Therapeutic Genome Editing

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Programmable Nucleases

Zinc Finger Nucleases (ZFNs)
- Nuclease (DNA-cutting) domain
- Custom-designed Zinc Finger DNA-Binding Domain

TAL Effector Nucleases (TALENs)
- Nuclease (DNA-cutting) domain
- Custom-designed TAL Effector DNA Binding Domain

Cas9

Target DNA
- 20nt
- 3' NGG

CRISPR/Cas-derived RNA-guided endonuclease (RGEN)
## Comparison of Programmable Nucleases

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

Challenges in Therapeutic Genome Editing

- Delivery
- Immunogenicity
- Mosaicism
- HDR vs. NHEJ
- Specificity: Off-target mutations
Genome Surgery

Ex vivo therapy

- Plasmid
- mRNA + gRNA
- RNP
- Viral vector

In vivo therapy

- Cationic lipid
- AAV
- Adenovirus
- T cells from HIV+ patients are treated with a programmable nuclease.
- CCR5-inactive T cells are delivered back to patients
Stem Cell Therapy: Gene Correction in iPS Cells

Patient’s somatic cells → Reprogramming → iPS cells → Gene correction → Gene-corrected iPS cells

Biopsy → Reprogramming

Patient → Transplantation

Gene-corrected cells
Hemophilia: The Royal Disease

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.
Inversion of the Hemophilia Gene

Normal

Patient

Z10 target site (Homology 1)

WT

..caaggagccccacctgagttggcgaaggtgccccag..140kbp..GTGGGCCCCACCTTTGCCCCAATGTTGCTCTCTTG..

..gttcctctggtgactcaaccggctg..140kbp..CAGCCGGGTTGAAAAACCGGTTGAGTCACCCAGAGGAAC..

Cleaved

..caaggagccccacctga gttggcgaaggtgccccag..140kbp..GTGGGCCCCACCTTTGCCCCAATGTTGCTCTCTTG..

..gttcctctggtgactcaac ccgtttccaccccgctg..140kbp..CAGCCGGGTTGAAAAACCGGTTGAGTCACCCAGAGGAAC..%

Flipped

..caaggagccccacctgagttggcgaaggtgccccag..140kbp..GTGGGCCCCACCTTTGCCCCAATGTTGCTCTCTTG..

..gttcctctggtgactcaacc cctgtttccaccccgctg..140kbp..CAGCCGGGTTGAAAAACCGGTTGAGTCACCCAGAGGAAC..

Breakpoint junction 1

..agtcgccccccccctttgccccaa---ctcagtgtggtctccttg.. (X6)

..agtcgccccccccctttgccccaa------------------------. (X1)

..agtcgccccccccctttgccccaa-----------------------. (X1)

..agtcgccccccccctttgccccaa------------------------. (X2)

Breakpoint junction 2

..agtcgccccccccctttgccccaa------------------------. (X6)

..agtcgccccccccctttgccccaa------------------------. (X1)

..agtcgccccccccctttgccccaa------------------------. (X1)

..agtcgccccccccctttgccccaa------------------------. (X2)

Lee et al. Genome Res. 22, 539 (2012)
Hemophilia Mice Treated w/ Gene-Corrected Cells

• CRISPR-Cas9 can revert large inversions in hemophilia iPSCs.
• Endothelial cells derived from corrected iPSCs rescue F8 deficiency in mice.

CjCas9: mini-Cas9 for AAV package

AAV-SpCas9 and AAV-sgRNAs-eGFP dual vectors system

AAV-SaCas9-sgRNAs single vector system

AAV-CjCas9-sgRNAs-eGFP all-in-one vector system

- CjCas9 is the smallest Cas9 ortholog reported to date
- Digenome-seq reveals that CjCas9 is more specific than SpCas9 or SaCas9
In vitro cleavage using Cas9 with its sgRNA

NGS library construction

PAM identification using NGS data

Adaptor

Index

CjCas9 PAM characterization

CjCas9 sgRNA optimization

AAVS1-TS2

\[
\begin{align*}
\text{GGGAGTAGAGGCGGCCACGACCTG} & \quad \text{GX23} \\
\text{GGAGTAGAGGCGGCCACGACCTG} & \quad \text{GX22} \\
\text{GAGTAGAGGCGGCCACGACCTG} & \quad \text{GX21} \\
g\text{TAGAGGCGGCCACGACCTG} & \quad \text{GX20} \\
g\text{TAGAGGCGGCCACGACCTG} & \quad \text{GX19} \\
\text{no sgRNA} & \\
\end{align*}
\]


AAVS1-TS3

\[
\begin{align*}
g\text{GCACCATTCTCACAAAGGGAGTT} & \quad \text{GX23} \\
\text{GCACCATTCTCACAAAGGGAGTT} & \quad \text{GX22} \\
g\text{ACCATTCTCACAAAGGGAGTT} & \quad \text{GX21} \\
g\text{CCATTCTCACAAAGGGAGTT} & \quad \text{GX20} \\
\text{no sgRNA} & \\
\end{align*}
\]

S. pyogenes : NGG

C. jejuni : NNNRNYAC

CjCas9 PAM characterization
In vivo Genome Editing with CjCas9

• CjCas9 targeted to Hif1a or Vegfa reduced the size of choroidal neovascularization
• Demonstrating the potential for treatment of age-related macular degeneration
Nuclease Off-target Effects

- ZFNs, TALENs, and CRISPR-Cas9 can cleave off-target sites
- Off-target mutations can
  - Inactivate essential genes
  - Activate oncogenes
  - Cause chromosomal rearrangements

Undetectable CRISPR Off-target Mutations

Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease

Seung Woo Cho\textsuperscript{1–3}, Sojung Kim\textsuperscript{1–3}, Jong Min Kim\textsuperscript{1,2} & Jin-Soo Kim\textsuperscript{1,2}

published online 29 January 2013; doi:10.1038/nbt.2507

- No detectable off-target mutations at sites with 2- or 3-nucleotide mismatches
- Confirmed by targeted deep sequencing in Cho et al. Genome Res. 2014
How to Assess Genome-wide Off-target Effects

- Whole genome sequencing: Limited by sequencing depth
- Digenome-seq: Nuclease-digested whole genome seq.
- Cell-based methods: GUIDE-seq, Translocation seq., BLESS

Overview of Digenome-seq

- After WGS, sequence reads were mapped to the reference human genome.
- Straight alignments of sequence reads are observed at the on-target site.
DNA Cleavage Score

Sequence reads with the same 5’ end are counted across the genome.

DNA cleavage scores are assigned to each nucleotide position.

Score at position $i =$

$$
\sum_{a=1}^{5} \frac{100(F_i - 1)}{D_i} \times \frac{100(R_{i-4+a} - 1)}{D_{i-4+a}} \times (F_i + R_{i-4+a} - 2)
$$

$$
+ \sum_{a=1}^{5} \frac{100(R_{i-1} - 1)}{D_{i-1}} \times \frac{100(F_{i-3+a} - 1)}{D_{i-3+a}} \times (R_{i-1} + F_{i-3+a} - 2)
$$

$F_i$: Number of forward sequence reads starting at position $i$

$R_i$: Number of reverse sequence reads starting at position $i$

$D_i$: Sequencing depth at position $i$
Genome-wide Cleavage Scores

Kim et al. Genome Res. (2016)
Off-target Sites Validated by Deep Sequencing

- 74 Digenome-captured sites were validated by targeted deep sequencing.
- Only five sites were mutated at frequencies ranging from 0.1 to 87%.
Digenome-seq vs Other Methods

**VEGFA 1**
- Digenome: 48
- GUIDE: 21
- HTGTS: 9

**EMX1**
- Digenome: 133
- GUIDE: 9
- HTGTS: 3

**RNF2**
- Digenome: 12
- GUIDE: 1

**HEK293-3**
- Digenome: 25
- GUIDE: 6

**GUIDE-seq**
- Cells
- DSB
- dsODN

**HTGTS**
- Cells
- DSB
- Prey
Digenome-seq Advantages

• Highly sensitive; Captures off-target sites w/ <0.1% indel freq.
• Not limited by chromatin accessibility
• Pinpoint off-target sites; no NHEJ-mediated indels in vitro
• Easy to carry out; no PCR steps prior to WGS
• Free form naturally-occurring DSBs in cells and PCR artifacts
• Compatible with RNA-guided programmable deaminases
Programmable Nuclease vs. Deaminase

CRISPR/Cas systems

Gene A

NHEJ

Targeted mutagenesis

Donor DNA

HR

Targeted gene replacement

dCas9 or D10A Cas9

Deamination of target C

Cystidine deaminase

Nishida et al. Science (2016)
Programmable Deaminases

First-generation base editor (BE1)

- APOBEC1–dCas9

Second-generation base editor (BE2)

- APOBEC–dCas9–UGI

Third-generation base editor (BE3)

- APOBEC–nCas9–UGI

DNA cleavage at uracil-containing sites

5'---GAGTCCGAGCAGAAGAAGAAGAAGAAGG---3'
3'---CTCAAGGCTCGTCTTCTTCTTCCC---5'

rAPOBEC1-Cas9(D10A)

5'---GAGTUUGAGCAGAAGAAGAAGAAGAAGG---3'
3'---CTCAAGGCTCGTCTTCTTCTTCCC---5'

Uracil DNA glycosylase (UDG)

5'---GAGT---GAGCAGAAGAAGAAGAAGAAGG---3'
3'---CTCAAGGCTCGTCTTCTTCTTCCC---5'

PCR product
rAPOBEC1-Cas9(D10A) - + - + +
USER - - + + +

DNA glycosylase-lyase Endonuclease VIII
Digenome-seq of BE3-treated genomic DNA
Genome-wide BE3 off-target sites

Kim et al. NBT (2017)

EMX1

Untreated (+) Base Editor

BE3

n = 13

Cas9

n = 143

HBB

Untreated (+) Base Editor

(+ Cas9 nuclease)

BE3

n = 3

Cas9

n = 20
Off-target sites validated in human cells

**HBB**

- tcaGCCCCCCACAGGGCAGTAA
  - Untreated: 0.3%
  - (+) BE3: 3%
- aTTGCCACgGGCAGTgA
  - Untreated: 0.2%
  - (+) BE3: 2%
- gcTGCCCCACAGGGCAGcAA
  - Untreated: 0.1%
  - (+) BE3: 1%
- tTgctCCCACAGGGCAGTAA
  - Untreated: 0.05%
  - (+) BE3: 0.5%
- CTTGCCCAACAGGGCAGTAA
  - Untreated: 0.2%
  - (+) BE3: 2%

Kim et al. NBT (2017)
Cas9 and BE3 Off-target Sites

**EMX1**

- **Cas9**
  - EMX1_5: GaaTCCaAG-AGAAGAAGAAATGG
  - EMX1_8: GtGTCCtAG-AGAAGAAGAAGGG
  - EMX1_12: GAGTCCacaCAGAAGAAGAAAGA
  - EMX1_13: GAGTCCaAG-AGAAGAAGTgAGG

- **BE3**
  - Bulge: RNA bulge
  - RNA bulge
  - x
  - RNA bulge

**HEK293-4**

- **Cas9**
  - HEK4_3: GGCACTGca-CTGGAGGTtGTTGG
  - HEK4_8: GGCACT-GGGCTGaAGGATaAGG
  - HEK4_19: GGCACTG-GGCTGGAGGcGcGGG
  - HEK4_24: GGCACTG-GGCTGGAGaTGGAGG

- **BE3**
  - Bulge: RNA bulge
  - RNA bulge
  - RNA bulge
  - RNA bulge
Reducing BE3 off-target effects via modified sgRNAs

5’-CTTGCCCACAGGCCAGTAA<sub>NGG</sub>-3’
5’-gGCCCAACAGGCCAGUAA-3’
5’-gUGCCCCACAGGCCAGUAA-3’
5’-gUGCCCCACAGGCCAGUAA-3’
5’-gCUUGCCCCACAGGCCAGUAA-3’
5’-ggCUUGCCCCACAGGCCAGUAA-3’

<sub>NGG</sub> target sequence

HBB target sequence

gX<sub>17</sub> sgRNA
gX<sub>18</sub> sgRNA
gX<sub>19</sub> sgRNA
gX<sub>20</sub> sgRNA
ggX<sub>20</sub> sgRNA

Specificity ratio (modified sgRNA / conventional sgRNA)

<table>
<thead>
<tr>
<th>ggX&lt;sub&gt;20&lt;/sub&gt;</th>
<th>gX&lt;sub&gt;20&lt;/sub&gt;</th>
<th>gX&lt;sub&gt;19&lt;/sub&gt;</th>
<th>gX&lt;sub&gt;18&lt;/sub&gt;</th>
<th>gX&lt;sub&gt;17&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>3.5</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>1.4</td>
<td>0.8</td>
<td>1.0</td>
<td>1.4</td>
<td>0.9</td>
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<tr>
<td>1.6</td>
<td>0.9</td>
<td>1.0</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>2.8</td>
<td>0.8</td>
<td>1.0</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>4.1</td>
<td>2.3</td>
<td>1.0</td>
<td>4.1</td>
<td>2.7</td>
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Base editing frequency (%)
Unexpected CRISPR Off-target Mutations In Mice?

<table>
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<tr>
<th></th>
<th>F05 vs F03</th>
<th>F03 vs F05</th>
<th>FVB vs F05</th>
<th>F05 vs FVB</th>
<th>FVB vs F03</th>
<th>F03 vs FVB</th>
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<tbody>
<tr>
<td>Strelka</td>
<td>1482</td>
<td>745</td>
<td>2759</td>
<td>2078</td>
<td>3251</td>
<td>2330</td>
</tr>
<tr>
<td>Mutect</td>
<td>2749</td>
<td>1957</td>
<td>5817</td>
<td>4026</td>
<td>6375</td>
<td>4348</td>
</tr>
</tbody>
</table>

Schaefer et al. (Nature Methods, 2017) did not validate off-target effects.

Neglected SNVs and indels unique to the “co-housed control” mouse.
How to Avoid Off-target Effects

• Choose a unique target site
• Use purified Cas9/Cpf1/BE proteins rather than plasmids
• Use modified guide RNAs
• Attenuated Cas9 proteins: eCas9 or Cas9 HF

Koo et al. Molecules and Cells (2015)
Do Off-target Effects Matter?

- No drugs are free from off-target effects, often leading to repositioning
- Etoposide, an anti-cancer drug, cleaves DNA randomly, inducing mutations
- CCR5-targeted ZFN has been proven safe in a clinical test (thus far)
- Biological consequences rather than mutations per se are more relevant
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