Cells of different tissue and cancer types have different success rates. For BC, papillary BC is more likely to survive and has a success rate up to 80%, while non-papillary BC has a success rate of less than 30% [24,25]. It is particularly important to optimize the medium and matrix. [26]. After organoids are established, they are characterized phenotypically, such as immunohistochemistry (IHC), flow cytometry, western-blot and proteomics, and genomically, such as exome sequencing, transcriptome sequencing and methylation profile. Mullecci et al. [26] optimized the culture conditions and established a variety of organoid models, including murine bladder basal cell organoids, murine ureter and suprabasal bladder organoids, human urothelial organoids. By comparing various biomarkers in mouse BC tissues and in organoids, such as basal (keratin 5, Ck5), intermediate (p63), and suprabasal/umbrella (keratin 20, Ck20, and UpkIII), it was found that organoids were able to retain the main features of mouse BC; by comparing different biomarkers of BC subtypes, such as basal (KRT5 and KRT6), luminal (KRT20, UPK1A, and UPK3A), and potential tumor-initiating cells (CD44), human BC organoids were found to retain these features of the human BC subtypes. Okuyama [23] analyzed E-cadherin, Ki67, uroplakin III (differentiation marker) and p63 (basal cell marker) of BC CTOSs and found that CTOSs retained the differentiation status of the primary tumors.

The tumorigenesis and progression of BC always involve the interaction between BC cells and its tumor microenvironment (TME). TME contains cellular components, such as tumor cells, normal epithelial cells, immune cells, stromal fibroblasts, etc., and non-cellular

components, such as cytokines, fibers, collagen, etc. Though the traditional co-culture technology can increase the communication among cells, tumor cells are very active in communicating with other components in TME in a 3-D space. So the 3-D culture can better simulate TME, provide better communication for the interaction between BC and TME [27]. For example, macrophages can be divided into tumor-suppressive (M1), a tumor-supportive (M2) andregulatory macrophages (Mreg) [28,29]. Cancer-associated fibroblasts (CAFs) affect the microenvironment by secreting a series of cytokines, which play important roles in tumorigenesis, progression, and resistance. Miyake et al [30] found that co-cultured CAFs and TAMs (tumor associated macrophages) promoted cell adhesion and interaction between these cells and BC cells by secreting the chemokine CXCL1 in 3D models; Compared to 2D cultures, tumor cells in a 3D culture showed higher cell survival rate and cell proliferation rate.

So far, extensive studies in organoids have been performed in BC. Lee et al established a BC organoid bio-bank by 3D culturing of primary and recurrent BC tissues from operation and biopsy *in vitro* [31]. These organoids highly retained the human BC mutational spectrum, tumor evolution, phenotypic stability, plasticity and some other characteristics. Xenograft models and organoids had similar resistance and mutational profiles. Mulves et al collected tissues from 53 BC patients and established an organoid bio-bank [26]. Furthermore, they established primary murine basal cell organoids and found that organoids had high fidelity of primary cancer tissues through analysis of cellar surface molecules and functional validation. Different organoids differed in sensitivity to the same chemotherapy drugs, indicating the heterogeneity of BC organoids.

Goulet et al. [32]extracted the urothelial cells, fibroblasts and endothelial cells from bladder biopsy tissues, and spread the fibroblasts on sheets to form 3D vesical stroma. Then non-invasive or invasive BC cells were seeded and cultured as compact spheroids. Invasive BC cells crossed the basement membrane and invaded the stromal compartment whereas non-invasive BC cells were confined to the urothelium. Thus the 3D culture model can possibly be used to study cancer behavior and drug effects.

Compared to the 2D cultures, organoids provide a 3D structure that partially retains the interaction of cancer cells with their TME. However, some of the drawbacks are obvious. First, even though vasculature can be established and maintained, organoids lack blood circulation and cells obtain nutrients mainly through concentration gradient and diffusion. Second, the culture medium supplemented with growth factors differs with that of the *in vivo* native TME and can change cancer cell behavior. Third, even though immune cells can be embedded, organoids lack the dynamic interaction of immune components in organoids with the immune system in the whole body.

2. 3D printing

3-D bioprinting deposits layers of bioinks, such as cells, extracellular matrix and supporting materials, in accordance with the specifications that are pre-designed and stored in a digital model to generate a spatially defined viable 3D constructs. In contrast to other 3D models that take advantage of the intrinsic properties of biological materials as seen in organoids, or that use extrinsic physical properties to aggregate biomaterials as seen in hanging drops,

ultra-low attachment plates and microchambers, 3D printing precisely deposits building materials, biological or synthetic, in spatially pre-defined manners.

Compared to other *in vitro* 2D and 3D models, there are several advantages of 3D printing. First, extracellular matrix properties are controllable. With pre-designed models, cellular composition and density, matrix stiffness, the concentration and gradients of bioactive molecules can all be controlled and adjusted to study cellular behavior in microenvironment. Extracellular mechanics plays important roles in cancer cell behaviors. Polyethylene glycol-based photocrosslinked scaffold with tunable stiffness has been used to study cancer cell migration [33] while precisely controlled spatiotemporal gradients of bioactive molecules mimic the physiochemical microenvironment of cancer cells and allow the study of cancer cell behavior in desired manners [34].

Second, high-throughput fabrication of replicable cancer models. While other cancer models usually rely on the natural growth/expansion of cancer models and generate heterogenicity among individual models with the same origin, identical cancer models can be rapidly fabricated with 3D bioprinting which allows rapid drug screening and biological studies with high precision.

Third, perfusable vasculature can be fabricated. All three bioprinting modalities, extrusion-, droplet- and laser-based, have been explored to fabricate vasculature in cancer models [35]. Both scaffold-based and scaffold-free bioprinting have been used to integrate vasculature in the models [36]. With scaffold-based approach, cells are bioprinted in an exogenous biomaterial (i.e., hydrogel or decellularized matrix components) resembling the target tissue structure while cell assembly and fusion/re-modeling are the driving mechanisms of vasculature formation in the cancer models [37]. Furthermore, to study cancer cell behavior including cancer invasion into vasculature, various biological factors, such as different cells, extracellular matrix and growth factors, can be programmed into cancer models [38].

Even though 3D bioprinting has been used to establish multiple cancer models [39], its application in BC research is very limited. While several reports on 3D printing of a bladder have been published [27,40], 3D printing in BC research is yet to be explored.

3. Other 3D Culture systems

In addition to organoids, several other *in vitro* 3D cultures have also been studied and reported. Amaral et al. [41,42] reported a hanging drop method and floating method using ultra-low attachment (ULA) plates. Drug sensitivity of BC cells using these methods is comparable to that using patient-derive xenografts.

Pump-less microfluid chambers or microchambers have recently been developed as a cheaper, convenient and highly efficient method to culture primary BC cells. Unlike most other *in vitro* culture methods of primary cells which usually need special medium supplemented with growth factors and/or feeding cells, microchambers culture cells in a restricted miniscule physical space with the height of 75 μm which prevents the dilution/diffusion of auto- and paracrine growth factors and allows the culture of difficult-to-culture cells, such as hepatocytes and stem cells, without the need of special medium [43].

Hepatocytes can be maintained in these microchamber devices without perfusion for up to three weeks with minimal loss of phenotype or function. In BC, primary cancer cell microchamber culture was developed in all six specimens [44]. Drug sensitivity to single drugs and combinations was comparable between microchambers and patient-derived xenografts, suggesting microchambers can possibly supplement patient-derived xenografts in screening for effective drug candidates.

IN VIVO MODELS

1. *In vivo* tumors from cell lines

In vivo tumors derived from cell lines are probably the most used models in cancer research. It is easy to manipulate, cheap and fast. Almost all cell lines can be used to generate *in vivo* models in BC. If cell lines are of human and dog origins, they are implanted in immunocompromised animals, such as nude mice, SCID (severe combined immune deficient) mice or nonobese diabetic (NOD).Cg- Prkdc^{scid}IL2rg^{tm1Wjl}/Sz (null; NSG) mice. If cell lines are of mouse and rat origins, they can be implanted in immunocompetent syngeneic mice or rats which have been widely used for research in immunotherapy as well as other researches.

Cells are usually implanted subcutaneously as it is easily accessible for measurement and manipulation. However, BC is unique in that over 70% of cases at diagnosis are at the NMIBC stages and are treated locally including intravesical instillation. Hence, subcutaneous models may not be applicable for research in NMIBC. Furthermore, the microenvironment of BC at the bladder may be different from that at the subcutaneous space. Hence, orthotopic cancer models are needed for BC research.

There are several approaches to establish orthotopic BC models. The most intuitive approach is to instill bladder cancer cells through urethra directly into the bladder cavity. Because of the tight junction of urothelial cells, cancer cells rarely attach to and develop orthotopic cancer. To increase cancer cell attachment to the urothelial layer and engraftment, the bladder is usually treated with another agent, such as trypsin, poly-L-lysine, HCl or even electrocautery [45,46]. Because of the structure of the urethra and bladder in mice and rats, female animals are preferred for easier access. Another approach is to expose the bladder through a lower abdominal incision and directly inject BC cells into the bladder with a needle [47,48]. To minimize surgical trauma, a minimally invasive ultrasound-guided intramural inoculation was used to establish orthotopic models with high success [49].

As BC is a wide spectrum of cancer, ranging from low-grade non-invasive cancer, to high-grade, locally advanced and metastatic cancer, orthotopic BC models provide unique opportunities to study and monitor cancer progression. Huebner et al. established a BC in-situ model by using fluorescein labeled highly invasive BC cell line UM-UC-3, and tracked the migration and invasion of BC cells in real time with bioluminescence imaging (BLI), magnetic resonance imaging (MRI), positron emission tomography (PET) and other technologies [50]. Lorenzatti et al labeled human BC cells with a variety of fluorescein markers to observe the invasion of BC in orthotopic models [48]. Erman et al used nanoparticles, immuno-fluorescein, and electron micrograph labeling technologies to

observe the early stage BC, and clearly observed the interaction and adhesion between BC cells and normal urinary epithelial cells [51].

2. Carcinogen-induced model

BC develops from urothelial cells that have direct contact with carcinogens in urine. Hence, chemicals, either carcinogenic directly or indirectly through *in vivo* metabolism, have been widely used to induce BC. After the initial report of carcinogen-induced BC in rats [52], BC has been induced with carcinogens in several other species (reviewed in [53]). N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) is one the first and most commonly used carcinogens. Several other carcinogens have been identified and used, including amines(such as N-nitrosamine) [54,55], anthracenes (such as 20-methylcholanthrene [56], and formamide FANFT(N-[57]-formamide) [57]. Most of the carcinogens have aromatic amine components. They are genotoxic that induces DNA damage in bladder urothelial cells [58,59].

BBN is clinically relevant as it can be detected in tobacco smoke, environment and infectious metabolites [60], and BC is induced through chronic feeding/exposure of BBN, similar to chronic exposure of carcinogens in human patients. Pathologically, BBN exposure induces progressively pathological changes from hyperplasia, dysplasia, cancer *in situ,* invasive cancer as well as metastasis, similar to the BC progression in human patients [60].

Genomically, carcinogen-induced BC has similar genetic alterations found in human BC. *TP53, KMT2D* and *KDM6A* are the most common mutated genes in human BC [61]. Yamamoto et al. analyzed mutational events of BBN-induced BC and found that BBN caused mutations of the *p53* gene heterogeneity at the early stage, and clonal *p53* mutations, commonly with C →T to G→A mutations, at the late stage [62]. Fantini et al. showed that the most common mutations were *Trp53* (80%), *Kmt2d* (70%), and *Kmt2c* (90%), which were similar to the mutation spectrum of human BC [63].

Gene expression profiling analysis suggests that the gene expression spectrum of BBN induced BC is similar to that of the human basal subtype [63,64]. Pathways that are consistently affected in human BC and carcinogen-induced rodent BC include cell cycle regulation, apoptosis, angiogenesis and MYC [65]. In human, BC can be classified into several subtypes based on the gene expression profiles [66]. Comparing genes with corresponding homologs across the species of humans and mice, most BBN-induced BCs can be clustered into the basal-like tumors of The Cancer Genome Atlas (TCGA; n=408) [13].

In summary, carcinogens can consistently induce BC across species. Carcinogen-induced BC resembles that of human BC based on the mechanisms of carcinogenesis, pathological features, genetical alterations and gene expression profiles. Since these cancers develop in immunocompetent mice, they can be used to study BC immunotherapy in addition to other aspects of cancer research.

Genetically Engineered Mouse Models (GEMM)—In GEMMs, mice that carry a cloned oncogene(s) or are knocked out a tumor suppressor gene(s) allow the investigation of the effects of an individual gene(s) or gene combinations on oncogenesis. Mice with germ line knock-in or knock-out can be used to study how alterations of a specific gene(s) affect

cancer development of the whole body and this study may not be feasible if perturbation of the gene(s) causes premature death or embryonic lethality. In order to study the effects of a gene(s) on specific organs/tissues, a tissue specific promoter is used to drive the expression of the target gene. In BC GEMM, the most used promoter that drives the bladder-specific expression of a target gene is the mouse Uroplakin II (*UpkII*) promoter. Uroplakins are membrane integral proteins expressed in urothelial cells. Multiple oncogenes under the control of the *UpkII* promoter has been widely studied in BC oncogenesis, such as *SV40 T, RAS,* cyclin D1 *(CCND1),* fibroblast growth factor receptor *(FGFR)* and epidermal growth factor receptor *(EGFR).* For example, expression of SV40 T antigen under the control of the *UpkII* promoter leads to the development of cancer *in situ* with low copy numbers and invasive to metastatic transitional carcinoma with high copy numbers [67]. Expression of *H-Ras* leads to urothelial hyperplasia to low-grade papillary non-invasive BC [68]. Nevertheless, double transgenic mice carrying both *SV40 T* and *H-Ras* develop high-grade invasive urothelial carcinoma within one month and succumb to this disease around 7 weeks of age [69].

To further increase the flexibility of gene manipulation, such as temporal and spatial control of gene expression and introduction of mutation, the *Cre-LoxP* system is widely used in generating GEMM. Cre is a recombinase that acts on palindromic sequences called LoxP sites that have been genetically engineered at specific sites in the mouse genome. Hence, when the expression of Cre is under the control of a tissue-specific promoter or an inducible promoter, the *Cre-LoxP* system can control gene expression or introduce mutation in target tissues/cells or when an inducer molecule is introduced. More recently, instead of controlling *Cre* expression with a tissue-specific promoter, adenovirus expressing Cre recombinase is delivered directly into the bladder cavity to temporally and spatially control gene expression [70].

GEMM has been making tremendous contribution in BC research, especially in studying how perturbation of specific genes and gene combinations affects the oncogenesis of BC. The major drawback of GEMM is that it does not recapitulate highly complicated oncogenic process. Cancers developed in GEMME lack heterogenicity seen in clinical BC. Because of the unique location and exposure of urothelium to carcinogens and their metabolites in urine, BC oncogenesis is a highly complicated process with integrated genetic and epigenetic alterations that cannot be replicated in GEMM. In fact, BC has the third highest mutation rate among all cancer types after skin melanoma and lung cancers [71].

3. Patient-derived xenograft (PDX) models

The PDX technique originated in the 1980s. Through continuous improvement, the success rate of transplantation gradually increases and the establishment time has also shortened [72]. Compared to other models, PDXs can better retain the characteristic of BC cells and its microenvironment *in vivo,* and have been widely used in the BC mechanism study, drug screening etc. [73,74].

The establishment and characterization of BC PDXs have been published (Figure 2) [74]. Briefly, fresh clinical BC tissues from patients are collected and minced into 3–5 mm^3 fragments, then transplanted subcutaneously into 4–5 weeks old NOD.Cg-*Prkdc*^{scid}

Il2rg^{tm1Wjl}*/SzJ* (aka, NSG) mice. Cancer tissues can also be digested into cell suspension and implanted into the bladder wall to establish orthotopic models. Mice are then monitored for tumor growth. For the first PDX establishment (P0), it usually takes 2–6 months. It takes much shorter time for subsequent implantation. The engraftment rate for P0 PDXs is around 40%. To improve the engraftment rate, Jager et al [75] implanted human BC tissue under the renal capsule of immunocompromised mice. All seven tumor tissues developed PDXs, and six out of seven could be successfully expanded in more mice. After PDXs are established, immunohistochemical, molecular and genomic characterizations should be performed. We found that both subcutaneous and orthotopic PDXs retained the morphological fidelity of their parental patient cancers, and that PDXs retained 92–97% of genetic alterations of parental patient cancers [74]. Occasionally, instead of PDXs, lymphoid tissue may grow out and need to be ruled out before using PDXs for further research. Use of the anti-CD20 antibody rituximab has decreased the development of lymphoid implant.

In addition to direct development from patient cancer tissues, PDXs and other patient-derived models of cancer can be inconvertible. For example, PDXs can be developed from CRC, microchamber cultures, hanging drop cultures and organoids, and PDXs and their corresponding models had comparable response to the same drugs [21,41,42,44]. Lee et al. also showed that drug response in organoids was recapitulated with their corresponding *in vivo* organoid-derived orthotopic BC PDXs [31]. Furthermore, PDXs and their derived CRC cultures shared similar genetic alterations [21], suggesting some of the *in vitro* patient-derived cultures can possibly complement PDXs in BC research.

Because PDXs morphologically and genomically recapitulate patient cancers, there are many potential applications in translational research in BC. First, PDXs can potentially be used to screen and select effective chemotherapy. In BC, the GC (gemcitabine and cisplatin/carboplatin) regimen is one of the two first-line chemotherapy regimens. It is assumed that these two drugs contribute to anti-cancer activity. However, when their activity is analyzed individually in PDXs, in six out of eight PDXs, one drug contributes most of the anti-cancer activity while the other one has very little activity, and the remaining two PDXs are resistant to both drugs [74]. To study the mechanisms of chemoresistance, Wei et al. [76] found that cisplatin resistance is associated with alterations in genes including *MLH1, BRCA2* and *CASP8* in BC PDXs derived from BC patients who are resistant to chemotherapy. Moreover, these samples highly expressed *SLC7A11, TLE4,* and *IL1A.* Martin et al. established that the levels of methionine adenosyltransferase 1a *(MAT1A)* gradually increased during GC treatment and overexpression of *MAT1A* increased tolerance to gemcitabine [77]. Instead of studying individual genes, novel biomarkers of chemotherapy-induced DNA adduct levels for chemosensitivity have been translated from preclinical studies in PDXs into clinical trials [78–84]. For example, platinum drugs (cisplatin, carboplatin and oxaliplatin) kill cancer cells mainly through induction of platinum-induced DNA damage/adducts while gemcitabine kills cancer cells through incorporation into DNA and termination of DNA replication. Hence measurement of platinum/gemcitabine-DNA adduct levels can potentially predict chemoresistance. PDX studies showed that platinum/gemcitabine-DNA adduct levels indeed correlate with drug sensitivity to platinum/gemcitabine chemotherapy which is further supported by clinical trials.

PDXs can also be used to screen for effective molecularly targeted therapy (MTT). Based on the BC TCGA (The Cancer Genome Atlas Program) database, over 70% of BCs harbor actionable genetic alterations [7]. However, in many cancers, targeted therapies matching to the underlying genetic alterations have low response rates [85,86]. The major reason is that many of the genetic alterations are not molecular drivers and current computational biology cannot distinguish drivers from passenger mutations. Similar findings can be observed in BC. PDXs can potentially be used to screen for effective therapies [74]. Cirone et al [87] used PI3K inhibitor PF-04691502 and MEK inhibitor PD-0325901 to inhibit the growth of BC PDXs, and the efficiency was similar to that of cisplatin at the maximum clinical dose, providing an experimental basis for preclinical targeted drug testing. *FGFR3* and *EGFR* are commonly altered in BC [61]. Mahe et al. [88] treated BC PDXs with FGFR3 and PI3K inhibitors, and these inhibitors significantly prevented the growth and progression of the BC cells highly expressing *FGFR3.* Similarly, our previous research results showed that EGFR/HER2 dual inhibitor lapatinib was also effective in preventing the growth of BC PDXs. The combination of a PI3K/AKT inhibitor and MAPK/ERK inhibitor was still effective on BC PDXs even if they developed secondary resistance to an EGFR/HER2 inhibitor [74]. Furthermore, Serial biopsies with deep sequencing can be used to decipher resistance mechanisms. For example, it was found that downregulation of lymphocyte-specific protein 1 (*LSP1*) was associated with resistance to a PI3K small molecule blocker pictilisib [89].

Another potential use of PDXs is for drug development. Traditionally drug development uses cell lines and their derived tumor implants. It has been shown that even a few passages of *in vitro* culture leads to irreversible genetic alterations [16]. Hence, it is not surprising that only 11% of drugs entering Phase I clinical trials are eventually approved by the Food and Drug Administration and it was less than 10% for oncology drugs [90]. The unique advantage of PDXs is that they are directly derived from clinical patient specimens and retain the morphology and genomic fidelity of their parental patient cancers [74]. Hence PDXs are being increasingly studied for drug development. While extensive data on how PDXs affect drug development are still missing, several PDX co-clinical trials showed promising results (reviewed at [91,92]). For example, Stebbing et al. showed a strong correlation between PDX model responses and clinical outcomes (81%, 13/16) [93]. In BC, PDXs have been used in several studies of drug development which has reached the pre-IND/IND (Investigational New Drug) stage [47,94–99].

Even though the PDX platform holds tremendous promise in translational research and drug development, there are several shortcomings. Compared to some of the *in vitro* models, PDXs are costly, difficult to manipulate cancer cells *in vivo,* take a long time to develop (4–6 months), and have altered tumor microenvironment. Some of the supporting cells and stroma are retained during the establishment of PDXs, but replaced with mouse ones during subsequent passaging. Another important factor, especially at the immunotherapy era, is lack of human immune system.

HUMANIZED MODELS

Lack of competent immune system in the host animals makes the PDX platform unsuitable for study of immunotherapy, a cornerstone therapy for cancer at modern era. Several

approaches have been taken to overcome this drawback. One approach is to combine PDX studies with immunocompetent mouse models. For example, based on the TCGA database, approximately 80% of BCs have alterations along the cyclin-dependent kinase (CDK) 4/6 pathway which can possibly be targeted for the treatment of BC [100]. After accomplishment of the PDX studies, a CDK 4/6 inhibitor was tested in an immunocompetent syngeneic mouse model with a mouse BC cell line and showed the synergistic anti-tumor activity with immunotherapy targeting the programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway. Another approach is to generate transgenic mice expressing human immune-oncology target molecules, such as human PD-1, PD-L1, CTLA4, TIGIT, etc. Even though triple and even tetra humanized immune-checkpoint transgenic mice have been generated and these mice respond to immunotherapeutic interventions against human targets, these mice and their syngeneic tumors are mouse origin and the findings in these mice may not apply to human patients.

To better recapitulate human cancer immune response, truly humanized mice have been studied in which both the immune cells and cancer cells are derived from humans. The first generation of humanized mice is established via direct transfusion of human peripheral blood mononuclear cells (PBMCs) into immunodeficient mice [101,102]. This humanized mouse model recapitulates the anti-cancer immune response when anti-human PD-1 antibody nivolumab is administrated. However, robust human xenograft versus host disease (xGVHD) develops within a few weeks that prevents long-term studies. To address this issue, humanized mice using human CD34+ hematopoietic progenitor and stem cells (HPSCs) were developed [103]. In this project, HPSCs are infused into 3-wk-old female NSG mice four hours after 140 cGy total body radiation [104,105]. B lymphocytes usually develop around 9 weeks after HPSC infusion followed by T cells three weeks later. The unique advantage of humanized mice from HPSCs is that xGVHD is usually mild, and we have kept those mice for over one year.

After human T and B lymphocytes have developed in humanized mice, PDXs can then be implanted for immune-oncology research. It has been shown that anti-human PD1 antibody pembrolizumab elicited anti-tumor response in the humanized NSG mice carrying human BC PDXs and induced CD8 T cell infiltration into PDXs [105]. Different responses are observed in the same batch of humanized mice that were developed from the same CD34 HPSCs and PDXs, similar to the heterogenous response observed among metastatic cancers in the same patients.

Some of the major drawbacks of humanized mice is that it is expensive and takes a long time to develop, almost 20 weeks from the time of HPSC infusion to the time of study. Furthermore, it is difficult to have HLA matched HPSCs and PDXs unless both of them are obtained from the same patients. However, it seems that HLA match is not needed from the published study [105]. Another major concern is whether immune cells in humanized mice are fully functional as the immune cells in human patients. One major reason is that NSG mice may not have a fully functional thymus which is needed for proper T cell development.

CONCLUSION

There are many *in vitro* and *in vivo* BC models. Each one has its own unique features. Here we summarize the pros and cons of each model (Table 1). For example, traditional BC cell lines and their derived *in vivo* models are economical and easy to operate and manipulate, and remain the most commonly used models in BC research, but may differ dramatically from human cancers in clinic; carcinogen-induced models and GEMM have competent immune system for research in immunotherapy, but the findings at the mouse background may not be applicable to that in human patients; patient-derived organoids and PDXs recapitulate patient cancers better than other models, but lack of immune system means that they have limited applications for research in immunotherapy; humanized mice carrying PDXs have both human cancer and immune system, but it is not clear how much the immune system in those mice differ from that in human beings. In summary, there is no single model that fits all the research needs. Researchers need to take into account their research needs to select the appropriate model(s).

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KEY POINTS

- Cell lines and their derived in vivo models are readily available and easy to manipulate, but may behave differently from human bladder cancer in clinic
- Carcinogen-induced models recapitulate the nature oncogenesis of human cancer;
- Genetically engineered mouse models have more uniform and defined genetic alterations, but lack of tumor heterogeneity
- Patient-derived models of cancer (organoids, conditionally reprogrammed cell cultures and xenografts) retains most of the genetic alterations of their parent cancers and have high concordance of drug response
- Humanized mice have human immune system in mice and can be implanted with human cancers for research in immunotherapy

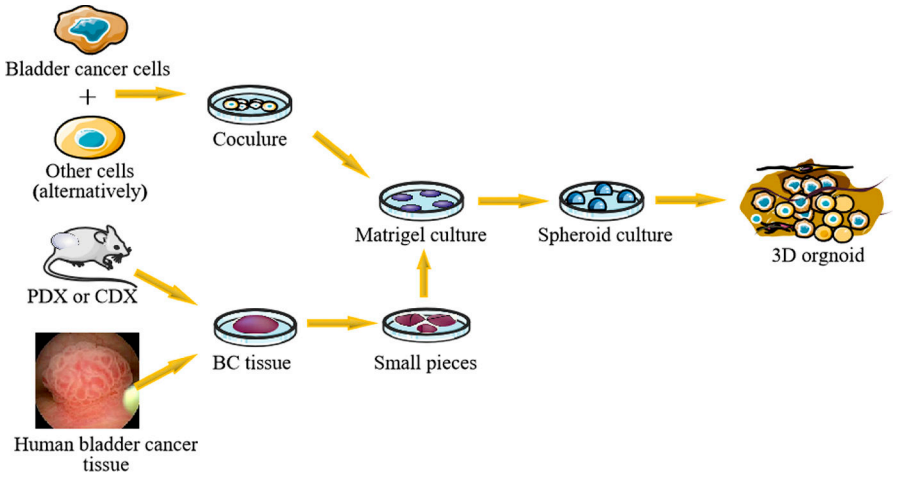

Figure 1. Organoid models:

Organoids can be developed from clinical cancer specimens, cell lines, or other models. After the initial pathology review and process (mincing and/or digestion), cells or tissues are seeded into culture dishes with supporting materials, such as Matrigel, which then grows in 3D structures and form spheroids. Organoids retain some of the 3D cancer structure and tumor microenvironment and recapitulate some of the cancer behaviors *in vivo.*

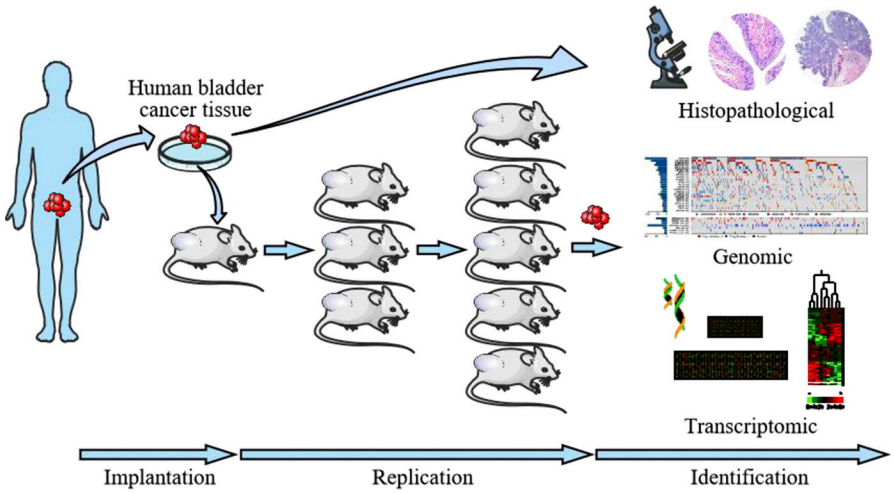

Figure 2. The establishment and identification of PDX.

BC tissues are collected from BC patients, and implanted into immune deficient mice to generate Passage 0 (P0) PDXs. After characterization and validation, P01 PDXs are re-implanted and expanded for cryopreservation and for research use.

Table 1

Preclinical systems to develop BC therapeutics

	Source	Advantage	Deficiency
In vitro 2D models	**Cell lines from human, mouse, rat and dog.**	• Easy to culture; • Economic; • Readily available; • Easy to manipulate; • Mouse and rat cell lines can be implanted into immunocompetent host to study immunotherapy; • The most widely used model in BC research.	• Different genetic and epigenetic compositions from those of human cancers because of long term *in vitro* culture; • Relatively pure cancer cell population • Lack of supporting cells and TME • Human cancer lines can only be implanted into immunocompromised mice and are not suitable to study immunotherapy
	Conditionally reprogrammed cell culture (CRC)	• Retention of genetic alterations of the parental cancer cells; • Capability to differentiate into the tissue that CRC is originally developed from; • High concordance of drug sensitivity with parental cancers; • Relatively high success rate in establishing CRC culture.	• Co-culture with feeding cells • Need to irradiate feed cells • Lack of 3D structure and tumor microenvironment • Lack of immune system
In vitro 3D models	**Organoids**	• 3D structure with supporting cells and TME • Similar genetic alterations as the parental cancer cells; • Relatively high concordance of drug sensitivity with parental cancers • Cheaper and easier than *in vivo* • models	• Lack of *in vivo* factors, such as blood circulation • Lack of dynamic immune system • Special medium with growth factor support
	3D printing	•3D structure with supporting cells and TME • Precise control of cancer cells, stromal cells and stroma • Rapid production of large numbers of tumor 3D printings	• Need for special 3D printer • Lack of *in vivo* factors • Difference of 3D structure in 3D printing compared to native cancers • Lack of dynamic immune system as in vivo
In vivo models	**Carcinogen induced model**	•Similar carcinogenesis process as human BC • Similar genetic alterations • Native cancer microenviroment • Competent immune system	•Time-consuming and expensive • Difficult to do large-scale tests • Random events • Unique cancer in each mouse
	GEMM	• Relative uniform cancers/mice • Generation of large identical mice • Native cancer microenviroment • Competent immune system	• Time-consuming and expensive • Lack of heterogenicity of cancer • Different carcinogenic mechanisms as human cancers
	PDX	• Closest replication of human cancers • Morphological and genetic fidelity of human cancers • High concordance of drug sensitivity with human cancers • 3D structure and TME	• Expensive, time-consuming • Lack of immune system • Difficulty to manipulate • Replacement with mouse stroma during establishment and passaging
	Humanized models	• Combination of human immune system with human cancer implants • Capability to study immunotherapy and other therapies	• Complicated, expensive and time-consuming • Possibly partially defective immune system with lack of fully functional thymus • Difficulty to obtain HLA-matched immune system and PDXs. • xGVHD