Editorial Commentary

Establishing and characterization of human and murine bladder cancer organoids

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Provenance: This is an invited article commissioned by Section Editor Xiao Li (Department of Urology, Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research & Nanjing Medical University Affiliated Cancer Hospital, Nanjing, China).

Comment on: Mullenders J, de Jongh E, Brousali A, *et al.* Mouse and human urothelial cancer organoids: A tool for bladder cancer research. Proc Natl Acad Sci U S A 2019. [Epub ahead of print].

Submitted May 17, 2019. Accepted for publication Jun 06, 2019.
doi: 10.21037/tau.2019.06.05
View this article at: http://dx.doi.org/10.21037/tau.2019.06.05

Bladder cancer is among the most common malignancies in the United States (4th for men and 11th for women) and worldwide (6th for men and 16th for women) (1,2). Bladder cancer is a heterogeneous disease with a large number of mutations in oncogenes and tumor suppressors (3). Bladder cancer is divided into two major categories with distinct treatment strategies, non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Tumors categorized as NMIBC include carcinoma *in situ* (CIS), papillary (Ta), and invasive into or beyond the lamina propria (T1). Once the tumor invades the muscularis propria (T2) or beyond (T3 and T4), it is considered MIBC. Patients with NMIBC have a relatively good prognosis, and are treated by transurethral resection of the bladder tumor (TURBT) and subsequent intravesical chemotherapy (Mitomycin C) or repeated courses of immunotherapy [bacillus Calmette-Guérin (BCG)] followed by life-long surveillance with cystoscopy and imaging. However, despite initial response, almost 50% of patients with NMIBC will progress to MIBC (4). For MIBC, the predominant treatment option is radical cystectomy with or without neoadjuvant chemotherapy (NAC) followed by chemo-radiation, or bladder sparing chemo-radiation (4). Patients with MIBC who fail first-line chemotherapy and radiation (either post-cystectomy or bladder-sparing therapies) have limited treatment options and poor prognosis. To date, there is no procedure or biomarker to stratify patients for any given therapeutic regimen or to predict therapeutic response (5). Limited advances in treatment of bladder cancer have been achieved in the last 30 years mainly because of the absence of model systems to study the normal and malignant bladder epithelium (urothelium) and faithfully recapitulate the biology of the normal and malignant urothelium and complexity of the disease. The urothelium is a specialized multi-layer epithelial lining that not only of the urinary bladder wall but also extends into the renal pelvis, ureters, as well as the urethra. The urothelium provides an impenetrable barrier for urine, and under physiological conditions, the urothelium exhibits slow proliferation and renewal relative to other tissues (6). The molecular signals that regulate renewal of the urothelium under physiological conditions are incompletely understood (7).

The current model systems to study bladder cancer include a number of established bladder cancer cell lines that are widely used. However, despite their widespread use, they do not recapitulate the natural history bladder cancer and have to be adapted in multiple models to investigate different aspects of the disease (8). Although cultures of primary mouse and human bladder cells were reported, their wide use is limited, due to their short lifespan (9,10). Genetic mouse models and orthotopic xenografts for bladder cancer have been created and they faithfully represent the clinical manifestation of the human bladder cancer, but are time-consuming and expensive to establish

and maintain (8,11).

Recently, three-dimensional (3D) cultures of primary bladder cancer cells have been increasingly reported using various culture techniques, media and supplements that can be passaged multiple times, massively expanded and characterized with respect to resemblance to the primary tumors and response to therapy (11-13). In this respect, Mullenders and colleagues (14) reported a novel approach to generate and maintain human and murine normal and malignant urothelial cells that exhibit the characteristics of basal and umbrella cells and can be passaged at reasonable time. To standardize the culture conditions and mitigate the impact of technical variabilities, they screened several culture media conditions, and included growth factors and inhibitors known to influence urothelium culture (7,14). They developed a defined bladder human and murine organoid media for normal and cancerous urothelia that is completely defined and devoid of any animal products to create a living biobank containing organoids grown from over 50 patient samples representing different stages of bladder cancer. The investigators followed a systematic comprehensive approach to characterize the organoids at the phenotypic, genetic, and molecular levels to confirm that the organoids closely resemble the tumor histology and heterogeneity, and can be maintained for a long time. Importantly, these organoids be used for drug screening and provide a platform for development of new drugs for the treatment of bladder cancer and personalized medicine.

Establishment and characterization of primary murine bladder organoids

Primary murine bladder organoids were established from digested bladders. Components of the culture media were optimized based on previous reports on primary bladder urothelium and published mouse organoid cultures (15-18). Murine bladder organoids appeared as dense structures of fast proliferating cells with a grape-like morphology and could be passaged on a weekly basis and propagated for prolonged periods (>2 year) with no gross chromosomal abnormalities as determined by karyotyping. Murine organoids were devoid of keratin 20 ($Ck20^+$) or uroplakin ($UpkIII^+$) positive cells at both protein and transcript levels. Confocal microscopy on whole-mount organoids confirmed the presence of $Ck5^+$ basal urothelial cells hence they were termed "basal bladder organoids". Most cells in mouse bladder organoids were positive for CD44, suggestive of stemness of the basal bladder organoids.

Interestingly, the organoids derived from entire dissociated bladders lacked suprabasal and umbrella cells. Organoids containing all cell types of the urothelium, were derived using two alternative methods using the same unique media used for bladder organoids. Urothelial cells were isolated from the urothelium of the ureter or by filling the bladder *ex vivo* with trypsin to enrich the extracted cells for suprabasal umbrella cells that were lacking from the basal organoid cultures. Both ureter and suprabasal mouse urothelial organoids not only were similar morphologically, but they could also be established efficiently and passaged frequently (every 2 weeks). Strikingly, these organoids contained uroplakin positive cells, indicating the presence of intermediate or umbrella cells, as well as keratin 5-positive cells indicating that these organoids contain both basal and suprabasal cells. Similar to basal organoids, the basal marker (Ck5) was observed predominantly on the outside of organoids, while CD44 appeared to have a broader pattern of staining. Transcriptional profiling of ureter and basal organoids by RNA sequencing revealed that ~1,200 genes were significantly differentially expressed when comparing organoids and primary urothelium, with overexpression of several basal markers (Keratins 4, 5, 6a, and 14) and absence of luminal cell markers (Krt20 and uroplakins) in basal bladder organoids.

Investigators then provided a proof of concept that primary murine organoids can be genetically manipulated for long-term cultures and to study the early events of transformation after genetic editing to create basal cells ($Ck5^+$) knocked out of the established tumor suppressor (Trp53) and one recently identified tumor suppressor in urothelial carcinomas (Stag2) using the CRISPR/Cas9 technology (19-21). Plasmids containing Cas9 and gRNAs for Tp53 and Stag2 were transfected into bladder organoids made into single-cell suspensions. Next, *Tp53*-inactivated cells were selected by adding an MDM2 inhibitor (Nutlin) to the culture media. Single organoids were picked and expanded, and the targeted genomic locus was confirmed by sequencing for mutation of both alleles of the *Tp53* and *Stag2* genes.

Establishment of an organoid biobank of human urothelial carcinoma

The investigators created an *in vitro* organoid-based culture system of urothelial carcinoma from the bladder tumor as well as macroscopically normal urothelium from the same patient undergoing radical cystectomy. Tissue pieces were

prepared into a cell suspension and grown as organoids in optimized culture media with a growth factor mix very similar to the mouse bladder culture media. Interestingly, several fibroblast growth factors (FGFs) namely FGF7 and FGF10 stimulated the proliferation of human bladder cancer organoids and were sufficient for their growth. Human urothelial organoids cultures could be established with around 50% efficiency and propagated for extended periods (>1 year). In addition, biopsies from TURBT procedures were used to establish organoid cultures that represent NMIBC. The investigators prospectively collected samples from 53 bladder cancer patients (42 cystectomies and 11 TURs) and established a biobank of 133 organoid cultures from the 53 patient samples. In most cystectomy cases, they started cultures from both normal and tumor tissue, and, in the case of large tumors, they established several lines of different parts of the tumor; and managed to culture several organoid lines for more than 30 passages.

Morphologically, cultured bladder cancer organoids, exhibited distinct morphological differences between samples derived from different patients. Immunophenotypic characterization revealed that all organoids contained active cycling cells as measured by Ki67 staining. Basal ($CK5^+$) cells were present in most organoid lines. TP63 staining showed that basal and intermediate cells were present in all organoids tested. Some of the organoids expressed markers of the luminal ($CK20^+$) and basal ($CK5^+$) subtypes of bladder cancer, respectively (22), with only one of the two organoids developed from same patient contained $CK20^+$ cells, and abundantly expressed $CD44^+$, a marker for urothelial tumor-initiating cells (23), thus representing tumor heterogeneity. Interestingly, immunofluorescence showed that luminal and basal markers are mutually exclusive that $CK5^+$ cells are never $CK20^+$, and *vice versa*.

To identify organoid lines that harbored *TP53* mutation (24), the MDM2 inhibitor Nutlin-3 was added to the culture media to select for cells with p53 mutation. Several organoids showed unimpeded growth in the presence of Nutlin-3, leading to the conclusion that these organoid lines have inactivated their p53 response. Similarly, the investigators screened human bladder organoids that with FGFR3 mutation (24) by culturing organoids in growth factors-deprived media, and selected organoid lines that managed to keep proliferating in the absence of growth factors. Targeted sequencing of the most frequent site of mutation in FGFR3 and confirmed that this mutation was indeed present in the growth factor-independent organoid lines. Investigators then assessed the gross genomic abnormalities in the bladder cancer organoids, karyotyping that revealed that 10 out of 11 organoid lines showed an abnormal number of chromosomes, confirming that these lines are of cancerous origin. Next, the investigators determined the expression levels of both basal (KRT5 and KRT6) and luminal (KRT20, UPK1A, and UPK3A) markers in human organoids, and identified several distinct basal and luminal subtypes in some organoids, whereas other organoids did not clearly identify with one subtype *vs.* the other.

Finally, the investigators exposed three random organoid lines from the human bladder cancer biobank to various concentrations of first-line chemotherapeutic agents for 5 days. They found that different organoids exhibited unique response patterns to the drug treatments. These experiments further proved that human bladder cancer organoids can be employed to determine response to anticancer drugs with potential to be used for screening of novel drugs and predicting the response of tumors to treatment options for personalized therapy.

Acknowledgments

This work was supported by R01 CA193437 to N.S.

Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Cite this article as: Said N. Establishing and characterization of human and murine bladder cancer organoids. Transl Androl Urol 2019;8(Suppl 3):S310-S313. doi: 10.21037/tau.2019.06.05

