Improving nonclinical research practices: way forward

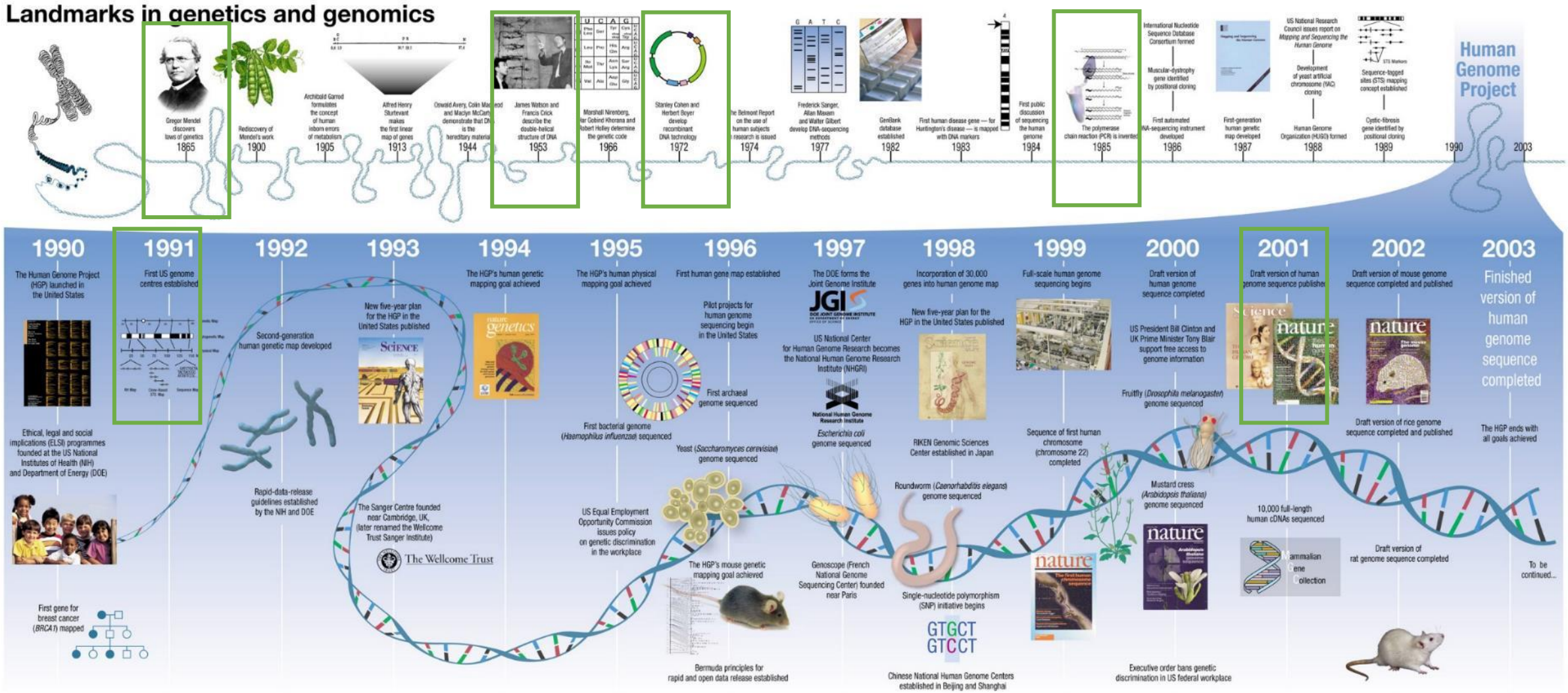
2022. LAS webinar series organized by CroLASA in collaboration with SLAS

CRISPR/Cas9 gene editing in animal models

Maja Sabol

Ruđer Bošković Institute

13.04.2022.

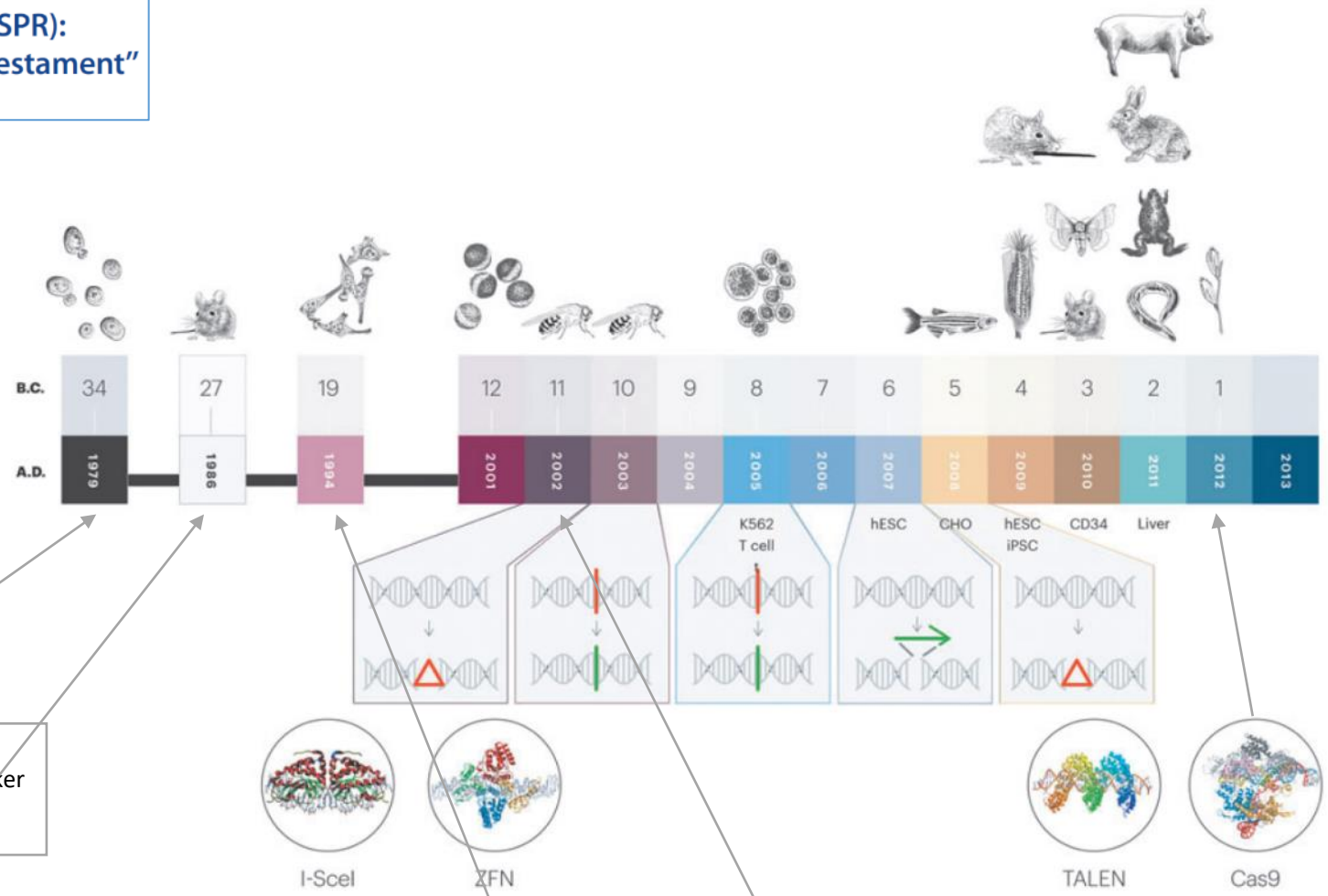


https://epochalscience.files.wordpress.com/2017/03/genomics-timeline.jpg

REVIEW ARTICLE

Genome Editing B.C. (Before CRISPR): Lasting Lessons from the "Old Testament"

Fyodor D. Urnov



Yeast
Target gene replaced by a selection marker
Homologous recombination
Scherer S, Davis RW. PNAS USA 1979: 76:4951-4955.

Mouse embryonic stem cells (ES)
Knock-out (replacement of a gene by a selection marker)
Homologous recombination
Thomas KR et al. Cell 1986: 44:419-428.

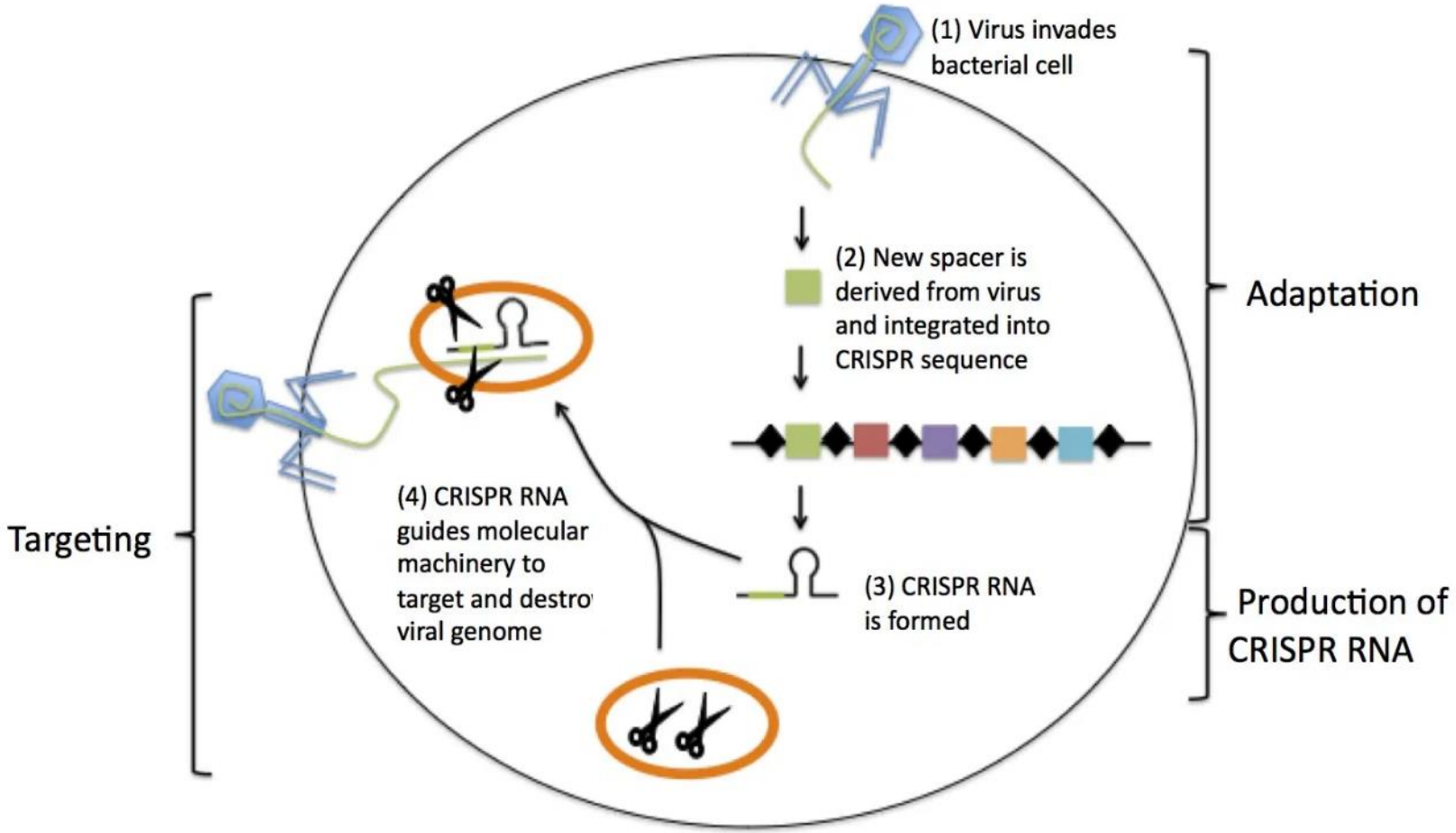
Fibroblast cell culture
Knock-out (replacement of a gene by a selection marker)
Homologous recombination
Brown JP et al. Science 1997: 277:831-834.

The use of AAV for delivery of construct
Hirata R et al. Nat Biotchenol 2002: 20:735-738.

Asn-Asn (NN)	guanine
Asn-Ile (NI)	adenine
His-Asp (HD)	cytozine
Asn-Gly (NG)	timine

Nobel prize for chemistry 2020

„Clustered regularly interspaced short palindromic repeats”



CRISPR provides acquired resistance against viruses in prokaryotes (*Streptococcus thermophiles)* – bacterial adaptive immune system (Barrangou et al, Science, 315 (2007)

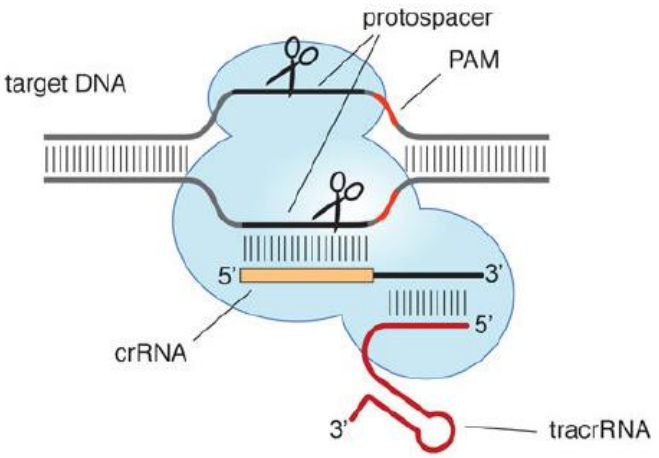
Barrangou, R. and Marraffini, Molecular Cell 54, 234-244 (2014).

RESEARCH ARTICLE

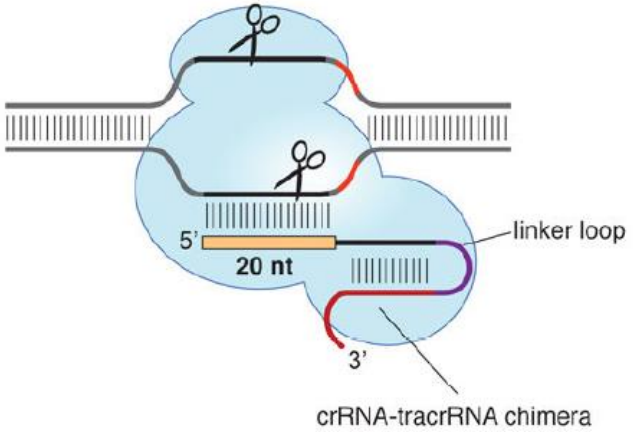
A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]† Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA

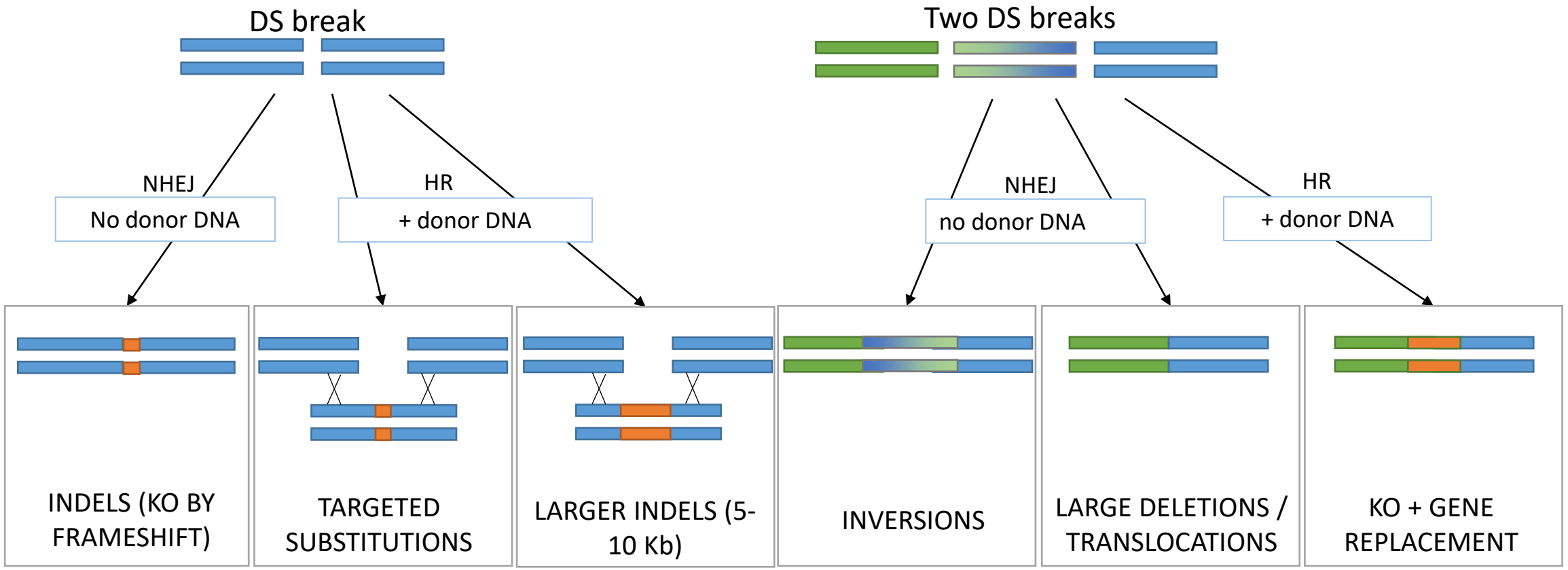


- Cas9 is a DNA endonuclease guided by two RNA molecules
- Targeting: CRISPR RNA (crRNA)
- Activation: trans-activating CRISPR RNA (tracrRNA)
- Chimeric RNA (crRNA-tracrRNA) can trigger the same effect → sgRNA

„We propose an alternative methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications."

Jinek et al, Science, 337 (2012)

What are the outcomes of a DS break?



Advantages and disadvantages

Simple and easy to use

Commercially available

Faster than traditional methods

Compatible with different delivery methods

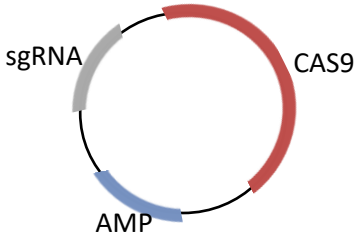
Possible off-targets

Targeting to multiple alleles

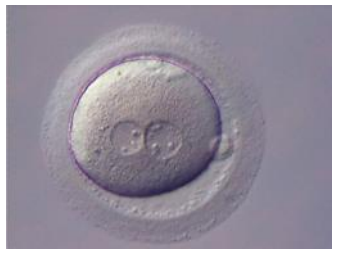
Ethical issues (manipulation of the human genome, misuse)

How do we do it?

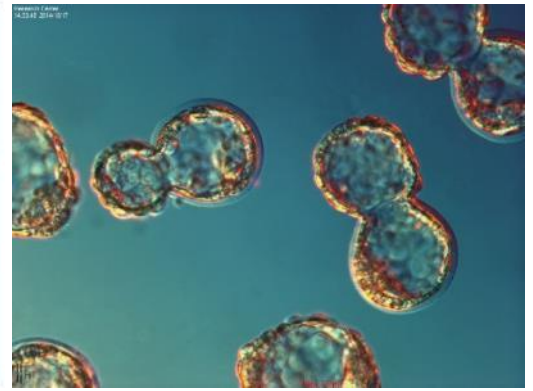
1 Design and clone your sgRNA
(online tools like http://crispor.tefor.net/)



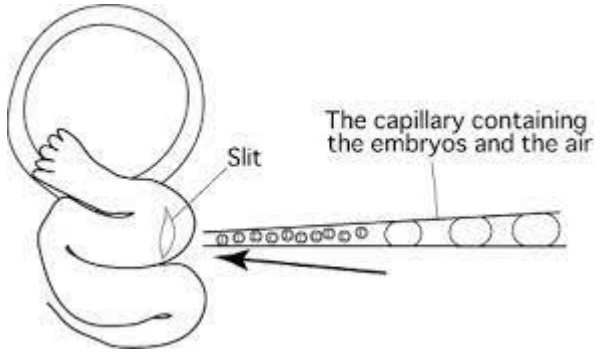
2 Breed mice and collect zygotes



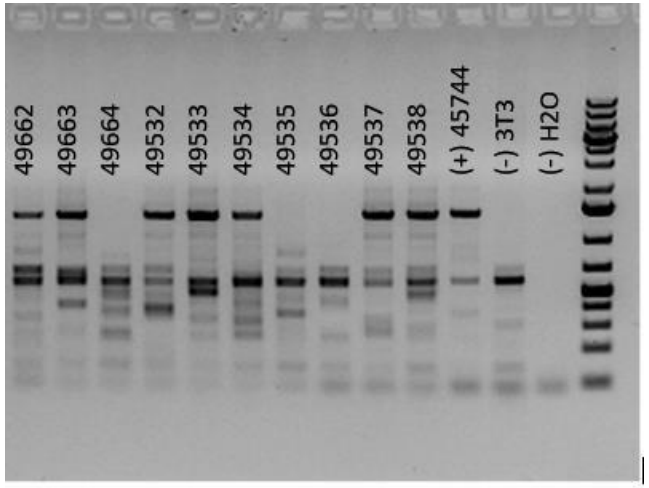
3 Microinject vectors into the pronucleus and maintain until blastocyst stage



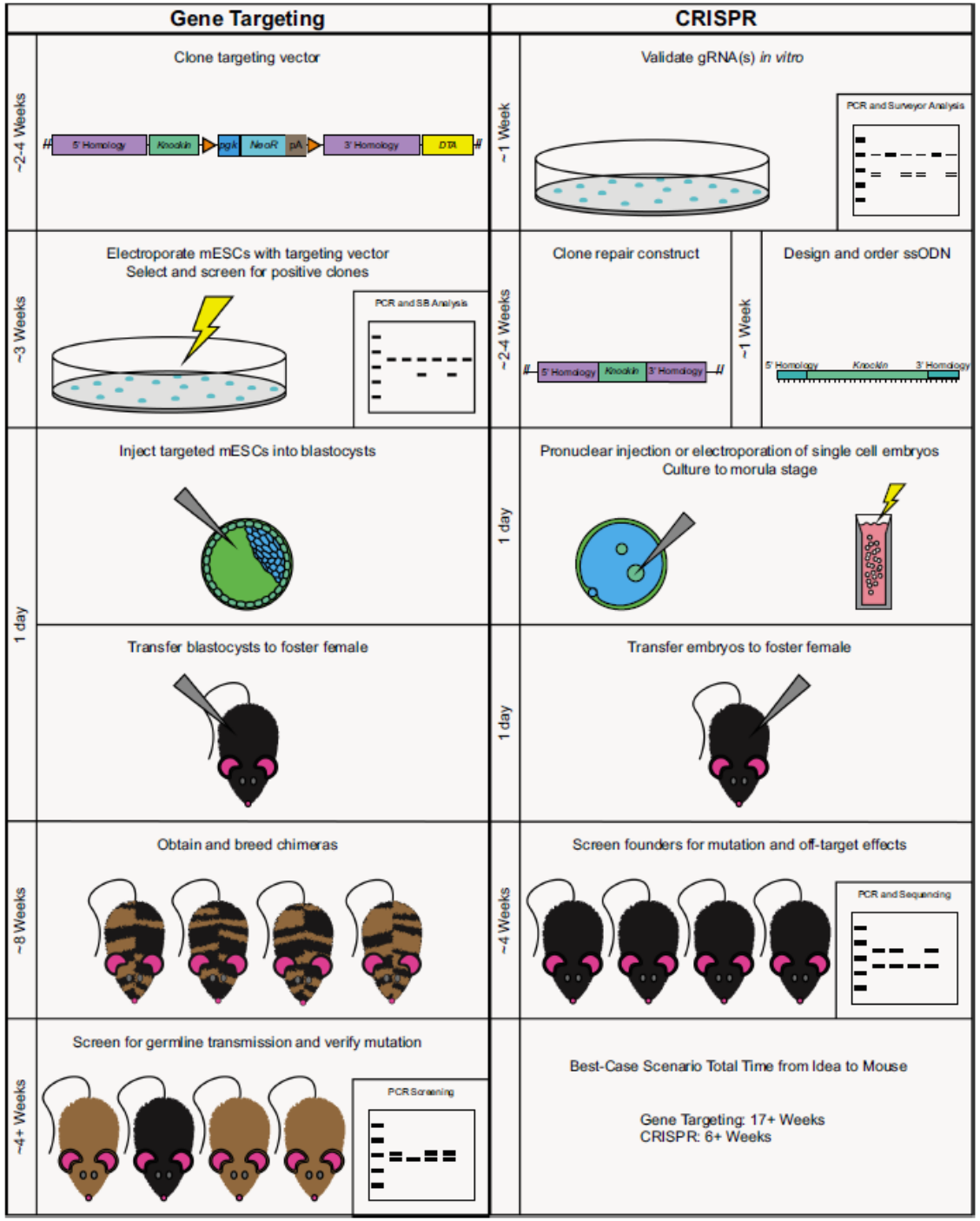
4 Implant into pseudopregnant mice and wait for offspring



5 Collect tail clippings and genotype



..and why?



Clark et al, Genes Dev, 34, 1256-1286 (2020)

CRISPR/Cas9 applications in animal models

- What is your scientific question?
  - Functional studies (gene KI/KO)
  - Disease models (introduction/removal of disease-causing mutations)
  - Preclinical research (humanization of specific proteins for drug testing)

- What modification do you need and what is the best approach for you?
  - Small targeted modifications vs. conditional alleles/humanization
  - Go through ES cells or microinject into zygotes?

Early applications of CRISPR/Cas9 system in animal models

Disease correction (embryo):

Correction of a dominant negative mutation of CRYGC gene (cataracts) – repaired by HDR repair from the healthy allele
(Wu Y et al, Cell Stem Cell 2013, 13:659-62.)

Correction of X-linked muscular dystrophy by correcting the mutation in the dystrophin gene – repaired by HDR using a donor template
(Long C et al, Science 2014, 345:1184-1188.)

Disease correction (adult organism):

Correction of hereditary tyrosinemia (mutation in fumarylacetoacetate hydrolase – FAH leads to accumulation of toxic metabolites, such as fumarylacetoacetate, in hepatocytes, resulting in severe liver damage): sgRNA and donor template injected into a tail vein – low efficiency of repair (1/250 cells) but disease was still corrected (strong positive selection and expansion of Fah+ hepatocytes)
(Yin H et al, Nat Biotchnol 2014, 32:551-553.)

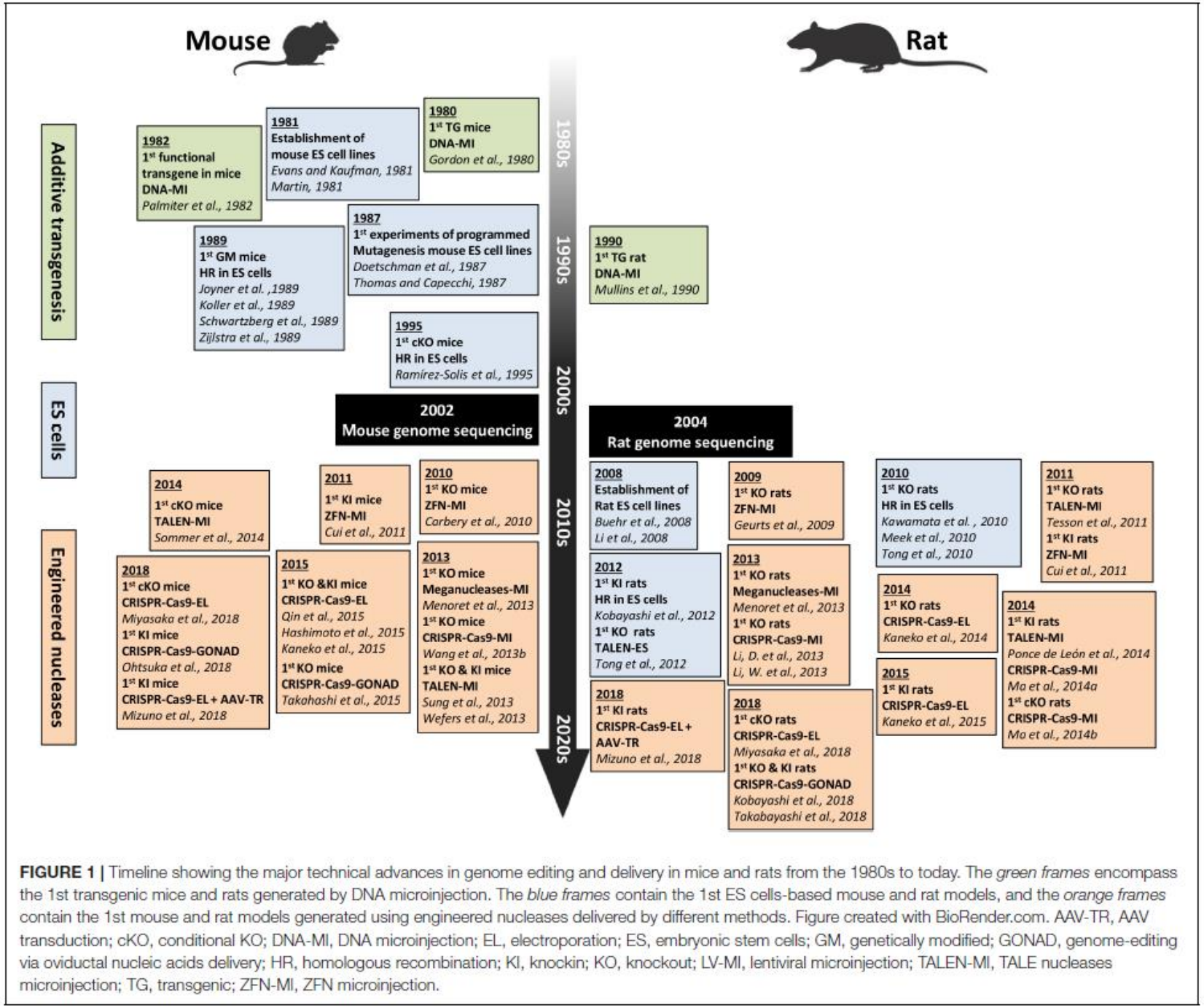


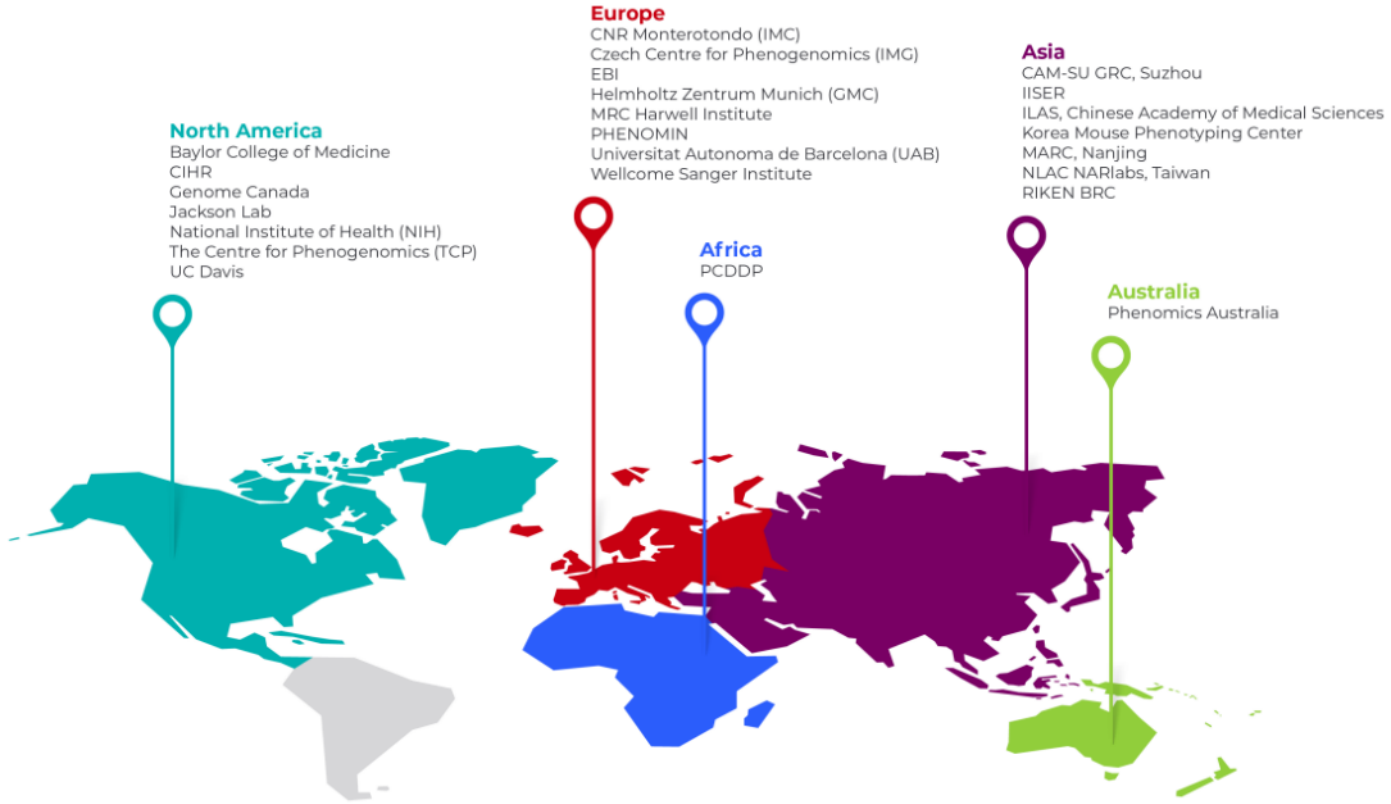
FIGURE 1 | Timeline showing the major technical advances in genome editing and delivery in mice and rats from the 1980s to today. The *green frames* encompass the 1st transgenic mice and rats generated by DNA microinjection. The *blue frames* contain the 1st ES cells-based mouse and rat models, and the *orange frames* contain the 1st mouse and rat models generated using engineered nucleases delivered by different methods. Figure created with BioRender.com. AAV-TR, AAV transduction; cKO, conditional KO; DNA-MI, DNA microinjection; EL, electroporation; ES, embryonic stem cells; GM, genetically modified; GONAD, genome-editing via oviductal nucleic acids delivery; HR, homologous recombination; KI, knockin; KO, knockout; LV-MI, lentiviral microinjection; TALEN-MI, TALE nucleases microinjection; TG, transgenic; ZFN-MI, ZFN microinjection.

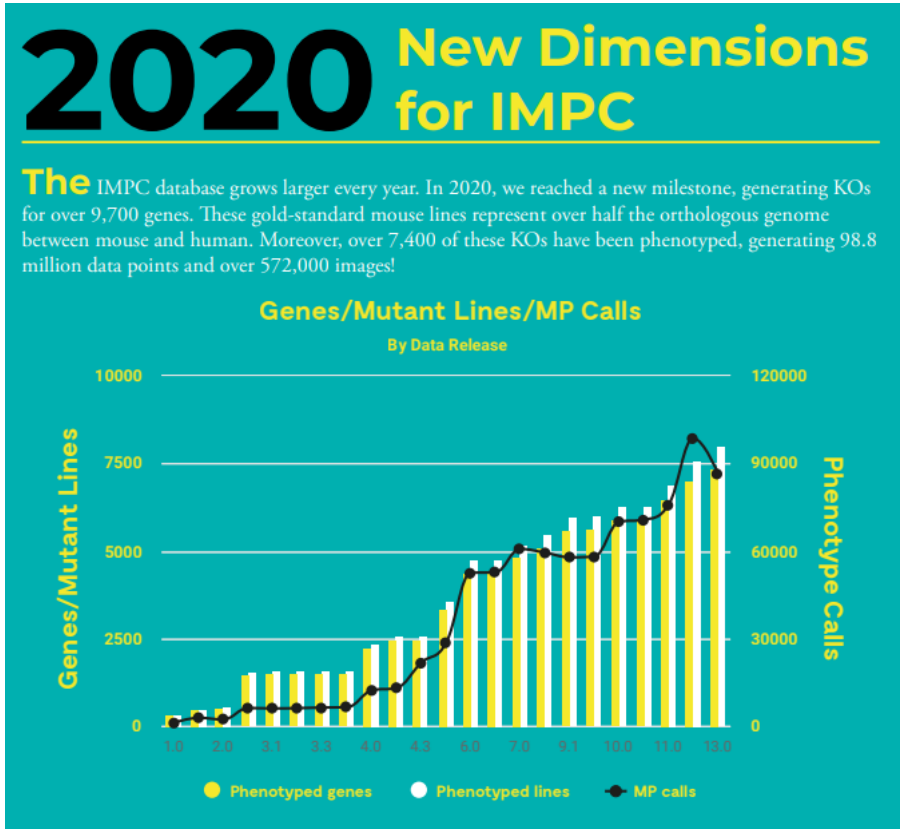
Chenouard et al, Front Genet, 12, 615491 (2021)

IMPC

International Mouse Phenotyping Consortium

The ultimate goal is to determine the function of every gene in the mouse genome. The mice are preserved in repositories and made available to the scientific community representing a valuable resource for basic scientific research as well as generating new models for human diseases..



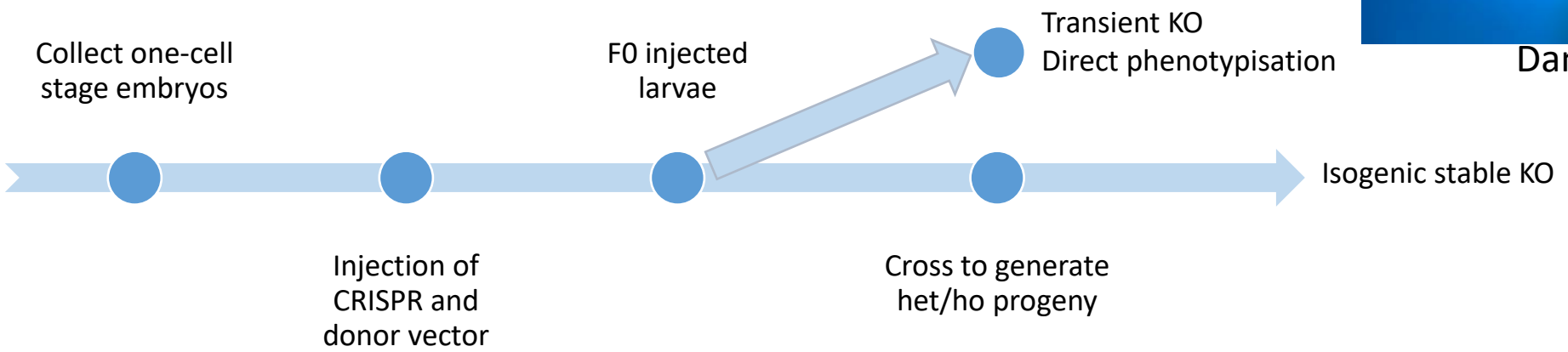


https://review.mousephenotype.org/files/IMPC-2020-Highlights.pdf

Zebrafish

- Microinjection of sgRNA and Cas9 into one-cell stage embryos

Danio rerio



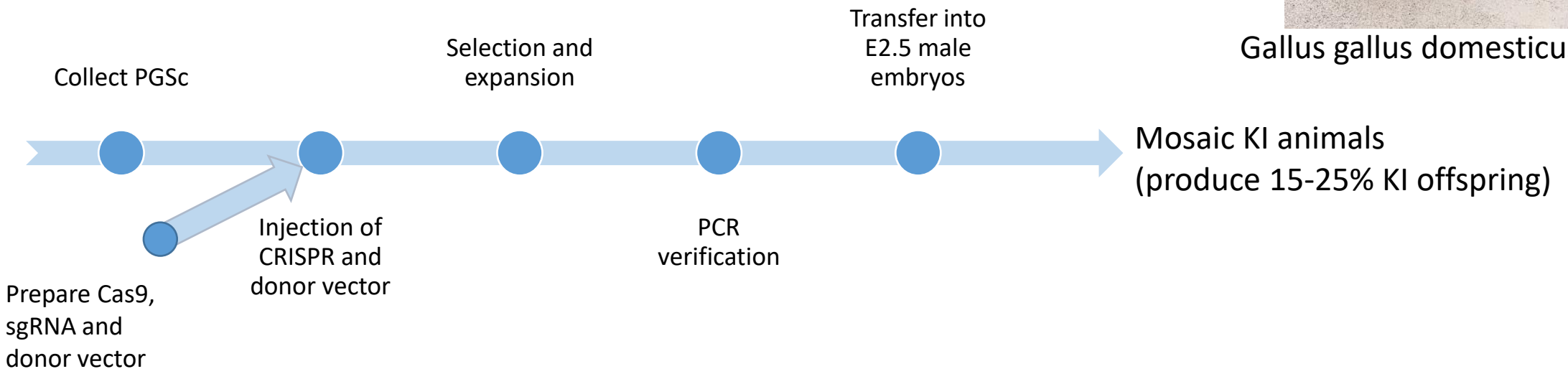
Advantages: large number of eggs, simple collection and fertilization, rapid embryonic development, maturation of 2-3 months

disease modeling in zebrafish represents a valuable approach – considering time and cost saving – to analyze the pathogenic effect of a given mutation or test a battery of candidate drugs before proceeding to further preclinical trials with mammalian animal models.

Chicken

- CRISPR targeting of E2.5 chicken embryos – in ovo electroporation
- Proof of principle study – PAX7 gene KO – 80% efficiency (Veron et al, Dev Biol, 407, 68-74 (2015)
- Specific mutations can be introduced into primoridal germ cells (PGSc), which can be easily isolated from embryonic blood and cultivated in vitro

Gallus gallus domesticus



- After CRISPR modification they are introduced into the blood of chick embryos at E2.5 – PGCs settle in the gonads and produce mosaic semen in adult roosters
- KI of human interferon beta (hIFN-β) into the chicken ovalbumin locus – production of hIFNβ in the egg white (Oishi et al, Sci Rep, 8, 10203 (2018)

Livestock (sheep, goat, pig, cow)

- Main issue: lack of stable ES cell lines
- Traditional approach: gene editing of cultured cells + somatic cell nuclear transfer
- CRISPR/Cas9: zygote microinjection or SCNT

MSTN disruption – improved meat production

human disease models (CF)

MSTN disruption – improved meat production

wool growth

multiple gene deletion – xenotransplantation

CD163 deletion – resistance to PRRSV virus

MSTN disruption – improved meat production

removal of allergenic components in milk

cashmere yield

polyclonal antibody production

hLYZ gene insertion – resistance to mastitis

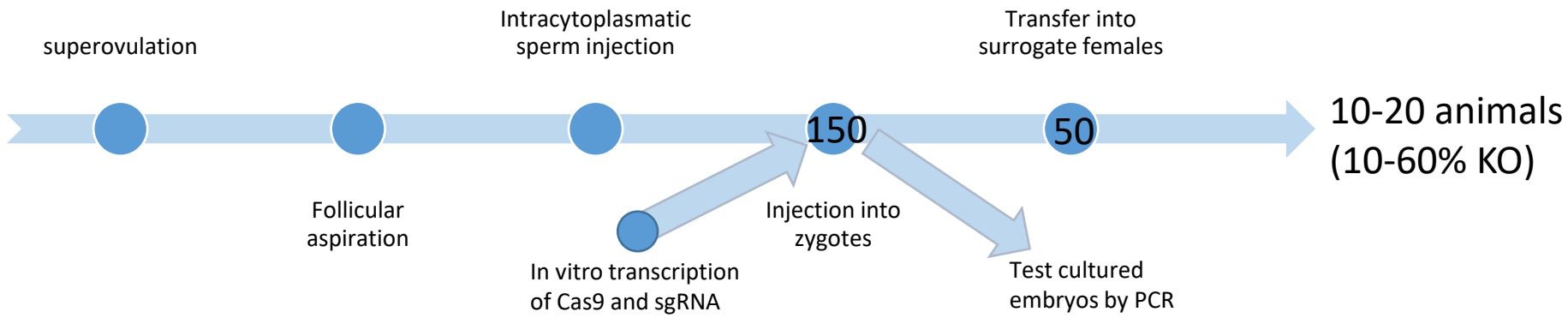
dairy cattle without horns

xenotransplantation

Perisse et al, Front Genet, 11, 614688 (2020)
Lee et al, Theriogeneology, 150, 229-235 (2020)

Nonhuman primate models

- More similar to humans in genetics, physiology, developmental biology, social behaviour and cognition



Macacca fascicularis

Macacca mulatta

Proof of concept study: KO of three targets (Nr0b1, Ppar-γ, Rag1) → efficiency 10-25%, some double KO

cultured embryos and founder animals showed multiple genotypes suggesting the CRISPR/Cas9-mediated cleavage had occurred multiple times at different stages of monkey embryogenesis and resulted in **mosaicism** (Niu et al, Cell, 156, 836-843 (2014)

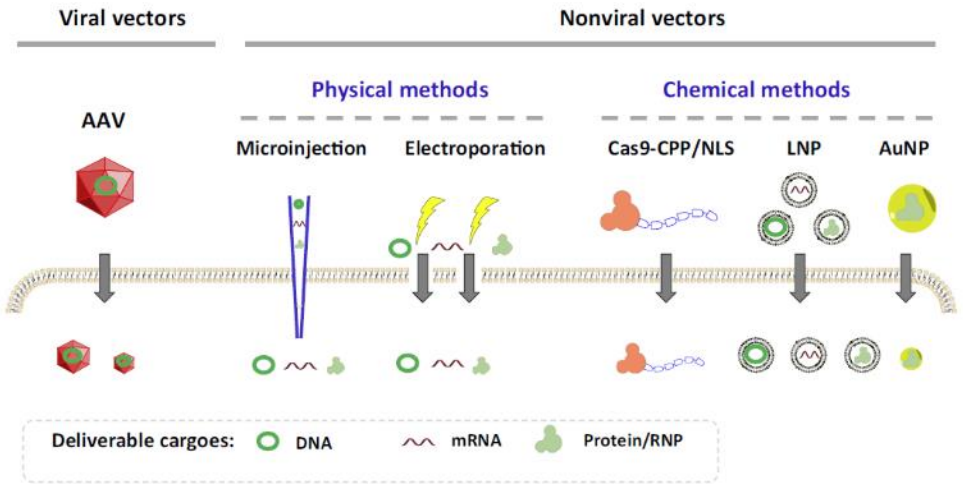
Biallelic KO of TP53 gene, mosaicism (Wan et al, Cell Res, 25(2), 258-261 (2015)

Macacca mulatta (rhesus monkey) – targeting the dystrophin gene hotspots (exons 4 and 46) to generate model of Duchenne muscular dystrophy (Chen et al, Hum Mol Genet, 24(13): 3764-3774 (2015)

But that's not all!
(modifications of the original system)

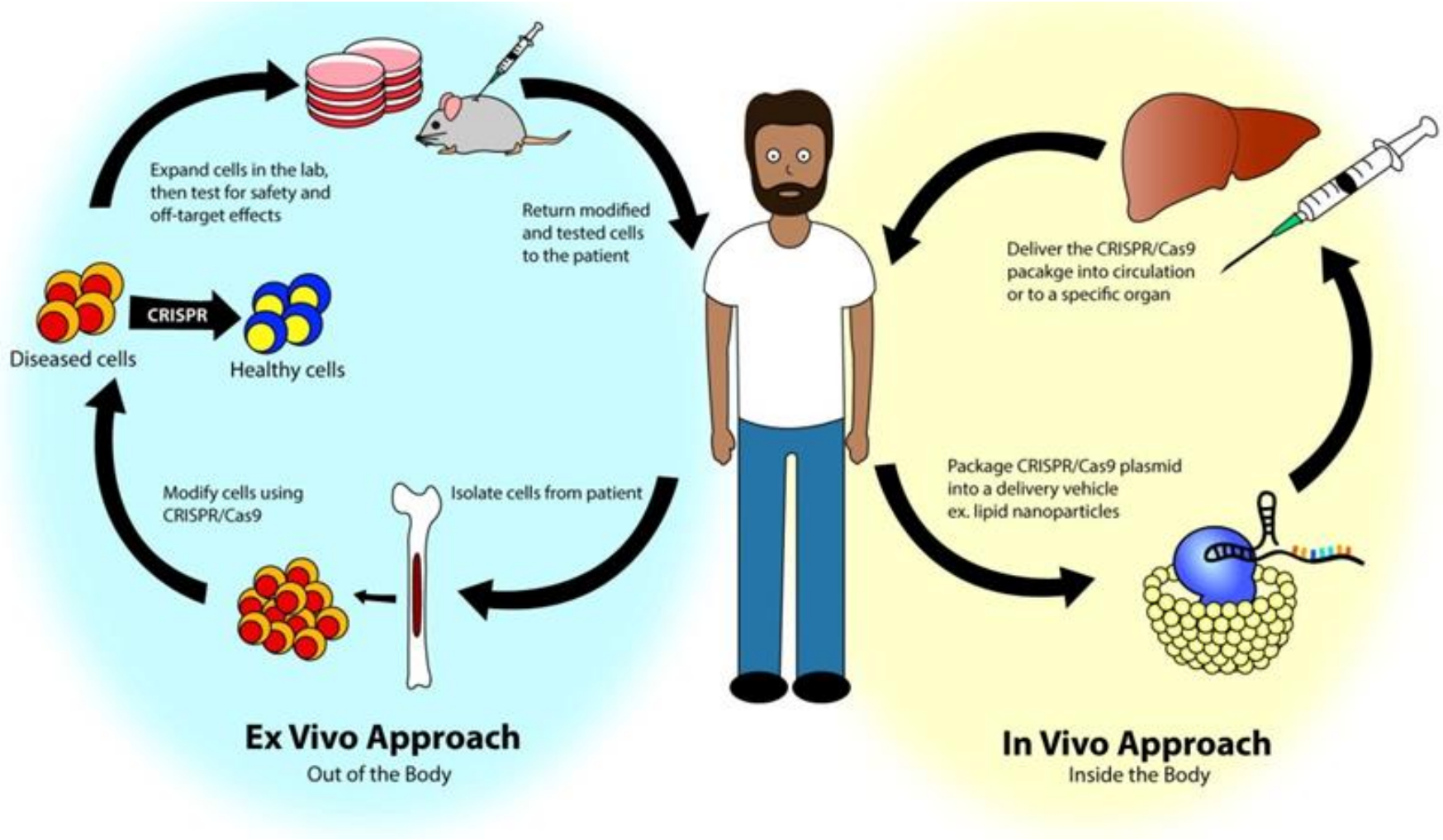
Application	Use of CRISPR	System Components	Anticipated Result(s)	References
Genome engineering	Generation of double-stranded DNA breaks	• Unmodified spCas9 • Gene-specific sgRNA	• Gene knockouts by NHEJ • HDR gene editing	• Jinek et al., 2012 • Cong et al., 2013 • Ran et al., 2013
	Generation of single-stranded DNA breaks	• Cas9 nickase (D10A or H841A) • Gene-specific sgRNA	• HDR gene editing with fewer off-target effects	• Cong et al., 2013 • Mali et al., 2013
	Generation of specific point mutations	• dCas9 linked to an AID ortholog • Gene-specific sgRNA	• Targeted C to T point mutations	• Nishida et al., 2016
		• dCas9 linked to an adenine base editor • Gene-specific sgRNA	• Targeted A to G point mutations	• Gaudelli et al., 2017
Screen	Genome-wide screen using double-stranded DNA breaks	• Unmodified spCas9 • sgRNA library	• Large-scale "drop-out" screens	• Shalem et al., 2014 • Wang et al., 2014
Direct regulation of gene expression	Disrupt gene expression	• dCas9 (D10A, H841A) • Promoter-specific sgRNA	• Gene knockdown	• Qi et al., 2013 • Chen et al., 2013
	Increase gene expression	• dCas9 linked to VP64 or SunTag/VP64 • Promoter-specific sgRNA	• Increased gene expression	• Cheng et al., 2013 • Tanenbaum et al., 2014
Imaging	Detection of specific sequences for imaging purposes	• dCas9 linked to fluorophore	• Imaging of target DNA sequences	• Chen et al., 2013
Epigenetic regulation of gene expression	Alter epigenetic modifications	• dCas9 linked to HAT domain • Region-specific sgRNA	• Increased gene expression	• Hilton et al., 2015
		• dCas9 linked to KRAB domain • Region-specific sgRNA	• Gene silencing	• Thakore et al., 2016
		• dCas9 linked to TET1 domain • Region-specific sgRNA	• Modest increase in gene expression	• Choudhury et al., 2016
SHERLOCK	Detection of disease-specific DNA in patient samples	• Cas13a • Disease-specific sgRNA	• Identification of target DNA sequence in sample	• Gootenberg et al., 2017

Delivery platforms:



Glass et al, Trends Biotech, 36 (2018)
Sanford and Weldon, JYI, 35 (2018)

Application of CRISPR/Cas9 in medicine



The main limitation: delivery to target tissues!

Table 4 Registered clinical trials for the treatment of malignant tumors using CRISPR/Cas9 modified adoptive cell therapy. Data from https://clinicaltrials.gov/ (last updated 14/12/2021)

Clinical Trial Number	Phase	Target Gene	Cancer Type	Cell Type	Sponsor/Country	Recruitment Status
NCT03747965	Phase 1	*PDCD1*-KO	Adult Solid Tumor	Mesothelin-directed CAR-T cells	China	Recruiting
NCT03044743	Phase 1/2	*PDCD1*-KO	Stage IV Gastric Carcinoma Stage IV Nasopharyngeal Carcinoma T-Cell Lymphoma Stage IV Stage IV Adult Hodgkin Lymphoma Stage IV Diffuse Large B-Cell Lymphoma	EBV-CTL cells	China	Recruiting
NCT03081715	Phase 1	*PDCD1*-KO	Esophageal Cancer	Primary T-cells	China	Completed
NCT02793856	Phase 1	*PDCD1*-KO	Metastatic Non-small Cell Lung Cancer	Primary T-cells	China	Completed
NCT04417764	Phase 1	*PDCD1*-KO	Advanced Hepatocellular Carcinoma	Primary T-cells	China	Recruiting
NCT04426669	Phase 1/2	*CISH*-KO	Gastrointestinal Epithelial Cancer Gastrointestinal Neoplasms Tract Cancer Gastrointestinal Cancer Colo-rectal Cancer Pancreatic Cancer Gall Bladder Cancer Colon Cancer Esophageal Cancer Stomach Cancer	TILs	The United States	Recruiting
NCT03057912	Phase 1	*CISH*-KO	Human Papilloma-virus-Related Malignant Neoplasm	TILs	China	Not yet recruiting
NCT03399448	Phase 1	*TRAC*, *TRBC*, *PDCD1*-KO	Multiple Myeloma Melanoma Synovial Sarcoma Myxoid/Round Cell Liposarcoma	NY-ESO-1 redirected autologous T cells	The United States	Completed
NCT03545815	Phase 1	*TRAC*, *TRBC*, *PDCD1*-KO	Solid Tumor	anti-mesothelin CAR-T cells	China	Recruiting
NCT03166878	Phase 1/2	*TRAC*, *TRBC*, *B2M*-KO	B Cell Leukemia B Cell Lymphoma	UCART019	China	Recruiting
NCT05037669	Phase 1	*B2M*, *CIITA*, *TRAC*-KO	Acute Lymphoblastic Leukemia Chronic Lymphocytic Leukemia Non-Hodgkin Lymphoma	CD19-specific CAR-T cells	The United States	Not yet recruiting
NCT04037566	Phase 1	*HPK1*-KO	Acute Lymphoblastic Leukemia in Relapse Acute Lymphoblastic Leukemia Refractory Lymphoma, B-Cell CD19 Positive	XYF19 CAR-T cells	China	Recruiting
NCT04557436	Phase 1	*CD52* and *TRAC*-KO	B Cell Acute Lymphoblastic Leukemia	CD19-specific CAR-T cells	The United Kingdom	Recruiting
NCT04976218	Phase 1	*TGFβR*-KO	Advanced Biliary Tract Cancer	CAR-EGFR T cells	Unknown	Not yet recruiting

Table 4 (continued)

Clinical Trial Number	Phase	Target Gene	Cancer Type	Cell Type	Sponsor/Country	Recruitment Status
NCT04767308	Early Phase 1	*CD5*-KO	$CD5^+$ Relapsed/Refractory Hematopoietic Malignancies Chronic Lymphocytic Leukemia Mantle Cell Lymphoma Diffuse Large B-cell Lymphoma Follicular Lymphoma Peripheral T-cell Lymphomas	CT125A cells	Unknown	Not yet recruiting
NCT04244656	Phase 1	Unknown	Multiple Myeloma	Anti-BCMA Allogeneic CRISPR-Cas9-Engineered T Cells (CTX120)	The United States	Recruiting
NCT04438083	Phase 1	Unknown	Renal Cell Carcinoma	Allogeneic CRISPR-Cas9-Engineered T Cells (CTX130)	The United States Australia Canada Netherlands	Recruiting
NCT04502446	Phase 1	Unknown	T Cell Lymphoma	Allogeneic CRISPR-Cas9-Engineered T Cells (CTX130)	The United States Australia Canada	Recruiting
NCT04035434	Phase 1	Unknown	B-cell Malignancy Non-Hodgkin Lymphoma B-cell Lymphoma Adult B Cell Acute Lymphoblastic Leukemia	CD19-specific CAR-T cells (CTX110)	The United States Australia Canada Germany Spain	Recruiting
NCT04637763	Phase 1	Unknown	Lymphoma Non-Hodgkin Lymphoma Relapsed Non-Hodgkin Lymphoma Refractory B-Cell Non-Hodgkin Lymphoma	CD19-specific CAR-T cells (CB-010)	The United States	Recruiting
NCT03398967	Phase 1/2	Unknown	B Cell Leukemia B Cell Lymphoma	Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cells	China	Recruiting
NCT05066165	Phase 1/2	Unknown	Acute Myeloid Leukemia	WT1-directed TCR T cells	The United States The United Kingdom	Not yet recruiting
NCT04244656	Phase 1	Unknown	Multiple Myeloma	Anti-BCMA Allogeneic CRISPR-Cas9-Engineered T Cells (CTX120)	The United States Australia Canada Spain	Recruiting

Wang et al, Molecular Cancer, 21:57, 2022.

15.000 years ago

The CRISPR Journal
Volume 5, Number 2, 2022
Mary Ann Liebert, Inc.
DOI: 10.1089/crispr.2021.0101

The CRISPR Journal

RESEARCH ARTICLE

Evolutionary Biology and Gene Editing of Cat Allergen, Fel d 1

Nicole F. Brackett,[1,*] Brian W. Davis,[2] Mazhar Adli,[3] Anna Pomés,[1] and Martin D. Chapman[1]

GOOD OR BAD?

SIMPLE
FAST
ADAPTIBLE
APPLICABLE IN MEDICINE

OFF-TARGET EFFECTS
SIDE EFFECTS
STILL NOT COMPLETELY UNDERSTOOD
EUGENICS