Zinc Finger Nuclease – The new Technology for Targeted Genome Modification in Animals

Dr. Rainer Ebel
Sales Development Manager
Emerging Technologies
Sigma-Aldrich Corporation
• Zinc Finger Nuclease Technology
• Targeted Knockout Zebrafish
• Targeted Knockout/-in Rats and Mice
• Targeted Knockout in Rabbits
• Sigma-Aldrich
What is a ZFN?

A reagent that creates a very targeted double strand break (DSB) in genomic DNA

www.sageresearchmodels.com
ZFP Design via Single Zinc Finger “Mix and Match” Strategy

Fingers:

Triplets:

3-finger ZFP:

9bp target site:

Link fingers of known subsite preference to yield protein with desired composite specificity
Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain

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

\textbf{Yang-Gyun Kim, Jooyeon Cha, and Srinivasan Chandrasegaran}\textsuperscript{*}

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Communicated by Thomas Kelly, October 3, 1995 (received for review April 13, 1995)

In most nucleases, DNA binding and nuclease functions are structurally integrated, making them difficult to engineer.
Each homologous arm contains five to six Zinc Finger Proteins

→ Gene specificity is 30 or 36 bases
ZFN-mediated Knockout

ZFNs

NHEJ

Error-prone repair

Error-free repair

Targeted Mutagenesis
Genotype of Three New DHFR -/- CHO Cell Lines

ZFN-mediated Knockin

Targeted Mutagenesis

Repair off sister chromatid

Repair off extra-chromosomal template

HR

Donor plasmid

HA-L insert HA-R

ZFN Cut Site

Chromosomally integrated insert
Creating endogenous gene reporters

**Direct Exonic Fusion**

- **GFP-tubulin**
- **GFP-actin**

ZFN Cut Site

Last Exon  Chromosome

GFP  polyA  Donor DNA

GFP-tubulin

GFP-actin
Agenda

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Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases

Yannick Doyon\textsuperscript{1,3}, Jasmine M McCammon\textsuperscript{2,3}, Jeffrey C Miller\textsuperscript{1}, Farhoud Faraji\textsuperscript{1}, Catherine Ngo\textsuperscript{1}, George E Katibah\textsuperscript{1}, Rainier Amora\textsuperscript{1}, Toby D Hocking\textsuperscript{1}, Lei Zhang\textsuperscript{1}, Edward J Rebar\textsuperscript{1}, Philip D Gregory\textsuperscript{1}, Fyodor D Urnov\textsuperscript{1,2} & Sharon L Amacher\textsuperscript{2}
Injected ZFN mRNA into single cell embryo

Scored phenotype after 2 days

Sequencing revealed broad range of deletions and insertions at targeted loci (single embryo alleles shown)

>60% of founders carry *ntl* mutations

Germline frequency 20% (average)
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Recombinant Knockout Technology

Utilizes selectable markers to identify recombination events

- 12 – 18 month timeframe for founder creation
- Well established technology in mice for specific strains

Knockout Rats via Embryo Microinjection of Zinc-Finger Nucleases

Aron M. Guerts,† Gregory J. Cost,‡ Yevgeniy Freyvert,‡ Bryan Zettler,‡ Jeffrey C. Miller,‡ Vivian M. Choi,‡ Shirin S. Jenkins,‡ Adam Wood,‡ Xiaoxia Cui,‡ Xiandong Meng,‡ Anna Vinceti,‡ Stephen Lam,§ Mieczyslaw Michalkiewicz,‡ Rebecca Schilling,‡ Jamie Foeckler,‡ Shawn Kalloway,‡ Hartmut Welle,‡,§ Séverine Menoret,‡ Ignacio Aragon,‡ Gregory D. Davis,‡ Lei Zhang,‡ Edward J. Rebar,‡ Philip D. Gregory,‡ Fyodor D. Urnov,‡ Howard J. Jacob,†,‡,§ Roland Buenez‡

The laboratory rat is a well-established model for the genetic dissection of human disease-related traits (1) despite the fact that targeted modification of its genome is largely intractable. We investigated the application of zinc-finger nucleases (ZFNs) to the generation of genetic disease models in the rat. ZFNs are engineered de novo to bind to a target site in the genome, typically containing a 20-bp protospacer adjacent motif (PAM) complementary to the 20-bp protospacer adjacent motif (PAM) complementary to the ZFN target site. ZFNs are then delivered to the zygote via microinjection, and the resulting embryos are amplified and characterized for the presence of mutations.

We delivered these ZFNs to 36 hamster strains (GFP-negative) via microinjection, followed by genome-wide screening for mutations. Nine strains were selected for further analysis based on the presence of mutations. The resulting mutant strains were used to generate transgenic lines by pronuclear microinjection, resulting in the production of homozygous mutant lines for 10 of the 36 strains.

References and Notes
4. Materials and methods are available as supporting material on Science Online.
8. M. Fink, K. Jacob, R. Hammes, P. Silverman, and three anonymous referees for helpful suggestions. D. Finkler and D. Finkler for technical assistance; and Colmar Life Sciences, incorporated for excellent service. Supported by NIH grants 1RO1GM074657 and 1RO1GM084612.
Pronuclear injection of ZFN against GFP into fertilized 1-cell stage embryos of GFP-rats

~ 12% efficiency

Mutant x WT: Confirmed germline transmission (4/13 pups)
ZFN Assembly Time: 6 weeks
ZFN Injection: week 7
Founders Born: weeks 10 – 11

– 15/58 Pups Born have Mutation
– Identify by PCR and sequence analysis

From Gene Sequence to Founder Identified in 12 weeks
p53 Validation by Western

(K) WT 731RP (L) KO 733RP

75
50
37
50
37

non-specific band
p53
Actin

(PAB240)

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### p53 Pathology

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (weeks)</th>
<th>Sex</th>
<th>Tumor Locus</th>
</tr>
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<tbody>
<tr>
<td>+/-</td>
<td>50</td>
<td>F</td>
<td>Brain</td>
</tr>
<tr>
<td>+/-</td>
<td>34</td>
<td>F</td>
<td>Brain</td>
</tr>
<tr>
<td>+/-</td>
<td>24</td>
<td>F</td>
<td>Brain</td>
</tr>
<tr>
<td>+/-</td>
<td>13</td>
<td>M</td>
<td>Lung/skeletal muscle of head</td>
</tr>
<tr>
<td>+/-</td>
<td>23</td>
<td>M</td>
<td>Brain</td>
</tr>
<tr>
<td>+/-</td>
<td>13</td>
<td>M</td>
<td>Abdomen</td>
</tr>
</tbody>
</table>

![Image a](image-a.png)

![Image b](image-b.png)

![Image c](image-c.png)
• ZFN Assembly Time: 6 weeks
• ZFN Injection: week of 4/21/09
• Founders Born: 5/12, 5/13
  – 34/44 Pups Have Mutation
  – Mutations Sequenced
  – Knockouts range from 3 to 695bps

9/9 Founders passed the knockout to F1s
Targeted integration in rat and mouse embryos with zinc-finger nucleases

Xiaoxia Cui, Diana Ji, Daniel A Fisher, Yumei Wu, David M Briner & Edward J Weinstein

Weinstein et al, 2010, Nat. Biotech
The Not I site was also integrated into the rat Mdr1a site using a similar donor construct.
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Efficient Immunoglobulin Gene Disruption and Targeted Replacement in Rabbit Using Zinc Finger Nucleases

Tatiana Flisikowska1, Irmgard S. Thorey2, Sonja Offner2, Francesca Ros2, Valeria Lifke2, Bryan Zeitler3, Oswald Rottmann1, Anna Vincent3, Lei Zhang3, Shirin Jenkins3, Helmut Niersbach2, Alexander J. Kind1, Philip D. Gregory3, Angelika E. Schnieke1*, Josef Platzer2

1 Chair of Livestock Biotechnology, Technische Universität München, Freising, Germany, 2 Pharma Research and Early Development, Roche Diagnostics GmbH, Penzberg, Germany, 3 Sangamo BioSciences Inc., Richmond, California, United States of America

• Cardiovascular disease
• AMD
• Dermal
• Joint
### Microinjection Statistics

<table>
<thead>
<tr>
<th></th>
<th>Session 1</th>
<th>Session 2</th>
<th>Session 3</th>
<th>Session 4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos Collected</td>
<td>207</td>
<td>119</td>
<td>120</td>
<td>111</td>
<td>557</td>
</tr>
<tr>
<td>Embryos Microinjected</td>
<td>196</td>
<td>98</td>
<td>96</td>
<td>75</td>
<td>465</td>
</tr>
<tr>
<td>Embryos Transferred</td>
<td>122</td>
<td>76</td>
<td>68</td>
<td>60</td>
<td>326</td>
</tr>
<tr>
<td>Rabbits Born</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>16</td>
</tr>
</tbody>
</table>

Embryos collected/rabbit born: 35
Embryos microinjected/rabbit born: 29
Embryos transferred/rabbit born: 20

5/16 founders were identified (mutations ranging from 8bp to ~300bp deletion (some with insertions).
• Zinc Finger Nuclease Technology
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• **CompoZr® Targeted Integration Kit → 2.125 €**
  - Human AAVS1
  - Murin Rosa26
    – Rapid Gene Insertion into the Human or Murine Cell Line of Your Choice
    – ZFNs engineered to target human AAVS1 or murine Rosa26 site

• **Knockout ZFN (human, rat, mouse) → 5.100 € (Plasmid only)**
  – Production time: 3 – 12 weeks

• **Custom ZFN against human, rat, mouse, hamster → 12.000 € (Plasmid only)**
  – Best performing custom validated ZFN against gene of interest
  – Production time: 9 – 12 weeks

• **Custom ZFN against “non-standard species” → 12.000 € (Plasmid only)**
  – Up to Top three ZFN against gene of interest
  – Production time: 9 – 12 weeks (up to 6 months)
• Priority access to catalog knockout rat models

  CNS
  • ApoE
  • Leptin
  • BDNF
  • DISC1
  • DJ-1
  • Lrrk2

  ADME/Tox
  • Mdr1a
  • PXR
  • Mrp1
  • Mrp2
  • Bcrp

  Immunodeficiency
  • Rag1
  • Rag 2
  • DNAPK
  • Foxn

  Carcinogenicity
  • p53

• Access to SAGEspeed™ custom model creation platform
  – Rat / Mouse / Rabbit
  – Founder animals (rat or mouse) produced in as little as 5 months
  – Limited researcher ‘slots’ available to customers per Year

• ModelShare™ Program
  – First of its kind rat model repository
Looking Beyond the Rodent.....

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