Generation of pulmonary neuroendocrine cells and tumors resembling small cell lung cancers from human embryonic stem cells

Weill Cornell Medicine
Joyce Chen
Arun Unni
Harold Varmus

Asaf Poran
Olivier Elemento

Columbia College of P&S
Sarah Huang
Hans Snoeck

bioRxiv 261461; doi: https://doi.org/10.1101/261461
Initiate oncogenesis in human ESC-derived differentiated cells to explore relationship between cell type and oncogenic genotype

--differentiate RU5ES-2 cells to lung lineage (with Hans Snoeck’s lab at Columbia)

--characterize cells for differentiation markers and for single cell transcriptomes (with Olivier Elemento’s lab at WCM)

--activate or induce mutations characteristic of common lung cancer types (LUAD, LUSC, SCLC)

--focus first on SCLC (neuro-endocrine cells, loss of RB1 and p53)
INDUCING PULMONARY NEUROENDOCRINE CELLS (PNECs) BY INHIBITION OF NOTCH AND RB DURING LUNG DIFFERENTIATION FROM A HUMAN ESC LINE

--LUNG DIFFERENTIATION VIA SNOECK PROTOCOL

--INHIBIT CLEAVAGE OF NOTCH RECEPTOR WITH DAPT

--KNOCK DOWN RB EXPRESSION WITH DOX INDUCED shRNA for RB1
DIFFERENTIATION OF RUES-2

A

hESCs (RUES2) → Endoderm induction → DE (C-kit+CXCR4+) → Lung differentiation

B

Lung progenitor cells (NKX2.1+ SOX2+ FOXA2+)

<table>
<thead>
<tr>
<th>NKX2.1</th>
<th>FOXA2</th>
<th>SOX2</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="nkx2.1.png" alt="Image" /></td>
<td><img src="foxa2.png" alt="Image" /></td>
<td><img src="sox2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

DAPI
Generation of PNEC-like cells with a NOTCH inhibitor (DAPT) that blocks gamma-secretase

**PNEC differentiation**

- hESC
- DE/AFE
- Lung progenitor (d15)
- Lung progenitor (d25)
- Mature lung cells (d50) + DAPT

**Graph:**
- DAPT(µM)
  - 5
  - 10
  - 0 (DMSO)

**Bar graph:**
- % of CGRP+ cells
  - DMSO
  - DAPT (5µM)

**Images:**
- CGRP
- DAPI
- NKX2.1
- CGRP/NKX2.1

**Results:**
- DMSO: 0.4 %
- DAPT (5µM): 8.4 %

**Note:**
- CGRP
- NKX2.1
- DMSO
- DAPT (5µM)
• **tSNE**: t-Distributed Stochastic Neighbor Embedding (tSNE) analyses to divide 1,200 single cells into distinct cell populations.

Clustering single lung cells by gene expression profiles

**tSNE of individual cells positive for PNEC markers**

**Putative Marker Genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold changes (Log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SST</td>
<td>0</td>
</tr>
<tr>
<td>ASCL1</td>
<td>5</td>
</tr>
<tr>
<td>SEC11C</td>
<td>10</td>
</tr>
<tr>
<td>CGRP</td>
<td>1</td>
</tr>
<tr>
<td>HES6</td>
<td>1</td>
</tr>
<tr>
<td>KLK12</td>
<td>1</td>
</tr>
<tr>
<td>GRP</td>
<td>1</td>
</tr>
<tr>
<td>SCG3</td>
<td>1</td>
</tr>
<tr>
<td>SCG2</td>
<td>1</td>
</tr>
<tr>
<td>MIAT</td>
<td>1</td>
</tr>
<tr>
<td>HMGB3</td>
<td>1</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>1</td>
</tr>
<tr>
<td>SOX2</td>
<td>1</td>
</tr>
<tr>
<td>ELF3</td>
<td>1</td>
</tr>
<tr>
<td>ABHD2</td>
<td>1</td>
</tr>
<tr>
<td>TAGLN3</td>
<td>1</td>
</tr>
<tr>
<td>FOXA2</td>
<td>1</td>
</tr>
<tr>
<td>BEX1</td>
<td>1</td>
</tr>
<tr>
<td>TPDS2</td>
<td>1</td>
</tr>
<tr>
<td>PTP4A3</td>
<td>1</td>
</tr>
<tr>
<td>PROX1</td>
<td>1</td>
</tr>
<tr>
<td>SYP</td>
<td>0</td>
</tr>
<tr>
<td>SCG5</td>
<td>0</td>
</tr>
<tr>
<td>SCNN1A</td>
<td>0</td>
</tr>
<tr>
<td>UCHL1</td>
<td>0</td>
</tr>
</tbody>
</table>
Knocking down RB with TetO-regulated shRNA
Knocking down RB increases the percentage of PNECs.
Reduced P53 or Mutated KRAS or EGFR do not induce or change percentage of PNECs

- TetO- shP53 transgenic RUES2 lines
- TetO-KRAS(G12V) or TetO-EGFR(L858R) transgenic RUES2 lines

hESCs \[\rightarrow\] DE/AFE \[\rightarrow\] Early progenitor (d15) \[\rightarrow\] late progenitor (d25) \[\rightarrow\] Lung cells (d55)

1. DAPT
2. DOX (shP53/KRAS G12V / EGFR L858R)
3. DAPT+ DOX

<table>
<thead>
<tr>
<th>Do x RB</th>
<th>GAPDH</th>
<th>P53</th>
<th>ES</th>
<th>LLP (D25)</th>
<th>LC (D55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do</th>
<th>GAPDH</th>
<th>KRAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>ES</td>
<td>LC (D55)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dox</th>
<th>KRAS G12V</th>
<th>EGFR L858R</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dox</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dox</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Reduced P53 or Mutated KRAS or EGFR do not induce or change percentage of PNECs
After inhibition of NOTCH, RB, and P53, hESC-derived lung cells form tumors in mice.

**Tumor formation SubQ in immunodeficient mice**

<table>
<thead>
<tr>
<th>Cells</th>
<th>% CGRP+ cells in total injected cells</th>
<th>Tumors / injection (≥ 250 mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental (DAPT alone)</td>
<td>7.6 ± 1.0</td>
<td>0/12</td>
</tr>
<tr>
<td>DAPT+shRB</td>
<td>39.6 ± 4.4</td>
<td>0/14</td>
</tr>
<tr>
<td>DAPT+shP53</td>
<td>8.0 ± 1.3</td>
<td>0/11</td>
</tr>
<tr>
<td>DAPT+shRB+shP53</td>
<td>41.9 ± 4.6</td>
<td>14/19 **</td>
</tr>
</tbody>
</table>
After inhibition of NOTCH, RB, and P53, hESC-derived lung cells form small SCLC-like tumors in mice sub Q.
SOME QUESTIONS:

--What accounts for increased proportion of PNECs after lowering RB levels? Replication vs differentiation?

--How similar are the tumors to clinical SCLC?

--Can additional genetic changes cause tumor progression?

--Are some PNECs more tumorigenic than others?

--Better tumorigenesis assays than xenografting subQ?

--What strategies can produce LUAD or LUSC from hESCs?

--Are other cells in the lung lineage or other lung cancer cell susceptible to changes that produce SCLC in PNECs?
THREE GENERAL OBSERVATIONS

• Then vs Now: transformation assays are still useful, but more sophisticated
THEN: HOW DOES A NORMAL CELL BECOME A CANCER CELL

LATTE STAGE CHICKEN EMBRYO

FIBROBLASTS IN CULTURE DISH

INFECT WITH RSV

TRANSFORMED CELLS (SARCOMA)

TEMIN AND RUBIN 1957
NOW: HOW DOES A NORMAL CELL BECOME A CANCER CELL?

**EARLY STAGE HUMAN EMBRYO**

Human Blastocyst

**EMBRYO STEM CELLS IN CULTURE DISH**

**HUMAN LUNG CELL PROGENITORS**

NKX2.1

FOXA2

**LUNG NEURO-ENDOCRINE CELLS**

CGRP/NKX2.1

**SMALL CELL LUNG CANCER IN MOUSE**

INACTIVATE TUMOR SUPPRESSOR GENES

INJECT CELLS UNDER MOUSE SKIN

CHEN ET AL 2018
THREE GENERAL OBSERVATIONS

• Then vs Now: transformation assays are still useful, but more sophisticated

• Developmental biology and cancer biology are increasingly intertwined and influenced by single cell biology
THREE GENERAL OBSERVATIONS

• Then vs Now: transformation assays are still useful, but more sophisticated

• Developmental biology and cancer biology are increasingly intertwined and influenced by single cell biology

• Internet-based communication of scientific results can (and should) be accelerated by pre-print servers
Not yet in a peer-reviewed journal, but available to all:

**Generation of pulmonary neuro-endocrine cells and tumors resembling small cell lung cancers from human embryonic stem cells**

Joyce Chen, Asaf Poran, Arun Unni, Sarah Huang, Olivier Elemento, Hans-Willem Snoeck, Harold Varmus

**bioRxiv 261461;**
doi: [https://doi.org/10.1101/261461](https://doi.org/10.1101/261461)

Encourage (or mandate) posting of preprints by members of the SCLC Consortium with alerts via email and web site.
cc
cc
REMINDEES ABOUT LUNG CANCER, ESPECIALLY SCLC

• Most common cause of cancer death worldwide

• Risks markedly increased by tobacco smoking

• Generally high mutation rate

• Three major forms---adenocarcinoma (CA), squamous CA, small cell (neuroendocrine) CA (SCLC)

• Characteristic genotypes

• Mouse models (Berns, Jacks) for SCLC
LUNG NE CELLS INDUCED BY INHIBITING NOTCH AND R...
Percentage of PNEC like (CGRP+) cells increased by blocking NOTCH signaling, augmented by knocking down RB1 RNA.
PUZZLES POSED BY GENOMIC RESULTS (& HOW USEFUL ARE MICE FOR SOLVING THEM)

Why are some mutations mutually exclusive?
Example: KRAS + EGFR in lung adenocarcinomas (LUA)

Why are some unexpected mutations oncogenic?
Example: splicing factor mutations in myeloid neoplasms

Why are certain patterns of mutations associated with cancers in certain lineages?
Examples: RB + P53 in small cell lung cancers (SCLC) KRAS pathway in LUAD
THREE EXAMPLES: THEN VS NOW

THEN = 20th Century (1970 on)  vs.  NOW = 21st Century (so far)

Most changes driven by technology, not new questions

Goals: (1) Identify and understand cancer genes

(2) Find meaning in mutational combinations

(3) Define conditions for making cancer cells in culture
Generation of lung cells by directed differentiation of human embryonic stem cells (hESCs) – RUES2 line

- **Lung differentiation**

  - **hESCs**
    - Definitive Endoderm (DE) \( \text{CXCR4}^+ / \text{C-KIT}^+ / \text{Ecad}^+ \)
    - Prior to day 1-3
    - BMP/TGF-b inhibition
    - Day 4-6
    - Anterior foregut endoderm (AFE) \( \text{CXCR4}^+ / \text{C-KIT}^+ / \text{Ecad}^+ \)
    - BMP4, FGF7, 10
    - Day 15-25
    - CHIR, Retinoic Acid
    - Day 25-50
    - Differentiated lung cells

  - **Lung Progenitor Cells** \( \text{NKX2.1}^+ / \text{SOX2}^+ / \text{FOXA2}^+ \)
    - Day 6-15
    - CHIR, FGF7, 10
    - Day 15-25
    - CHIR, FGF7, 10, cAMP
    - Differentiated lung cells

  - **Ectoderm**
    - CXCR4+/C-KIT+/Ecad+
    - hESCs
    - Activin/BMP4/bFGF
    - Day 1-3

  - **Mesoderm**
    - BMP/TGF-b inhibition
    - Day 1-3

Themes...

Transformation assays

Development and cancer genotypes

Information exchange