Genome Editing with Programmable Nucleases

Jin-Soo Kim
Department of Chemistry
Seoul National University
Method of the Year 2011: Engineered Nucleases

ZFNs and TALENs. Schematic (not to scale) of a ZFN pair (top) and a TALEN pair (bottom) to illustrate the DNA-binding principles of these enzymes.
RNA-guided Cas9 Endonuclease

NEWS & VIEWS

Rewriting a genome

A bacterial enzyme that uses guide RNA molecules to target DNA for cleavage has been adopted as a programmable tool to site-specifically modify genomes of cells and organisms, from bacteria and human cells to whole zebrafish.

NEWS AND VIEWS

RNA guides genome engineering

Claudio Mussolino & Toni Cathomen

The Cas9 endonuclease is reprogrammed by RNAs for site-specific modification of eukaryotic and microbial genomes.

RNA-guided gene editing

Targeted gene modification can be guided by programmable RNA in bacteria, zebrafish and mammalian cells. Humble creatures, prokaryotes and viruses, have an illustrious history of providing biologists with molecular tools. Where, after all, would molecular biology—or for that matter, all genomics—be without restriction endonucleases or adjacent motif (PAM), which takes the form NGG, immediately following the 20-base pair target sequence. In contrast to current tools, zinc finger nucleases and transcription activator-like effector nucleases (TALENs), targeting of a new sequence with CRISPR-Cas requires the design of only a new RNA guide and not a new pair of enzymes. The efficiency of
FokI and the First ZFN

Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain

(\textit{Flavobacterium okeanokoites}/chimeric restriction endonuclease/protein engineering/recognition and cleavage domains)

\textbf{YANG-GYUN KIM, JOOYEUN CHA, AND SRINIVASAN CHANDRASEGARAN*}

- FokI is a type IIS restriction enzyme.
- FokI consists of two modular domains; DNA-binding domain and nuclease.
- DNA-binding domain of FokI can be replaced w/ other DNA-binding domains.
Targeted mutagenesis in Drosophila using ZFNs

Enhancing Gene Targeting with Designed Zinc Finger Nucleases
Marina Bibikova,* Kelly Beumer, Jonathan K. Trautman, Dana Carroll†

2 MAY 2003 VOL 300 SCIENCE

- Zinc finger-FokI nuclease fusion proteins function as site-specific endonucleases.
- Two 3-finger proteins could recognize a unique site (18 bp) in a genome.
- ZFNs induce site-specific DSBs in cells.
- DSBs are repaired by endogenous mechanisms, HR or NHEJ.

Dana Carroll
Cys$_2$-His$_2$ Zinc Finger

- Found in fungi, plants, animals and human
- 6,500 zinc finger sequences exist in the human genome.
- Modular head-to-tail arrangement
- A single zinc finger module interacts w/ a 3-bp subsite.


Carl Pabo
Figure 1. In vivo screening system and zinc 'fingerprints'. (A) Zinc-finger screening system in yeast. Zinc-finger domain 'A' recognizes the 3-bp target.
Modular Assembly of Zinc Finger Arrays

Zinc finger-DNA interaction directory

<table>
<thead>
<tr>
<th>3-bp subsite</th>
<th>Zinc finger</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAA</td>
<td>QSNR</td>
</tr>
<tr>
<td>GAC</td>
<td>CSNR</td>
</tr>
<tr>
<td>GAG</td>
<td>RSNR</td>
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<td>RDER</td>
</tr>
<tr>
<td>GCT</td>
<td>VSTR</td>
</tr>
</tbody>
</table>

Modular assembly

Zinc fingers recognizing diverse 3-bp subsites were isolated from the human genome.


• Zinc fingers recognizing diverse 3-bp subsites were isolated from the human genome.
Zinc Finger Transcription Factor Library

A

Random shuffling

Zinc fingers → ZFPs → ZFP-TF library

B

ZFP-TF vector library + Mammalian or microbial cells

Transformation or transfection

ZFPs expressed in cells

Screening

Altered cellular phenotype

Preassembled zinc-finger arrays for rapid construction of ZFNs

Seokjoong Kim\textsuperscript{1,3}, Mi Jung Lee\textsuperscript{2,3}, Hyojin Kim\textsuperscript{1}, Mijin Kang\textsuperscript{2} & Jin-Soo Kim\textsuperscript{1}

\textsuperscript{1}Department of Chemistry, Seoul National University, Seoul, South Korea. \textsuperscript{2}ToolGen, Inc., Biotechnology Incubating Center, Seoul National University, Seoul, South Korea. \textsuperscript{3}These authors contributed equally to this work.

e-mail: jskim01@snu.ac.kr
Targeted Gene Knockout in Animals

Prof. CH Kim at CNU

- RNA transcripts encoding ZFNs were injected into one-cell embryos.
- >20 genes were successfully targeted using our ZFNs in zebrafish, medaka, and frogs.

sam2 in zebrafish habenula (adult)

Novel C-C chemokine
ZFNs are associated with cytotoxicity

• ZFN-induced mutations often disappear at day 9 post-transfection.
• Mammalian cell lines are much more susceptible to ZFN cytotoxicity.
• >100 clones need to be analyzed to obtain a single KO clone.

Kim et al. (2009) Genome Res. 19, 1279.
Transcription Activator-Like (TAL) Effectors

J. Boch et al. Science 2009
M.J. Moscou et al. Science 2009
Optimized TALEN Architectures

TALEN-L

Variable fusion junctions
8 bp
9 bp
10 bp
11 bp
12 bp
13 bp
14 bp
15 bp
16 bp
17 bp
18 bp
19 bp
20 bp
21 bp

TALEN activity (% of GFP+ cells)

TALEN-L

Optimized TALEN Architectures

Variable fusion junctions

- L1
- L2
- L3
- L4
- S+28
- S+63

- 8 bp
- 9 bp
- 10 bp
- 11 bp
- 12 bp
- 13 bp
- 14 bp
- 15 bp
- 16 bp
- 17 bp
- 18 bp
- 19 bp
- 20 bp
- 21 bp
Genome-Scale Assembly of TALENs

- One-step Golden Gate cloning system using 424 TALE arrays and 8 FokI plasmids.
- 18,740 TALENs that target every protein-coding gene in the human genome.

>99% Success Rate of TALENs

- Mutation frequencies are measured using T7 endonuclease I.
18,740 pre-assembled TALENs; 169,362 designed TALENs for 18,740 human genes

Since March 2013, we provided SNU TALENs to >80 labs around the world.

http://www.talenlibrary.net
Surrogate reporters for enrichment of cells with nuclease-induced mutations

Hyojin Kim¹, Eunji Um², Sung-Rae Cho³, Chorong Jung⁴, Hyongbum Kim⁴ & Jin-Soo Kim¹

Zinc-finger nucleases (ZFNs) and TAL-effector nucleases (TALENs) are powerful tools for creating genetic modifications in eukaryotic cells and organisms. But wild-type and mutant cells that contain genetic modifications induced by these programmable nucleases are often phenotypically indistinguishable, hampering isolation of mutant cells. Here we show that transiently transfected episomal reporters encoding fluorescent proteins can be used as surrogate genes for the efficient enrichment of endogenous gene-modified cells by flow cytometry.
Gene KO Cells Created Using TALENs

Wild type
- 292bp
- 282bp
- 261bp
- 288bp

WT KO
TNFR1  TNFR1
IL1R1  IL1R1

aattgatgTTCGTCCCTGTCCTCTTAAC
ccaaatgaacacAAAGGCACTATAACTTGGTA
aattgatgTTCGTCCCTGTCCTCT

TECGTCCCTGTCCTCTTAAC
ccaaatgaacacAAAGGCACTATAACTTGGTA

aattgatgTTCGTCCCTGTCCTCTTAAC
ccaaatgaacacAAAGGCACTATAACTTGGTA

aattgatgTTCGTCCCTGTCCTCTTAAC
ccaaatgaacacAAAGGCACTATAACTTGGTA

TNFR1

GAPDH

Relative luciferase activity

TNFα
IL-1β
(-)

TNFR1 SI
IL1R1 KO

WT
TNFR1 KO

WT
TNFR1 KO

- siCTRL
- siTNFR1
- siIL1R1
- TNFR1 KO
- IL1R1 KO
- TNFR1 / IL1R1 KO

WT
TNFR1 KO

WT
TNFR1 KO

WT
TNFR1 KO

WT
TNFR1 KO

WT
TNFR1 KO

- siCTRL
- siTNFR1
- siIL1R1
- TNFR1 KO
- IL1R1 KO
- TNFR1 / IL1R1 KO

WT
TNFR1 KO

TNFR1 KO

IL1R1 KO

TNFR1 / IL1R1 KO

TNFR1

GAPDH

- siCTRL
- siTNFR1
- siIL1R1
- TNFR1 KO
- IL1R1 KO
- TNFR1 / IL1R1 KO
TNFR1 KO cells created using TALENs

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<th>TNFR1 WT</th>
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<th>TNFR1 KO</th>
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<tr>
<td></td>
<td>TNF-α(-)</td>
<td>TNF-α(+)</td>
<td>TNF-α(-)</td>
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<td><img src="image14" alt="Merge" /></td>
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Targeted Gene Knockout in Mice

• RNA transcripts encoding ZFNs/TALENs were injected into the embryos.
• 49 to 77% of F0 contained mutations.
Queen Victoria and her royal family

- British Queen Victoria was a carrier of the hemophilia gene.
- Almost half of the severe form of hemophilia A is caused by DNA inversion.
ZFN-induced Inversion of the Hemophilia Gene

**Breakpoint junction 1**

![](image1)

**Breakpoint junction 2**

![](image2)

**Patient gDNA**

**ZFN 10**

**Empty vector**

(F1+F2) (R1+R2)

Lee et al. (2012) Genome Res. 22, 539.
Stem Cell Therapy: Gene Correction in iPS Cells

- Patient somatic cells
- Reprogramming
- iPS cells
- Gene correction via Nucleases
- Gene-corrected iPS cells
- Differentiation
- Gene-corrected cells
- Transplantation
- Biopsy
CRISPR: Adaptive Immune System in Prokaryotes

- Clusters of Regularly Interspaced Palindromic Repeats
- Cas9/crRNA/tracrRNA: RNA-guided programmable restriction enzymes
RNA-Guided ENdonuclease (RGEN)
Cloning-Free Gene Knockout in Human Cells

**CCR5**

<table>
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<tr>
<th>Cas9 plasmid</th>
<th>Guide RNA</th>
<th>Indels (%)</th>
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<tr>
<td>-</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13</td>
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**C4BPB**

<table>
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<th>Cas9 plasmid</th>
<th>Guide RNA</th>
<th>Indels (%)</th>
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<tr>
<td>-</td>
<td>-</td>
<td>9.3</td>
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<tr>
<td></td>
<td>+</td>
<td>15</td>
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<tr>
<td></td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>23</td>
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CAATCTATGACATCAATTATTATA-\textcolor{red}{\textbullet}CATCGGAGGCTGCACAAAATCAA \hspace{1em} \text{WT}

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} \text{WT}

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +1

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +2

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +3

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +4

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +6

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +9

CAATCTATGACATCAATTATTATATACATGAGGGCAGCAATCGGAGCCAG \hspace{1em} +12

**Cloning-Free Gene Knockout in Human Cells**

- Synthetic guide RNA rather than plasmids encoding guide RNA were used.
RNA-Guided Gene KO in Animals

- Recombinant Cas9 complexed w/ guide RNA was tested in vitro first.
- Active ribonucleoproteins were injected into one-cell embryos.
- Up to 93% of newborn mice carried mutations.
- RGEN proteins induced mutations in zebrafish, fruitfly, C. elegans, etc.

Prof. Han-Woong Lee at Yonsei Univ.  
*Genetics in press; Genome Res. in press*
Paired ZF Nickase

• Two adjacent nicks produced by paired nickases yield a composite DSB.
• Paired nickases induce mutations at high frequency in human cells.

Kim et al. (2012) Genome Res. 22, 1327.
## Comparison of Engineered Nucleases

<table>
<thead>
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<th>ZFN</th>
<th>TALEN</th>
<th>RGEN</th>
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<tbody>
<tr>
<td>Success rate</td>
<td>~24%</td>
<td>&gt;99%</td>
<td>~90%</td>
</tr>
<tr>
<td>Average mutation rate</td>
<td>&lt;10%</td>
<td>~20%</td>
<td>~20%</td>
</tr>
<tr>
<td>Length of target site</td>
<td>18 to 36 bp</td>
<td>30 to 40 bp</td>
<td>23 bp</td>
</tr>
<tr>
<td>Restriction in target site</td>
<td>Guanine-rich</td>
<td>Start with T and end with A</td>
<td>End with GG (PAM)</td>
</tr>
<tr>
<td>Design density</td>
<td>One per ~100 bp</td>
<td>One per every bp</td>
<td>One per 8 bp</td>
</tr>
<tr>
<td>Off-target effects</td>
<td>High</td>
<td>Low</td>
<td>Low?</td>
</tr>
<tr>
<td>Size</td>
<td>2 x ~2 kbp</td>
<td>2 x ~3 kbp</td>
<td>4.2 kbp + gRNA</td>
</tr>
</tbody>
</table>
Engineered Nucleases: “Restriction enzymes” in the post-genomic era

- Gene knock-out and knock-in
- Genome rearrangements: deletion, duplication, inversion, translocation

- Gene knockout studies in cell lines
- Targeted mutagenesis in research organisms: zebrafish, C. elegans etc.
- Plant and animal biotechnology: applications in agriculture, fishery etc.
- Stem cell research and cell/gene therapy