

Modeling of Patient-Derived Xenografts in Colorectal Cancer

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Abstract

Developing realistic preclinical models using clinical samples that mirror complex tumor biology and behavior are vital to advancing cancer research. While cell line cultures have been helpful in generating preclinical data, the genetic divergence between these and corresponding primary tumors has limited clinical translation. Conversely, patient-derived xenografts (PDX) in colorectal cancer are highly representative of the genetic and phenotypic heterogeneity in the original tumor. Coupled with high-throughput analyses and bioinformatics, these PDXs represent robust preclinical tools for biomarkers, therapeutic target, and drug discovery. Successful PDX engraftment is hypothesized to be related to a series of anecdotal variables namely, tissue source, cancer stage, tumor grade, acquisition strategy, time to implantation, exposure to prior systemic therapy, and genomic heterogeneity of tumors. Although these factors at large can influence practices and

patterns related to xenotransplantation, their relative significance in determining the success of establishing PDXs is uncertain. Accordingly, we systematically examined the predictive ability of these factors in establishing PDXs using 90 colorectal cancer patient specimens that were subcutaneously implanted into immunodeficient mice. Fifty (56%) PDXs were successfully established. Multivariate analyses showed tissue acquisition strategy [surgery 72.0% (95% confidence interval (CI): 58.2–82.6) vs. biopsy 35% (95% CI: 22.1%–50.6%)] to be the key determinant for successful PDX engraftment. These findings contrast with current empiricism in generating PDXs and can serve to simplify or liberalize PDX modeling protocols. Better understanding the relative impact of these factors on efficiency of PDX formation will allow for pervasive integration of these models in care of colorectal cancer patients. *Mol Cancer Ther*; 16(7): 1435–42. ©2017 AACR.

Introduction

Integrated application of patient-derived xenograft (PDX) modeling has emerged as a key approach to more effective biomarker, therapeutic target, and drug discovery (1, 2). Traditionally, preclinical evaluation of therapeutic targets in colorectal cancer has been performed using either well-established cell lines in tissue culture or tumors xenografted using these cell lines into

immunocompromised mouse strains (e.g., NOD-SCID mice). As established colorectal cancer cell lines are most often phenotypically and genetically uniform due to extended selection or passage, they often fail to account for the heterogeneity of the original tumors from which they were derived. Thus, cell line-based preclinical "in vivo" models have been criticized for their modest genetic and molecular diversity (3). These cell lines propagated *in vitro* demonstrate genetic departure from their corresponding primary tumors (4). Furthermore, by virtue of their *in vitro* selection in tissue culture, colorectal cancer cell lines generally tend to display poorly differentiated and more aggressive characteristics relative to more heterogeneous human colorectal tumors. Although the uniformity of colorectal cancer cell lines is very useful for studying cell signaling pathways, these factors have limited translation into the real world effectiveness of colorectal cancer cell line-based models in cancer drug discovery and therapeutic marker/target identification, with few predictive achievements and many notable failures (5).

On the basis of this unmet need for more representative heterotypic disease-related translational research tools, newer approaches involve the direct implantation of patient tissue into immunocompromised mouse strains, so called PDX modeling. As this approach actually incorporates the original heterogeneous tumor, its use has increased considerably during the last decade (6–14). This shift in preclinical models was influenced by notable studies showing the advantage of PDX models relative to traditional xenografts in recapitulating the complex biology of human disease (9, 15). Indeed, PDX models demonstrate genetic and

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molecular diversity that are fundamental characteristics of human tumors (15–17). In addition, immediate passages preserve a component of the human stromal compartment (18–22), shown to play a key role in disease progression and development of resistance to therapy. In consideration of these unique characteristics, numerous research groups focused on developing PDX models that represent the morphologic and the biologic spectrum of the disease (20, 23, 24). To this end, xenografts derived from primary tumors, metastatic sites, and treatment-naïve tumors and heavily treated tumors are gaining acceptance as a key preclinical platform to study cancer behavior and to improve new drug development (7–10, 25–29).

Nevertheless, the successful engraftment of patient samples into PDX models is not universal for all tumor types. In the case of one colorectal cancer study, approximately 70% of patient samples grafts developed a PDX (22). Anecdotal practices, speculative factors, and limited experimental findings in other tumor types support the notion that engraftment rates are affected by several factors, including tumor type, tissue source, cancer stage, tumor grade, tumor molecular characteristics, acquisition strategy, exposure to prior radiation or systemic chemotherapy, and technical manipulations during grafting (such as time to implantation; refs. 30–33). In this study, we established a large cohort of PDX models derived from both surgical and biopsy specimens to systematically examine whether these factors effect engraftment in PDX models. Our results are vital to understanding the role of genetic characteristics in the ability of colorectal cancer tumors to establish as a PDX and the fidelity of these models in recapitulating human disease. Moreover, we discuss novel strategies for the optimization of engraftment rates.

Materials and Methods

Patient characteristics

Patients enrolled on the study were treated at The University of Texas MD Anderson Cancer Center (MDACC, Houston, TX) between April 2012 and September 2014. Patient data were collected prospectively and included gender, age, stage of the disease, prior chemotherapy, prior targeted therapies, and tumor genetic mutation status (Table 1). The study was carried out under an Institutional review board–approved protocol and an informed consent was obtained from each patient.

Collection of colorectal cancer human specimens

Colorectal cancer biospecimens collected included both surgery and biopsy specimens. Immediately after collection, two tumor pieces were snap-frozen and stored in liquid nitrogen for molecular characterization; two pieces were fixed in formalin and then embedded in paraffin (FFPE) for hematoxylin and eosin staining (H&E) and IHC analysis. In addition, 2–6 tumor pieces were placed in DMEM media supplemented with 1% antibiotic, 10% FBS, and 1% nonessential amino acids prior to engraftment.

Tumor engraftment

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice from The Jackson Laboratory (JAX) were maintained in the animal facilities of MDACC and JAX following standard animal regulation and strict health controls. Tumor specimens were collected at MDACC and were engrafted into NSG mice at MDACC and JAX using similar mouse host and method for implantation. Tumor specimens engrafted at MDACC constituted the immediate engraftment

Table 1. Baseline characteristics of patients involved in study (N = 90)

Characteristics	Value (%)
Age (years)	
Median ± SD	54 ± 13.35
Range	20–86
Gender	
Female	41 (45.6%)
Male	49 (54.4%)
Method of tumor acquisition	
Surgery	50 (55.6%)
Biopsy	40 (44.4%)
Site of sampling	
Primary	15 (16.7%)
Metastatic	75 (83.3%)
Time to engraftment	
0–2 hours (immediate)	59 (65.6%)
12–24 hours (delayed)	31 (34.4%)
Parental tumor mutational status	
KRAS mutant	47 (52.2%)
BRAF mutant	14 (15.6%)
PIK3CA mutant	21 (23.3%)
Tumor grade	
Moderately differentiated	69 (76.7%)
Poorly differentiated	16 (17.8%)
Prior therapy	
Neoadjuvant therapy	74 (82.2%)
No neoadjuvant therapy	13 (14.4%)
Preoperative bevacizumab	47 (52.2%)

cohort and were engrafted within 2 hours. Tumors engrafted at JAX constituted the delayed engraftment cohort and were engrafted between 12 and 24 hours of collection at MDACC. Rodent care and housing were in accordance with institutional guidelines and regulations as well as according to Institutional Animal Care and Use Committee approved animal protocols. Tumor fragments (~50 mm³) were subcutaneously placed into the flanks of mice anesthetized by 2%–4% isoflurane/O₂ inhalation. One piece of tumor was implanted per mice. Tumor growth was monitored and documented twice a week, with date of first palpable growth noted. Mice were euthanized when tumor burden was reaching 1,500 mm³.

Pathologic characterization

Samples were formalin-fixed, processed, paraffin-embedded, sectioned, and stained with hematoxylin/eosin using standard pathology techniques. Dual-color FISH assays were performed by the Molecular Pathology Shared Resource Cytogenetics Laboratory at the University of Colorado Cancer Center. Unstained slides with formalin-fixed paraffin-embedded tissue (FFPE) sections from human primary tissue or PDX were subjected to a dual-color FISH assay using the Human (SR)/Mouse (SG) DNA probe set. The Human and Mouse probes were prepared by labeling 1 µg of Cot-1 DNA of each species (commercial reagents obtained from Invitrogen), respectively, with SpectrumRed- and SpectrumGreen-conjugated dUTPs (Abbott Molecular) using the Vysis Nick translation kit (Abbott Molecular), according to manufacturer's instructions. The assays were performed according to standard laboratory protocol using reagents from FFPE pretreatment kit IV (Abbott Molecular). The samples were incubated at 56°C for 4 hours, soaked in CitriSolv series 3 times for 5 minutes each followed by dehydration in 100% ethanol and then allowed to air dry. The tissue areas to be hybridized were marked with a diamond pen. The slides were incubated in pretreatment solution at 80°C for 10 minutes and in the protease solution at 37°C for 18

minutes. After the specimens were washed and dehydrated in EtOH series, the slides were allowed to air dry. Probe was applied to the selected hybridization areas using 50 ng each of Human Cot-1 and Mouse Cot-1 per 12-mm circular area and then covered with a glass coverslip and sealed with rubber cement. DNA denaturation was performed in a thermocycler for 5 minutes at 75°C and hybridization was allowed to occur at 37°C overnight. Posthybridization wash was performed through incubations in wash buffer I at 74°C and wash buffer II at room temperature for 2 minutes each, followed by dehydration. Finally, DAPI/anti-fade (0.3 µg/mL in Vectashield mounting medium) was applied to the slide and the area covered with a coverslip. The quality of the preparation regarding nuclear morphology and intensity of the fluorescence signals was verified on epifluorescence microscope using single interference filters sets for green (FITC), red (Texas red, TR), and blue (DAPI). Monochromatic images were captured in each channel and merged using CytoVision application (Leica Microsystems).

Molecular characterization

Mutational status of the parental tumor performed as a standard of care under CLIA (Clinical Laboratory Improvement Amendments) environment was collected and included mutation status of *KRAS*, *BRAF*, and *PIK3CA* genes. Tumor cellularity was determined by H&E staining of sections adjacent to those used for DNA extraction. Standard laboratory procedures were used to isolate sectioned tissue and then extract, purify, and quantify DNA. The gene analysis was performed as a standard of care at MDACC as described previously (34). Ion Ampliseq Cancer Panel (Life Technologies) was used to identify hotspot mutations found in 46 genes, which was expanded to 50 genes by adding the following genes: *EZH2*, *IDH2*, *GNA11*, and *GNAQ* (34). Sequence reading, alignment, and base calling were conducted using Torrent Suite software V2.0.1 (Life Technologies) with reference standards consisting of Human Genome Build 19 (Hg19). Genomic variant detection was performed using Torrent Variant Caller software V1.0 (Life Technologies)

Statistical analysis

Statistical analysis was performed to determine the association of PDX establishment with covariates. The PDX establishment was treated as a binary outcome and time to PDX establishment was treated as a time-to-event outcome for the analysis. Fisher exact test and logistic regression analysis were used to determine the association of PDX establishment as a binary outcome with covariates, and the log-rank test and Cox proportional hazards model was used to determine the association of PDX establishment as a time-to-event outcome with covariates.

Results

Tissue acquisition strategy affects PDX establishment

The majority of previous PDX establishment studies relied on surgical samples (Table 1). Nevertheless, the use of biopsy samples can extend the application of the PDX models to a wider range of patients and improve the ability to study changes in tumor biology during the evolution of the disease (2). As a key focus, we examined whether the method of parental specimen acquisition affects PDX establishment. A total number of 50 (55.6%) surgical and 40 (44.4%) biopsy specimens were transferred to mice and used for analysis. We found that the PDX development success rate was higher in surgical (36/50 = 72%) than biopsy (14/40 =

35%) specimens [OR = 4.78; 95% confidence interval (CI): 1.79–13.0; $P = 0.001$]. This difference remained statistically significant even after adjusting for primary versus metastatic and immediate versus delayed implantation (OR = 3.63, $P = 0.048$, Table 2). Within surgical specimens, we found no association between the size (maximal dimension) of the tumor resected during surgery and rate of PDX establishment ($P = 0.279$; Supplementary Data).

Immediate and delayed implantations are feasible and equally effective for PDX establishment

Prior studies and current practices in PDX development were performed in collaboration with a specialized institution and have focused on the need for early implantation (15, 35). However, little is known about the effect of delayed implantation in PDX engraftment rates in colorectal cancer. To answer this question, we perform both immediate and delayed implantation. Immediate implantations were performed within 2 hours, whereas delayed implantations were performed between 12 and 24 hours from tissue acquisition. This decision was based on logistic factors, including the time of day that the procedure was completed. Overall 59 (65.6%) specimens were grafted immediately and 31 (34.4%) were grafted within 24 hours (Table 2). We recorded a development of PDX in 28 of 59 (47.5%) of immediate and 22 and 31 (72%) of delayed implantations. While this difference was statistically significant in univariate analysis (OR = 0.37; $P = 0.045$), there was an imbalance of surgical and biopsy samples with more surgical samples undergoing delayed implantation. As a result, this effect was not maintained in the multivariate model that included surgery versus biopsy (OR = 0.98, $P = 0.970$; Table 2).

Source of tissue has little influence on colorectal cancer PDX establishment

Next, we examined the influence of tissue acquisition site in PDX establishment. A total number of 15 (16.7%) primary tumors and 75 (83.3%) metastatic tumors were implanted. Overall, 13 of 15 (86.7%) primary and 37 of 75 (49.3%) metastatic specimens developed PDXs (Table 2). While this was significantly different in univariate analysis (OR = 6.68, $P = 0.010$), after correcting for confounding variables, this difference was not maintained (OR = 3.87, $P = 0.104$; Table 2).

Previous therapy affects time to establishment of PDX but not overall establishment rates

Another factor that potentially affects PDX engraftment is previous chemotherapy, as this may induce tumor volume shrinkage and tumor cell death, affecting the number of tumor cells that are engrafted alive. However, previous therapy also induces molecular changes, such as epithelial to mesenchymal transition and regional hypoxia that might also affect engraftment. For this reason, we evaluated the role of any neoadjuvant chemotherapy and anti-VEGF therapy specifically (bevacizumab) in PDX establishment. Overall, 74 (85.1%) patients received neoadjuvant cytotoxic chemotherapy prior to implantation and 47 (62.7%) patients received anti-VEGF therapy. As shown in Table 2, neither prior chemotherapy nor bevacizumab specifically affected the overall rate of PDX establishment. However, PDX models established from patients with previous neoadjuvant therapy grew significantly slower than those without previous therapy (median time to establishment: 12.2 months vs. 3.8 months; HR = 0.38 (0.16–0.89); $P = 0.026$; Table 3). This effect was not attributed to prior bevacizumab therapy.

Table 2. Univariable and multivariable analyses to determine the association between covariates and PDX establishment

Covariates	Univariate analysis		OR (P)
	No	Yes	
Gender			
Female	20 (48.8%)	21 (51.2%)	1.38 (0.525)
Male	20 (41.8%)	29 (59.2%)	
Method of tumor acquisition			
Surgery	14 (28.0%)	36 (72.0%)	4.78 (<0.001)
Biopsy	26 (65.0%)	14 (35.0%)	
Site of sampling			
Primary	2 (13.3%)	13 (86.7%)	6.68 (0.010)
Metastatic	38 (50.7%)	37 (49.3%)	
Prior therapy ^a			
Neoadjuvant therapy	37 (50.0%)	37 (50.0%)	0.30 (0.129)
No neoadjuvant therapy	3 (23.1%)	10 (76.9%)	
Preoperative bevacizumab ^a			
Yes	23 (48.9%)	24 (51.1%)	1.39 (0.633)
No	16 (57.1%)	12 (42.9%)	
KRAS mutant ^a			
Yes	10 (40.0%)	15 (60.0%)	1.71 (0.329)
No	25 (53.2%)	22 (46.8%)	
BRAF mutant ^a			
Yes	8 (57.1%)	6 (42.9%)	0.72 (0.765)
No	26 (49.1%)	27 (50.9%)	
PIK3CA mutant ^a			
Yes	9 (42.9%)	12 (57.1%)	1.48 (0.589)
No	20 (52.6%)	18 (47.4%)	
Time to engraftment			
<2 hours (immediate)	31 (52.5%)	28 (47.5%)	0.37 (0.045)
12–24 hours (delayed)	9 (29.0%)	22 (71.0%)	
Tumor grade ^a			
Poorly differentiated	7 (43.8%)	9 (56.3%)	0.88 (1.000)
Moderately differentiated	28 (40.6%)	41 (59.4%)	
	Multivariate analysis		
Covariates	OR	95% CI	P
Surgery vs. biopsy	3.63	1.0–13.0	0.048
Primary vs. metastatic	3.87	0.8–19.8	0.10
Immediate vs. delayed	0.98	0.3–3.70	0.97

^aVariables with missing values.

PDX models represent the broad spectrum of human colorectal cancer genetic alterations

The ability of PDX models to represent with fidelity the colorectal cancer biology relies on recapitulating the spectrum of genetic alterations found in human disease. Thus, we examined the role of clinically relevant genetic alterations (36) in the establishment of PDX models. Specifically, we analyzed the status of *KRAS*, *BRAF*, and *PIK3CA* genes found in parental tumors and correlate these results with PDX establishment. As shown in Tables 2 and 3, genetic alterations found in parental tumors did not affect either the rate of establishment or time to establishment of PDX. These results suggest that, PDX models represent the broad spectrum of genetic alterations found in human disease.

PDX establishment is not affected by the parental tumor differentiation state

Equally important is the ability of recapitulating the morphologic spectrum of the disease. Thus, we examined whether the differentiation status in parental tumors affects PDX engraftment. Pathology report was available for 85 cases. A total number of 69 (81.2%) moderately differentiated and 16 (18.8%) poorly differentiated tumors were implanted into mice. Overall, 41 of 69 (59.9%) moderately differentiated and 9/15 (56.4%) poorly

differentiated specimens developed a PDX (Table 2). These results show that the extent of parental tumor cell differentiation does not affect the establishment of a PDX line. Furthermore, the differentiation state also remains similar between the human primary tissues and the established PDX (Supplementary Fig. S1).

Mouse stromal tissue in PDX growth

The human/mouse species-specific hybridization probe set recognized human chromatin (red) and mouse chromatin (green). Patient original tissue carried only cells of human origin (Fig. 1A). In contrast, consistent with prior reports, PDX specimens displayed a mixture of human and mouse cells by passage 2 (Fig. 1B and C). Mouse stromal in tissue migrates into the growing human xenograft tissue and displayed a similar pattern of behavior in multiple PDX samples examined.

Discussion

Preclinical evaluation of investigational agents has been inefficient, which is reflected by the high rates of clinical trial failures. Specifically, only 5% of chemotherapeutics and targeted agents that qualify *in vivo* preclinical evaluation show efficacy in late-stage clinical trials. These failures highlighting the need for novel strategies of predicting clinical efficacy before drugs enter the clinic (37). One such limitation is the use of animal models that

Table 3. Log-rank test and multivariable cox model to compare time to PDX establishment

Univariate analysis				
Covariates	N ^a	Event	Median time to PDX (months) (95% CI)	P
	88	41	12.2 (8.1-NA)	
Method of tumor acquisition				
Surgery	51	31	8.1 (5.5-14.4)	0.053
Biopsy	37	10	NA	
Site of sampling				
Primary	12	11	3.8 (2.5-NA)	0.15
Metastatic	76	30	12.2 (11.97-NA)	
Prior therapy				
Neoadjuvant	74	31	12.2 (11.9-NA)	0.007
No neoadjuvant	10	7	3.8 (2.5-NA)	
Preoperative bevacizumab				
No	26	10	21.9 (8.1-NA)	0.64
Yes	50	20	12.2 (11.9-NA)	
KRAS mutant				
No	47	18	12.2 (11.9-NA)	0.81
Yes	25	12	14.4 (5.7-NA)	
BRAF mutant				
No	54	24	12.2 (11.9-NA)	0.26
Yes	11	2	NA	
PIK3CA mutant				
No	38	14	12.2 (11.9-NA)	0.66
Yes	20	11	14.4 (3.7-NA)	
Time to engraftment				
<2 hours (immediate)	51	19	13.8 (12.2-NA)	0.0004
12-24 hours (delayed)	37	22	5.5 (3.8-NA)	
Tumor grade				
Moderately differentiated	68	35	12.2 (6.3-NA)	0.86
Poorly differentiated	12	6	11.9 (3.8-NA)	
Multivariate analysis				
Covariates	HR (95% CI)			P
Surgery vs. biopsy	0.98 (0.26-3.67)			0.98
Neoadjuvant therapy vs. No	0.38 (0.16-0.89)			0.026
Immediate vs. delayed	0.30 (0.09-1.01)			0.053

^aTime to implantation was not available for some models.

fail to represent the molecular biology of the cancers that will be enrolled in the proof-of-principle clinical trials. To overcome this barrier, basic and translational research is trending toward the development of animal models that recapitulate the molecular and genetic complexity of human disease with more heterogenic tumor target fidelity relative to traditional cell lines. Currently, four major categories of mouse models have been developed, including carcinogenesis models, genetically engineered mouse models (GEMM), cultured tumor cell xenografts and patient-derived xenograft (PDX) models (14, 38-41). Each approach has specific advantages and disadvantages. The most common carcinogenesis model involves azoxymethane (AOM) initiation and dextran sodium sulfate (DSS) promotion (40, 41). This approach recapitulates the various steps of tumor progression, especially inflammation-associated colorectal cancers. The AOM/DSS approach using immunocompetent mice is reproducible and recapitulates the spontaneous adenoma-carcinoma sequence, but is typically more appropriate for chemoprevention studies not therapeutic discovery for advanced disease (40, 41). Similarly, rodent GEMM models utilize genetically enforced expression of cancer predisposition properties or deletion of suppressor genes, resulting in spontaneous tumor formation (38, 39). Rodent GEMM models typically examine a single gene or limited series of genetic lesions that in many cases do not capture the complexity or diversity of the human heterogeneous colorectal cancer disease

state. As another simplistic mainstay human/rodent model by contrast, subcutaneous or orthotopic injection of colorectal cancer tumor cells has long been used for preclinical drug testing (42, 43), but, as mentioned, lacks the molecular and genetic complexity found in colon tumors (6-14). Orthotopic studies in colorectal cancer have been limited by reproducibility and complexity of the cecal injections, and are rarely used in qualifying novel therapies for clinical trial development. The power of a tumor cell-centric focus is harnessed by restricted genetic manipulation of the human cellular component in xenograft models, which holds a significant advantage for altering specific signal transduction pathways during drug testing but is heavily influenced by a limited genetic context. These xenograft models using immunocompromised mice inherently do not include complete immune responses. Lack of human immune or stromal responses can in some cases be partly addressed through the use of humanized mouse models (44-46).

Our studies endeavor to overcome certain deficiencies associated with testing drugs using other mouse models by developing a significant cohort of PDX models to represent the broad biologic and clinical spectrum of colorectal cancer. This achievement was feasible using patient tumor samples from both surgery and guided biopsies. In this discovery setting, we were able to shed light on poorly understood intrinsic or extrinsic factors that affect PDX engraftment rates. This is particularly important to understand whether transfer of human colorectal cancer samples into the murine background selects for specific tumor characteristics that might not be relevant to human disease.

We systematically addressed these questions by examining the role of (i) tumor acquisition method, (ii) site of sampling, (iii) time to engraftment, (iv) parental tumor mutational status, (v) degree of tumor cells differentiation, and (vi) previous therapy in the development of a PDX model. To our knowledge, this is the first such systematic study that focuses on whether these parameters influence colorectal cancer PDX engraftment rates from both surgical and biopsy specimens.

Our analyses identified three factors affecting engraftment: method of tumor acquisition, site of sampling, and previous chemotherapy. Specifically, we showed that surgery specimens have higher rates of engraftment relative to biopsy specimens. The engraftment rate of surgery specimens was approximately 70%, in agreement with previous reports (22, 25). In contrast, only 35% of biopsy specimens led to a PDX model. In the multivariate model, this corresponded to a 4.8-fold lower odds of engraftment compared to the surgical specimens. This result was expected due to differences in tissue sample size, percentage of tumor cells relative to normal tissue and viability of tumor cells between surgical and biopsy samples that have been previously documented (23, 47). Variations in the tissue bulk, technical challenges of tissue collection and tumor initiating cell content within the samples may also account for some of these differences. Nevertheless, the proportion of biopsy specimens leading to the development of a PDX model remains high enough for feasibility for many of the proposed uses. Utilizing biopsy specimens is crucial to increase the range of disease biology that is represented in PDX models. For example, serial biopsy-derived models can recapitulate changes that occur within the same tumor during the evolution of the tumor with treatment and time (48, 49). Recent studies have shown that metastatic foci have a large percentage of necrosis; however, tissue in the periphery of these lesions maintains high levels of viability (50-52). Thus, guided biopsies targeting the

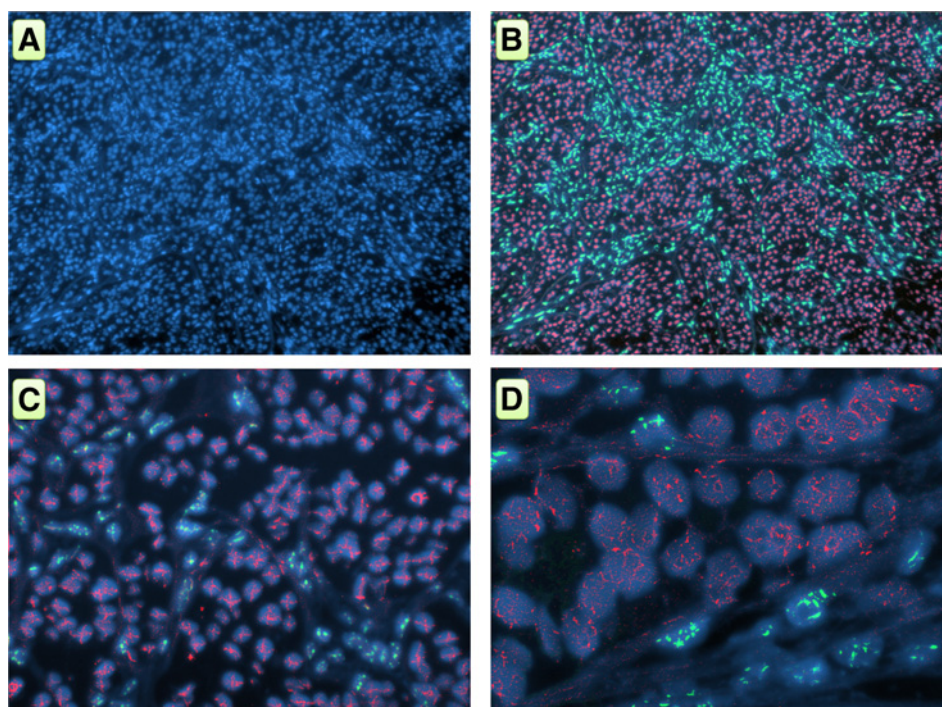


Figure 1.

Dual-color FISH on FFPE tissue sections from established PDXs shows mixture of human and mouse cells. **A**, Single DAPI filter showing nuclear chromatin; **B–D**, Merged DAPI, FITC, and TR filters highlighting the nuclei from human (painted in red) and from mouse (painted in green) origin. **A** and **B**, low magnification microscope field (10× objective); **C**, high magnification (40× objective); **D**, high magnification (100× objective).

periphery of metastatic foci might increase the possibility of acquiring living tissue and improve the engraftment rates of the biopsy group.

Although we expected that metastatic tumors are more aggressive biologically and would adapt more successfully as subcutaneous implants than primary tumors, this was not the case. Primary tumor samples engrafted PDX lines more effectively relative to metastatic tumors in our cohort, with an almost 7-fold higher odd of model establishment. We believe that this difference is related to tissue accessibility and viability of tumor cells engrafted. Indeed, improving biopsy techniques would be important to access live tumor cells from metastatic sites and development of a PDX line. Sample bulk and the tumor-initiating cell content within these different samples may also account for these differences. Notably, time to implantation did not adversely affect rate of PDX establishment. This finding is encouraging as it allows for a more liberal time in protocols from biopsy to implantation. This can promote studies with remote biopsy acquisition without the concern of decreased rate of PDX establishment.

Interestingly, previous therapy significantly delayed the time to engraftment of PDX model, without any significant effect in overall engraftment rate. Untreated tumors are more likely to be actively proliferating and have higher tumor–cell content relative to stroma. We hypothesize that this advantage may be offset by a greater resistance to environment stress and reduced metabolic requirements. Furthermore, the impact of therapy on dormancy, or senescence, might influence the sample and thereby require additional time for the emergence of tumor-initiating cells during engraftment (53–55). Nevertheless, our anecdotal association of delayed development with rapid growth after that point suggests that PDX models from pretreated tumors might be more aggressive once established. To answer this question, we suggest studying the growth rates of the next PDX generations and comparing the two groups. Importantly, none of the clinically relevant

genetic mutations tested (KRAS, BRAF, and PIK3CA) affected the development of a PDX line. This result is critical for the further use of PDX models as an unbiased preclinical research tool, and specifically as a screening strategy for the development of novel investigational agents. However, we acknowledge that these three genes are not sufficient to fully recapitulate the spectrum of colorectal cancer biology, and additional undetected selection bias may still exist.

The main limitation of our study is that although we have looked at key factors, there are additional factors that may impact rate of model establishment, such as tumor viability, proliferative rate, time from last dose of chemotherapy, time from vascular clamping to tissue harvesting in surgical specimens, postcollection preservation techniques, among others, which remain unaccounted for and merit further investigations. In addition, as size (and weight) of biopsy specimens are often lower than those obtained by surgery, we cannot reliably rule out the possibility of similar PDX establishment rate with biopsy specimens of equivalent size.

In conclusion, our study provides evidence supporting that genetic selection during the transfer of human tumors into the murine background does not appear to occur. In addition, we identified three factors that affect the PDX engraftment rates and our ability to develop biologically relevant tools for the preclinical evaluation of investigational agents. More important, these factors are amendable to improvement through better sampling technique and manipulations throughout tissue transfer. Furthermore, as both PDX and humanized mouse model technologies are emerging simultaneously, one might envision combining them in the future. A potential scenario might call for establishing PDX tumors initially and/or subsequent placement into a mouse with the appropriate human stroma for even more effective drug discovery and testing. This would be particularly critical for combining chemotherapy and immunotherapy in an adjuvant

setting. For now, we submit that our PDX platform is the most immediate and effective approach forward for uncovering more active therapeutic agents as we target heterogeneous colorectal cancer disease. Ultimately, the inherent tumor heterogeneity found in PDX modeling constitutes a better representation of the original tumor and thereby is expected to provide a more biologically relevant platform for testing therapeutic drugs.

Disclosure of Potential Conflicts of Interest

G. Powis is a founder/SAB at PHusis Therapeutics, a CEO at Enscye Biosciences, has received speakers bureau honoraria from Roche, and is a consultant/advisory board member for PHusis Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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