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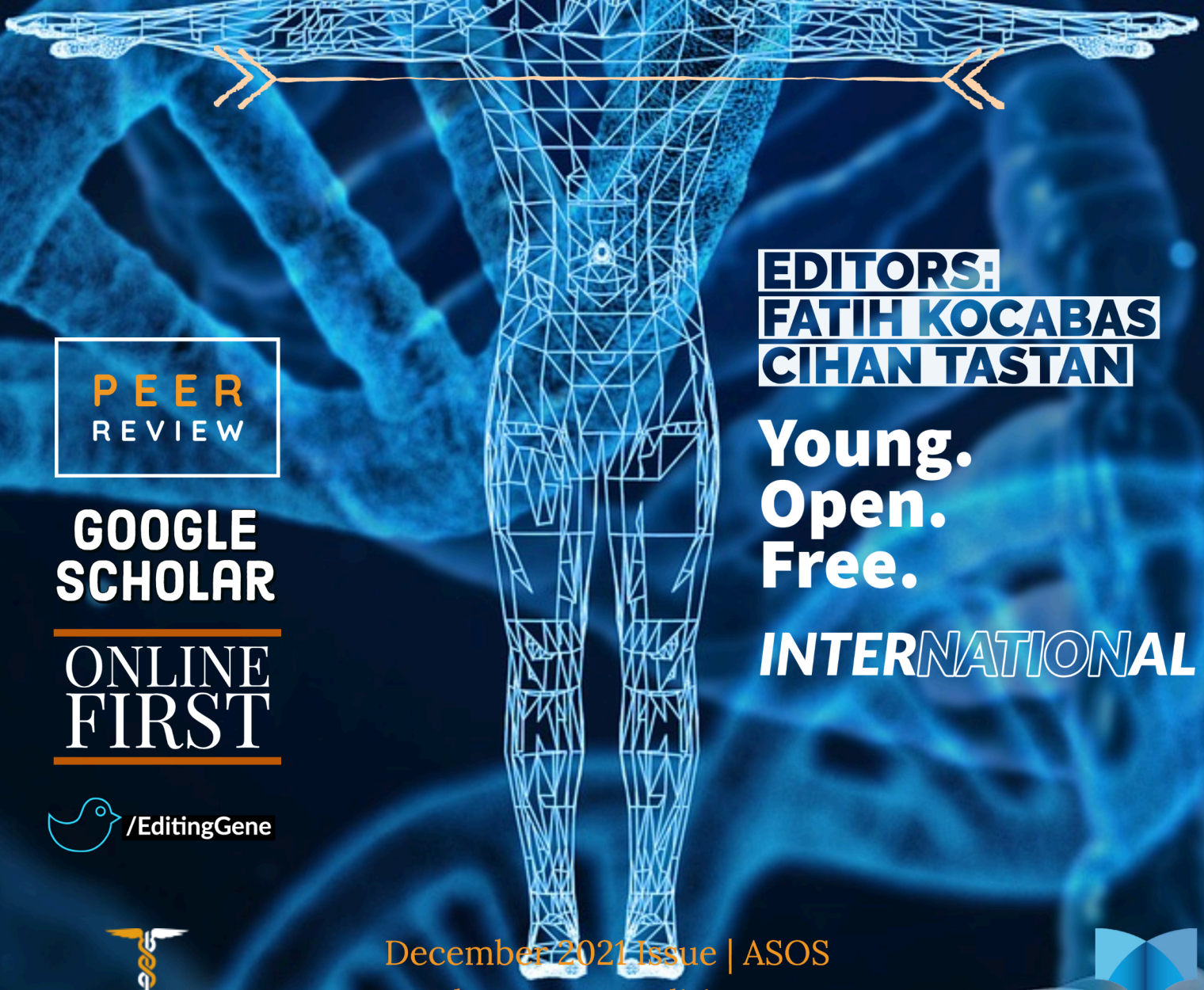

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Contents

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Gene Therapy Products Reached to Market by 2021

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Abstract: Cell and gene therapy are targeted treatments for genetic illnesses that reduce life quality and shorten lifespan. Cell and gene therapy differ from traditional treatments in that they directly target the malfunctioning gene and can cure it in a single dose. Approvals for cell and gene therapy products have increased rapidly in recent years, particularly since 2017, and investment in this sector has increased. When we look at it, cell and gene therapy products continue to be the most expensive pharmaceuticals. The high cost of R&D technologies, the length of regulatory processes, and the difficulties of mass manufacturing are some of the causes of this high cost. The cell and gene therapy product market, which was $11.9 billion in 2021, is expected to reach $43.7 billion by 2024. This target is dependent on the FDA's announcement that it expects to approve 10-20 new cell and gene therapy products per year until 2025. We have compiled a list of 25 gene therapy products that have been approved by any institution through 2021. Some of these products were withdrawn from the market after they were launched for various reasons, which we have also mentioned here.

Key words: gene therapy, cell therapy, future gene therapy market, genetic disease

INTRODUCTION

Gene therapy products entered the pharmaceutical industry when Vitravene, which was produced for the treatment of cytomegalovirus retinitis, received approval from both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 1998 [1,2]. The market for cell and gene therapy products is expanding as the number of FDA-approved medicines grows. Gene therapy modifies or repairs DNA to give function to dysfunctional or malfunctioning genes [3]. In doing so, it uses viral vectors (Adeno-Associated Virus, Adenovirus Type5, Herpes simplex virus type 1, Retrovirus), antisense oligonucleotide, RNA-Aptamer, plasmid or polynucleotide. On the other hand, in cell therapy, cells taken from the patient or donor are used to perform the functions of genes. Cell and gene therapy have the potential to treat illnesses that conventional medications cannot cure [3]. There are several cell and gene therapy treatments on the market that have been authorized. After the FDA announced that it will approve 10-20 items every year through 2025, research in this field has accelerated [4]. In 2021, cell and gene therapy products will be priced around $11.9 billion (According to 2019 March, EvaluatePharma Report). We summarized 25 gene therapy products that have been authorized by any institution up to 2021 in this review. These products are classified as iv vivo and ex vivo according to their application techniques (**Figure 1**). At the same time, we compiled the approval dates, the institutions they gained permission from, the diseases they target, their prices, and manufacturers (**Table 1**).

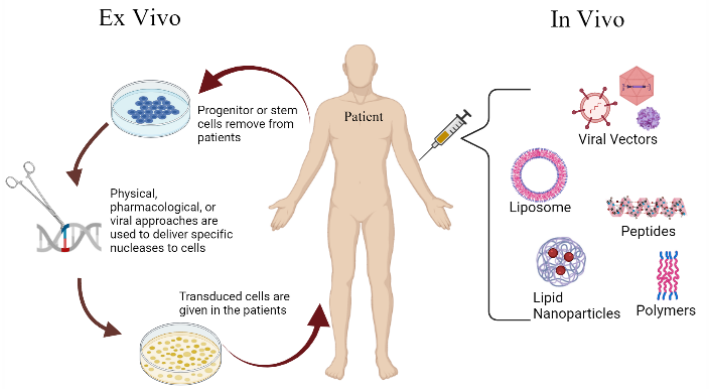

Figure 1. Genome editing in vivo and ex vivo for clinical treatment. Ex vivo, stem cells are first harvested from the patient, then specific modifications are made to these cells, and the mutant gene is fixed and returned to the patient in the corrected form. For in vivo, gene therapy items that have been approved are given by injection into the targeted tissue or organ. Either viral or non-viral approaches are applied.

GENE THERAPY PRODUCTS

Vitravene (Fomivirsen)

Vitravene (Fomivirsen) was the first gene silencing antisense oligonucleotide therapy authorized by the FDA in 1998 and the EMEA in 1999. It was created in conjunction with Isis Pharmaceuticals and Novartis Ophthalmics for the treatment of cytomegalovirus (CMV) retinitis patients [5].

Cytomegalovirus is a member of herpes-type virus that causes cytomegalovirus retinitis. Cytomegalovirus retinitis (CMV-R) is known as retinal inflammation in the eye and leads to blindness. This disease, especially seen in people with weakened immune systems, mostly occurs in AIDS individuals [6]. One in every three children in the US gets infected with this virus by the age of five, and more than half of adults are infected by the age of forty [7]. Fomivirsen is an antisense oligonucleotide containing phosphorothioate links that complement cytomegalovirus immediate-early 2 (IE2) mRNA and inhibits viral replication. Its sequence that contain 21 based oligonucleotide in the 5'-3' direction is provided below [8];

GCGTTTGCTCTTCTTCTTGCG

The Highly Active Antiretroviral Therapy (HAART) approach was created to inhibit HIV replication and enhance the immune system when Vitravene was performing its phase 3 clinical trials. With this method, the number of patients affected by CMVR decreased by 75%. While HAART has been used as standard for a year, Vitravene has just hit the market. During this time the number of patients appeared to be decreasing. As a result, sales were substantially lower than expected. Therefore, Novartis has pulled the drug from Europe in 2002 and from America in 2006 [9,10].

Gendicine (Recombinant Human p53 Adenovirus)

Gendicine was approved for clinical use in humans by the State FDA of China in 2003 as the first gene therapy product for the treatment of head and neck squamous cell carcinoma (HNSCC). Gendicine was produced by Shenzhen SiBiono GeneTech [11]. By January 2006, there were around 1020 authorized gene therapy clinical trials globally, with 66% of these clinical trials being used for cancer treatment. In 58 of these clinical studies for cancer therapy, recombinant adenovirus expressing the human p53 tumor suppressor gene was employed (named rAd-p53). Shenzhen SiBiono GeneTech created a recombinant human adenovirus-p53 injection. In October 2003, the SFDA approved Gendicine for the treatment of HNSCC, and it was formally released in April 2004, after all clinical studies were finished [12].

Gendicine is a human serotype 5 adenovirus recombinant with a human wild-type p53 expression cassette in replacement of the E1 region. The recombinant adenovirus is created in a bioreactor utilizing HEK 293 (Human embryonic kidney) cells. The therapeutic p53 gene and its delivery vehicle, a recombinant adenoviral vector, are combined to form Gendicine. With the gene, the virus attacks cancerous cells, causing them to express more tumor-suppressing genes and immune response proteins [13]. The stability of the expressed protein results in an increase of p53 gene expression at the post translational level. Cell cycle arrest or apoptotic cell death arise from the activation of the p53 gene. In around 50 percent to 70 percent of human cancers, the p53 gene is altered or deleted (null). Mutant variants of the p53 gene are not always dormant, and they can acquire oncogenic activities that aid tumorigenicity [14].

Overall, whether Gendicine was taken in conjunction with chemotherapy, radiation, or other typical treatment regimens, the response rates were significantly higher than when standard treatments were used alone. In several studies, it was discovered that Gendicine combination regimens had longer progression-free survival periods than conventional therapy alone. Gendicine has been used in China for more than 10 years to treat advanced malignancies in patients other than HNSCC [15].

Macugen (Pegaptanib Sodium)

Macugen (pegaptanib sodium), developed together by Eyetech Pharmaceuticals, Inc. and Pfizer, Inc., was the first aptamer-based gene therapy medicine authorized by the FDA in December 2014. This gene therapy medication was created to treat all kinds of wet age-related macular degeneration (AMD). AMD is a primary cause of visual loss in those over the age of 55. This condition is characterized by a deficiency in the center of the retina caused by blood vessel leaking [16]. Macugen uses an RNA aptamer to inhibit VEGF-165, an angiogenic regulating protein, preventing blood vessels from becoming too big underneath the retina and thereby curing the illness. AMD is an irreversible advanced-age disease that causes visual loss in over 500,000 sufferers each year. This condition is predicted to reach around 1 million individuals in the United States during the next five years, posing a major health risk. It is well recognized that it has a significant negative effect on the living quality of the older persons who are affected [17].

This condition causes vision loss as a consequence of injury to the macula, the middle region of the retina of the eye. In AMD, abnormal new blood vessels grow in the central region of the retina, causing it to bleed and leak. This can degrade or entirely eliminate center vision, which is the macula's role. Macugen attempts to inhibit the production of aberrant blood vessels in order to avoid bleeding and leaking from newly created vessels. In all AMD patients, it is utilized to address new aberrant blood vessel development [18]. The molecular formula of 28 base-RNA aptamer for treating the AMD is C294H342F13N107Na28O188P28(C2H4O)2n, (n=900), molecular weight 50 kDa and biological half-life of 10 days [19]. A single dose of Macugen contains 0.3 mg of pegaptanib sodium, administered by the physician to the patient by intravitreal injection every six weeks for one to two years (9 times a year).

Macugen's sales were good between 2004-2011. However, with the introduction of Lucentis (ranibizumab; Novartis), Macugen's revenues dropped to $12 million per year, remaining low in the face of intense competition. And this Novartis-marketed medication looks to be more effective than Macugen. With this competition, Macugen still has a relatively limited and largely uncontrolled market share for the AMD therapy as of 2017 [20].

Oncorine (rAD-H101)

Oncorine (rAD-H101) is the world's first oncolytic viral product, authorized by the Chinese CFDA in November 2005. It was manufactured by Shanghai Sunway Biotech Co., Ltd. Oncorine is an anti-cancer gene therapy treatment that is used in conjunction with cisplatin chemotherapy for advanced head and neck cancer [21]. Oncolytic viruses are effective anticancer medicines because they were designed to proliferate solely in cancer cells and destroy cancer cells throughout their normal life cycle. Oncorine is the first Chinese FDA-approved anti-cancer gene therapy medication that employs oncolytic viruses to target the p53 gene. Following the successful use of Oncorine, research on oncolytic viruses has increased [22].

Loss of T3 gene function is linked to chemotherapy resistance and significantly lowers survival in most cancers. As a result, TP53 gene therapy has shown promise. In Oncorine adenovirus, the E1b-55KD gene, which is responsible for p53 inactivation, is fully deleted. As a result, Oncorine infects and blocks replication cancer cells lacking P53 [23]. Oncolytic virus-based cancer treatment studies and clinical trials are carried out in other cancer types. Combined treatments with radio or chemotherapeutic protocols are also ongoing [24].

Rexin-G (Mx-dnG1)

Epeius Biotechnology developed Rexin-G, a retroviral vector that carries a mutant version of the cyclin G1 gene. The first tumor-targeted injectable retroviral vector, Rexin-G, is a possible therapeutic for metastatic pancreatic cancer [25]. It was licensed for usage in the Philippines in December 2007, and phase 3 studies are currently underway in the US. It was approved in 2010 by the FDA [26].

Suppressing the expression of the CCNG1 gene (specifically the dnG1 protein) enhances the susceptibility of hepatocellular carcinoma (HCC) cells to doxorubicin, laying the groundwork for cyclin-blocking therapy for HCC. Rexin-G is the first tumor-targeted gene therapy product, since it encodes a dominant mutant construct of the CCGN1 gene and depends on selective inhibition of cyclin G1-dependent pathways [27]. In malignant cells, Rexin-G inhibits the cell cycle during the G1 phase, causing apoptosis and cell death. Rexin-G produced in 293T cells contains a hybrid LTR promoter and neomycin resistance [28]. In 2008, the FDA recognized Rexin-G as an orphan medication for soft tissue sarcoma and osteosarcoma. Rexin-G phase 1 and phase 2 clinical trials were completed successfully in 2010. As a consequence of this research, it was shown that Rexin-effectiveness G's in tumor suppression was dose-dependent. Survival and cure rates in sarcoma and pancreatic cancer were raised, particularly in higher dosage studies [29].

Neovasculgen (pCMV-vegf165)

On December 7, 2011, Neovasculgen, the first gene therapy medicine produced by Human Stem Cells Institute (HSCI), was approved in Russia for the treatment of atherosclerotic Peripheral Arterial Disease (PAD), which restricts arteries in the legs, resulting in restricted blood flow [30].

In Russia, an estimated 5 million people have been diagnosed with PAH, with 145,000 suffering from the disease's severe stage, Critical Limb Ischemia. Amputation is performed on around two-thirds of these individuals, and an average of 25% die. Because some individuals with CLI are unable to undergo surgery, Neovasculgen is their sole option [31].

Neovasculgen is a highly purified supercoiled version of the plasmid pCMV-VEGF165, which encodes a VEGF under the control of a promoter. This recombinant plasmid DNA consists of the following components: part of the regulatory region (22 nucleotide pairs) that determines the transcription of the 165 amino acid VEGF minigene, during which the VEGF isoform is synthesized, splicing signal, polyadenylation signal and SV40 transcriptional terminator, synthesis of mature RNA gene and producer of E. coli. It provides the helper sites necessary for the efficient biosynthesis of plasmid DNA in cells of the strain. When molecules of this plasmid enter mammalian cells, VEGF is produced, stimulating endothelial cells leading to enlargement of blood vessels (vascularization) at the injection site [32].

Neovasculgen is a medication that stimulates blood vessel development and expansion through a pre-programmed procedure. Neovasculgen, a plasmid vector carrying the VEGF gene, induces the formation of collateral blood vessels (angiogenesis). According to the drug's clinical trial results, Neovasculgen enhances the number of working capillaries in ischemic tissues, improves blood flow, and lowers the amputation rate, particularly in patients with lower limb ischemia who cannot be operated on [33].

Glybera (Alipogene Tiparvovec)

Glybera, also known as alipogene tiparvovec, was the first gene therapy medicine authorized by the European Union in 2012 to treat the genetic condition Lipoprotein Lipase Deficiency (LPLD). After the sale of this drug, developed by Amsterdam Molecular Therapeutics (AMT), to Uniqure, all sales rights passed to this company. The drug was never approved in the US [34]. Glybera is one of the 3 gene therapy drugs that use gene therapy using adeno-associated viral vectors, the other two are Luxturna and Zolgensma [35].

LPLD is a genetic illness in which a person has a faulty gene for lipoprotein lipase, resulting in extremely high triglycerides, stomach discomfort, and fat deposits beneath the skin, which can lead to pancreas and pancreatic issues, as well as liver damage, which can lead to diabetes. The condition can only develop if a kid receives the faulty gene from both parents [36].

Glybera, which was the world's most expensive drug and sold for 1 million dollars in a single dose in 2019, remained on the market for two years (2015-2017) and in 2017, it was removed off the market after a single industrial sale during this period. The Canadian Research Council (NRC) has decided to reintroduce the drug in question, and over a 7-year period, approximately 80 Millions of dollars have been provided by the Canadian federal government for six different projects aimed at creating a low-cost version of Glybera as well as low-cost alternatives to other gene and cell therapies [37]. Glybera has been used to treat a total of 31 individuals, and these have been for trial purposes without paying before the drug was released on the market [38].

Kynamro (Mipomersen)

Kynamro, a human apoB antisense inhibitor, is the first treatment approved as an addition to lipid-lowering drugs and diet to reduce low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (apoB), total cholesterol (TC), and non-HDL-C in individuals with homozygous genetic metabolic disorders [39]. Kynamro is a manufactured ASO that targets the mRNA of apoB-100, a key component of LDL particles and other atherogenic lipoproteins. ASOs bind to target mRNAs and cause them to degrade, resulting in lower quantities of the associated protein. Different indications have also been explored, including Kynamro, high-risk hypercholesterolemic, homozygous and heterozygous FH. Recent phase II and III clinical trials have revealed that mipomersen reduces LDL-C levels by 25-47 percent in patients [40,41].

Mipomersen is a polynucleotide of the 2nd generation consisting of 20 base sequences. The relevant messenger involved in Apo B synthesis binds to RNA, making it double-stranded. The messenger RNA, which has lost its structural feature, cannot fulfill its task and ribosomal Apo B synthesis is prevented. A decrease in Apo B levels results in a decrease in all lipoproteins carrying Apo B. This suggests not just LDL cholesterol but also all other atherogenic lipoproteins such as lipoprotein a, chylomicron residues and VLDL residues are reduced. Thus, all atherogenic particles, which are considered as residual risk and are held responsible for increased CV risk despite reaching the LDL-C target, are reduced [42].

In January 2013, it was FDA-approved for use as an adjunct to maximal treatment and diet in HoFH. This approval, which was given before the side effect profile was seen, was given to treat HoFH patients with high mortality and high need for new treatment. However, the FDA has monitored the risk of side effects under the name of "risk evaluation and mitigation strategy (REMS)". Fatty liver was detected in 10% of those who used the drug as a side effect, which is an expected side effect in all Apo B-targeted therapies [43].

Imlygic (Talimogene laherparepvec)

Imlygic (Talimogene laherparepvec) is being developed as an oncolytic viral therapy agent for use in different cancer types. Finally, in October 2015, Talimogene laherparepvec became the first oncolytic viral therapy medicine to be licensed by FDA for the treatment of melanoma patients with cutaneous, subcutaneous, and nodal lesions that cannot be cured surgically [44]. Oncolytic viruses are live viruses that can replicate in host cells, but this replication is selective for tumor cells because of genetic modification. Herpes simplex virus type 1 (HSV-1) is ideal as an oncolytic viral vector as it can infect a wide variety of cell types, as it is a large virus containing many non-essential genes that can be deleted or modified to increase therapeutic efficacy, and this virus causes cancer cell lysis following viral replication [45].

Imlygic has been genetically engineered to multiply within tumors and create the immune-boosting protein granulocyte-macrophage colony stimulating factor (GM-CSF). When injected into melanoma tumors, this medication promotes tumor cell lysis and the release of tumor-derived antigens that, when paired with virally generated GM-CSF, can activate an anti-tumor immune response [46]. HSV-1 has been genetically engineered to reproduce in cancer cells and may elicit an anticancer immune reaction using recombinant DNA technology. The deletion of two copies of the infected cell protein (ICP) 34.5 genes increases Imlygic unique replication in tumor tissue. The ICP47 gene was also deleted and replaced with the GM-CSF coding sequence. With these modifications, the lytic cycle activity of the virus in tumor cells was increased, and at the same time, the activation of systemic anti-tumoral immunity was ensured thanks to the

transgene that triggered the antitumoral immunity. Therefore, T-VEC is also defined as an "oncolytic vaccine" that provides "oncolytic immunotherapy" [47,48].

Amgen, the Imlygic developer, did not publish Imlygic revenues in its quarterly results, but EvaluatePharma projected average analyst expectations of $45 million and $250 million for global Imlygic sales in 2016 and 2022, respectively [49,50].

Exondys 51 (Eteplirsen)

Eteplirsen is a synthetic antisense oligonucleotide used to treat Duchenne muscular dystrophy by causing faulty exons to be skipped during the production of the dystrophin gene that drug developed by Sarepta Therapeutics and approved by FDA in September, 2016 [51]. Eteplirsen is a specific mutation-targeting drug that can be used to cure roughly 14% of DMD cases [52].

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular illness that affects around one in every 3,500-5,000 male births. This is due to loss-of-function mutations in the DMD gene, which codes for dystrophin (a cytoskeletal protein that helps protect the plasma membrane of muscle fibers). DMD is inherited as an X-linked recessive trait. Exondys 51 is a kind of antisense treatment that stimulates dystrophin production by restoring DMD's translational reading frame in defective gene variants by skipping exon 51 [53,54]. Eteplirsen is a 30 base pair antisense oligonucleotide known as a phosphorodiamidate morpholino oligomer (PMO), with the sequence CTCCAACATCAAGGAAGATGGCATTTCT [55,56]. Eteplirsen is an antisense oligomer that causes exon 51 to be removed from the dystrophin RNA transcript during pre-mRNA splicing.

The EMA evaluated the medicine in 2018, however it was not authorized [57]. Following the approval of eteplirsen, the FDA granted provisional approval to two more medications of a similar nature, Golodirsen and Viltolarsen, for the treatment of persons with a verified dystrophin gene [58,59].

Spinraza (Nusinersen)

In 2012, a partnership agreement was signed between the United States-based Biogen pharmaceutical company and Ionis Pharmaceuticals for the development of this drug. After human trials, the drug, which was named Spinraza as a commercial label, received approval from FDA in 2016. Thus, this drug became the first treatment approved and licensed for SMA disease in the world, also licensed by EMA in 2017, making it the first treatment approved for SMA in Europe [60]. Both FDA and EMA institutions have approved Nusinersen Sodium active ingredient Spinraza for SMA patients of all ages and types and Spinraza must be taken throughout the individual's lifetime.

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by progressive muscle atrophy and weakness. With rates ranging from 8.5 to 10.3 per 100,000 live births [61], the disease is one of the leading causes of infant mortality. SMA is generated by a homozygous deletion or mutation in the survival motor neuron 1 (SMN1) gene, resulting in reduced SMN protein synthesis and motor neuron degeneration in the spinal cord and brainstem anterior horn cells. The SMN2 gene, which is homologous to the SMN1 gene, also encodes the SMN protein, but owing to improper splicing, it is unable to fully compensate for the loss of the SMN1 gene, resulting in inadequate SMN protein [62]. The first medicine approved for SMA was Nusinersen, an ASO that promotes the inclusion of exon 7 in SMN2 mRNA transcripts. Nusinersen binds to an intronic splice-silencing site in SMN2 intron 7 and inhibits the activity of other splicing factors, enabling exon 7 to be integrated into RNA [63]. Thus, it is aimed to produce SMA protein.

This potentially life-saving treatment costs a lot of money because of Biogen, which licensed it from Ionis (formerly Isis). Spinraza costs $750,000 for the first year of therapy ($125,000 each injection) and $350,000 for each subsequent year, according to Biogen [64]. For the treatment of SMA, three types of medicines have been researched and authorized. Spinraza, which is only one of these drugs in Turkey, is paid for by SGK. Apart from Spinraza, which is utilized in Turkey, the FDA and EMA have also authorized Zolgensma and Risdiplam. Families want to get Zolgensma because it is a one-time drug unlike Spinraza, which has to be used for life [65].

Defitelio (defibrotide sodium)

Jazz Pharmaceuticals produced Defitelio (defibrotide sodium), an injectable medication that is a combination of single-stranded oligonucleotides for the treatment of hepatic veno-occlusive disease (VOD). This condition affects adults and children who have renal or pulmonary failure following hematopoietic stem cell transplantation (HSCT) [66]. VOD occurs when blood vessels in the liver become blocked, which reduces blood flow and can cause liver damage. Defibrotide works by increasing the breakdown of clots in the blood. In 2016, the FDA authorized Defitelio as a therapy for hepatic VOD, and the EC awarded marketing authorization in 2013, with Australia following suit in July 2020 [67,68].

Hepatic VOD produces swelling and decreased blood flow inside the organ as a result of a blockage of a portion of the liver

arteries, resulting in liver injury. The patient is more likely to suffer from renal and lung failure in the most severe version of this disease. Hepatic VOD affects less than 2% of patients following HSCT, but it kills 80% of those patients [69].

By enhancing the enzyme activity of plasmin, this medicine promotes the hydrolysis of fibrin clots and lowers the expression of adhesion molecules on endothelial cells by creating prostaglandin 12. The drug's specific action is uncertain, however it is suspected to protect endothelium cells against damage induced by chemotherapy, tumor necrosis factor, and serum deprivation. Defibrotide sodium enhances tissue plasminogen activator expression while lowering plasminogen activator inhibitor-1 expression to minimize endothelial cell activation and boost endothelial cell-mediated fibrinolysis [70].

Since its discovery as a premium oligonucleotide 50 years ago, defibrotide has been extensively studied. Despite the fact that many parts of its specific mechanism of action are yet unclear, defibrotide appears to have profibrinolytic, anti-thrombotic, anti-inflammatory, and angio-protective properties, which include protecting the EC from injury and boosting its activities. Defibrotide is presently the sole authorized medication for patients with VOD/SOS in the US and for post-HSCT patients with severe VOD/SOS in the EU [71].

Luxturna (Voretigene Neparvovec-rzyl)

Spark Therapeutics' Luxturna is the first FDA-approved directly administered gene therapy to address an illness caused by mutations in a specific gene. Luxturna is an adeno-associated virus (AAV) vector-based gene therapy used to treat retinal degeneration in individuals with viable retinal cells and a biallelic RPE65 mutation. The FDA authorized this medicine in 2017 and the EMA approved it in 2018 to treat children and adults with genetic vision loss that can lead to blindness [72].

Luxturna works by directly delivering a normal copy of the RPE65 gene to retinal cells, allowing the retinal cells to make the normal protein and cure the retinal dystrophy caused by this RPE65 mutation. Luxturna employs an adeno associated virus derived from recombinant DNA methods and containing a normal copy of the RPE65 gene [73]. AAV vectors have emerged as the most effective gene delivery vehicle for gene therapy and immunization, with three AAV-based gene therapy medications now on the market. The FDA and EMA authorized Glybera, Luxturna, and Zolgensma for inherited lipoprotein lipase deficiency (LPLD), hereditary retinal disease (IRD), and spinal muscular atrophy (SMA) [74,75]. Of these treatments, Glybera was removed off the market for financial reasons.

A clinical development program was conducted with 41 patients aged 4 to 44 years who had confirmed biallelic RPE65 mutations to look into the safety and effectiveness of Luxturna. Group receiving Luxturna showed significant improvements compared to the control group. Common side effects of luxyurna include cataracts, retinal detachment, and eye redness. It is currently priced at $850,000 ($425,000 each eye) [76,77].

Patisiran (Onpattro)

Patisiran (Onpattro) is one of two authorized siRNA medicines on the market, created by Alnylam Pharmaceuticals. Patisiran is a lipid nanoparticle formulation containing ribonucleic acid interference (RNAi) that can reduce TTR production in the treatment of transthyretin (TTR) amyloidosis [78]. Hereditary transthyretin-mediated (hATTR) amyloidosis is a hereditary, quickly progressing, neurological, and potentially fatal illness caused by TTR amyloid deposition in the peripheral nervous system, heart, and other organs. With 5–15 years of survival following diagnosis, patients develop neuropathy, cardiomyopathy, and ambulation problems [79]. Patisiran, the only FDA-authorized RNA interference (RNAi) medicine targeting polyneuropathy, was approved by the FDA and EMA in August 2018 [80,81].

Patisiran is an RNA interference therapy that contains a double-stranded, formulated new siRNA (ALN-18328) encapsulated in a nanoparticle made of lipids and is designed to block the synthesis of hepatic transthyretin protein. Patisiran interacts to a genetically conserved sequence in the 3' untranslated region of mutant and wild-type transthyretin (TTR) mRNA, causing degradation and a reduction in TTR protein deposits in blood and tissue. Patisiran siRNA (ALN-18328) reduces blood levels of TTR protein by suppressing the mRNAs of both wild-type and mutant TTRs in hepatocytes [82].

As of 2020, there were 1050 patients worldwide taking Onpattro, resulting in a net income of $65.5 million for the manufacturer [83].

Zolgensma (Onasemnogene Abeparvovec)

Onasemnogene abeparvove, marketed under the brand name Zolgensma, is a treatment that uses an adeno-associated viral vector technology to transfer a copy of the healthy motor neuron gene from people with spinal muscular atrophy (SMA). AveXis, a Novartis subsidiary, developed it, and it was licensed in the US in May 2019 for the treatment of 2-year-old children patients with SMA and biallelic mutations in the SMN1 gene [84].

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder that affects motor nerve cells in the brainstem and leads them to lose function over time. This disease is brain and anterior horn, located at the base of the spinal cord and controlling the movements of the voluntary muscles leads to the gradual destruction of cells (lower motor neurons) [85]. It occurs in 1 in 5000 to 10,000 births in Europe. However, the carrier frequency is 1 in 50. There are several varieties of SMA, and their severity levels vary depending on the genes that cause SMA, but SMA type I (SMA-I) accounts for 50-60% of SMA cases and is the most severe, frequently resulting in death before the age of two. More than 80% of people with SMA have SMA I or SMA II, both of which are fatal [86].

SMA is a neuromuscular disorder caused by a mutation in the SMN1 gene, which reduces the amount of SMN protein in the body. Zolgensma is an SMN1gene replacement therapy based on a non-replicating adeno-associated virus capsid (scAAV9) that effectively distributes wild-type SMN1gen to motor neuron cells. This construct, made up of an AAV9 vector and SMN1 complementary recombinant DNA, has the potential to cross the blood-brain barrier and promote long-term SMN protein synthesis [87,88].

The key advantages of this method are that, unlike Spinraza, it just requires a single injection, resulting in systemic SMN protein expression. It is the most expensive drug with a market price of over 2 million dollars [89].

Givlaari (Givosiran)

Givosiran (Givlaari) is one of 2 approved siRNA therapeutics products manufactured by Alnylam Pharmaceuticals [90]. The other one, Patisiran, is the siRNA therapeutic product used for the treatment of transthyretin (TTR) amyloidosis, also produced by this company. Givosiran was approved by the FDA in 2019 and by the EMA in 2020 for the treatment of acute hepatic porphyria (AHP) in children aged 12 years and older [91].Porphyrias are a group of uncommon disorders caused by abnormalities in genes that code for haem production. AHP is identified by the overexpression of hepatic -aminolevulinic acid synthase 1 (ALAS1), which results in the overproduction of heme precursors or the major clinical symptoms. Furthermore, it causes the deposition of neurotoxic haem intermediates, -aminolevulinic acid (-ALA), and porphobilinogen (PBG) in the liver. ALAS1 regulation is important in the normal hepatic heme production pathway, offering insight into the pathophysiologic processes and prospective therapeutic targets for the treatment of porphyrias [92].

Haem accumulated in the body due to the mutation in the ALAS1 gene causes severe abdominal pain, nausea and nervous system disorders in AHP patients [4]. Givosiran lowers the quantity of accumulated intermediates such as neurotoxic heme intermediates and porphobilinogen by downregulating the ALAS1 gene. When the givosiran group was compared to the placebo group, the rate of compound porphyria attacks dramatically decreased, as were pain levels [93].

Givlaari appeared to do much better than placebo in reducing annual porphyria attacks. In a study of 94 patients, the group taking Givlaari had 3 attacks per year, while the group taking placebo had 13 attacks. The dose of use varies according to the weight of the patient and is administered by subcutaneous injection once a month [94]. At a list price of $39,000 each treatment, the yearly cost per patient would be $575,000 on average .

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Table1: Approved Gene Therapy Products

Product Name	Developer Company	Approved	Therapeutic Target	Mechanism of Action	Price (2021)	Route of administration (Vectors)
Vitravene (Fomivirsen)	Isis Pharmaceuticals, Inc. with Novartis Phar.	USA FDA (1998) EMA (1999)	Treatment of cytomegalovirus (CMV) retinitis	Antisense Oligonucleotide (ASO) Inhibition of the IE-2 mRNA molecule	Withdrawn from EU (2002) US (2006)	In vivo- 21 base antisense oligonucleotide
Gendicine (recombinant human p53 adenovirus)	Shenzhen SiBionoGeneTech	China FDA (October 2003)	Head and neck squamous cell carcinoma (HNSCC)	Adenovirus contains wildtype p53 suppressor gene	$360 per injection	In vivo- viral adenovirus vector
Macugen (Pegaptanib Sodium)	Eyetech Pharmaceuticals and Pfizer. Inc	USA FDA (2004)	Treatment of age-related macular (ARM) degeneration	RNA aptamer - inhibition VEGF165 isoform	One dose (0.3 mg) is around $783	In vivo-28 base pair RNA aptamer
Oncorine (rAD-H101)	Shanghai Sunway Biotech	China FDA (2005)	Treatment for nasopharyngeal carcinoma	Recombinant adenovirus oncolytic virus designed to express wild-type p53, E1B-55 kDa gene-deleted replication-selective adenovirus	-	In vivo- viral adenovirus vector
Rexin-G (Mx-dnG1)	Epeius Biotechnologies	Philippine FDA (2007) USA FDA (2010)	All solid tumors, specifically stage IV pancreatic cancer	Retroviral vector contains cyclin G1. Causes cell death by blocking the cell cycle event in G1 phase and promoting apoptosis in cancer cells	$500,000 per treatment	In vivo, viral retrovirus vector
Neovasculgen (Pcmv-VEGF165)	Human Stem Cells Institute	Russian Ministry of Healthcare (2012)	Atherosclerotic Peripheral Arterial Disease (PAD), including Critical Limb Ischemia (CLI) intramuscular transfer (calf muscles)	Plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (pCMV - VEGF165)	$6,600 for treatment	In situ-intramuscular injection, non-viral
Glybera (Alipogene tiparvovec)	Amsterdam Molecular Therapeutics (AMT) and Qniqure	European Commission (EC) - 2012	Lipoprotein Lipase Deficiency (LPLD) treatment	AAV1- include human Lipoprotein Lipase (LPL) genes, based on an AAV-vector to replace the gene responsible for the expression of LPL	Withdraw (2017) from European Union	In vivo- viral adeno associated virus

Kynamro (Mipomersen)	ISIS Pharmaceuticals	USA FDA (2013)	Treatment of homozygous familial hypercholesterolemia	Antisense oligonucleotide - Inhibition of the synthesis of apoB100	$6,910 for 1-ml vial	In vivo- 20 base antisense oligonucleotide
Imlygic (Talimogene laherparepvec)	Amgen	USA FDA (2016) EMA (2015)	Multiple solid tumors (Melanoma treatment)	HSV-1oncolytic virus with deletions in the γ34.5 and α47 regions, which GM-CSF gene inserted into the deleted γ34.5 loci.	$65,000 per treatment	In vivo, viral herpes simplex virus
Exondy51 (Eteplirsen)	Sarepta Therapeutics	USA FDA (2016)	Treatment of Duchenne Muscular Dystrophy (DMD)	Phosphomorpholidate Morpholino Oligomer (PMO)	€18,348.00 For 1 vial of 100 mg/2 mL	In vivo, 30 base pair antisense oligonucleotide
Spinraza (Nusinersen)	Biogen Ionis Pharmaceuticals, Inc.	USA FDA (2016) EMA (2017)	Spinal Muscular Atrophy (SMA)	A modified 2'-O-2-methoxyethyl phosphorothioate ASO	$125,000 per Injection, and then $350,000 for each subsequent year for life	In vivo, antisense oligonucleotide
Defibrotide (defibrotide sodium)	Jazz Pharmaceuticals plc	EMA (2013) USA FDA (2016)	SOS/VOD with multiorgan dysfunction	Single-stranded oligodeoxyribonucleotides	$9,994 for a supply of 25 milliliters	In vivo, single stranded oligonucleotide
Luxturna (Voretigene Neparvovec-rzyl)	Novartis Inc	USA FDA (2017) EMA (2018)	Inherited Retinal Dystrophies (IRD), retinal pigment epithelial (RPE) cells	AAV2 is laboring a normal copy of the RPE65 gene	$425,000 per eye	In vivo- viral adeno associated virus
Patisiran (Onpattro)	Alnylam Pharmaceuticals Inc	USA FDA (2018) EMA (2018)	Familial amyloid polyneuropathy (FAP), Liver, Peripheral nerves Heart, Kidney, Gastrointestinal tract	dsRNA encapsulated in a liposome	$345,000 per 2 mg/ml	In vivo- non viral lipid complex (siRNA)
Zolgensma (Onasemnogene Abeparvovec)	AveXis/ Novartis	USA FDA (2019)	Pediatric individuals less than 2 years of age diagnosed with SMA having bi-allelic mutations in SMN1 gene	Non-replicating rAAV9 containing cDNA of the human SMN gene under the control of CMV enhancer/chickenβ-action hybrid promoter	$2.125 per treatment	In vivo- viral adeno associated virus
Givlaari (Givosiran)	Alnylam Pharmaceuticals Inc	USA FDA (2019) EMA (2020)	Treatment of acute hepatic porphyria (AHP) in children aged 12 years and older	Double-stranded siRNA that degrades ALAS1 mRNA in hepatocytes, lowering high levels of ALAS1 mRNA in the liver.	$39,000 each treatment, the yearly cost per patient would be $575,000 on average	In vivo- non viral covalently linked ligand complex (siRNA)
Strimvelis (GSK2696273)	GlaxoSmithKline (GSK)	EMA (May 2016)	ADA-SCID	Autologous HSC expressing ADA gene	$648 per patient	Ex vivo- autologous (viral retrovirus vector)
Zalmoxis (Nalotimagene Carmaleucel)	MolMed	EMA (2016)	Hematopoietic Stem Cell Transplantation Graft vs Host Disease	Allogenic T cell expressing HSV-TK suicide gene	withdrawn from the market in 2019	Ex vivo- allogeneic (viral retrovirus vector)
Kymriah (Tisagenlecleucel)	Novartis Pharmaceuticals	USA FDA (2017)	Patients up to 25 years of age with B-cell precursor ALL	Autologous CART cell targeting CD19	$475,000	Ex vivo- autologous (viral lentiviral vector) CAR-T

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Yescarta (axicabtagene ciloleucel)	Kite Pharma	USA FDA (2017)	Diffuse large B-cell lymphoma (DLBCL), B-cell lymphoma, high grade B-cell lymphoma, and DLBCL arising from follicular lymphoma	CD19 antagonist	$373,000 per treatment	Ex vivo- autologous (viral retrovirus vector) CAR-T
Invossa (TissueGene-C)	KolonTissueGene	South Korea (2017)	Osteoarthritis (OA)	Allogeneic non-transformed (HC cells) and retrovirally transduced chondrocytes that overexpress TGF-1 (TC cells) in a 3:1 ratio.	5 million won ($4,400) to 8 million won($7000) for single dose injection	Ex vivo-allogeneic (viral retrovirus vector) CAR-T
Zynteglo (Betibeglogene autotemcel)	BlueBirds Bio Company	EMA (2019)	Beta thalassemia	Autologous CD34+ HSC that have been ex vivo transduced with the lentiviral vector, which contains functional copies of an altered -globin gene	$1.8 million for treatment	Ex vivo-viral lentiviral vector
Libmeldy (Atidarsagene autotemcel, OTL-200)	Orchard Therapeutics	EMA (2020)	Children with metachromatic leukodystrophy (MLD)	CD34+ cells to make ARSA using lentivirus	-	Ex vivo- viral lentivirus vector
Tecartus (Brexucabtagene Autoleucel)	Kite Pharma Inc	FDA (July 2020) EMA (December 2020)	Mantle cell lymphoma (MCL) and acute lymphoblastic leukemia (ALL)	Tecartus attaches to CD19-expressing cancer cells and causes them to die.	$373,000 for a therapy	Ex vivo- autologous viral retrovirus vector CAR-T
Skysona (Elivaldogene Autotemcel)	BlueBirds Bio Company	EMA (2021)	Treatment of early cerebral adrenoleukodystrophy (CALD) for patients 18 and younger	A lentivirus is used to introduce a gene that allows them to manufacture ALDP into the CD34+ cells	Withdraw from EU 2021	Ex vivo- viral lentivirus vector

GENE-MODIFIED CELL THERAPY PRODUCTS

Strimvelis (GSK2696273)

GlaxoSmithKline (GSK) created Strimvelis, the first ex vivo stem cell EMA-approved gene therapy, for the treatment of severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID). Strimvelis is notable for being the first autologous ex vivo gene therapy to be licensed, and Fondazione Telethon, a charity organization that contributes to rare disease research, took part in its production. The product was approved in May 2016 and treated its first patient in March 2017 [95]. GSK has approved the application of the treatment only at its center in Milan, Italy, since the application of the treatment requires special environmental conditions, and stated that the patients in the European Union member countries can access the treatment within the framework of the cross-border health care program [96]. Adenosine deaminase (ADA) deficiency, which affects 1 to 9 persons in every million births, causes toxic metabolites to accumulate, destroying the immune system and resulting in severe combined immunodeficiency (ADA-SCID). Strimvelis is a gene therapy authorized by EMA for ADA-SCID patients who do not have a compatible bone marrow donor (more than 75% of patients) [97].

Strimvelis is a one-of-a-kind therapy that uses the patient's own bone marrow cells. It works by inserting a new gene into bone marrow stem cells, allowing them to express ADA genes. Hematopoietic stem cells (HSCs) are isolated from the patient and purified such that only CD34-expressing cells remain. These cells are cultivated with cytokines (SCF, Flt3, TPO, IL-3) and growth factors. These cells were then transduced with a gammaretrovirus encoding the human adenosine deaminase gene before being reinfused into the patient. These cells establish themselves in the person's bone marrow, reproducing and generating cells that have the adenosine deaminase protein. Patients using Strimvelis should avoid future donations of blood, organs, tissues, or cells for transplantation [98,99].

The cost of Strimvelis is €594,000 per patient. While Strimvelis is not the most costly gene treatment available, it is quite expensive due to the fact that ADA-SCID is an orphan illness. Only roughly 350 persons worldwide are affected by ADA-SCID [100]. As a result, because there are few people affected by this disease and the medicine cannot be mass produced, the cost per patient is exorbitant.

Zalmoxis (Nalotimagene Carmaleucel)

Zalmoxis, developed by MolMed, is an add-on medication for individuals who have had a partially matched haematopoietic stem cell transplant (HSCT). Patients with significant blood malignancies, such as some leukemias and lymphomas, Zalmoxis is administered to patients who have received haploidentical HSCT. Before having an HSCT, the patient would have received therapy to remove existing cells from the bone marrow, such as cancer cells and immune cells. Zalmoxis is administered to the patient after the transplant to help the patient's immune system recuperate. Zalmoxis is produced by isolating T cells from the HSCT donor from the rest of the cells in the transplant. After that, the T cells have been genetically modified to include a "suicide gene" [101]. EMA recognized Zalmoxis as a "orphan medication" in 2003, due to the small number of patients who undergo haploidentical HSCT. MolMed S.p.A. developed Zalmoxis, which received 'conditional clearance' from EMA in 2016. This means further proof about the drug will be forthcoming, which the firm is compelled to give.

When taken following a transplant, Zalmoxis aids in the recovery of the patient's immune system and thus aids in the prevention of infections. However, Zalmoxis' T cells might occasionally assault the patient's body, resulting in graft-versus-host disease. Zalmoxis T cells carry a suicide gene, making them vulnerable to the antiviral drugs ganciclovir and valganciclovir. It was withdrawn from the market in 2019 due to financial difficulties.

Kymriah (Tisagenlecleucel)

Kymriah, developed by Novartis, is the anti-cancer FDA-approved gene therapy for refractory or second- or later-stage relapsed B-cell acute lymphoblastic leukemia under the age of 25. Kymriah is a CAR-T therapy that retrains the body's immune cells to detect and attack malignant cells. Lymphocytes from the patient's serum are collected and changed in the lab to attack cancer cells before being reintroduced to the patient. This procedure takes approximately 22 days. The product's efficacy was tested 63 pediatric and young adult patients took part in a multicenter clinical study, with an 83 percent remission rate three months following therapy. However, life-threatening adverse effects like cytokine release syndrome and neurological problems have been reported [102].

Unlike traditional small molecule or antibody therapy, the quantities of CAR-T cells increase over time and can take months or even years. Tisagenlecleucel is a CAR-T cell immunotherapy that employs patient-derived T cells that have been genetically modified ex vivo by lentiviral transduction to create a CD19-targeted CAR (CAR19) that activates T cells [103].

Kymriah uses a second generation CAR, a 4-1BB stimulant domain with significant effects in terms of persistence and efficacy. Yescarta; Similar to Kymriah, it uses a single-chain variant CD19 binding strand, but unlike Kymriah it contains a

CD28 excitatory signaling domain [104]. Medicare and Medicaid Services in the US announced a program for Kymriah, a $475,00 one-time treatment for a subtype of acute lymphoblastic leukemia. Only if a patient demonstrates proof of a clinical response after one month the agency will pay the manufacturer [105].

Yescarta (Axicabtagene Ciloleucel)

CAR-T cell therapy is a promising new therapeutic option for hematologic cancers. The FDA authorized two CAR T-cell products for clinical use in 2017: tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta) [106]. Axicabtagene ciloleucel, anti-cancer gene therapy, was the first CAR-T cell therapy approved by FDA on 18 October, 2017. Kite Pharma developed it and sold it as Yescarta [107]. It is used to treat adult patients with large B-cell lymphoma who have had two or more lines of systemic therapy, including DLBCL.

Yescarta binds to both healthy and cancerous B cells that express CD19. The medication targets downstream signaling cascades in CD19-expressing cancer cells. When CAR T cells contact with CD19-expressing target cells, T-cell proliferation is triggered, leading in the generation of inflammatory cytokines. As a result of this chain of events, CD19-expressing cells undergo apoptosis. After reinfusion, anti-CD19 CAR T cells detect and kill target cells that express CD19 [108]. Yescarta's active component is axicabtagene ciloleucel, a genetically modified autologous T-cell immunotherapy treatment that extracts a patient's own T cells and genetically transforms them ex vivo using a retroviral vector to produce a chimeric antigen receptor. Anti-CD19 CAR T cells are transduced and cultured in vitro before being injected back into the patient to detect and destroy CD19-expressing cells. Yescarta is made up of human blood cells that have been genetically modified using a replication-incompetent retroviral vector [109].

In a multicenter clinical trial including 100 patients, the treatment efficacy and safety were assessed. After treatment, 51 percent of patients had a complete response. Yescarta has the potential to induce major adverse effects, the most serious of which being cytokine release syndrome. Excessive cytokine release may occur as a result of proliferation and activation of CAR-T-modified T lymphocytes. This can cause high fever, flu-like symptoms, and neurological toxicities. When cytokine release syndrome is not taken under control, it leads to life-threatening situations. The drug called Tocilizumab (Actemra) is used against this type of side effect [110]. CAR-T cell therapies are developed with advanced genetic engineering technology. For this reason, the price of the drug is quite high. The cost of the treatment for just one patient is $373,000. The most important feature that distinguishes Yescerta from previous products is that it is a treatment that affects a much larger number of patients. It is estimated that there are 7,500 patients each year for this treatment in the USA.

Invossa (TissueGene-C)

TissueGene-C, marketed as Invossa, is a new cell and gene therapy product for osteoarthritis treatment that drugs combines allogeneic non-transformed (HC cells) and retrovirally transduced chondrocytes that overexpress TGF-1 (TC cells) in a 3:1 ratio. Invossa has attained marketing approval in Korea, 2017 by KolonTissueGene and its phase 3 trials have been completed in the USA [111]. The most prevalent form of arthritis, osteoarthritis (OA), is characterized by cartilage degradation, discomfort, and inflammation in the joints. Existing drugs can help with the symptoms, but their effects on the disease's progression are limited. TissueGene-C reduced pain, everyday activities, sports functions, and cartilage structure in individuals with OA, according to the results of a phase III clinical trial [112].

Osteoarthritis is a degenerative illness that affects about 250 million individuals globally. The incidence of osteoarthritis is predicted to rise significantly as the world population ages, obesity rates rise, and new disease-modifying osteoarthritis medications become available (DMOADs). Chondrocytes control the generation and degradation of extracellular matrix components like proteoglycans and collagens, which help to keep articular cartilage in good shape. This equilibrium is broken in osteoarthritis, resulting in net articular cartilage deterioration and increased production of inflammatory cytokines and matrix-degrading enzymes. Many ongoing drug development efforts for osteoarthritis try to regulate chondrocyte function in order to halt or reverse cartilage deterioration. Transforming growth factor (TGF), an anti-inflammatory cytokine, regulates chondrocyte proliferation and differentiation, as well as extracellular matrix deposition, to maintain metabolic homeostasis and the structural integrity of articular cartilage. As a result, TGF- has been proposed as a major contender for rebuilding damaged cartilage in osteoarthritis patients' articular joints [113].
Depending on the facility, a single Invossa injection might cost anywhere from 5 million won ($4,400) to 8 million won.

Zynteglo (Betibeglogene autotemcel)

Zynteglo is a gene addition therapy medication that is only used once to treat beta thalassemia, a blood condition that requires monthly blood transfusions in individuals aged 12 and up. FDA designated Zynteglo as a breakthrough therapy in February 2015, and EMA approved it for medical use in May 2019. It has not, however, been authorized in the US [114,115].

Transfusion-dependent -thalassemia (TDT) is a rare, severe genetic condition caused by mutations in the hemoglobin subunit beta (HBB) gene. It is characterized by decreased or nonexistent production of the -globin protein in adult hemoglobin, resulting in insufficient red blood cell formation and significant anemia. Zynteglo treatment aims to correct defective erythropoiesis and allow for lifelong, steady synthesis of functional adult hemoglobin at levels adequate to eliminate the requirement for blood transfusions. A single infusion of Zynteglo, which addresses the fundamental cause of TDT, has the potential to eliminate the requirement for lifelong transfusions. The Zynteglo therapy involves the transplantation of autologous CD34+ HSC that have been ex vivo transduced with the lentiviral vector, which contains functional copies of an altered -globin gene with a threonine to glutamine change at position 87 [116].

Zynteglo therapy requires the collection of stem cells from the patient's blood that cells are transformed by a virus that introduces functioning copies of the beta-globin gene within the cells. When the patient receives these changed cells, the cells start producing healthy red blood cells that produce beta-globin. The effects of Zynteglo are likely to last the remainder of the patient's life. In two major studies, Zynteglo has been shown to lessen the need for blood transfusions in patients with beta thalassemia who required frequent blood transfusions. In these investigations, 11 of the 14 patients who did not have total beta-globin deficiency and were given Zynteglo had enough red blood cells to avoid needing blood transfusions for at least a year following treatment [117].

Soon after receiving European certification for severe beta thalassemia in June 2019, Bluebird announced a price of $1.8 million for its gene treatment, Zynteglo.

Libmeldy (Atidarsagene autotemcel)

Libmeldy (also known as atidarsagene autotemcel, OTL-200) is a gene therapy medicine developed by Orchard Therapeutics that is used to treat children with metachromatic leukodystrophy (MLD) who have a mutation in the human arylsulfatase A (ARSA) gene. The European Commission approved it in December 2020 for the treatment of MLD in qualifying early-onset patients with biallelic mutations in the ARSA gene, resulting in decreased ARSA enzymatic activity in children [118].

MLD is a rare and potentially fatal genetic condition of the body's metabolic system that affects around one in every 100,000 births. MLD arises when sulfatides build in the brain, liver, gallbladder, kidneys, or spleen as a result of ARSA gene abnormalities. Their nerve systems deteriorate over time, resulting in neurological issues such as motor, behavioral, and cognitive deterioration, severe spasticity, and seizures. Patients with MLD who have these neurological issues gradually lose their ability to move, talk, swallow, eat, and see, finally leading to death. There is currently no authorized therapy for MLD [119].

Libmeldy's active component is stem cells taken from the patient's own bone marrow or blood that have been engineered to carry a copy of the gene required to manufacture normal ARSA and may proliferate to produce other types of blood cells. For Libmeldy treatment, CD34+ cells are taken from the patient's blood or bone marrow and their own hematopoietic stem cells are identified. A gene is then added into the genome of the patient's own stem cells, allowing CD34+ cells to generate ARSA using a genetically engineered virus called lentivirus. Gene-edited HSCs have the ability to pass the blood-brain barrier and migrate to the brain, where they can produce functional enzymes and permanently fix the genetic state. The genome-modified stem cells are injected back into the patient's vein, where they create ARSA, which aids in the breakdown of sulfatides in the surrounding cells, therefore managing the disease's symptoms [120].

Libmeldy is approved in the European Union, UK, Iceland, Liechtenstein and Norway however in the US it is still an investigational therapy. The most common side effect of Libmeldy is the development of antibodies to ARSA, seen in at least one in 10 people, but this doesn't seem to affect how well Libmeldy works. The San Raffaele-Telethon Gene Therapy Institute (SR-Tiget) in Milona, Italy, collaborated on the development of Libmeldy [121].

Tecartus (Brexucabtagene Autoleucel)

Tecartus (Brexucabtagene Autoleucel) is a CAR-T cell medication that is used to treat mantle cell lymphoma (MCL) and acute lymphoblastic leukemia (ALL). It is a CD19-directed genetically engineered autologous T cell immunotherapy. Tecartus, developed by Kite Pharma Inc, was approved by the FDA in July 2020, and by EC on December 14, 2020. Tecartus is a T-cell product that, in terms of production and CAR structure, is identical to Yescarta; but, it is the first and only CAR-T cell treatment for adults with MCL [122,123].

Tecartus is made up of the patient's own T cells that have had the gene for a protein called chimeric antigen receptor (CAR) put into them , allowing them to recognize and destroy cancer cells. The gene is put into the cells through a retrovirus that has been engineered to carry the gene and does not cause disease. The CAR protein is then produced by the transformed T cells and displayed on their surface. CAR can bind to CD19, a protein

found on the surface of cancer cells. When the patient receives Tecartus, the modified T cells bind to and kill cancer cells, assisting in the removal of the cancer from the body [124].

The FDA's clinical safety evaluation was based on an examination of 88 patients who were treated in a trial (NCT02601313), a phase II multicenter research for the treatment of MCL that is presently being conducted at many sites in the US and Europe with a total of 105 patients. The clinical study has already yielded encouraging results, with 93 percent of patients responding to a single Tecartus infusion and 67 percent obtaining a complete response. In the two phase I/II multicenter studies, over 100 patients with B-ALL or B-NHL will be treated with Tecartus (NCT02614066, NCT02625480). The research locations are located throughout the United States and Europe [125].

More than half of the patients in the clinical research experienced significant adverse effects. Cytokine release syndrome (a potentially life-threatening condition characterized by fever, vomiting, shortness of breath, pain, and low blood pressure), encephalopathy and infections are the most common serious side effects [124]. The one-time therapy will be sold for $373,000 by Kite Pharma (a Gilead-owned cell therapy firm).

Skysona (Elivaldogene Autotemcel)

On 16 July 2021, the EMA approved elivaldoge autotemcel (Skysona) for the treatment of early cerebral adrenoleukodystrophy (CALD) for patients 18 and younger. Skysona is a gene therapy developed by BlueBirds Bio Company for treatment of CALD. CALD is a rare, inherited genetic disease with mutations in the ABCD1 gene. This X-linked genetic disease mostly (even completely) affects males. This drug is given to patients who cannot find a donor for haematopoietic stem cell transplantation. The mutation hinders the development of an enzyme known as ALDP (adrenoleukodystrophy protein), which is responsible for the breakdown of fatty compounds in the body known as very long-chain fatty acids (VLCFAs) [126]. As a consequence, VLCFAs accumulate, causing inflammation and myelin breakdown .

Skysona is formed of CD34+ cells taken from the patient's own bone marrow or blood that have been engineered to incorporate a copy of the gene to build a functioning ALDP and may proliferate to produce other types of blood cells. Skysona uses CD34+ cells collected from patients' blood or bone marrow. A lentivirus is used to introduce a gene that allows them to manufacture ALDP into the CD34+ cells [127]. This genetically modified cell produces ALDP, which aids in the breakdown of fatty compounds in the surrounding cells and the regulation of illness symptoms.

One month after the medicine was authorized, the firm chose to withdraw from the European market in order to focus on the American market. BlueBirds Bio has previously removed another gene treatment, Zynteglo, from Germany after failing to reach an agreement on a market price of $1.8 million. The firm, together with Skysona, announced that it will cease operations in Europe [128].

Cell and Gene Therapy Product Market

Cell and gene therapy products differ from conventional therapies and medications in that they directly alter the gene responsible for the condition, often curing sickness and treating uncommon disorders with a single application. Aside from the fact that these products are one-of-a-kind, the challenges in manufacturing contribute to their high price. These difficulties may be; different individual responses, the difficulty of mass production, the length time of clinical trial and approval, the inability to reverse existing damage (only stabilizing the effects of the disease).

There are currently more than 450 pharmaceutical companies working for cell and gene therapy products worldwide, and these companies conduct more than 800 registered clinical trials (ARM Q3 update). According to FDA estimates, they expect to receive 200 new applications as IND (investigational new drugs) every year. And they estimate they will approve between 10 and 25 cell and gene therapy products each year by 2025 [129]. After this statement of FDA, investments in this field have increased. It is seen that the market for cell and gene therapy products is growing day by day. Luxturna used for the treatment of retinal dystrophy produced by Spark company is $850.000, and Zolgensma is the drug used for the treatment of SMA produced by Novartis is $2.2M. According to the EvaluatePharma March 2019 report, sales of cell and gene therapy products are expected to increase by almost 33 times to $43.7B in 2024, from $1.3B in 2017 (**Figure 2**).

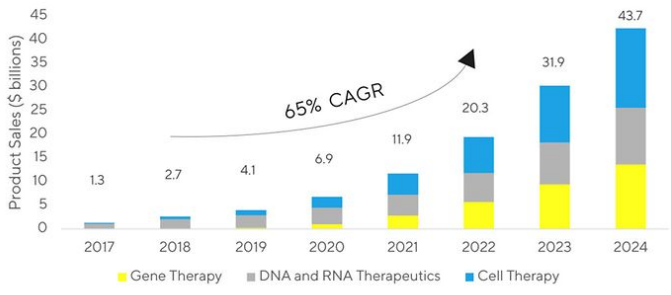

Figure 2. Sales Growth Trends of Cell and Gene Therapy Products from 2017 to 2024. Source: EvaluatePharma, March 2019.

Currently, about 100 companies compete in the cell and gene therapy market. For example, Gilead's 2017 acquisition of Kite Pharma for $12 billion is just one example of market competition and billion-dollar deals. In September, 2021, AbbVie and Regenxbio formed a strategic partnership combining eye care and gene therapy expertise to develop RGX-314, an investigational gene therapy for age-related macular degeneration, diabetic retinopathy, and other chronic retinal diseases. Regenxbio will receive $370 million in prepayment and a total of $1.38 billion in payments at necessary steps throughout the production process (https://regenxbio.gcs-web.com/) . While most of the companies work in the field of both cell and gene therapy, some of them have focused on a single field. When we look at cell and gene therapy products, we see that most of the products are not produced by a single company, but they make strategic collaborations among themselves. These strategic collaborations can take place in the form of a production agreement, license agreement or service procurement. And while the number of active collaborations was 42 in 2012, it is over 444 in 2018, that is, it has increased 10 times in 5 years [130].

CONCLUSION

Cell and gene therapy products are used to treat a wide range of disorders, including cancer, blindness, aging, hereditary and uncommon diseases. Cell and gene therapy medications are manufactured and approved in a different manner than regular pharmaceuticals, allowing for tailored treatment. One of the most difficult obstacles for cell and gene treatments is that they frequently target illnesses that affect only a small number of people. It explains why it was removed from the market after receiving clearance from the appropriate organizations for this sale. Glybera is one such case. Glybera, the most expensive medicine in the world with a $1 million price tag, was only available to one individual once it was authorized. Its creators withdrew from the European market in 2017, preferring not to extend the therapy's regulatory authorization following commercial failure in Europe and challenges in accessing the US market. Skysona is another example that we will provide shortly. The producer (BlueBirds Bio Company) pulled it from the market because they couldn't agree on a price barely one month after it was authorized in Europe. BlueBirds Bio Company has withdrawn Zynteglo, another drug that Skysona had previously approved and marketed in Europe, from the market for the same reasons. After Skysona, the company stopped all its activities in Europe and aimed to reach the American market. When we look at the market size, we can see that since the FDA stated their aim to approve 10-20 gene therapy medications every year through 2025, spending in this sector has skyrocketed. According to the EvaluatePharma March 2019 study, revenues of cell and gene therapy products are predicted to nearly triple to $43.7 billion in 2024, up from $1.3 billion in 2017. The number of cell and gene therapy medications available increases year after year, as do investments in this research.

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Development of Gene Editing Strategies for CCR5 Gene in Endothelial Cells

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Abstract: The CRISPR system has been adapted as a promising gene editing technology that provides robust and efficient DNA alterations. It was demonstrated that it has a high adaptability and great potential to cure acquired immune deficiency syndrome (AIDS) through gene disruptions. Endothelial cells are an essential component of the vascular system, and CRISPR has emerged as a promising candidate for AIDS therapies by removing the human immunodeficiency virus (HIV) reservoir in endothelial cells. CCR5, an essential receptor for HIV infection, is expressed by endothelial cells. We developed CCR5 gRNAs and tested their effectiveness in gene targeting. We established a basic and successful transfection system, as well as on-target CCR5 mutagenesis using the endonuclease assay. These findings imply that endothelial diseases could be targeted using the CCR5 gRNA/CRISPR system, which would be a novel approach. Endothelial diseases caused by genetic mutations or HIV infection could be treated in endothelial cells using the CRISPR/Cas9-based gene therapy platform by targeting CCR5.

Key words: Endothelial cells, AIDS, CRISPR, CCR5.

1. Introduction

Acquired immune deficiency syndrome (AIDS) was reported firstly in 1980s and caused by the human immunodeficiency virus type 1 (HIV-1) [1, 2]. HIV-1 infection mainly destroys primary CD4+ T cells processed by binding of the viral protein called gp120 to the CD4 receptor of the cells with either co – receptors CCR5 or CXCR4 [3, 4]. It was reported that CCR5 serves a main coreceptor for HIV-1 virus infection [5]. In addition, it is stated that individuals who are resistant to HIV-1 infection have a homozygous mutation as 32 base pair deletions in CCR5 locus which also results in lack of function of CCR5 receptor [6-8]. These findings led to discover the novel therapies to prevent the HIV-1 infection based on the knowledge that lack of function in CCR5 protect from HIV infection [6-9]. Further studies with transplantation HSCs verified that HIV-1 infection treatment through transplantation from a naturally occurred CCR5-delta 32 donor in 2007 [10, 11]. Therefore, therapeutic power of CCR5 disruption stands out for HIV infection treatment based on this finding.

Previous studies aimed to target CCR5 and HIV by ZFN, and TALEN technologies in CD4+T cells and HSCs [12-15]. CRISPR-Cas9 technology as a latest introduced genome engineering technique is easier and more effective technique [16]. It is applicable in various researches because of its basic system including Cas9 endonuclease protein dual 20 base pair RNA sequences [12, 16]. Moreover, its system based on the using cells homologous recombination which are the homologous directed repair (HDR) or non-homologous end joining (NHEJ) [12-14, 17-19]. It was reported that CRISPR-Cas9 has been used *in vitro* and *in vivo to* disrupt CCR5 in HSCs and CD4+T cells [20-25]. Thus, it has been evaluated that CRISPR-Cas9 has been potential to prevent effect of HIV-1.

Latest researches indicate that there is a correlation between HIV infection and cardio vascular diseases (CVDs), especially atherosclerosis, which elevated in HIV infected individuals without traditional cardiovascular traditional risks such as an increase endothelial permeability, pro inflammatory cytokine levels and adhesion molecule expression [26-41]. It was reported that HIV RNA levels and HIV viral protein levels correlate with endothelial dysfunction [42-44]. It was stated that HIV viral proteins cause aortic endothelial dysfunction in mouse and with reduced of endothelial vasomotor function in humans. It was observed that HIV based endothelial dysfunction caused through direct infection of endothelial cells by virus and indirect infection of endothelial cells by released HIV proteins into the environment. There is gp120 a glycoprotein of HIV is essential for virus infection and presented not only on the surface of the virus but also in body fluids as soluble form. It interacts with CD4 receptor and co-receptors CCR5 and CXCR4 in bound or soluble form [45, 46]. It was stated that it interacts with endothelials through co- receptors and cause dysfunction human coronary ECs, human umbilical vein ECs (HUVECs) and brain microvascular ECs (BMVECs) [47-51].

In this study, we established CRISPR/Cas9 gene knockout and non-viral transfection system in endothelial cells. In addition, we achieved CCR5 disruption evaluated in T7 endonuclease assay *in vitro*.

2. MATERIALS and METHODS

2.1. Cell lines and culture conditions

Human embryonic kidney cell line (HEK293T), human brain microvascular endothelial cells (HBMEC), and human umbilical vein endothelial cells (HUVEC) were obtained originally from ATCC (American Type Cell Collection). Human Embryonic Kidney Cells (HEK293T) (ATCC, CRL-3216) were cultured in Dulbecco's Modified Eagle Medium (Gibco DMEM High Glucose, Pyruvate). Human Brain Microvascular Endothelial Cells (HBMECs) were cultured in Dulbecco's Modified Eagle Medium (Gibco DMEM Low Glucose, Pyruvate). Both mediums were supplemented with 10% FBS (Lonza) 1% Penicillin-Streptomycin (Gibco).

2.2. Design of gRNA for target locus

The genomic location of CCR5 was determined by using National Centre for Biotechnology Information (NCBI) gene database. SnapGene tool was used to locate exon and intron sites. Online gRNA design tool (http://crispr.mit.edu/, by Zhang Lab, MIT) was used to determine gRNA sequence which listed based on-target and off-target scores. gRNA with the highest score was chosen and it was obtained as 20 bp length (Table 1).

Table 1: gRNA sequences designed for CCR5 targeting

Name	Sequence (5'-3')	PAM
CCR5-F	TAATAATTGATGTCATAGAT	GGG
CCR5-R	ATCTATGACATCAATTATTA	GGG

As a final step in design of gRNA oligos, adapter sequences were added to gRNA oligos tofacilitate cloning as given in table and ordered (Table 2).

Table 2: The final forms of CCR5 gRNA sequences

Name	Sequence (5'-3')	PAM
CCR5-F	**CACCG**TAATAATTGATGTCATAGAT	GGG
CCR5-R	**AAAC**ATCTATGACATCAATTATTA**C**	GGG

2.3. Annealing of oligonucleotides

gRNA oligos which are ssDNA oligos, were phosphorylated and annealed by a reactionmixture as forward oligo (100 µM) 1 µl, reverse oligo (100 µM) 1 µl, 10X T4 Ligation Buffer 1 µl, T4 Polynucleotide Kinase 1 µl, and Nucleotide Free Water 6 µl. Phosphorylation and annealing reaction were performed by using thermal cycler as 37°C for 30 min, 95°C for 5 min, ramp down from 95 to 25°C with 0.1°C/sec cooling. Than oligos, which was annealed, was diluted by using nuclease free water in 1:200 ratio andwere stored in -20ºC until use.

2.4. Digestion of the plasmid and purification of backbone

pSpCas9(BB)-2A-GFP plasmid was digested by using BpiI restriction enzyme as 2 µl of 10X Fast Digest buffer, plasmid DNA up to 5 µg, 1 µl of BpiI Fast Digest Enzyme, and completed to 20 µl with nuclease free water. Restriction was performed at 37ºC for 30 minutes at thermal cycler and then dephosphorylation was performed by using Alkaline Phosphatase as 3 µl of Alkaline Phosphatase, 3 µl of 10X Fast AP Buffer, 20 µl of digested reaction, and 3 µl of nuclease free water. Dephosphorylation performed at 37ºC for 20 minutes and then at 80ºC for 15 minutes. Agarose gel was prepared for separation of digested plasmid fragments in 1%(w/v) in TBE buffer. Agarose gel electroporation was conducted for 1 hour at 150 V after loading of plasmid mix with dye and ladder. Visualization was performed with the Biorad Chemidoc XRS device and image was captured. Backbone band, which is around 9,5 kb determined, then it was dissected by using a scalper. It was placed into eppendorf tube and weight was measured before plasmid purification. Purification was performed with NucleoSpin® Gel and PCR Clean-up Kit. Solubilization of the gel was performed with NTI buffer (200 µl / 100 mg) at 50°C for 10 minutes. Sample was transferred into collection tube and washing performed by centrifugation at 11,000 x g for 30 seconds and 700 µl of NT3 Buffer according to manual. Additionally, one more centrifugation step performed at 11,000 x g for 1 minute to dry up the membrane. Elution was performed by using 30 µl NE bufferand centrifugation at 11,000 x g for one minute. Nanodrop was used to measure concentration.

2.5. Ligation of the plasmid backbone with gRNA

Ligation was performed by using T4 DNA ligase as 50 ng of purified pSpCas9(BB)-2A-GFP plasmid backbone, 1 µl of annealed gRNA Oligos (1:200), 1 µl of T4 DNA Ligase, 2 µl of 10X T4 DNA Ligase Buffer, and completed up to 2u µl with nuclease free water. Ligation was performed at room temperature for 60 minutes.

2.6. Bacterial transformation and plasmid isolation

5 µl of ligated plasmid was mixed with 50 ul of DH5α competent cell after thawing on ice and were incubated on ice for 30 minutes. Then it was performed heat schock as 90 seconds for the samples by using heat block adjusted to 42°C. Samples immediately incubated on ice again for two minutes. It was added 950 µl of LB broth to each tube and cells were incubated at 37°C, 150 rpm for 150 minutes. After centrifugation step 100 µl inoculum was spreaded on agar plates containing Ampicilin and incubation were performed at 37°C overnight. It was observed colonies next day on plates.

It was picked colonies to culture for plasmid isolation and cultured with 100 ml LB Broth containing Ampicilin. It was incubated overnight at 37°C, 150 rpm for overnight. The next day glycerol stock was prepared as final in 25% glycerol which stored in -80°C and were continuedto purification. It was started to purification with centrifugation step at 6,000 x g, 10 minutes at 4°C which followed by resuspension of pellet with 8 ml of RES

buffer of the PureLink Genomic DNA Mini Kit (Invitrogen, K182001). The cells were lysed with 8 ml of LYS bufferat RT for 5 minutes. Equilibration buffer was used to equilibrate column filter with 12 ml. NEUbuffer was used for neutralization of lysed cells as 8 ml. Cells transferred to column and 5 ml of EQU buffer and 8 ml of Wash buffer was used to wash the column. Purification wasperformed with 5 ml of ELU buffer and then 0.7 volumes of isopropanol was added into theeluted which followed with the incubation step at RT for 2 minutes. Secondary purification step was performed with finalizer and samples transferred to the syringe which attached to thefinalizer. The washing steps were performed with 2 ml of %70 ethanol and purification were performed with 500 µl of TRIS buffer. Concentration measurement was performed by using Nanodrop.

2.7. Polymerase chain reaction (PCR) verification

The PCR amplification was used to test presence of gRNA on the plasmids. The primers listed in table 3 was used as 10 µM stock concentration. The forward primer was U6 promoter primer and the reverse primer was the original reverse oligo.

Table 3: PCR reaction primer sequences.

Primer Name	**Sequence 5'-3'**
U6 Cloning Forward	GACTATCATATGCTTACCGT
CCR5 gRNA Reverse	AAACATCTATGACATCAATTATTAC

Reaction was prepared by using Taq2X master mix (NEB) as 10 µl of 2X master mix, 1 µl of plasmid DNA, 1 µl of 10µM forward primer and 10µM reverse primer, 7 µl of nuclease free water. The running condition was performed in thermocycler as 95 °C for 60 s, 30 cycles of polymerase chain reaction (Denaturation at 95 °C for 15 s, and annealing at 55 °C for 15 s, Extension at 68 °C for 10 s), and final extension at 68 °C for 2 min. 10 µl of PCR products was run on 2% agarose gel prepared using TBE buffer at 150 V for 50 minutes. Biorad Chemidoc XRS device was used. Visualization was performed with the Biorad Chemidoc XRS device and image was captured.

2.8. Mammalian cell transfection optimization

HEK293T were seeded in 6 well plate with $3x10^5$ density, cultured in 2 ml DMEM high glucose supplemented by 10% FBS, 1% Penicillin-Streptomycin, and incubated overnight at 37oC, 5% CO2. The next day medium was changed with the antibiotic free medium. Transfection mixture were perepared by mixing 1 µg pSpCas9(BB)-2A-GFP-sgRNA with 2 µg and 3 µg of PEI (1 mg/ml stock) in 200 µl serum-free DMEM to obtain 1:2 and 1:3 of DNA: PEI ratio and incubated at room temperature for 15 minutes. After adding the mixture to the wells, cells incubated for four hours at 37oC, 5% CO2. The medium was changed with the medium including antibiotics. GFP expression was checked by using Cytell fluorescent microscope (GE Healthcare Life Sciences) and cells incubated for 48 hours additionally. Cells were used for downstream applications.

2.9. Screening of the transfected HEK293T cell line

Transfection efficiency was checked with the Cytell fluorescent microscope (GE Healthcare Life Sciences) with GFP expression 24 hours after transfection. FITC filter wasused for imaging and it was performed with 10X magnification and recorded in TIFF format.

Then, HEK293T were harvested by trypsin to measure transfection efficiency by using Beckman Coulter's CytoFLEX flow cytometry device with GFP expression. Cells were washed with PBS and dissolved in 200 µl PBS at final. Acquisition was set to 1 $x10^5$ events. Forward and side scatter plots was used to determine live population. FITC-A / FSC-A dot plot wasused to determine GFP expression. Results were recorded as histogram and analyzed.

2.10. Indel detection of gRNA

Genomic DNA isolation was performed with the PureLink Genomic DNA Mini Kit (Invitrogen, K182001). Cells were harvested by tripsinization and resuspended in 200 µl PBS. Cells lysed with 200 µl of Lysis/Binding Buffer at 55°C for 10 minutes after incubation with 20 µl of Proteinase K and 20 µl of RNase A at RT for 2 minutes. 200 µl of absolute ethanol was added, mixed and lysate transferred to the column. Washing step were performed with the 500 µl Wash Buffer 1 and 500 µl Wash Buffer 2 respectively. Elution step were performed with 50 µl of Genomic Elution Buffer. Concentration measurement was performed by using NanoDrop.

The PCR amplification were performed to amplify the target region. The primers listed in Table 4 was used as 10 µM stock concentration. The forward and reverse primers were produced 1 kb product.

Table 4: PCR reaction primer sequences of target region.

Primer Name	**Sequence 5'-3'**
CCR5 Target Forward	CTGAGCTGCACCATGCTTGA
CCR5 Target Reverse	TCCCGAGTAGCAGATGACCA

Reaction was prepared by using Taq2X Master Mix (NEB) as 10 µl of 2X master mix, 500 ng of plasmid DNA, 1 µl of 10µM forward primer and 10µM reverse primer, and up to 20 µl of nuclease free water. The running condition was performed in thermocycler as 95 °C for 60 s, 35 cycles of polymerase chain reaction (Denaturation at 95 °C for 15 s, and annealing at 55 °C for 15 s, Extension at 68 °C for 10 s), and final extension at 68 °C for 1 min.

Another PCR reaction was set for control template, which supported by the kit, as 10 µl of 2X master mix, 2 µl of control

template and primer mix, and 8 µl of nuclease free water. The running condition was performed in thermocycler as 98 °C for 30 s, 35 cycles of polymerase chain reaction (Denaturation at 98 °C for 10 s, and annealing at 55 °C for 15 s, Extension at 68 °C for 30 s), and final extension at 68 °C for 1 min.

5 µl of PCR products of control and target locus was run on 1% agarose gel prepared using TBE buffer at 150 V for 50 minutes. Visualization was performed with the Biorad Chemidoc XRS device and image was captured.

2.10.1 T7 endonuclease assay

5 µl of PCR products of control and target was used to form heterodublex as 2 µl of 10X NEBuffer 2, 5 µl of PCR product, and 12 µl of nuclease free water. The running condition was performed in thermocycler as denaturation at 95 °C for 5 min, annealing at 95–85 °C at 2 °C/sec ramp rate for 5 s, annealing at 85–25 °C at 0.1 °C/sec ramp rate for 5 s and hold at 4 °C until use.

Then, 1 µl of T7 Endonuclease I enzyme added to the mixture and incubation performed at 37 C for 15 minutes. 5 µl of heteroduplex products was runned on 1% agarose gel prepared using TBE buffer at 150 V for 50 minutes. Visualization was performed with the Biorad Chemidoc XRS device and image was captured.

2.11. Transfection of HBMEC cell line with pSpCas9(BB)-2A-GFP, screening andknockout control

HBMECs were seeded in 6 well plate with $3x10^5$ density, cultured in 2 ml DMEM low glucose supplemented by 10% FBS, 1% Penicillin-Streptomycin, and incubated overnight at 37oC, 5% CO2. The next day medium was changed with the antibiotic free medium. Transfection mixture were prepared by mixing 1 µg pSpCas9(BB)-2A-GFP-sgRNA with2 µg of PEI (1 mg/ml stock) in 200 µl serum-free DMEM to obtain 1:2 of DNA: PEI ratio and incubated at room temperature for 15 minutes. The medium was changed with the medium including antibiotics. GFP expression was checked by using Cytell fluorescent microscope (GE Healthcare Life Sciences) and cells incubated for 48 hours additionally.

Transfection efficiency were checked with the Cytell fluorescent microscope (GE Healthcare Life Sciences) with GFP expression 24 hours after transfection. FITC filter wasused for imaging and it was performed with 10X magnification and recorded in TIFF format.

Genomic DNA isolation were performed with the Genomic DNA Mini Kit (Invitrogen, K182001), and PCR amplification were performed by using CCR5 target forward and reverse primers (Table 4.). Heteroduplex analysis performed through T7 endonuclease assay as defined.

2.12. Flow cytometry analysis

HBMECs were harvested by trypsin to measure transfection with GFP expression and knockout efficiency with CCR5 expression by using Beckman Coulter's CytoFLEX flow cytometry device. Cells were washed with PBS and dissolved in 200 µl PBS at final. Acquisition was set to 1 $x10^5$ events. Forward and side scatter plots was used to determine live population. FITC-A / FSC-A dot plot was used to determine GFP expression. APC-A/ FSC-A dot plot was used to determine GFP expression. Results were recorded as histogram and analyzed.

3. RESULTS

3.1. Verification of sgRNA insertion into pSpCas9(BB)-2A-GFP

PCR amplification were performed to confirm gRNA insertion into into pSpCas9(BB)-2A- GFP plasmids. It was used CCR5 gRNA reverse oligo as reverse primer and U6 Promoter as forward primer because of U6 promoter located in the upstream of the gRNA site. It wasexpected to detect 100 bp in PCR product in precense of gRNA. PCR product would not be visualized in the absence of gRNA due to oligo would not anneal to plasmid DNA. Visualization of PCR reaction was determined by Biorad Chemidoc XRS device after running samples with agarose gel electrophesis (Figure 1).

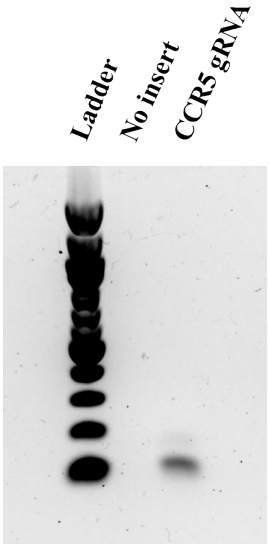

Figure 1: Agarose gel image of PCR products with 50 bp 50bp DNA Step Ladder(Promega) DNA ladder for size determination.

Figure 1 indicates that successful insertion of gRNA into pSpCas9(BB)-2A-GFP because of the 100 bp DNA product in CCR5 gRNA sample while plasmid with no insert does not have any band.

3.2. Optimization and Screening of Cell Transfection

HEK293T cells were transfected as 300,000 cell per well with 1 µg of pSpCas9(BB)-2A- GFP plasmid in same amount and with PEI in different amount to obtain most efficient concentration. DNA: PEI ratio was the 1:2 and 1:3 as 2 µg and 3 µg of PEI respectively and cells was checked for GFP expression after 24 hours (Figure 2).

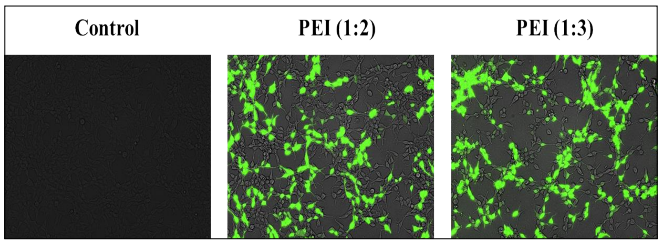

Figure 2: Transfection of HEK293T cells with with pSpCas9(BB)-2A-GFP plasmidusing different PEI concentrations.

It was figured out that PEI has given successful results in the manner of transfection. Instead of providing successful transfection by different PEI concentrations as shown in figure 2, higher concentration of PEI caused more death than lower concentration. Therefore 1:2 DNA: PEI concentration was chosen for transfection.

It was assessed the flow cytometry experiment with the same protocol in HEK293T transfection with PEI for flow cytometry analysis. Cells were collected 48 hours after transfection and GFP expression was analyzed by flow cytometry.

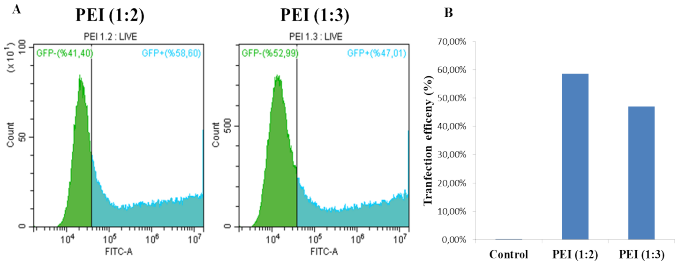

Figure 3: GFP expressing transfected HEK293T cells. A; histogram graph representation, B; GFP+ HEK293T cell line analysis.

It was observed that 1:2 DNA:PEI ratio provide higher transfection efficiency than the 1:3 ratio. In addition, it was suggested that PEI transfection is efficient to work for HEK293T cell lineas can be seen from figure 3.

3.3. Validation of Gene Knockout

T7 Endonuclease Assay

Mutation detection on target locus was performed by using T7 Endonuclease Assay. Two cell lines which are HEK293T and HBMEC lines were transfected with pSpCas9(BB)-2A- GFP plasmid without insert as a control and another group transfected with pSpCas9(BB)- 2A-GFP plasmid including CCR5 gRNA. Genomic DNAs were isolated and target locus was amplified by PCR reaction. Primers designed to produce one band as 1 kb product in PCR reaction and two bands as 400 bp and 600 bp in the manner of heterodublex reaction by T7 Endonuclease. PCR products of target locus for HEK293T and HBMEC cell lines screened by agarose gel electrophesis (Figure 4 and Figure 5).

The result given in figure 4 and figure 5 shows that 1 kb products obtained successfullyin PCR of target locus in both cell lines. It was not observed any unspecific products, and then it was continued to T7E1 assay.

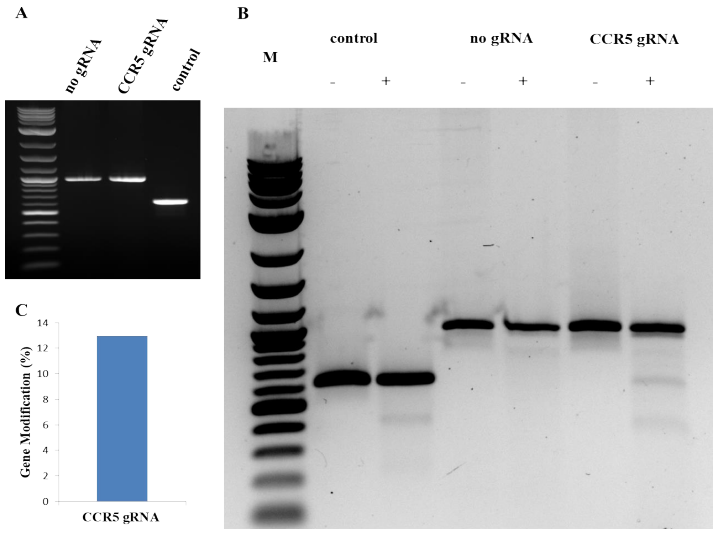

Figure 4: CRISPR/Cas9 activity with T7E1 assay in HEK293T cell line. A; representativegel image of PCR products amplified from target sites of pSpCas9(BB)-2A-GFP only control, pSpCas9(BB)-2A-GFP + CCR5 gRNA and PCR control, B; representative gel image of T7E1 treated PCR products C; estimated % modification analyzed by imageJ.

It was observed that CCR5 gRNA successful activity of cleavage on target sequence, which detected by the T7E1 assay in HEK293T cell line as 13% ranging between 10% and 18%. pSpCas9(BB)-2A-GFP only control did not show any cleavage on target sequence due to lack of gRNA as expected. We used the same amount of genomic DNA to evaluate the groups.

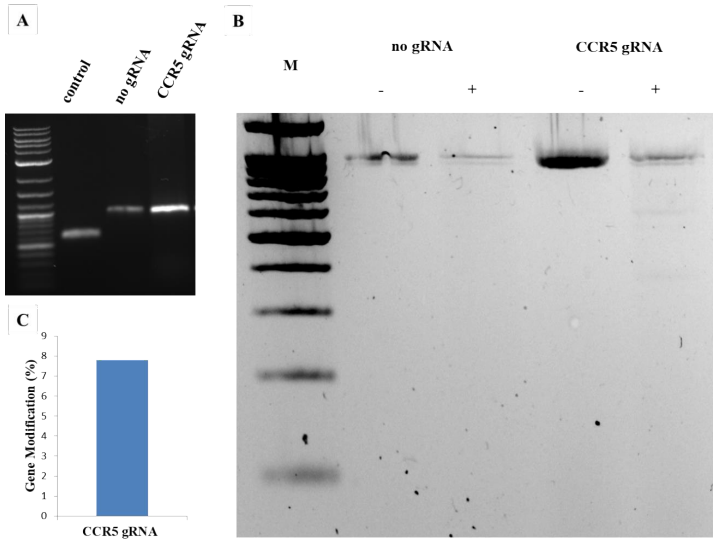

Figure 5: CRISPR/Cas9 activity with T7E1 assay in HBMEC cell line. A; representativegel image of PCR products amplified from PCR control and target sites of pSpCas9(BB)- 2A-GFP only control, pSpCas9(BB)-2A-GFP + CCR5 gRNA respectively, B; representative gel image of T7E1 treated PCR products C; estimated % modification analyzed by imageJ.

It was observed that CCR5 gRNA successful activity of cleavage

on target sequence, which detected by the T7E1 assay in HBMEC cell line about 7.5%. pSpCas9(BB)-2A-GFP only control did not show any cleavage on target sequence due to lack of gRNA as expected. We used the same amount of genomic DNA to evaluate the groups.

Flow Cytometry Analysis

Knockout ratio of CCR5 for target locus was performed by using anti CCR5 antibody with flow cytometric assay. HBMEC lines were transfected with pSpCas9(BB)-2A-GFP plasmid without insert as a control and another group transfected with pSpCas9(BB)-2A- GFP plasmid including CCR5 gRNA. After the incubation for 48 hours, cells trypsinized and incubated with anti CCR5 antibody. FITC-A and APC-A plots were used for analysis as shown (Figure 6)

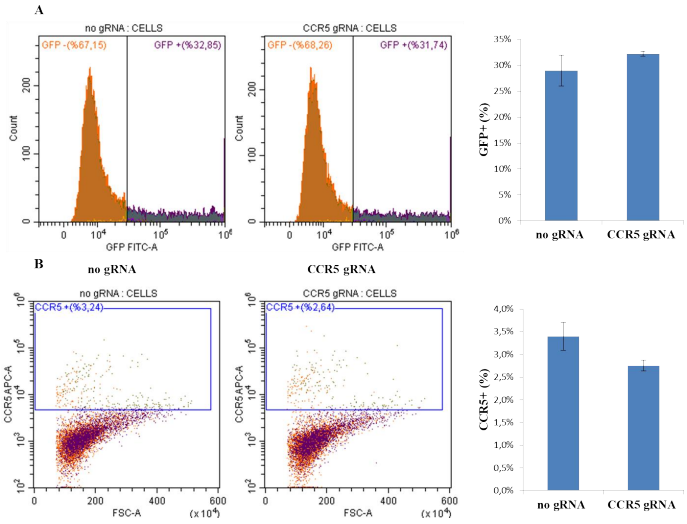

Figure 6: Flow cytometry analysis of transfected HBMEC cells only pSpCas9(BB)-2A- GFP plasmid and pSpCas9(BB)-2A-GFP + CCR5 gRNA plasmid. A; GFP+ HBMECs histogram representation and analysis, B; CCR5+ HBMECs histogram representation and analysis.

It was observed around 30 % transfection efficiency of pSpCas9(BB)-2A-GFP plasmid in HBMECs. There is no essential difference between two group transfection efficiencies (Figure 6A). In addition, instead of not being huge, there is a decrease in CCR5+ HBMEC cells ratio, which suggest knockout efficiency of system provided (Figure 6B).

4. DISCUSSION

The main objective of this study was to suggest an optimized protocol to disrupt CCR5 gene on endothelial cells and so we reported gene modification in HEK293T and HBMEC mammalian cells through CRISPR/Cas9 system.

CRISPR-Cas9 technology is introduced recently and RNA mediated genome engineering technique. Its basic design system through Cas9 endonuclease and changeable short RNA sequence made it easier, more robust and more effective technique to introduce knock-ins, knockouts, translocations and base changes rather than other endonuclease-based systems which are ZFNs and TALENs [12, 16]. Nowadays, it becomes a standard experimental strategy in research labs to apply *in vitro* and *in vivo* for plants and animals [52-54]. In latest decade, so it was showed that genome engineering has a great potential to cure disease and generating models for drug therapies [55-60].

Improvement in genome engineering provides a way to us to cure the diseases permanently. There are ongoing researches to cure HIV viral disease by the CRISPR-Cas9 technology. This disease caused through interaction of virus with cells through glycoprotein of virus and receptors of cells, which followed by integration of virus into the cells [3-5]. Therefore, it was proposed that disruption of the receptors on cell surface would prevent the HIV infection. Although it has not been reached 100% targeting efficiency, studies reported successful prevention of HIV by disrupting CCR5 receptor of HSCs by using CRISPR/Cas9 technology [20-22, 24-25]. As the latest research showed that HIV virus cause infection also on the endothelials through same way, which results in endothelial dysfunction as atherosclerosis [47-51]. In this study we aimed to target knockout CCR5 in endothelials using CRISPR/Cas9 and mammalian vector transfer system. To target human CCR5 gene, we designed a gRNA with 20 base pair lengths.

In this study decided to use HUVEC endothelial cell line and HBMEC primary endothelial cell line which are characterized endothelial cells to use in endothelial researches [61-63]. Additionally, HEK293T cells used for targeting verification in first due to being easy to transfect [64]. We used non-viral delivery system pSpCas9(BB)-2A-GFP mammalian plasmid instead of the viral systems as lentivirus vectors. Also, it was used PEI reagent for transfection, which is composed of cationic polymer [65]. To this end, we performed transfection optimization, and on-target mutagenesis analysis through T7 endonuclease assay in HBMECs and HUVECs. Although, it was planned to use HUVECs as endothelials, we decided to not continue with it due to being not transfected neither with Lipofectamine 3000 not with PEI (Data not presented). Therefore, it was decided to continue with HEK293T and HBMEC cells for downstream applications. Two different doses of PEI were used for transfection optimization and it was decided to use 1:2 DNA to PEI ratio because of exhibiting higher efficiency and lower toxicity rather than 1:3 DNA to PEI ratio. Transfection efficiencies were evaluated using flow cytometry and it was indicated that average transfection efficiency obtained for HEK293T as 55% and for HBMECs as 30%. It was observed decreased transfection efficiency in endothelial cell line HBMEC compared to HEK293T. On-target mutagenesis analysis were evaluated using T7 endonuclease assay showed that sgRNA gives nearly 13% gene modification in HEK293T. After this targeting verification with HEK293T, T7 endonuclease assay performed with HBMECs. On-target mutagenesis analysis using T7 endonuclease assay showed that sgRNA gives nearly 8% gene modification in HBMEC. Also, It was observed a decrease ingene modification efficiency in HBMEC compared to HEK293T. Flow cytometry results evaluate that there is a decrease in receptor

ratio in the manner of transfection with gRNA.

To sum up, successful gene modification was verified using T7 endonuclease assay and flow cytometry, despite observed low gene modification in both cell line. It needed further optimizations to increase gene modification efficiency and use of different gRNA could solve this problem. Different gRNA was not used due to limited time of this study;however, this study can be performed using another gRNA with optimized protocol for endothelials performed in this study. It was suggested that the protocol performed has been successful to perform knockout in endothelials and can be modified to target other genes.

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Diagnosis and Treatment of Fanconi Anemia: Special Focus on the Nuclease-Mediated Gene Therapy

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Abstract: Fanconi anemia is the most frequently reported group of rare hereditary bone marrow failure syndromes. The pathogenesis of the disease includes progressive bone marrow failure, developmental abnormalities, and enhanced predisposition of cancer. The main reason for childhood death in Fanconi anemia patients is bone marrow failure. Unfortunately, most children with Fanconi anemia later develop acute myeloid leukemia or myelodysplastic syndrome. In addition, adult patients are more prone to additional malignancies. To date, 22 different genes have been reported to cause Fanconi anemia. In the treatment of the disease, mainly androgens, and hematopoietic stem cell transplantation (HSCT) applications are included. However, androgen is not a definitive treatment, and its side effects can be severe. Although allogenic HSCT has some side effects, including the high risk of graft-versus-host (GVHD) and difficulty in finding donors, it is used compulsorily since there is no alternative therapy. For these reasons, there is a need for more specific, personalized, and effective solutions for the treatment of Fanconi anemia. Recent advances have been made in gene editing approaches from research for the treatment of single-gene diseases. There are different gene-editing methods available today; yet, the current diagnosis and treatment methods and nuclease-based gene-editing methods of Fanconi anemia will be discussed in this review.

Key words: HSCT, Fanconi Anemia, Gene Editing, Nuclease

1. Introduction

Fanconi anemia (FA), first described by Swiss pediatrician Guido Fanconi in 1927 is a genetically inherited hematologic disease (Fanconi 1927). He described three brothers from the same family who had bone marrow failure and different ranges of physical abnormalities including short stature, hyperpigmentation, and hypogonadism (Risitano, et al. 2016; Wu 2013). Since then, notable progresses have been achieved in our understanding of this biologically and clinically complex disease (Nalepa and Clapp 2018; Solanki, et al. 2017). The incidence of FA range from 1 in 100000 to 250000 births (Anurogo, et al. 2021).

1.1. Molecular Basis and Clinical Features Fanconi Anemia

FA is caused by a mutation in some of the DNA repair pathways genes (Cheung and Taniguchi 2017). It is a genetically heterogeneous disease and there are about 22 (A to W) genes responsible for disease progression (Moreno, et al. 2021). Most of the cases are inherited in autosomal recessive fashion except for two conditions including a heterozygous pathogenic variant in RAD51, known to cause autosomal dominant inheritance, and a hemizygous pathogenic variant in FANCB, recognized to cause X-linked recessive form of FA. Different complementation groups, different mutations within the same gene, modifier genes, and other genetic and environmental factors could affect the clinical expression of FA (Faivre, et al. 2000). Congenital malformations, susceptibility to cancer, and bone marrow failure that manifests as pancytopenia are the main clinical presentations of the FA (Moreno, et al. 2021).

1.2. Diagnosis of Fanconi Anemia

Although there is no definitive treatment for FA other than allogeneic HSCT, the early diagnosis of the disease is important because of the advantages such as avoiding the uncertainty of the diagnosis of the patients, cancer follow-up, genetic counseling, changing the way of life (no harmful habits), modification of radiation and chemotherapy protocols (Pilonetto, et al. 2009).

The first test to be used in the diagnosis of FA is the chromosomal breakage test. When performing this test, the lymphocytes obtained from the patient are first stimulated for proliferation, then they are treated with mitomycin C (MMC) or deoxybutane (DEB), which stimulates inter cross-links (ICL) between DNA strands, and induces chromosomal breakages in FA patients while in healthy patients the FA pathway repair those ICLs (Auerbach 2009). In most cases, an accurate diagnosis could be done with a careful history, physical examination, and a positive chromosomal breakage test of lymphocytes (Soulier 2011).

Some of the issues to be considered when performing this test are as follows: a healthy group should be tested together with the patient group. In addition, as the breakage rates between the

groups may be different, the patient group should be tested without treatment with MMC or DEB to setup baseline chromosomal breakage. Finally, if the suspicion of FA is strongly supported in patients with negative chromosomal breakage test in peripheral blood, a chromosomal breakage test should be performed in fibroblasts tissue of this group to exclude somatic mosaicism (Castella, et al. 2011).

Somatic mosaicism, detected in nearly 25% of FA patients, is described by the presentation of two different lymphocyte populations in the blood sample of patients (Ten Foe, et al. 1997). It exhibits itself by the presence of both lymphocytes that have crosslink sensitivity and that lost the crosslink sensitivity, appearing wildtype (WT). The reversion of an inherited mutation or the introduction of a de novo variant with a result of decreasing or excluding the deleterious nature of the inherited variant could cause somatic mosaicism (Asur, et al. 2018).

In some laboratories, cell cycle kinetics is used instead of DEB or MMC-induced chromosomal breakage test. Here, peripheral blood lymphocytes obtained from patients and healthy controls are treated with mitogen and DEB or MMC, followed by cell cycle analysis by flow cytometer. As the cell cycle progresses in healthy individuals, the DEB or MMC-induced DNA ICLs in FA patients cannot be repaired, and the majority of these lymphocytes (> 40%) are retained in the G2 phase of the cell cycle (Seyschab, et al. 1995).

Another FA diagnostic method is created based on the FA signaling pathway. The monoubiquitination of the FANC-D2 and following translocation to nuclear foci involve the utilization of multiple upstream FA genes. Western blot of FANC-D2 (both isoforms monoubiquitinated and non-ubiquitinated could be seen), offers a simple and quick assay for the integrity of the FA pathway (Pilonetto, et al. 2009; Shimamura, et al. 2002).

After successful identification of FA disease, the genetic mutation needs to be described. Characterization of mutation is important for prenatal diagnosis, disease progression, and identifying rare mutations or founder mutations (Frohnmayer, et al. 2014; Solanki, et al. 2017). Starting from the most mutated group (FANC-A), Sanger sequencing can be performed to identify the specific mutations (Soulier 2011). Another mutation screening method NGS is a cheap and fast method compared to Sanger sequencing. Five hundred different genes can be screened at the same time by making a panel for FA and related diseases with Next-generation sequencing (NGS). It should be noted that the NGS method is insufficient to detect large deletions or duplications and in such cases, the MLPA or array-CGH method is recommended. After all, it is good to perform bone marrow cytogenetic analysis that is an important sign of hematological malignancy to find out whether a clonal abnormality is present or not. For this reason, G-banding and FISH tests are recommended for FA-positive patients (Frohnmayer, et al. 2014).

Diseases that should be taken into account for differential diagnosis of FA, involving Bloom Syndrome, Dyskeratosis Congenita (DC), Shwachman-Diamond syndrome (SBDS), Xeroderma Pigmentosum (XP), Nijmegen breakage syndrome (NBS), Saethre-Chotzen syndrome (SCS), Diamond-Blackfan anemia (DBA), Baller Gerold syndrome (BGS), Thrombocytopenia absent radius syndrome (TAR), Dubowitz syndrome, Velocardiofacial syndrome (VFCS), Holt-Oram syndrome (HOS), VATER/VACTERL Association and VACTREL-H syndrome that are sharing some of the basic phenotypes of FA including short stature, hematological malignancies, chromosomal breakage and skeletal abnormalities (Auerbach 2015; Nalepa and Clapp 2018).

2. Treatment of Fanconi Anemia

Clinical heterogeneity of FA needs a comprehensive evaluation of patients at diagnosis for better treatment. Since it is a disease of multisystem, a multidisciplinary clinical approach is required for better management of FA. For this reason; hematology, oncology, endocrinology, ultrasonography, gastroenterology, orthopedia, plastic surgery are all need to work together for better outcomes in FA patients' treatment (Nalepa and Clapp 2018).

Treatment options for FA do not include many alternatives and get more difficult after malignancy develops. Administration of androgen, which increases bone marrow production that induced blood cell precursors; in times of crisis transfusion of red blood cells and platelets to replace blood cells; or replacing the diseased bone marrow with allogeneic hematopoietic stem cell transplantation could be beneficial to maintain hematological findings of the disease (Feben, et al. 2015). Disease management, in FA patients with aplastic bone marrow and without an HLA-matched sibling donor, is challenging (Azik, et al. 2010). In addition, granulocyte colony-stimulating factor (G-CSF) is used to increase the neutrophil count (Calado and Clé 2017). A chemotherapy drug Fludarabine is often used for the treatment of chronic lymphocytic leukemia. Combination of fludarabine and T-cell depletion in recipients of unrelated donors, decrease the rate of GVHD, increase the rate of hematopoietic recovery, and enhances both early and overall survival (Chaudhury, et al. 2008).

2.1. Limitations of Current Treatment Methods of Fanconi Anemia

Although there are some treatment options for FA, they have considerable limitations and serious side effects when both their permanence and effectiveness are taken into account. For example, androgens stimulate hematopoiesis in patients with FA, but it is also linked with risks of benign and malignant liver tumors. Therefore, HSCT is considered as first-line therapy for bone marrow failure in FA with available human leukocyte antigen (HLA) matched family donors (Moreno, et al. 2021; Shafqat, et al. 2021). However, patients with FA who will undergo HSCT, need low-intensity chemotherapeutics due to the hypersensitivity to DNA-damaging chemotherapeutics (Deviren 2018; Nalepa and Clapp 2018). In addition, although HSCT is the only applicable approach for hematological complications of FA including MDS and AML, not all patients have a suitable donor, reasons such as failed transplants and solid tumors, graft failure, and especially GVHD and opportunistic infections make the treatment of the disease difficult (Bagby 2018; Feben, et al. 2015; Song 2009). Finally, in a recent literature review, oral cancer was

diagnosed in 121 individuals affected by FA, and it was reported that HSCT may increase the risk of oral cancer development, especially 5 years after transplantation (Furquim, et al. 2018). Despite these applications, unfortunately, the disease cannot be completely controlled and patients are dying at an early age. For these reasons, there is clearly a need for a less toxic and more successful form of treatment for FA.

2.2. Nuclease-Mediated Treatment Studies on Fanconi Anemia

The side effects of androgens, the inability to find an effective donor for HSCT, and the risk of developing GVHD, are important limitations of current treatment methods for FA. Therefore, it is inevitable to develop new treatment strategies for FA.

Gene editing technologies have achieved great momentum in recent years and are being tested in many diseases and models. Possible situations in which gene therapy is applied are recessive single-gene diseases and inherited genetic disorders. There are different molecular tools developed and used to implement these regulations (Hussain, et al. 2019).

Currently, there are five major classes of gene regulatory nucleases used in genome editing, including Homing endonucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Mega Nucleases (MNs), and lastly, the Guide RNA (gRNA) Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-Associated protein 9 (Cas9/gRNA) system (Dever and Porteus 2017). Although each nuclease system is at different stages of its clinical applications, the Cas9/gRNA system is used as the most preferred engineered nuclease for clinical treatments in genome editing (Dever and Porteus 2017).

In order to translate hematopoietic stem cell-based genome editing technologies into the clinic, it is important to find genome editing reagents that are efficient, scalable, specific, and non-toxic. For this purpose, significant progress has been made in the stages of programmable nucleases, nuclease delivery, and homologous donor template delivery (Dever and Porteus 2017).

Gene therapy and gene editing studies show great promise in the treatment of FA to identify new FA genes and avoid the negative effects of HSCT (Che, et al. 2018). Nuclease-based gene correction studies in FA are started in the 2010s and the first study was reported in 2014. In these studies, gene correction of several complementation groups of FA including "FANC-A, C, D1, D2, F, and I" were accomplished (**Figure 1**).

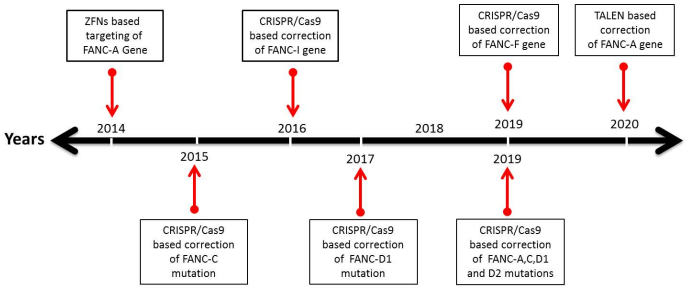

Figure 1: Timeline of Nuclease Based Gene-Corrections in FA

Most of the studies were done with CRISPR/Cas9 Nuclease System. In 2014, a group of researchers reported that they were successful in gene editing using ZFNs in the FANC-A gene (Rio, et al. 2014). Diez et al., on the other hand, were not only successful in gene editing using ZFNs in the FANC-A gene, but were also able to deliver it to CD34+ hematopoietic cells of FANC-A mutant patients (Diez, et al. 2017). A group in the United States has demonstrated that CRISPR/Cas9 technology corrects FANC-C associated mutations that cause FA disease. In addition, they compared the activities of Cas9 enzyme (nuclease), which can cut both DNA chains, and Cas9 enzyme (nickase), which can cut only one DNA chain, and reported that Cas9 nickase performed better (Osborn, et al. 2015). Another group has shown gene editing in the FANC-I gene using Cas9 nickase (Osborn, et al. 2016). The FANC-D1 gene mutation was corrected with the CRISP/Cas9 method using primary patient fibroblasts (Skvarova Kramarzova, et al. 2017). Slightly different from these studies, gene silencing was performed in zebrafish by targeting 17 FA and two FA-related genes with the multiple CRISPR/Cas9 system. The main purpose of this study is to test the effect of multiple gene inhibition on the FA pathway (Ramanagoudr-Bhojappa, et al. 2018). CRISPR/Cas9-mediated gene editing methodology in the FANC-F gene was also reported (van de Vrugt, et al. 2019). FA-corrected embryonic stem cells without genotoxicity gained quite a good proliferative advantage over uncorrected cells(van de Vrugt, et al. 2019). Non-homologous end joining (NHEJ)-mediated repair of targeted CRISPR-Cas9-induced DNA breaks and gene editing is shown in both FA lymphoblastic cell lines and primary CD34+cells derived from multiple FA complementation groups, including FA-A, FA-C, FA-D1, and FA-D2 (Román-Rodríguez, et al. 2019). A therapeutic gene editing with TALE nucleases, into the mouse Mbs85 orthologous locus in fibroblasts and HPCs of a mouse model of FA-A, were also accomplished by a group of scientists (Pino-Barrio, et al. 2020). On the other hand, one of the interesting findings is that multiple components of the FA DNA repair pathway are necessary for Cas9-mediated single-stranded template repair (SSTR) but not non-homologous end joining (NHEJ) (Richardson, et al. 2018).

3. Conclusion

Studies about gene editing in FA show the feasibility of these technologies. On the other hand, one of the interesting findings is that multiple components of the FA DNA repair pathway are necessary for Cas9-mediated single-stranded template repair (SSTR) but not NHEJ, which limits the use of this technology (Richardson, et al. 2018). As a result, more studies should be performed to get a better treatment strategy for FA.

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The Development of Recombinant hSpCas9 Production System in E. coli

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Abstract: The development of CRISPR/Cas9 system has revolutionized and accelerated the era of programmable nuclease mediated gene editing technologies. The CRISPR/Cas9 system consists of a Cas9 nuclease and a synthetic chimeric RNA known as guide RNA (gRNA). Cas9 endonuclease cleaves target DNA at a sequence-specific site with the help of designed sgRNA. The utilization of Cas9 nuclease protein as an important molecular tool in gene editing makes a great emphasis on the production of recombinant Cas9 protein. Due to Cas9 protein's large size and its complexity, recombinant Cas9 protein production can be challenged. Here, we subcloned a sequence encoding human codon-optimized Streptococcus pyogenes Cas9 (hSpCas9), and recombinant protein production of hSpCas9 was evaluated in different E. coli strains at different induction settings. The expression of recombinant hSpCas9 protein was determined within BL21(DE3) host strain at 25 °C induction temperature for four hours and following overnight induction. The purification of HisTagged recombinant hSpCas9 protein was performed by affinity chromatography. These findings imply that recombinant hSpCas9 protein could be produced on a large scale for potential utilization in the biotechnology market. The production of recombinant hSpCas9 nuclease protein could provide a significant tool for the advancement in the ribonucleoprotein complex utilization in CRISPR/Cas9 technology as well as the improvement of pharmaceutical-based approaches to modulate Cas9 nuclease protein activity for precise gene editing.

Key words: Cas9 nuclease, recombinant protein production, CRISPR/Cas9

1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) is represented as CRISPR/Cas9 (Jinek et al., 2012, 2014). This CRISPR/Cas9 system is originally RNA mediated adaptive immune system found in bacteria and archea for fighting against viruses and foreign invaders under genomic attacks (Philippe & Rodolphe, 2010). This defence mechanism has been repurposed and developed into a powerful tool to be used for genome editing in mammalian cells (Jinek et al., 2012; Liu et al., 2017; Prashant et al., 2013). The CRISPR/Cas9 system enables to manipulation of DNA sequence provides high specificity and efficiency in gene editing contrast to other genome editing methods such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Thus, it also ensures a programmable capacity for efficient gene targeting. The CRISPR/Cas9 system is compeised of Cas9 endonuclease and synthetic chimeric RNA molecules which is single guide RNA (sgRNA) (Cho et al., 2013; Mali et al., 2013; Uslu et al., 2021). The Cas9 protein operates DNA cleavage which is directed by sgRNA to target a specific sequence of DNA (Jinek et al., 2012) where a double-stranded breaks (DSBs) are induced by Cas9 nuclease. These DSBs at specific site of genome are repaired via endogenous homologous recombination (HR) or nonhomologous end joining (NHEJ) DNA repair processes that lead to desired sequence modifications including gene insertion, correction, deletion, and chromosomal rearrangement (Sander & Joung, 2014).

The strength of CRISPR/Cas9 system is that Cas9 nuclease can be adjusted with an ease to specifically target any region in the genome via simply redesign of sgRNA sequence. Thus, multiple sequences can be targeted by this technology for genome editing. Compared to ZFN and TALEN, there is no need to change nuclease protein for each new target. Given new designs of sgRNA within same Cas9 protein is able to cleave desired new target sites. As a most current technology in gene editing, it has been extensively used in vitro and in vivo studies since it was developed (Chira et al., 2018; Ran et al., 2015; Uslu et al., 2021; Yang et al., 2017) and it possesses the potential power to be used in therapeutical applications. It has been stated that a study conducted in China reported the first clinical trial on the editing of the ex vivo immune cells using CRISPR/Cas9 technique for a patient suffering from lung cancer in 2016 (Cyranoski, 2016).

In CRISPR/Cas9 based genome editing, it has to be transported directly to target cell nucleus for the therapeutical applications (Glass et al., 2018). CRISPR/Cas9 system delivery can be performed with several strategies including transfection of plasmids or transduction of viral vector encoding sgRNA and Cas9 protein, microinjection of Cas9 mRNA sgRNA and ribonucleoprotein complex (RNP) with Ca9 protein and sgRNA (Chu et al., 2016; S. M. Kim et al., 2018; Ran et al., 2015). There are advantages and disadvantages belonging to all delivery systems. Although, plasmid-based strategies especially viral vectors provide the delivery of the stable expression, it also

causes the possible random integration of plasmid into host genome (Li et al., 2018; Maddalo et al., 2014). This strategy leads to prolonged time of Cas9 protein and sgRNA expression that can elevate the off-targeting effect (Liang et al., 2015). Since mRNA is not highly stable as DNA, the usage of Cas9 mRNA and sgRNA has been provided transient expression with low off-target effects. However, delivery of Cas9 mRNA in genome editing studies showed no high efficiency (Carmignotto & Azzoni, 2019; Chu et al., 2016; Li et al., 2018). As another delivery method, the ribonucleoprotein (RNP) complexes containing in vitro transcribed gRNA and recombinant Cas9 protein has been demonstrated promising results in decrease off-target effect (S. Kim et al., 2014; S. M. Kim et al., 2018; Liang et al., 2015). This CRISPR/Cas RNP complexes do the cleavage of target DNA immediately after delivery with no any undesired integration. Besides, this strategy provides a better control over the protein and RNA component intracellular concentration, more quick activity, rapid degradation in the cells and low toxicity (DeWitt et al., 2017; S. Kim et al., 2014). Since the direct delivery of CRISPR/Cas RNP possesses these clear-cut advantages on the CRISPR/Cas mediated genome editing, the recombinant Cas9 production has been gain attention on developing Cas RNP based gene therapeutics.

Cas9 protein is derived from bacterium Streptococcus pyogenes. This large protein composed of 1368 amino acids and 158 kDa molecular weight (Carmignotto & Azzoni, 2019). It has been showed that this protein is convenient for heterologous expression system within E. coli strain (Jinek et al., 2014; Ramakrishna et al., 2014). However, the enormous size of Cas9 and existence of 137 rare codons in related gene might cause serious difficulties during recombinant protein production.

Owing to significance and robust potency of Cas9 protein in genome editing studies, we aimed to cloning and expression of Cas9 proteins in E. coli. By using different E. coli bacterial strains, we determined the impact of these strains in protein production, different temperatures as well as time of induction for the Cas9 protein production in E. coli.

2. Materials and methods

2.1. Plasmids and bacterial culture strains

LentiCRISPv2 encoding hSpCas9 gene and pET-26b (+) (Novagen, 69862-3) vector were used for the subcloning procedure. LentiCRISPv2 vector was kindly given a gift from Dr. Tamer Onder at Koç University. LentiCRISPv2 (14873 bp) contains S. pyogenes Cas9 gene (4200bp size) with a FLAG tag in C terminal and ampicillin resistance gene. In order to tag hSpCas9 with 6X Histidine, hSpCas9 gene was subcloned into pET-26b (+) (5360bp) which contains histidine tag in C terminal and kanamycin resistance gene. The BL21(DE3), Rosetta and Origami bacterial strain were used as bacterial expression strain. These bacterial strains were freshly grown in Lysogeny broth (LB) medium and stored within glycerol stock at -80 °C.

2.2. Subcloning of hSpCas9 into pET26b (+) vector

The gene of interest in this study is human codon-optimized Streptococcus pyogenes Cas9 (hSpCas9) which is commercially available and encoded in LentiCRISPv2 vector. To subclone of hSpCas9, the hexahistidine tag at the upstream of hSpCas9 gene and suitable restriction enzyme sites were designed to add for the sequence of hSpCas9 located in LentiCRISPv2 vector. To do this, two pair sets of primers were designed for the amplification of hSpCas9 (Table 1) and lentiCRISPv2 vector was used as a template. Then, PCR reaction mixture was prepared by using Q5® High-Fidelity 2X Master Mix (NEB, M0492S) as in Table 2 and PCR reaction was performed with the thermocycler condition shown in Table 3. After PCR reaction, hexahistidine (6XHis) tag sequence (HisTagged) attached to the C terminus of the coding sequence of Cas9. Due to Cas9 coding sequence flanked by two restriction enzymes which are NdeI and NcoI, hSpCas9 gene were cut with NdeI (NEB, R0111S) and NcoI (NEB, R0193S) restriction enzymes. pET26b (+) was also linearized by cutting with NdeI and NcoI restriction enzymes. After cutting with restriction enzymes at 37 °C for 10 hours, the inhibiton of enzymes was subsequently done at 80°C for 20 minutes (min). Then, they were run on 0.7% agarose gel and gel purification was done using NucleoSpin Gel and PCR Clean-up kit according to the manufacturer's protocol (Macherey-Nagel, 740609.50). Linearized hSpCas9 inserted into pET26b (+) vector by using InFusion HD clonning kit according to the manufacturer's protocol (Clontech, 639650). Briefly, mixture was prepared with linearized hSpCas9 insert and pET26b (+) with 5X In-Fusion HD Enzyme Premix. After incubation at 50 °C de 15 min and 1 min on ice, it was transferred to stellar competent cells provided within In-Fusion HD cloning kit. Then, transformation was done with heat shock procedure at 42 °C de 45 sec and 1 min on ice. 450ul of SOC medium was added and incubated at 37°C by shaking 250 rpm for 1 hours. After that, they were inoculated on the LB Agar containing kanamycin and incubated at 37°C overnight. Day after overnight incubation, colonies were determined and selected for colony PCR. Selected colonies were culture in LB broth for plasmids isolation by using NucleoSpin Plasmid miniprep kit (Macherey-Nagel, 740588.50). To check whether transformation is proper, plasmids were cut with Nco1 and Nde1 restriction enzymes and run with %0.7 agarose gel electrophoresis. Nucleotide sequences confirmed by DNA sequencing through using universal T7 promoter and T7 terminator primers.

Table 1: Primer sets for subcloning of hSpCas9 gene

Set1-Forward primer	5'TAAGAAGGAGATATACATATGATGCATCATCATCATCATCATATGGACAAGAAGTACAGCAC
Set1-Reverse primer	5'AATTAATTCCGATATCCATGGTCATTTCTTCTTCTTAGCCTGTCC
Set2-Forward primer	5'-TAAGAAGGAGATATACATATG
Set2-Reverse primer	5'-AATTAATTCCGATATCCATGG
Set3-Forward primer	5'ATGCATCATCATCATCATCATATGGACAAGAAGTAC
Set3-Reverse primer	5'TCATTTCTTCTTCTTAGCCTGTCCAGCCTTCTTTG

Set4-Forward primer	5'TAAGAAGGAGATATACATATGATGCATCATCATC
Set4-Reverse primer	5'AATTAATTCCGATATCCATGGTCATTTCTTCTTC

Table 2: PCR reaction for amplification of hSpCas9 gene

2x Q5® High-Fidelity 2X Master Mix	10 µl
Vector	2 µl
Forward primer	0.6 µl
Reverse primer	0.6 µl
ddH_2O	6,8 µl
Total	20 µl

Table 3: PCR thermocyler condition

Initial denaturation	98°C	1 min
35 cycles	98 °C	10 sec
	60 °C	15 sec
	72 °C	1 min
Final extension	72°C	5 min
Hold	+4 °C	∞

2.3. Expression of recombinant Cas9 protein in different E. coli strains

For the expression of recombinant Cas9 protein, HisTagged hSpCas9 inserted into pET-26b (+) was transformed into different E. coli strains which are BL21(DE3), Origami, Rosetta and Rosetta Gami. HisTagged hSpCas9 protein was expressed in these E. coli strains by using IPTG (Isopropyl ß-D-1-thiogalactopyranoside) at different temperatures such as 22°C, 25°C, 37 °C. A single colony was transferred was inoculated into 5 ml culture broth containing lysogeny broth (LB) broth and kanamycin (1:1000) as a starter culture and incubated at 37 °C and 200 rpm overnight. Then, the culture was transferred to 250ml culture medium at 37 °C and 250 rpm until the OD600 reached 0.6-0.8. 1 ml of bacterial culture was taken as non-induced control. IPTG was added at the final concentration of 1 mM in the culture and the temperature was changed to 22°C, 25°C, 37 °C, separately. The optimum temperature of induction and different times of induction such as 1 hour (1h), 2 hours (2h), 3 hours (3h), 4 hours (4h) and overnight were determined. Protein expression was also visualized by using SDS-PAGE electrophoresis further staining with coomassie brilliant blue (CBB) R250 solution for IPTG induced samples and compared with uninduced control samples.

2.4. Purification of recombinant Cas9 protein

Bacterial culture samples were centrifuged at 6000rpm for 15 minutes at 4 °C. Pellets were weighted and 10 ml lysis buffer was added each 1g of pellet. Mobile phase solutions including lysis, wash, equilibrium and binding buffer was prepared as 6M Guanidine hydrochloride, 500 mM NaCl, 50mM phosphate buffer, 20mM immidazole, 14,1µM β-mercaptoethanol. Elution buffer recipe was different regarding the immidazole concentration. It includes 500mM immidazole instead of 20mM along with 6M Guanidine hydrochloride, 500 mM NaCl, 50mM phosphate buffer and 14,1µM β-mercaptoethanol. All buffers were filtered before usage. After dissolving pellet with lysis buffer, sonication was done for 30 seconds at three times with 1 min interval. Then, sample was diluted up to 30 ml and centrifugation was done at 10000g 30 minutes. Protein supernatant was filtered by 0.45 µm filters and ÄKTA prime liquid chromatography system affinity chromatography system (GE Healthcare) was used. His-Tag column (HisTrap™ HP, GE Healthcare) was connected to ÄKTA prime plus drop-by-drop flow. 5ml/min flow rate was used for this system. SDS-PAGE gel electrophoresis was performed and the protein concentration was quantified by Bradford assay.

2.5. Desalting of recombinant Cas9 protein

To remove salt from protein environment which is found highly in elution buffer, 100K Amicon Ultra-0.5 Centrifugal filter unit (Millipore, UFC5100BK) were used to separate salt from protein and make it more concentrated according to the manufacturer's protocol.

3. Results

3.1. Subcloning of hSpCas9 into pET26b (+) vector

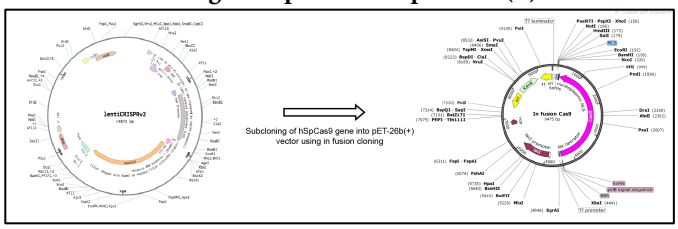

Figure 1. Schematic representation of hSpCas9 subcloning by using SnapGene® software. LentiCRISPv2 encoding hSpCas9 gene (shown in orange color) subcloned into pET-26b (+) vector by using in-fusion cloning technique (shown in pink color). Primers were designed for hSpCas9 to add 6XHisTag and restriction enzyme sites for PCR reaction. Then, hSpCas9 inserted into pET26b (+) vector by using InFusion HD clonning kit. Histagged hSpCas9 within pET26b (+) vector shown in pink color.

The lentiviral CRISPR/Cas9 systems have been extensively used in the genome editing studies. The LentiCRISPv2 construct that used in this study was included within two expression cassettes which are human Streptococcus pyogenes Cas9 gene (hSpCas9) and the chimeric guide RNA. The size of this lentiviral vector is 14873 bp and it includes hSpCas9 in 4200bp size with a FLAG tag in C terminal. To add 6xHisTag in the C terminal end of hSpCas9 for the recombinant protein production, hSpCas9 gene was subcloned into pET-26b (+) plasmid by using InFusion cloning technique. LentiCRISPv2 construct was virtually

subcloned and all the restriction enzymes were also depicted by using SnapGene® software (Figure 1). Primers corresponding with hSpCas9 gene region in LentiCRISPv2 with 15 bp extensions complementary to vector ends were designed. NdeI and NcoI restriction enzyme cutting site as well as 6XHisTag were also added to this hSpCas9 through PCR reaction by using these designed primer sets. The design of hSpCas9 gene subcloning post PCR reaction was shown in (Figure 2). Histidine tags (6XHisTag) was added upstream of the hSpCas9 gene and the restriction sites for NdeI and NcoI enzymes were also included along with the 15 bp extensions which are complementary to vector ends for InFusion HD cloning.

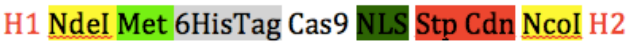

Figure 2. Representative figure for the design of hSpCas9 subcloning after PCR reaction. The sets of primers were designed for hSpCas9 gene within lentiCRISPRv2 to add the restriction sites to be cut separately by NdeI and NcoI enzymes as well as histidine tag (6HisTag) in the C terminal of hSpCas9. H1 and H2 showed the 15 bp extensions complementary to vector ends for InFusion cloning procedure. Met refers to methionine start codon and stp cdn which highlighted in red color showed stop codon. NLS is nuclear localization signal in lentiCRISPRv2 plasmid.

3.2. Validation of hSpCas9 subcloning into pET26b (+) vector

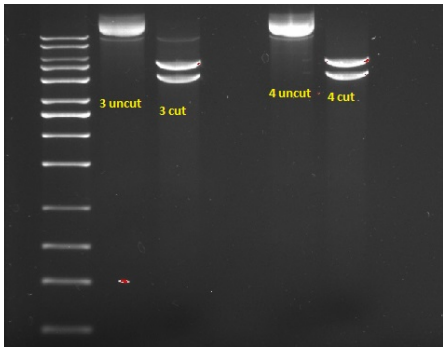

Figure 3. Validaiton of hSpCas9 subcloning into pET-26b (+) vector. HisTagged hSpCas9 inserted into pET-26b (+), which is previously cut by NcoI and NdeI, by using InFusion HD clonning kit. Colonies were validated by using colony PCR. Selected colonies (#3 and #4) cut with Nco1 and Nde1 restriction enzymes and run with %0.7 agarose gel electrophoresis. 3 uncut: uncut plasmid belonging to 3rd colony, 3 cut: isolated plasmid from 3rd colony cut with NcoI and Nde1, 4 uncut: 4th colony uncut plasmid, 4 cut: 4th colony cut plasmid with NcoI and Nde1, marker: 1kb DNA ladder (Axygen).

After insertion of HisTagged hSpCas9 into pET-26b (+) by using InFusion HD cloning technique, bacterial transformation was performed and colonies were validated by using colony PCR. In order to validate the subcloning of hSpCas9, colonies were selected and isolated plasmids were cut with Nco1 and Nde1 restriction enzymes and run with %0.7 agarose gel electrophoresis. The uncut plasmid and cut with these restriction enzymes were compared (Figure 3). The expected size of pET-26b (+) is 5360 bp and 4218 bp is for hSpCas9. Compared to uncut subcloned plasmid that isolated from selected colony, two bands were obtained with expected size in base pairs according to the 1 kb DNA marker (Figure 3).

Figure 4. DNA Sequencing validation of hSpCas9 subcloning into pET-26b (+) vector. Multiple sequence alignment was done with ClustalW2 software for subcloned HisTagged hSpCas9.

DNA sequencing validation was also performed to determine the sequence of hSpCas9 gene post subcloning and multiple sequence alignment was analyzed with original sequence of hSpCas9 and subcloned one using ClustalW2 software (Figure 4). The Amino acid sequence of hSpCas9 protein was also were deduced from the nucleotide sequence by using ExPasy translate tool. Molecular weight of hSpCas9 protein was determined as 161.2 kDa (Figure 5).

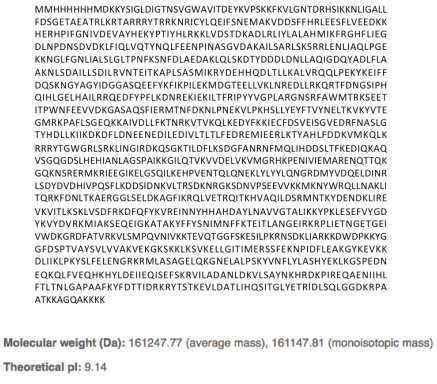

Figure 5. Amino acid sequence analysis of hSpCas9. Amino acid sequence of hSpCas9 protein were deduced from the nucleotide sequence by using ExPasy translate tool.

3.3. Evaluation of Cas9 protein expression in different E. coli strains

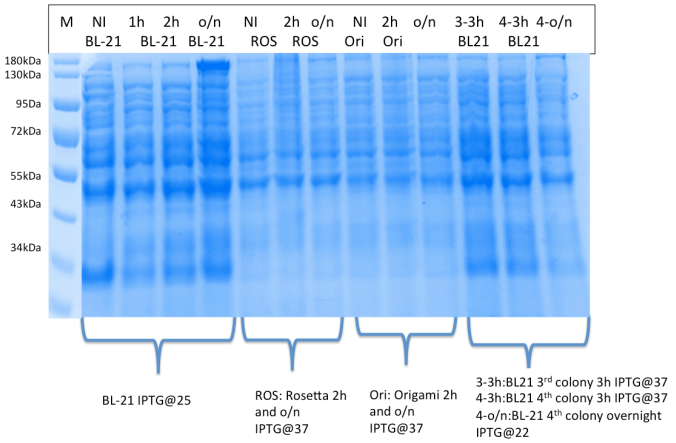

Figure 6. Optimization of hSpCas9 protein expression. Different bacterial strains and conditions were used for hSpCas9 protein expression. M: Protein Ladder; PageRuler Prestained Protein Ladder (Thermo Scientific), NI: Non-induced (before IPTG induction)

The recombinant hSpCas9 protein expression was evaluated by using different E. coli strains which are BL21 (DE3), Rosetta and Origami. The expression of hSpCas9 protein in these host strains was also compared at different temperature settings for different times of induction. Upon 1mM IPTG induction, the protein expression was analyzed with the induction temperature of 22 °C, 25 °C and 37 °C for the induction time of 1h, 2h, 3h, 4h and overnight. Among the different induction temperature for BL21(DE3) strain, the expression of recombinant hSpCas9 protein was determined within BL21(DE3) strain using 1mM concentration of IPTG at 25 °C induction temperature for overnight (Figure 6). There was no enough recombinant protein expression in Rosetta and Origami E. coli bacterial strains by IPTG induction at 37 °C temperature for overnight induction time (Figure 6). Non-induced controls for the each of bacterial strains which were taken before IPTG induction were used to compare their protein expression.

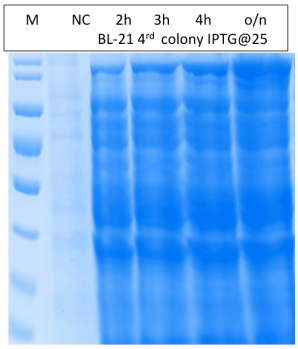

Figure 7. hSpCas9 protein expression within BL-21(DE3) *E. coli* strain. After IPTG induction, total bacterial lysate loaded and run onto %8 SDS-PAGE. NC: Empty BL-21(DE3) strain (BL21(DE3) without plasmid), 2h: 2-hours IPTG induction @25; 3h: 3-hours IPTG induction @25, o/n: overnight IPTG induction @25.

After optimization of recombinant hSpCas9 protein expression in different E. coli strains, the following step was to confirm the expression of hSpCas9 protein in BL21(DE3) strain at defined temperature and condition. The recombinant hSpCas9 protein expression was induced using 1mM IPTG at 25 °C induction temperature for the induction time of 0, 1h, 2h, 3h, 4h and overnight. The hSpCas9 protein induction was gradually increased by the prolonged time of induction through the overnight (Figure 7). Empty BL-21(DE3) strain which is BL21(DE3) strain that having no transferred hSpCas9 plasmid was used as control to ensure the protein expression of hSpCas9. As it was expected, empty BL-21(DE3) strain had no hSpCas9protein expression whereas BL-21(DE3) strain containing hSpCas9 plasmid harboured the recombinant protein expression at 25 °C post IPTG induction (Figure 7).

3.4. Purification of HisTagged hSpCas9 recombinant protein

Recombinant hSpCas9 protein was tagged with six consecutive histidines through subcloning and it was easily purified from bacterial cell lysate by using HisTrap™ HP affinity columns in ÄKTA prime liquid chromatography system (Figure 8). This system provided us to perform rapid and efficient protein purification. The UV monitor of ÄKTA prime displayed the chromatogram helped to track the purification process and which elution fraction contained our expressed protein. The each of fractions such as flow-through, wash and different elusions was also separately collected through purification and run with the SDS-PAGE gel electrophoresis was used to determine the efficiency of protein production. The HisTagged hSpCas9 protein concentration was quantified as 1mg/ml post protein induction and purification.

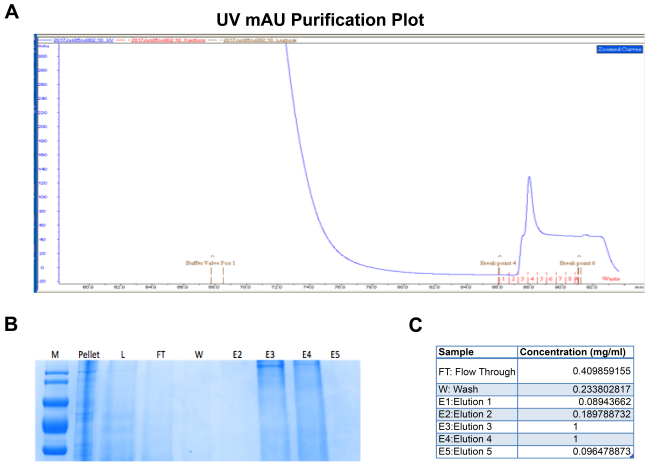

Figure 8. HisTagged hSpCas9 protein purification. After IPTG induction, hSpCas9 protein was purified using HisTag affinity columns and ActaPrime (GE Healthcare). **A)** Representative UV mAU purification plot, **B)** SDS-PAGE electrophoresis after purification, **C)** Quantification of protein concentration post purification by using Bradford assay. M: Protein Ladder; PageRuler Prestained Protein Ladder (Thermo Scientific)

4. Discussion

The genome editing has been reformed by the discovery of CRISPR/Cas9 system. The ability of engineering of Cas9 endonuclease with simple design of sgRNA in CRISPR/Cas9 system put an emphasis on the control of Cas9 nuclease activity for gene targeting and editing. Recombinant DNA technology and protein engineering platforms have been gained tremendous

importance in the further development and application of these programmable nuclease mediated gene editing technologies especially the modulation of nuclease protein activity.

Taking advantages of recombinant DNA technology, a sequence encoding hSpCas9 was prepared by PCR amplification using LentiCRISPv2 vector as a template and then subcloned into pET-26b (+) vector by using InFusion cloning technique (Figure 1). There are different E. coli expression vectors such as pET, pUC, pBAD and pQE with different fusion tags including hexahistidine (6x His-tag), glutathione S-transferase (GST) and maltose binding protein (MBP) to be available for the production of recombinant protein (Tripathi, 2016). pET vectors possessing T7 promoter system is the commonly preferred for the recombinant protein production with the high protein expression. Besides, IPTG is utilized to induce the recombinant proteins expression under the T7 promoter (Tripathi, 2016). Thus, pET-26b (+) vector was preferred to used in this study.

The desing of Histagged hSpCas9 subcloning was depicted in (Figure 2). After the design of primer sets corresponding to hSpCas9 gene within lentiCRISPRv2, restriction sites for NdeI and NcoI enzymes and histidine tag (6HisTag) in the C terminal of hSpCas9 sequence was added along with 15 bp extensions complementary to vector ends which is needed for InFusion HD cloning procedure through PCR reaction (Figure 2). Since the sequence of hSpCas9 gene was very huge enough, four different sets of primers were designed. While primer set1 and set2 were used together, primer set3 and set4 were used together within subsequent PCR reactions in order to add these extra needed sites on this long big enough sequence of hSpCas9. Primer set3 was designed to add firstly hexahistidine tag in the hSpCas9 sequence with one PCR reaction. Primer set4 was designed to add restriction sites and 15 bp extensions complementary to vector ends (H1 and H2) for this previously amplified PCR product that primer set3 used in the reaction. Primer set1 was designed at very long sequence which is unwanted criteria for primer design but it included all these needed sites together to be amplified by PCR. Primer set 2 was used to amplify the PCR product in which set1 primer used. All sets of primers were successfully amplified the desired sequence (data not shown).

The subcloning of Histagged hSpCas9 was validated by cutting with Nco1 and Nde1 restriction enzymes and running onto agarose gel electrophoresis. Compared to the uncut plasmid, two bands were observed as expected in size of 5360 bp for pET-26b (+) and 4218 bp is for hSpCas9 (Figure 3). Besides, DNA sequencing validation of hSpCas9 subcloning into pET-26b (+) was also determined by using T7 universal primers (Figure 4).

It has been stated that Cas9 nuclease protein consists of 1390 amino acids with a 158kDa molecular weight (Jinek et al., 2014). We found that the molecular weight of this subcloned hSpCas9 was 161.2 kDa by the analysis of amino acid sequence of hSpCas9 protein (Figure 5).

Escherichia coli has been extensively utilized for the expression of recombinant protein studies owing to several advantages such as rapid cell growth, its availability as a molecular tool, easily manipulation, well-understood genetics and metabolism of E. coli (Tripathi, 2016). Nevertheless, it has been stated that Cas9 protein coding sequence consists of substantial number of rare codons that are present in E. coli. Thus, the existence of rare codons can cause obstacles for the production of protein expression. During translation, the available rare tRNA in low amount can diminish the quality and quantification of the protein yield. The excess of these codons even leads to translational troubles (Carmignotto & Azzoni, 2019; Kane, 1995). It has been stated that Cas9 protein has 137 rare codons in the gene sequence even if the heterologous expression in E. coli is stated to be available (Carmignotto & Azzoni, 2019). To do this, we also checked the rare codon analysis for hSpCas9 input sequence within the length of 4218 nucleotide and the number of total rare codons for arginine (CGA, CGG, AGG, AGA), isoleucine (AUA), glycine (GGA, GGG) and proline (CCC) were analyzed through Caltor program. To overcome this obstacle, Rosetta E. coli strain is a commonly preferred one for the expression of genes harbouring rare codons due to having another plasmid which is pRARE encodes these rare tRNAs (Carmignotto & Azzoni, 2019; Tegel et al., 2010). Several studies reported Cas9 protein expression in the Rosetta E. coli host strain by using different bacterial culture growth medium supplemented with additional antibiotic such as chloramphenicol (Jinek et al., 2012; Lin et al., 2014; Ma et al., 2015; Pawluk et al., 2016; Staff, 2014).

In this study, we compared the expression of hSpCas9 protein by using different E. coli host strains which are BL21(DE3), Rosetta and Origami. In contrast to study findings about the use of Rosetta E. coli strain for Cas9 protein expression, hSpCas9 protein expression was obtained using BL21(DE3) as host strain by using the large volume of LB broth growth culture including kanamycin (Figure 5). Among different induction temperatures using same concentration of IPTG (1mM), the induction of hSpCas9 protein expression was observed at 25 °C for BL21(DE3) host strain (Figure 5). Considering to Cas9 protein harbouring a lot of rare codons in E. coli, it was an unexpected result. Similarly, one recent study showed that higher expression of Cas9 protein in BL21(DE3) strain rather than BL21(DE3) Rosetta strain within the culture of LB broth (Carmignotto & Azzoni, 2019). The induction conditions of the Cas9 protein expression have been stated as the use of 0.5mM IPTG for four-hour induction at 30 °C. The reason of this low expression level in Rosetta strain has been addressed with a hypothesis on the probable impact of rare codons on the protein translation kinetics and folding. Another possibility has been also proposed that pRARE plasmid in Rosetta strain can cause the excessive metabolic side effects leading to diminished expression of recombinant protein (Carmignotto & Azzoni, 2019). To confirm recombinant hSpCas9 protein expression in BL21(DE3) host strain, the induction was repeated using 1mM IPTG at the induction temperature of 25 °C and different induction time settings with 0, 1h, 2h, 3h, 4h and overnight were followed. The more protein induction was obtained with the gradually increased time of induction through the overnight protein induction (Figure 7). Since empty BL-21(DE3) strain without hSpCas9 plasmid was used as control to compare the protein expression of hSpCas9, empty BL-21(DE3) strain showed no

hSpCas9protein expression while BL-21(DE3) strain containing hSpCas9 plasmid demonstrated the recombinant protein expression at 25 °C post IPTG induction (Figure 7).

Immobilized metal affinity chromatography is used for the purification of polyhistidine tagged protein using nickel-charged resins (Karakus et al., 2016). Recombinant protein with six consecutive histidine (6XHis) tag has a high affinity for nickel metal ions. The bounded 6XHisTagged proteins can be eluted by reducing pH of buffer or increasing imidazole concentrations for the displacement of the imidazole ring of histidine from metal ions (Karakus et al., 2016).

Since our recombinant hSpCas9 protein possesses 6XHisTag at the C-terminus end, the purification of recombinant hSpCas9 protein by using HisTrap™ HP affinity columns in ÄKTA prime liquid chromatography system. The purification was assessed by SDS-PAGE and the gel indicated the presence of a band which is between 180kDa and 130kDA in the collected elution fractions during purification process. This band was corresponded to the recombinant hSpCas9 protein which is 161 kDa (Figure 8). The concentration of recombinant hSpCas9 protein was quantified as 1mg/ml in the elution fractions that are collected during elution steps in the purification setting (Figure 8).

To sum up, we evaluated the subcloning of hSpCas9 nuclease into pET-26b (+) vector by using LentiCRISPv2 vector as a template. Recombinant hSpCas9 nuclease expression was compared with different host the E. coli BL21(DE3) strains and the optimum protein expression was determined in the E. coli BL21(DE3) host strain. Then, HisTagged Recombinant hSpCas9 nuclease was purified by using HisTrap™ HP affinity columns in ÄKTA prime liquid chromatography system. In this study, we could not evaluate the enzymatic activity of our recombinant hSpCas9 nuclease protein. Therefore, hSpCas9 nuclease activity should be functionally evaluated by using in vitro cleavage assay. Besides, the purification of recombinant hSpCas9 protein could be optimized to yield the purity and recovery of recombinant protein. This recombinant hSpCas9 nuclease protein expression in E. coli BL21(DE3) host strain could be scaled-up by using a bioreactor for a large-scale production to be used as a commercial product in the biotechnology industry. Overall, the production of recombinant hSpCas9 nuclease protein could enable researchers to advancement of the existing approaches on the utilization of the ribonucleoprotein complex delivery in the application of CRISPR/Cas9 technology. This also could provide the development of small molecule based pharmaceutical strategies on precise Cas9 protein nuclease activity for its potential therapeutical approaches in gene targeting and editing.

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Development of CXCR4 Gene Editing in Human Umbilical Vein Endothelial Cells Using the CRISPR/CAS9 System

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Abstract: Infection with the Human Immunodeficiency Virus (HIV) is associated with the progressive loss of CD4+ T cells, either through destruction or decreased production. When an individual is infected, HIV is propagated in CD4+ T cells using only the blood pathway or the transmission site. In this regard, gene targeting by the CRISPR system, which allows for genetic editing via gene inactivation, is preferred because it appears to produce significantly lasting effect than existing methods. Thus, the CRISPR-Cas9 system was tested to induce knock-out of CXCR4 gene in HUVECs, which could make the inner surface of blood vessels resistant to HIV infection and forms a separating surface. We have tested four different gRNAs and demonstrated their efficacy at genome level by T7 endonuclaase assay and protein level by CXCR4 flow cytometer. Three out of four gRNAs against CXCR4 demonstrated the efficacy in gene modification as well downregulation of CXCR4 proteins in HUVEC plasma membrane. This study demonstrates the use of the CRISPR-Cas9 system to generate gene-edited endothelial cells resistant to HIV infections by silencing the CXCR4 gene on human umbilical vein endothelial cells.

Key words: HIV, CRISPR/Cas9, Gene editing, knock-out CXCR4 gene

1. Introduction

Preclinical studies of potential gene therapy for AIDS have focused primarily on making cells resistant to human immunodeficiency virus (HIV) infections (J. J. Rossi and N. Sarver, 1992). According to the World Health Organization (WHO), around 36.7 million individuals were living with HIV globally in 2015, and about 2.2 million individuals died of AIDS, while over 2.1 million new patients were diagnosed with HIV infection. More obviously, despite important progress in the prevention and therapy of HIV infection, HIV/acquired immunodeficiency syndrome (AIDS) continues to be a major health risk. The main reason for HIV/AIDS persistence is the inability of current therapies to clear or eradicate the various reservoirs of HIV that occur in the human body (Siliciano JD, et al. 2003).

Particles of the HIV virus communicate on cell surfaces with several receptors. In some infected people, variants that switch to CXCR4 and possibly other co-receptors develop and have modified tropism and pathogenic characteristics (Michael NL, 1999; Benkirane M, 1997; Michael NL, et al. 1998; Scarlatti G, et al. 1997). In this way, Endothelial cells can undergo chemotaxis, proliferation and angiogenesis in response to certain CXC chemokines (Strieter RM, et al. 1995). CXCR4 expression in endothelial cells indicates that HIV could infect endothelial cells by a CD4-independent pathway. CXCR4 is abundantly expressed on the cell surface of HUVEC. Furthermore, CXCR4 ligand, SDF-1, can evoke a broad range of human endothelial cell reactions and CXCR4 on endothelial cells can serve as a receptor for HIV isolates that can use chemokine inhibitors in the absence of CD4 (E. Oberlin, et al. 1996; C.C. Bleul, et al. 1996).

Gene editing is a major technological advancement that enabled researchers to modify the DNA of an organism. CRISPR/Cas9 is a flexible system that enables genetic editing through inactivation (knock-out gene) among other methods due to its simplicity and precise comparability to other methods (Ormond KE, et al. 2017; Gupta RM and Musunuru K, 2014). These reagents enable for accurate modifications to their DNA locations, which are acknowledged and processed via the non-homologous end joining (NHEJ) or homologous recombination (HR) repair pathways through the cleavage of the two DNA strands to produce double-stranded breaks (DSBs) (Capecchi MR, 1989). In addition to that delivering Cas9 protein together with the preformed sgRNA as an Ribonucleoprotein (RNP) complex avoids many of the pitfalls associated with mRNA or viral delivery, provides advantages as being a transient genome editing and reduces the off-target effects (S. Kim, et al. 2014; S. Lin, et al. 2014).

In this study, we established CRISPR-Cas9 RNP system and non-viral transfection system to induce knock-out CXCR4 gene in human umbilical vein endothelial cells (HUVEC) to obtain resistant cells against HIV infection through applying different methods for the creation of synthetic vessels in further future. In addition, we achieved CXCR4 disruption evaluated by T7 endonuclease assay and by flow cytometric analysis in vitro.

2. MATERIALS and METHODS

2.1. Cell lines and culture conditions

HUVEC (ATCC® CRL-1730™) and HEK293T (ATCC® CRL-3216™) cell lines were utilized in transfection were previously frozen and kept at -80°C. Human Embryonic Kidney Cells (HEK293T) and Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from ATCC. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml kanamycin was utilized to maintain these cell lines.

2.2. Design of the sgRNAs for target locus and transcription

The genomic location of CCR5 was determined by using National Centre for Biotechnology Information (NCBI) gene database. SnapGene tool was used to locate exon and intron sites. Online gRNA design tool (Synthego's design tool) was used to determine gRNA sequence, which listed, based on-target and off-target scores. gRNA with the highest score was chosen and it was obtained as 20 bp length (Table 1).

Table 1: sgRNAs used in transfection

sgRNAs	Sequence	Yield nmol
CXCR4-136115952	U*G*G*GUUAAUGCUUGCUGA*A*U*	0.6
CXCR4-1261155891	C*A*C*UUCAGAUAACUACAC*C*G*	0.6
CXCR4-136115878	U*A*C*ACCGAGGAAAUGGGC*U*C*	0.6
CXCR4+136115799	A*A*G*AUGAUGGAGUAGAUG*G*U*	0.6

Synthego's CRISPR kit was utilized and Synthego's chemically modified CRISPR sgRNAs were the best choice for the highest editing efficiencies in any cell type. Furthermore, sgRNA can be made by in vitro transcription (IVT) in two steps as template synthesis by assembly PCR followed by vitro transcription and purification of the sgRNA.

2.3. Cas9 protein expression

Our experiment was performed with the Streptococcus pyogenes Cas9 nuclease expressed in *Escherichia coli* from a bacterial expression vector that is available from Synthego. The construct includes two nuclear localization signals (NLSs), an HA epitope tag and a hexa histidine tag (His6 tag). Also, Synthego's CRISPR kits offer high quality and synthetic guide RNAs to achieve the best editing in any cell lines.

2.4. Ribonucleoprotein (RNP) assembly and transfection of cells

The RNP complex was produced by mixing Cas9 and one or more sgRNAs into an Opti-MEM™. sgRNAs and Cas9 were diluted to 3µM as working stock concentration. To avoid solubility problems that arise if there is an excess of protein over RNA during mixing, we utilize a final 1.3-fold molar excess of sgRNA and add Cas9 to the sgRNA slowly with stirring. Lipofectamine 3000 Reagent was used for high-efficiency transfection on HUVEC and HEK293T cell lines. This was done with 3 pmol (1µL) of Cas9, 3.9 pmol (1.3µL) of sgRNA, 25 µL of Opti-MEM and 1µL of Lipofectamine Cas9 Plus Reagent in a total of 28.3 µL of RNP complex for 24-well plate. The mixtures were incubated at room temperature for about 5 minutes. Then, RNP complex and transfection solution were mixed gently by pipetting and incubated at room temperature for 20 minutes. After approximately 2-3 days, the cells can be screened by flow cytometer and T7 endonuclease assay can be applied.

2.5. Genomic DNA isolation

The PureLink® Genomic DNA Kit was utilized to provide rapid and efficient purification of genomic DNA. The PureLink® Genomic DNA Kits are based on the selective binding of DNA to silica-based membrane. For adherent cells as HEK293T cells, growth medium was removed from the culture plate and harvested cells by trypsinization and centrifuged the cells at 250×g for about 5 minutes to pellet cells. Supernatant was removed and the cells were re-suspended with 200 µL of PBS. Then, 20 µL of Proteinase K was added into the sample for efficient lysis of cells. In the same way, 20 µL of RNase A was added to degrade RNA present in the sample and minimize RNA contamination in the purified DNA sample. Samples were mixed well with vortexing and incubated at room temperature for 2 minutes. For lysis and binding the DNA, 200 µL of PureLink® Genomic Lysis/Binding Buffer was added and mixed with vortexing to obtain a homogenous solution. Heat Block was set up at 55°C and cells incubated in heat block for about 10 minutes to promote protein digestion. After incubation, 200 µL of 96–100% ethanol into the lysate and mixed with vortexing for 5 seconds. PureLink® Spin Column was placed in a PureLink® Collection Tube. Prepared lysate was added to the spin column and centrifuged at 10,000xg for 1 minute at room temperature. Collection tube was removed and the spin column was placed into a new collection tube. For Washing DNA, 500 µl of Wash Buffer I was added onto the spin column and centrifuged at 10,000xg for 1 minute at room temperature. Then, the collection tube was removed and the spin column was placed into a new collection tube. 500 µL of Wash Buffer II was added and centrifuged at maximum speed for 3 minutes at room temperature. For Eluting DNA, the collection was removed and 1.5 mL microcentrifuge tube was placed. 60 µL of PureLink® Genomic Elution Buffer was added onto the column and incubated at room temperature for 2 minutes and then, centrifuged at maximum speed for 1 minute. The column was removed and the micro-centrifuged tube contained purified genomic DNA. Purified DNA was kept at –20°C for use in the next experiment.

2.6. Indel detection of gRNAs

Polymerase chain reaction (PCR) was used to amplify specific the target region. The primers listed in Table 2 was used as 10 µM stock concentration which were produced 1 kb product.

Table 2: Primers used in PCR of target region.

Primers	Sequence	Tm
CXCR4-F	TGGGAGTGGCCTCTTTGTGTG	56°C
CXCR4-R	GGCAGGATAAGGCCAACCAT	54°C

The reaction was prepared by using 10 µL of Q5 Stark High Fidelity 2X Master Mix, 500 ng of genomic DNA, 1 µL of CXCR4-F primer, 1 µL of CXCR4-R primer and up to 20 µL of dH_2O. Th running condition were performed by using Bio-RAD MyCycler ™ Thermal Cycler. PCR steps were applied as initial denaturation at 95°C for about 30 seconds, 35 cycles of polymerase chain reaction (denaturation at 95°C for about 15 seconds, annealing at 65°C for 30 seconds, elongation at 68°C for 1 minute) and final elongation of the polymer was done at 68°C for 5 minutes.

Then, 5 µL of PCR products were run on a 1% agarose gel containing TBE and ethidium bromide. Power supply was applied at 150V for about 40 minutes. Then, PCR samples were visualized under UV illumination by using molecular imager Bio-Rad ChemiDoc XRS$^+$.

2.7. Analysis of Indel Frequency by T7 Endonuclease Assay

The targeted region was amplified by PCR used to generate an amplicon containing the targeted region and to form of heteroduplexes. For recognizing heteroduplexes, 5 µL of PCR products were mixed with 2 µL of 10X NEB Buffer II and 12 µL of water. The running condition was performed in thermal cycler as denaturation at 95 °C for 5 min, annealing at 95–85 °C at 2 °C/sec ramp rate for 5 s, annealing at 85–25 °C at 0.1 °C/sec ramp rate for 5 s and hold at 4 °C until use.

After applying decrement PCR, 1 µl of T7 Endonuclease I enzyme (NEB, 10 µg/µl) added to the mixture and incubation performed at 37 C for 30 minutes. 1 µl of Proteinase K was used to stop the reaction and incubation performed at 37°C for about 5 minutes. 5 µl of heteroduplex products was run on a 1% agarose gel containing TBE and ethidium bromide at 150V for about 50 minutes. Visualization was performed with the Biorad Chemidoc XRS device under UV illumination and image was captured.

2.8. Flow cytometry analysis

Endothelial cells were harvested enzymatically by trypsinization for flow cytometry analysis by using Beckman Coulter's CytoFLEX to measure knockout efficiency. CD184 (CXCR4) Monoclonal Antibody and Anti-Mouse IgG as the secondary antibody were used for labelling and measurement. Acquisition was set to 1 x10^5 events. Forward and side scatter plots was used to determine live population. APC-A / FSC-A dot plot was used to determine knockout efficiency. Results were recorded as histogram and analyzed.

3. RESULTS

3.1. Validation of gene knockout by T7 endonuclease assay

We performed the T7E1 assay to detect the mutation on target locus and measure the insertion/deletion (indel) efficacy at each target site of CXCR4. HEK293T cells were transfected as one group with RNP complex of control Rela sgRNA and as other groups with RNP complexes of CXCR4 sgRNAs. Genomic DNAs were isolated and target locus was amplified by PCR reaction. The result given in figure 1 shows that 1 kb products obtained successfully in PCR of target locus in both cell lines. It was not observed any unspecific products, and then it was continued to T7E1 assay. Primers designed to produce one band as 1 kb product in PCR reaction, two bands as 400 bp and 600 bp for sgRNA1, sgRNA2 and sgRNA3 and one band as 500 bp for sgRNA4 in the manner of heteroduplex reaction by T7 Endonuclease. PCR products of target locus for HEK293T cells screened by agarose gel electrophesis (Figure 1).

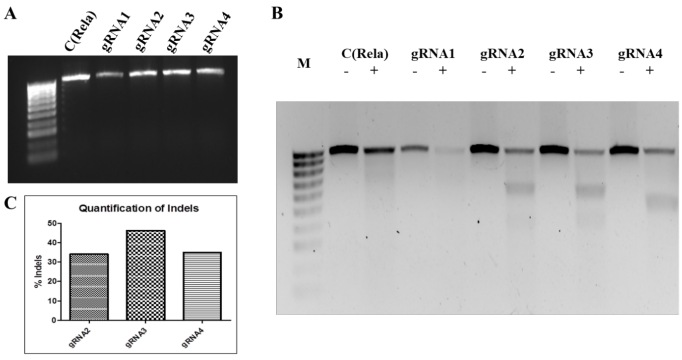

Figure 1: CRISPR/Cas9 RNP activity with T7E1 assay in HEK293T cell line. A; representative gel image of PCR products amplified from target sites of RNP Rela sgRNA control, and RNP CXCR4 sgRNA samples, B; representative gel image of T7E1 treated PCR products C; estimated % modification analyzed by ImageJ.

It was observed that CXCR4 targeted RNP complex was successful in the activity of cleavage on target sequence, which was detected by the T7E1 assay in HEK293T cell line depending on the sgRNA samples. The results revealed that the indel mutation rate of sgRNA2, sgRNA3 and sgRNA4 were 33.96, 46.20, and 35.01% respectively, while there is no cleavage observed with sgRNA1. The cleavage efficacy from T7E showed that sgRNA3 combination could specifically and efficiently disrupt the specific targeted gene. RNP of Rela sgRNA control did not show any cleavage on target sequence due to lack of gRNA as expected. The same amount of genomic DNA was used to evaluate the groups.

3.2. Validation of gene knockout by flow cytometric analysis

Expression of CXCR4 gene in HUVEC cell line transfected with CRISPR-Cas9 by Lipofectamine transfection reagent was analyzed with flow cytometry. Knockout ratio analysis of CXCR4 for target locus was performed by using anti CXCR4 antibody with flow cytometric assay. HUVEC cell lines were transfected with RNP Cas9 protein without sgRNA as a control and other groups transfected with RNP complexes of CXCR4 sgRNAs. After the incubation for 48 hours, cells trypsinized and incubated with anti CXCR4 antibody. APC-A plot were used for analysis as shown in Figure 2.

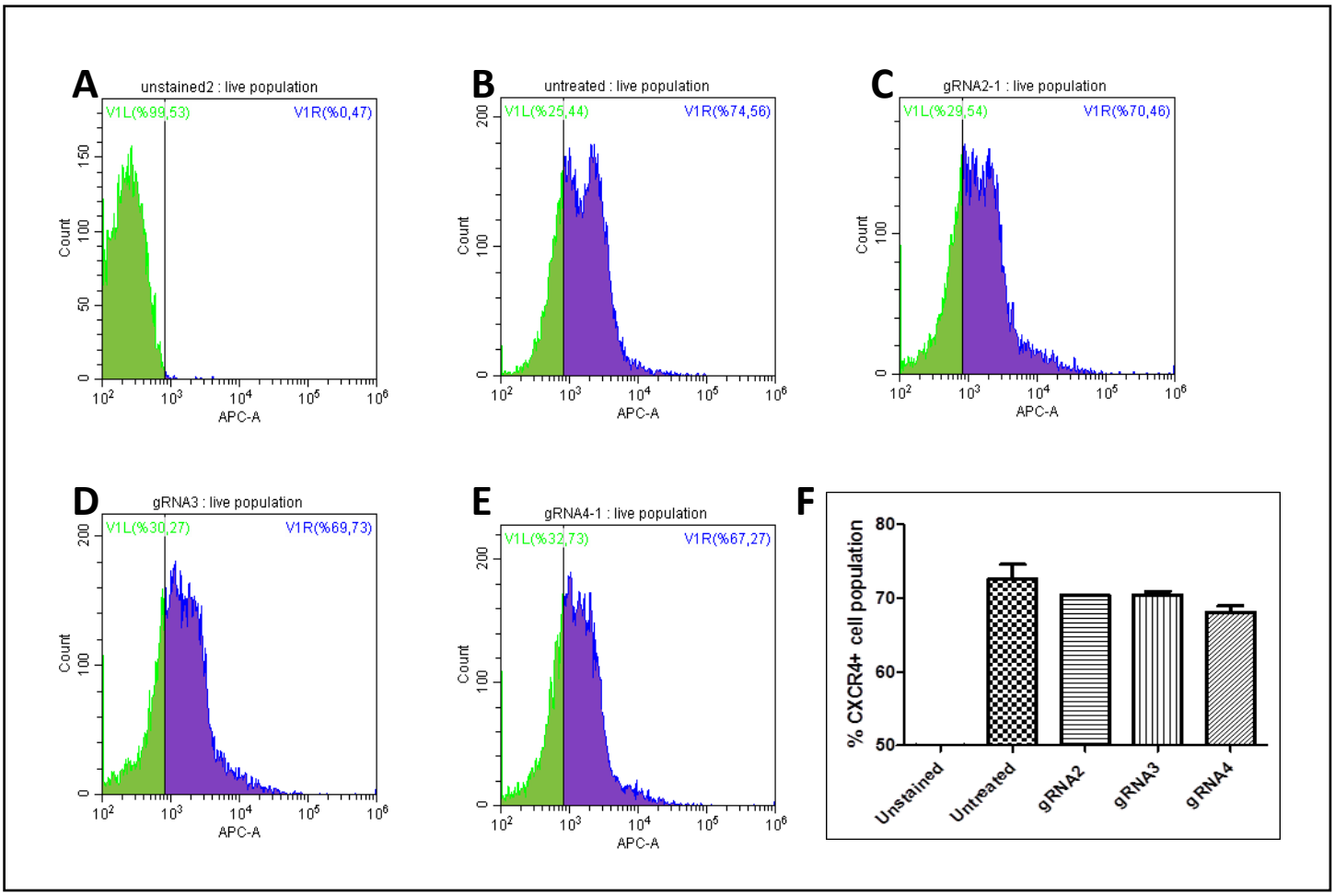

Figure 2: Flow cytometry analysis of transfected HUVEC cells with the RNP complexes. Representative CXCR4 analysis of HUVECs by cytometer for A; unstained, B;untreated/control, C; gRNA2, D; gRNA3, and E; gRNA4. F; CXCR4+ HUVECs and their quantification.

The knockout efficacy at the genome level was analyzed and found that CXCR4 genes were specifically edited as expected. This diagram showed that the knockout efficacy ratio as a percentage was obtained as shown in figure 2F. While there are 74.56% CXCR4+ cells in control treatment, it decreases to 70.46% when treated with RNP of sgRNA2, 69.73% when treated with RNP of sgRNA3, and 67.27% when treated with RNP of sgRNA4. Thus, we observed that CXCR4 deletion with gRNA2, gRNA3 and gRNA4 resulted in knockout efficacy of 4.1, 4.86 and 7.31% respectively, as compared to negative control.

4. DISCUSSION

In this study, we demonstrated that CRISPR/Cas9 could efficiently disrupt the HIV co-receptor CXCR4 using sgRNAs targeting CXCR4 gene sequences. From previous studies, HIV-1 enters cells with co-receptor CCR5 at the early stage of infection and many studies involving CCR5 disruption were performed with CRISPR/Cas9 system (Tebas P, et al. 2014: Perez EE, et al. 2008). However, co-receptor CXCR4 at late-stage entry accelerates the progress of the disease. In our study, we further confirmed that knock-out of CXCR4 by CRISPR/Cas9 with sgRNAs could function in HUVECs since they are the main component in the formation of the vasculature.

The applications in gene therapy were revolutionized with transfection studies. RNP based editing can be accomplished with higher efficiencies to generate targeted indels. To disrupt the HIV co-receptor of CXCR4 at genome level, we amplified and inserted various sgRNAs of CXCR4 along with Cas9 protein into cultured cells. For efficacy and safety concerns, multiple combinations of sgRNAs of CXCR4 was assessed. The RNP complex that included Cas9 protein and sgRNAs was administered by Lipofectamine transfection reagent to HEK293T

cells as a control to demonstrate transfection efficiency and to check theoretically designed 1000bp nucleotide chain primers.

To evaluate CRISPR-Cas9 mediated knockout efficacy at the genome level, CXCR4 targets containing fragments were amplified with PCR. sgRNAs labelled as Rela, 1, 2, 3 and 4 were successfully transfected onto HEK293T cells as RNP complex. The result demonstrated that CXCR4 genes at the genome level were disrupted in the CRISPR-Cas9 edited HEK293T cells, depending on expected band formation around 1000bp since we have designed the primers having 1000 bp nucleotide chains. sgRNA-Rela was used as a control in transfection and mutation detection. In addition, expected 1000 bp band formation was detected with high intensity in sgRNA-3, 4 and 5. However, a low intensity band was observed in sgRNA-1. Thus, theoretically designed primers were checked in the CRISPR-Cas9 edited HEK293T cells and confirmed in an experimental process.

After the targeted region was amplified by PCR using appropriate primers to generate an amplicon containing the targeted region it allowed the formation of heteroduplexes between PCR products. We performed the T7E1 assay to cleave heteroduplex DNA and to measure the insertion/deletion (indel) efficacy at each target site of CXCR4 on HEK291T cells. Fragments were analyzed on agarose gel to demonstrate expected two band formation as 400bp and 600bp and one band formation for sgRNA4 as 500bp. sgRNA1 product were not cleaved. Depending on the previous result, it may be unaffected by the enzyme due to low intensity. Also, sgRNA2 and sgRNA3 products were cleaved into the size of 600bp and 400bp. DNA sequence related to sgRNA4 was cleaved into only 500bp. The table of indel frequency for knockout CXCR4 gene was developed. This result demonstrated that the indel mutations ratio as a percentage was obtained based on the intensity of the related bands. The results revealed that the indel mutation rate of gRNA2, gRNA3 and gRNA4 were 33.96, 46.20, and 35.01% respectively and found that CXCR4 genes were potentially mutated as expected. In this way, we designed and studied four different sgRNA combinations and then, the cleavage efficacy from T7E showed that specifically sgRNA2, sgRNA3 and sgRNA4 could efficiently disrupt the targeted gene.

To observe the changes in protein expression level on the cell surface, the treated cells were analyzed by flow cytometry. Human umbilical vein endothelial cells were screened by flow cytometer after Lipofectamine transfection reagent. This analysis was performed easily due to high expression of CXCR4 on the cell surface of HUVEC. We observed that CXCR4 editing with gRNA2, gRNA3 and gRNA4 resulted in knockout efficacy of 4.1, 4.86 and 7.31% respectively, as compared to negative control. The results of flow cytometry indicated that the level of knockout CXCR4 genes on cell surface were not robustly down-regulated in HUVEC. Depending on these findings, in order to increase knockout efficacy, the amount of gRNAs should be increased during the delivery of RNP complex. In transfection trials, electroporation method was also used instead of using Lipofectamine transfection reagent. However, electroporation did not yield efficient results due to the limited amount of ribonucleoprotein complex components sgRNA and Cas9. If it was used with a higher amount, the knockout efficacy will also increase. While maintaining experimental procedures, optimization should be done and the changes in total protein levels of CXCR4 should also be detected by western blot. Subsequently, TA cloning strategy could be used for directly inserting the products into a plasmid vector after seeing the efficient knockout effect. Finally, successful ligations could be isolated and a sanger sequencing method could be utilized for analyzing the nucleotide sequence of edited DNA. In addition, it should be noted that CXCR4 has a critical role in sustaining normal physical function of hematopoietic stem cells and other cells, thus in vivo studies that target CXCR4 should be done in caution.

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The Effect Of Molecular Genetic Mechanisms On Drug Addiction And Related New Generation CRISPR Gene Engineering Applications

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Abstract: Drug addiction causes alterations in gene expression, synaptic function, and nerve flexibility in brain reward regions. Up to date improvements in genome editing technologies, suchlike the CRISPR-related endonuclease Cas9, have accelerated the development of neuroscience by rapidly and efficiently manipulating the endogenous genome of various cell types. CRISPR for the first time generated three epigenome editing platforms at the molecular, cellular, circuit-behavioral levels and performed single chromatin modifications on certain genes in specific cell populations. DNA methylation, histone modification and genes related to reward systems are effectors of the epigenome that have effects on the expression of small RNAs found in various pathways such as aging, memory and cardiovascular disease. Previous attempts to alter one or more neurotransmitter receptors have had restricted achievement, and so far, no FDA-confirmed drugs are in hand to treat addiction disorders such as cannabis, methamphetamine, and cocaine use disorders. In the near future, clinically effective therapy could be possible with the CRISPR/Cas9 systems. The advancement of in vivo neural epigenome editing tools and administrations has been explored to reveal contribution of epigenetics to the pathophysiology of the brain. To date, the drugs that measure phenotypes and epigenetic effects are rather small, and further investigation of these effects is necessary to fully understand the outcomes of developmental exposure to drugs. In this review, we discussed how histone acetylation affects gene expression of brain reward pathways. Recent advances that contribute to drug addiction include epigenetic mechanisms and CRISPR/Cas9 systems to develop new therapeutics for future addiction treatments.

Key words: CRISPR/Cas9, neuroscience, drug addiction, brain reward center

INTRODUCTION

CRISPR/Cas9 system is the recent technology to the genome engineering tool of RNA/protein complex which has been inherited from the immune system of prokaryotes (Hryhorowicz et al., 2017). CRISPR/Cas9 technology facilitates the generation of double-strand breaks mediated endonuclease Cas9 and guides RNA (sgRNA) in a desired region of the genome (Wu et al., 2014). Genome editing is performed by using homologous repair (HR) and DNA repair mechanisms (Nambiar et al., 2018). CRISPR/Cas9 technology is used in a wide range areas suchlike identification of bacterial strains, determination of gene and miRNA functions, addition/deletion of DNA fragments to the genome, transcriptional and epigenetic targeting or generation of disease models (Rodriguez et al., 2019; Li et al., 2020). CRISPR/Cas9 technology has started a new era in genome editing with its feasibility in mammalian cells (Liu et al., 2019). Today, scientists can easily edit genomes, using CRISPR/Cas9-mediated tools (Hsu et al., 2014).

Since understanding drug addiction today, genome engineering has become an important tool for treatment opportunities such as more effective and personalized treatment. CRISPR/Cas9 technology, which has a wide range of areas such as disease models, genome editing, and epigenetic regulation, has emerged as a result of functional targets in the treatment of drug addiction.

Drug dependence is a psychiatric disorder with a complicated aetiology that involves the interaction of intrinsic and extrinsic elements (Bevilacqua and Goldman, 2009). Evidence shows that epigenetic changes in the genome, involving DNA methylation and histone modifications, are necessary to understand the underlying mechanism of drug addiction and neurobiological responses to addictive substances (Nielsen et al., 2012). CRISPR/Cas9 systems may contribute to molecular genetic mechanisms in the treatment of drug addiction.

CRISPR/Cas9 Applications

Over the years, CRISPR/Cas technology have arised as crucial tools for genetic investigation in clinical treatments, elucidating genetic pathways and metabolic engineering contexts and have been widely used in many aspects of modern biology (Nidhi et al., 2021). Prior to the emergence of the CRISPR/Cas system, scientists have tried to understand the neuron circuits and its

functions (Sandoval et al., 2020). Clarification of the mammalian nervous system has been studied with conventional techniques combined with Next Generation Sequencing and cell-specific targeting systems. (Handel et al., 2013; Sandoval et al., 2020; Zhao et al., 2020). Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) ensured targeting specific DNA sequence feasibilities. However, in practice, extensive protein engineering requirements did not make them suitable (Li et al., 2020). With the invention of the CRISPR/Cas systems, a comprehensive target specific nuclease era began (Ishino et al., 1987). In 2012 CRISPR/Cas technology for the first time was redesigned in mammalian genes (Gasiunas et al., 2012; Jinek et al., 2012). CRISPR/Cas technology offered the opportunity to easily edit Cas9 endonucleases. Thus silencing, activating or editing anywhere in the genome has become possible. With the help of this specificity to understand the nervous system and neuropathy related therapeutic interventions have been raised (Bonnerjee and Bagh, 2021).

CRISPR applications in neuroscience

Coordinated nerve regeneration in the peripheral system requires organized function within neurotrophic elements and neuronal cells (Hsu et al., 2019). CRISPR activation (CRISPRa) is a potent tool using inactive Cas9 (dCas9), single guided RNA (sgRNA) and transcription activator for gene regulation (Casas-Mollano et al., 2020). CRISPR/Cas related genome editing tools, increase the acceleration of biological research and enable genetic investigation in organisms and cells (Xiong et al., 2016). The advent tools present an opportunity for the improvement of new systems to study the nervous system complexity, comprising *in vitro* and *in vivo* models (Li et al., 2019). Accurately gene editing with the help of CRISPR/Cas technology has the capability to speed up neuroscience research (Hsu et al., 2019).

The last improvements in genome editing technologies have accelerated neuroscience by enabling accurate replacement of endogenous genomes in various cell types (Nishiyama, 2019).
Investigations over the years have emerged of epigenetic mechanisms associated with psychiatric and neurological diseases (Ptak and Petronis, 2010). So far, investigating chromatin regulatory proteins did not identify roles for epigenetic modifications in certain genes using overexpression or knockout approaches (Yim et al., 2020). For the first time, molecular, cellular and behavioral levels have been regulated with a single chromatin modification using the CRISPR/Cas system by epigenome editing of a particular gene in a given cell population (Pulecio et al., 2017) Advances in tools in the field of neuro epigenome editing and administrations to demonstrate epigenetic contributions to the pathophysiology of the brain has been investigated (Hamilton et al., 2018). Further, *in vivo,* neuro epigenome editing experiments have been performed to investigate chromatin markers by manipulation of chromatin topology (Xie et al., 2018; Fukushima et al., 2019; Peter et al., 2019). These studies suggested that the induction of epigenetic modifications in multiple genes may have potential clinical applications for *in vivo* neuro epigenome editing (Yim et al., 2020). Also, these may help to understand brain pathology.

Applications of CRISPR/Cas Systems in Neuroscience

The rapid regeneration of genome editing in somatic cells *in vivo* seems to make it possible to treat genetic damage-associated human diseases (Nishiyama, 2019). The limitations of these technologies in terms of efficiency and applicability in the brain, especially in post-mitotic neurons, limit their use in neuroscience (Nishiyama, 2019; Uddin et al., 2020). With advances in new technologies, these difficulties can be overcome and genetic functions and damage can be studied in mitotic and mature post-mitotic neurons *in vitro* and *in vivo* (Straub et al., 2014; Heidenreich and Zhang, 2016; Hana et al., 2021).

CRISPR/Cas based genome editing tools have accelerated targeted research in every organism and cell. These tools provide opportunities for the development of new models and for investigating the complication of the nervous system (Heidenreich and Zhang, 2016).

CRISPRa systems make it possible to investigate multigene relationships that regulate the expression of genes. CRISPR/Cas activation systems offer possibilities in regulating the activity of transcription of target genes in eukaryotes. CRISPRa may be able to create new predictable and metabolite-responsive artificial gene regulation (Fontana et al., 2020).

A hybrid Baculovirus vector has been developed to transfer the CRISPR system that can perform multiplex activation of three neurotrophic element genes. The Adipose-derived Stem Cell (ASC) sheets designed by CRISPRa demonstrated the potentials of the CRISPRa system delivered with hybrid Baculovirus vector (Hsu et al., 2019).

Applicability of CRISPR/Cas system on submandibular gland nerve growth factor

Nerve growth factor (NGF) is an important trophic factor in the nervous systems for neuron growth and survival. Mouse NGF has long been used for neuronal and non-neuronal disorders. With the help of the CRISPR/Cas9 system, transgenic mice were generated that express human NGF (hNGF) especially in their submandibular glands (Gu et al., 2020).
CRISPR/Cas-based genome editing tools may provide an important solution in the future for understanding and defining neuronal circuits and set light to mysteries of complex neurological disorders (Heidenreich and Zhang, 2016).

Neurodevelopmental, Neurological and Behavioral Regulation with CRISPR/Cas System

Pathological changes trigger various neurodevelopmental disorders (NDDs) during early embryonic development (Parenti et al., 2020). Patient-derived iPSCs, production of functional neurons and glia through directed differentiation protocols for

CRISPR/Cas9 technology, provided an opportunity to solve of brain development for rare neurodevelopmental disorders (Du et al., 2015; Bell et al., 2017; Trujillo et al., 2018; Yeh et al., 2018). With this study possible therapeutic opportunities demonstrated cellular and molecular phenotypes for neurological disorders (Sabitha et al., 2021).

A study indicated that endogenous homeostatic mechanisms can re-establish normal neuronal function following cocaine-induced neuroadaptations (Huang et al., 2011). Although no molecular identification has yet been made, such mechanisms could be used for next-generation developments in the treatment of cocaine addiction (Carpenter et al., 2020). Mouse gene expression has been profiled in the course of cocaine deprivation to define assumed regulators of neural homeostasis. Cocaine activated the Nuclear receptor 4A1 (*Nr4a1*) transcription factor and the target gene protein (*Cartpt)*. Continuous activation of *Cartpt* in late deprivation was combined with depletion of the suppressive histone modification, and enrichment of activating markers (Yu et al., 2017; Carpenter et al., 2020). Using CRISPR and transcription factor *Nr4a1* activation, the direct causal role of *Nr4a1* in *Cartpt*'s permanent activation and attenuation of cocaine-induced behavior has been demonstrated (Carpenter et al., 2020). The results present evidence that homeostatic gene expression caused by deprivation is a possible therapeutic target in cocaine addiction (Carpenter et al., 2020).

In another study of epigenetics in the organizing of inherited and potentially reversible changes in gene expression that occur without changes in DNA sequence (Gibney and Nolan, 2010; Nielsen et al., 2012). Epigenetic modifications can occur suddenly or stepwise and can be inherited to subsequent generations by mitosis and meiosis. The relationship of drug addiction with epigenetics mechanisms had been revealed as a result of studies. Considering genetics, epigenetics can play an important role in addiction pathogenesis. Although scientific studies have found associations with epigenetic changes (Nielsen et al., 2012; Nestler, 2014) direct causal evidence is lacking to suggest that these changes alter addiction vulnerability or occur as a result of particular drug use. Illumination of the epigenetic modifications, with CRISPR/Cas9 mediated technology in the improvement of drug addiction or in the course of deprivation can broaden our knowledge of the addiction mechanisms.

Epigenetics is a genome-related mechanism (Bastle and Neisewander, 2016; Wei et al., 2016). DNA methylation, as an epigenetic marker, occurs in CpG dinucleotides and serves to control gene regulation and transcription (Moore et al., 2012; Riancho et al., 2016). The epigenome is especially susceptible to dysregulation caused by toxic substances or stressors during the mitotic stage distribution of epigenetic markers and patterns that can lead to disease phenotypes in adulthood (Wanner et al., 2019).

Epigenetics changes cause prolonged cellular memories in the brain that translate the mechanisms and response to environmental stimuli (e.g. drug exposure) (Zovkic et al., 2013; Kim and Kaang, 2017). Thus predisposing individuals to environmental factors associated with drug-taking behavior. Emerging evidence contributes significantly to the susceptibility of epigenetic factors to the disease (Wong et al., 2010).

Epigenetics has enabled a new point of view for studying the structure of drug addiction (Nielsen et al., 2012). Multiple studies have demonstrated the important role of epigenetics in drug addiction. MicroRNAs (miRNAs), one of the important methods of epigenetic regulation.(Osella et al., 2014; O'Brien et al., 2018). With the help of this mechanism, microRNAs can deactivate or inhibit the translation of their target genes (Xu et al., 2020).

In the case of drug addiction, interactions between genotype and environmental factors attribute a crucial role in epigenetic modifications in the response to drugs and the initiation of addiction (Ajonijebu et al., 2017). This epigenetic point of view is convenient with the longevity of psychiatric conditions and the obstacle of developing pharmacotherapeutic interventions to effectively treat chronic behavioral disorders.

For example, it is theoretically possible to use CRISPR/dCas9 to target multiple genes within the same cells and thus manipulate experimentally drug-regulated gene networks (Li et al., 2020). Using these approaches together with animal addiction samples such as self-medication, researchers will be capable of isolating the specific epigenetic incompatibilities that trigger the most damaging elements of drug addiction (Cadet, 2016).

Epigenetic studies so far have been based on a link between epigenetic markers and the gene expression pattern (Vojta et al., 2016). Emerging technologies in epigenome editing allow direct examination of exact epigenetic modifications and the functional relevance of gene editing (Nakamura et al., 2021). Reversibility of epigenetic modifications has already been used to remodel the abnormal epigenetic patterns that have been reached non-selectively using epigenetic inhibitors (Cheng et al., 2019). At specific loci, epigenetic regulation demonstrates a novel attempt that can change gene expression selectively and inherently. CRISPR/Cas9 mediated tool has been developed for certain DNA methylation, with inactivated dCas9 nuclease and a guide RNA to any DNA sequence. Targeted CpG methylation by the fusion protein in a broad region has been demonstrated. DNA methylation activity is particular to the targeted site and is inherited between mitotic divisions. Eventually directed DNA methylation of a broader promoter region of target loci is shown. The aim of this research is to associate CRISPR/Cas9 technology with drug addiction and to demonstrate that CRISPR technology is a new therapeutic treatment for drug addiction (Vojta et al., 2016).

Epigenetic drugs, most of which have completed phase clinical studies and are confirmed by the FDA, showing that they can be tolerated in humans (Sartor, 2019).Alteration of gene expression provides potential therapeutic approaches. Therefore CRISPRoff consists of dCas9 fusion protein introduced. Temporary

CRISPRoff expression initiates rather particular DNA methylation and gene suppression (Nunez et.al., 2021). Comparing CRISPRoff with genome-wide screens and analysis of chromatin markers sets guidelines for inherited gene silencing. Single guide RNAs (siRNAs) have the ability to silence the greater part of genes, as well as those non-canonical CpG islands, were identified, revealing a broad targeting perspective extending on the further side of annotated CpG islands (Nunez et al., 2021). Long-standing aspects of addiction may be key modulators of drug-dependent gene expression of epigenetic modifications. This is because epigenetic changes can maintain and sustain permanent structural chromatin adaptations. This situation has directed investigators to attempt to explain the epigenetic mechanisms related to psychostimulant dependence (Godino et al., 2015).

Although epigenetic mechanisms have been frequently studied in cellular differentiation and development, they also play a role in the longevity of postmitotic cells. Therefore, along with the learning and memory mechanisms of epigenetic changes, it can also regulate the brain's reward center and drug addiction (Tuesta and Zhang, 2014).

The key regions of the brain which have the capability to accumulate or remove specific epigenetic markers in confined gene regions within targeted cell types contribute to researchers on epigenetic molecular mechanisms related to neuropsychiatric syndromes and causality related to drug addiction. The modularity and flexibility of neuroepigenetic regulation approaches make them optimal and appropriate tools for solving *in vivo* (Cadet, 2016; Hamilton and Nestler, 2019).

Transcriptional activity of an epigenetic modulated single gene that links subsequent reward behavior. Lately, epigenetic mechanisms have been linked to inherited drug-induced expression of gene networks in specific brain nuclei (Sadri-Vakili, 2015).

There is rising evidence that epigenetic modulation plays a role in sustaining addiction vulnerability as well as behavioral adaptations caused by substance addiction. Thus, these mechanisms are usually first reactions to a dynamic environment and regulate various processes, including the gene. With a specific focus on DNA methylation and histone modifications, examining genome-wide epigenetic modifications in the brain subsequent drugs of abuse in humans, primates, or rodent models. A special point has been placed on critical factors to consider when evaluating neuro epigenetic reprogramming. It is discussed whether mechanisms or biomarkers shared in epigenetic programming have been identified following drug exposure. A study of the structural rearrangement of chromatin, could widen our comprehension of these molecular adjustments in addictive behaviors, explain the permanent phenotypic impacts of drugs, and especially their vulnerability to relapse (Nogueira et al., 2019).

Within the scope of this review, particular epigenetic mechanisms suchlike DNA methylation, histone acetylation, remodelling of chromatin through methylation and microRNAs are discussed as the mechanism that regulates gene networks and cocaine addiction (Sadri-Vakili, 2015).

With the aim of this article, one question needed to clarify is whether epigenetic changes increase vulnerability to developing a drug addiction, or the addiction itself creates an epigenetic reaction to these drugs (Nielsen et al., 2012).

Effectors of the epigenome that have effects on various pathways. The number of drugs and phenotypes which affect epigenetic mechanisms is correlatively small so far, in addition to research of these effects will be crucial to fully understand the results of developmental exposure to drugs (Wanner et al., 2019).

Based on a study of the mammalian central nervous system, it has been shown that Excitatory Amino Acid Transporter 2 (EAAT2) is the dominant astrocyte glutamate transporter related to the reuptake of synaptic glutamate in the mammalian central nervous system (CNS) (Alam and Datta, 2019). Gene expression can be regulated without altering DNA sequences through epigenetic mechanisms (D'Addario and Maccarrone, 2016). This highlights the evidence for the role of epigenetics in the regulation of EAAT2 in different neurological disorders and discusses the current pharmacological approaches used to induce EAAT2 expression with CRISPR/Cas9 technology, and the potential use of new therapeutic approaches (Alam and Datta, 2019).

HISTONE METHYLATION

Studies have shown that chronic cocaine and stress are effective in the dynamic regulation of histone methylation (Covington et al., 2011). Especially, recurring cocaine usage decreases G9a expression and strengthening of H3K9me2 in the NAc, inducing derepression in many genes, including Fosb (Heller et al., 2014). Histone methylation is thought to suppress gene expression through the aggregation of supplemental methyltransferases and HP1 that can cause the generation of silent heterochromatin (Heller et al., 2014).

G9a studies indicated that G9a is a histone methyltransferase constantly upregulated in human HCCs. Functionally, it demonstrated G9A inactivation by RNAi knockdown. CRISPR/Cas9 knockout and pharmacological inhibition were significantly removed from H3K9 demethylation and suppressed by both in vitro and in vivo models of HCC cell proliferation and metastasis (Nestler et al., 2010). It has been shown that frequent G9a regulation in human HCCs refers to increasing gene copy numbers on chromosome 6p21. Besides that, microRNA-1 has been identified as a negative regulator of G9a (Nestler et al., 2010). The loss of microRNA1 alleviated post-transcriptional pressure on G9a and contributed to its up-regulation in human HCC. Using RNA sequencing, the tumor suppressor *RARRES3* was identified as a crucial target of G9a (Nestler et al., 2010). The

roles of enzymes that control histone methylation have not yet been investigated.

The prominent cellular mechanism that combines various environmental inducers with altering in gene expression is chromatin remodelling. Signal-linked enzymes can change the structure of chromatin at certain gene loci to facilitate the activation or suppression of particular gene transcription (Renthal and Nestler, 2010).

Studies for H3 lysine could be a new promise in the future with CRISPR/Cas9 technology in drug addiction treatment; technologies that facilitate targeted manipulation of epigenetic markers can be used to exactly control cell phenotype or to examine the correlation between the epigenome and transcriptional control. This study describes a programmable CRISPR/Cas9 mediated acetyltransferase comprising the dCas9 protein integrated with human acetyltransferase p300 (Thakore et al., 2016). The fusion protein performs acetylation of H3 histone lysine 27, resulting in transcriptional activation of distal and proximal enhancers from the promoter of the target gene. Gene activation by targeted acetyltransferase is unique to the genome. Unlike prior dCas9 mediated activators, acetyltransferase activates genes with a guide RNA from enhancer regions (Thakore et al., 2016).

Chromatin's highly dense concentrated structure supplies unparalleled chromatin control over gene expression by providing transcriptional activators entry to DNA (Urnov and Wolffe, 2001; Renthal and Nestler, 2010). The chromatin structure has various mechanisms that help to physically loosen the chromatin (e.g. histone acetylation) or remodel (e.g. nucleosome remodelling related to SWI-SNF) or provide insertion sites for additional transcriptional co-activators. Such alterations comprising histone acetylation, phosphorylation, and methylation that co-impact the activity of the gene (Urnov and Wolffe, 2001; Renthal and Nestler, 2010). Dimethylation of histone H3 lysine 9 (H3K9me2), a significant type of chromatin modification, is checked by a multiplex interaction between histone methyltransferases, (Shinkai and Tachibana, 2011).

Data from postmortem human brain tissue as well as animal models have also shown that histone methylation plays a role in the spread of depressive-like behaviors and other psychiatric syndromes (Covington et al., 2011).

Conditions that increase histone acetylation expression make it sensitive to cocaine stress, and pain. While conditions that decrease histone acetylation reduce sensitivity to these inducers has crucial impacts on the pathogenesis of the brain (Nestler and Renthal, 2010). Eventually, the prominent role of histone acetylation is to increase the transcription, or transcription of genes that alter neural function (Nestler and Renthal, 2010). Therefore, any study of chromatin modifications has a relationship with the underlying mechanism of gene activity. In this direction, CRISPR/Cas9 studies will reveal new treatments for drug addiction.

GENE EXPRESSION

As epigenetic mechanisms, DNA methylation and histone modifications continue to be investigated biochemically (Miller and Grant, 2012; Sartor, 2019). Efforts to perform locus-specific histone acetylations demonstrate the lack of experimental tools to be used. Cas9 nuclease is capable of performing epigenetic modifications but is lacking in synthetic epigenome remodelling (Kwon et al., 2017). Two different cell types were analyzed to research the range of activity, specificity and target gene expression depending on cellular content were evaluated. The results show that the chromatin environment is a crucial consideration when using this synthetic histone deacetylase (HDAC) (Kwon et al., 2017).

DNA methylation is associated with behavioral disorders. It may reflect a person's lifestyle and environmental conditions according to epigenetic changes (Alegria-Torres et al., 2011). Therefore, epigenetic changes can be used as biomarkers indicating metabolic dysfunction. Epigenetic changes perform to reduce or activate genes, but they may also have other functions which they are not fully understood (Gibney and Nolan, 2010). One of the most studied epigenetic mechanisms is DNA methylation (Moore et.al., 2012). It involves adding a methyl group to the C5 position of cytosine with DNA methyltransferase (DNMT) to form 5-methylcytosine (5-mC) (Moore et al., 2012). Methylation changes chromatin packaging, making it more condensed and therefore less available to transcription factors (Masiak et al., 2020). The regulation of DNA methylation has been examined in animal models across a range of physiological and behavioral phenotypes. It has been shown to play a role especially in neuronal and brain development. (Masiak et al., 2020).

Using CpG dinucleotides, CRISPR/Cas9 technology could shed light on drug addiction. With studies obtained, results demonstrated that developments in sequencing technology assists investigators to map genome-wide alters in the progression of disease in DNA methylation (McDonald et al., 2016). However, experimental tools for site-specific manipulation of DNA methylation to discriminate functional results are lacking. CRISPR/Cas9 DNA methyltransferase 3A (DNMT3A) fusion was developed to stimulate DNA methylation at particular loci in the genome (McDonald et al., 2016). These tools allow the role of guiding molecular processes that define cellular fate with DNA methylation (Masiak et al., 2020). Epidemiological studies have shown potential cooperation between leukocyte DNA methylation and certain cancers or schizophrenia (Masiak et al., 2020).

Transcriptional activation of many genes promotes cocaine uptake, analyzing genes regulated by such mechanisms with CRISPR/Cas9 technology that will widen our knowledge of the complex biological basis of drug addiction and could assist more efficient treatments for addictive disorders.

BRAIN REWARD CENTER

The brain reward center, which develops for survival, is affected by cocaine addiction (Dackis and O'Brien, 2001). In studies related to this; Optogenetic stimulation of Locus coeruleus (LC)-NE (norepinephrine) neurons triggers instant transitions from sleep to waking up. To question the role of LC-derived NE in regulating wakefulness, applied cell-type-specific CRISPR/Cas9 technology *in vivo* to selectively disrupt the dopamine beta-hydroxylase (*dbh*) gene in adult LC-NE neurons (Yamaguchi et al., 2018). Single-acting *dbh* gene impairment removed the sudden stimulation subsequent optogenetic stimulation of the LC. The bilateral LC-specific *dbh* impairment significantly decreased NE concentration in the LC projection regions and decreased the waking length even in the presence of noticeable inducers (Yamaguchi et al., 2018). These consequences propose that CRISPR/Cas9 gene editing in adult neurons can be used to change gene function in genetically defined neuronal circuits related to drug addiction (Yamaguchi et al., 2018).

Abuse of drugs activates dopamine signals with molecular and cellular mechanisms in the mesocorticolimbic circuit. DA neurons in the ventral tegmental area (VTA) are the main substrates of abuse drugs and have played a role in addiction-associated behaviors (DeBacker et al., 2021).
Disorganization of brain reward pathways is associated with a drastic increase in drug-seeking behavior and intake (Haas et al., 2009).

Studying gene function in the mammalian brain could help in treating drug addiction by methods of manipulating the genome of neurons in vivo. CRISPR/Cas9 from *Streptococcus pyogenes* (SpCas9) can be used to edit genes in the replication of eukaryotic cells (Swiech et al., 2015). Herein the effects of genome editing in postmitotic neurons *in vivo* Adeno-associated viral (AAV) mediated SpCas9 and guide RNA delivered to target MECP2 in the adult mouse brain. The results show that AAV-based SpCas9 genome editing can facilitate genomic alteration of gene function in the brain (Swiech et al., 2015).

Synaptic organization in brain regions related to motivation and reward (such as the striatum) centers may change as a result of continuous exposure to abused drugs, together with in the brain regions involved in the inhibitory control of judgment and behavior (such as the prefrontal) (Samaha et al., 2004). These brain regions related to maladaptive responses to addictive drugs are also needed for several forms of cognitive processing (Robinson et al., 2006). Recurring cocaine exposure has also been shown to raise histone acetylation in the brain region, which has been shown to increase the rewarding, enhancing and locomotor activating features of the drug (Covington et al., 2011). The DNA methylation mechanism in reward-related brain regions undergoes changes after exposure to cocaine(Camila et al., 2019). CREB (cAMP response element-binding protein) and ΔFosB are both activated in reward centers of the main brain reward region, but mediate different points of the addictive state (Nestler, 2004).

ΔFosB is a highly consistent unique transcription factor found in the course of the brain and stimulated by chronic neuronal activity (Covington et al., 2011) that plays a crucial role in long-term adaptive alterations in the brain related to various conditions such as drug addiction (Self et al., 2001). To overcome this limitation, the focus has been on the transcription factor ΔFosB, (Heller et al., 2014). ΔFosB is induced by chronic cocaine exposure in rodents and human cocaine addicts but its expression is decreased by certain forms of chronic stress in rodents and depressed humans (Heller et al., 2014).

The relationship of ΔFosB with stress in drug addicts has also been proven with the CRISPR/Cas9 technology. Within the studies, ΔFosB has a well-known feature of mediating stress sensitivity in the nucleus accumbens (NAc), which regulates synaptic and intrinsic effects of medium spiny neurons and as well as needed for learning in the dorsal hippocampus (dHPC). ΔFosB is stimulated in the course of HPC by stress and antidepressant treatment, and vHPC CA3 ΔFosB is crucial for the prophylactic effects of ketamine on stress responses (Heller et al., 2014). This demonstrates that ΔFosB is a crucial modulator of vHPC function and can regulate long-term alterations in gene expression fundamental depressive and anxiety disorders. Herein, circuit-specific CRISPR/Cas9 gene editing is used to reveal in advance the unknown role of ΔFosB in vHPC neurons reflecting NAc. It has also been shown that ΔFosB regulates the excitability of this circuit and identifies possible downstream gene targets underlying stress resistance in this area (Heller et al., 2014).

It indicated that the degradation of G9a expression in the nuclear accumulator (NAc), with the coincident decrease in H3K9me2, was crucial in mediating high levels of transcriptional and behavioral responses to recurred cocaine (Covington et al., 2011). In comparison to cocaine suppression of G9a and H3K9me2 in the NAc, histone acetylation in this brain region causes the opposite effect of cocaine stimulation and maintains animals from the harmful results of chronic stress (Covington et al., 2011).

The Ras-checked Raf-MEK-ERK protein kinase signalling cascade has been specified as a crucial tool of both cognitive processing and the neurobiological effects of psychostimulant drugs (Robinson et al., 2006). The pharmacological blockade of the Raf-MEK-ERK cascade also appears to attenuate the improvement of a conditional location choice such as cocaine, amphetamine, and modulate progress (Robinson et al., 2006).
According to the data obtained, the studies conducted for ERK1 and ERK2 will also guide drug addiction (Lopez-Guerrero et al., 2017). Endoplasmic reticulum Ca2 + sensor STIM1, which modulates the activity of plasma membrane Ca2 + channels, phosphorylates at ERK1 / 2 target sites during Ca2 + storage consumption induced by thapsigargin or epidermal growth factor (EGF). This ERK1 / 2 dependent phosphorylation regulates STIM1 localization and dissociation from microtubules, and it is known to increase binding to ORAI1, a

store-operated Ca 2+ input (SOCE) channel, causing to activation of Ca 2+ (Lopez-Guerrero et al., 2017).

Disruption of the brain reward system including DNA methylation dynamics interferes with various mechanisms. The drugs produce long-term brain alterations that underlies addiction, in part by activating certain genes. These changes are very durable, understanding how they are determined and preserved will provide treatment with new studies to be made in this area with next-generation CRISPR/Cas9 technology.

PHARMACOTHERAPY

Epigenetic changes may result from inheritance through genomic imprinting, life events, chronic drug use, or pharmacotherapies for addictions. It is thought that the pharmacotherapeutic management network of DNA methylation and histone modifications will be used in the treatment in the future (Nielsen et al., 2012).

Cocaine becomes addictive very quickly and creates serious medical, psychosocial and psychological problems. Rejection protects dependent individuals from their situation and should be considered during treatment. Clinicians who lack pharmacological choices should depend entirely on psychosocial attempts (Dackis and O'Brian, 2001).

There is no proven pharmacotherapy for cocaine addiction (Haas et al., 2009). However, since cocaine addiction is due to a pharmacologically induced decrease in the neuroplasticity of brain circuits that mediate normal reward learning, new pharmacotherapies that target the biological pathology of addiction should be in place.
New pharmacotherapies that directly target the biological pathology of addiction should be applicable. Advances in the neurobiology of cocaine addiction have provided investigators to define drugs that can help patients initiate deprivation and prevent relapse. Several such drugs and a vaccine have demonstrated strengthening consequences in controlled clinical trials with cocaine-abused individuals (Haas et al., 2009).

Pharmacotherapies offered for the treatment of abuse and addiction disorders are used to succeed certain main purposes: 1. Decrease the risk of addiction, 2. Reduction in high-risk behavior, 3. Decrease in morbidity and mortality from addiction, 4. Reduction in drug use and/or the triggering of deprivation, 5. Remission of withdrawal symptoms and 6. Prevention of return (Klein, 1998).

Pharmacological manipulation of specific epigenetic associated proteins in animal models measuring contextual reward processing for substance use disorder (SUD) is sufficient to improve drug-induced alterations. For instance, the nonselective histone deacetylase (HDAC) inhibitors trichostatin A and phenylbutyrate dose-dependently reduced cocaine self-application (Sartor, 2019). Class III histone deacetylase (sirtuin) inhibitor, sirtinol, reduced cocaine conditioned place preference, while the sirtuin agonist resveratrol had the reverse effect. Subsequent recurred cocaine administration, the histone acetylation protein BRD4 expression increases in the nucleus accumbens. Pharmacological inhibition of BRD4 reduced transcriptional and behavioral reactions to cocaine and heroin (Sartor, 2019).

Identifying new therapeutics for the treatment of drug addiction is a field of intensive research. Strategies for neurotransmitter receptor replacement have had limited success, and FDA-approved drugs for the treatment of cocaine, methamphetamine and marijuana use disorders are currently not available. Further investigation needs to be done in this field. Therefore, the possibility of clinically effective treatments in the near future may occur as advances in epigenetic pharmacotherapies and CRISPR/Cas9 technology.

In recent years, our comprehension of how drugs of abuse impress transcription factors to initiate plasticity-related gene expression in epigenetics, histone methylation, gene expression, and other addiction-related brain regions has increased dramatically. Appearing technologies such as CRISPR/Cas9 are likely to further restructure our comprehension of how addictive drugs remodel brain reward and motivation areas. In the development of new therapeutics that can modulate or even reverse the transcriptional actions of drug abuse in the brain to enable withdrawal effects and fight addiction. Researching new approaches with CRISPR/Cas9 technology need to be done to express these important insights into new therapeutics to combat drug addiction.

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