Genome Editing and Engineering
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Module - 08
Applications of genome editing in treating human diseases
Lecture - 31
Human cell engineering in diseases: Severe combined immunodeficiency (SCID) -Part B

Welcome to my course on Genome Editing and Engineering. We are discussing about Severe Combined Immunodeficiency Disease.

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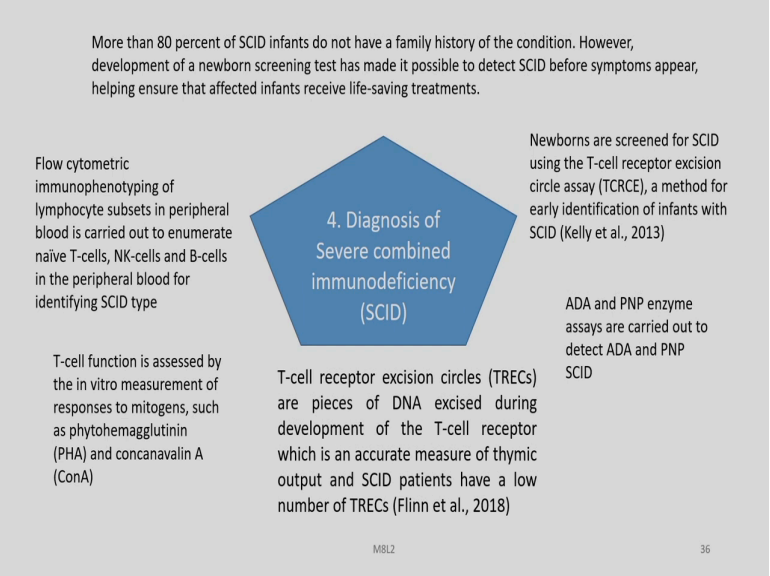

So, in this part, we are going to discuss about the diagnosis of severe combined immunodeficiency diseases. In general, more than 80 percent of SCID infants do not have a family history of the condition. However, development of a newborn screening test has made it possible to detect SCID before symptoms appear, helping ensure that affected infants receive a life-saving treatments.

Some of the existing diagnostic procedures for detecting SCID are flow cytometric immunophenotyping of lymphocyte subsets in peripheral blood, which is carried out to enumerate naive T-cells and NK-cells and B-cells in the peripheral blood of identifying the SCID type. On the other hand, newborns are screened for SCID using the T-cell receptor

excision circle assay or TCRCE, which is a method for early identification of infants having SCID.

Other diagnostic methods include ADA and PNP enzyme assays which are carried out to detect these particular enzymes. Then, we have T-cell receptor excision circles or TRECs. These are pieces of DNA excised during development of the T-cell receptor which is an accurate measure of thymic output and SCID patients have a low number of TRECs. Lastly, T-cell function is assessed by the in vitro measurement of responses to mitogens, such as phytohemagglutinin and concanvalin A.

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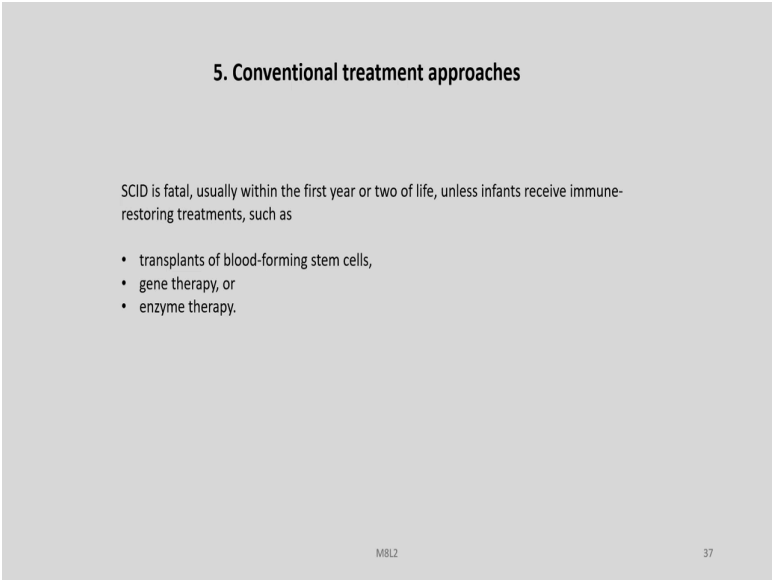

Now, let us discuss about the conventional treatment approaches for SCID. SCID is a fatal disease, usually within the first year or two of life, unless infants receive immuno restoring treatments which may be as follows. Transplants of blood-forming stem cells, gene therapy or enzyme therapy.

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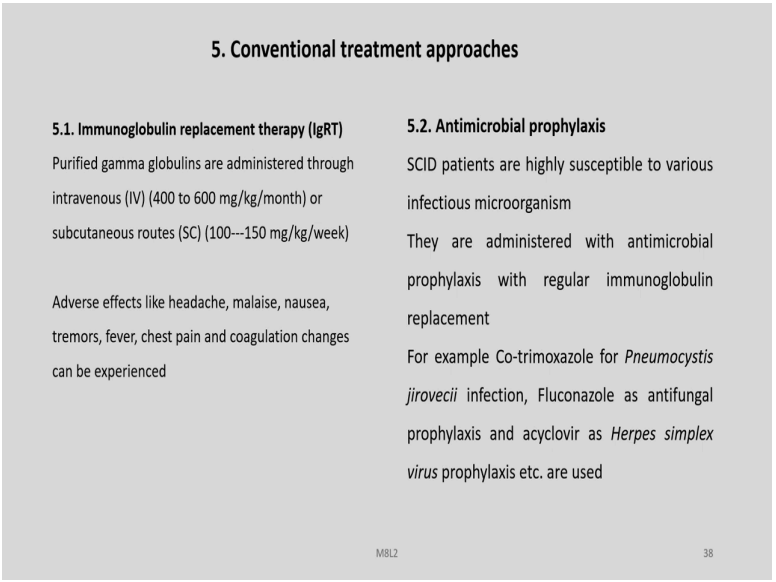

One of the conventional treatment approach is immunoglobulin replacement therapy, IgRT. Here purified gamma globulins are administered through intravenous or subcutaneous routes. However, there are certain adverse effects like headache, malaise, nausea, tremors, fever, chest pain and coagulation changes experienced by the patient.

Another is the antimicrobial prophylaxis; SCID patients are highly susceptible to various infectious microorganisms. They administered with antimicrobial prophylaxis with regular immunoglobulin replacement. For example, Co-trimoxazole for Pneumocystis infection, Fluconazole as antifungal prophylaxis and acyclovir as Herpes simplex virus prophylaxis are used.

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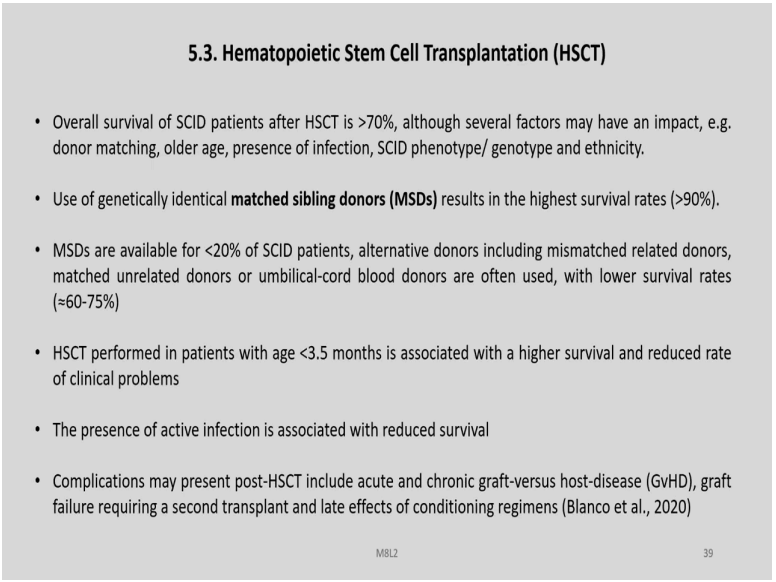

Another treatment approach is hematopoietic stem cell transplantation HSCT. Overall survival of SCID patients after receiving HSCT is about for 70 percent, although several factors may have an impact, example donor matching, older age, presence of infections, SCID phenotype, genotype and ethnicity.

Use of genetically identical matched sibling donors MSDs, results in the highest survival rates as high as 90 percent or more. MSDs are available for about 20 percent of SCID patients; alternative donors including mismatched related donors, matched unrelated donors or umbilical-cord blood donors are often used, with lower survival rates.

HSCT performed in patient with age around 3.5 months is associated with a higher survival and reduced rate of clinical problems. The presence of active infection is associated with reduced survival. Complications may present post-HSCT and include acute and chronic graft-versus host-disease, graft failure requiring a second transplant and late effects of conditioning regimens.

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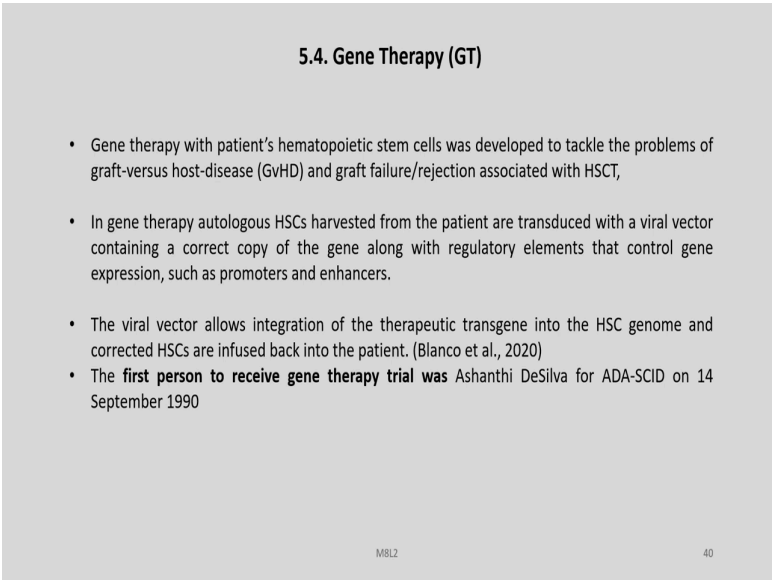

Gene therapy with patient's hematopoietic stem cells was developed to tackle the problems of graft-versus host disease and graft failure, rejection associated HSCT. In gene therapy autologous hematopoietic stem cells harvested from the patient are transduced with a viral vector containing a correct copy of the gene along with regulatory elements that control gene expressions, such as promoters and enhancers.

The viral vector allows integration of the therapeutic transgene into the hematopoietic stem cell genome and corrected HSCs are infused back into the patient. The first person to receive gene therapy trial was Ashanthi DeSilva for ADA-SCID on 14th September 1990.

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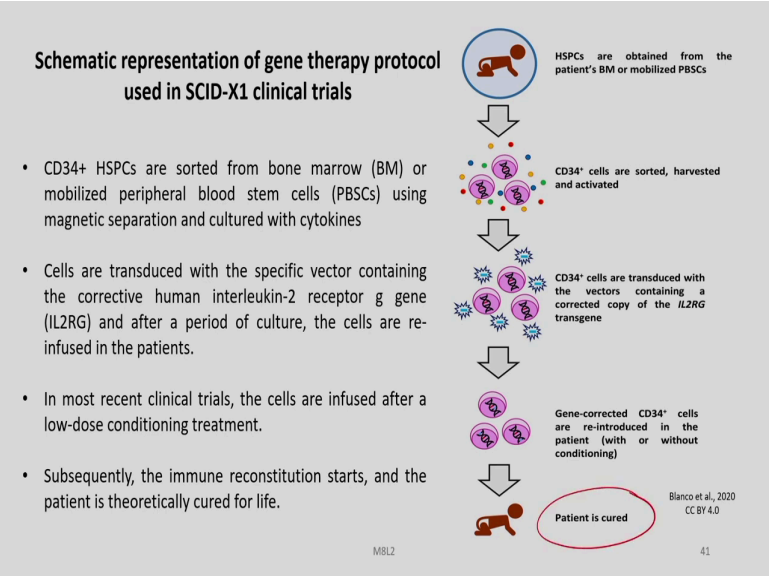

Here is the schematic for gene therapy protocol used in SCID-X1 clinical trials. So, here HSPCs are obtained from the patient's bone marrow or mobilized PBSCs, then CD34 plus cells are sorted, harvested and activated. So, CD34 HSPCs are sorted from bone marrow or mobilized peripheral blood stem cells using magnetic separation and cultured with cytokines.

The cells are transduces with the specific vector containing the corrective human interleukin-2 receptor g gene, IL2RG, and after a period of culture the cells are re-infused into the patient. In most recent clinical trials, the cells were infused after a low-dose conditioning treatment. Subsequently, the immune reconstitution starts, and the patient is theoretically cured for life.

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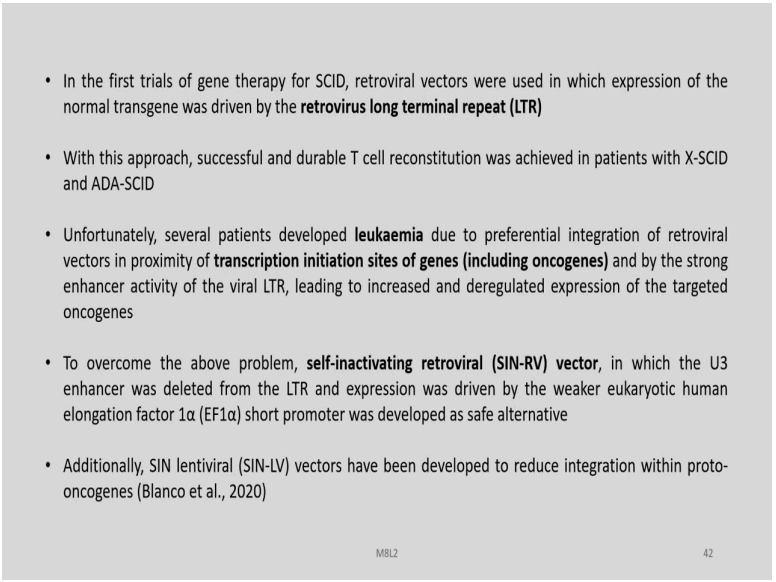

In the first trials of gene therapy for SCID, retroviral vectors were used in which expression of the normal transgene was driven by the retrovirus long terminal repeat or LTR. With this approach, successful and durable T-cell reconstitution was achieved in patients with X-SCID and ADA-SCID.

Unfortunately, several patients developed leukaemia due to preferential integration of retroviral vectors in proximity of transcription initiation sites of genes including oncogenes, and by the strong enhancer activity of the viral LTR, leading to increased and deregulated expression of the targeted oncogenes.

To overcome the above problem, self-inactivating retroviral vector SIN-RV, in which the U3 enhancer was deleted from the LTR and expression was driven by the weaker eukaryotic human elongation factor 1 alpha, EF1 alpha short promoter was developed as safe alternative. Additionally, SIN lentiviral vectors have been developed to reduce integration within proto oncogenes. Some viral vectors used in gene therapy of SCID-X1 patients.

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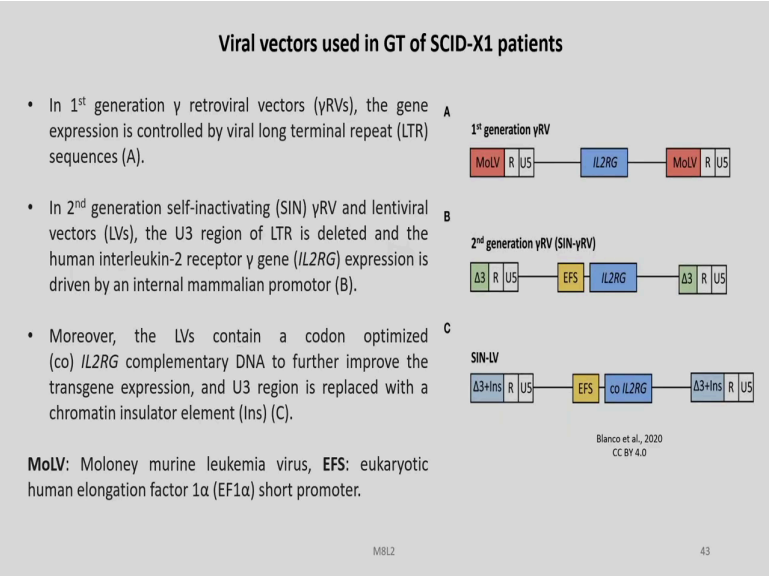

In first generation gamma retroviral vectors, gamma RVs, the gene expression is controlled by a long terminal repeat sequence. In second generation, self-inactivating gamma RV and lentiviral vectors, the U3 region of LTR is deleted and the human interleukin-2 receptor gamma gene IL2RG as shown in the figure expression is driven by an internal mammalian promoter.

Moreover, the LVs contain a codon optimized IL2RG complementary DNA to further improve the transgene expression, and U3 region is replaced with a chromatin insulator element called Ins. MoLV stands for maloney murine leukemia virus; EFS stands for eukaryotic human elongation factor 1 alpha short promoter, as shown in this picture by Blanco et al.

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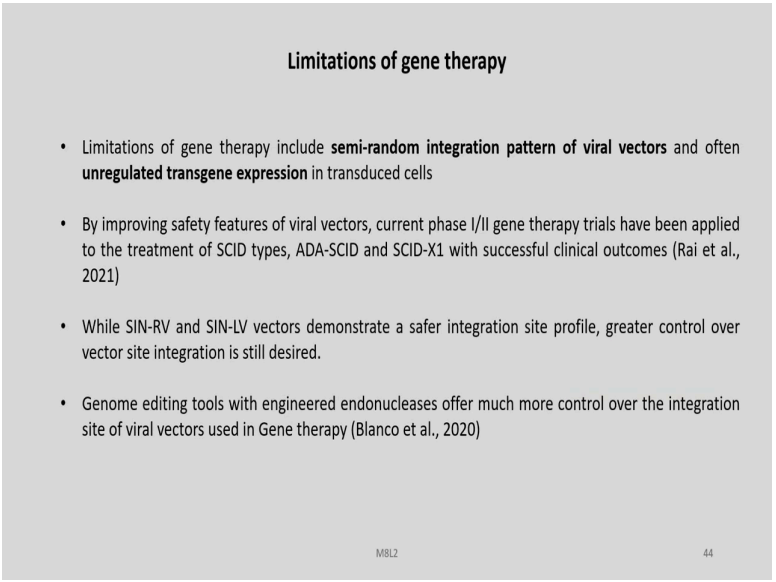

However, gene therapy has certain limitations. What are the limitations of gene therapy? They include semi-random integration pattern of viral vectors and often unregulated transgene expression in transduced cells. And by improving safety features of viral vectors, current phase 1, 2 gene therapy trials have been applied to the treatment of SCID types, ADA-SCID and SCID-X1 with successful clinical outcomes as reported by Rai et al.

While SIN-RV and SIN-LV vectors demonstrate a safer integration site profile, greater control over vector site integration is still desired. Genome editing tools with engineered endonucleases offer much more control over the integration site of viral vectors used in Gene therapy, as suggested by Blanco et al in 2020.

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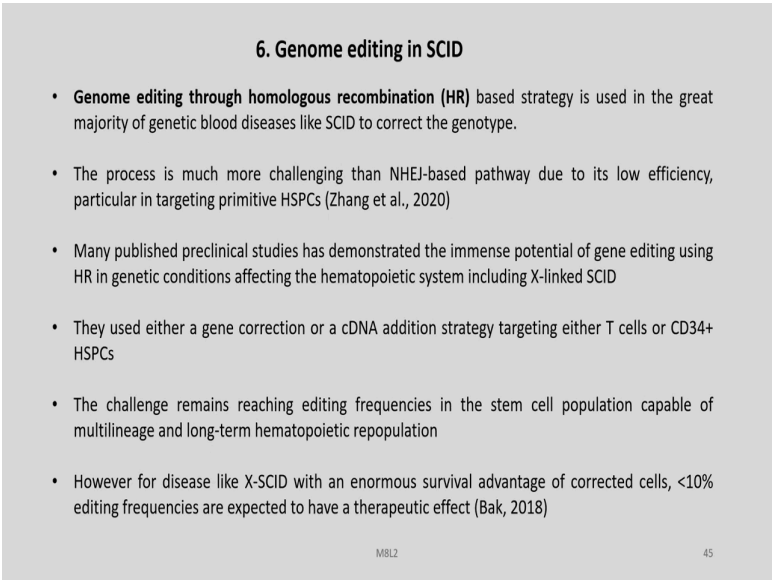

So, the latest therapeutic approach is the genome editing in SCID. Genome editing through homologous recombination based strategy, is used in the great majority of genetic blood diseases like SCID to correct the genotype. The process is much more challenging than NHEJ-based pathways due to its low efficiency, particularly in targeting primitive HSPCs.

Many published preclinical studies has demonstrated the immense potential of gene editing using homologous recombination in genetic conditions affecting the hematopoietic system including X-linked SCID. Researchers have used either a gene correction or a cDNA addition strategy targeting either T-cells or CD34 plus HCPCs.

The challenge however, remains in reaching editing frequencies in the stem cell population capable of multi-lineage and long-term hematopoietic repopulation. However, for diseases like X-SCID with an enormous survival advantage of corrected cells, more than 10 percent editing frequencies are expected to have a therapeutic effect.

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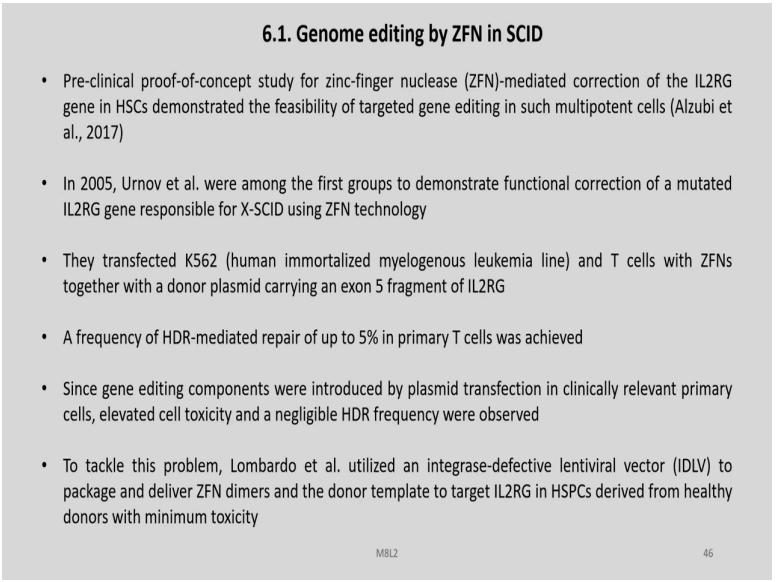

One of the gene editing technologies that is used in SCID is ZFNs. Here pre-clinical proof-of-concept involving ZFN mediated correction of the IL2RG gene in HSCs have been demonstrated and the feasibility of targeted gene editing in such multi-potent cells have been tried.

In 2005, Urnov et al were among the first groups to demonstrate functional correction of a mutated IL2RG gene responsible for X-SCID using ZFN technology platform. They transfected K562, human immortalized myelogenous leukaemia line, and T-cells with ZFNs together with a donor plasmid carrying an exon 5 fragment of IL2RG.

A frequency of HDR mediated repair of up to 5 percent in primary T-cells was achieved. Since, gene editing components were introduced by plasmid transfection in clinically relevant primary cells; elevated cell toxicity and a negligible HDR frequency were observed.

To tackle this problem, Lombardo et al utilized an integrase-defective lentiviral vector to package and deliver ZFN dimers and the donor template to target IL2RG in HSPCs derived from healthy donors with minimum toxicity.

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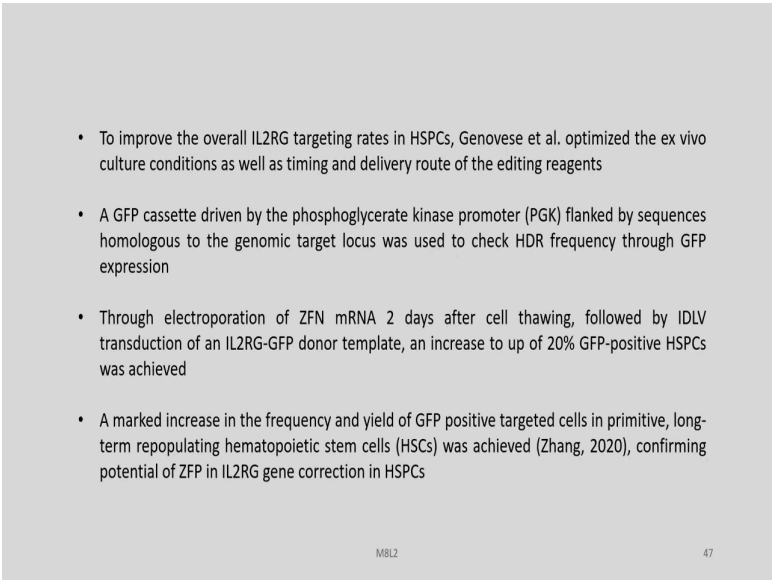

To improve the overall IL2RG targeting rates in HCPCs, Genovese et al has optimized the ex vivo culture conditions as well as timing and delivery route of the editing reagents. A GFP cassette driven by the phosphoglycerate kinase promoter flanked by sequences homologous to the genomic target locus was used to check HDR frequency through GFP expression.

Using electroporation ZFN mRNA were introduced 2 days after cell thawing followed by IDLV transduction of an IL2RG-GFP donor template, and an increase of up to 20 percent GFP-positive HSPCs was achieved.

There was a marked increase in the frequency and yield of GFP positive targeted cells in primitive, long-term repopulating hematopoietic stem cells as reported by Zhang. And it was confirmed there upon the potential of ZFP in IL2RG gene correction in the HCPCs.

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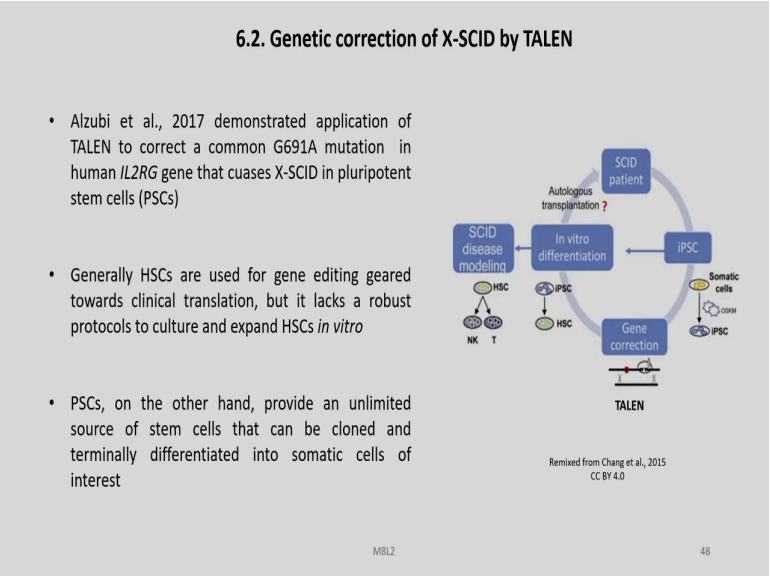

Let us now discuss a little bit about the genetic correction of X-SCID by TALEN, which is another promising genome editing technology. In 2017, Alzubi demonstrated the application of TALEN to correct a common G691A mutation in human IL2RG gene that causes X-SCID in pluripotent stem cells. Generally, hematopoietic stem cells are used for gene editing a geared towards clinical translation, but it lacks a robust protocol to culture and expand hematopoietic stem cells in vitro.

Pluripotent stem cells, on the other hand, provide an unlimited source of stem cells that can be cloned and terminally differentiated into somatic cells of interest. So, in this picture or illustration taken from Chang et, al you can see the schematics of the SCID disease modeling. And the SCID patient from which the cells has been obtained, and induced pluripotent stem cells have been developed and gene correction being done with the help of TALEN, gene editing technologies. And these are reintroduced into the SCID patients.

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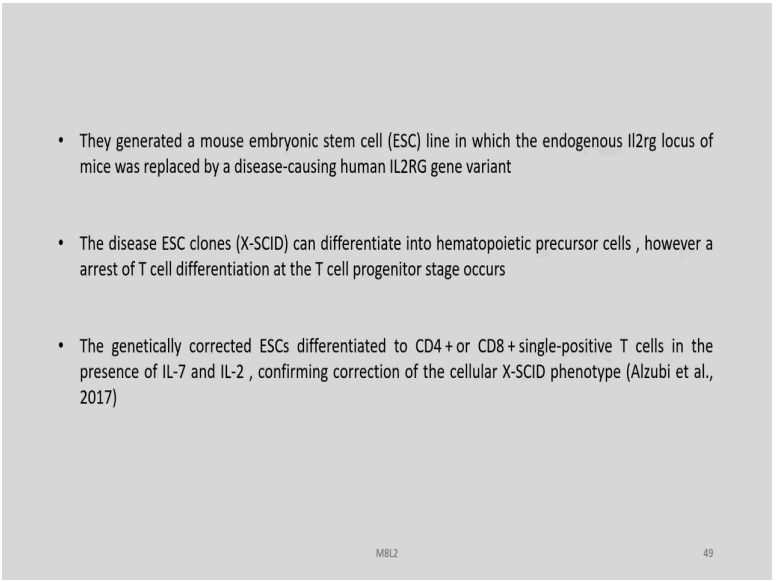

In this case, the researchers generated a mouse embryonic stem cell line in which the endogenous 2Il2rg locus of mice was replaced by disease-causing human IL2RG gene variant. The disease ESC clones X-SCID can differentiate into hematopoietic precursor cells.

However, arrest of T-cell differentiation at the T-cell a progenitor stage occurs. The genetically corrected ESCs differentiated to CD4 plus or CD8 plus single positive T-cells in the presence of IL-7 and IL-2, confirming correction of the cellular X-SCID phenotype.

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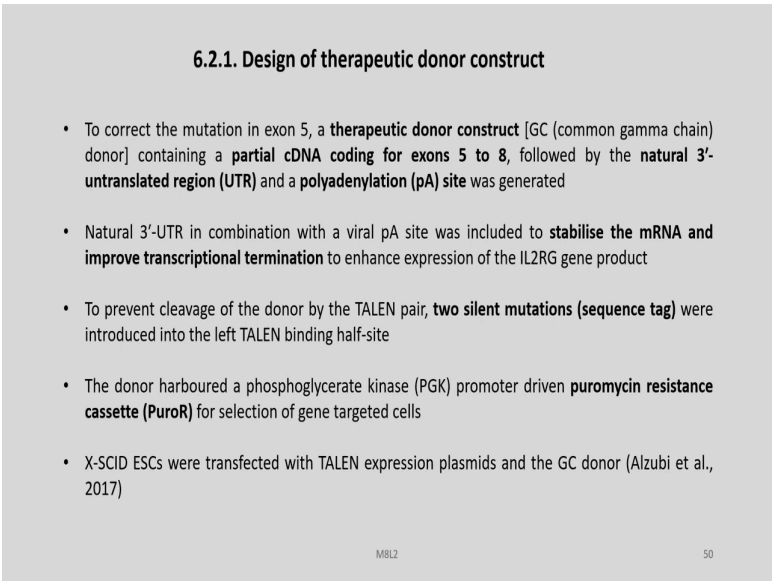

It is important to design the therapeutic donor construct. So, for correcting the mutation in exon 5, a therapeutic donor construct GC common gamma chain, containing a partial cDNA coding for exons 5 to 8 followed by the natural 3 prime untranslated region UTR and a polyadenylation site was generated.

Natural 3 prime UTR in combination with a viral pA site was included to stabilize the mRNA and improve transcriptional termination to enhance expression of the IL2RG gene product. To prevent cleavage of the donor by the TALEN pair, two silent mutations were introduced into the left TALEN binding half-site.

The donor harboured a phosphoglycerate kinase promoter driven puromycin resistance cassette for selection of gene targeted cells. X-SCID ESCs were transfected with TALEN expression plasmids and GC donor, as reported by Alzubi et al in 2017.

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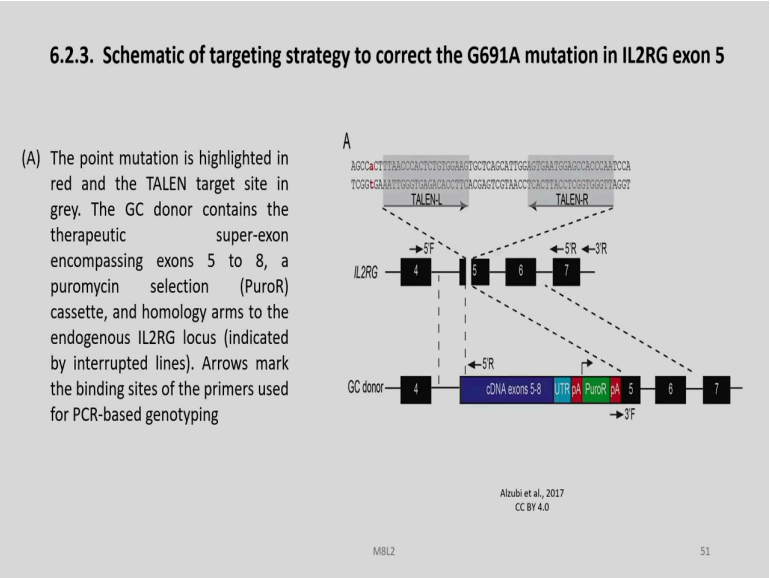

Here is a schematic of targeting strategy to correct the G691A mutation IL2RG exon 5. So, here is the left TALEN and this is the right TALEN, and this is the IL2RG with different exons including exon 5, as you can see in this diagram. And this is the GC donor. And the point mutation here is highlighted in red in this figure over here.

The GC donor contains the therapeutic super exons, encompassing exons 5 to 8, a puromycin selection PuroR cassette. And homology arms to the endogenous IL2RG locus indicated by

the interrupted lines. Arrows mark the binding sites of the primers used for PCR-based genotyping.

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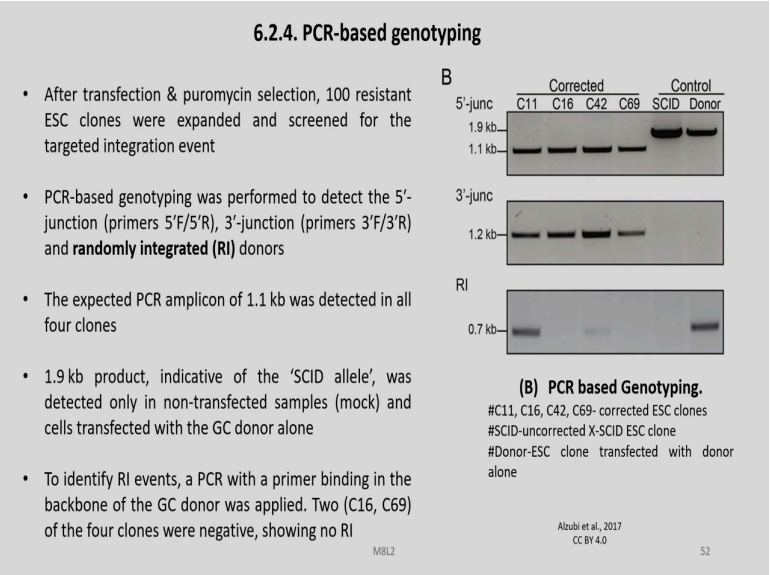

So, in a PCR-based genotyping, after transfection and puromycin selection, 100 resistant ESC clones were expanded and screened for targeted integration event. PCR-based genotyping was performed to detect the 5 prime junction, 3 prime junction and randomly integrated donors. The expected PCR amplicon of 1.15 kb was detected in all the 4 clones.

1.9 kb product indicative of the SCID allele was detected only in non-transected samples or the mock samples and cells transfected with the GC donor clone alone. To identify RI events, a PCR with a primer binding in the backbone of the GC donor was applied. Two, C16, C69 of the 4 clones were negative, showing no RI.

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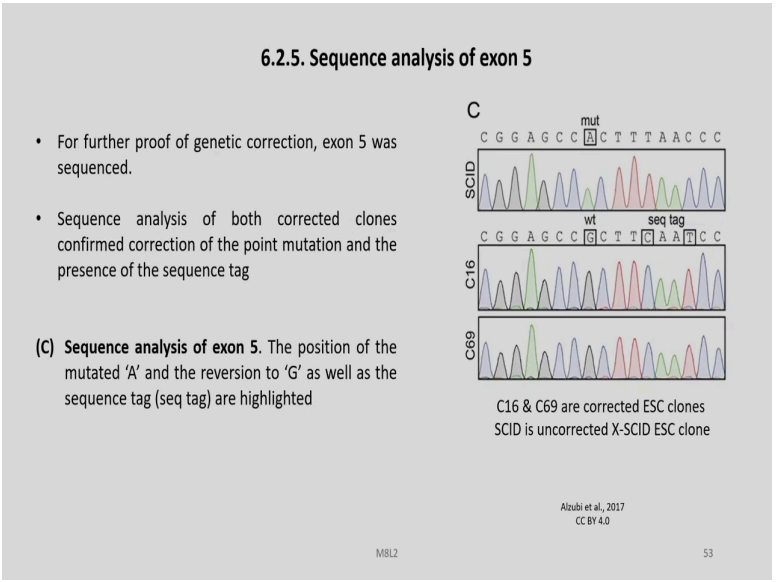

Further, sequence analysis of the exon 5 was done, as a proof of genetic correction. Sequence analysis of both corrected clones confirmed correction of the point mutation and the presence of the sequence tag. And in this figure C, you can see the sequence analysis of exon 5. The position of the mutated 'A' and the reversion to 'G' as well as a sequence tag seq tag are highlighted in this figure.

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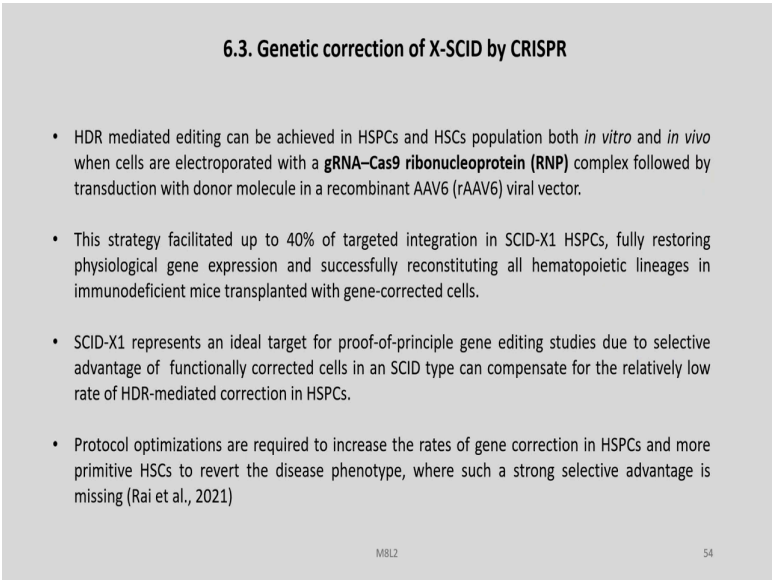

Genetic correction of X-SCID by CRISPR genome editing technology is also now a highly developed method apart from the editing by ZFN and TALEN technologies. Here HDR

mediated editing is achieved in HSPCs and HSCs population both in vitro and in vivo, and with the help of electroporation with a gRNA-Cas9 ribonucleoproteins complex followed by transduction with donor molecules in a recombinant AAV6 viral vector.

In this strategy up to 40 percent of targeted integration of SCID-X1 HCPCs were observed, fully restoring physiological gene expression and successfully reconstituting all hematopoietic lineages in immunodeficient mice transplanted with gene-corrected cells.

SCID-X1 represents an ideal target for proof-of-principle gene editing studies due to selective advantage of functionally corrected cells in an SCID type can compensate for the relatively low rate of HDR-mediated in HSPCs. Protocol optimizations are required to increase the rates of gene correction in HSPCs and more primitive HSCs to revert the disease phenotype, where such a strong selective advantage is absent.

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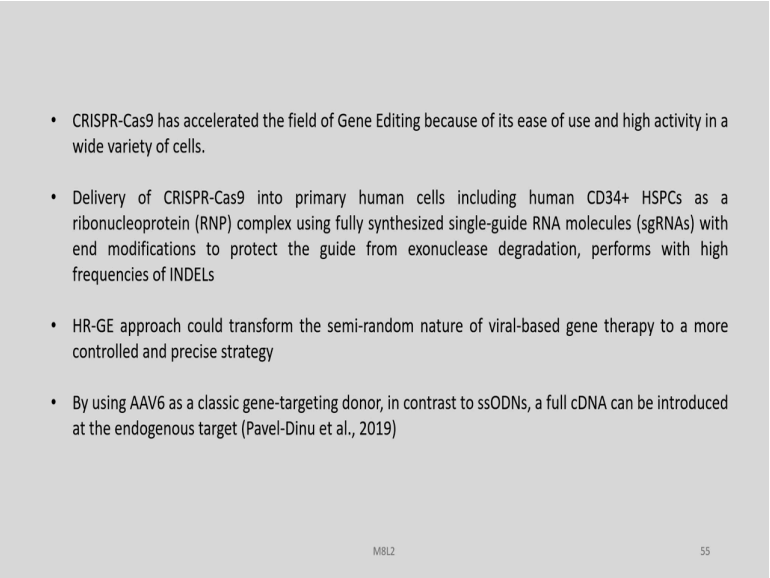

CRISPR-Cas9 has accelerated the field of Gene Editing because of its ease of use and high activity in a wide variety of cells, and also in the application of Gene Therapy. Delivery of CRISPR-Cas9 into primary human cells include human CD34 plus HSPCs as a ribonucleoprotein complex using fully synthesized single-guide RNA molecules with end modifications to protect the guide from exonucleaus degradation, performs with high frequencies of INDELs.

HR-GE approach could transform the semi-random nature of viral-based gene therapy to a more controlled and precise strategy. By using AAV6 as a classic gene-targeting donor in contrast to ssODNs, a full cDNA can be introduced at the endogenous target.

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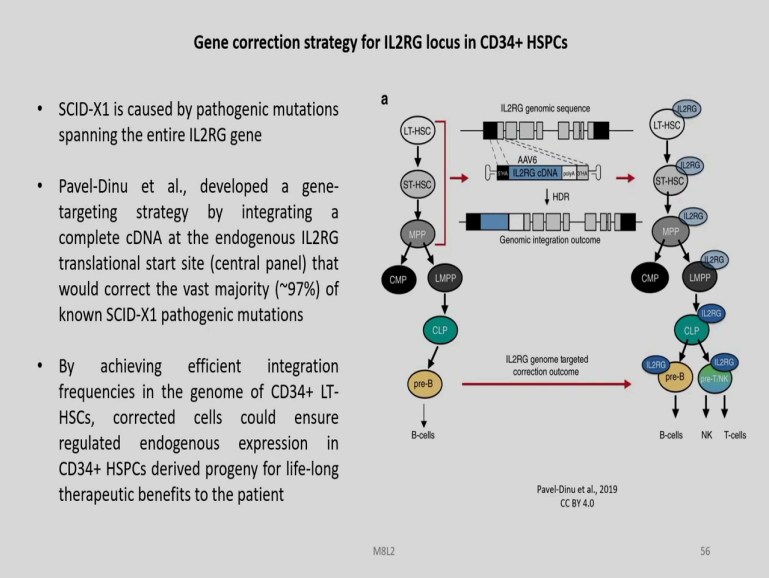

In the schematic, you can see the gene correction strategy for IL2RG locus in CD34 plus HSPCs. SCID-X1 is caused by pathogenic mutations spanning the entire IL2RGene, which we have discussed now and then in this lecture. Pavel-Dinu et al developed the gene-targeting strategy by integrating a complete cDNA at the endogenous IL2RG translational start site.

You can see these in the central panel that would correct the vast majority of over 97 percentage of known SCID-X1 pathogenic mutations. By achieving efficient integration frequencies in the genome of CD34 plus LT-HSCs, corrected cells could ensure related endogenous expression in CD34 plus HSPCs derived progeny for lifelong therapeutic benefits to the patients.

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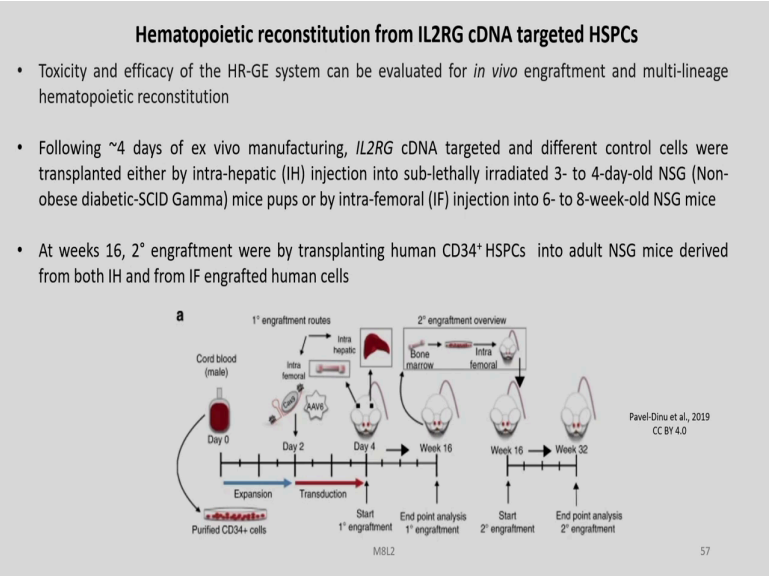

Hematopoietic reconstitution from IL2RG cDNA targeted HSPCs. The toxicity and efficacy of the HR-GE system can be evaluated for in vivo engraftment and multi-lineage hematopoietic reconstitution. Following around 4 days of ex vivo manufacturing, IL2RG cDNA targeted and different control cells were transplanted either by intra-hepatic injection into sub-lethally irradiated 3 to 4 days old NSG, non-obese diabetic SCID gamma, mice pups or intra-femoral injection into 6 to 8 week old NSG mice.

At week number 16, which is the end point analysis of primary engraftment, secondary engraftment were done by transplanting human CD34 plus HSPCs into adult NSG mice derived from both IH and from IF engrafted human cells.

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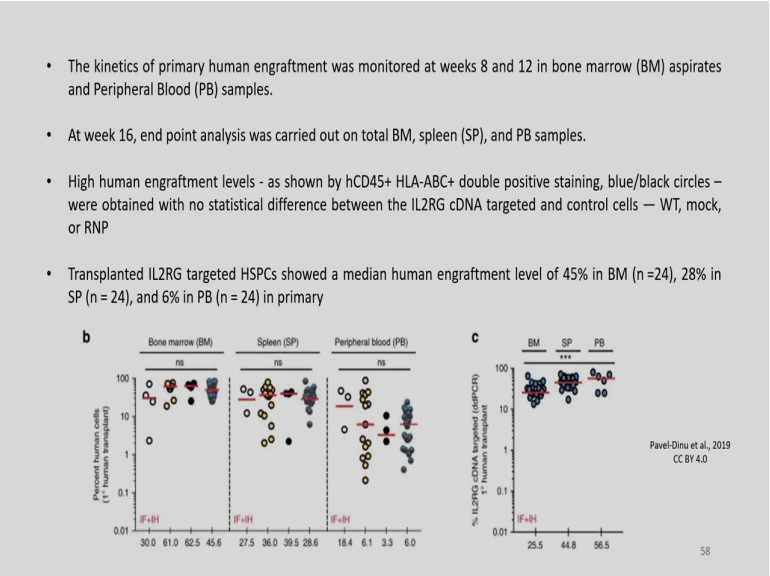

The kinetics of primary human engraftment was monitored at week number 8 and 12 in bone marrow aspirates and Peripheral Blood samples. At week 16, end point analysis was carried out on total bone marrow, spleen, and peripheral blood samples. High human engraftment levels as shown by hCD45 plus HLA minus ABC plus double positive staining, blue black circles were obtained with no statistical difference between the IL2RG cDNA targeted and control our cells which are wild-type, mock, and RNP or RNP.

Transplanted IL2RG targeted HSPCs showed a median human engraftment level of 45 percent in BM, 28 percent in SP and 6 percent in peripheral blood samples.

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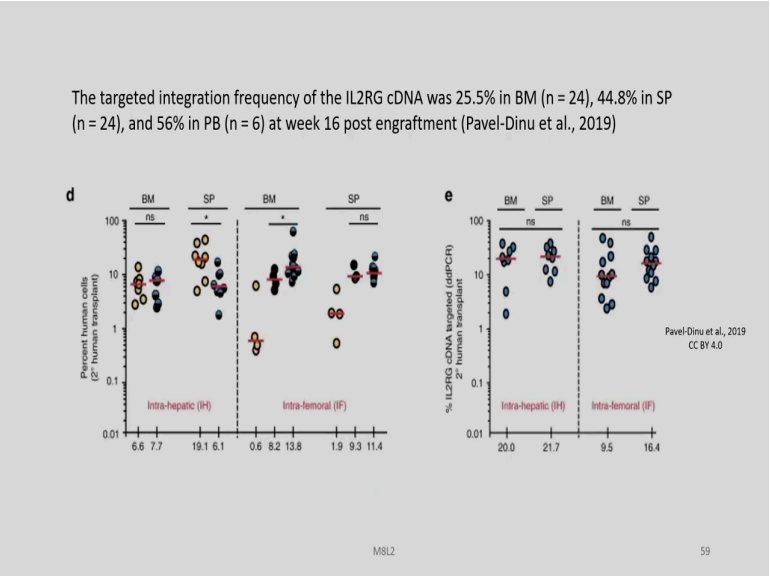

The targeted integration frequency of the IL2RG cDNA was 25.5 percent in BM, 44.8 percent in SP and 56 percent in PB at week 16 post engraftment, as reported by Pavel-Dinu.

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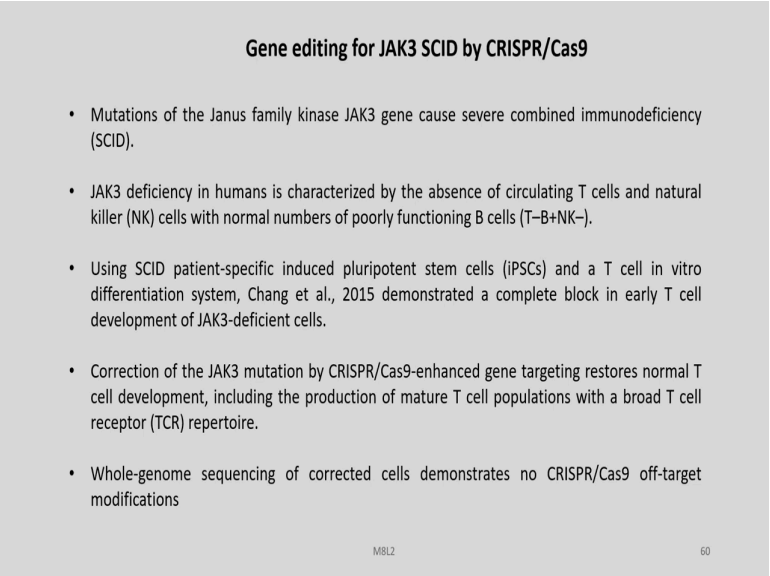

Let us now discuss about gene editing for JAK3 SCID by CRISPR-Cas9. Mutations of the Janus family kinase gene cause severe combined immunodeficiency syndrome. JAK3 deficiency in humans is characterized by the absence of circulating T-cells and natural killer cells with normal numbers of poorly functioning B-cells.

Using SCID patient-specific induced pluripotent stem cells and a T-cell in vitro differentiation system, Chang et al, demonstrated a complete block in early T-cell development of JAK3 deficient cells. Correction of the JAK3 mutation by CRISPR-Cas9 enhanced gene targeting restores normal T-cell development, including the production of mature T-cell populations with a broad T-cell receptor repertoire. Whole-genome sequencing of corrected cells demonstrates no CRISPR-Cas9 off-target modifications and is considered as an advantage in this case.

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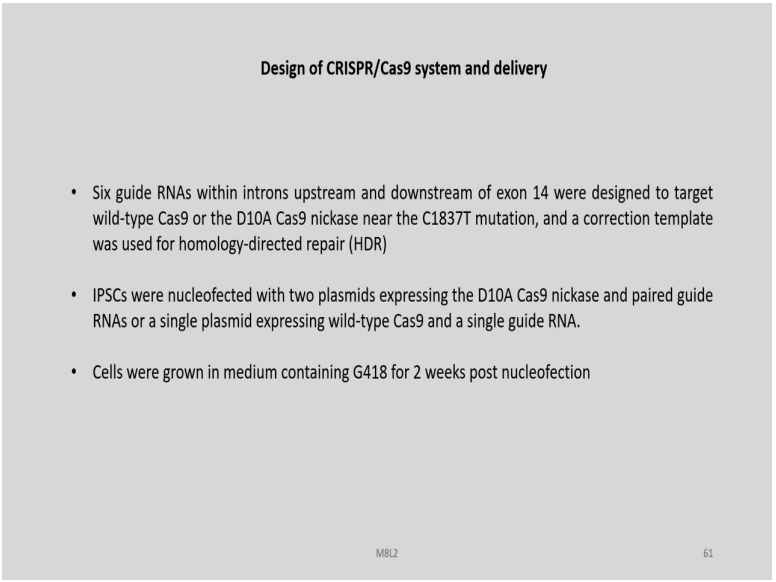

Design of the CRISPR-Cas9 system and delivery: Six guide RNAs with introns upstream and downstream of exon 14 were designed to target wild-type Cas9 or D10A Cas9 nickase nickase near the (Refer Time: 28:40) design of CRISPR-Cas9 system and delivery.

Six guide RNAs with introns upstream and downstream of exon 14 were designed to target wild-type Cas9 or the D10A Cas9 nickase near the C1837T mutation, and a correction template was used for homology directed repair.

IPSCs were nucleofected with two plasmids expressing the D10A Cas9 nickase and paired guide RNAs or a single plasmid expressing wild-type Cas9 in a single guide RNA. Cells were grown in medium containing G418 for 2 weeks post nucleofection.

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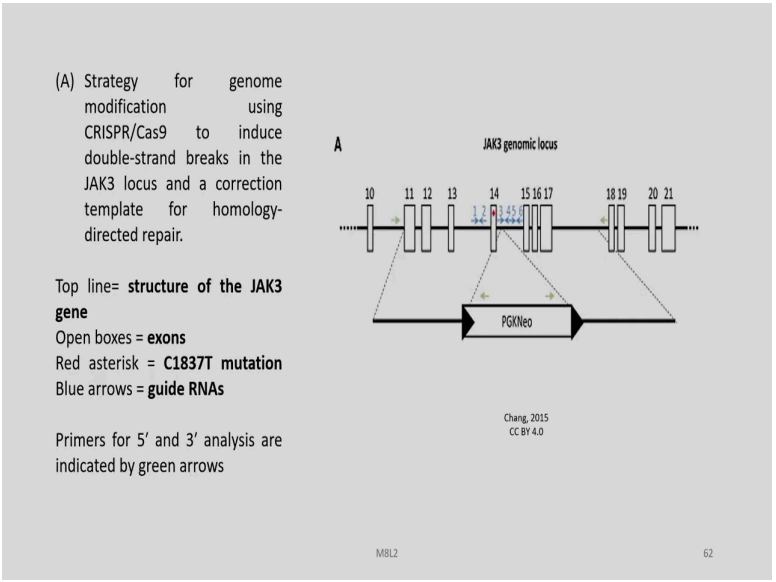

A strategy for genome modification using CRISPR-Cas9 to induce double-strand breaks in the JAK3 locus and a correction template for homology directed repair, as shown in this diagram. In the top line, we can see structure of the JAK3 gene, where the open boxes represents the various exons, and red asterisks is the C1837T mutation. The blue arrows shows the guide RNAs. And the primers for 5 prime and 3 prime analysis are indicated by the green arrows in these diagram.

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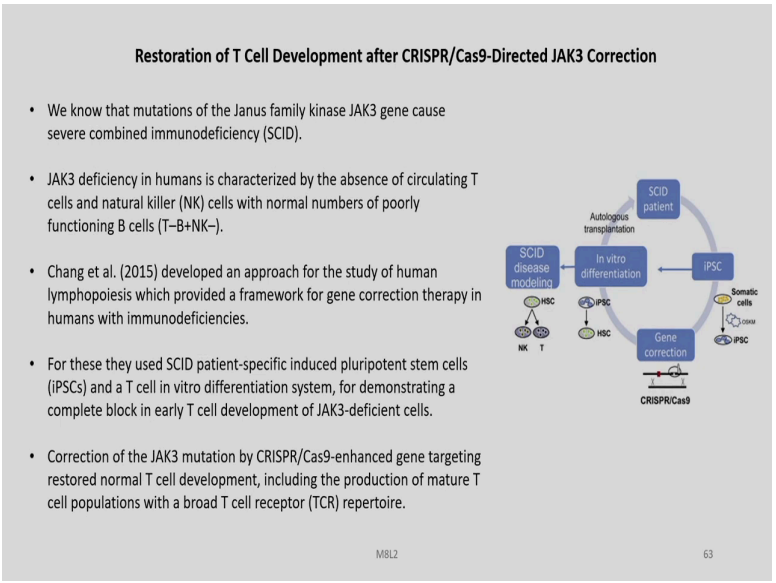

Restoration of T-cell development after CRISPR-Cas9 directed JAK3 correction. It is known to you that mutations of the Janus family kinase gene causes severe combined immunodeficiency disease or syndrome. JAK3 deficiency in humans is characterized by the absence of circulating T-cells and natural killer cells which are normal numbers of poorly functioning B-cells.

Chang et al developed an approach for the study of human lymphopoiesis which provided a framework for gene correction therapy in humans with immunodeficiencies. For these they used SCID patient-specific induced pluripotent stem cells and a T-cell in vitro differentiation system, for differentiating a complete block in early T-cell development of JAK3 deficient cells.

Correction of the JAK3 mutation by CRISPR-Cas9 enhanced gene targeting restored normal T-cell development, including production of mature T-cells populations with a broad T-cell receptor repertoire.

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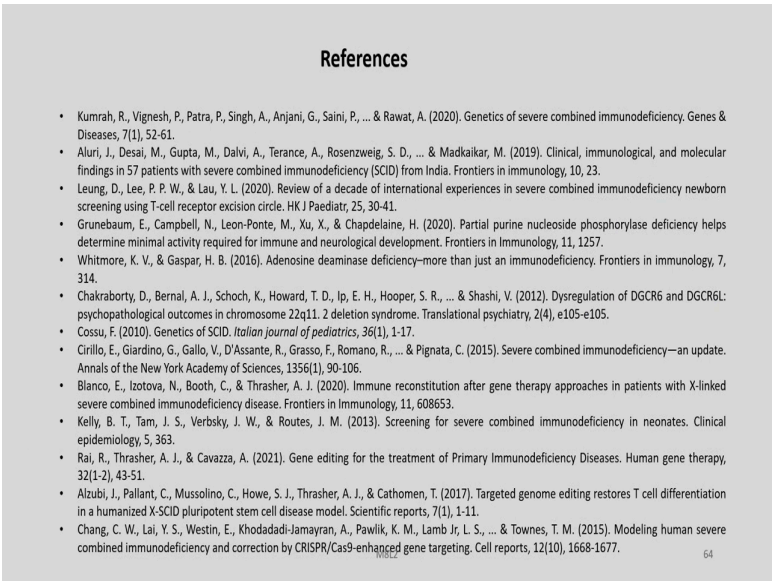

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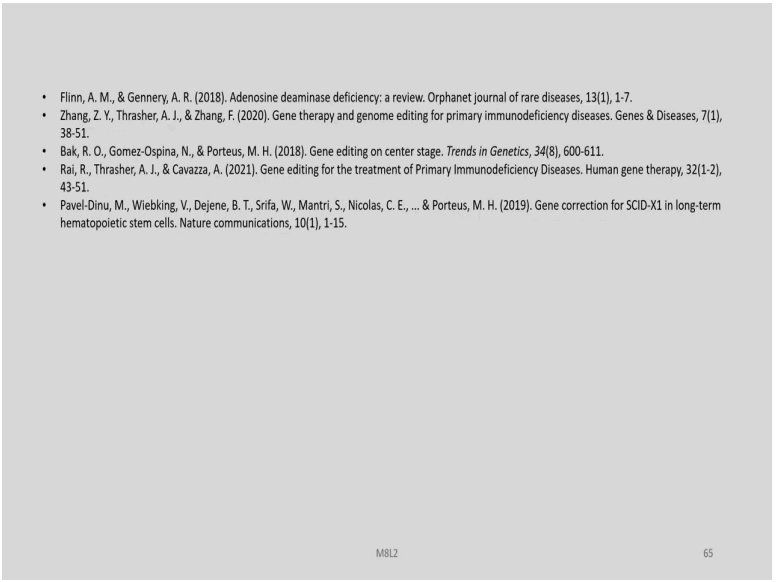

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Though, see these are some of the references, which were consulted for preparing this lecture. And for details of the various techniques, and also some of the genome editing used for SCID therapy can be found in the literacy cited over here.

Thank you for your kind attention.