Chapter 12

In Vivo Pharmacology Models for Cancer Target Research

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Abstract

Experimental animal tumor models have been broadly used to evaluate anticancer drugs in the preclinical setting. They have also been widely applied for drug target discovery and validation, which usually follows four experimental strategies: first, assess the roles of putative drug targets using in vivo tumorigenicity and tumor growth kinetics assays of transplanted tumors, engineered through gain-of-function (GOF) by overexpressing transgene or knock-in (KI) or loss-of-function by gene silencing using knockdown (KD) or knockout (KO) or mutation via mutagenesis procedures; second, similarly genetically engineered mouse models (GEMM), through either germline or somatic cell procedures, are used to test the roles of potential targets in spontaneous tumorigenicity assays; third, patient-derived xenografts (PDXs), which most closely resemble patient genetics and histopathology, are used in tumor inhibition assays for evaluating target-/pathway-specific inhibitors, including large and small molecules, thus assessing the drug target; and fourth, the targets can be assessed in population-based trials, mouse clinical trials (MCT), so that the validation can be generally meaningful as performed in human clinical trials. This chapter outlines the commonly used protocols in cancer drug target research: the first four sections describe four sets of different, specific pharmacology protocols used in the respective cancer modeling stages, with the last section summarizing the common protocols applicable to all four pharmacology modeling steps.

Key words Xenograft, PDX, Homograft, GEMM, Tumorigenesis, Tumor growth kinetics, Tumor growth inhibition, Transgene, Knock-in, Knockout, Knockdown

1 Introduction

One of the straightforward strategies for assessing a gene target is to overexpress or silence the gene of interest in a human cell line. This can be performed through transgene overexpression or gene knock-in (KI) (gain-of-function, or GOF), by knockdown (KD or silencing)/knockout (KD/KO), or by other means of loss-of-function (LOF), thus impacting tumorigenicity (tumor formation in vivo) or tumor growth kinetics. Methods of cellular genetic

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manipulations including transgene, gene KI, gene KO, mutation introduction (e.g., mutagenesis or gene editing such as CRISPR-Cas9) [1, 2], and RNAi-mediated gene silencing (e.g., shRNA) [3] have been extensively described elsewhere and are described in more detail in other chapters of this book. Due to the fact that the majority of drugs are inhibitors of a given target, rather than activators, the more relevant experimental approaches to validate a cancer drug target are LOF of the gene target (usually "oncogenes"), instead of transgene expression.

It is worth mentioning that when investigating a drug target for in vivo assessment, silencing many oncogenes may be embryonically lethal or lead to developmental defects, therefore hindering the evaluation of such targets in adulthood. Therefore, in many instances, a conditional (induced) KD/KO in vivo becomes necessary (e.g., by tet-on or tet-off system). A brief outline of tumorigenicity and tumor growth kinetics studies are summarized as follows: the engineered tumor cell lines are implanted subcutaneously (SC) or orthotopically into immunocompromised mice (e.g., nude, NOD/SCID, NSG®, NOG®) [4]. Tumor growth is monitored by cage-side observation and by measuring tumor volume (TV) using calipers and/or imaging methods. An experiment management software such as Studylog® can be utilized to directly measure and store the TV and body weight (BW) data. Tumor growth kinetics are determined by the TV and duration of the study. A negative impact on tumorigenicity and growth kinetics means that the targets are "oncogenes" and that they can be further investigated as potential drug targets (Fig. 1).

When utilizing inducible expression or silencing vectors [5], the engineered cells are implanted as described above, followed by subsequently randomly grouping into treatment and control groups and subjecting to induction at desired time points in vivo. In many situations, the control vector-engineered cells are processed in parallel as additional controls. Tumor growth is then monitored and compared among the groups.

Recently, libraries of shRNA using an RNAi approach or sgRNA using a CRISPR-Cas9 approach of genome-wide gene target validation have been introduced into cell populations [6–8], so that genes which negatively impact tumorigenicity/growth kinetics could be identified as potential drug oncogene targets. It is worth mentioning that negative selection/identification is usually much more challenging than positive selection [9–12].

The cell lines used for engineering can be human or mouse cell lines. Engineered human cell lines can be xenografted into immunocompromised mice, while engineered mouse cell lines can be homografted into syngeneic mouse strains, most of which are immunocompetent, therefore enabling certain immuno-oncology (I/O) targeting.

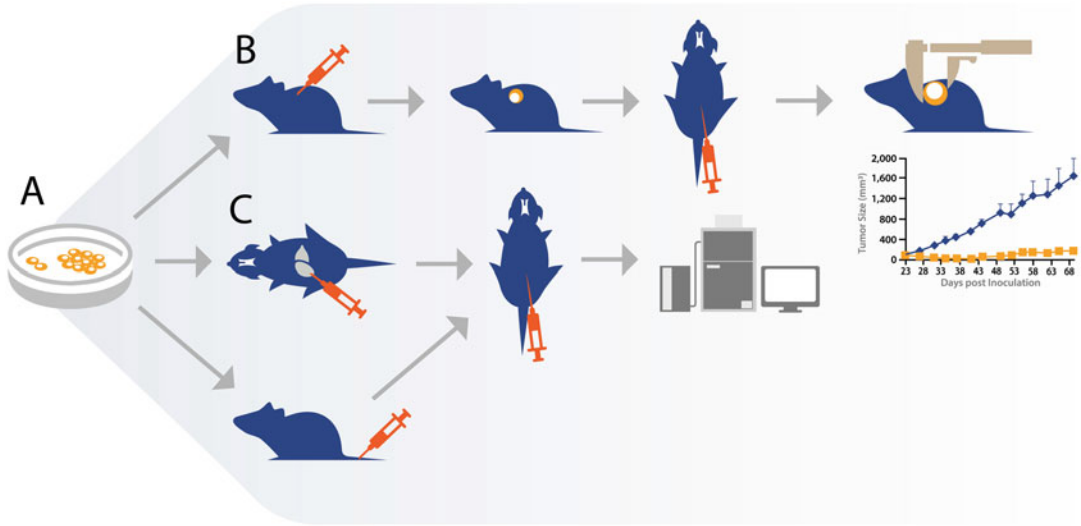

Fig. 1 Different tumor animal modeling. A: Genetically engineered tumor cell culture ± induction. B: SC inoculation, followed by induction if induced system is involved or ± pharmacological treatment. TV is measured by caliper. C: Orthotopic implantation (e.g., liver, spleen, mammary fat pad) or intravenously. Tumor burden is measured by imaging or autopsy

For over three decades, genetically engineered mouse models (GEMMs), based on germline or somatic cell gene alterations of key oncogenic/tumor-suppressor pathways, have enabled numerous insightful mechanistic findings on tumor onset, progression, and metastasis and also enabled evaluation of potential drug targets [4, 13]. LOF of tumor suppressors or GOF of oncogenes are introduced to produce GEMMs [14] where conditional expressing/silencing is achieved in a constitutive manner or tissue-specific/inducible regulation, resulting in spontaneous tumor development. Utilizing CRISPR/Cas9 technology for direct in vivo targeting, or targeting embryonic stem cells, has greatly improved the efficiency of GEMM creation [15]. In particular, multiplex Cas9-mediated genome editing [16, 17] enables simultaneous modeling of a multigene tumorigenesis process to recapitulate the complex combinations of genetic lesions in patients. Compared to transplanted tumors, where fully developed tumors are implanted into a naïve host, tumorigenesis in GEMMs represents de novo tumor onset and progression, mimicking tumorigenesis in patients and accompanied by escape from immune surveillance.

Tumorigenesis in GEMMs can be used to evaluate potential drug targets according to the introduced genetic alterations in mice, similarly as described above for transplanted tumors [18, 19]. Gross-necropsy or imaging methods (e.g., micro MRI) are usually required to monitor tumorigenesis [20–22]. GEMM cancer models have an advantage over xenografts in that mouse

immunity is intact, and therefore GEMM models are suitable for evaluating I/O targets [4]. These targets are only mouse surrogates of human targets, and therefore caution is needed for I/O target evaluation as mouse tumor/immunity may differ from human patients [23, 24].

On a separate note, genetic engineering techniques are also used to create chimeric GEMMs, which KI human targets, e.g., immune checkpoint genes, such as CTLA-4 or PD-1. This partially humanized GEMM, which we have named "HuGEMM™", can have mouse tumor homografts from syngeneic cell lines transplanted for facilitating evaluation of human I/O targets and therapeutics [4]. The study design is similar to the efficacy studies described in Subheading 4.

When a highly specific small molecule inhibitor or antibody is available against a potential gene target, it can directly be used to evaluate targeting by treating xenograft and homograft models. Patient-derived xenografts (PDX) are patient tumors grown in immunocompromised mice [4] which are known to maintain the patient tumor histopathology and molecular pathology [25, 26]. PDX are considered to be the model most predictive of the original patient response to treatment. PDX may not be readily used for I/O target evaluation due to its immunocompromised environment [4], but are particularly relevant for common target research. Selecting adequate PDX models per genetic characteristics, e.g., specific mutations or expression levels of one or a set of genes, is critical to testing hypotheses on potential drug targets [27–30]. The negative impact on tumor growth kinetics caused by specific agents can suggest that these genetic alternations might be oncogenic drivers or potential drug targets.

Blood cancer xenografts are the "liquid version" of PDX. Patient leukemia cancer cells are systemically inoculated into immunocompromised mice, resulting in the development of leukemia [30], where leukemic tumor load is usually determined by measuring human $CD45^+$ cells in peripheral blood using flow cytometry at various time points and in bone marrow, spleen, or other infiltrated organs at the terminal time point.

Homografts of spontaneous or induced murine primary mouse tumors (the "mouse version of PDX") [4], or simply syngeneic cell lines that grow in syngeneic immunocompetent mice, can be developed to evaluate mouse surrogate I/O therapeutics and therefore also their targets. Similar to PDX models, GEMM tumor-derived homografts recapitulate their original mouse disease significantly better than traditional, in vitro immortalized cell line-derived syngeneic models. This is observed for PDX vs. cell line-derived xenografts: maintaining heterogeneous histopathology as a typical cancer stem cell-driven disease [13]. Homografts derived from GEMM tumors with specific driver mutations are also a useful tool to assess potential targetability. The derived models also have

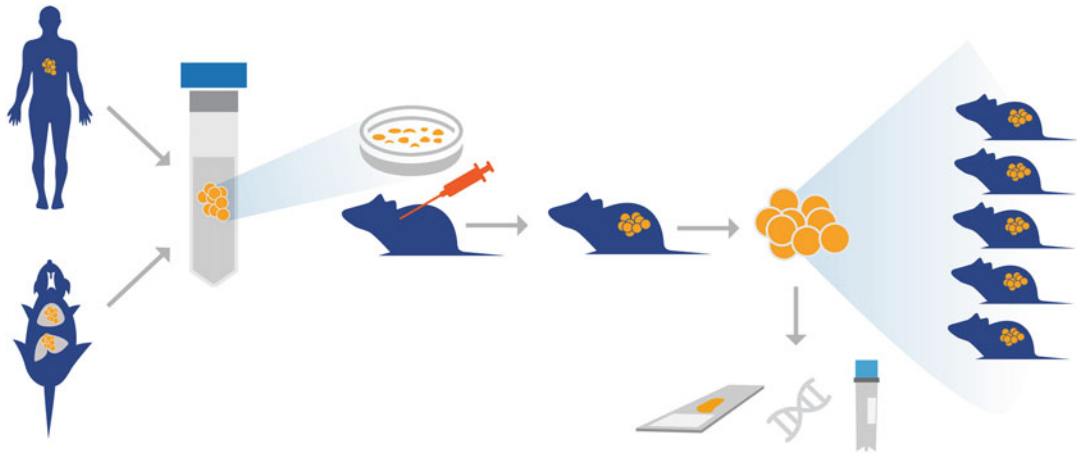

Fig. 2 PDX and mouse homograft tumor modeling. PDX or mouse primary tumors (e.g., spontaneous tumors from GEMM) are collected and cut into pieces of 2–3 mm in diameter. Chunks of these fragments are transplanted into mice using a trocar. Tumor growth is monitored and examined. Tumors are harvested when their volume reaches approximately 500–1200 mm^3 for downstream processes, including efficacy studies, cryopreservation banking, histopathology, and molecular pathology characterization

superiority and simplicity in operational handling as compared to the original GEMM tumors — due to their short duration and synchronized tumor development. The methods and usage of allograft models are rather similar to PDX (as described above), including profiling, characterization, etc. The process for PDX or homograft modeling is summarized in Fig. 2.

Alternatively, syngeneic cell lines derived from mouse tumor models are the most commonly explored models for preclinical I/O investigation. Most checkpoint inhibitors proof of concept (POC) was first confirmed in syngeneic models, e.g., PD-1 antibodies using MC38 tumors. Now, many syngeneic models have been extensively profiled genomically, immunologically, and for I/O agent efficacy by various laboratories. Furthermore, syngeneic tumor cell lines or mouse tumor homografts can be inoculated into humanized chimeric GEMM mouse to evaluate human-specific therapeutics directly [4].

This chapter provides some basic protocols that have been used in cancer pharmacology for animal models and which are applicable for cancer target research. These protocols intend to provide an elementary scope of the commonly used procedures for readers new to the field but are by no means comprehensive and detailed. Readers will need to generate more detailed protocols based around their own specific research objectives prior to conducting their investigations. *See* Table 1 for frequently used abbreviations.

Table 1
Frequently used abbreviations

Abbreviation	Definition
AFP	Alpha-fetoprotein
AUC	Area under curve
BME	Basement membrane extract
BW	Body weight
CRISPR	Clustered regularly interspaced short palindromic repeats
CV%	Standard deviation of TV/average TV
DOX	Doxycycline
ECM	Extracellular matrix
FFPE	Formalin-fixed paraffin-embedded
GEMM	Genetically engineered mouse model
GFP	Green fluorescence protein
GOF	Gain of function
IF	Immunofluorescence
IHC	Immunohistochemistry
I/O	Immuno-oncology
IP	Intraperitoneal(ly)
IV	Intravenous(ly)
IVC	Individual ventilated cages
IVIS	In vivo imaging system
KD/KO	Knockdown/knockout
KI	Knock-in
LOF	Loss of function
MCT	Mouse clinical trials
MRI	Magnetic resonance imaging
MTVR	Maximum tumor volume reduction
PD	Pharmacodynamic
PDX	Patient-derived xenograft
PO	Oral(ly)
POC	Proof of concept
QC	Quality control
RNAseq	RNA sequencing or transcriptome sequencing

(continued)

Table 1
(continued)

Abbreviation	Definition
SC	Subcutaneous(ly)
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SOC	Standard of care drug
SPF	Specific pathogen-free
STR	Short tandem repeats
TCGA	The Cancer Genome Atlas
TV	Tumor volume

2 Assay Tumorigenicity and Growth Kinetics of Transplanted Tumors Derived from GEMM Tumors or Engineered Cell Lines of a Gene(s) of Interest

2.1 Materials

The common and general materials and reagents are described in Subheading 6.

2.1.1 Project-Specific Materials

Doxycycline, Sigma, MO, USA.

Pancreatin, Sigma, MO, USA.

Trypsin.

DMEM.

BioCoat™ Collagen 25 cm^2 rectangular canted neck cell culture flask with vented cap.

2.1.2 Animals

Immunocompromised mice: NOG/NSG, NOD-SCID, B/C nude, or nu/nu mice.

Immunocompetent mice: C57BL/6, Balb/c, FVB/N, and other strains.

Mice are purchased from local suppliers in the major markets.

2.1.3 Cell Lines

The cell lines used for engineering can be human cancer cell lines and mouse tumor cell lines, many of the cell lines can be obtained from the ATCC and other depositories.

2.2 Methods

1. Inducible vectors are transferred into the tumor cell line.

2.2.1 Study Design

2. Inoculate the engineered cell line and vector control cell line into mice for tumor development.

3. Monitor the tumor growth for tumorigenicity after inoculation, or after induction of the gene KD/KO, by cage-side observation.
4. After tumors become palpable, begin TV measurement.
5. Compare the difference in tumor growth kinetics between engineered and control cell lines.

2.2.2 Culture and Prepare Tumor Cells

The genetically engineered cancer cell lines are expanded in vitro via tissue culture prior to inoculation. As the cell number per injection site varies greatly between different injection methods or cell types (normally around 10^4 to 10^6 cells per site), the scale of culture will be considered accordingly.

1. Tumor cell lines are grown in complete medium and maintained as monolayer cultures.
2. Once the cell lines are growing in the exponential growth phase and reach 70% confluence, they are harvested for inoculation.
3. Remove the medium and rinse the cells briefly with PBS.
4. Digest the cells with 0.25% trypsin until they detach from the flask, shake the flask and stop trypsinization by adding FBS-containing medium, and then slowly pipette to resuspend cells.
5. Spin down the cells at 4 °C by centrifugation at 1000 × *g* for 5 min.
6. Resuspend the cell pellet in cold PBS by slowly pipetting the cell pellet using a 1 mL pipette.
7. Count the cells with a particle counter or hemocytometer, and assess the cell viability using trypan blue staining. Cell viability should be >95% to ensure successful inoculation.
8. The cell suspension is re-pelleted via centrifugation, followed by resuspending in serum-free medium to obtain the cell suspension in the required concentration. The volume of each inoculation should be around 100–200 μL.

2.2.3 Model Establishment

General methods for the establishment of transplant tumors will be described in Subheading 7 General Protocols, including animal housing, tumor cell inoculation, and BW/TV monitoring.

2.2.4 Induction of Gene Silencing or Transgene Expression

1. DOX (doxycycline) will be given via drinking water that contains 5% sucrose [5] or chow.
2. The treatment is initiated on the day the cells are inoculated [5, 31] or when tumor volume reaches a predefined size (e.g., ~100 mm^3) [32], thus allowing assessment of the staged tumor response to treatment.

3. The animals are monitored for tumor growth and overall health during the induction period or until study endpoint.
4. Tumor-bearing mice are euthanized, and tumors are excised and weighed before sampling.

2.2.5 Efficacy Readout

The difference in growth kinetics is calculated based on the TV measurement, which is described in Subheading 7.

3 Assay Spontaneous Tumorigenesis of Mice with Target Gene KO/KI

3.1 Materials

The common and general materials and reagents are described in Subheading 7. GEMM and immunocompetent mouse strains can be obtained from various vendors in the different markets.

3.2 Methods

3.2.1 Study Design

Spontaneously tumorigenic GEMMs develop tumors; however, this usually takes a long period of time, which varies from model to model (general range, 2–6 months). This leads to the requirement of a large number of study animals to ensure statistically significant results. The mice are randomized into different treatment groups per BW, followed by tumor formation monitored via clinical observation, necropsy, and/or MRI.

3.2.2 Clinical Observation

GEMM mice spontaneously developing tumors, which may also be accompanied by certain clinical signs, which need to be closely monitored (daily). The method of clinical observation is described in Subheading 7.

3.2.3 Mouse MRI

In contrast to superficially implanted tumors (e.g., SC-transplanted) that can be monitored by standard caliper measurement, autochthonous tumor growth in GEMMs must be monitored by longitudinal imaging strategies, e.g., MRI. The MRI method is described in Subheading 7.

3.2.4 Necropsy

In many cases, tumor-bearing mice with certain clinical symptoms are sacrificed for necropsy. Tumors and organ tissues are collected as formalin-fixed paraffin-embedded blocks (FFPE) or snap frozen samples for pathology and genetic analysis. GEMM can have organ preference for tumor development. For instance, KRAS-G12D GEMM result in spontaneous tumorigenesis in the intestines or the lung [33]. Therefore, those involved organs are usually examined for gross and microscopic pathology. The necropsy and pathology evaluation is normally only focused on targeted organs. Necropsy and sample collection methods are described in Subheading 7.

4 Assay Tumor Growth Kinetic Changes Caused by Target-Specific Agents: Small Molecular Inhibitors or Antibodies

4.1 Materials

4.1.1 Specific Reagents

1. RPMI-1640 with 2× antibiotic and antimycotic.
2. RPMI-1640.
3. Ficoll-Paque™ PREMIUM.
4. FITC antihuman CD45 antibody BioLegend®, CA, USA.
5. Red blood cell lysis buffer (generic).
6. EDTA routine blood tube 2 mL.
7. TaKaRa Taq™ (Taq DNA polymerase) Takara Bio.
8. dNTPs (generic).

4.1.2 Animals

Immunocompromised animals for xenografts: NOG/NSG, NOD-SCID, B/C nude, or *nu/nu* mice.

Immunocompetent animals for homografts: C57/B, chimeric GEMM mouse.

4.2 Methods

4.2.1 Study Design

1. Usually 5–10 mice per arm are used in treatment studies.
2. Model selection can be based on the cancer type, pathology diagnosis, genetic profile, growth features, and/or standard of care treatment (SOC) efficacy.
3. Dosing is similar to that described for other models above.
4. For blood cancer, the tumor growth kinetics are determined by human $CD45^+$ levels in blood.

4.2.2 Model Establishment

Solid Tumor PDX

1. The patient tumor is collected by surgery or biopsy.
2. The collected patient tumor is kept in a sterile 50 mL tube containing 30 mL 4 °C transfer media immediately postsurgery and transferred to an animal facility within 6 h.
3. Tumors are cut into chunks of 2 mm in diameter.
4. Inoculate the tumor chunk on the right flank site of immunocompromised mice (further details found within Subheading 7). The mice are observed each week for tumor development.
5. No palpable tumor developed within 120 days is considered unsuccessful engraftment.
6. Any developed tumor will be serially transplanted into new immunocompromised mice and preserved for banking and characterization.

Leukemia PDX

1. The patient bone marrow sample is collected into a sterilized 50 mL tube containing 4 °C 30 mL transfer media and transferred to an animal facility within 6 h.

2. Mononuclear cells are isolated by Ficoll-Plaque per product instructions.
3. The isolated cells are IV injected into immunocompromised mice (NOG/NSG), per the method described in Subheading 7.
4. BW of mice is measured weekly.
5. The mice are sacrificed when $CD45^+$ cells are >70%, and tumor (observed in some models), bone marrow, and spleen are collected. $CD45^+$ cells are measured by flow cytometry; a detailed method is described in Subheading 7.
6. Tumor, bone marrow, and spleen are harvested at termination and digested to prepare single-cell suspensions. The spleen samples also need to be treated with the red blood cell lysis buffer.
7. The collected cells can be used for characterization, serially passed through engraftment, or cryopreserved.

Mouse Tumor Homograft

1. GEMM mice are bred for 4–6 months to allow spontaneous tumor development.
2. Mice are observed daily for clinical symptoms of tumor development; the mouse is sacrificed for necropsy when such clinical symptoms become obvious.
3. The primary organ where the spontaneous tumor was expected to arise is collected and checked visually.
4. Tumor nodules are collected for transplantation, following the same protocol as PDX model establishment. The inoculation method is described in Subheading 7.
5. Part of the organ containing the spontaneous tumor is collected and embedded as FFPE for pathology examination.

Model Quality Control (QC)

The consistency of PDX, patient-derived blood cancer, and homograft models with their original tumors (patient or mouse) is critical. Thus QC of these models needs to be applied before their use. Short tandem repeat (STR) genotype genetic fingerprints, molecular pathology, and histopathology methods are often used for model QC.

STR Genotype Genetic Fingerprint QC of PDX Models

STR are regions with short repeat units (usually 2–6 base pairs in length), the number of which are highly variable among individual people. STR are therefore very useful in identifying the models in banks, different passages, and different studies, thereby reducing the risk of error. The simple protocol that we used is as follows:

1. 50 mg tumor tissue is collected for DNA extraction, as described before.

Table 2
STR QC PCR reaction system

Reagent	Volume (μL)	Final concentration
10× PCR buffer	2.5	1×
10 M forward primer mixture	0.5	0.2 M
10 M reverse primer mixture	0.5	0.2 M
2.5 mM dNTPs	2	0.2 mM
TaqE (5 U/μL)	0.5	0.1 U
Template (DNA)	1	50–100 ng
Sterilizing water	19	
Total	25	

2. Primer pairs of eight chosen STR loci, D12S217, D7S820, TPOX, FGA, CSF1PO, D16S539, tyrosine hyderoxylase (TH01), and VWA, are prepared into three sets of mixtures per size for PCR reaction.
3. The PCR reaction system is detailed in Table 2.
4. Cycling program (steps detailed as below):

   Step 1: 95 °C for 11 min.

   Step 2: 96 °C for 1 min.

   Step 3: 94 °C (temperature ramp rate 100%) for 30 s.

   Step 4: 60 °C (temperature ramp rate 29%) for 30 s.

   Step 5: 70 °C (temperature ramp rate 23%) for 45 s.

   Step 6: Go to Step 3, nine times.

   Step 7: 90 °C (temperature ramp rate 100%) for 30 s.

   Step 8: 60 °C (temperature ramp rate 29%) for 30 s.

   Step 9: 70 °C (temperature ramp rate 23%) for 45 s.

   Step 10: Go to Step 7, 19 times.

   Step 11: 60 °C for 30 min.
5. The PCR product from the original patient tumor is used as a control template.
6. PCR products of test tumors are compared alongside the control templates using 10% non-denaturing PAGE electrophoresis.
7. Mismatch with the patient tissue suggests mistakes in the model. Sometimes weak signaling might suggest high mouse contents.

Histology Pathology QC

Model histopathology is usually performed for each PDX and compared with the hospital-derived pathology diagnosis of the original patient, as another important QC procedure. The method of this QC is a routine pathology process: a model FFPE slide is prepared and H&E stained, followed by histopathology examination performed by pathologists. Additional immunohistochemistry (IHC) of different tissue markers is also needed to confirm disease type on a case-by-case basis.

Molecular Pathology QC

To further confirm the accuracy of PDXs' pathology obtained from hospitals, we have recently developed a new molecular pathology algorithm for PDX diagnosis, which is able to accurately diagnose the pathology of PDX tumors using transcriptome sequences [26]. The RNAseq profiling data will be used for this QC process.

4.2.3 Tumor Implantation Methods

Transplanted tumors are the most commonly used animal models in cancer pharmacology, including target research. There are several common practices utilized in the laboratories per usages. A detail protocol is described in Subheading 7.

4.2.4 Grouping Methods

Randomized Enrollment of Study Mice

TV is the most commonly used parameter for randomization during grouping. Biomarkers whose levels are correlated to TV can sometimes also be used as a parameter for randomization, when tumor volume cannot be readily measured (e.g., AFP in orthotopic HCC models).

1. When there are sufficient mice with TV of approximately 100–200 mm^3, the mice with close TV and BW value are selected and randomly assigned to respective treatment groups.
2. CV% of TV in each group should be less than 40%.
3. Treatment is initiated immediately after grouping. Each model should follow the same dosing regimen.
4. For blood cancer models, mice where the % $hCD45^+$ cells reach 2–10% are enrolled for randomized grouping.

Clinical Trial-Style Enrollment

Another grouping process mimics the enrollment method utilized in clinics, often used when there are insufficient animals within the close range of grouping parameters for simultaneous enrollment and synchronized dosing.

1. TV and BW of tumor-bearing mice are measured weekly.
2. Mice that reach the enrollment criteria (e.g., TV approximately 150–200 mm^3, BW >20 g) are enrolled and randomized into treatment groups.
3. The treatment of an individual will be initiated immediately following enrollment, and the enrollment will continue until

sufficient numbers of each group are enrolled at the set parameter.

4. All individual animals will follow respective dosing regimens, which are not synchronized, according to the protocol.

4.2.5 Tumor Growth Kinetics Readout

Tumor growth kinetics based on timely readouts of TV or tumor weight (TW) are the most common measurement reflective of tumor growth or tumor growth inhibition (efficacy). The detailed protocol is described in Subheading 7.

4.2.6 Pharmacodynamic (PD) Readouts, Beyond Tumor Response

Pharmacodynamic (PD) readouts are usually required to evaluate the inhibition of drug targets and to investigate the mechanism of antitumor activity *via* a putative target. A common PD analysis includes dephosphorylation of kinases. Particularly for tyrosine kinase inhibitors (TKIs), samples need to be harvested at 2, 6, 16 h posttreatment *via* Western blot and/or IHC. mRNA expression level changes are also evaluated by RNAseq at desirable time points. The sample collection method is described in Subheading 7.

4.2.7 I/O PD Readout

For I/O therapeutic target assessment, tumor baseline immunophenotype and/or PD change of immunophenotype resulting from I/O treatment is key to studying I/O targeting. The most common approach is to measure tumor-infiltrating immunological markers, reflective of common and key immune cells, e.g., different subtypes of T cells, macrophages, NK cells, etc. The common methods of these analyses are multicolor flow cytometry (FACS) [4]. Detailed methods are described in Subheading 7.

5 Evaluate Targets in Population-Based Mouse Clinical Trials (MCT) Using Specific Agents

Used as avatars of cancer patients, PDXs provide the ideal experimental platform for clinical trial-like population-based investigations, also called mouse clinical trials (MCT) to evaluate drugs, but also for the discovery of new targets (hypothesis generation) or target validation (hypothesis testing) [28, 29, 34–36]. The principle of an MCT project is shown in Fig. 3. This type of trial has recently been enabled by the establishment of large PDX libraries of diverse cancer types, with full genomic annotations [26] based on characterization using some common assay types summarized in Table 3. The design of population-based studies and their data analysis have been previously described [36]. The protocol for running each mouse in an MCT is similar to that for single PDX model studies.

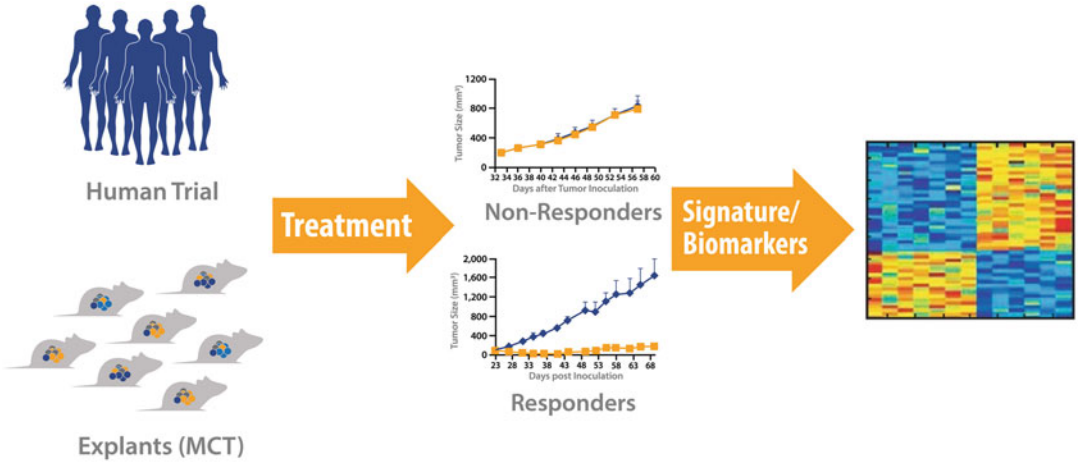

Fig. 3 Schematic illustration of mouse clinical trial (MCT). A large cohort of PDXs reflective of the heterogeneity of the patient population can be enrolled into a mouse clinical trial. The trial results can be potentially classified into two groups, responders and non-responders. The model efficacy data and genomic information are used to identify predictive biomarkers using machine learning methods

Table 3
Assays and applications commonly used in target research

Assays	Applications
Transcriptome sequencing (RNAseq)	• Genome-wide expression profile. • Mutation data at transcript level. • Gene fusion.
Whole exome sequencing (WES)	• Exonic mutation data. • Gene copy number.
Real-time PCR	• Target expression determination. • GCN determination. • SNP genotyping. • Mutation allele frequency analysis.
Immunohistochemistry (IHC)	• Protein expression. • Phosphorylation. • Microscopic localization.
Tissue microarray (TMA)	An array of tumor tissues on the same chip, for biomarker screening
Flow cytometry	• Hematological or immune cell phenotyping: surface/intracellular markers. • Receptor density quantification. • PD readouts: proliferation, apoptosis, cell cycle, differentiation.

6 Preclinical Cancer Pharmacology General Protocols

There are many common procedures that can be applied to different small animal modeling studies of cancer targeting. To avoid redundant description in each pharmacology section, here we summarize the common cancer pharmacology protocols that are used in the specific cancer pharmacology sections above.

6.1 Materials

6.1.1 Equipment

1. IVC system.
2. Biohazard hood.
3. Caliper, Sylvac S-Cal pro.
4. Portable liquid nitrogen tank.
5. IVIS bioluminescent imaging system.
6. Flow cytometer LSRFortessa X-20, BD, NJ, USA.
7. Centrifuge, Thermo ST16R.
8. Cell counter, Nexcelom Bioscience Cellometer® Auto T4.
9. gentleMACS™ Octo Dissociator, Miltenyi Biotec, Germany.
10. Studylog® Studylog Systems, Inc. CA, USA.

6.1.2 Consumables

1. 70 μm cell filters, one for each tissue.
2. Miltenyi C-tubes (one for each tumor).
3. Miltenyi gentleMACS with heater blocks (one for every eight tumors), Miltenyi Biotec, Germany.
4. EDTA routine blood tube 2 mL.
5. Trocar 20# (inner diameter 2 mm).

6.1.3 Specific Regents

1. Ficoll-Paque PREMIUM.
2. FITC antihuman CD45 antibody BioLegend, CA.
3. Cultrex® High Protein Concentration (HC20+) BME, Path-Clear Trevigen MD or BD Matrigel™ Basement Membrane Matrix High concentration, BD Bioscience NY.
4. Red blood cell lysis buffer.
5. Tumor Dissociation Kit.
6. Phosphate-buffered saline (PBS) 50 mL.
7. RPMI-1640.
8. Iodophor swabs.
9. 10% formalin.
10. RNAlater® Thermo Fisher Scientific.
11. Murine tumor dissociation kit, Miltenyi Biotec, Germany.
12. Flow cytometry buffers:

Mincing buffer (for tissues such as tumor-draining lymph node or spleen).

- 5000 U/mL (10×) DNAse I, Roche in RPMI-1640.
- Dilute immediately before use; store at 4 °C for up to 1 month.

FACS wash buffer.

- 10% FBS; azide-free
- 40 mM EDTA 0.5 M pH 7.4 stock
- Sterile Ca + and Mg + free PBS (1×, stock).

13. Brilliant staining buffer, BD, NJ.
14. Purified rat anti-mouse CD16/CD32, mouse BD Fc Block™ BD, NJ.
15. Foxp3 Fix/Perm kit, eBioscience Thermo Fisher Scientific.
16. PBS sterile, Hyclone.
17. UltraComp eBeads, eBioscience, Thermo Fisher Scientific.

7 Regulatory Compliance in Animal Experimentation

All of the protocols and amendment(s) or procedures involving the care and use of animals need to be reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC) prior to the conduct of studies. The care and use of animals will be conducted in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International guidelines as reported in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011).

All animal experimental procedures will be under sterile conditions at SPF (specific pathogen-free) facilities and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals from different government institutions (e.g., the National Institutes of Health). The protocol will need to be approved by the Committee on the Ethics of Animal Experiments at the facility institution (e.g., institutional IACUC).

7.1 Methods

All animal experiments are conducted in SPF facilities.

7.1.1 Animal Housing

1. Mice are housed in individual ventilated cages.
2. Temperature: 20–26 °C; humidity 30–70%; lighting cycle: 12-h light and 12-h dark.
3. Corncob bedding is used and changed weekly.
4. Diet: irradiation sterilized dry granule food during the entire study period.
5. Water: animals have free access to sterile drinking water.

7.1.2 Tumor Inoculation

Tumor Cell SC Inoculation

1. Mix the cell suspension with an equal volume of ECM (extracellular matrix, e.g., Matrigel® which can sometimes significantly increase tumor take rate [37]), and keep on ice during transportation to the vivarium.
2. Draw ECM-cell mixture slowly into a chilled 1 mL syringe, and keep the filled syringes on ice to avoid ECM solidification.
3. Immobilize the mouse, and inject 100–200 μL of ECM-cell mixture subcutaneously to the right flank of the recipient mouse; hold syringes for >3 s to allow complete ECM solidification.
4. Normally 5–10 mice are inoculated per group for tumor growth monitoring. When using tet-inducible cells, animals are randomly enrolled into two groups of 5–10 mice each and treated ± induction agents (e.g., DOX).
5. Mice are monitored 24-h postinoculation for procedure-related abnormalities.

Tumor Cell IV/IP Inoculation

For non-superficial implantation, e.g., IP and IV inoculation, tumors may not be readily and macroscopically visible. Therefore, IVIS or other imaging approaches are required to monitor tumorigenesis. Some of these imaging procedures require the engineered cell lines to have reporter gene expression (e.g., luciferase or GFP).

1. Luciferase- or GFP-engineered cells are suspended in serum-free DMEM and transported to the vivarium.
2. Gently mix the cells and then draw the mixture slowly into a 1 mL syringe. For IV injection, go to **steps 3** and **4**; for IP injection go to **steps 5** and **6**.
3. For IV injections: first immobilize the mouse, and then inject cells into the tail vein.
4. Gently constrict the injection point for 3–5 s to prevent bleeding or leakage. Move to **step 7**.
5. For IP injections: inject the cell suspension into the abdominal cavity of immobilized mice.
6. Rotate the needle 90° and retract to prevent leakage.
7. Check the mice 24-h postinjection for any procedure-related abnormalities.

Tumor Chunk Inoculation

1. Monitor BW and TV of tumor-bearing donor mice. When TV reaches 500–1200 mm^3, the animal is euthanized in a biohazard hood as per protocol (shave the mouse around the tumor if necessary, e.g., when NOD-SCID mice are used).
2. Sterilize the skin around the tumor using iodophor swabs.
3. Surgically remove the tumor and place in a petri dish containing 20 mL PBS.

4. Cut the tumor in half, removing any extra skin, vessels, calcification, and/or necrosis.
5. If there is contaminating blood, transfer the tumor into another petri dish, and wash the tumor with PBS.
6. Cut approximately 50 mg of tumor tissue, and place it into a 2 mL cryopreserved tube. Snap freeze using liquid nitrogen for QC.
7. Put the rest of the tumor into a sterile 50 mL centrifuge tube, add 20 mL transport medium, and then transport the tube to a separate animal room for pharmacology studies.
8. Cut the tumors into 2 mm diameter pieces using a scalpel, putting 1 chunk into each trocar.
9. Sterilize the recipient mouse using iodophor swabs.
10. Inoculate the tumor chunk on the right flank site of the mouse. The mice will be kept for weekly observations of tumor development.

Blood Cancer Model Inoculation and Dissociation of Solid Tumor to Single-Cell Suspension

1. Tumors, bone marrow, or the spleen of donor mice are collected and put in digestion media in one well of a sterile 6-well plate.
2. Hold the tissues in place with sterile tweezers/forceps, and slice them into small pieces (~1 mm^3) with a scalpel.
3. Place tissue pieces into a C-tube, and use the remaining digestion buffer to wash the plate. Transfer the fluid into the C-tube, which is placed on ice until digestion.
4. Digest tumors using the desired gentleMACS program, and turn on the gentleMACS Octo Dissociator with Heaters.
5. Attach the tumor dissociation C-tubes upside down to the sleeves of the free tube positions, and alter the status of the tube positions from “free” to “selected”.
6. Select a dissociation program (37_c_m_TDK_1) [38], and press the Folder icon to select the required folder. The list of gentleMACS programs in the respective folder will be displayed.
7. After termination of the program, detach the C-tubes from the gentleMACS Dissociator, and perform a short spin down at 300 *g* to collect the sample at the bottom of the tubes.
8. Resuspend the samples, and apply the cell suspension to a cell strainer placed above a 50 mL tube.
9. Spleen cells need to be subjected to red cell lysis using the RBC lysis solution.
10. Wash the cells through the cell strainer with 10 mL wash buffer to obtain a single-cell suspension.

11. Centrifuge the tubes at 300 × *g* for 5 min, discard the supernatant, and resuspend the cells with 5 mL wash buffer.
12. Tumor cells are then inoculated IV following the instructions in the tumor cell IV/IP inoculation procedure as described above.

7.1.3 Tumor-Bearing Mice Health Monitoring

The health of the recipient mice is monitored daily.

1. Check the water and food consumption.
2. Check the mouse appearance for an ungroomed hair coat, lumps, thinness, abnormal breathing, and ascites.
3. Palpate the abdomen to check if there are spontaneous tumors on the liver or spleen.
4. Weigh mice weekly using a balance.
5. If any of the following clinical signs are observed, the mice are sacrificed for sample collection and necropsy: BW loss >20%; impaired mobility (not able to eat or drink); unable to move normally due to significant ascites and enlarged abdomen; effort respiration; and dying.

7.1.4 Tumor Burden Determination

1. The length and width of the tumor are measured by calipers after tumors become palpable.

SC Tumor TV Measurement

2. Immobilize the mouse; measure the longest diameter and the diameter perpendicular to the longest diameter using calipers.
3. TV is calculated using the formula $\mathrm{TV} = a \times b^2 \times \frac{\pi}{6}$, where a is the longest diameter of the tumor and b is the shorter diameter of the tumor.
4. Data are automatically analyzed and stored on the Studylog® database.

IVIS Bioluminescent Imaging System (Firefly Luciferase as an Example)

1. Mice are anesthetized using 5% isoflurane mixed with oxygen and their skin sterilized with 70% ethanol.
2. 150 mg/kg of luciferin is IP injected.
3. Place the mice on a disinfected imaging chamber of a commercial imaging system.
4. Maintain anesthesia using 1% isoflurane mixed with oxygen through a nose cone.
5. Take the first image by exposing for 5 min and check the signal intensity. Adjust the exposure time to optimal signal versus background.

Mouse MRI

1. Restrain animal, and induce anesthesia with isoflurane at 5%. Maintain anesthesia by nose cone at 1%.

2. Set up animal on animal holder, and place the animal in the correct position on the MRI.
3. Scan animals.
4. Remove the animals and stop the isoflurane, and then return the animals to cage.

Blood Cancer Model Tumor Burden Determination

1. In blood cancer models, % human $CD45^+$ ($hCD45^+$) cells are measured to reflect tumor growth status.
2. Whole blood (50–100 μL) is collected by facial vein bleeding weekly in a BD EDTA routine blood tube, followed by lysis using red blood cell lysis buffer.
3. The mononuclear cells are stained by fluorescence-labeled $huCD45^+$ antibody and analyzed using flow cytometry (for the flow cytometry method, *see* Subheading 7.1.7 within this section). % $huCD45^+$ cells to total cells are calculated.
4. At the termination of the experiment, bone marrow and spleen are examined for $huCD45^+$ ratio. For the cell digestion and flow cytometry methods, *see* Subheading 7.1.7 in this section.

Tumor Growth Kinetics

Tumor growth kinetics or its inhibition are the key readouts in preclinical tumor pharmacology. A frequently used analysis procedure is as follows.

1. The tumor growth curve is generated by entering TV data into a software tool; a typical tumor growth curve is shown in Fig. 4.
2. ΔT/ΔC% and tumor growth inhibition (TGI) are the most commonly used readouts/endpoints of preclinical cancer pharmacology efficacy assessment. In ΔT/ΔC%, T and C are the mean tumor volume of the treated and control groups, respectively, on a given day. $TGI\% = 1 - \Delta T/\Delta C\%$.
3. We recently developed a new efficacy calculation endpoint called the median AUC ratio of a growth curve (Guo et al., unpublished): the median AUC ratio is calculated in three steps. First, the normalized AUC of each animal in the vehicle and treatment groups are calculated; second, each mouse in the treatment group is paired with any other mouse in the vehicle group, and the AUC ratio of each pair is calculated; third, the median of all AUC ratios is obtained. There are two advantages of the median AUC ratio: (1) It reflects the treatment efficacy over a whole period of treatment, whereas in the calculation of ΔT/ΔC% or TGI%, only the tumor volume on the selected day was considered; and (2) it is based on the median which is not skewed as much by the extremely large or small values that are frequently seen in PDX or I/O efficacy studies due to the tumor heterogeneity on growth rate and mouse immune status.

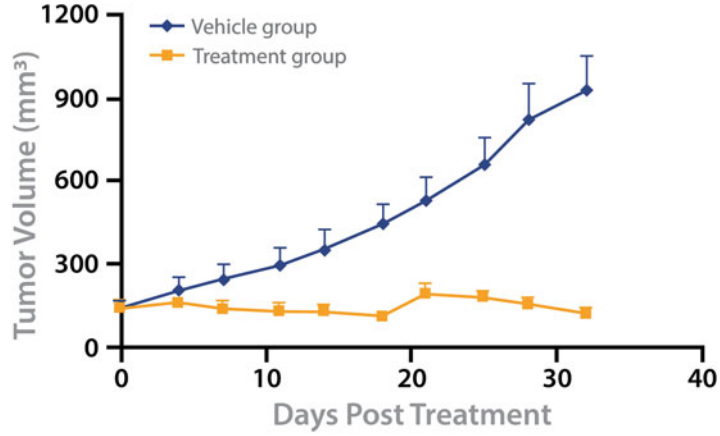

Fig. 4 Typical tumor growth kinetic curves with and without treatment

7.1.5 Necropsy

At the termination of studies, mice are sacrificed for necropsy. Tumors and organ tissues are collected as FFPE or snap frozen tissue for pathology and genetic analysis.

1. Animal is anesthetized with 5% isoflurane and maintained by nose cone at 1%.
2. Visual and palpation inspection occurs for the presence of palpable tumors.
3. Animals are euthanized per approved protocol.
4. Open the abdomen and visually examine target organs for tumors.
5. Harvest tumor or target organ samples.

7.1.6 Sampling

Tumors or organs are collected per various purposes. Sample collection should follow the proper sequence: FFPE > snap frozen tissue for DNA/RNA (RNAlater) > tumor tissue for transplantation or cell suspension for other analysis, e.g., flow cytometry.

1. Animal is anesthetized with isoflurane at 5%, and anesthesia is maintained by nose cone at 1%.
2. The mice are euthanized per approved protocol, and tumors removed by surgery and added to cold PBS.
3. Cut ~300–500 mm^3 of a tumor using a scalpel, and place in a 15 mL centrifuge tube with 10 mL room temperature 10% formalin.
4. Remove the blood, blood vessels, calcification, and necrosis.
5. Cut ~50 mg tumor and place into a 2 mL cryopreserve tube. Snap freeze by liquid nitrogen, and/or put the tumor into a 2 mL cryopreservation tube containing 1.5 mL RNA later.

6. The remaining tumor tissues are transferred into RPMI-1640 and kept on ice before transplantation or cell digestion.
7. Tissues should be collected immediately after mice are euthanized. If the tumor/mouse needs to be photographed, which is required at the same time as the mouse is euthanized, then it is recommended to not collect the tumor.

7.1.7 Flow Cytometry

Hematological markers (or immunological markers) are important for the characterization of hematological cancers and tumor-infiltrating immune cells and the immune components of the tumor microenvironment [38]. Multicolor FACS and/or immunofluorescence (IF) (or IHC) analysis are commonly performed to monitor these immune markers. Flow cytometry is most commonly used to monitor blood tumor growth or tumor-infiltrating immune cells in I/O animal modeling for I/O targets. Alternately, IF and IHC can reveal the location information of immune cells within tumors.

Tumor Dissociation

The first step for flow analysis is the dissociation of tumors into single-cell suspensions using various protocols, optimized for different tumors allowing the greatest yield of viable immune or leukemic cells [38]. However, the general simplified steps can be represented as follows.

1. Wash the tumor in PBS, and remove normal tissues attached to the tumor (e.g., blood vessel, fat, fascia, etc.).
2. Digest the tumor pieces as per the method described in Subheading 7 (Blood Cancer Model Inoculation).
3. Count cells using a cell counter, and adjust cell concentration to 1×10^6 cells per tube or per sample.

Staining

1. Fc block sample cells: resuspend cells in 200 μL staining buffer with 1 μg/mL Fc block (Mouse BD Fc Block™), followed by incubation on ice or 4 °C refrigeration for 15 min in the dark.
2. Stain cells using the desired antibody/fluorescence panels (e.g., T-cell panel, macrophage panel, etc.): add the antibody mixture diluted in Fc blocking buffer to each sample; stain for at least 30 min on ice in the dark.
3. Add 1 mL of ice-cold PBS to each tube, and gently resuspend the cells, followed by centrifugation at $300 \times g$ for 5 min. Discard the resulting supernatant.
4. Repeat **step 3** to wash the cells a total of twice.
5. Stain for intracellular markers if needed, following **steps 6–10**, otherwise proceed to **step 11**.
6. Resuspend the cell pellet by pulse vortex, and add 200 μL of prepared fixation/permeabilization working solution for each

sample. Pulse vortex again, and then incubate at 4 °C overnight (preferred) or 30 min at room temperature in the dark.

7. Pellet cells and remove the supernatant.
8. Wash twice by adding 1 mL of 1× permeabilization buffer (made from 10× permeabilization buffer, diluted with distilled H_2O) followed by centrifugation and decanting of supernatant.
9. Add intracellular marker antibody in 1× permeabilization buffer, and incubate at room temperature for 30 min in the dark.
10. Wash cells twice with 1 mL of 1× permeabilization buffer. Centrifuge and decant supernatant.
11. Resuspend cells in 150 μL of staining buffer and analyze on cytometer. Due to the fixation and permeabilization procedure, the FSC (forward-light scatter)/SSC (sidelight scatter) distribution of the cell population will be different to live cells. Therefore, the gate and voltages will need to be modified.
12. The data are analyzed by Flowjo v10 software.

FMO Controls (Fluorescence Minus One Control)

Multicolor flow cytometry is centrally important for today's I/O tumor-infiltrating immune cell (TILs) analysis, requiring a way to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. FMO controls or Fluorescence Minus One control is an important approach for this purpose. To this end, additional mice should usually be included in each Rx group for FMO controls (at least two per Rx) and processed individually for each tissue. After tissue dissociation, tissues should be pooled. For example, in a study with four Rx groups, eight additional tumors should be processed individually and then pooled into one sample for FMO's.

Flow Instrument Setup

1. Prepare compensation beads while the machine is warming up (at least 20 min).
2. Use BD's CS&T beads to check performance.
3. Voltage and compensation settings: use eBioscience's UltraComp beads, and vortex the UltraComp beads thoroughly before use.
4. Label a separate 12 × 75 mm sample tube (BD Falcon™) for each fluorochrome-conjugated antibody.
5. Add 100 μL of staining buffer (e.g., BD Pharmingen Stain (FBS)) to each tube.
6. Add 1 drop (approximately 60 μL) of the UltraComp eBeads to each tube.

7. Add antibodies, and perform the staining procedure as per the sample process stated in Subheading 7.
8. Resuspend the bead pellets by adding 0.5 mL of staining buffer to each tube. Vortex thoroughly.
9. Set the flow cytometer instrument PMT voltage settings using the target tissue for the given experiment.
10. Run each tube separately on the flow cytometer. Gate on the singlet bead population based on FSC and SSC characteristics.
11. Adjust flow rate to 200–300 events per second.
12. Create a dot plot for the given fluorochrome-conjugated antibody as appropriate (i.e., to set compensation for a fluorescein (FITC)-conjugated antibody, use an FL1 vs. FL2 dot plot).
13. Place a quadrant gate such that the negative bead population is in the lower left quadrant and the positive bead population is in the upper or lower right quadrant. Adjust the compensation values until the median fluorescence intensity (MFI) of each population (as shown in the quadrant stats window) is approximately equal (i.e., for FL2-%FL1, the FL2 MFI of both bead populations should be approximately equal when properly compensated).
14. Repeat **steps 12** and **13** for each of the experimental tubes.
15. Proceed to acquiring the actual staining experiment.

Run the compensation wizard, and save the settings with the format "date experiment your initials."

7.2 General Consideration of Tumor Selection

Appropriate tumor model(s) are chosen dependent on the specific objectives of the target investigation (Table 4). Common parameters include species-specific targeting (e.g., human vs. mouse), conventional targeting vs. I/O targeting, tumor growth kinetics vs. tumorigenicity (e.g., initiation), etc.

8 Notes

In order to use adequately different cancer models, one needs to fully understand the nature of these models, or in other words, the full-annotation of these models should be available for facilitating specific study design and model selection. The common annotations include genomic profile, pathology, driver mutations, as well as tumor microenvironment [38] particularly when an I/O study is planned.

Using PDX as an example, certain criteria usually need to be met for optimal utilization of PDX, including (1) comprehensive genomic profiling (gene copy number (GCN), mRNA expression, mutation profile, gene rearrangements, etc.), where transcriptome

Table 4
Different tumor models for target research

Tumor model	Comments	Examples
Spontaneous tumor	No transplantation; suitable for mouse targeting and I/O targeting; for studies involving tumor initiation	GEMM tumors
Autograft	Transplanted tumors from one location to another location of the same individual (identical MHC)	Skin transplantation of the same person (similar to syngeneic transplantation)
Homograft	Donor and recipient are different individuals but belong to the same inbred of strain or identical twin (identical MHC); suitable for mouse targeting and I/O targeting	Syngeneic tumors, MuPrime™ (murine version of PDX)
Allograft	Donor and recipient are different individuals (usually unmatched MHC)	Common organ donor
Xenograft	Transplanted between species; for human targeting	PDX, cell line-derived xenograft, rat tumor in mice

sequencing (RNAseq) and whole exome sequencing (WES) have been commonly used; (2) adequate/stable tumor take rate and growth kinetics to support consistent and reliable results; and (3) sufficient banking of early passages to support relevant and reproducible investigations. Due to the potential changes during continuous passages, some models may drift in their growth properties (usually faster at later passage) [39], their genomic profile, and response to drugs. We therefore recommend to use passages after three, but fewer than ten, for relatively stable properties in these aspects. We also recommend, if possible, establishing a master bank of cryopreserved tumors at early passages.

For xenograft model growth in mice, spontaneous mouse tumors occasionally occur and contaminate human tumor or completely take over, which would result in misleading observations. A QC process to quantify mouse/human DNA ratio could help to determine if there is mouse tumor contamination.

Another key process vital to the success of cancer pharmacology program is the unique identification (ID) of each models, since the morphology of tumor or even growth will not be able to distinguish individual models. Most common technologies used for xenografts (PDX, cell line-derived xenografts) are STR described in this chapter and HLA typing, superior to genomic profile in cost and efficiency. However, mouse tumor ID cannot usually and readily use these methods, due to that most mouse tumors are derived from inbred and difference from those of human. To this end, usually certain genomic methods have been custom-developed (Cai et al., unpublished).

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