Genome Editing and Engineering
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Module - 08
Applications of genome editing in treating human diseases
Lecture - 29
Human cell engineering in diseases: Thalassemia - Part B

Welcome to my course on Genome Editing and Engineering. We are discussing Applications of genome editing in treating human diseases. In the last part, we are discussing about Human cell engineering in diseases with respect to Thalassemia.

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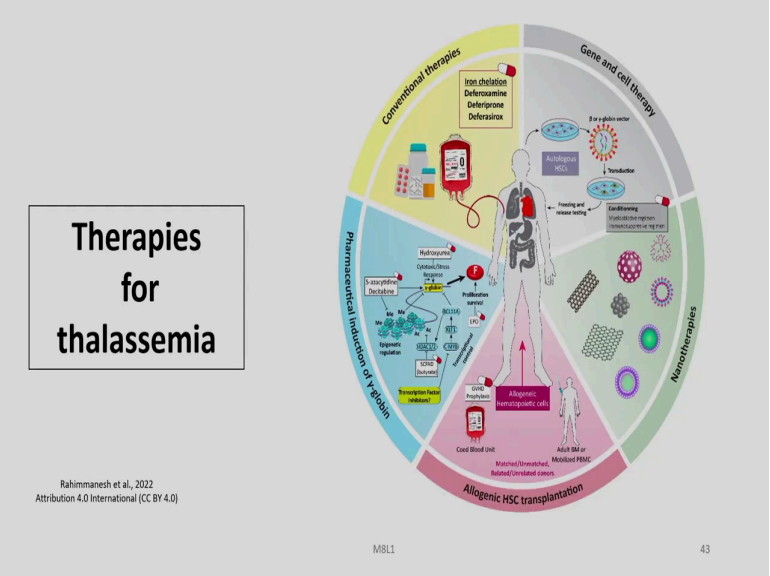

In this part, we are continuing thus that discussion. So, you now know about the various basic aspects of the disease and today, we are discussing here some of the therapies, which are available for thalassemia or which are under development.

So, let us begin with the conventional therapies where you have iron chelation, then you have pharmaceutical induction of gamma globin; then some of the advanced therapies includes allogenic hematopoietic stem cell transplantation and also many research papers has been published in potential nano therapies for thalassemia.

One of the important domain that is undergrowth in this area is gene and cell therapy for thalassemia. We will be trying to discuss some of the gene editing technologies and some research which has been going on in this respect.

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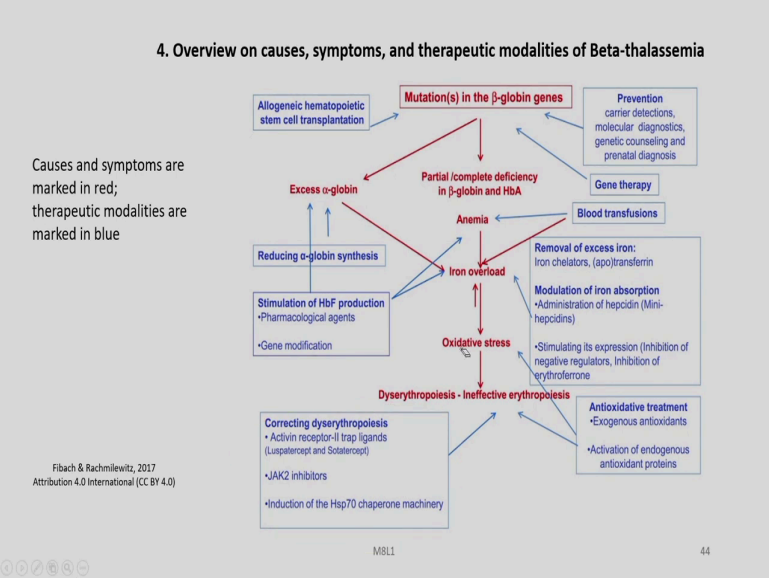

Before that let us go on to discuss some of the symptoms, the causes and therapeutic modalities of beta thalassemia. So, in the center we have these printed in red.

So, these are the causes and symptoms and the various therapeutic modalities are marked in blue. So, we know that they have certain mutations which lead to the disease and in this case you can see the mutations in the beta-globin genes which may partial or complete deficiency the beta-globin and HBA and it causes anaemia and then there is iron overload leading to oxidative stress and so on.

Accordingly, we have various therapeutic approaches. Number 1 is the prevention – carrier detections, molecular diagnostics, genetic counselling and prenatal diagnosis. These are thought to be very very important for the control of this disease in a population.

Then we have options for gene therapy as already shown in the earlier slide. And, then whenever this blood transfusions we have to go for anemia sorry, whenever there is anemia we have to go for blood transfusions, and then we have already shown you about the allogenic hematopoietic stem cell transplantation and then in certain cases wherever there is excess alpha globin, we have to go for reducing the alpha globin synthesis.

And, if there is iron overload, we have to emphasize on the removal of excess iron using iron chelators, apo transferring; then modulation of iron absorption, and then also apply anti-oxidative treatments by supplying exogenous antioxidants or activation of endogenous antioxidant proteins.

Then in certain cases, to control excess alpha globin, we go for the stimulation of HbF production using pharmacological agents and gene modification. Then certain corrections using activin receptor-II trap ligands or JAK2 innovators and also induction of the Hsp70 chaperone machinery is used.

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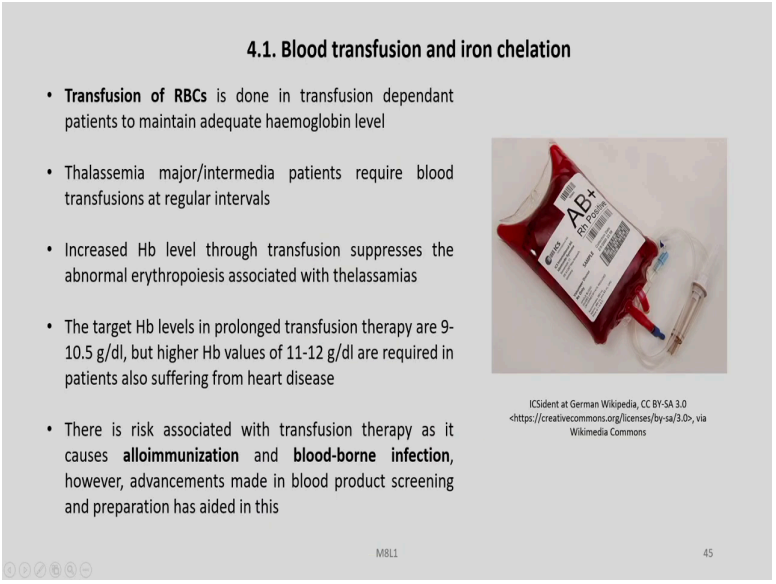

So, these are some of the strategies for the therapies. So, we have spoken about the blood transfusion and iron chelation. Transfusion of red blood cells is done in transfusion dependent patients to maintain adequate haemoglobin level. Thalassemia major and intermedia patients require blood transfusions at regular intervals.

Increased Hb level through transfusion suppresses the abnormal erythropoiesis associated with thalassemias. The target Hb levels in prolonged transfusion therapy are 9 to 10.5 gram per deci liters, but higher Hb values 11 to 12 are required in patients which are also suffering from heart diseases.

There is risk associated with transfusion therapy as we know it causes a low immunization and blood-borne infection, however, advancements made in blood product screening and preparation has help us in overcoming these shortcomings.

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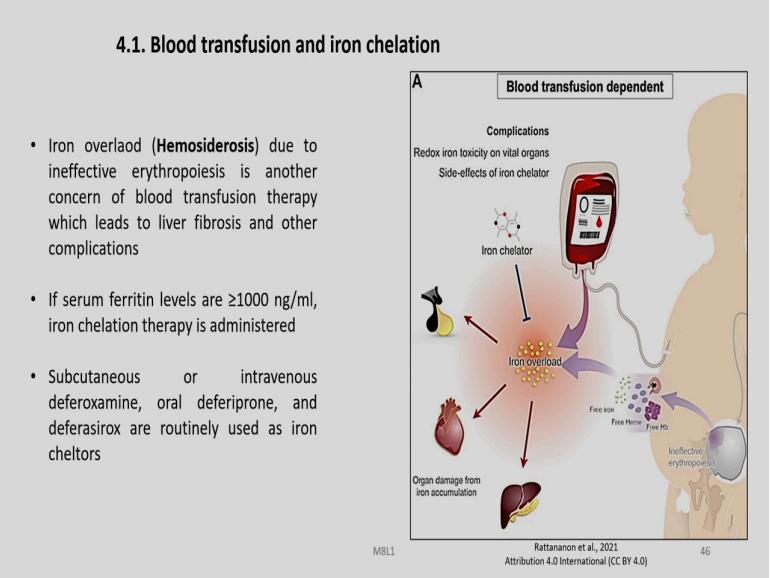

Iron overload due to ineffective erythopoiesis is another concern of blood transfusion therapy which leads to liver fibrosis and other complications. If serum ferritin levels are more than 1000 nanogram per millilitre, iron chelation therapy is administered. Subcutaneous or intravenous deferoxamine, oral deferiprone and deferasirox are routinely used as iron chelators.

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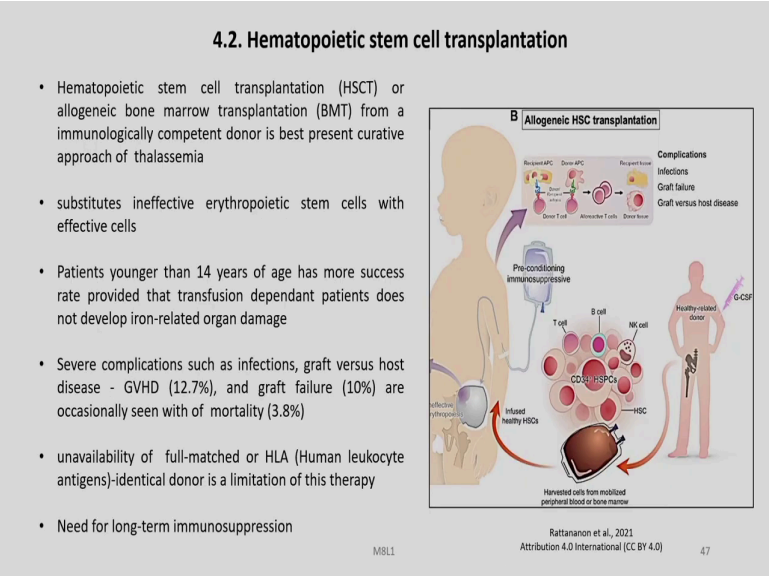

Hematopoietic stem cell transplantation is another approach as we have already told you in the beginning of the slides. Here allogenic bone marrow transportation BMT from immunologically component donor is the best present curative approach for thalassemia. Substitutes ineffective erythropoietic stem cells with effective cells is the approach in this case. Particularly, patients younger than 40 years of 14 years of age has more success rate provided the transfusion dependent patients does not develop iron-related organ damage.

Severe complications such as infections, graft versus host disease and graft failure are occasionally seen with some mortality amounting to around roughly 4 percent, then unavailability of fully matched or HLA human leukocyte antigens identical donor is a limitation of this therapeutic approach. Need for long term immunosuppression is also another disadvantage.

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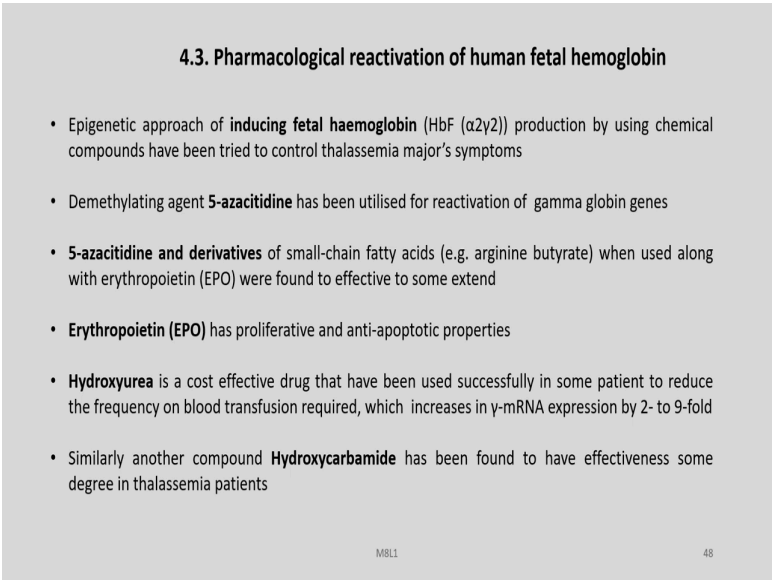

And, the pharmacological reactivation of human fetal haemoglobin is also one of the practical approaches for therapeutic approaches for thalassemia. Epigenetic approach of inducing fetal haemoglobin production by using chemical compounds have been tried to control thalassemia majors symptoms.

Demethylating agent 5-azacitidine has been utilized for reactivation of gamma-globin genes. 5-azacitidine and its derivatives of small chain fatty acids when used along with erythropoietin were found to be effective to some extent. EPO has proliferative and anti apoptotic properties.

Hydroxyurea is a cost effective drug that has been used successfully in some patient to reduce the frequency of blood transfusion required which increases in gamma-mRNA expression by 2-to-9 folds. Similarly, another compound hydroxycarbamide has been found to be effective to some degree in certain thalassemia patients.

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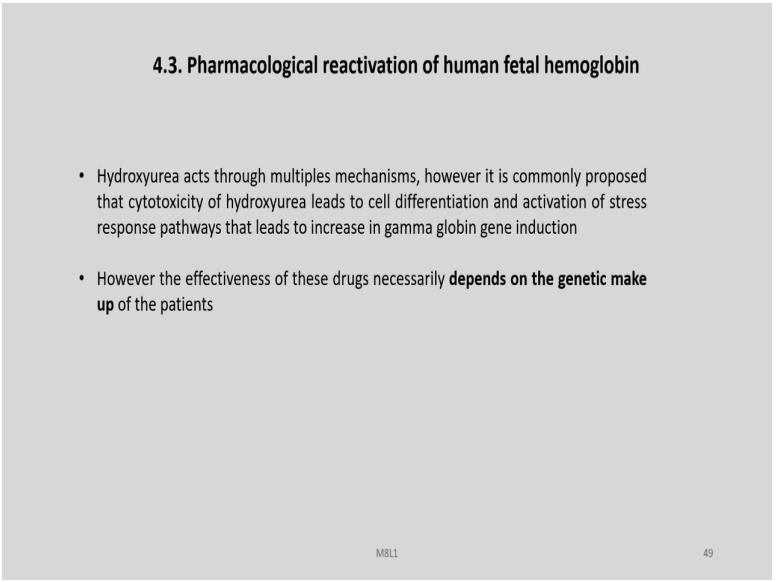

Hydroxyurea acts through multiple mechanisms, however, it is commonly proposed that cytotoxicity of hydroxyurea leads to cell differentiation and activation of stress response pathways that leads to increase in the gamma-globin gene induction. However, the effectiveness of these drugs necessarily depends on the genetic makeup of the patients which varies from individual to individual.

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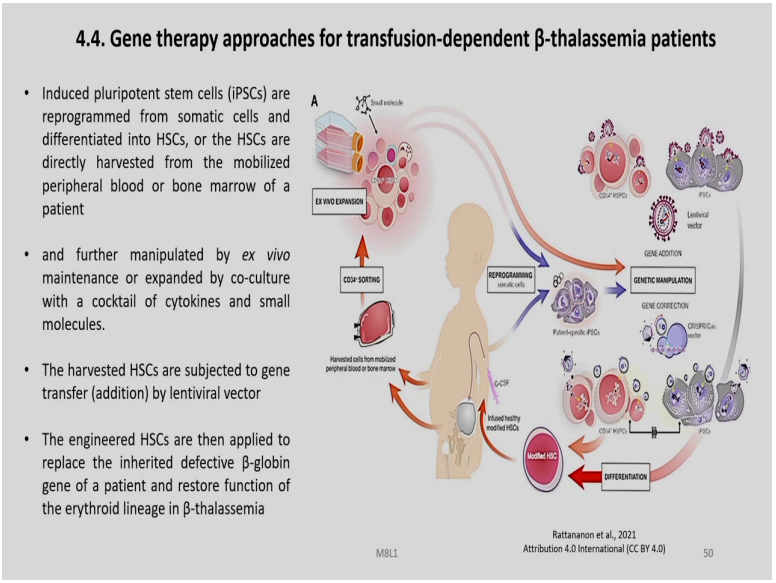

One of the most interesting therapy is the gene therapy approach for transfusion dependent beta thalassemia patients. So, induced pluripotent stem cells iPSCs are reprogrammed from

somatic cells and differentiated into HSCs, or HSCs are directly harvested from the mobilized peripheral blood or bone marrow of a patient.

So, these are depicted in the picture as you can see and these are further manipulated by ex vivo maintenance or expanded by co-culture with a cocktail of cytokines and small molecules. The harvested hematopoietic stem cells are subjected to gene transfer addition by lentiviral vector. The engineered stem cells or HSCs are then applied to replace the inherited defective beta-globin gene of a patient and restore function of the erythroid lineage of beta-thalassemia.

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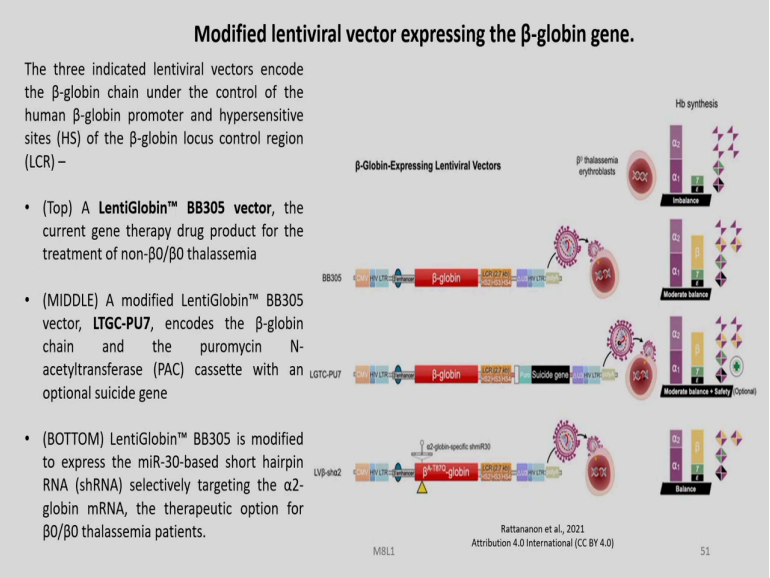

The three indicated lentiviral vectors encode the beta-globin chain under the control of the human beta-globin promoter and hypersensitive sites called HS of the beta-globin locus controlled region or LCR as shown in this picture. On the top you can see a lentiglobin BB305 vector followed by LTGC-PU7, in the middle and LV-beta sh alpha 2 in the bottom.

So, these are a commercial entities as you can see from the trademarks over here. So, on the top you see these vector where the current gene therapy drug product of the treatment of non beta 0 beta 0 thalassemia. And in the middle is the modified vector which encodes the beta-globin chain and the puromycin N-acetyltransferase cassette with an optional suicide gene as you can see over here.

And, in the bottom is the vector lentiglobin BB305 which is modified to express the miR-30-based short hairpin RNA or shRNA selectively targeting the alpha2 globin mRNA, the therapeutic option for beta 0 beta 0 thalassemia patients.

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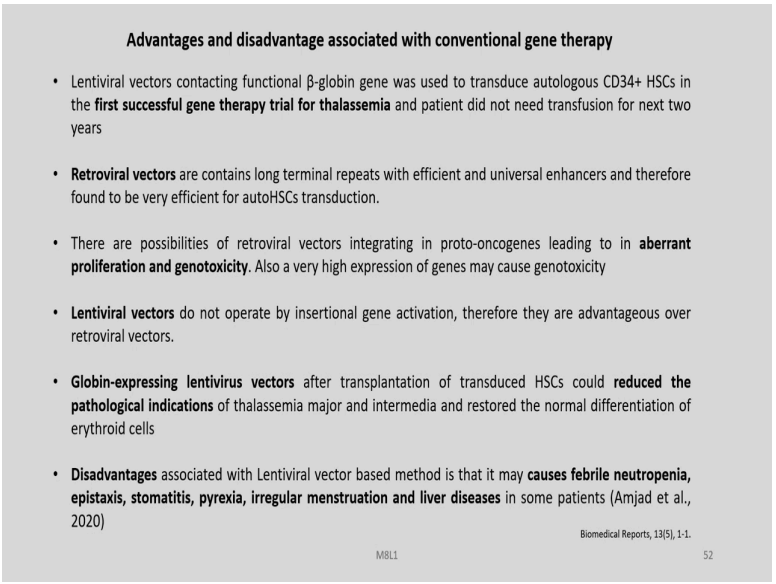

What are the advantages and disadvantages associated with conventional gene therapy. Lentiviral vectors contacting functional beta-globin gene was used to transduce autologous CD34 plus HSCs in the first successful gene therapy trial for thalassemia and patient did not need transfusion for the next two years.

Retroviral vectors contains long terminal repeats with efficient and universal enhancers and therefore, are found to be very efficient for autoHSCs transduction. There are possibilities of retroviral vectors integrating in proto oncogenes leading to in aberrant proliferation and genotoxicity. Also a very high expression of genes may cause genotoxicity.

Lentiviral vectors do not operate by insertional gene activation and therefore, advantageous over retroviral vectors. Globin-expressing lentiviral vectors after transplantation of transduced HSCs could reduce the pathological indications of thalassemia major and intermedia and restored the normal differentiation of erythroid cells. Disadvantages associated with lentiviral vector is that it may cause febrile neutropenia, epistaxis, stomatitis, pyrexia, irregular menstruation and liver diseases in certain patients.

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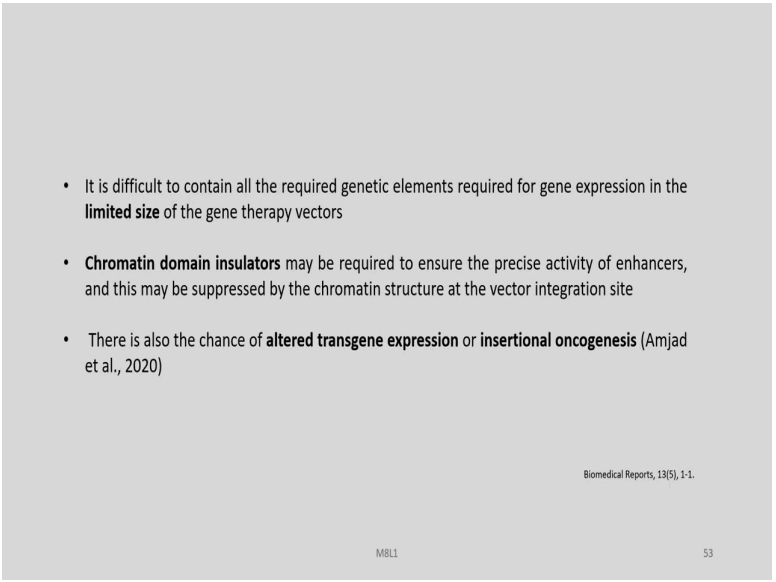

It is difficult to contain all the required genetic elements required for gene expression in the limited size of the gene therapy vectors. The chromatin domain insulators may be required to ensure the precise activity of enhancers, and this may be suppressed by the chromatin structure at the vector integration site. There is also the chance of altered transient expression in insertional oncogenesis.

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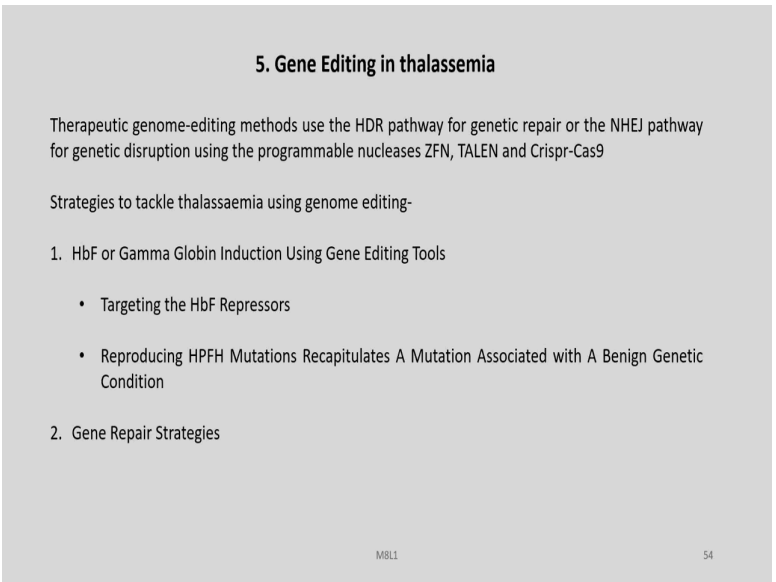

Let us now discuss about gene editing in thalassemia. Therapeutic genome editing methods use the HDR pathway for genetic repair or the NHEJ pathway for genetic disruption using the programmable nucleases like ZFN, TALEN and Crispr-Cas9.

Some of the strategies to tackle thalassemia using genome editing include: number 1 – HbF for gamma-globin induction using gene editing tools like targeting the HbF repressors. Reproducing HPFH mutations recapitulates a mutation associated with a benign genetic condition; and, the second approach is the gene repair strategies.

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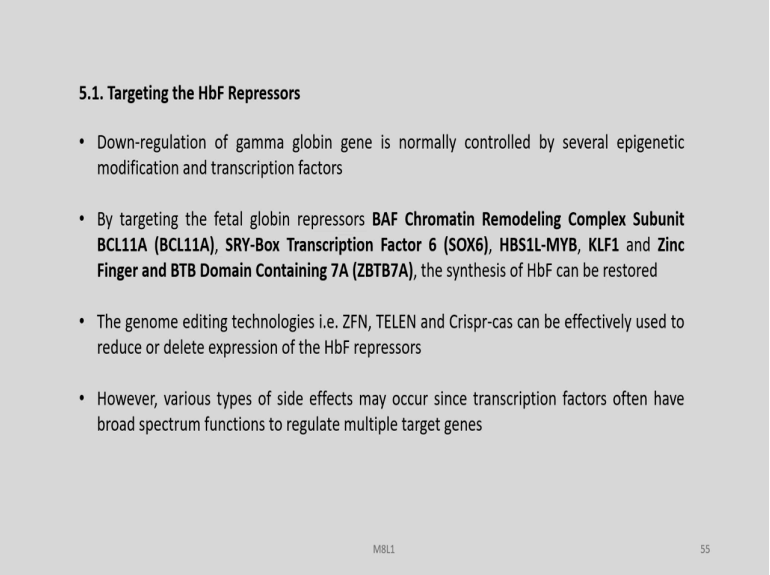

Let us discuss about targeting the HbF repressors. Down regulation of gamma-globin gene is normally controlled by several epigenetic modification and transcription factors. By targeting the fetal globin repressors BAF Chromatin Remodeling Complex Subunit BCL11A, SRY-Box Transcription Factor 6 or SOX6, HBS1L-MYB, KLF1 and Zinc Finger and BTB Domain containing 7A ZBTB7A, the synthesis of HbF can be restored.

The genome editing technologies like ZFN, TELEN and Crispr-cas can be effectively used to reduce or delete expression of the HbF repressors. However, various types of side effects may occur since transcription factors often have broad spectrum functions to regulate multiple target genes.

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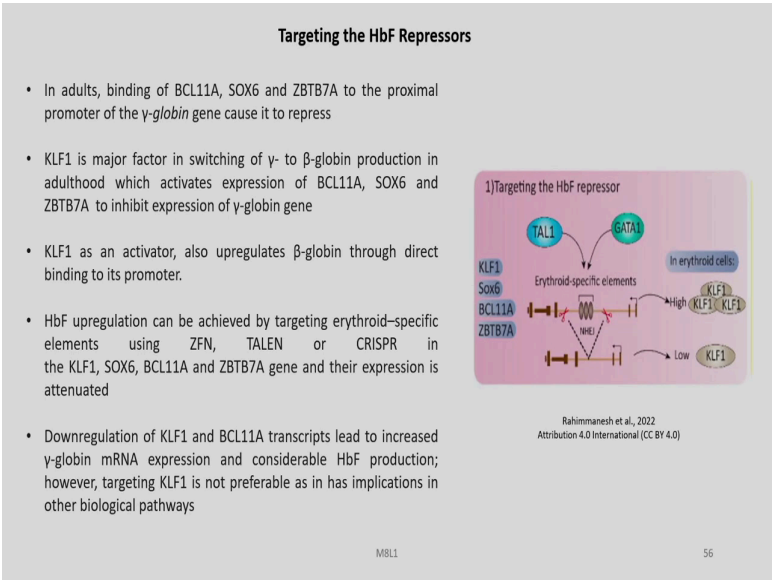

Here you can see a schematic diagram for the targeting of HbF repressor and in adults, binding of BCL11A to the proximal promoter of the gamma-globin gene cause repression. KLF1 is a major factor in switching of gamma to beta-globin production in adulthood which activates expression of BCL11A, SOX6 and ZBTB7A to inhibit expression of gamma-globin gene.

KLF1 as an activator, also up regulates beta-globin through direct binding to its promoter. HbF up regulation can be achieved by targeting erythroid-specific elements using ZFN, TALEN and CRISPR in the KLF1, SOX6, BCL11A and ZBTB7A gene and their expression is attenuated.

Down regulation of KLF1 and BCL11A transcripts lead to increased gamma-globin mRNA expression and considerable HbF production; however, targeting KLF1 is not preferable as it has implications in other biological pathways.

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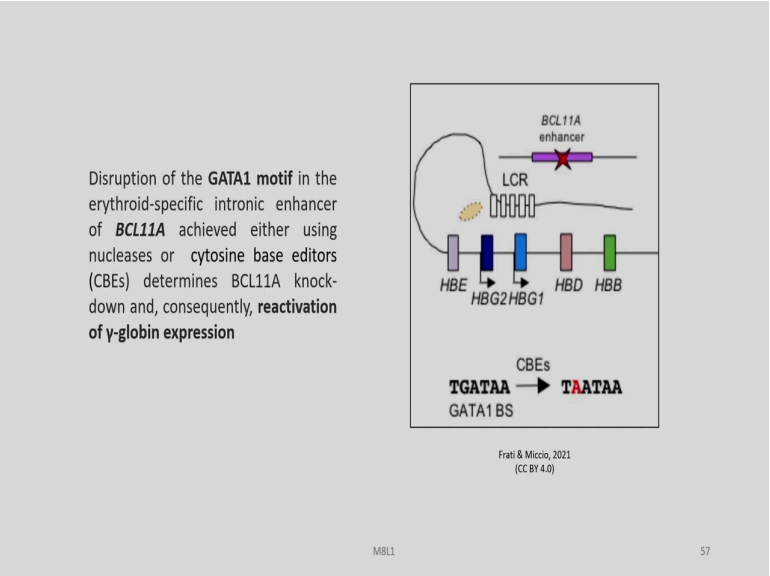

Disruption of GATA1 motif in the erythroid-specific intronic enhancer of BCL11A is achieved either using nucleases or cytosine based editors or CBEs and it determines BCL11A knockdown and consequently reactivation of gamma-globin expression.

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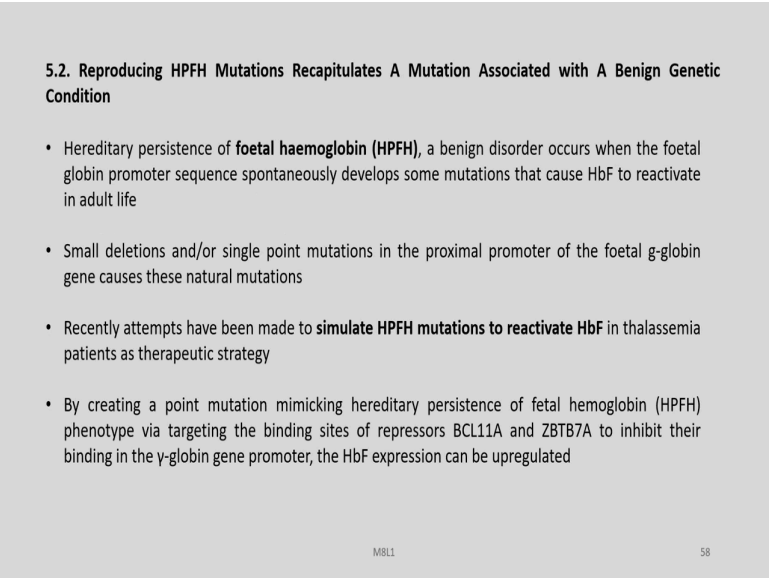

Reproducing HPFH mutations recapitulate a mutation associated with a benign genetic condition. Hereditary persistence of foetal haemoglobin, a benign disorder occurs when a foetal globin promoters sequence spontaneously develops some mutations that cause HbF to reactivate in adult life.

Small deletions and or single point mutations in the proximal promoter of the foetal g-globin gene causes these natural mutations. Recently attempts have been made to simulate HPFH mutations to reactivate HbF in thalassemia patients as therapeutic strategy.

By creating a point mutation mimicking hereditary persistence of fetal haemoglobin phenotype via targeting the binding sites of repressors BCL11A and ZBTB7A to inhibit their binding in the gamma-globin gene promoter, the HbF expression can be upregulated.

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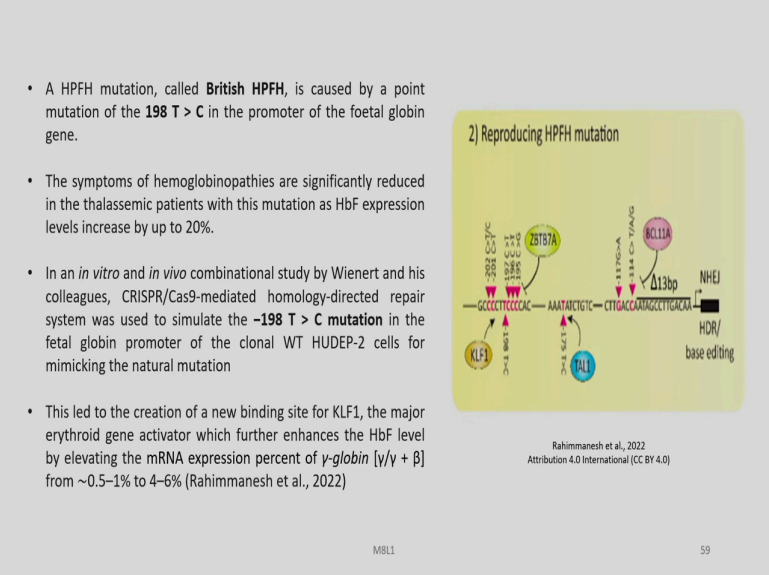

So, here in this picture you can see the reproducing HPFH mutation. HPFH mutation called British HPFH is caused by a point mutation of the 198 position T to C in the promoter of the foetal globin gene. The symptoms of hemoglobinopathies are significantly reduced in the thalassemic patients with this mutation as HbF expression levels increase by up to 20 percent.

In an in vitro and in vivo combinational study by Wienert and his colleagues Crispr Cas9 mediated homology-directed repair system was used to simulate the 198 T to C mutation in the fetal globin promoter of the clonal wild type HUDEP-2 cells for mimicking the natural mutation.

This led to the creation of a new binding site for KLF1, the major erythroiod gene activator which further enhances the HbF level by elevating the mRNA expression percent of gamma-globin from 0.5 to 1 percent to around 4 to 6 percent.

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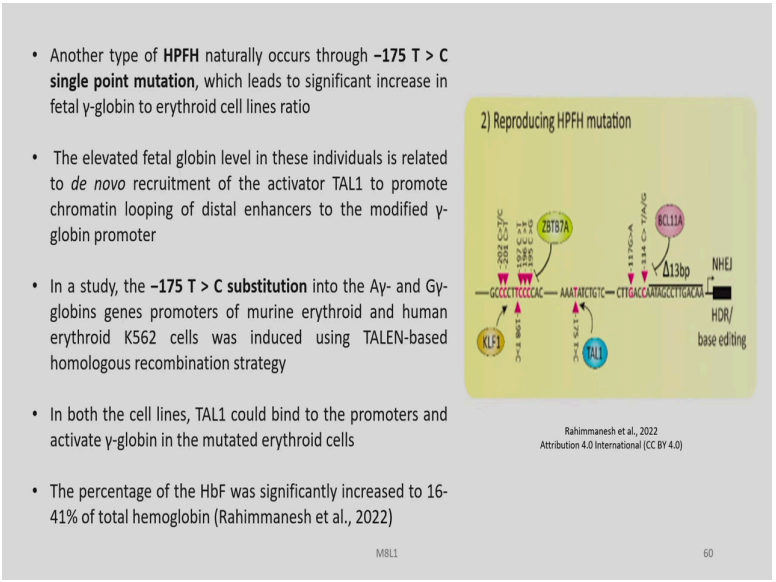

Another type of HPF naturally occurs through 175 T to C single point mutation which leads to significant increase in fetal gamma-globin to erythroid cell lines ratio. The elevated fetal globin level in these individuals is related to de novo recruitment of the activator TAL1 to promote chromatin looping of distal enhancers to the modified gamma-globin promoter.

In a study, the 175 T to C substitution into the A gamma and G gamma-globin genes promoters of murine erythroid and human erythroid K562 cells was induced using TALEN-based homologous recombination strategy. In both the cell lines, TAL1 could bind to the promoters and activate gamma-globin in the mutated erythroid cells. The percentage of the HbF was significantly increased to 16 to 41 percent of total haemoglobin.

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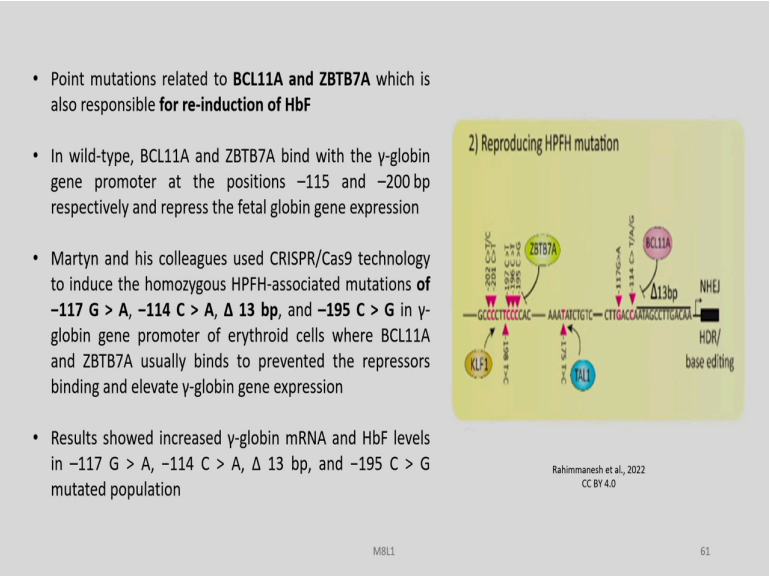

Point mutations related to BCL11A and ZBTB7A which is also responsible for re-induction of HbF. In the wild-type, BCL11A and ZBTB7A bind with the gamma-globin gene promoter at the positions 115 and 200 base pairs respectively and replace the fetal globin gene expression.

Martyn and his colleagues used CRISPR Cas9 technology to induce the homogeneous HPFH associated mutations of 117 G to A, 114 C to A, delta 13 base pair and minus 195 C to G in gamma-globin gene promoter of erythroid cells where BCL11A and ZBTB7A usually binds to prevent the repressor binding and alleviate gamma-globin gene expression. Results from such experiment showed increased gamma-globin mRNA in HbF levels in 117 G to A, 114 A and delta 13 base pair and 195 C to G mutated population.

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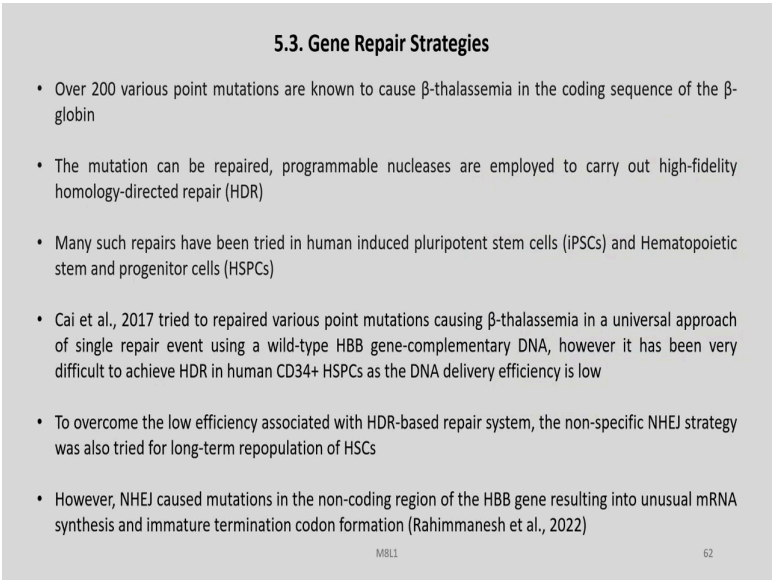

Another strategy for thalassemia therapy is the gene repair strategy. Over 200 various point mutations are known to cause beta-thalassemia in the coding sequences of the beta-globin. The mutation can be repaired, programmable nucleases are employed to carry out high-fidelity homology-directed repair. Many such repairs have been tried in human induced pluripotent stem cells and hematopoietic stem and progenitor cells.

Cai et al., in 2017 tried to repair various point mutations causing beta-thalassemia in an universal approach of single repair event using a wild-type HBB gene-complementary DNA, however, it has been very difficult to achieve HDR in human CD34 plus HSPCs as the DNA delivery efficiency is low.

To overcome the low efficiency associated with HDR-based repair system, the non-specific NHEJ strategy was also tried for long term repopulation of HSCs. However, NHEJ caused mutations in the non-coding regions of the HBB gene resulting into unusual mRNA synthesis and immature termination codon formation.

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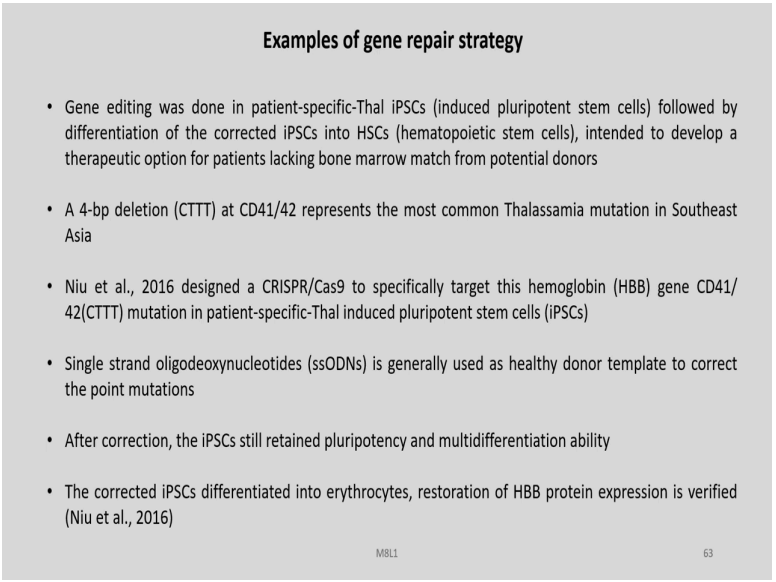

Let us discuss some examples of gene repair strategy. Gene editing was done in patient-specific Thal iPSCs thalassemia iPSCs followed by differentiation of the corrected iPSCs into hematopoietic stem cells, intended to develop a therapeutic option for patients lacking bone marrow match from potential donors.

A 4-base pair deletion C triple T at CD41 42 represents the most common thalassemia mutation in Southeast Asia. Niu et al., 2016 designed a Crispr Cas9 to specifically target this hemoglobin gene CD41 oblique 42 mutation in patient-specific thalassemia induced pluripotent stem cells or iPSCs.

Single strand oligodeoxynucleotides ssODNs is generally used as healthy donor template to correct the point mutations and after correction, the iPSCs retained pluripotency and multidifferentiation ability and the corrected iPSCs differentiated into erythrocytes, restoration of HBB protein expression is also verified.

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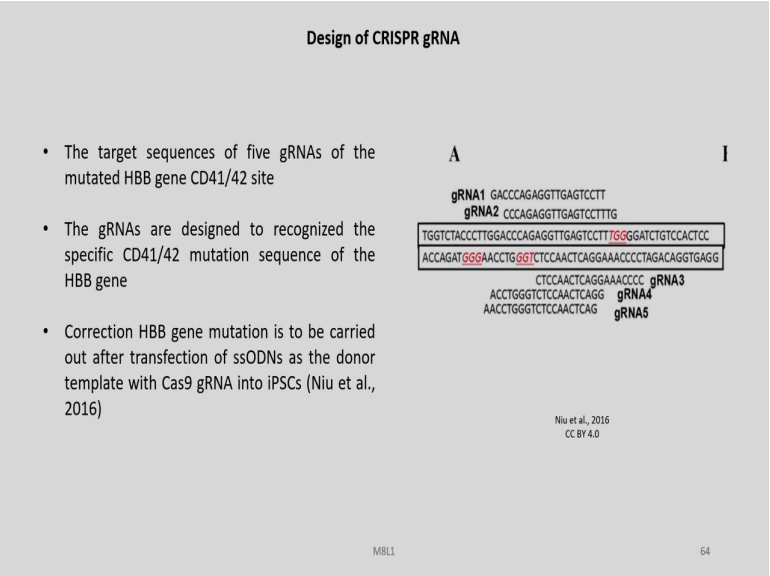

And, for this the CRISPR guide RNA designing plays an crucial role. Here you can see 5 guide RNAs – gRNA 1, 2, 3, 4, 5 designed for specific positions and these are the target sequences of the five gRNAs of the mutated HBB gene CD41 oblique 42 site. The gRNAs are designed to recognize the specific mutation sequence of the HBB gene. Correction of HBB gene mutation is carried out after transfection of ssODNs as the donor template with Cas g9 guide RNA into iPSCs.

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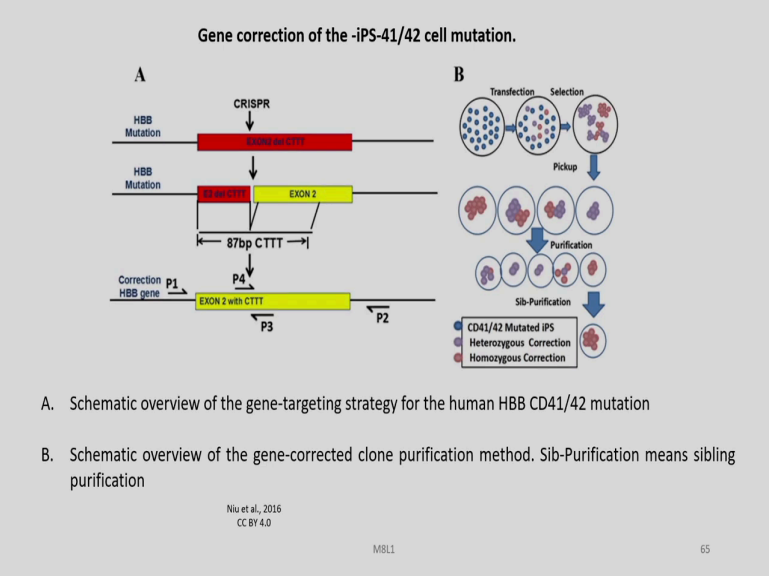

Correction of the genes of the iPS-41/42 cell mutations; so, here you can see in figure A the CRISPR targeting exon 2 del CTT and then in the following figure you can see in B that we go for the transfection, selection, pickup, purification and so on. This is the overall schematic view of the gene targeting strategy for the human HBB CD41/42 mutation where we have these correction of the HBB gene. In the B, we have the schematic overview of the gene-corrected clone purification method where Sib-purification is sibling purification.

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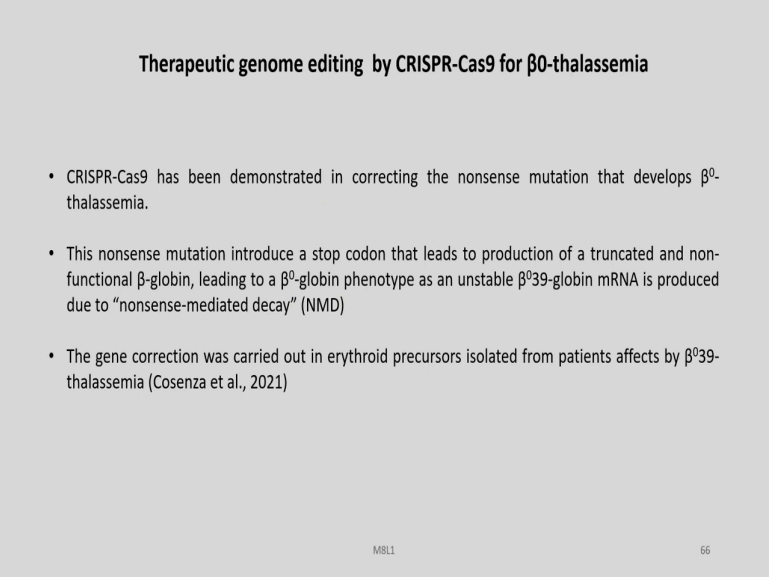

Therapeutic genome editing by CRISPR-Cas9 for beta 0 thalassemia. CRISPR-Cas9 has been demonstrated for correcting the nonsense mutation that develops beta 0 thalassemia.

This nonsense mutation introduces stop codon that leads to production of a truncated and non-functional beta-globin leading to a beta-globin phenotype as an unstable beta 0 39-globin mRNA is produced due to nonsense mediated decay or called NMD in brief. The gene correction was carried out in erythroid precursors isolated from patients affected by these beta 0 39-thalassemia.

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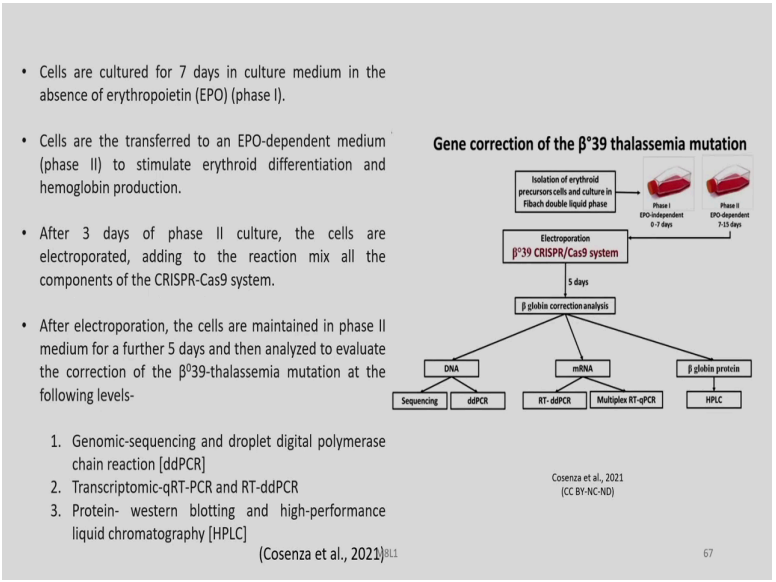

Cells are cultured for 7 days in culture medium in the absence of erythropoietin and then they are transferred to an EPO-dependent medium in phase II to stimulate erythroid differentiation and haemoglobin production. After 3 days of phase II culture as seen in these picture, the cells are electroporated, adding to the reaction mixer all the components of Crispr Cas9 system.

After electroporation, the cells are maintained in phase II medium for a further 5 days and then analyzed to evaluate the correction of the beta 0 39-thalassemia mutation at various levels by genome sequencing and droplet digital polymerase chain reaction ddPCR; then transcriptomic qRT-PCR and RT-ddPCR; protein-western blotting and high performance liquid chromatography.

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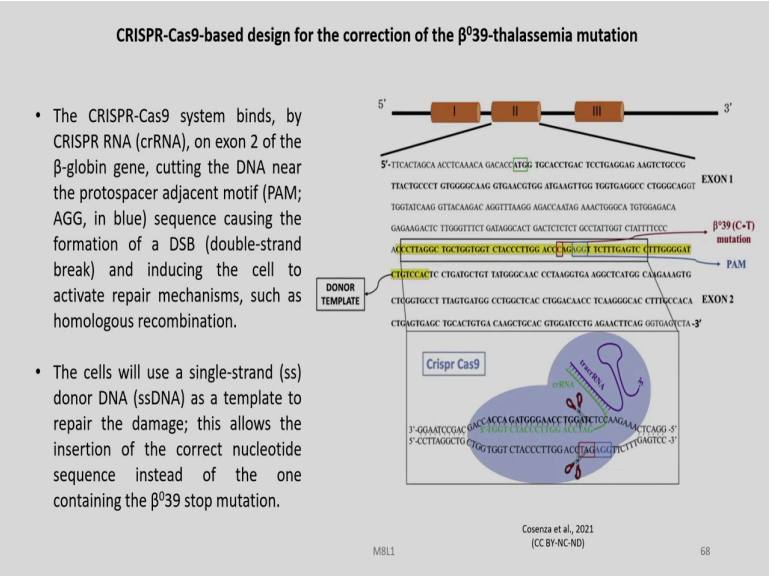

The CRISPR-Cas9 based design for the correction of beta 0 39 thalassemia mutation is shown in this figure, where you have this donor template and these are the two exons 1 and 2 and the CRISPR-Cas9 construct along with the tracer RNA, which targets these correction.

The CRISPR-Cas9 system binds by CRISPR RNA or exon 2 of the beta-globin gene, cutting the DNA near the proto spacer adjacent motif causing the formation of double-strand break and inducing the cell to activate repair mechanisms, like homologous recombination. The cells will use a single-strand donor DNA as a template to repair the damage. This allows the insertion of the correct nucleotide sequence instead of the one containing the beta 0 39 stop mutation.

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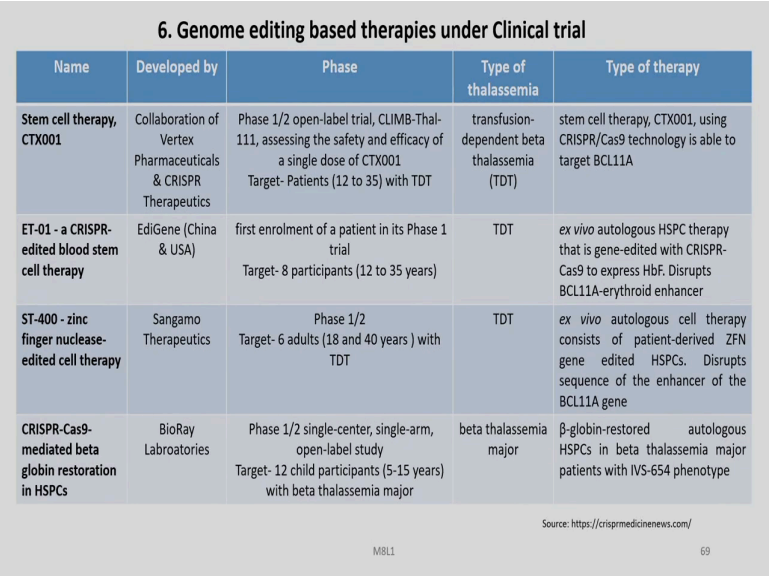

There are several genome editing based therapies for thalassemia which are under clinical trial currently developed by various companies. For example, stem cell therapy CTX001 is developed by a collaborative effort between vertex pharmaceuticals and CRISPR therapeutics. And, it is in phase 1 and 2, where open level trial.

And, then ET-01-a CRISPR-edited blood stem cell therapy developed by EdiGene China and USA, there is a first enrolment of a patient in its phase 1 trial and it target 8 participants between the age range of 12 to 35 years. Similarly, you have ST-400 – zinc finger nuclease edited cell therapy, developed by Sangamo Therapeutics and CRISPR-Cas9 mediated beta-globin restoration in HSPCs developed by BioRay Laboratories.

And, you can see the various phases they are 1 and 2 and targeting around 6 adults between the age range of 18 to 40 years and the type of thalassemia being targeted by each of these cell therapies are listed in this fourth column. The first one is targeting transfusion dependent beta-thalassemia and so on the other two.

CRISPR-Cas9 mediated beta-globin restoration is targeting beta thalassemia major. So, they also all belong to different types of therapies for example, the first one belongs to stem cell therapy. The second one belongs to the ex vivo autologous HSPC therapy and the fourth one belongs to ex vivo autologous cell therapy and the last one belongs to beta-globin restored autologous HSPCs in beta-thalassemia major patients and so on.

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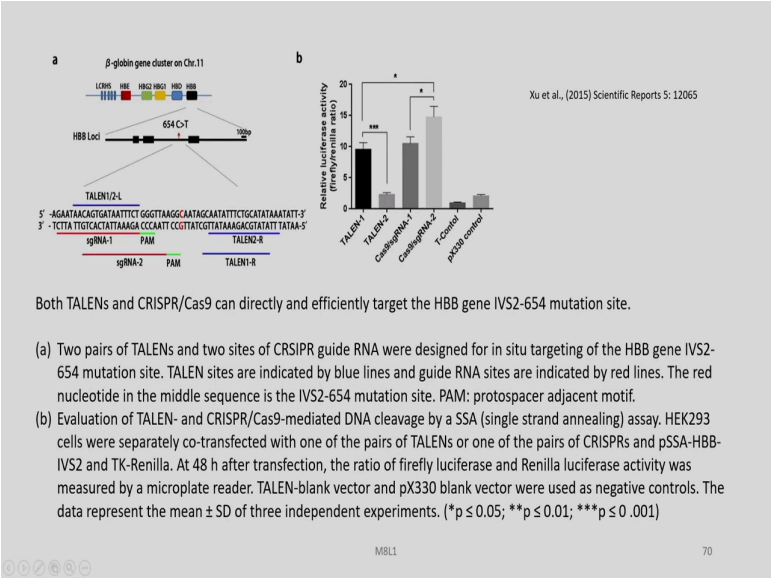

So, we now know that all the gene editing technologies like ZFN, TALEN and CRISPR have the potential to be utilized for therapy, therapeutic applications in thalassemia. In certain experiments by Xu et al., they found both TALEN and CRISPR-Cas9 can directly and efficiently targeted target the HBB gene IVS2-654 mutation site as shown in this picture.

For these they took two pairs of TALENs and two sites of CRISPR guide RNA designed for in situ targeting of the HBB gene. TALEN sites are indicated by the blue lines and guide RNA sites are indicated by the red lines. The red nucleotide in the middle sequence here is the IVS2-654 mutation site and you can see the PAM adjacent motif as well.

Evaluation of TALEN and CRISPR-Cas9 mediated DNA cleavage by a single strand annealing assay is being shown in picture 9. Here HEK293 cells were separately co-transfected with one of the pairs of TALENs or one of the pairs of CRISPR-Cas9 and pSSA-HBB-IVS2 and TK-Renilla.

After 48 hours of transfection, the ratio of firefly luciferase and Renilla luciferase activity was measured by a microplate reader. TALEN-blank vector and px330 blank vector were used as negative controls in this experiment and that represent the mean of plus minus SD of three independent experiments.

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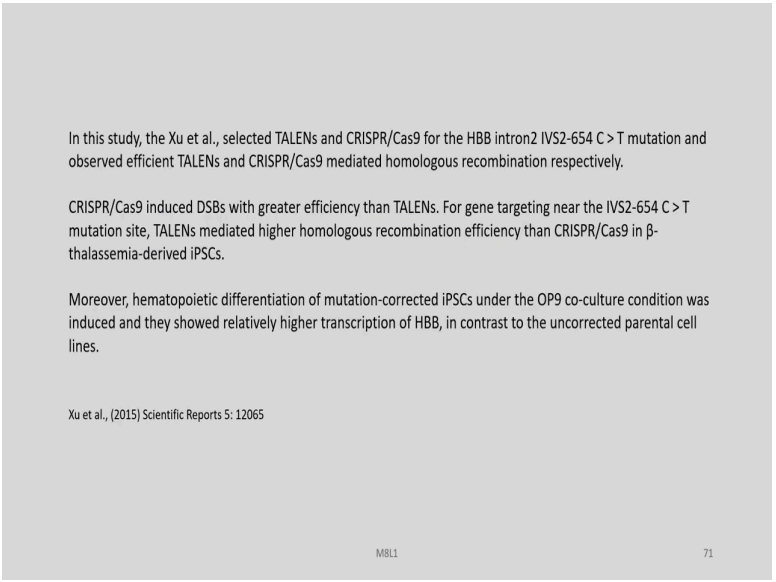

Throughout this study Xu et al., selected TALENs and CRISPR-Cas9 for the HBB intron 2 IVS2-654 C to T mutation and observed efficient TALENs and CRISPR-Cas9 mediated or homologous recombination are respectively. Crispr Cas9 induced double strand breaks with greater efficiency than TALENs. For gene targeting near the IVS2-654 C to T mutation site, TALENs mediated higher homologous recombination efficiency than CRISPR-Cas9 in beta-thalassemia-derived induced pluripotent stem cells.

Moreover, hematopoietic differentiation of mutation-corrected iPSCs under the OP9 co-culture condition was induced and they showed higher transcription of HBB, in contrast to the uncorrected parental cell lines. So, we see that the gene editing technologies has big scope in treating thalassemia through gene editing technologies.

(Refer Slide Time: 36:10)

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Thank you for your patient hearing. Here some of the references with the help of which this part of the lecture and this whole lecture was prepared.

Thank you.