Genome Editing and Engineering
Prof. Utpal Bora
Department of Bioscience and Bioengineering
Indian Institute of Technology, Guwahati

Module - 09
Genome Engineered Disease Modelling
Lecture - 01
Animal models - Part B

Welcome back to my course on Genome Editing and Engineering. We are discussing about Genome engineered Disease models, and in part A, we have discussed the basics and importance of Animal models, and this is a continuation of that lecture.

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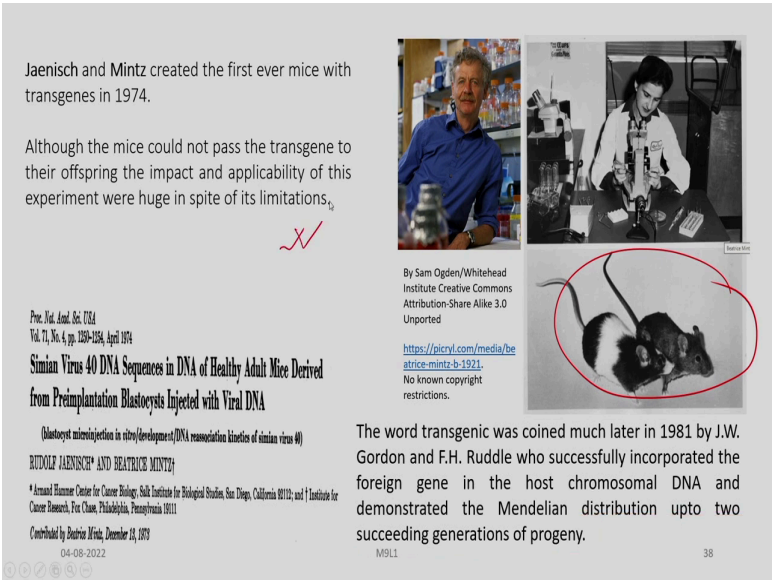

As long as in 1974 Jaenisch and Mintz, you can see here published this paper in PNS. They were the first to create mice with transgenes. And these are some of the pictures of those transgenic mice and as well as Beatrice Mintz and Rudolf Jaenisch. However, the mice they develop could not pass the transgene to the offspring. The impact and applicability of this experiment were however, huge in spite of this limitation.

However, at the time the word transgenic was not known. It was coined in 1981 by J W Gordon and F H Ruddle who successfully incorporated the foreign gene in the host chromosomal DNA, and demonstrated the mendelian distribution up to two succeeding generations of progeny, unlike in this earlier case.

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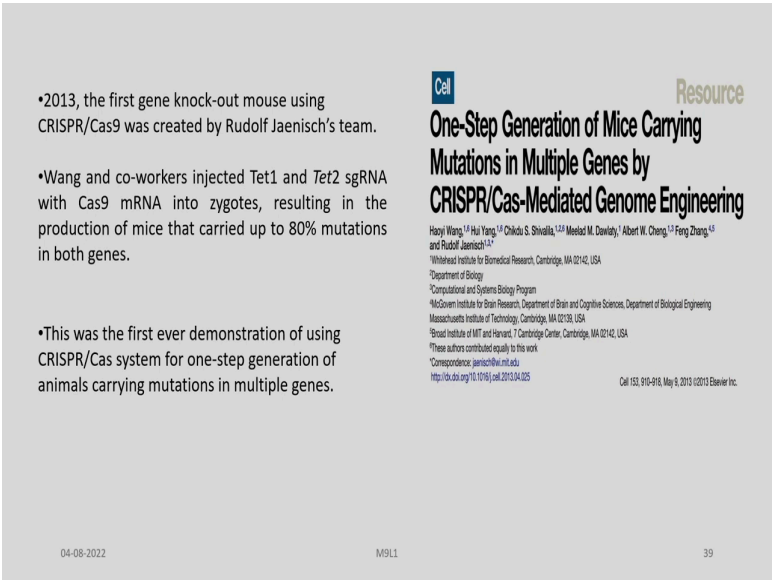

Many years later in 2013, Rudolf Jaenisch again led a team and he was the first to develop gene knockout mouse using CRISPR Cas9. And this paper, you can see in Cell where he reports about a single step generation of mice carrying mutations in multiple genes by CRISPR Cas mediated genome engineering. Wang and co-workers injected Tet 1 and Tet 2 single guide RNA with Cas9 mRNA into zygotes resulting in the production of mice that carried up to 80 percent mutations in both the genes.

And this was the first ever demonstration of using CRISPR Cas system for one step generation of animals carrying mutations in multiple genes.

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One of the important thing that is critical in animal transgenesis and genome editing is the delivery methods. By this time you know about many delivery methods we have discussed in the past lectures. However, we can develop them into the following 3 categories like ex-vivo approaches and you have under them various methods like pronuclear injection, viral vectors, receptor mediated uptake, in vitro electroporation, liposomal transfection, blastocyst microinjection. Then sperm mediated gene transfer and intracytoplasmic sperm injection mediated gene transfer.

And you can see here where they are used. For example, pro-nuclear injection, it is the most commonly used method followed by thousands of labs for over 3 decades. Viral vectors are used by a few because it has limited success. And receptor mediated uptake in the year this paper was compiled, I had only one such report. And novel approach electroporation was reported to be a novel approach at that time and also proven using in the CRISPR Cas system

Whatever progress has been made you can see here the germ line transmission potential. In certain cases it is very high, in certain cases it is very low and in certain cases data or proof is yet to come.

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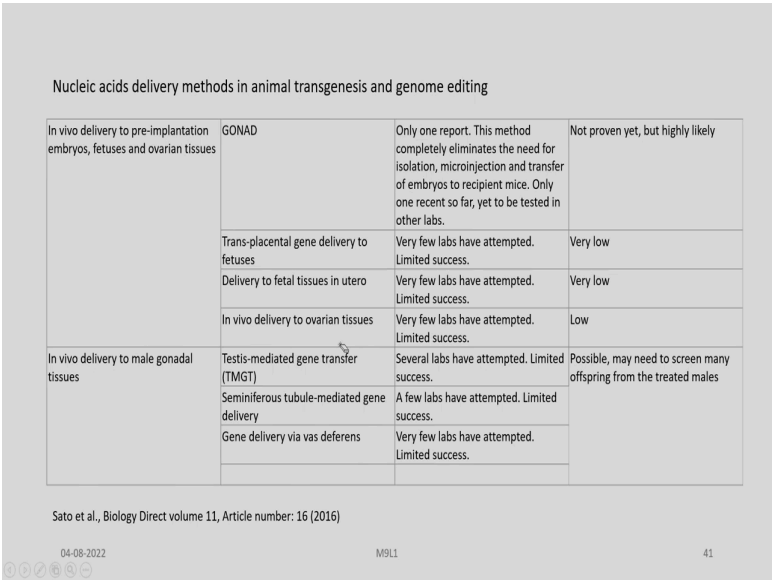

So, another category of nucleic acid delivery is the in vivo delivery to pre implantation embryos foetuses and ovarian tissues. So, here one of the method is the GONAD, we are going to discuss about it. Then, trans-placental gene delivery to foetuses, then delivery to foetal tissues in utero, in vivo delivery to ovarian tissues. And you can see here in the remarks column, GONAD not proven yet, but highly likely to be favourable. In other cases, they are ranged from low to very low.

Then the third category is in vivo delivery to male gonadal tissues and here testis mediated gene transfer method, the seminiferous tubule mediated gene delivery and gene delivery via vas deferens are adopted.

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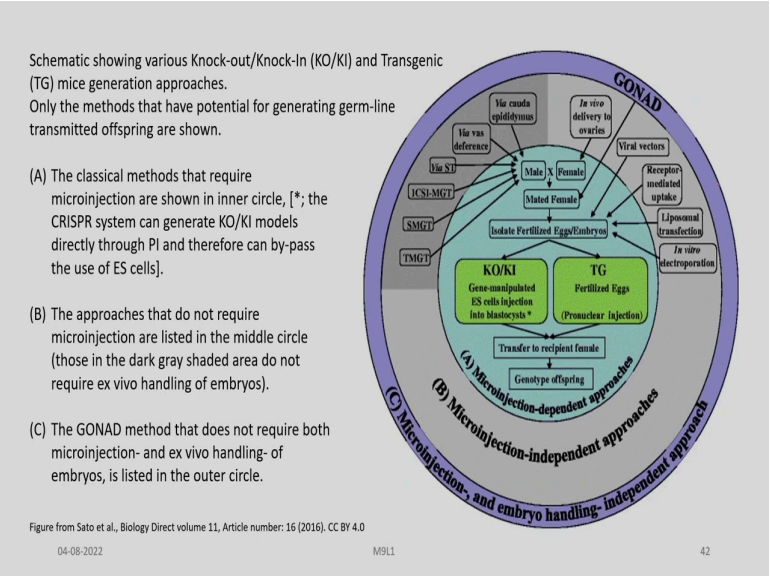

And here you can see a schematic of some of the methods for knockout and knock in in transgenic mice generation. Here only the methods that the potential for generating germ line transmission offspring are shown. For details of all these methods you can visit Sato et al, Biology Direct volume 11, article 16, published in 2016, from where these figure has been adapted.

So, here you can see the number one or a the classical method that require micro injection and these are shown in the inner circle and CRISPR system can generate knock out, knock in models directly through PI and therefore, can bypass the use of embryonic stem cells.

In this zone, you have the approaches which do not require micro injection and they do not require ex vivo handling of the embryos. For example here you have in vitro electroporation liposomal transfection, receptor mediated uptake, then viral vectors, in vivo delivery to ovaries and so on.

In the third approach lies the GONAD method that does not require both micro injection and ex vivo handling of embryos which are listed in this outer circle.

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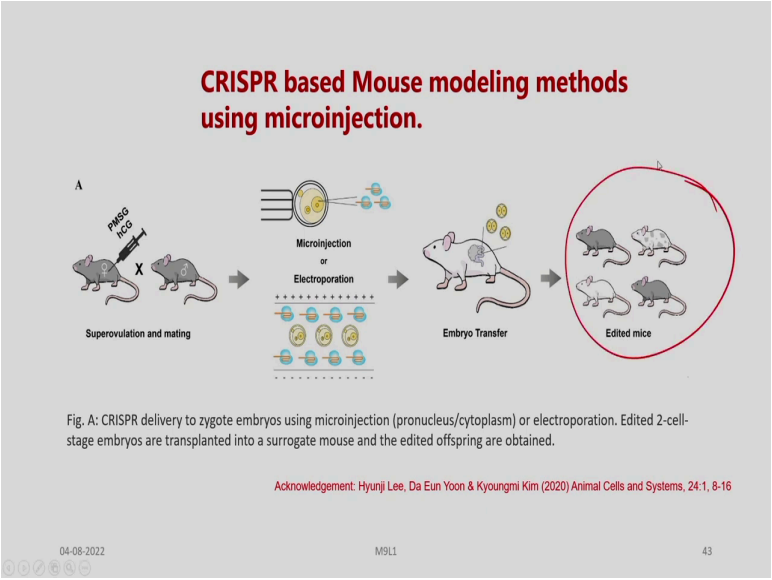

Let us look into the CRISPR based mouse modelling methods using micro injection. So, here you see CRISPR delivery to zygote embryos using micro injection or electroporation. Edited 2-cell stage embryos are transplanted into a surrogate mouse and the edited offsprings are obtained.

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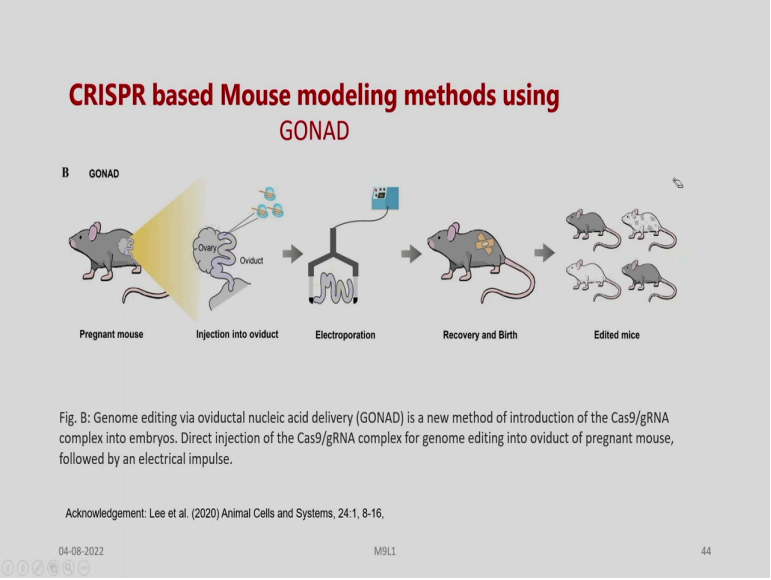

This is the CRISPR based mouse modelling method which uses GONAD. This is genome editing via oviductal nucleic acid delivery. And this is a new method of introduction of the Cas9 guide RNA complex into embryos. Direct injection of the Cas9 DNA complex for

genome editing into oviduct or pregnant mouse is done, followed by an electrical impulse as you can see over here. After which there is recovery and birth, which results to edited mice progeny. And here you can see some chimeras as well.

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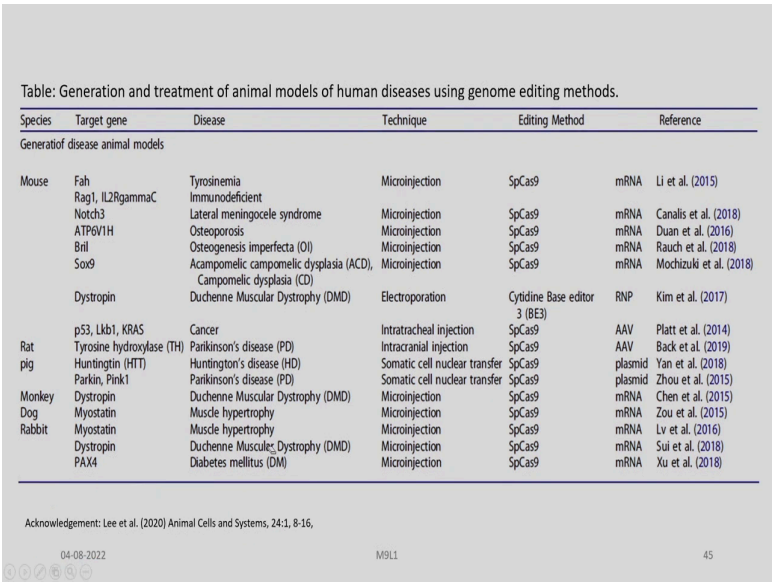

Table: Generation and treatment of animal models of human diseases using genome editing methods.

Species	Target gene	Disease	Technique	Editing Method		Reference
Generatiof disease animal models						
Mouse	Fah	Tyrosinemia	Microinjection	SpCas9	mRNA	Li et al. (2015)
	Rag1, IL2RgammaC	Immunodeficient				
	Notch3	Lateral meningocele syndrome	Microinjection	SpCas9	mRNA	Canalis et al. (2018)
	ATP6V1H	Osteoporosis	Microinjection	SpCas9	mRNA	Duan et al. (2016)
	Bril	Osteogenesis imperfecta (OI)	Microinjection	SpCas9	mRNA	Rauch et al. (2018)
	Sox9	Acampomelic campomelic dysplasia (ACD), Campomelic dysplasia (CD)	Microinjection	SpCas9	mRNA	Mochizuki et al. (2018)
	Dystropin	Duchenne Muscular Dystrophy (DMD)	Electroporation	Cytidine Base editor 3 (BE3)	RNP	Kim et al. (2017)
	p53, Lkb1, KRAS	Cancer	Intratracheal injection	SpCas9	AAV	Platt et al. (2014)
Rat	Tyrosine hydroxylase (TH)	Parikinson's disease (PD)	Intracranial injection	SpCas9	AAV	Back et al. (2019)
pig	Huntingtin (HTT)	Huntington's disease (HD)	Somatic cell nuclear transfer	SpCas9	plasmid	Yan et al. (2018)
	Parkin, Pink1	Parikinson's disease (PD)	Somatic cell nuclear transfer	SpCas9	plasmid	Zhou et al. (2015)
Monkey	Dystropin	Duchenne Muscular Dystrophy (DMD)	Microinjection	SpCas9	mRNA	Chen et al. (2015)
Dog	Myostatin	Muscle hypertrophy	Microinjection	SpCas9	mRNA	Zou et al. (2015)
Rabbit	Myostatin	Muscle hypertrophy	Microinjection	SpCas9	mRNA	Lv et al. (2016)
	Dystropin	Duchenne Muscular Dystrophy (DMD)	Microinjection	SpCas9	mRNA	Sui et al. (2018)
	PAX4	Diabetes mellitus (DM)	Microinjection	SpCas9	mRNA	Xu et al. (2018)

Acknowledgement: Lee et al. (2020) Animal Cells and Systems, 24:1, 8-16,

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This table gives us some idea about the generation and treatment of animal models of human diseases using genome editing methods and the various species that are used. This tells us about the generation of disease animal models. Here are the species like mouse, rat, pig, monkey, dog and rabbit. And certain genes are being targeted to create the disease model. I will not go into details of these genes, but that particular gene will depend on the type of disease for which we are trying to create the disease model.

For example in Duchenne muscular dystrophy, our target is dystropin. And for osteoporosis our target is ATP6V1H. And then you have notch 3 for lateral meningocele syndrome. Similarly, in rat, we are trying to target the Huntingtin for creating a disease model for Huntington disease or Parkin and Pink1 for creating a disease model of Parkinson's disease and so on and so forth as you can see in this table.

Now, for creating this disease model, the delivery mode is also very very important. In the majority of cases you can see micro injection is used, but in certain cases we also have electroporation or intra tracheal injection. And other techniques like somatic cell nuclear transfer as well. And here are the editing methods. You use here streptococcus pyogenes Cas9 or SpCas9 in the majority of cases.

But in certain cases for Duchenne muscular dystrophy, we are using cytidine base editor. And those who are interested for further details about these disease models and the techniques and the editing methods, you can visit these references which are available in Lee et al, 2020, Animal Cells and Systems, volume 24:1 and page number 8 to 16.

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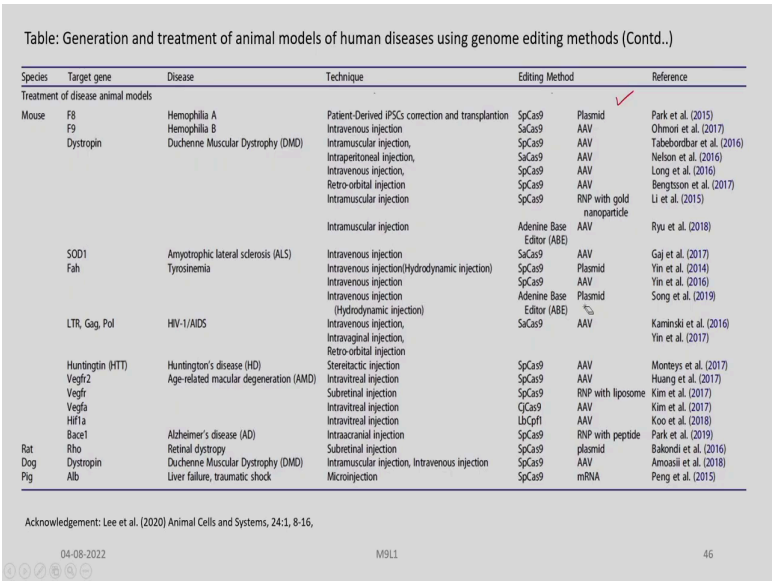
Table: Generation and treatment of animal models of human diseases using genome editing methods (Contd..)

Species	Target gene	Disease	Technique	Editing Method		Reference
Treatment of disease animal models						
Mouse	F8	Hemophilia A	Patient-Derived iPSCs correction and transplantion	SpCas9	Plasmid	Park et al. (2015)
	F9	Hemophilia B	Intravenous injection	SaCas9	AAV	Ohmori et al. (2017)
	Dystropin	Duchenne Muscular Dystrophy (DMD)	Intramuscular injection,	SpCas9	AAV	Tabebordbar et al. (2016)
			Intraperitoneal injection,	SaCas9	AAV	Nelson et al. (2016)
			Intravenous injection,	SpCas9	AAV	Long et al. (2016)
			Retro-orbital injection	SpCas9	AAV	Bengtsson et al. (2017)
			Intramuscular injection	SpCas9	RNP with gold nanoparticle	Li et al. (2015)
			Intramuscular injection	Adenine Base Editor (ABE)	AAV	Ryu et al. (2018)
	SOD1	Amyotrophic lateral sclerosis (ALS)	Intravenous injection	SaCas9	AAV	Gaj et al. (2017)
	Fah	Tyrosinemia	Intravenous injection(Hydrodynamic injection)	SpCas9	Plasmid	Yin et al. (2014)
			Intravenous injection	SpCas9	AAV	Yin et al. (2016)
			Intravenous injection (Hydrodynamic injection)	Adenine Base Editor (ABE)	Plasmid	Song et al. (2019)
	LTR, Gag, Pol	HIV-1/AIDS	Intravenous injection, Intravaginal injection, Retro-orbital injection	SaCas9	AAV	Kaminski et al. (2016) Yin et al. (2017)
	Huntingtin (HTT)	Huntington's disease (HD)	Stereitactic injection	SpCas9	AAV	Monteys et al. (2017)
	Vegfr2	Age-related macular degeneration (AMD)	Intravitreal injection	SpCas9	AAV	Huang et al. (2017)
	Vegfr		Subretinal injection	SpCas9	RNP with liposome	Kim et al. (2017)
	Vegfa		Intravitreal injection	CjCas9	AAV	Kim et al. (2017)
	Hif1a		Intravitreal injection	LbCpf1	AAV	Koo et al. (2018)
	Bace1	Alzheimer's disease (AD)	Intraacranial injection	SpCas9	RNP with peptide	Park et al. (2019)
Rat	Rho	Retinal dystropy	Subretinal injection	SpCas9	plasmid	Bakondi et al. (2016)
Dog	Dystropin	Duchenne Muscular Dystrophy (DMD)	Intramuscular injection, Intravenous injection	SpCas9	AAV	Amoasii et al. (2018)
Pig	Alb	Liver failure, traumatic shock	Microinjection	SpCas9	mRNA	Peng et al. (2015)

Acknowledgement: Lee et al. (2020) Animal Cells and Systems, 24:1, 8-16,

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This is a continuation of the earlier table where we are having some details on the treatment of disease models. And you can see here similarly the species mouse, rat, dog and pig and the target gene over here F8, 9 and dystropin and the corresponding diseases. And here is the technique which is briefly described over here. In case of mouse, target gene F8, disease haemophilia A, the technique used is patient derived iPSCs correction and transplantation.

And in majority of cases you have intravenous injection or intramuscular injection or in certain cases you have subretinal retinal injection and intramuscular and also micro injections. And the editing method mostly dominated by SpCas9. And in certain cases we have the plasmids or the AAVs or in certain cases even RNP with gold nano particle has been deployed.

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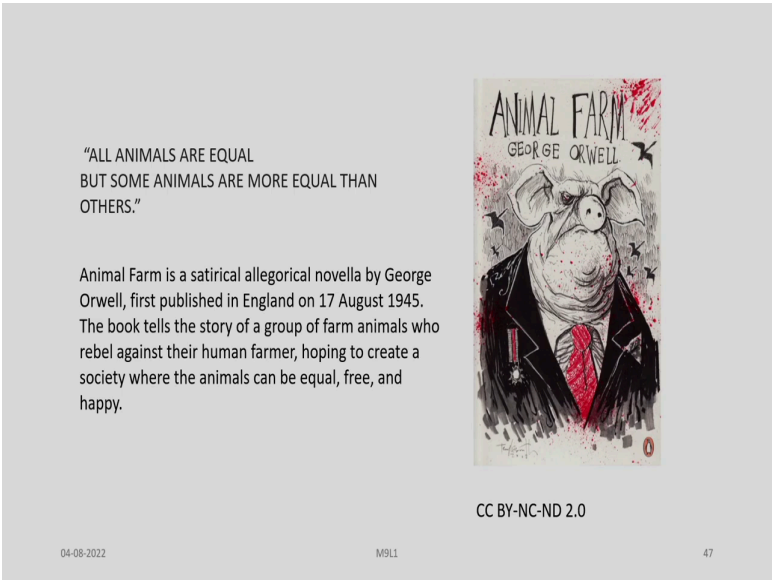

Let us now discuss a little bit something different from the earlier concepts. So, this is a famous novel by George Orwell called Animal Farm. Basically, it is a satirical allegorical novella. And this book tells the story of a group of farm animals who rebel against their human owner farmer and hoping to create a society where the animals can be equal, free and happy.

And at the end of this novel you will find that many of these pigs started becoming like their human farmer. And they even started behaving whether in terms of dressing, eating and even social behaviour more like humans. So, this is one of the famous quotes in the book, "All animals are equal, but some animals are more equal than others." So, the whole idea of taking you to the story framework of these particular novel is that the idea about humanization of animals.

I mean whether there are techniques to convert a animal into a humans which has been of course, used in a very satirical way by George Orwell. The answer is possible. So, we are having interest in humanized animals from the point of view of animal disease models. So, we know that animals are not exact replica of humans, and we also know about many of the human and animal genes are similar, but not exactly 100 percent same. So, if we can transform those genes in the animals to be more like human by changing the sequences, we may be having better animal models.

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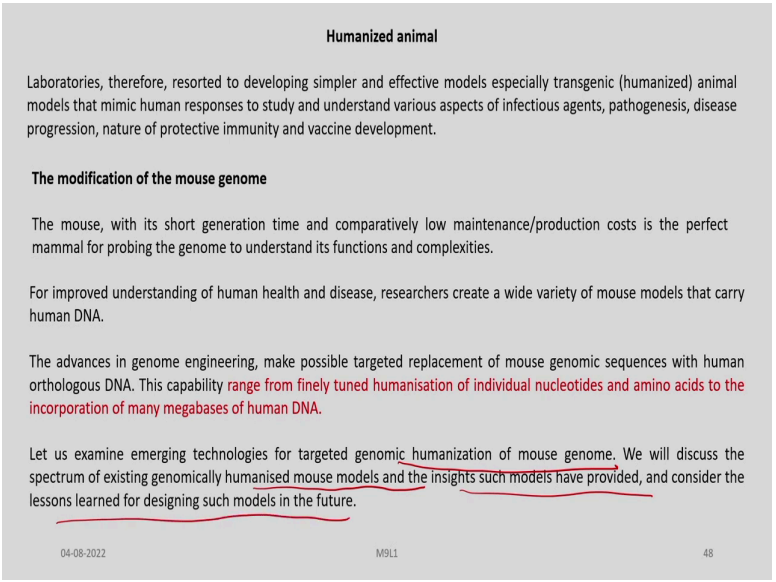

So, here you can see that, laboratories have resorted to developing simpler and effective models especially transgenic, animal models that mimic human responses to study and understand various aspects of infectious agents, pathogenesis, disease progression, nature of protective immunity and vaccine development. The modification of the mouse genome: The mouse, with its short generation time and comparatively low maintenance and production cost is a perfect mammal for probing the genome to understand its functions and complexities.

And it is being used for improved understanding of human health and disease, for which researchers have created a wide variety of mouse models that carry human DNA. The advances in genome engineering, make possible targeted replacement of mouse genomic sequences with human orthologous DNA. This capability range from finely tuned humanization of individual nucleotides and amino acids to the incorporation of many mega bases of human DNA.

We will examine the emerging technologies for targeted genomic humanization of a mouse genome and discuss the spectrum of existing genomically humanized mouse models and the insights such models have provided, and considered the lessons learned for designing such models in future.

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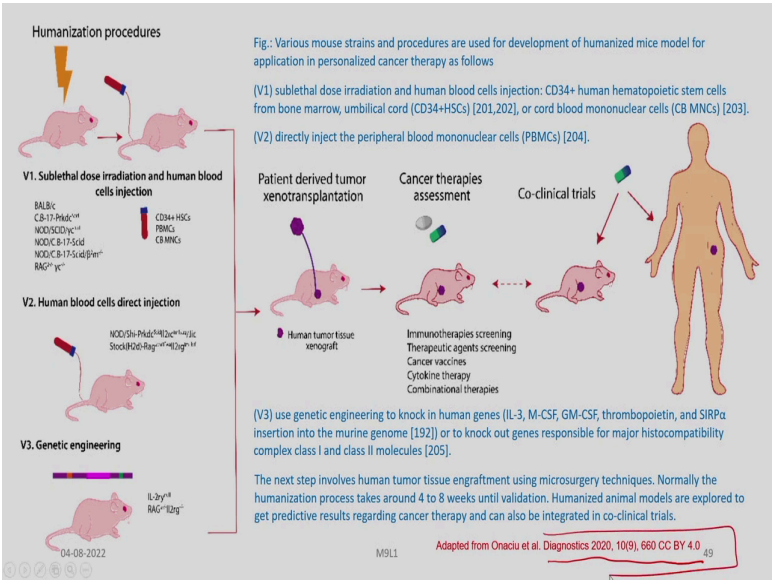

Let us examine some of the humanization procedures which are being used in various mouse strains for the development of humanized mice models for application in personalized cancer therapy. The first one is the sublethal dose irradiation and human cell injection CD34 plus human hematopoietic stem cells from bone marrow, umbilical cord or cordless mononuclear cells are utilized for creating these kind of models.

In the second approach, there is a direct injection of the peripheral blood mononuclear cells. And in the third approach they used the genetic engineering to knock in human genes, like IL3, M-CF, GM-CSF thrombopoietin etcetera. Auto knockout genes responsible for major histocompatibility complex class 1 and class 2 molecules. The next step involves human tissue engraftment using microsurgery technique. Normally, the humanization procedure takes around 4 to 8 weeks until validation.

Humanized animal models are explored to get predictive results regarding cancer therapy and can also be integrated into co clinical trials. So, this figure is adapted from this Diagnostics 2020, 10 (9), 660. For further details about these procedures you can refer to this paper by Onaciu.

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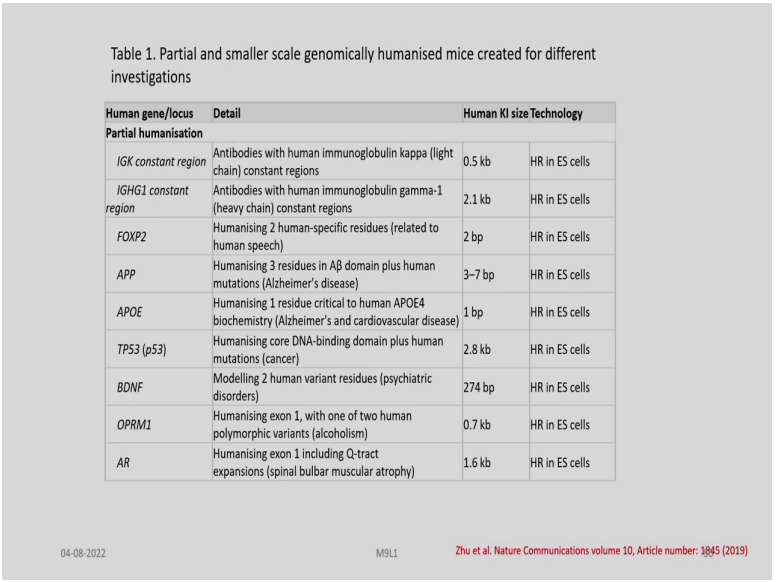

Table 1. Partial and smaller scale genomically humanised mice created for different investigations

Human gene/locus	Detail	Human KI size	Technology
Partial humanisation			
IGK constant region	Antibodies with human immunoglobulin kappa (light chain) constant regions	0.5 kb	HR in ES cells
IGHG1 constant region	Antibodies with human immunoglobulin gamma-1 (heavy chain) constant regions	2.1 kb	HR in ES cells
FOXP2	Humanising 2 human-specific residues (related to human speech)	2 bp	HR in ES cells
APP	Humanising 3 residues in Aβ domain plus human mutations (Alzheimer's disease)	3–7 bp	HR in ES cells
APOE	Humanising 1 residue critical to human APOE4 biochemistry (Alzheimer's and cardiovascular disease)	1 bp	HR in ES cells
TP53 (p53)	Humanising core DNA-binding domain plus human mutations (cancer)	2.8 kb	HR in ES cells
BDNF	Modelling 2 human variant residues (psychiatric disorders)	274 bp	HR in ES cells
OPRM1	Humanising exon 1, with one of two human polymorphic variants (alcoholism)	0.7 kb	HR in ES cells
AR	Humanising exon 1 including Q-tract expansions (spinal bulbar muscular atrophy)	1.6 kb	HR in ES cells

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This table gives us an idea about the partial and smaller scale genomically humanized mice created for different investigations. Here we have the human gene locus and the partial humanization. You can see the IGK constant region, and this is associated with the antibodies with human immunoglobin kappa constant region. Here is another candidate like FOXP2. This is the humanization of 2 human-specific residues. And then APP is the humanizing 3 residues in a beta domain plus human mutations related to Alzheimer's disease.

Similarly, we have APOE, which is the humanization of 1 residue critical to human APOE4 biochemistry for Alzheimer's and cardiovascular diseases. Similarly, we have BDNF which is the modelling of 2 human variant residues connected to psychiatric disorders. Overall, we can see a large number of researchers are working in the area of partial or small scale humanization of the mice genome whereby human genes are tried to be; tried to be cloned.

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Table 1. Partial and smaller scale genomically humanised mice created for different investigations

Human gene/locus	Detail	Human KI size	Technology
Partial humanisation			
HTT	Humanising exon 1 including Q-tract expansions (Huntington disease)	250–500 bp	HR in ES cells
TNFSF11	Humanising single exon; target of monoclonal antibody drug (bone disorders)	0.4 kb	HR in ES cells
FUS	Recapitulating FUS splice site mutation ('delta14'), plus human frameshifted C-terminus (ALS)	1 bp + 9 bp	HR in ES cells
IKAP	Humanising exon 20 + flanking introns, including human mutation (familial dysautonomia)	1.5 kb	HR in ES cells
FKTN	Humanising exon 10 ± human-specific SVA retrotransposon insertion (Fukuyama muscular dystrophy)	6 + 3 kb	RMCE in ES cells
PRNP	Humanising prion protein gene with mutation, minus signal peptide (prion diseases)	0.8 kb	HR in ES cells
CTLA4	Humanising gene minus signal peptide, to study anti-human CTLA4 antibody efficacy (cancer)	3.2 kb	HR in ES cells
DMPK	Humanising exons 13–15 plus CTG repeats (myotonic dystrophy)	1.5–1.7 kb	HR in ES cells
Humanising non-coding variants			
eQTL rs2277862; T	To model lipid-functional non-coding human variant	5 bp	CRISPR/Cas9-assisted HR in zygotes

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And we have many other such candidates like HTT, TNFS11, and FUS and IKAP and so on. We also have some people working in humanizing the non-coding variance where you see this eQTL rs2277862. And it is used to model lipid functional non-coding human variants. And in majority of these cases you can see the technology that is being used mostly HR in embryonic stem cells, and in this case, we are using CRISPR Cas9 assisted HR in the zygotes.

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Table 2. Whole gene and larger-scale genomically humanised mice created for different investigations

Human gene/locus	Detail	Human KI size	Technology	References
				Whole gene humanisation
APOE	APO-E2, -E3, -E4 human variants (Alzheimer's and cardiovascular disease)	4.1 kb	HR in ES cells	39,40,–41
IL3, TPO, CSF1, CSF2, IL15, SIRPA	Supporting the human cellular component of chimaeric animals with a human immune system in immunodeficient mice	8.5–17.5 kb	HR in ES cells	44,45,46,47,–48
C5AR1	To generate and study anti-human C5AR1 antibody efficacy (inflammation)	1 kb	HR in ES cells	49
PXR, CAR	Xenobiotic sensors; predicting human drug responses	4.3 kb and 7 kb	HR in ES cells	52, 53
GCGR	Humanising target of monoclonal antibody drug (diabetes)	4.7 kb	HR in ES cells	62
SIRPA and *CYP2D6*	Testing ssODN-mediated end joining	199.5 and 6.2 kb	ssODN-mediated end joining in rat zygotes	124
KMT2D	Human cancer gene KI; testing CRISPR/Cas9-assisted HR in ES cells and homology arm lengths	42 kb	CRISPR/Cas9-assisted HR in ES cells	128
RHO	Whole Rhodopsin gene plus GFP tag (retinal degeneration)	7.4 kb	Both HR and RMCE in ES cells	106

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And there many such whole gene and large scale genomically humanized mice created for different investigations as well with various human gene locus. And different technologies

has been used for this, like HR in ES cells or ssODN mediated end joining in rat zygotes or CRISPR Cas9 assisted HR in ES cells or both HR and RMCE in ES cells. So, overall, today we have many developments using homologous recombination as well as CRISPR Cas9 for the humanization of a mice genome.

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Table 2. Whole gene and larger-scale genomically humanised mice created for different investigations

Human gene/locus	Detail	Human KI size	Technology	References
Humanising large loci and gene clusters				
IGH and *IGK* variable regions	Antibodies with human variable regions	2.6 + 3 Mb	Iterative HR in ES cells	56, 57
IGH, IGK, and *IGL* variable regions	Antibodies with human variable regions	917 + 838 + 932 kb	S-RMCE in ES cells	55
α globin cluster	Proof-of-principle for large humanisation and to study α globin gene expression	117 kb	RMGR	58
β *globin* genes	To study β thalassaemia	8.7–11.7 kb	HR in ES cells	94, 97,98,–99
CYP3A4/CYP3A7	To study cytochrome P450-mediated drug metabolism	~100 kb	RMCE in ES cells	51
Transchromosomic mice				
Chromosome 21	Transchromosomic mouse model for Down syndrome	42 Mb	MMCT	54, 60, 61, 70, 71, 107
Immunoglobulin loci transchromosomic mice	Transchromosomic mouse model for human monoclonal antibody production	1.5 + 2 Mb	MMCT	102, 129

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And they are these are some of the additional information in these progress.

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Compared to the large number of classic transgenic mouse strains that mostly carry human DNA as randomly inserted, multicopy transgenes, the number of targeted genomically humanised mouse strains is less (Tables 1 and 2 showed).

The latter are designed to have greater physiological relevance than their classic transgenic counterparts as,

- they maintain the correct genomic context of a gene of interest to preserve physiological expression levels and
- correct spatiotemporal expression patterns.
- the translated protein is expected to display the unique biochemical properties of the human gene, including potentially unique deleterious properties when mutated.
- where non-coding human sequences are incorporated, human-specific regulation and human gene splice isoforms may be maintained.

Zhu et al. Nature Communications volume 10, Article number: 1845 (2019)

Compared to the large number of classic transgenic mouse strains that mostly carry human DNA as randomly inserted, multicopy transgenes, the number of targeted genomically humanized mouse strains is less, as we can see in the earlier tables. The latter are designed to have greater physiological relevance than their classic transgenic counterparts as, they maintained the correct genomic context of a gene of interest to preserve physiological expression levels. And correct spatiotemporal expression patterns.

The translated protein is expected to display the unique biochemical properties of the human gene, including potentially unique deleterious properties when mutated. When non-coding human sequences are incorporated human-specific regulations and human gene splice isoforms may be maintained.

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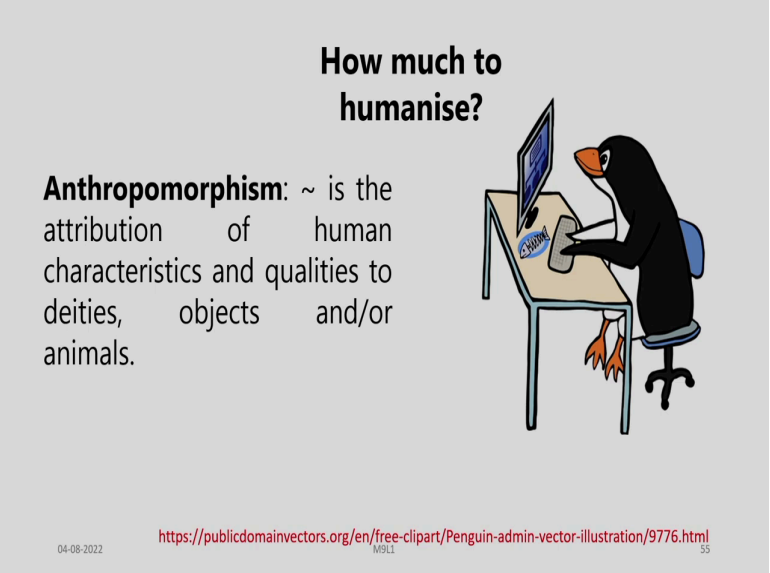

So, today, we have the technology, not only the classical transgenic technologies of random insertion of human genes into mice genome or other animals which may be pig as well, we also deploy the genomic ending technologies for humanizing mouse or animal models. Now, a question may arise, how much to humanize? If we transfer all the human genes and maybe soon in the future also the non-coding sequences, would there be some kind of a catastrophe like that mentioned in Animal Farms by George Orwell.

The pigs becoming almost like humans. And if some kind of ethical problems may arise because we have humanized, the mouse too much, these are all hypothetical questions and there are no any direct answers to these in the current time. This is a term called

anthropomorphism. This is the attribution of human characteristics and qualities to deities, objects and or animals. So, our humanization of the mice may result in some opportunity or challenge or disadvantage of this nature.

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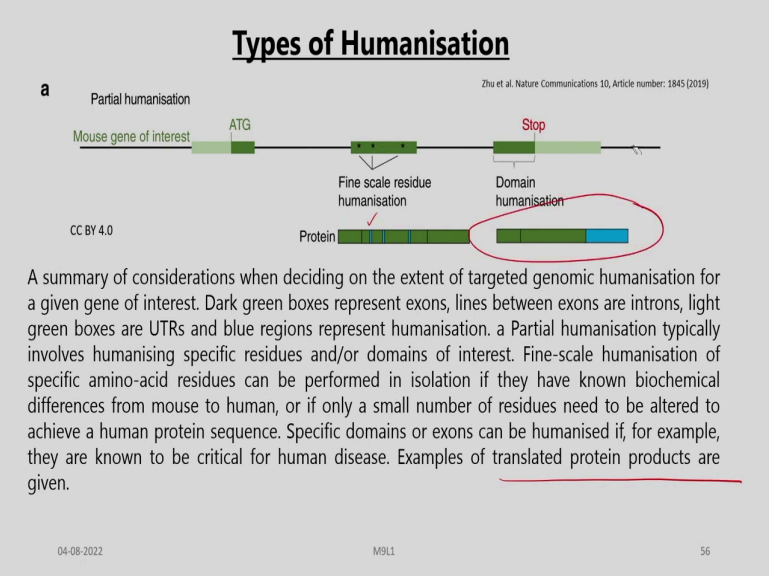

Anyway let us try to find out what are the different kinds of humanization that is technically available or feasible as of now. We have discussed in table 1 and table 2 about some partial and whole humanization. So, we have the technologies for partial humanization, so here you have a mouse gene of interest and you can see here a ATG sequence.

And you have here a stop sequence, and then you have fine scale residue where you are doing the humanization and we may also go for a full domain humanization over here.

So, a summary of considerations when deciding on the extent of targeted genomic humanization for a given gene of interest. The dark green boxes represent the exons, lines between exons are introns, light green boxes are UTRs and blue regions represent the humanization. So, here you can see the limited humanization and here is a full domain humanization.

Fine-scale humanization of specific amino-acid residues can be performed in isolation if they have known biochemical differences from mouse to human, or if only a small number of residues need to be altered to achieve a human protein sequence. Specific domain or exons

can be humanized if, for example, they are known to be critical for human disease. So, here is the example of translated protein products are given.

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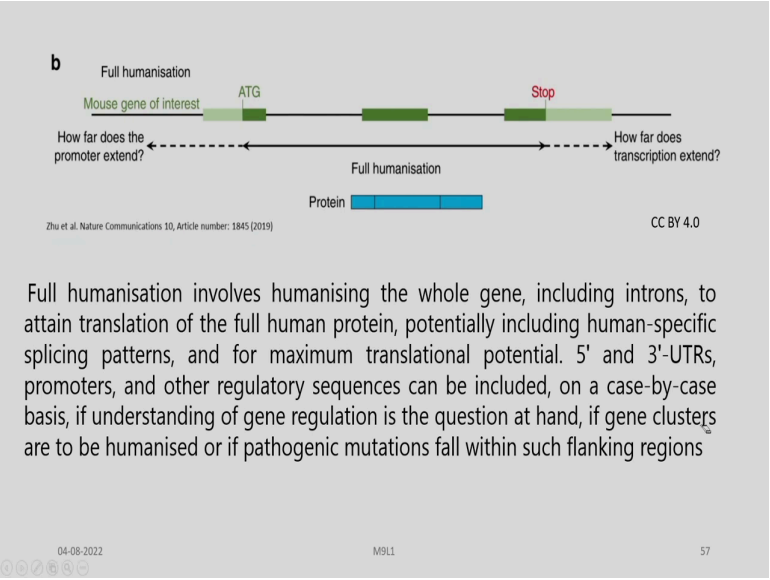

The option 2 is the full humanization. You have here the mouse gene of interest and then you have the start and the stop codon, and this is the protein, and you can see here it has been fully humanized. So, full humanization involves humanizing the whole gene including introns to attain translation of the full human protein, potentially including human-specific splicing patterns, and for maximum translational potential.

5 prime and 3 prime UTRs promoters, and other regulatory sequences can be included, on a case by case basis, if understanding of gene regulation is the question at hand, if gene clusters are to be humanized or if pathogenic mutations fall within such flanking regions. For more information on these on this partial and full humanization, you can refer to this paper by Zhu et al in Nature Communications.

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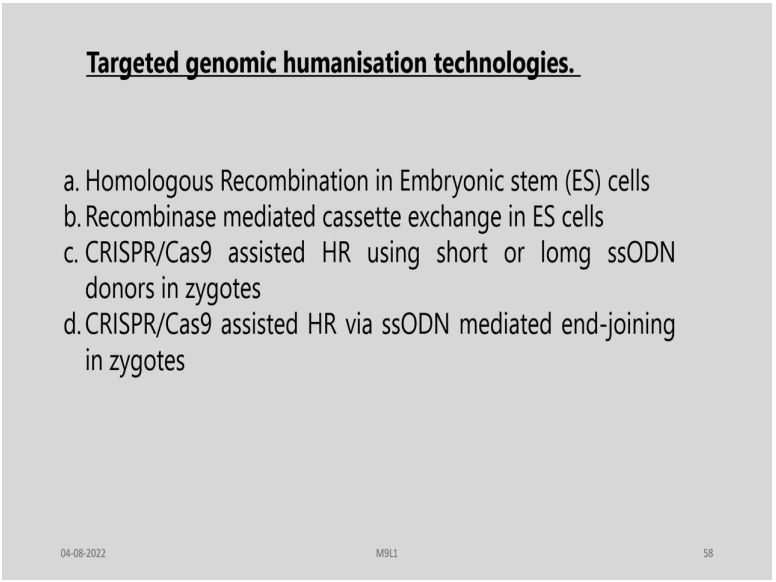

Let us now study about the targeted genomic humanization technologies, where we have homologous recombination in embryonic stem cells or recombinase mediated cassette exchange in embryonic stem cells. Then, CRISPR Cas9 assisted homologous recombination using short or long ssODN donors in zygotes. Then, CRISPR Cas9 assisted homologous recombination via ssODN mediated end-joining in zygotes.

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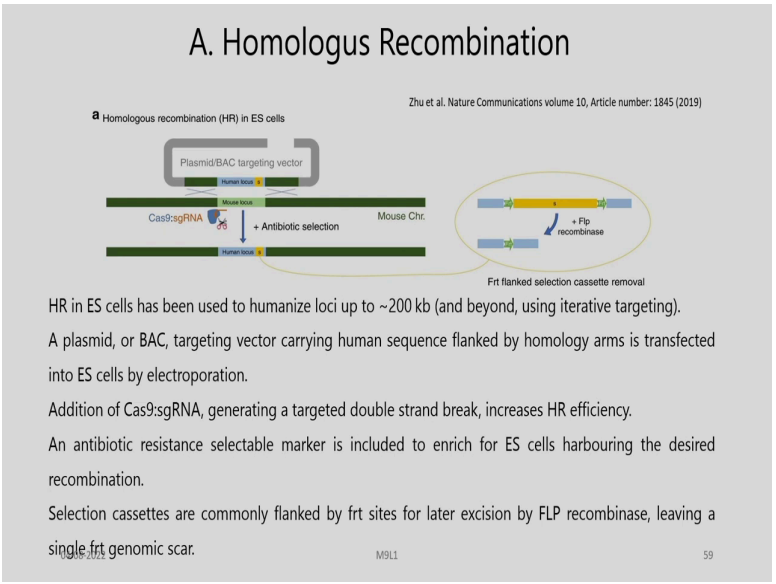

Here we are showing a picture of homologous recombination in ES cells and this has been used to humanize loci up to around 200 kb and beyond using iterative targeting. A plasmid or BAC, targeting vector carrying human sequence flanked by homology arms is transferred

into ES cells by electroporation. Addition of Cas9 single guide RNA, generating a targeted double strand break increases the HR efficiency.

An antibiotic resistance selectable marker is included to enrich for ES cells harbouring the desired recombination. Selection cassettes are commonly flanked by frt sites for later excision by FLP recombinations, leaving a single frt recombination genetics genomic scar.

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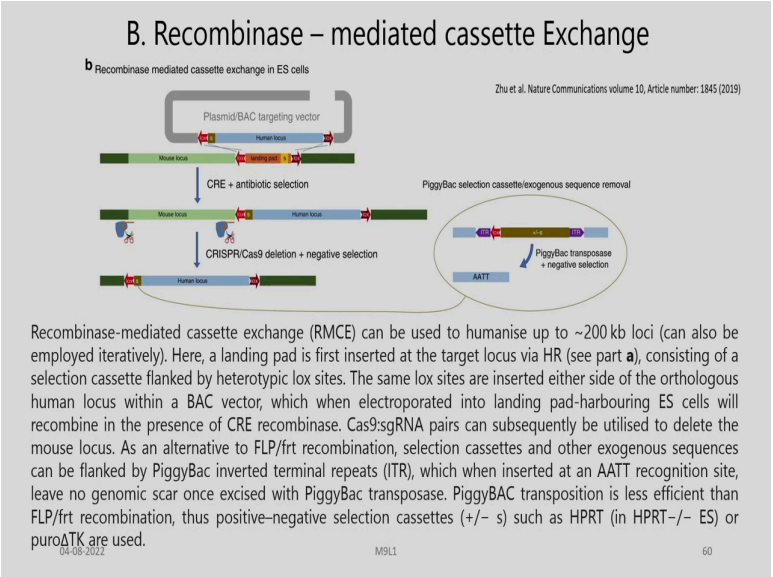

In method B, where we use recombinase mediated exchange cassettes to humanize up to around 200 kilo base loci. Here, landing pad is first inserted, at the locus target locus via HR, consisting of a selection cassette flanked by heterotypic lox sites. The same lox sides are inserted either side of the orthologs human locus within a vector which when electroporated into landing pad-harbouring ES cells recombine in the presence of CRE recombinase.

Cas 9 single gate RNA pairs can subsequently be utilized to delete the mouse locus. As an alternative to FLP/frt recombination, selection cassettes and other exogenous sequences can be flanked by PiggyBac inverted terminal repeats, which when inserted at an AATT recognition site, leave no genomics scar once excised with PiggyBac transposes. PiggyBac transposition is less efficient than FLP/frt recombination, thus positive-negative selection cassettes such as HPRT or puro del TK are used.

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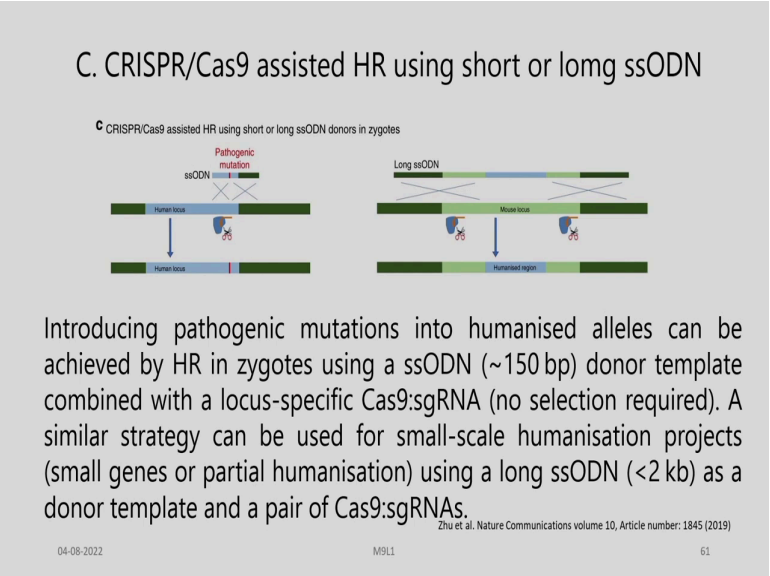

In the third method, we use CRISPR Cas9 assisted homologous recombination using short or long ssODN. Here introducing pathogenic mutation into humanized alleles can be achieved by homologous recombination in zygotes using a ssODN about 150 base pairs in size donor templates combined with a locus specific Cas9 sgRNA where no selection is required. A similar strategy can be used for small scale humanization process using a long ssODN as a donor template and a pair of Cas9 single guide RNAs.

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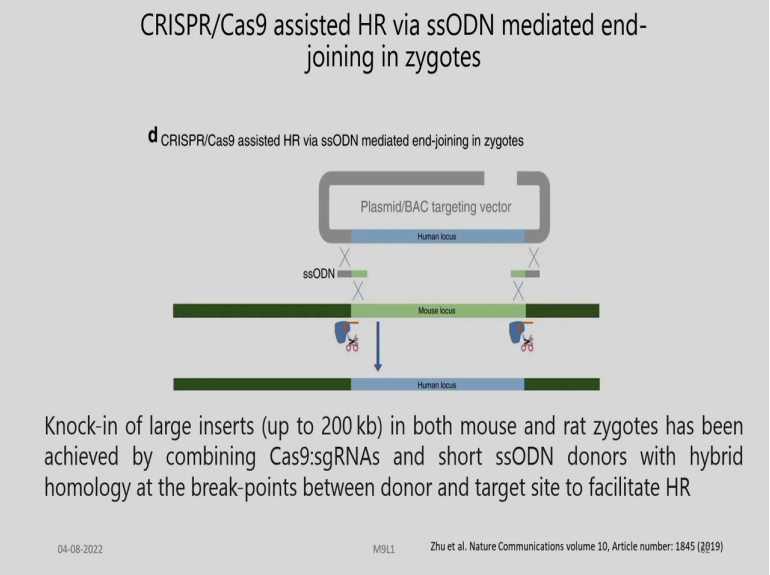

In the 4th method, we use CRISPR Cas9 assisted HR via ssODN mediated end joining in zygotes. Here, knock in of large inserts up to 200 kb in both mouse and rat zygotes has been achieved by combining Cas9 single guide RNAs, and short ssODN donors with hybrid homology and the break-points between donor and target site to facilitate the homologous recombination.

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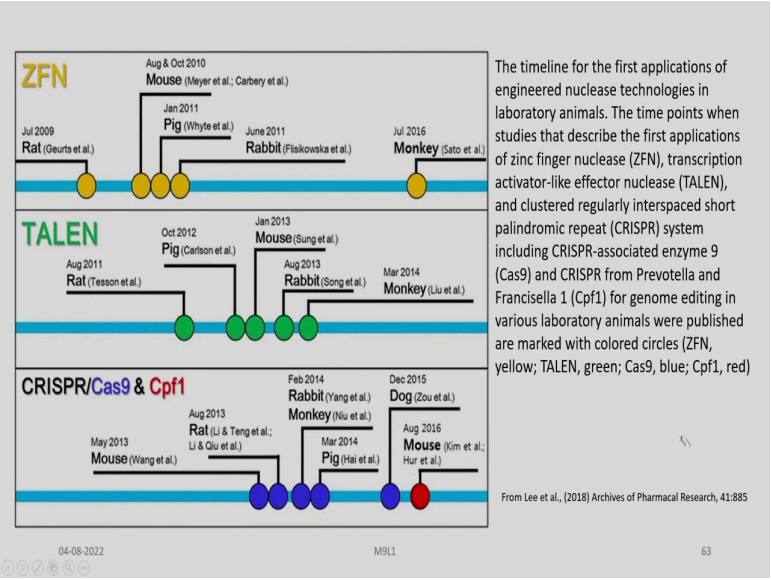

So, overall, let us have a look on the timeline for the first applications of engineered nuclease technologies in laboratory animals. The first time points when studies that describe the first application of zinc finger nucleases transcription activator like effector nucleases TALEN and clustered regularly interspaced short palindromic repeat CRISPR system including CRISPR from Prevotella and Francisella 1 Cpf 1 and Cas9 for genome editing in various laboratory animals are shown in this timeline.

So, you can see here in the development of the ZFN technologies in early 2000 middle of 2009, Geurts et al has applied these in rat followed by Meyer et al in 2010 in mouse and Whyte et al in 2011 in pig, and rabbit by Flisikowska et al in June, the same year. And as late as 2016 ZFN technology continues to be popular and Sato et al developed these monkeys using ZFN technologies.

And you can see that onset of TALEN is somewhere in 2011 where Tesson et al used these technology for rat. And then Carlson et al use it in 2012 in a pig, followed by Sung et al in mouse 2013 and Sung et al in rabbit the same year and in monkey by Liu et al in 2014. But

with time these two technologies are now slowly losing their popularity. TALEN of course, continues to have certain specific interest groups and advantages for which it is being still pursued. ZFN seems to have slowed down drastically.

And what has become popular today is the CRISPR Cas9 and say other like CRISPR Cpf1 technologies as well as d Cas9 base editors in the development of animal models as well as other experimental methods and also including humanization of mice models. The first application of CRISPR Cas9 for engineering animals was used by Wang et al in mouse in 2013, followed by Li et al in rat in 2013, and also by Li and Qiu.

And in rabbit by Yang et al in 2014, and the same year monkey and pig it was also used for developing, the engineering of the particular animal genomes. And then it has been also used in dog by Zou et al and of course, once again by Kim et al in 2016 they have used this technology for engineering certain traits in mouse.

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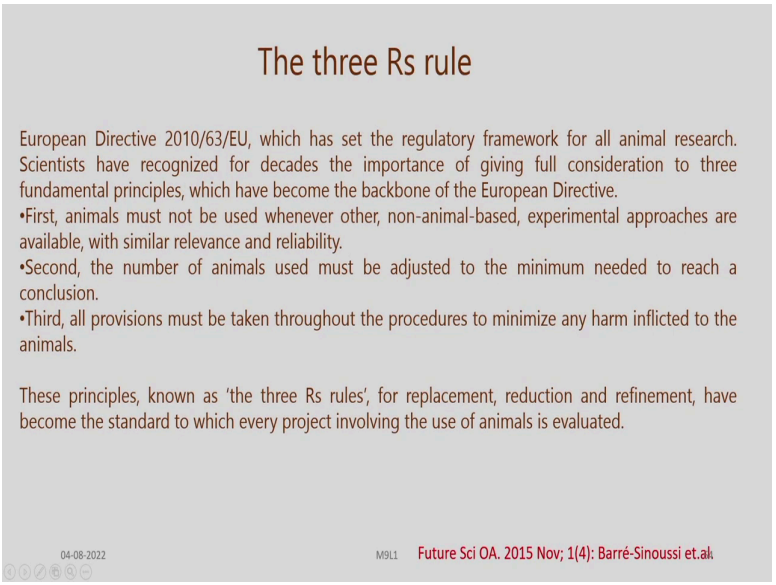

Before we end we need to focus on certain things. For example, animal models are not the exact replicas of human disease models we have to remember. They have many advantages and as well as many disadvantages. They are at the best human surrogates of human disease model. The European directive has set the regulatory framework for all animal research. And scientists have recognized for decades the importance of giving full consideration to 3 fundamental principles, which have become the backbone of these European directive.

Number one is, animals must not be used whenever other non-animal based experimental approaches are available with similar relevance and reliability. We know that the genome engineering and editing technology has huge potential for developing animal models which can in future almost become like humans or like humanized counterparts. But even with technological advances if we have other non-animal based models available, we should not opt for animal models.

Secondly, the number of animals used must be adjusted to the minimum needed to reach a conclusion. In the advantages of small animals, we have highlighted that because of bigger litter sizes in rats and mice and some of the other animals, we can get statistically relevant data, but that does not mean that we will be using large number of animals. We have to restrict the number of animals to be used if at all we have to go for animal experimentation to look for new knowledge and new therapies.

The third point is all provisions must be taken throughout the procedures to minimize any harm inflicted to the animals. We should not indulge in any kind of cruelty except for the predefined approved procedures by the animal ethics committee. So, these 3 principles need to be kept all the time in researcher's mind, and to respect the animals because otherwise it will be a violation of a set of ethics or regulatory framework guidelines, which have been recognized by scientist for many decades.

These principles, in brief known as 'the three R rules', stands for replacement. If you have a alternative model for a instead of a animal disease model, we should replace it with the non-animal model. Second one is reduction, do not use excessive number of animals, use the minimal number of animals with which you get a good quality data for your studies. And the third one is refinement and these have become the standard to which every project involving the use of animal is evaluated. With these, we come to end of this lecture on Animal Models.

Thank you.