Gene editing for medical applications

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Gene editing is a technique where DNA is inserted, replaced or removed from a genome using artificially engineered nucleases

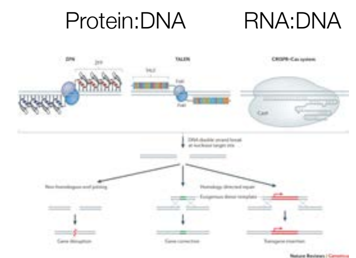
A toolbox for clinical gene editing

- Delivery of gene editing tools to the target cells
- Induction of double-stranded DNA break in correspondence of a desired sequence
- Stimulation of repair through either NHEJ or HDR

Gene editing technology

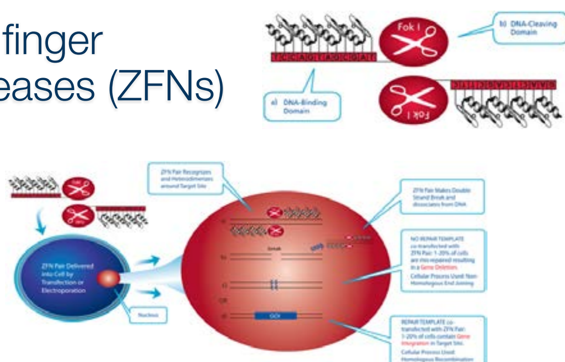
- zinc finger nucleases (ZFNs)
- transcription activator-like effector nucleases (TALENs)
- clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system

Protein:DNA RNA:DNA

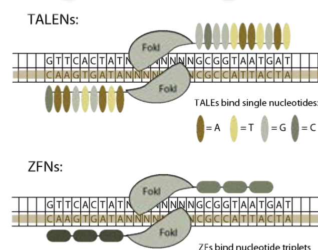


Nature Reviews Genetics 15, 541–555 (2014)

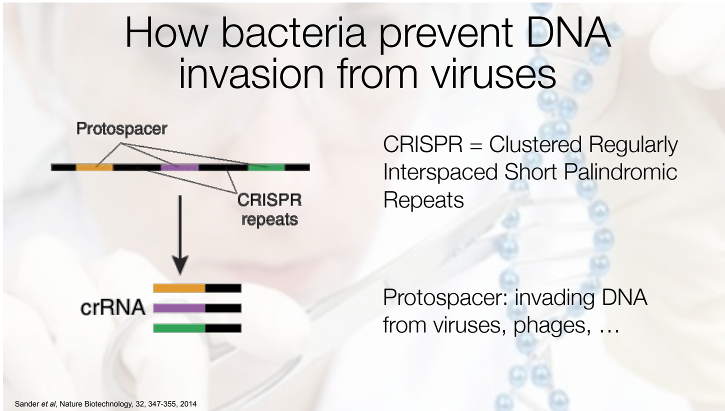
Zinc finger nucleases (ZFNs)

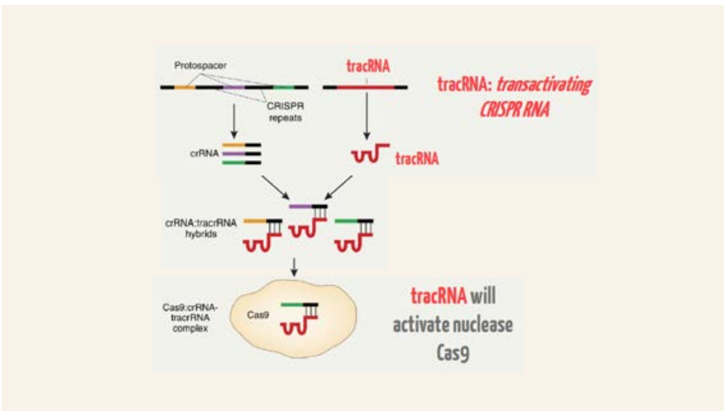


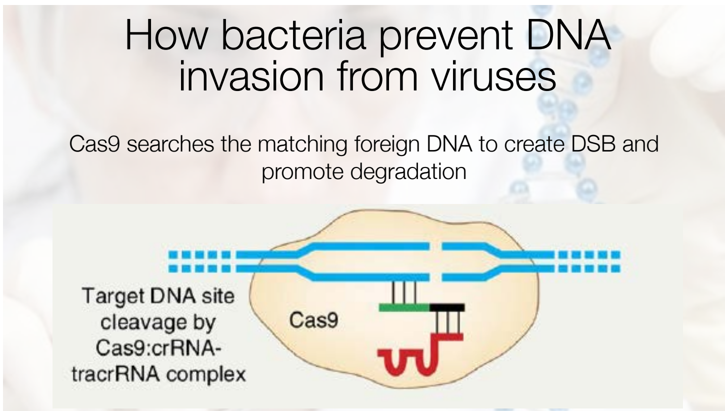
Transcription activator-like effector nucleases (TALENs) are more precise as they recognise single nucleotides

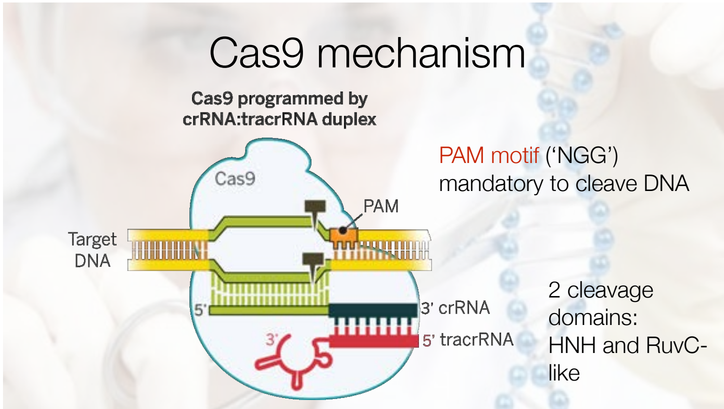


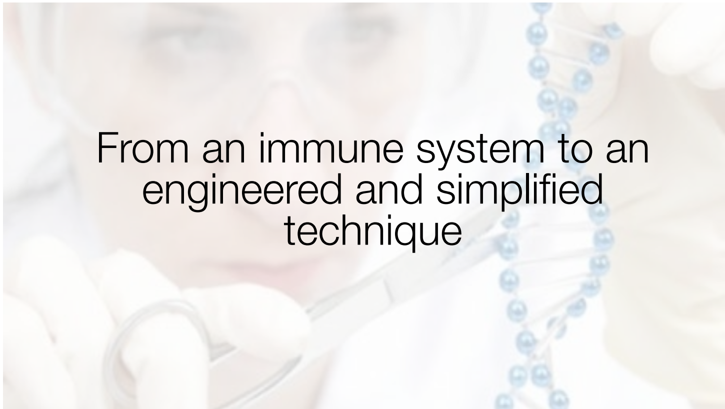
CRISPR/Cas9
a clever immune system


How bacteria prevent DNA invasion from viruses
Protospacer
CRISPR repeats
crRNA
CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats
Protospacer: invading DNA from viruses, phages, …
Sander et al, Nature Biotechnology, 32, 347-355, 2014

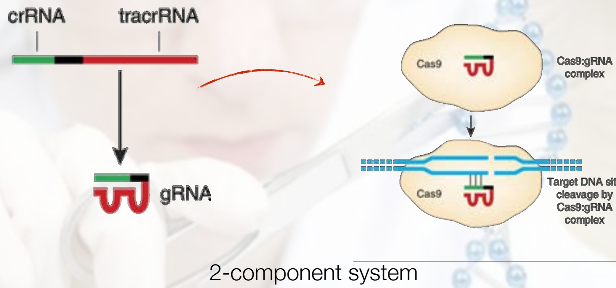

Protospacer
tracRNA
tracRNA: transactivating CRISPR RNA
CRISPR repeats
crRNA
tracRNA
crRNA:tracrRNA hybrids
Cas9:crRNA-tracrRNA complex
Cas9
tracRNA will activate nuclease Cas9


How bacteria prevent DNA invasion from viruses
Cas9 searches the matching foreign DNA to create DSB and promote degradation
Target DNA site cleavage by Cas9:crRNA-tracrRNA complex
Cas9

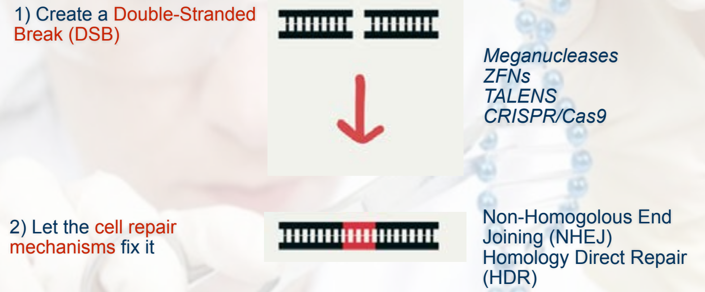

Cas9 mechanism
Cas9 programmed by crRNA:tracrRNA duplex
Cas9
PAM
Target DNA
5'
3'
3' crRNA
5' tracrRNA
PAM motif ('NGG') mandatory to cleave DNA
2 cleavage domains:
HNH and RuvC-like


From an immune system to an engineered and simplified technique

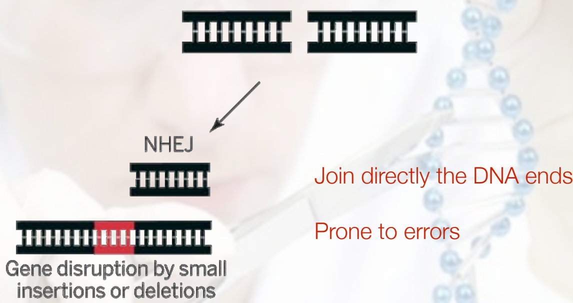
Fusion of crRNA and tracrRNA to a single guide RNA



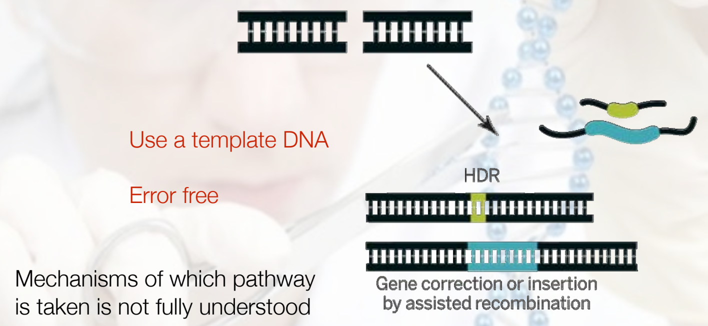
Two phases



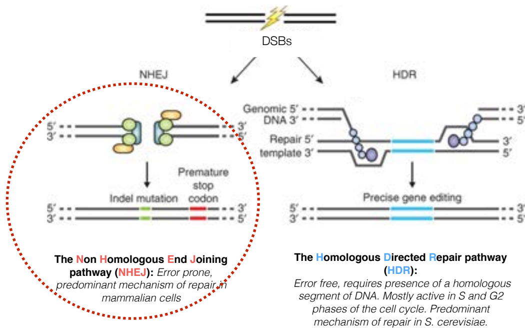
DSB repair mechanisms



DSB repair mechanisms

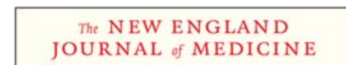


How cells repair dsDNA breaks (DSBs)

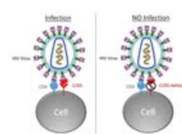


Adapted from Ran et al. 2013. Nat Protoc.

Genome editing for human therapy



Gene Editing of CCR5 in Autologous CD4 T Cells of Persons Infected with HIV



ZFNs have been used to disrupt CCR5 (C-C motif chemokine receptor type 5) expression in human T cells, and later also in HSCs (phase I/II trial ongoing), to render these cells resistant to HIV infection.

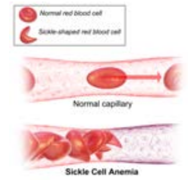
Ex vivo gene editing

Haemoglobinopathies

Red blood cells use hemoglobin to carry oxygen from the lungs to all the tissues of the body. Mutations in a gene that encodes part of the hemoglobin molecule cause two different genetic disorders: sickle cell disease (SCD) and beta thalassemia.

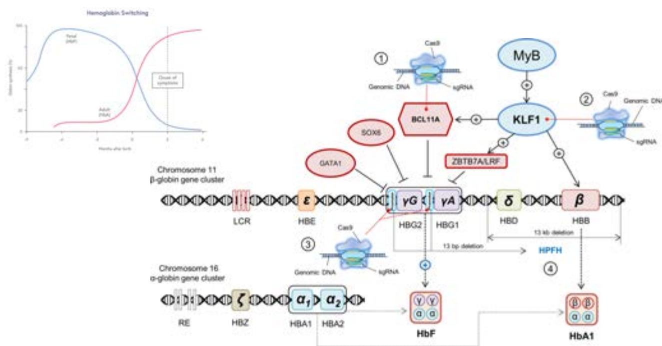
In sickle cell disease (SCD), red blood cells are misshapen. Their crescent or "sickle" shape makes them block blood vessels, slowing or stopping blood flow. This causes sudden, severe pain. Complications include chronic pain, organ damage, strokes, and anemia.

In beta thalassemia, patients do not make enough hemoglobin. This leads to anemia and fatigue. In more severe cases, patients have organ damage, especially to the liver, bones, and heart. Both diseases can be fatal.



There are some treatments available, but often, patients still suffer severe symptoms and complications from their diseases. Patients with more severe SCD and beta thalassemia need frequent blood transfusions. Bone marrow transplant can be curative; however, this can only be done when a healthy, matching donor can be found. This is not an option for most SCD or beta thalassemia patients.

Ex vivo gene editing for haemoglobinopathies



Ex vivo gene editing for haemoglobinopathies

- CTX001 is an investigational ex vivo CRISPR gene-edited therapy for patients suffering from Transfusion-Dependent β-Thalassaemia (TDT) or severe Sickle Cell Disease (SCD).
- Haematopoietic stem cells are engineered to produce high levels of fetal hemoglobin (HbF; hemoglobin F) in red blood cells.
- Partnership between CRISPR Therapeutics and Vertex Pharmaceuticals Inc (Zurich and Boston).
- CTX001 was granted Fast Track Designation by the U.S. Food and Drug Administration for the treatment of SCD in January 2019.
- Two Phase 1/2 studies, one in β-thalassemia and one in Sickle Cell Disease, to assess the safety and efficacy of a single dose of CTX001 in patients ages 18 to 35. In both studies, the first two patients are treated sequentially and, pending data from these initial two patients, the trial will open for broader concurrent enrolment.
- Trial on β-thalassemia conducted at multiple clinical trial sites in Canada and Europe, with future addition of the United States. Trial on Sickle Cell Disease conducted at clinical trial sites in the United States.

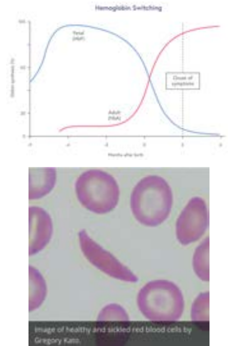
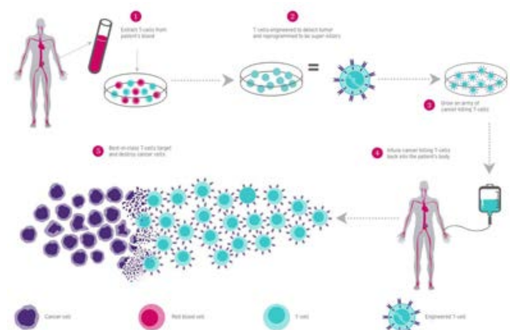


Image of healthy and sickled red blood cells by Gregory Kato.

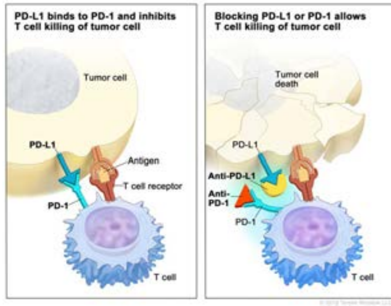
Victoria Gray, the first patient with SCD treated with CRISPR in July 2019

https://innovativegenomics.org/multimedia-library/meet-victoria-gray/

Immunotherapy for cancer



Immune checkpoint inhibitors to treat cancer



Approved checkpoint inhibitors

Name	Target	Approved
Ipilimumab	CTLA-4	2011
Nivolumab	PD-1	2014
Pembrolizumab	PD-1	2014
Atezolizumab	PD-L1	2016
Avelumab	PD-L1	2017
Durvalumab	PD-L1	2017
Cemiplimab	PD-1	2018

Chimeric Antigen Receptor (CAR)-T cells

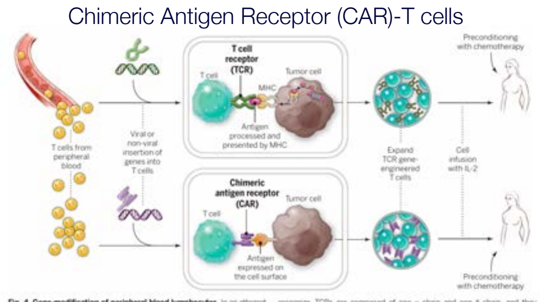


Fig. 4. Gene-modification of peripheral blood lymphocytes. In an attempt to broaden the reach of ACT to other cancers, techniques are being developed to introduce antitumor receptors into normal T cells that could be used for therapy. The top panel shows the insertion of a conventional TCR into a patient's T lymphocytes, followed by the expansion and infusion back into the patient. The bottom panel shows the insertion of a CAR into a patient's T cell, followed by the expansion of these cells and their re-infusion. TCRs and CARs are fundamentally different in their structures and in the structures that they recognize. TCRs are composed of one α chain and one β chain, and they recognize antigens that have been processed and presented by one of the patient's own MHC molecules. CARs are artificial receptors that can be constructed by linking the variable regions of the antibody heavy and light chains to intracellular signaling chains (such as CD3-zeta, CD28, 41BB) alone or in combination with other signaling moieties. CARs recognize antigens that do not need to be MHC-restricted, but they must be presented on the tumor cell surface.

66 3 APRIL 2015 • VOL 348 ISSUE 6230 sciencemag.org SCIENCE

THE CRISPR REVOLUTION

First U.S. Patients Treated With CRISPR As Human Gene-Editing Trials Get Underway

NY-ESO-1-redirected CRISPR (TCRendo and PD1) Edited T Cells (NYCE T Cells)
ClinicalTrials.gov Identifier: NCT03399448

- First CRISPR-based therapy trial that combines CAR-T and PD-1 immunotherapy
- University of Pennsylvania with the Parker Institute
- Autologous T cells transduced with a lentiviral vector to express a TCR with affinity to NY-ESO-1 and electroporated with CRISPR guide RNA/Cas9 to disrupt expression of endogenous TCRα, TCRβ and PD-1 (NYCE T Cells)
- Patients with late-stage cancers (multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma) - 18 patients
- Two patients treated, one with relapsed multiple myeloma and one with relapsed sarcoma

NEWS

Genome editing seems safe suggests first study in US patients

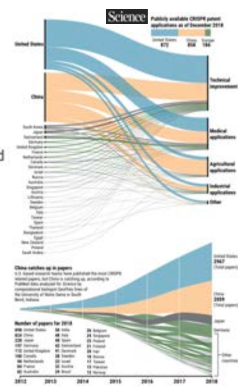
11 November 2019

Seven active or recruiting trials in China are listed on the *ClinicalTrials.gov* clinical trial database.

TREATMENTS

Doctors In China Lead Race To Treat Cancer By Editing Genes

With its CRISPR revolution, China becomes a world leader in genome editing



ARTICLES
https://doi.org/10.1038/s41591-020-0840-5

nature medicine

Check for updates

Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer

You Lu[1,14], Jianxin Xue[1,14], Tao Deng[2,14], Xiaojuan Zhou[1,14], Kun Yu[2,14], Lei Deng[3], Meijuan Huang[1], Xin Yi[4], Maozhi Liang[5], Yu Wang[6], Haige Shen[6], Ruizhan Tong[1], Wenbo Wang[7], Li Li[1], Jin Song[4], Jing Li[4], Xiaoxing Su[8], Zhenyu Ding[1], Youling Gong[1], Jiang Zhu[1], Yongsheng Wang[1,5], Bingwen Zou[1], Yan Zhang[1], Yanying Li[1], Lin Zhou[1], Yongmei Liu[1], Min Yu[1], Yuqi Wang[4], Xuanwei Zhang[1], Limei Yin[1], Xuefeng Xia[4], Yong Zeng[2], Qiao Zhou[9], Binwu Ying[10], Chong Chen[11], Yuquan Wei[11], Weimin Li[12] and Tony Mok[13]

Clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 editing of immune checkpoint genes could improve the efficacy of T cell therapy, but the first necessary undertaking is to understand the safety and feasibility. Here, we report results from a first-in-human phase I clinical trial of CRISPR–Cas9 *PD-1*-edited T cells in patients with advanced non-small-cell lung cancer (ClinicalTrials.gov NCT02793856). Primary endpoints were safety and feasibility, and the secondary endpoint was efficacy. The exploratory objectives included tracking of edited T cells. All prespecified endpoints were met. *PD-1*-edited T cells were manufactured ex vivo by cotransfection using electroporation of Cas9 and single guide RNA plasmids. A total of 22 patients were enrolled; 17 had sufficient edited T cells for infusion, and 12 were able to receive treatment. All treatment-related adverse events were grade 1/2. Edited T cells were detectable in peripheral blood after infusion. The median progression-free survival was 7.7 weeks (95% confidence interval, 6.9 to 8.5 weeks) and median overall survival was 42.6 weeks (95% confidence interval, 10.3–74.9 weeks). The median mutation frequency of off-target events was 0.05% (range, 0–0.25%) at 18 candidate sites by next generation sequencing. We conclude that clinical application of CRISPR–Cas9 gene-edited T cells is generally safe and feasible. Future trials should use superior gene editing approaches to improve therapeutic efficacy.

NATURE MEDICINE | VOL 26 | MAY 2020 | 732–740 | www.nature.com/naturemedicine

- The treatment was safe to administer and had acceptable side effects like fever, rash, and fatigue.
- The desired edit was found in a median of 6% of T cells/patient before infusion back into the patient.
- Off-target effects — unwanted changes at various places in the genome — were observed at a low frequency and were mostly in parts of the genome that don't code for proteins. On-target effects — unwanted changes at the target site — were more common (median of 1.69%).
- Edited T cells were found in 11 out of 12 patients two months after the infusion, although at low levels. Patients with higher levels of edited cells had less disease progression.

In vivo gene editing

TheScientist
EXPLORING LIFE, INSPIRING INNOVATION

Man Receives First In Vivo Gene-Editing Therapy

The 44-year-old patient has Hunter syndrome, which doctors hope to treat using zinc finger nucleases.

Nov 15, 2017
KERRY GRENS

Hunter syndrome, or mucopolysaccharidosis II (MPS II), is a lysosomal storage disease caused by a deficient (or absent) enzyme, iduronate-2-sulfatase (I2S). When the enzyme is defective or missing, the sugars build up and can cause developmental delays, organ problems, brain damage, and early death.

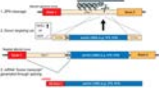
GENE THERAPY

In vivo genome editing of the albumin locus as a platform for protein replacement therapy

Rajiv Sharma, Xavier M. Anguela, Yannick Doyon, Thomas Wechsler, Russell C. DeKelver, Scott Sproul, David E. Paschon, Jeffrey C. Miller, Robert J. Davidson, David Shivak, Shangzhen Zhou, Julianne Rieders, Philip D. Gregory, Michael C. Holmes, Edward J. Rebar, and Katherine A. High

Key Points

- AAV- and ZFN-mediated targeting of the albumin locus corrects disease phenotype in mouse models of hemophilia A and B.
- Robust expression from the albumin locus provides a versatile platform for liver-directed protein replacement therapy.



BLOOD, 8 OCTOBER 2015 · VOLUME 126, NUMBER 15

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How does the treatment work?

Insertion of a replacement copy of the gene, using gene editing to snip the DNA helix of liver cells in a specific place near the promotor for the albumin gene - NOT GENE CORRECTION

The cells fix the damage by inserting the DNA for the new gene, supplied along with the ZFNs, and the gene's activity is then controlled by the powerful albumin promoter.

FDA has approved 3 clinical trials exploiting these modified liver cells into a factory delivering the factor IX gene for hemophilia B (NCT02695160), the a-L-iduronidase gene for mucopolysaccharidosis I (NCT02702115), and the iduronidate-2-sulfatase gene for mucopolysaccharidosis II (MPS II, Hunter syndrome) (NCT03041324).

This targeted approach should avoid the risks of insertional mutagenesis.
Because the body doesn't need much of the enzyme, modifying just a small fraction of the liver's cells should be enough to treat the disease.

Although Hunter syndrome patients often receive weekly infusions of the missing enzyme, their blood levels drop within a day. The hope is that the one-time gene-editing treatment—given as a 3-hour intravenous infusion—will allow the liver to keep making the enzyme at a steady rate for years.

Caveat: the I2S enzyme does not cross the blood-brain barrier, so the new treatment may not stop the brain damage that can occur in Hunter syndrome (as for replacement therapy).

A human has been injected with gene-editing tools to cure his disabling disease. Here's what you need to know

By Jocelyn Kaiser | Nov. 15, 2017, 6:00 PM

Science

Brian Madeux, who has Hunter syndrome, has received a treatment aimed at editing the genome of his liver cells.

SB-913: 3 AAV6 vectors

1. intact IDS gene
2. ZFN binding upstream of the target site
3. ZFN binding downstream of the target site

i.v. infusion

low dose is not effective:
represents a de facto placebo arm

approval upon efficacy demonstrated on clinical endpoints: six-minutes walk and lung function

In vivo gene editing LCA10 Leber Congenital Amaurosis

- Leber Congenital Amaurosis (LCA) is the most common cause of inherited childhood blindness. LCA10 is the most common form of LCA. It causes severe vision loss or blindness within the first few months of life.
- Due to mutations in the centrosomal protein 290 kDa gene (CEP290, MIM610142). Defects in this gene are also associated with Joubert syndrome and nephronophthisis. As of today, 35 different mutations in CEP290 are responsible for causing LCA.
- In the retina, CEP290 is mainly located to the connecting cilium of photoreceptors, where it plays an essential role in both cilium assembly and ciliary protein trafficking.
- Of the CEP290 mutations that result in LCA10, the most recurrent one, accounting for up to 15% of all LCA cases in many Western countries, is a deep intronic mutation (c.2991+1655A > G) in intron 26 of the CEP290 gene (hereafter referred to as "IVS26 mutation" or "IVS26 splice mutation").

EDIT-101 Aims to Rescue Vision in LCA10

Degeneration of outer segment but cell body remains intact

EDIT-101 subretinal injection to remove disease-causing mutation

Restoration of full-length protein and rebuilding of outer segment

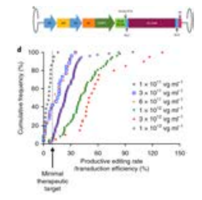
PERSPECTIVE

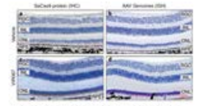
LCA10 trial of CRISPR genome editing treatment initiated

July 31, 2019

Single Ascending Dose Study in Participants With LCA10
ClinicalTrials.gov Identifier: NCT03872479

- First in vivo gene editing trial - the Brilliance trial
- AAV5 vector carrying *S. aureus* Cas9 and a guide targeting CEP290 intron 26.
- Patients receive a single subretinal injection in one eye following vitrectomy - 18 patients in up to five cohorts across three dose levels
- Editas Medicine in collaboration with Allergan - currently recruiting patients volunteers throughout the US.





NATURE MEDICINE | VOL 25 | FEBRUARY 2019 | 229–235

Patent War

Jennifer Doudna at the University of California, Berkeley, and Emmanuelle Charpentier, now at Umeå University in Sweden and the Max Planck Institute for Infection Biology in Berlin.

Cas9 enzyme can be directed to cut specific sites in isolated DNA

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek, Krzysztof Chylinski, Ines Fonfara, Michael Hauer, Jennifer A. Doudna, Emmanuelle Charpentier

17 AUGUST 2012 VOL 337 SCIENCE www.sciencemag.org

Patent application initiated on 25 May 2012

Feng Zhang at the Broad Institute of MIT and Harvard in Cambridge, Massachusetts

CRISPR–Cas9 can be applied and used in mammalian cells

Multiplex Genome Engineering Using CRISPR/Cas Systems

Le Cong, F. Ann Ran, David Cox, Shuailiang Lin, Robert Barretto, Naomi Habib, Patrick D. Hsu, Xuebing Wu, Wenyan Jiang, Luciano A. Marraffini, Feng Zhang

www.sciencemag.org SCIENCE VOL 339 15 FEBRUARY 2013

Patent application initiated on 12 December 2012

Although the Berkeley team filed first, the Broad team submitted its application to an expedited review programme, and was awarded the patent in April 2014.

THE NOBEL PRIZE IN CHEMISTRY 2020

Emmanuelle Charpentier

Jennifer A. Doudna

"for the development of a method for genome editing"

THE ROYAL SWEDISH ACADEMY OF SCIENCES

Human monogenic disease

Human protein-coding genes*

19,950

GENCODE

Genes with mutations causing disease**

3947

50 YEARS OMIM

*http://www.gencodegenes.org/stats/current.html
** https://www.omim.org/statistics/geneMap

Cardiovascular disorders with Mendelian inheritance

Table 1. Selected Examples of Mendelian Diseases and Insights Derived from the Study of Causal Genes

Mendelian Condition	Causal Genes	Key Biological and Clinical Insights	References
Severe hypercholesterolemia	LDLR, APOB, ABCG5, ABCG8, ARH, PCSK9	(1) Receptor-mediated endocytosis, (2) receptor recycling, (3) feedback regulation of receptors, (4) molecular mechanism of intestinal cholesterol absorption and biliary cholesterol secretion, (5) high LDL cholesterol is sufficient to cause MI.	(Abifadel et al., 2003; Brown and Goldstein, 1986; Garcia et al., 2001; Lehrman et al., 1985)
Familial hypobetalipoproteinemia	APOB, PCSK9, ANGPTL3	(1) Lifelong low LDL cholesterol (from loss-of-PCSK9 function) is sufficient to protect from MI despite other coronary risk factors.	(Cohen et al., 2006; Musunuru et al., 2010a; Soria et al., 1989)
Mendelian forms of low and high blood pressure	SLC12A3, SLC12A1, KCNJ1, CLCNKB, NR3C2, SCNN1A, SCNN1B, SCNN1G, CYP11B2, CYP11B1, HSD11B2, NR3C2, SCNN1B, SCNN1G, WNK1, WNK4, KLHL3, CUL3	(1) Genes converge on a final common pathway of altering net renal sodium handling and balance; (2) identification of new targets for the treatment of blood pressure.	(Boyden et al., 2012; Chang et al., 1996; Geller et al., 2000; Geller et al., 1998; Hansson et al., 1995; Lifton et al., 1992a, 1992b, 2001; Mune et al., 1995; Shimkets et al., 1994; Simon et al., 1996a, 1996b, 1996c, 1997; Wilson et al., 2001)
Hypertrophic cardiomyopathy	MYH7, TNNT2, TPM1, TNNI3, MYL2, MYBPC3, ACTC, MYL3	(1) Mutations have expanded knowledge of the molecular mechanisms of heart muscle contraction; (2) mutations may cause increased TGF-β signaling in the myocyte with subsequent effects on neighboring fibroblasts, leading to fibrosis and scarring.	(Bonne et al., 1995; Carrier et al., 1993; Geisterfer-Lowrance et al., 1990; Kimura et al., 1997; Olson et al., 2000; Poetter et al., 1996; Seidman and Seidman, 2001; Thierfelder et al., 1994; Watkins et al., 1995)
Marfan's syndrome	FBN1	(1) Aneurysm formation is likely due to perturbations in cytokine signaling cascades and the smooth muscle contractile apparatus rather than defects in the extracellular matrix; (2) unexpected role for TGF-β pathway in disease.	(Dietz et al., 1991; Lindsay and Dietz, 2011)
Atrial or ventricular septal defects	NKX2-5, GATA-4, TBX5	(1) These transcription factors, originally discovered in flies and mice, are critical for proper heart development in humans and function in a common complex.	(Basson et al., 1997; Garg et al., 2003; Schott et al., 1998)
Bicuspid aortic valve, Calcific aortic valve disease	NOTCH1	(1) NOTCH1 functions to repress a default osteoblast fate of the valve mesenchymal cells; (2) NOTCH1 mutations likely result in a derepression of this fate choice and subsequent differentiation of valve cells into an osteoblast-like phenotype.	(Garg et al., 2005)

S. Kathiresan & D. Srivastava. 2012. Cell 148, 1242

How cells repair dsDNA breaks (DSBs)

DSBs

NHEJ

HDR

5' 3'

Genomic DNA 5' 3'

Repair template 5' 3'

Indel mutation

Premature stop codon

Precise gene editing

The Non Homologous End Joining pathway (NHEJ): *Error prone, predominant mechanism of repair in mammalian cells*

The Homologous Directed Repair pathway (HDR): *Error free, requires presence of a homologous segment of DNA. Mostly active in S and G2 phases of the cell cycle. Predominant mechanism of repair in S. cerevisiae.*

Adapted from Ran et al. 2013. Nat Protoc.

Cell, Vol. 33, 25–35, May 1983, Copyright © 1983 by MIT 0092-8674/83/050025-11 $02.00/0

The Double-Strand-Break Repair Model for Recombination

Review

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Summary

Gene conversion is the nonreciprocal transfer of information from one DNA duplex to another; in meiosis, it is frequently associated with crossing-over. We review the genetic properties of meiotic recombination and previous models of conversion and crossing-over. In these models, recombination is initiated by single-strand nicks, and heteroduplex DNA is generated. Gene conversion is explained by the repair of mismatches present in heteroduplex DNA. We propose a new mechanism for meiotic recombination, in which events are initiated by double-strand breaks that are enlarged to double-strand gaps. Gene conversion can then occur by the repair of a double-strand gap, and postmeiotic segregation can result from heteroduplex DNA formed at the boundaries of the gap-repair region. The repair of double-strand gaps is an efficient process in yeast, and is known to be associated with crossing-over. The genetic implications of the double-strand-break repair model are explored.

a b c d e f

non-crossover crossover

Figure 8. A Double-Strand-Break Repair Model

(a) A double-strand cut is made in one duplex, and a gap flanked by 3' single strands is formed by the action of exonucleases. (b) One 3' end invades a homologous duplex, displacing a D loop. (c) The D loop is enlarged by repair synthesis until the other 3' end can anneal to complementary single-stranded sequences. (d) Repair synthesis from the second 3' end completes the process of gap repair, and branch migration results in the formation of two Holliday junctions. Resolution of two junctions by cutting either inner or outer strands leads to two possible noncrossover (e) and two possible crossover (f) configurations. In the illustrated resolutions, the righthand junction was resolved by cutting the inner, crossed strands.

Cell, Vol. 51, 503–512, November 6, 1987, Copyright © 1987 by Cell Press

Site-Directed Mutagenesis by Gene Targeting in Mouse Embryo-Derived Stem Cells

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Summary

We mutated, by gene targeting, the endogenous hypoxanthine phosphoribosyl transferase (HPRT) gene in mouse embryo-derived stem (ES) cells. A specialized construct of the neomycin resistance (*neo*ʳ) gene was introduced into an exon of a cloned fragment of the *Hprt* gene and used to transfect ES cells. Among the G418ʳ colonies, 1/1000 were also resistant to the base analog 6-thioguanine (6-TG). The G418ʳ, 6-TGʳ cells were all shown to be *Hprt*⁻ as the result of homologous recombination with the exogenous, *neo*ʳ-containing, *Hprt* sequences. We have compared the gene-targeting efficiencies of two classes of *neo*ʳ–*Hprt* recombinant vectors: those that replace the endogenous sequence with the exogenous sequence and those that insert the exogenous sequence into the endogenous sequence. The targeting efficiencies of both classes of vectors are strongly dependent upon the extent of homology between exogenous and endogenous sequences. The protocol described herein should be useful for targeting mutations into any gene.

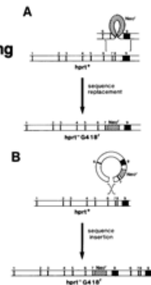
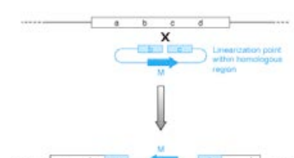


Figure 1. Disruption of the Hprt gene by Gene Targeting

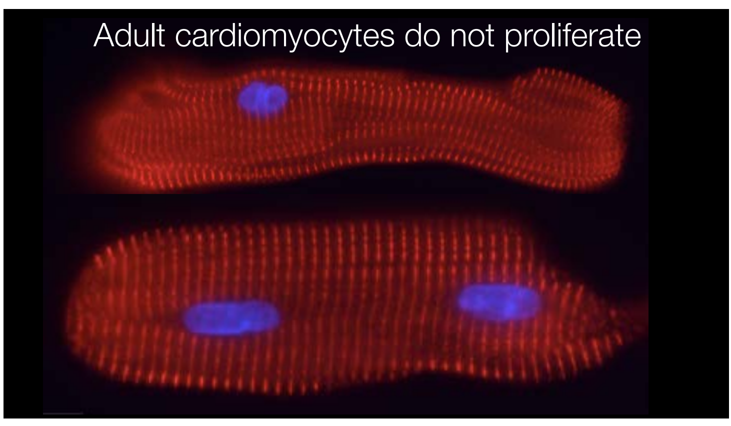
Gene targeting by homologous recombination in ES cells can be used to produce mice with a mutation in a predetermined gene

Gene targeting typically involves introducing a mutation by homologous recombination in mouse ES cells: once a mutation has been engineered into a specific mouse gene within the ES cells, the modified ES cells can then be injected into the blastocyst of a foster mother and eventually a mouse can be produced with the mutation in the desired gene in all nucleated cells

Homologous recombination in mammalian cells is a very rare occurrence and its frequency is increased when the degree of sequence homology between the introduced DNA and the target gene is very high
To assist identification of the desired homologous recombination events, the targeting vector (often a plasmid vector) contains a marker gene, such as the *neo* gene, which permits selection for cells that have taken up the introduced DNA.



The gene in question is selectively inactivated, producing a **'knock-out' mouse**, and the effect of the mutation on the development of the mouse is monitored



Factors that enhance HDR?

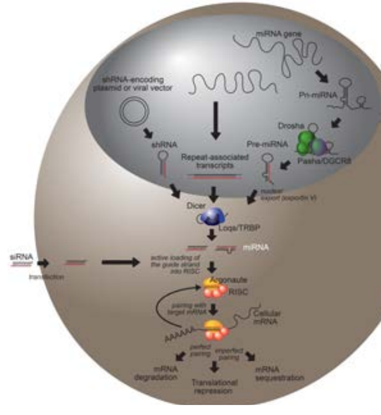
Luca Braga

High content RNAi functional screenings: from large libraries to functional hits

Arrayed Libraries

1 well —> 1 Factor

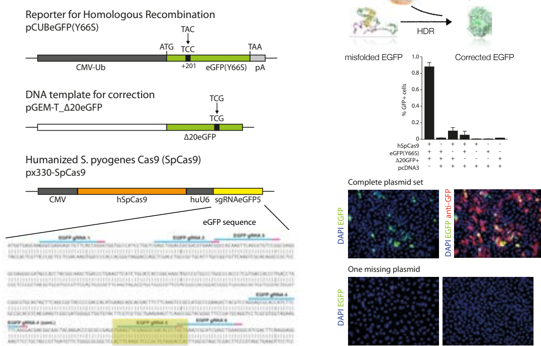
- Human/Mouse whole Genome siRNAs
- Human synthetic microRNA mimics (*2042 mature sequences, miRBase v. 19.0*)
- Human miRCURY LNA inhibitors (*1972 molecules*)
- FDA approved small molecules (*1280 molecules*)
- Custom cherry-picked human and mouse siRNAs
- Mouse secreted factors (*1202 cDNAs*)



The microRNA network

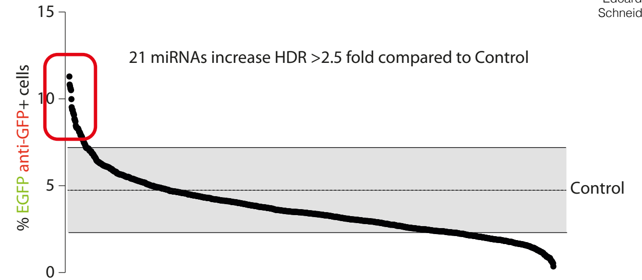
- Many, but not too many! 1917 precursors, 2654 mature miRNAs in humans (*miRBase 22, March 2018*)
- Pleiotropic. Each miRNA targets tens or hundreds of transcripts. Ideal to target complex functions
- Small. Easy to deliver in vivo. Can be dosed and used as small molecules
- Can be screened for function

HTS for miRNAs enhancing HDR

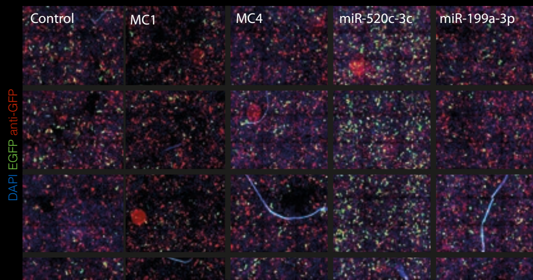


HTS for miRNAs enhancing HDR

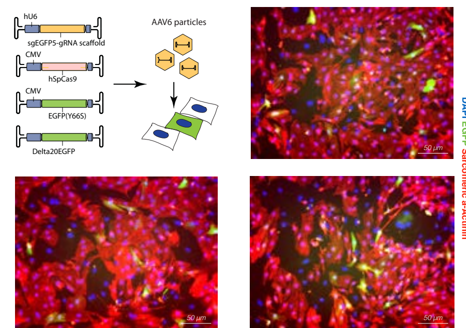
Edoardo Schneider



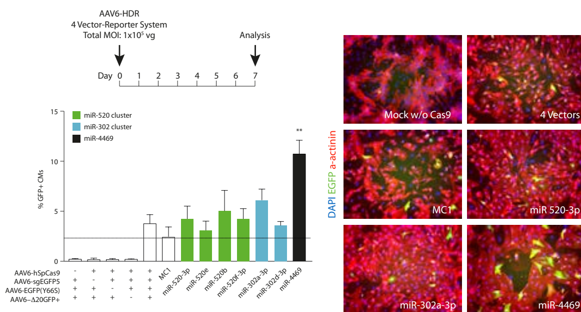
HTS for miRNAs enhancing HDR

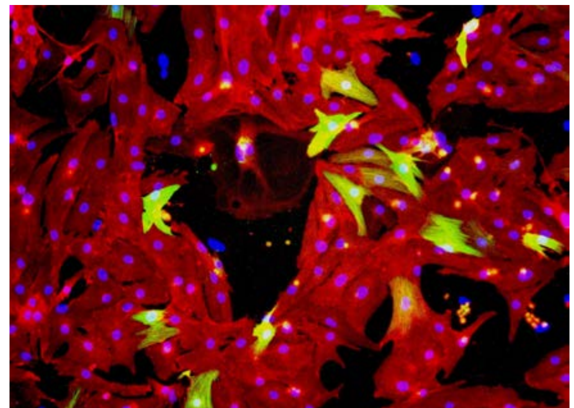


An AAV-based assay to measure HDR in primary neonatal cardiomyocytes



hsa-miR-4469 improves HDR efficiency in neonatal rat cardiomyocytes





In vivo

A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9

Kelli J. Carroll[a], Catherine A. Makarewich[a], John McAnally[a], Douglas M. Anderson[a], Lorena Zentilin[b], Ning Liu[a], Mauro Giacca[b], Rhonda Bassel-Duby[a], and Eric N. Olson[a,1]

[a]Department of Molecular Biology and the Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390-9148; and [b]Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology, I-34149 Trieste, Italy

Contributed by Eric N. Olson, December 7, 2015 (sent for review November 21, 2015; reviewed by Leslie A. Leinwand and Joseph M. Miano)

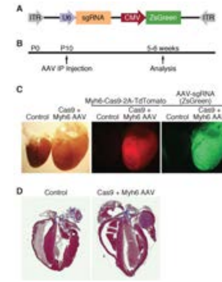
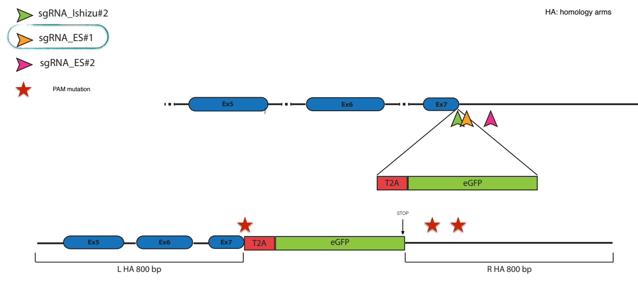
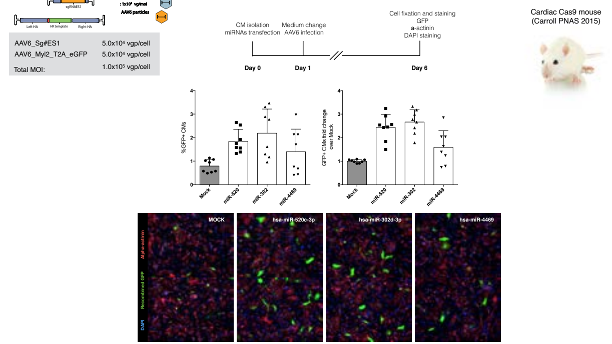


Fig.2. AAV9-driven expression of sgRNA. **A.** sgRNA under the control of the U6 promoter was cloned into an AAV9 backbone, together with a CMV-driven ZsGreen reporter. **B.** Animals were injected intraperitoneally at postnatal day 10 (P10) and subsequently analyzed 5–6 wk later. **C.** An Q:33 example of a Myh6-Cas9-2A-TdTomato heart (red, Center) that also received AAV-sgRNA against Myh6 exon 3 (green, Right). Compared with a littermate control animal, hearts from animals that received both Cas9 and sgRNA against Myh6 displayed extreme cardiac dilation and hypertrophy. **D.** Histological section of a control heart and a heart that contained both Cas9 and AAV-sgRNA against Myh6 exon 3. Edited hearts displayed thinning of the ventricular walls and massive dilation of both the atria and ventricles.

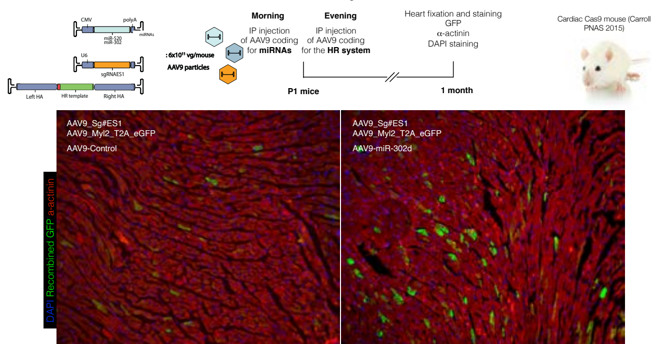
HR to promote integration into the mouse Myl2 locus

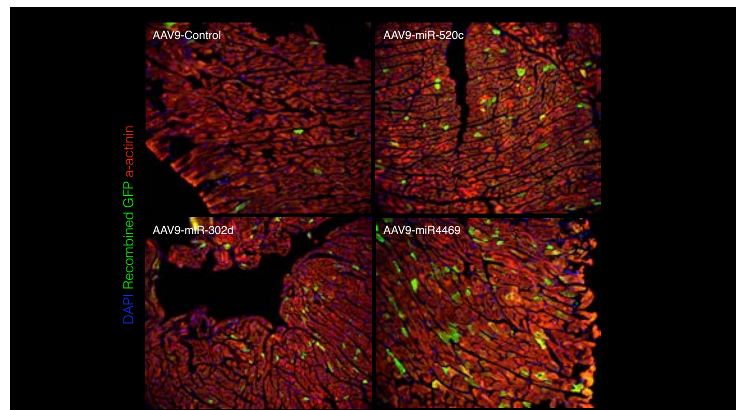


HR to promote integration into the mouse Myl2 locus



HDR to promote integration into the mouse Myl2 locus





A toolbox for cardiac gene editing

- Delivery of gene editing tools to the heart *FEASIBLE*
- Precise dsDNA break in correspondence of a given sequence *FEASIBLE*
- In vivo gene inactivation *PROBABLY*
- In vivo precise gene eding *PERHAPS*

10 genes that could be gene edited to improve appearance, disease risk or performance

1. A variant coding for **extra-strong bones** (LRP5 G171V/+)
2. A variant coding for **lean muscles** (MSTN)
3. A variant rendering people **less sensitive to pain** (SCN9A)
4. A variant associated with **low odor production** (ABCC11)
5. A variant rendering people **more resistant to viruses** (CCR5, FUT2)
6. A variant connected to a **low risk of coronary disease** (PCSK9)
7. A variant associated with a **low risk of Alzheimer's disease** (APP A673T/+)
8. A variant associated with a **low cancer risk** (GHR, GH)
9. A variant associated with a **low risk of type 2 diabetes** (SLC30A8)
10. A variant associated with a **low risk of type 1 diabetes** (IFIH1 E627X/+)

BUSINESS INSIDER

A conversation with George Church on Genomics & Germline Human Genetic Modification

Genome editing in human embryos

RESEARCH ARTICLE

CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

Puping Liang, Yanwen Xu, Xiya Zhang, Chenhui Ding, Rui Huang, Zhen Zhang, Jie Lv, Xiaowei Xie, Yuxi Chen, Yujing Li, Ying Sun, Yaofu Bai, Zhou Songyang, Wenbin Ma, Canquan Zhou, Junjiu Huang

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Received March 30, 2015 Accepted April 1, 2015

Attempt to correct the human β-globin (HBB) gene in 'non-viable' embryos (β-thalassaemia)

- 7 of 86 embryos were successfully mutated
- much higher rates of off-targeting

Raise huge ethical concerns…

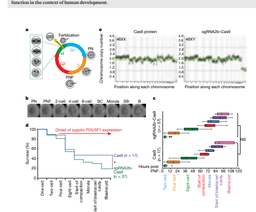
Protein Cell 2015, 6(5):363-372
DOI 10.1007/s13238-015-0153-5

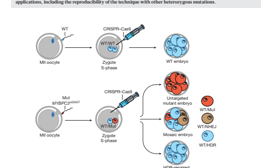
Genome editing in human embryos

In February 2016, the Human Fertilization granted limited permission for researchers in the UK to genetically modify human embryos, with the hope of elucidating which genes are necessary for successful embryological development.

Although Dr. Kathy Niakan and her team at the Francis Crick Institute are only allowed to use the embryos for 14 days, and may not implant a modified embryo in the womb, this permission crossed a frontier in genetic research.
It is the first time human embryonic genetic modification is authorized.

Frederik Lanner at the Karolinska Institute in Sweden, got the go-ahead on a project that will also involve gene editing in human embryos.





Germline gene editing

2018: announcement of the birth of twin girls with edited genomes

- Lack of definitive evidence
- Strategy: engineering mutations, inducing resistance to HIV (silencing of CCR5), into human embryos (requiring IVF)
- The major problem is not gene editing itself but lack of safety testing (other mutations, increased sensitivity to other diseases), lack of standard procedures for recruiting, HIV people should not undergo IVF

Germline gene editing

Role of other scientists: which is the authority to report possible abuse? Need for an international advisory board/ registry to identify commonalities and differences between countries (i.e. international committee by WHO)

International Regulatory Landscape

Survey on 39 countries (2014)

- 29 countries ban germline gene modification (China, India, Ireland, and Japan forbid it based on guidelines that are less enforceable than laws, and are subject to amendment)
- 9 countries are ambiguous about the legal status of the modification
- in the US FDA regulates the clinical trial, whereas the NIH restricts the application of germline gene modification.

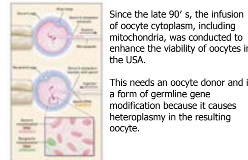
This regulatory landscape suggests that human germline gene modification is not totally prohibited

Israel, which explicitly bans germline gene modification, but has possible exemptions in the relevant law may permit it upon the recommendation of an advisory committee. This Israeli law has been temporary legislation until May 23, 2016. Now, the country might permit human germline gene modification.

In the UK, the DH will consider the timing of the regulations to permit mitochondrial replacement that is currently illegal for mtDNA alternation in the germline. Taking into consideration that there is no legal ban on research on the human germline gene modification as long as the Human Fertilisation and Embryology Authority (HFEA) licenses such research in the UK, the legalization of medical use of mitochondrial replacement is likely to lead to legal permission for the modification of germline nuclear genome that can be readily changed by genome editing technology.

Two legal approaches are similar to germline genetic modification

Ooplasmic transfer and low

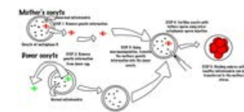


Since the late 90' s, the infusion of oocyte cytoplasm, including mitochondria, was conducted to enhance the viability of oocytes in the USA.

This needs an oocyte donor and is a form of germline gene modification because it causes heteroplasmy in the resulting oocyte.

Although ooplasmic transfer led to more than 30 childbirths, the Food and Drug Administration (FDA) decided to regulate this procedure owing to potential health risk to progeny

Mitochondrial replacement



Currently proposed to prevent maternal transmission of serious mitochondrial diseases that result from aberrant mitochondrial DNA (mtDNA) in patient' s oocyte. Mitochondrial replacement is also a form of germline gene modification because this procedure involves altering the mtDNA content of human oocytes or embryos.

Mitochondrial replacement as well as ooplasmic transfer require **oocyte donation** which could potentially cause ovarian hyperstimulation syndrome in female donors.

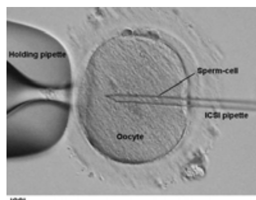
The US FDA allows mitochondrial replacement under certain conditions.

The UK Department of Health (DH) has lifted the ban of mitochondrial replacement, which is now legal.

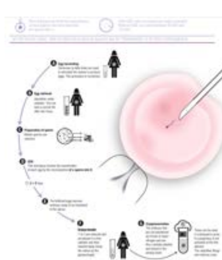
Such regulatory changes in a few, but major countries, may impact the international regulatory landscape that prohibits human germline gene modification.

Germline gene editing during IVF

Targeted gene modification is frequently carried out by simply microinjecting of genome editing system which consists of the nuclease mRNAs (or plasmids harboring the nuclease gene), single guide RNAs (sgRNAs for Cas9), and a homology-containing donor DNA template (if necessary) into animal embryos made by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).



ICSI



This microinjection process resembles assisted reproductive technology (ART) to facilitate fertilization in fertility clinics. Thus, **genome editing is more likely to develop into medicine for preventing a genetic disease if integrated into assisted reproductive technology**, including IVF and ICSI.
Importantly, germline gene correction by genome editing <u>does not require cell donation</u> such as oocyte donation that is needed for ooplasmic transfer and mitochondrial replacement.

Germline gene editing

Role of other scientists: which is the authority to report possible abuse? Need for an international advisory board/ registry to identify commonalities and differences between countries (i.e. international committee by WHO)

Off-target effects

Gene-gene interactions

Benefit to risk ratio depends on real need: PGD exists

Corrective genome editing integrated into ART would be preventive medicine rather than therapy

- it aims at prevention of transmission of a genetic disease to offspring, not at the treatment of existing patients

- potential subjects: those with congenital anomalies caused by chromosomal, **monogenic**, multifactorial or environmental/teratogenic factors

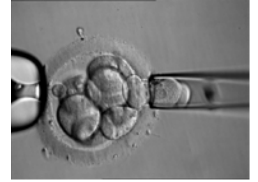
- candidate diseases: autosomal recessive disease in which both parents are homozygous (e.g. cystic fibrosis, phenylketonuria) or an autosomal dominant disease where at least one parent is homozygous (e.g. Huntington' s disease, familial adenomatous polyposis)

- preimplantation genetic diagnosis (PGD) may circumvent an affected pregnancy by selecting IVF embryos with no off-target mutations

Should affected parents not use such a risky genetic intervention and instead use donor gametes or donor embryos (or consider prenatal diagnosis, termination of a pregnancy and adoption)?

The use of CRISPR/Cas9 works currently worse than in-vitro fertilization with preimplantation genetic diagnosis for obtaining healthy embryos in cases where the parents are carriers of a mutation. Considering that in-vitro fertilization would be required to apply these techniques in the germline, there does not really appear to be any justification for its application for this purpose.

Pre-implantation genetic diagnosis in ART: cleavage-stage vs trophectoderm biopsy



The PGD entails the opening of the zona pellucida and the removal of embryonic cell(s) from an embryo. It implies that the embryo undergoes physical interventions twice, namely, microinjection of the genome editing system, and the biopsy for PGD. If ICSI is used to increase a success rate of fertilization and avoid polyspermy, three interventions are conducted. Such physical interventions might affect the subsequent development of the embryos in vitro or in vivo.

Accurate genetic testing depends on biopsied embryonic cell(s). Since a cleavage-stage embryo is composed of six to eight cells, a single cell biopsy is widely used for PGD.
However, mosaicism which affects 15-80% of embryos may impact the interpretation of PGD results. Meanwhile, in the blastocyst stage, the embryo consists of approximately 130 cells in the inner cell mass which subsequently develops into the fetus and the surrounding trophectoderm. Trophectoderm cells have been recently biopsied from a blastocyst for PGD in order to avoid damaging the embryo. Although mosaicism remains at the blastocyst stage, the result of a recent randomized clinical trial supports that a single cell biopsy at the cleavage-stage is more significantly damaging to the embryo than biopsy at the blastocyst stage, and resulted in poorer clinical outcomes. Therefore, sufficiently optimized, **trophectoderm biopsy-based PGD may be effective in the zygote approach**.

Germline gene editing and ethics

Inform consent

Enhanced prejudice towards disable people

Enhanced health inequalities → ART is generally expensive and creates disparities in access to this infertility services even in a country or a state with insurance coverage. Would the insurance coverage. Would this access to this preventive medicine be completely confined to the wealthier segment?

Non-health-related enhancement purposes

Gene editing and eugenics

The prospect of human gene editing inevitably recalls past abuses of human rights involving the biological sciences, and especially the history of eugenics in the first half of the 20th century.

Eugenics was not only an ideology but was embraced by physicians, mental health professionals, and scientists. Eugenics posited that unfit human traits known as criminality, feeble-mindedness, and pauperism were inherited genetically in the same way as physical characteristics. At the time, eugenic ideas led to widespread forced sterilization and immigration restrictions for individuals and groups thought to be genetically inferior. Only when the Nazis took eugenic ideas to horrific extremes was the concept thoroughly discredited.

Though eugenics is no longer a powerful movement, several of the forces that animated the eugenics movement a century ago remain vital

- **economic forces to reduce health care costs could put pressure on people to change genetic sequences associated with disease**
- the belief that genes influence particular behaviors or other complex traits could lead to pressures to change those genes in future generations. And **consumer demand for particular attributes in offspring could lead people to pursue private sector options for human gene editing that are difficult to regulate**

A survey of 1700 women who formed their families using donor spermatozoa

Neroli Sawyer [a], Eric Blyth [b], Wendy Kramer [c], Lucy Frith [d,*]

Other than health, women wanted to know the intelligence, height and ethnicity of sperm donors.

The position(s) of patient advocacy groups

Patient advocacy groups are extremely heterogeneous:

"Ban editing of human germline genome because of the moral status of the embryo / human dignity"
"hell yes"
"we need to look at this scientifically"
"WE NEED TO LOOK AT THE ETHICS"
"*let's talk about this when the scientists have all the technology straight*"
"**Gene editing will be acceptable when its benefits, both to individuals and to the broader society, exceeds its risks, though the relevant risks and benefits and levels of acceptable risk are today uncertain**"
"GENE EDITING PROVIDES A MEANS OF EVOLVING BY A PROCESS MORE RATIONAL AND MUCH QUICKER THAN DARWINIAN EVOLUTION"

Members of patient communities are fighting hard to eliminate diseases while also working to change physical and social environments so that all people can live productive and fulfilling lives.
The line between diversity and disability is fuzzy. Biomedical researchers can overlook and thereby reinforce stigma and social disparity by treating certain conditions as disabilities that need to be "fixed" through biomedical interventions.

Governance is becoming increasingly international and participatory, especially given the role that the public now plays in shaping policies. It's no longer possible to control technologies by the laws of one country. If there is a demand for a technology, people will go to whichever country has it.

A major component of the National Academy of Sciences and the National Academy of Medicine's Human Gene-Editing Initiative is an international summit that took place December 1-3 in Washington, D.C. Co-hosted with the Chinese Academy of Sciences and the U.K.'s Royal Society, the summit convened experts from around the world to discuss the scientific, ethical, and governance issues associated with human gene-editing research.
After these three days of thoughtful discussion, the organizing committee for the summit issued a statement on human gene-editing research and its potential applications, including uses that could alter the human germline.

The summit brought together more than 500 people from around the world for three days of presentations and deliberations on the scientific, ethical, legal, social, and governance issues associated with human gene editing, while an additional 3,000 people watched the summit online.

Opening remark

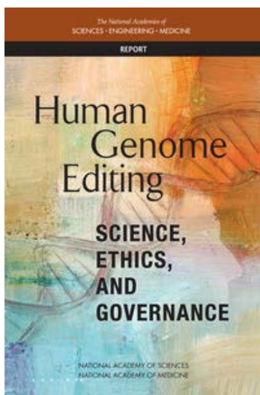
We could be on the cusp of a new era in human history. Today, we sense that we are close to being able to alter human heredity. Now we must face the questions that arise. How, if at all, do we as a society want to use this capability?

Innovation *vs* Precaution?

Innovation and precaution do not need to be mutually exclusive.

Innovation *and* Precaution?

They can be complementary, with public understanding and effective oversight creating the confidence needed to support risk-taking and novel technologies.



Basic Science Research

Basic research involving both somatic and germline cells is essential to the advancement of science and should continue with existing regulatory structures.

Somatic Cell Editing for Treatment and Prevention of Disease and Disability

There is no single standard for somatic genome editing efficiency or specificity—and no single acceptable off-target rate—that can be defined at this time, as this must be evaluated in light of the particular intended use and technique.

Potential Use of Genome Editing for "Enhancement"

Somatic genome editing for purposes other than treatment or prevention of disease and disability should not proceed at this time.

Germline Editing for Treatment or Prevention of Disease or Disability

Criteria under which heritable germline editing could be permitted:
- absence of reasonable alternatives
- restriction to preventing a serious disease or condition;
- restriction to editing genes that have been convincingly demonstrated to cause or strongly predispose to that disease or condition;
- restriction to converting such genes to versions that are prevalent in the population and are known to be associated with ordinary health with little or no evidence of adverse effects;
- availability of credible pre-clinical and/or clinical data on risks and potential health benefits of the procedures;
- during the trial, ongoing, rigorous oversight of the effects of the procedure on the health and safety of the research participants;
- comprehensive plans for long-term multigenerational follow-up that still respect personal autonomy;
- maximum transparency consistent with patient privacy;
- continued reassessment of both health and societal benefits and risks, with broad, ongoing participation and input from the public;
- reliable oversight mechanisms to prevent extension to uses other than preventing a serious disease or condition.

Current deficiencies in CRISPR-Cas9 technology

- may fail to induce a biallelic modification in an animal, thereby resulting in only an animal with a monoallelic modification
- could cause off-target mutations other than desired gene modification in a target sequence (tolerance of Cas9 to mismatches in the RNA guide sequence), which could inactivate essential genes, activate cancer-causing genes, or cause chromosomal rearrangements (many drugs cause off-target effects but are still effective)
- can induce mosaic modifications in which wild-type cells, including germline cells, and genetically modified cells coexist in the same organism
- can generate immune responses if introduced into the body
- limited by PAM motif

High-fidelity CRISPR-Cas9 nuclease variants

High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects

Rationally engineered Cas9 nucleases with improved specificity

Ian M. Slaymaker, Linyi Gao, Bernd Zetsche, David A. Scott, Winston X. Yan, Feng Zhang

sciencemag.org SCIENCE

A highly specific SpCas9 variant is identified by *in vivo* screening in yeast

Antonio Casini, Michele Olivieri, Gianluca Petris, Claudia Montagna, Giordano Reginato, Giulia Maule, Francesca Lorenzin, Davide Prandi, Alessandro Romanel, Francesca Demichelis, Alberto Inga & Anna Cereseto

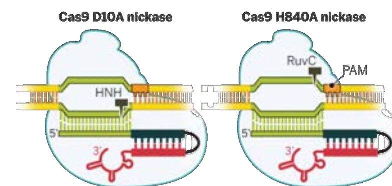
NATURE BIOTECHNOLOGY ADVANCE ONLINE PUBLICATION

Enhanced proofreading governs CRISPR–Cas9 targeting accuracy

Janice S. Chen, Yavuz S. Dagdas, Benjamin P. Kleinstiver, Moira M. Welch, Alexander A. Sousa, Lucas B. Harrington, Samuel H. Sternberg, J. Keith Joung, Ahmet Yildiz & Jennifer A. Doudna

408 | NATURE | VOL 550 | 19 OCTOBER 2017

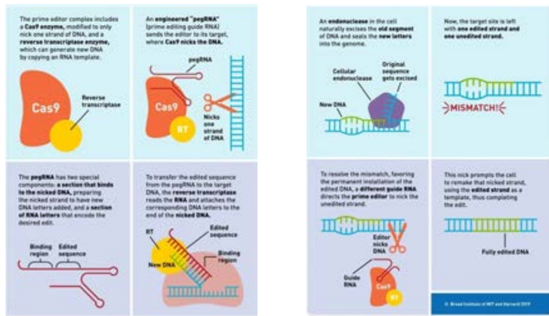
Variants of the Cas9 systems



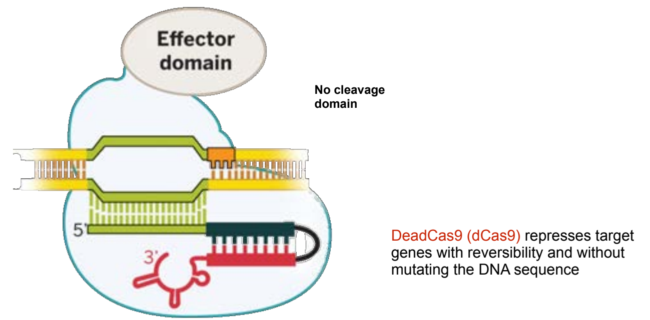
Only one strand of the DNA will be cut

two properly targeted Cas9n molecules are required to efficiently create DSBs at the target locus, which greatly enhances specificity compared to wild-type SpCas9

Prime Editing

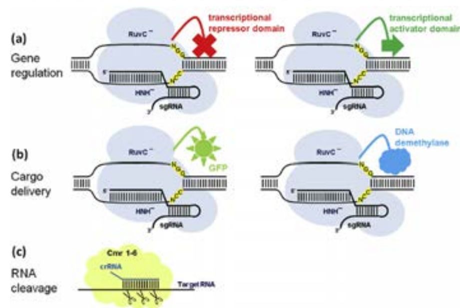


Variants of the Cas9 systems



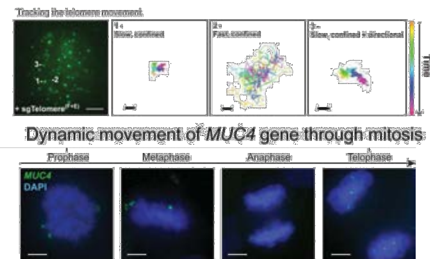
DeadCas9 (dCas9) represses target genes with reversibility and without mutating the DNA sequence

Fusion of dCas9 with activator/ repressor/fluorescent domains



Dynamic Imaging of genomic loci

GFP attached to a nuclease-deficient Cas9 (dCas9)

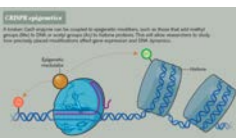


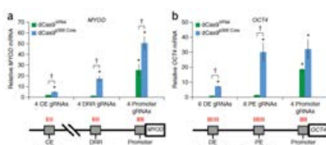
Chen *et al.*, Cell, 2013, Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system

Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers

Isaac B Hilton[1,2], Anthony M D'Ippolito[2,3], Christopher M Vockley[2,4], Pratiksha I Thakore[1,2], Gregory E Crawford[2,5], Timothy E Reddy[2,6] & Charles A Gersbach[1,2,7] 17 (2015)

In the past few years, millions of dollars have been poured into cataloguing epigenetic marks in different human cells, and their patterns have been correlated with everything from brain activity to tumour growth.
But without the ability to alter the marks at specific sites, researchers were unable to determine whether they cause biological changes…





The dCas9p300 Core fusion protein activates transcription of endogenous genes from distal enhancer regions.

The human *MYOD* locus is schematically depicted with corresponding gRNA locations in red. CE, MyoD core enhancer; DRR, MyoD distal regulatory region.

The human OCT4 locus is schematically depicted with corresponding gRNA locations in red. DE, Oct4 distal enhancer; PE, Oct4 proximal enhancer.

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CRISPR CODE CRACKING

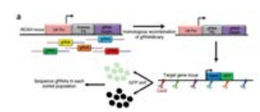
annotation of the non-coding genome

- More than 98% of the human genome does not code for proteins.
- Some of it codes for RNA molecules — such as microRNAs and long non-coding RNAs — that are thought to have functions apart from making proteins.
- Other sequences are 'enhancers' that amplify the expression of the genes under their command.
- Most of the DNA sequences linked to the risk of common diseases lie in regions of the genome that contain non-coding RNA and enhancers.

High-throughput mapping of regulatory DNA

Nisha Rajagopal[1], Sharanya Srinivasan[1,2], Kameron Kooshesh[2,3], Yuchun Guo[1], Matthew D Edwards[1], Budhaditya Banerjee[2], Tahin Syed[1], Bart J M Emons[2,4], David K Gifford[1] & Richard I Sherwood[2]

Quantifying the effects of *cis*-regulatory DNA on gene expression is a major challenge. Here, we present the multiplexed editing regulatory assay (MERA), a high-throughput CRISPR-Cas9–based approach that analyzes the functional impact of the regulatory genome in its native context. MERA tiles thousands of mutations across ~40 kb of *cis*-regulatory genomic space and uses knock-in green fluorescent protein (GFP) reporters to read out gene activity. Using this approach, we obtain quantitative information on the contribution of *cis*-regulatory regions to gene expression. We identify proximal and distal regulatory elements necessary for expression of four embryonic stem cell–specific genes. We show a consistent contribution of neighboring gene promoters to gene expression and identify unmarked regulatory elements (UREs) that control gene expression but do not have typical enhancer epigenetic or chromatin features. We compare thousands of functional and nonfunctional genotypes at a genomic location and identify the base pair–resolution functional motifs of regulatory elements.



VOLUME 34 NUMBER 2 FEBRUARY 2016 NATURE BIOTECHNOLOGY

Search for Cas9 relatives

Article

Cell

Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System

Graphical Abstract

Authors

Bernd Zetsche, Jonathan S. Gootenberg, Omar O. Abudayyeh, ..., Aviv Regev, Eugene V. Koonin, Feng Zhang

Correspondence

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In Brief

Cpf1 is a RNA-guided DNA nuclease that provides immunity in bacteria and can be adapted for genome editing in mammalian cells.

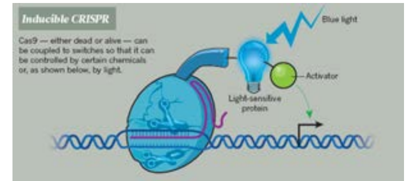
Cell 163, 759–771, October 22, 2015 ©2015 Elsevier Inc.

Highlights

- CRISPR-Cpf1 is a class 2 CRISPR system
- Cpf1 is a CRISPR-associated two-component RNA-programmable DNA nuclease
- Targeted DNA is cleaved as a 5-nt staggered cut distal to a 5' T-rich PAM
- Two Cpf1 orthologs exhibit robust nuclease activity in human cells

… but few alternative enzymes found so far work as well as the most popular Cas9

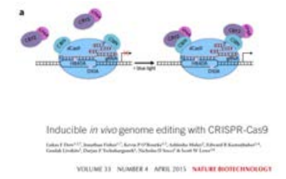
Inducible Cas9



A light-inducible CRISPR-Cas9 system for control of endogenous gene activation

Lauren R Polstein & Charles A Gersbach

NATURE CHEMICAL BIOLOGY | VOL 11 | MARCH 2015 | www.nature.com/naturechemicalbiology



Photoactivatable CRISPR-Cas9 for optogenetic genome editing

Yuta Nihongaki, Fuun Kawano, Takahiro Nakajima & Moritoshi Sato

NATURE BIOTECHNOLOGY

Inducible *in vivo* genome editing with CRISPR-Cas9

VOLUME 33 NUMBER 4 APRIL 2015 NATURE BIOTECHNOLOGY

Other uses of the technology

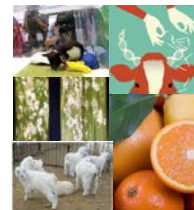
Biology		Biotechnology		Biomedicine
Cell lines	**Model organisms**	**Crop plants**	**Fungi**	Organoids
HEK293	Mice	Rice	*Kluyveromyces*	hESCs
U2OS	Rats	Wheat	*Chlamydomonas*	iPSCs
K562	Fruit flies	Sorghum		
	Nematodes	Tobacco		
	Arabidopsis			
	Salamanders			
	Frogs			
	Monkeys			

Gene editing vs GMOs

Process-based or product-based GMO regulations

Traceability

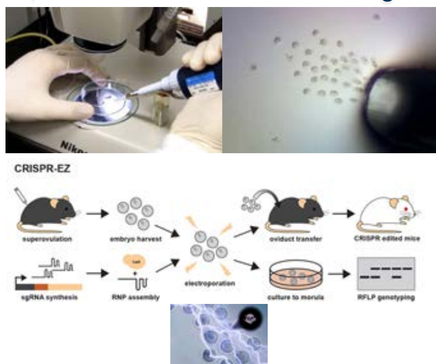
Reversibility



CRISPR on the farm

- petite pigs
- disease-resistant wheat and rice
- dehorned cattle
- disease-resistant goats
- vitamin-enriched sweet oranges

Faster, more efficient CRISPR editing in mice



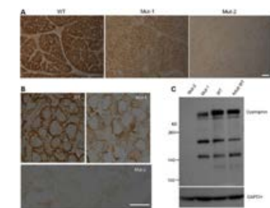
ORIGINAL ARTICLE

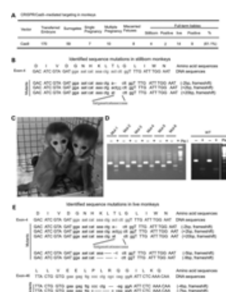
Human Molecular Genetics, 2015, Vol. 24, No. 13 3764–3774

Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9

Yongchang Chen[1,3,5,†], Yinghui Zheng[2,†], Yu Kang[1,3,5,†], Weili Yang[2,†], Yuyu Niu[1,3,5], Xiangyu Guo[2], Zhuchi Tu[2], Chenyang Si[1,5], Hong Wang[1,5], Ruxiao Xing[2], Xiuqiong Pu[1,5], Shang-Hsun Yang[6], Shihua Li[4], Weizhi Ji[1,3,5,*] and Xiao-Jiang Li[2,4,*]

[1]Yunnan Key Laboratory of Primate Biomedical Research, Kunming 650500, China, [2]State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, 10010, China, [3]Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, 650500, China, [4]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, 30322, USA, [5]Kunming Biomed International and National Engineering Research Center of Biomedicine and Animal Science, Kunming, 650500, China and [6]Department of Physiology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan





CRISPR and gene drive

nature
biotechnology

OPEN

A CRISPR–Cas9 gene drive targeting *doublesex* causes complete population suppression in caged *Anopheles gambiae* mosquitoes

Kyros Kyrou[1,2], Andrew M Hammond[1,2], Roberto Galizi[1], Nace Kranjc[1], Austin Burt[1], Andrea K Beaghton[1], Tony Nolan[1] & Andrea Crisanti[1]

In the human malaria vector *Anopheles gambiae*, the gene *doublesex* (*Agdsx*) encodes two alternatively spliced transcripts, *dsx-female* (*AgdsxF*) and *dsx-male* (*AgdsxM*), that control differentiation of the two sexes. The female transcript, unlike the male, contains an exon (exon 5) whose sequence is highly conserved in all *Anopheles* mosquitoes so far analyzed. We found that CRISPR–Cas9-targeted disruption of the intron 4–exon 5 boundary aimed at blocking the formation of functional AgdsxF did not affect male development or fertility, whereas females homozygous for the disrupted allele showed an intersex phenotype and complete sterility. A CRISPR–Cas9 gene drive construct targeting this same sequence spread rapidly in caged mosquitoes, reaching 100% prevalence within 7–11 generations while progressively reducing egg production to the point of total population collapse. Owing to functional constraint of the target sequence, no selection of alleles resistant to the gene drive occurred in these laboratory experiments. Cas9-resistant variants arose in each generation at the target site but did not block the spread of the drive.

Received 6 April; accepted 3 August; published online 24 September 2018; doi:10.1038/nbt.4245

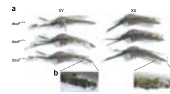
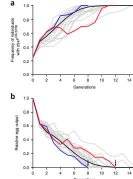
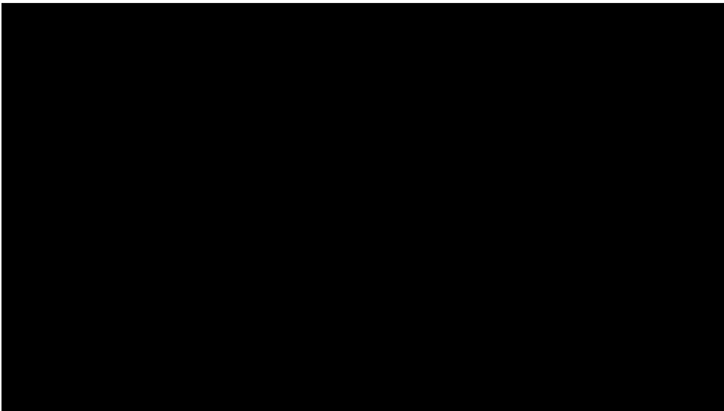


Figure 2 Morphological analysis of homozygous $dsxF^{-/-}$ mutants. (**a**) Morphological appearance of genetic males and females heterozygous ($dsxF^{+/-}$) or homozygous ($dsxF^{-/-}$) for the exon 5 null allele. This assay was performed in a strain containing a dominant RFP marker linked to the Y chromosome, whose presence permits unambiguous determination of male or female genotype. Anomalies in sexual morphology were observed only in $dsxF^{-/-}$ genetic female mosquitoes. This group of XX individuals showed male-specific traits, including a plumose antenna (red arrowhead) and claspers (blue arrowheads). This group also showed anomalies in the proboscis and accordingly they could not bite and feed on blood. Representative samples of each genotype are shown. (**b**) Magnification of the external genitalia. All $dsxF^{-/-}$ females carried claspers, a male-specific characteristic. The claspers were dorsally rotated rather than in the normal ventral position.





CRISPR and gene drive