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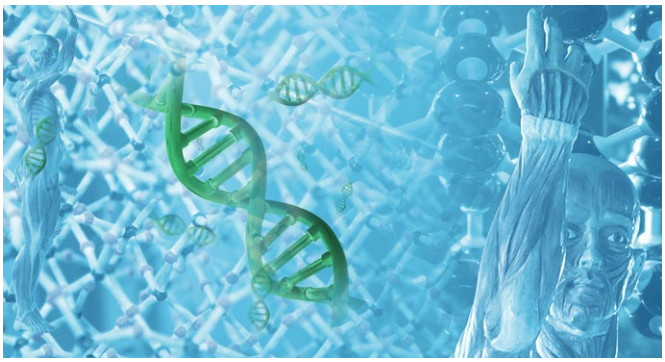
EDITORIAL MISSION: CRISPR TARGETS ANIMAL MODELS



CRISPR: State of the Science

In the world of transgenic animal model systems, hundreds of different disease states have been recreated using the first-of-its-kind knockout mouse model developed by Mario Capecchi, Martin Evans, and Oliver Smithies in 1989. Their work was also recognized with the Nobel Prize in Physiology in 2007. However, today, the genome editing technology known as CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) enables the creation of a whole new selection of physiologically relevant disease models. A vast array of animal species, including *Danio rerio, Caenorhabditis elegans, Drosophila, Bos taurus, Apis mellifera*, and nonhuman primates have been edited in a number of ways for wide-ranging research purposes. Transgenic mice, however, continue to dominate the *in vivo*, human-disease modeling arena. With CRISPR genome-editing technology, researchers can now easily tailor the genome of an organism to suit their needs with minimal animal model development experience required.

In 2012 at the University of California, Berkeley, biochemist Jennifer Doudna and Swedish molecular biologist Emmanuelle Charpentier published their landmark paper announcing the programmable capability of bacterial CRISPR/Cas9 for genome editing.[1] Several months later, Broad Institute's Feng Zhang published his success in adapting the CRISPR/Cas9 genome-editing principle to eukaryotic cells, and thus potentially humanity.[2] What followed was the realization of the importance of this technology across all fields of science and medicine. This fact is not only reflected in the complicated patent battle that has ensued, but by its incredibly quick adoption, development, and refinement in both academia and industry.

Success in Challenge

To date, the translational potential of CRISPR/Cas9 has been realized in its ability to preclinically abolish a number genetic diseases and identify genes involved in the pathogenic and metabolic processes of others. For example, scientists have successfully applied the technology to repair mutations that cause cystic fibrosis, hereditary tyrosinemia, and furthermore have even eliminated the HIV genome from latently infected cells.[3-5] CRISPR/Cas9 also has potential as an antimicrobial tool, where specific artificial arrays have been designed to target antibiotic resistance or virulence genes of various pathogenic bacterial strains.[6,7] Moving forward, expanding the technology to editing of the epigenome holds great promise in revealing novel site-specific modifications responsible for the regulation of genes critical in a variety of diseases.

The advancement of CRISPR-based genomic medicine is met with two primary challenges: the reliable generation of site-specific double-stranded breaks (DSBs) and nonviral cell-specific component delivery. Although Cas9 can overcome some degree of undesirable guide RNA (gRNA) and target-sequence mismatches, off-target effects have been reported.[8,9] This has prevented CRISPR's immediate application in the clinic, but tremendous efforts to improve its specificity and efficacy are underway, including the recent successful use of a gold particle (CRISPR-Gold) to nonvirally deliver Cas9, gRNA, and donor DNA in a mouse model of Duchenne muscular dystrophy.[10]

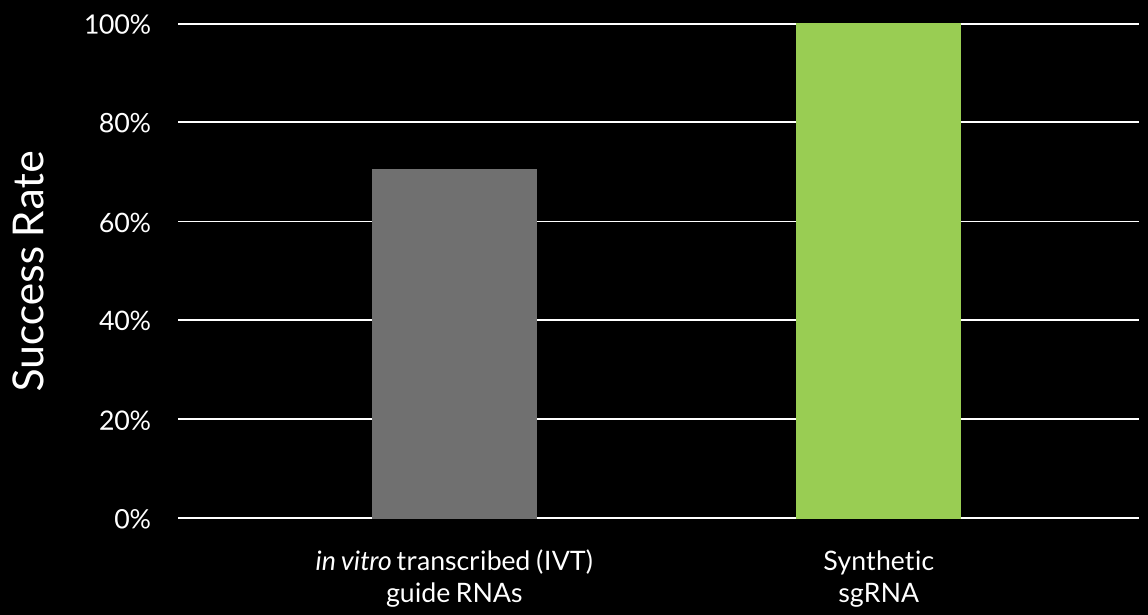
"Every time we unlock one of nature's secrets, it signals the end of one experiment—and the beginning of many others." – Jennifer Doudna

References

1. E. Charpentier, et al., "A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity," *Science* 337:816-821, 2012.
2. L.A. Marrafini, F. Zhang, et al., "RNA-guided editing of bacterial genomes using CRISPR-Cas systems," *Nat Biotechnol* 31:233-239, 2013
3. G. Schwank, et al., "Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients," *Cell Stem Cell* 13:653-658, 2013.
4. H. Yin, et al., "Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype," *Nat Biotechnol* 32:551-553, 2014.
5. W. Hu, et al., "RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection" *Proc Natl Acad Sci* 111:461-411, 2014.
6. D. Bikard, et al., "Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials," *Nat Biotechnol* 32:1146-1150, 2014.
7. A.A. Gomaa, et al., "Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems," *mBio* 5, e00928-13, 2014.
8. F. Fu, et al., "High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells," *Nat Biotechnol* 31:822-826, 2013.
9. P.D. Hsu, "DNA targeting specificity of RNA-guided Cas9 nucleases." *Nat Biotechnol* 31:827-832, 2013.
10. K. Lee, et al., "Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair," *Nat Biomed Eng* doi:10.1038/s41551-017-0137-2, 2017.

SAVE TIME & MONEY WITH HIGH SUCCESS RATES

Successfully Develop Modified Animals Using Synthetic sgRNA



Use of IVT sgRNA guides led to a 70% ratio of successful projects, with 30% failures (measured by how often micro-injection of about 100 zygotes led to successful generation of at least one live born pup with the appropriate edit), whereas use of synthetic sgRNAs led to a 100% success rate.

Data shown are from 13 projects using IVT sgRNAs and 7 projects using synthetic sgRNAs. Data provided courtesy of our collaborators in an academic transgenic facility.

TRY NOW

SYNTHEGO

CRISPR WORKFLOWS:
A GUIDE TO EFFICIENT AND TARGETED EDITING

As gene-editing technologies continue to evolve, better ways to develop transgenic animal models have emerged. Experimental design is the key to your success. Follow along to see if you have the tools in place for rapidly generating your next genetically-modified animal model system.

CAS9/GRNA COMPLEX GENERATION

Gene-targeting vector and synthetic gRNA components are constructed and synthesized

Synthetic gRNAs are compatible with both microinjection and embryonic stem (ES) cell transfection approaches

COMPONENT DELIVERY VIA MICROINJECTION

MICROINJECTION

Micropipette containing Cas9/gRNA complex is injected into the pronuclei of fertilized eggs

EMBRYO TRANSFER

Female surrogate is implanted with the modified blastocyst

BIRTH OF F0 GENERATION

Subsequent mating to produce F1 generation

GENOTYPIC SCREENING AND CONFIRMATION

Positive (PCR) screening of F1 generation harboring genetic mutation

Desired mutant clone obtained at this step

GENERATION OF HOMOZYGOUS MUTANTS

Positive mutant offspring are selected for further breading

COMPONENT DELIVERY VIA TRANSFECTION OF EMBRYONIC STEM CELLS

ELECTROPORATION

Gene-targeting vector components are introduced into the stem cells

ANTIBIOTIC SELECTION & FLUORESCENT REPORTER ENRICHMENT

Positive drug selection occurs in culture, where mutant cells are identified via GFP fluorescence

Desired mutant clone obtained at this step

EXPANSION

Positive cell clones are expanded and stored for future use

INJECTION INTO EMBRYO

Female surrogate is implanted with the modified blastocyst

BREEDING FOR DESIRED MUTANT POPULATION

Successful germ-cell genetic integration will produce offspring harboring the genetic modification

Transgenic Toolkit: CRISPR's Capabilities

Transgenic Toolkit: CRISPR's Capabilities

To assemble an active CRISPR/Cas9 complex, your toolkit must contain a protein component and an RNA component. The protein, Cas9, is an RNA-dependent endonuclease responsible for the cutting of double-stranded DNA at a particular location. Small exogenous gRNAs find and bind the complementary region of target DNA, guiding the cutting activity of Cas9. Guide RNAs can either be a two-component RNA, CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA), or a one-component RNA, a single guide RNA (sgRNA) made up of a single molecule that has both crRNA and tracrRNA sequences. As a hybrid product, the gRNA is capable of binding to and directing Cas9 to the complementary target DNA sequence, which must be adjacent to a protospacer-adjacent motif (PAM).[1] The PAM sequence, a short stretch of nucleotides, is the final and required binding signal for Cas9.

The Genome under Repair

CRISPR exploits two DNA repair mechanisms required for the mending of DSBs, namely non-homologous end joining (NHEJ) and homology-directed repair (HDR). HDR can be adapted to repair a break using an exogenous sequence of DNA, thereby introducing precise genetic alterations.[2] The NHEJ pathway is useful due to the high frequency of random indels (nucleic-acid base insertions or deletions) that occur at the Cas9-targeted break site. If the break is within a gene's protein coding region, the resulting frameshift mutations produce the desired genetic knock-out.[2] As such, NHEJ creates loss-of-function genetic models, whereas the HDR mechanism can be used to create a variety of genetic and genomic modifications, ranging from gene tagging to subtle modifications of non-coding DNA.

Several groups have reported various ways in which either NHEJ or HDR can be swayed experimentally to produce the sought after, genetically-modified *in vivo* model.[3-5] The successful repair of DSBs by either NHEJ or HDR facilitates CRISPR's utility in the study of various human diseases where accurate *in vivo* modeling is dependent on the manipulation of multiple genetic factors. Indeed, the creation of complex disease models driven by a combination of calculated genomic inversions, translocations, deletions, and insertions is now possible.[2]

In addition to dictating the method of DSB repair, improving the specificity with which your predesigned gRNA finds and binds its homologous site match in the genome can enhance editing accuracy.[6] Tolerance for mismatch varies between different gRNAs, but the launch of DNA-free, synthetic gRNA has provided superior editing efficiency and success when compared with *in vitro* transcribed or vector based gRNA.[7]

Cas9-Targeted Breeding of Animals

CRISPR/Cas9 has been used to engineer animals of various genetic backgrounds for use as *in vivo* research models, physiologically relevant drug discovery tools, and for the development of gene drives for applications as diverse as infectious disease prevention (e.g., malaria) and sustainable agriculture. Germline-modified animals are generated by either editing the embryonic genome, followed by embryo transfer, or via the genetic manipulation of embryonic stem cells for transfer. Alternatively, the animal genome can be directly edited by co-injection of in vitro-transcribed Cas9 mRNA and gRNA into the cytoplasm of zygotes during the pronuclear stage.[8] This has allowed for the rapid development of double knock-out models and does not require experience in handling embryonic stem cells.

With the ongoing refinement of the CRISPR toolkit and the emergence of novel applications by researchers, it is easy to envision a near-term *in vivo* modeling landscape that is exclusively CRISPR drive.

References

1. S. Chira, et al., "CRISPR/Cas9: Transcending the reality of genome editing," *Mol Ther Nuc Acids* 7:211-222, 2017.
2. E.L. Dow, "Modeling disease in vivo with CRISPR/Cas9," *Trends Mol Med* 21:609-621, 2015.
3. V.T. Chu, et al., "Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells," *Nat Biotechnol* 33:538-542, 2015.
4. T. Maruyama, et al., "Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining," *Nat Biotechnol* 33:538-542, 2015.
5. C. Yu, et al., "Small molecules enhance CRISPR genome editing in pluripotent stem cells," *Cell Stem Cell* 16:142-147, 2015.
6. P. Mali, et al., "RNA-guided human genome engineering via Cas9," *Science* 339:823-826, 2013.
7. L.M. Kelley, et al., "Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing," *J Biotechnol* 233:74-83, 2016.
8. F.D. Tschaharganeh, "Using CRISPR/Cas to study gene function and model disease in vivo," *FEBS J* 283:3194-3203, 2016.

Guiding Bright: The Blazing Trails of CRISPR Pioneers

CRISPR genome-editing technology is only five years old, but has already allowed for remarkable strides to be made in biomedical, microbial, and agricultural research. Previously, researchers were equipped with the tools and information to only genetically modify one gene at a time for *in vivo* study, primarily using mice. However, CRISPR affords scientists the ability to edit multiple genes at once in any extant organism. This is especially relevant when generating *in vivo* model systems for diseases like cancer, where multiple mutations drive the disease. We've seen its profound effects in biomedical research already, and it's worth reflecting on its recent humble beginnings to better understand where the future may lead.

Editing the Mouse Genome: A Timeline

In 2013, shortly after the separate but equally trailblazing inventive work done by Doudna and Zhang, the Jaenisch lab was the first to show the speed with which novel animal models could be produced using CRISPR.[1] More specifically, they created mutations in up to eight alleles from a single embryonic stem cell transfection.[1] Almost simultaneously, Malina et al published a proof-of-principle study showing that CRISPR-based germline mutations for disease modeling can be created through ex vivo modification of stem and progenitor cells and transplanted into a syngeneic recipient.[2] The following year, this was validated *in vivo* by using pooled CRISPR lentivirus to target eight genes routinely mutated in myeloid cancers.[3]

CRISPR genome-editing technology holds much promise in the complex study of cancer-driving genes, and in 2015 Chen et al were first to use the methodology for genome-wide screening of cancer-causing and -promoting genes, rather than the conventional gene-by-gene analysis in mice.[4] Recognizing the importance of specificity when modeling human disease, a few groups have been instrumental in upgrading the technique to direct *in vivo* delivery. The CRISPR/Cas9 activated complex can be successfully dispatched to specific tissue types, as opposed to the multi-step CRISPR-modified cell transplantation approach.[5-7] To account for the not-so-readily-available tissues for direct delivery of DNA and/or virus, tissue-specific and conditionally inducible Cas9 transgenic models are also in use. These models are either Cre-dependent or doxycycline (dox)-inducible.[8,9]

Gene Hacking in non-Mouse Animals

Besides the mouse, Cas9-mediated genome editing has generated other genetically modified organisms. For example, CRISPR has been used to elucidate the function of various genes of the European honeybee (A. mellifera) in both agricultural research and for the study of the multifaceted mechanisms governing its social behaviors.[10] Recently, the use of the technology in porcine animal models has received much attention, due to the extraordinary promise of organ xenotransplantation to human beings. In particular the work of Yang et al, where she was first to successfully create a model with a complete knockout of the PERV pol gene.[11] Finally, another notable example is the precise gene-targeting achievement by Chen et al to coinject the Cas9/gRNA complex in cynomolgus monkey embryos, the preferred animal model for physiologically-relevant human neurodegenerative disease research.[12]

References

1. H. Wang, et al., "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering," *Cell* 153:910-918, 2013.
2. A. Malina et al., "Repurposing CRISPR/Cas9 for in situ functional assays," *Genes & Dev* 27:2602-2614, 2013.
3. D. Heckl, et al., "Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing," *Nat Biotechnol* 32:941-946, 2014.
4. S. Chen, et al., "Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis," *Cell* 160:1246-1260, 2015.
5. L. Sweich, et al., "In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol* 33:102-106, 2015.
6. F.J. Sanchez-Rivera, et al., "Rapid modelling of cooperating genetic events in cancer through somatic genome editing," *Nature* 516:428-431, 2014
7. R. Cheng, et al., "Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9," *FEBS Lett* 588:3954-3958, 2014.
8. L.E. Dow, et al., "Inducible in vivo genome editing with CRISPR-Cas9," *Nat Biotechnol* 33:390-394, 2015.
9. R.J. Platt, et al., "CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling," *Cell* 159:440-455, 2014.
10. H. Kohno, et al., "Production of knockout mutants by CRISPR/Cas9 in the European Honeybee, Apis mellifera L.," *Zoological Sci* 33:505-512, 2016.
11. L. Yang, et al., "Genome-wide inactivation of porcine endogenous retroviruses (PERVs)," *Science* 350:1101-1104, 2015.
12. Y. Chen, et al., "Generation of Cynomolgus Monkey Chimeric Fetuses using Embryonic Stem Cells," *Cell Stem Cell* 17:116-124, 2015.