Tampereen yliopisto

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Generation of isogenic human iPSC line from a DCM patient carrying the Finnish founder mutation (p. S143P) in LMNA gene using the CRISPR/Cas9 system

Faculty of Medicine and Health Technology
Master's thesis
November 2019

PRO GRADU-TUTKIELMAN TIIVISTELMÄ

Paikka: Tampereen yliopisto, Lääketieteen ja terveysteknologian tiedekunta
Tekijä: Heini Auri
Tutkinto-ohjelma: Bioteknologian tutkinto-ohjelma
Otsikko: Ihmisen isogeenisen iPS-solulinjan tuottaminen CRISPR/Cas9 systeemillä DCM-potilaalta, joka kantaa LMNA geenissä suomalaista perustajamutaatiota (p.S143P)
Sivumäärä: 78
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Tarkastajat: professori, LT, FT Katriina Aalto-Setälä ja professori, FT Heli Skottman
Päiväys: 11.11.2019

Tutkimuksen tausta ja tavoitteet: CRISPR (clustered regularly interspaced short palindromic repeats) systeemi on osa useiden bakteerien ja arkkien adaptiivista immuunipuolustus-järjestelmää vieraita geneettisiä tekijöitä vastaan. CRISPR teknologiaa on käytetty eukaryooteilla tehokkaaseen ja täsmälliseen genomin muokkaukseen. CRISPR teknologian avulla voidaan esim. korjata mutatoituneita alleeleita potilaslinjoissa, joka mahdollistaa ns. isogeenisten soluparien analysoinnin. Täten on mahdollista tutkia yksityiskohtaisesti poikkeavuuksia molekyyli- ja solufenotyypeissä. CRISPR/Cas genominmuokkauskokeet on toistaiseksi suoritettu käyttämällä ihmisen solulinjoja (esim. HEK293 solut). Näitä solulinjoja on helppo käsitellä ja transfektoida. Tämän tutkimuksen tavoitteena oli korjata laajenevaa eli dilatoivaa kardiomyopatiaa (DCM) aiheuttava LMNA geenin suomalainen perustajamutaatio (p. S143P) käyttämällä CRISPR/Cas9 geeninmuokkausteknologiaa.

Menetelmät: Isogeeninen, geenikorjattu solulinja saatiin luotua hyödyntämällä CRISPR/Cas9 teknologiaa. CRISPR/Cas9 systeemi koostuu Cas9:n, ohjaaja RNA:n ja donor plasmidin muodostamasta kompleksista. Donor plasmidi tarjoaa homologisen juosteen sisältäen mutaation korjauskohdan. T7 Endonukleeasia käytettiin havaitsemaan kolmen sgRNA näytteen pilkkomistehokkuus HEK293 soluilla. HEK293 solut transfektoitiin käyttämällä tiettyä kaupallista transfektio-reagenssia yhdessä Cas9-endonukleaasin ja sgRNA:n kanssa. Agaroosigeelielektroforeesilla mitattiin Cas9-välitteinen pilkkoutumistehokkuus. Positiivisten iPS-solukloonien seulontaa varten sekä eksogeenisen restriktioentsyyminkohdan liittämiseksi donor plasmideihin suoritettiin ns. quikchange mutageneesi. Potilaan iPS-solulinjan, UTA.12619.LMNA, nukleofektio suoritettiin kaupallisen kitin avulla. Resistentit solupesäkkeet seulottiin antibiootin avulla. Transfektoidut iPS-solupesäkkeet poimittiin mekaanisesti kasvamaan 96-kuoppalevylle. Onnistunut geeninmuokkaus varmistettiin DNA eristyksellä, PCR:llä ja Sanger sekvensointimenetelmällä. Lopuksi iPS-solut erilaistettiin sydänsoluiksi käyttämällä pienmolekyylierilaistusmenetelmää.

Tutkimustulokset: Nukleofektioon valittiin paras sgRNA näyte T7 Endonukleeasi analyysin perusteella ja sen PAM-sekvenssi sijaitsi lähellä pistemutaatiota. EcoR1-restriktiokohta saatiin onnistuneesti insertoitua L_2 donor plasmidiin korjattujen iPS-solupesäkkeiden seulontaa varten. Nukleofektion tehokkuus arvioitiin Cas9-plasmidin EGFP ekspressiolla. Isogeenisen solulinjan luominen osoitettiin sekvensoimalla resistentit solupesäkkeet.

Johtopäätökset: CRISPR/Cas9 tekniikkaa hyödyntämällä saatiin korjattua toivottu pistemutaatio isogeenisen solulinjan luomiseksi eli uudessa solulinjassa ei tuota mutaatiota enää ollut. Pistemutaation korjaustehokkuus jäi kuitenkin heikoksi, joten menetelmän tehokas optimointi ja off-target ilmiön vaikutusten minimointi ovat keskeisiä tulevaisuuden tutkimuksen tavoitteita ennen kuin CRISPR/Cas9 voidaan hyödyntää terapeuttisiin sovelluksiin.

ABSTRACT OF MASTER'S THESIS

Place: Tampere University, Faculty of Medicine and Health Technology
Author: Heini Auri
Degree programme: Master's degree programme in Biomedical Technology
Title: Generation of isogenic human iPSC line from a DCM patient carrying the Finnish founder mutation (p. S143P) in LMNA gene using the CRISPR/Cas9 system
Pages: 78
Supervisor: PhD Reeja Maria Cherian
Reviewers: Professor, PhD, MD Katriina Aalto-Setälä and Professor, PhD Heli Skottman
Date: 11.11.2019

Background and aims: CRISPR (clustered, regularly interspaced, short, palindromic repeats) system is an adaptive immune system against foreign genetic elements that has been found in various bacterial and archaeal species. CRISPR system has been used efficiently and precisely to edit diverse genomes of eukaryotes. It has provided an ability to repair mutated alleles in patient lines that enable the analysis of isogenic cell pairs that differ in a single genetic change, allowing a detailed study of the molecular and cellular phenotypes that result from this abnormality. So far, CRISPR/Cas genome editing experiments have been primarily performed using human immortalized cell lines (e.g. HEK293T cells). These cell lines are easy to manipulate and transfect. While only little information is available considering gene correction in human iPS cells. The purpose of this study was to precisely correct the Finnish founder mutation (p. S143P) in the LMNA gene that causes Dilated cardiomyopathy (DCM) using CRISPR/Cas9 genome editing technique.

Methods: Isogenic gene-corrected cell line was obtained using the CRISPRs/Cas9 system that comprises a Cas9 and a guide RNA plasmid in combination with a donor plasmid serving as a homologous template covering the site of the mutation. T7 Endonuclease assay was used to detect the cleavage activity of three selected sgRNAs in HEK293 cells. For that, HEK293 cells were transfected using Fugene HD Transfection reagent with Cas9 plasmid and sgRNA plasmid. Agarose gel electrophoresis was used to observe the efficiency of Cas9-mediated cleavage. Further, quick change mutagenesis analysis was performed to insert an exogenous restriction enzyme site in the donor plasmid template for enabling the screening of positive CRISPR iPSC clones. Nucleofection of the patient iPSC line, UTA.12619.LMNA carrying S143P mutation in LMNA gene, was performed using the P1 primary cell 4D-Nucleofector X kit. Antibiotic was used to select the resistant cell clones. The transfected iPSC clones were mechanically picked into the wells of 96-well plate. Successful gene engineering was confirmed by DNA isolation, PCR and Sanger sequencing. Finally, iPSCs were differentiated into cardiomyocytes using small molecule differentiation method.

Results: Based on the T7 endonuclease assay results, the best sgRNA with the highest cleavage activity and the PAM sequence located close to the point mutation site was selected for nucleofection. The restriction site EcoR1 was successfully introduced into the L_2 donor plasmid for screening the corrected iPSC clones. The efficiency of the iPSC nucleofection was confirmed by EGFP expression by Cas9 plasmid. The analysis of each resistant clone by sequencing using a primer close to the corrected locus validated the generation of isogenic cell line.

Conclusions: The correction of a point mutation to obtain isogenic iPSC line utilizing CRISPR/Cas9 system was successful. However, the correction efficiency remained low, so the efficient optimization of the CRISPR method and off-targets effects minimization would be the main goals of future research work before utilizing CRISPR system in therapeutic applications.

Acknowledgements

This thesis work was carried out in Heart Group, Faculty of Medicine and Health technology, Tampere university. First of all, I would like to thank you the leader of the Heart group, Professor, MD, PhD Katriina Aalto-Setälä for this opportunity. I would like to express my gratitude to my supervisor PhD Reeja Maria Cherian. I'm thankful for all the help, unconditional support, valuable advice and suggestions I get during my thesis project. I take this opportunity to thank Laboratory Technologist, Bachelor of Laboratory Services Henna Lappi. I am profoundly grateful to her for her continuous encouragement and helpful advice.

I would also like to extend my gratitude to all my dear friends and family for many delighted moments and just being there for me. Special thanks to my parents Lisse and Jukka Auri, for the unwavering support, help and encouragement throughout my life and this thesis project.

Tampere 11.11.2019
Heini Auri

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck -ohjelmalla.

Originality of this publication has been checked with Turnitin OriginalityCheck program.

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Abbreviations

AAV	Adeno-associated virus
AC	Arrhythmogenic
BSA	Bovine serum albumin
Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DCM	Dilated cardiomyopathy
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DSB	Double strand break
EB	Embryoid body
E. coli	Escherichia coli
END-2 cells	Endoderm-like cells
FBS	Fetal bovine serum
fCas9	Fusion protein of catalytically inactive Cas9 and *Fok* I nuclease
FDA	Food and Drug Administration
GFP	Green fluorescent protein
gRNA	Guide RNA
HCM	Hypertrophic cardiomyopathy
HEK293	Human embryonic kidney cells
HiPSC	Human induced pluripotent stem cells
HR	Homologous recombination
ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
KLF	Kruppel-like factor
LV	Left ventricle
MYC	Myelocytomatosis viral oncogene homolog
NDS	Normal donkey serum
NHEJ	Non-homologous end joining
OCT	Octamer-binding factor
PAM	Protospacer adjacent motif
PB	Phosphate buffer
PB	PiggyBac
PBS	Phosphate-buffered saline
PSCs	Pluripotent stem cells
RGEN	RNA-guided endonucleases
RNP	Ribonucleoprotein complexes
ROCK	Rho-associated protein kinase
sgRNA	Single guide RNA
SOX	Sex determining region Y-box 2
SpCas9	Streptococcus pyogenes Cas9
SSN	Site-specific nuclease
TALEN	Transcription activator-like effector nuclease
tru-gRNAs	Truncated gRNAs
WT	Wild type
ZFN	Zinc-finger nuclease

1. Introduction

Shinya Yamanaka, MD, PhD made a remarkable discovery in 2006 by introducing four transcription factors; Oct3/4, Klf4, c-Myc and Sox2, which are essential for reprogramming somatic cells into pluripotent stem cells. These cells were named as induced pluripotent stem cells (iPSCs) that have the capacity to differentiate into a variety of specialized cell types originating from the three germ layers (ectoderm, endoderm and mesoderm) of the embryo. (Hamilton et al. 2009; Okita et al. 2007) iPSC technology has the potential to overcome many challenges including the ethical concerns related to the use of embryos and the risk of immune rejection after transplantation (Menon et al. 2016). iPSCs are commonly used to generate patient-specific iPSCs, which are becoming an increasingly valuable tool for disease modeling, drug development and future patient-specific cellular therapies (Singh et al. 2015).

Genome editing refers to different methods comprising insertion, deletion and replacement to modify DNA at a specific site in the genome of an organism. Previously, genome editing was performed by using engineered nucleases, such as transcription activator-like effector nucleases (TALEN) and zinc finger nucleases (ZFN). Drawbacks of these methods are low efficiency, poor specificity and high cost. The recently developed clustered regularly interspaced short palindromic repeats (CRISPR) technology is a powerful genome editing tool to precisely and efficiently modify the genome of eukaryote with advantages of high editing efficiency and low cost. CRISPR is an adaptive immune system of many bacteria and most archaea against invading genetic elements, containing plasmids and viruses. (Barrangou and Marraffini 2014; Hryhorowicz et al. 2017)

The method of CRISPR system is based on the engineered Cas9 nuclease that uses a specific single guide RNA (sgRNA) for generating a double-stranded break (DSB) at the targeted genomic site. DSB is repaired by DNA repair pathways, where the DNA sequence of host can be modified by the introduction of small deletion or insertion mutations via non-homologous end joining (NHEJ) or by the insertion of additional sequences via homologous recombination (HR) pathways. (Sander and Joung 2014) Immortalized cell lines (e.g. human neonatal fibroblast and HEK293T cells) are commonly used for achieving CRISPR/Cas genome editing in human cells due to the easiness of manipulation and transfection. While only little information is available considering gene correction in human iPS cells. (Grobarczyk et al. 2015)

In this master's thesis, an isogenic iPSC line was generated using CRISPR/Cas genome editing method. The feasibility of CRISPR components were studied under controlled conditions and the most effective and suitable components to perform genome editing were chosen and delivered into iPSCs. Positive colonies harboring efficient recombining were selected and were expanded for validation analysis of CRISPR gene editing. Finally, precise genome editing was confirmed by PCR and Sanger sequencing method.

2. Literature Review

2.1 Stem cells

Stem cells are defined by their capacity for self-renewal and the ability to differentiate into various cell types in the body (Chagastelles and Nardi 2011; Sun et al. 2016). A term self-renewal indicates to the ability of the cell to undergo various divisions while maintaining an undifferentiated state. Potency refers to the capacity of the cell to differentiate down numerous different cell lineages. (Menon et al. 2016)

Stem cells can be classified according to their source of origin into two major classes (Figure 1). Embryonic stem cells can form all three embryonic germ layers containing ectoderm, endoderm and mesoderm. (Sobhani et al. 2017) Adult stem cells (also called as somatic stem cells) are undifferentiated cells present in various tissues and organs for maintaining and repairing the function of tissues within the adult organism (Pekovic and Hutchison 2008). There exist four types of stem cells that are classified according to the differentiation potential (Mahla 2016). Totipotent stem cells have an exceptional capacity to limitlessly divide. They have also the potential to differentiate into all germ layers as well as extra-embryonic tissues or placental cells. A few days after fertilization, totipotent stem cells of the morula, mature and form the most primate stem cells called as pluripotent stem cells (PSCs). They have the ability to generate all the cell types in the body. Multipotent and unipotent stem cells have a limited capacity for self-renewing. (Singh et al. 2016) Non-embryonic adult stem cells are multipotent stem cells, that have the potential to evolve into numerous specialized cell types, whereas unipotent stem cells can differentiate only to one cell lineage. (Menon et al. 2016)

Nearly 40 years ago, in 1981 embryonic stem cells were isolated from early embryos of mice at the first time to develop stem cell line *in vitro* (Evans and Kaufman 1981; Gail 1981). Seven years later in 1998 James Thomson and his colleagues isolated human embryonic stem cells from blastocyst of human embryo, which was produced by *in vitro* fertilization (Vazin and Freed 2010). The use of human embryos and oocytes in medical research has induced ethical concerns, which caused the urge of having a different source of stem cells to replace the human embryos. The production of pluripotent cells directly from the patients' somatic cells could represent the solutions for this concern. In 2006 researchers Takahashi and Yamanaka presented the idea of direct reprogramming, which provide an efficient way to generate human pluripotent stem cells from adult somatic cells. The term induced pluripotent stem cells was established for the first time. (Takahashi & Yamanaka 2006)

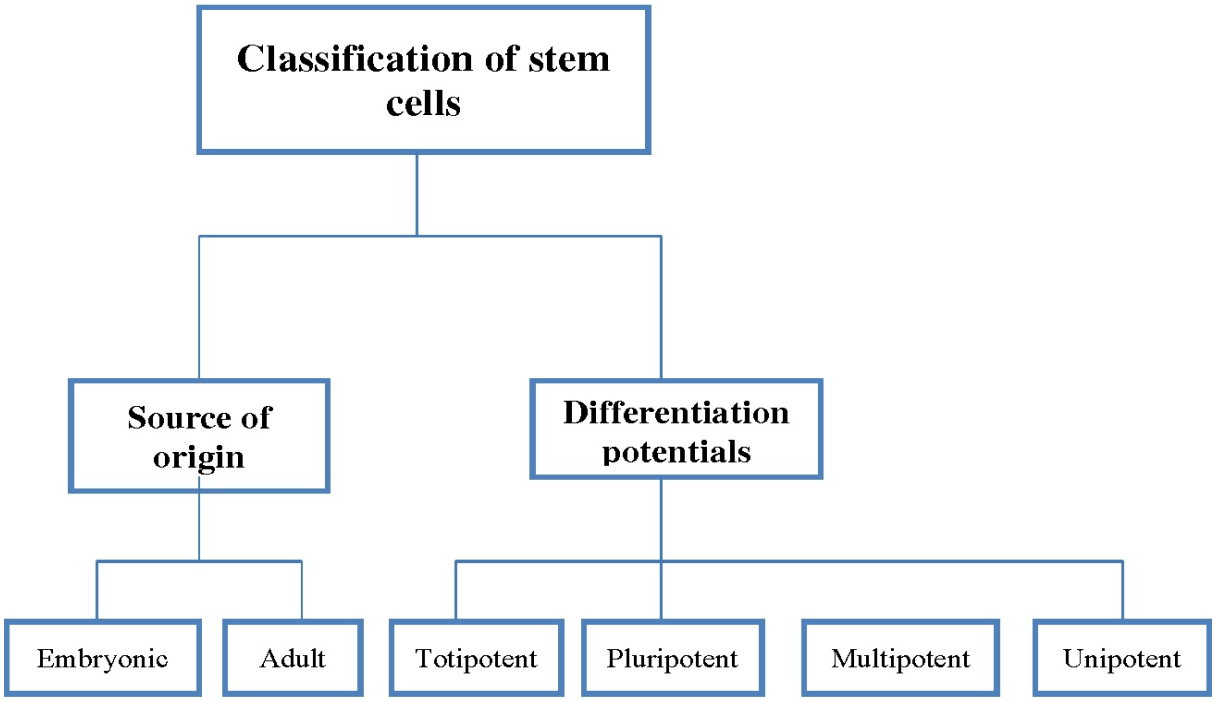


Figure 1: Classification of stem cells. Stem cells can be classified according to their source of origin into two classes and four classes according to differentiation potentials. Figure modified from Sobhani et al. 2017.

2.2 Human induced pluripotent stem cells

Induced pluripotent stem cells can be defined as a type of pluripotent stem cells that are produced from somatic cells by reprogramming through the introduction of specific pluripotent-associated genes. iPSCs can be produced using cells from different sources such as fibroblasts, blood and liver cells as well as cancer cells. (Menon et al. 2016; Omole and Fakoya 2018) Embryonic stem cells derived from the inner cell mass (ICM) of preimplantation embryos were the most well-known pluripotent stem cells before the discovery of iPSCs (Omole and Fakoya 2018). The generation of iPSCs can be summarized in three main phases. The first of them is initial phase, second phase is called as maturation and the last one is stabilization phase (Singh et al. 2015).

iPSCs share similar properties with embryonic stem cells containing morphology, expression of surface markers, feeder dependence and *in vivo* teratoma formation efficiency (Narsinh et al. 2011; Omole and Fakoya 2018). Therefore, iPSCs offer a limitless source of proliferating cells, which are able to differentiate almost any human cells types needed for medical or therapeutic applications (Ebert et al. 2012). The main goals of iPSC technology are creating individualized,

patient- and disease-specific stem cell treatments as well as to improve the understanding of the stem cells potential (Takahashi & Yamanaka 2006). Researchers Takahashi and Yamanaka presented the hypothesis at the first time in 2006 that fully differentiated somatic cells (e.g. fibroblasts) can be reprogrammed to iPSCs by retroviral-mediated introduction of four transcription factors: Octamer-binding factor 4 (Oct4), Kruppel-like factor 4 (Klf4), myelocytomatosis viral oncogene homolog (c-Myc) and sex determining region Y-box 2 (Sox2) (Figure 2) (Takahashi & Yamanaka 2006).

The combination of these four genes is also known as OSKM factors or "Yamanaka Factors". Yamanaka factors are known to be essential in early embryonic development and vital for embryonic stem cell identity. (Omole and Fakoya 2018) Nowadays scientists can generate iPS cells, expand and then differentiate them into numerous cell types such as neural cells, adipocytes and cardiomyocytes, which can be utilized for regenerative medicine, modeling of complex diseases and drug screening studies (Figure 2) (Ebert et al. 2012; Menon et al. 2016).

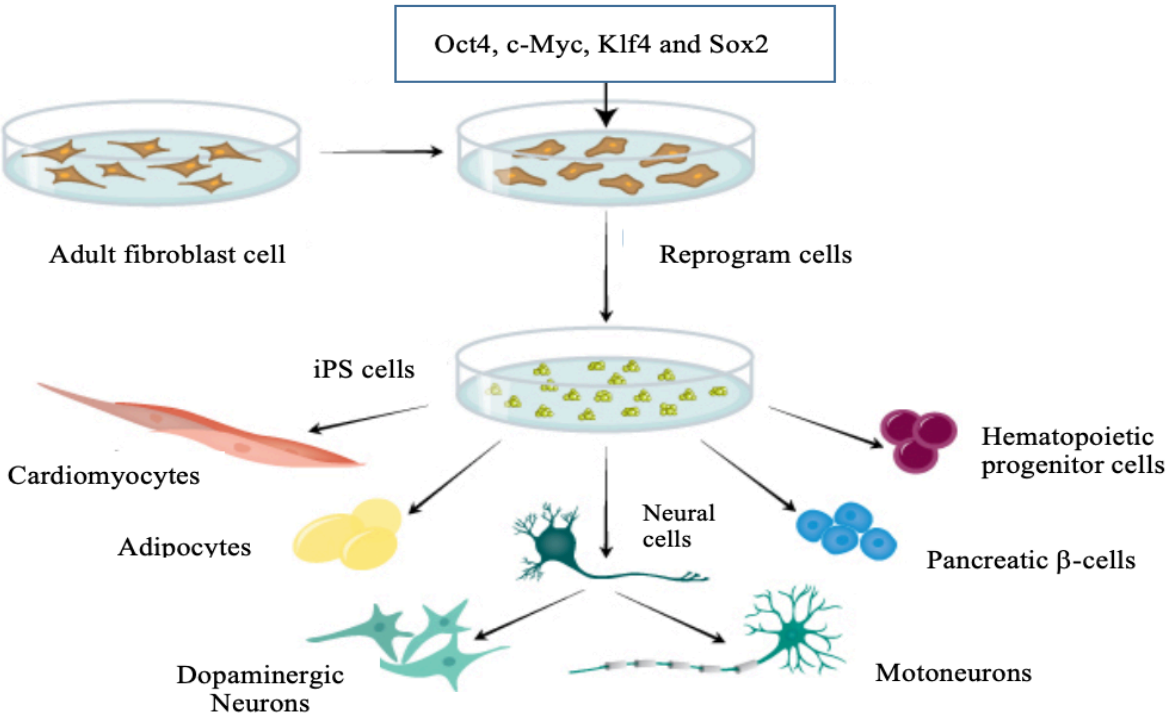


Figure 2: Generation of induced pluripotent stem cells. Induced pluripotent stem cells can be generated by introducing four transcription factors: Octamer-binding factor 4 (Oct4), Kruppel-like factor 4 (Klf4), myelocytomatosis viral oncogene homolog (c-Myc) and sex determining region Y-box 2 (Sox2) via different transfection methods. Induced pluripotent stem cells may further differentiate into various types of cells. Figure modified from Amabile and Meissner 2009.

2.2.1 Reprogramming techniques

Methods for the introduction of reprogramming factors for generation of iPSCs can be classified into two categories based on integrating capacity. These methods are integrating system and non-integrative system. Integrating system involves the integration of exogenous genetic element into the host cell genome, while non-integrating system refers to method in which the genetic element has not incorporated into the host cell DNA. The integrative delivery methods can be performed with the use of viral vectors (e.g. retrovirus and lentivirus) or non-viral vectors (e.g. transposons and linear/plasmid DNA fragments). Correspondingly, the non-integrative delivery methods contain the use of viral vectors such as adenovirus and Sendai virus and non-viral vectors such as episomal DNA vectors, mRNA, and proteins. (Brouwer et al. 2016; Omole and Fakoya 2018)

First studies were carried out by using the integrating systems (retroviral and lentiviral) due to the effectiveness and easiness of these delivery systems. Diverse cell types, such as adipose cells, blood cells and human fibroblast have been used to derive iPSCs through retroviral transduction. The reprogramming efficiency remains low (0.01-0.02 %) by using human cells. Slightly higher (0.1–2 %) reprogramming efficiency can be achieved by using lentiviral system instead of retroviral system. The main disadvantage of retrovirus-mediated reprogramming method is that the cells have to be actively dividing and slowly dividing cells (e.g. neurons) cannot be utilized. Additionally, integration of retroviral DNA into the host genome may result in the expression of transgenes. This may lead to an increased risk for insertional mutagenesis and cause cancer. (Menon et al. 2016; Omole and Fakoya 2018; Singh et al. 2015) Overall, integrative delivery systems always contain the risk of insertional mutagenesis, and due to the lack of safety, their potential use in therapeutic application may be limited (Omole and Fakoya 2018).

Most widely used delivery system for transcriptional factors utilize non-integrating method to avoid throwbacks associated with integrating methods (Menon et al. 2016; Omole and Fakoya 2018). Numerous of new non-integrating virus-mediated iPSCs reprogramming techniques have been currently studied, including the transgene excision method that significantly reduce the risk caused by the integration of viral vectors. Another simple and sustainable non-integrating method for reprogramming is the piggyBac (PB) transposon. PB technique uses a movable genetic element that is able to effectively transposes between vectors and chromosomes through the mechanism called as “cut and paste”. Other non-integrating methods

currently available are adenoviral vectors, non-viral minicircle DNA vectors and episomal plasmids. (Menon et al. 2016)

In summary, while comparing the delivery systems of transcriptional factors, it has been shown that integrative delivery method has a higher reprogramming efficiency than non-integrating method, although integrative method is less safe. Hence, the use of non-integrating methods may be more desirable choice for iPSC generation and use in a clinical application. Taken together, depending on the purpose of generated iPSCs the appropriate delivery method has to be carefully selected. (Brouwer et al. 2016; Omole and Fakoya 2018)

2.2.2 Applications of hiPSCs

HiPSCs offer tremendous opportunity for generating patient- and disease specific stem cells to model human diseases, development and screening of new drugs and individualized cell therapies (Omole and Fakoya 2018). HiPSCs are excellent candidate for research due to their ability to adopt the patient characteristics of multiple cell types, containing disease-relevant cells. In addition, iPSCs can preserve specific genetic and epigenetic memories of the patient from which they derived. This factor allows the specific simulation of the patient's own disease under laboratory conditions. Nowadays, patient-specific iPSCs can be generated from somatic cells by reprogramming method. Different gene editing methods can be used to correct iPSCs with the genetic defects. These iPSCs can be differentiated into different target cells that can be utilized for disease modeling of numerous disorders. (Diecke et al. 2014) Disease models can be used for testing the toxicity for evaluating the safety of new drugs (Singh et al. 2015). Although therapeutic applications of iPSCs are promising, safety and availability issues regarding iPSC-based therapies are the two key concerns for their clinical use (Diecke et al. 2014).

2.2.3 Disease modeling

Due to the intrinsic properties of self-renewing and its potential to differentiate into almost all cell types in the human body, patient-specific iPSCs can be used for modeling and studying diseases from patients with complex genetic defects (Ebert et al. 2012) . Generation of disease

specific cell types from patient specific iPSCs is preferred over other cell transfer therapies due to the lower harm and risk to the patient (Diecke et al. 2014).

Previously, animal models and immortalized cell lines were utilized to screen therapeutics for various human disorders that lacked success due to the difference between genetic background (Sayed et al. 2016). iPSCs based disease models may overcome issues related to the use of animal models. Many scientists have reported the differences between animal disease and human disease models. The major weakness is the fact that animal models never completely mimic the true human disease phenotype. (Inoue et al. 2014; Shi et al. 2017) In addition, poor corresponding between human and animal drug toxicity response has been observed. Toxicity studies of iPSCs are low-cost compared to ordinary testing method. (Omole and Fakoya 2018)

Disease modeling with iPSCs has also associated with other limitations and concerns. Cell culture conditions and cellular artifacts have impact on the diversity of iPSC lines and *in vitro* disease modelling. Researchers have observed undesired heterogeneous cell populations after differentiation of iPSCs. (Inoue et al. 2014) Advantages that support the research and application of patient-specific iPSCs for drug screening and disease modeling are summarized to safety, accuracy and inexpensiveness. Furthermore, generation of patient and disease-specific iPSCs is a great leap towards personalized medicine. (Diecke et al. 2014)

2.2.4 Drug screening studies

The research and development path of new drug contains a time-consuming and inefficient step through essential research, invention of the medicine, preclinical development analyses, complex clinical trials with humans, and regulatory approval by the Food and Drug Administration (FDA) (Hughes et al. 2011). Safety and efficiency problems are two main reasons why more than 90 % of drug candidates fail to get approved as drugs (Moore 2003).

Due to the unique genetic characteristics, all the patients don't respond in the same way to specific medications. Two different patients with similar symptoms might have different underlying causes (etiologies) for the disease. Despite the difference etiologies the patients will receive the same drug according to their symptoms. To overcome these challenges, iPSC-based drug screening methods provide precision medicine that enables the development of personalized therapy for every patient. (Sayed et al. 2016)

The iPS cell platform can be used for the production of human disease-specific cell types, which enable better prediction of therapeutic response and toxicology of the developing drug. In addition, an enormous amount of various iPS cell lines for the same human disease can be produced offering insight into the genetic and potentially epigenetic variation of a wide section of the population. (Liam et al. 2010)

Although iPSC technology offers great promises for screening and toxicity testing of new drugs, produced cell types remain partially immature resembling fetal cells compared to adult cells. The usage of these cells for disease modelling and drug studies can be inaccurate and of limited utility. Different maturating strategies for iPSCs derived cell types are developed for directing these cells towards more mature phenotype and allowing more precise disease modeling. (Machiraju and Greenway 2019)

2.2.5 Regenerative medicine

Regenerative medicine is a new, developing and interdisciplinary field of medicine focusing on repairing or replacing injured or degenerated tissues after cellular transplantation. New tissues can be generated in the laboratory with the help of iPSCs and then transferred to the site of injury. (Mahla 2016; Mao and Mooney 2015; Singh et al. 2015) Researchers have studied new alternative methods alongside the organ transplantation due to the lack of organ availability and observed difficulties with immune rejection. iPSC-based replacement methods have several advantages compared to allogenic donor sources of tissues. The ability of iPSCs to self-renewal and differentiate into almost any cell types in the human body enables the replacement of dysfunctional cells. (Yu et al. 2008) In addition, patient specific iPSCs treatment is free of the risk of transmitted donor-derived diseases (Sackett et al. 2016).

The recent innovations in genome editing technology now enables the introduction of genetic changes into iPSCs in a site-specific manner. The repairing of disease-causing gene mutations in patient-derived iPSCs can be done by the researchers. iPSC-based cell therapy utilized genetically healthy human iPSCs lines generated in the laboratory. Recent genome editing methods also permit the precise introduction of specific mutation into non-disease affected wild type (WT) iPSCs for generating isogenic iPSC lines. (Omole and Fakoya 2018) Isogenic cell lines can be used in modelling of diseases of interest, to understand the cellular effects of disease

mutations, screening of pathological mechanisms of the disease to develop more efficient medication to prevent or treat studied diseases (Bassett 2017; Omole and Fakoya 2018).

Efficiency and easy of accurate gene editing in the human genome have significantly enhanced due to the genome editing techniques involving zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and more lately, clustered regularly interspaced short palindrome repeats (CRISPR). Genome editing methods have usually been associated with disease modeling due to the ability of generating mutations or production of the isogenic control cell line using patient derived iPSCs. IPSCs-based cell therapy combines the iPSC platform with genome editing methods for creating a powerful tool for a therapeutic approach to treat human genetic diseases. (Ho et al. 2018; Omole and Fakoya 2018)

2.2.6 Genome editing in hiPSCs

There exist two main methods for genetic modifications. Random insertion refers to nonspecific insertion of genetic material into a host genome and site-specific targeting methods based on the insertion of genetic material in a specific locus. In addition, widely used retroviruses vectors, lentiviruses vectors and transposons are also successfully utilized in random insertions. Random insertion method has several deficiencies. The risk of inducing mutagenesis exists by inserting segments in the random position of the genome, additionally the expression level of host cell genes compared to randomly inserted genes may vary a lot. Site-specific integration is an ideal method overcome these challenges offering greater stability and allowing controllable and reproducible insertion of DNA. (Chang et al. 2018)

Recent genome editing techniques are based on the use of site-specific nucleases (SSN) that are able to create a double strand break (DSB) at desired position in the genome (Gaj et al. 2013). Non-homologous end joining (NHEJ) and homology directed repair (HDR) are two main endogenous pathways to repair damaged DNA. Genome-editing technology has become a powerful tool to introduce site-specific modification in the genome due to the ability to efficiently introduce a DSB at a target site. The repairing method of NHEJ is based on the re-ligating the broken DNA strand directly or inserting or delating bases at the ends of the break without the homologous DNA template. DSB ends are combined in an error-prone process, which results the formation of indels (insertions and deletions). In contrast, HDR always occurs

in the presence of homologous DNA template to direct a precise repair. (Shrock and Güell 2017)

Currently, two commonly used SSNs for genome editing are ZFN and TALENs (Chang et al. 2018; Singh et al. 2015). ZFNs and TALENs ability for gene editing is based on the formation of functional dimeric nuclease through the combination of sequence-specific DNA-binding domains and a nonspecific DNA cleavage domain, Fok1. Even if ZFN and TALEN are effective tools for genome engineering, the disadvantages of these methods are the need to reconstruct each editing site and complex to use for multiple genome edits. CRISPR technology has overcome these disadvantages and considerably enhanced the simplification and ease of genome editing. (Diecke et al. 2014; Parekh et al. 2017)

2.3 CRISPR method

2.3.1 History of CRISPR

CRISPR is an adaptive immune system of many bacteria and most archaea against invading genetic elements, such as plasmids and viruses (Barrangou and Marraffini 2014; Hryhorowicz et al. 2017). More than 30 years ago, in 1987 the typical repeats of CRISPR system were detected at the first time in Escherichia coli (E. coli) bacteria by Japanese biologist and scientist Yoshizumi Ishino. While studying the gene sequence coding an alkaline phosphatase enzyme that is responsible for conversion of aminopeptidase, he detected unusual repeat sequence downstream of the gene. (Ishino et al. 1987) In addition, to E. coli strains, analogous repetitive regions have also been observed in other enterobacteria (e.g. Shigella dysenteriae and Salmonella enterica) that are closely related to E. coli (Ishino et al. 1987).

Six years later, in 1993 archaeal CRISPR repeats were observed by a Spanish microbiologists Francisco JM Mojica. Mojica and his colleagues were studying the impact of salinity on the growth of Haloferax mediterranei, which is a species of halophilic archaea found principally in the Mediterranean Sea. They detected a long DNA sequence in the genome of Haloferax mediterranei that was comprised of regularly spaced repeats. Instead of observed similarity between regularly spaced repeats, no sequence similarity in E. coli and Haloferax mediterranei was detected. (Mojica et al. 1993) Due to these findings numerous sequences of archaea and bacteria were analyzed. The results revealed that unusual repeat arrangement is common in

prokaryotes. According to estimates CRISPR loci exist in nearly 90 % genomes of archaea and 40 % genomes of all bacteria. (Han and She 2017; Jansen et al. 2002; Touchon and Rocha 2010) Ruud Jansen with his colleagues proposed the term CRISPR in 2002. Due to the wide collection of genomic sequences, researchers were able to study and compare the genomic arrangement of CRISPR locus in various organisms. Researchers observed four conserved genes that often present nearby CRISPR region. These CRISPR-associated genes were named Cas genes 1 to 4. At the same time the foreign origin of spacer was observed as well as identification of the so called protospacer adjacent motif (PAM) element. (Jansen et al. 2002)

The significant observation about spacer regions containing the homologous sequences with bacteriophages, prophages and plasmids indicated that the plasmids and phages are not able to infect host strains containing the homologous spacer sequences in the CRISPR. After a comprehensive analysis, scientist deduced that acquired immunity system of prokaryotes is a result of collaboration of CRISPR and Cas proteins (products of Cas genes). This immunity system protects bacteria and archaea against invading exogenic elements in the same way that the RNA interference (RNAi) does eukaryotic system. (Bolotin et al. 2005)

The function of the CRISPR repeats remain the mystery until 2007. The scientist Rodolphe Barrangou and his colleagues confirmed the defense mechanism of CRISPR system for the first time demonstrating that Streptococcus thermophilu*s* bacteria can gain adaptive immune system against a bacteriophage by integrating a DNA sequence of an infectious virus into its CRISPR locus. By this way bacteria can generate a cellular memory of past invaders. This study provided the important evidence that similarity between spacer and protospacer sequences is a key for CRISPR immunity. (Barrangou et al. 2007)

2.3.2 CRISPR mechanism

The immune system is a defense system of the host containing several biological components and processes, which are able to protect against the pathogens. The immune system can be divided into two overlapping mechanism; innate and adaptive immune systems. Non-specific innate immune response is the first line defense mechanism against invading pathogens, containing of physical, chemical and cellular defenses. On the other hand, second line defense mechanism is called as specific adaptive immunity, because of the ability to recognize specific features of pathogens. (Chaplin 2003; Rath et al. 2015)

The discovery of an adaptive immune system in prokaryotes was unexpected due to the complexity of that system. The immune defense system of prokaryotes is based on the different regions of DNA termed as CRISPR. (Rath et al. 2015) The CRISPR locus contains a DNA control component (Leader), which follows an array of short (23 – 47 bp), highly conserved and identical repeat sequences called as repeats and the foreign, invader derived DNA sequences called as spacers (Figure 3). This foreign DNA is usually derived from plasmids and viruses, which refers to CRISPR´s part of an anti-virus system. New and different viruses are able to be recognized by transferring new spacers to the CRISPR locus. Typically, new spacers are placed in a chronological record at the one side of CRISPR locus reflecting the previous virus infections of the organism. (Richter et al. 2012) A set of genes that are able to code CRISPR-associated (Cas) proteins, crucial to the immune response, are usually placed next to the leader sequence (Rath et al. 2015; Touchon and Rocha 2010; Wei et al. 2015).

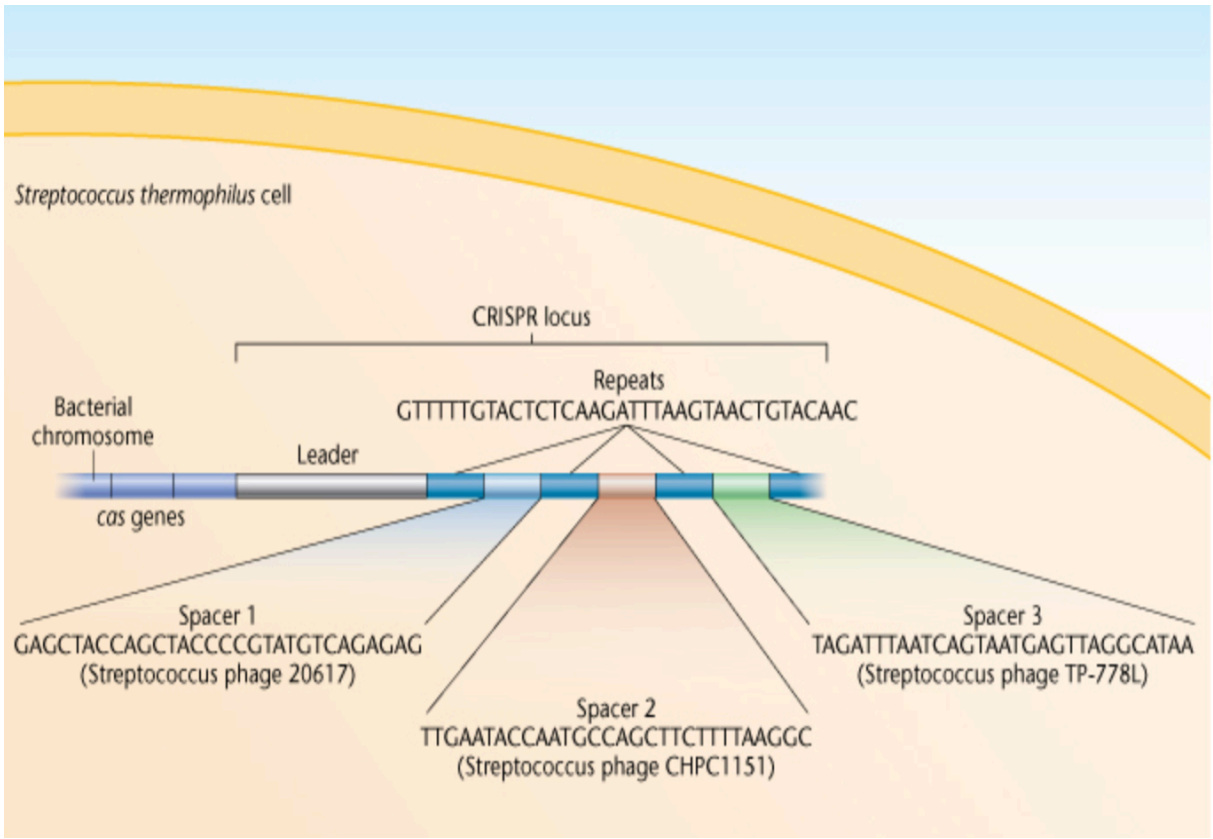


Figure 3: The typical structure of a CRISPR locus. Cas genes are located next to the leader region and are crucial for the function of CRISPR system. The CRISPR locus contains an array of alternating spacers sequences and palindromic repeat sequences. Repeat sequences are usually identical and highly conserved, whereas spacer sequences include DNA from foreign invaders serving the molecular memory of previous invaders. Figure modified from https://www.increiblefotos.com/imagenes/memory-cell-attacking-bacteria-e7.html; 15.6.2019.

CRISPR immunity system consists of a few distinct phases. First of them is an adaption or acquisition stage, second crRNA biogenesis and third is called as interference with invading DNA (Figure 4). During the first stage, Cas nuclease cut the invading virus or plasmid DNA into small pieces, called as protospacer sequences. Short DNA sequences, new spacers are inserted into the CRISPR locus of the prokaryotes. The protospacers selection depends on the part in the specific recognition of protospacer adjacent motifs (PAMs) present within the viral genome. Protospacer sequence, which is inserted into the CRISPR locus does not include PAM sites. (Rath et al. 2015)

At the second phase, the CRISPR locus is transcribed producing a long RNA molecule called as Pre-CRISPR RNA (Pre-crRNA). Trans–activating CRISPR RNA (tracrRNA) refers to a second RNA from a genomic locus upstream of the CRISPR locus, which has been also transcribed. The tracrRNA combines with the newly transcribed pre–crRNA generating a double-stranded RNA, which is then cleaved by RNaseIII, which results in the formation of a crRNA:tracrRNA complex. This complex combines with a single or multiple Cas proteins to form an active ribonucleoprotein complexes (RNP). At the final stage (interference phase) RNP is able to recognize and form specific base pairs between invader's DNA and RNP, which lead to the cleavage of invading nucleic acid sequences (interference). If there exist any differences between target DNA and spacer or if PAM contains any mutations the cleavage process does not occur. (Parekh et al. 2017; Rath et al. 2015; Thurtle-Schmidt and Lo 2018)

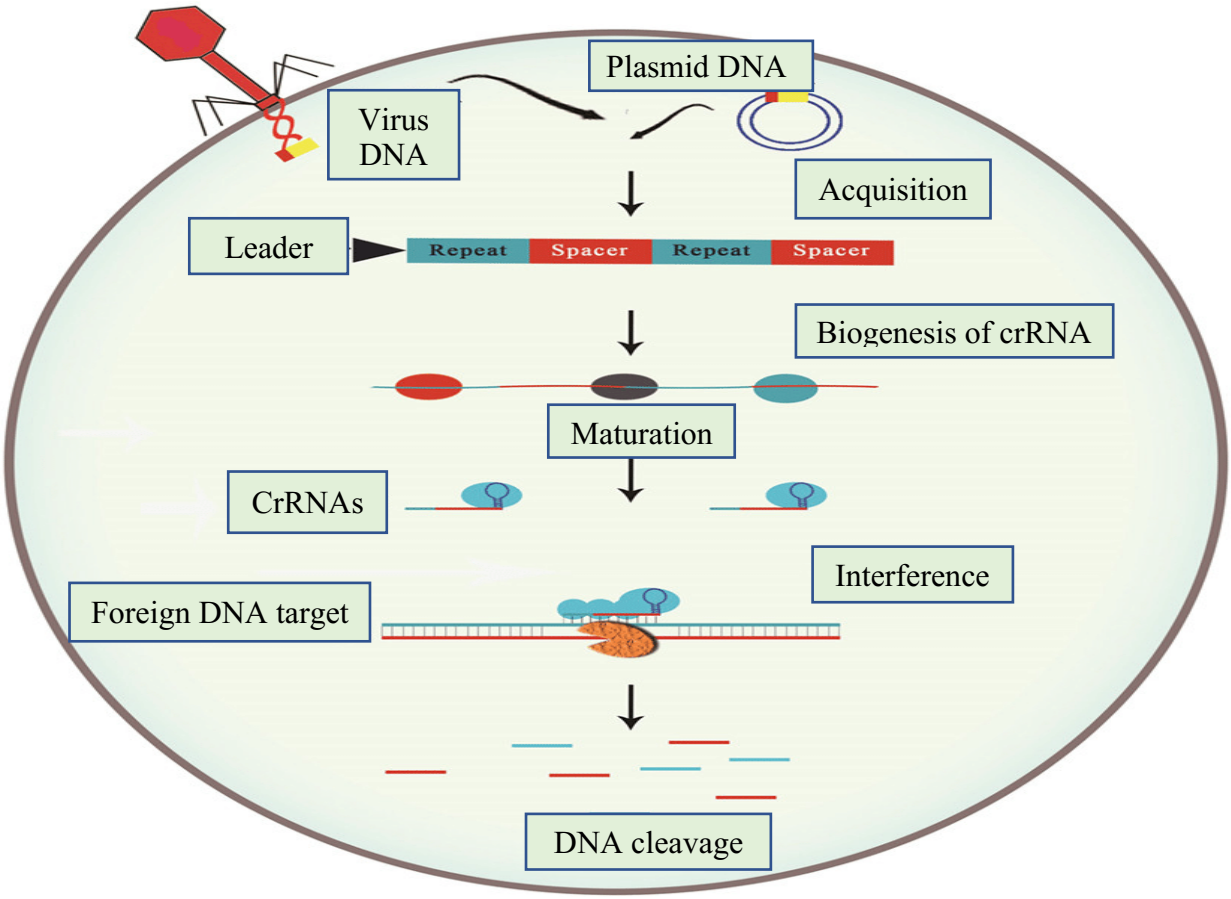


Figure 4: Three major phases of CRISPR immunity system. During the adaption phase, invading foreign DNA is cut and inserted into the CRISPR locus. In the second phase (crRNA biogenesis) CRISPR locus is transcribed into Pre–CRISPR RNA. At the final stage crRNAs guide the Cas proteins to recognize the foreign DNA sequence and the cleavage is triggered by nuclease enzymes. Figure modified from Shabbir et al. 2016.

CRISPR/Cas systems in bacteria and archaea are classified into three categories based on the differences between the structure and the sequence of Cas proteins. Each CRISPR type can be divided into several subgroups. Types I and III are not used in genome editing due to the complicated mechanism of immunity. Whereas type II is commonly used for genome editing due to its straightforwardness. (Thurtle-Schmidt and Lo 2018) Different types of CRISPR system can be recognized according to the presence of Cas genes. The type I system is identified by the existence of Cas 3 gene with DNase and helicase domains, which together are responsible for destroying the target. Six subtypes of the type I system are defined at the moment. The type II CRISPR system is able to encode Cas proteins 1, 2 and 9. Cas9 works and assists in adaption phase, attends in crRNA processing and is able to cleave the target DNA. Three subtypes (II-A, II-B and II-C) are recently identified. The type III system contains Cas10 gene, whose function is unclear. (Rath et al. 2015)

2.3.3 Targeting of iPSCs with CRISPR/Cas9 system

After the significant discovery of CRISPR immune system in prokaryotes, scientists started to study the potential opportunities to exploit CRISPR for precise genome editing in eukaryotes (Thurtle-Schmidt and Lo 2018). Cell-intrinsic DNA repair mechanisms are activated after the Cas9 nuclease generates a double strand break at the desired sequence site. Lack of a homologous sequence, which usually provide a template for repairing process, diverse repairing pathways containing single-strand annealing, microhomology-mediated end-joining (MMEJ) or NHEJ are activated as a result of DSB. NHEJ repairing pathway is the most common repairing pathway that is activated by the CRISPR/Cas9 system. (Parekh et al. 2017; Thurtle-Schmidt and Lo 2018)

Various errors (mutations) may occur during NHEJ pathway leading to the continuous activity by Cas9 nuclease. As a result of generated point mutations or indels (insertions or deletions) shifts in reading frame and disruption in expression of proteins may occur. NHEJ pathway can be utilized to study the function of various genes in a specific process by using multiple sgRNAs targeting different genes simultaneously. In addition, NHEJ pathway can be used to remove larger sections by using two sgRNAs targeting sequences that can flank the section to be removed. (Parekh et al. 2017; Thurtle-Schmidt and Lo 2018)

Alternatively, the DSB can be repaired through the HDR pathway. HDR utilizes a homologous template to complete the repair. This pathway utilizes exogenous templates for the introduction of precise genomic alterations. Template functions as a model for correction of the mutant gene or generating of an isogenic cell line. CRISPR/Cas9 editing through HDR is used to generate of point mutation and small alterations in the genome by insertion of single strand DNA template or plasmid donor vectors, along with plasmids for sgRNA and Cas9 expression. The insertion of larger sequences can be done by employing donor templates with larger homology arms, even if the efficiency remains low using hiPSCs. (Parekh et al. 2017; Thurtle-Schmidt and Lo 2018)

HDR based methods are beneficial for different application with patient-derived iPSCs including the correction of disease associated mutations and studying alongside with the mutant counterpart or the potential source for *in vivo* cell-replacement therapies. Through HDR modification hPSC lines can be engineered with stable expression of drug and antibiotic selection markers, fluorescent proteins and so on. In addition, it is possible to create reporter

cell lines by using different cell lines during the hPSCs differentiation process. (Parekh et al. 2017; Thurtle-Schmidt and Lo 2018)

2.3.4 Design of gRNA

Currently, there are several guidelines for the creation of an efficient guide RNA with less off-targets. The following facts have to be taken into consideration while designing a gRNA. In the case of SpCas9 (Streptococcus pyogenes Cas9), the target region or gene (usually 20 nucleotide) of the gRNA have to be located nearby to a PAM sequence 5'-NGG-3' where "N" refers to any nucleobase followed by two guanine nucleobases (Figure 5). Next, the genomic region containing a desired mutation have to be identified for the generation of selected 20-nucleotide target region that is located nearby to a PAM site. For achieving the optimal results, the PAM site should be located as close to the site of the desired mutation as possible. *(*Parekh et al. 2017; Thurtle-Schmidt and Lo 2018)

	Sequence to be targeted **PAM**	
5′	TCTCAGTGAGAAGCGCACGCTGG	3′
3	AGAGTCACTCTTCGCGTGCGACC	5′

Figure 5: 20 nucleotide sequence of a targeted genomic locus used in this study and located next to the PAM (5'-NGG-3') site.

Different computational CRISPR design tools and software are available to assist the design of gRNA. Online design tool can easily verify the specificity of a target sequence for reducing off-target effects. The low specificity of target sequence can result in the unwanted binding and cutting of Cas9 that could confuse experimental results. In addition, the efficiencies of gRNA can vary a lot for unknown reasons, but the presence of A or G nucleotides at the 3′ end of the 20-nucleotide target may increase the effectiveness. (Thurtle-Schmidt and Lo 2018)

It is essential to consider intended application, e.g. knockout via non-homologous end joining (NHEJ), CRISPR activation (CRISPRa), knock-in or CRISPR interference (CRISPRi) while designing of gRNA. Applications have different limitations and requirements, for example suitable position of gRNA for CRISPRa or CRISPRi applications can be different than the appropriate position of gRNA planned to be used in creation of a knockout or knock-in allele.

Further, it is recommended to design several different gRNAs for a single target. For the designing of effective gRNA, it is vital to exclude the terminator sequence for the promoter from the gRNA sequence. (Mohr et al. 2016)

2.3.5 Off-target effects

Nonspecific and unwanted genetic modifications, which may occur through the use of CRISPR/Cas9 method are called off-target genome editing. Commonly used CRISPR/Cas9 nucleases may induce off-target mutations at the genomic sites, which resembles the intended target sequence. Genome editing performed with RNA-guided endonucleases (RGEN) is simple, fast and enables the effective modifications of endogenous genes in wide range of essential types of cells that have been generally difficult to genetically engineered. (Sander and Joung 2014) On the other hand, RGEN have a very high frequency of off-target activity, which refers to possibility to induce mutations at undesired locations. These off-target effects are one of the major cause of concern for potential use of CRISPR in therapeutic applications. (Kleinstiver et al. 2015; Zhang et al. 2015)

To overcome the challenges related to off-target genome editing, researchers have started to study different approaches to reduce RGEN off-target effects. Streptococcus pyogenes Cas9 is the most commonly used nucleases to reduce off-target cutting. First method utilizes the modified gRNA sequences to reduce off-target effect. Sequences of gRNA have been shortened at the 3′ ends or two extra guanine nucleotides have been attached to the 5′ end. These shortened gRNA are called truncated gRNAs (tru-gRNAs), consisting of 17-19 nucleotides instead of general amount of 20 nucleotides. Tru-gRNA has been observed to function as efficiently as full-length gRNAs but expressing reduced mutagenic effects at off-target sites. (Sander and Joung 2014; Zhang et al. 2015)

Second, possible approach for decreasing off-target effect is to reduce the concentrations of the Cas9-sgRNA complex. A balance between off-target effects and on-target cleavage efficiency is essential, because decreased amount of transfected DNA may increase the specificity, but it causes also a drop in on-target cleavage. (Sander and Joung 2014; Zhang et al. 2015) The third strategy for enhancing the cutting specificity is called paired nicking, containing the mutant version (D10) of Cas9 paired with two sgRNA enabling to cut only one DNA strand. Compared

to previous approach this method does not sacrifice on-target cleavage activity and the reduction in off-target activity is significant. (Sander and Joung 2014; Zhang et al. 2015)

The fourth approach for improving the specificity of DNA cleavage is the generation of a fusion protein of catalytically inactive Cas9 and FokI nuclease (fCas9). DNA cleavage by fCas9 demands association of two fCas9 monomers that bind at the same time to target sites. While this method improves specificity and reduces off-target cutting, the number of potential target sites is lower due to PAM and other sgRNA design restrictions. (Sander and Joung 2014; Zhang et al. 2015)

One of the major challenges in the genome editing field is a highly sensitive and versatile detection of off-target sequences. T7 endonuclease I assay was the first method, which was used to identify off-target mutations. The limitations of this assay are high-cost in large-scale screening and poor sensitivity. Currently, more sophisticated methods for detecting off-targets have been developed and widely accepted, containing deep sequencing method, ChIP-seq and web-based prediction tools. On the contrary, while using the modified sgRNA, on-target genome editing has been detected to decrease. (Zhang et al. 2015)

2.3.6 Advantages and disadvantages of CRISPR

Simplicity, efficiency and low-cost are advantages of CRISPR method compared to other available genome editing technologies (Khan 2019). Another benefit of CRISPR method is the possibility to alter specific genomic locus, in other words, perform targeted genome editing in a wide range of organisms by the creation a double-stranded break at the genomic locus to be modified (Sander and Joung 2014).

Nowadays CRISPR method is utilized in the treatment of genetic disorders in animals, but in the near future it will be used for curing human diseases caused by a single point mutation. Recently, the ability of CRISPR/Cas9 to modify pathogenic genes to treat inherited diseases has been proven and reported. The potential of CRISPR/Cas9 can be expanded for prevention and treatment of diseases that contains the modification of somatic cells. Recently-approved clinical trial presented the method whereby genetically modified cells of the immune system were used as a cancer therapy in cancer patient. Currently, CRISPR technology is used in a number of human clinical trials against the cancers of renal, lung and prostate. In addition,

genome editing capacity of CRISPR is also applied in the treatment of primary infection of HIV by destroying the CCR5 co-receptor. (Kang et al. 2017; Tebas et al. 2014)

Significant finding has been recently done by two research groups demonstrating that homologous recombination alongside CRISPR/Cas9 method can be applied to correct the genome of early human tripronuclear (3PN) zygotes and 2PN zygotes. These findings provide the potential opportunity to prospective parents, who are carrying genetic mutations that will lead to diseases, through preventing the transfer of these mutations to their children. (Kang et al. 2017; Tang et al. 2017)

In addition, CRISPR technology has opened up a wide range of potential applications that can be utilized across diverse fields such as biotechnology, medicine and agriculture. Despite the great potential of CRISPR technology, there exist several disadvantages related to CRISPR method including various ethical concerns that have to be addressed. One of the issues is the big size of the Cas9 protein. The difficulties and challenges may occur while delivering large size of Cas9 molecule via viral vectors or as an RNA molecule. Adeno-associated viruses (AAV) are ideal for somatic gene therapy due to the several reasons. They are able to target cells, which are not dividing, they are not pathogenic and additionally they are able to induce week and mild immune response. The size limit of cargo for AAV is less than 5 kb meaning that AAV is not able to accommodate Cas9 complex. (Gupta and Musunuru 2014; Kang et al. 2017)

Off-target effects related to use of CRISPR/Cas9 system is one major concern. Genomes may include identical DNA sequences with target sequence, which lead to non-specific cutting induced by Cas9 at undesired positions of the genome, resulting in the development of new mutations. Depending on where the mutation is formed, it can cause the cell death or transformation. Different strategies have been developed to reduce the effect of off-targets and several of these are listed in chapter 2.3.5 (off-target effect). (Gupta and Musunuru 2014)

Last presented obstacle is genetic mosaicism, which refers the presence of more than one genotype in one individual. Mosaicism can be caused by natural (e.g. mutation) or by manipulation factor (e.g. genome editing) (Mehravar et al. 2019). Despite the introduced challenges related to CRISPR-mediated genome editing, this technology has shown fast-paced progress with significant achievement (Kang et al. 2017).

2.3.7 Future perspective

In a short period of time CRISPR/Cas9 system has managed to become one of the leading and most significant scientific discovery in the field of genome engineering. Scientists from around the world have realized the potential of CRISPR/Cas9 system. Even though CRISPR enables the tailoring of medical treatment to the individual characteristics of each patient (personalized medicine) and holds great promises for the cure of complex genetic diseases that currently lack efficient treatment. There still exist many challenges and ethical concerns that have to be resolved before considering the use of CRISPR in clinical and therapeutic applications. (Lino et al. 2018)

One of the problems is unwanted off-target cutting. Different strategies have been developed to reduce this non-specific DNA binding, containing Cas9 nickases and mutants. Different software have been developed to design the structure of sgRNA and predict the potential binding site, despite this, understanding of off-target effects remain poor. (Lino et al. 2018) This branch of CRISPR needs new and continued studies to improve the specificity and understanding of off-target mechanism.

Another obstacle related to use of CRISPR/Cas9 system is gene cargo delivery system. Several different delivery systems have been evolved, but an all-purpose system is still under progress. Every system has both advantages and disadvantages. Currently, there exists more delivery choices for small-molecule cargo systems compared to larger molecule complexes. In the future new and more effective delivery systems for RNP will have significant effect to the field. Additionally, researchers will be studying alternative options that ensure the specificity and safety of delivery systems. A delivery system that enables high-specificity targeting in the desired cells will also reduce off-target effects and increase safety. Currently, there exists limited amount of information related to long term exposure of nanoparticles delivery systems that are transported in the body. Long term studies for safety assessment and evaluating the toxicity are necessary area of study. (Lino et al. 2018)

Despite the described disadvantages, CRISPR/Cas9 system offers an exciting new potential for therapeutic applications. The field of CRISPR has developed at an astoundingly fast pace and comprehensive research has been done on the altering of cell line genotypes and phenotypes using this gene editing system. However, scientists are still in the earliest stages of exploiting

the full potential of CRISPR/Cas9 system. In the future, if CRISPR technology develops into a highly precise and efficient genome editing tool with increased overall safety, it will be an extremely versatile technology that could be a huge benefit for humankind. (Lino et al. 2018)

2.4 Differentiation of hiPSCs into cardiomyocytes

Cardiovascular disorders are the leading reason of mortality in worldwide. Human cardiomyocyte sources are restricted due to lack of donors as well as the limited proliferation frequency of adult cardiomyocytes. Due to this fact alternative sources of human cardiomyocytes for regenerative therapies, disease modeling as well as drug toxicity studies are extremely desired. iPSCs hold great potential for offering an unlimited source of cardiac cells by reprograming adult somatic cells into iPSCs and after that differentiating these into cardiomyocytes. (Batalov and Feinberg 2015) At the first time in 2009 the successful differentiation of hiPSCs into cardiomyocytes *in vitro* was reviewed. The scientists observed the similarities between cardiomyocytes derived from human iPSCs and ESCs. Expression patterns of cardiac genes, sarcomeric organizations and proliferation resembled each other. (Csöbönyeiová et al. 2015)

Numerous different protocols for cardiac differentiation from human induced pluripotent stem cells are available nowadays. Currently, differentiation methods can be classified into three different categories. The 2D monolayer culture system, the embryoid body (EB) differentiation in suspension and co-culture with mouse endoderm-like cells (END-2 cells) are widely used (Csöbönyeiová et al. 2015; Mummery et al. 2012). EB differentiation method is based on the physical and chemical signals that modify pathways of cell signaling and induce differentiation of iPSCs into cardiomyocytes. EBs consist of spherical aggregates of PSCs that resemble early embryonic development. The differentiation efficiency of PCS into cardiomyocytes varies a lot. The percentage of EBs containing beating cardiomyocytes may range between 5-70 % depending on selected protocol and differentiation method. (Batalov and Feinberg 2015) Other disadvantages related to EB method are immature cardiomyocyte phenotype and the production of cardiomyocytes usually remains poor (< 1 %). Due to these inconsistent and unreliable results, researchers have evolved a monolayer method to overcome these challenges. (Ban et al. 2017)

Monolayer differentiation method depends on various growth factors and small molecules, which trigger differentiation down the cardiac lineage. The main difference between EB and monolayer differentiation is that cells grow in 2D monolayer instead of 3D culture. Furthermore, monolayer method possesses many advantages compared to EB method including more mature cardiomyocytes, subtype specification and increased yield of cardiomyocytes (85-95 %). (Batalov and Feinberg 2015)

The inductive culture system is based on the idea of co-culturing mouse endoderm-like cells (END-2 cells) with pluripotent stem cells. The END-2 cells are extracted from P19 Mouse embryonal carcinoma. (Rajala et al. 2011) The END-2 cells can produce different signaling molecules that induce the iPSCs differentiation into cardiomyocytes. A couple of disadvantages have been perceived by using this method, including a low cardiomyocyte yield (less than 10 %, but usually around 1 %) and additionally an immature cardiomyocyte phenotype. Furthermore, the mechanism of differentiation isn't properly characterized resulting in the production of unknown factors by the END-2 cells. (Batalov and Feinberg 2015) On the other hand, it has been shown that the addition of ascorbic acid promotes the cardiac differentiation and improves the efficiency of differentiation along with serum-free conditions (Passier et al. 2005; Rajala et al. 2011). Over 20 % improvement in the efficiency of cardiomyocyte yield has achieved by adding of p38 MAPK inhibitor. Simplicity and low costs are the benefits of END-2 cell co-culture methods. (Batalov and Feinberg 2015)

2.5 Dilated cardiomyopathy

Cardiomyopathies refer to intrinsic diseases of heart muscle in which the structure and functionality of the heart is abnormal. Morphologically described subtypes contain hypertrophic (HCM), dilated (DCM), arrhythmogenic (AC) and left ventricular noncompaction (LVNC) cardiomyopathies. (McNally and Mestroni 2017)

DCM is a disease of the heart muscle characterized by an enlarged and poorly contractile left ventricle (LV) and/or reduced systolic function. DCM can be due to genetic and nongenetic reasons containing valve disease, hypertension, inflammatory/infectious causes and toxins. It has been shown that an individual's genetic profile may also influence to these "nongenetic" forms of cardiomyopathy. (Lakdawala et al. 2013; McNally and Mestroni 2017) DCM is the most common form of cardiomyopathy among children and adults. In Finland the prevalence

of DCM cases 1:2500 has been registered. Furthermore, DCM is the main reason for heart transplantation and a comparatively common cause for heart failure and sudden cardiac death. (Akinrinade et al., 2015)

At the moment, more than 40 genes have been classified as causative gene related with hereditary DCM. The main purpose of those genes is mainly encoding two major cardiac proteins, called as sarcomere and cytoskeletan. Genes MYBPC3, LMNA, and MYH7 were the most often identified ones. (Park 2017) Mutations in the LMNA gene causes around 6 % of all the DCM cases (McNally and Mestroni 2017; Tesson et al. 2014). LMNA mutations are inherited most commonly in an autosomal dominant manner, even if autosomal recessive, X-linked, and mitochondrial inheritance are also studied and reported (Tayal et al. 2017).

LMNA gene is located on chromosome 1q11-q23 and it is comprised of 12 exons (Taylor et al. 2003). The first ten exons are identical between intermediate filament proteins lamins A and C. LMNA gene can encode lamin A and lamin C by alternative way of splicing in exon 10. The first 566 amino acids are the same, but the C-terminal sequences are different due to the splicing process. (Florwick et al. 2017) Lamins A and C are involved in various cellular processes, including regulating gene expression, mechanosensing, DNA replication and also play a vital role in cytoplasmic transportation. Exons 11 and 12 are unique to Laminin A. Laminin A is associated with various types of human diseases, which are classified into primary and secondary laminopathies. Mutation on laminin A results in primary laminopathies while secondary laminopathies are caused by mutation occurred in laminin B. (Kang et al. 2018)

3 Aims of the study

The main aim of this thesis work was to precisely correct a point mutation in human iPSC line using genome editing method called as CRISPR/Cas9 system. UTA.12619.LMNA patient cell line (p. S143P mutation in LMNA gene) was used to obtain the isogenic hiPSC line. The main aim can be divided into intermediate goals.

The first goal of this project was to study and engineer all the plasmids–Cas9 plasmid, sgRNA plasmid and the donor plasmid carrying the HDR DNA template, which were necessary to perform genome editing by CRISPR/Cas9. HDR template included the corrected sequence and enabled the precise alteration in the genome at the DSB site.

Second aim was successfully delivering those plasmids and HDR DNA template into iPS cells using transfection method known as nucleofection and selection of positive clones by antibiotic treatment.

The last aim was to screen the potential positive clonal iPSC line and to validate the corrected isogenic hiPSC line by sequencing and differentiating into cardiomyocytes using small molecule differentiation method.

4 Materials and Methods

4.1 Cell lines

Cell line used in this study was newly reprogrammed from a female patient with cardiac disorder, carrying the p. S143P mutation in the LMNA gene. Patient-derived fibroblasts used in this study for iPSC induction were harvested from skin biopsies. Cell line UTA.12619.LMNA was produced by reprogramming the fibroblast with Sendai virus. The first three numbers of the cell line refer to ID code of patient and the following numbers refer to the used cell colony.

The Ethical Committee of Pirkanmaa Hospital District (R08070) approved the collection of skin biopsies used in this study for generating the human induced pluripotent stem cell lines and a written approbation was obtained from the patient.

4.2 Human iPS cell culture

iPS cells were cultured in feeder-free conditions using Geltrex matrix (Thermo Fisher Scientific, Waltham, Massachusetts, USA). iPS cells were grown on sterile six-well plates in 4 ml mTeSR1 (Stemcell Technologies, Waterbeach, Cambridge, UK) per well, half of the cell culture medium was changed every other day until the iPS colonies with defined edges and uniform morphology appeared.

After iPSCs reached the confluence of 70-90 %, passage was carried out. New Geltrex coated well-plates were prepared by diluting Geltrex into DMEM/F12 medium (Thermo Fisher Scientific) in the ratio of 1:100. iPSCs were detached from the bottom of the well-plate by using Versene (Thermo Fisher Scientific). iPSCs were treated with 1ml Versene per well for 5-10 minutes at +37 °C, 5 % CO_2. After incubation, cells were detached from each other (inspected with light microscopy). Versene was aspirated gently and iPSCs were suspended using fresh mTesr1 medium (Stemcell Technologies). Cell suspension was then transferred to new Geltrex coated well-plate.

The cells were kept in an incubator at +37 °C, 5 % CO_2 during cell culturing. The viability of iPS cells was verified every second day under a light microscope (Carl Zeiss Axiocam ERc 5s).

4.2.1 Freezing of iPSCs

iPSCs can be cryopreserved when the cells reach 80 % confluence. The culture media was aspirated, and cells were dissociated with Versene and incubated at +37 °C for 10 minutes. Versene was aspirated and 0.5 ml fetal bovine serum (FBS, Gibco by Life Technologies, Carlsbad, California, USA) was added to each well to harvest the cells. Colonies were dissociated by pipetting up and down to a uniform cell suspension. 50 µl of DMSO (Dimethylsulfoxide, Sigma Aldrich, Saint Louis, Missouri, USA) was pipetted to a 2.0 ml freezing tubes for preventing formation of ice crystals. 0.5 ml of cell suspension was quickly transferred to each labelled freezing tube. Freezing tubes were placed into a freezing container to achieve a cooling rate of 1 °C / min. Freezing container was then directly transferred to -80 °C isopropanol freezer for overnight. Next day freezing tubes were removed from the container and immediately placed in the liquid nitrogen storage tank.

4.3 HEK293 cell culture

Human embryonic kidney cells (HEK293 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10 % FBS, 1 % L-glutamax (Gibco by Life Technologies) and 1 % penicillin streptomycin (Pen/Strep, Sigma Aldrich). For cell quantification, mixed cell suspension (10 µl) was mixed with Trypan blue solution (10 µl) (Lonza) in an Eppendorf tube. Stained cells (10 µl) were pipetted to cell counter chamber and the number of living cells were reviewed using a countess II FL Automated Cell Counter device (Thermo Fisher Scientific). Cells with more than 90% viability were plated in a sterile Geltex-coated 12-well plate at the seeding density of 130 000 cells/well a day before transfection. After transfection, HEK293 cells were plated to a new 12-well plate. Trypsin (Lonza) was used to detach the HEK293 cells before harvesting. Cells were incubated with trypsin for 5 minutes at +37 °C, 5 % CO_2. HEK293 cells were maintained in an incubator at +37 °C and 5 % CO_2 during culturing.

HEK293 cells were harvested 72 h after transfection. Cells were detached with Trypsin and suspended in fresh DMEM-medium. Cell suspension was then gently transferred to a falcon tube. Cells were pelleted by centrifugation at 250 x g for 5 minutes at room temperature. After centrifugation, supernatant was discarded and 80 µl of Lysis buffer T1 (Macherey Nagel, Dueren, Germany) was added to the cell pellet for DNA isolation.

4.4 Functional validation of the sgRNA specificity

4.4.1 Fugene HD Transfection

HEK293 cells were transfected (process of introducing exogenous DNA/RNA into eukaryotic cells) using Fugene HD Transfection reagent (Promega, Madison, Wisconsin, USA) with Cas9 encoding plasmid and single quide RNA plasmid. The Cas 9 and sgRNA were delivered into HEK293 cells to induce DNA cutting and following NHEJ events. This step was performed in HEK293 cells for the observing of these NHEJ events.

HEK293 cells were plated a day before transfection in a way that cells are approximately 80 % confluent on the day of transfection. The optimal volumes of DMEM medium and plasmid DNAs (Cas9 + sgRNA) were calculated beforehand (Table 2). The ratio of Fugene HD Transfection Reagent to DNA used in this experiment was 3:1 according to Fugene HD Transfection Reagent user manual. In this study, the optimal amount of DNA was 2 µg and optimal volume of Fugene HD Transfection Reagent was 6 µl.

Plasmid DNA concentrations were measured with NanoDrop (Thermo Fisher Scientific) according to NanoDrop manufacturer instructions. For validating the specificity, four potential sgRNAs named as LG1-LG4 that were designed to target the S143P mutation in the LMNA gene were used in Fugene HD Transfection (Table 1).

Table 1: Designed sgRNA and PAM sequences

Sample	sgRNA sequence (5`to 3`)	PAM
LG1	TCTCAGTGAGAAGCGCACGC	TGG
LG2	GGCGAGCTGCATGATCTGCG	GGG
LG3	GCCGAGCCTGAGCAGCTATC	AGG
LG4	GCCGAGCCTGAGCAGCTATC	GGG

Table 2: FuGENE HD Transfection Reagents

Sample name	SgRNA (µl)	Cas9 (µl)	DMEM medium (µl)	Sample concentration (ng/µl)
LG1	7.34	3.52	89	109
LG2	9.31	3.52	87	85.9
LG3	5.11	3.52	91	156.6
LG4	7.58	3.52	89	105.6

On the day of transfection, the total volume of DNA mixture was diluted to DMEM medium in an eppendorf tube. Next, Fugene™ HD Transfection Reagent was added to the solution, mixed gently and incubated at room temperature for 12 minutes. After incubation, 100 µl of medium + Fugene transfection reagent + DNA mixture was added per well of a 12-well plate containing 1 ml DMEM growth medium. Cells were returned to the incubator for 72 h. In this study, a reporter gene, GFP (green fluorescent protein) that was expressed in Cas9 plasmid, was used to determine the percentage of cells that have received and were expressing the foreign DNA sequence. Transfection efficiency was evaluated under the fluorescence microscopy.

4.4.2 T7 Endonuclease 1 assay

Insertions and deletions may occur in the genome after NHEJ events. T7 endonuclease is a structure-selective enzyme, which can recognize and cleave these mismatches. T7 endonuclease 1 assay was used to detect the specificity of guide RNA mediated Cas9 gene editing. Cas9 plasmid together with each selected Guide RNA plasmid were transfected into the cells. The genomic DNA of each transfection was extracted and the DNA fragment surrounding the target locus was amplified by PCR. PCR product was then denatured and re-annealed, which allowed the mutant and wild-type strands to form heteroduplex DNA. Digested DNA products were analyzed by gel electrophoresis (Figure 6). The detection of cleaved products indicates the presence of a heteroduplex formed by a mismatch.

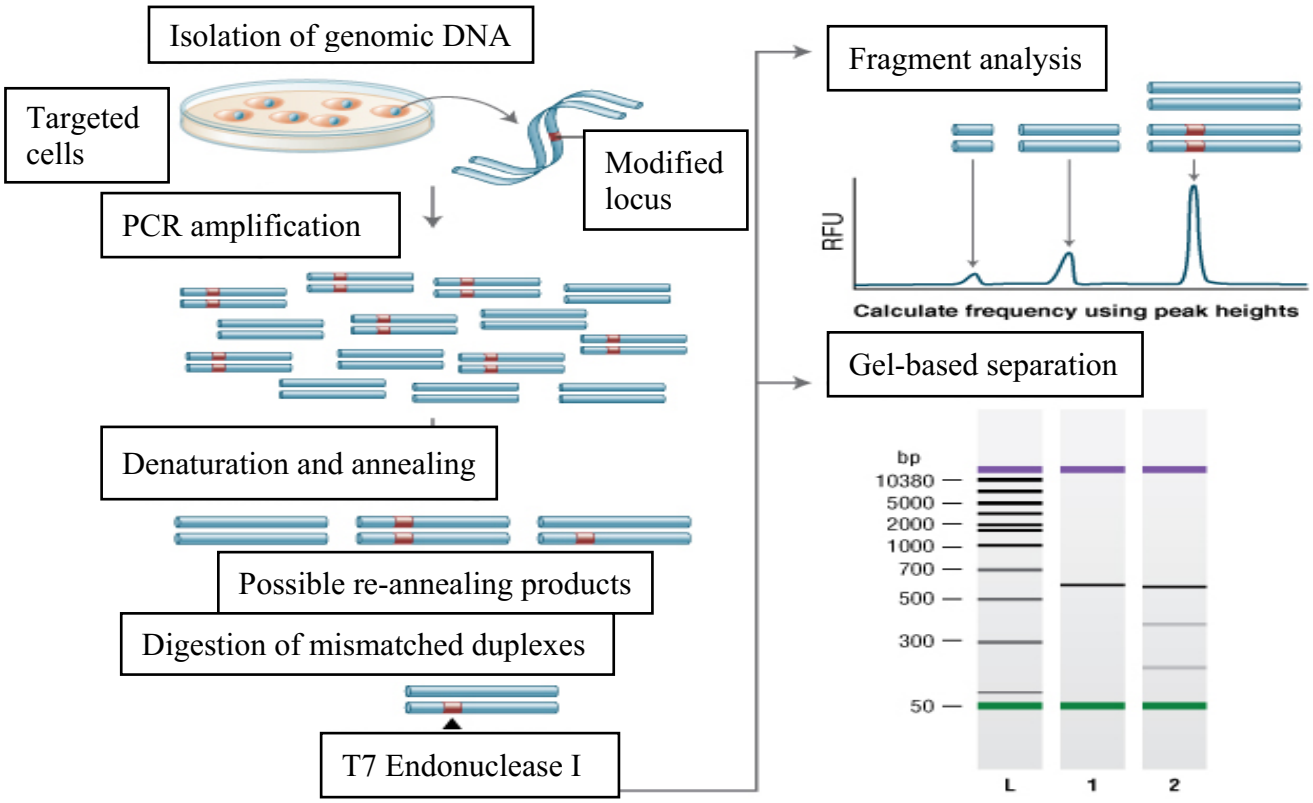


Figure 6: Method of T7 Endonuclease assay.
Figure modified from https://international.neb.com/applications/genome-editing/measuring-targeting-efficiency-with-the-t7-endonuclease-i-assay; 8.5.2019.

Genomic DNA from the transfected HEK cells was extracted using the NucleoSpin® Tissue kit (Macherey Nagel). DNA was isolated according to NucleoSpin® Tissue kit user manual. DNA concentration were measured with NanoDrop. Isolated DNA was eluted with 20 µl of elution buffer from the kit and stored at −20 °C.

Genomic target site was amplified by PCR using the reaction composition and conditions described below (Table 3 and 4). Primers (forward and reverse) were designed based on the target sequence. Master mix for LMNA samples consisted of component 1-6 shown in Table 4. The total volume of PCR mix used in this study was 50 µl. First master mix without the template was prepared on ice. Templates were added to the appropriate PCR tubes and PCR mixture was gently mixed by vortexing and briefly spinning to collect all components to the bottom of the tube. PCR tubes were transferred from ice to T100 PCR machine (BioRad) and PCR was performed using following thermal cycling conditions (Table 3).

Table 3: PCR program

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	2.5 min	1
Denaturation	95	0.5 min	30
Annealing	59	0.5 min	30
Extension	72	50 sec	30
Final Extension	72	10 min	1
Hold	4	∞	

Table 4: Reaction compositions in PCR using DreamTaq DNA Polymerase

Component	50 µl reaction	Final volume (µl)
1) 10X DreamTaq™ Buffer	5 x 5 µl	25
2) 10 mM dNTPs	5 x 1.2 µl	6
3) LMNA T7 forward primer 1	5 x 2.5 µl	12.5
4) LMNA T7 reverse primer 1	5 x 2.5 µl	12.5
5) DreamTaq DNA Polymerase	5 x 0.8 µl	4
6) Nuclease-free water	5 x 36.5 µl	182.5
7) Template DNA	1.5 µl	

Agarose gel electrophoresis was used for separating DNA fragments of varying sizes (PCR products). Agarose gel (1 %) was prepared in TAE-buffer beforehand. Gelred (Biotium, Fremont, California, USA) is a fluorescent nucleic acid dye used in this study for staining DNA instead of toxic ethidium bromide. First, the loading buffer (6 x DNA loading dye, Thermo Fisher Scientific) was added to each DNA samples. Loading dye helps to detect the rate of migration, and also allows the sample to sink into the gel. Then samples were carefully pipetted into the wells of the gel. GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific) was used as a standard. The gel was run at 95 V for 50 minutes. The bands were visualized with imagelab software (BioRad) and the images were captured using the ChemiDoc XRS+ (BioRad) system after the run.

PCR products were purified, and the DNA was extracted using Nucleospin gel and PCR clean-up kit (Machery Nagel). The DNA extraction was performed according to Nucleospin

Gel and PCR Clean-up kit user manual excluding a few exceptions. With the help of sterile scalpel DNA fragments were excised from an agarose gel and 700 µl of NTI buffer was added to the gel. The DNA was eluted in 20 µl of NE buffer from the kit.

4.4.3 T7 Endonuclease I digestion

DNA mix was prepared in PCR-tubes according to Table 5 for T7 digestion. Hybridization reaction was run in a PCR cycler according to the conditions presented in Table 6.

Table 5: DNA mix components for hybridization reactions

Component	19 µl Annealing reaction
Purified PCR product	200 ng
10 X NEBuffer 2	2 µl
Nuclease-free water	To 19 µl

Table 6: Hybridization conditions

Cycle step	Temperature (°C)	Ramp rate	Time (min)
Initial denaturation	95		5
Annealing	95-85	-2 °C/ second	
	85-25	-0.1 °C/ second	
Hold	4		

After hybridization, 1 µl of T7 endonuclease I was added to each annealed PCR products. The re-annealed PCR products were then digested at +37 °C for 15 minutes. Reaction was stopped by adding 2 µl of 0.25 M EDTA to the solution. For visualization, the digested products were run with 10 X DNA loading dye (Thermo Fisher Scientific) on a 2 % TBE agarose gel at 90 V for 60 minutes.

4.5 Plasmids for CRISPR/Cas9 genome editing

The CRISPR genome editing system consists of three plasmids that express the Cas9, gRNA and the donor plasmid providing the homologous template for correcting the mutation.

4.5.1 Quick change mutagenesis of DNA template

Quick change mutagenesis analysis was performed to insert an exogenous silent restriction enzyme site and also to mutate the PAM site in the donor plasmid template. The restriction site enables the screening of the positive clones and mutating the PAM site in the HR template avoids the donor plasmid being degraded by Cas9. This method is based on mutagenic primers (Table 7) that introduce the specific mutations in the donor plasmids. The mutagenic oligonucleotide primers, LMNA_ECOF and LMNA_ECOR could create a silent EcoR1 restriction site in the targeting location and the primers LMNA_PAMF and LMNA_PAMR could change the PAM site without disturbing the amino acid sequence.

Table 7: PCR primers

Reverse primer LMNA_ECO
AGTGCGGCCTCCTTGGAATTCAGCAGAGC
Reverse primer LMNA_PAM
TCATGCAGCTCGCCCTCGAGCGTGCGCT
Forward primer LMNA_ECO
TGGAGGCTCTGCTGAATTCCAAGGAGG
Forward primer LMNA_PAM
TGAGAAGCGCACGTCGAGGGCGAGCT

Reaction mixture for PCR was prepared in 500 µl PCR tubes as indicated below (Table 8). Pfu Turbo DNA polymerase was added just before the PCR reaction. PCR was performed using the cycling conditions outlined in Table 9. After PCR, 0.5 µl of DpnI (a methylation-dependent endonuclease) restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA) was added directly to each sample tube. Each reaction was incubated at +37 °C overnight to digest the parental supercoiled dsDNA.

Table 8: Reaction mixture for quick change-mutagenesis by PCR using Pfu Turbo DNA polymerase

Component	Amount per reaction (µl)
10 X Pfu reaction buffer	2.5
DNA template	0.5
Forward primer	1.0
Reverse primer	1.0
dNTPs mix	0.5
Pfu Turbo DNA polymerase	0.5
Distilled water (dH_20)	19.5
Total reaction volume	25

Table 9: PCR cycling parameters for Pfu Turbo DNA polymerase

Step	T (°C)	Time (min:sec)	Cycles
Initial denaturation	95	0:30	1
Denaturation	95	0:30	15
Annealing	55	1:00	15
Extension	68	7:00	15
Final extension	68	5:00	1
Cooling	4	Forever	

4.5.2 Transformation of XL1 blue supercompetent cells with DpnI- digested product

At the beginning sterile SOC medium (Appendix 9.3) was preheated to +37 °C and three LB-ampicillin agar plates were transferred to room temperature to dry. 30 µl of XL1 Blue supercompetent cells were thawed on ice. After that 1 µl of DpnI-digested product was added to the cells. Samples were gently mixed and incubated on ice for 30 minutes. Then the samples were treated by a heat pulse for 45 s at +42 °C and returned to the ice for 2 minutes. 500 µl of preheated SOC medium was added to samples. Tubes were gently inverted to mix and incubated at +37 °C for 1 hour with shaking at 225 rpm. 100 µl of cells were plated on 10 cm LB ampicillin agar plates and incubated at +37 °C overnight.

4.5.3 Plasmid DNA Purification

Next day, five potential colonies were picked from selective plates for inoculation. 5 ml of LB medium (containing 10 µl of ampicillin) (Appendix 9.2) was pipetted to bacterial culture tubes. Five single colonies were scraped from the agar plate using a sterile pipette tip. The tip was dropped into the culture tube and bacterial culture was incubated at +37 °C for 16 h in a shaking incubator at 225 rpm. Next day the growth of bacteria was observed by a cloudy haze in the media. Bacterial culture was harvested by centrifugation for 2 min at 8000 rpm at room temperature. Rest of the centrifugation were carried out at 13 200 rpm. Plasmids were purified using GeneJet plasmid Miniprep Kit (Thermo Scientific) according to the manual provided by the manufacturer.

4.5.4 Sequencing

Sequencing PCR was carried out using PTC-100 programmable thermal controller (MJ Research). Master mix for PCR was prepared in 500 µl PCR tubes according to Table 10. Template DNA was added to the individual tubes containing the reaction mix and then sequencing PCR was run with the cycling program presented in Table 11.

Table 10: Components for Sequencing PCR

Component	Amount per reaction
BigDye terminator sequencing buffer	1 µl
BigDye	1 µl
Primer	1 µl
dH_20	3 µl
DNA template	4 µl

Table 11: Sequencing PCR program

Step	T (°C)	Time (min:sec)
Initial denaturation	95	1:00
Denaturation	95	0:10
Annealing	50	0:10
Extension	60	4:00
Final extension	60	10:00
Cooling	4	Forever

PCR samples were removed from the thermal cycler and briefly span down. 1 µl 3 M Na-acetate and 22 µl EtaxA was added to each sample tube. Samples were incubated at room temperature for 15 minutes and centrifuged at 13 200 rpm for 20 minutes at room temperature. Supernatant was removed and pelleted DNA was washed with 200 µl of 70 % EtOH. Samples were centrifuged at 13 200 rpm for 5 minutes and supernatant was removed. Sample tubes were incubated for 10 minutes at +94 °C to dry. 15 µl of HI-DI enzyme was added to each tube and DNA was let to elute for 10 minutes at +94 °C. Samples were then sent for sequencing.

4.5.5 Endotoxin free plasmid purification for nucleofection

Inoculation was performed the day before the EF plasmid purification by pipetting of LB medium into the sterile separate Erlenmeyer flasks containing the appropriate selective antibiotics and bacterial cultures described in Tables 12 and 13. Flask were placed on a shaking incubator at +37 °C, at 225 rpm for 16 h.

Table 12: Inoculation for guide plasmid

Components	Amount
LB medium	150 ml
Kanamycin	200 µl
LG_2 guide plasmid	1 ml

Table 13: Inoculation for donor plasmid

Components	Amount
LB medium	150 ml
Ampicillin	150 µl
L_2 donor plasmid	1 ml

For pelleting, the cells were centrifuged at 5000 g for 10 minutes at +4 °C. Plasmid DNA for nucleofection was isolated with the NucleoBond Xtra midi EF kit (Macherey Nagel) as described by the manufacturer instruction. In the final stage (reconstitution) DNA pellet was dissolved in 150 µl of endotoxin-free buffer TE-EF instead of H_20-EF. DNA concentrations were measured using the Nanodrop.

4.6 Genome editing in human iPS cells

4.6.1 Nucleofection of LMNA cell lines

Genome editing plasmids were delivered into the human iPSCs using the P1 primary cell 4D-Nucleofector X kit (Lonza). Selected optimal gRNA was introduced along with Cas9 and donor (repair) template to drive HDR events. Cas GFP I- complex included a GFP-linked expression cassette for Cas9, which ensured that all necessary CRISPR/Cas components (e.g. Cas9 and gRNA coding sequences) are successfully delivered into GFP positive cells. Geltrex-coated 10 cm petri dishes were prepared one hour before nucleofection by diluting Geltrex 1:100 in cold DMEM and by covering the whole surface of culture dishes with Geltrex solution. After dissociating iPSCs into single cells, apoptosis is immediately triggered. The ROCK (Rho-associated protein kinase) inhibitor Y-27632 was added to the cell medium to prevent cell death.

For nucleofection, nucleofector solution P3 was combined with supplement according to the protocol (Lonza) (Figure 8, step 2). Nucleofection reaction was prepared by diluting and combining the DNA mixtures (presented in Table 14) in Nucleofector solution P3 (with supplement) to a final volume of 10 µl. Each nucleofection reaction included 2 µg of guide plasmid, donor plasmid and Cas9 expressing plasmid. For nucleofection, UTA.12619.LMNA iPSCs culture was detached by Versene treatment and suspended in mTesr1 media with rock inhibitor. The cell number and viability of the cell suspension was determined using a countess II FL Automated Cell Counter device. Recommended number of cells per nucleofector reaction

was 4-5 x 10^5. The required volume of cell suspension was centrifuged at 110 g for 3 minutes. Supernatant was carefully aspirated, and the cell pellet was resuspended in 10 µl of nucleofector solution P3. The DNA mix was mixed with the resuspended cells and the final 20 µl sample was pipetted to a nucleocuvette for nucleofection. Nucleofection program CB-150 was run (Figure 8, steps 2 and 3). Immediately after pulsing, 80 µl of prewarmed mTesr1 with rock inhibitor was added to the cuvette and resuspended. Each nucleofection reaction was transferred to own geltex-coated petri dishes containing 10 ml of mTesr1 with rock inhibitor (Figure 8, step 4). Cells were incubated 24 h at +37 °C, 5 % CO_2.

Table 14: Volumes of DNA mix for Nucleofection

Component	Volume (µl)
Cas GFP I	0.9
LG_2 guide plasmid	0.4
L2 donor plasmid	0.9

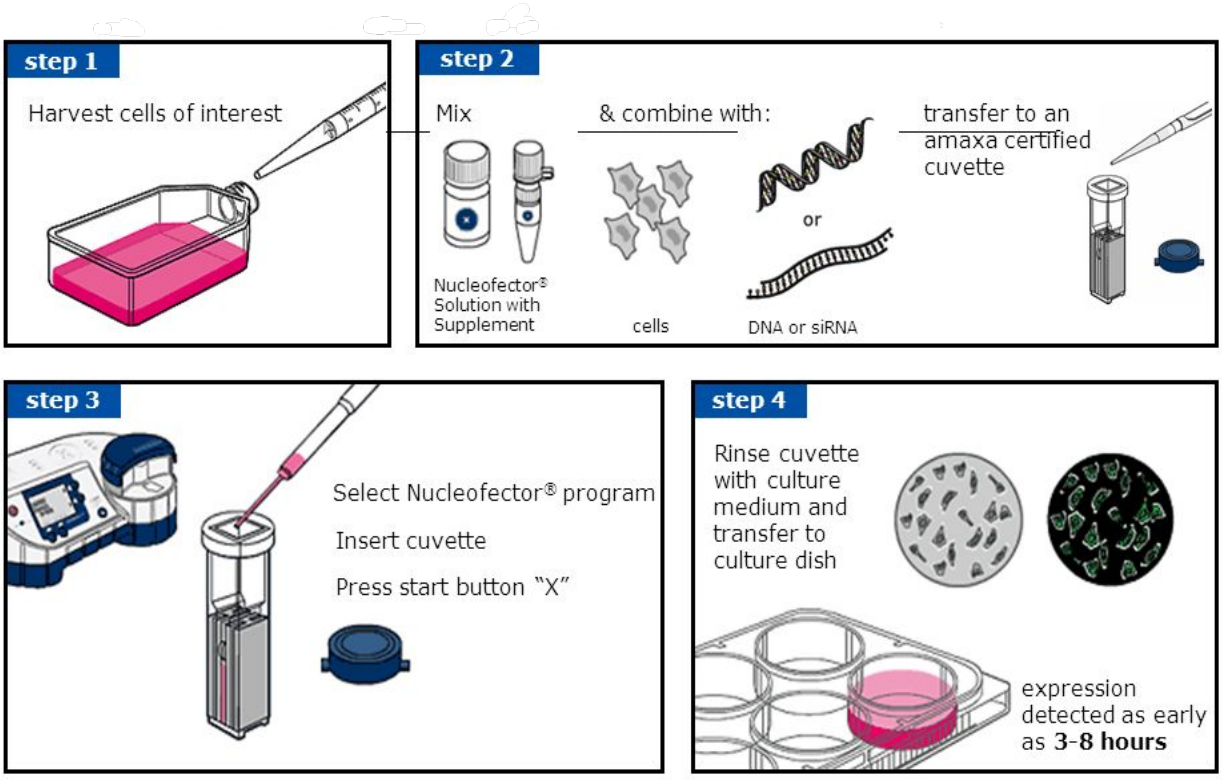


Figure 8: Nucleofection protocol for induced pluripotent stem cells. Figure modified from http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Tips_to_Get_More_Out_of_Your_Nucleofection.pdf; 1.8.2019.

4.6.2 Antibiotic selection

The rock inhibitor in the culture was removed the next day by changing the medium to mTesr1 without rock inhibitor. G418 (Sigma Aldrich) commonly known as geneticin (50 mg/ ml), a selective antibiotic that was added to the cells 48 h after nucleofection. By adding G418 to the cell culture, the non-transfected antibiotic- sensitive cells will be eliminated as the guide RNA plasmid contains a Neomycin/G418 resistance gene. After 24 h, all the medium containing G418 was removed and fresh, prewarmed mTesr1 medium without antibiotic was added to the cells. Human iPS cell colonies usually appear between 7 and 15 days.

4.6.3 Colony picking and expanding of iPS cells

Eight days after Geneticin selection, iPS cell colonies appeared large enough for colony picking. Geltrex-coated 96-well plate was prepared an hour before the picking by diluting Geltrex 1:100 in cold DMEM and dispensing 100 µl of coating solution into each well of 96-well plates. After an hour incubation (at +37 °C, 5 % CO_2), Geltrex was aspirated and 200 µl of prewarmed mTesr1 medium was dispensed into each well. iPS cell plate was placed in a laminar hood under a microscope to select undifferentiated, healthy colonies. Colonies were picked by using a 10 µl pipette tip and transferred into individual wells of the 96-well plate. 96-well plate was placed in an incubator after colony picking. Half of the culture medium was replaced every other day until the culture reached the confluence of 70 %.

4.6.4 Splitting colonies into two 96-well plates

Once iPS cell colonies become visible to the naked eye in a majority of wells, they were split from one 96-well plate to two 96-well plates. The 96-well plates were prepared by coating with Geltrex (100 µl per well) for 1h at +37 °C. After an hour, Geltrex was aspirated and 100 µl of prewarmed mTesr1 medium was added per well using a multichannel pipette. Culture medium was aspirated, 35 µl Versene was added to each well and incubated at +37 °C for 10 minutes. Versene was aspirated after incubation and cells were dissociated with 200 µl prewarmed mTesr1 medium. 90 µl of cell suspension was transferred to the new plate A and 100 µl of cell suspension was transferred to the plate B. The cells were cultured until most wells of plate B have become 90 % confluent under the conditions described before.

4.6.5 Genomic DNA isolation from 96-well plate

This extraction method was chosen, because it is a rapid and cost-effective way of generating genomic DNA of appropriate quality to enable screening of large number of iPSC colonies.

Genomic DNA from culture plate was extracted once it reached 90 % confluence. Media (mTeSR1) was aspirated with a multichannel pipette and cells were washed once with 100 µl PBS. PBS was aspirated and 60 µl Direct PCR reagent (Viagen Biotech) together with proteinase k (20 µg/ml) was added per well. The plate was incubated on a rocking plate overnight at +55 °C in a wet chamber. The plate was incubated at +85 °C for 1.5 h to inactivate Proteinase K. The plate was cooled down at room temperature and vortexed for a few seconds.

4.6.6 PCR of clones in 96-well plate

This PCR reaction was performed to screen the clones by amplifying the genomic region surrounding the target site. PCR reactions were assembled on ice according to Table 15 by pipetting reagents in the indicated order with a multichannel pipette. PCR was performed according to conditions presented in Table 16.

Table 15: PCR composition

Reagents	Amount (µl) / reaction	Total amount (µl)
dH_20	17.8	63 x 17.8 = 1121.4
Dreamtaq Buffer	2.5	63 x 2.5 = 157.5
dNTPs	0.8	63 x 0.8 = 50.4
LMNA T7 forward primer	1.2	63 x 1.2 = 75.6
LMNA T7 reverse primer	1.2	63 x 1.2 = 75.6
Dreamtaq polymerase	0.5	63 x 0.5 = 31.5
DNA template	1	

Table 16: PCR program

Step	Temperature °C	Time	Number of cycles
Initial denaturation	95	2.5 min	1
Denaturation	95	0.5 min	30
Annealing	59	0.5 min	30
Extension	72	50 sec	30
Final Extension	72	10 min	1
Hold	4	∞	

4.6.7 Restriction and agarose gel electrophoresis

Reaction components were combined at room temperature in the order indicated in Table 17. 15 µl of reaction mixture was dispensed to each well of 96-well plate and incubated at +37 °C for 2 h.

Table 17: Components for Fast digestion of PCR product

Reagent	Volume (µl) / reaction
MQH_20	12
10 X FastDigest Green Buffer	2
PCR product	15
EcoR1 restriction enzyme	1
Total Volume	30

The FastDigest Green Buffer (Thermo Scientific) was used as a loading buffer for electrophoresis. 1 % agarose gel was prepared by dissolving 2 g of agarose to 200 ml TAE-buffer and by adding 20 µl of Gelred to solution. Digested DNA samples were carefully pipetted to the wells of the gel. 5 µl of GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific) was used as a standard. The gel was run at 110 V for 50 minutes. The bands were visualized with imagelab software (BioRad) and the images were captured using the ChemiDoc XRS+ (BioRad) system after the run.

4.7 Validation of the corrected human Isogenic iPS cell line

Three potential LMNA clones were chosen for expansion based on the restriction analysis. The corresponding clones in the culture plate A was expanded for further validation by DNA isolation, PCR and Sanger sequencing.

4.8 Differentiation of hiPSCs into cardiomyocytes

The differentiation of hiPSCs into cardiomyocytes was performed by using small molecule method. HiPSCs were cultured on three wells of Geltrex-coated six-well plate at 80 % confluence. Four days before differentiation, cells were seeded into six wells of a 12-well plate precoated with Geltrex. Half of the cell culture medium was refreshed every other day. Typically, four days after the seeding, the undifferentiated cells reach the confluence of 100 %

and the small molecule differentiation is started. RPMI/B27-insulin, (along with 8 µM CHIR99021) medium was prepared according to Table 18. Insulin-free B27 is estimated to increase differentiation efficiency because insulin has negative effects on cardiomyocyte differentiation. The day when CHIR99021 treatment started was differentiation day 0.

After 24 h, CHIR99021 was withdrawn and the cells were cultured with RPMI /B27- insulin medium alone for 48 h (Figure 9). On the third day 1 ml of culture medium was collected from cell culture plate and combined with 1 ml fresh RPMI /B27- insulin medium. IWP4 at 5µM was added to the solution for 48 h. On the fifth day the culture medium was aspirated to get rid of the IWP4 and replaced with fresh RPMI /B27- insulin medium. Half of the medium was also changed on the seventh day. The RPMI /B27- insulin medium was aspirated on the tenth day and replaced with RPMI medium shown in Table 19. RPMI/B27+insulin medium was changed every other day. Cells were visualized under the light microscope every other day to observe new spontaneously beating aggregates.

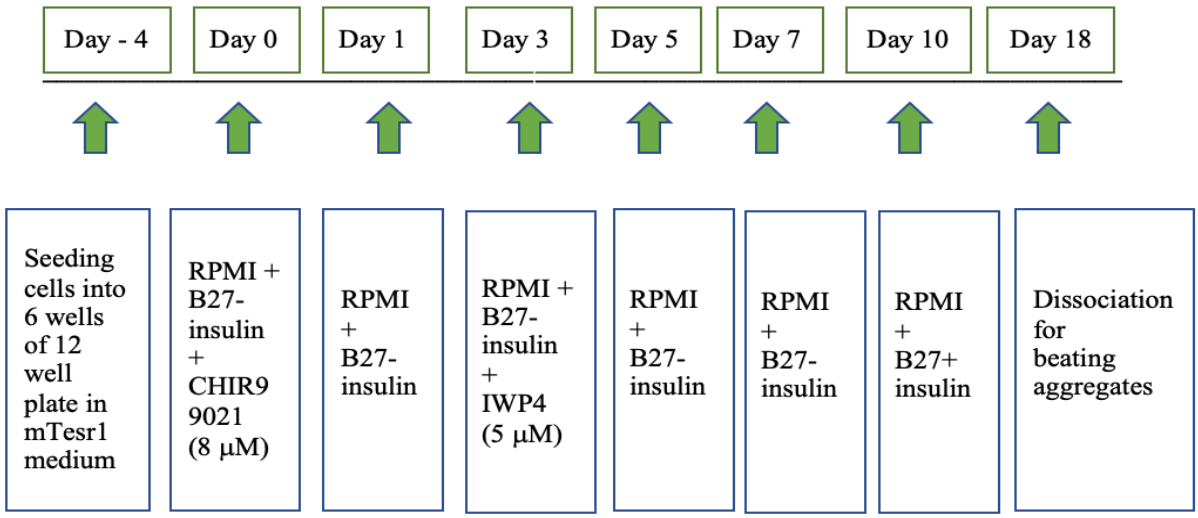


Figure 9: Timeline for differentiation of hiPSCs into cardiomyocytes.

Table 18: RPMI/B27-insulin medium

Components	Volume
RPMI (Fischer Scientific)	48.75 ml
Pen/Strep (Fischer Scientific) (50 U/ml)	0.25 ml
B27-insulin (Fischer Scientific)	1 ml
CHIR99021 (TOCRIS bioscience)	1.6 µl / 2 ml of medium

Table 19: RPMI/B27+insulin medium

Components	Volume (ml)
RPMI (Fischer Scientific)	48.75
Pen/Strep (Fischer Scientific)	0.25
B27+insulin (Fischer Scientific)	1

4.8.1 Dissociation protocol for beating aggregates

On the 18th day of differentiation beating aggregates were isolated with a microscalpel under a stereo microscope and transferred to one well of 24-well plate. Beating aggregates were washed in low calcium buffer 1 (Appendix 9.1) for 30 minutes at room temperature. After incubation buffer 1 was aspirated and replaced with enzyme buffer 2 (Appendix 9.1), containing collagenase A (1mg/ml). Cells were incubated in Enzyme buffer 2 at +37 °C for 45 minutes to achieve the loosening of the cell junctions. Then buffer 2 was aspirated and cells were treated with KB buffer 3 (Appendix 9.1), containing 10 µl glucose/500 µl at room temperature for one hour. During the incubation new 24-well plate was coated with 0.1 % gelatin (Sigma) at room temperature. After this, aggregates were resuspended by pipetting up and down to break up clumps and transferred to gelatin coated 24-well plate containing prewarmed EB medium.

4.8.2 Immunostaining of cardiomyocytes

Immunostaining of cardiomyocytes was performed one week after dissociation. Cells were rinsed twice with PBS before fixation using 4 % PFA for 20 minutes at room temperature. After washing twice with PBS, cells were incubated at room temperature for 45 minutes in permeabilization and blocking solution containing 10 % Normal donkey serum (NDS), 0.1 % Triton X-100 and 1% Bovine serum albumin (BSA). Cells were rinsed once after permeabilization and blocking with washing solution containing 1 % NDS, 1 % BSA in PBS and 0.1 % Triton X-100. Primary antibody incubation was performed overnight at +4°C by diluting primary antibodies to washing solution. Troponin T (goat) antibody (Abcam, catalog # ab33589) at a dilution of 1:2000 and α-actin (mouse) antibody (Sigma, catalog # A7811) at a dilution of 1:1500 in a washing solution were pipetted to cells. Next morning cells were washed

thrice for 5 min with 1 % BSA in PBS before secondary antibody treatment. After washing, secondary antibody mixture combined with 1 % BSA in PBS was added to cells and incubation was performed at room temperature for 1h. The secondary antibody solution included a dilution of 1:800 of Alexa Fluor 568 nm Donkey anti-goat (Invitrogen, catalog # A11057) and Alexa Fluor 488 nm Donkey anti-mouse (Invitrogen, catalog # A21202) antibodies. Cells were then washed thrice for 5 min with PBS and twice with phosphate buffer (PB). After washing, cells were mounted and cover slipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Cells were imagined under the Olympus IX51 fluorescence microscope.

5 Results

5.1 LG2 guide RNA was selected for its high targeting efficiency

Designing the optimum gRNA sequence is important for high editing efficiency by CRISPR/Cas9 system. It is important to ensure on-target activity of guide RNA and minimize gRNA off-target effects. For effective knock-in via the donor template, it's often necessary to design and screen multiple gRNAs against the target gene. In this study, four potential gRNAs were tested for their targeting efficiency by T7 endonuclease assay (Figure 10).

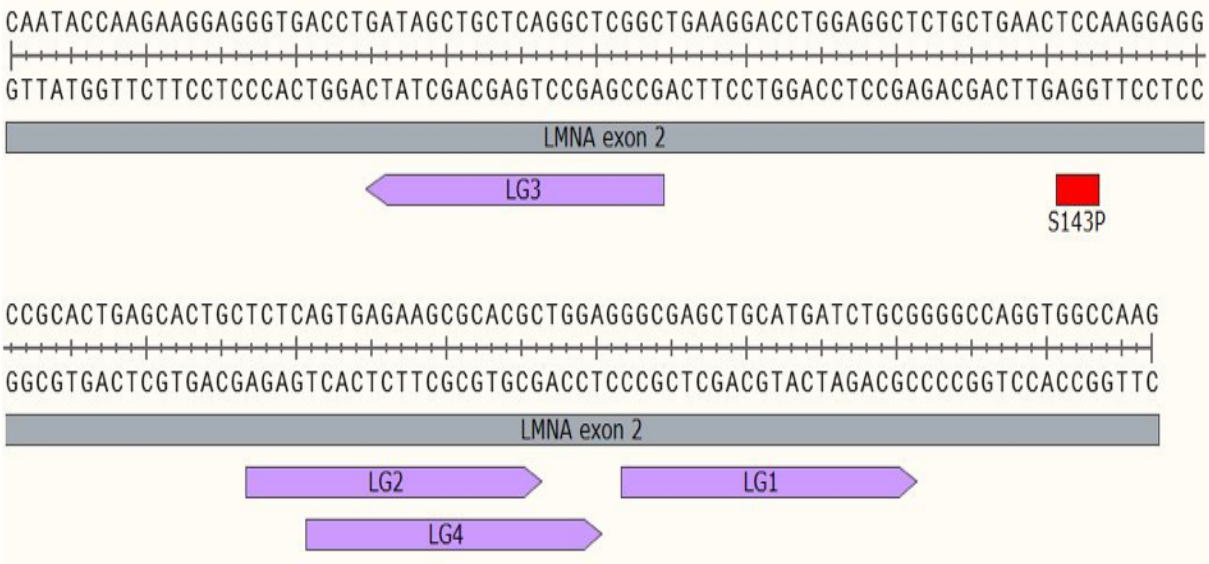
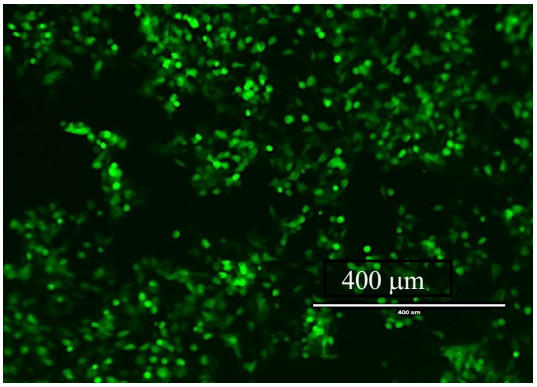


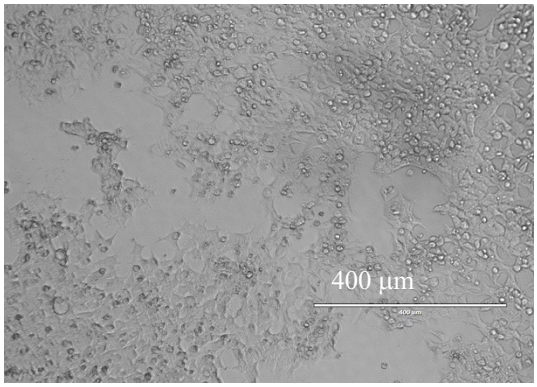
Figure 10: S143P mutation within exon 2 in LMNA gene. SgRNA sequences LG1-LG4 are marked in purple arrows.

T7 Endonuclease 1 assay in HEK293 cells was performed to determine the targeting efficiency of the four selected guide RNA. HEK293 are ideal cells to estimate activity of sgRNA due to their high transfection efficiency and proliferation ability (Li et al. 2016). HEK293 cells were transfected with purified single guide RNA and a conjugated Cas9GFP plasmid for analyzing the functionality and cleavage activity of chosen sgRNA. Presence of GFP indicated that the transfection was successful (Figure 11).

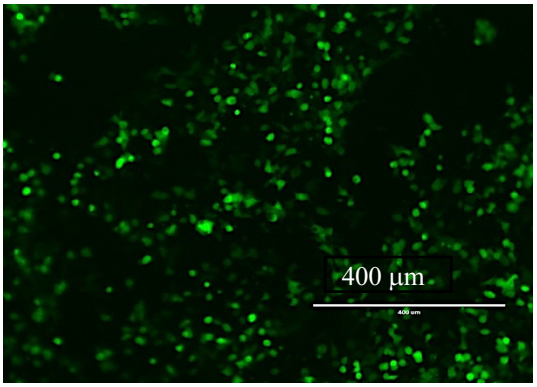
GFP **Phase contrast**

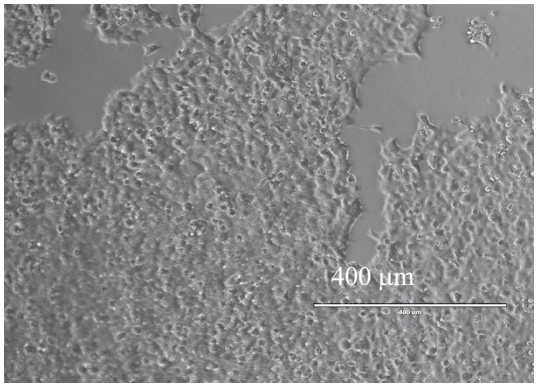
LG1



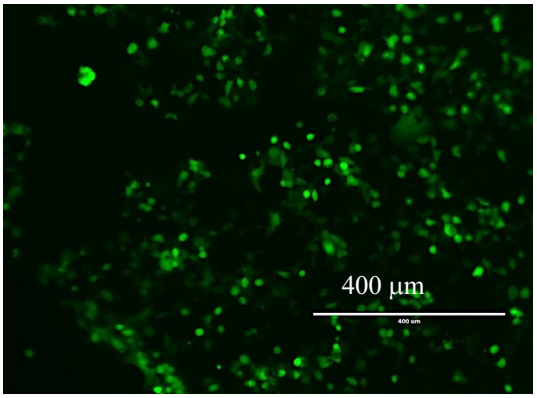


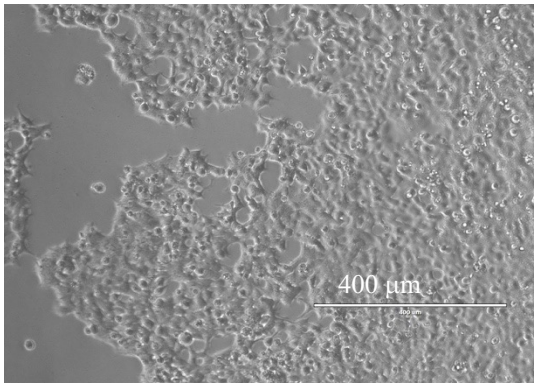
LG2



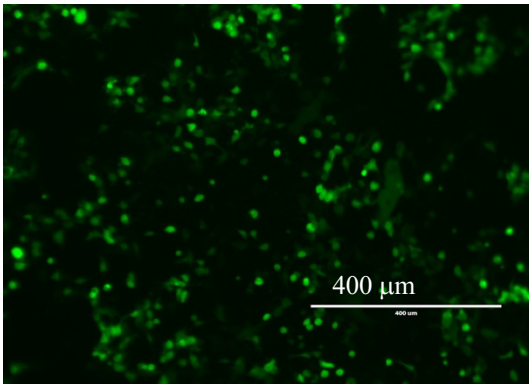


LG3





LG4



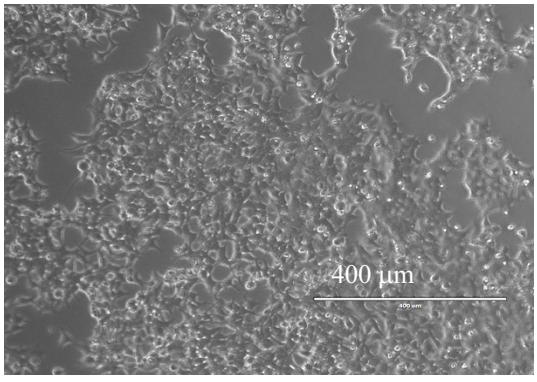


Figure 11: Transfection efficiencies were visualized under the fluorescence microscopy after 24 hours. Pictures on the left side are fluorescence images and right-side pictures were captured with bright field microscopy. Scale bar 400 μm.

A 776 bp DNA fragment spanning the target sequence was amplified from the transfected HEK293 genomic DNA for the T7 assay. The PCR reactions were analyzed by gel electrophoresis in order to verify appropriate amplification and confirm the size of PCR products. The expected sizes of PCR products are shown in Figure 12.

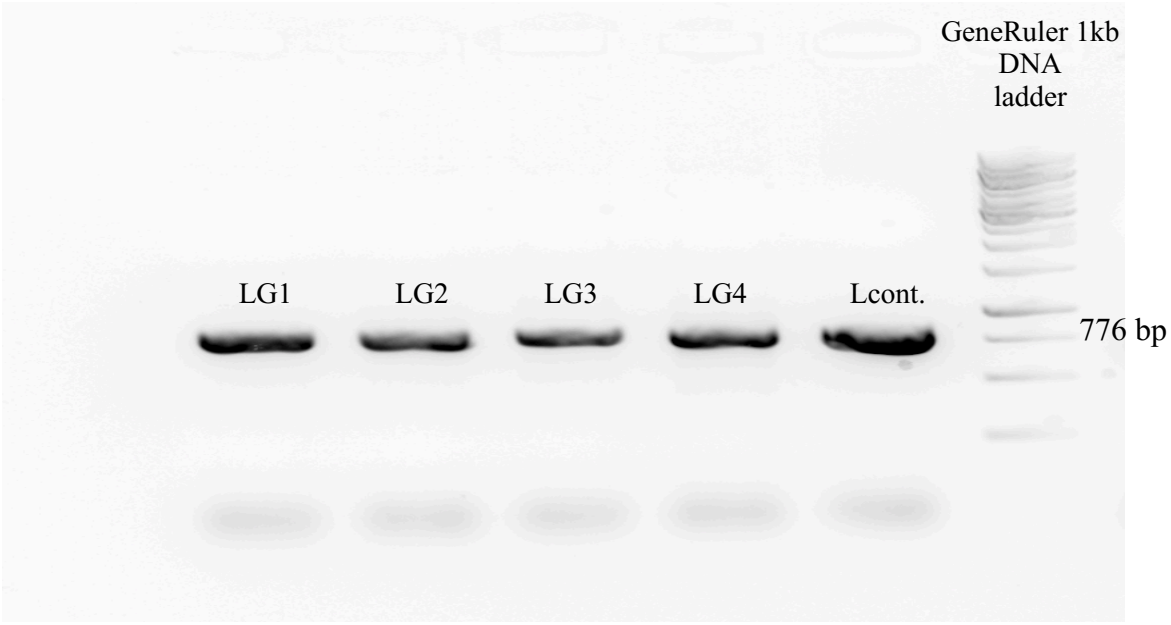


Figure 12: PCR reactions were run on 1 % agarose gel to verify appropriate amplification and confirm the sizes of PCR products.

The PCR-amplified target regions were treated with T7 Endonuclease for cleaving the indels formed due to Cas9 cleavage. The agarose gel image presented in Figure 13 shows the cleavage products and the full-length PCR product. The sum of cleaved bands should be equal to the full-length PCR fragment. Samples LG2-LG4 produced mismatched bands and showed high cleavage activity by the T7 Endonuclease 1 assay in HEK293 cells, whereas sample LG1 remain undigested (cleavage bands were not detected). The control sample (Lcont.) has only one band corresponding to the size of the PCR product.

SgRNA 2 (LG2 sample) was selected for further experiment due to its highest targeting activity and its closeness to the point mutation. In addition, LG2 sample has the fewest potential off-targets.

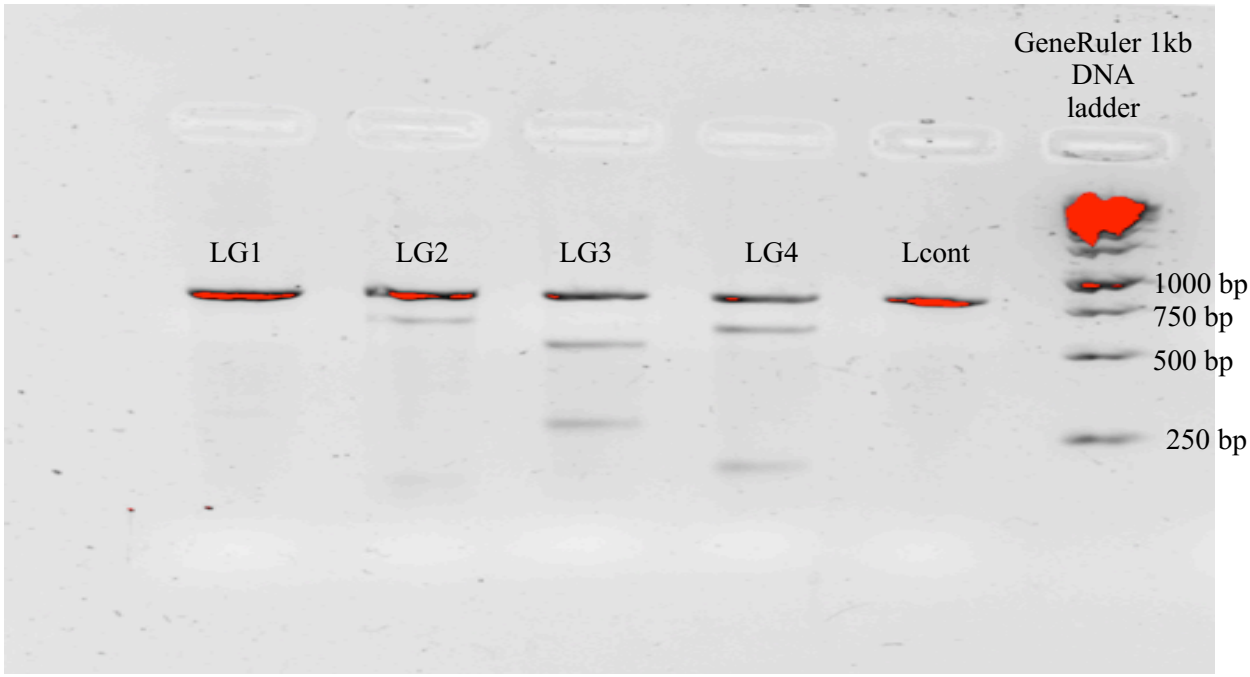


Figure 13: HEK293 cell transfection by Fugene HD transfection reagent with Cas9 and sgRNAs. Order of samples left to right: LG1, LG2, LG3, LG4, Lcont., Generuler 1kb DNA Ladder.

5.2 EcoR1 silent restriction site and PAM site mutation was successfully engineered in the donor template.

To enable the screening of positive isogenic iPSC clones, an exogenous silent EcoR1 restriction site was successfully incorporated in the donor template near to the point mutation (Figure 14). This will ease and reduce the cost of screening the positive clones. The PAM site of the LG2 guide RNA was also mutated, without disturbing the amino acid sequence, to prevent the Cas9 induced donor template cleavage (Figure 15).

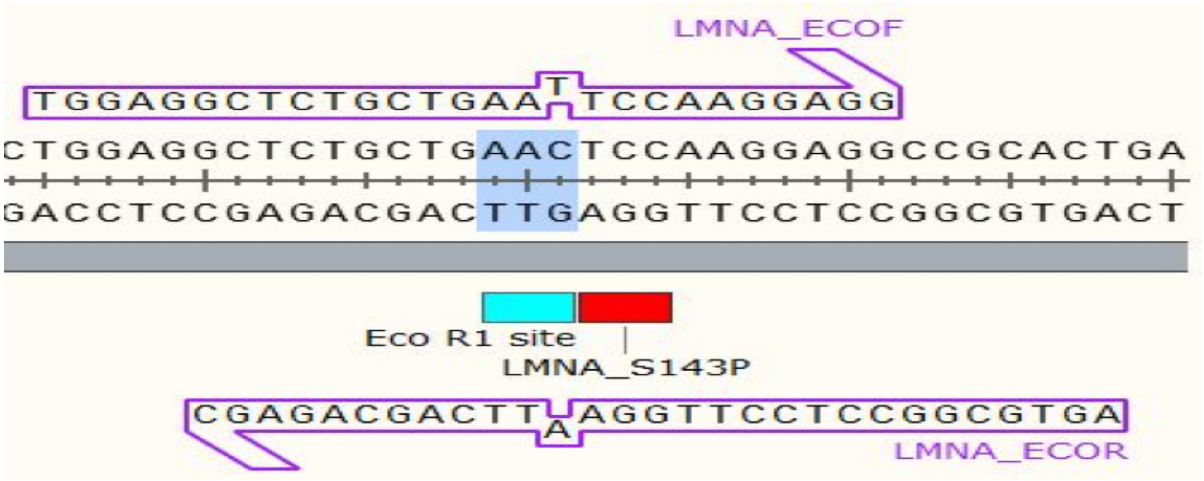


Figure 14: An exogenous silent EcoR1 restriction site in the donor template located close to the point mutation. The primers LMNA_ECOF and LMNA_ECOR generate the EcoR1 site by changing the codon AAC to AAT, both encoding amino acid Asparagine.

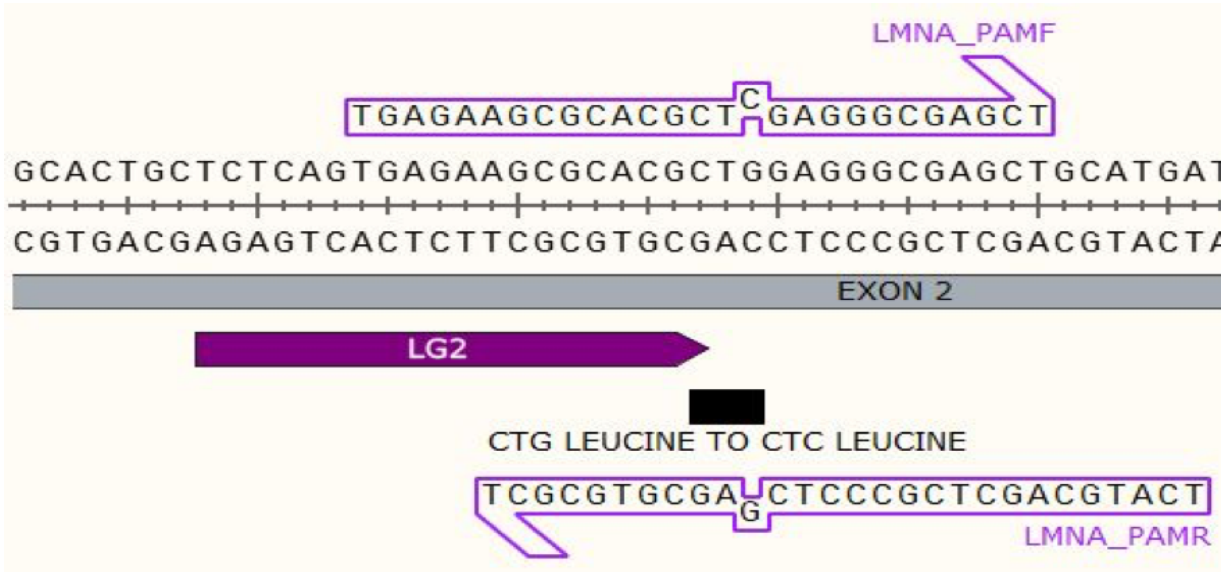


Figure 15: The PAM site of the LG2 guide RNA. The primers LMNA_PAMF and LMNA_PAMR changes the PAM site of LG2 guide RNA by changing CTG to CTC, both encoding amino acid Leucine. The PAM site TGG is changed to TCG to prevent the cleavage of donor template by CAS9.

5.3 Nucleofection of LMNA cell line

The effective transfection of Cas9 and sgRNA into the cells of interest is crucial for the efficient cleavage of DNA at the desired site. There exist various transfection methods currently available. Nucleofection (Nucleofector, Lonza) is commonly used genome editing technique in human iPSC. It is an electroporation-based method selected for this study due to the great transfection efficiency. On the other hand, this method induces massive cell death during the experiment, reported in numerous cell lines. (Li et al. 2018) Delivery efficiency of CRISPR components inside the iPSCs was weak, which can be observed by the low number of GFP-expressing cells, presented in figure 16.

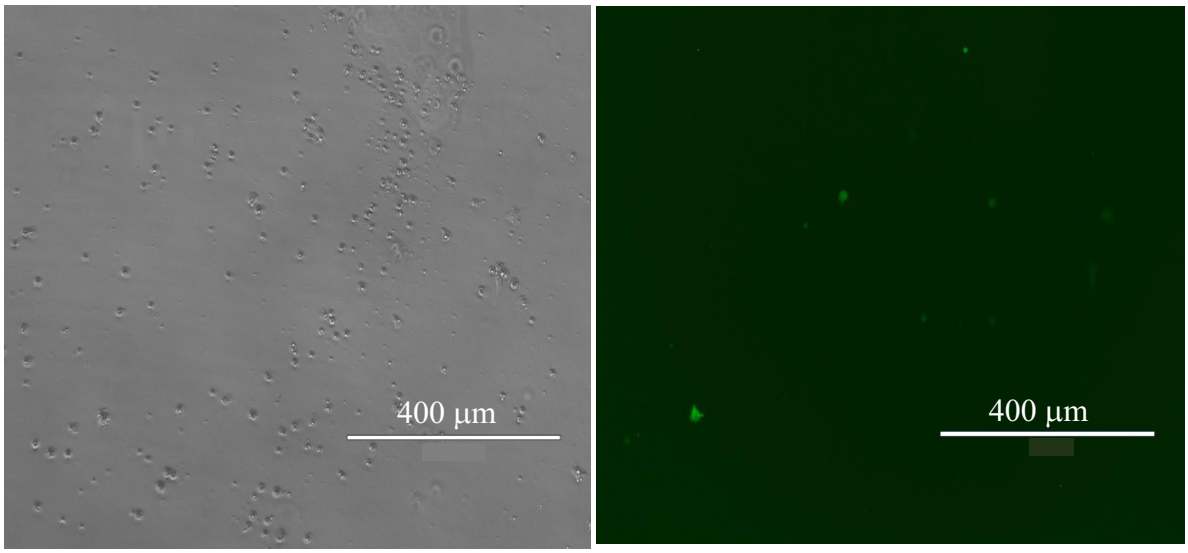


Figure 16: Nucleofection efficiency of iPSCs. Cells were nucleofected with selected sgRNA, donor template and Cas9-GFP plasmid using program CB-150. Expression of GFP protein was detected next day after post-transfection by fluorescence microscopy. Scale bar 400 μm.

Due to the low number of GFP-positive iPSCs, FACS were not used to sorting cells (induce a high rate of cell death that decreases the efficiency of the experiment) instead 60 iPSC colonies resistant to G418 selection drug were manually picked after reaching appropriate colony size (Figure 17).

Morphology of iPSC colonies was visualized under a light microscope. Colonies were compact, containing distinct borders and well-defined edges (shown in Figure 17). Colonies were formed of cells with a big nucleus and relatively less cytoplasm.

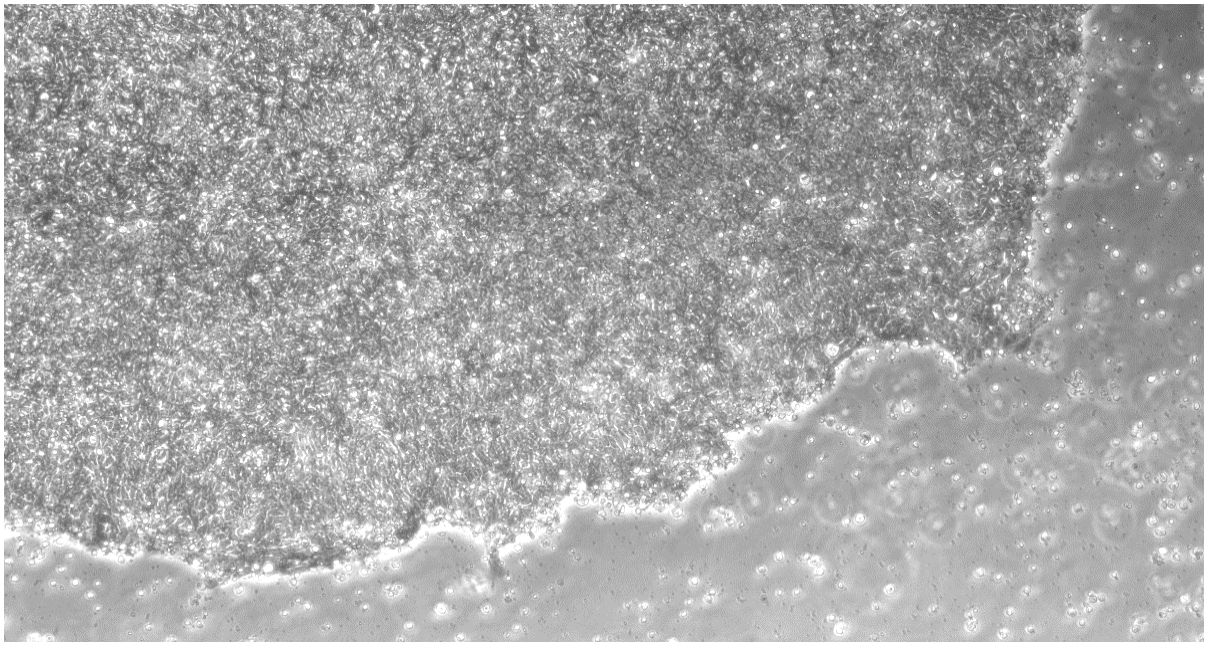


Figure 17: Phase contrast image of the morphology of iPSC colony in Geltrex-coated dish 8 days after nucleofection. iPSC colony is typically flat, round shaped, tightly packed and contains well-defined border.

5.4 Screening the potential isogenic UTA.12619.LMNA iPSC clones with corrected S143P mutation

DNA was extracted from each cell colony and the CRISPR targeting site was amplified by PCR. The PCR product (776 bp) was digested with EcoR1, the artificial silent restriction site incorporated in the donor template, for clonal screening. The digested PCR products was run on an agarose gel for detecting the presence of cleavage fragments. The expected band size of the EcoR1 cleaved products are 549 and 227 bp (Figure 18).

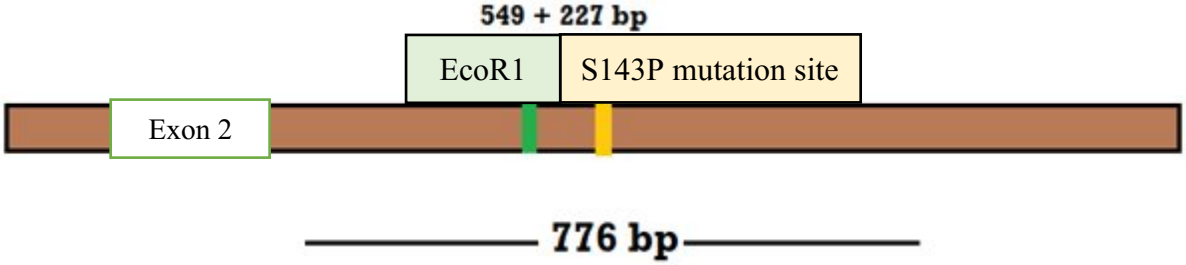


Figure 18: S143P mutation site and EcoR1 restriction site within exon 2 in LMNA gene

The gel was imaged using the imageJ software. The restriction assay results are shown in Figure 19. The samples that have only one band correspond to the size of the target PCR product (undigested, 776 bp). The sum of target cleavage band sizes have to be equal to the size of the PCR product.

Samples are named as A1-10, B1-10, C1-10, D1-10, E1-10 and F1-10. Three black arrows indicate the digested samples (potential clones: named as A4, C7 and D3) that were selected for clonal expansion.

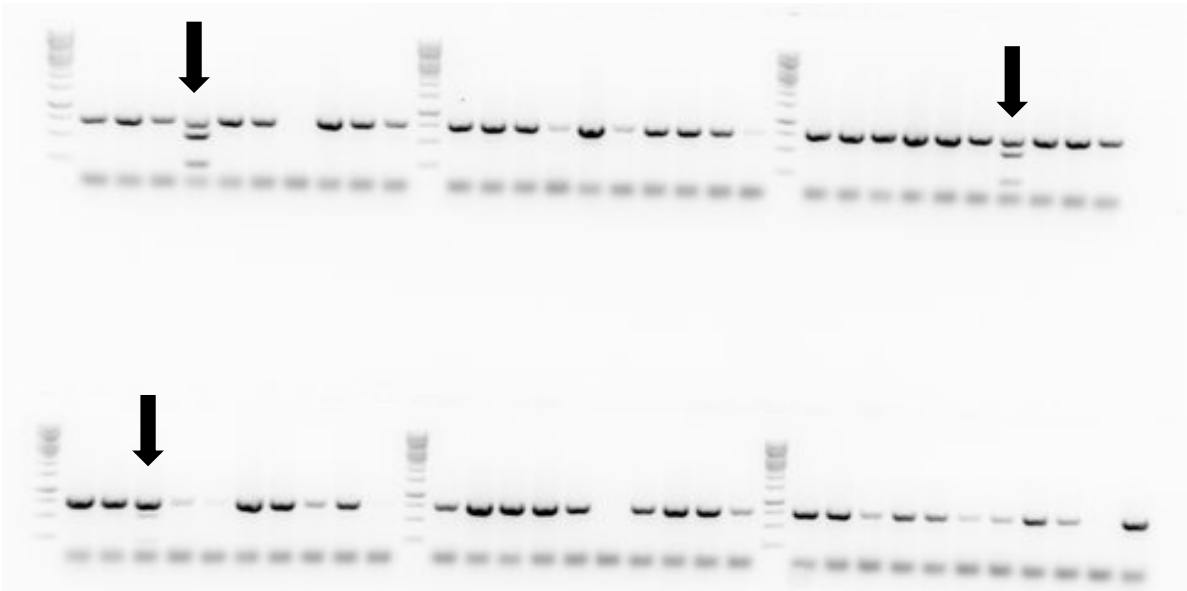


Figure 19: Restriction digestion assay to detect potential positive clones.

5.5 Sanger sequencing of potential positive clones

The patient line carries the heterozygous point mutation S143P in the exon 2 of LMNA gene. In one of the alleles, amino acid codon TCC (encoding serine) is mutated to CCC (encoding Proline). As the mutation is heterozygous, it is important to verify that the correct allele carrying the mutation is corrected in the potential positive isogenic clones. For that, three possible clones were screened through a simplified process of genomic DNA extraction, followed by direct PCR amplification of the region of interest and Sanger sequencing of the PCR product. Based on the sequencing results, sample A4 was selected for iPSC differentiation into cardiomyocytes due to the successful gene editing, on the other hand samples C7 and D3 were corrected on the wrong allele by CRISPR/Cas9 method.

Figure 20 presents sequencing results of A4 clone. Wild type sequence is aligned with LMNA A4 (above) and parental line 12619 LMNA (below) sequences to identify the similarities between DNA sequences and to detect possible modification in genes. Figure 20 and 21 show CRISPR editing, which results in the correction of CCC (Proline) to TCC (Serine) in the amino acid codon of the LMNA-A4 Isogenic line of UTA.12619.LMNA.

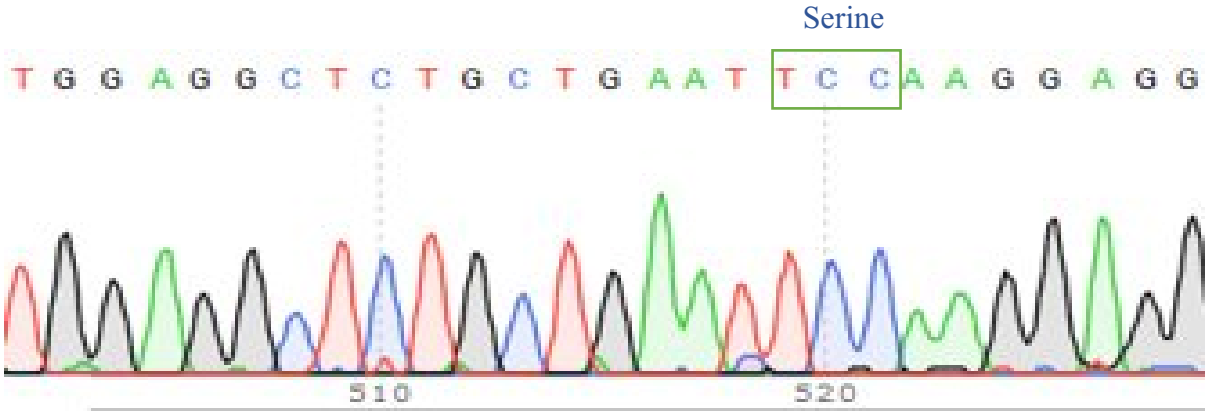


Figure 20: LMNA A4 Sequencing result.

Figure 21: Top panel shows the LMNA A4 alignment with the original sequence. The point mutation is corrected (red boxed codon TCC encoding serine) and the sequence aligns with the original sequence. The red boxed T is the point mutation that we incorporated to generate the EcoR1 restriction site for screening, this mutation is silent as both AAC and AAT encodes for Asparagine. The bottom panel shows the patient line 12619 LMNA alignment with original sequence. Instead of T, the sequencing shows Y (Y is either C or T) confirming the heterozygous mutation in the patient line.

5.6 Differentiation of LMNA-A4 isogenic line into cardiomyocytes

Successful differentiation of iPS cells into cardiomyocytes was determined by two different methods. Spontaneously beating cells inspected in a cell culture on the ninth day of differentiation process were the first sign of successful cardiomyocytes differentiation. In addition, expression of cardiomyocyte-associated gene expression is essential to confirm the specific cell type. Cardiomyocyte-specific proteins expression was estimated by immunofluorescence staining of cardiac troponin and α-actin. Immunostaining analysis is beneficial to evaluate the expression of certain markers and also to obtain information on their subcellular localization. Red fluorescence labeled cardiac troponin protein and green fluorescence labeled sarcomeric α-actin were detected in the differentiated cardiomyocytes. Binucleate cardiomyocytes (two nuclei in each of the cells) are presented in Figures 22 and 23.

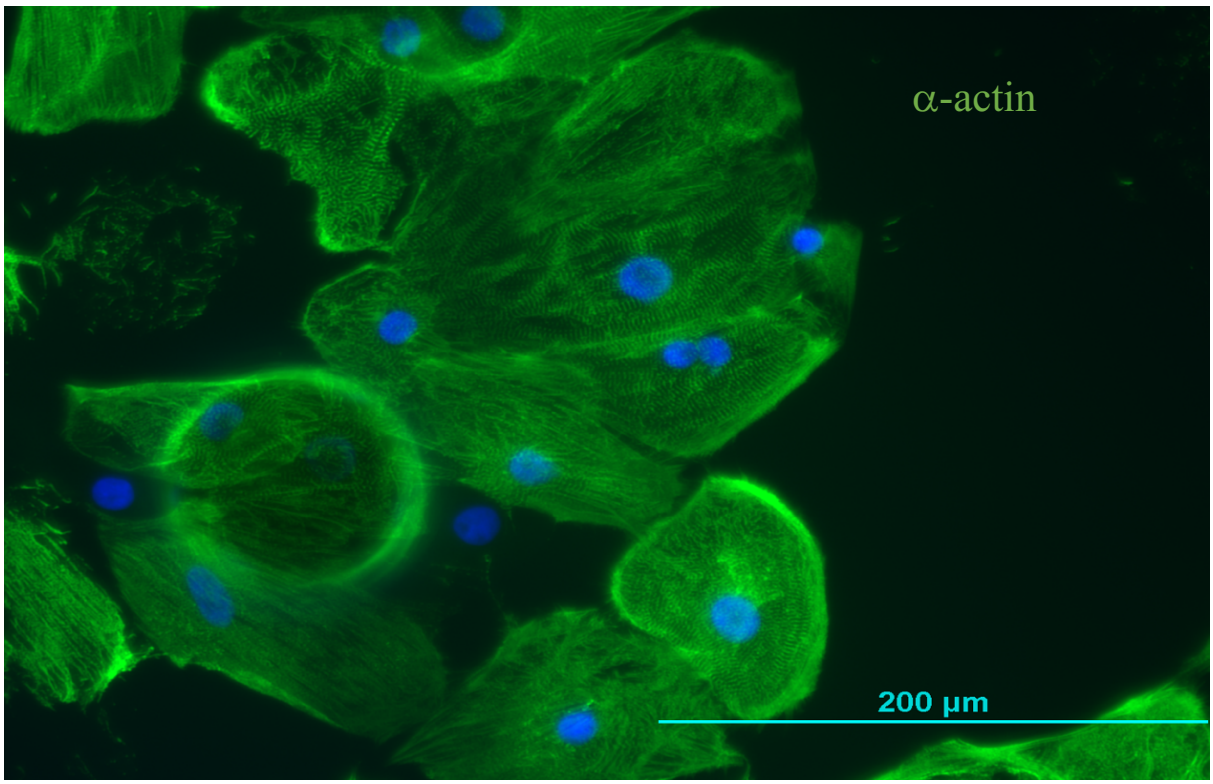


Figure 22: immunofluorescence staining of cardiomyocytes derived from iPSCs: α-actin expression in cardiomyocytes. Image was taken at a 20X magnification. Scale bar 200 µm.

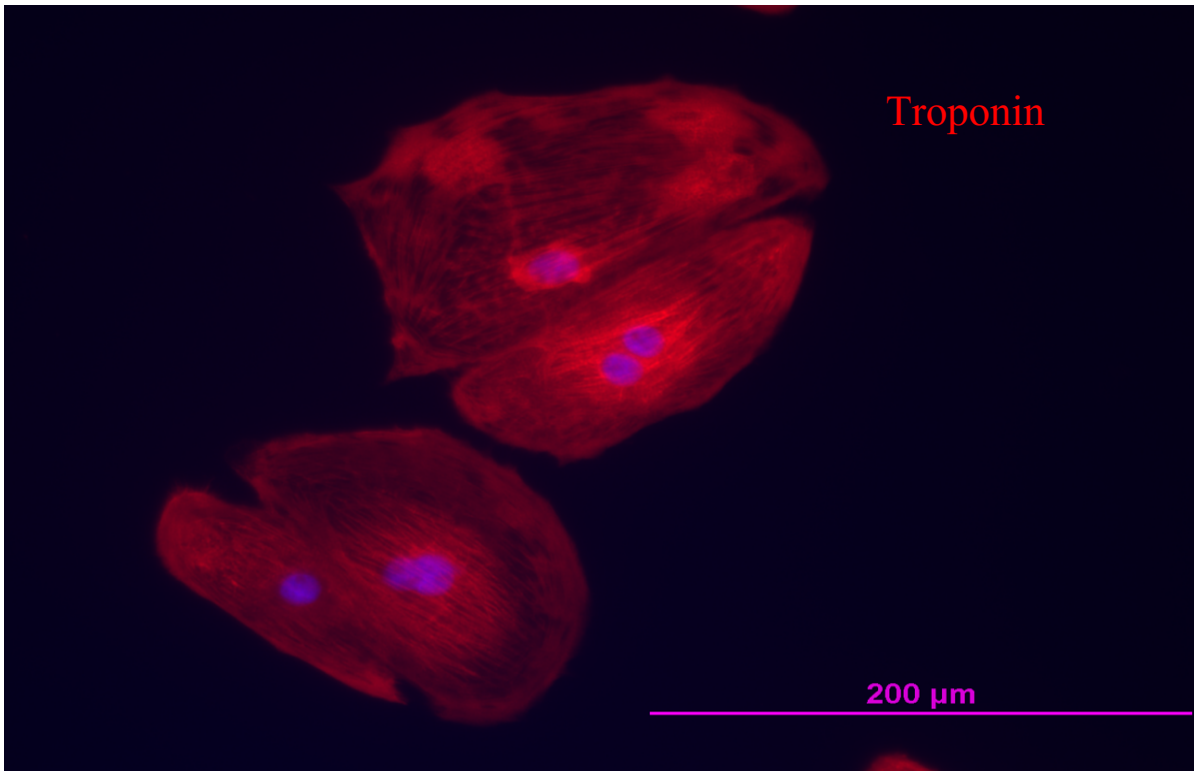


Figure 23: immunofluorescence staining of cardiomyocytes derived from iPSCs: troponin expression in cardiomyocyte. Image was taken at a 20X magnification. Scale bar 200 µm.

6 Discussion

The main aim of this study was to generate precisely corrected isogenic human iPSC line by genome editing using CRISPR/Cas9 system. Furthermore, it helped to acquire comprehensive knowledge related to precise gene correction in human iPSCs, because currently very few data are available in the generation of isogenic lines. Isogenic cell lines are valuable tools in iPSC research for validating the molecular and cellular phenotype of a particular mutation

6.1 Selection of highly efficient sgRNA

CRISPR system depends on sgRNA to direct its specificity, through base pairing of its first 20 nucleotide with the corresponding target DNA sequence in the genome. Choice of guide RNA is very important in CRISPR experiments to minimize off-target mutagenesis. SgRNA specificity is vital for efficient function of CRISPR system. Repeat sequences are really common is human genome. Such repeat sequences should be avoided while designing sgRNA. It is recommended to identify a unique and specific target sequence with minimal sequence similarity (off target sites) elsewhere in the genome. Currently, there are many sgRNA design online tools available, but none of them can accurately predict off-target risk or cleavage activity. Due to this inaccuracy, it is essential to test the specificity of every guide RNA in simpler host systems before starting the CRISPR experiment. In our study, four different sgRNA were tested and analyzed.

T7 Endonuclease 1 assay was employed to recognize and cleave heteroduplexes formed by hybridization of wild-type and CRISPR/Cas9 mutant DNA sequence, otherwise stated to select the most efficient sgRNA for nucleofection. All targeting sequences were located to 20 nucleotides upstream of the PAM (Figure 11) and three out of four sgRNA samples were digested after T7 endonuclease assay. By adding extra “GC” bases (i.e. > 80 % or < 20 %) in the target sequence can have an effect on DNA cleavage activity (Li et al. 2016).

In order to achieve efficient transcription, the initial nucleotide of the sgRNA is proposed to be changed to “G”. It has been reported that the alteration of the initial nucleotide to “G” has insignificant effect on target specificity due to the bendable distal region of sgRNA. In this study, the initial nucleotide of sgRNA 1 was T instead of G, and no cleavage activity was observed. On the other hand, sgRNA 2-4 sequences started with G nucleotide and clear digested

band were detected in each sample. Furthermore, the target sequence of sgRNA should not contain a poly “T” stretch, because “TTTTT” sequence function as a terminator signal. Our study chose the best guide RNA which could successfully target the point mutation in the patient line.

6.2 Delivery of CRISPR/Cas9 system into human iPS cells

Genome editing plasmids were delivered into the human iPS cells by nucleofection that can ensure transfection efficiencies of 60 % to 70 % in iPSC. In order to ensure the successful transfection of CRISPR knock-in components into the target cell line, a Cas9 plasmid carrying a green fluorescent protein (GFP)-linked expression cassette was employed. The transfection efficiency in our experiment was low based on the GFP expression after nucleofection (Figure 16). This could be due to the Cas9 induced cell toxicity and also cell death occurring, during and following nucleofection.

Higher transfection efficiency in human iPSCs is possible to achieve by affecting the following factors. The growth state and confluency of cells can be determined before nucleofection. It is recommended to culture iPSCs under a healthy undifferentiated state and at a logarithmic growth state. Excessive cell densities or obtained stationary phase can negatively effect on the viability of the cells. Actively dividing cells have the ability to take up external nucleic acid more efficiently than slowly dividing quiescent cells. High cell confluency may also influence on contact inhibition, which results in low uptake of nucleic acids and reduced expression of nucleofected gene (Li et al. 2016).

The plasmid DNA used for nucleofection should be prepared with high purity. Plasmids were purified using an endotoxin-free column purification system (Macherey Nagel) and finally dissolved in TE buffer. 260/280 and 260/230 ratios were slightly greater than expected values 1.8 and 2.2. Values evaluate the purity of DNA measured with Nanodrop. The recommended speed for centrifugation provided by the manufacturer (Lonza) is 90 g instead of 110 g used in this study, causing extra stress for cells. In addition, samples remained in a nucleofector cuvette for a short period of time after nucleofection. During the nucleofection pulsing process cytotoxic aluminum ions can release from the electrodes of nucleofector device and may damage the cells (Lonza). These factors may have an effect on decreased transfection efficiency (Figure 16).

6.3 Screening and selecting the isogenic clones

Selection of successfully edited hiPSCs from the population of cells requires screening hundreds of hiPSC clones which can be laborious. For targeted "knock-in" gene insertions it must be ensured by sequencing that the point mutation has been incorporated into the genome, and also that both homology arms have been recombined in the correct site, which is time consuming and costly. A faster alternative is to incorporate an exogeneous restriction site near the target site without altering the amino acid sequence. In our study, we have introduced an EcoR1 mutation near to the point mutation in the donor template.

Three samples out of sixty were digested after EcoR1 restriction digestion assay, indicating 5 % efficiency of Cas9 induced target modification. Low editing efficiency has been reported previously among several research group. Miki and colleagues presented 2.68 % and Wattanapanitch et al. showed 2.9 % gene correction efficiency though CRISPR/Cas9 method using iPSCs (Miki et al. 2019; Wattanapanitch et al. 2018). Three potential colonies (A4, C7 and D3) were analyzed by sequencing using a primer close to the corrected locus (ideally no further than 150–200 bases). One clone (A4) contained the corrected gene editing based on the sequencing results.

Gene editing via HDR pathway has a low efficiency compared to NHEJ pathway. HDR occurs only in the late S and G2 phases, although NHEJ is active throughout the cell cycle. Due to this, several various approach to improve the efficiency of HDR mediated repair process or decrease the efficiency of NHEJ have been studied. Small molecules that are able to inhibit the NHEJ pathway such as brefeldin A, KU-0060648, L-755,507, NU7441 have been observed to improve the efficiency of HDR. Further, inhibition of key enzymes of NHEJ pathway may increase the efficiency of HDR. Another method may be the promotion of HDR using small interfering RNA, which is able to inhibit the expression of KU protein. Ku protein is a crucial protein for NHEJ repairing pathway. (Liu et al. 2019) In further experiments it would be interesting to utilize different methods described above to overcome limitations related to low HDR efficiency.

6.4 Differentiation of iPSCs into cardiomyocytes

The differentiation of hiPSCs into cardiomyocytes was performed using small molecule method. Characterization of these cells was carried out by evaluating the morphology, observing spontaneously beating aggregates and immunostaining several cardiac proteins. Maturation of cardiomyocytes can be evaluated by analyzing expression levels of sarcomeric proteins such as troponin and α-actin (Yang et al. 2014). Cardiac specific proteins troponin and α-actin were expressed in all the samples (Figure 22 and 23) confirming that hiPSCs had successfully differentiated into cardiomyocytes. However, relatively immature cardiomyocytes may express sarcomeric proteins. Due to this observation detection of sarcomeric genes should not be used as the only estimation of maturation (Yang et al. 2014).

Cardiomyocyte differentiation was also evaluated based on the spontaneously beating aggregates observed on the 9th day of differentiation process. Sharma et al. 2015 reported that spontaneously beating cardiomyocytes usually emerged between days eight to ten. The typical morphological changes during hiPSCs differentiation process towards cardiomyocytes were assessed to confirm successful differentiation of hiPSCs into cardiomyocytes. Specific morphological features for mature cardiomyocytes such as elongated, rod-shaped, larger size and branch-like structures were detected during differentiation. A typical banding pattern of organized sarcomeres and presence of multinucleated cardiomyocytes visualized after immunofluorescence staining under fluorescence microscope support the previous findings of successful differentiation. To characterize the functional properties of cardiomyocytes, more methods should be employed.

Currently available methods such as flow cytometry, which reveals the purification of cardiomyocytes, RT-PCR that indicates differences between gene expressions and electrophysiological characterization (e.g. patch-clamp and calcium imaging) that enables the study of electrophysiological properties of cardiomyocytes, would be essential to confirm the molecular and cellular phenotype of the mutation under study.

6.5 Study limitations

Although this study was successful in generating isogenic human iPSC line using the CRISPR/Cas9 system, study limitations need to be taken into consideration before drawing any

valid conclusion based on the results. This study was carried out for the first time by using UTA.12619.LMNA cell line (p. S143P mutation in LMNA gene), so previous study results were unavailable. Further, it is important to do the functional characterization studies like patch clamping to confirm the pathogenicity of the mutation.

Another important limitation was the Cas9 induced cell death indicated by low GFP expressing cells. Therefore, it would be beneficial to test other Cas9 plasmids or other delivery methods that can reduce the cell death. This could improve the success rate of CRISPR knock-in.

Next step would be the determination the optimal number of cells for transfection to achieve the highest efficiency and lowest mortality. After reaching the highest transfection efficiency using iPSCs, FACS sorting can be used to isolate separate populations of cells from heterogeneous cell pools. FACS method is also useful due to its ability to plate one cell per well. In this study cell colonies were picked manually by using a pipette tip and transferred into individual wells of the 96-well plate. FACS method is faster and more efficient way to isolate and sort cells compared to manual picking method. By achieving the high transfection efficiency, the probability to perform successful gene editing by CRISPR increases.

6.6 Future challenges

CRISPR/Cas9 technology is a powerful new genome-editing tool, which demands progressive enhancement. Thoroughly optimizing various components of the CRISPR/Cas9 system is essential for reducing the off-target activity without suffering on-target cleavage efficiency. Furthermore, different methods for detecting off-target mutations may enable the detection of rare off-target sites before utilizing CRISPR/Cas9 system in gene or cell therapy. (Zhang et al. 2015)

A main aim of the future CRISPR research will be to enhance the accuracy of predictive models by combining additional features. Various currently available methods that are used to predict target efficiency and specificity are based on the sequence of the target site, even though it has been reported that epigenetic features are able to modulate gene expression and change phenotypes (Li et al. 2016). In 2016 several researchers demonstrated the relationship between chromatin and CRISPR/Cas activity. The results showed how the presence of nucleosomes at the target site prevented the access of CRISPR/Cas9 and decreased total activity. Furthermore,

same CRISPR/Cas9 target site may induce different activities across cell lines due to the differences in chromatin environment. (Wilson et al. 2018) By adding epigenetic information to future predictive models, the accuracy and efficiency of CRISPR method will improve.

Another obstacle related to the use of CRISPR system is the absence of all-purpose delivery system for gene cargo delivery. Though, several methods have been developed for delivering CRISPR to cells, every method has both benefits and drawbacks. The challenge for future research is the development of new and efficient delivery system that will make a significant impact in the field of CRISPR. (Lino et al. 2018) Also, safety and specificity of chosen delivery system have to be taken into consideration. Additionally, delivery system that is able to target the desired cells with high-specificity will also limit off-target effects and increase safety. By combining currently available and improved methods and knowledge of CRISPR system, it may be possible in the future to manipulate and further correct many various disease related gene mutations in a way that is simple, efficient, reliable, cost effective and precise.

7 Conclusion

The reprogramming of adult somatic cells to pluripotent cells has become a useful technology to study human diseases. Importantly, these cells contain all the genetic aspects of the patient from which they were derived, enabling the development of new disease models. Inherited cardiovascular disorders were one of the first diseases for which hiPSC lines were derived from patients. Such models can enable the understanding of the pathogenesis of many inherited cardiac diseases and also lead to development of new drugs and therapies. One of the major drawbacks of studies on disease modeling using patient hiPSCs is the lack of a suitable control. Genetic differences (single-nucleotide polymorphisms) could enhance or hide the disease phenotype when comparing patient hiPSCs to a different hiPSC line.

Isogenic cell lines that have identical genetic background and differ in only a single genetic change from the parent line allows a thorough assessment of the molecular and cellular phenotypes that result from any disease mutation. The true impact of the mutation that could be masked by variations in cellular phenotype due to the different genetic backgrounds can be detected by using isogenic lines. The use of such isogenic lines to understand the cellular consequences of mutations in iPS disease modelling will be a huge benefit.

Genome editing is an essential element of such studies, to revert or introduce desired genetic mutations enabling us to understand their function by comparison with a fully isogenic background. The advent of CRISPR/Cas9 has vastly increased the ability to introduce defined mutations within the genomes of human cells. This can offer exciting possibilities for personalized genetic medicines in the near future.

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9 Appendices

9.1 Components for Dissociation buffers 1-3

Component	Buffer 1: Low-Ca	Buffer 2: Enzyme	Buffer 3: KB
NaCl	12 ml (1M)	12 ml (1M)	-
$CaCl_2$	-	3 µl (1M)	-
K_2HPO_4	-	-	3 ml (1M)
KCL	0.54 ml (1M)	0.54 ml (1M)	8.5 ml (1M)
Na_2ATP	-	-	2 mmol/L
$MgSO_4$	0.50 ml (1M)	0.50 ml (1M)	0.50 ml (1M)
EGTA	-	-	0.1 ml (1M)
Na Pyruvate	0.50 ml (1M)	0.50 ml (1M)	0.50 ml (1M)
Glucose	2 ml (1M)	2 ml (1M)	2 ml (1M)
Creatine	-	-	5 ml (0.1M)
Taurine	20 ml (0.1M)	20 ml (0.1M)	20 ml (0.1M)
Collagenase A	-	1 mg/ml	-
HEPES	1 ml (1M)	1 ml (1M)	-
pH correction	NaOH	NaOH	
pH	6.9	6.9	7.2

9.2 LB Media components

Component	Volume/Weight
dH2O	Up to 1l
NaCl	10 g
yeast extract	5 g
tryptone	10 g

PH was adjusted to 7.0 with 1 N NaOH.

LB media was sterilized by autoclaving.

Prepared LB media was stored at room temperature.

9.3 SOC Medium components

Component	Weight/Volume
Yeast extract	5 g
Tryptone	20 g
NaCl (5 M)	2 ml
KCl (1 M)	2. 5 ml
$MgCl_2$ (1 M)	10 ml
$MgSO_4$ (1 M)	10 ml
Glucose (1 M)	20 ml

SOC medium was sterilized by autoclaving.

Prepared SOC medium was stored at room temperature.