Principle and Practice to explore cancer stem cell

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Two general models of heterogeneity in solid cancer cells

Clonal selection and expansion

Tumour cells are heterogeneous, but most cells can proliferate extensively and form new tumours

Cancer stem cell model

Tumour cells are heterogeneous and only the cancer stem cell subset (CSC; yellow) has the ability to proliferate extensively and form new tumours

Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001 Nov 1;414(6859):105-
Die hard: Are cancer stem cells Bruce Willises of tumor biology?

Cytometry A. 2009; 75:67–74
Clonal evolution?

CSC marker positive
Marker negative

Cancer

Clonal evolution?

CSC

Stem cells

Progenitors

Differentiated Cells

Normal tissue

in vivo tumorigenicity

1: loss of regulated quiescence
2: regained long term self renewal potential
3: acquired self renewal, proliferation and multilineage capabilities

Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001 Nov 1;414(6859):105-
Stemness in Cancer Stem Cells

Principle and practice to identify CSC

- **Self renewal**
  - Serial colony forming unit assay
  - Tumor sphere
  - In vivo injection (exclusion of secondary aggregates)

- **Asymmetric division**
  - Phenotype of CSC vs. non-CSC sorting
  - In vitro proliferation
  - In vitro migration/invasion

- **Stemness**
  - Intratumoral (multiclonal) heterogeneity
  - Stemness gene activation
  - Drug resistance (Side population by Hoechst dye SP with Verapamil)
List of stem cell markers with frequently used synonym

1. **CD24** heat stable antigen (HSA)
2. CD29 β1 integrin chain
3. CD31 platelet endothelial cell adhesion molecule 1 (PECAM-1)
4. **CD44** Pgp-1
5. CD49b α2 integrin chain
6. CD49c α3 integrin chain
7. CD49d α4 integrin chain
8. CD49f α6 integrin chain
9. CD45 leukocyte common antigen, T200, B220
10. CD54 intercellular adhesion molecule 1 (ICAM1)
11. CD62E E-selectin
12. CD62L L-selectin
13. CD62P P-selectin
14. CD87 uPAR (urokinase plasminogen activator receptor)
15. CD90 Thy1
16. CD105 endoglin
17. **CD117** c-kit, stem cell growth factor receptor
18. **CD133** Prominin, AC133
19. CD156b ADAM17, TACE
20. CD156c ADAM10, Kuzbanian protein homologue
21. CD166 ALCAM (activated leukocyte cell adhesion molecule)
22. CD184 CXCR4, fusin, CXCL12 receptor
23. CD243 MDR-1 (Multidrug resistance-1), P glycoprotein 1
24. CD326 EpCAM (epithelial cell adhesion molecule), ESA
25. **CXCL12** stroma derived factor 1 (SDF1)
26. Sca-1 stem cell antigen 1, Ly6A/E
Least identified CSC in Ovary

- Few matched with gross findings
  - Too large, cystic and necrotic
  - Too contaminated
  - Avoid the burden of tumor, focus their edges

- Often ITH encountered
  - Too variable, mixed or unclassified
  - Strong evidence of existence of CSC

- Often recurrent even looking CR
  - Pseudo CR ~15%
  - Powerful evidence of CSC

- Lists-up of CSC candidates-relatively fewer
  - CD133 (*BMC Ca* 2009)
  - NAC1 (*Oncogene*, 2009)
  - CD44 (*Cell cycle*, 2009)
  - SP
HOX genes are related with ITH of EOCs

<table>
<thead>
<tr>
<th>HOX Gene</th>
<th>Location</th>
<th>Image 1</th>
<th>Image 2</th>
<th>Image 3</th>
<th>Image 4</th>
<th>Image 5</th>
<th>Image 6</th>
<th>Image 7</th>
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Nat Med 2005; 11:531
Principle to identify CSC

• **Stemness**
  – Intratumoral (multiclonal) heterogeneity
  – Stemness gene activation
  – Drug resistance (Hoechst dye SP with Verapamil)

• **Self renewal**
  – Serial colony forming unit assay
  – Tumor sphere
  – In vivo injection for exclusion of secondary aggregates

• **Asymmetric division**
  – Phenotype of CSC vs. non-CSC sorting
  – In vitro proliferation
  – In vitro migration/invasion
## Materials and Methods

### Protocol to identify CSC

#### Stemness

**Intratumoral heterogeneity**

- Surface markers expression heterogeneity
- Cell Proliferation and Viability heterogeneity
  - Propotional heterogeneity of SP
  - Genetic aneuploidy heterogeneity

**Network and gene ontology**

#### Self renewal

**CSC characterization**

- Chemotherapeutic sensitivity assay
- Determination of potential ovarian CSC marker
- Properties of purification of candidates in vitro
- Isolation of multiple surface markers + cells

**In vivo recapitulation**

**Asymmetric division**

Cell growth property, cell invasion, migration and adhesion assay to compare between CD24+ and CD24-

**Validation of CD24+ as ovarian CSC in vitro**
Isolation and characteristics of heterogeneous cell clones from human ovarian tumors. – Slow proliferation and less cell density in front zone.

Ovarian mucinous carcinoma, well differentiated with very focally differentiated to endometrioid, serous and sarcomatous phenotypes.
Expression of surface markers and stemness genes in front zones, F1

<table>
<thead>
<tr>
<th>Surface markers</th>
<th>Stemness genes</th>
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<tr>
<td>ABCG2</td>
<td>Nestin</td>
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<tr>
<td>CD24</td>
<td>Bmi-1</td>
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<tr>
<td>CD117 (c-kit)</td>
<td>Oct3/4</td>
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<tr>
<td>CD44</td>
<td>Oct4</td>
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<tr>
<td>EpCAM</td>
<td>SMO</td>
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<td>Notch-1</td>
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Surface markers:
Relative mRNA expression level
$P<0.05$

Stemness genes:
Relative mRNA expression level
$P<0.05$
Front zone has more sub G1 fraction and G2/M delay, with cis-platin resistance.
The proportion of double surface markers in outnumbered in F3
Side Populations are abundant in F1
Side population in CD24+ sorting cells are increased up to 5 folds in F1.
Sampling Tip:
potential stem cell niche

Ensure where CSC niche is along the tumor edge!
Microenvironment surrounding CSC is critical in niche
Take Home Message

• Exclusion Criteria
  – Avoid too large size
  – Avoid totally replaced tumor
    • Interface zone between tumor and normal
  – Avoid cystic mass
  – Avoid varigated hemorrhagic foci
  – Avoid too soft or too hard mass

Well Begun is Half Done

• Inclusive criteria
  – Smaller, more solid mass with fewer chance of hemorrhage or necrosis
  – Expansile invasive pattern with partial replacement
    • Interface zone along the tumor edge
  – Microscopically confirmed ITH, simultaneously or later
Chromosomal heterogeneity is more frequent in SP

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<td>q24.3, q24.3</td>
<td>NEURL</td>
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Cancer Lett 2011 [n Press]
Stemness
**Self Renewal**

**CD24+ 5x10^3 In Vivo Recapitulation**

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**Tumorigenicity of CD24+ cells in nude mice.**

(a) A representative tumor in a mouse at the SQ injection site of \(5 \times 10^3\) CD24+ cells, and no tumor formation at the injection site of equivalent CD24- cells.

(b) Representative H&E staining sections of primary patient tumor and xenograft tumor. Well-formed glands with mucin were distributed (b1; 40\(\times\)) and tumor cells were basally located and bland-looking (b2; 200\(\times\)) in primary tumor. Identical mucinous adenocarcinoma was formed in the subcutaneous fat tissue and back muscle of the nude mouse; mucinous glands were found in the xenograft tumor mass, and xenograft tumor cells were bizarre, hyperchromatic and pleomorphic. Immunohistochemistry staining of anti-CD24 antibody in xenograft tumor, with brown staining indicating the presence of specific antigen.

**CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells.** *Oncogene*, 2010, 29: 2672-80
CD24 positive tumor
Growth properties of purified CD24+ and CD24- cells in vitro

- CD24+ cells lower proliferation than CD24- cells
- SPF higher and G2M delay than CD24- cells
- Higher expression of stemness-related genes than CD24- cells
STEMNESS

Growth properties of purified CD24+ and CD24− cells in vitro

- CD24+ cells are differentiated to CD24− cells
- More resistant to cisplatin

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<thead>
<tr>
<th></th>
<th>IC$_{50}$</th>
<th>P</th>
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<tr>
<td>CD24+</td>
<td>18.75 ± 2.90</td>
<td>&lt;0.01</td>
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<tr>
<td>CD24−</td>
<td>8.60 ± 2.70</td>
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</table>
CD24+ cells enhance cell migration/invasion.

Migration Assay (4x10^5 cells seeding)

CAOV3

CD24+

CD24-

OV90

CD24+

CD24-
Stemness

Invasion Assay

Cell Adhesion Assay

CAOV3

OV90

Invaded cells

CD24+ cells

CD24- cells

A. 550nm

BSA

FN

Vn

*P<0.05
Stemness

A

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<tr>
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<tr>
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<td>CD24</td>
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<td>CXCR4</td>
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B

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Graph shows migration of cells under different conditions.
Stemness

Invasive Property in CD24 + sorting cells

Pre-sorting

Post-sorting

CAOV3 OV90

CD24

β -Actin

CAOV3

CD24

E-cadherin

Vimentin

Snail

Twist

β -Actin

MMPs

CD24

MMP2

MMP9

β -Actin

CAOV3

Integrin

CD24

Integrin β1

Integrin β3

Integrin αv

Integrin β4

β -Actin

CAOV3

Integrin downstream

CD24

C-Src

Rab25

β -Actin
CD24+ as candidate of CSC in ovarian CSC

- **CD24+ cell possess some properties of stemness**
  - Clone derivation with different ITH characteristics
    - Different growth rate
    - Different cell cycle distribution
    - Different gene patterns of stem cell markers
    - Different protein expression patterns of stem cell markers

- **CD24+ cell possess some properties of self renewal**
  - In vivo: CD24+ cells have strong capacity of tumorigenicity in nude mice

- **CD24+ cell possess some properties of asymmetric division**
  - CD24+ cells keep quiescent
  - CD24+ could differentiate into CD24- cells
  - CD24+ cells are chemotherapeutic resistance to cisplatin
  - Cell cycle distribution was different in CD24+ and CD24- population
  - CD24+ cells preferentially expressed stem cell genes
Conclusion

- CSC niche could be enriched in tumor peripheral margin.
- A subset of CD24⁺/CD44<sup>high</sup>/EpCAM<sup>high</sup> cells possess CSCs' properties of relative quiescence, chemoresistance, preferential expression of stem cell genes, and tumorigenicity in nude mice.
- CD24⁺ in ovarian ca cell lines demonstrated farther migration and more invasion and adhesion to fibronectin.
- Ovarian cancers show high levels of inter/intratumoral heterogeneity by evidences of acquisition of distinct genetic abnormality and chromosomal alterations.
- CSC could be strongly a/w EMT in terms of dynamic migration.
- TME in a viewpoint of interface zone adjacent to the tumor margin is an important soil for CSC.
Ongoing Project

• CSC targeted therapy
  – Anti-CD24/ anti-CD24-related miRNA
  – Anti-Igb1/b4/ILK targeted therapy
• CSC-niche enriched molecular margin
• CSC niche-dependent TME
  – Paracrine cell-matrix interaction system biology
  – Integrin switching
  – Cross-talking between ligands-receptors or downstream signals
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