Genome Editing and Engineering
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Module - 05
Zinc Finger Nuclease (ZFN) Technology
Lecture - 13
Applications of Zinc Finger Nucleases - PART B

Welcome to part B of Applications of Zinc Finger Nucleases. Here we will be discussing about the Applications of Zinc Finger Nucleases as therapeutic approaches for curing human diseases.

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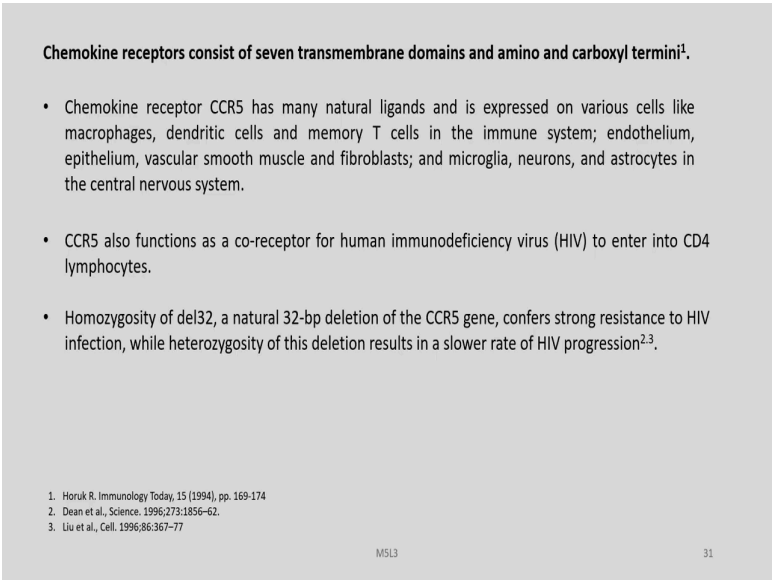

First, let us take up the case of application in HIV treatment. So, before going to the exit applications, let us try to understand some of the basic concepts related to the pathology of HIV. So, here certain molecular players are involved in these particular disease. For example, the chemokine receptors play an important role. And these are having around 7 transmembrane domains and amino and carboxyl termini.

The chemokine receptor CCR5 has a many natural ligands and is expressed in various cells like macrophages, dendritic cells, and memory T cells in the immune system; endothelium, epithelium, vascular smooth muscle and fibroblasts; microglia, neurons, astrocytes in the central nervous system.

These particular receptor, CCR5 also functions as a co-receptor for human immuno-deficiency virus to enter into CD4 lymphocytes. So, homozygosity of del32, which is a deletion mutant which is and a natural 32 base pair deletion of this particular gene CCR5, confers strong resistance to HIV infection while heterozygosity of this deletion results in slower rate of HIV progression.

So, a person having a homozygous phenotype of this del32 mutant of CCR5 will be naturally resistance to HIV. And it is reported that about 1 percent of the Scandinavian population carries this mutation and they are naturally immune to HIV.

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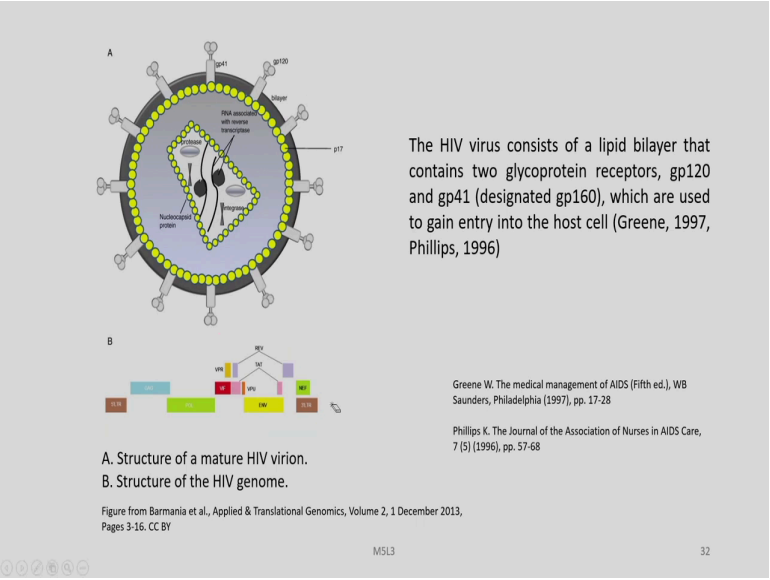

And this is the simple structure of the HIV virus which is required to understand how it enters the human cells. So, you have lipid layer and it contains two glycoprotein receptors, gp120 and gp41. And these are used to gain entry into the host cells.

So, this is the structure of a mature HIV virion and structure of these HIV genome, which produces the various components of this particular virus.

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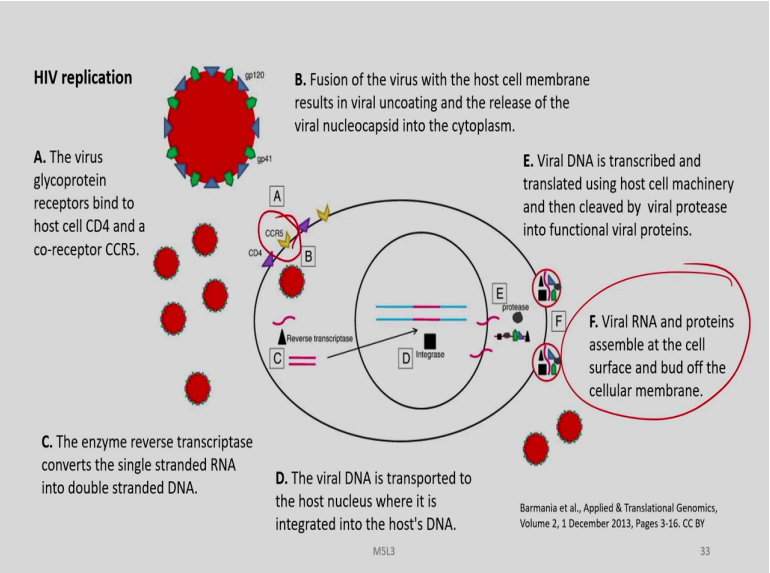

So, these glycoprotein receptors are important for the virus to gain entry into the host cells. So, this is a simplified diagram of this HIV virus showing the gp120 and gp41. And this is a human cell, ok. And these human cell has CD4 receptor and CCR5 receptors.

So, here these virus glycoprotein will bind to the host cell CD4, here, and you can see this matching, and these peg like matching here and a co-receptor CCR5. So, these two are important to which the virus glycoprotein receptors will bind.

The fusion of the virus with the host cell membrane results in viral uncoating and the release of the viral nucleocapsid into cytoplasm. So, here first it will bind and then this is internalized as you can see here, the B.

So, in the next step, this is releasing its nucleic acids. The enzyme reverse transcriptase converts the single stranded RNA into double stranded DNA, ok. You can see here. And these viral DNA is transported to the host nucleus where it is integrated into the host DNA through the integase. And you know about this integration mechanism which we have discussed in the preliminary lectures.

Finally, the viral DNA is transcribed and translated using host cell machinery and then cleaved by viral protease into functional viral proteins. And then some of them are released, which go and bind to other nearby cells through the CD4 and CCR5 receptors. And so, the proliferation happens by this way.

These viral RNA and proteins will assemble at the cell surface and bud off the cellular membrane.

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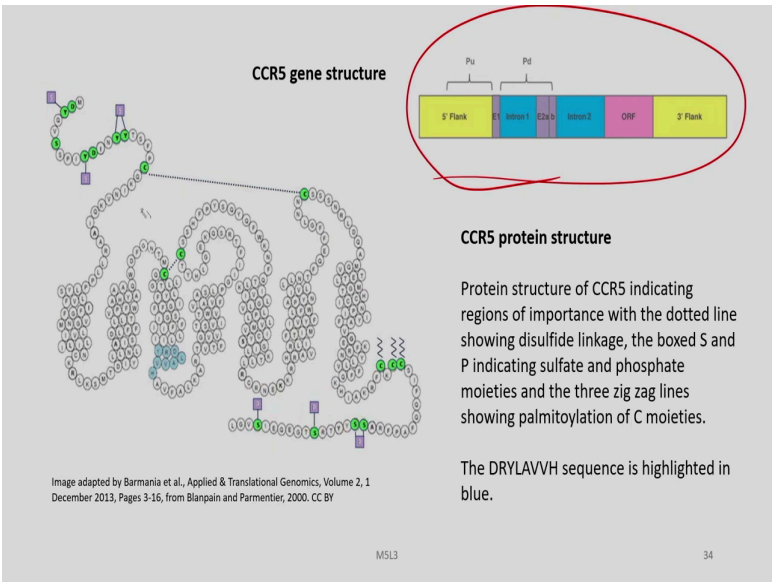

Now, let us discuss a little bit about the structure of these CCR5 protein and the gene. As we have discussed already that this is a trans-membrane protein having a certain domains. And this is the gene structure, you can see over here. So, the protein structure of these CCR5 indicates regions of importance with the dotted line showing disulfide linkages here, ok. The boxed S and P, this is the S and the P here, you can see, indicating sulfate and phosphate moieties and the 3 zigzag lines showing palmitoylation of the C moieties.

The DRYLAVVH sequences highlighted in blue here, ok. So, these are the gene structure and the protein structure of CCR5 gene.

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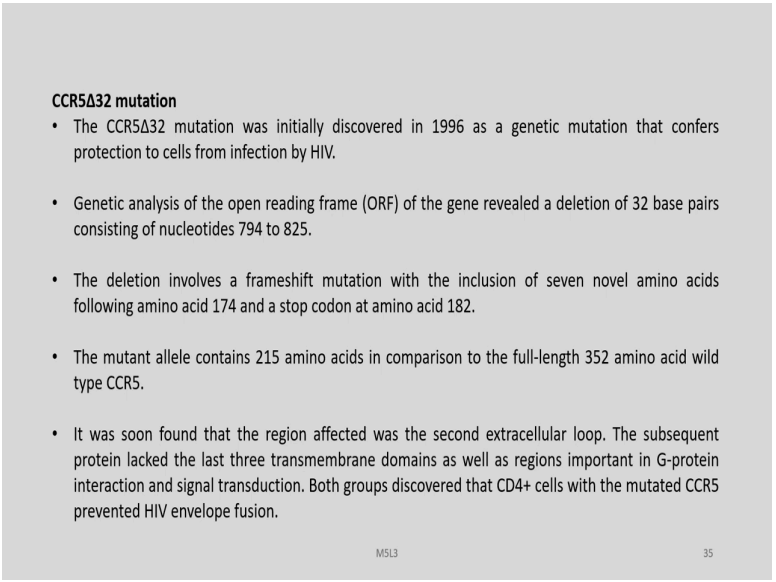

Now, these particular gene undergoes a mutation which is known as the CCR5 delta32 mutation. And this was initially discovered in 1996 as a genetic mutation that confers protection to cells from infection by HIV. And we have already discussed about the immunity to HIV, if it is homozygous and if it is heterogeneous, it delays the infection of HIV.

And genetic analysis of the ORF of this gene revealed the deletion of around 32 base pairs from 794 to around 825, that is why this is known as del32. And these deletion involves a frame shift mutation with the inclusion of 7 novel amino acids, following amino acid 174 and a stop codon at amino acid 182. The mutant allele contains 215 amino acids in comparison to the full length 352 amino acid wild type CCR5.

It was soon found out that the region affected was the second extracellular loop. The subsequent protein lacked the last 3 transmembrane domains as well as regions important in G-protein interaction and signal transduction. Both groups discovered that CD4 plus cells with the mutated CCR5 prevented HIV envelope fusion.

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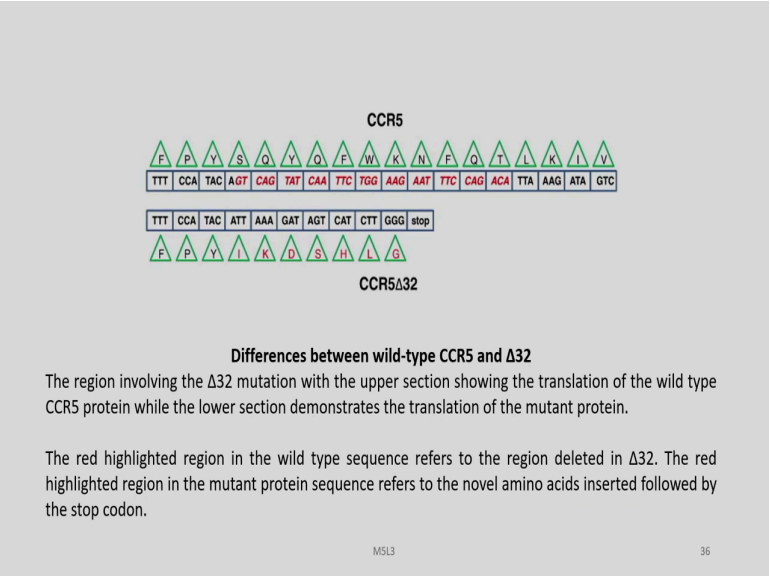

So, this is the CCR5 wild type and this is the CCR5delta32. So, what are the difference between these wild type and the deletion mutants?

The region involving the deletion 32 mutation with the upper section showing the translation of the wild type CCR5 protein, while the lower section demonstrates the translation of the mutant proteins. The red highlighted regions in the wild type sequence refers to the regions deleted in delta32. The red highlighted regions in the mutant protein sequence refers to the novel amino acids inserted followed by the stop codon.

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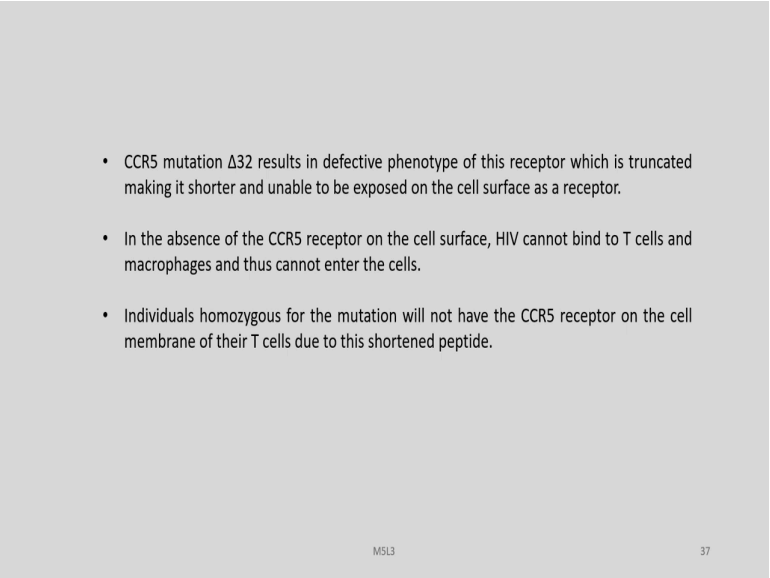

The CCR5 mutation results in defective phenotype of this receptor which is truncated making it shorter and unable to be exposed to the cell surface as a receptor. It becomes a half receptor a part of it will remain inside the cell attached to near the cell membrane.

In the absence of the CCR5 receptor on the cell surface, due to this mutation, the HIV cannot bind to T cells and macrophages and thus cannot enter the cells. Individuals homozygous for this mutation will does not have the CCR5 receptor on the cell membrane of their T cells due to this shortened peptide.

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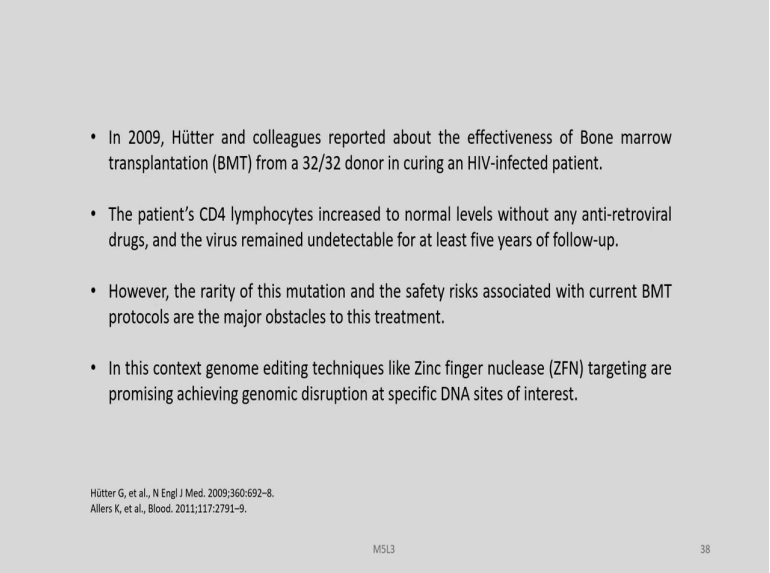

So, around in 2009, Hutter and colleagues reported about the effectiveness of a Bone Marrow Transplantation from a homogeneous 32 del 32 donor in curing an HIV-infected patient. And this becomes a kind of a breakthrough and then people are now trying to create this kind of deletions by genome editing.

In this particular case, the patient CD4 lymphocytes increased to normal levels without any anti-retroviral drugs, and the virus remain undetected for at least 5 years of follow-up. However, the rarity of this mutation and the safety risk associated with current Bone Marrow Transplantation protocols are the major obstacles to this treatment. And in this context genome editing techniques like Zinc Finger Nucleases are promising alternatives for genomic disruptions at specific DNA sites of interest and in this particular case the CCR5 delta 32 sequence.

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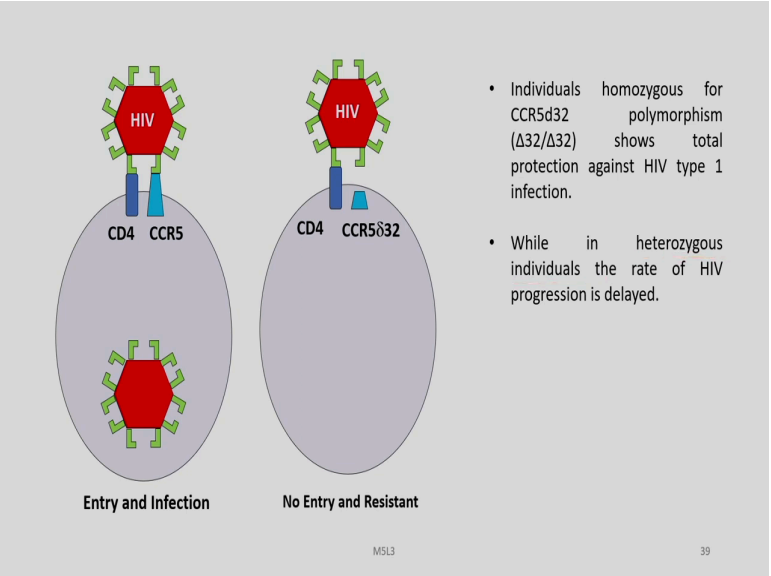

So, here you can see that you have the full receptor where you have the external domain as well expressed and which is exposed outside the cell. So, HIV binds not only to CD4, it also binds to the CCR5 because it's domains are available for binding. And this helps in the entry and finally, infection and proliferation.

But in the case of the CCR5delta32 mutation, now this is a half receptor and its trans-membrane domains are not exposed to the outside of the wall. So, HIV can bind to CD4, but it will not be able to enter and infect the cells. So, therefore, this kind of cells or individuals will be resistant to HIV.

So, I mean, again we have emphasized that in case of homozygous individuals, we will have full resistance. But in the case of heterozygous individuals, the resistance will not be full, but the rate of progression of HIV will be delayed. So, this has become an important application area of ZFN technology.

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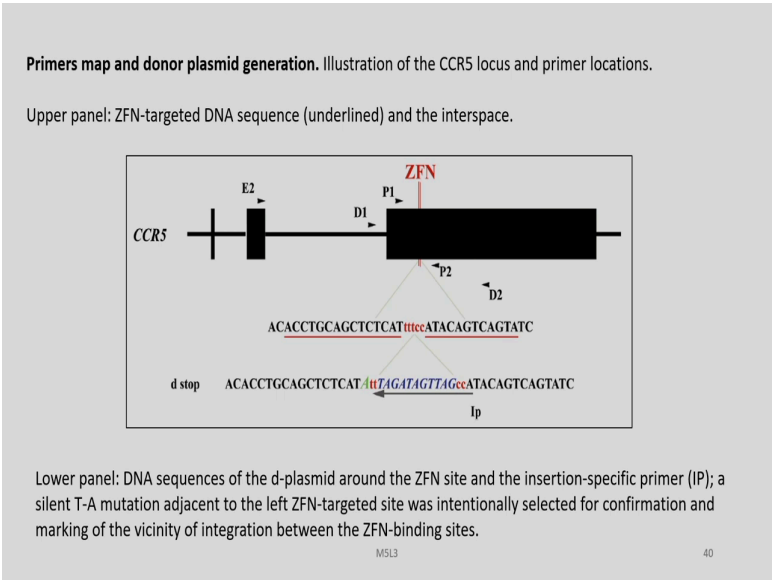

So, for doing these we need primers and donor plasmid generation. And you can see here in the upper panel the ZFN targeted DNA sequence and the interspace. And then in the lower panel, we can see DNA sequence of the d-plasmid around the ZFN site in the insertion-specific primer IP. A silent T-A mutation adjacent to the left ZFN target site was intensively selected for confirmation and marking of the vicinity of the integration between the ZFN binding sites.

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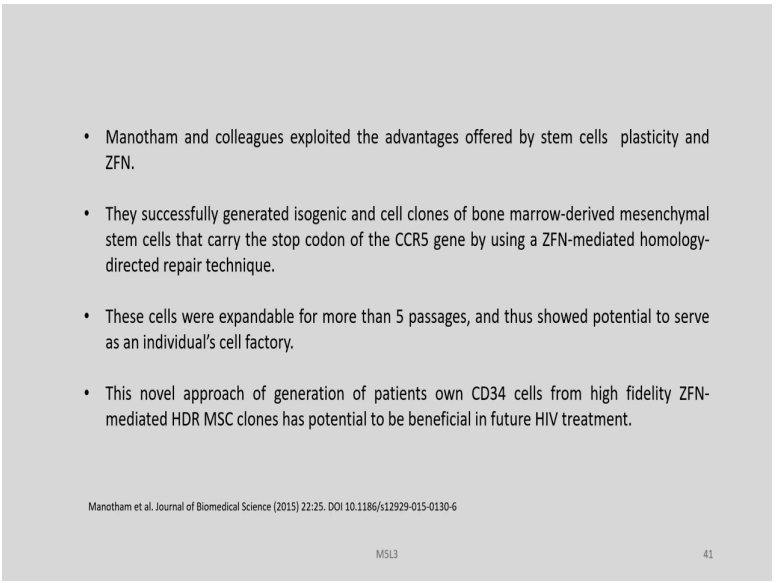

And Manotham and colleagues exploited the advantages offered by the stem cells plasticity and ZFN. They successfully generated isogenic and cell clones of bone marrow derived mesenchymal stem cells that carry the stop codon of the CCR5 gene by using ZFN-mediated homology directed repair technique.

These cells were expandable for more than 5 passages, and thus showed potential to serve as an individual's cell factory. And these approach of generation of patients own CD4 cells from high fidelity ZFN-mediated HDR MSC clones has potential to beneficial in future HIV treatment.

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Let us now discuss about another application of ZFN technology. As the case of HIV therapy, ZFN technology has huge potential for cancer therapy. And many workers have been trying to use it in diverse kinds of cancers.

In our lecture, we will be discussing about one such cancer only due to paucity of time. The application here we discuss is the application of ZFN technologies in cervical cancer as a model example.

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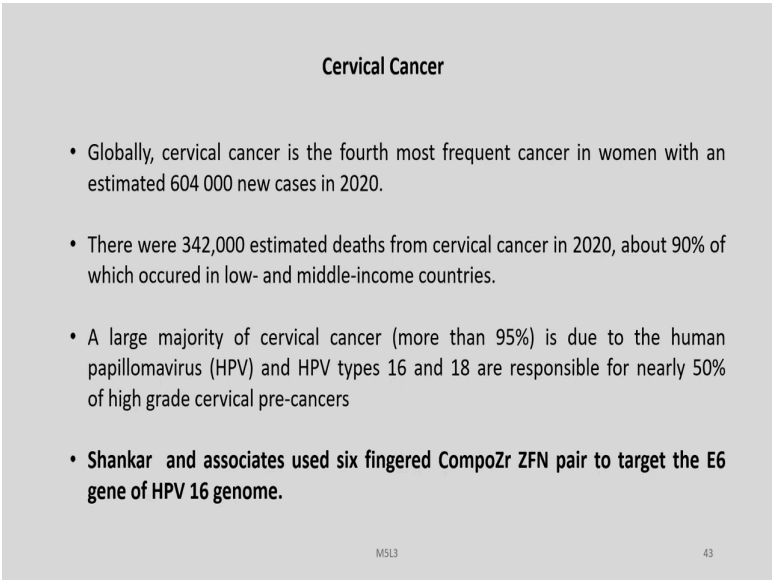

So, let us try to learn about a little bit about the cervical cancer, its incidence rates. The globally, this cancer is the 4th most frequent cancer in women with an estimated 604,000 new cases in 2020. And there were 342,000 estimated deaths from cervical cancer in 2020, about 90 percent of which occurred in the low and middle income countries.

A large majority of cervical cancers more than 95 percent is due to the human pepillomavirus HPV, and particularly HPV types 16 and 18 are responsible for nearly 50 percent of high grade cervical pre-cancers. Shankar and associates used 6 fingered CompoZr ZFN pair to target the E6 gene of HPV 16 genome.

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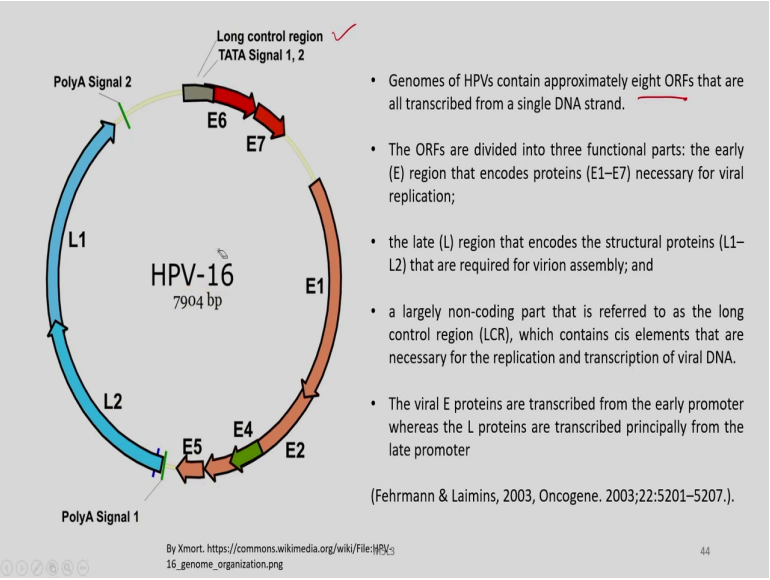

So, this is the HPV-16 genomic map. You can see here various genes. So, let us study them one by one. The HPV genome contains approximately around 8 open reading frames, and these are all transcribed from a single stranded DNA. These ORFs are divided into 3 functional parts, the early region, that encodes proteins E1 to E7, ok, and this is necessary for viral replication. Then, there are the late region that encodes the structural proteins L1 to L2 that are required for virus assembly.

And there is a largely non-coding part that is referred to as the long control region which contains cis elements that are necessary for the replication and transcription of viral DNA. The viral E proteins are transcribed from early promoters whereas, the L proteins are transcribed principally from the late promoters. And this is the 7904 is the total number of base pairs which is the size of these HPV-16 genome.

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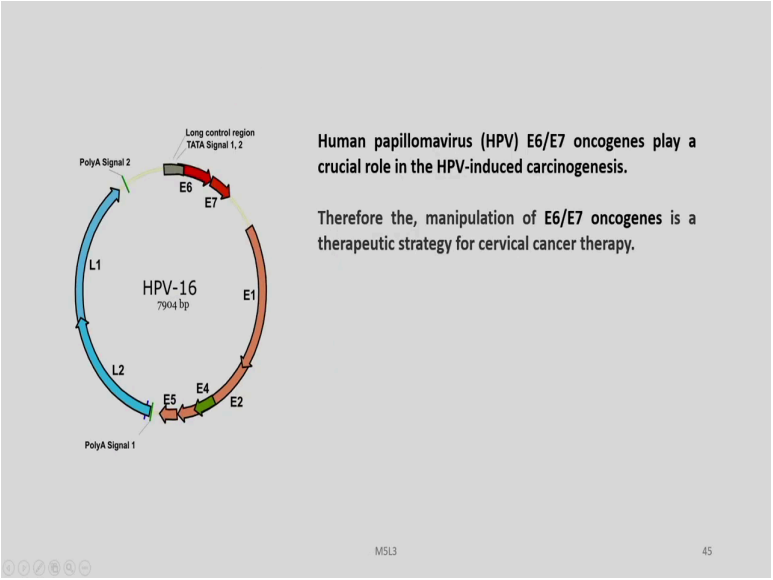

The HPV E6 and E7 oncogenes play a crucial role in the HPV induced carcinogensis. So, if we can manipulate these genes, can silence them, E6 and E7, we can develop some kind of a therapeutic strategy or cancer, cervical cancer therapy.

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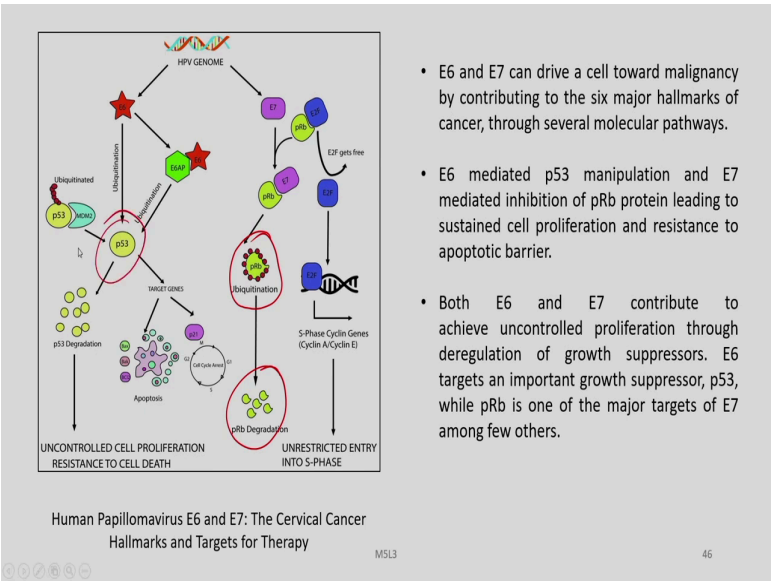

So, this is the HPV genome and it produces E6 and E7 which are oncogenes and which play crucial role in the development of this disease. And both these genes products can drive a cell towards malignancy by contributing to the 6 major hallmarks of cancer, through several

molecular pathways. You can see over here so many different pathways. We will try to understand these pathways one by one.

The E6 mediated p53 manipulation and the E7 mediated inhibition of pRb protein leading to sustained cell proliferation and resistance to apoptotic barriers. Both these E6 and E7 contribute to achieve uncontrolled proliferation through deregulation of growth suppressors. E6 targets an important growth suppressor p53, while pRb is one of the major targets of this E7 among others.

So, here you can see this ubiquitination leads to the pRb degradation. And here the ubiquitination of p53 will also lead to the degradation of these particular proteins.

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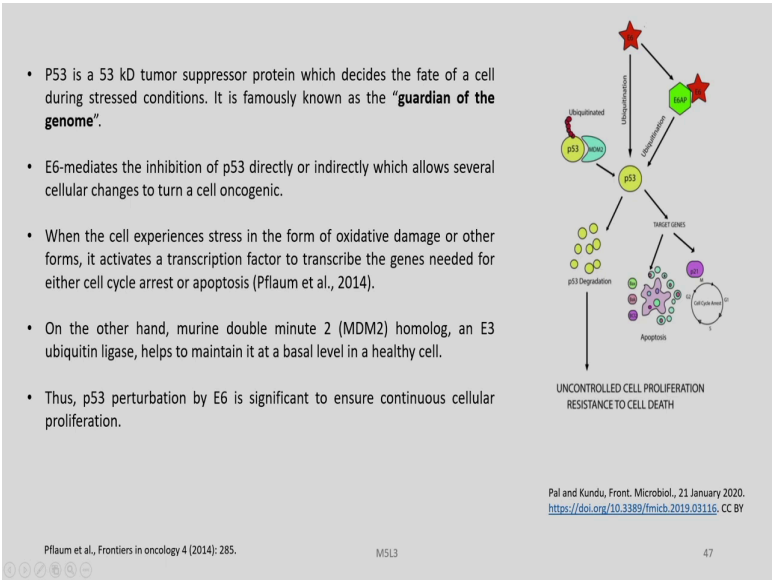

These P53 is famously known as the guardian of the genome. This is a 53 kilo delton tumor suppressor protein which decides the fate of a cell during conditions of stress. This E6-mediates the inhibition of p53 directly or indirectly, here you can see it is direct and here it is indirect which allows several cellar changes to turn a cell into a oncogenic state.

When the cell experiences stress in the form of oxidative damage or other forms, it activates a transcription factor to transcribe the genes needed for either cell cycle arrest or apoptosis.

On the other hand, the murine double minute 2, MDM2, here you can see, an E3 ubiquitin ligase, helps to maintain it at a basal level in the healthy cell. Thus, p53 perturbation by E6 is significant to ensure continuous cellular proliferation.

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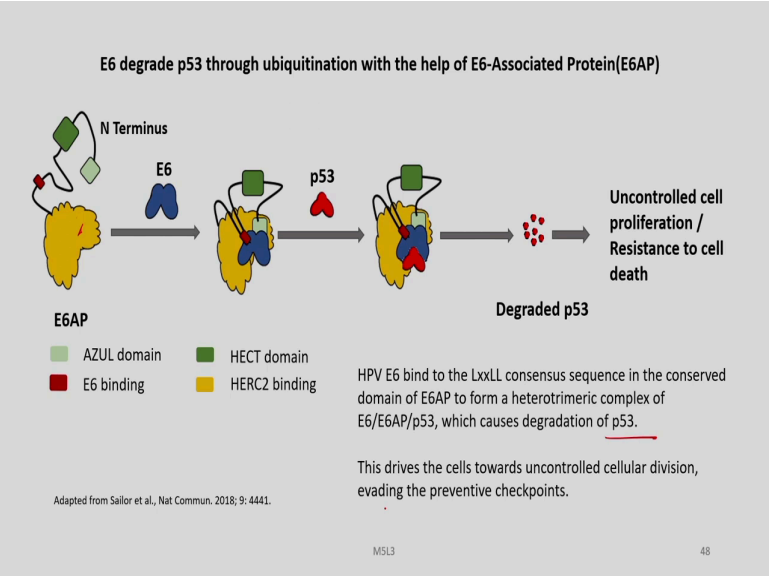

The E6 degrade p53 through ubiquitination which I have already told you with the help of E6 associated protein, ok. So, here you can see here E6 associated protein, and it has certain domains called the AZUL domain, and the E6 binding domain, then HECT domain, and HERC2 binding. So, this is in brief the structure of the structural domains of E6 associated protein, and this is the E6 which will bind and form a partnership with the E6 associated protein here, ok.

And soon these two partners will go and find out a p53 molecule and they will form a trimer over here. And since these are all 3 different proteins, we call it as a heterotrimeric complex. And then, as a result of these heterotrimeric complex formation, the p53 will be degraded, and then this leads to the uncontrolled cell proliferation and resistance to cell dead.

So, in brief HPV E6 bind to the LxxLL consensus sequence in the conserved domain of E6 associated protein and forms a heterotrimeric complex of E6, E6AP, and p53 which causes the degradation of p53. And drives the cells towards uncontrolled cellular division evading the prevailing checkpoints.

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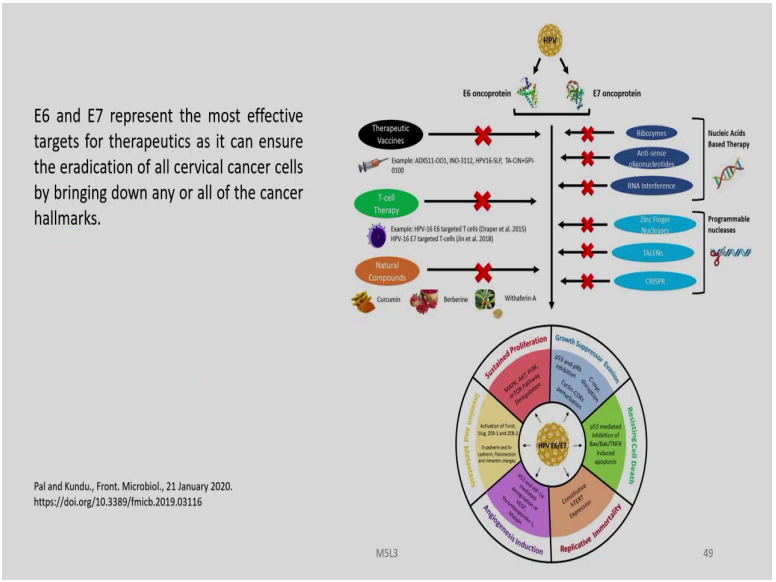

So, this is HPV and you have this E6 oncoprotein and E7 oncoprotein and their various approaches for cervical cancer therapy, like vaccines, T cell therapy, using natural compounds, or ribozyme mediated, anti-sense oligonucleotides, RNA interference, which comprises the nucleic acid based therapies. And now the latest in these therapeutic arsenal are the programmable nucleases like Zinc Finger, TALENs and CRISPR. We will discuss about zinc finger nucleases.

So, the E6 and E7 represent the most effective targets for therapeutics in cervical cancer, as it can ensure the eradication of all such cancers, cancer cells by bringing down any or all of the cancer hallmarks.

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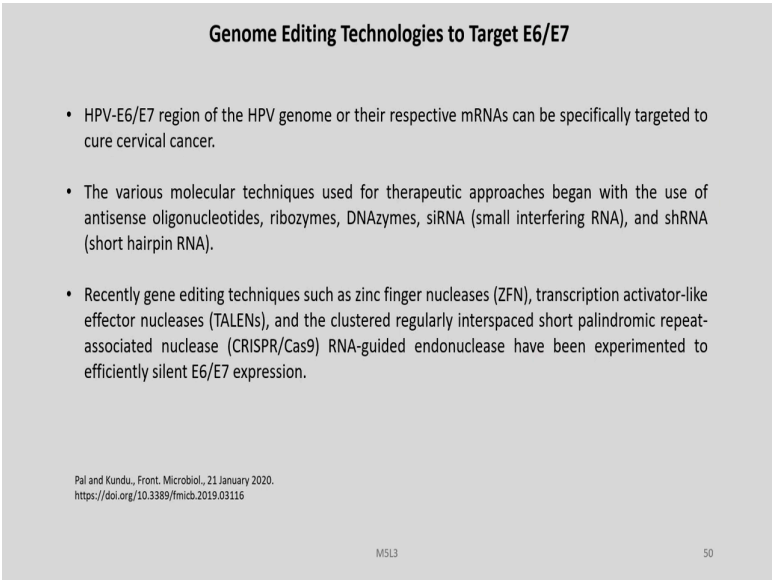

So, genome editing technologies to target these oncogenes E6 and E7. The HVP-E6 E7 region of the HPV genome or their respective mRNAs can be specifically targeted to cure cervical cancer.

The various molecular techniques used for therapeutic approaches begin with the use of antisense oligonucleotides types, ribozymes, DNAzymes, siRNA and siRNA as already discussed in the previous slide. And recently genome editing technologies are being utilized like the ZFNs, TALENs and CRISPRs to efficiently silent the E6 E7 expression.

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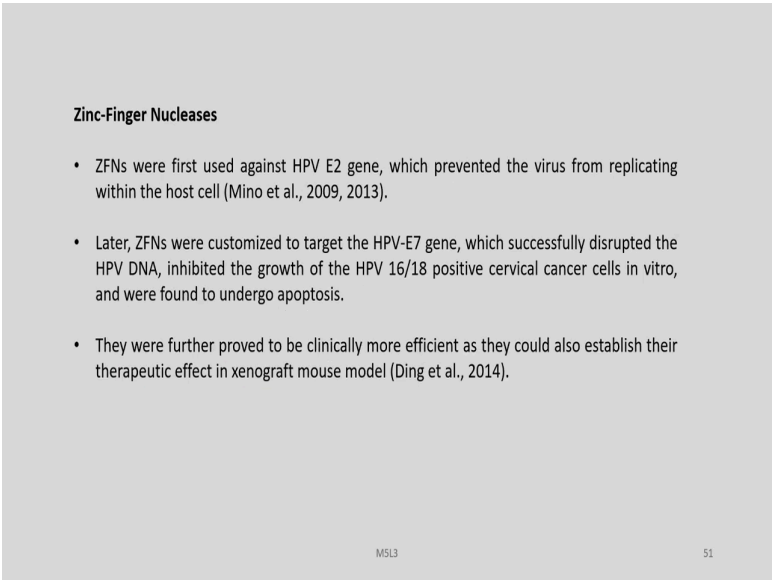

ZFNs were first used against HPV E2 gene which prevented the virus from replicating within the host cell. Later, the ZFNs were customized to target the HPV-E7 gene, which successfully disrupted the HPV DNA, inhibited the growth of the HPV-16 18 positive cervical cancer cells in vitro, and were found to undergo apoptosis.

And these were harder proved to be clinically more efficient as they could also establish their therapeutic effect in xenograft mouse model.

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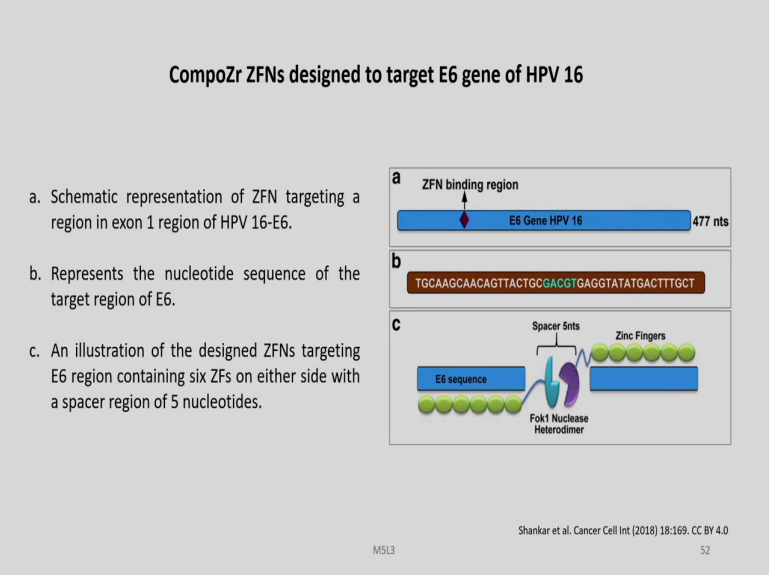

And here CompoZr ZFNs were designed to target E6 gene of HPV-16. And this is the schematic representation of ZFN targeting. A reason in axon, one region of HPV-16 E6. So, this is the ZFN binding site.

And here this represents the, number b) will represent the nucleotide sequence of the target region of the E6. And c) is the illustration of the designed ZFNs targeting E6 regions containing 6 zinc fingers on either side 1, 2, 3, 4, 5, 6, and 6 in either side. And there is a special region of around 5 nucleotides and the Fok1 nucleases will dimerize and do the genome editing and follow the recombination pathway.

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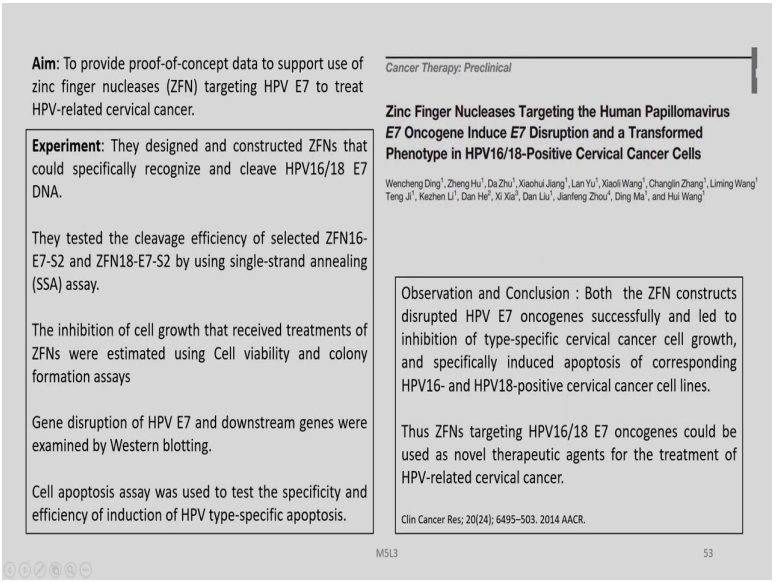

So, this is another work on the application of zinc finger nucleases targeting the HPV E7 oncogene. In the earlier case, we have studied about the targeting of E6 gene, and this is another study where the E7 oncogene was targeted or disrupted. And here, Ding and his collaborators started the work with an aim to provide a proof of concept, to support the use of ZFNs to target HPV E7 to treat HPV related cervical cancer.

And in the experiment, the designed and constructed ZFNs that could specially recognize and cleave the HPV-16 or 18 E7 DNA. And once the design was over, they tested the cleavage efficiency of selected ZFNs16-E7-S2 and ZFN18-E7-S2 by using single stranded annealing assay.

The inhibition of the cell growth that received treatments of ZFNs were estimated using cell viability and colony formation assays. And gene disruption of HPV E7 and downstream genes were examined by methods like Western blotting. Cell apoptosis assay was used to test the specificity and efficiency of induction of HPV type specific apoptosis.

And they finally, concluded that both the ZFN constructs they designed and synthesized disrupted HPV E7 oncogenes successfully and led to innovation of type specific cervical cancer growth and specifically induced apoptosis of corresponding HPV-16 and HPV-18 positive cervical cell lines.

Therefore, ZFNs targeting HPV-16 or HPV-18 E7 oncogenes could be used as novel therapeutic agents for the treatment of HPV related cervical cancer.

There are many other examples where ZFNs has been used as models for cancer therapy or proof of concepts. We have lectures in the next few classes where we will be discussing the use of genomic editing technologies for generating cancer disease models. There we will discuss some of the remaining examples both in the case of ZFN, TALEN as well as CRISPR (Refer Time: 31:05).

Thank you.