Cell sorting strategies for the isolation of cancer cells and associated fibroblasts from tumor biopsies, surgical resections and patient-derived xenografts.

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Introduction

The ability of early passage patient-derived tumor xenografts (PDXs) to recapitulate human tumor biology makes them invaluable tools for pre-clinical drug development. Similarly, clinically-annotated human tumor cell lines of low passage have potential as scalable biosimilar reagents for *in vitro* study. Here, we describe an integrated platform for the purification, expansion, and quality control of tumor cells and cancer-associated fibroblasts (CAFs) from patient biopsies, surgical resections and mouse PDX models. Following enzymatic and mechanical dissociation of solid tumors, cells are cultured on Matrigel® in fibroblast conditioned media containing *Y*-27632 (a Rho-associated kinase inhibitor). Cells are then analyzed by FACS using a panel of species- or cell type-specific antibodies. This panel (anti-mCD9, hCD9, hCD24, hCD90, MHC, HLA, and hEPCAM) permits quantitation of the percentage of mouse/human cells, and the percentages of tumor cells and CAFs in each patient specimen. From these data, a cell sorting strategy can be selected (e.g. mCD9 and hCD90 with hEpCAM or CD24) that will permit purification of both tumor cells and CAFs, along with removal of any mouse fibroblast contamination (for PDX tumors). Samples are sorted multiple times until target populations reach 99.99% purity (typically 2-3 rounds). Purified cells are then returned to culture, expanded over several weeks, QC'd and frozen at low passage.

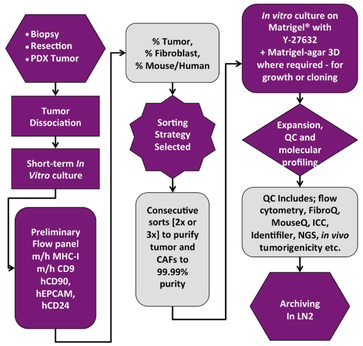

Figure 1: Workflow for the generation of purified tumor cultures and CAFs.

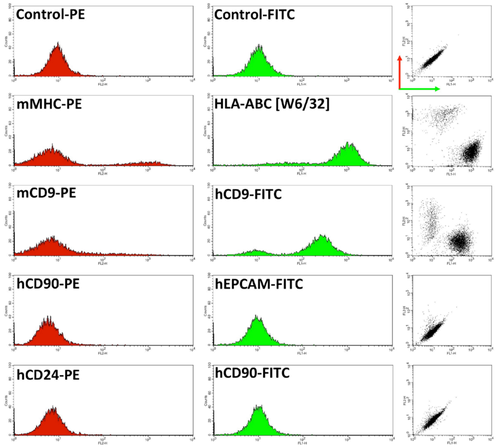

Figure 2: An example of preliminary FACS panel data. Here, dissociated cells from a melanoma PDX model [serially passaged in mice] were analyzed. Two populations were detected – mCD9+ murine cells and HLA+/hCD9+/CD90- human tumor cells.

Murine Fibroblasts	mCD9 (+++)	mMHC (+++)	
Human Fibroblasts	HLA-A,B,C (+++)	hCD9 (+++)	hCD90 (+++/-)
Tumor	EPCAM or CD24 (+++)	hCD9 (+/-)	hCD90 (+/-)

Figure 3: common outcomes from the preliminary cytometry assay

Materials and Methods

Antibodies used in preliminary panel and cell sorting were as follows; Anti-mouse CD9-PE (eBioscience, eBioKMC8), Anti-human CD9 FITC (eBioscience, eBioSN4), Anti-human CD24-PE (eBioscience, eBioSN3), Anti-human EPCAM-FITC (R&D systems, 158206), Anti-mouse MHC class I-PE (eBioscience, 28-14-8), Anti-human HLA-ABC FITC (eBioscience, W6/32), Control Mab FITC (eBioscience, eBR2a), Control Mab PE (eBioscience, eBR2a). Preliminary FACS analysis performed using a BD FACScalbur cytometer and all sorting performed using a FACSAria II.

Results and Discussion

Classical cell line generation is a time consuming process that favors the development of the fast growing, media-adapted clones. The platform presented here (Figure 1) provides an approach to preserve both clonal heterogeneity and the stromal component for any given tumor. Initially, considerable effort was expended developing a panel of antibodies capable of differentiating mouse from human cells and also distinguishing human fibroblasts from tumors of diverse histological origin (Figure 2 and 3).....

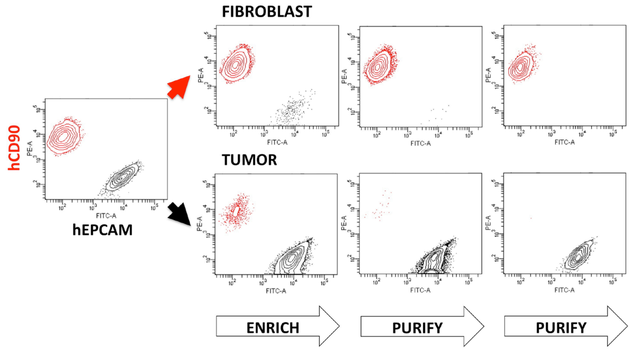

Figure 4: A representative sorting strategy for a sample presenting with roughly equal percentages of human tumor and fibroblasts. This sample was taken through gross enrichment followed immediately by two rounds of purification, resulting in cell populations of 99.9% purity.

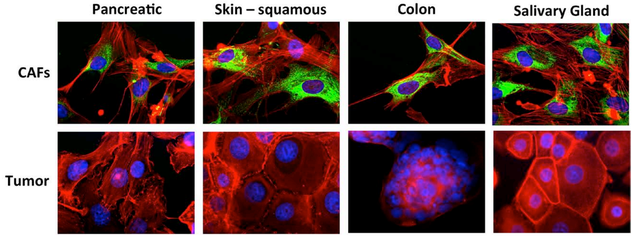

Figure 5: Examples of diverse tumor and CAF pairs derived using this approach. Green = TE7^{A488} [Fibroblast marker], Red = Phalloidin^{A555}, Blue=DAPI

.....Following this, after evaluating a range of technologies including immunomagnetic isolation, complement-mediated lysis and toxin-mediated immunodepletion; It became clear that FACS was the only technology offering the flexibility to derive cell populations with the required purity (>99%). Central to this was the ability to perform multiple rounds of sorting on the same sample to enhance purity, combined with the ability to track (and troubleshoot) performance throughout purification. Concerns were initially raised regarding whether multiple rounds of sorting had an adverse effect on cell viability. However, tests showed that cells remained viable after 3 concurrent sorts. One drawback was that cell recovery often declines by 50% with every round of sorting. In this regard input cell number is an important in determining whether cells can be sorted using this approach. A representative 2-way 3x sort is shown in Figure 4 and immunocytochemistry of four CAF/tumor pairs is shown in Figure 5. Sorted cells are then returned to culture, expanded and subjected to QC (See Figures 1, 6 and 7) at specific passage intervals.

Figure 6: Quality control of sorted cells using a Fibroblast qRT-PCR Array. Data shown is a heat map of control fibroblasts vs. cells derived from either CD90+ or CD24+ sorts. Correlation coefficients [R] of control data vs. CD90+ [0.91] or CD24+ [0.03] sorts confirmed fibroblast or tumor origin, respectively.

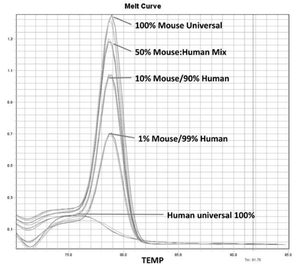

Figure 7: Mouse detection RT-PCR. For sorts involving the removal of mouse cells from PDX-derived cultures a further layer of QC was performed. Here, cDNAs from post-sort cultures were analyzed by RT-PCR using primers directed against a mouse-specific region of the housekeeping gene β-2-Microglobulin. Melt curve analysis was then used to determine extent of mouse contamination.

	Gene Symbol	DCT Control CAFs	DCT CD90+ Sort	DCT CD24+ Sort
High	FN1	0.3	2.0	7.3
	COL1A1	2.7	3.2	16.8
	VIM	3.9	3.3	13.2
	GREM1	4.2	4.4	13.2
	COL1A2	4.3	5.3	15.6
	LOX	5.5	6.4	10.3
	ACTA2	5.8	9.2	14.8
	COL3A1	6.1	4.8	19.3
	CD248	6.3	7.2	11.8
	KRT18	6.9	7.4	5.7
	KRT16	7.1	11.1	3.9
	CD44	7.3	5.0	3.4
	KRT8	7.5	6.9	8.0
	ERBB2	7.9	11.0	10.5
	CD24	8.0	10.5	3.1
	EGF	8.1	10.9	12.7
	CDH2	8.4	10.4	14.2
	KIT	8.8	12.5	14.2
	KRT1	8.8	12.7	5.1
	IGF1	8.9	12.5	16.1
	CD45	8.9	12.9	15.3
	ERBB3	9.1	11.0	8.3
	MUC1	9.2	12.8	15.1
	KRT5	9.8	13.9	-0.2
	EPCAM	10.1	14.0	9.6
	KRT7	10.1	8.3	14.1
	KRT19	10.1	11.6	5.2
	CD46	10.3	9.5	9.5
	KRT14	10.7	15.1	0.0
	VCAM-1	10.7	11.9	20.7
	HGF	10.8	8.3	17.2
	EGFR	11.0	9.6	6.1
	CD34	11.3	15.5	17.9
	PECAM1	11.4	15.2	16.5
	KRT4	11.6	11.5	6.9
	CEACAM5	11.6	15.4	19.0
	KRT9	11.7	16.3	13.8
	MUC4	11.9	16.4	19.1
	KRT15	11.9	15.9	6.5
	CDH1	12.1	15.3	8.2
	KRT13	12.3	15.8	7.0
	KRT3	13.9	18.0	15.5
	KRT10	14.1	17.3	3.0
	KRT20	14.2	19.2	15.8
	CD133	14.3	14.9	16.9
Low	KRT17	14.6	19.1	2.1
			R=0.91	R=0.03

Conclusions

While PDX models are gaining popularity as research tools, little emphasis has been placed on generating *in vitro* reagents with similar advantages. The approach outlined here provides all the tools necessary to derive highly purified heterologous tumor cell populations from almost any patient-derived material. Furthermore, autologous CAF cultures can be generated within the same workflow.