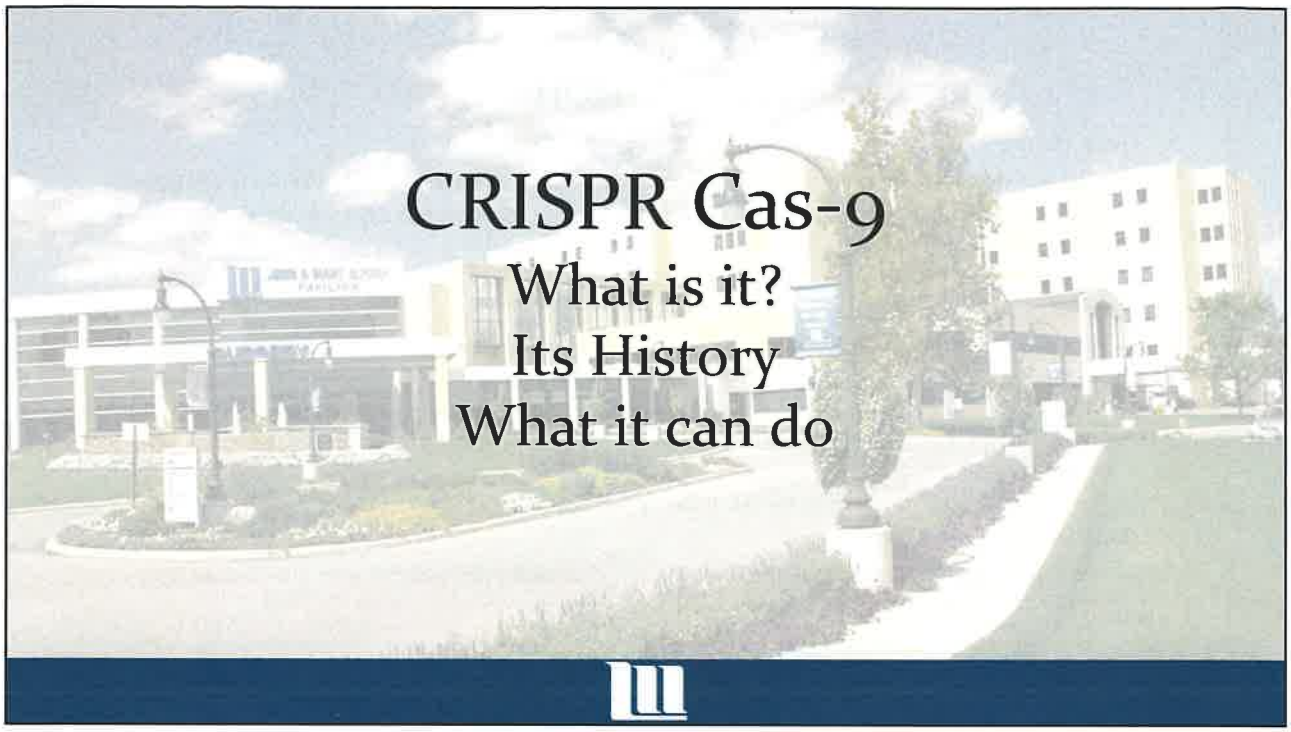
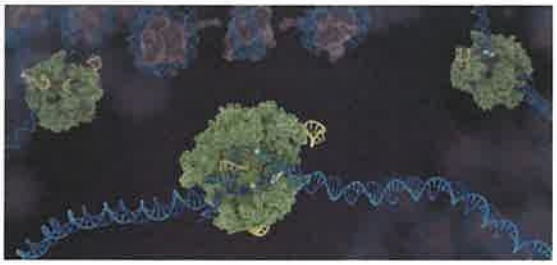
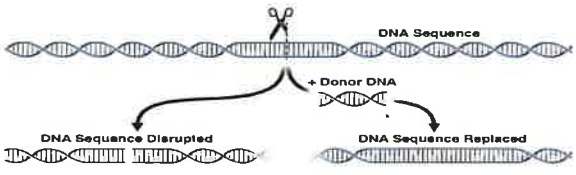


What is it?

- An RNA-guided genetic engineering tool that uses a CRISPR sequence of DNA and its associated protein to edit the base pairs of a gene.
  - An improved innovation; simpler, and more efficient with respect to both time and money over other gene-editing systems:
    - Engineered meganucleases
    - Zinc-finger nucleases (ZFN)
    - Transcription activator-like effector nucleases. (TALEN)
  - These systems are protein-guided and more time-consuming and less efficient that CRISPR.





Definitions

- Gene Therapy
  - The introduction of an exogenous gene or genes into one or more autologous or allogeneic cell types.
  - Example: V*ortigene neparvovec*, wildtype human RPE65 complementary DNA via Lentivirus to patients with Leber's congenital amaurosis.
    - ≈ 1000-3000 prevalence at $850,000 per one time treatment.
- Gene Silencing
  - Does not add or alter the primary genetic information in patients' cells, but uses molecular methods to reduce the expression of one or more genes.
  - Example: *Inotersen,* an "antisense oligonucleotide" that inhibits the hepatic production of transthyretin, a protein that causes the polyneuropathy of *hereditary transthyretin-mediated amyloidosis.*
    - *Prevalence 50,000 patients worldwide* at 284 mg/wk. = $10,691.40 /wk. =$555,952.80 / year.
- Gene Editing
  - Sequence-specific alterations in the DNA of a cell using molecular methods with site-directed DNA repair after strand breakage.

What is it?

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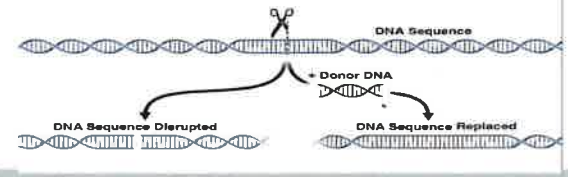
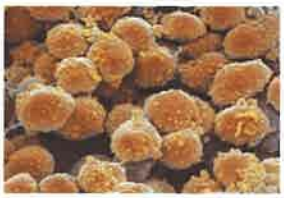


Discovery

- Studied Archaea from the marshes of Santa Pola on Spain's Costa Blanca
  - Mojica, F.J., Juez, G. & Rodríguez-Valera, F. (1993): Transcription at different salinities of Haloferax mediterranei adjacent to partially modified PstI sites. Molecular Microbiology, Vol. 9, Nr. 3, page 613-621, 1993



Archaea (Haloferax mediterranei)

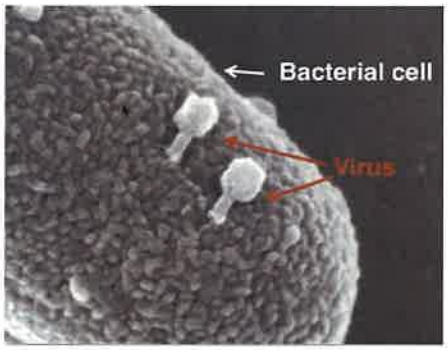
Francisco Mojica

Molecular biologist at the University of Alcante in Spain

Clustered Regularly Interspaced Short Palindromic Repeats

- He found multiple copies of near-perfect, palindromic, repeated sequences of 30 bases, separated by spacers of roughly 36 bases that did not resemble any family of repeats known in microbes.
- By 2000 he had found CRISPR loci in 20 different prokaryotic microbes: Mycobacterium tuberculosis, Clostridium difficile, and Yersina pestis, and E. coli
- He focused on the "spacer sequences" that separated the CRISPR and discovered a gene sequence from a viral phage that infected E. Coli.
- He then studied 4,500 spacers and discovered that two-thirds had sequences of viral or plasmid organisms that infected these bacteria.

Mojica, Francisco et.al. "Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements". Journal of Molecular Evolution. 60 (2): 174–182. 2005.



He concluded that these sequences were a prokaryotic "immune system to protect bacteria form these invading phages

Ruud Jansen

- Utrecht University (Netherlands)
- Collaborated with Mojica to report genes that were associated with *CRISPRS*, which encoded directions for making an enzyme that he labeled "*CRISPR-associated*" or *Cas* enzymes.

Identification of genes that are associated with DNA repeats in prokaryotes;; Jansen, JDA Embden, W Gaastra... - Molecular ..., 2002 -

Luciano Marraffini

- Demonstrated that Bacterial DNA, not RNA interference was the target of CRISPR bacterial immunity and that CRISPR is a programmable system with the potential for genomic editing in heterologous systems.

"Here we show that CRISPR interference prevents conjugation and plasmid transformation in *S. epidermidis*. Insertion of a self-splicing intron into *nickase* blocks interference despite the reconstitution of the target sequence in the spliced mRNA, indicating that the interference machinery targets DNA directly. We conclude that CRISPR loci counteract multiple routes of HGT and can limit the spread of antibiotic resistance in pathogenic bacteria."

Marraffini and Sontheimer : Science. 2008 Dec 19;322 (5909) 1843-1845

Luciano Marraffini Northwestern University

Food Science Contribution

- Starter cultures (*Streptococcus thermophiles*) for cheese and yogurt ≈ $40 billion annually.
  - Viral phages are a serious economic threat
- Bacteria following a large phage attack had new spacers with sequences from these viruses that was now a part of the bacteria's DNA and transmitted to future generations and conferred resistance to these viruses.
  - Confirmation of Mojica's work.

Barrangou, Moineau, Horvath, et.al., "CRISPR Provides Acquired Resistance against Viruses and Prokaryotes," Science Mar. 23, 2007.

Phillipe Horvath Danisco Laboratory

Barrangou and Horvath

- Three CRISPR systems with a cascade of endonucleases.
- Their studies focused on type 2 based on Cas-9 and when Cas-9 was eliminated resistance disappeared.
  - *Barrangou and Horvath:"A Decade of Discovery:CRISPR Functions and Applications,"Nature microbiology, 2017 - nature.com*

Table 1. Classification and Examples of CRISPR Systems

Class	Type	Subtype	Hallmarks	Example effector	Example organism	Studies Cited
Class 1	Type I		multisubunit effector complex; Cas3	Cascade	*E. coli*	Brouns et al., 2008
	Type III	III-A	multisubunit effector complex; Csm effector module; DNA targeting	Csm10-Csm	*S. epidermidis*	Marraffini and Sontheimer, 2008
		III-B	multisubunit effector complex; Cmr effector module; RNA targeting	Cmr	*P. furiosus*	Hale et al., 2009
Class 2	Type II		single protein effector; tracrRNA	Cas9	*S. thermophilus*	Bolotin et al., 2005; Barrangou et al., 2007; Sapranauskas et al., 2011; Gasiunas et al., 2012
					S. pyogenes	Deltcheva et al., 2011; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013
	Type V		single protein effector; single-RNA guided	Cpf1	*F. novicida*	Zetsche et al., 2015

CRISPR systems are currently organized into two overarching classes: Class 1, which contain multi-subunit effectors, and Class 2, which contain single protein effectors. These classes are subdivided into five types (Makarova et al., 2015), with type IV remaining a putative type within Class 1. Although only Class 2 systems have been adapted for genome engineering, the results described in this review emerged from studying a diversity of CRISPR-Cas systems. (Type III-B systems are not discussed but represent an unusual system that targets RNA rather than DNA (Hale et al., 2009).)

Lander, Cell 164, January 14, 2016

John Van der Oost

- Demonstrated that the CRISPR-Cas-9 system uses an RNA-guided mechanism (Cas-RNA), a small segment of RNA that contained genetic coding from a virus that had attacked the bacteria in the past, to target DNA.
- His team created an artificial CRISPR array of four genes in a lambda (λ) phage and inserted it into a bacterium that showed resistance to the λ phage following inoculation. (a flu shot for bacteria).

Van der Oost et. al. (2014) "Unraveling the Structural and Mechanistic Basis of CRISPR;" Cas-9 Systems. Nat. Rev. Microbiol. 12 479- 492

John Van der Oost Wageningen University Netherlands

Emmanuelle Charpentier

- Discovered that an additional RNA segment, trans-activating RNA (tracrRNA) was necessary for CRISPR to work.
  - It facilitates the making of crRNA, the sequence that carries the memory of the virus that had previously attacked the bacteria.
  - It serves as a handle to latch on to the invading virus so that crRNA can target the correct locus for the Cas-9 enzyme to cleave.

Deltcheva, Chylinski, Vogel, and Charpentier. "CRISPR RNA maturation by trans-coded small RNA and host factor RNase III; Nature 2011 Mar 31;471 (7340) 602-7.

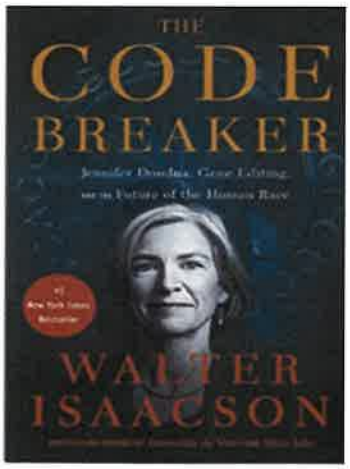
Feng Zhang

- Background in eukaryotic genetic engineering:
  - Engineered meganucleases, zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALENS)
- Applied CRISPR to eukaryotes and mammalian cells.

Ran,Hsu, Wright, Agarwala, Scott, and Zhang. "Genome engineering using the CRISPR-Cas9 system;" Nat Protoc 2013 Nov;8(11); 2281-2301.

Feng Zhang
Broad Institute of MIT and Harvard

Collegial Rivalry



Cell

Leading Edge
Perspective

The Heroes of CRISPR

Eric S. Lander[1,2,3,*]
[1]Broad Institute of MIT and Harvard, 415 Main Street, Cambridge, MA 02142, USA
[2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[3]Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA
[*]Correspondence: lander@broadinstitute.org
http://dx.doi.org/10.1016/j.cell.2015.12.041

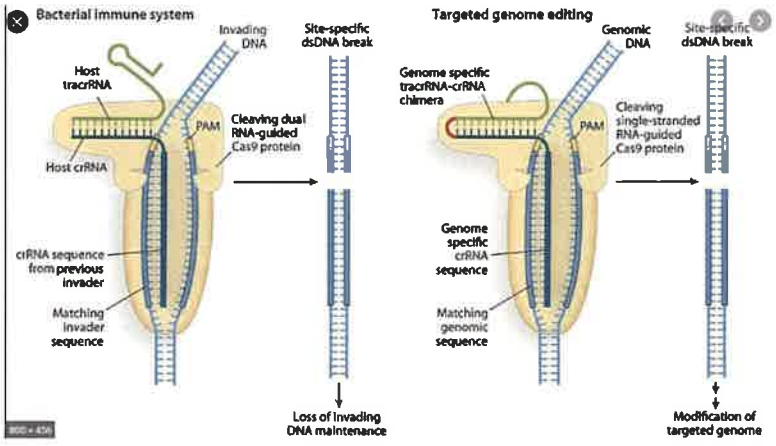
Three years ago, scientists reported that CRISPR technology can enable precise and efficient genome editing in living eukaryotic cells. Since then, the method has taken the scientific community by storm, with thousands of labs using it for applications from biomedicine to agriculture. Yet, the preceding 20-year journey—the discovery of a strange microbial repeat sequence; its recognition as an adaptive immune system; its biological characterization; and its repurposing for genome engineering—remains little known. This Perspective aims to fill in this backstory—the history of ideas and the stories of pioneers—and draw lessons about the remarkable ecosystem underlying scientific discovery.

Doudna and Charpentier

- Two landmark innovations:
  1. tracrRNA guiding crRNA and coordinating it with Cas-9 to target and cut a specific locus on DNA.
  2. Constructing fusion of both these RNA fragments into a single-guide RNA to make the process simpler, more ecconical, and more precise.

Science 2012 August 17;337(6096): 816-821

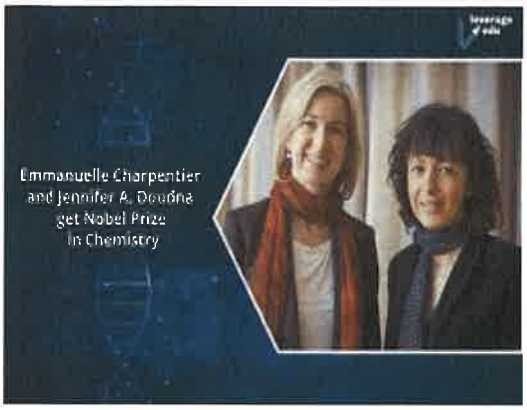
Emmanuelle Charpentier, Jennifer Doudna, Martin Jinek, and Krzysztof Chylinski



Gene Editing with a single RNA chimera

Single-guide RNA with cleavage by Cas-9 endonuclease targeting DNA at the protospacer adjacent motif (PAM) and editing a new strand of DNA

2020 Nobel Prize in Chemistry



So What Can CRISPR Do?

Good, Bad, and Ugly

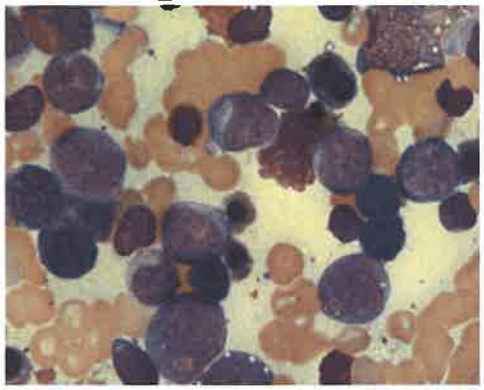
Alterations in over 3000 human genes are known to be associated with diseases:

BioMed Research International. 2019 Oct 7: pp 1-15.

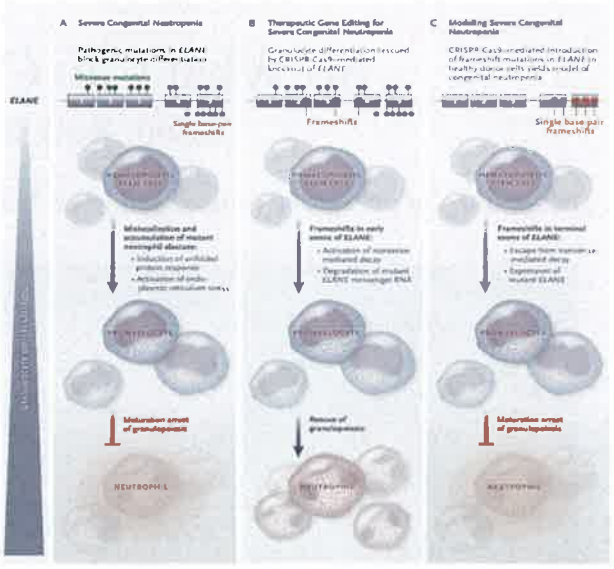
Monogenic Disorders	Multifactorial Diseases
Cystic Fibrosis	HPV Cancers
Sickle cell Anemia	PD-1 Cancers
B Thalassemia	P53 Cancers
Huntington's Disease	Diabetes
Duchenne Muscular Dystrophy	ASCVD (PCSK-9, ApoE and LDLR)
Chronic Granulomatous Disease	Congenital Neutropenia

Congenital Neutropenia

- Neutropenia present at birth affecting the myeloid series:
  - Schwachman-Diamond Syndrome
  - Chediak-Higashi syndrome
  - Kostmann Syndrome
  - Severe Congenital Neutropenia
    - 2-3 cases /million population
    - ANC < 200/microL and ↑monocytes (30-50%)
    - Treatment with G-CSF and HSCT
    - Mortality 0.81%/yr. with cumulative incidence of death at 15 years of therapy with G-CSF 10% [95CI] (6-14%)
    - ↑myeloid and lymphoid cancers with survival



Normal to decreased cellularity with myeloid arrest at the promyelocyte/myelocyte stage. Often with atypical nuclei and cytoplasmic vacuolization.



Severe Congenital Neutropenia

Genetic transmission

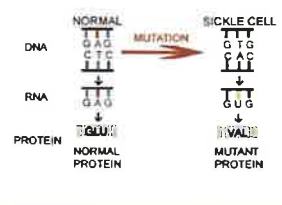
- 50%-60% with autosomal dominant inheritance
- Genetically heterogeneous disorder, which is caused by mutations in more than 30 genes.
  - ≈ 50% of patients have a mutation of the *ELANE* gene that encodes for neutrophil elastase.

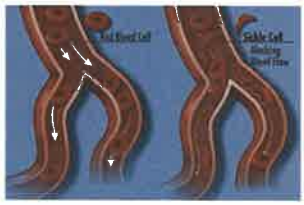
➢ Pre-clinical in-vitro study at Tübingen Univ. introduced frame-shift mutations of *ELANE* in HSCs from patients with SCN via CRISPR-Cas-9, which degraded mutant *ELANE* mRNA with restoration of granulopoeisis.

Skokowa. NEJM; May 20, 2021, pp 1956-1958

Sickle Cell Disease

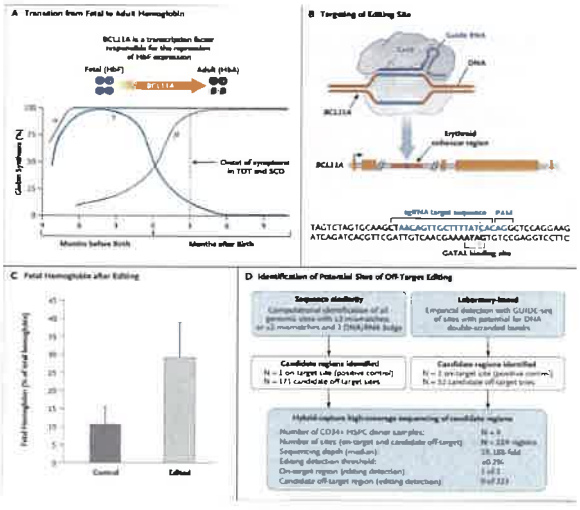
- Most common monogenic disease worldwide
  - 300,000 new cases/yr. with 100,000 new cases/yr. in U.S.
- Caused by a point mutation in the hemoglobin β subunit gene (*HBB*) that replaces glutamic acid with valine at amino acid position 6.
- Multi-organ involvement with painful vaso-occlusive crises and life expectancy shortened by thirty years.





Victoria Gray

- 33-year-old female with SCD (βS/βS and a single ά-globin deletion)
- In the 2-years preceding treatment she had averaged:
  - 7 severe vaso-occlusive episodes per year.
  - 3.5 SCD-related hospitalizations per year.
  - 5 RBC transfusions per year.
  - She applied for bone marrow transplant, but did not have an HLA-matched donor.
- She was treated with CRISPR Cas-9 gene editing at HCA TriStar Centennial Hospital in Nashville Tennessee in 2019.
  - Hematopoietic stem and progenitor cells (HSPCs) at the erythroid-specific enhancer region of *BCL11A* to reduce *BCL11A* expression in erythroid-lineage cells, restore γ-globin synthesis, and reactivate production of fetal hemoglobin.



Frangoul, NEJM 384;3 Jan 21,2021

Germline Genomic Editing

- Human Embryonic Genomic Studies.
  - Francis Crick Institute (London)
  - Studied donated viable supernumerary human fertilized IVF zygotes to measure blastogenesis with CRISPR mediated OCT 4 (octamer-binding transcription factor 4) from the POU5F1 gene.
    - *"WE conclude that CRISPR-Cas9 mediated genome editing is a powerful method for investigating gene function in the context of human development."*

      Fogarty et. al. Nature 2017 Oct 05; 550(7674): 67-73
    - Study obtained ethical approval, but ethics have been questioned:

      Niemiec E, Howard H. Computational and Structural Biotechnology Journal; (18) 21 March 2020: 887-896.

- Questions:
  - *"Medical research involving human subjects may only be conducted if the importance of the objective outweighs the risks and burdens to the research subjects. (...) All medical research involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and groups involved in the research in comparison with foreseeable benefits to them and to other individuals or groups affected by the condition under investigation."*

    Declaration of Helsinki
  - *"The requirement that the results of an experiment be susceptible to analysis and characterization before further applications are undertaken cannot be met with human germ-line modification with current methods, because the results of any such manipulation could not be analyzed or understood for decades or generations—a situation incompatible with ethical imperatives and with the scientific method."*

    The American Society for Gene and Cell Therapy

Lulu and Nana

- Professor at Southern University of Science and Technology in Shenzhen, China
- He used CRISPR-Cas9 for germline editing of the CCR5 gene in human embryos to delete the receptor for the HIV virus.
- Viable twin girls were delivered in November 2018.
- This was globally condemned by the scientific community.
  - His study was refused for publication.
  - He was fired from his position
  - He was fined three million yuan ($430,000) and imprisoned for 3 years.

He Jiankui

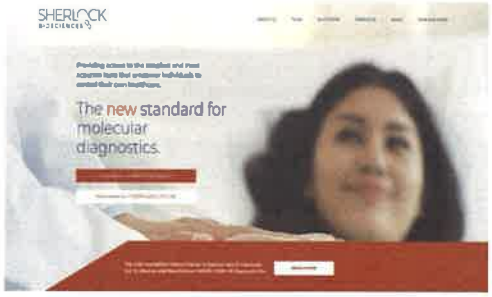
BBC News

Treatments vs. Enhancements

- Huntington's Disease
  - Autosomal Dominant
  - Single gene mutation (IT15) on chromosome 4 that codes for Huntington protein (Huntingtin).
- Sickle Cell Disease
  - Autosomal Recessive
  - Point mutation in the hemoglobin β subunit gene (*HBB*) on chromosome 11.
- Cystic Fibrosis
  - Autosomal recessive
  - Mutations in CFTR gene on chromosome 7
- Sports Performance
  - Muscle strength
    - MSTN gene affects myostatin.
  - Eero Mäntyranta 4-time Olympic champion (1960-1972)
    - Primary familial and congenital polycythemia (autosomal dominant with EPOR gene mutation)
  - ACTN3 gene prevalent in champion distance runners.
- Height
  - CDKN1C gene
- Cognitive performance
  - Memory enhanced in mice with editing of genes for NMDA receptors in nerve cells.
- Skin Color
  - SLC24A5

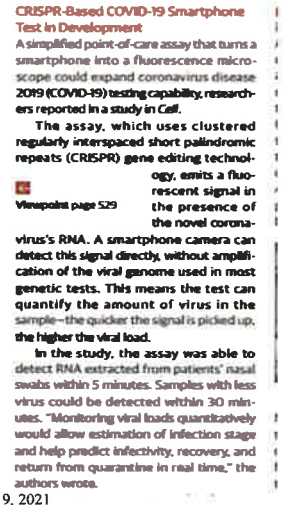
Other Applications

Diagnostic Testing



SHERLOCK

- *Specific high-sensitivity enzymatic reporter unlocking*
- Combined Cas12 and Cas13 to detect both viral DNA and RNA suitable for lateral flow technology



CRISPR-Based COVID-19 Smartphone Test in Development

A simplified point-of-care assay that turns a smartphone into a fluorescence microscope could expand coronavirus disease 2019 (COVID-19) testing capability, researchers reported in a study in *Cell*.

The assay, which uses clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology, emits a fluorescent signal in the presence of the novel coronavirus's RNA. A smartphone camera can detect this signal directly, without amplification of the viral genome used in most genetic tests. This means the test can quantify the amount of virus in the sample—the quicker the signal is picked up, the higher the viral load.

In the study, the assay was able to detect RNA extracted from patients' nasal swabs within 5 minutes. Samples with less virus could be detected within 30 minutes. "Monitoring viral loads quantitatively would allow estimation of infection stage and help predict infectivity, recovery, and return from quarantine in real time," the authors wrote.

Viewpoint page S29

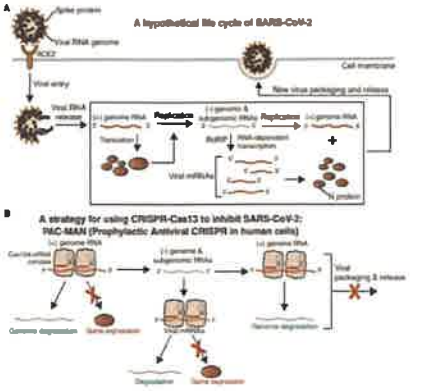
JAMA February 9, 2021

Cell 184, 323-333, January 21,2021

Other Applications

Vaccines

- PAC-Man
  - Prophylactic Antiviral CRISPR in human cells
  - Pre-clinical with research at Berkley and Stanford:
    - Stanley Qi, Timothy Abbot, and Ross Wilson
  - Uses Cas13d as the endonuclease to target:
    - RNA dependent RNA polymerase
    - Nucleocapsid protein
  - Eliminates the potential for the emergence of mutant strains.
  - Technology is applicable to all Coronaviruses both human and zoonotic.
    - Abbott et.al. Development of CRISPR as an antiviral Strategy to Combat SARS-COV-2 and Influenza; *Cell 181, 865-876 May 14, 2020*

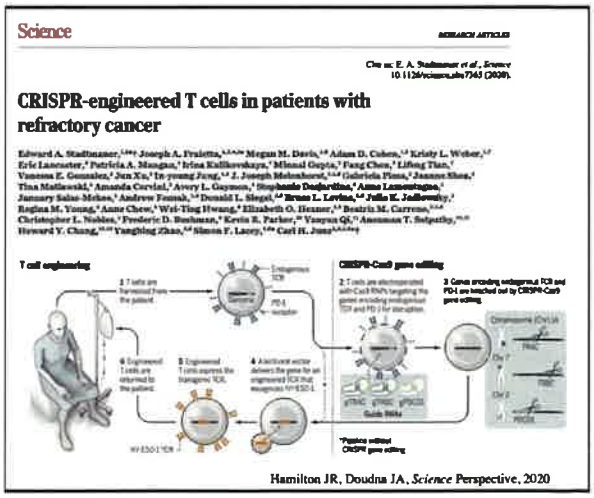


Cell 181, 865-876 May 14, 2020

Other Applications

Cancer

- Phase one trial of 3 patients (2 with refractory myeloma and 1 with metastatic sarcoma) at the Univ. of Pennsylvania.
  - Ex vivo engineered T cells (adoptive T-cells) edited with CRISPR Cas9 to disable PD-1 were infused following lympho-depleting chemotherapy.
  - Transgenic TCR-T-cells were used rather than CAR-T to avoid cytokine storm.
  - A 62-year-old female with advanced myeloma died. Unrelated to her treatment.
  - The 66-year-old sarcoma patient showed a 50% reduction in tumor mass.
  - The 66-year-old female with advanced myeloma did not have progression of her disease over 4 months.
  - No adverse effects from the treatment were cited.


Science

CRISPR-engineered T cells in patients with refractory cancer

Hamilton JR, Doudna JA, *Science* Perspective, 2020

Stadtmauer et. al., Science 367: 28 Feb 2020

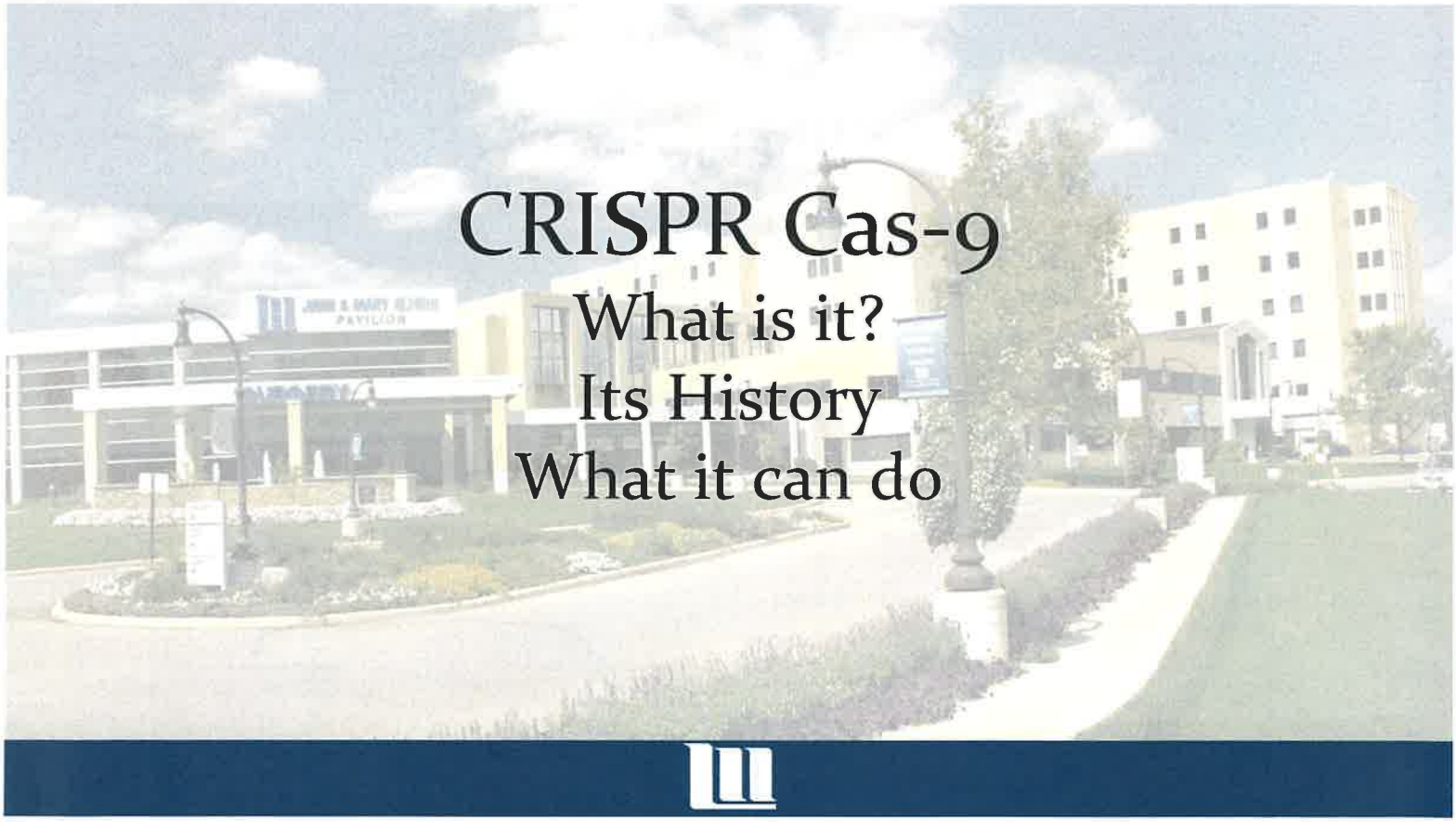
"For first of all we must prepare a Natural and Experimental History, sufficient and good; and this is the foundation of all; for we are not to imagine or suppose, but to discover, what nature does or may be made to do."

(*Priom enim paranda est Historia Naturalis et Experimentalis, suffidens et bona; quod fundamentum rei est; neque enim fingendum, aut excogitandum, sed inveniendum, quid natura faciat aut ferat.)*

Francis Bacon (1561-1626)
In Novum Organum Book 2, Aphorism 10

"Our sole responsibility is to produce something smarter than we are; any problems beyond that are not ours to solve."

Ray Kurzweil (1948-)
The Singularity is Near: When Humans Transcend Biology



CRISPR Cas-9

What is it?

Its History

What it can do

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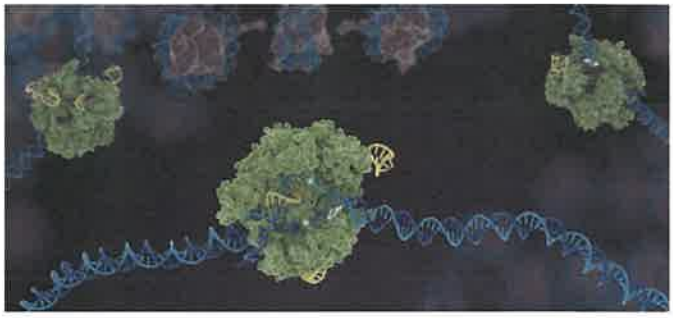

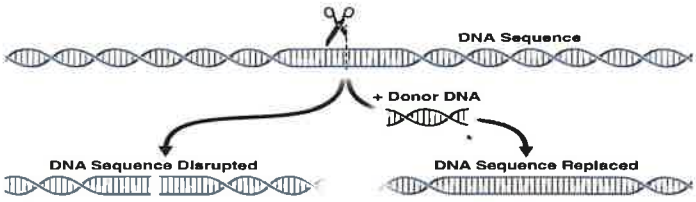

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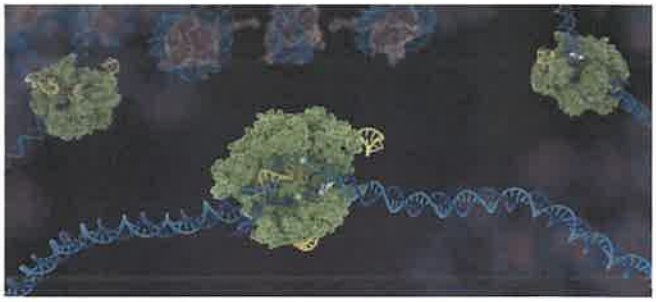
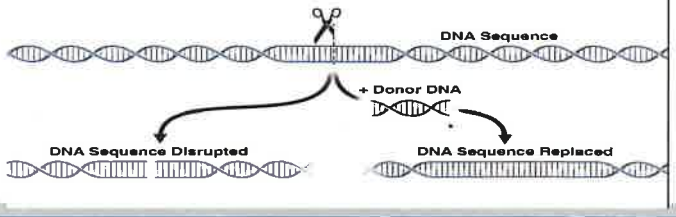
- Gene Silencing
  - Does not add or alter the primary genetic information in patients' cells, but uses molecular methods to reduce the expression of one or more genes.
  - Example: *Inotersen,* an "antisense oligonucleotide" that inhibits the hepatic production of transthyretin, a protein that causes the polyneuropathy of *hereditary transthyretin-mediated amyloidosis.*
    - *Prevalence 50,000 patients worldwide* at 284 mg/wk. = $10,691.40 /wk. =$555,952.80 / year.
- Gene Editing
  - Sequence-specific alterations in the DNA of a cell using molecular methods with site-directed DNA repair after strand breakage.

What is it?

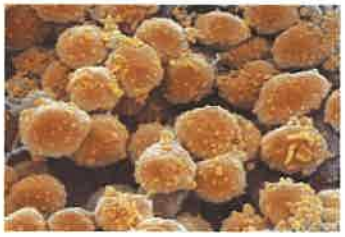
- An RNA-guided genetic engineering tool that uses a CRISPR sequence of DNA and its associated protein to edit the base pairs of a gene.
  - An improved innovation; simpler, and more efficient with respect to both time and money over other gene-editing systems:
    - Engineered meganucleases
    - Zinc-finger nucleases (ZFN)
    - Transcription activator-like effector nucleases. (TALEN)
  - These systems are protein-guided and more time-consuming and less efficient that CRISPR.





Discovery

- Studied Archaea from the marshes of Santa Pola on Spain's Costa Blanca
  - Mojica, F.J., Juez, G. & Rodríguez-Valera, F. (1993): Transcription at different salinities of Haloferax mediterranei adjacent to partially modified PstI sites, Molecular Microbiology, Vol. 9, Nr. 3, page 613-621. 1993



Archaea (Haloferax mediterranei)

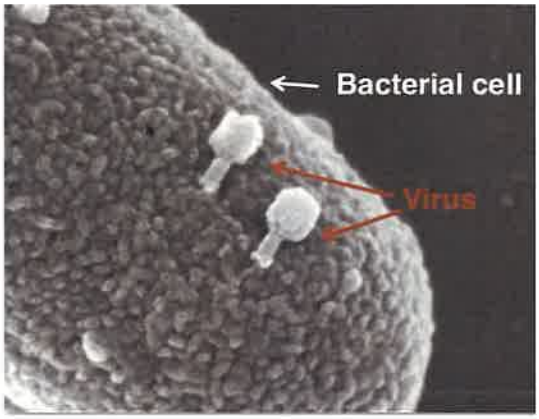
Francisco Mojica

Molecular biologist at the University of Alcante in Spain

Clustered Regularly Interspaced Short Palindromic Repeats

- He found multiple copies of near-perfect, palindromic, repeated sequences of 30 bases, separated by spacers of roughly 36 bases that did not resemble any family of repeats known in microbes.
- By 2000 he had found CRISPR loci in 20 different prokaryotic microbes: Mycobacterium tuberculosis, Clostridium difficile, and Yersina pestis, and E. coli
- He focused on the "spacer sequences" that separated the CRISPR and discovered a gene sequence from a viral phage that infected E. Coli.
- He then studied 4,500 spacers and discovered that two-thirds had sequences of viral or plasmid organisms that infected these bacteria.

*Mojica, Francisco et.al. "Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements". Journal of Molecular Evolution. **60** (2): 174–182. 2005.*



He concluded that these sequences were a prokaryotic "immune system to protect bacteria form these invading phages

Ruud Jansen

- Utrecht University (Netherlands)
- Collaborated with Mojica to report genes that were associated with *CRISPRS*, which encoded directions for making an enzyme that he labeled "*CRISPR-associated*" or *Cas* enzymes.

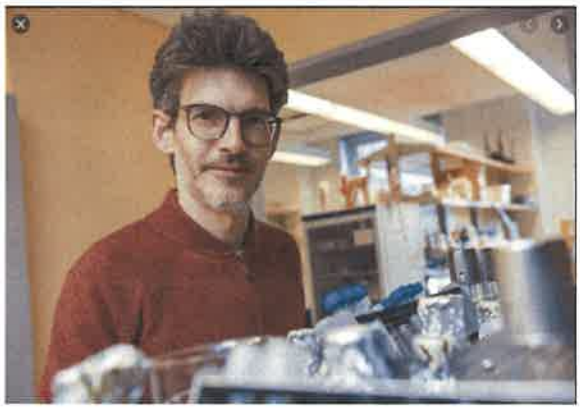
Identification of genes that are associated with DNA repeats in prokaryotes;; Jansen, JDA Embden, W Gaastra... - Molecular ..., 2002 -

Luciano Marraffini

- Demonstrated that Bacterial DNA, not RNA interference was the target of CRISPR bacterial immunity and that CRISPR is a programmable system with the potential for genomic editing in heterologous systems.

"Here we show that CRISPR interference prevents conjugation and plasmid transformation in *S. epidermidis*. Insertion of a self-splicing intron into *nickase* blocks interference despite the reconstitution of the target sequence in the spliced mRNA, indicating that the interference machinery targets DNA directly. We conclude that CRISPR loci counteract multiple routes of HGT and can limit the spread of antibiotic resistance in pathogenic bacteria."

Marraffini and Sontheimer : Science. 2008 Dec 19;322 (5909) 1843-1845



Luciano Marraffini Northwestern University

Food Science Contribution

- Starter cultures (*Streptococcus thermophiles*) for cheese and yogurt ≈ $40 billion annually.
  - Viral phages are a serious economic threat
- Bacteria following a large phage attack had new spacers with sequences from these viruses that was now a part of the bacteria's DNA and transmitted to future generations and conferred resistance to these viruses.
  - Confirmation of Mojica's work.

    Barrangou, Moineau, Horvath, et.al., +CRISPR Provides Acquired Resistance against Viruses and Prokaryotes," Science Mar. 23, 2007.

Phillipe Horvath Danisco Laboratory

Barrangou and Horvath

- Three CRISPR systems with a cascade of endonucleases.
- Their studies focused on type 2 based on Cas-9 and when Cas-9 was eliminated resistance disappeared.
  - *Barrangou and Horvath:"A Decade of Discovery:CRISPR Functions and Applications,"Nature microbiology, 2017 - nature.com*

Table 1. Classification and Examples of CRISPR Systems

Class	Type	Subtype	Hallmarks	Example effector	Example organism	Studies Cited
Class 1	Type I		multisubunit effector complex; Cas3	Cascade	*E. coli*	Brouns et al., 2008
	Type III	III-A	multisubunit effector complex; Csm effector module; DNA targeting	Cas10-Csm	*S. epidermidis*	Marraffini and Sontheimer, 2008
		III-B	multisubunit effector complex; Cmr effector module; RNA targeting	Cmr	*P. furiosus*	Hale et al., 2009
Class 2	Type II		single protein effector; tracrRNA	Cas9	*S. thermophilus*	Bolotin et al., 2005; Barrangou et al., 2007; Sapranauskas et al., 2011; Gasiunas et al., 2012
					S. pyogenes	Deltcheva et al., 2011; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013
	Type V		single protein effector; single-RNA guided	Cpf1	*F. novicida*	Zetsche et al., 2015

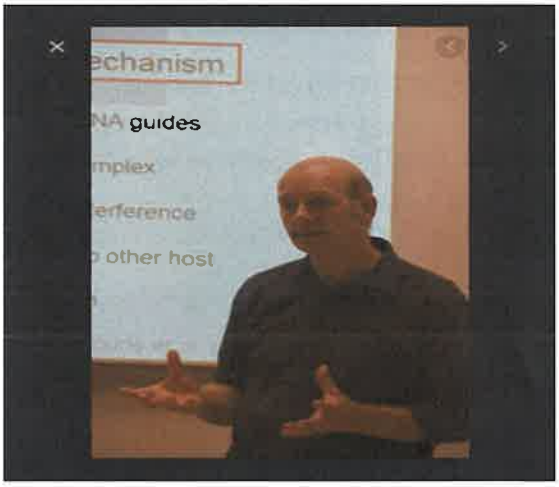
CRISPR systems are currently organized into two overarching classes: Class 1, which contain multi-subunit effectors, and Class 2, which contain single protein effectors. These classes are subdivided into five types (Makarova et al., 2015), with type IV remaining a putative type within Class 1. Although only Class 2 systems have been adapted for genome engineering, the results described in this review emerged from studying a diversity of CRISPR-Cas systems. (Type III-B systems are not discussed but represent an unusual system that targets RNA rather than DNA [Hale et al., 2009].)

Lander, Cell 164, January 14, 2016

John Van der Oost

- Demonstrated that the CRISPR-Cas-9 system uses an RNA-guided mechanism (Cas-RNA), a small segment of RNA that contained genetic coding from a virus that had attacked the bacteria in the past, to target DNA.
- His team created an artificial CRISPR array of four genes in a lambda (λ) phage and inserted it into a bacterium that showed resistance to the λ phage following inoculation. (a flu shot for bacteria).

Van der Oost et. al. (2014) "Unraveling the Structural and Mechanistic Basis of CRISPR;" Cas-9 Systems. Nat. Rev. Microbiol. 12 479- 492



John Van der Oost Wageningen University Netherlands

Emmanuelle Charpentier

- Discovered that an additional RNA segment, trans-activating RNA (tracrRNA) was necessary for CRISPR to work.
  - It facilitates the making of crRNA, the sequence that carries the memory of the virus that had previously attacked the bacteria.
  - It serves as a handle to latch on to the invading virus so that crRNA can target the correct locus for the Cas-9 enzyme to cleave.

Deltcheva, Chylinski, Vogel, and Charpentier. "CRISPR RNA maturation by trans-coded small RNA and host factor RNase III; Nature 2011 Mar 31;471 (7340) 602-7.

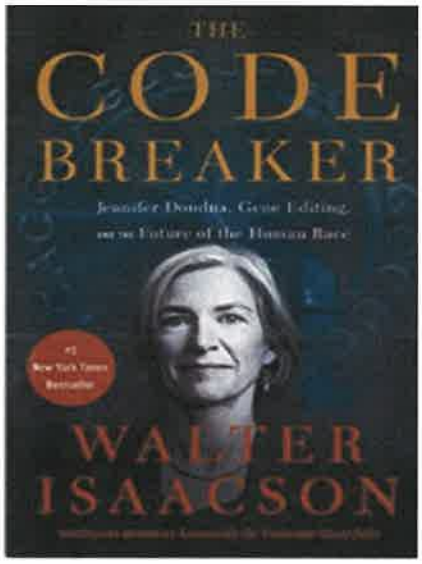
Feng Zhang

- Background in eukaryotic genetic engineering:
  - Engineered meganucleases, zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALENS)
- Applied CRISPR to eukaryotes and mammalian cells.

Ran,Hsu, Wright, Agarwala, Scott, and Zhang. "Genome engineering using the CRISPR-Cas9 system;" Nat Protoc 2013 Nov;8(11); 2281-2301.

Feng Zhang
Broad Institute of MIT and Harvard

Collegial Rivalry



Cell

Leading Edge
Perspective

The Heroes of CRISPR

Eric S. Lander[1,2,3,*]
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http://dx.doi.org/10.1016/j.cell.2015.12.041

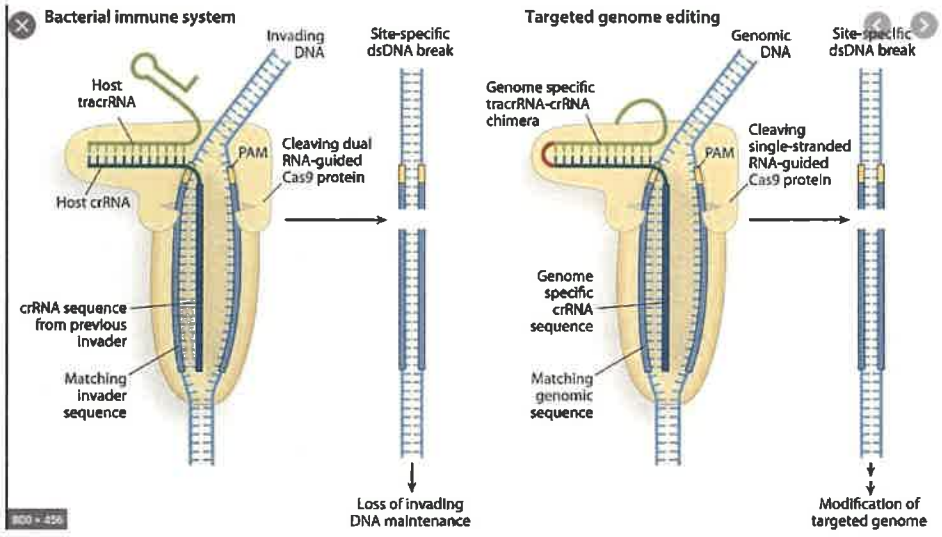
Three years ago, scientists reported that CRISPR technology can enable precise and efficient genome editing in living eukaryotic cells. Since then, the method has taken the scientific community by storm, with thousands of labs using it for applications from biomedicine to agriculture. Yet, the preceding 20-year journey—the discovery of a strange microbial repeat sequence; its recognition as an adaptive immune system; its biological characterization; and its repurposing for genome engineering—remains little known. This Perspective aims to fill in this backstory—the history of ideas and the stories of pioneers—and draw lessons about the remarkable ecosystem underlying scientific discovery.

Doudna and Charpentier

- Two landmark innovations:
  1. tracrRNA guiding crRNA and coordinating it with Cas-9 to target and cut a specific locus on DNA.
  2. Constructing fusion of both these RNA fragments into a single-guide RNA to make the process simpler, more ecconical, and more precise.

Science 2012 August 17;337(6096): 816-821

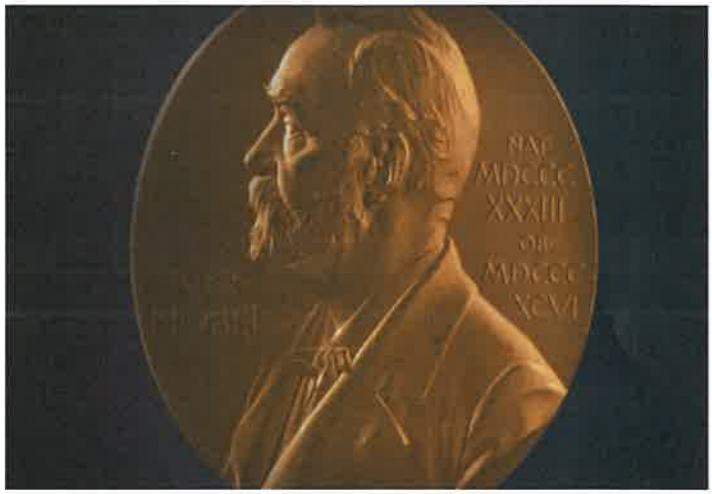
Emmanuelle Charpentier, Jennifer Doudna, Martin Jinek, and Krzysztof Chylinski



Gene Editing with a single RNA chimera

Single-guide RNA with cleavage by Cas-9 endonuclease targeting DNA at the protospacer adjacent motif (PAM) and editing a new strand of DNA

2020 Nobel Prize in Chemistry



So What Can CRISPR Do?

Good, Bad, and Ugly

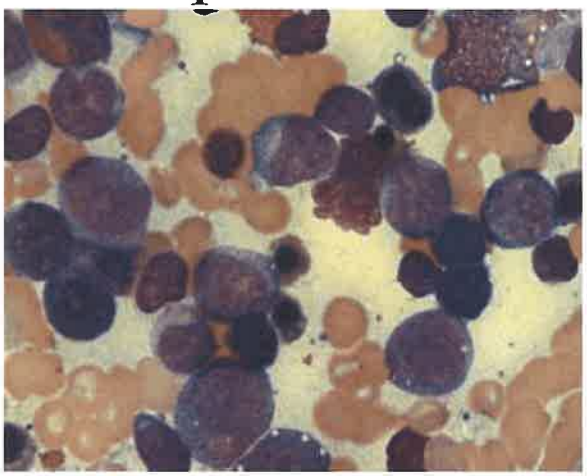
Alterations in over 3000 human genes are known to be associated with diseases:

BioMed Research International. 2019 Oct 7: pp 1-15.

Monogenic Disorders	Multifactorial Diseases
Cystic Fibrosis	HPV Cancers
Sickle cell Anemia	PD-1 Cancers
B Thalassemia	P53 Cancers
Huntington's Disease	Diabetes
Duchenne Muscular Dystrophy	ASCVD (PCSK-9, ApoE and LDLR)
Chronic Granulomatous Disease	Congenital Neutropenia

Congenital Neutropenia

- Neutropenia present at birth affecting the myeloid series:
  - Schwachman-Diamond Syndrome
  - Chediak-Higashi syndrome
  - Kostmann Syndrome
  - Severe Congenital Neutropenia
    - 2-3 cases /million population
    - ANC < 200/microL and ↑monocytes (30-50%)
    - Treatment with G-CSF and HSCT
    - Mortality 0.81%/yr. with cumulative incidence of death at 15 years of therapy with G-CSF 10% $^{.95CI}$ (6-14%)
    - ↑myeloid and lymphoid cancers with survival



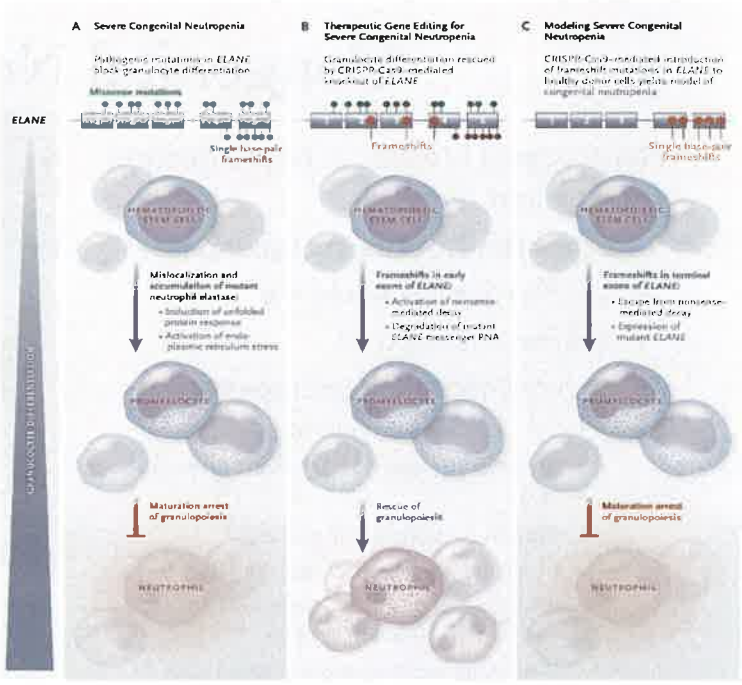
Normal to decreased cellularity with myeloid arrest at the promyelocyte/myelocyte stage. Often with atypical nuclei and cytoplasmic vacuolization.

Severe Congenital Neutropenia

Genetic transmission

- 50%-60% with autosomal dominant inheritance
- Genetically heterogeneous disorder, which is caused by mutations in more than 30 genes.
  - ≈ 50% of patients have a mutation of the *ELANE* gene that encodes for neutrophil elastase.

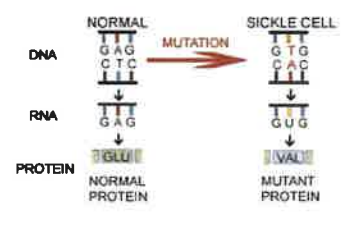
➢ Pre-clinical in-vitro study at Tübingen Univ. introduced frame-shift mutations of *ELANE* in HSCs from patients with SCN via CRISPR-Cas-9, which degraded mutant *ELANE* mRNA with restoration of granulopoeisis.



Skokowa. NEJM; May 20, 2021, pp 1956-1958

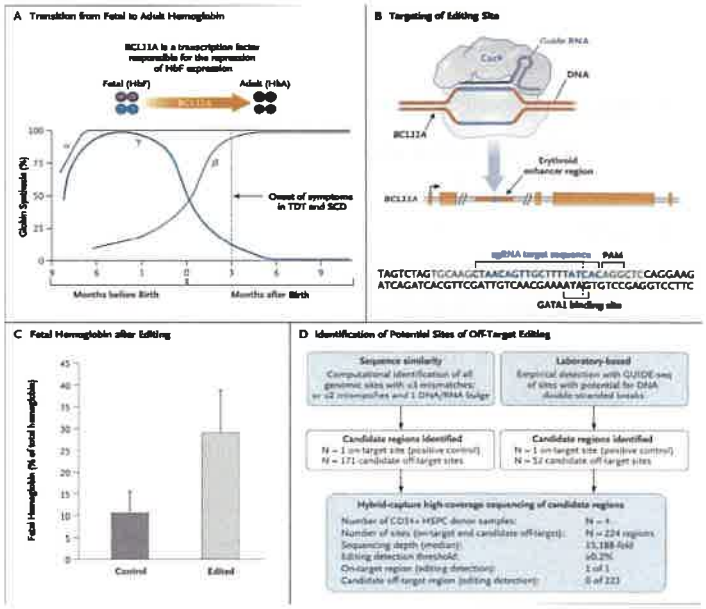
Sickle Cell Disease

- Most common monogenic disease worldwide
  - 300,000 new cases/yr. with 100,000 new cases/yr. in U.S.
- Caused by a point mutation in the hemoglobin β subunit gene (*HBB*) that replaces glutamic acid with valine at amino acid position 6.
- Multi-organ involvement with painful vaso-occlusive crises and life expectancy shortened by thirty years.



Victoria Gray

- 33-year-old female with SCD (βS/βS and a single ά-globin deletion)
- In the 2-years preceding treatment she had averaged:
  - 7 severe vaso-occlusive episodes per year.
  - 3.5 SCD-related hospitalizations per year.
  - 5 RBC transfusions per year.
  - She applied for bone marrow transplant, but did not have an HLA-matched donor.
- She was treated with CRISPR Cas-9 gene editing at HCA TriStar Centennial Hospital in Nashville Tennessee in 2019.
  - Hematopoietic stem and progenitor cells (HSPCs) at the erythroid-specific enhancer region of *BCL11A* to reduce *BCL11A* expression in erythroid-lineage cells, restore γ-globin synthesis, and reactivate production of fetal hemoglobin.



Frangoul. NEJM 384;3 Jan 21,2021

Germline Genomic Editing

- Human Embryonic Genomic Studies.
  - Francis Crick Institute (London)
  - Studied donated viable supernumerary human fertilized IVF zygotes to measure blastogenesis with CRISPR mediated OCT 4 (octamer-binding transcription factor 4) from the POU5F1 gene.
    - *"WE conclude that CRISPR-Cas9 mediated genome editing is a powerful method for investigating gene function in the context of human development."*

      Fogarty et. al. Nature 2017 Oct 05; 550(7674): 67-73
    - Study obtained ethical approval, but ethics have been questioned:

      Niemiec E, Howard H. Computational and Structural Biotechnology Journal; (18) 21 March 2020: 887-896.

- Questions:
  - *"Medical research involving human subjects may only be conducted if the importance of the objective outweighs the risks and burdens to the research subjects. (...) All medical research involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and groups involved in the research in comparison with foreseeable benefits to them and to other individuals or groups affected by the condition under investigation."*

    Declaration of Helsinki
  - *"The requirement that the results of an experiment be susceptible to analysis and characterization before further applications are undertaken cannot be met with human germ-line modification with current methods, because the results of any such manipulation could not be analyzed or understood for decades or generations—a situation incompatible with ethical imperatives and with the scientific method."*

    The American Society for Gene and Cell Therapy

Lulu and Nana

- Professor at Southern University of Science and Technology in Shenzhen, China
- He used CRISPR-Cas9 for germline editing of the CCR5 gene in human embryos to delete the receptor for the HIV virus.
- Viable twin girls were delivered in November 2018.
- This was globally condemned by the scientific community.
  - His study was refused for publication.
  - He was fired from his position
  - He was fined three million yuan ($430,000) and imprisoned for 3 years.

He Jiankui

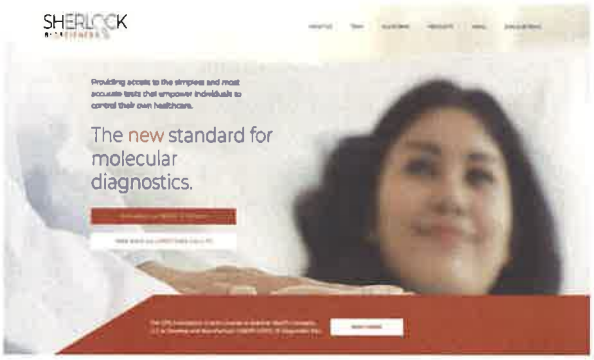
BBC News

Treatments vs. Enhancements

- Huntington's Disease
  - Autosomal Dominant
  - Single gene mutation (IT15) on chromosome 4 that codes for Huntington protein (Huntingtin).
- Sickle Cell Disease
  - Autosomal Recessive
  - Point mutation in the hemoglobin β subunit gene (*HBB*) on chromosome 11.
- Cystic Fibrosis
  - Autosomal recessive
  - Mutations in CFTR gene on chromosome 7
- Sports Performance
  - Muscle strength
    - MSTN gene affects myostatin.
  - Eero Mäntyranta 4-time Olympic champion (1960-1972)
    - Primary familial and congenital polycythemia (autosomal dominant with EPOR gene mutation)
  - ACTN3 gene prevalent in champion distance runners.
- Height
  - CDKN1C gene
- Cognitive performance
  - Memory enhanced in mice with editing of genes for NMDA receptors in nerve cells.
- Skin Color
  - SLC24A5

Other Applications

Diagnostic Testing



SHERLOCK

- *Specific high-sensitivity enzymatic reporter unlocking*
- Combined Cas12 and Cas13 to detect both viral DNA and RNA suitable for lateral flow technology

CRISPR-Based COVID-19 Smartphone Test in Development

A simplified point-of-care assay that turns a smartphone into a fluorescence microscope could expand coronavirus disease 2019 (COVID-19) testing capability, researchers reported in a study in *Cell*.

The assay, which uses clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology, emits a fluorescent signal in the presence of the novel coronavirus's RNA. A smartphone camera can detect this signal directly, without amplification of the viral genome used in most genetic tests. This means the test can quantify the amount of virus in the sample—the quicker the signal is picked up, the higher the viral load.

Viewpoint page 529

In the study, the assay was able to detect RNA extracted from patients' nasal swabs within 5 minutes. Samples with less virus could be detected within 30 minutes. "Monitoring viral loads quantitatively would allow estimation of infection stage and help predict infectivity, recovery, and return from quarantine in real time," the authors wrote.

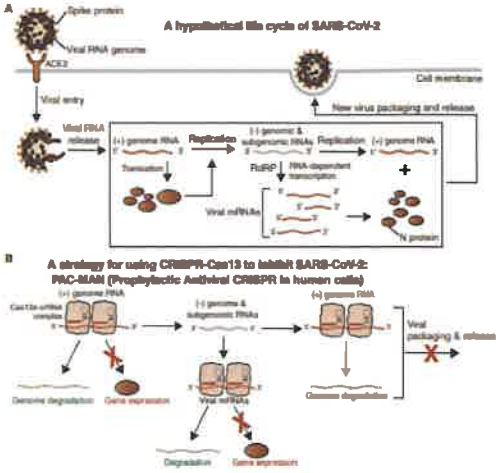
JAMA February 9, 2021

Cell 184, 323-333, January 21,2021

Other Applications

Vaccines

- PAC-Man
  - Prophylactic Antiviral CRISPR in human cells
  - Pre-clinical with research at Berkley and Stanford:
    - Stanley Qi, Timothy Abbot, and Ross Wilson
  - Uses Cas13d as the endonuclease to target:
    - RNA dependent RNA polymerase
    - Nucleocapsid protein
  - Eliminates the potential for the emergence of mutant strains.
  - Technology is applicable to all Coronaviruses both human and zoonotic.
    - Abbott et.al. Development of CRISPR as an antiviral Strategy to Combat SARS-COV-2 and Influenza; *Cell 181, 865-876 May 14, 2020*

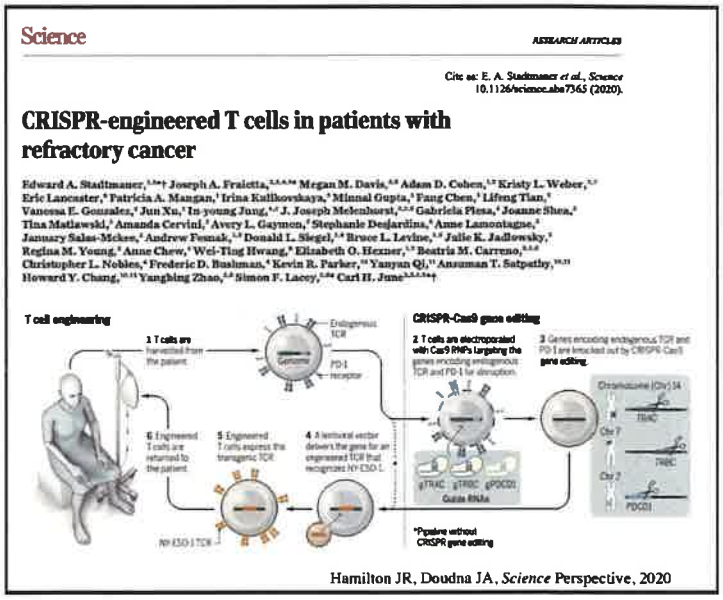


Cell 181, 865-876 May 14, 2020

Other Applications

Cancer

- Phase one trial of 3 patients (2 with refractory myeloma and 1 with metastatic sarcoma) at the Univ. of Pennsylvania.
  - Ex vivo engineered T cells (adoptive T-cells) edited with CRISPR Cas9 to disable PD-1 were infused following lympho-depleting chemotherapy.
  - Transgenic TCR-T-cells were used rather than CAR-T to avoid cytokine storm.
  - A 62-year-old female with advanced myeloma died. Unrelated to her treatment.
  - The 66-year-old sarcoma patient showed a 50% reduction in tumor mass.
  - The 66-year-old female with advanced myeloma did not have progression of her disease over 4 months.
  - No adverse effects from the treatment were cited.



Science

RESEARCH ARTICLES

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CRISPR-engineered T cells in patients with refractory cancer

Edward A. Stadtmauer,[1,2*†] Joseph A. Fraietta,[3,4,5*†] Megan M. Davis,[3,6] Adam D. Cohen,[1,2] Kristy L. Weber,[2,7] Eric Lancaster,[8] Patricia A. Mangan,[1] Irina Kulikovskaya,[3] Minnal Gupta,[1] Fang Chen,[1] Lifeng Tian,[9] Vanessa E. Gonzalez,[3] Jun Xu,[1] In-young Jung,[3,4] J. Joseph Melenhorst,[3,4,5] Gabriela Plesa,[4] Joanne Shea,[2] Tina Matlawski,[2] Amanda Cervini,[2] Avery L. Gaymon,[2] Stephanie Desjardins,[4] Anne Lamontagne,[2] January Salas-McKee,[4] Andrew Fesnak,[3,5] Donald L. Siegel,[3,4] Bruce L. Levine,[3,4] Julie K. Jadlowsky,[3] Regina M. Young,[3] Anne Chew,[3] Wei-Ting Hwang,[8] Elizabeth O. Hexner,[1,2] Beatriz M. Carreno,[3,4,5] Christopher L. Nobles,[4] Frederic D. Bushman,[4] Kevin R. Parker,[10] Yanyun Qi,[10] Anuraman T. Satpathy,[10,11] Howard Y. Chang,[10,11] Yanghing Zhao,[4,5] Simon F. Lacey,[3,5*] Carl H. June[3,4,5*†]

Hamilton JR, Doudna JA, *Science* Perspective, 2020

Stadtmauer et. al., Science 367; 28 Feb 2020

"For first of all we must prepare a Natural and Experimental History, sufficient and good; and this is the foundation of all; for we are not to imagine or suppose, but to discover, what nature does or may be made to do."

(*Priom enim paranda est Historia Naturalis et Experimentalis, suffidens et bona; quod fundamentum rei est; neque enim fingendum, aut excogitandum, sed inveniendum, quid natura faciat aut ferat.)*

Francis Bacon (1561-1626)

In Novum Organum Book 2, Aphorism 10

"Our sole responsibility is to produce something smarter than we are; any problems beyond that are not ours to solve."

Ray Kurzweil (1948-)

The Singularity is Near: When Humans Transcend Biology