Genome editing at TIGM: Production of various mutant alleles via CRISPR/Cas9

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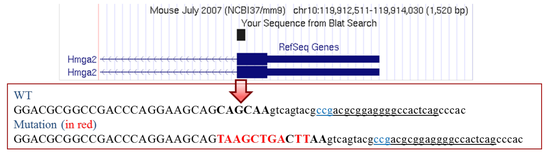
Abstract

Texas A&M Institute for Genomic Medicine (TIGM) has been housing the world's largest library of knockout C57BL/6N ES cells and providing transgenic mice for researchers worldwide. As interest in the gene trap lines declines, TIGM is repositioning itself as a provider of basic transgenic core services to researchers within the Texas A&M system and external customers as well. Among the most recent additions to our array of services is the successfully adopted production of mouse lines by CRISPR/Cas9 genome editing including indels, point mutations, deletions and conditional-ready (floxed) mutants. Here we are presenting technical details on the most recent successful editing projects many of which have been produced with the help of Sigma-Millipore CRISPR Core Partnership.

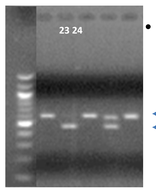
HDR Inserting STOP codons: Hmga2



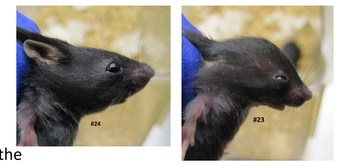
- sgRNA Designed by SIGMA: HMGA2-1_0_62_CCG, CTGAGTGGCCCCTCCGCGT CGG
- Donor oligo carrying 3xSTOP in different frames: 77 nt each arm, 180 nt total
- Mix: Cas9 mRNA (Sigma):sgRNA:ssDNA @ 100:50:100 (ng/ul) + RNasin
- Denatured RNAs prior to mixing, injected into cytoplasm; results:

Embryos injected	Embryos survived injection	2-cell	Pups born	Positive	Targeting efficiency
151	126	89	17	6	35%

- Germline confirmed
- Some mutants looked like homs in genotyping:



- And displayed clear phenotypical characteristics (smaller body, craniofacial defects):

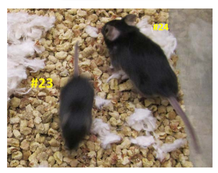


- This was consistent with the original KO phenotype described [1]:

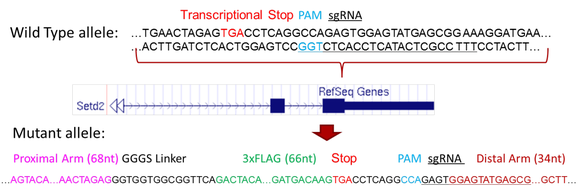
Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C

Xianjin Zhou, Kathleen F. Benson, Hena R. Ashar & Kiran Chada

Homozygous *Hmgi-c*$^{-/-}$ mice revealed the classical features of the pygmy phenotype, including reduced birth weight, craniofacial defects (shortened head), and an adult body weight of approximately 40% (39.8 ± 2.9) of that of wild-type littermates[19].



HDR Insertion of a 3xFlag marker at the C-terminus of a gene



... into a transgenic line with mutation on the same chromosome

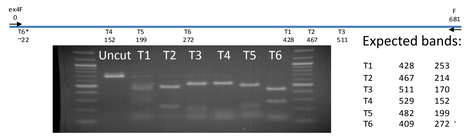
- Cryopreserved sperm from the transgenic line
- Performed IVF with C57BL/6 N (CRL) donors early in the morning
- Injected fertilized zygotes later in the afternoon (cytoplasmic)
- Mix: Cas9 mRNA (ThermoFisher):sgRNA:ssDNA @ 100:20:100 (ng/ul)

Results:

injected	survived injection	2-cell	1-cell	Pups	Positives
First 4 rounds					
399	305	243	8	3	0
Additional 3 rounds					
530	283	227	39	36	3

NHEJ Deleting an exon of a gene: Ifnar1

- sgRNAs produced via GeneArt™ Precision gRNA Synthesis Kit (Invitrogen)
- In vitro test: amplified target region, digested with sgRNA and Cas9:



- Mix: Cas9 protein:sgRNAs @ 120-300:50:50 (ng/ul); pronuclear inj.

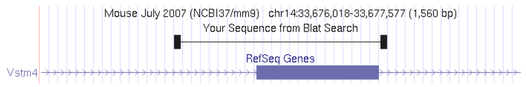
Results:

injected zygotes	# survived injection	2-cell	# Pups	Positives
596	526	428	39	0

- Target region has been amplified and re-sequenced, but no mutations
- Also genotyped expanded embryos, no mutations either

NHEJ Deleting an exon of a gene: Vstm4

- sgRNAs designed/validated by SIGMA:
  mmVstm4up_0_33 CTAAAGTAAAATAAGACGA AGG
  mmVstm4down_0_5 AACGCTGTTGGCATCTCGG AGG



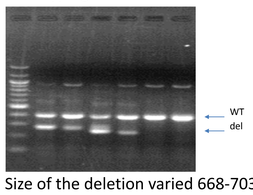
- Mix: Cas9 mRNA/Sigma:sgRNA @ 100:50 (ng/ul) +/- RNasin/Promega

Results:

Embryos injected	Embryos survived injection	2-cell	1-cell	Pups born	Positive	Targeting efficiency
170	130	56	74	22	10	45% *

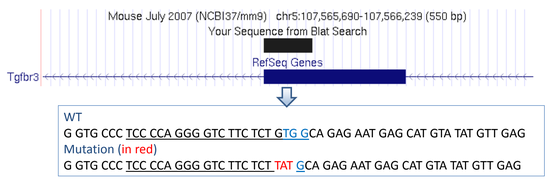
*No significant difference between 1-cell and 2-cell transfers or +/- RNasin

- Genotyping confirmation:



- Size of the deletion varied 668-703 bp

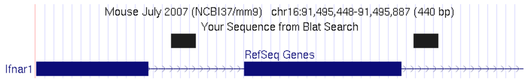
HDR Inserting point mutation: Tgfbr3 V612Y



- Designed by SIGMA: Tgfbr_0_29_TGG TCCCCAGGGGTCTTCTCTG TGG
- Donor oligo: 60 nt each arm, 124 nt total
- Mix: Cas9 mRNA (Sigma):sgRNA:ssDNA @ 200:100:100 (ng/ul) + RNasin
- Denatured RNAs prior to mixing, injected into cytoplasm; results:

Embryos injected	Embryos survived injection	2-cell	1-cell	Pups born	Positive	Targeting efficiency
74	43	24	17	1	1	100%

- sgRNAs produced and validated by Sigma:
  Downstream_0_11_CCG TCGGCTACGGAATCTAACA CGG
  Upstream_0_21_AGG TTTGATGTGAGAGAAATAG AGG

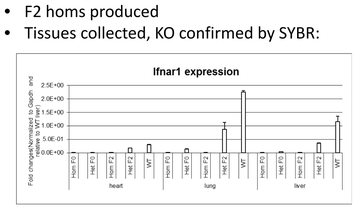


- Mix: Cas9 mRNA/Sigma : sgRNA @ 166:83:83 (ng/ul); also added RNasin® Ribonuclease Inhibitors (Promega).
- Injected into cytoplasm

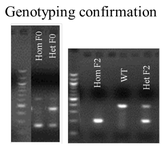
Results:

injected zygotes	# survived injection	2-cell	# Pups	Positives
126	51	44	12	10

- Targeting efficiency: 83%; possible F0 homs (by genotyping)
- F2 homs produced
- Tissues collected, KO confirmed by SYBR:

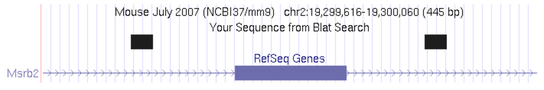


Genotyping confirmation



NHEJ Deleting an exon of a gene: Msrb2

- sgRNAs designed/validated by SIGMA:
  mmMsrb2_0_158_GG ATTACTTCTCCAGGTGATA GGG
  mmMsrb2_0_419_CCT GTGCACGTGTGAGCGTCGT AGG

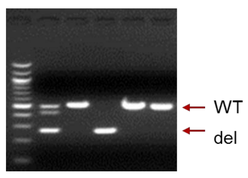


- Used several mixes; the best combination that produced the most positives was:
  Cas9 mRNA/Sigma:sgRNAs @ 140:70:70 (ng/ul) + RNasin/Promega
- Denatured RNAs prior to mixing, injected into cytoplasm
- Results:

Embryos injected	Embryos survived injection	2-cell	1-cell	Pups born	Positive	Targeting efficiency
478	309	156	149	23	7	30% *

*No significant difference between 1-cell and 2-cell transfers

- Genotyping confirmation:



HDR Production of floxed mutations: Irf3

- All work was done according to protocols described by Rudolf Jaenisch's group [2]
- Injection mix:
  - 2 sgRNAs produced via GeneArt™ Precision gRNA Synthesis Kit (Invitrogen)
  - 2 donor oligos: 60 nt each arm, 180 nt total
  - Cas9: started injecting as RNA, switched to protein, both pronuclear and cytoplasmic
- Injected almost 1300 embryos; produced 56 pups;
- Most single LoxP insertions were produced from Cas9 RNA injections
- The only double LoxP insertions was produced from the Cas9 protein (targeting efficiency: ~2%)
  - Both alleles have been targeted
  - Additional mutations were found
  - Breeding out the non-specific mutations failed
  - Cre-mediated recombination produced strange results

Conclusion: most likely both LoxP sites were correctly inserted, but the whole floxed region was excised and inserted at a random location; other recombination events might have taken place. The allele was deemed unsuitable for conditional KO

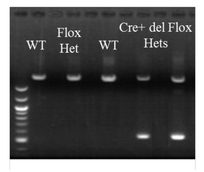
HDR Production of floxed mutations: Phactr1

- Designed, verified and produced by SIGMA
- Injection mix:
  - 2 sgRNAs (50 ng/ul each)
  - 2 donor oligos: 70 nt each arm, 180 nt total (100 ng/ul each)
  - Cas9 mRNA (Sigma) (100 ng/ul),
  - added RNAsin (Promega) to some mixes; injected cytoplasmic
- Results:

Embryos injected	Embryos survived injection	2-cell	1-cell	Pups born	Positive	Targeting efficiency
331	189	93	94	42	1	2%

- 3-5 with single LoxP insertions (those injected with RNAsin); one had both LoxP on the same chromosome (no RNasin)
- Germline confirmed
- Passed the test for Cre-mediated excision:

Testing for Cre-based excision:



Conclusions

1. **Commercial design and production of CRISPR reagents provides the best and most consistent results**
2. **Cas9 mRNA cytoplasmic injections work the best; RNAsin helps sometimes**
3. **CRISPR/ Cas9: NHEJ-mediated deletions**
   - 30-80% success rate after optimization
4. **CRISPR/ Cas9: HDR-mediated insertions**
   - Works well, though efficiency varies
   - Produced small knock-ins and point mutations and managed to target both alleles
   - Produced conditional KO

References

1. Nature. 1995 Aug 31;376(6543):771-4.
2. Yang, et al., Nature Protocols 9, 1956–1968 (2014)

For more information please visit www.tigm.org or contact us at info@tigm.org.