CRISPR/Cas9 Mouse Production

Emory Transgenic and Gene Targeting Core

http://cores.emory.edu/tmc

Tamara Caspary, Ph.D. Scientific Director
Teresa Quackenbush --- Lab Operations and Communications Coordinator
Yao Huang --- Animal Husbandry Coordinator
Karolina Piotrowska-Nitsche, Ph.D.---Technical Coordinator
E-mail: mousecore@emory.edu
Before you make a model, see what is available

mousephenotype.org
Catalogs the International Mouse Knockout Project’s 19,000 alleles.
ES cells and live mice available (Core can generate mice from ES cells)
Phase 2 is performing systematic phenotyping using IMPRESS protocols

www.informatics.jax.org
Mouse Genome Informatics site curated by The Jackson Labs
Thorough information source for every gene
From homepage, click “Phenotypes & Mutant Alleles and follow instructions
Traditional methods to generate novel alleles

**Transgenesis**

1. Fusion gene construct
2. Superovulated female mouse
3. Microinjection
4. Birth of transgenics
5. DNA analysis
6. Breeding positives to establish transgenic lines

**Targeted homologous recombination**

(Knockouts and Knockins)

ES cells

Targeting vector introduced by electroporation

Positive-negative selection

Rare cell carrying targeted gene
Traditional methods to generate novel alleles

Transgenesis

✦ Integrate exogenous DNA fragment in genome

✦ About $5K and 3 months to founders, 6 months to germline

✦ Need to analyze at least three lines

✦ Caveats can include promoter used, position effect, copy number
Generation of Transgenic Mice

1. Fusion gene construct
2. Superovulated female mouse
3. Microinjection
4. Birth of transgenics
5. DNA analysis
6. Breeding positives to establish transgenic lines
Traditional methods to generate novel alleles

Targeted homologous recombination (Knockouts and Knockins)

- Precise manipulation of endogenous gene
- Over 20K alleles available, including 19K through consortia efforts (~$7000)
- Custom alleles $25K and up
- 8-12 months to chimeras, 11-15+ to germline
- Partnered with Ingenious Targeting Laboratory (seed grants available)
A. Gene targeting of embryonic stem cells

- Mouse blastocyst
- ES cells
- Targeting vector introduced by electroporation
- Rare cell carrying targeted gene
- Positive-negative selection
- Pure population of targeted ES cells
B. Generation of gene targeted mice

Targeted ES cells are injected into blastocysts...

...which are implanted into foster mothers

...which give birth to chimeric mice

Mating between chimeric mouse and normal mouse.

Egg

Sperm

Egg

Sperm

Gene targeted mice

Normal mice
CRISPR/Cas9 editing of the genome
CRISPR/Cas9 editing in ES cells

A

Multiple Gene targeting in ES cells

ESC

Transfection

Colony Picking

Genotyping

Quintuple Targeted Clones
Editing in the zygote accelerates generation of mouse alleles

One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering

Haoyi Wang, Hui Yang, Chikdu S. Shivalila, Meelad M. Dawlaty, Albert W. Cheng, Feng Zhang, Rudolf Jaenisch

Cell
153 (4): 910-918 (May 2013)
DOI: 10.1016/j.cell.2013.04.025

One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering

Hui Yang, Haoyi Wang, Chikdu S. Shivalila, Albert W. Cheng, Linyu Shi, Rudolf Jaenisch

Cell
154 (6):1370-1379 (September 2013)
DOI: 10.1016/j.cell.2013.08.022
Editing in the zygote accelerates generation of mouse alleles

One Step Generation of Mice With Mutiple Mutations

Targeted Mutations (Deletion / Insertion)

Predefined Precise Mutations
Editing in the zygote accelerates generation of mouse alleles
NHEJ at the **Rosa26** locus

![Sequence Diagram]

1  2  3  4  1b 2b 3b 4b  5  387 bp
Modified Sample Result

Sequence Deconvolution


1GGGGAGAAGGCCGCACCCTTCTCCGGAGGGG

2GGGGAGAAGGCCGCACCCTTCTCCGGAGGGG

RGGGGAGAAGGCCGCACCCTTCTCCGGAGGGGAGGGGAGTGTTGCAATACCTTTCTGGGAGTTCTCTGCTGCCTCCTGGCTTC

Step1

Step2
Allele 1 has a 34bp deletion
Allele 2 has a 27bp deletion
ROSANA results

REF TCTCTGCTGCCTCCCTGGCTTCTGAGGAACCGCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
1 TCTCTGCTGCCTCCCTGGCTTCTGAGGAACCGCCTGAGGGCCTTCCCTCCCTCCCTCCCTGCTGATCTGCAACTCCAGTC
(UNABLE TO DETERMINE IF/HOW CRISPR CUT)
2 (FORWARD AND REVERSE SEQUENCES HAVE SOME BASES WITH 3 CALLS)
3 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
4 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
5 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
6 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
7 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
8 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
9 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
10 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
11 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
12 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
13 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC
14 TCTCTGCTGCCTCCCTGGCTTCTGAGGACCGCCCTGAGGTTCCTGGAATCCCTTCCCTCCCTCTCGTGATCTGCAACTCCAGTC

PAM sequence is BOLD
deletions identified by -
Guide RNA is underlined base changes in gray
duplications in orange
<table>
<thead>
<tr>
<th>Injection session</th>
<th>Injected zygotes</th>
<th>Transferred embryos</th>
<th>Recipient moms</th>
<th>Pups born</th>
<th>Number with two edited alleles</th>
<th>Number with one edited allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>137</td>
<td>77</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>22</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
<td>55</td>
<td>2</td>
<td>11</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

57.5% of alleles edited
6/6 mice tested went germ line in F1 generation
Have successfully edited another locus

<table>
<thead>
<tr>
<th>Editing CRISPR project #</th>
<th># Zygotes injected</th>
<th># Embryos implanted (%)</th>
<th># Pups born (%)</th>
<th>NHEJ</th>
<th>HR</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>116</td>
<td>90 (78)</td>
<td>14 (16)</td>
<td>12</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
Editing in the zygote accelerates generation of mouse alleles

One Step Generation of Mice With Mutiple Mutations

Targeted Mutations (Deletion / Insertion)

Predefined Precise Mutations

1/20

1/60-
1/180
Workflow

How to Make a Mouse Model using CRISPR with Emory’s Transgenic Mouse Core Facility

Sigma form
Sigma Quote
Oligo/Plasmid
Crispr Delivered
Injection
Genotype Toe
Confirm + Genotype
Transfer Founders

Week 1
Week 9
Week 10
Week 14
Week 15
Week 18

If project requires an oligo
Sigma will produce the oligo and ship to the Mouse Core

If project requires a plasmid
Sigma provides the plasmid donor sequence
Cloning and prep performed by Emory Integrated Genomics Core
~$300
Transgenic Mouse Core
Workflow

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Week 1
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Week 10
Week 14
Week 15
Week 18

Sigma package- $2000
CRISPR design
Top 3 designs experimentally validated
CRISPR and CAs9 RNA purification
Oligo design and synthesis
Or plasmid design

Zygote injection- per session
FVB or DB6F2: $1299.25
C57BL/6: $1330.75
## Cost Calculation

<table>
<thead>
<tr>
<th>Frequency (per live pup)</th>
<th>Probable # of injection sessions</th>
<th>Cost/session</th>
<th>Injection costs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHEJ, no donor</td>
<td>1/20</td>
<td>2</td>
<td>$1330*</td>
</tr>
<tr>
<td>Oligo-based KI (50 bp)- HR</td>
<td>1-60- 1/180</td>
<td>4-12</td>
<td>$1330*</td>
</tr>
</tbody>
</table>

*Cost in pure C57/BL6, $30 less in FVB or DB6F2

+ CRISPR package (Sigma) ~$2000
CRISPR PROJECTS

METHODS USED TO DELIVER CRISPR PRODUCTS

PRONUCLEAR or CYTOPLASMIC INJECTION

ELECTROPORATION

- pulse power supply
- lid
- cuvette
- electrodes
- electrical contacts
- cells in suspension
Electroporation versus pronuclear injection

We have modified the zygote electroporation of nuclease (ZEN) protocol published by Qin et al. 2015, Genetics.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Embryos transferred</th>
<th>Mice born</th>
<th>Mutant mice</th>
<th>Mutant percentage (%)</th>
<th>Embryos transferred</th>
<th>Mice born</th>
<th>Mutant mice</th>
<th>Mutant percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd69</td>
<td>59</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cd226</td>
<td>61</td>
<td>19</td>
<td>4</td>
<td>21</td>
<td>20</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clec16a</td>
<td>57</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>25</td>
<td>16</td>
<td>2</td>
<td>13</td>
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<tr>
<td>Cyp27b1</td>
<td>64</td>
<td>23</td>
<td>12</td>
<td>52</td>
<td>28</td>
<td>20</td>
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<tr>
<td>Fut2</td>
<td>64</td>
<td>25</td>
<td>7</td>
<td>28</td>
<td>29</td>
<td>25</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td>Ormd13</td>
<td>62</td>
<td>19</td>
<td>17</td>
<td>89</td>
<td>26</td>
<td>15</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Rgs1</td>
<td>62</td>
<td>18</td>
<td>8</td>
<td>44</td>
<td>28</td>
<td>19</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>Tlr7</td>
<td>66</td>
<td>22</td>
<td>6</td>
<td>27</td>
<td>30</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tlr8</td>
<td>60</td>
<td>15</td>
<td>1</td>
<td>7</td>
<td>29</td>
<td>22</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Tnfsf9</td>
<td>61</td>
<td>21</td>
<td>15</td>
<td>71</td>
<td>25</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>616</td>
<td>185</td>
<td></td>
<td></td>
<td>296</td>
<td>175</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Live birth rate 30%*  
*Live birth rate 59%*  

Qin et al. 2015, Genetics
## Testing electroporation conditions

<table>
<thead>
<tr>
<th>Session #</th>
<th>Zygotes electroporated</th>
<th>Zygotes implanted (%)</th>
<th>Blastocysts developed (%)</th>
<th>Pups born (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>NA</td>
<td>15 (94)</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>20 (95)</td>
<td>NA</td>
<td>9 (45)</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>26 (93)</td>
<td>NA</td>
<td>12 (46)</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>24 (83)</td>
<td>NA</td>
<td>12 (50)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>94</strong></td>
<td><strong>70 (90)</strong></td>
<td><strong>15</strong></td>
<td><strong>33 (47)</strong></td>
</tr>
</tbody>
</table>

AT – 20/30 seconds; Voltage – 30V; Pulse – 100ms
Editing in the zygote accelerates generation of mouse alleles

Injection or electroporation

Plasmids- Injection recommended

Currently not recommending (most common results are single loxP or deletion)
JAX labs

Indel KO- NHEJ
- No donor DNA
- 12-14 weeks + 14-16 weeks
- ~$13,500

Deletions KO (<5 kb)- NHEJ
- No donor DNA
- 12-14 weeks + 14-16 weeks
- ~$20,500

Oligo-based KI (50 bp)- HR
- Oligo used as donor DNA
- 12-14 weeks + 14-16 weeks
- ~$27,000

Plasmid-based KI- HR
- DS plasmid used as donor DNA
- 19-21 weeks + 14-16 weeks
- ~$38,000

Note that pricing does not include a guarantee
## Cost Comparison

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<th>JAX</th>
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<td>~$4660</td>
<td>$13,500</td>
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<tr>
<td>Deletions KO (&lt;5 kb)</td>
<td>$4000 (2 CRISPRs) + $5320 (4 sessions) ~$9320</td>
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<td>Plasmid-based KI- HR</td>
<td>Pilot ongoing so unknown $2000-$4000 (CRISPRs)+ $1330 X needed injection sessions</td>
<td>$38,000</td>
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Conclusions

• CRISPR editing works and is a cost-effective option for certain projects
• HR is less efficient than NHEJ
• Large donors are less efficient than small ones

Cost reduction Possibilities

• Higher rate embryo survival for electroporated zygotes versus pronuclear injection may enable us to reduce costs for subset of CRISPR projects
• If synthetic sgRNA works that could save time and cost of each project significantly
Contact Information

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