Genome Editing and Engineering
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Module - 07
Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology
Lecture - 02
CRISPR/Cas9 in Genome Editing-Part A

Welcome to my course on Genome Editing and Engineering, in this lecture we are going to discuss about CRISPR Cas9 in Genome Editing. In the last lecture you have learnt about the basics of CRISPR Cas9 systems and various other similar systems. Today we are going to learn how these systems are used in genome editing.

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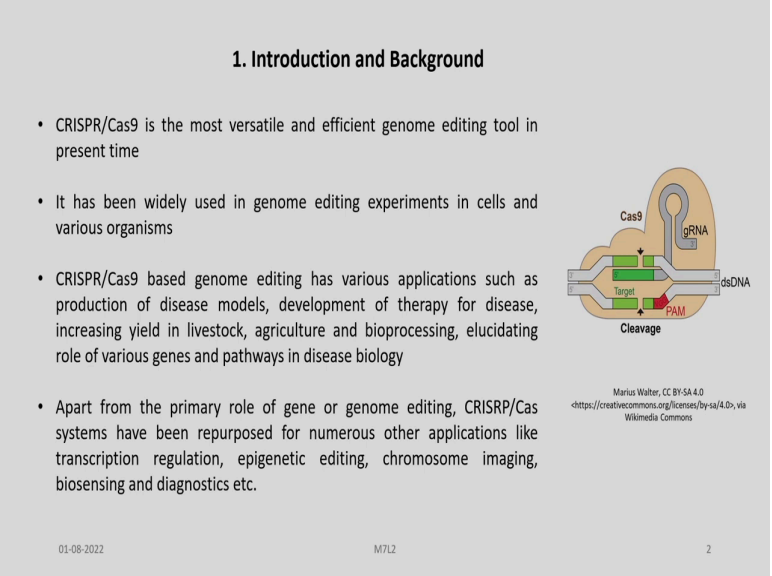

So, you can see this picture where you have a big protein molecule called Cas9 and then you have one guide RNA and then there is a sequence called PAM sequence and this is the target DNA. So, when this CRISPR Cas9 system along with the guide RNA binds to a target DNA it will cleave the DNA. So, this is known to you. Now today we are going to discuss a little bit more about the other facets of CRISPR Cas9 which you know is a now the most versatile and efficient genome editing tool currently and it has been used widely for genome editing experiments ranging from cells to various organisms.

This CRISPR Cas9 based genome editing has various applications, such as in the production of disease models for which we will have a lecture later on. Then development of therapy for diseases or gene therapy, then increasing yield in livestock, agriculture as well as bioprocessing and elucidating the role of various genes and pathways in disease biology.

Apart from the primary role of gene or genome editing CRISPR Cas9 systems has been now repurposed for numerous other applications like transcription regulation, genetic epigenetic editing, chromosome imaging, biosensing and diagnostics.

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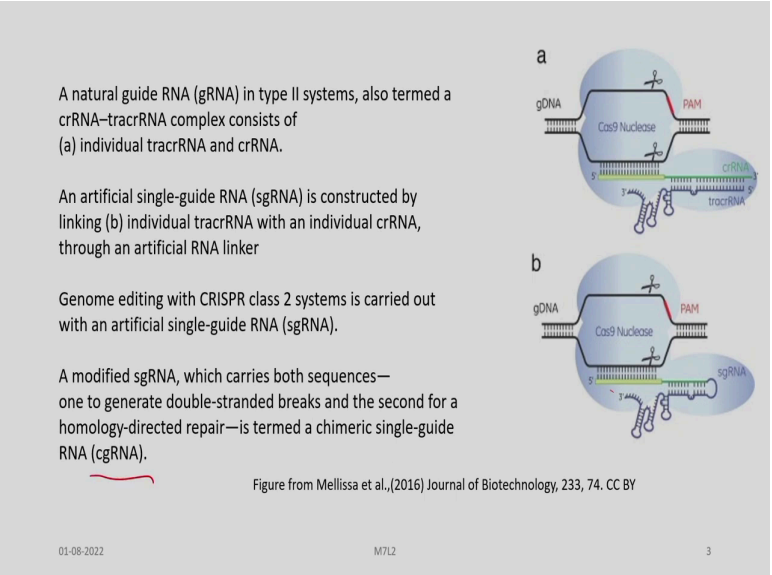

Let us see a little bit closer the difference between a natural system and a artificial CRISPR Cas9 system. So, in figure (a) you can see there is a guide DNA or the target DNA with a PAM sequence and then you have the CRISPR Cas9 nuclease and you can see here the CRISPR RNA and then you can see here the tracrRNA.

So, in this natural guide RNA type 2 system, which is also termed as the CRISPR RNA tracrRNA complex, it consist of individual tracrRNA and CRISPR RNA along with a Cas9. While if you look into the lower picture (b) you can see there is no any CRISPR RNA and tracrRNA, but there is a single guide RNA, but you can see some sequences are very similar to CRISPR RNA and tracrRNA as shown by the color codes.

So, this is an artificial single guide RNA which is constructed by linking individual crRNA with a tracrRNA through a linker molecule. So, artificial guide RNA is constructed by linking individual tracrRNA with an individual crRNA, through an artificial RNA linker.

Genome editing with CRISPR class 2 systems is carried out with artificial single-guide RNA systems. A modified RNA sgRNA which carries both sequences, one to generate double stranded breaks and the second for a homology directed repair is termed a chimeric single guide RNA or cgRNA.

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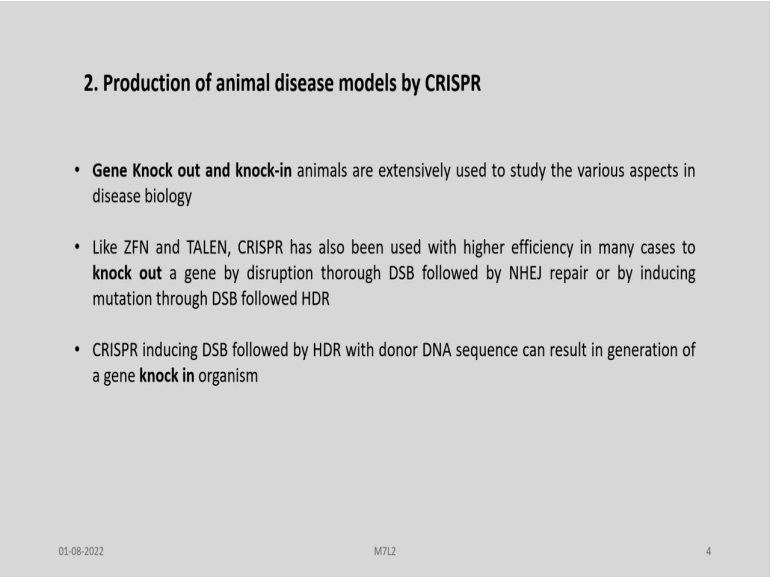

So, some of the things we can do with CRISPR Cas9 is that we can use them for producing animal disease models. We have some lectures in one of the future modules where we will discuss in detail about these applications, but this is just to brief you that gene knockout and knock in animals are extensively used to study the various aspect of disease biology.

And similar to ZFN and TALEN, CRISPR has also been used with high efficiency in many cases to knock out a gene by disruption through double strand breaks followed by non-homologous adjoining repair or by inducing mutation through DSB followed by HDR.

A CRISPR inducing double strand breaks followed by HDR with donor DNA sequences can result in generation of a knock in organism.

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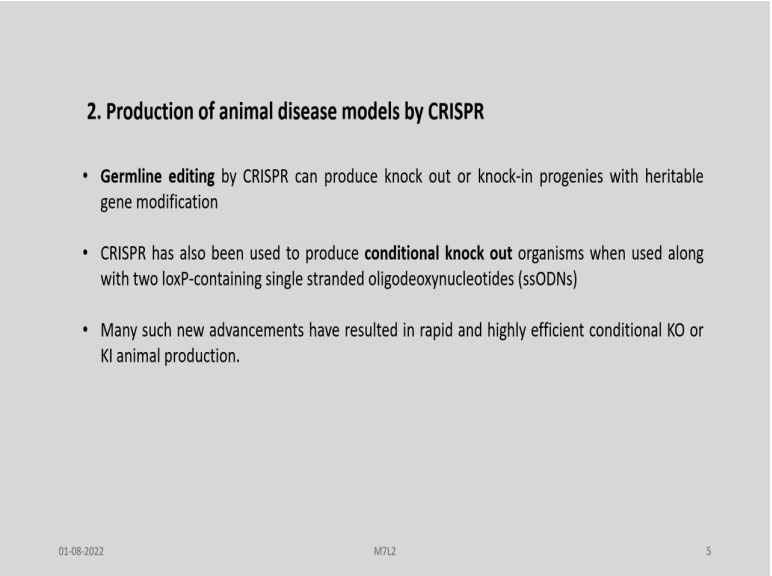

Germline editing by CRISPR can produce knockout or knock-in in progenies with heritable gene modification. A CRISPR has also been used to produce conditional knock out organisms when used along with two loxP-containing single stranded oligodeoxynucleotides. Many such new advancements have resulted in rapid and highly efficient conditional knockout or knock in animal production.

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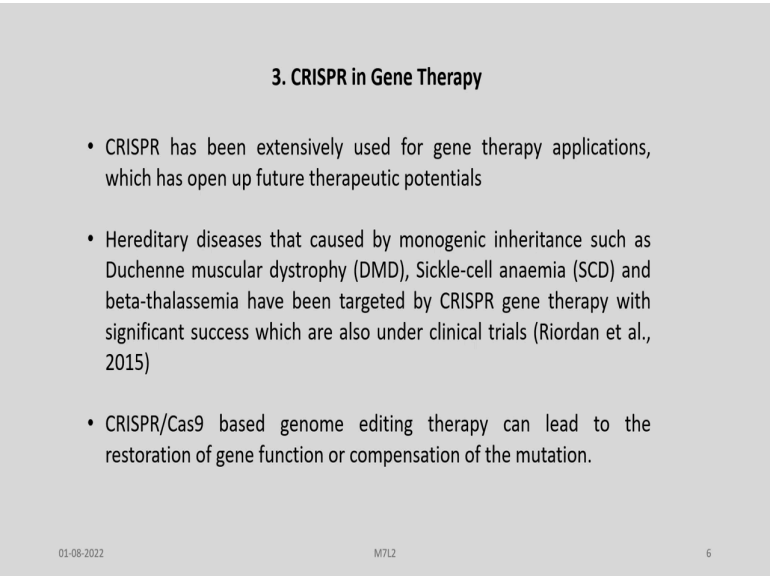

CRISPR is also being used in gene therapy increasingly and various experiments are in different stages of development including some in clinical trial stage. So, it has used

therapeutic potentials in future. We know that heredity diseases are caused by monogenic inheritances or polygenic inheritances or genes. Those caused by monogenic inheritances such as Duchene muscular dystrophy or DMD, Sickle- cell anemia and beta-thalassemia have been targeted by CRISPR gene therapy with significant success and are currently under clinical trials.

A CRISPR Cas9 based genome editing therapy can lead to the restoration of gene function or compensation of the mutation which causes the genetic disease.

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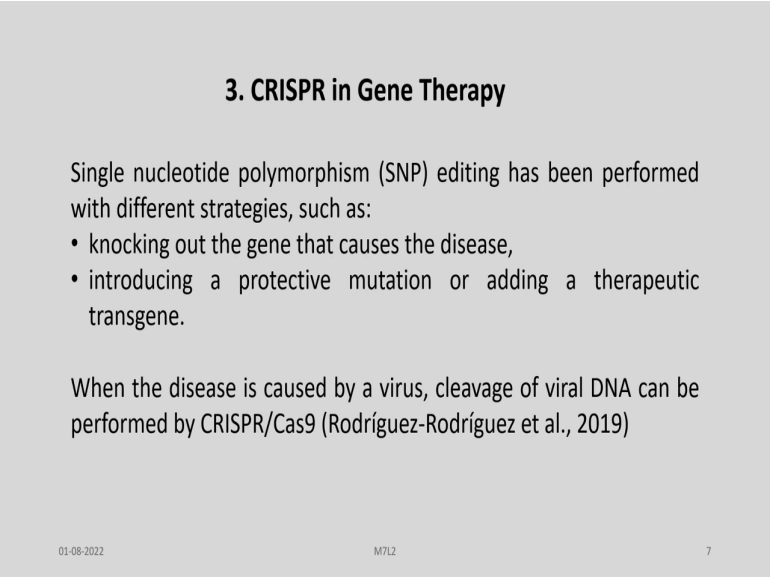

SNP editing has been performed with different strategies as well such as knocking out the gene that causes the disease or introducing a protective mutation or adding a therapeutic transgene. When the disease is caused by a virus, cleavage of viral DNA can be performed by CRISPR Cas9 as reported by Rodriguez in 2019.

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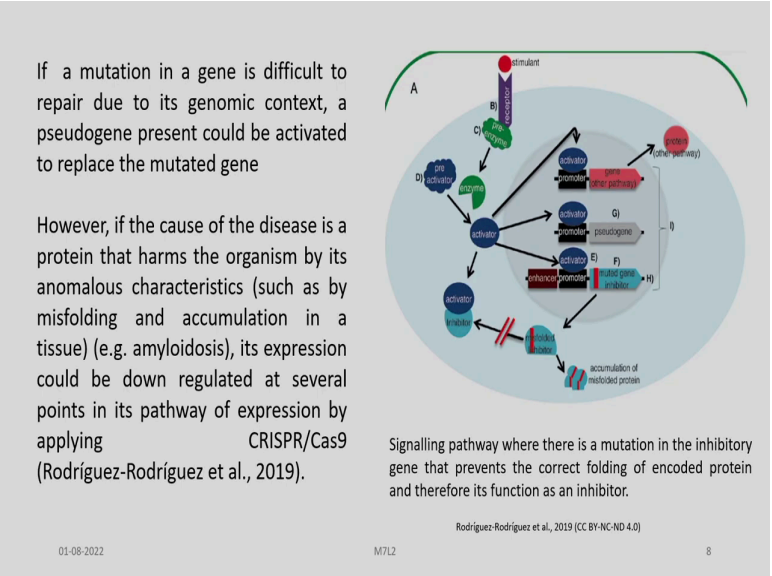

Now, let us have a closer look into this figure and you can see here there is a mutated gene inhibitor and this is the mutation as shown in red color which has happened, due to this mutation when these gene is transcribed and translated it may become misfolded and as it is misfolded, if it is a inhibitor protein it would not be able to act like an inhibitor to the activator and therefore, it will accumulate inside the cell system.

So, if a mutation in a gene is difficult to repair due to its genomic context, a pseudogene present could be activated to replace this mutated gene. However, the cause of the disease is a protein that harms the organism by its anomalous characteristics such as by misfolding and accumulation as shown here in a tissue. For example, amyloidosis its expression could be down regulated at several points in its pathway of expression by applying CRISPR Cas9 technology.

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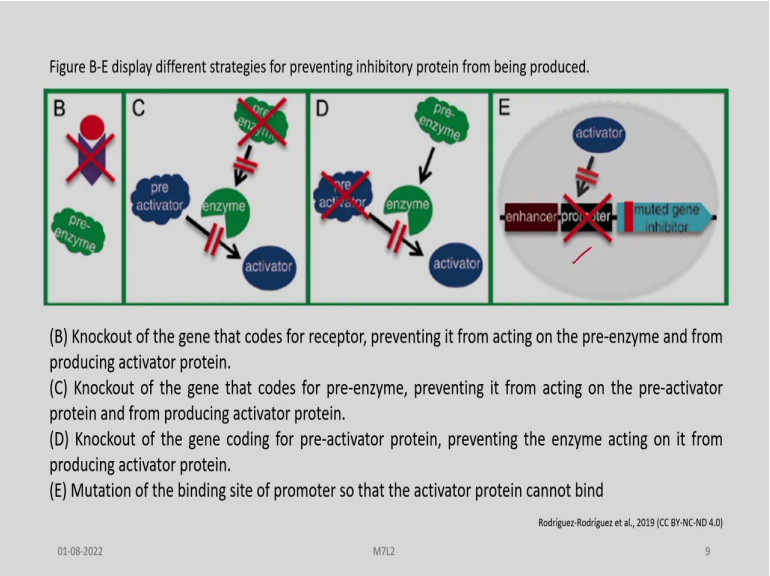

So, these are various representations by which we can deploy different strategies for preventing inhibitory protein from being produced. Let us study them one by one. In the case of B, we can knock out the gene that codes for receptor, preventing it from acting on the pre-enzyme and from producing activator protein.

In C, we knock out the gene that codes for pre-enzyme, preventing it from acting on the pre-activated protein and from producing activator protein. In strategy D, we knock out the gene which codes for pre-activated protein, preventing the enzyme acting on it from producing activator protein. And in E, we have mutation of the binding site of promoter so that the activator protein cannot bind.

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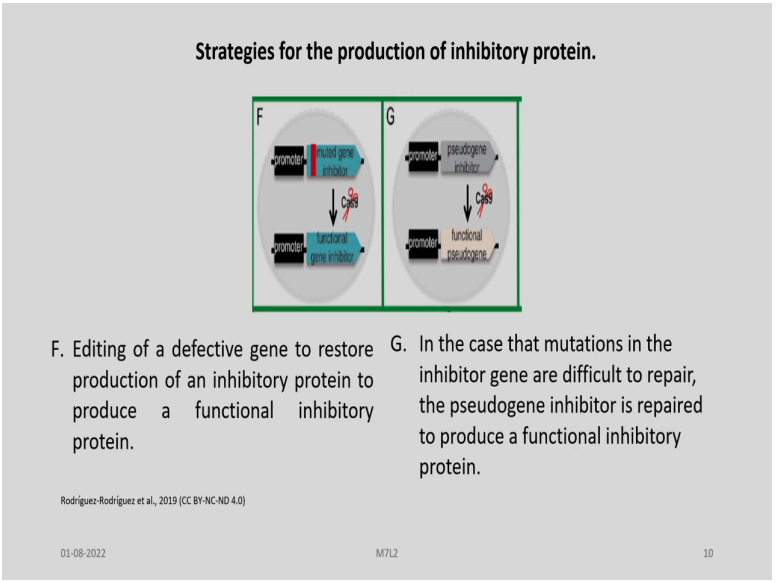

There are two other strategies as well, like strategy F, where editing of a defective gene is done to restore the production of an inhibitory protein to produce a functional inhibitory protein. And in case of strategy G, the mutations in the inhibitory gene are difficult to repair, the pseudogene inhibitor is repaired to produce a functional inhibitory protein.

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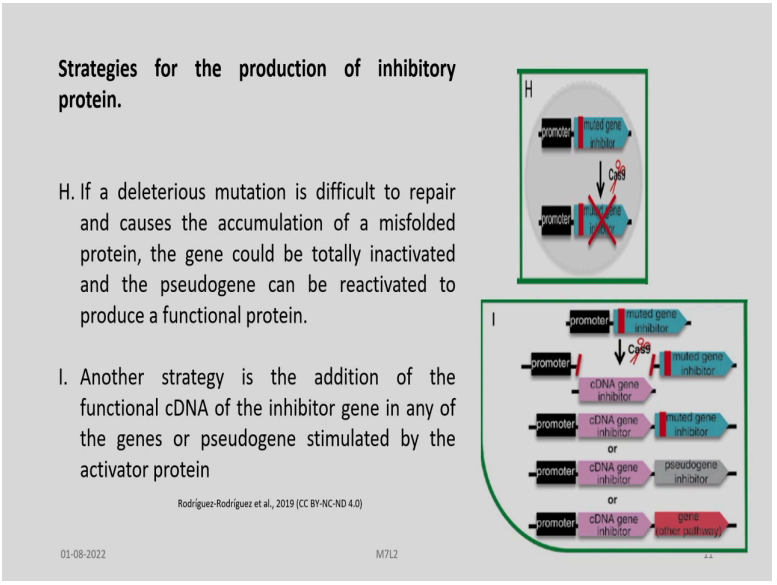

Another strategy involves the strategy as shown in H for example, if a deleterious mutation is difficult to repair and cause the accumulation of a misfolded protein, the gene could be totally inactivated, and the pseudogene can be reactivated to produce a functional protein. In yet

another strategy the addition of the functional cDNA of the inhibitor gene in any of the genes or pseudogenes was stimulated by the activator protein shown in this picture.

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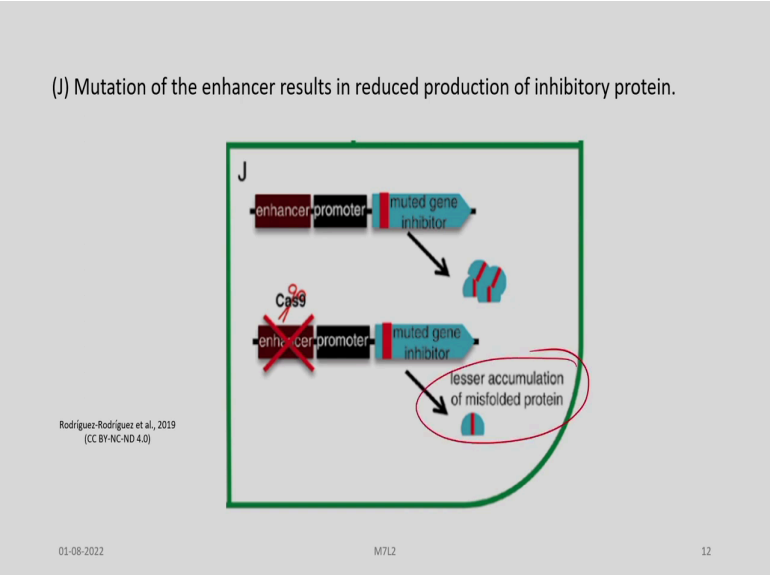

In this strategy J, you can see the mutation of the enhancer which results in reduced production of the inhibitor protein or lesser accumulation of the misfolded protein finally.

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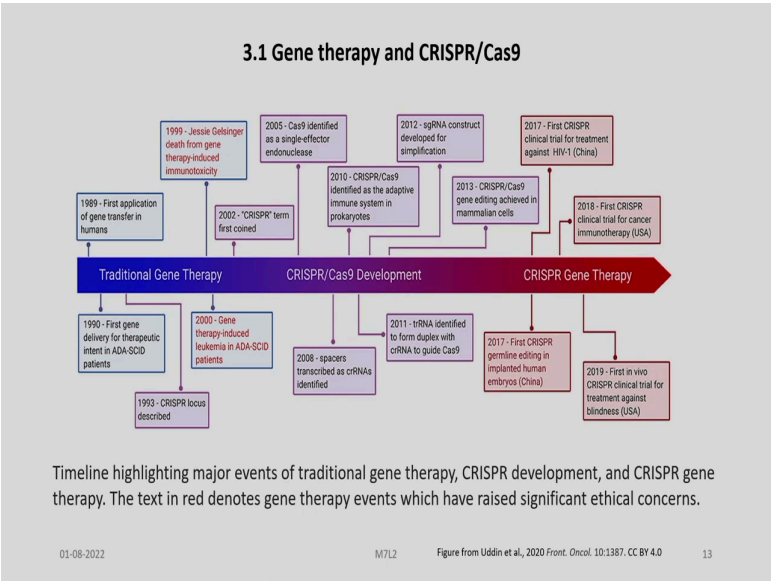

Let us investigate the various events or developments in the field of gene therapy and CRISPR Cas9 highlighting the major events of traditional gene therapy and then CRISPR gene therapy along with the development of the CRISPR Cas9 technology.

So, the first application of gene transfer started in 1989 and in the next year 1990 the first gene delivery for therapeutic intent in ADA-SCID patients was completed. In 1993, the CRISPR locus was described as we have discussed about this earlier and in 1999 Jessie Gelsinger's death occurred from gene therapy induced immunotoxicity which was an unfortunate event. Also, in 2000 gene induced gene therapy induced leukemia in ADA- SCID patients were reported.

So, these two events were a setback in the progress of gene therapy, and it took many years to bring a gene therapy back into clinical trials due to these events. So, by 2002 the term CRISPR was developed as you all know and in 2005 the Cas9 was identified as a single effector endonuclease, and the discovery of spacers transcribed as crRNAs was in 2008. In 2010 the CRISPR Cas9 was identified as a adaptive immune system in prokaryotes.

And in 2012 tracrRNA identified to form duplex with CRISPR RNA to guide the Cas9. By 2012, single guide RNA constructs were developed for simplification which we have discussed in the earlier slides and in 2013 CRISPR Cas9 gene editing was achieved in mammalian cells.

In 2017, the first CRISPR clinical trial for treatment against HIV-1 in China was conducted and in the same way the first CRISPR germline editing in implanted human embryos were also conducted and we have discussed about these controversial experiments in the development of CRISPR Cas9 technology earlier.

In 2018, the first CRISPR clinical trial for cancer immunotherapy it was carried out in USA and in 2019 first in vivo CRISPR clinical trial for treatment against blindness in USA happened. So, this is in brief, the timeline highlighting the major events of gene therapy starting from the traditional gene therapy to the CRISPR based gene therapy.

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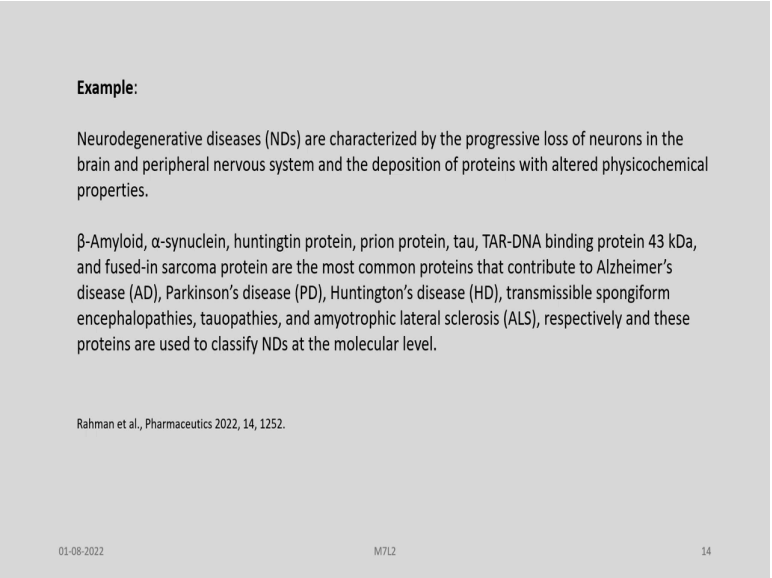

So, CRISPR Cas9 technology is a versatile technology as you know. So, it can be used in various kinds of gene therapies particularly those controlled by monogenic genes but as this technology is quite versatile and flexible it is also possible to transfer multiple genes and so, theoretically it is possible to also attempt for gene therapy which involves polygenic factors.

Let us take some examples of potential areas where gene therapy through CRISPR Cas9 can be undertaken. For example, the neurodegenerative disease area or NDs are some of the potential areas in which CRISPR Cas9 can be play a very critical role. So, these neurodegenerative diseases are characterized by the progressive loss of neurons in the brain as well as in the peripheral nervous system and the deposition of proteins with altered physicochemical properties.

So, certain proteins like beta amyloid, synuclein huntingtin protein, prion, tau and so on are the most common proteins that contribute to diseases like Alzheimer's, Parkinson's, Huntington's, and transmissible spongiform encephalopathies, tauopathies, amyotrophic lateral sclerosis etc, and these proteins are used to classify the NDs or the neurodegenerative disease at the molecular level.

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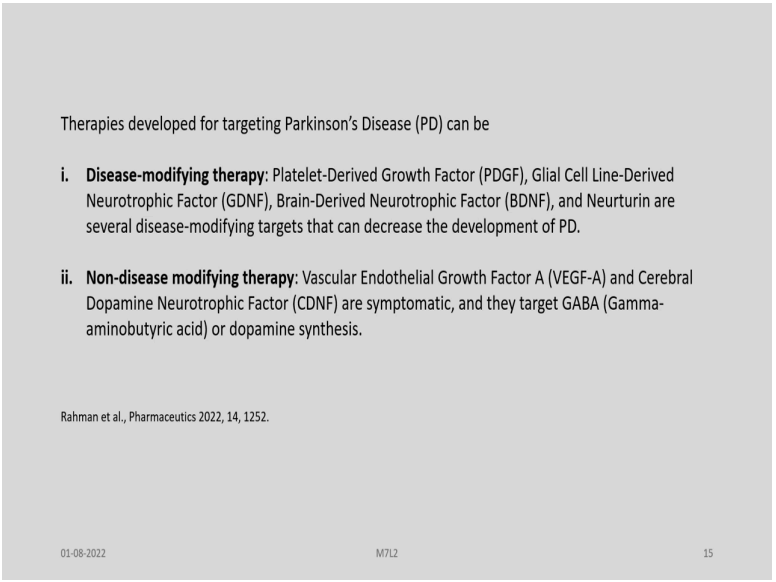

Let us focus on the targeting of Parkinson's disease and from the point of gene therapy. So, the therapies developed for targeting Parkinson's disease can be of two types - the disease modifying therapy or the non-disease modifying therapy.

In disease modifying therapy the Platelet - Derived Growth Factors, Glial Cell Line-Derived Neurotrophic Factors, then Brain-Derived Neurotrophic Factor and Neurturin are several disease modifying targets that decrease the development of the Parkinson's Disease.

While in the second type, factors like VEGF-A and Cerebral Dopamine Neurotrophic Factor are symptomatic and they target GABA or dopamine synthesis.

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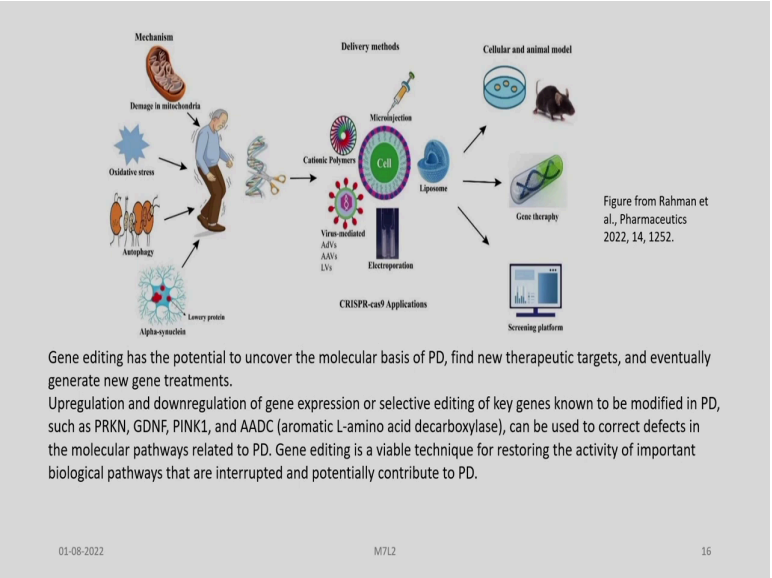

So, this is just a schematic of Parkinson disease, you can see here there is damage in the mitochondria and then oxidative stress autophagy and proteins like alpha synuclein involve in this disease.

So, we can develop certain targeted therapy against Parkinson's disease and for delivering these therapies we may have various delivery methods like micro injection, cationic polymers, or you can use virus or electroporation for the delivery of CRISPR Cas9 based targeted gene therapy in Parkinson diseases. And then also for studying this particular disease we may create cellular models or animal models and we can also develop genetic therapy for PD as discussed.

So, gene editing has the potential to uncover the molecular basis of Parkinson's disease and find the new therapeutic targets and eventually generate new genetic therapy. The upregulation and down regulation of gene expression or selective editing of key genes are known to be modified in PD such as PRKN, GDNF, PINK 1 and AADC, can be used to correct defects in the molecular pathway related to Parkinson's disease.

Gene editing is a viable technique, particularly the CRISPR Cas9 technology for restoring the activity of the important biological pathways that are interrupted and potentially contribute to development of Parkinson's disease.

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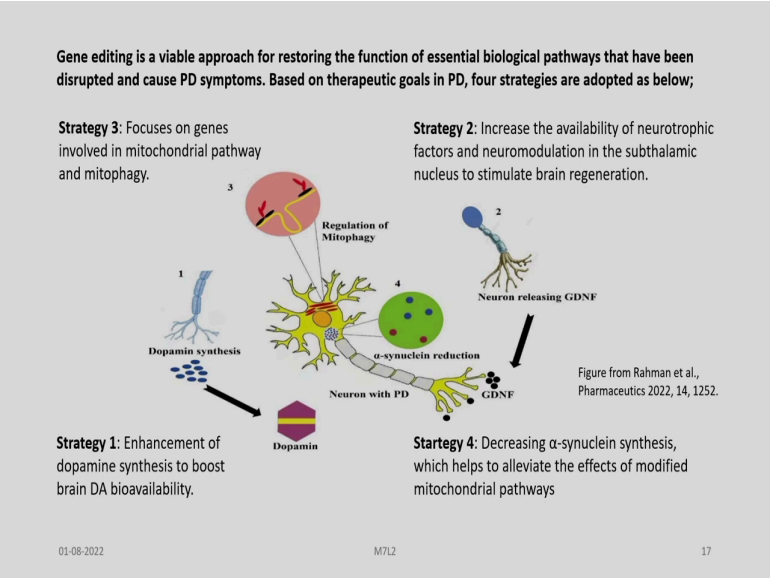

So, now we know that gene editing is a viable approach for restoring the function of essential biological pathways that have been disrupted and has resulted in Parkinson's disease symptoms. Based on the therapeutic goals in Parkinson's disease, four strategies are adopted as described below:

The strategy number 1 is enhancement of dopamine synthesis to boost brain DA bioavailability. Strategy number 2 is to increase the availability of neurotrophic factors and neuromodulation in the subthalamic nucleus to stimulate brain regeneration. And strategy number 3 is to focus on genes which are involved in mitochondrial pathway and mitophagy. The last strategy focuses on decreasing on alpha synuclein synthesis, which would help to alleviate the effects of modified mitochondrial pathways.

Now, all these different strategies would involve different kind of CRISPR Cas9 methods in certain cases we would like to induce certain mutations, in other cases we would like to replace DNA fragments or sequences. So, using CRISPR Cas9 there is a huge potential of developing therapies for Parkinson's disease.

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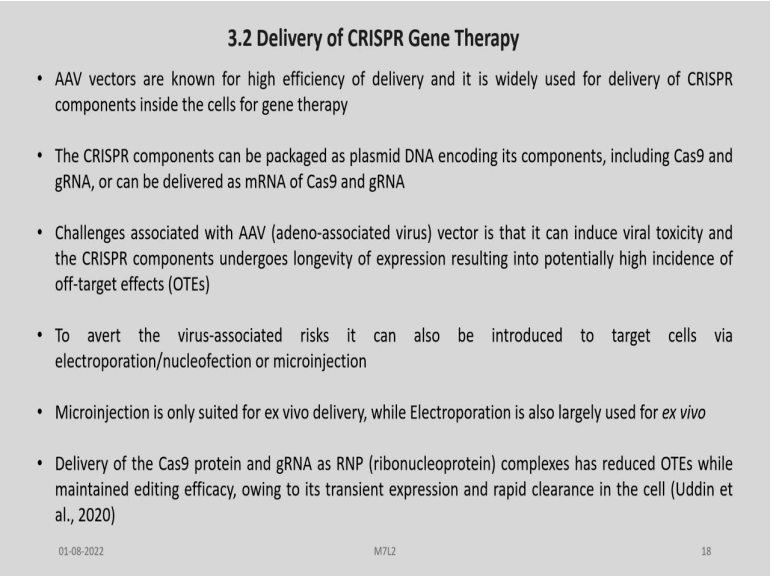

Let us now discuss a little bit about the delivery of CRISPR gene therapy, while discussing the development of Parkinson's disease we have come across some of the delivery methods. Now we will have a detailed discussion on some of them and in additionally a few more.

So, we know that virus vectors can be used for delivery of gene therapy. AAV vectors are known for high efficiency of delivery, and it is widely used for delivery of CRISPR components inside the cells for gene therapy. The CRISPR components can be packaged as plasmid DNA encoding its components, including Cas9 and guide RNA or this can be delivered as mRNA of Cas9 and gRNA.

The challenges associated with AAV vector is that it can induce viral toxicity and the CRISPR components undergo longevity of expression resulting into potentially high incidence of off-targets. So, we will have some discussion how to take care of these off -targets in one of the sections later.

To avert the virus associated risk it can also be introduced to target cells via electroporation, nucleofection or direct microinjection. Microinjection is only suited for ex-vivo delivery, while electroporation is also largely used for ex vivo and not in vivo delivery. The delivery of the Cas9 protein and guide RNA as ribonucleoprotein complexes has reduced off target effects while maintaining editing efficacy, owing to its transient expression and rapid clearance in the cell.

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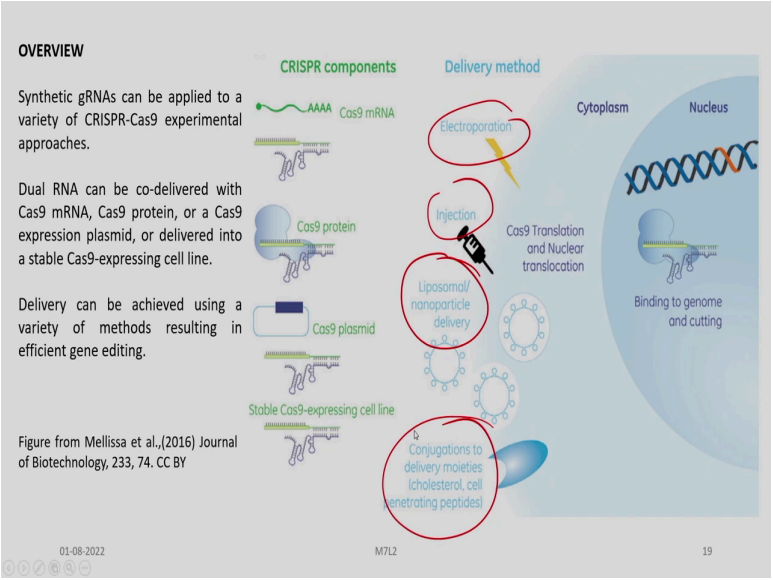

So, here you can see in brief the overview of the various delivery methods and here you can see the various components and the various delivery methods and once these are delivered they will end up in the nucleus where they bind to the genome and carry out the desired genome editing or engineering.

So, the synthetic guide RNAs can be applied to a variety of CRISPR Cas9 experimental approaches. Dual RNA can be co - delivered with Cas9 mRNA, Cas9 protein or Cas9 expression plasmid or delivered into a stable Cas9- expressing cell line. So, these are various approaches.

Delivery can be achieved using a variety of methods like electroporation, injection, liposomal, nanoparticle delivery, then conjugations to delivery moieties etc.

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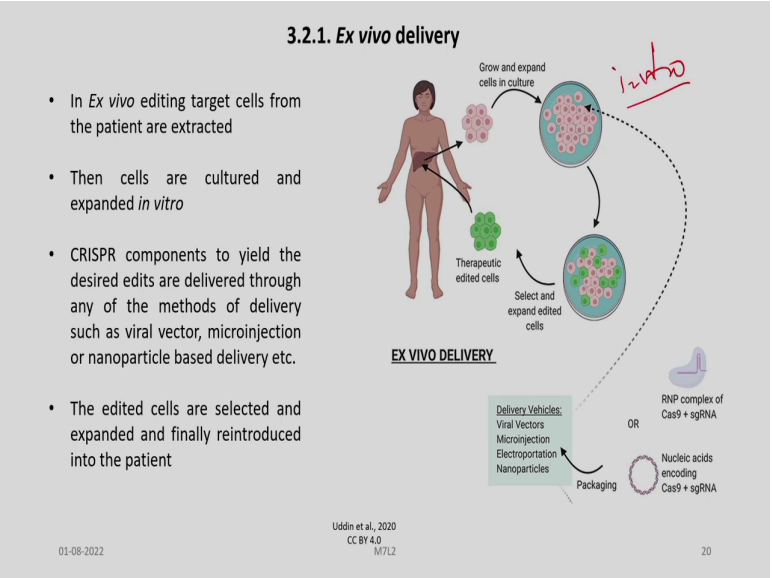

Let us focus a little bit on Ex vivo delivery. So, what do you do in Ex vivo delivery? In Ex vivo editing, target cells from the patients are extracted. We take out the cells from the patient in whom we are going to carry out the genome editing whether for gene therapy or for other cases.

These cells are cultured and multiplied or expanded in vitro. The CRISPR components to yield the desired edits are delivered through any of the methods of delivery which we have discussed earlier including viral delivery. The edited cells are selected and expanded and finally, reintroduced into the patient cell.

So, these are the CRISPR Cas9 components along with the various delivery vehicles which help us in delivering the CRISPR Cas9 components into these cultured cells which are expanded, and the therapeutically edited cells are reimplanted into the patients.

So, this is the process by which the Ex vivo delivery of genome edited constructs are being done.

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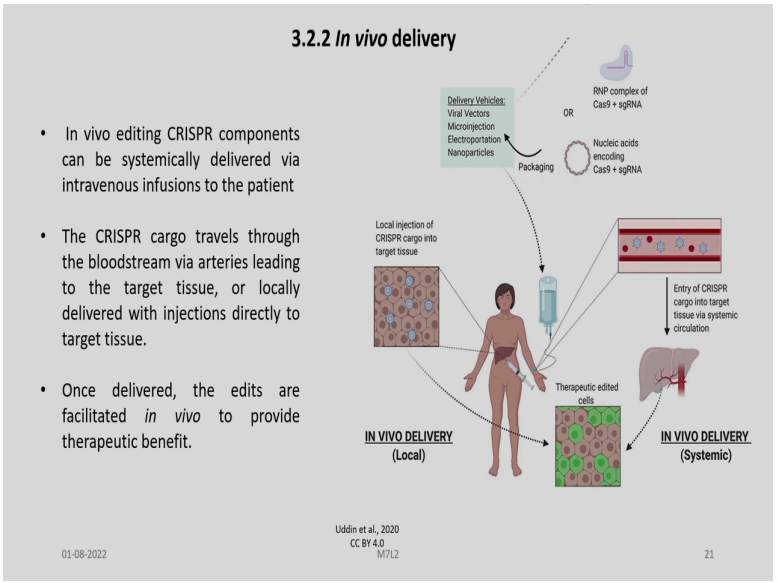

In vivo delivery as the name suggest it is direct delivery into the body. So, cleared components can be systematically delivered via intravenous infusion to the patient. The CRISPR cargo travels through the bloodstream via arteries leading to the target tissue or locally delivered with injections directly to the target tissue. Once delivered, the edits are facilitated in vivo to produce therapeutic delivery.

So, these are the components, and these are packaged and this is being delivered directly through injection into the patient's arteries and it travels and lands up in the desired tissue.

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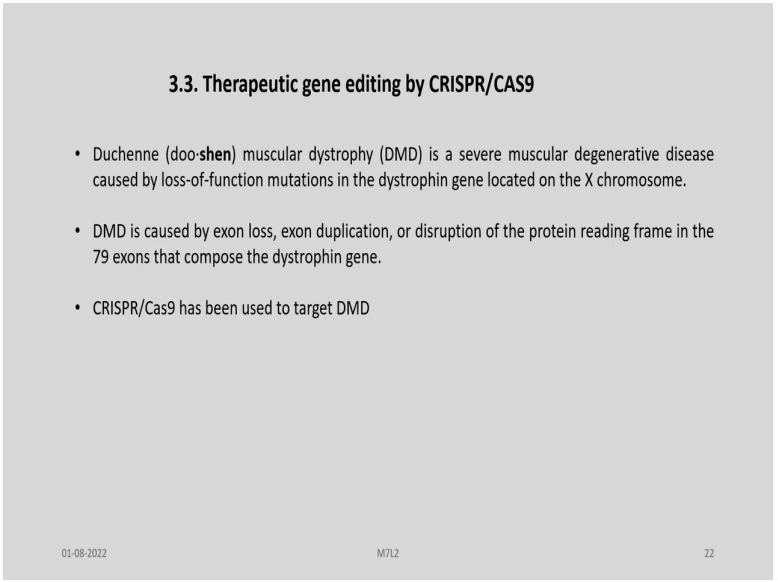

Let us now discuss in specific, the therapeutic gene editing by CRISPR Cas9 taking some examples like doo-shen muscular dystrophy, which is a severe muscular degenerative disease, and it is caused by loss-of-function mutations in the dystrophin gene located on the X chromosome.

A DMD is caused by exon loss, exon duplication or disruption of the protein reading frame in the 79 exons that compose the dystrophin gene. CRISPR Cas9 has been used successfully to target DMD.

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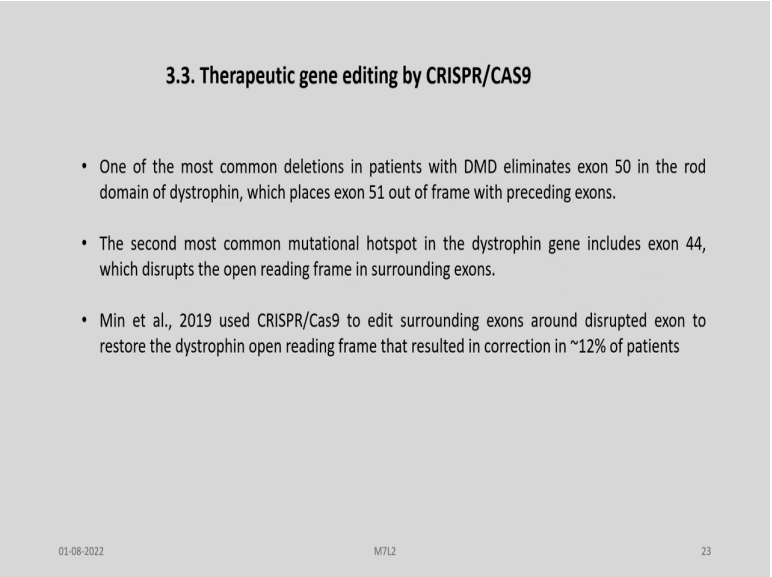

One of the most common deletions in patients with DMD eliminates exon 50 in the rod domain of dystrophin, which places exon 51 out of the frame with preceding exons.

The second most mutational hotspot in the dystrophin gene includes the exon 44, which disrupts the open reading frame in surrounding exons. Min et al., used CRISPR Cas9 to edit surrounding exons around disrupted exon to restore the dystrophin open reading frame that resulted in correction in about 12 percent of the patients.

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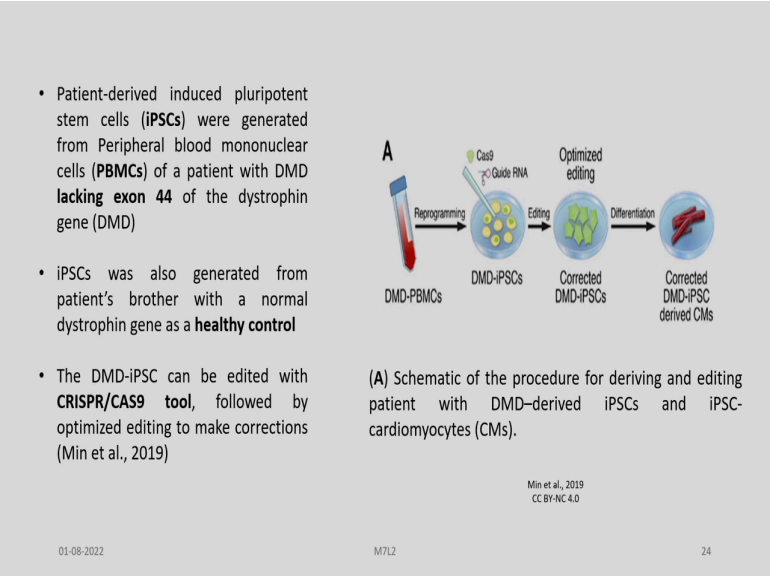

So, as already discussed, we take the harvest the cells from the patient. So, here we are taking patient-derived induced pluripotent stem cells. So, these are generated from the peripheral blood mononuclear cells of a patient with DMD lacking exon 44 of the dystrophin gene.

So, here this is the semantic of the procedure for deriving and editing patient with DMD derived iPSCs and iPSC cardiomyocytes. So, here the cells are reprogrammed, and this is the DMD- iPSCs and we do the editing, and these are corrected DMD- iPSCs. They are then allowed to differentiate resulting in the corrected DMD- iPSC derived CMs.

iPSCs was also generated from patients' brother with a normal dystrophin gene as a healthy control. The DMD-iPSC can be edited with CRISPR Cas9 tool followed by optimized editing to make corrections.

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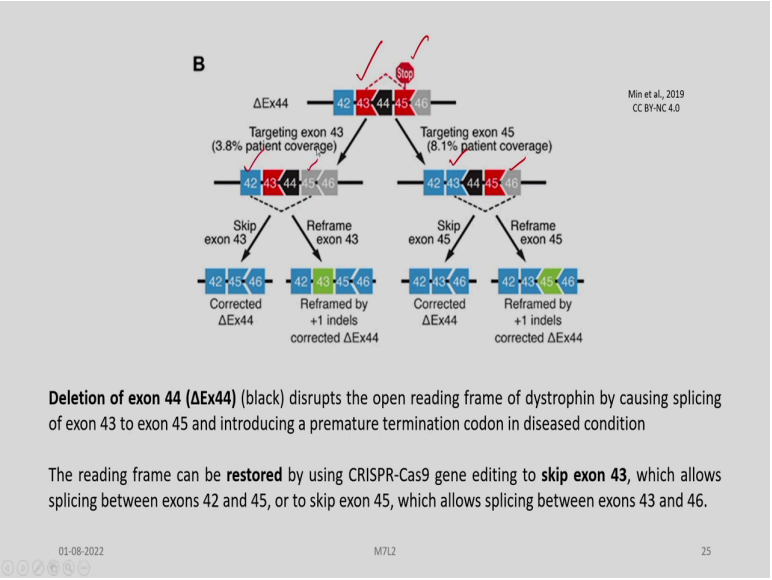

So, you can see here deletion of exon 44, which we refer to as del Ex 44 which is shown in black here. So, these del Ex 4 disrupts the open reading frame of dystrophin by causing splicing of exon 43 to exon 45 and introducing a premature termination codon in diseased condition. The reading frame can be restored by using CRISPR Cas9 gene editing to skip exon 43, which allows splicing between exons 42 and 45 or to skip exon 45, which allows splicing between exons 43 and 46. You can see here 43 and 46 and in the first strategy the exon 42 and the exon 45.

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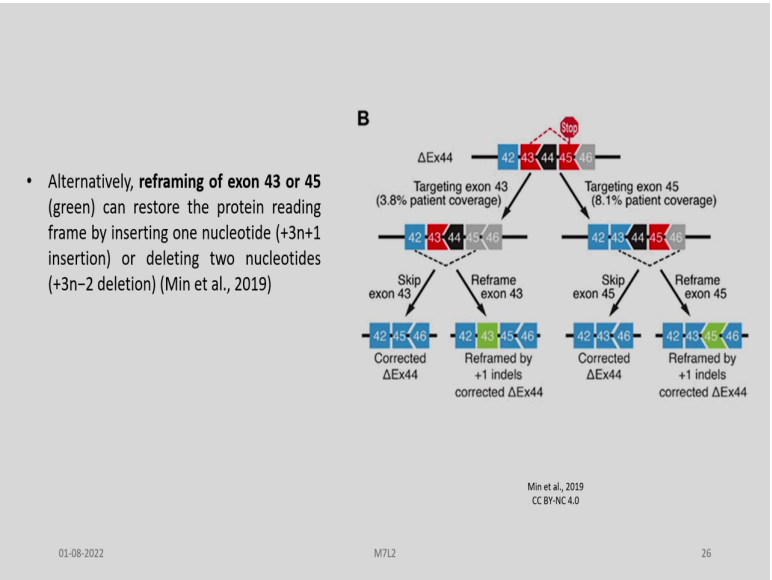

Alternatively, reframing of exon 43 or 45, you can see in green color here, can restore the protein reading frame by inserting one nucleotide or deleting two nucleotides. So, you have plus 3 and minus 2 deletion, or plus 3 andn plus 1 insertion in these cases.

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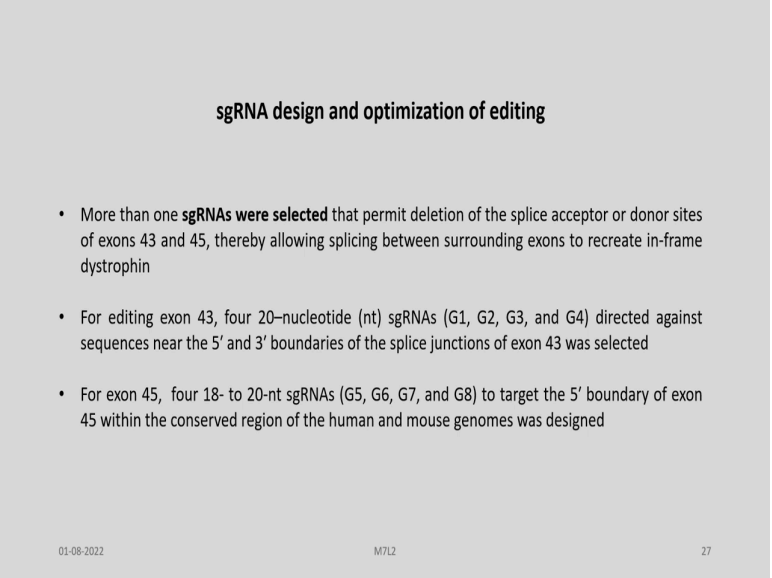

sgRNA design and optimization of editing. More than one sgRNAs were selected by the researchers which permit deletion of the splice acceptor or donor sites of exons 43 and 45 and these allows splicing between the surrounding axons to recreate in-frame dystrophin.

For editing exon 43, four 20-nucleotide single guide RNAs named G1, G2, G3 and G4 directed against sequences near the 5’ and 3’ boundaries of the splice junctions of exon 43 was selected. And for exon 45, four 18 to 20 nucleotides single guide RNAs named as G5, 6, 7 and 8. to target the 5’ boundary of exon 45 within the conserved region of the human and mouse genome was designed.

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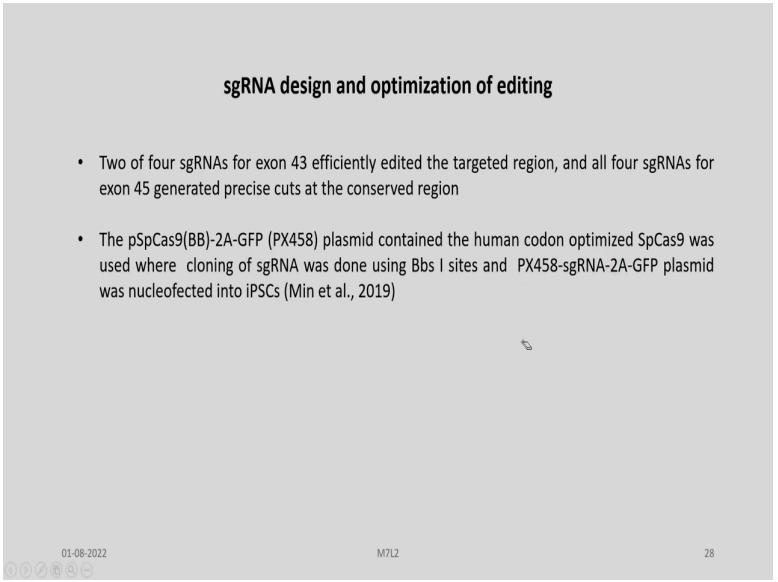

Two of the four single guide RNAs for exon 43 efficiently edited the targeted region and all four single guide RNAs for exon 45 generated precise cuts at the conserved region. The pSpCas9 BB-2A-GFP plasmid contained the human codon optimized SpCas9 was used where cloning of single guide RNA was done using Bbs 1 site and PX458-sgRNA- 2A- GFP plasmid was nucleofected into iPSCs.

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In a figure C you can see, the sequence of single guide RNAs targeting exon 43 splice acceptor and donor sites in the human DMD gene.

The protospacer adjacent motif PAM denoted as the red nucleotide of the single guide RNA is located near the exon 43 splice junctions.

Exon sequence is represented by letters in bold upper case. The intron sequences are represented by letters in the lower case. You can see the arrowheads they show the sites of Cas9 DNA cutting with each single guide RNA. Splice etcetera and donor sites are shaded in yellow as see you can see in the picture.

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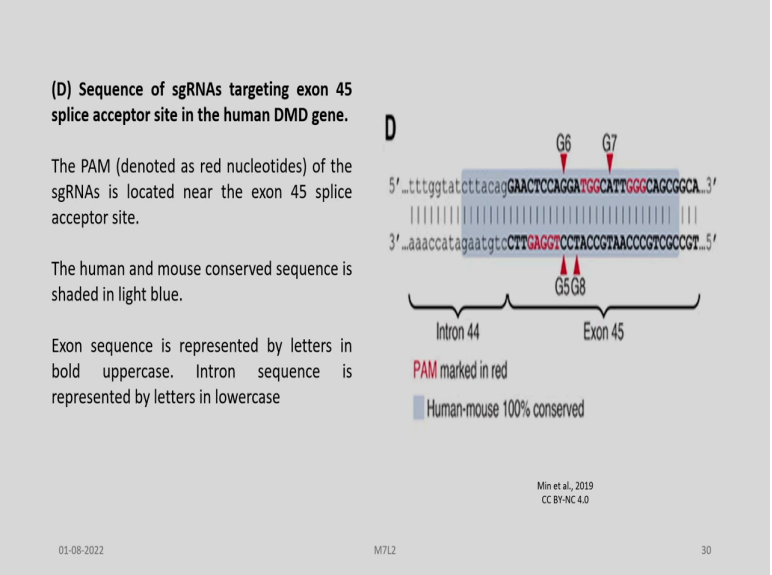

Figure D shows the sequence of single guide RNAs targeting exon 45 splice acceptor site in the human DMD gene. The palm denoted as red nucleotides of the single guide RNAs is located near the exon 45 splice acceptor site. The human and mouse conserved sequence is shaded in light blue color. The exon sequence is represented by letters in bold case and the intron sequence is again represented by letters in the lower case.

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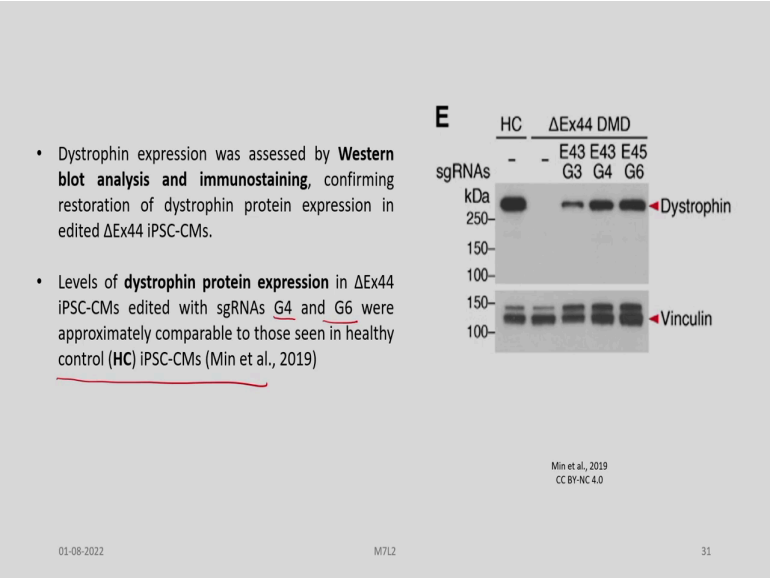

In this figure E you can see the dystrophin expression was analyzed by Western blot and blotting and immunostaining to confirm restoration of dystrophin protein expression in the edited del Ex 44 iPSC-CMs. The levels of dystrophin protein expression in these mutants edited with single guide RNAs G4 and G5, G6 were approximately comparable to those seen in healthy control, as you can see in this gel picture. So, with these we come to the end of part A of this lecture. We will continue the discussion of various aspects of CRISPR Cas9 technology in the next part.

Thank you.