Identification and Characterization of Stem-like Cells in Human Esophageal Adenocarcinoma and Normal Epithelial Cell Lines

No disclosures / conflict of interest

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CANCER BIOLOGY

CLONAL EVOLUTION

- Multistep process
- Accumulation of genetic mutations
- Modulated by oncogenes, tumor suppressor genes, epigenetic factors etc.

Does not entirely explain:
- latency
- heterogeneity
- cellular de-differentiation
CANCER BIOLOGY

CLONAL EVOLUTION vs. STEM CELL MODELS

(Till & McCulloch, 1963; Dick 1997)

- Multistep process
- Accumulation of genetic mutations
- Modulated by oncogenes, tumor suppressor genes, epigenetic factors etc.

Does not entirely explain:
- latency
- heterogeneity
- cellular de-differentiation

DEPARTMENT OF SURGERY

Cell surface markers (flow cytometry)
- AML: CD34+ CD38-
- Myeloma: CD19+ CD138-
- Glioma: CD133+ nestin*
- Colon / GI: CD133+ CD166+ EpCAM^high Lgr5

Functional assays
- in vitro: sphere formation in cell culture
- soft agar assays (clonogenicity, drug resistance)
- in vivo: xenograft formation in immunodeficient mice
EVIDENCE FOR STEM CELLS IN BARRETT
ESOPHAGUS & ESOPHAGEAL MALIGNANCY

Reviewed in Barbera & Fitzgerald: Biochem. Soc. Trans 2010

Murine tissues:
• Integrin α6 / CD71 (Croagh et al Stem Cells 2007)
• CD43 (Kalabis et al J. Clin Invest 2008)

Human tissues & cell lines:
• GPR49 (Lgr5) (Yang et al Proc. AACR, abstract, 2009)
• None (CD24, CD29, CD34, CD44, CD133, CD166, EpCAM, β-catenin) (Grotenhuis et al J. Pathol 2010)
• Musashi-1 (Bobryshev et al Dis. Esoph 2010)
• Mid-IR spectrum (Zhao et al Analyst 2010)
• Bone Marrow-derived cells (Hutchinson et al Stem Cells Dev. 2011)
• CD44 / CD24 (OE19, OE33 cell lines) (Honing et al Proc. AACR, abstract, 2012)
RATIONALE, OBJECTIVES, MODEL

• Changing epidemiology / poor outcomes associated with esophageal malignancy

• Improved understanding of esophageal tumor biology, specifically to identify subpopulations of cancer stem-like cells

• Human esophageal cell lines derived from:

  Primary esophageal adenocarcinomas

  OE33  (Rockett et al Br. J. Cancer 1997; ECCC, Porton Down UK)

  JH-EsoAd1  (Alvarez et al Cancer Biol. Ther. 2008; Johns Hopkins)

  Immortalized normal esophageal epithelium

  Het-1A  (Stoner et al Cancer Res 1991; ATCC, Manassas VA)
METHODS

Esophageal cells grown in serum-free media to form spheres (enriched with stem-like cells)

Flow cytometry, cell sorting
(CD24, CD34, CD44, CD71, CD133, EpCAM, integrin α6, Musashi-1, Oct4)

Soft-agar colony formation assays (clonogenicity)

Xenotransplantation (tumorigenicity, immunohistochemistry)

Cell proliferation / MTT assays (chemosensitivity to Cisplatin & 5-Fu)

PCR array (mRNA expression of 253 cancer stem cell related genes; SABiosciences)

Western Blot analysis (protein expression of selected genes)
RESULTS: Clonogenicity

- All Het-1A, OE33 and JH-EsoAd1 cells (parent & spheroids) formed colonies in soft agar.

- Spheroids exhibited significantly enhanced clonogenicity compared with parent cells.

- Estimated frequencies were quantitated using Extreme Limiting Dilution Analysis (ELDA) by Hu & Smyth *J. Immunol. Methods* 2009.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PARENT CELLS Median frequency x 10^5 (95% CI)</th>
<th>SPHEROID CELLS Median frequency x 10^5 (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Het-1A</td>
<td>1 / 51.5 (1 / 92.8 – 1 / 28.5)</td>
<td>1 / 8.1 (1 / 15.2 – 1 / 4.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>OE33</td>
<td>1 / 49.4 (1 / 85.8 – 1 / 28.5)</td>
<td>1 / 15.1 (1 / 27.5 – 1 / 8.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>JH-EsoAd1</td>
<td>1 / 196.2 (1 / 409.0 – 1 / 94.2)</td>
<td>1 / 72.4 (1 / 124.0 – 1 / 42.2)</td>
<td>&lt;0.05</td>
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</table>
RESULTS: Tumorigenicity

No xenograft tumors from Het-1A cells (parent or spheroids)

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<th>SPHEROID</th>
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<tbody>
<tr>
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<td>Number of Tumors (%)</td>
<td>Median tumor volume (mm³)</td>
</tr>
<tr>
<td>OE33 (29 weeks)</td>
<td>2 / 15 (13%)</td>
<td>1.7</td>
</tr>
<tr>
<td>JH-EsoAd1 (13 weeks)</td>
<td>6 / 15 (40%)</td>
<td>275</td>
</tr>
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All xenografts were adenocarcinomas (spheroids higher grade)

CD71 & CD133 negative; Positive staining for CD44 & Integrin α6

Increased frequency of tumors resulting from spheroids (Kaplan-Meier & ELDA)

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<tr>
<td></td>
<td>Median frequency x 10⁵ (95% CI)</td>
<td>Median frequency x 10⁵ (95% CI)</td>
</tr>
<tr>
<td>OE33</td>
<td>1 / 36.1 (1 / 145.0 – 1 / 8.9)</td>
<td>1 / 8.7 (1 / 20.5 – 1 /3.7)</td>
</tr>
<tr>
<td>JH-EsoAd1</td>
<td>1 / 4.6 (1 / 10.8 – 1 / 1.9)</td>
<td>1 / 0.4 (1 / 1.1 – 1 / 0.1)</td>
</tr>
</tbody>
</table>
RESULTS: Chemosensitivity

Compared with parent cells, spheroids:

- had lower proliferation rates
- were resistant to 5-Fu

For Cisplatin (CDDP):

- Het-1A & OE33 spheroids were resistant
- JH-EsoAd1 spheroid and parent cells exhibited similar chemosensitivity
RESULTS: Cell surface markers - 1

<table>
<thead>
<tr>
<th>NEGATIVE:</th>
<th>CD24</th>
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<tr>
<td></td>
<td>CD34</td>
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<td></td>
<td>CD133</td>
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| HIGH POSITIVITY: | CD44          |
|                  | Oct 4         |

| MODERATE POSITIVITY: | Musashi-1 |
|                     | CD71        |

**Integrin α6**
(regulates cell adhesion in the ECM)

| Het-1A (5.0%) | OE33 (0.2%) |
|              | JH-EsoAd1 (44%) |

**Integrin α6**

| Het-1A (0.11%) | OE33 (0.01%) |
|                | JH-EsoAd1 (0.21%) |
**RESULTS:** Cell surface markers - 2

**Integrin α6**<sup>bright</sup> / **CD71**<sup>dim</sup>

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<th>Het-1A</th>
<th>OE33</th>
<th>JH-EsoAd1</th>
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<tbody>
<tr>
<td>Parent</td>
<td>0.11%</td>
<td>0.01%</td>
<td>0.21%</td>
</tr>
<tr>
<td>Spheroid</td>
<td>21.3%</td>
<td>5.73%</td>
<td>11.3%</td>
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Subpopulations of Het-1A, OE33 and JH-EsoAd1 cells expressing **Integrin α6**<sup>bright</sup> / **CD71**<sup>dim</sup> were found to have enhanced clonogenicity and spherogenicity.

Reported to enrich stem-like progenitor cells in:
- Human keratinocytes (Terunuma et al Stem Cells 2007)
- Murine esophageal basal cells (Croagh et al Stem Cells 2007)

**Musashi-1:** No differences between parent cells or spheroids
RESULTS: mRNA expression profiles & Western analysis

Ascl2 (Achaete-schute complex homolog 2)

- direct transcriptional target of Wnt
- regulates cell proliferation by modulating cell cycle progression at G2/M
- overexpressed in colorectal adenocarcinomas
- implicated in maintenance of human intestinal stem cells & drug resistance (5-Fu)

Lgr5 expression was consistently negative in all cell lines (parent & spheroids) and xenografts
SUMMARY & CONCLUSIONS

1. Human esophageal cells grown in serum-free media to form spheroids exhibited increased clonogenicity, tumorigenicity and drug resistance, reflecting enrichment of stem-like cell populations.

2. Esophageal cells enriched for \textit{Integrin $\alpha_6^{\text{bright}}$ / CD71 $\text{dim}$} and/or overexpressing \textit{Ascl2} would appear to represent a subpopulation of stem-like cells.

3. Limitations of human cell line / spheroid models. These findings should be considered preliminary, and require confirmation in human tissues.

4. The role of stem cells in human solid tumors remains controversial. Future studies will lead to an improved understanding of fundamental esophageal tumor biology with potential to direct future therapeutic strategies.

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