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Applications of Organoids for Cancer Biology and Precision Medicine

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Abstract

Organoid technologies enable the creation of *in vitro* physiologic systems that model tissues of origin more accurately than classical culture approaches. Seminal characteristics, including three-dimensional structure and recapitulation of self-renewal, differentiation, and disease pathology, render organoids eminently suited as hybrids that combine the experimental tractability of traditional 2D cell lines with cellular attributes of *in vivo* model systems. Here, we describe recent advances in this rapidly evolving field and their applications in cancer biology, clinical translation and precision medicine.

Introduction

Over the past decades, preclinical cancer biology studies have commonly relied on immortalized 2D cancer cell lines *in vitro*, as well as xenografted or transgenic animal models[1]. Although these approaches have contributed enormous insights, extensively passaged cell lines may not accurately represent the biology and pathophysiology of the original parent tumor, and animal models are costly and time consuming.

Alternatively, newly developed 3D organoid methods now facilitate the robust culture of healthy human tissues and their cognate tumors, thus representing an independent *in vitro* approach to studying cancer. To be classified as an organoid, the culture should retain the identity of the modeled organ, contain multiple cell types, preserve some physiological aspects of the organ and self-organize according to the same principles as the organ[2,3]. Furthermore, organoids maintain several properties of primary tissues, such as self-renewal, multilineage differentiation, signaling nodes and histology. Once established, organoids can often be cultured long-term, expanded, cryopreserved, and genetically manipulated similar to traditional 2D cell lines. As such, organoids combine the tractability of *in vitro* systems with the 3D architecture and differentiation of *in vivo* model organisms. Accordingly, recent years have witnessed an exponential growth in the use of organoids as a replacement for

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Competing interests

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immortalized cell lines, allowing the use of primary human cells for the *in vitro* modeling of biological processes, including normal physiology, stem cell biology and diverse pathophysiologic states. As relevant to this review, the singular attributes of organoids have led to their rapid adoption as powerfully exploitable cancer models.

Organoid methodologies

In general, organoids can be classified not only by their tissue of origin, but also by their generation from adult tissues[4,5] versus embryonic stem cells[6]. Organoids can be further distinguished by whether they solely contain epithelium[4], or also possess stromal cells[5]. These distinct classes of organoids can be reproducibly generated by correspondingly diverse techniques (Figure 1).

Submerged culture.

Many seminal organoid studies utilized intestine as starting material with growth within solid gels of extracellular matrix (laminin-rich Matrigel or equivalents, such as BME-2), submerged beneath tissue culture media. Intestinal organoids can be established from either purified adult small intestinal crypts or sorted single $LGR5^+$ intestinal stem cells (ISCs) in an elegant growth factor-defined culture condition that mimics the stem cell niche[4]. This culture condition generally includes Wnt pathway ligands such as Wnt3a and/or R-spondin, epidermal growth factor (EGF), and the bone morphogenetic (BMP) inhibitor Noggin that allow ISCs to undergo long-term self-renewal and differentiation into all cell lineages[4]. As a result, the ISCs form highly polarized 3D epithelial structures that represent intestinal crypt-villus compartments. Variations of this submerged organoid culture system use specialized growth factor cocktails adapted for many different organs, including small and large intestine[4,7-9], stomach[10,11], esophagus[9,12], salivary gland[13,14], taste buds[15], pancreas[16-19], liver[20-23], airway[24-27], breast[28], prostate[29,30], kidney[31], fallopian tube[32], ovary[33,34], endometrium[35] and skin[36]. As discussed below, this method has also been used extensively for cancer modeling and to grow tumor biopsies. Because of the necessity for cellular disaggregation during processing, engendering deleterious Rho kinase (ROCK)-dependent anoikis as well as stress[37] and injury responses[38], ROCK inhibitors have greatly increased the efficiency of organoid generation[4]. Typically, submerged organoids are polarized with the apical surface facing a central lumen, although this polarity can be deliberately inverted[39]. A defining characteristic of submerged organoids is their possession of exclusively epithelial cells, and absence of stroma.

Air-liquid interface (ALI).

A parallel method utilizes air-liquid interface (ALI) culture to grow organoids that contain both epithelial cells and surrounding stroma as a cohesive unit directly from tissue fragments. In ALI, mechanically dissociated tissues are grown into a type I collagen matrix on top of an inner transwell insert with culture medium provided through a permeable membrane[5,40,41]. The top of the transwell directly contacts air, thereby facilitating oxygen diffusion[42], which may underlie the ability of ALI to grow large multicellular organoids that preserve native tissue architecture, such as epithelium, *en bloc* with endogenous stromal cells without reconstitution. In ALI, the stromal cells are sufficient to support organoid

growth without growth factor supplementation, likely by producing essential endogenous niche factors[5]. The ALI method grows intestine, stomach and pancreas organoids as cystic structures containing both epithelium and mesenchymal stroma, displaying expansile growth, stem cell populations, multilineage differentiation and even intestinal peristalsis[5,41,42]. These features render ALI a complementary method to submerged organoids, particularly for inclusion of stromal populations, which has been exploited for cancer oncogene engineering[41] and tumor microenvironment modeling[28] as detailed below.

Induced pluripotent stem cells (iPSC) or embryonic stem cells (ESC).

In contrast to submerged and ALI methods, the generation of iPSC-derived organoids obligately entails directed differentiation to the target tissue of interest. Diverse iPSC-derived organoids have been described, such as brain[43-46], small and large intestine[6,47], stomach[48,49], esophagus[50], liver[51], airway[52-55], thyroid[56,57], kidney[58,59], pituitary gland[60,61], inner ear[62-64] , retina[65], hair follicle[66] and blood vessels[67]. These efforts have been greatly aided by bespoke tissue-specific differentiation protocols, typically requiring weeks to months, to reprogram iPSCs to the desired organ type. Of note, some tissues, like brain, cannot be grown in submerged or ALI culture, making iPSC organoids a unique platform for studying the timing of development of neural tissues. A salient feature of endodermal iPSC models is the frequent co-development of epithelium alongside mesenchymal stroma[6,47-49]. Compared with adult cell types, molecular characteristics of iPSCs-derived organoids are more similar to immature fetal cells and *in vivo* transplantation can enhance maturation[68-72]. iPSC organoids have been broadly deployed for disease modeling, including cancer, as reviewed elsewhere[73,74].

Bottom-up organoid approaches

The genetic manipulation of primary wild-type organoids provides a unique opportunity to model human tumor initiation and progression in a tissue-specific fashion that closely recapitulates the oncogenic process (Figure 2). Conventionally, interrogating the function of a candidate oncogene or tumor suppressor *in vitro* has utilized 2D transformed cell lines, whose underlying genetic complexity both precludes true de novo cancer initiation and may contain significant modifier loci that could mask oncogenic effects. Analogously, *in vivo* examination of oncogenes and tumor suppressors has required transgenic manipulation, typically in a mouse context. In contrast, systematic introduction of oncogenic loci into wild-type tissue organoids provides an attractive method for “bottom-up” tumor initiation into normal cells possessing a non-mutated genetic background, yielding a hybrid combining the tissue architecture, stem cells and differentiation of *in vivo* mouse models with the facile genetic manipulation of transformed human cancer cell lines.

This tractability in turn renders wild-type organoids amenable to forward genetic approaches such as oncogene engineering and functional genomics.

Modeling oncogenic loci in wild-type organoids

A first proof of concept for bottom-up organoid modeling of cancer exploited ALI organoids for robust *in vitro* transformation of adult wild-type intestinal organoids to

adenocarcinoma[41] Organoids were generated from adult transgenic mice containing floxed alleles, thus allowing combinatorial CreER-mediated deletion of *Apc, Tp53* and/or CreER activation of $Kras^{G12D}$ expression, in concert with *Smad4* shRNA knockdown. The addition of tamoxifen induced CreER-dependent progressive transformation depending on whether 1, 2, 3 or 4 genetic lesions were present. *In vivo* growth as tumors required 4 mutations[41], recapitulating the classical multihit "Vogelgram" model of colon tumorigenesis[75]. Thereafter, two independent studies using submerged Matrigel organoids successfully transformed normal human colonic organoids to adenocarcinoma by CRISPR-based sequential engineering of common alterations in colon cancers, including $APC^{-/-}$, $KRAS^{G12V/D}$, $SMAD4^{-/-}$, $PIK3CA^{E545K}$, and $TP53^{-/-}$ [76,77]. Importantly, colon organoids with quadruple mutations grew *in vivo* when implanted subcutaneously into mice, confirming oncogenic transformation[76,77]. To compensate for the inefficiency of CRISPR, these human organoid studies used selective media to enrich for mutants. For example, *APC* mutations can be selected for by growing organoids in medium without Wnt/R-spondin, whereas selection for *TP53* mutations is possible using medium with the MDM2-P53 complex inhibitor Nutlin-3[76,77]. In addition, SMAD4-mutated organoids can survive in medium lacking Noggin and the transforming growth factor-β (TGFβ) inhibitor A83-01, but containing TGFβ, whereas oncogenic $KRAS^{G12V/D}$ and $PI3KCA^{E545K}$ CRISPR knock-in allows organoid growth without EGF[76,77].

Engineered human colon organoids can also be used to study premalignant colorectal polyposis[78]. CRISPR/Cas9-engineered $BRAF^{V600E}$ human colon organoids in combination with TGFβ signaling manipulation induce a mesenchymal phenotype consistent with sessile serrated adenomas (SSA)[79], supporting prior hypotheses of $BRAF^{V600E}$ function[80]. Furthermore, CRISPR/Cas9 introduction of an *EIF3E-RSPO2* fusion gene in human colon organoids modeled progression of traditional serrated adenomas (TSA) *in vitro* with histologic recapitulation of SSA following *in vivo* orthotopic xenotransplantation[81]. Superimposed expression of the mesenchymal bone morphogenetic protein antagonist GREM1 in *EIF3E-RSPO2;* $BRAF^{V600E}$ organoids induced TAS-like morphological aberrations[81], consistent with the phenotype of *Grem1* transgenic mice[82].

The paradigm of oncogenic conversion of colon organoids has now been expanded to additonal studies in microsatellite instability[83].

Oncogene engineering studies have rapidly progressed from early work in colorectal cancer (CRC) to many prevalent solid tumor types. *In vitro* conversion of normal mouse gastric and pancreatic organoids to adenocarcinoma has been achieved by deletion of *Tp53* and $Kras^{G12D}$ activation in ALI organoids, thereby allowing *in vitro* dysplasia and *in vivo* tumorigenicity upon transplantation and recapitulating features such as desmoplasia and epithelial-mesenchymal transition (EMT)[41]. Further, *Tgfbr2* deletion enhances tumorigenicity of $Cdh1^{-/-}$; $Tp53^{-/-}$ ALI gastric organoids[84]. Similar mouse transformation of colon[85] and pancreatic[16] studies have been performed using submerged Matrigel models. CRISPR/Cas9 oncogene editing of wild-type human organoids has allowed oncogenic conversion of human gastric[86], pancreatic [87,88], esophagus[89], breast[90] and liver[91] tissue. In human stomach organoids, deletion of *TP53* or *CDH1* confers transformation with a diffuse gastric cancer phenotype in the latter[86]. Normal pancreatic organoids harboring four

oncogenic driver mutations, including *KRAS*^{G12V}, *CDKN2A, TP53*, and *SMAD4*, acquired niche factor independency and histologically resembled PanIN-like lesions and invasive pancreatic ductal adenocarcinomas (PDACs)[87,88]. Normal esophageal organoids lacking *APC* quickly developed Barrett esophagus-associated neoplasia[89]. Similarly, engineered breast organoids carrying mutations in four tumor suppressor genes, *TP53, PTEN, RB1* and *NF1*, recapitulate breast tumorigenesis[90].

Additionally, iPSC-derived organoids model neoplastic progression. Generation of iPSC from PDAC tumors, followed by redifferentiation to pancreatic lineages, results in rare lines recapitulating early PanIN lesions[92]. Early neoplastic changes are observed upon colonic differentiation of iPSC, followed by expression of mutant *APC*[93], or upon mutant *KRAS* and *TP53* expression in iPSC induced to pancreatic lineages[94]. Engineered iPSC-derived cerebral organoids carrying oncogenic mutations exhibit many features of tumor malignancy[95]. iPSC-derived organoids may find particular utility in modeling germline cancer predisposition syndromes, as with *APC* mutation in Familial Adenomatous Polyposis[93] or *TP53* mutation in Li-Fraumeni syndrome[96]. However, potential drawbacks to cancer modeling using iPSCs include their fetal context compromising the study of adult cancers.

Organoids as functional genomics platforms for new cancer gene discovery

A significant potential application of the bottom-up organoid strategy is the functional validation of novel oncogenic alterations. Numerous examples exist, including confirmation of miR-483 as a driver oncogene amplified at 11p15 in colon cancer through overexpression in colon organoids[41], as well as demonstration of the oncogenicity of *EIF3E-RSPO2* fusions[81,97]. Expression of *Rhoa* mutant alleles in gastric organoids showed that *Rhoa*^{Y42C} acts as an oncogene through the activation of focal adhesion kinase in *Cdh1*-mediated diffuse gastric cancer[98]. CRISPR/Cas9-engineered liver organoids have validated the function of a previously uncharacterized tumor suppressor gene, *BAP1*, in hepatic carcinogenesis[91]. In these various cases, mutation of the test oncogene or tumor suppressor generally elicits *in vitro* and *in vivo* tumorigenic phenotypes.

Engineered human organoids also provide tractable platforms for functionally studying interactions between cancer genotypes and phenotypes. For example, recent landmark human cancer genomic projects, such as The Cancer Genome Atlas (TCGA), have molecularly characterized four distinct subtypes of primary gastric cancer, including Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), chromosomal instability (CIN), and genomically stable (GS) tumors[99]. Cre-lox-mediated deletion of *Cdh1*, recurrently mediated in the GS subtype of gastric cancers, along with *Tp53*, induced signet ring cells characteristic of diffuse gastric cancers and enhanced *in vivo* tumorigenicity in mouse ALI gastric organoids[84]. CRISPR/Cas9 deletion of *CDH1* and *RHOA* in normal human gastric organoids elicited striking morphological changes and vigorous migratory features characteristic of diffuse-like gastric cancers. Interestingly, RHOA inhibition reverses these phenotypes and sustains *CDH1* knockout organoid cells, supporting the concurrency of *CDH1* and *RHOA* mutations in gastric cancer[86].

Thus, many comprehensive tumor models with specific mutations have been established using organoid-based “bottom up” genetic approaches, allowing functional assessment of small numbers of genetic alterations commonly seen in patient tumor samples. The 3D properties of organoids may be especially beneficial in modeling tumor suppressor loci. For instance, lung cancer cell lines display stronger growth phenotypes in response to CRISPR-mediated knockout of tumor suppressor loci when grown as 3D spheroids versus 2D monolayer[100]. Such benefits may also extend to 3D organoid systems. Moving forward, substantial potential exists for exploiting organoids for broader-based unbiased oncogene screens leveraging substantial genetic and epigenetic data from genome-scale sequencing studies, for instance using sgRNA or shRNA barcoded approaches.

Studying tumor genomic evolution

Previous studies have identified mutational signatures and driver mutations by whole genome sequencing in seemingly normal colonic glands[101], and clonal evolution in breast cancer has been assessed by single cell DNA sequencing[102]. The success in establishing organoids from normal and tumor tissue allows biomass expansion from single cell-derived clones, thus increasing fidelity of whole genome sequencing[103] and extension to multi-omics sequencing[104], to examine mutational processes in normal[103,105,106] or tumor tissue[104]. Whole genome sequencing of mouse clonal organoids from single stomach glands, colon crypts or prostate cells revealed distinct mutational signatures amongst individual clones[105]. A similar approach demonstrated that human adult stem cells (ASCs) accumulated approximately 36 mutations per year but exhibited different turnover rates and tissue-specific mutational processes[103].

Analogous studies of forward genetic oncogenic transformation of human wild-type organoids can similarly elaborate the evolutionary dynamics and driving forces underlying tumor initiation and progression. Loss of *APC* and *TP53* in normal colon organoids stimulates abnormal chromosome segregation and extensive aneuploidy, suggesting a key driving force of chromosome instability, a hallmark of tumor progression[77]. Accumulation of total mutational burden was quickly observed after depletion of *MLH1*, a key DNA repair gene, in normal colonic organoids[83]. These studies indicated the potential for longitudinal tumor evolution studies in human organoids. For example, the chemotherapeutic drug 5-Fluorouracil (5-FU) was shown to accelerate tumor evolution with rapid T>G transversions after treatment[107]. Additionally, ongoing chromosomal instability (CIN) in colorectal tumor organoids has been documented by single-cell karyotype sequencing, further demonstrating the prevalence of genomic heterogeneity in human cancer[108].

Organoid modeling of cancer stem cells

In vivo lineage tracing in engineered mouse models is a powerful tool to study stem cell properties in adult mammalian tissues[109], as evidenced by inducible Cre recombinase lineage tracing of long-lived adult Lgr5$^+$ ISCs[110]. Similarly, lineage tracing and clonal analysis in organoids represents a parallel strategy to study human cancer stem cells[111,112]. Following *Apc* loss in mice, Lgr5$^+$ ISCs, but not other cell types, give rise to intestinal adenomas[113] reminiscent of the cell hierarchy of normal intestine[114] and consistent with Lgr5$^+$ ISCs representing a cancer stem cell (CSC) population[115]. Analogously, this mouse

lineage tracing strategy was subsequently applied to primary human organoids to demonstrate cancer stem cell activity[112,116]. Human LGR5$^+$ cells exhibited multipotency and self-renewal in colon organoids harboring inducible CRISPR/Cas9 knock-in lineage tracing alleles of *LGR5*[116], which following transplantation self-renewed and differentiated to generate heterogeneous progeny in tumors[112]. Remarkably, specific ablation of LGR5$^+$ CSCs within tumors did not induce long-term regression of primary tumors, which instead exhibited dramatic plasticity where other cell types replenished the LGR5$^+$ CSC population[112]. Surprisingly, in a parallel transgenic mouse study[117], ablating Lgr5$^+$ CSCs did not induce primary tumor regression, but formation and maintenance of colon cancer-derived liver metastases was reduced, suggesting that targeting Lgr5$^+$ CSCs could selectively eradicate colon cancer metastases. Upon mouse transplantation of oncogene-engineered colon organoids, the majority of metastases were seeded by circulating Lgr5$^-$ colon cancer cells which could regenerate functional Lgr5$^+$ CSCs, indicating potential prevention of metastases by targeting cellular plasticity[118]. In humans, it remains unclear whether LGR5$^+$ cells serve as functional CSCs in human colon cancer metastases, where LGR5 expression is downregulated[38,119]. Similar cellular plasticity of Lgr5$^+$ ISCs has also been described previously in normal mouse intestinal epithelium[120,121]. Implicit to cancer stem cell analysis in organoids are cell of origin questions for particular cancers. Indeed, successful oncogenic transformation of a particular lineage in organoids may represent *prima facie* evidence that a given population serves as a cell of origin for the cognate cancer.

Studying oncogenic pathogens

A lack of appropriate primary culture systems has previously constrained the exploration of numerous human pathogens. Organoid culture technology has filled numerous emergent needs in the field of infectious diseases pathogenesis. Co-cultures of organoids with pathogens, such as parasites[122], bacteria[11,49,123-129], and viruses[130-132], have modeled host-pathogen interactions and pathogenic-induced oncogenesis. Microinjection of gastric cancer-associated *Helicobacter pylori* into the lumen of normal gastric organoids stimulates a robust inflammatory response[11] and epithelial hyperproliferation[49,128,129]. A causal relationship between chronic *Salmonella enterica* infection and gallbladder cancer was revealed in a gallbladder organoid model[124]. The oncogenic *Salmonella*-infected organoids acquired growth factor independency and exhibited histological characteristics of cellular transformation, including loss of cellular polarity and increased nuclear pleomorphism. Various colorectal cancer-associated bacterial species are enriched in patients, and therefore the intestinal microbiome has been suggested to facilitate tumorigenesis[133]. Accordingly, colibactin-producing *pks$^+$ Escherichia coli* directly induced DNA mutagenesis in a long-term organoid co-culture system. Whole exome sequencing data indicated that organoids exposed to *pks$^+$ Escherichia coli* exhibited a characteristic single thymine (T) deletion signature, as well as increased numbers of single base substitution, preferably T>N substitutions. Interestingly, these *genotoxic Escherichia coli*-induced mutational signatures were identified in a subset of CRC patients, suggesting a possible cause of tumorigenesis[127].

Top-down organoid approaches

Human patient-derived tumor organoids (PDOs) can be directly established from clinical cancer biopsies in a tissue-specific fashion. As opposed to the “bottom-up” approach of engineering oncogenic alterations into wild-type tissue organoids, the direct organoid culture of fresh tumor tissue is a “top-down” strategy of studying pre-established malignancies (Figure 3). Attempts to propagate fresh tumor specimens as *in vitro* conventional 2D cancer cell lines *or in vivo* patient-derived xenografts (PDX)[134,135], while successful, have been historically fraught with inefficiency. However, success rates for tumor-derived organoids and PDOs have been typically higher than conventional 2D culture and PDX and while varying for different tissues, can be robust for commonly used tumor organoid systems such as pancreatic and colon cancer (Table 1). PDOs have been established from surgical resections, tissue biopsies, circulating tumor cells, and ascitic fluid[34,136-138]. General features of PDO cultures, and indeed essential quality control metrics, are their accurate preservation of histologic and genetic features of the parent tumor, the latter extending to truncal alterations, such as copy number variations (CNV) and single-nucleotide variations (SNV). Furthermore, PDOs are extremely tractable from an experimental standpoint, undergoing facile lentiviral transduction, CRISPR-based gene editing and *in vivo* xenografting. These properties are significantly abetted by their ready expansion which renders PDOs potentially useful as preclinical models for drug screening or functional genomics with throughput greater than PDX studies. On the other hand, PDO cultures have until recently generally lacked important *in vivo* attributes such as stroma or immune components[40]. As such, tumor organoids containing exclusively epithelium generally exhibit transcriptional concordance with the original cancers[86,139-141], except for downregulation of blood-, immune- and extracellular matrix-related genes[139,140].

Recently, many organoid biobanks have emerged that contain large numbers of PDOs from different cancer types, including colon[136,137,141-145], rectum[146,147], stomach[86,140,148,149], pancreas[16,88,94,150,151], bladder[152,153], prostate[138,154], ovary[33,34,155], esophagus[156,157], breast[28], oral mucosa[158], endometrium[159], liver[160-162] , kidney[163] , airway[164] and brain[139] typically utilizing the submerged Matrigel culture method, although ALI PDOs have also been described[40]. The Human Cancer Models Initiative (HCMI) seeks to biobank thousands of new PDO models, replete with genetic sequencing and clinical annotations[165]. Overall, the breadth of successful PDO generation provides an exciting new bridge between basic and translational cancer research.

Recapitulation of sensitivity to targeted therapies

PDOs are now used to identify new therapeutic strategies via discovery of gene-drug interactions, correlating between drug sensitivity to genomic alterations. One proof-of-concept for small molecular compound screening of PDOs was performed in an early biobank of colon cancer organoids[136]. By connecting drug sensitivity data and genetic information of PDOs, a positive correlation between oncogenic *KRAS*-mutated organoids and resistance to anti-EGFR inhibitor cetuximab was identified, recapitulating previous clinical observations[166]. Similarly, androgen receptor (AR) amplification in prostate cancer organoids conferred sensitivity to the AR antagonist enzalutamide. In agreement with

previously identified synthetic lethality[167], a recent biobank revealed poly (ADP-ribose) polymerase (PARP) inhibition as a therapeutic vulnerability of *BRCA1/2*-deficient breast cancer organoids[28]. A bladder cancer organoid biobank indicated that organoids harboring gain-of-function *FGFR3* mutations exhibited significant sensitivity to the MEK inhibitor trametinib and the ERK inhibitor SCH772984[153]. These results illustrate the ability of PDOs to accurately recapitulate clinically observed therapeutic vulnerabilities to archetypal genetic alterations.

Dissecting tumor growth factor dependencies

Organoid cultures can dissect the growth factor "niche" dependency of tumor cells within their microenvironment, examining if cancers exhibit altered or reduced growth factor requirements. Conventionally, tumor organoid cultures have been grown in rich conditions replete with signals for Wnt, R-spondin, EGF, BMP and other pathways, while conversely, omission of such growth factors has enabled functional selection of CRISPR-induced oncogenic mutations[76,77]. However, alternation of niche requirements can be crucial for the selective growth advantage of certain subtypes of cancers and for establishing cognate PDOs. The use of diverse conditions allowed successful PDO generation from many rare colon cancer subtypes previously refractory to PDO culture, such as sessile serrated adenoma, tubulovillous adenoma, mucinous adenocarcinoma and neuroendocrine carcinoma, preserving pathological features of the original tumors both *in vitro* and following *in vivo* kidney capsule xenotransplantation[144]. Despite differences in genetic background often translating to specific growth dependencies of PDOs, the functional classification of tumor subtypes based on distinct niche dependencies instead of genetic signatures may lead to identification of new molecular mechanisms underlying inter-tumor heterogeneity[144]. Previously unappreciated *GATA6*-mediated Wnt signaling dependencies identified in organoids have defined three functional subtypes of PDACs[88]. Similarly, gastric cancer organoids harboring simultaneous *TP53* and *CDH1* mutations gained R-spondin independency whereas combined CRISPR/Cas9 double knockout of *TP53* and *CDH1* in wild-type gastric organoids was sufficient to allow culture without R-spondin[86].

Organoid modeling of the tumor microenvironment

Cancers grow within a complex microenvironment where tumor epithelial cells are invested by diverse stromal cellular components, including fibroblasts, endothelial and immune cells[168-170]. The crucial roles of endothelial and immune components during tumorigenesis is particularly illustrated by the clinical efficacy of anti-angiogenic and immunotherapies, respectively[171,172]. Nowhere has the need for holistic tumor microenvironment culture been more acute than for tumor immunology and the accompanying need to study interactions between cancer cells and their veritable ecosystem of co-habitating immune cells. However, conventional organoid models of cancer have typically only represented tumor epithelium, and holistic culture of cancer cells alongside endogenous stromal elements has been elusive. In the absence of culture systems allowing tumor cells and stroma to be preserved alongside each other, numerous studies have reconstituted heterologous cell types along with organoids containing solely tumor epithelium. Examples include "tumor-only" submerged organoids with addition of distinct cancer-associated fibroblast (CAF) subtypes[173,174], or supplementation with chimeric antigen receptor (CAR) natural killer (NK) cells to study

tumor cytotoxicity[175]. Of note, multiple rounds of co-culture of PDOs with matched autologous peripheral blood lymphocytes were sufficient to select for tumor-reactive T cells, which performed specific killing of tumor cells but not matched normal organoids[176].

As typical PDOs only contain tumor epithelium, there has been a singular lack of robust *in vitro* culture systems that retain cancer cells alongside infiltrating endogenous immune stroma and recapitulate *in vivo* checkpoint inhibition responses. However, recent ALI tumor organoid studies have robustly preserved the complex cellular diversity and physical architecture of both endogenous tumor and stroma compartments. PDOs in ALI preserved cancer-associated fibroblasts, numerous infiltrating immune cells, such as T cells (cytotoxic, helper, regulatory and exhausted), B cells, NK cells and macrophages over 1-2 months[40]. Importantly, subsets of ALI PDOs from non-small cell lung cancer, melanoma, renal cell carcinoma and bladder contained functional tumor-infiltrating lymphocytes (TILs) that rapidly exhibited clonal expansion, activation and/or cytotoxic responses upon short-term PD-1/PD-L1 checkpoint blockade treatment. Additionally, mouse tumors from syngeneic immunocompetent hosts could be similarly cultured in ALI with anti-PD-1/PD-L1-dependent T cell responses[40]. Although this type of holistic tumor microenvironment culture is in its infancy, potential applications include mechanistic immunooncology studies, screening of novel immunomodulatory agents and precision medicine.

Studying tumor heterogeneity

Cancer organoid cultures present an opportunity to capture the intratumoral heterogeneity (ITH) of human cancer for *in vitro* investigation. Generally, tumor organoids retain mutations from the tissue of origin[16,34,142,150,162], as well as copy number status [108,140]. However, PDOs can undergo dynamic changes in culture. Many studies only compare early passage organoids with the tumor tissue but following serial passaging subclonal mutations can be gained or lost, although truncal mutations tend to be retained[153,164]. Long-term culture of CRC organoids for >6 months revealed that MSI lines with mismatch repair deficiency developed 75 and 82 *de novo* non-synonymous mutations respectively, whereas a microsatellite stable (MSS) was comparatively unchanged[144]. Liver tumor organoids retained around 92% of the tissue mutations after <2 months and >80% of mutations after >4 months in culture[162]. Thus, organoid cultures show strong correlation with tissue mutational status at early passages, but genetic drift and selection can occur. In addition, any dynamic PDO genetic or epigenetic changes in culture could be biased by culture media that may not fully reflect *in vivo* conditions.

Since organoids can only represent their originating tumor biopsy, faithful recapitulation of tumor heterogeneity may require culture from multiple sites of a single tumor or from distinct metastatic sites. Several examples of organoid cultures from multiple tumor regions indicate that these “sibling” cultures can manifest distinct drug sensitivity profiles. For example, organoids from different metastases from a PDAC patient showed similar response to three chemotherapy drugs, but different responses to 5-FU[150]. In CRC, five sibling PDO cultures from multiple regions of the same tumor exhibited up to 30 fold differences in drug response, suggesting that a single tumor biopsy may not be enough to capture all ITH[143]. A study of 27 liver cancer organoids from 5 patients indicated that most tested drugs possessed

low intra-patient variation, but several drugs, including targeted therapies such as tyrosine kinase inhibitors, displayed high intra-patient variability[160]. The most extensive ITH study performed genomic, transcriptomic and epigenetic analysis of 78 clonally derived CRC PDOs across multiple tumor regions from 3 previously untreated patients[104]. Clonal organoids from the same region shared common driver mutations but could still exhibit substantial differences in overall mutational content (40%) and sensitivity to chemotherapeutics and targeted drugs (3 log variation in IC_{50}). In this study, principal component analysis of methylation and transcriptional state showed that while clones from the same patient clustered together, subclones, (for example *TP53* WT vs *TP53* mutant) clustered separately. Overall, organoid ITH studies indicate that although concordance exists, even clonal organoids derived from the same tumor region can exhibit highly variable mutations, transcriptomes, epigenomes and drug responses. Additionally, PDOs can model tumor mutational evolution with shared truncal mutations leading to subclonal divergence.

Organoid-based functional precision medicine

Precision Medicine is a treatment approach that seeks to exploit patient-specific individualized therapeutic strategies[177]. Given their tractability and potential to capture patient and tumor type diversity, organoids are well-suited for the development of personalized therapeutic approaches.

Drug screening against organoid biobanks

The diversity of cancers captured by organoid biobanks has created numerous applications for therapeutics screening. Such drug sensitivity profiling can be particularly powerful when combined with genomic and/or transcriptomic organoid characterization, identifying specific drug response profiles for different tumor subtypes, or alternatively, defining sensitive and resistant PDOs. PDOs can also be used for synthetic lethality analysis, identifying drugs with selective efficacy against organoids with specific mutation(s).

Numerous examples include screening a liver cancer organoid biobank of 6 liver PDOs against 29 anti-cancer drugs, which nominated ERK inhibitors as a potential therapeutic agent for a subset of liver cancers[162]. Similarly, exposure of 27 liver cancer organoid lines from multiple tumor regions of 5 patients to 129 FDA-approved anti-cancer drugs revealed 13 drugs with broad cytotoxicity including inhibitors of histone deacetylase (HDAC), proteasome, DNA topoisomerase II, protein translation and RNA synthesis, as well as several novel agents. Comparison of organoid drug response with publicly available drug response databases demonstrated separate clustering of organoids versus traditional liver cancer cell lines, indicating that primary 3D PDOs may respond to drugs differently than conventional 2D-cultured cells[160]. Systematic exposure of a genetically characterized 66 patient pancreatic cancer PDO biobank to five clinically used chemotherapeutic agents such as gemcitabine, revealed that 33% of PDAC samples were resistance to all five drugs, but half of the multi-resistant lines were sensitive to a targeted therapy when tested against a panel of 21 such agents, suggesting alternative treatment strategies. In this case, correlations between PDO gene expression and drug sensitivity revealed a gemcitabine drug sensitivity gene signature that significantly correlated with improved progression-free survival (PFS)

upon post-surgical adjuvant gemcitabine treatment in a 55 patient cohort[150]. Large-scale drug screening of PDOs may also identify unexpected treatments or combinations thereof. Evaluation of gastric cancer PDOs against a 37 compound library has indicated genotype-specific vulnerabilities[140]. Further, generation of matched tumor and normal PDOs from the same patient was exploited to discover the gemcitabine as a potentiator of the EZH2 inhibitor UNC1999 combination in PDAC PDOs[94].

Drug sensitivities between sibling pairs of PDOs and PDXs have been compared in CRC models[141]. In a panel of 46 PDOs and 59 PDXs, the 19 tumors captured in both systems displayed fairly concordant responses to 16 tested drugs. However, different patterns were observed for individual drug sensitivity and RNA signatures for hypoxia, EMT, G2/M checkpoints, proliferation, stemness and metabolism. Since both PDOs and PDXs recapitulate many key genetic and phenotypic features of their parental tumors but possess distinct drawbacks[135], future studies combining the strengths from these preclinical models could provide clinically relevant metrics in precision medicine.

The large number of developing organoid biobanks provides a unique opportunity for targeted drug development[178]. Compound screening against large, well defined organoid repositories has the potential to identify drugs efficient against a subsets of tumors with a specific genetic composition. While such screens have been perfomed extensively for cells grown in 2D monolayers[179,180], emerging evidence suggests that cells grown in 3D often respond differently to drugs[160], including increased chemoresistance[181]. For example, 2D versus 3D drug responses in ovarian cancer found correlation for cytotoxic but divergence for cytostatic drugs[182]. Conceivably, 3D organoid biobanks could lower false positives in large compound screens, which in turn could reduce the extent of expensive confirmatory preclinical mouse studies. In addition, organoids can be grown from corresponding healthy tissue which would give an early indication of compound toxicity, as well inform underlying biology[94,183]. Organoid-based models to fully replace mouse experiments for drug discovery have yet to emerge, but there are encouraging developments including *en bloc* organoid culture with tumor and infiltrating immune cells[40] and integrated multi-organoid body- on-a-chip systems to study drug metabolism and toxicity[184]. Positive compound hits will still need to be verified in xenograft experiments in the foreseeable future.

Overall, current nascent efforts with PDO biobanks represent a promising approach to screening therapeutics against different molecular subtypes within distinct solid tumors. The rigorous genetic characterization of such PDOs, combined with small molecule screening, has potential to identify new patient-relevant gene-drug interactions and synthetic lethal vulnerabilities, yielding novel insights versus traditional cancer cell lines and PDX models. Additionally, the biobank paradigm has broad application beyond cancer, such as with recently established cystic fibrosis organoid resources[185].

Precision medicine and personalized therapy design

Precision Oncology efforts have traditionally exploited predictive mutational biomarkers for targeted therapies. However, such biomarkers are often lacking[145]; for instance, whole exome sequencing on a mixed cohort of tumors only identified actionable mutations with cognate FDA-approved drugs for 3 of 737 patients (0.4%)[177]. Even when targetable

biomarkers are discovered, patients do not always respond to targeted therapy, raising a pressing need for strategies to predict efficacy or identify alternative options[177].

PDO technologies represent a tantalizing opportunity for functional therapeutic response prediction in living cells. Several studies have reported that PDOs can forecast patient anticancer drug responses for a limited number of cases (n<5)[28,34,140,151,186]. A relatively few number of studies have more cases (n>5 and <10)[146,150,158]. Generally, published tumor organoid drug responses correlate with patient outcome. For example, in an organoid biobank of well-characterized pancreatic cancer PDOs eight out of nine organoids responded similarly to the cognate patients when exposed to chemotherapy, giving a match rate of 89%[150]. To date, only a handful of studies have measured patient and organoid drug correlations for more than 10 patients. For example, patient-organoid drug response across different gastrointestinal tumor types and drug regimens indicated the matched organoids could correctly predict responders and non-responders in 20 of 21 patients[142]. Similarly, comparison of metastatic colorectal patient versus PDO responses indicated relative success for irinotecan monotherapy (n=10), and the combination of irinotecan and 5-FU (n=12), but not for a combination of 5-FU and oxaliplatin (n=10). The authors speculated that the failure of PDOs to predict oxaliplatin response may be due to the lack of an immune system and stroma in organoids[145].

Two recent publications have concluded that organoids can accurately portend responses to neoadjuvant chemoradiation (NACR) in rectal cancer. A large study established 80 PDOs from biopsies of treatment-naive patients with locally advanced rectal cancer (LARC) from a phase III clinical trial of neoadjuvant radiation combined with 5-FU with or without irinotecan[147]. Organoids were treated with radiation, 5-FU or irinotecan separately but not in combination. Considering the organoids as sensitive to NACR when sensitive to at least one of the three treatments (radiation, 5-FU, irinotecan), the study correctly predicted outcome in 85% of patients. Since many organoids were only sensitive to single or double agents, the authors hypothesized that not all three therapies are necessary in combination, and that organoid screening can identify ineffective treatments and thus avoid overtreatment. Another study, also studying NACR in rectal cancer, indicated varying sensitivity of rectal PDOs to *ex vivo* radiation, which correlated with clincal response in a cohort of 19 patients[146].

Taken together, functional precision medicine, where individualized predictions are made based on a cellular drug response test instead of identification of a biomarker, is an intriguing prospect for PDOs. The success rate of establishing cultures in current biobanking efforts varies substantially between tumor types, but can be high (>80%), suggesting the feasibility of organoid-based functional precision medicine. From these few initial studies, some preliminary observations can be made. Despite tumor heterogeneity being a significant potential confounding variable for extrapolating therapeutic response from a single biopsy region, functional prediction appears to be feasible at least in the specific tumor types and drugs tested. However, the current lack of stromal and immune compartments in most organoid-based therapy prediction systems may preclude universal applicability. Negative results have not been described, raising the issue of publication bias, and perhaps most significantly, studies to date have trained predictive metrics based on foreknowledge of clinical outcome. Although current studies depict high (80-95%) rates of concurrency, it will

be necessary to observe if initial successes persist in larger prospective independent validation cohorts of patient-organoid pairs where response metrics are fixed in advance.

Challenges and Perspectives

Organoid culture technologies have rapidly exerted a transformative impact on cancer research, with widespread adoption for both basic and translational applications. However, despite promising studies to date, numerous challenges remain. Although tumor PDOs often preserve the genetic composition of the original tumor at early passages, the extent of genetic drift, clonal selection and continued tumor evolution at later passage and after freeze-thaw cycles has not been well documented and may be tumor-specific[144]. Similar caveats apply to forward oncogene engineering of normal tissue as well. Differences in seemingly normal cells with regard to telomere length, genetic and epigenetic state could potentially influence phenotypic outcome. In addition, the ordering of mutations in serial genetic engineering, the exact location, alteration and duration of CRISPR knock-out modifications, genetic bottlenecks introduced by selection of rare transformed clones, and presumed ongoing evolution may all produce variable results. This may account for differences in invasive behavior between two recent organoid models of colorectal cancer that engineered the inclusion of the same set of mutations[76,77].

Another key limitation of conventional organoid methods is a demonstrated lack of endogenous tumor-associated stromal components, particularly immune cells and fibroblasts. Thus, the continued development of organoid systems that more holistically represent the tumor microenvironment is an urgent need. Ultimately, PDOs incorporating immune and other stromal components may open new research directions such as immuno-oncology and help actualize the promise of precision cancer therapies.

Although essential culture components for growing organoids from different normal tissues and cancers have been widely investigated, costs are substantial and reagents can vary between laboratories, abetted by use of conditioned medium containing factors such as Wnt3a, R-spondin, and Noggin. Further, the presence of serum in conditioned media may disadvantage long-term culture[88]. Recently developed artificial water-soluble Wnt agonists[187] or afamin-stabilized Wnt3a[188] may facilitate serum-free alternatives to conventional Wnt conditioned media[4] and enable economical, fully defined and recombinant culture reagents. A practical challenge is reproducing the potency of organoid media across different batches and laboratories, which may provide a selection pressure for a subset of cells that might not be representative of original tumors. At the same time, the evaluation of numerous different culture conditions can improve success rates of PDO generation[144].

Current CRISPR/Cas9-mediated oncogene engineering in primary human organoids has been inefficient and largely restricted to selected genes in which mutational pathway activation allows bespoke media-based functional selection strategies to enrich for gene knockouts. Future approaches may bypass such screening requirements, perhaps by allowing phenotypic screening, increasing efficiency or using selectable knock-in cassettes. Opportunities also exist for large-scale genetic and chemical perturbations of organoids. Improved, highly efficient editing methods[185,189,190] could facilitate screening of a broader

range of target loci, extending to high-throughput genome-scale functional screens[191,192]. Similarly, small molecule screening in organoids has been restricted to modest panels of therapeutics, which could be enlarged to more extensive compound libraries by ultra-high throughput miniaturized cultures[193]. The scale of both genetic and small molecule screens could be substantially augmented by methods to expand organoid biomass to much larger degrees than currently practiced.

Perhaps the most revolutionary potential applications of organoids for oncology reside in therapeutic prediction. Numerous studies suggest the feasibility of PDO-based functional testing of patient drug responses, which now require prospective confirmation in validation cohorts. Optimally, biologic organoid testing will require rapid turnaround in clinically actionable timeframes, evidence of enhanced survival advantage and conceivably, extension to modalities such as immunotherapy. Additionally, the creation of ever-enlarging PDO biobanks, for example with the HCMI initiative[165], may establish large collections representing diverse cancer subtypes and genotypes, whose systematic drug screening could establish ready-made therapeutic response patterns that are possibly relevant to patients with similar tumor genotypes. Such transformation of prior descriptive metrics into organoid-based biological predictive assays using living cells would be a capstone of the rapid progress with cancer organoids to date, portending additional advances in both basic biology and clinical translation.

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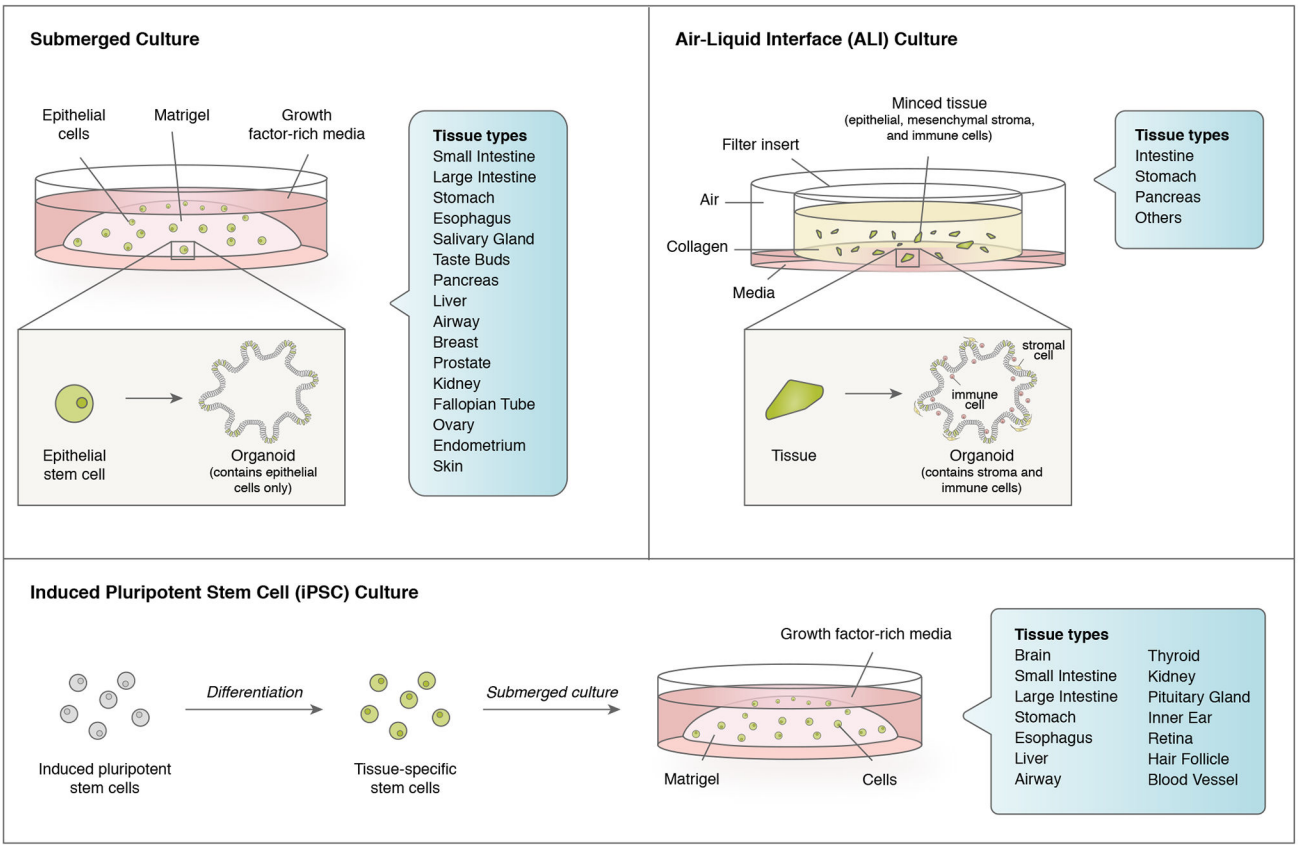

Figure 1. Organoid methodologies.

Currently employed organoid methodologies include submerged culture, which typically solely includes epithelial cells, and air-liquid interface (ALI) culture, which is a more organotypic method including epithelial cells alongside integrated stroma and immune cells. As opposed to submerged and ALI methods which utilize adult tissues as starting material, organoids can be generated from uncommitted induced pluripotent stem cells (iPSCs) by a series of differentiation steps resulting in generation of the desired tissue type, often with accompanying stroma. Organoids can be additionally classified by their tissue of origin for all three methods.

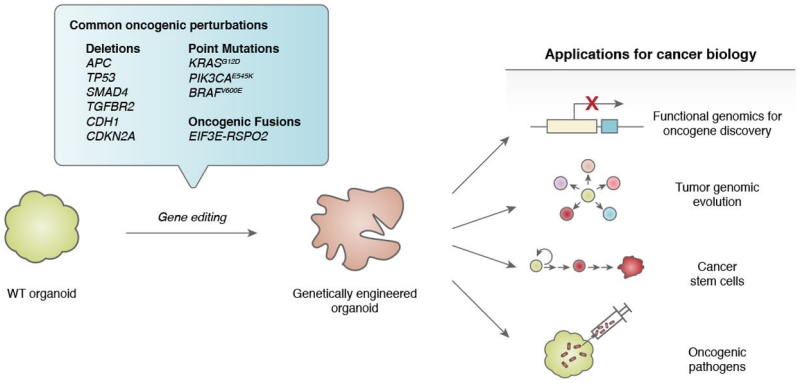

Figure 2. Bottom-up cancer modeling in wild-type organoids.

Wild-type tissue organoids can be engineered with tumorigenic alterations to model cancer initiation, evolution, initiating cells and oncogenic pathogens.

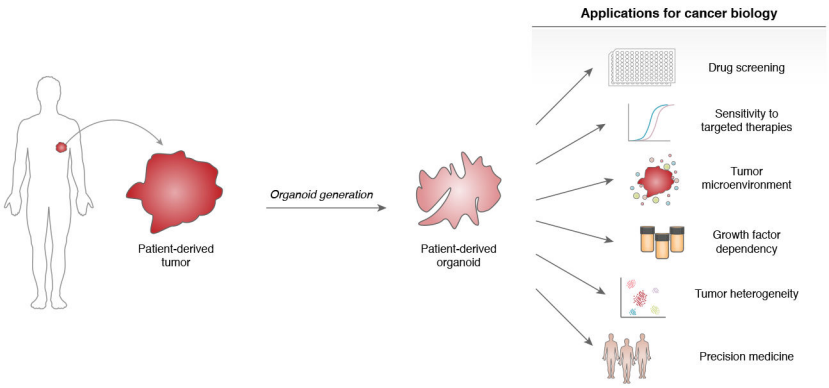

Figure 3. Top-down cancer modeling in patient-derived tumor organoids.

Human patient-derived tumor organoids possess numerous applications for translational cancer research. Potential uses include novel therapeutic drug screening, the study of the tumor microenvironment and tumor heterogeneity, as well as personalized medicine and response prediction.

Table 1.

Overview of Human Patient-derived Tumor Organoid (PDO) Biobanks

Tumor Site	N	Histological Subtype	Sampling	Success Rate	Drug Testing	Correlation to Patient Outcome	Ref
Colorectum	22	Colorectal carcinoma	Surgical resection	90%	Yes (83 drugs)	No	136
Colorectum	55	Neuroendocrine carcinoma Premalignant lesions Adenocarcinoma Metastases of adenocarcinoma	Surgical resection Endoscopic biopsy	100%	No	No	144
Colorectum	10	Colorectal adenocarcinoma Metastatic colorectal adenocarcinoma	Tissue biopsies	71%	No	No	137
Colorectum	91	Colorectal adenocarcinoma (primary and metastatic specimens)	Surgical resection Synchronous multi-regional sampling PDX-derived	58%	Yes (10 drugs)	No	143
Colorectum	46	Colorectal adenocarcinoma (sibling pairs of PDOs versus PDXs)	Surgical resection	60%	Yes (16 drugs)	No	141
Colorectum	40	Metastatic colorectal adenocarcinoma	Tissue biopsies	63%	Yes	Yes (N=29)	145
Rectum	65	Rectal adenocarcinoma	Tissue biopsies	77%	Yes	Yes (N=13)	146
Rectum	96	Rectal adenocarcinoma Rectal mucinous adenocarcinoma	Tissue biopsies	86%	Yes	Yes (N=21)	147
Pancreas	8	Ductal adenocarcinoma	Surgical resection	75%-80% normal 75%-83% cancer	No	No	16
Pancreas	39	Ductal adenocarcinoma	Surgical resection EUS-FNA* Ascites puncture	80%	No	No	88
Pancreas	114	Ductal adenocarcinoma (primary and metastatic specimens)	Surgical resection Tissue biopsies	75%	Yes	Yes (N=8)	150
Pancreas	17	Ductal adenocarcinoma	Surgical resection	85%	Yes	No	94
Pancreas	52	Ductal adenocarcinoma	Surgical resection Tissue biopsies	62%	Yes (76 drugs)	Yes (N=4)	151
Stomach	37	Gastric adenocarcinoma	Surgical resection Endoscopic biopsy Ascites puncture	55%-75%	No	No	86
Stomach	63	Normal, dysplastic, and cancer Lymph node metastases	Surgical resection	>90% normal 50% cancer	Yes (37 drugs)	Yes (N=3)	140
Stomach	20	Gastric adenocarcinoma	Surgical resection	N/A	Yes	No	149
Stomach	7	Gastric adenocarcinoma	Sleeve gastrectomies	N/A	Yes	No	148

Tumor Site	N	Histological Subtype	Sampling	Success Rate	Drug Testing	Correlation to Patient Outcome	Ref
Liver	7	Hepatocellular carcinoma Cholangiocarcinoma	Surgical resection	100%	Yes (29 drugs)	No	162
Liver	10	Hepatocellular carcinoma	Tissue biopsies	26%	Yes (only Sorafenib)	No	161
Liver	27	Hepatocellular carcinoma Cholangiocarcinoma	Surgical resection	N/A	Yes (129 drugs)	No	160
Bladder	20	Urothelial carcinoma Squamous cell carcinoma	Cystectomy Endoscopic biopsy Cold loop resection	70%	Yes (40 drugs)	No	153
Bladder	77	Urothelial carcinoma (both basal and luminal subtypes)	Radical cystectomies Transurethral resection (TUR)	N/A	Yes (6 drugs)	No	152
Prostate	7	Adenocarcinoma metastases Circulating tumor cells	Tissue biopsies	15%-20%	Yes (2 drugs)	No	138
Prostate	4	Neuroendocrine prostate cancer	Tissue biopsies	16%	Yes (129 drugs)	No	154
Ovary	33	High-grade serous carcinoma	Ascitic or pleural fluid	80%-90%	Yes (4 drugs)	Yes (N=2)	34
Ovary	56	Borderline tumors Clear cell carcinoma Endometrioid carcinoma Mucinous carcinoma Serous carcinoma	Surgical resection Ascitic or pleural fluid	85%	Yes (7 drugs)	No	33
Breast	95	Ductal adenocarcinoma Lobular adenocarcinoma	Surgical resection	>80%	Yes (6 drugs)	Yes (N=2)	28
Esophagus	15	Esophageal squamous cell carcinoma Oropharyngeal squamous cell carcinoma	Tissue biopsies	71%	Yes (only 5-FU)	No	156
Esophagus	10	Esophageal adenocarcinoma	Surgical resection	31%	Yes (24 drugs)	No	157
Oral Mucosa	31	Head and neck squamous cell carcinoma	Surgical resection	65%	Yes (8 drugs)	Yes (N=7)	158
Endometrium	72	Normal, endometriosis, hyperplasia, low- and high-grade carcinomas	Tissue biopsies	N/A	Yes (5 drugs)	No	159
Mixed	56	Tumors from prostate, breast, colorectal, esophagus, brain, pancreas, lung, small intestine, ovary, uterus, soft tissue, bladder, ureter, and kidney	Surgical resection Tissue biopsies	39%	Yes	No	177
Mixed	23	Metastatic colorectal cancer Gastroesophageal cancer	Tissue biopsies	70%	Yes (55 drugs)	Yes (N=21)	142

* Endoscopic ultrasound guided-fine needle aspiration (EUS-FNA)