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Review

Prostate organoid technology – the new POT of gold in prostate stem cell and cancer research

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Abstract: Organoids are self-organized cellular clusters in three-dimensional culture, which can be derived from a single stem cell, progenitor or cell clusters of different lineages resembling *in vivo* tissue architecture of an organ. In the recent years, organoids technology has contributed to the revolutionary changes in stem cell and cancer fields. In this review, we have briefly overviewed the emerging landscape of prostate organoid technology (POT) in prostate research. In addition, we have also summarized the potential application of POT in the understanding of prostate stem cell and cancer biology and the discovery of novel therapeutic strategies for prostate cancer. Lastly, we have critically discussed key challenges that lie in the current state of POT and provided a future perspective on the second-generation of POT, which should better recapitulate cellular behaviors and drug responses of prostate cancer patients.

Key words: organoids; three-dimensional cell culture; tumor model; prostate stem cells or progenitors; prostate cancer

前列腺类器官技术(POT)——前列腺干细胞和癌症研究中的“新金桶”

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摘 要：类器官是由单个干细胞、祖细胞或不同谱系的细胞簇在三维培养条件下自发形成类似体内组织结构的细胞簇。近年来，类器官技术为干细胞和癌症领域带来了革命性的变化。在这篇综述中，我们简要地概述了前列腺类器官技术(prostate organoid technology, POT)在前列腺研究中的进展。此外，我们也总结了POT在探究前列腺干细胞和癌症生物学以及探索前列腺癌治疗策略方面的潜在应用。最后，我们就当前POT在应用过程中的技术短板进行了讨论，并为第二代POT在研究前列腺癌细胞行为和患者对药物反应中的用途提供了全新的视角。

关键词：类器官；三维细胞培养；肿瘤模型；前列腺干细胞或祖细胞；前列腺癌
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Designing an *ex-vivo* model that can recapitulate human prostate has been of interest to the scientific community to understand prostate biology. An early study from the Wilson Laboratory has discovered the ability of sponge cells to undergo re-aggregation upon tissue dissociation [1]. This observation has subsequently driven the development of three-dimensional (3D) culture using primary tissues. Notably, the Brugge's group has elegantly demonstrated the use of 3D culture to study how oncogenic induction could affect morphological changes in mammary epithelium [2]. Using the similar strategy, the Witte Laboratory has successfully established spherical structure from mouse prostate epithelial cells cultured in Matrigel matrices [3]. However, most 3D culture models established at that time did not possess long-term propagating ability under *in vitro* conditions. In 2009, the Clevers's group invented a novel method that enabled the growth of intestinal crypt-villus organoids from Lgr5-expressing stem cells [4]. In particular, the resulting Lgr5 stem cells-initiating intestinal organoids possessed long-term *in vitro* propagating ability under the well-defined epidermal growth factor (EGF), Noggin, R-spondin 1 (termed ENR hereafter) culture condition [4]. After the initial publication from the Clevers's group, subsequent studies from the same group and others including us have shown organoid-forming ability in various epithelial tissues and cancer types, including liver [5], stomach [6], pancreas [7, 8], colon [9, 10] and prostate [11–13]. Although older publications typically equate an "organoid" to organelle in the cells [14] or a tumor with complex tissue-like structure [15], the accuracy of these definitions is now in doubt. More recently, the term "organoid" is used to refer to a resemblance of a 3D structure that contains more than one type of cell lineages, recapitulates specific organ functions and is capable of self-organizing into organ-mimic structure [16].

Prior to the establishment of prostate organoid technology (POT), most preclinical cancer models used in prostate cancer research were cell lines derived from human prostate tumors or metastases [17, 18]. Notably, most prostate cancer cell lines do not express intact and functional androgen receptor (AR) protein, making them non-ideal models to study androgen and AR signalling in prostate cancer [17, 18]. To overcome such shortcoming, the Sawyers's group has inserted functioning AR into LNCaP [19], which is a prostate cancer cell line that expresses endogenous AR with mutated ligand-binding domain [20]. Using the established LNCaP/AR cell line, the Sawyers's group has subsequently identified Enzalutamide as a novel second-generation antiandrogen for castration-resistant prostate cancer (CRPC) with elevated AR expression [19]. Since most prostate cancer cell lines were derived from sub-population of a tumor, the resulting cell lines are typically clonal and relatively homogenous [21]. Moreover, continuous passaging prostate cancer cell lines on monolayer cell culture condition can exacerbate sub-clonal selection process, thus further hampering the use of these model systems to study prostate cancer heterogeneity [21]. Nevertheless, prostate cancer cell lines are by far the most popular choice for preclinical prostate cancer research, particularly in signalling pathway investigation due to its great affordability and minimal technical requirements.

In view of the limitations of prostate cancer cell lines in recapitulating structural complexity of human prostate tumor, various animal models have emerged as an alternative option for the study of prostate cancer etiology, prevention and treatment [22, 23]. In particular, the major types of *in vivo* models of prostate cancer include patient-derived xenografts (PDX) and genetically-engineered mouse (GEM) models of prostate cancer [22, 23]. In recent years, various PDX models were established from tumor specimens of patients that encompass a spectrum of different pathologies and molecular characteristics [24–27]. However, some major disadvantages of PDX models include relatively low establishment rate, being time consuming and laborious and the high cost involved for their continuous maintenance [24–27]. Moreover, PDX models are grown and propagated in immunodeficient mice, making them an imperfect system to study cellular interaction between tumor and immune compartments.

In comparison, many GEM models of prostate cancer are generated through gene manipulation of the mouse genome [22, 23, 28–30]. In particular, introduction of various oncogenes or perturbation in tumor suppressor genes can be achieved through pronuclear injection or embryonic cell-based gene targeting [31, 32]. Notably, GEM models of prostate cancer have significant advantages over PDX models because they reflect tumor progression over time in an intact immune system environment [22, 23]. Moreover, incorporation of chemical-inducible *Cre* recombinase in gene targeting strategy has enabled the initiation of prostate carcinogenesis in GEM models at desired time points [28–30]. Although generation of GEM model using conventional approaches can be very time

consuming, the inclusion of CRISPR/Cas9 gene editing approach has greatly reduced the experimental duration [33]. More recently, the Lowe Laboratory has used prostate-targeted electroporation approach to introduce relevant genetic alterations in prostate cancer patients into the mice [34]. Using this approach, tumors were generated within a shorter period of time, while the resulting tumors recapitulated phenotypic features of traditional germline models [34]. In addition, the Greenberg's group has successfully derived cancer cell lines from the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which could form tumors upon injection back into the C57BL/6 hosts [35]. Such "GEM-cell lines-syngeneic model establishment" strategy could be potentially utilized to assess the function of genes of interest in prostate cancer progression in an immune-intact host. Together, these results have implied that the time required for the generation of GEM models of prostate cancer can be greatly shortened with the advancement of more cutting-edge gene editing approaches. Although various GEM mouse models may provide some insights into the mechanisms of prostate cancer initiation and progression, translation of the research findings from mouse to human remains a great challenge for prostate cancer research community as mouse and human prostate are known to be anatomically and structurally different [36]. Nevertheless, the recently established single-cell atlas for mouse prostate epithelium has greatly improved our understanding on prostate biology [37], and may potentially lead to better development of therapeutic agents for prostate cancer using the data arisen from mouse studies.

In this review, we provide an overview of the emerging landscape of POT as one of the newest tumor models for prostate cancer research in recent years. In addition, we also review the current state of POT, particularly on its application for the study of prostate biology as well as tumor initiation and progression. Lastly, we discuss on various key challenging issues when using POT and provide our vision for the next-generation prostate organoid models, which should better recapitulate the *de novo* cellular dynamic interactions and preserve key molecular characteristics of the cells in the prostate.

1 The emerging landscape of POT

Prior to the development of POT, prostate research was hindered by the lack of a robust *in vitro* model system that could preserve the *de novo* lineage hierarchy, as well as tumor heterogeneity. Although publication on organoids was dated back as early as 1946 [38], the first publication on POT only emerged in 1965 [39], as shown by the search term "Prostate Organoid" in PubMed (Fig. 1). The success rate of organoid establishment from prostate tumors was much lower compared to breast, colorectal or pancreatic tumors, again highlighting the difficulty of establishing prostate cancer cell lines [40]. This observation was also in line with the relatively low number of publications with the "Prostate Organoid" as a search term in the PubMed (Fig. 1). Although POT-related publications are far from dominating the overall development of organoid research, there has been a tremendous increase in the number of POT-related publications since 2014. Notably, three initial studies have independently demonstrated the capability of long-term maintenance and propagation of prostate epithelial progenitors and cancer cells in culture as organoids [11–13]. In particular, the Clevers's group modified the ENR organoid protocol to culture normal prostate epithelial progenitors, which were capable of producing both basal and luminal progeny [11]. In collaboration with Sawyers and Chen's Laboratories, they extended the use of this protocol for prostate tumor organoid derivation from patients' specimens [12]. During the same period, we developed a different POT protocol that favored the growth of prostate luminal progenitors, luminal and cancer cells (hereafter termed Luminal-Favoring or LF condition) [13]. Detailed formulation of the organoids culture media (Table 1) as well as the experimental procedures for the POT protocols were published following the initial reports to facilitate researchers venturing into the POT [41, 42].

There are several differences between the ENR and LF protocols. In particular, the ENR condition requires the use of advanced DMEM/F12 as the basal medium, together with three major components, including EGF, Noggin and R-spondin 1. For human prostate organoid culture, additional growth factors, small molecules and supplements are added (detailed in Table 1) [11, 12, 41]. Under the ENR-based POT, cells or cell clusters were embedded in high concentration growth factor-reduced Matrigel and the solidified "embedded buttons" carrying the cells are then covered with complete medium for continuous culture [11, 12, 41]. In comparison, the LF protocol was developed based in part on the importance of hepatocyte medium for prostate epithelial cell cultures [43], together with the supplementation of EGF and heat-inactivated charcoal-stripped fetal bovine serum

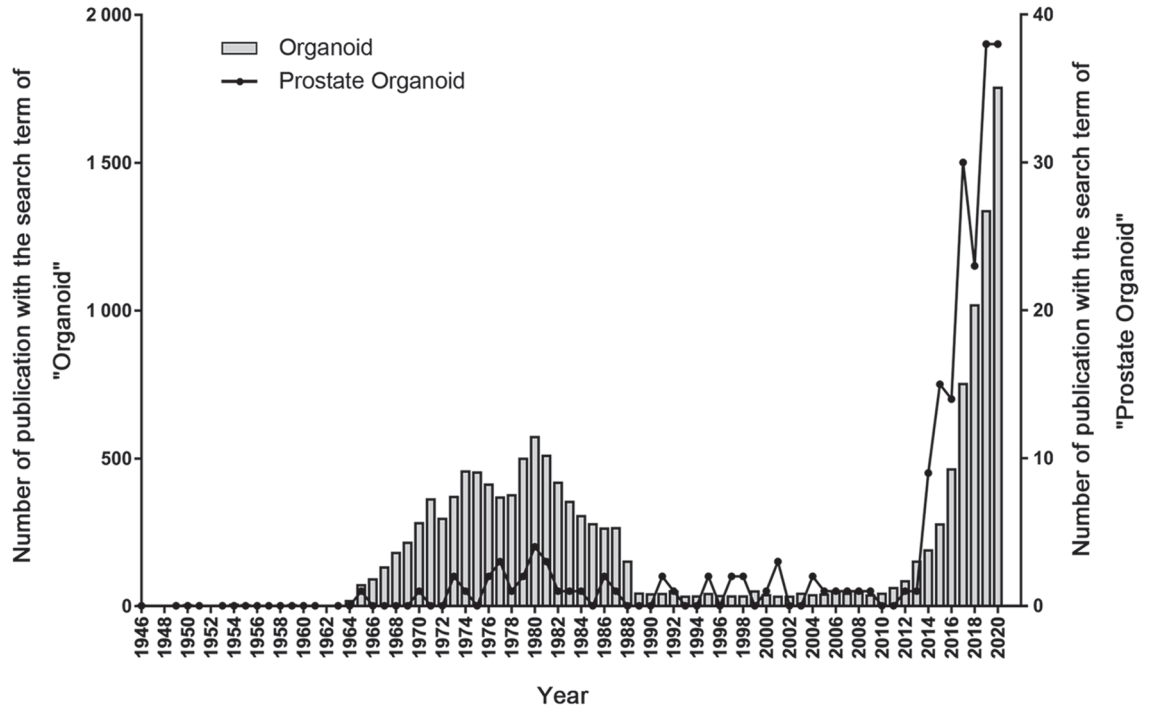

Fig. 1. Comparison of the number of publications between the search terms of "Organoid" and "Prostate Organoid" in the PubMed. The graph indicates the number of articles with the search terms of "Prostate Organoid" (black line, right Y-axis) and "Organoid" (grey bar, left Y-axis) since 1946.

Table 1. Comparison of customised culture conditions for prostate organoids

	LF condition [42]	ENR condition [41]
Type of organoid	Mouse	Mouse, Human
Culture medium	Hepatocyte medium	Advanced DMEM/F12
Glutamine	GlutaMAX™	GlutaMAX™
Growth factor	EGF	EGF
		FGF10 (Human organoid only)
		FGF2 (Human organoid only)
		Prostaglandin E2 (Human organoid only)
Dihydrotestosterone	Yes	Yes
ROCK inhibitor	Y-27632	Y-27632
Extracellular matrix	Matrigel	Matrigel
R-spondin-1	No	In conditioned media
Noggin	No	In conditioned media
TGF-β inhibitor	No	A83-01
Antioxidant	No	B-27™
		N-acetyl-*L*-cysteine
Antibiotic	Optional	Penicillin
Antimycotic	Optional	Streptomycin
Serum	Heat-inactivated charcoal-stripped FBS	No
p38 kinase inhibitor	No	SB202190 (Human organoid only)
Nicotinamide	No	Yes (Human organoid only)

DMEM/F12: Dulbecco's modified eagle medium/Ham's F12; EGF: Epidermal growth factor; FBS: Fetal bovine serum; FGF: Fibroblast growth factor; ROCK: Rho-associated protein kinase; TGF-β: Transforming growth factor-β. LF: Luminal-Favoring; ENR: EGF, Noggin, R-spondin 1.

(FBS) [13, 42]. Under LF protocol, cells were mixed in a complete medium containing 5% Matrigel, in which organoids were formed in a "floating" condition [13, 42], similar to the previous report on 3D culture of mammary epithelium [44]. Notably, both conditions have included Rho-associated protein kinase (ROCK) inhibitor, which could promote cell survival through re-expression of E-cadherin in the dissociated cells, thus contributing to higher organoid-forming efficiency [45]. Interestingly, prostate organoids grown under the ENR condition required less dihydrotestosterone (DHT) for growth maintenance compared to those grown under the LF condition (ENR, 0.1–1 nmol/L; LF, 1–100 nmol/L, Table 1), indicating that they were more sensitive to DHT [11, 13, 41, 42]. Notably, the use of growth factor-reduced Matrigel and FBS-free condition in the ENR protocol might be the possible contributing factors for this phenomenon [11, 12, 41]. Nevertheless, organoids derived from both protocols retained AR expression and androgen responsiveness, as well as possessed prostate ductal forming ability upon re-implantation back to the mice [11, 13]. More importantly, tumor organoids derived from GEM models of prostate cancer and human prostate specimens could recapitulate *de novo* cancer phenotype and genotype, as well as drug response to anticancer agents [11, 13].

The formation of prostate epithelial organoids is generally more efficient under the ENR condition, but luminal cells are favored to grow under the LF condition [11, 13]. However, it remains unclear whether the ENR and LF conditions enforce differential selection pressure on different prostate cell populations in culture. Notably, a recent study by the Shen Laboratory has compared the ability of various epithelial cell clusters to generate organoid using both the ENR and LF conditions [37]. Although prostate basal epithelial cells or cell clusters expressing basal markers demonstrated greater organoid forming efficiency under the ENR condition, only rare luminal progenitors could initiate organoid formation under the LF condition [37]. Moreover, the Wang's group discovered that prostate basal stem cells selected from prostatesphere assay were preferentially differentiated into AR- and CK18-expressing luminal population in the LF condition, and with a lesser extend in the ENR condition [46], indicating the superiority of POT in promoting luminal characteristic in prostate epithelial cells. These results have implied that the exclusively luminal characteristic of prostate tumors might enable the continuous maintenance and propagation of prostate cancer cells in these assays.

2 Applications of POT

2.1 Identification of cell populations with stem cell or progenitor properties

For many years, tissue recombination assay has been considered the gold standard for validating stem cell or progenitor potential of a prostate epithelial cell population [47, 48]. However, POT has gradually emerged as another major assay for stem cell or progenitor properties determination in recent years [11, 13]. In particular, an earlier study by the Clevers's group discovered that prostate luminal population could generate organoids carrying both luminal and basal cells, a phenotype that closely resembled prostate glands, indicating the existence of bipotent luminal progenitors in the prostate [11]. In line with this observation, we also demonstrated that CARNs, a luminal progenitor population was capable of exhibiting bipotentiality and responsiveness to androgen when cultured as organoids [13]. Importantly, CARNs-derived organoids could form prostate ducts in a tissue recombination assay, suggesting the retention of prostate epithelial progenitor property in the cultured organoids [13]. Since the inception of these two major POT studies [11, 13], subsequent analyses by other groups have used these assays to verify various novel stem cell or progenitor populations in the prostate, including Sca1-positive luminal progenitors [49], Ly6d-positive luminal progenitors [50], Zeb1-positive basal cells [51], Luminal P population and PrU population that co-expressing luminal and basal markers [37] as well as Luminal C population that expressing *Tacstd2*, *Ck4* and *Psca* [52]. Notably, these newly identified stem cell or progenitor populations were highly efficient in prostate organoid and duct formation using both POT and tissue recombination assay, respectively [37, 49–52], suggesting the feasibility of using either assay to verify stem cell or progenitor potential of prostate epithelial subpopulations. Interestingly, organoids derived from different prostate epithelial stem cells or progenitors exhibited different morphology, such as round translucent, round compact, irregular compact or irregular with multi-elongated structures [11, 13, 37, 49–53], suggesting different degree of intra-organoid organization due to different composition and arrangement of basal and luminal cells. With the blossoming of single-cell RNA sequencing results of the prostate epithelium [37, 50–53], it is anticipated that POT can serve as a powerful first-line platform for the identification of novel prostate epithelial stem cell or progenitor populations.

2.2 Assessment of gene function in prostate cancer initiation and progression

With the advancement of gene editing approaches, gene manipulation can be easily performed in organoids derived from wild type prostate epithelium or prostate cancer from GEM models, thus facilitating the functional analysis of a gene or gene combination in different epithelial lineages or subpopulations in the prostate during tumor initiation and progression. For example, the Clevers's group has engineered *TMPRSS2-ERG* gene fusion, one of the most common early genetic events in prostate cancer in mouse prostate epithelial organoids using CRISPR/Cas9-based gene editing strategy [54]. Since the establishment of organoids from human primary prostate cancer has not been achieved, the resulting organoids can potentially serve as a tool to study the role of *TMPRSS2-ERG* during early-stage of prostate cancer [54]. Moreover, the Pietrzak's group has shown an integrated approach involving GEM model, lentivirus-based gene manipulation and POT to assess the functional role of *Tip5* during prostate cancer initiation and progression [55]. In particular, deletion of *Tip5* affected the ability of prostate luminal cells to initiate cancer in the context of *Pten* deletion as evidenced by the generation of more translucent and bi-layered organoids [55]. Interestingly, reversing the order of *Tip5* and *Pten* deletion in prostate luminal cells did not revert *Pten* deletion-mediated oncogenic transformation, implying the importance of temporal order of oncogenic events [55]. In another two separate studies, the Barbieri's group has demonstrated the feasibility of using POT to study the functional role of *Spop* mutation in prostate cancer progression [56, 57]. Notably, *SPOP-F133V* in combination with *Pten* deletion resulted in the formation of invasive prostate adenocarcinomas that were PI3K/mTOR- and AR-dependent but ERG-independent [56, 57]. Lastly, using POT, three independent research groups have identified BAF, FOXA1 and ERG, respectively as important regulators for the maintenance of luminal identity in prostate cancer, indicating that POT could serve as an assay to study cellular differentiation [58–60]. Comparing to other prostate cancer models, these results have highlighted the advantages of POT in gene function analysis, including being time- and cost-efficient as well as easy to manipulate in a temporal fashion.

2.3 Investigation of the role of different cells of origin or cancer cell subtypes in prostate cancer

Previous studies have also demonstrated the use of POT to study the role of different cell populations in prostate cancer initiation and progression [13, 61–63]. In our earlier study, we showed that luminal cells could serve as a cell of origin for prostate cancer upon oncogenic transformation through *Pten* deletion and $Kras^{G12D}$ activation in organoid culture assay [13]. Interestingly, in the presence of identical oncogenic events, early passage of luminal cell-initiated tumor organoids exhibited less aggressive phenotype compared to CARN-initiated tumor organoids, indicating that CARNs might serve as a more efficient target for oncogenic transformation [13]. In addition, the Witte's group has adopted the ENR-based POT to grow c-Myc and myrAKT1-transduced human prostate luminal and basal cells [61]. Upon re-grafting the resulting oncogenic transformed organoids into immunodeficient mice, the transformed luminal organoids were found to produce well-differentiated adenocarcinoma whereas transformed basal cell-initiated organoids generated histologically more aggressive tumor phenotype, with loss of acinar structure and minimal AR and PSA expression [61]. Moreover, the Abdulkadir Laboratory has isolated prostate epithelial cells from African American men and transduced the cells with lentiviruses carrying MYC, shPten, shP53 and/or AR [62]. Notably, the lentivirus-transduced organoids carrying either MYC and/or in combination with other oncogenic events exhibited a greater rate of basal-to-luminal differentiation, indicating the cellular plasticity-inducing ability of MYC oncogene [62]. These results have highlighted the power of POT to rapidly model common genetic events in prostate cancer. More importantly, these observations have also implied the feasibility of using POT to understand the contribution of different cells of origin and genetic alterations in conferring prostate cancer cell aggressiveness. Lastly, POT was also used to test stem cell or progenitor property of different luminal marker-expressing cancer cells from *Pten*- and *Tp53*-deleted prostate tumors [63]. Interestingly, tumor organoids generated from different cancer cells exhibited heterogeneous phenotypes *in vitro*, which could then produce distinctive tumor histopathology *in vivo* [63]. Taken together, this line of evidences supports the use of POT in understanding prostate tumor heterogeneity.

2.4 Representative prostate tumor modeling

For decades, prostate cancer research is hampered by the lack of representative *in vitro* culture system. Notably, the invention of POT has facilitated the establishment of novel prostate tumor organoid lines from both

human and mouse sources [12, 13]. In particular, Gao and colleagues have generated different prostate tumor organoid lines from biopsy specimens of prostate cancer metastases as well as circulating tumor cells from patients with advanced disease [12]. Notably, the derived tumor organoid lines recapitulated molecular diversity of human prostate cancers and expressed common genetic alterations in prostate cancer, such as *TMPRSS2-ERG* gene fusion, *SPOP* mutation, *SPINK1* overexpression, *CHD1* deletion and loss of both *p53* and *RB* genes [12]. Using the ENR-based POT approach, the Beltran Laboratory has derived and characterized tumor organoid lines from neuroendocrine prostate cancer (NEPC), implying the feasibility of using POT to model rare prostate cancer phenotypes [64]. In addition, we also discovered that various types of tumor organoids could be derived from different GEM models of prostate cancer, which encompassed different disease stages [13]. Importantly, the resulting GEM models-derived prostate tumor organoid lines exhibited distinctive morphology and histopathology, again highlighting the preservation of tumor characteristic in the POT culture condition [13]. More recently, the Kelly's group attempted to derive tumor organoids lines from the LuCAP PDX series using a modified ENR condition [65]. Unfortunately, not all LuCAP lines tested could propagate continuously in the organoid culture assay, indicating the urgent need for further optimization of the existing culture condition [65]. Nevertheless, this proof-of-principle study has implied the potential use of POT for the establishment of representative tumor models from the existing and well characterized PDX series [24–27].

2.5 Drug response evaluation in aggressive prostate tumors

By accurately preserving the genetic and phenotypic properties of human prostate tumors, POT generated tumor organoid lines could be applied for prospective drug sensitivity and testing study. As a proof-of-principle study, prior works from ours and others have suggested the feasibility to recapitulate the synergistic effect of dual inhibition of PI3-K/AKT and AR pathways using *Pten*-deleted prostate tumor organoids derived from GEM model as well as human prostate tumor organoid lines [12, 13]. Compared with conventional preclinical studies using *Pten* loss GEM model of prostate cancer or PDX models [66], the POT approach has greatly reduced the time and cost required for drug efficacy assessment. Similarly, Beshiri and colleagues could recapitulate the efficacy of PARP inhibitor olaparib on prostate cancer patients with *BRCA2* loss using the organoid culture system [65], again highlighting the advantage of using POT to yield timely treatment options to the patients. In addition, various groups have utilized POT to evaluate cytotoxic effect of targeted therapies on different genetic alterations in CRPC and NEPC, including EZH2 [64], *AR* mutations and amplification, as well as the expression of *AR-V7* [67, 68], *SPOP* mutation [69, 70] and Aurora A [71]. More importantly, several independent studies have also demonstrated the feasibility of using tumor organoid models for drug sensitivity assessment [72–74]. For examples, the Wei Laboratory showed that prostate tumor organoids-carrying *SPOP* mutation were more resistant to BET inhibitors [72]; Abdulkadir and colleagues discovered that *ALK* F114C-expressing tumor organoids exhibited variable responses to various ALK inhibitors [73]; while the Beltran's group showed that *SLFN11* expression status could predict platinum-based chemotherapy in CRPC-derived prostate tumor organoids [74]. Moreover, POT could also be used for the identification of novel drug candidates as a single treatment modality [75] or combinatorial treatment regimen [76, 77]. In particular, the Schreiber's group has used prostate tumor organoid models to demonstrate that targeting GPX4, an important regulator for neuroendocrine differentiation in prostate cancer, caused ferroptotic cell death [75]. Moreover, the Watt's group demonstrated that dual targeting of fatty acid uptake and *de novo* lipogenesis pathways could substantially inhibit the growth of prostate tumor organoids derived from PDX models [76], while the Marzi's group discovered synergistic effects of indenoisoquinoline TOP1 inhibitors and olaparib in homologous recombination-deficient and SLFN11-positive prostate tumor organoids [77]. Taken together, these results have highlighted POT as an efficient *in vitro* drug testing platform to catalyse the discovery of potential pharmacological modalities for AR-independent prostate cancer.

3 Key challenges and perspective in prostate organoid research

3.1 Non-optimal dissociation protocol

For decades, prostate cancer researchers have acknowledged the difficulties of establishing prostate cancer cell line [78]. Although POT inventions have greatly improved the success rate of prostate cancer cell lines derivation under 3D culture condition [11–13], establish-

ing human prostate tumor organoid lines from primary cancers is still not achievable. There are a couple of possible explanations for this phenomenon. Counterintuitively, we have discovered that non-transformed prostate epithelial cells could grow better than prostate cancer cells in the organoid assay (unpublished observation). Consequently, a mixture of non-transformed prostate epithelial cells and prostate cancer cells as starting populations would eventually lead to the dominance of non-transformed cells in the culture during the continuous propagation. Moreover, non-optimal dissociation procedures could reduce organoid forming efficiency of prostate cancer cells. Notably, cell dissociation efficiency was expected to be lower in specimens with a high cell density and collagen content [42]. For example, papillary urothelial carcinomas provided a better quality starting material for tumor organoid establishment than muscle invasive bladder cancers, because they were typically less collagenous and with less stroma content, and thus could be easily dissociated to produce high yield of viable cells within a shorter time frame [79]. In comparison, primary prostate tumors have demonstrated greater interpatient heterogeneity, particularly in respect to stroma and extracellular matrices (ECM) contents [80]. Therefore, when dealing with different primary prostate tumor specimens, adjusting the dissociation protocol might be crucial to ensure optimal yield of viable cells for successful organoid establishment. Taken together, these results have highlighted the importance of tailoring dissociation protocols based on the stroma-to-cancer ratio as well as collagen and/or other ECM contents of prostate tumor specimens.

3.2 The importance of integrating stroma in POT

Most prostate cancer drug investigative studies were conducted using the available POT models, which were primarily epithelial in nature without stromal components, such as immune cells, fibroblasts, smooth muscle cells and endothelial cells [64, 65, 67–77]. Undoubtedly, epithelial-stromal interactions play a crucial role during prostate development and carcinogenesis, and possibly in drug response modulation [81–86]. Recent study by the Nonn's group discovered that incorporation of native prostate stromal cells promoted branching of non-malignant epithelial organoids and enhanced the expression of AMACR and the survival of tumor organoids. Such observation suggested an indispensable role of stroma in the maintenance of normal and malignant prostate phenotypes [80]. By adopting the ENR-based organoid protocol, the Brugge Laboratory has noticed a significant phenotypic differences between breast epithelial organoids and the corresponding primary tissues, namely the loss of CD10 and increased CD44 expression in the breast epithelial organoids [87]. The authors attributed this phenomenon to the loss of stroma-interacting cells or innate tissue architecture upon the culture establishment [87]. Interestingly, Sachs and colleagues attempted to alter the ENR culture condition in order to enhance proliferation capacity of breast tumor organoid lines in the absence of stromal cells, but had indeed caused the loss of 3D structures in the tumor organoid lines [88]. These results have again implied the importance of "check and balance" in the cellular composition in order to maintain an intact tissue architecture and proliferation capacity, by which stroma is an essential integrated component.

Integrating stromal components into prostate organoid culture remains a challenging task for prostate cancer researchers. Of note, we found that urogenital mesenchymal cells did not grow well in Matrigel, a crucial component for both ENR and LF culture conditions [41, 42]. Thus, the discovery of a culture condition that favors the growth of both stromal and epithelial cells could be a critical breakthrough in POT. In addition, other related critical issues include (i) the identification of the ratio of different stromal populations to the epithelial or cancer compartment, (ii) the type and composition of stroma and ECM, (iii) the ECM stiffness, and (iv) the introduction of oxygen and blood supply. With the blossoming of single-cell RNA sequencing analyses of the prostate epithelium [37, 50–53] and possibly prostate tumors in the near future, we could understand better the cellular composition and hierarchy in the prostate during normal homeostasis and malignant transformation. Together with our understanding on the genomic and metabolomic profiles of prostate cancer [89, 90], the future development of POT should consider incorporating and preserving the relevant molecular characteristics of cancer along with the stroma compartment. Such inclusive second-generation POT models will certainly facilitate a better understanding of disease progression and accelerate the development of novel anticancer drugs.

3.3 Overcoming the failure to recapitulate physiological conditions in POT

Undoubtedly, POT remains as an *in vitro* culture system with the absence of *in vivo* physiological processes.

Consequently, animal models is still irreplaceable by any *in vitro* models, including POT in the study of angiogenesis, metastasis and tumor dormancy [23]. Moreover, *in vivo* models are still the gold standard for monitoring pharmacokinetics and pharmacodynamics, adverse reaction or toxicity exerted by a tested drug [91, 92]. Interestingly, recent study has demonstrated the feasibility of using intestinal organoids for pharmacokinetic evaluation in drug development studies [93]. In this proof-of-principle experiment, the research group derived intestinal organoids from induced pluripotent stem cells (iPSCs) and discovered that the resulting organoids expressed drug transporters and possessed efflux transporter activity as well as the ability to induce drug-metabolizing enzyme CYP3A4 when treated with nuclear receptors ligands [93]. These results implied that on the one hand, we could assess the efficacy of potential anticancer drugs using POT, while on the other hand we could simultaneously evaluate pharmacokinetics and toxicity of the drug using relevant organs-derived organoid systems. The implementation of such approaches could greatly reduce the use and reliance of laboratory animals.

3.4 Generation of syngeneic animal models using mouse prostate organoids

To overcome the issues of stroma integration and physiological relevance of POT, we could consider grafting mouse organoids directly into host mice with the same genetic background to generate a syngeneic model. Such approach was adopted successfully by two independent research groups using colorectal tumor organoids as a model [94, 95]. Notably, two-thirds of the host mice that were inoculated with GEM model of colorectal cancer-derived tumor organoids showed evidence of tumor engraftment within 6 weeks, local invasion in 11–12 weeks and distant metastasis in approximately 20 weeks, indicating that the method was a highly efficient approach for preclinical investigation [94]. In addition, oncogenic-transformed colon organoids through CRISPR/Cas9 gene editing approach could also provide similar results upon re-implanted orthotopically in immune-intact host mice, highlighting the power of combining gene editing approach and organoid technology in the generation of representative *in vivo* tumor models [95]. Similarly, the Greenberg's group had successfully generated a prostate cancer syngeneic model using TRAMP model-derived prostate cancer cell line [35]. Because prostate tumor organoids derived from different GEM model could preserve androgen responsiveness and distinctive *in vivo* phenotypic characteristics [13], we anticipate that the resulting "GEM-organoid-syngeneic model establishment" strategy should enable the establishment of highly representative tumor models for preclinical studies of prostate cancer. Nonetheless, we have to put in more efforts to resolve various technical challenges, including determination of the optimal number of transplanted cells or organoids, efficiency of various implantation routes and sites, as well as tumor rejection issue prior to having a stable, repeatable and efficient methodology.

3.5 Generating non-malignant prostate epithelial models using POT

Prostate cancer researchers do not have representative non-malignant prostate epithelial models for the study of tumor initiation. Notably, prostate abnormalities, such as benign prostate hyperplasia and prostate cancer, are rarely found in young men. Consequently, clinicians will only consider investigating prostates of the young men when these young men encounter male fertility issues [96], leading to insufficient starting material to generate non-malignant prostate epithelial organoids for the study of prostate biology. To address the issue involving the shortage of normal prostatic tissues, various groups have attempted to use the trans-differentiation approach to generate prostatic tissues from iPSC [97, 98]. In particular, by applying a computational approach, the Shen's group identified candidate driver genes for prostate specification, namely *FOXA1*, *NKX3.1* and *AR* [97]. Interestingly, iPSCs expressing these genes were capable of generating prostatic tissues in a tissue recombination assay [97]. Importantly, the resulting prostatic tissues showed epithelial and stromal marker expressions that were similar to native prostatic tissues [97]. Using the similar approaches, the Heer's group has independently demonstrated the ability to generate prostatic tissue using human iPSC [98]. All in all, these studies have highlighted the potential use of iPSC-differentiated prostatic tissues for the derivation of normal prostate epithelial organoid models, which should be valuable for a longitudinal study on prostate cancer initiation and progression.

4 Concluding remarks

In the most ideal scenario, an improved version of POT

should recapitulate the tumor biology of patients. Consequently, such data could lead to a better personalized genomic assessment, and subsequently, a personalized treatment decision aiming for a more desirable clinical outcome. However, the above-discussed challenges and limitations need to be resolved prior to harnessing the maximum impact of POT in offering personalized medicine. In the meantime, we propose an integrated POT system that involves the use of existing tumor models for the study of prostate tumor initiation and progression (Fig. 2). Firstly, we could derive normal prostate epithelial organoids from mouse prostates or human iPSC, which are subjected to oncogenic transformation through gene editing approach. Such approach should delineate the ability of different epithelial subpopulations to serve as the cell of origin for prostate cancer, and at the same time, provide novel tumor models for cancer study. Secondly, we could also establish tumor organoid lines from GEM and/or PDX models, or directly from prostate cancer metastases. Such models could be genetically modified to assess gene function, treatment resistance and metastasis. Notably, the influence of stromal components could be assessed either through *in vitro* co-culture system or by injecting mouse organoids into host mice with identical background strain. Taken together, our proposed integrated strategy should serve as a powerful tool for prostate cancer researchers while the field is progressing into the era of second-generation POT. We envision that the second-generation POT would be a promising system to support personalized medicine for prostate cancer patients in the near future.

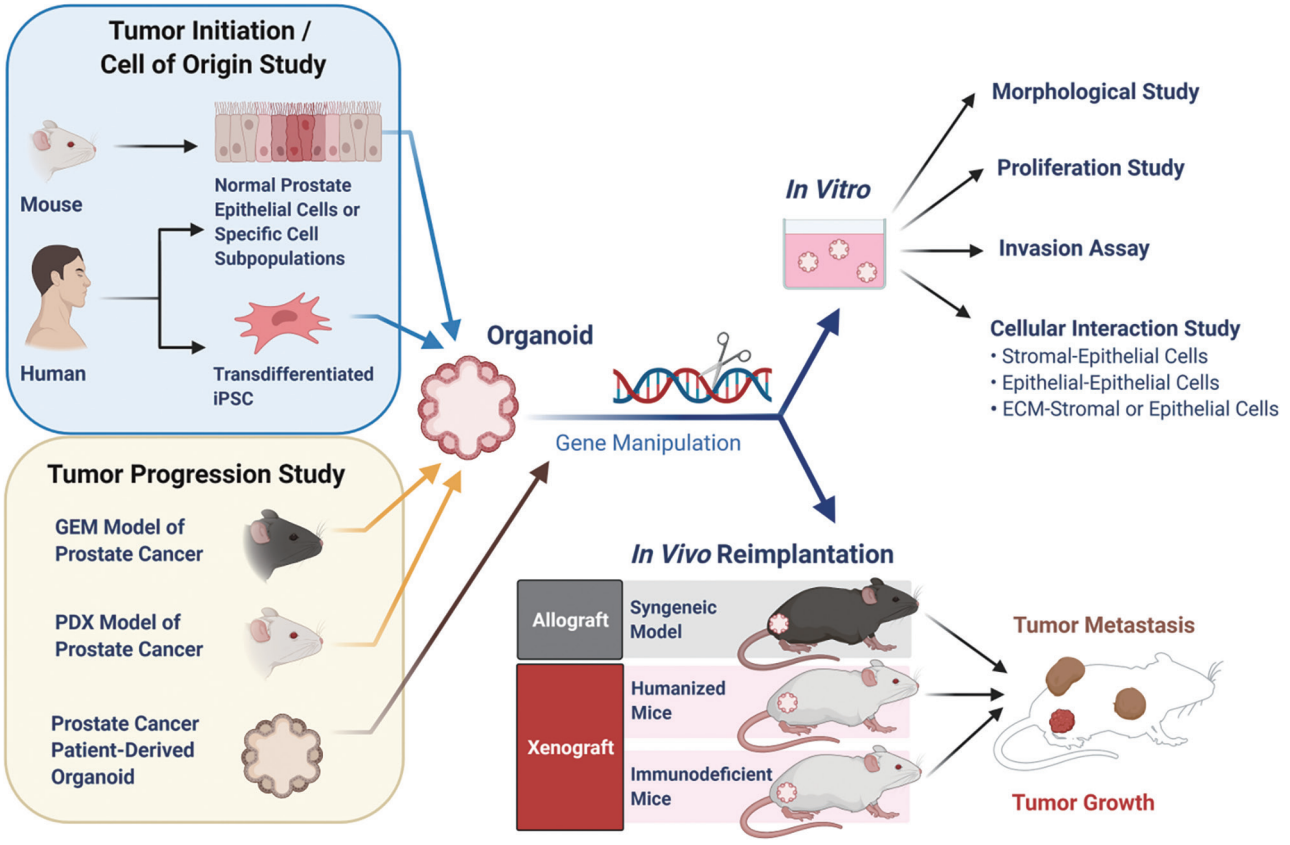

Fig. 2. A comprehensive prostate cancer research model. Different models could be applied based on the research hypothesis. To generate organoids for tumor initiation study, mouse or human samples could be collected to isolate normal prostate epithelial cells or to generate transdifferentiated induced pluripotent stem cells (iPSC). While for tumor progression study, besides prostate cancer patient-derived organoids, genetically-engineered mouse (GEM) or patient-derived xenograft (PDX) of prostate cancer could be used to generate organoids. These organoids could be subjected to gene manipulation for further *in vitro* studies or *in vivo* reimplantation for functional studies. This figure was created with BioRender.com.

* * *

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