CRISPR: A Simple Tool for Answering Complex Questions

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- Small crRNAs transcribed from CRISPR loci & Cas proteins
- Prokaryotic adaptive immune response, sequence-specific detection and silencing of foreign nucleic acids.
- Via base pairing with the crRNA guide sequence, leading to Cas protein-mediated DNA cleavage.
- Three main types (I, II, and III).
Type II CRISPR-Cas9 System

Guide RNA

PAM

Cas9 (SpCas9)

Target DNA

20nt

NGG

crRNA

tracrRNA

Guide RNA

5'
Applications of CRISPR-Cas9

• Knockout (KO): indels, deletions

• Knock in (KI): point mutations, epitope tags, reporter sequences

• CRISPRi: transcription repression (*inactive Cas9 fused to transcription repression domain*)

• CRISPRa: transcription activation (*inactive Cas9 fused to transcription activation domain*)
Using CRISPR-Cas9 for Genome Editing

Knockout (KO)

NHEJ

Indels
Using CRISPR-Cas9 for Genome Editing

Knock in (KI)
# Practical Considerations

<table>
<thead>
<tr>
<th></th>
<th>RNAi (shRNA/siRNA)</th>
<th>CRISPR/Cas9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target</strong></td>
<td>Transcript</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td><strong>Loss of function</strong></td>
<td>Reversible KD</td>
<td>Irreversible KO</td>
</tr>
<tr>
<td><strong>Type of phenotype</strong></td>
<td>hypomorphic</td>
<td>Null</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>Short</td>
<td>Long</td>
</tr>
<tr>
<td><strong>Ease</strong></td>
<td>Easy</td>
<td>More difficult</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td><strong>Off-targeting</strong></td>
<td>High</td>
<td>Low*</td>
</tr>
</tbody>
</table>
Generating CRISPR KO/KI Cell Lines

- Cells of interest. Gene of interest
- Cas9
- sgRNA (crRNA + tracrRNA)
- Donor template (KI) (ssODN/fragment)

Delivery
- Transfection (plasmids, RNA, protein/RNA)
- Injection (DNA, RNA)
- Viral transduction

Target DNA

Plasmid(s)
RNA/mRNA
RNP

- All-in-one
- Split
- Inducible
- Viral
Generating CRISPR KO/KI Cell Lines

Cell cloning

- To obtain monoclonal cell populations
  - Limiting dilution
  - Single-cell sorting

Clone analysis

- Isolate and expand individual clones
- Confirm KO/KI
  - PCR
  - Sequencing
  - Western blotting
  - Immunostaining
  - ELISA
Scientific Examples

(1) Using a CRISPR KI breast cancer cell line to study estrogen receptor (ER) mutations and metastatic breast cancer

Guowei Gu and Suzanne Fuqua

(2) Using inducible CRISPR KO cancer cell lines to study telomere dysfunction

Hyeung Kim and Zhou Songyang
Estrogen Receptor (ER) Mediates Gene Transcription

ON- Estrogen Dependent

OFF

ON- Estrogen Independent

Gene Transcription?

Metastatic Tumor Growth?

Resistance?

Primary Tumor Growth?

Response

San Antonio Breast Cancer Symposium – December 6-10, 2016

Zhang 1997

Gene Transcription

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Mutations in the Gene Encoding ERα (ESR1) and Metastatic Breast Cancer (MBC)

• 20-40% of MBC contain ESR1 gene mutations, acquired during treatment of MBC with aromatase inhibitors (AI)

• An AI alone may be countraindicated in a MBC patient with ESR1 mutations

• Fulvestrant is effective in MBC with ESR1 mutations
Y537S ESR1 Mutation Model

Cells homozygous knocked in for the Y537S mutation (YS1 and YS30)

T7 assay for Cas9 cleavage efficiency

gRNAs

Genomic DNA sequencing

ESR1

Cas9, gRNA-2, ssODN donor

MCF-7

exon 8

MW

1 2 3

*
**Y537S ESR1 Mutation Model**

**Estrogen Regulated Genes**

<table>
<thead>
<tr>
<th>P/WT</th>
<th>YS1</th>
<th>YS30</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Myc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCDN1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
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</table>

**Estrogen Independent Transcription**

- **Pronounced Tamoxifen Resistance**
- **Responsive to Fulvestrant**

**Growth Assay (Fold)**

- **Tamoxifen**
- **Fulvestrant**

![Guowei Gu](image)
Y537S ESR1m Induces an Epithelial to Mesenchymal Transition (EMT)

• Lose contact inhibition and cell polarity
  e.g., enhanced migratory capacity, invasiveness, and resistance to apoptosis.

EMT – a mechanism for the initiation of invasive and metastatic epithelial cancers
Cell Mixing Experiment

Mixing WT + YS1(M)

Primary tumor growth
Metastases (macro-met and micro-met)
The Y537S ESR1 Mutant Has Competitive Proliferative Advantage and ‘Drives’ Metastasis

Mutant Frequency (ddPCR)

Primary Metastases

<table>
<thead>
<tr>
<th>Frequency</th>
<th>1%</th>
<th>10%</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutant Frequency vs. Metastasis Frequency

Macro-Met

Lung Micro-Met

* indicates significant difference
ESR1 Mutation Summary and Clinical Implications

- Mutations are a bona-fide mechanism of acquired hormone resistance
- First preclinical evidence that *ESR1* mutations also enhance tumor progression and metastatic behavior
• Telomeres are repetitive sequences at the ends of linear chromosomes – buffers. (TTAGGGG)

• Telomere shortening is associated with aging.

• Telomere dysfunction has been linked to aging-related diseases and cancer.
The Telosome Maintains Telomere length and Integrity

Telosome/Shelterin

Telomere loss
DNA damage

Telomere

T-loop
D-loop
5' 3'

Hyeung Kim
Generating Inducible CRISPR KO Cells to Study Telomere Regulation by the Telosome

Inducible Cas9 (lentiviral)↓Drug Selection↓-dox

Dox (µg/ml) 0 0.3 0.5 1 2

-Flag(Cas9)

α-Actin

HeLa cells expressing inducible Cas9
Generating Inducible CRISPR KO Cells to Study Telomere Regulation by the Telosome

Inducible Cas9 (lentiviral)

↓ Drug Selection

-gRNA(s)-

↓

TIN2
TRF2
TRF1
TPP1
RAP1
POT1

-drug
-drug
-drug
-drug
-drug
-drug

↓ Drug Selection

±drug
±drug
±drug
±drug
±drug
±drug
Generating Inducible CRISPR KO Cells for All Six Telosome Subunits

- **TRF1**: acidic (24), TRFH, Myb (395)
  - Trf1_g1
  - Trf1_g2

- **TRF2**: basic (73), TRFH, Myb (490)
  - Trf2_g1
  - Trf2_g2

- **RAP1**: BRCT (105), Myb (321)
  - Rap1_g1
  - Rap1_g2

- **TPP1**: OB fold (137), RD, S-rich (508)
  - Tpp1_g1
  - Tpp1_g2

- **TIN2**: MIS, TBM (11), OB2 (273)
  - Tin2_g1
  - Tin2_g2

- **POT1**: OB1 (82), OB2 (273)
  - Pot1_g1
  - Pot1_g2
Dual gRNAs Lead to More Complete KO
Inducible CRISPR KO Cell Lines for All Six Telosome Subunits
Using Inducible CRISPR KO Cells to Study Essential Genes
KO Individual Telosome Subunits Disrupts Telosome Targeting to Telomeres

Individual telosome subunits were IPed +/- doxycycline
The amount of co-precipitated telomere DNA was quantified
KO Individual Telosome Subunits Disrupts Telosome Targeting to Telomeres (cont’d)

<table>
<thead>
<tr>
<th>KO cell lines</th>
<th>TRF1</th>
<th>TRF2</th>
<th>RAP1</th>
<th>TPP1</th>
<th>TIN2</th>
<th>POT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF1</td>
<td>0.12</td>
<td>0.34</td>
<td>0.65</td>
<td>0.23</td>
<td>0.37</td>
<td>0.40</td>
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<tr>
<td>TRF2</td>
<td>0.96</td>
<td>0.01</td>
<td>0.23</td>
<td>0.43</td>
<td>0.48</td>
<td>0.60</td>
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<tr>
<td>RAP1</td>
<td>0.92</td>
<td>1.05</td>
<td>0.21</td>
<td>0.90</td>
<td>0.95</td>
<td>0.93</td>
</tr>
<tr>
<td>TPP1</td>
<td>0.81</td>
<td>0.77</td>
<td>0.71</td>
<td>0.18</td>
<td>0.39</td>
<td>0.18</td>
</tr>
<tr>
<td>TIN2</td>
<td>0.63</td>
<td>0.71</td>
<td>0.34</td>
<td>0.06</td>
<td>0.13</td>
<td>0.23</td>
</tr>
<tr>
<td>POT1</td>
<td>0.61</td>
<td>0.59</td>
<td>0.66</td>
<td>0.35</td>
<td>0.33</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Low ChIP ratios, reduced binding to telomeres
KO Individual Telosome Subunits Disrupts Telomere Maintenance

<table>
<thead>
<tr>
<th>KO Individual Telosome Subunits</th>
<th>Telomere fusions</th>
<th>Telomere loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF1-DG (-Dox)</td>
<td>0</td>
<td>0.7%</td>
</tr>
<tr>
<td>TRF1-DG (+Dox)</td>
<td>0</td>
<td>7.1%</td>
</tr>
<tr>
<td>TRF2-DG (-Dox)</td>
<td>0.1%</td>
<td>1.2%</td>
</tr>
<tr>
<td>TRF2-DG (+Dox)</td>
<td>73.1%</td>
<td>-</td>
</tr>
<tr>
<td>TIN2-DG (-Dox)</td>
<td>0.3%</td>
<td>0.9%</td>
</tr>
<tr>
<td>TIN2-DG (+Dox)</td>
<td>8.3%</td>
<td>19.6%</td>
</tr>
<tr>
<td>TPP1-DG (-Dox)</td>
<td>0</td>
<td>1.1%</td>
</tr>
<tr>
<td>TPP1-DG (+Dox)</td>
<td>0</td>
<td>3.5%</td>
</tr>
<tr>
<td>POT1-DG (-Dox)</td>
<td>0</td>
<td>2.7%</td>
</tr>
<tr>
<td>POT1-DG (+Dox)</td>
<td>0</td>
<td>7.8%</td>
</tr>
<tr>
<td>RAP1-DG (-Dox)</td>
<td>0</td>
<td>1.1%</td>
</tr>
<tr>
<td>RAP1-DG (+Dox)</td>
<td>0</td>
<td>9.6%</td>
</tr>
</tbody>
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Jun Xu

Hyeung Kim

Zhou Songyang

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