Genome-wide CRISPR-based screen to study cancer evolution in vivo

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3.10.2017
Outline

CRISPR background

Cancer and Evolution

Genome-wide CRISPR-based screens to study cancer evolution *in vivo*

Conclusion
CRISPR background
CRISPR Cas9 mechanism

A

1st infection

Foreign fragment acquired to the CRISPR locus

DNA repeats

tracrARN

Cas9

Cas1, 2...

tracrARN

Cas genes

CRISPR locus

2nd infection

RNA/Cas9 complex formation

Monitoring Recognition

Cleavage of foreign DNA

Bacterial resistance to the virus

Foreign DNA

Fusion of crRNA + tracrRNA

sgRNA


Sander & Joung, 2014
DSB reparation

End processing
subsequent ligation
Imprecise

NHEJ
Nuclease-induced
Double-strand Break

HDR
Homology directed repair

end resection
produces a 3’ ss end

Strand invasion

new strand is synthesized
using the intact, homologous DNA strand as a template

Deletion
Insertion
Variable length indels

Precise insertion or modification

Donor template
CRISPR applications

- Gene editing/knockout
- Transcription repression/activation
- Epigenetic modifications

CRISPR/Cas9

Large-scale genetic screen
- sgRNA library
- Screening
- Before
- After

Generation of animal models

Genomic imaging
- Fluorescent protein

Lineage tracing

Trends in Cell Biology
Cancer and Evolution

How does a normal cell become a cancer cell?

Somatic Evolution
Accumulation of mutations in the cells of a body during a lifetime, and the effects of those mutations on the fitness of those cells. Important in aging as well as the development of some diseases, such as cancer.
Cancer and Evolution

Natural selection in cancer
The differential survival and reproduction of individuals due to differences in phenotype (6 hallmarks). It is a key mechanism of evolution, the change in heritable traits of a population over time.
Metastasis

Primary tumor formation → Local invasion → Intravasation → Extravasation → Arrest at a distant organ site → Survival in the circulation → Clinically detectable macroscopic metastases

Micrometastasis formation → Metastatic colonization

Lungs, Liver, Brain, Bones

Valastyan & Weinberg 2011 cell
Aim:

Systematic phenotypic measurement to identify causal genes in various hallmarks of cancer progression.

Mutagenized a non-metastatic mouse cancer cell line using a genome-scale library with 67,405 sgRNAs.
Method

Cell line from a mouse non-small cell lung cancer (NSCLC)

- With KPD mutations =
  oncogenic \textit{Kras} \textit{Kras}^{G12D/ +}
  GTPase and is an early player in many signal transduction pathways.  
  the mutation of a KRAS gene is an essential step in the development of many cancers

- homozygous \textit{p53} \textit{p53−/−}
  Tumor suppressor, activates DNA repair, arrest growth, initiate apoptosis, 
  senescence response in telomers

- heterozygous \textit{Dicer1} loss of function \textit{Dicer1+/−}
  Mutations in the DICER1 gene are associated to pleuropulmonary blastoma

Cells are capable of inducing tumors when transplanted into immunocompromised mice
clonal Cas9-GFP KPD cell line provides genetic and cellular homogeneity for subsequent screens

pooled genome-wide mouse sgRNA library

67,405 sgRNAs targeting 20,611 protein-coding genes and 1,175 microRNA precursors in the mouse genome

1,000 control sgRNAs (termed non-targeting sgRNAs) with minimal homology to sequences in the mouse genome

They transduced the CRISPR library with three independent infection replicates and each replicate with more than 400x cells per lentiviral CRISPR construct (library coverage).

carcinogenic metastases

Method

transduction

Lung

transduction Days after transplan

F

Untransduced
Immunocompromised *Nu/Nu* mice, hairless and athymic, T cell deficient, lack CD4$^+$ and CD8$^+$ T Cells are capable of inducing tumors when transplanted into immunocompromised mice.
Both Cas9-GFP KPD cells transduced with CRISPR library and untransduced, formed tumors at the injection site.

Like most subcutaneously transplanted tumors, these tumors were poorly differentiated.

The primary tumors induced by CRISPR library transduced cells grew slightly faster than tumors from the untransduced cells at an early stage, but at late stages all tumors were similar in size.
Results

10 20 30 40

1 week

Lung  Liver  Kidney  Spleen

3 x 10^7 transduced and into flanks of NU/Nu mice

3 mice

micro-computed tomography (μCT) a fluorescent stereoscope
Results

At 6 weeks post-transplantation, imaging using micro-computed tomography ($\mu$CT)

Untransduced

Transduced with CRISPR library

Tumors in mice transplanted with CRISPR library cells

No tumors in mice with cells without CRISPR library
Results

At 6 weeks post-transplantation, imaging using fluorescent stereoscope at 6x magn.

80% of their lung lobes positive for metastases

None (0/3) of the control mice developed detectable metastases in the lung

These data indicate that CRISPR library-mediated mutagenesis promotes metastasis
Results

Dynamic evolution of sgRNA library representation during tumor growth and metastasis

Investigate the sgRNA representation through different stages of tumor evolution
identify genes where LOF confers a proliferative or metastatic phenotype

To read out the sgRNA representation, do **deep sequencing** of different stages of tumor evolution:

**Plasmid**: CRISPR input plasmid library

**Cells**: the pre-transplantation Cas9-GFP KPD cells

**Early primary**: early stage primary tumors (two weeks post transplantation, one mouse from each infection replicate)

**Late primary**: late stage primary tumor (6 weeks post transplantation)

**Lung mets**: three random lobes from the lung of each of the nine CRISPR mice
The sgRNA representation of cell samples highly correlates with the plasmid representation, forming a cell-plasmid clade.

Early primary tumor samples also clustered with each other and then with the cell-plasmid clade.

Late tumors and lung metastases clustered together in a distinct group.
Late 1° tumors only a share a small fraction of sgRNAs (< 4% of all sgRNAs, < 8% of sgRNAs in the early primary tumor of the corresponding replicate)

Late 1° tumors retain few sgRNAs (on average 813 sgRNAs, n = 9 mice)

The lung samples retained ≤ 0.4% of all sgRNAs in the CRISPR library, or ≤ 1.1% of sgRNAs found in the early 1° tumor of the corresponding replicate, with a subset of highly enriched sgRNAs

Dynamic evolution of sgRNA library representation during tumor growth and metastasis
Results

Identify enriched sgRNAs in late primary tumors

False discovery rate: sgRNAs enriched compared to the distribution of the 1,000 non-targeting sgRNAs

24 candidate genes that were targeted by two or more independent sgRNAs enriched in late primary tumors

These genes were found to be mutated in patients in many previously reported cancer sequencing studies

Several candidates were well-known tumor suppressors and apoptosis pathways
Results

Identify enriched sgRNAs in lung metastases

False discovery rate: enriched sgRNAs compared to the 1,000 non-targeting sgRNAs

In each lobe, the sgRNA representation is dominated by one or a few sgRNAs

In each mouse, the lung sgRNA representation is also dominated by a small number of sgRNAs (on average, 3.4 ± 0.4 sgRNAs)

➔ metastases were seeded by a small set of cells, which grew to dominance over this time scale
Results

Identify enriched sgRNAs in lung metastases

False discovery rate: enriched sgRNAs compared to the 1,000 non-targeting sgRNAs

Non-targeting sgRNAs were occasionally detected in the metastases, but never observed at high frequency.

These observations are consistent with our finding that un-transduced tumors are not metastatic

→ specific sgRNA-mediated mutations led to metastasis
Results

Identify enriched sgRNAs

sgRNA representations in the **lung metastases** are similar to those in the **late stage primary tumors**
- the detected sgRNAs in lung samples significantly overlap with those in late tumor samples
- the number of sgRNAs detected in lung samples correlates, albeit weakly, with the number of sgRNAs detected in late primary tumor samples

> mutants with preferential ability to proliferate in late primary tumors are more likely to dominate the metastases
Results

Identify enriched sgRNAs

→ 147 sgRNAs enriched in more than one lobe

→ 105 sgRNAs enriched in the lung of more than one mouse
Four candidate genes, were targeted by two independent sgRNAs enriched in lung metastases.

Validation *in vivo* using individual sgRNAs with

- 4 candidate genes
- the top two scoring microRNAs (*miR-152* and *miR-345*)
Results

Validation *in vivo* using single sgRNAs

Deep sequencing to examine the distribution of indels at the target site

- 6 hits
  - 6 sgRNAs for each protein-coding gene
  - 4 sgRNAs for each miRNA

![Transduction](image1.png)

![3d Puro](image2.png)

![Nu/Nu](image3.png)

E: Trim72

F: miR345, miR162
Results

Validation *in vivo* using single sgRNAs transduction

Histograms of indel sizes at the genomic locus targeted by sgRNA for each gene/miRNA

For **protein coding genes** > 80% of indels are out-of-frame, which potentially disrupt the protein functions

For **miR-152** and **miR-345** the sgRNAs generated mostly deletions (> 90%)
Inject cells targeted with CRISPR sublibrary into mice
Examine the primary tumor and lungs after 5 weeks
Validation *in vivo* using single sgRNAs: examine lung metastases after 6 weeks

> all genes tested showed increased lung metastasis formation compared to controls

H&E staining of lung lobes, scale bar: 10 μm

% of lung lobes with metastases after 6 weeks

→ loss-of-function mutations in any of *Nf2*, *Pten*, *Cdkn2a*, *Trim72*, *Fga*, *miR345* or *miR-152* are sufficient to accelerate the rate of metastasis formation in this genetic background.

Ctrl ing

Subcutaneous transplant

Examine lung metastases

Cas9-EGFP NSCLC cell line

+ lentiviral sgRNA targeting one gene or microRNA
Most genes targeted by single sgRNAs also contributed to accelerated primary tumor growth compared to controls.

miRNAs show no acceleration in 1° tumor growth

\[\text{Number of lobes with lung metastases correlates strongly with the 1° primary tumor volume}\]

\[\rightarrow \text{mutant cells at a single gene level with a stronger ability to promote primary tumor growth, generate metastases faster.}\]

\[\rightarrow \text{Check on multiple gene level}\]

Validation \textit{in vivo} using single sgRNAs: examine 1° tumor and lung metastases after 6 weeks

\% of lung lobes with 1° tumor after 6 weeks for the mice

1°tumor growth curve of \textit{Nu/Nu} mice transplanted with NSCLC cells
Competitive dynamics of top hits assessed using an sgRNA minipool

Understand the relative metastatic potential of multiple genes from the genome-wide screen.

Design a targeted pooled screen with a smaller library = minipool

Validation minipool

524 sgRNAs targeting 53 genes which had highly enriched sgRNAs in lung metastases in the genome-wide screen with 10 sgRNAs per gene for most genes.

100 non-targeting sgRNAs

control minipool = size-matched library containing 624 non-targeting sgRNAs
Examine 1° tumors and lung metastases after minipool transduction and injection

Primary tumor growth curve of *Nu/Nu* mice transplanted with Cas9 vector + % of lung lobes with metastases

<table>
<thead>
<tr>
<th>C = control minipool</th>
<th>V = validation minipool</th>
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Both induced primary tumor growth at a similar rate

Mice transplanted with validation minipool cells had a *dramatically elevated rate of lung metastasis formation*
Competitive dynamics of top hits assessed using an sgRNA minipool

Deep sequencing

plasmids

C

D

late tumor mets

lungs

Examine metastases and sequencing
sgRNA representation in the cell sample strongly correlated with the plasmid.

Late primary tumors retained less than half of the sgRNAs.

Metastases in the whole lung only retained a small fraction (2 – 7%) of all sgRNAs.
Competitive dynamics of top hits assessed using an sgRNA minipool

Deep sequencing

are dominated by a few sgRNAs, suggesting these sgRNAs outcompete others during tumor growth and metastasis

Check which genes are expressed in lung metastases

sgRNA representations are highly correlated between late 1° tumors and lung metastases
Genes are expressed in lung metastases

10 sgRNAs / gene
Ranked genes according to enriched sgRNAs

Nf2: Neurofibromin 2 = tumor suppressor protein involved in Neurofibromatosis type II

Pten: Phosphatase and tensin homolog = tumor suppressor that is mutated in a large number of cancers at high frequency

Trim72: Tripartite Motif Containing 72 regulates myogenesis

Ube2g2: Ubiquitin-conjugating enzyme E2 G2
Conclusion

Individual sgRNAs and a small pool of 624 sgRNAs targeting the top hit genes from the primary screen dramatically accelerate metastasis. In all of these experiments, the effect of mutations on 1° tumor growth positively correlates with the development of metastases.

The validation minipool screen reveals the dynamics of multiple competing mutants chosen from the 1° screen hits → mutants with strong pro-growth effects tend to enhance metastases
Discussion

Pooled mutagenesis in a metastasis model

In this study, cancer cells transplanted into the flanks of mice and form 1° tumors \textit{in situ} cells from this mass undergo the intravasation – circulation - extravasation - clonal growth cascade to form distal metastases

The initial lung cancer cell line has little capacity to form metastases After being mutagenized with the CRISPR genome-scale Cas9 knockout library, the cell population form highly metastatic tumors

$\rightarrow$ These mutations accelerate metastasis
$\rightarrow$ The effect of mutations on metastasis strongly correlates with their abundance in late stage primary tumors.
Discussion

sgRNA dynamics during tumor evolution

The dynamics of the sgRNA population changed dramatically over the course of tumor development and metastasis, reflecting the selection and bottlenecks of cellular evolution in vitro and in vivo.

Cells retained most of the sgRNAs present in the plasmid library
Same number of non-targeting control sgRNAs and targeting genes
→ selective pressure of in vitro culture alone does not radically alter sgRNA representation
Discussion

Relevance of screen hits to human cancer

Genes that are significantly enriched in lung metastases largely overlap with those found in abundance in the late 1° tumor. Several of these hits were validated in vivo using multiple individual sgRNAs, including Nf2, Pten, Cdkn2a, Trim72, Fga, miR-152 and miR-345.

*Nf2 and Pten* are well-known tumor suppressor genes.

Intriguingly, the *NF2* locus is only mutated at a 1% frequency in primary tumors of human Non-Small Cell Lung Cancer patients. Maybe *NF2* mutations influence metastases more than 1° tumor growth, but this awaits metastasis genomics from patient samples.

*PTEN* was found mutated at 8% in adenocarcinoma patients

*Trim72* an E3 ubiquitin ligase: little information associating it to metastasis or cancer.

Studies have shown that *miR-152* and *miR-345* are associated with cancer and metastasis (Cheng et al., 2014; Tang et al., 2011)
Discussion

Future \textit{in vivo} functional genomic screens

\textit{In vivo} pooled screens are challenging due to many factors, such as
- the complexity of the library, limitations of virus delivery and/or cell transplantation
- uniformity of viral transduction at a low multiplicity of infection (MOI)
- the complex dynamics and interactions of cells in animal

Most cancer sequencing studies involve samples from primary tumors of patients. In the clinic, metastases are rarely sampled. Future patient sequencing directly from metastases may further connect genes identified in the mouse model to those mutated or silenced in clinical metastases.
Discussion

Future *in vivo* functional genomic screens

The study demonstrates Cas9-based screening as a robust method to systematically assay gene phenotypes in cancer evolution *in vivo*.

Future studies can take advantage of this model to explore other oncogenotypes, delivery methods or metastasis target organs.

 Genome-scale CRISPR screening is feasible using a transplant model with virtually any cell line or genetic background. Other cell delivery methods, such as intravenous injection or orthotopic transplantation, may help identify genes regulating extravasation and formation of new clones.

Targeted drug therapies or immunotherapies can be applied in conjunction with the *in vivo* screening strategy to identify genes involved in acquired resistance.

Cas9-mediated activation may be used to identify metastasis-regulating factors that act in a gain-of-function manner.
Questions regarding genome-wide CRISPR-based screen to study cancer evolution \textit{in vivo}?

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3.10.2017
(A) A barcode with ten Cas9 target sites (gray bars), as well as flanking primer sequences (green) is introduced into the genome of interest.

(B) During this cell’s lifetime Cas9 introduces an insertion (target 3 blue), a mark that will be passed onto all progeny cells. The pattern of shared edits between many thousands of cells can be used to trace lineage.