Flow cytometry on disaggregated tissues: Detecting cancer stem cell candidates in epithelial tumors

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This tutorial will review the cancer stem cell controversy: Does it make sense to think of clonogenic self-replicating tumor cells as stem cells? Do clonogenic self-replicating tumor cells have unique phenotypic markers that distinguish them from nontumorigenic tumor-derived cells? Do clonogenic self-replicating tumor cells share other tissue stem cell characteristics, such as the ability to persist in a dormant state and resist cytotoxic drugs and radiation? Must a cell be rare or multipotent to be considered a stem cell? What is the difference between a stem cell and a progenitor cell and does it matter when discussing cancer? What markers have been used to detect stem cells in normal and malignant epithelial tissues and do they define unique populations? This discussion will be accompanied by a technical tutorial in which we will provide practical suggestions for disaggregation of tumors and normal tissues into single cell suspensions. We will focus on avoiding some of the technical pitfalls encountered when performing multi-color flow cytometry on disaggregated tissues. Specifically, these include recognizing sources of bias in cell recovery, use of doublet discrimination, use of DAPI to detect and remove hypodiploid events, coping with autofluorescence, and optimal use of dump gates.
Does it make sense to think of clonogenic self-replicating epithelial tumor cells as stem cells?

- Like normal tissues, tumors are heterogeneous with respect to stromal cells, vascular cells, infiltrating immune cells and the epithelial cells themselves.
- Depending on tumor grade, many of the tumor cells may be non-clonogenic progeny of clonogenic cells.
- Clonogenicity and self-replication are primary attributes that we associate with normal stem cell behavior.
- The thought that in some cases, tumors may derive from mutated adult tissue stem cells (chronic myelogenous leukemia), even embryonic stem cells (developmental tumors) or germ cells is an additional link between clonogenic tumor cells and stem cells.
- Caveat: A tumor cell does not have to derive from a mutated stem cell to acquire stem cell attributes. Mutation and epigenetic reprogramming can produce any phenotype that offers selective advantage to the cancer cell.
Do clonogenic self-replicating tumor cells have unique phenotypic markers that distinguish them from nonclonogenic tumor-derived cells?

• The answer has two parts:
  
  • First, the majority of cells within a tumor are often not tumor cells per se. They are stromal cells, immune cells, reactive cells, vascular cells, normal and dysplastic epithelial cells. These cells are clearly non-tumorigenic. With the exception of normal and dysplastic epithelial cells within the tumor mass, they are easily distinguished from clonogenic tumor cells and can be eliminated in experimental preparations by cell sorting if necessary.

  • Second, Yes. A variety of markers have been proposed which enrich clonogenic tumor cells in an experimental preparation. The caveat is that any marker that depletes the cells described above will “enrich” for clonogenicity. Correctly designed experiments divide the epithelial component of the tumor into those positive and negative for the marker of interest. CD44, CD133, CD90, CD117, CD34, CD49, aldehyde dehydrogenase activity and MDR activity (side population) have all been proposed to identify (sometimes exclusively) the clonogenic, self-renewing tumor cell. Caveat Emptor!
Do clonogenic self-replicating tumor cells share other tissue stem cell characteristics, such as the ability to persist in a dormant state and resist cytotoxic drugs and radiation?

• The principal distinctions between stem and progenitor cells is that the former retain the ability to self-renew throughout the life of the organism, and characteristically persist in a dormant state. Dormancy means either out of cell-cycle, or balanced with respect to proliferation and death or differentiation. Dormancy is a characteristic of tumors which may persist in a subclinical state after therapy and reactivate at a later time.

• Normal tissue stem cells, and a subset of epithelial tumor cells share a battery of protective mechanisms. In normal stem cells, the expression of multiple drug resistance transporters, detoxifying pathways, and other protective mechanisms is inducible. At any given time some stem cells are positive and some negative, and negative cells can give rise to positive cells and vice versa. These mechanisms contribute to the stability of the normal stem cell pool. Caveat: None of these mechanisms are unique to stem cells. SP+, ALDH+ and GSH+ cells serve important functions in differentiated tissues too.

• Cancer is dysregulation and the mechanisms which protect stem cells against toxic injury are hijacked to protect tumor as well.
Must a cell be multipotent or rare to be considered a stem cell?

• Self-renewal and self-protection are the major attributes of normal adult tissue stem cells and the degree of multipotency is specific to a given tissue. Hematopoietic stem cells are self-renewing and self-protecting and give rise to 4 lineages, MSC give rise to more. Breast stem cells are reported to be bipotent. Memory B cells and T cells are quintessentially lineage committed, but self-renew and self protect. Cancer cells can be multipotent (e.g CML in blast crisis), but need not be.

• In normal tissues stem cells tend to be one the rare side. Hematopoietic stem cells (CD34+/CD38-) are less then 1% of bone marrow, BM-MSC are even more scarce. However, ADSC in the SVF of adipose tissue are quite prevalent (35% of non-hematopoietic). In well-differentiated tumors clonogenic (tumorigenic) cells tend to be rare. In poorly differentiated tumors they can be very prevalent. In extreme cases, virtually all tumor cells are clonogenic. Are they stem cells?
What is the difference between a stem cell and a progenitor cell and does it matter when discussing cancer?

<table>
<thead>
<tr>
<th>Adult Stem Cells</th>
<th>Progenitor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary (niche)</td>
<td>Transiting</td>
</tr>
<tr>
<td>Resting</td>
<td>Proliferating</td>
</tr>
<tr>
<td>Self renewing</td>
<td>Limited self renewal</td>
</tr>
<tr>
<td>Some are protected (MDR, ALDH, GSH)</td>
<td>Limited protection</td>
</tr>
<tr>
<td>More rare</td>
<td>More prevalent</td>
</tr>
<tr>
<td>Relatively undifferentiated</td>
<td>Some lineage commitment</td>
</tr>
</tbody>
</table>

- In cancers the distinction is blurred, and sometimes obscured altogether. In high grade malignancies, self-renewal, self-protection, high proliferative capacity and mobility are all present in the same stem/progenitor cell.
What markers have been used to detect stem cells in normal and malignant epithelial tissues and do they define unique populations?

- In breast, lung, and esophageal cancers we have experience with CD44, CD117, CD90, CD133, ABCG2 and ABCB1 used in conjunction with epithelial differentiation antigens cytokeratin, EpCAM, E-Cadherin, MUC-1.
- With the exception of metastatic pleural effusions, it is usually not possible to distinguish normal and malignant tissue on the basis of prevalence of these markers.
- There is a suggestion that discordant expression of these markers may uniquely identify clonogenic tumor cells.
Technical Aspects

Technical pitfalls encountered when performing multi-color flow cytometry on disaggregated tissues:

- Collection of viable tissue
- Mechanics of tissue digestion
- Sources of bias in cell recovery
- Use of doublet discrimination
- Use of DAPI to detect and remove hypodiploid events
- Coping with autofluorescence
- Optimal use of dump gates
Tissue Collection: keep it fresh
Tissue Collection

- Weigh tissues for yield calculations
- Reserve tissue for paraffin and frozen sections
- Maintain sterility. **Can dip nonsterile tissues** in bleach and rinse in sterile PBS (esophagus, vertebral bodies, foreskin)
Immunohistochemical Detection of Antigens in Solid Tissues

Donnenberg et al, Clinical Cytometry 2010

Understanding histologic location of markers aids in interpretation of antigen localization
Allows extrapolation from normal to abnormal (tumor)
One antigen/section (no co-expression), fixed tissue
Why to do flow cytometry on solid tissues?

- Simultaneously detect multiple parameters on rare cells without pre-enrichment
- Understand heterogeneity of tumor and normal tissue
- Identify and sort tumorigenic/clonogenic cells
- Quantify metastatic tumorigenic cells
- Determine whether tumorigenic cells circulate
- Isolate and purify these cells for further studies
Use enzymatic and mechanical disaggregation; include DNase with collagenases

Examine microscopically: Count cells, check viability by trypan blue exclusion, check for undigested cell clumps

Tissue Digestion

Donnenberg et al. International Drug Discovery 2009
### Cell recoveries from Fat, NB, BrCA, NL, NSCLCa

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cells/Gram</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Breast</td>
<td>$8.89 \pm 1.67 \times 10^5$</td>
<td>$71.3 \pm 0.6%$</td>
</tr>
<tr>
<td>AdenoCa Breast</td>
<td>$72.0 \pm 28.6 \times 10^5$</td>
<td>$66.2 \pm 6.3%$</td>
</tr>
<tr>
<td>Normal Lung</td>
<td>$32.9 \pm 4.57 \times 10^5$</td>
<td>$62.8 \pm 15%$</td>
</tr>
<tr>
<td>NSC Ca Lung</td>
<td>$226 \pm 60.3 \times 10^5$</td>
<td>$72.8 \pm 3.2%$</td>
</tr>
<tr>
<td>Whole Adipose</td>
<td>$2.98 \pm 0.87 \times 10^5$</td>
<td>$57.6 \pm 6.8%$</td>
</tr>
</tbody>
</table>
Multiparameter Staining

9-10 color flow cytometry (3-laser CyAn and Gallios cytometers):

Surface markers: EpCAM/HEA, CD44, CD90, CD133, CD117

Lineage (hematopoietic): CD14, CD33, Glycophorin, CD45, CD31

Intracellular markers: Cytokeratin nuclear stain – DAPI transcription factors

DAPI: (200μg/ml: 8μl of stock to 5 million cells in 200μL: [8μg/mL])

This dataset: 13-16 parameters
Multiparameter Staining Design

Remove Sources of Artifact
Doublets: FS pulse height vs FS area
DNA content cell debris/apoptotic cells <2N DNA (nuclear stain – DAPI, log scale)
Clean denominator “healthy cell scatter of all populations”

Classifiers: Known expression identifies populations of interest
CD45, CD31 and hematopoietic lineage CD14, CD33, GlyA
intracellular cytokeratin, extracellular HEA/EpCAM/CD326

Outcomes: Measurement made on populations of interest
   Know something about them but may not know their exact level of expression (isotype controls): Cytokeratin, DNA content (linear), CD44, CD90
   Unknown profile: morphology FS , SSC, DNA content, CD133, CD117, transcription factors and their localization
Efficient Flow Cytometric Staining

Cell suspension to be stained 15mL conical

Centrifuge cell suspension down to a pellet

Remove and discard supernatant

Block NSB by adding 5uL of neat mouse serum

Incubate 5 minutes, spin down and remove residual liquid

Add MAB 2uL/antibody, dimmest and lowest abundance epitope add first

Incubate 15 minutes, add buffer 100uL/condition and split

BULK staining: antibodies common to all tubes

OUTCOME staining: antibodies unique to each tube

FMO
Data Analysis

“We must learn to love complexity”
George Klein, Karolinska Institute

Elimination of sources of artifact
Remove doublets, hypodiploid cells and cell debris, autofluorescent/NSB events

Classifiers
Identify population(s) of interest (known marker expression profile)

Outcomes
Measurements made on populations of interest
Case Studies: NB002

NB002

40x
Case Studies: NB002

**Artifact Removal**

- **Pulse Width**
  - FS: 60.2%

- **Gate Singlets**
  - B: 36.02%

- **Gate A&B**
  - CD14-33-GlyA PE-Cy7: 41.8%
  - Ly 7.6%
  - CD45 APC-Cy7
Case Studies: NB002

Lymphocyte Gate

DAPI Lin

SS

FS
**Artifact: Gated on Doublets/Clusters**

In this example 93% of clusters are low FSc and hypodiploid.
**Hypodiploid Cells/Debris**

These events have little or no DNA but appear to bind antibody.
Case Studies: NB002

**Primary Classifier: Clean Heme Lineage Negative**
Case Studies: Adipose stromal vascular fraction

Selection of singlets, nucleated cells, elimination of debris

FatSC04-12-07 singlets AC

Selection of non hematopoietic cells, FITC dump gate

AJ: 76.43% J: 4.63%
K: 68.44%
AD: 18.84%

3 first channels autofluorescence

ABC G: 30.40% ABC H: 12.44% ABC l: 15.30%

Remaining channels Artifacts and off scale (too bright) events

ABC D: 17.17%
ABC E: 0.06%
ABC F: 3.10%

Pulse Width

FS Lin - FS Violet 2 FS Lin - FS

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme w tooB

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme without dump

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme

CD3 FITC

CD146 PE CD34 ECD CD34 ECD

Non heme
Using “SP” to Identify and Isolate Tumor SC as is done for Tissue Stem Cells

MDR has been used for tissue SC identification and isolation in the absence of specific tiss.SC markers: Any tissue contains a rare population of “SP” cells. When sorted these cells are highly clonogenic (Margaret Goodel, 1998)

Caution: Not all SC have MDR activity and not all MDR active cells are SC! (MDR in BBB, kidney, small gut, T cells)

Use of Inhibitors to Confirm Transporter Specificity

K562 Parent

No Inhibitor CsA Vincristine Fumitr Vera No Inhibitor CsA Vincristine Fumitr Vera

K562 G185

Real Time PCR
MRP1 BCRP1 MDR1
ABC C1 ABC G2 ABC B1
Samples Average Δ Ct Values
K562 P 8.4 14.9 14.4
K562 G 7.3 14.4 0.4

0.71% 98%INH 0%INH 66%INH
3.36% 74%INH 0%INH 80%INH

96% INH 99%INH 0%INH 0%INH
3.5% 96.5% 0%INH 0%INH
Tumorigenicity and SP

Donnenberg et al JCR2007

If cells are sorted on MDR (SP), no net increase in tumorigenicity.

If cells are sorted on MDR (SP), increase in tumorigenicity.

Untreated primary

0.82% SP

Hoechst 33342

Blue 420-480 nm

TSCsort017

untreated PE

51% SP

Hoechst 33342 Red 660-680 nm

+Fumitromargin +CsA: No SP

TSCsort020 PE42
• We have characterized a subset of highly tumorigenic breast cancer cells expressing CD90, CD44 and resting morphology.

• These cells were detected in previously untreated primary tumors.

• *Which cells survive a “partial response” to chemotherapy?*
Analysis of Tumor Cell Survival After Therapy

Which Cells are Targeted by Cytotoxic Therapy?

Cancer Progenitor

Genetic Instability

Drug Selection

Constitutive MDR

Acquired MDR

Drug Resistant Bulky Tumor

CHEMOTHERAPY Shrinks the tumor!