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Beyond organoids: restoring the value of patient-derived two-dimensional colorectal cancer models

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Abstract

Background Two-dimensional (2D) cell lines derived from colorectal cancer (CRC) are inexpensive, scalable and well suited to drug testing, but derivation from patient tissue has been inefficient. We asked how often defined, serum-free media yield stable lines from primary tissue and from patient-derived xenografts (PDX), and whether the clinical record predicts which tumors will grow.

Methods We analyzed 193 culture attempts on 158 CRC specimens from 136 patients (95 primary-tissue and 98 PDX-derived attempts; 35 specimens by both routes) from a single biobank, 2015 to 2023. Twelve conditions were compared: three defined serum-free media with and without the ROCK inhibitor Y-27632, and six serum-containing historical conditions. A culture counted as established after at least three passages with stable growth. Eleven clinical and histopathological parameters and six molecular markers were tested against establishment with Bonferroni correction. Overall and relapse-free survival by establishment status were analyzed in an exploratory Cox model.

Results PDX-derived cultures established more often than primary cultures (57.7% vs 39.1%; odds ratio [OR] 2.13, P = 0.013). Defined media outperformed historical conditions in both routes (P < 0.001); the three defined media did not differ from each other. In paired comparisons, Y-27632 converted 13 failed cultures to successful ones and the reverse occurred 3 times (OR 4.33, P = 0.021). When both routes were attempted, 81.8% of specimens gave a line. In total, 90 authenticated lines were obtained from 74 specimens of 65 patients. No parameter predicted establishment after correction; the lowest P value was for alcohol use (P = 0.017; corrected α = 0.0045). At a median follow-up of 65.8 months, establishment was associated with shorter survival after adjustment for stage and resection status (overall survival hazard ratio [HR] 1.77, 95% CI 1.03–3.06; relapse-free survival HR 1.95, 1.11–3.42). Unadjusted differences were not significant.

Conclusions With defined serum-free media, Y-27632 and a PDX backup route, patient-derived 2D CRC lines can be obtained at rates close to those reported for organoids. Clinical, histopathological and molecular data do not predict which tumors will grow, so the panel is not biased toward a clinical subgroup. Whether ex vivo growth marks tumors with poorer outcome needs testing in an independent cohort.

Keywords Colorectal cancer; Patient-derived models; Two-dimensional cell culture; Patient-derived xenograft; ROCK inhibitor; Biobank; Precision oncology

Background

Colorectal cancer (CRC) is among the most common and lethal cancers, and models that reflect individual patients remain a bottleneck in translational research [1]. Patient-derived two-dimensional (2D) adherent cell lines are cheap to maintain, easy to scale and compatible with almost any assay, which makes them useful for functional studies and drug screening [2]. Their use has been limited by low success rates when lines are derived directly from tumor tissue.

CRC is molecularly heterogeneous, with four consensus molecular subtypes (CMS1 to CMS4) and five CRC intrinsic subtypes (CRIS-A to CRIS-E) that differ in biology and drug response [3]. Patient-derived lines that keep these features allow drug responses to be studied against the molecular class of the source tumor.

Passaging tumor tissue in immunodeficient mice as patient-derived xenografts (PDX) enriches viable tumor cells and removes most of the microbial load of colonic tissue. Secondary cultures from PDX tissue therefore establish more often than cultures from fresh patient tissue [4]. Culture media have also changed. Defined, serum-free formulations aim to support epithelial tumor cells more selectively than classic DMEM- or RPMI-based media with fetal bovine serum [5–7], and the ROCK inhibitor Y-27632 reduces anoikis and improves attachment of primary epithelial cells, including CRC cells [8].

Here we report a multi-year comparison of twelve culture conditions in a CRC biobank with both primary and PDX-derived material [9]. We had three aims: to measure 2D establishment rates under defined media with and without Y-27632, to derive a practical protocol, and to test whether establishment can be predicted from routine clinical, histopathological and molecular data. During follow-up of the cohort we also examined whether establishment status is associated with patient outcome.

Methods

Patients and biobank

All specimens were collected in the Hanse-Rostock CRC (HROC) biobank at the University Medical Center Rostock, which prospectively stores primary tumor, metastatic and blood samples from patients undergoing resection or biopsy, together with clinical follow-up. The Ethics Committee of the University of Rostock approved biobanking and all procedures (A 2018-0054 and A 2019-0187). The study followed the Declaration of Helsinki, and all patients gave written informed consent. The culture cohort comprised 158 specimens from 136 patients: 95 primary-tissue and 98 PDX-derived culture attempts, with 35 specimens processed by both routes (193 culture experiments). One PDX specimen from an external

partner had no patient data and was excluded from clinical analyses, leaving 157 specimens from 136 patients (Table 1).

PDX establishment and tissue processing

All animal experiments were authorized by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF M-V) under permit numbers 7221.3-2-020/17 and 7221.3-2-023/20. All experimental protocols were approved by this authority, and all methods were carried out in accordance with the relevant guidelines and regulations, including the German Animal Welfare Act (Tierschutzgesetz) and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The study is reported in accordance with the ARRIVE guidelines. PDX models were established as described [9]. Nude and NSG mice were bred in-house from breeding pairs obtained from Janvier Labs (Le Genest-Saint-Isle, France). Mice were euthanized by carbon dioxide (CO_2) inhalation with a displacement rate of 30–40% chamber volume per minute using the equipment of the Animal Core Facility of the University Medical Center Rostock, followed by cervical dislocation as a confirmatory secondary method. Fresh tumor tissue was implanted subcutaneously into NSG or nude mice, engraftment was confirmed by caliper measurement and histology, and PDX tissue from passages 1 to 5 was used for culture. For primary cultures, tissue was transported on ice in PBS and processed within 2 h of resection: it was minced with crossed scalpels, passed through a 100 µm strainer, centrifuged (300 × g, 5 min) and resuspended in the respective medium [4]. PDX tissue was processed in the same way.

Culture media and conditions

Twelve conditions were evaluated (Table 2). The historical conditions were MOITUM (DMEM/F12 1:1 with 0, 5, 10 or 20% fetal bovine serum [FBS Gold, PanBiotech], non-essential amino acids, insulin-transferrin-selenite, BME vitamins, 2 mM L-glutamine, penicillin, streptomycin, gentamicin and amphotericin B) and DMEM/F12 with 1% human AB serum or 1% autologous patient serum. The defined, serum-free media were Sphero Medium, Pancreas TumorMACS™ (Miltenyi Biotec, Bergisch Gladbach, Germany; 130-127-484) and Colon TumorMACS™ (Miltenyi Biotec; 130-127-169). Sphero Medium is an in-house formulation adapted from organoid protocols: DMEM/F12 (1:1) with 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL Primocin, 1× B-27, 1.25 mM N-acetyl-L-cysteine, 10 mM nicotinamide, 50 ng/mL EGF, 100 ng/mL Noggin, 500 ng/mL R-spondin-1, 50% (v/v) Wnt-3A conditioned medium, 500 nM A83-01, 10 µM SB202190, 10 nM [Leu15]-gastrin I and 10 nM prostaglandin E2. The TumorMACS™ compositions are proprietary. Where indicated, Y-27632 dihydrochloride (MedChemExpress, HY-10583) was added at 10 µM at every medium change until stable growth.

Culture, authentication and definition of establishment

Six-well plates were coated with type I collagen (calf skin; Sigma-Aldrich, C9791) and type IV collagen (human placenta; Sigma-Aldrich, C5533) and air-dried. Cells were seeded at densities set by the available material and kept at 37°C and 5% CO_2. Medium was changed about every 3 days, where possible by replacing half the volume, and cultures were inspected by phase-contrast microscopy at least twice weekly. Cultures were passaged at 70–80% confluence, or when colonies began to disintegrate, with TrypLE Express (Thermo Fisher Scientific, 12605010) or trypsin-EDTA (PanBiotech, P10-023100). A culture counted as established after at least three consecutive passages with sustained proliferation and stable morphology. All established lines were authenticated by short tandem repeat profiling against patient blood or normal-tissue DNA and tested for mycoplasma by PCR or PlasmoTest (InvivoGen, rep-pt1). Stocks were frozen in FBS with 10% DMSO in a controlled-rate freezer and stored over liquid nitrogen [4]. Culture attempts were made between 2015 and 2023; the systematic media comparison took place mainly in 2019 to 2021.

Clinical data and follow-up

Clinical, histopathological and molecular data were taken from the biobank records. Age at collection, date of collection and date of death came from the patient registry; BMI, smoking and alcohol use from the clinical questionnaire, with BMI recorded per surgery. Outcome dates and tumor status at last contact came from the curated follow-up file, updated with cancer registry information until June 2026. Overall survival (OS) and relapse-free survival (RFS) were calculated in months from the date of specimen collection, which equals the date of surgery. Death from any cause was the OS event. For RFS, relapse or persistent disease at last contact, or death, counted as an event; patients alive and tumor-free were censored at last contact. Specimens from patients who were never tumor-free after surgery (R2 resection, unresected metastases or perioperative death) were excluded from RFS. Three specimens with conflicting or missing dates were excluded from survival analyses.

Statistical analysis

Analyses were done in Python 3.12 with SciPy and statsmodels. Establishment rates are positive cultures divided by evaluable attempts; contaminated cultures were treated as not evaluable. Binary outcomes were compared with Fisher's exact test (unpaired) and McNemar's exact test (paired, on specimens tested under both conditions). The Y-27632 effect was tested with a pooled McNemar test over the six medium × route combinations (181 pairs). Odds ratios (OR) with 95% confidence intervals (CI) were calculated by Woolf's method (unpaired) or exact binomial inversion (paired). Differences among the six defined conditions were tested with Cochran's Q test in specimens evaluable under all six.

For predictor analyses a specimen counted as established if any route gave a line (combined cohort); the primary-tissue and PDX-derived routes were also analyzed separately. Continuous variables (age, BMI, tumor volume, UICC stage as an ordinal score) were compared by Mann-Whitney U test with Cliff's δ as effect size. Categorical variables (sex, smoking, alcohol, tumor budding as MG vs LG and MG vs HG, and tumor site right vs left/rectum, restricted to primary-tumor specimens) were compared by Fisher's exact test, and UICC stage in four categories by χ^2 test. These eleven comparisons per cohort were corrected by Bonferroni ($\alpha = 0.05/11 = 0.0045$). The molecular markers MSI-H, KRAS, BRAF, APC, TP53 and PIK3CA were analyzed by Firth penalized logistic regression because of small cell counts. A multivariable logistic model included UICC stage, MSI, KRAS and BRAF in complete cases. In a sensitivity analysis contaminated cultures were counted as failures. Tumor factor by medium interactions were tested in each of the six defined conditions and both routes. Detectable effect sizes were estimated for a binary predictor with 50% prevalence at 80% power.

OS and RFS by establishment status were estimated by the Kaplan-Meier method and compared by log-rank test. Hazard ratios (HR) came from Cox models, unadjusted and adjusted for UICC stage IV and for stage IV plus R status (R1/R2 vs R0), with standard errors clustered by patient. A sensitivity analysis used one specimen per patient. Median follow-up was estimated by the reverse Kaplan-Meier method. The survival analysis was not planned at the start of the study and is reported as exploratory. Code that reproduces all statistics is available (see Availability of data and materials).

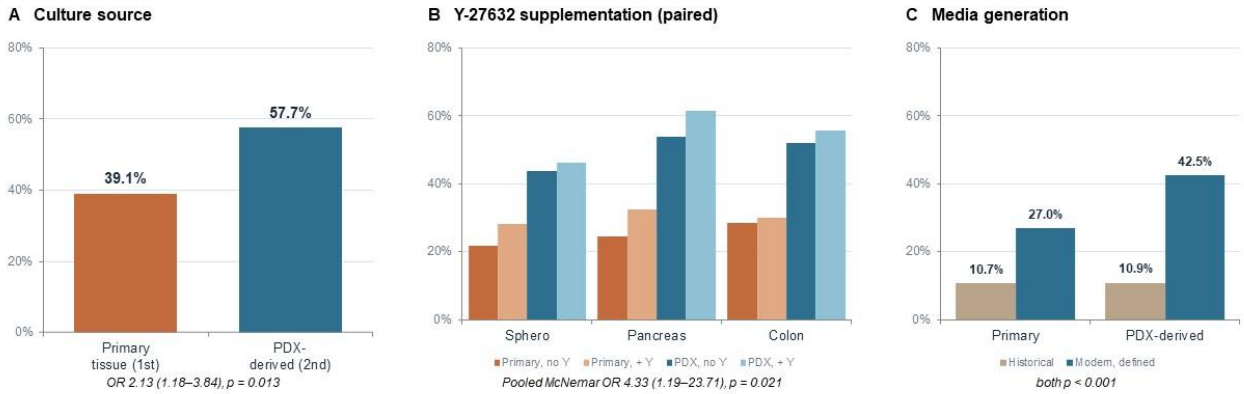
Results

Cohort and overall establishment

The clinical cohort comprised 157 specimens from 136 patients (97 male, 60 female; median age 71 years, range 30 to 93; 13 patients younger than 50 years; 50.3% UICC stage IV; Table 1). Establishment rates were 39.1% (34/87 evaluable) for primary cultures and 57.7% (56/97 evaluable) for PDX-derived cultures. These 90 positive cultures correspond to 90 stable lines from 74 specimens of 65 patients; 16 specimens gave a line by both routes (Fig. 1, Table 2).

Figure 1

Establishment of 2D CRC cultures by source, medium supplementation, and media generation



PDX-derived cultures establish more often than primary cultures

PDX-derived cultures established more often than primary cultures (57.7% vs 39.1%; OR 2.13, 95% CI 1.18–3.84, P = 0.013; Fig. 1a, Table 3a). Contamination was a problem mainly in primary cultures. Nine specimens were lost to contamination, eight of 95 primary-tissue specimens (8.4%) and one of 98 PDX-derived specimens (1.0%; OR 0.11, 95% CI 0.014–0.91, P = 0.017; Table 3d). At the level of single conditions, 64 primary and 8 PDX-derived cultures were contaminated. One specimen lost to contamination in primary culture later gave a line from PDX tissue.

Defined media and Y-27632

Defined media outperformed historical conditions in both routes (primary: 27.0% vs 10.7%, OR 3.09, 95% CI 1.57–6.09, P < 0.001; PDX-derived: 42.5% vs 10.9%, OR 6.07, 95% CI 3.77–9.77, P < 0.001; Fig. 1c, Table 2). The three defined media did not differ. Cochran's Q test showed no difference among the six defined conditions (primary P = 0.100, PDX-derived P = 0.747), none of the 15 pairwise comparisons approached significance (smallest P = 0.25 primary, 0.52 PDX-derived), and comparisons of the base media pooled over ± Y-27632 gave P ≥ 0.31 throughout (Additional file 1: Tables S7a–c).

Because most specimens were tested with and without Y-27632 in the same base medium, the supplement was evaluated by paired analysis (Table 3b). Y-27632 raised establishment by 4.2 to 12.5 percentage points in primary cultures and by 2.6 to 7.7 percentage points in PDX-derived cultures. Across 181 paired observations, it converted a failed culture into a successful one 13 times; the reverse happened 3 times (McNemar P = 0.021; OR 4.33, 95% CI 1.19–23.71; Fig. 1b). The effect pointed the same way in every medium and in both routes, but no single route reached significance on its own (primary 7 vs 1, P = 0.070; PDX-derived 6 vs 2, P = 0.289; Additional file 1: S6). Pancreas TumorMACS™ with Y-27632 had the highest

rates (primary 32.5%, PDX-derived 61.5%) without being significantly better than the other defined conditions.

Matched primary and PDX-derived cultures

Thirty-three specimens were evaluable in both routes (Table 3c). In this subset primary cultures established in 57.6% (19/33) and PDX-derived cultures in 72.7% (24/33); at least one route succeeded in 81.8% (27/33). Sixteen specimens established in both routes and 6 in neither. Only the PDX route succeeded in 8 specimens (24.2%) and only the primary route in 3 (9.1%; McNemar P = 0.227). Of the 56 specimens established from PDX tissue, 46 (82%) grew at the first attempt. In the most recent series, in which every specimen was tested under all six defined conditions, PDX-derived cultures established in 73.1% (19/26), compared with 52.1% (37/71) in earlier PDX specimens (OR 2.49, 95% CI 0.93–6.67, P = 0.10).

Eighteen patients contributed more than one specimen (primary and metastatic tissue, several metastases or two primary tumors). Among patients with an evaluable culture, at least one line was obtained in 13 of 16 patients with several specimens and in 51 of 115 patients with a single specimen (P = 0.007). Part of this difference is expected from the larger number of attempts per patient.

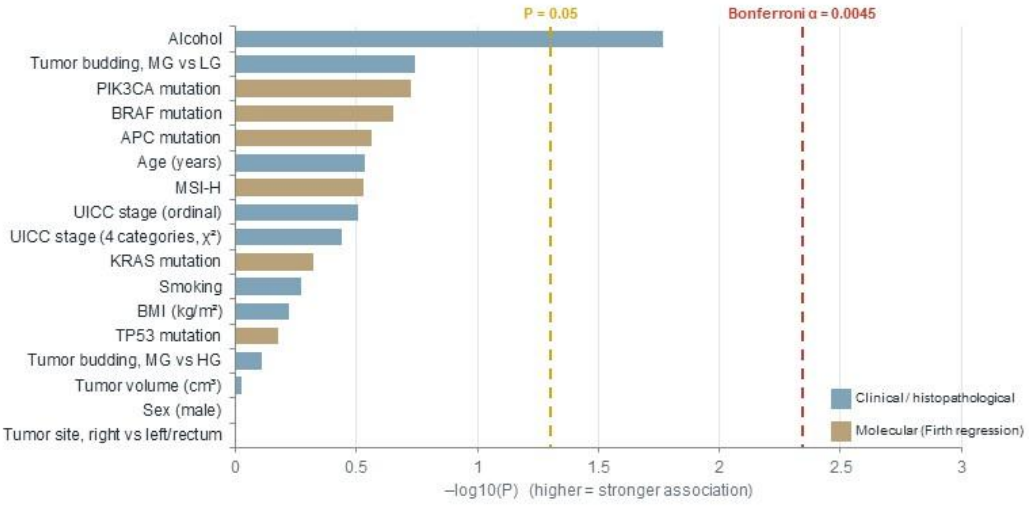
Establishment is not predicted by clinical, histopathological or molecular data

No parameter predicted establishment after Bonferroni correction in any cohort (Fig. 2, Table 4). In the combined cohort (n = 150; 73 established, 77 failed) the lowest P value was for alcohol use (65% vs 43% established; OR 2.50, 95% CI 1.18–5.32, P = 0.017), which did not reach the corrected threshold of 0.0045 and was not significant in either route alone (primary P = 0.086, PDX-derived P = 0.18). UICC stage, tumor volume, budding, tumor site, age, sex, BMI and smoking were far from significance (all P ≥ 0.18). None of the six molecular markers was associated with establishment (Firth regression, all P ≥ 0.14 in every cohort). The multivariable model with UICC stage, MSI, KRAS and BRAF explained almost none of the variation (n = 132; pseudo-R^2 = 0.022; likelihood-ratio P = 0.40). Counting contaminated cultures as failures did not change these results (alcohol P = 0.029; all other P ≥ 0.11; Additional file 1: Table S3). With 150 specimens, a binary predictor of 50% prevalence would have been detected with 80% power at an OR of about 3.4 at the corrected threshold and about 2.5 at P = 0.05.

We also asked whether any tumor factor favored a particular medium. Of 144 factor × condition tests, 13 had P < 0.05, against 7.2 expected by chance; none passed the corrected threshold (α = 0.00035; smallest P = 0.0030; Additional file 1: Table S8).

Figure 2

Clinical, histopathological and molecular parameters tested against 2D establishment (either route)



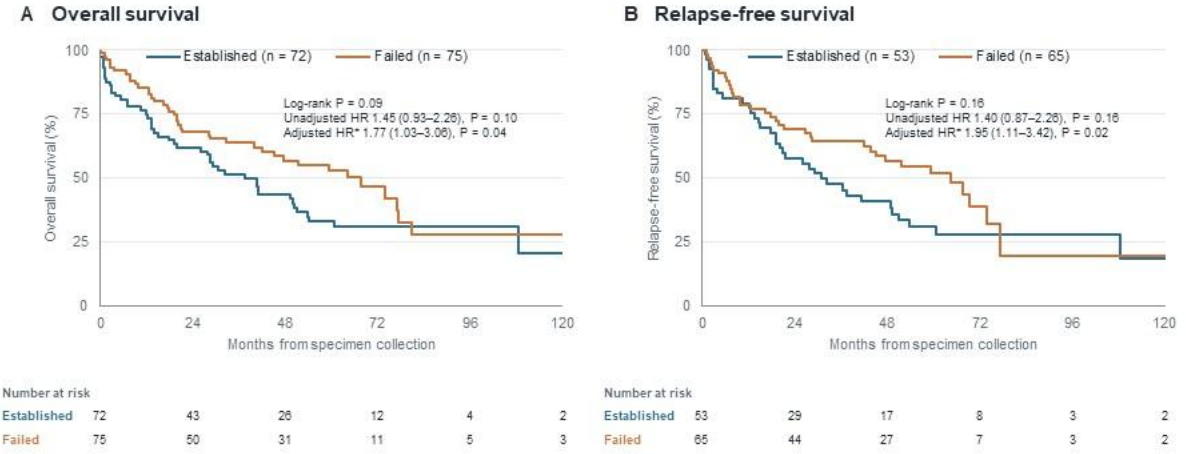
Combined cohort, n = 150 specimens. Eleven clinical and histopathological comparisons; Bonferroni α = 0.05/11. Molecular markers are shown for reference. No parameter crosses the corrected threshold.

Establishment status and outcome (exploratory)

Follow-up data were available for 154 specimens, with 91 deaths and a median follow-up of 65.8 months; 125 specimens were evaluable for RFS (76 events). Median OS was 37.6 months when the tumor established and 67.6 months when it failed (log-rank P = 0.086; Fig. 3a, Table 5). Median RFS was 30.8 and 64.4 months (log-rank P = 0.16; Fig. 3b). Unadjusted Cox models were not significant (OS HR 1.45, 95% CI 0.93–2.26; RFS HR 1.40, 0.87–2.26). After adjustment for stage IV and R status, establishment was associated with shorter OS (HR 1.77, 1.03–3.06, P = 0.039) and RFS (HR 1.95, 1.11–3.42, P = 0.020). Results were similar with one specimen per patient (Additional file 1: Table S4) (OS HR 2.28, 1.27–4.10; RFS HR 2.16, 1.20–3.90). The association was stronger for the primary-tissue route (OS HR 2.27, 1.22–4.24, unadjusted) than for the PDX-derived route (OS HR 1.29, 0.79–2.11). Only 20 relapses were documented separately from death, so the RFS result is driven mainly by deaths.

Figure 3

Overall and relapse-free survival by 2D establishment status (either route)



**Cox model adjusted for UICC stage IV and R status (R1/R2 vs R0), complete cases; standard errors clustered by patient. Relapse-free survival counts relapse or death as events; specimens never tumor-free after surgery are excluded. Exploratory analysis.*

Discussion

In this series of 158 specimens, defined serum-free media gave stable 2D CRC lines in 39.1% of primary and 57.7% of PDX-derived cultures. In the most recent series the PDX-derived rate reached 73.1%, and with both routes 81.8% of specimens gave a line. These rates are in the range reported for patient-derived organoids [10]. The idea that adherent CRC lines cannot be derived efficiently from patient material therefore no longer holds when current media are used.

Three practical points follow. First, the move from serum-containing to defined media mattered most, with a three- to six-fold increase in the odds of establishment. Which defined medium is used matters much less: Sphero Medium and the two TumorMACS™ media did not differ, and no tumor characteristic favored one of them. Colon and Pancreas TumorMACS™ performed alike despite their different target tissues, probably because both support EGF, Wnt and R-spondin signaling; the proprietary compositions do not allow a firmer statement. Second, Y-27632 helped in the paired analysis, where each specimen serves as its own control and time trends cancel out. The pooled effect was clear, the effect within each route was small and imprecise, which fits its known action on attachment and anoikis in the first passages [8]. Third, PDX tissue gave more lines and far less contamination than fresh tissue, and in a quarter of matched specimens only the PDX route succeeded. Where PDX models exist, a PDX backup raises the yield considerably. Serial passaging in mice can shift the clonal composition [12,13], which needs to be considered when PDX-derived lines are characterized.

The main analytical result is negative. Neither stage, tumor volume, budding, sidedness, age, sex, BMI and smoking, nor MSI, KRAS, BRAF, APC, TP53 or PIK3CA status predicted establishment. Alcohol use reached nominal significance in the combined cohort, but not after correction and not within either route, and we see no biological reason to pursue it. The study had good power for large effects only. Strong predictors, with an OR above about 3.4, can be excluded; weak ones cannot. This has two consequences. The lines are not drawn from a clinically or genetically distinct subgroup, which supports their use as a cross-section of the disease. And no laboratory can predict from the chart which tumors are worth the culture effort, so every specimen should be attempted.

The survival analysis adds a qualification. Tumors that grew in culture came from patients with somewhat shorter survival once stage and resection status were taken into account. The direction is plausible, because tumors that proliferate without their microenvironment may also behave more aggressively in patients. The finding is, however, weak. It was not planned, the unadjusted tests were not significant, several models were fitted, and the RFS result rests mainly on deaths. The stronger effect in the primary route than in the PDX route fits the fact that PDX-derived cultures come from tumors already selected by engraftment, but could also be chance. The effect is modest, and our cohort is too small to confirm it. We therefore suggest testing the association in larger, independent cohorts, ideally with culture outcome recorded before follow-up, to see whether it holds and reaches significance.

Reported establishment rates for patient-derived CRC models vary with method and with the definition of success. Permanent 2D lines from fresh primary tissue have been obtained in roughly 10 to 15% of attempts, median CRC PDX engraftment is around 70%, and organoid derivation succeeds in about 50 to 80% [14–16]. Comparisons are only meaningful when the success criterion is stated; ours, three passages with stable growth, is strict and excludes transient outgrowth.

The 90 authenticated lines cover all UICC stages, primary tumors and synchronous and metachronous metastases, including matched pairs from the same patient. Within the biobank they are linked to PDX models and, where available, organoids, so in vitro and in vivo results can be compared directly. Mutation status and, for part of the panel, CMS and CRIS subtypes are known [4], which allows drug responses to be interpreted against molecular class. The lines are available through the biobank and from a commercial distributor.

The study has limitations. It is observational, and media were introduced at different times, so the comparison of historical and defined media is confounded by time; the paired Y-27632 analysis is not affected by this. Only tumors that engrafted in mice entered the PDX route, which selects for proliferative tumors; resection status differed between the routes accordingly.

The establishment criterion may underestimate the initial take rate. The predictor analysis can exclude only strong effects. The cohort comes from one center, molecular subtype data are incomplete, and 2D lines lack stroma, immune cells and extracellular matrix [17].

Conclusions

Defined serum-free media, Y-27632 and a PDX backup route make the derivation of patient-derived 2D CRC lines efficient, with success in four of five specimens when both routes are available. Establishment could not be predicted from clinical, histopathological or molecular data, so the resulting panel represents the disease without obvious clinical bias. The possible association between ex vivo growth and shorter survival should be tested in other cohorts.

Abbreviations

2D: two-dimensional; CI: confidence interval; CMS: consensus molecular subtype; CRC: colorectal cancer; CRIS: CRC intrinsic subtype; FBS: fetal bovine serum; HR: hazard ratio; IQR: interquartile range; MSI-H: high microsatellite instability; MSS: microsatellite stable; OR: odds ratio; OS: overall survival; PDX: patient-derived xenograft; RFS: relapse-free survival; ROCK: Rho-associated protein kinase; UICC: Union for International Cancer Control.

Declarations

Ethics approval and consent to participate Approved by the Ethics Committee of the University of Rostock (Ethikkommission an der Universitätsmedizin Rostock; A 2018-0054 and A 2019-0187). All patients gave written informed consent. The study followed the Declaration of Helsinki. Animal experiments for PDX establishment were approved by the responsible state authority as described [9].

Consent for publication Not applicable.

Availability of data and materials The de-identified specimen-level dataset (Additional file 2), a de-identified table of all culture attempts and the analysis code are deposited at Zenodo (https://doi.org/10.5281/zenodo.23340697) and reproduce all reported statistics. Dates and other identifying clinical data are held in the HROC biobank and are available from the corresponding author under a data transfer agreement. Cell lines, PDX models and matched tissue are available for academic research under a material transfer agreement; the 2D lines are also distributed by Cytion (Eppelheim, Germany).

Competing interests The University Medical Center Rostock holds a licensing agreement with Cytion (Eppelheim, Germany) for the distribution of HROC cell lines, under which the university receives royalties. The authors declare no other competing interests.

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Authors' contributions ML: conceptualization, methodology, formal analysis, writing of the original draft, supervision, funding acquisition. MEH: formal analysis, writing of the original draft, review and editing. MP: data curation, review and editing. MK and ED: methodology, investigation. XZ and ZS: formal analysis. CS: resources. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 Establishment of 2D CRC cultures by source, Y-27632 supplementation and media generation. a Primary-tissue vs PDX-derived cultures (39.1% vs 57.7%; OR 2.13, 95% CI 1.18–3.84, P = 0.013). b Each defined medium with and without Y-27632 in both routes (pooled paired McNemar OR 4.33, 95% CI 1.19–23.71, P = 0.021). c Defined vs historical media in both routes (both P < 0.001). Rates are positive/evaluable; contaminated cultures excluded.

Fig. 2 Clinical, histopathological and molecular parameters tested against establishment by either route (n = 150). Bars show $-\log_{10}(P)$: Mann-Whitney U test for continuous variables, Fisher's exact test for categorical variables, χ^2 test for UICC stage in four categories, and Firth logistic regression for molecular markers. Dashed lines mark P = 0.05 and the Bonferroni threshold for eleven clinical and histopathological comparisons ($\alpha = 0.0045$). No parameter crosses the corrected threshold.

Fig. 3 Overall (a) and relapse-free (b) survival from specimen collection by establishment status (either route). Kaplan-Meier estimates with numbers at risk. Hazard ratios from Cox models, unadjusted and adjusted for UICC stage IV and R status, with standard errors clustered by patient. Relapse-free survival counts relapse, persistent disease or death as events and excludes specimens from patients never tumor-free after surgery. Exploratory analysis.

Additional files

Additional file 1 Supplementary methods and results: predictor effect sizes by route (Table S1), detectable effect sizes (S2), contaminated cultures counted as failures (Table S3), sensitivity Cox models (Table S4), sources of follow-up data (S5), Y-27632 by route (S6), comparison of the defined media (Tables S7a–c) and tumor factor × medium interaction (Table S8).

Additional file 2 De-identified specimen-level dataset.

Tables

Table 1 Clinical, histopathological and molecular characteristics of the culture cohort

Characteristic	All specimens (n = 157)	Primary-tissue cohort (n = 95)	PDX-derived cohort (n = 97)
Patient characteristics			
Sex, n (%)			
M	97 (61.8)	64 (67.4)	57 (58.8)
F	60 (38.2)	31 (32.6)	40 (41.2)
Age at collection, years, median (IQR)	71 (63–79)	69 (60–78)	73 (61–80)
Early-onset (<50 years), n (%)	13 (8.3)	9 (9.5)	9 (9.3)
BMI, kg/m², median (IQR)	26.5 (23.6–29.9)	26.8 (24.0–29.9)	26.5 (23.6–29.0)
Current/former smoker, n (%)			
yes	31 (20.0)	17 (18.3)	21 (21.9)
no	124 (80.0)	76 (81.7)	75 (78.1)
Not available	2	2	1
Regular alcohol use, n (%)			
yes	42 (27.1)	25 (26.9)	29 (30.2)
no	113 (72.9)	68 (73.1)	67 (69.8)
Not available	2	2	1
Specimen characteristics			
Specimen origin, n (%)			
Primary tumor	111 (70.7)	58 (61.1)	72 (74.2)
Metastasis / other	46 (29.3)	37 (38.9)	25 (25.8)
Primary tumor site*, n (%)			
Right	64 (57.7)	31 (53.4)	42 (58.3)
Left	36 (32.4)	21 (36.2)	24 (33.3)
Rectum	11 (9.9)	6 (10.3)	6 (8.3)
Site of non-primary specimen†, n (%)			
Liver	29 (63.0)	20 (54.1)	19 (76.0)
Lung	6 (13.0)	6 (16.2)	2 (8.0)
Peritoneum	4 (8.7)	4 (10.8)	1 (4.0)
Other	7 (15.2)	7 (18.9)	3 (12.0)
Tumor characteristics			
UICC stage, n (%)			
I	16 (10.2)	10 (10.5)	7 (7.2)
II	33 (21.0)	18 (18.9)	18 (18.6)
III	29 (18.5)	15 (15.8)	21 (21.6)
IV	79 (50.3)	52 (54.7)	51 (52.6)
T category, n (%)			
T1-2	20 (15.3)	12 (16.4)	10 (11.8)
T3	70 (53.4)	36 (49.3)	46 (54.1)
T4	41 (31.3)	25 (34.2)	29 (34.1)
Not available	26	22	12
N category, n (%)			
N0	56 (43.1)	33 (45.8)	28 (33.3)
N+	74 (56.9)	39 (54.2)	56 (66.7)
Not available	27	23	13
M category, n (%)			
M0	78 (49.7)	43 (45.3)	46 (47.4)
M1	79 (50.3)	52 (54.7)	51 (52.6)
Grading, n (%)			
G1	4 (3.0)	2 (2.7)	2 (2.4)
G2	93 (70.5)	52 (70.3)	59 (69.4)
G3	35 (26.5)	20 (27.0)	24 (28.2)

Characteristic	All specimens (n = 157)	Primary-tissue cohort (n = 95)	PDX-derived cohort (n = 97)
Not available	25	21	12
Resection status, n (%)			
R0	97 (78.2)	60 (89.6)	56 (70.9)
R1	7 (5.6)	2 (3.0)	5 (6.3)
R2	20 (16.1)	5 (7.5)	18 (22.8)
Not available	33	28	18
Tumor volume, cm³, median (IQR)	56.6 (19.4–121.0)	49.3 (18.1–121.0)	60.0 (26.3–114.8)
Not available	9	7	5
Tumor budding, n (%)			
LG	30 (34.5)	13 (27.1)	22 (38.6)
MG	28 (32.2)	16 (33.3)	19 (33.3)
HG	29 (33.3)	19 (39.6)	16 (28.1)
Not available	70	47	40
Follow-up			
Deaths during follow-up, n (%)	91 (59.1)	49 (53.3)	66 (68.0)
Median follow-up, months (reverse Kaplan-Meier)	65.8	58.6	84.0
Molecular characteristics			
MSI status, n (%)			
MSS	114 (73.5)	74 (79.6)	69 (71.1)
MSI-L	2 (1.3)	0 (0.0)	2 (2.1)
MSI-H	39 (25.2)	19 (20.4)	26 (26.8)
Not available	2	2	0
KRAS, n (%)			
wt	95 (66.0)	53 (64.6)	60 (63.8)
mut	49 (34.0)	29 (35.4)	34 (36.2)
Not available	13	13	3
BRAF, n (%)			
wt	105 (75.0)	58 (74.4)	69 (74.2)
mut	35 (25.0)	20 (25.6)	24 (25.8)
Not available	17	17	4
APC, n (%)			
wt	74 (52.1)	37 (45.7)	52 (55.3)
mut	68 (47.9)	44 (54.3)	42 (44.7)
Not available	15	14	3
TP53, n (%)			
wt	81 (57.0)	46 (56.8)	55 (58.5)
mut	61 (43.0)	35 (43.2)	39 (41.5)
Not available	15	14	3
PIK3CA, n (%)			
wt	121 (87.1)	67 (85.9)	80 (87.9)
mut	18 (12.9)	11 (14.1)	11 (12.1)
Not available	18	17	6

Data for 157 specimens with patient-level data (136 patients). One PDX specimen from an external partner contributed to the culture experiments only. The primary-tissue and PDX-derived cohorts overlap: 35 specimens were processed by both routes. Percentages refer to specimens with available data. *Primary-tumor specimens only; right = cecum, ascending and transverse colon; left = descending colon and sigmoid; rectum includes rectosigmoid. †Metastasis-derived and other non-primary specimens. Follow-up from specimen collection; deaths among specimens with follow-up data. IQR interquartile range; LG/MG/HG low/medium/high-grade budding; MSI microsatellite instability; MSS microsatellite stable.

Table 2 Culture conditions and establishment per condition

Condition	Serum	Defined	Y-27632	Primary, pos/eval (%)	PDX-derived, pos/eval (%)
Historical: MOITUM (DMEM/F12)					
MOITUM 0% FBS	–	No	No	3/19 (15.8)	5/25 (20.0)
MOITUM 5% FBS	5% FBS	No	No	4/18 (22.2)	8/58 (13.8)
MOITUM 10% FBS	10% FBS	No	No	0/17 (0.0)	4/56 (7.1)

Condition	Serum	Defined	Y-27632	Primary, pos/eval (%)	PDX-derived, pos/eval (%)
MOITUM 20% FBS	20% FBS	No	No	1/22 (4.5)	5/56 (8.9)
Historical: human serum (DMEM/F12)					
1% AB serum	1% AB	No	No	0/9 (0.0)	2/22 (9.1)
1% autologous serum	1% autologous	No	No	3/18 (16.7)	0/4 (0.0)
Defined: Sphero Medium (in-house, serum-free)					
Sphero Medium	–	Yes	No	14/64 (21.9)	33/97 (34.0)
Sphero Medium + Y-27632	–	Yes	10 µM	16/57 (28.1)	18/39 (46.2)
Defined: Pancreas TumorMACS™					
Pancreas TumorMACS™	–	Yes	No	13/53 (24.5)	37/96 (38.5)
Pancreas TumorMACS™ + Y-27632	–	Yes	10 µM	13/40 (32.5)	16/26 (61.5)
Defined: Colon TumorMACS™					
Colon TumorMACS™	–	Yes	No	10/35 (28.6)	23/49 (46.9)
Colon TumorMACS™ + Y-27632	–	Yes	10 µM	12/40 (30.0)	15/27 (55.6)
Pooled					
All historical conditions (6)		No		11/103 (10.7)	24/221 (10.9)
All defined conditions (6)		Yes		78/289 (27.0)	142/334 (42.5)

Condition-level counts; a specimen can contribute to several conditions. Contaminated cultures (64 primary, 8 PDX-derived) excluded. Historical and defined media were used mostly in different periods, so their comparison is confounded by time. FBS fetal bovine serum.

Table 3 Culture performance by source, Y-27632, matched pairs and contamination

a Primary-tissue vs PDX-derived culture

Culture source	Positive	Evaluable	Rate	OR (95% CI)	P
Primary tissue	34	87	39.1%	Reference	–
PDX-derived	56	97	57.7%	2.13 (1.18–3.84)	0.013

Fisher's exact test; Woolf 95% CI.

b Paired effect of Y-27632

Route	Medium	Pairs	% without Y	% with Y	b	c	P
Primary	Sphero Medium	41	22.0	26.8	1	3	0.625
Primary	Pancreas TumorMACS™	24	25.0	37.5	0	3	0.250
Primary	Colon TumorMACS™	24	29.2	33.3	0	1	1.000
PDX-derived	Sphero Medium	39	43.6	46.2	0	1	1.000
PDX-derived	Pancreas TumorMACS™	26	53.8	61.5	1	3	0.625
PDX-derived	Colon TumorMACS™	27	51.9	55.6	1	2	1.000
	Pooled (six comparisons)	181			3	13	0.021

b, specimens established without but not with Y-27632; c, the reverse. Pooled McNemar exact test: OR 4.33 (95% CI 1.19–23.71). Y-27632 favorable in 13 of 16 discordant pairs.

c Matched primary and PDX-derived cultures (33 specimens evaluable in both routes)

	PDX-derived positive	PDX-derived negative	Total
Primary positive	16	3	19
Primary negative	8	6	14
Total	24	9	33

McNemar exact P = 0.227; OR 2.67 (95% CI 0.64–15.6). At least one route successful in 27/33 specimens (81.8%).

d Specimens lost to contamination

Culture source	Lost to contamination	Specimens	Rate	P
Primary tissue	8	95	8.4%	–
PDX-derived	1	98	1.0%	0.017

Fisher's exact test; OR 0.11 (95% CI 0.014–0.91).

Table 4 Clinical, histopathological and molecular parameters and 2D establishment

Variable	Established	Failed	Effect size (95% CI)	Combined P (n = 150)	Primary P (n = 87)	PDX-derived P (n = 96)
Clinical and histopathological (Bonferroni α = 0.0045)						
UICC stage (ordinal)	IV	IV	Cliff's δ = +0.09	0.31	0.39	0.21
UICC stage (4 categories, χ^2)			χ^2 = 3.18	0.37	0.40	0.65
Tumor volume (cm^3)	58.3	56.6	Cliff's δ = -0.01	0.95	0.25	0.42
Tumor budding, MG vs LG	59%	38%	OR 2.38 (0.81–6.96)	0.18	0.24	0.21
Tumor budding, MG vs HG	59%	52%	OR 1.34 (0.45–4.02)	0.78	0.71	0.51
Tumor site, right vs left/rectum†	44%	45%	OR 0.97 (0.44–2.10)	1.00	1.00	1.00
Age (years)	70.0	72.0	Cliff's δ = -0.10	0.29	0.64	0.11
Sex (male)	49%	48%	OR 1.03 (0.53–2.00)	1.00	0.10	1.00
BMI (kg/m^2)	26.4	26.5	Cliff's δ = -0.05	0.60	0.50	0.67
Smoking	43%	50%	OR 0.75 (0.33–1.72)	0.53	1.00	0.45
Alcohol	65%	43%	OR 2.50 (1.18–5.32)	0.017	0.086	0.18
Molecular (Firth logistic regression)						
MSI-H	40%	50%	OR 0.66 (0.31–1.43)	0.29	0.22	0.29
KRAS mutation	46%	52%	OR 0.77 (0.38–1.57)	0.48	0.61	0.20
BRAF mutation	39%	52%	OR 0.61 (0.27–1.35)	0.22	0.76	0.34
APC mutation	55%	45%	OR 1.45 (0.74–2.85)	0.28	0.95	0.15
TP53 mutation	47%	51%	OR 0.86 (0.44–1.69)	0.66	0.14	0.33
PIK3CA mutation	65%	47%	OR 2.03 (0.71–5.83)	0.19	0.20	0.93

Established and failed columns refer to the combined cohort and show medians (continuous variables) or the percentage established within the exposed group vs the reference group (binary variables). Mann-Whitney U test with Cliff's δ, Fisher's exact test with Woolf odds ratio, χ^2 test for UICC stage in four categories. †Primary-tumor specimens only. Molecular markers by Firth penalized logistic regression. Multivariable logistic model (UICC stage, MSI, KRAS, BRAF; n = 132): pseudo-R^2 = 0.022, likelihood-ratio P = 0.40.

Table 5 Establishment status and survival (exploratory)

Endpoint and cohort	n (events)	Log-rank P	Unadjusted HR (95% CI)	P	Adjusted for stage IV, HR (95% CI)	P	Adjusted for stage IV and R status, HR (95% CI)	P
Overall survival, either route	147 (86)	0.086	1.45 (0.93–2.26)	0.10	1.45 (0.91–2.31)	0.12	1.77 (1.03–3.06); n = 114 (56)	0.039
Overall survival, primary-tissue route	84 (44)	0.006	2.27 (1.22–4.24)	0.010	2.16 (1.17–3.99)	0.014	4.20 (1.67–10.59); n = 56 (19)	0.002
Overall survival, PDX-derived route	96 (65)	0.32	1.29 (0.79–2.11)	0.31	1.28 (0.76–2.14)	0.35	1.36 (0.76–2.44); n = 78 (48)	0.30
Relapse-free survival, either route	118 (71)	0.16	1.40 (0.87–2.26)	0.16	1.67 (1.04–2.68)	0.034	1.95 (1.11–3.42); n = 97 (51)	0.020
Relapse-free survival, primary-tissue route	62 (32)	0.14	1.70 (0.78–3.71)	0.18	2.05 (0.92–4.60)	0.080	3.11 (1.05–9.14); n = 46 (17)	0.040
Relapse-free survival, PDX-derived route	76 (53)	0.29	1.34 (0.80–2.27)	0.27	1.52 (0.89–2.59)	0.12	1.51 (0.82–2.79); n = 66 (43)	0.18

Hazard ratios compare established with failed specimens. Cox models with standard errors clustered by patient; adjusted models in complete cases. Relapse-free survival counts relapse, persistent disease or death as events and excludes specimens from patients never tumor-free after surgery. With one specimen per patient and adjustment for stage IV and R status: overall survival HR 2.28 (1.27–4.10), relapse-free survival HR 2.16 (1.20–3.90).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- JECCRAdditionalfile1.docx
- JECCRAdditionalfile2dataset.xlsx