



Organoid culture of bladder cancer cells

An organoid is a miniature three-dimensional (3D), multicellular, “stem-cell derived genetically-encoded self-assembly programmed structure” which exhibits characteristics, e.g., cell-cell interactions, tissue polarity, hypoxia, drug penetration, and nutrition gradients, that recapitulate the *in vivo* state of the original tissue better than cell lines that are maintained in two-dimensional (2D) adherent cultures. Because precursor stem cells needed to form organoids can be edited to express desired genetic variants or gene mutations or can be derived from complex genetic backgrounds, organoids become powerful tools to model diseases-from Zika virus infection and single gene disorders like cystic fibrosis to complex conditions like cancer. Patient-derived organoids (PDOs) have recently emerged as robust preclinical models for their abilities to recapitulate patient drug responses in the clinic, and they might therefore be integrated into precision medicine programs. PDO cultures have been established for many types of cancer, including colorectal, prostate, breast, pancreas, lung, and liver cancers. As for urothelial cancer, our group succeeded in preparing PDOs from surgically resected human bladder cancer tissue by applying the cancer-tissue originated spheroid (CTOS) method [1]. In this editorial, we introduce our recent findings and those of others using bladder cancer organoids and discuss future applications of them for discovery and precision medicine.

ORGANOIDS RETAIN CHARACTERISTICS OF THEIR PARENT TUMORS

The CTOS method can yield organoids efficiently from human bladder cancers by maintaining minimal cell-cell contact throughout tissue dissociation [1]. Organoids prepared by the CTOS method retain similar expression profiles of Ki67, uroplakin III, and p63 to those of the original parent tumors. We also used the CTOS method to generate organoids from N-methyl-N-nitrosourea (MNU)-induced bladder tumors developed in rats [2]; one of the

attractive features of MNU is that it acts directly on the bladder urothelium without a requirement for metabolic activation. After 4 rounds of local instillation of MNU into the bladder, rats developed bladder tumors that progressed over time from dysplasia, carcinoma *in situ*, non-muscle invasive tumors, and ultimately muscle-invasive tumors. The MNU-induced bladder tumors protruded into bladder lumen and exhibited papillary histological feature. When MNU-induced bladder tumors were partially digested and cultured *ex vivo* based on the CTOS method, organoids composed of highly purified tumor cells were successfully obtained. Quantitative real-time polymerase chain reaction analysis and immunohistochemistry revealed that luminal and basal markers were generally maintained in the organoids in patterns that were comparable to those observed in the original tumors. We also established a cell line from these organoids that could be maintained in adherent culture and named it T-MNU-1. When T-MNU-1 was compared to the original organoids in terms of its expression of differentiation markers, luminal markers (uroplakin Ia, FOXA1, and PPAR γ) were generally downregulated and basal markers (CK5 and CK14) were markedly upregulated, suggesting that T-MNU-1 became more “basal” in tissue culture. These findings reinforce the conclusion that organoids preserve the differentiation state of the parent tumor better than cell lines in 2D culture. Organoids may be particularly important for modeling luminal bladder cancer subtypes.

HEREGULIN AND CHIR99021 PROMOTE THE GROWTH OF BLADDER CANCER ORGANIDS

Supplemental components like growth factors are critical for the propagation of organoids in culture. In our characterization of bladder cancer PDOs, we observed that heregulin promoted their growth more efficiently than the other growth factors we tested [1]. Heregulin is a ligand

for human epidermal growth factor receptors 3 (HER3) and 4, which are receptor tyrosine kinases that transduce mitogenic signals. Heregulin induced phosphorylation of HER3 and its downstream molecules Akt and S6 in the bladder cancer organoids, while LY294002, a PI3K inhibitor, and RAD001, an mTOR inhibitor, suppressed heregulin-induced growth of the organoids and inhibited heregulin-induced phosphorylation of S6 [1]. Consistent with the central importance of the AKT pathway, we also recently observed that CHIR99021, a GSK3 inhibitor, enhanced the growth of bladder cancer PDOs [3]. CHIR99021 stimulates the canonical Wnt pathway by inhibiting GSK3 and subsequent phosphorylation and degradation of β -catenin. Consequently, we observed translocation of β -catenin into nuclei and upregulation of AXIN2 expression when bladder cancer organoids were incubated with CHIR99021 as compared to DMSO controls, indicating that CHIR99021 caused activation of canonical Wnt signaling. Notably, there was remarkable variability among patient samples with regard to the growth responses induced by both heregulin and CHIR99021, demonstrating the presence of inter-tumor heterogeneity. In aggregate, heregulin/HER3 signaling and the Wnt/ β -catenin pathway play important roles in promoting the proliferation of primary bladder cancer cells in organoid culture, but their relative impact appears to vary among tumors.

FUTURE PERSPECTIVES

Methods for preparing and culturing bladder cancer PDOs need further optimization. The CTOS method is not currently applicable to non-papillary bladder tumors, which make up most of advanced bladder cancer. Success rates for preparing organoids from non-papillary bladder tumors were less than 30% using the CTOS method as compared to ~80% success with papillary tumors [4]. The low success rate is probably related to the lower levels of homotypic adhesion in non-papillary bladder tumors, since maintaining cell-cell contact is crucial to the success of the CTOS method. Recently, another group used a different method to generate bladder cancer PDO lines from 20 individual patient tumors with a success rate of 70% [5]. Notably, however, 17 of the 20 PDO lines were derived from either papillary or non-muscle invasive tumors. The group also used genetic, transcriptome, and immunohistochemical analyses to compare the molecular characteristics of the PDOs and their parental tumors, which revealed that 64% of the organoid lines switched molecular subtype from that of their parental tumors, and effects that were reversed again when the organoids were used to propagate orthotopic tumors in immunodeficient

mice [5]. Therefore, further effort is required to identify strategies to maintain levels of differentiation in the PDOs *ex vivo*. Interestingly, most phenotypic changes observed in organoid culture were associated with a transition towards a more basal phenotype. Now that the field of bladder cancer-derived organoids is growing [6], additional protocols for preparation and culture will be introduced, and we can expect rapid improvement of the methodologies in the near future.

The unique characteristics of the 3D structure of organoids that are not present in conventional 2D cell line cultures can provide the context to unravel new aspects of cancer cell biology. For example, there is growing evidence that metastasis requires the collective invasion of tumor cells traveling together as clusters (“collective invasion”) as opposed to invasion and migration as single cells. Organoids have been used as excellent models to study collective invasion [7,8].

There is also strong enthusiasm for using PDOs to refine predictions of drug sensitivity and resistance in cancer patients. Despite recent technological advances that have produced promising predictive biomarkers, DNA and RNA analyses of patient tumors cannot yet predict drug sensitivities with absolute certainty. Genomic measurements are static and do not take adaptive mechanisms into account. In contrast, PDOs provide a means of monitoring dynamic responses of live tumor cells to therapeutic agents. However, even PDOs possess several limitations that need to be addressed before their full potential for informing precision medicine can be realized. First, PDOs lose the stromal cells that were present in their parental tumors, and there is a large body of work demonstrating that analyzing intrinsic drug sensitivities of tumor cells in isolation does not take these important microenvironmental signals into account. Second, PDOs are generated using preselected exogenous supplements, and we need to be aware of the effects these supplements might have in changing the molecular characteristics of the parental tumor. As introduced above, several supplements are suitable for supporting the growth and maintenance of PDOs, and it will be important to be aware of the potential effects of these additives on tumor drug responses as we optimize culture media so as not to overlook effective drugs. Lastly, our ability to exploit PDOs as barometers of drug sensitivity assay for precision medicine requires prospective validation in clinical trials, even though positive correlations between clinical response in patients and drug sensitivity assay using PDOs were reported in retrospective case series as a part of methodology papers. A landmark study about drug sensitivity assay using

PDOs was recently described [9], in which Vlachogiannis et al. [9] prepared PDOs from colorectal and gastroesophageal cancer patients who were enrolled in phase I/II clinical trials, and compared the drug responses of the PDOs to those of the actual patient tumors they were derived from. They were able to evaluate the predictive value for the PDOs for molecular-targeted agents or chemotherapy in 21 cases, finding 100% sensitivity, 93% specificity, 88% positive predictive value, and 100% negative predictive value in predicting response to therapeutic drugs in patients. The results were remarkably promising, warranting further clinical trials to validate decision-making process using PDO sensitivity assay to select appropriate treatment drugs.

In conclusion, organoids have become standard tools for investigating cancer biology, complementing conventional cancer cell lines and animal models. Organoids carry an excellent potential to augment existing approaches for defining cancer vulnerabilities and improving treatment responses. Protocols for preparing bladder cancer PDOs still need to be refined for more efficient preparation and effective maintenance of characteristics of the original tumors. As clinical trials evaluate the predictive value of DNA- and RNA-based genomic testing, the possible utility of incorporating PDO drug sensitivity assays into precision medicine platforms should be evaluated in prospective clinical trials.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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