Genome Editing and Engineering
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Module - 09
Genome Engineered Disease Modelling
Lecture - 02
IPSc models - Part B

Welcome to my course on genome editing and engineering we are discussing about IPSc models currently. So, now, today we are going to discuss about the ZFNs and how they are used in creating IPSc models.

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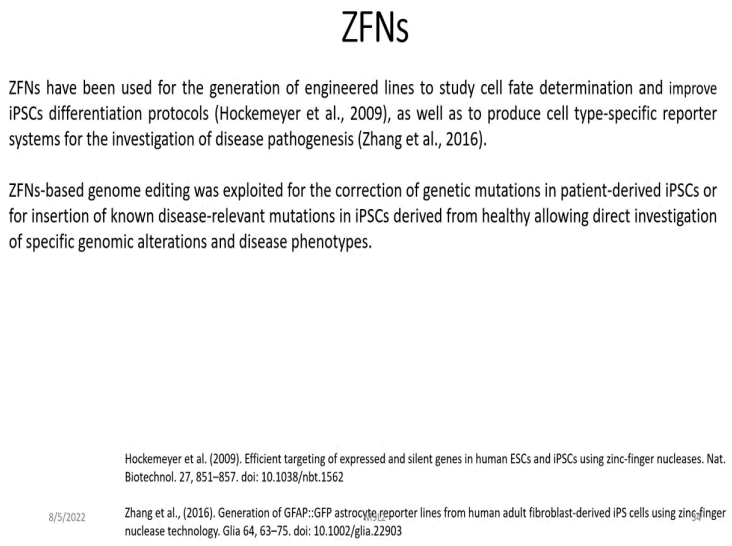

ZFNs have been used for generation of engineered lines to study cell fate determination and improve iPSCs differentiation protocols. As well as to produce cell type specific reporter systems for the investigation of disease pathogenesis. ZFN based genome editing was exploited for the correction of genetic mutation in patient derived iPSCs or for insertion of known disease relevant mutations in iPSCs derived from healthy patients allowing direct investigation of specific genomic alterations and disease phenotypes.

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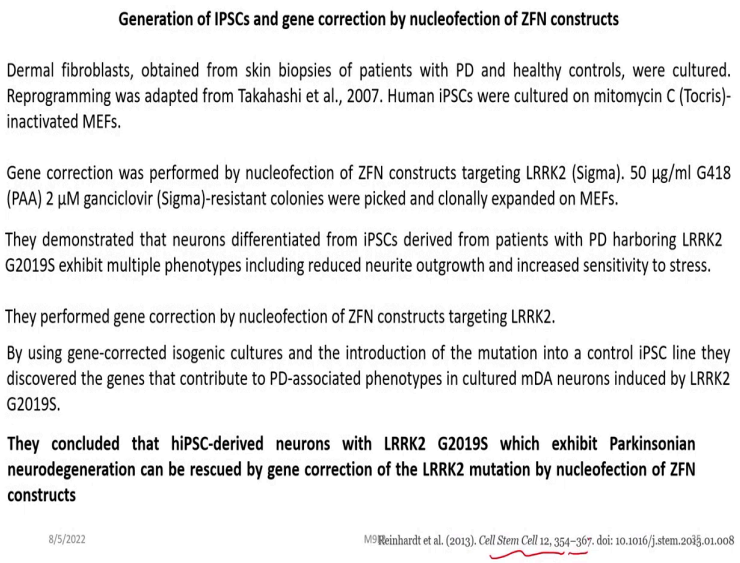

The generation of iPSCs and gene correction by nucleofection of ZFN constructs. Dermal fibroblasts, obtained from skin biopsies of patients with a PD and healthy control were cultured, reprogramming was adapted from Takahashi et al. Human iPSCs were cultured on mitomycin C inactivated MEFs.

Gene correction was performed by nucleofection by which ZFN constructs targeting LRKK 2. They demonstrated that neurons differentiated from iPSCs derived from patients with Parkinson's disease harboring LRKK 2, G2019S exhibit multiple phenotypes including reduced neurite outgrowth and increased sensitivity to stress.

They performed the gene correction by nucleofection of ZFN constructs targeting this LRKK 2 gene. By using gene corrected isogenic cultures and the introduction of the mutation into a control iPSC line, they discovered the genes that contribute to Parkinson's disease associated phenotypes in cultured mDA in neurons induced by LRKK2 G2019S.

And after all these probing, they concluded that human induced pluripotent stem cell derived neurons with LRKK G2019S which exhibit parkinsonian neuron neuro degeneration can be rescued by gene correction of the LRKK2 mutation by nucleofection of ZFN constructs. For a detailed methods and procedure for conducting these experiment, you may refer to cell stem cell 12 page number 354.

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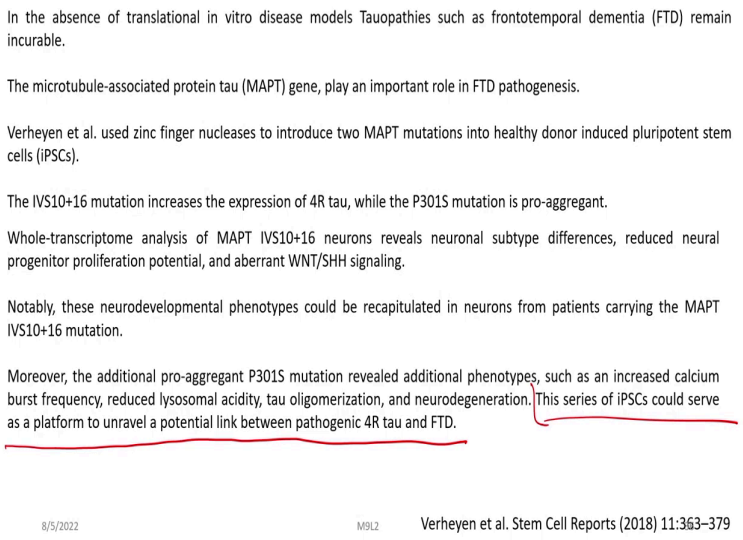

In the absence of translational in vitro disease models Tauopathies such as frontotemporal dementia remain incurable, the microtubule associated protein tau or MAPT gene plays an important role in these frontotemporal dementia pathogenesis. Verheyen has used zinc finger nucleus is to introduce two MAPT mutations into healthy donor induced pluripotent stem cells iPSCs. The IVS 10 plus 16 mutation increases the expression of 4R tau while the P301S mutation is pro-aggregant.

Whole transcriptome analysis of MAPT IVS 10 plus 16 neurons reveals neuronal subtype differences, reduce neural progenitor proliferation potential and aberrant WNT SHS signaling. Notably, these neurodevelopmental phenotypes could be recapitulated in neurons from patients carrying the MAPT mutation.

Over the additional pro-aggregant P301S mutation revealed additional phenotypes such as an increased calcium burst frequency, reduced lysosomal activity, tau oligomerization, and neurodegeneration. This series of iPSCs could serve as a platform to unravel a potential link between the pathogenic 4R tau and FTD and this is an important development for a disease which is incurable as of now.

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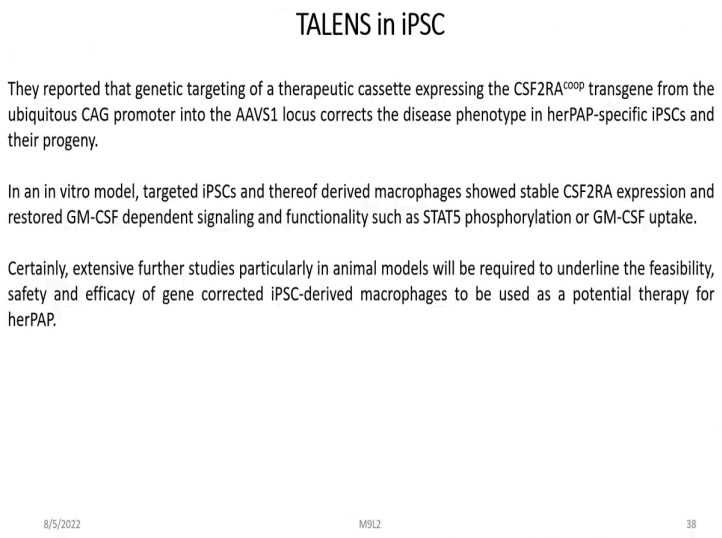

Let us now discuss about a TALENs in iPSc, the genetic targeting of a therapeutic cassette expressing the CSF2RA coop transgene from the ubiquitous CAG promoter into the AAVS1 locus corrects the disease phenotype in herPAP-specific iPSCs and their progeny.

In an in vitro model targeted iPSCs and thereof derived macrophages showed stable CSF2RA expression and restored GM-CSF dependent signaling and functionality such as STAT5 phosphorylation or GM-CSF uptake. Certainly, extensive studies further particularly in animal models will be required to underline the feasibility, safety, and efficacy of gene corrected iPSCs derived macrophages to be used as a potential therapy for a herPAP.

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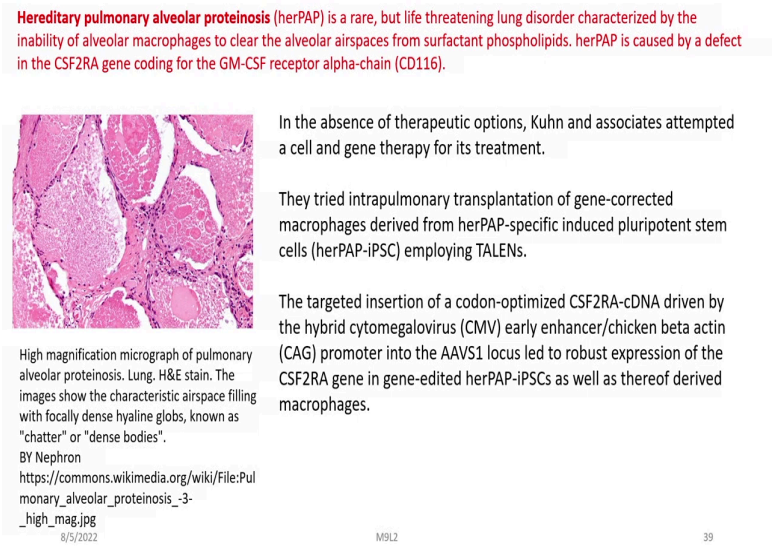

So, herPAP or hereditary pulmonary alveolar proteinosis is a rare, but life-threatening lung disorder which is characterized by the inability of alveolar macrophage to clear the alveolar airspaces from surfactant and phospholipids. HerPAP is caused by a defect in the CSF2RA gene coding for the GM-CSF receptor alpha chain or CD116.

So, here you can see high magnification micrograph of pulmonary alveolar proteinosis. The images show the characteristics airspace filling with focally dense hyaline globs known as chatter or dense bodies. In the absence of therapeutic options, Kuhn and associates attempted a cell and gene therapy for its treatment. They tried intrapulmonary transplantation of gene corrected macrophages derived from herPAP specific induced pluripotent stem cells employing TALENs.

The targeted insertion of a codon optimized CSF2RS-cDNA driven by the hybrid cytomegalovirus CMV early enhancer chicken beta actin CAG promoter into the AAVS1 locus led to robust expression of the CSF2RA gene, in gene edited herPAP iPSCs as well as thereof derived macrophages.

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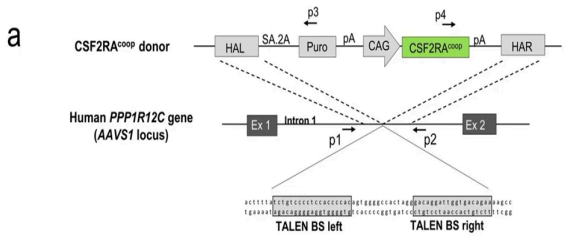

Generation and genotyping of gene edited PAP patient-derived iPSCs. (a) Scheme of the AAVS1 target site and the CSF2RAcoop donor plasmid. The donor plasmid is used as template for homologous recombination at the intended target site. The puromycin selection cassette and the CAG-driven CSF2RA coop gene are flanked by AAVS1-specific homology arms. The AAVS1-specifc TALEN binding sites are located in intron 1 of the PPP1R12C gene (AAVS1 locus).

Kuhn et al. Scientific Reports (Sci Rep) Article number: 15195 (2017) ISSN 2045-2322 (online)

So, here is the schematic for the generation and genotyping of gene edited PAP patient derived iPSCs. You can see the scheme of the AAVS1 target site and the CSF2RA coop donor plasmid. The donor plasmid is used as template for homologous recombination at the intended target side. The puromycin selection cassette and the CAG-driven CSF2RA coop genes are flanked by AAVS1 specific homology arms. The AAVS1 specific TALEN binding sites are located in intron 1 of the PPP1R12C gene or AAVS1 locus.

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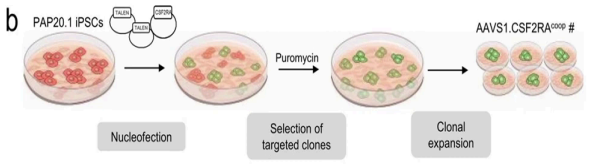

(b) Experimental scheme for the generation, selection and expansion of clones with CSF2RA coop integration within the AAVS1 locus of herPAP iPSCs.

Briefly this study provided a proof-of-concept, that TALEN-mediated integration of the CSF2RA gene into the AAVS1 safe harbor locus in patient-specific iPSCs represents an efficient strategy to generate functionally corrected monocytes/macrophages, which in the future may serve as a source for an autologous cell-based gene therapy for the treatment of herPAP.

Kuhn et al. Scientific Reports (Sci Rep) Article number: 15195 (2017) ISSN 2045-2322 (online)

In b we can see the experimental scheme for the generation, selection and expansion of clones with CSF2RA coop integration within the AAVS1 locus of herPAP iPSCs. Briefly this study provided a proof of concept that TALEN mediated integration of the CSF2RA gene into the AAVS1 safe harbor locus. In patient specific iPSCs represent an efficient strategy to generate functionally corrected monocytes or macrophages which in the future may serve as a source for on autologous cell based gene therapy for the treatment of herPAP.

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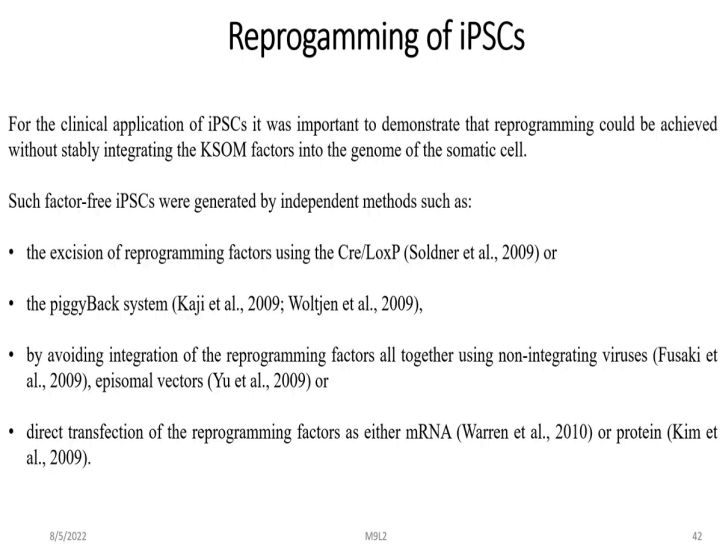

Reprogramming of iPSCs, for the clinical application of iPSCs it was important to demonstrate that reprogramming could be achieved without stably integrating the KSOM factors into the genome of the somatic cell. Such factor free iPSCs were generated by independent methods such as the excision of programming factors using the Cre/LoxP, the piggyBack system by avoiding integration of the programming factors all together using non integrating viruses and direct transfection of the reprogramming factors as either mRNA.

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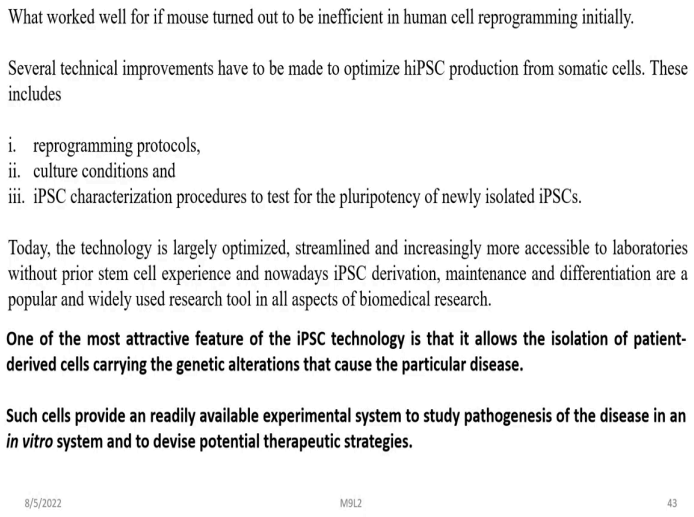

What worked well for a mouse however turned out to be inefficient in human cell reprogramming initially. Several technical improvements have to be made to optimize human induced pluripotent stem cell production from somatic cells. This included reprogramming protocols, culture conditions, and iPSC characterization procedures to test for the pluripotency of newly isolated iPSCs.

Today the technology is largely optimized, streamlined and increasingly more accessible to laboratories without prior stem cell experience and nowadays iPSC derivation, maintenance and differentiation are a popular and widely used research tool in all aspects of biomedical research.

One of the most attractive features of the iPSC technology is that, it allows the isolation of patient derived cells carrying the genetic alterations that cause the particular disease. Such cells provide a readily available experimental system to study pathogenesis of the disease in an in vitro system and to devise potential therapeutic strategies.

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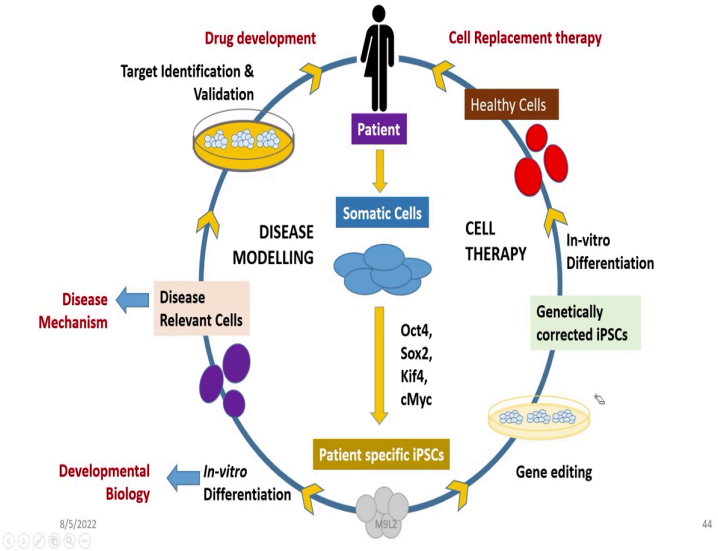

So, in this picture we can see a patient from which the somatic cells are isolated and then these are reprogrammed using the OSKM factors; and thereby, we can get patient specific induced pluripotent stem cell. This may be either taken for in-vitro differentiation and used for developmental biology studies or for disease relevant cells for understanding the disease mechanisms.

And used further for target identification and validation in drug development and the knowledge would be applied finally on the patient. On the left-hand side, you can see that these patient specific iPSCs can be used for gene editing.

And then we may genetically correct the iPSCs and go for in-vitro differentiation and generate healthy cells which can be used for cell replacement therapy. So, in either case we have a huge potential of iPSCs in association with CRISPR Cas9 technologies, we may also develop animal models using these technologies.

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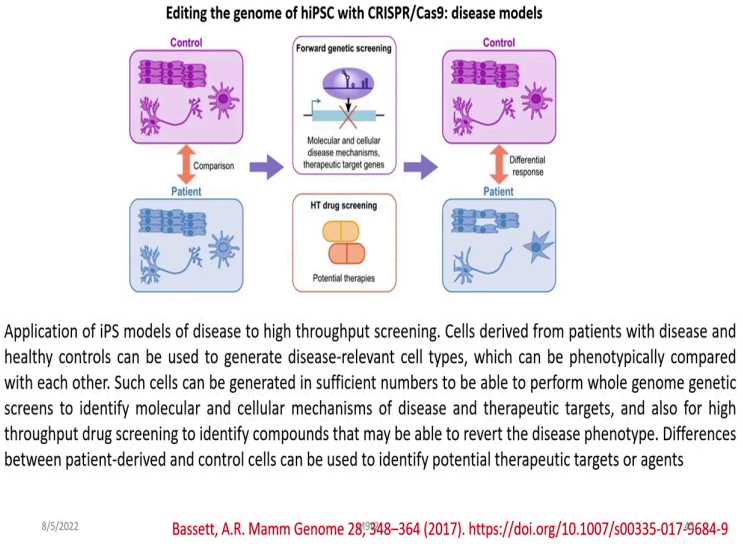

Application of iPS models of disease to high throughput screening. Cells derived from patients with disease and healthy controls can be used to generate disease-relevant cell types, which can be phenotypically compared with each other. Such cells can be generated in sufficient numbers to be able to perform whole genome genetic screens to identify molecular and cellular mechanisms of disease and therapeutic targets, and also for high throughput drug screening to identify compounds that may be able to revert the disease phenotype. Differences between patient-derived and control cells can be used to identify potential therapeutic targets or agents

8/5/2022 Bassett, A.R. Mamm Genome 28, 348–364 (2017). https://doi.org/10.1007/s00335-017-9684-9

So, let us discuss about the editing the genome of human induced Putin human induced pluripotent stem cells with CRISPR Cas9 for developing disease models. So, application of the induced pluripotent stem cell model of disease to high throughput screening is possible. Cells derived from patients with disease and healthy control can be used to generate disease relevant cell types which can be phenotypically compared with each other.

Such cells can be generated in sufficient numbers to be able to perform whole genome genetic screens to identify molecular and cellular mechanisms of disease and therapeutic targets. And also, for high throughput drug screening to identify compounds that may be able to revert the disease phenotype. Differences between patient derived and controlled cells can be used to identify potential therapeutic targets or agents.

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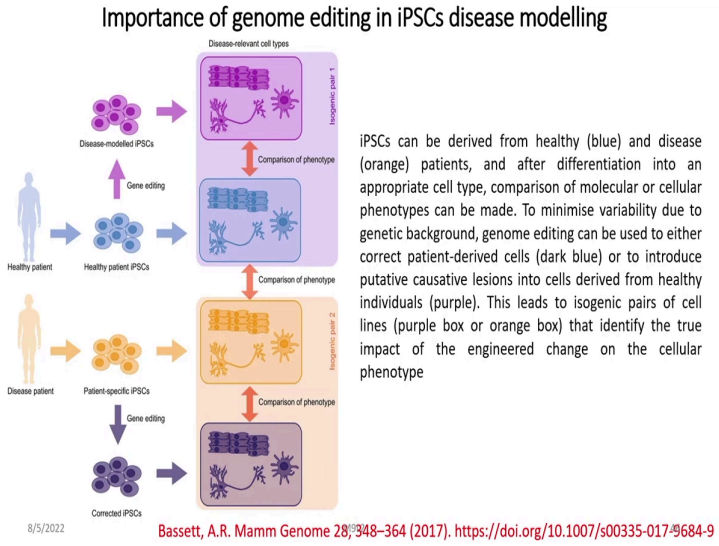

So, we see that genome editing in iPSC for disease modelling has a lot of scope and importance. Here, we have a healthy patient and a diseased patient giving rise to healthy patient iPSCs and patient specific iPSCs from with the help of gene editing we can go for disease model iPSCs or we can go for corrected iPSCs.

And then in the case of disease model iPSCs, we can go for disease relevant cell types and go for comparison of the phenotypes. Similarly, also in the case of corrected iPSC we can compare between the two populations. So, iPSCs can be derived from healthy as well as a diseased patient as shown over here.

And to minimize the variability due to genetic background genome editing can be used to either correct the patient derived cells or to introduce putative causative lesions in the cells derived from healthy individuals. This leads to isolating pairs of cell lines that identify the true impact of the engineered change on the cellular phenotype.

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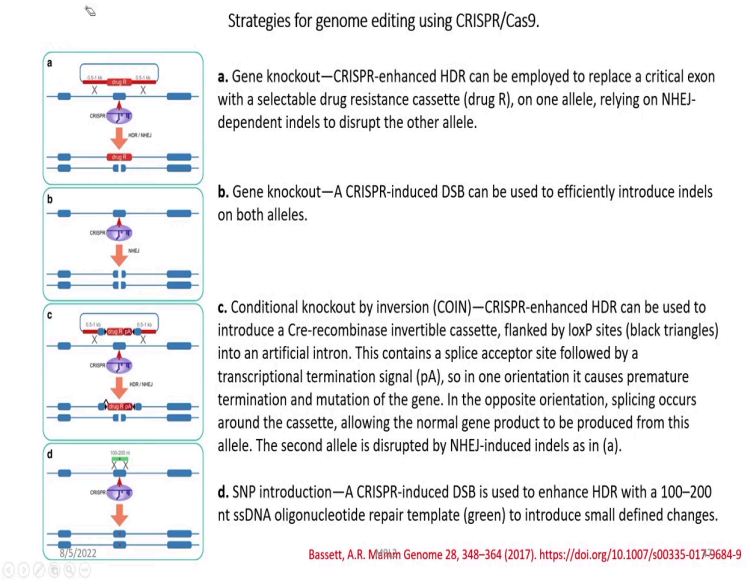

What are the various strategies for genome editing using CRISPR Cas9 these are all known to you we can go for a gene knockout or gene knockin or conditional knockouts by inversion (COIN). So, this is a CRISPR enhanced HDR can be used to introduce a Cre-recombinase invertible cassette flanked by loxP sites into an artificial intron.

This contains a splice acceptor site followed by a transcriptional termination signal PA. So, in one orientation it causes premature termination and mutation of the gene, in the opposite orientation splicing occurs around the cassette allowing the normal gene products to be produced from this allele. The second allele is disrupted by non-homologous and joining induced indels as in the figure a.

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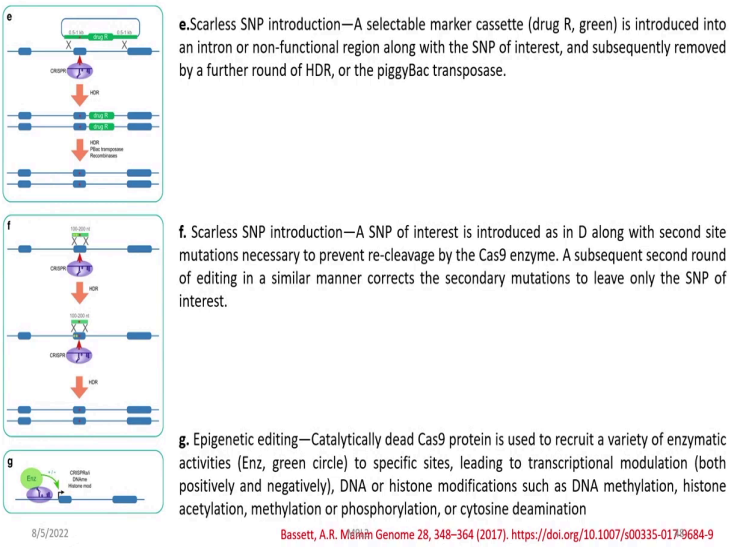

We can also go for SNP introduction using CRISPR Cas9 technology and also with advancements we can today produce scarless SNP introduction or epigenetic editing using date Cas9. These are all these known to you from prior discussions, what is important using iPSCs is the quality of the iPSCs.

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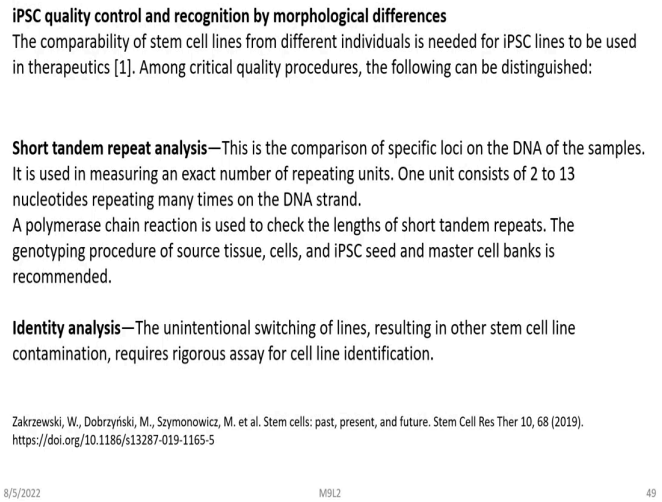

So, we have to have quality control of iPSCs and recognition of the morphological differences. The comparability of stem cell lines from different individuals is needed for iPSC lines to be used in therapeutics among critical quality procedures the following are

important or distinguishable Short tandem repeat analysis. This is the comparison of specific loci on the DNA of the samples it is used in measuring an exact number of repeating units.

One unit consists of 12 to 13 nucleotides repeating many times on the DNA strand, A polymerase chain reaction is used to check the lengths of short tandem repeats. The genotyping procedures of source tissues cells and iPSC seed and master cell banks is recommended. Identity analysis is also one important quality control aspect, the unintentional switching of lines resulting in other stem cell line contamination requires rigorous assay for cell line identification.

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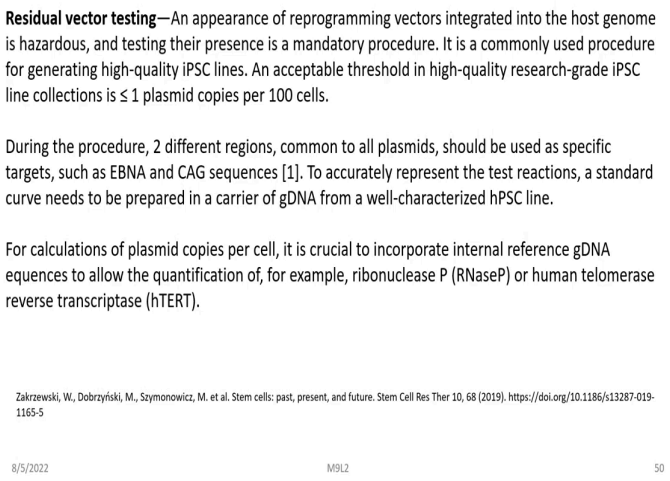

Residual vector testing is also very very important an appearance of reprogramming vectors integrated into the host genome is hazardous, and testing their presence is a mandatory procedure. It is a commonly used procedure for generating high quality iPSC lines an acceptable threshold in high quality research grade iPSC line collection is less than or maximum equal to 1 plasmid copies per 100 cells; so, it is less than 1 percent.

During the procedure 2 different regions common to all plasmids should be used as specific targets such as EBNA and CAG sequences. To accurately represent the test reactions, a standard curve needs to be prepared in a carrier of CR GDNA from a well characterized hPSC line. For calculations of plasmid copies per cell it is crucial to incorporate internal reference GDNA sequences to allow the quantification of, for example, ribonuclease P or human telomerase reverse transcriptase's.

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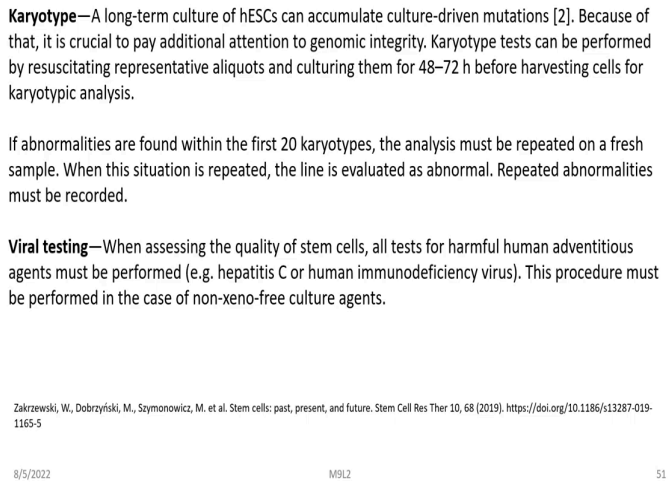

Another important quality control parameter is the karyotype, a long-term culture of stem cells can accumulate culture driven mutations because of that it is crucial to pay additional attention to genomic integrity. Karyotype tests can be performed by resuscitating representative aliquots and culturing them for 48 to 72 hours before harvesting cells for karyotype analysis.

If abnormalities are found within the first 20 karyotypes the analysis must be repeated on a fresh sample. When the situation is repeated, the line is evaluated as abnormal, repeated abnormalities must be recorded.

Viral testing, when assessing the quality of stem cells all tests for harmful human adventitious agents must be performed. This procedure must be performed in the case of non-xeno free culture agents.

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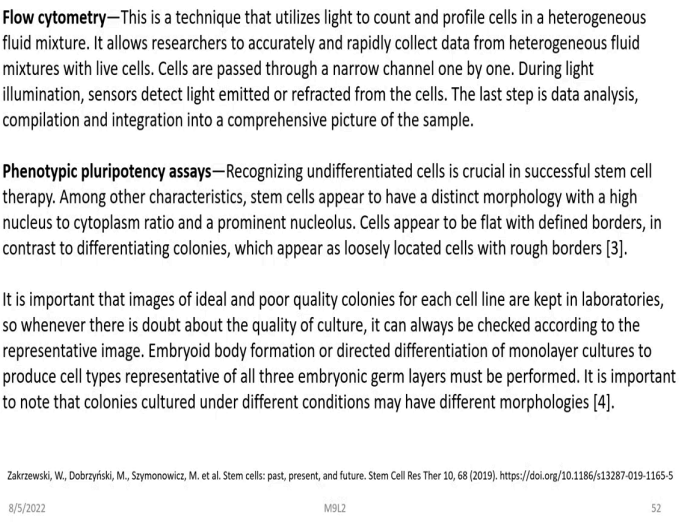

Using of Flow cytometry, this is a technique that utilizes light to count and profile cells in a heterogeneous fluid mixture. It allows researchers to accurately and rapidly collect data from heterogeneous fluid mixtures with live cells. Cells are passed through a narrow channel one by one. During light illumination, sensors detect light emitted or reflected from the cells. The last step is data analysis compilation and integration into a comprehensive picture of the sample. A flow cytometry can be used to assess the quality of the cells.

Phenotypic pluripotency assays, recognizing undifferentiated cells is crucial in successful stem cell therapy among other characteristics. Stem cells appear to have a distinct morphology with a high nucleus to cytoplasm ratio and a prominent nucleolus. Cells appear to be flat with defined borders in contrast to differentiating colonies which appear as loosely located cells with rough borders.

It is important that images of ideal and poor-quality colonies for each cell line are kept in laboratories. So, whenever there is a doubt about the quality of culture, it can always be checked according to the representative image. Embryoid body formation or directed differentiation of monolayer cultures to produce cell types. Representative of all three embryonic germ layers must be performed, it is important to note that colonies cultured under different conditions may have different morphologies.

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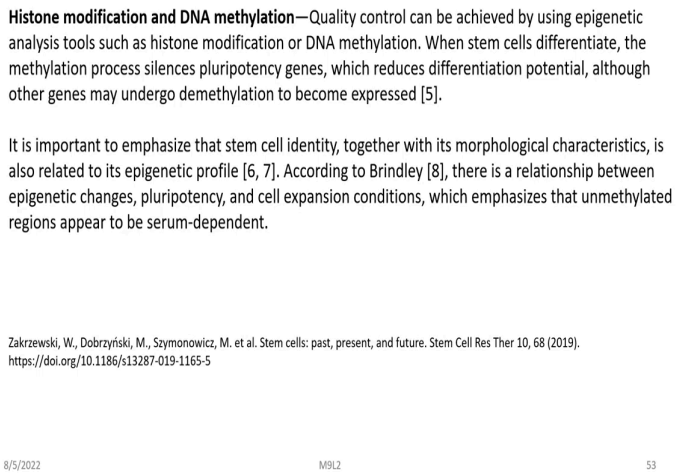

Histone modification and DNA methylation, quality control can be achieved by using epigenetic analytic tools such as histone modification or DNA methylation. When stem cells differentiate, the methylation process silence pluripotency genes, which reduces differentiation potential. Although other genes may undergo demethylation to become over expressed.

It is important to emphasize that stem cell identity together with its morphological characteristics is also related to its epigenetic profile. According to Brindley, there is a relationship between epigenetic changes pluripotency and cell expansion conditions which emphasizes that unmethylated region appear to be serum dependent.

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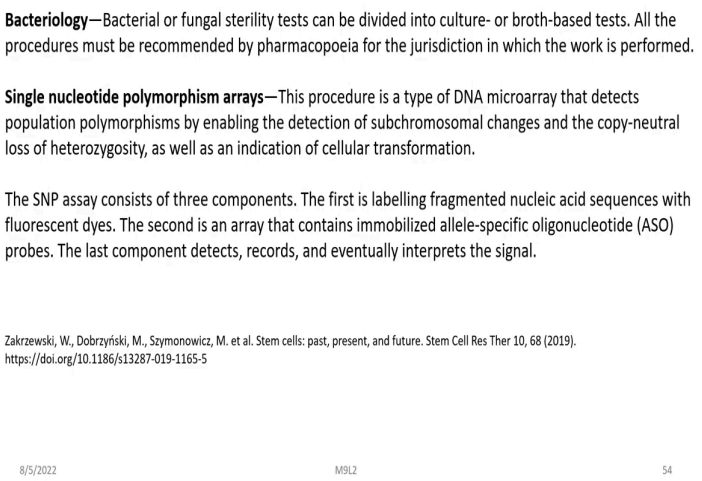

Other important parameters are the presence of bacteria or single nucleotide polymorphisms. Bacterial or fungal sterility test can be divided into cultures or broth-based tests. For all the procedures must be recommended by pharmacopoeia for the jurisdiction in which the work is performed. In SNP polymorphism arrays, we detect population of polymorphisms by enabling the detection of sub chromosomal changes and the copy neutral loss of heterozygosity as well as an indication of cellular transformation.

The SNP assay consists of three components, first is labelling fragmented nucleic acid sequences with fluorescent dyes, second is an array that contains immobilized allele- specific oligonucleotide probes. And the last component detects records and eventually interprets the signal.

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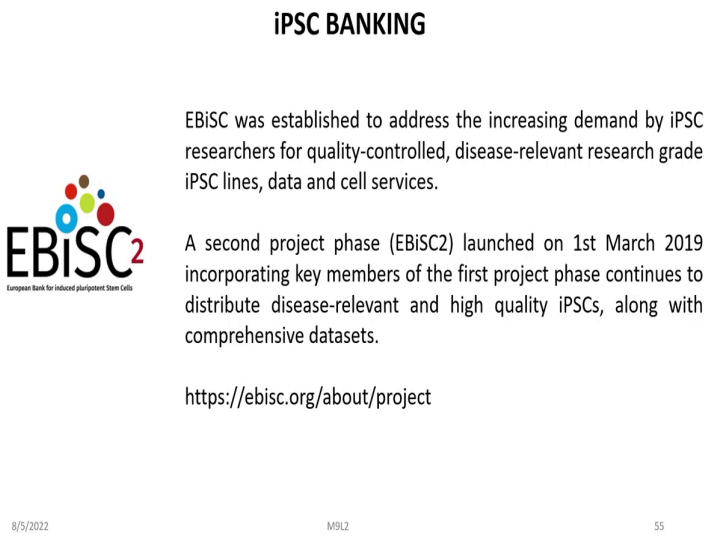

iPSC banking, European bank for induced pluripotent stem cells was established to address the increasing demand by iPSC researchers. For quality control disease relevant research grade iPSC lines, data and cell services. This kind of banking services reduces the work of researchers to a large extent. A second project phase was launched on March 2019 incorporating key members of the first project phase.

And it continues to distribute this is relevant in high quality iPSCs along with comprehensive dataset. The quality control of iPSCs are very very important which we have discussed in the earlier section. And this European bank for induced pluripotent stem cells try to maintain the highest quality of iPSCs and provide to the researcher network.

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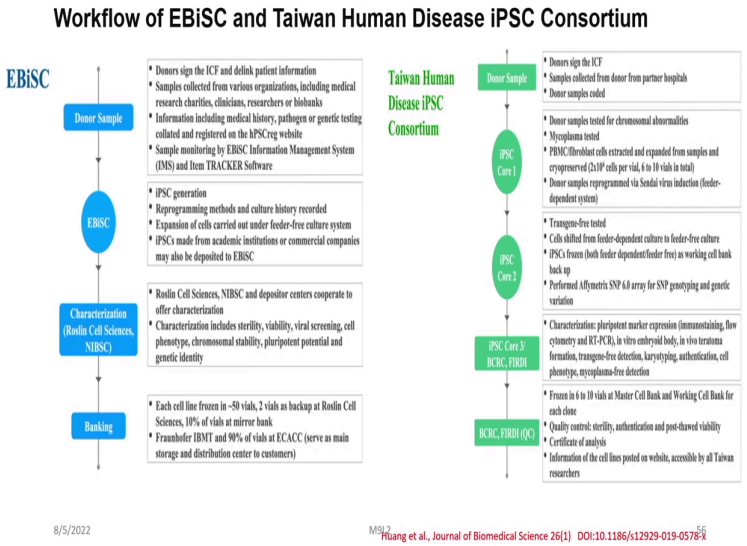

So, similar to this European bank for induced pluripotent stem cells there is also a bank in Taiwan called the Taiwan Human Disease iPSC consortium. And you can see the workflow of both the repositories in these flow diagram. They are donor samples and then the iPSC generation takes place in the second phase.

There is a programming, reprogramming methods and the culture history are recorded. Expansion of the cells are carried out under feeder free culture system, then iPSCs made from academic institutions commercial companies may also be deposited to this European bank.

Then characterizations are there Roslin Cell Sciences, NIBSC and depositor center cooperate to offer the characterizations which include the sterility, viability, viral screening, cell phenotype, chromosomal stability, pluripotent potential, and the genetic identity which we discussed at length in the earlier slides. And then finally, these are sent for banking or for the repository. A cell line is frozen in about 50 vials, 2 vials as backup at Roslin Cell Sciences, 10 percent of vials at mirror banks, there are many other similar banks.

And Fraunhofer IBMT and 90 percent of highest at EC ACC is serve as main storage and distribution center to the customers. In Taiwan human disease iPSC consortium similar approach is there with the donor samples which are coded. And then these are tested for chromosomal abnormalities, and then they are reprogrammed by a Sendai virus induction. And then transgene free tested cell shifted from feeder dependent culture to feeder free culture, iPSC is a frozen as working cell bank backup.

Then performed Affymetrix SNP 6.0 array for SNP genotyping and genetic variation. Then these are also characterized using various methods and they are frozen in 6 to 10 vials at master cell bank and working cell bank for each clone. A quality control a sterility authenticity and post thawed viability. And then certificate of analysis is also issued, informational cell lines posted on website and these are accessible by all researchers in Taiwan.

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Table 2 Brief overview of iPSC banks worldwide

Bank Name	Location	Profit Type	Ownership	Types of Diseases	Number of iPSC Lines	Investment (USD)	Related Publication
California Institute for Regenerative Medicine (CIRM)	USA	Non-profit	Government-owned	23	1556	$3000,000	[51–53]
Coriell Institute for Medical Research (Coriell)[a]	USA	Non-profit	Government-owned	40	91	$4,250,000 from NIH $10,000,000 from CIRM	[54–56]
Fujifilm Cellular Dynamics International (FCDI)[b]	USA	Profit	Owned by Fujifilm	N/A	N/A	$16,000,000 and shared grant of $6,300,000 from CIRM	[57–61]
Center for iPS Cell Research and Application (CiRA)	Japan	Non-profit	Government-owned	10	22	$27,383,000	[62–67]
European Bank for induced pluripotent Stem Cells (EBiSC)	Europe	Non-profit	European Commission, private enterprises	36	815	EBiSC project- $38,423,189 EBiSC2 project- $9,931,047	[68–71]
Human Induced Pluripotent Stem Cell Initiative (HipSci)	UK	Non-profit	Medical Research Council, Wellcome Trust	15	835	$20,500,000	[72–77]
Human Disease iPSC Consortium Resource Center (Taiwan Human Disease iPSC Consortium)	Taiwan	Non-profit	Government-owned	20	78	N/A	[78–94]
Institute of Physical and Chemical Research (RIKEN)	Japan	Non-profit	Government-owned	68	480	$24,862,180 for all departments	[95–98]
Korean National Stem Cell Bank (KSCB)	Korea	Non-profit	Government-owned	0	15	N/A	[99, 100]
WiCell Research Institute (WiCell)	USA	Non-profit	University of Wisconsin-Madison	58	1316	N/A	[101–104]

So, there are many such iPSC banks worldwide and some of these are accessible to researchers globally. Majority of them are government owned and they are also private owned, like fuji film cellular dynamics international which is owned by Fujifilm. And there are also certain owned by certain universities like the university of Wisconsin and you can see the huge investment involved in building up of these iPSC banks.

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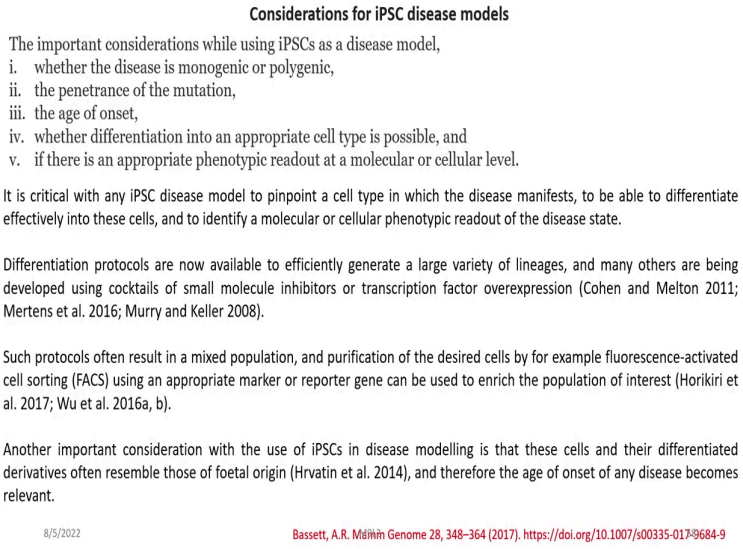

We have to take into consideration certain things for generating iPSC disease models. these are the following important considerations for using iPSC as a disease model. Number one whether the disease is monogenic or polygenic, if the penetration of the mutation is known, the age of onset. Whether differentiation into an appropriate cell type is possible, and if there is an appropriate phenotypic read out at a molecular or cellular level. It is important for any iPSC disease model to pinpoint a cell type in which the disease manifests to be able to differentiate effectively into these cells and to identify a molecular or cellular phenotypic read out of the disease state. Differentiation protocols are now available to efficiently generate a large variety of lineages and many others are being developed using cocktails of small molecule inhibitors or transcription factor over expression.

Such protocols often result in a mixed population and purification of the desired cells. For example, by fluorescence activated cell sorting using an appropriate marker or reporter gene can be used to enrich the population of interest. Another important consideration with the use of iPSCs in disease modeling is that these cells and their differentiated derivatives often resemble those of foetal origin and therefore, the age of onset of any disease becomes relevant.

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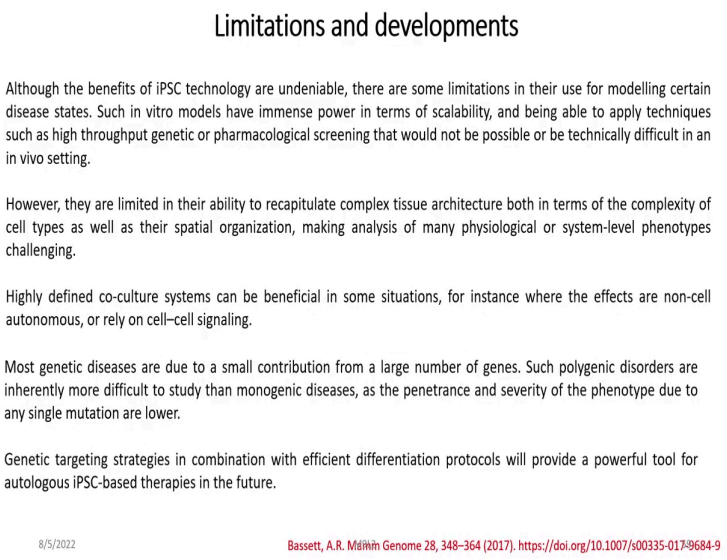

In spite of these various developments there are certain limitations, the benefit of iPSC technology are undeniable. As you can see from the various potential and the discussion we have made regarding certain diseases. There are some limitations in the use for modeling certain disease states as well. Such in vitro models have immense power in terms of scalability and being able to apply techniques such as high throughput genetic or pharmacological screening that would not be possible technically and difficult in an in vivo setting.

However, they are limited in their availability in their ability to recapitulate complex tissue architecture both in terms of the complexity of cell types as well as their spatial organization. Making analysis of many physiological or system level phenotypes challenging. Highly defined co culture systems can be beneficial in some situations for instance where the effects are non-cell autonomous or rely on cell-cell signaling.

Most genetic diseases are due to a small contribution form from a large number of genes. Such polygenic disorders are inherently more difficult to study than monogenic diseases as the penetrance and severity of the phenotype due to any single mutations are lower. Genetic targeting strategies in combination with efficient differentiation protocols will provide a powerful tool for autologous iPSC-based therapies in the future.

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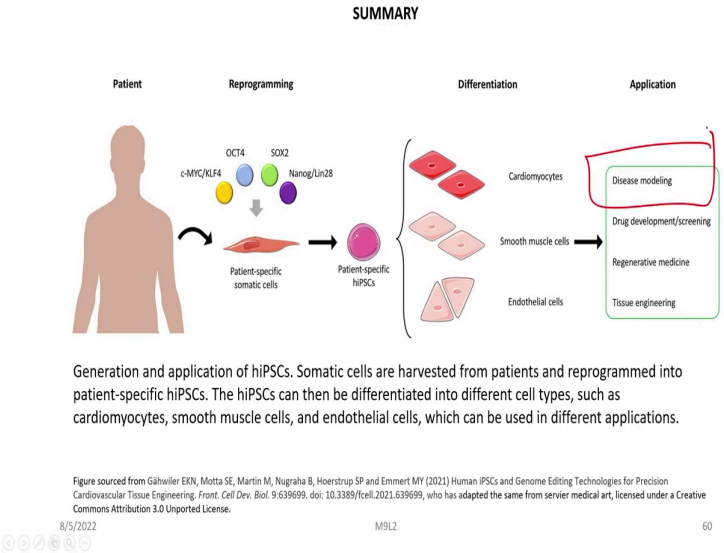

To sum up we can see here a patient from whom the patient specific somatic cells has been isolated and reprogrammed using various factors whose which may be OSCM or also including Nanog, Lin 28. And it generates patient specific human induced pluripotent stem cells.

And these may be further differentiated into different type of cells and used for various applications like tissue engineering, regenerative medicine, drug development screening. And disease modeling which is the focus of these particular major focus of this particular lecture. So, the potential applications of human induced pluripotent stem cells and gene editing combined together is quite huge as we have discussed in these particular lectures.

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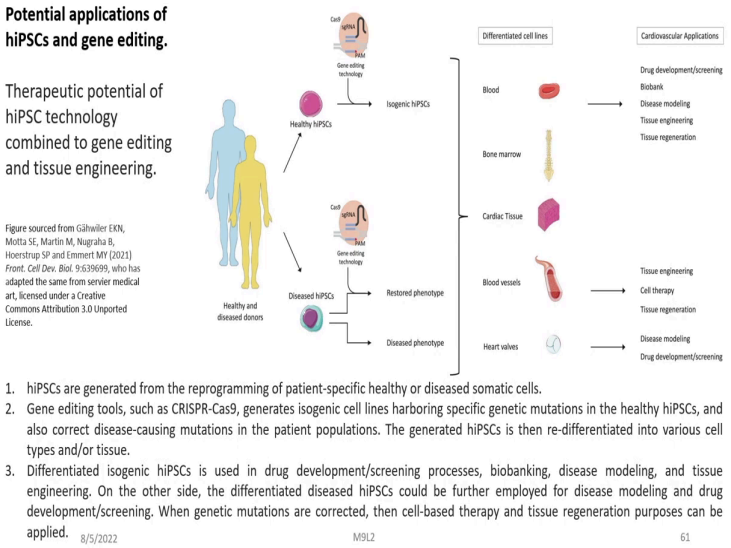

1. hiPSCs are generated from the reprogramming of patient-specific healthy or diseased somatic cells.
2. Gene editing tools, such as CRISPR-Cas9, generates isogenic cell lines harboring specific genetic mutations in the healthy hiPSCs, and also correct disease-causing mutations in the patient populations. The generated hiPSCs is then re-differentiated into various cell types and/or tissue.
3. Differentiated isogenic hiPSCs is used in drug development/screening processes, biobanking, disease modeling, and tissue engineering. On the other side, the differentiated diseased hiPSCs could be further employed for disease modeling and drug development/screening. When genetic mutations are corrected, then cell-based therapy and tissue regeneration purposes can be applied.

In conclusion we need to remember that human induced pluripotent stem cells are generated from the reprogramming the patient specific healthy or diseased somatic cells. Gene editing tools like CRISPR Cas9, ZFN, TALENs, generate isogenic cell lines, harboring specific genetic mutations in the healthy induced pluripotent stem cells. And also can correct disease causing mutations in the patient population, the generated human induced pluripotent stem cells are then re differentiated into various cell types and or tissues.

Differentiated isogenic human iPSCs are used in drug development screening process, disease modeling and tissue engineering and they are also preserved in biobanks for supply to various researchers. On the other side the differentiated disease human iPSCs could be further employed for disease modeling and drug development screening. When genetic mutations are corrected, then cell based therapy and tissue regeneration purposes can be also achieved.

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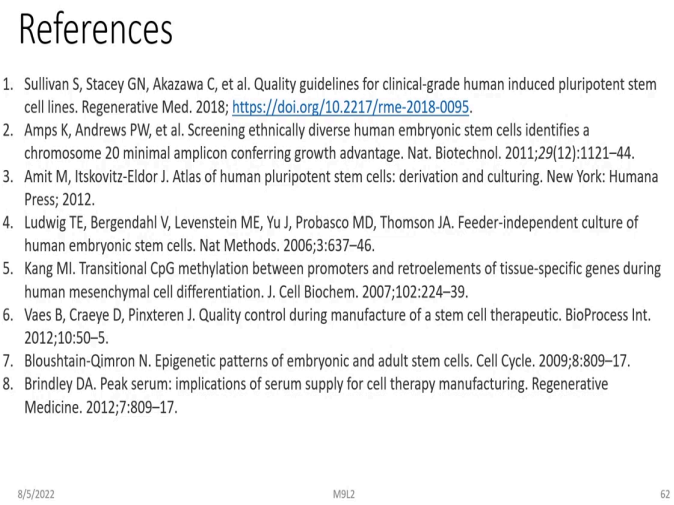

These are some of the references from which we have included various texts as well as figures for details, you can read them. With this we come to the end of this lecture thank you for your patient hearing.