CRISPR and human fertility

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History of Genome Engineering

CRISPR makes for crispier news!

Designer babies? US scientists swap DNA in embryos, replacing mutation that causes heart problems

Ricki Lewis | August 2, 2017 | Genetic Literacy Project

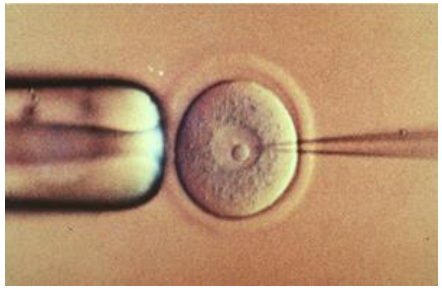
Human embryos edited to stop disease

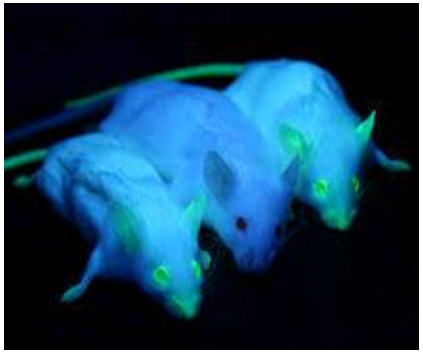
By James Gallagher
Health and science reporter, BBC News website

In a first, scientists have edited the DNA of human embryos that could turn into people using CRISPR

Genetic Engineering: A 40 year human effort to alter the building blocks of life

- In 1974 transgenic mice were made by injecting DNA into zygotes
- Goats making spider silk in their milk, 2012
- Giant mice expressing growth hormone transgene
- Mice expressing fluorescent proteins, 2009



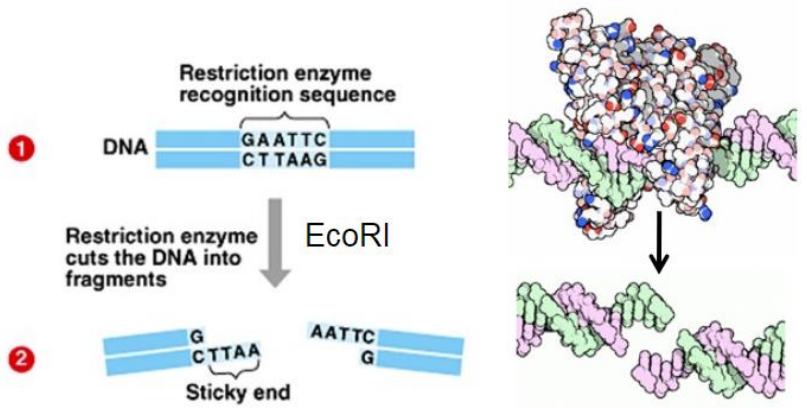


Foundations of genetic engineering and molecular biology

- **Plasmids**- cytoplasmic, small circular DNA from bacteria or protozoa
- **Restriction Endonucleases**- enzymes mainly derived from bacteria that cleave DNA molecules with specificity
- **DNA ligases**-catalyze phosphodiester bond to join DNA strands
- **Polymerase chain reaction**- technique to amplify DNA across several magnitudes

Restriction endonucleases

- An enzyme that cleaves DNA into fragments at or near specific recognition sites, called as restriction sites
- Originally discovered in bacteria and archae
- Have evolved as a defense mechanism against invading viruses



Most genetic engineering in the beginning performed on episomal DNA

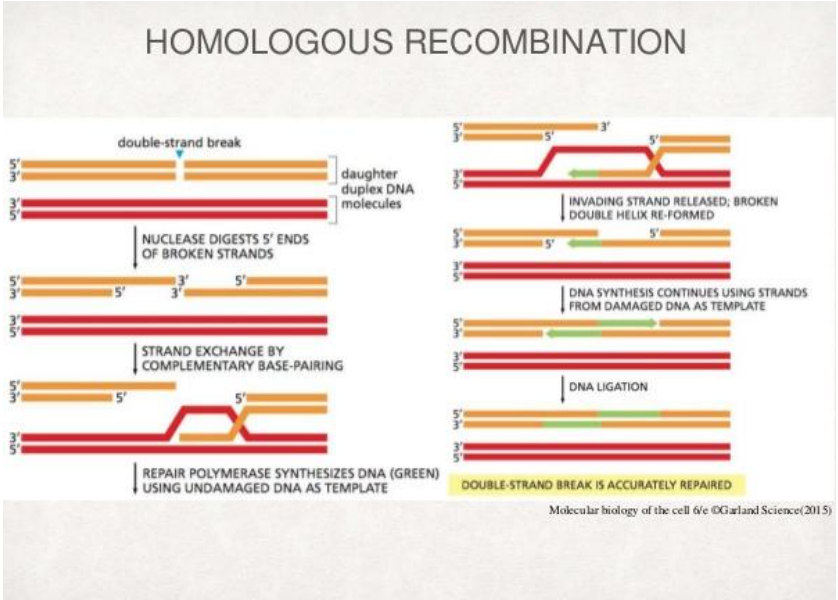
- Typical restriction endonucleases recognize 4-8bp
- A unique 4/6/8 bp cutting site would occur once every 256 (4^4), 4096(4^6),65536(4^8)
- This makes it infeasible to work with conventional REs to modify large genomes such as Human that is 3 billion bp
- Several limitation pertaining to the size of DNA that can be manipulated in vitro : usually around 20kb

Efforts towards improved efficiency have given rise to the next generation of genome engineering techniques

- Homologous recombination
- Artificial Restriction Enzymes such as Zinc Finger nucleases and Transcription activator-like effector nucleases (TALEN)
- CRISPR/Cas9

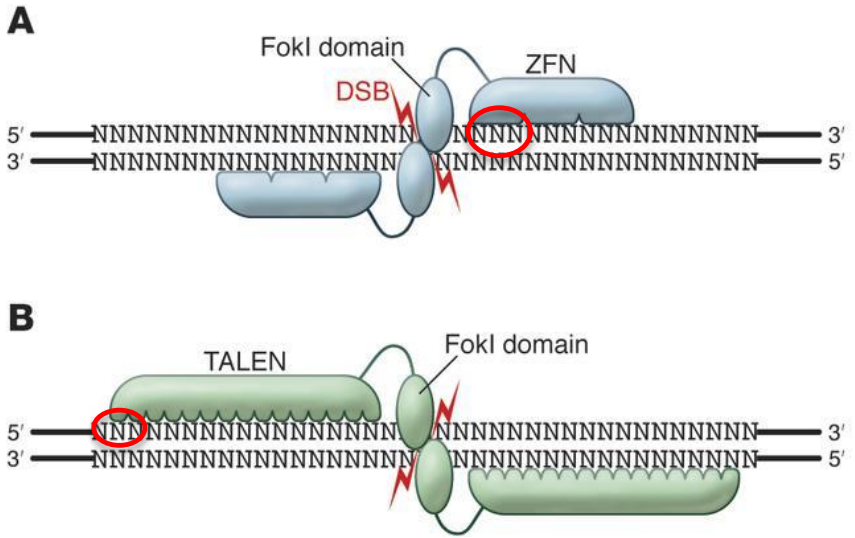
Homologous recombination

- Best studied in yeast
- Most widely used by cells to accurately repair harmful double stranded breaks of DNA
- Limitations: Time consuming, low efficiency
- Could be used in conjunction with other genome editing methods



Zinc Finger nucleases (ZFN) and TALENS

- Artificial restriction enzymes
- ZF and TALE DNA binding domains are fused to DNA cleavage domains (non-specific nucleases) to create these genome editing tools
- Multiple ZF attached together for high codon level specificity
- TALEs are specific to nucleotides, multiple TALEs can be fused to target a specific sequence



1. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering Gaj, Thomas et al. Trends in Biotechnology , Volume 31 , Issue 7 , 397 – 405
2. Rajat M. Gupta, Kiran Musunuru Published October 1, 2014 Citation Information: J Clin Invest. 2014;124(10):4154-4161. doi:10.1172/JCI72992.

Drawbacks of ZFN and TALENS

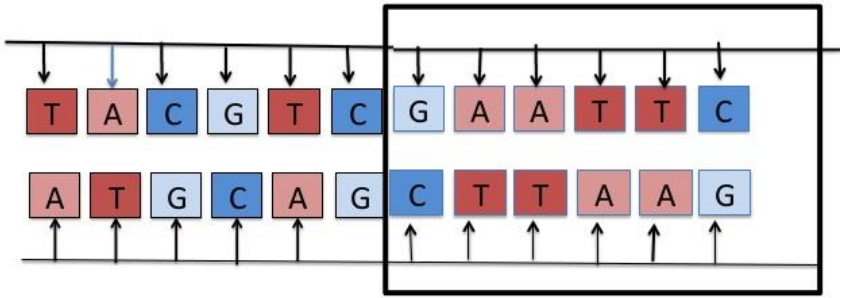
- Both methods are costly and time consuming
- Additionally they require a lot of expertise to prepare these
- While TALEN recognizes single nucleotides, it is much harder to create interactions between Zinc Fingers and their target codons
- Both require direct protein-DNA interactions to enable editing
- CRISPR a lot easier to work with

What is CRISPR/Cas9?

A bacterial immune mechanism

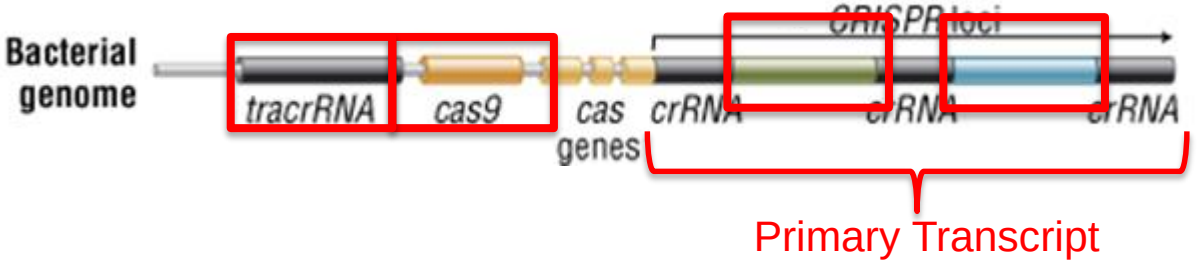
CRISPR discovered as repeat sequences in bacterial genome

- Originally reported by Ishino et. Al in 1987 as 'exotic Junk DNA'
- Stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats (CRISPR)
- Serves as an **adaptive** immune mechanism to protect from bacteriophages and plasmids
- Present in most archae and bacteria



Jansen, R. , Embden, J. D., Gaastra, W. and Schouls, L. M. (2002), Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology, 43: 1565-1575. doi:10.1046/j.1365-2958.2002.02839.x

Topography of CRISPR locus on the bacterial genome



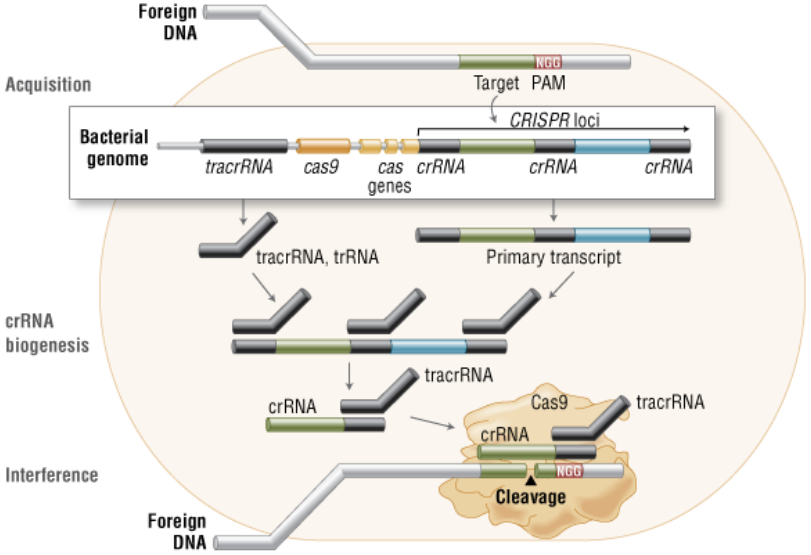
- CRISPR locus on the bacterial genome consists of arrays of short repeats from the invading genetic material and clusters of Cas genes
- Type II CRISPR systems developed from *Streptococcus pyogenes* are commonly used in the lab
- Type I CRIPSR systems observed in *Clostridium difficile* are uncommon in labs and require a multi-protein (cas3, cas6) proteins
- Similar basic principle for both systems

CRISPR associated Protein (Cas)

- Proteins evolved alongside CRISPR to evade viruses
- Cas proteins typically exhibit helicase (DNA unwinding) and nuclease (cleavage) motifs
- Cas9 is a large protein with nuclease activity that was discovered in Streptococcus thermophilus
- Cas9 is a RNA-dependent programmable DNA endonuclease

Mechanism of CRISPR/Cas9 mediated cleavage as observed

- CRISPR repeat array is transcribed that included trans activating RNA (tracrRNA)
- Active complex now consists of crRNA-tracrRNA complex and the cas9 protein.
- crRNA acts as a guide RNA to bind to complimentary sequences binding at the protospacer associated motif (PAM)



https://www.neb.com/applications/genome-editing

Adaptation of a bacterial immune system for genome editing

- CRISPR/Cas9 systems contribute to specific target cleavage.
- Cleavage was the first step required in genome editing for cell repair machinery to be activated
- Re-engineering the system, provided the correct elements were present, CRISPR/Cas9 could work in virtually all cell types
- *Streptococcus pyogenes* CRISPR/Cas9 used to achieve targeted cleavage in 293FT cells

Cong L, Ran FA, Cox D, et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science (New York, NY)*. 2013;339(6121):819-823. doi:10.1126/science.1231143.

Can we do this in our own lab?

Yes, it is surprisingly easy

- Reason for popularity is due to the ease of adapting this in a molecular biology lab
- Performing basic CRISPR/Cas9 experiments is not equipment intensive
- Few basic lab equipment such as a PCR machine, cell culture hoods, pipettes and centrifuges required
- Basic knowledge of DNA structure, and genetics is sufficient

Genome editing workflow

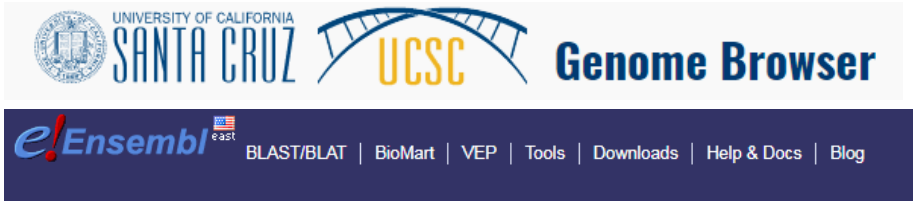
Identify target sites, design guides

Deliver Editing tools with highest transfection efficiency

Confirm gene editing efficiency

gRNA synthesis begins by identifying targets

- Define a target of interest for your lab & obtain target sequence from Ensembl or UCSC genome browsers
- Select appropriate species on the browser and search for gene of interest
- For example: Homo sapien, HPRT1, exon1
- Copy-paste the sequence from the genome browser in a FASTA format

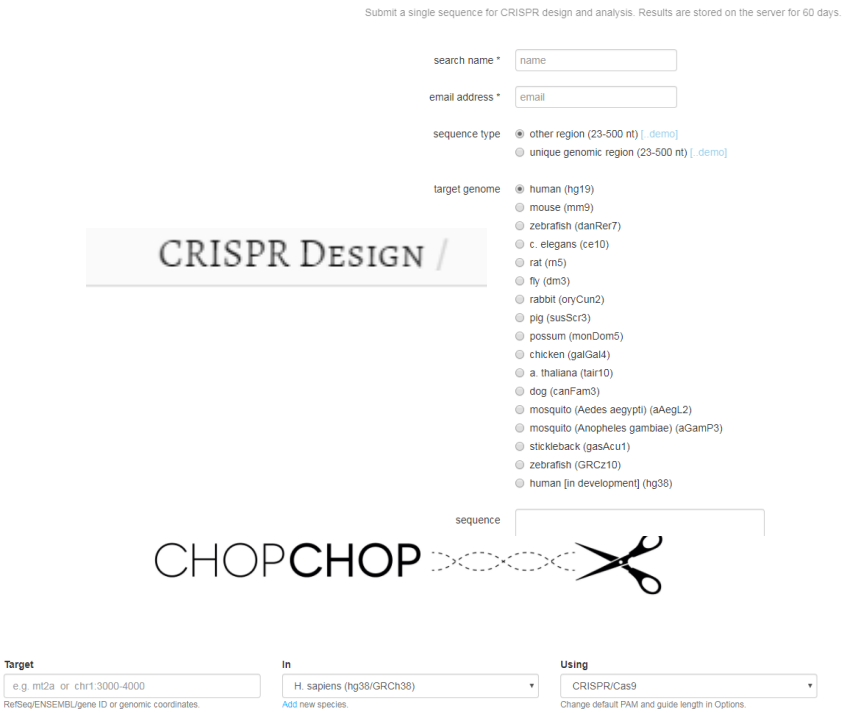


Gene: HPRT1 ENSG00000165704

```
>chromosome:GRCh38:X:134459553:134521113:1
TGGCCAGGCTGGTCTCGTACTCCTGACCTTGAGTGATCCACCTGCCTCGGCCTCCCAAAG
TGCTGGGATTACAGGCATGAGCCACCGCGCCGCGGGCTAATATGCTCATTTTAGTGAGGCAA
AAATAGAGGCTCAGAGTCTGATTTGTACAAAACTACAGAGCAGTTAAGTGTCCTCTCAGA
TGTGTACCCTGATCTGGGTGACTCTAGGACTCTAGGTCTCAACTGTTACAACCAGTTAAG
GGTTTGGGGAAGCACTGGGCCAAGAGTCAGGAAAATGGAAGCCACAGGTAGTGCAAGGTC
TTGGGAATGGGACGTCTGGTCCAAGGATTCACGCGATGACTGGAACCCGAAGAGCCGGGG
CCCGGTTTACGGCCGCCATGAAGCAACGCGCGCCGGTAGGTTTGGGAATCAGGGAGCCCT
CTGAATAGGAGACTGAGTTGGGAGGGAAAGGGGCTTCGCTGGGGGAGCCTCGGCTTCTTC
TGGGAGAAAATTCCCACGGCTACCTAGTGAGCCTGCAAACTGGTAGGCGCCGGCGTAGGC
GCGCGGGCGGGGCCGGGGGCGGGGCCTGCGGGGCGTGGCGGGGCGGGCAGAGGGCGGGGC
CTGCTTCTCCTCAGCTTCAGGCGGCTGCGACGAGCCCTCAGGCGAACCTCTCGGCTTTCC
CGCGCGGCGCCGCCTCTTGCTGCGCCTCCGCCTCCTCCTCTGCTCCGCCACCGGCTTCCT
CCTCCTGAGCAGTCAGCCCGCGCGCCGGCCGGCTCCGTTATGGCGACCCGCAGCCCTGGC
GTCGTGGTGAGCAGCTCGGCCTGCCGGCCCTGGCCGGTTCAGGCCCACGCGGCAGGTGGC
GGCCGGGCCCTGAGGCGCGGGATCCGCAGTGCGGGCTCGGCGGCCGGGCCCAGGGAACC
CCGCAGGCGGGGGCGGCCAGTTTCCCGGGTTCGGCTTTACGTCACGCGAGGGCGGCAGGG
AGGACGGAATGGCGGGGTTTGGGGTGGGTCCCTCCTCGGGGGAGCCCTGGGAAAAGAGGA
```

gRNA design tools available online

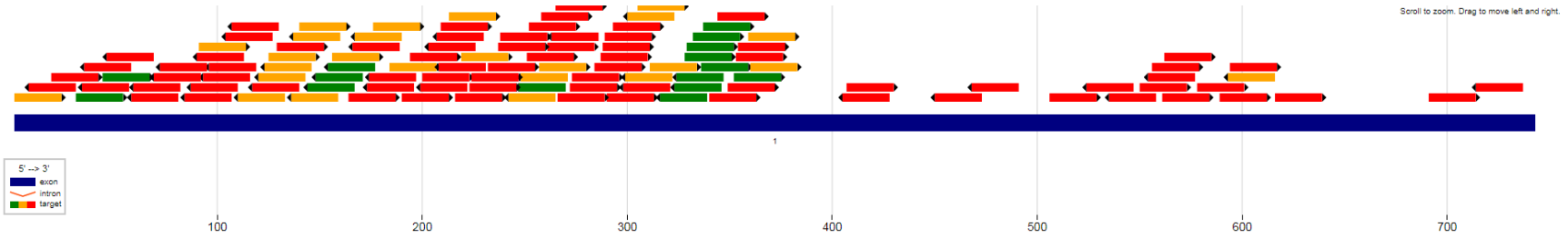
- Several free CRISPR design tools are available online
- CRISPR Design from the Zhang lab at MIT is the original
- Paste the FASTA sequence of interest from the browser, select the appropriate species
- The tool will then calculate all available guide RNA targets available for the region of your choice



http://crispr.mit.edu/
http://chopchop.cbu.uib.no/

gRNA results from CHOPCHOP Exon 1 of HPRT1

sequence



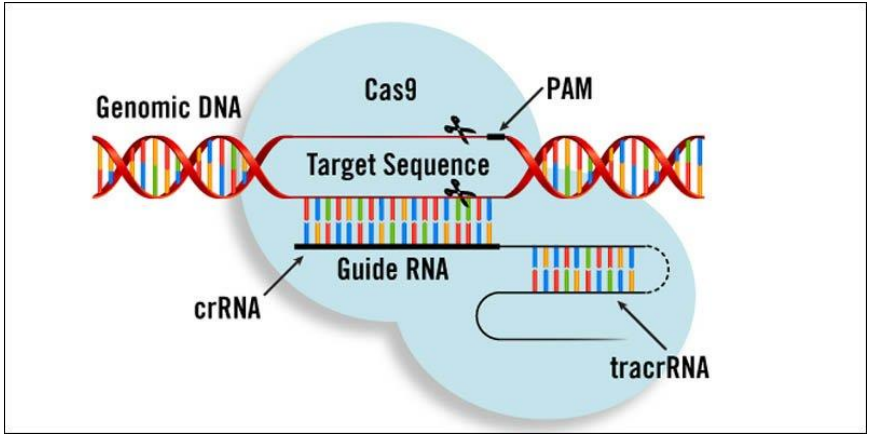
Download results: Please select one

Ranking	Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
							0	1	2	3	
1	TTCGGCTTTACGTCACGCGAGGG	>:330	1	+	55	0	1	0	0	0	0.74
2	GTTCGGCTTTACGTCACGCGAGG	>:329	1	+	60	0	1	0	0	0	0.68
3	CGTGACGTAAAGCCGAACCCGGG	>:324	1	-	60	1	1	0	0	0	0.65
4	GGCTTTACGTCACGCGAGGGCGG	>:333	1	+	65	0	1	0	0	0	0.63

- Multiple targets (20 bp) are usually found in any given region
- Off-target effects are listed based on 0,1,2 or 3 mismatches
- For example, we could select rank 1 target here

Almost ready for genome editing

- The preferred gRNA can be ordered in a ready to transfect gRNA format from several providers or gRNA synthesis kits can be used to assemble the gRNA-tracrRNA complex
- The cas9 nuclease can be transfected as mRNA or is also available in a purified protein format
- These tools can be chosen depending on the efficiency required



You are now almost a CRISPR expert

Applications & the use of CRISPR in human embryos

Wide- reaching effects of CRISPR- Agricultural applications

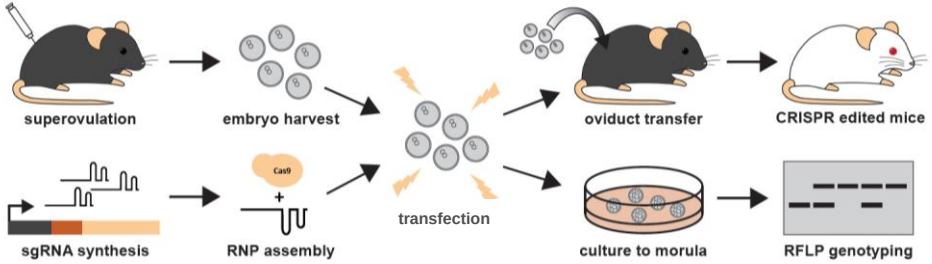
- Rapid trait improvement in livestock is possible
- Protection against viruses, hornless cows
- Trait improvement in plants and other foods. Eg. Mushrooms that do not brown
- Production of medical products or tissues. Eg. Human albumin into Pig albumin

Wide- reaching effects of CRISPR- Antibacterial and antiviral

- Antibiotic resistance a big threat
- Design of CRISPR systems to program bacterial death
- Can target precise clinical genotypes and epidemiological isolates
- CRIPSR therapies against human viruses including HIV, Hep B

Wide- reaching effects of CRISPR- Animal models

- Flexibility to work in all cell types
- Makes it easier to generate disease models
- For example, It would take 8-12 months to establish a knockout mouse model before CRISPR, with no guarantee of result
- It takes about 3 months to generate a founder pair with CRISPR, with almost a certain guarantee



Wide- reaching effects of CRISPR- Cell therapy applications

- Establish *in- vitro* (cell culture), *in-vivo* (model animal) systems are established
- *Ex -vivo* editing of genes and then introduction of the corrected cells back *in-vivo* into humans
- For example, Cystic fibrosis, DMD, ASD etc. corrected cells that differentiated into mature airway epithelial cells *in vitro* can be introduced *in-vivo*

CRISPR/Cas9 use in human embryos tried for the first time in 2015

- Germline changes made early enough could theoretically lead to a disease free human
- Junjiu Huang from Sun Yat-sen University in Guangzhou performed these experiments for the first time in human embryos
- Attempted to modify the gene responsible for β-thalassaemia
- Low-efficiency and a high number of "off-target" effects reported

Protein & Cell

May 2015, Volume 6, Issue 5, pp 363–372

CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

Authors | Authors and affiliations

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

Open Access | Research article

First Online: 18 April 2015

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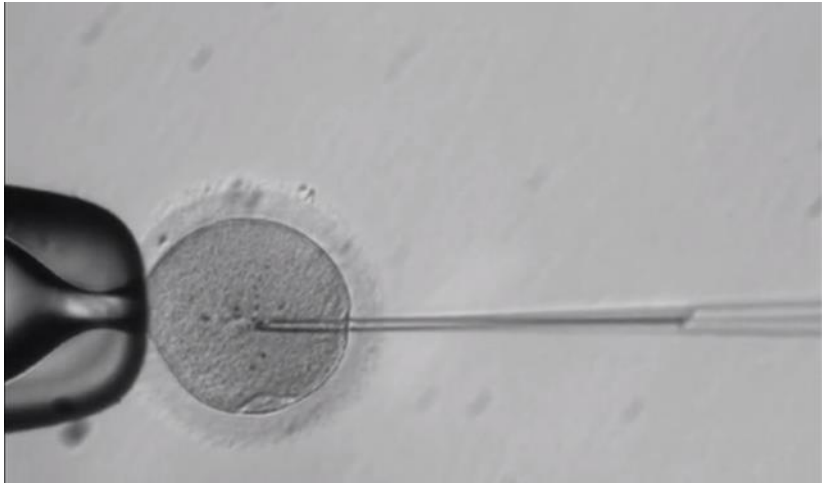
What was done in Oregon

- This was done at Oregon Health and Science University by Shoukhrat Mitalipov.
- Involved changing the DNA of a large number of one-cell embryos with CRISPR in a gene called *MYBPC3*
- None of the embryos were allowed to develop for more than a few days- no intention of implanting
- Mitalipov and his colleagues are said to have convincingly shown that it is possible to avoid both mosaicism and "off-target" effects



Correction of a pathogenic gene mutation in human embryos Hong Ma, Nuria Marti-Gutierrez[…]Shoukhrat Mitalipov
Nature volume 548, pages 413–419 (24 August 2017)doi:10.1038/nature23305

Other efforts in human embryos

- UK Scientists gained a license in 2016 from HFEA to edit genomes in human embryos
- First experiment involved blocking the activity of a 'master regulator' gene called *OCT4*, which is active in cells that go on to form the fetus.
- Swedish scientists also performing similar experiments

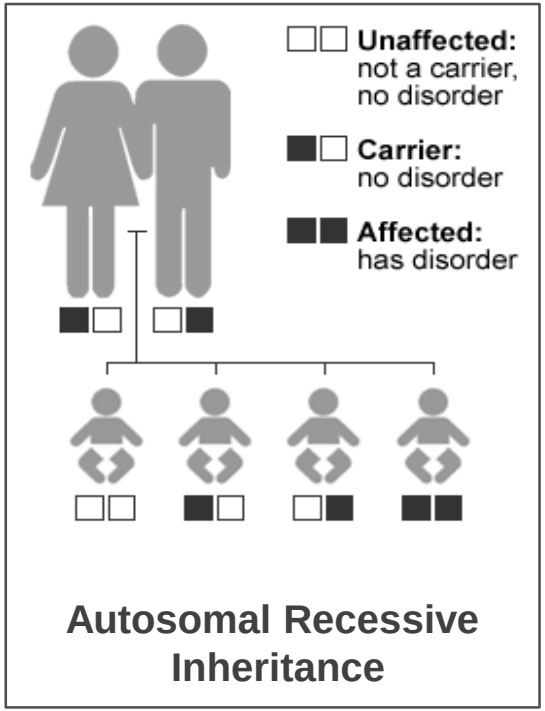
Genetic tests for infertility

Indication	Test couples		Action
	Genetic Test	Result	
Maternal age	-	≥35	**PGS**
RPL translocation	Karyotype	translocation carrier	**PGS**
RPL idiopathic	-	≥2 miscarriages	**PGS**
Gene defect	**Carrier Screen (Recombine)**	both affected	**PGD + PGS**
Infertility idiopathic	**Endometrial receptivity**	altered	change transfer day
	sperm epigenetics	altered	????
	Fertility map	genetic component	change treatment
Paternal age	-	≥40	PGD de novo mutations

Complexity of the human genome

- 3.5 billion pairs of bases
- 88 million (2.5%) are different **single nucleotide polymorphisms (SNPs)** – they make us different from one another
- 3% are coding genes (about 35,000 genes)
- 97% is “junk” DNA with regulatory activity over genes

The Burden of genetic disease



- **>6000 single gene disorders**
- **Combined incidence:** 1/300 births (U.S.)
- **Everyone carries 14-18 severe recessive mutations** that can cause genetic conditions
- **Carriers may not have a family history** or symptoms of a genetic disease
- Hence, **carrier screening is the only way to determine carrier status**

1. Bell, C.J., Dinwiddie, D.L., Miller, N.A., Hateley, S.L., Ganusova, E.E., Mudge, J., Langley, R.J., Zhang, L., Lee, C.C., Schilkey, F.D., Sheth, V., Woodward, J.E., Peckham, H.E., Schroth, G.P., Kim, R.W., Kingsmore, S.F., 2011. Carrier testing for severe childhood recessive diseases by next-generation sequencing. Sci Transl Med. Jan 12;3(65):65ra4
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PGD for single gene disorders

We can do PGD for any monogenic disease provided the mutation is known

Diseases tested: Acetyl Co-Oxidase type I deficiency, Adrenoleucodistrophy, Alpha-thalassemia, Alport syndrome, Autosomal Dominant Polycystic Kidney Disease (ADPKD), Autosomal Recessive Polycystic Kidney Disease (ARPKD), Beta-thalassemia, Branchio-Oto-Renal syndrome (BOR), BRCA1 breast cancer predisposition, BRCA2 breast cancer predisposition, Canavan Charcot-Marie-Tooth type IA (CMT1a), Choroideremia, Congenital adrenal hyperplasia (CAH), Congenital neutropenia, Connexin 26 hearing loss, Cystic fibrosis, Duchenne/Becker Muscular Dystrophy (DMD), Ectrodactyly, Ectodermal dysplasia, and Cleft lip/palate syndrome (EEC1), Fabry Disease, Familial adenomatous poliposis coli (FAP), Familial dysautonomia, Familial intrahepatic cholestasis 2, Fanconi anemia, Fragile site mental retardation , Gangliosidosis type 1 (GM1), Gaucher disease, Glomuvenous malformations (GVM), Glycogen-storage disease type I (GSD1), Glycosylation type 1C, Hemoglobin SC disease, Hemophilia A, Hemophilia B, Hereditary nonpolyposis colon cancer (HNPCC), Hereditary pancreatitis, HLA matching Huntington disease, Hurler syndrome, Hypophosphatasia, Incontinential pigmenti, Krabbe disease (Globoid cell leukodystrophy), Long QT syndrome, Marfan syndrome, Meckle gruber, Metachromatic leukodystrophy (MLD), Methylmalonic aciduria cblC type (MMACHC), Myotonic Dystrophy 1, Myotubular myopathy, Neurofibromatosis 1, Neurofibromatosis 2, Niemann-Pick Disease, Noonan syndrome, Oculocutaneous albinism 1 (OCA1), Ornithine carbamoyltransferase deficiency (OTC), Osteogenesis Imperfecta 1, Rapp Hodgkin ectodermal dysplasia, Retinitis pigmentosa, Retinoblastoma, Sickle Cell Anemia, Smith-Lemli-Opitz syndrome (SLOS), Spinal bulbar muscular atrophy (SBMA), Spinal Muscular Atrophy Type 1 (SMA1), Tay Sachs, Tuberous sclerosis 1 (TSC1), Tuberous sclerosis 2 (TSC2), Von Hippel-Lindau Syndrome (vHL), X-linked dominant Charcot–Marie–Tooth (CMTX), etc…… *(see review Gutierrez et al. (2008))*

PGD procedures performed

Cases performed: 9,277

Disease genes tested: 778

Top 10 diseases:

Cystic fibrosis
Fragile X
Huntington disease
Sickle cell anemia
Spinal Muscular atrophy
Beta Thalassemia
Breast cancer (BRCA1, BRCA2)
Myotonic dystrophy
Polycystic Kidney Disease (PKD1)
Neurofibromatosis type 1

Monogenic diseases could potentially be eradicated

- Theoretically CRISPR/Cas9 gives us the ability to correct any genetic mutation to a healthy genotype.

- Provided treated early enough, genome editing can be performed on gametes or early stage embryos to prevent the disease from propagating to the next generation

Ethical questions surrounding CRISPR use in humans

Treatment versus Enhancement

- **Making changes beyond ordinary, established human capabilities , or anything outside of treatment/prevention of diseases and disability**
- Significant public concern about fairness, if available only to some people, and about creating pressure to seek out enhancements
- But many other kinds of enhancements are in fact tolerated or even encouraged: Nutrition, education, cosmetic procedures
- Potential uses of genome editing beyond therapy
  - For example: curing muscular dystrophy versus becoming stronger than normal
  - Range of possible uses of approved therapies for enhancement seems limited
- At this time enhancement is doubtful to offer benefits sufficient to offset risks

Other concerns

- Genetic Changes may be inherited by the next generation: This has been usually viewed as unacceptable in the past
- Multigenerational risks (but also possible benefits)
- Need for (and possible difficulty) for long term follow-up
- Lack of consent by affected persons (future child, generations)
- The degree of intervention in nature
- Affecting acceptance of children born with disabilities

Limitations of CRISPR

A few pitfalls observed

- Not perfectly accurate- Substantial amount of off-target mutagenesis predicted
- Need more data based on WGS to estimate true effect of off-target effects
- Different design tools consider different parameters, gRNA might have to be modified to attain maximum efficiency
- Better delivery vehicles need to be designed to maximize efficiency
- New fragment integration not a part of the CRISPR/Cas9 system

Questions

Reprogenetics℠ Recombine℠ Genesis Genetics℠