Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online) DOI: 10.7176/ALST
Vol.76, 2019
www.iiste.org

Application of Biotechnology in Diagnosis and Treatment of Human Genetic Disorders

TILAYE ATNAF ASHINE[1] TESFAYE SISAY TESEMA[2]
1.College of Natural and Computational Science Department of Biology (Stream of Medical Biotechnology), Madda Walabu University, PO box 47, Bale Robe, Ethiopia
2.Unit of Health Biotechnology, Director, Institute of Biotechnology, Addis Ababa University

Abstract
Biotechnology is the application of biological molecules to make useful products that can be used to solve problems in different sectors. Medical biotechnology in particular aims to know the root causes of genetic disorders and develop therapeutic strategies. Although human diseases are caused by different factors, those caused by genetic disorders are increasing in prevalent worldwide. Early detection and treatment of these condition is paramount important. Chemical treatments that cure only the symptom of genetic disorders were practiced so far to manage the effect. However, as a result of advances in understanding of biological molecules, treatment of the cause of the disease called gene therapy has been developed more popularity nowadays. Moreover, most research centers abandoned the classical techniques that are prone to error, and began to use high throughput technologies to correct genetic disorders. Among these, CRISPR Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats Associated Cas9) is the most easy to use and allows precise gene editing in higher organisms to repair disease-causing genes by homologous directed repair. The recent development of modified versions CAs9 Nickase that reduce off target effects and the "base editor" that preform without double strand DNA break and without the need to template indicated its application for therapeutic genome editing in man in the future.
Keywords: Biotechnology, CRISPR Cas9, gene therapy, Nickase
DOI: 10.7176/ALST/76-02
Publication date:September 30th 2019

Introduction

The goals of biotechnology in medicine include identifying the root cause of diseases, production of drugs and therapeutics, analysis of the genes in genetic disease and correction of genetic defects (Zahid *et. al*., 2016). Therefore, biotechnology aims to target the causes of diseases and not the symptoms. And that is why biotechnology offers one of the strongest hopes for patients to treat diseases. The concept of treating a genetic disorder by altering the function of the responsible underlying gene rather than its effect had been emerged in early 1970s. This change in thinking was due partly to understanding the structure of DNA, the nature of genes as segments of DNA that encode protein products, the nature of mutations and their role in human disease, and the development of methods to isolate specific genes, to "splice" molecules of DNA together to create entirely new combinations of genetic information (Friedmann and Schneider, 2006).

Among the approximately 25,000 annotated genes in the human genome, mutations in over 3,000 have already been linked to disease phenotypes (Benjamin, Cox, Platt, and Zhang, 2015; Mahdieh and Rabbani, 2013). Genetic disorders are caused by the mutations, permanent changes in the genetic material of a cell. Mutations can be caused by copying errors in the genetic material during cell division and by exposure to radiation, chemicals, or viruses, or can occur deliberately under cellular control during the processes such as meiosis. Different types of mutations like single nucleotide mutation, chromosomal aberration, mitochondrial genome change and also environmental factors play a role in inducing these genetic disorders in humans (Badizadegan, 2003,Uppangala, 2010).

A new generation of genome engineering technologies based on the class of RNA-guided endonucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9, and their rapid applications are now bringing a further revolution in biology and medicine (Yang, 2015). This review, therefore, focuses on the application of recent biotechnology techniques in correcting human genetic disorders and their future promises and challenges.

Genetic Disorders

Genetic disorders are caused by mutations or abnormalities that occur in a chromosome or genome. These abnormalities may appear phenotypically at any time of a human life. It is being estimated that there are around more than 4,000 genetic disorders which affect the human life(Uppanqala, 2010). But this number keeps changing as we know more about our gene and genome. Genetic disorders are generally classified into four main types: chromosomal abnormalities, single-gene defects, mitochondrial genetic disorder and multifactorial conditions (Dayal, 2015).

Chromosomal disorders are due to chromosomal aberrations including numerical and structural damages (Mahdieh & Rabbani, 2013). When small portion or entire chromosome are duplicated, altered or some portion of chromosome is missing then a condition known as chromosomal genetic disorder occur. Chromosome anomalies usually occur when there is an error in cell division following meiosis or mitosis. There are many types of chromosome anomaliesthat can be organized into two basic groups, numerical and structural anomalies. For example an extra copy of chromosome 21 leads to the condition known as Down's syndrome. This extra copy of DNA will lead to more production of proteins. This in turn will disturb the normal functions of the body (Friedmann and Schneider, 2006).

Single Gene Disorders or Mendelian disorders result from errors in DNA sequence of a gene and include autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XR), X-linked dominant and Y-linked (holandric) disorders (Mahdieh & Rabbani, 2013). When mutations or change in DNA sequence of a single gene leads to a disorder, then this condition is known as single gene disorder. The changes in the gene will lead to the formation of non-functional proteins in the body. This non-functional protein will lead to a disorder or genetic condition. For example Cystic fibrosis is an example for single gene disorder. Cystic fibrosis condition is caused by a mutation in chromosome 7 gene known as cystic fibrosis transmembrane conductance regulator (CFTR) (Schneider, 2006). The mutation in the gene CFTR produces the abnormal non-functional protein, which affect the movement of sodium chloride in the body cell. This leads to the formation of abnormal thick mucus in the lungs. This in turn makes it difficult for patients to breathe and digest. This is also an example for recessive genetic disorder. Recessive genetic disorders are the ones when both the alleles of a gene are defective then only the condition occurs phenotypically. That is both the parents should pass the defective gene to their offspring. Dominant genetic disorders are expressed when only one allele of the gene is replaced by the defective code. Example for this condition is Marfan syndrome.

Mutations can also be categorized on the basis of the function: 1) The loss-of-function mutations cause a decrease or a loss of the gene product or the activity of the gene product; 2) The gain-of-function mutations cause an increase in the amount of gene product or its activity, and sometimes create a new property, leading to a toxic product responsible for a pathological effect (Benjamin et al., 2015; Mahdieh & Rabbani, 2013). Mitochondrial genetic disorder occurs due to the genetic mutations that occur within the mitochondrial DNA. Mutations in mitochondrial DNA (mtDNA) are present in the oocyte. Mitochondrial diseases are caused by a mutation in a proportion of the mtDNA molecules present in the patient's cells, a phenomenon called heteroplasmy. Mutations in the mtDNA are transmitted exclusively via the oocyte, which can carry between 10 000 and 100 000 mtDNA copies. Leber Optic Atrophy is an example for mitochondrial genetic disorder that causes degeneration of optical nerve cells (Tuppen et al., , 2010; Vassena et al., 2016).

Multifactorial genetic disorders are caused by combination of many factors like gene mutation, small variations in the chromosome structure and also environmental factors. Cancer, heart conditions or disease are best examples for this type of genetic disorder (Mahdieh & Rabbani, 2013). For instance, a single mutation in the gene will not induce skin cancer. Rather multiple DNA mutations caused by environmental factors like X-ray or UV rays may increase the chances of developing skin cancer. This type of genetic disorders is very difficult to treat as modified cells would be out competed by their diseased counterparts, causing the benefit of treatment to be low(Benjamin et al., 2015)

Mutations may act as dominant or recessive when the amount of product from one allele is not sufficient for a complete function (Haploinsufficiency), e.g. mutations in the low-density lipoprotein receptor (LDLR) leading to haploinsufficiency in familial hypercholesterolemia (FH) (Pastinen et al., 2016). If the product of the defective allele interferes with the product of normal allele (Dominant negative) it affects the function of normal protein. A mutated allele may gain a new or excessive activity (gain of function) e.g. mutations of fibroblastic growth factor receptor 3 (FGFR3) in achondroplasia (Vassena et al., 2016).

Techniques Used in Detection of Genetic Disorders

Genetic testing is a type of medical test that identifies changes in chromosomes, genes, or proteins. The results of a genetic test can confirm or rule out a suspected genetic condition or help determine a person's chance of developing or passing on a genetic disorder. More than 1,000 genetic tests are currently in use, and more are being developed. In the past, the main genetic tests searched for abnormal chromosome numbers and mutations that lead to rare, inherited disorders. Today, tests involve analyzing multiple genes to determine the risk of developing certain more common diseases such as heart disease and cancer (Genetics Home Reference, 2016) . The identification of disease-causing mutant genes helps to understand how genes contribute to pathogenesis, aid in developing strategies aimed at the treatment of the disease and may also result in the development of genetic tests. With the development of new technologies for more accurate understanding of the genome and potential gene therapies, the detection of mutations has an increasingly central role in various areas of genetic diagnosis including newborn screening used just after birth to identify genetic disorders that can be treated early in life, diagnostic testing used to identify or rule out a specific genetic or chromosomal condition, Carrier testing used to

identify people who carry one copy of a gene mutation that, when present in two copies, causes a genetic disorder, Prenatal testing used to detect changes in a fetus's genes or chromosomes before birth by amniocentesis that could be done only after 18 weeks or later in pregnancy or during 6 to 8 weeks of pregnancy by performing a biopsy of trophoblastic villi which form the external part of the embryo (Friedmann and Schneider, 2006), Preimplantation testing, also called preimplantation genetic diagnosis (PGD), a specialized technique that can reduce the risk of having a child with a particular genetic or chromosomal disorder (Dayal, 2015) and Predictive and presymptomatic types testing used to detect gene mutations associated with disorders that appear after birth, often later in life (Mahdieh & Rabbani, 2013; Nollau & Wagener, 1997).

Karyotype Analyses

A chromosome anomaly, abnormality, aberration, or mutation is a missing, extra, or irregular portion of chromosomal DNA (Dayal, 2015). It can be from an atypical number of chromosomes or a structural abnormality in one or more chromosomes. Chromosome mutation was formerly used in a strict sense to mean a change in a chromosomal segment, involving more than one gene. A karyotype refers to a full set of chromosomes from an individual that can be compared to a "normal" karyotype for the species via genetic testing(Mahdieh & Rabbani, 2013).

Establishing the karyotype of cells provides knowledge about syndromes caused by gross chromosomal aberrations. Chromosome identification depends on the use of various staining techniques. Staining is responsible for the alternating dark and light bands on the chromosomes. The most routinely used technique stains the metaphase chromosomes with Giemsa. Each chromosome pair stains with its own characteristic banding pattern. The bands (G-bands) correlate approximately with the DNA sequence underlying it. AT-rich areas stain darkly, GC-rich areas lightly(Mahdieh & Rabbani, 2013).

There are several other staining techniques used for more specialized purposes. One, called Q-banding, stains chromosomes with quinacrine mustard and views them fluorescently. The bright Q bands correspond almost exactly with the dark bands seen with Giemsa. A second method, called R-banding, treats chromosomes in such a way that the dark and light G bands are reversed (Mahdieh & Rabbani, 2013).

Fluorescence In Situ Hybridization /FISH /

The most recent, as well as revolutionary, is the FISH (fluorescence in situ hybridization) technique (Dayal, 2015). FISH deploys DNA probes specific for each chromosome (or sub chromosomal region or single locus). These probes are fragments of DNA or RNA, usually 100 to 1,000 bases long, used to detect the presence of nucleotide sequences that are complementary to the sequence in the probe. The probes are labeled with modified nucleotides that fluoresce under particular conditions. By using different fluorochromes, a karyotype can be “painted”. FISH is used for the determination of sex for X-linked diseases, chromosomal abnormalities, and aneuploidy screening (Mahdieh and Rabbani, 2013).

Comparative Genomic Hybridization /CGH/

A human cell contains 23 pairs of chromosomes; however, FISH analysis allows accurate assessment of only 7-9 chromosomes in each biopsied cell. Consequently, many abnormal embryos, incapable of forming a successful pregnancy, remain undetected and may be transferred. Comparative Genomic Hybridization /CGH/ enables enumeration of all 23 pairs of chromosomes and also provides a more detailed picture of the entire length of the chromosome which may detect imbalance of chromosomal segments (Dayal, 2015).

Using CGH, however, the embryo nucleus is labeled with a fluorescent dye and a control cell is labeled using another color (i.e., red or green). The two cells are then cohybridized onto a control metaphase spread, and the ratio between the two colors is compared. If the chromosomal analysis shows an excess of red, the embryo nucleus contains an extra chromosome. If an excess of green is apparent, then the embryo nucleus is missing one of these chromosomes. CGH enables not only enumeration of all 23 chromosomes but provides a more detailed picture of the entire length of the chromosome which may detect imbalance of chromosomal segments either using array CGH, an accelerated CGH protocol providing results in 24 hours for all chromosomes, or by using polar body biopsy. Few laboratories currently offer this technology (Mahdieh and Rabbani, 2013, Dayal, 2015).

Enzyme Assay

In most genetic disorders, the presence of defective genes results in production of defective proteins or enzymes. Sometimes the enzyme is not produced at all. Most of these proteins have already been identified. The presence of the proteins can be detected by using the appropriate substrate or using antibodies that specifically recognize that protein(Prajnya and Rehder, 2011; Singh et al., 2010).

Polymerase Chain Reaction

PCR, sometimes called DNA amplification, is a technique in which a particular DNA sequence is copied many

times in order to facilitate its analysis. PCR is used for the diagnosis of single gene defects, including dominant and recessive disorders(Greber, Tandara, Lehrach, & Himmelbauer, 2005).

PCR is a relatively fast and convenient way to test DNA. The method has been used in a variety of preimplantation genetic testing protocols. However, it requires sufficient amounts of a pure, high-quality sample of DNA. It requires controlled laboratory environment and well trained technicians to avoid all types of outside interferences. Errors in PCR can result in misdiagnoses leading to an affected embryo being transferred or the discarding of a normal embryo. The preferential amplification of one allele over another during the PCR process is mainly a problem for preimplantation genetic diagnosis of dominant disorders or when 2 different mutations are carried for a recessive disorder and only one mutation is being analyzed (Sermon et al., 2015, Mahdieh & Rabbani, 2013).

The different versions of PCR, and their applications in diagnosis of genetic defects, include:

(a). Reverse transcriptase PCR (RT-PCR): In this version, a strand of RNA molecule is transcribed reversely into its complementary DNA (cDNA) using the reverse transcriptase enzyme. This cDNA is then amplified by PCR. RT-PCR is applied to study the mutations at RNA level.

(b). Multiplex PCR: In this technique, multiple selected target regions in a sample are amplified simultaneously using different pairs of primers.

(c). Nested PCR: It includes two successive PCRs; the product of the first PCR reaction is used as a template for the second PCR. This type of PCR is employed to amplify templates in low copy numbers in specimens. It has the benefits of increased sensitivity and specificity.

(d). Amplification refractory mutation system (ARMS) PCR: it is a general technique for the detection of any point mutation or small deletion. The genotype (normal, heterozygous and homozygous states) of a sample could be determined using two complementary reactions: one containing a specific primer for the amplification of normal DNA sequence at a given locus and the other one containing a mutant specific primer for amplification of mutant DNA. ARMS-PCR has been used to check the most common mutation in GJB2 gene, 35delG mutation among deaf children. Typically, AS-PCR is well known as a simple, fast and cost-effective method in mutation detection and polymorphism genotyping, but the biggest limitation is that the allele-specific primer can often be non-specifically extended by most DNA polymerases, even if there is a mismatch with the template at its 3'-end, which results in false positive results(Hao, Fan, Chen, Chen, & Zhang, 2015). As a new kind of allele discrimination strategy, proofreading PCR (PR-PCR) was first developed for mutation detection in 1998 (Hao et al., 2015). This technique exploits the 3'-5' exonuclease (proofreading) activity of high-fidelity DNA polymerase to remove the blocked 3'-terminal nucleotide from the inert primer when the blocked nucleotide mismatches with the template and then to extend the primer with a free 3'-hydroxyl group (-OH). If the inert primer with a blocked 3'-OH matches with the template completely, it will not be extended since the blocked 3'-terminal nucleotide is unable to be removed. However, this approach has still not been widely applied due to its low efficiency and sensitivity in allele discrimination.

As a result Hao et al.(2015) has reported a novel modified PR-PCR method that uses a dideoxynucleotide (ddNTP) blocked primer and a mixture of DNA polymerases with and without the 3'-5' proofreading function. The new method exhibits better performance in allele discrimination and higher sensitivity in comparison with the conventional PR-PCR and can be used to rapidly and accurately detect variations, especially those at lower frequency. In the new strategy, the ddNTP-blocked primer, which appears to have the best blocking effect, was selected as the allele-specific primer to improve the specificity of PR-PCR and the amount of high fidelity DNA polymerase was decreased to avoid the non-specific primer extension.

(e). Real time PCR:

In this technique, the amplified DNA is detected as the PCR progresses. It is commonly used in gene expression studies and quantification of initial copy number of the target (Mahdieh & Rabbani, 2013).

Restriction Fragment Length Polymorphism (RFLP)

Analysis of RFLP makes use of the fact that in certain genetic disorders, the gene mutation changes the recognition site for restriction enzyme. It can either create a new site or delete the normal recognition site. The RFLP thus obtained is detected by southern blotting using a small sequence of the specific gene as the probe. For example the recognition site for the restriction enzyme MstII is "CCTNAGG" in the normal beta globin gene of haemoglobin. In case of sickle cell anaemia the mutation of "GAG" to GTG" deletes this recognition site. DNA of the fetal cells is digested with MstII and electrophoresed on gel and probed with a sequence of the beta globin gene. Parallely normal beta globin gene DNA is also subjected to the same process. If the test sample shows bands comparable to normal DNA it means the test individual has a normal beta globin gene. In case of a sickle cell mutant beta globin gene the pattern of the bands will differ from the normal gene in a detectable way. RFLP can be used only in genetic disorders where the mutation leads to a change in restriction sites and thus is not applicable in detection of all disorders. RFLP is used to detect mutations occurring in restriction sites (Mahdieh & Rabbani, 2013)

Oligonucleotide Probes

This is a general approach of detection. This method uses radiolabelled oligonucleotide probes. A set of two probes is used. One probe is complementary to the normal gene and the other has complimentary sequence to the mutant gene (Pastinen et al., 2016). These probes can differentiate the normal and the defective genes using the southern blot technique. Sickle cell anaemia has been detected using these probes. A set of two 19 base long probes have been employed to detects sickle cell anaemia. One of the probe carries complimentary nucleotides to that of a normal beta globin(beta-a) gene while the other has complimentary nucleotides to a mutant beta globin gene(beta-s).The southern blots of normal individuals will hybridize only with beta-a while those of sickle cell homozygote hybridize with beta-s only, and those with heterozygote sickle cell hybridize with both. Another application of this method is the detection of alpha-antitrypsin gene implicated in pulmonary emphysema. However, oligonucleotide polymers can be used as detection tool only when the genetic sequence of both the normal and the mutant gene is known (Pastinen et al., 2016).

DNA Microarray

DNA “chips” or microarrays have been used as a possible testing for multiple mutations. In this technology, single DNA strands including sequences of different targets are fixed to a solid support in an array format. On the other hand, the sample DNA or cDNA labeled with fluorescent dyes is hybridized to the chip. Then using a laser system, the presence of fluorescence is checked; the sequences and their quantities in the sample are determined(Mahdieh & Rabbani, 2013). Gene specific arrays will also be of value for detecting single nucleotide polymorphisms (SNP) (Ohbayashi et al., 2005) that induce specific diseases such as factor V Leiden in deep vein thrombosis, apolipoprotein E4 in Alzheimer's disease, and the recently identified susceptibility gene for Crohn's disease, NOD2. Microarray based expression profiles combined with linkage analysis led to identification of new disease genes (Hoopes, 2008; Cojocaru1, 2001).

Single Strand Conformational Polymorphism (SSCP)

SSCP is one of the simplest screening techniques for detecting unknown mutations (microlesions) such as unknown single-base substitutions, small deletions, small insertions, or microinversions. A DNA variation causes alterations in the conformation of denatured DNA fragments during migration within gel electrophoresis. The logic is comparison of the altered migration of denatured wild-type and mutant fragments during gel electrophoresis. In this technique, briefly, DNA fragments are denatured, and renatured under special conditions preventing the formation of double-stranded DNA and allowing conformational structures to form in single-stranded fragments . The conformation is unique and resulted from the primary nucleotide sequence. Mobility of these fragments is differed through nondenaturing polyacrylamide gels; detection of variations is based on these conformational structures. PCR is used to amplify the fragments, called PCR-SSCP, because the optimal fragment size can be 150 to 200 bp. About 80–90% of potential point mutations are detected by SSCP (Nollau & Wagener, 1997).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE has been used for screening of unknown point mutations. It is based on differences in the melting behavior of small DNA fragments (200-700 bp); even a single base substitution can cause such a difference(Mccammon et al., 2005). In this technique, DNA is first extracted and subjected to denaturing gradient gel electrophoresis. As the denaturing condition increases, the fragment completely melts to single strands. The rate of mobility in acrylamide gels depends on the physical shape of the fragment. Detection of mutated fragments would be possible by comparing the melting behavior of DNA fragments on denaturing gradient gels. Approximately less than 100% of point mutations can be detected using DGGE. Maximum of a nearly 1000 bp fragment can be investigated by this technique(Mahdieh & Rabbani, 2013).

Heteroduplex Analysis

A mixture of wild-type and mutant DNA molecules is denatured and renatured to produce heteroduplices. Homoduplices and heteroduplices show different electrophoretic mobilities through nondenaturing polyacrylamide gels. In this technique, fragment size ranges between 200 and 600 bp is required. Nearly 80% of point mutations have been estimated to be detected by heteroduplex analysis(Mahdieh & Rabbani, 2013).

Next Generation DNA Sequencing

In recent years, newer technologies for DNA sequencing in a massive scale have been emerged that are referred to as next-generation sequencing (NGS). High speed and throughput, both qualitative and quantitative sequence data are allowed by means of NGS technologies so that genome sequencing projects can be completed in a few days(Kamps et al., 2017). NGS systems provide several sequencing approaches including whole-genome sequencing (WGS), whole exome sequencing (WES), transcriptome sequencing and methylome. The coding

sequences compromises about 1% (30Mb) of the genome. More than 95% of the exons are covered by WES; on the other hand, 85% of disease-causing mutations in Mendelian disorders are located in coding regions. Sequencing of the complete coding regions (exome) therefore, could potentially uncover the mutations causing rare, mostly monogenic, genetic disorders as well as predisposing variants in common diseases and cancer (Kamps et al., 2017; Mahdieh & Rabbani, 2013).

Application of Biotechnology in Gene Therapy of Genetic Diseases

Gene therapy is altering DNA within cells in an organism to treat or cure a disease. Gene therapy is a very different type of genetic biotechnology application which involves the manipulation of genetic material, and the genetic manipulation of organisms, in the attempt to cure specific diseases. Gene therapy involves using normal, unmutated genes to replace copies of defective disease-causing genes, or in some cases to initiate or booster an immune response to a specific disease (such as cancer) using viral vectors such as retroviral, lentiviral, and adeno-associated virus (AAV) or nonviral vectors such as liposomes (Friedmann and Schneider, 2006). It is one of the most promising areas of biotechnology research. Gene therapy is a technique for correcting defective genes responsible for disease development in which one of these approaches can be used.

1. A normal gene may be inserted into nonspecific location within the genome to replace a nonfunctional gene.
2. An abnormal gene could be swapped for a normal gene through homologous recombination.
3. The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.

Most of the human genetic diseases are caused by single-gene recessive mutation. Though most of these diseases are manageable, there is no cure for them except for gene therapy which is fast emerging.

New genetic therapies are being developed not only to treat monogenetic diseases but also non-monogenetic diseases that appear later in life such as cancers (Benjamin et al., 2015; Keener, Kevin & Balasubramanian, 2016; Yang, 2015)

The Classical Gene Therapy

Knowledge of the genetic basis of disease has improved our understanding of disease mechanisms and pointed toward potential therapeutic strategies. Emerging therapeutic strategies that can modify nucleic acids within disease affected cells and tissues have potential for the treatment of rare monogenic, highly penetrant diseases, such as severe combined immunodeficiency (SCID), hemophilia and nonmonogenic disorders as safe and effective alternative treatments (Yang, 2015).

Two of the most powerful genetic therapeutic technologies developed thus far are gene therapy, which enables restoration of missing gene function by viral transgene expression, and RNA interference (RNAi), which mediates repression of defective genes by knockdown of the target mRNA (Benjamin et al., 2015; Cooper et al., 2009; Doudna & Gersbach, 2015). **F**or gene therapy of inherited diseases, integrating vectors, such as retroviral, lentiviral, and adeno-associated virus (AAV) vectors, have been used to introduce a therapeutic gene into random sites on target cell chromosomes (Benjamin *et.al*., 2015). The classical gene therapy has been used to successfully treat monogenic recessive disorders affecting the hematopoietic system, such as SCID, by semi-randomly integrating functional genes into the genome of hematopoietic stem/progenitor cells. However, random integration has potential problems, such as insertional mutagenesis and possible effects on gene expression (Flynn et al., 2015; Ohbayashi et al., 2005; Schneider, 2006). RNAi has been used to repress the function of genes implicated in cancer, age-related macular degeneration and transthyretin (TTR)-related amyloidosis (Mann and Mattiske, 2012). Despite promise and recent success in gene therapy RNAi have limitations that preclude their utility for a large number of diseases. Meanwhile, the use of RNAi is limited to targets for which gene knock down is beneficial. Also, RNAi often cannot fully repress gene expression and is therefore unlikely to provide a benefit for diseases in which complete ablation of gene functions is necessary for therapy. RNAi may also have poor specificity, posing potential safety concerns and sometimes decreasing the effectiveness of treatment (Benjamin et al., 2015).

The main difficulty in the early attempts at genome editing was the poor targeting efficiency of the techniques, which was highly dependent on the cell line and the specific locus to be targeted(Lockye, 2016). The attempts to improve targeting efficiencies were focused on the development of negative selectable markers and the identification of proteins of the endogenous homologous recombination machinery. However, a significant breakthrough was achieved when researchers found that a dramatic increase in targeting efficiency could be obtained when double strand breaks (DSBs) adjacent to the integration sites were generated (Benjamin,2015, Flynn et al., 2015).

Homologous recombination (HR) is an ideal strategy for gene therapy of inherited diseases because it has the potential to repair mutant genes. It also can be applied to introduce a gene into a safe and transcriptionally active chromosomal site to obtain a predictable level of expression. For instance , HR between electroporated exogenous DNA and chromosomal loci in mammalian cells has been extensively applied in mouse ES cells to

make gene knockout mice (Ohbayashi et al., 2005).

Gene Therapy Based On the New Classes of Genome Editing Tools

The possibility of editing the genome of cells in a targeted manner has its basis in the experimental observation that DNA constructs harboring stretches of homology are able to interact and eventually integrate at the target site in the genome, assisted by the endogenous homologous recombination machinery of the cells (Vassena et al., 2016).The new classes of genome editing tools that can be used for gene therapy include: engineered zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS) and clustered regularly interspaced short palindromic repeats (CRISPR cas9) (Lockye, 2016). These are also called sequence-specific nucleases (SSNs). The SSNS have been developed to enable site-specific genome editing (Benjamin et al., 2015; Vassena et al., 2016). These tools enable precise genome editing by introducing DNA double-strand breaks (DSBs) at specific genomic loci. DSBs recruit endogenous repair machinery of the cell for either non-homologous end-joining (NHEJ) in the absence of a homologous DNA template, which is an error-prone process that causes small insertions or deletions or homology-directed repair (HDR), in the presence of a synthetic repair template which enables the introduction of any desired base pair changes, which can be exploited with the use of an exogenously introduced double-strand or single-strand DNA repair template to knock in or correct a mutation in the genome (Daneshvar, 2015; Natasa and Savic, 2016; Weterings and Chen, 2008). SSNs opened the possibility of achieving genome edition in more therapeutic contexts, such as in vivo genome editing of hepatocytes in living animals or muscle of dystrophin knockout mice (Benjamin et al., 2015; Vassena et al., 2016). These nuclease systems can also be broadly classified into two categories based on their mode of DNA recognition: ZFNs and TALENs achieve specific DNA binding via protein DNA interactions, whereas Cas9 is targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions (Natasa and Savic, 2016).

"CRISPR" derives from the "clustered regularly interspersed short palindromic repeats" in the bacterial genome where the gene editing machinery was found, and Cas9 is an endonuclease protein that breaks up and thus deactivates the DNA of invading viruses. CRISPR/Cas9 endonuclease belongs to a prokaryotic adaptive immune system. The adoption of CRISPR gene editing for use in research, and for developing novel medicines, has been remarkably rapid, in part because it is so easy to apply to higher organisms including, in principle, humans (Lundberg & Novak, 2015).

The recent discovery of the CRISPR/Cas9 complex from the type II adaptive immune system of *Streptococcus pyogenes* has provided researchers an invaluable tool to target and modify any genomic sequence with high levels of efficacy and specificity. From the three types of CRISPRS, CRISPR/Cas9 has been engineered as a simple tool for genome editing (Oostrom et al., 2013; Vassena et al., 2016, Yena et al.,2014). This engineered system consists of two parts: a single-guide RNA (sgRNA) and the Cas9 nuclease. The sgRNA is a chimeric molecule, containing the CRISPR RNA (crRNA) and the transacting RNA (tracrRNA). A 20-nucleotide region within the crRNA is designed to be complementary to the desired target DNA sequence containing a protospacer adaptor motif (PAM) of sequence "NGG", conferring specificity whereas the tracrRNA is necessary for RNA-Cas9 complex formation. By changing the gRNA sequence, virtually any gene sequence with an adjacent PAM can be targeted by the CRISPR/Cas9 system, enabling the possibility of systematic targeting of sequences on a genomic scale (Wang et al., 2013; Yang, 2015). CRISPR-Cas9 has been shown to be an effective approach for genome editing in human cells. The simplicity of the CRISPR-Cas9 system has enabled widespread applications of this system for efficient genome engineering in various species (Yang, 2015).

The simplicity of the sgRNA in the CRISPR-Cas9 system makes this approach very attractive as a genome editing tool compared to ZFNS and TALENs (Natasa, Savic, 2016). For instance, superior performance of CRISPRs than TALENS was observed in human pluripotent stem cell genome editing (Dinget.al., 2013). Moreover, the Cas9 protein is invariant and can be easily re-targeted to new DNA sequences by changing a small portion of the sequence of the sgRNA. Another potential advantage of Cas9 is its ability to introduce multiple DSBs in the same cell via expression of distinct guide RNAs (Wang *et.al.*,2016). Thus this system has been rapidly adopted for use to modulate disease-causing alleles in vivo in various model organisms and ex vivo in somatic and induced pluripotent stem cells, raising hope for therapeutic genome editing (Benjamin et al., 2015; Natasa and Savic, 2016; Schwank et al., 2013; Tu *et al.*, 2015).

Application of CRISPR Cas 9 in Gene Therapy

CRISPR-Cas9 is a powerful new technique that allows for precise editing of DNA(Lockye, 2016). One of the most exciting applications of the CRISPR-Cas9 is the possibility of curing genetic diseases. Several studies have raised high hopes for CRISPR/Cas9-mediated gene therapy, which aims to repair disease-causing genes by changing the DNA sequence at the exact location on the chromosome(Fig.1) (Lundberg and Novak, 2015; Natasa and Savic, 2016).

CRISPR Case 9 genome editing technology based gene therapy of genetic disorders can be achieved

through a number of approaches including correction or inactivation of deleterious mutations, introduction of protective mutations, and addition of therapeutic transgenes (Benjamin et al., 2015; Flynn et al., 2015; Natasa and Savic, 2016).

Pathogenic mutations that cause genetic disorders in man can be broadly classified as causing either gain or loss of function in a gene product (Lockye, 2016). A gain-of-function mutation, such as those found in the *HTT* gene in Huntington disease and in *FGFR3* in achondroplasia, results in the expression of a pathogenic gene product and may be treated by using NHEJ-mediated mutations to specifically inactivate the mutant allele while leaving the wild-type allele intact on the homologous chromosome (Pankowicz, *et al*., 2017). Additionally, it may be possible to treat nucleotide expansion disorders, such as spinocerebellar ataxia, Huntington disease and Friedriech ataxia, by NHEJ-based deletion of the pathogenic insertion via the creation of two DSBs on both sides of the expansion(Benjamin et al., 2015).

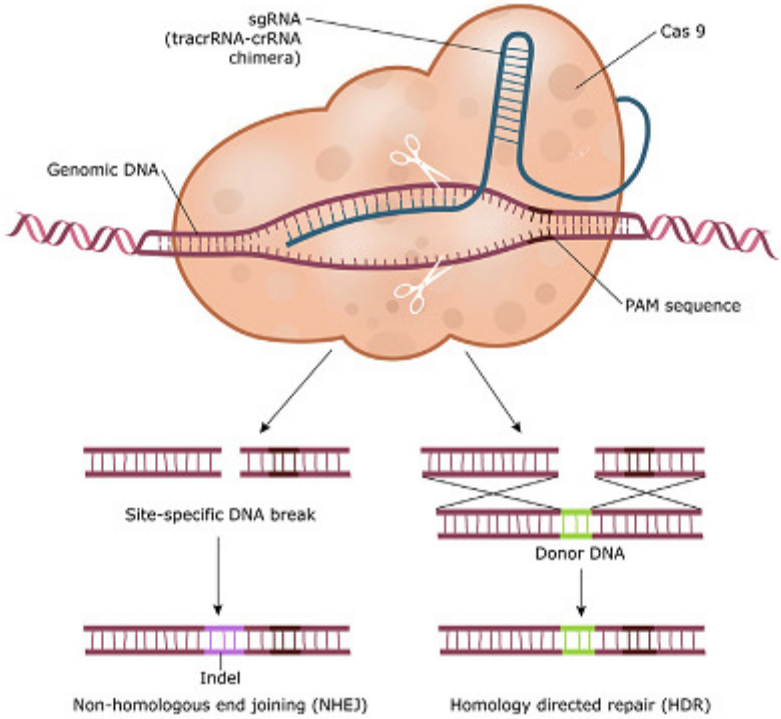

Figure 1 CRISPR/Cas9-mediated genome editing (Savic and Schwank 2016)

A combination of DSBs may also be used to edit multiple loci to achieve a therapeutic effect (Wang *et.al*.,2016). However, some gain-of-function mutations, such as the superoxide dismutase 1 (*SOD1)* G93A mutation found in some individuals with amyotrophic lateral sclerosis (ALS) are point mutations, which may not be sufficiently different from the functioning allele on the homologous chromosome to be distinguished by the current generation of programmable nucleases, potentially leading to an undesirable complete loss of protein function if the mutation is targeted using NHEJ. In such cases HDR could instead be used to change the gain-of-function allele to the wild-type sequence, restoring gene function and eliminating pathogenic activity while preserving physiological levels of gene expression. Similarly, loss-of-function mutations, such as those found in Tay-Sachs disease, would necessitate precise sequence changes to eliminate pathogenicity, requiring HDR gene correction to revert the loss-of-function mutation to the wild-type sequence (Ran et al., 2013). For deleterious loss-of-function mutations and protective gain-of-function mutations, a therapeutic effect may also be achieved by introducing a copy of the wild-type gene or gain-of-function mutant, respectively (Pankowicz, *et al*., 2017). The therapeutic transgene may be inserted into a new locus, including identified ‘safe harbor’ loci regions of the genome whose disruption does not lead to discernible phenotypic effects to restore missing gene function. Gene insertion may also be used to stably confer on cells novel functions that protect against disease, as with the insertion of chimeric antigen receptors (CAR) into T cells to target certain leukemia (Benjamin et al., 2015).

Currently, there are two approaches*: Ex vivo* versus *in vivo* editing therapy (Fig. 2). In *ex vivo* editing therapy, cells are removed from a patient being treated, edited and then reintroduced. *In vivo* therapy involves genome editing of cells *in situ* (Benjamin et al., 2015).

Ex vivo approaches that aim to modify somatic stem or progenitor cells in culture with subsequent transplantation back into the patient is a promising strategy in the future gene therapy in human. Schwank et al. (2013) have isolated and expanded adult intestinal stem cells from two CF patients and corrected the mutant

F508 del allele using the CRISPR/Cas9 mediated homologous recombination, which provides a potential strategy for future adult stem cell gene therapy in patients. The Study of Flynn et al. (2015) showed gene correction results in restoration of oxidative burst function in induced pluripotent stem (iPS) derived phagocytes from chronic granulomatous disease (CGD) patients by reintroduction of a previously skipped exon in the cytochrome b-245 heavy chain (CYBB) protein by using CRISPR-Cas9. This study provides proof-of-principle for a gene therapy approach to chronic granulomatous disease (CGD) treatment using CRISPR-Cas9.

Another proof-of-concept studies for ex vivo CRISPR/Cas9-based gene therapy was done in iPSCs and tackled the genetic blood disorder b-thalassemia, which is caused by mutations in the hemoglobin beta gene that reduce the production of hemoglobin. Currently, the only cure for b-thalassemia is by transplantation of healthy, donor-derived histocompatible hematopoietic stem cells. Corrected patient-derived iPSCs, however, might provide an alternative source for generating transplantable hematopoetic stem cells. IPSCs were established from fibroblasts of a patient homozygous for b-thalassemia and transfected them with CRISPR/ Cas9-based vectors targeted to the disease-causing allele together with a DNA template for HDR (Xie *et al*.,2014). Homologous recombination events were identified by selecting for a cointegrated antibiotic resistance cassette, which was later excised through transposase mediated recombination. Corrected iPSCs were then differentiated into fully functional red blood cell precursors, which in the future could potentially be used for transplantations (Natasa and Savic, 2016).

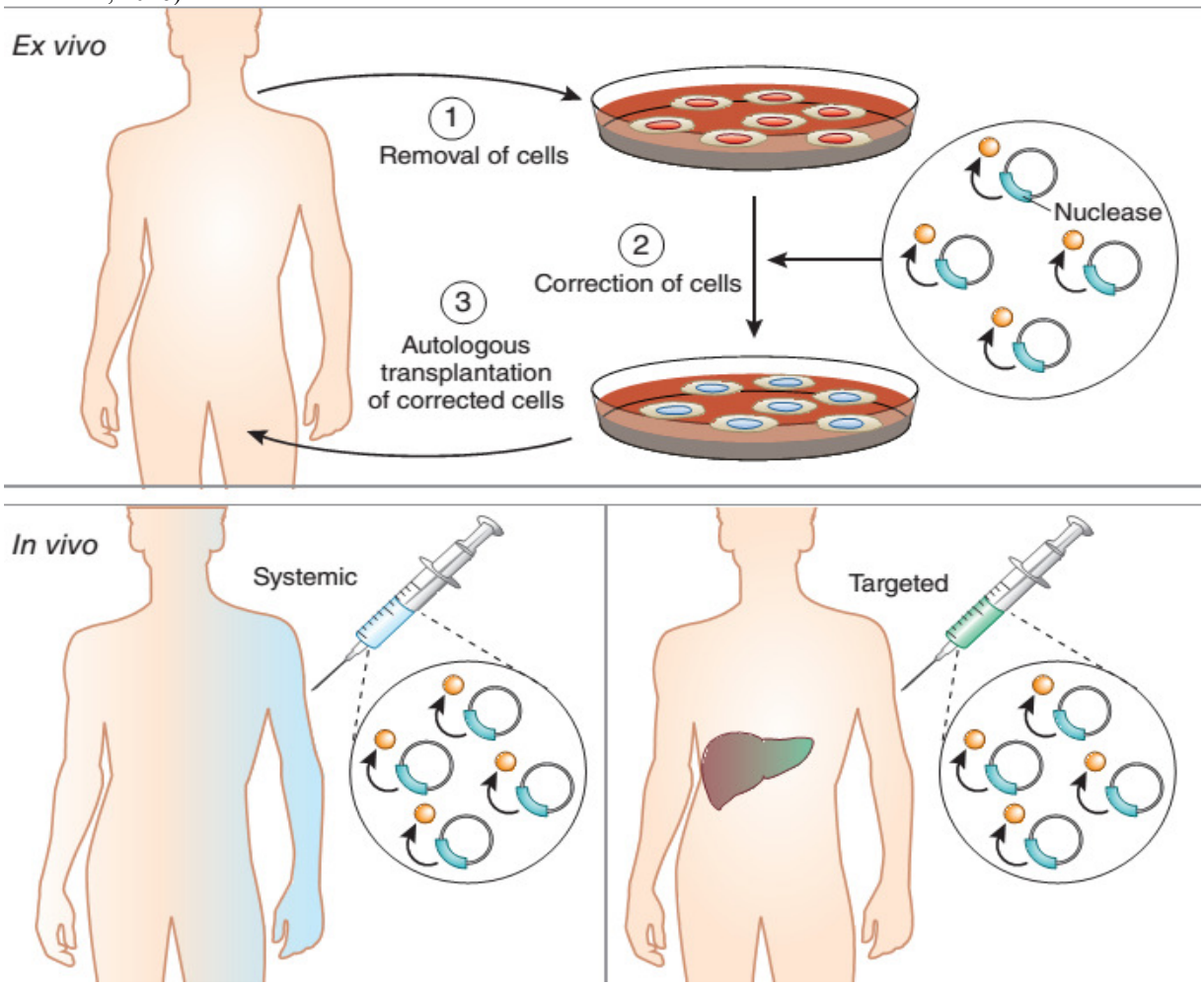

Figure 2.Ex vivo versus in vivo editing therapy (**Benjamin *et al.*, 2015)**

Two independent ex vivo studies used CRISPR/ Cas9 to correct alleles causing Duchenne muscular dystrophy (DMD) in patient–derived iPSCs and immortalized cell lines. In the first study, CRISPR/Cas9 was used in combination with a donor template to restore the full-length dystrophin gene by homologous recombination in iPSCs derived from DMD patients that lacked exon 44. Repaired iPSCs were selected and differentiated into skeletal muscle cells, in which the wild-type dystrophin protein was expressed. In the second study single and multiple exon deletions were generated to restore the reading frame of the dystrophin gene in immortalized myoblasts derived from DMD patients. Importantly, multiplex gene editing allowed to create a large deletion that excises exons 45–55, a mutational ‘‘hot spot,’’ harboring more than 60% of disease-causing DMD mutations (Natasa and Savic, 2016).

One of the first studies that successfully corrected a genetic disease in postnatal animals by in vivo CRISPR/Cas9 mediated genome editing was conducted by Yin et al., (2015) in a mouse model for type I tyrosinemia. Hereditary type I tyrosinemia is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase (FAH), leading to cytotoxic metabolite accumulation and cell death of hepatocytes. Through hydrodynamic tail vein injection the authors delivered vectors encoding for Cas9 and the specific sgRNA, along with a DNA oligo for HDR directly into the mouse liver. This resulted in the correction of the mutant fumarylacetoacetate hydrolase allele and stabilization of the protein, leading to reduced hepatocellular toxicity and a rescue in weight loss of mice.

Another in vivo study focused on the disruption of the gene encoding for the proprotein convertase subtilisin/ kexin type 9 (PCSK9) in the mouse liver. PCSK9 is secreted into the plasma by hepatocytes, and limits low-density lipoprotein (LDL) cholesterol uptake and degradation by functioning as an LDL receptor antagonist. Naturally occurring loss-of-function mutations in PCSK9 therefore decrease blood cholesterol levels. To mimic this condition, PCSK9 was disrupted using adenoviral CRISPR/Cas9 vectors in the mouse liver. This led to a decrease in PCSK9 protein levels, an increase in hepatic LDL receptor levels, and subsequently decreased plasma cholesterol levels. Importantly, because this approach is based on the disruption of a gene function by NHEJ, it was possible to reach editing efficiencies of up to 50%, which might already be sufficient for clinical application (Lundberg & Novak, 2015).

Injection of CRISPR/ Cas9 components into the zygote or early stage embryo allows modifying the genome in all cells of the organism, including the germ line. Several studies proved that genetic alteration of germ line by direct manipulation of human embryos is feasible (Natasa and Savic, 2016). Thus, this approach results in permanent changes that can be passed on to subsequent generations, offering the possibility to eliminate a genetic disease from an entire family. Wu et al., (2014) were one of the first to use this approach to repair a disease-causing mutation in the mouse embryo. They focused on a dominant loss-of-function mutation in the Crygc gene, which causes cataract. Injection of Cas9 mRNA and the sgRNA targeting the dominant Crygc allele into the zygote corrected mutation, with the wild-type allele on the homologous chromosome acting as a template. Another study that used CRISPR/Cas9-mediated genome editing in the mouse embryo corrected a mutation in the gene dystrophin, responsible for inherited disease, X-linked Duchenne muscular dystrophy (DMD). In this case, the Cas9 mRNA and specific sgRNA together with a single stranded DNA oligo was injected into the zygote and achieved HDR from the exogenous DNA template. Although only partially corrected mosaic mice were obtained because of the editing occurring after the zygote stage, selective advantage of the corrected skeletal muscle cells still led to a complete rescue of the phenotype (Lundberg and Novak, 2015) .

Direct injection of CRISPR-Cas9 system into zygotes could not produce healthy progeny at an efficiency of 100% and could potentially generate off-target modifications. As a possible alternative to the zygote approach, the CRISPR/Cas9 system could be used on immature oocytes or sperm to generate gene corrected mature sperm or oocytes to overcome genetic conditions in the following generation. In the female germ line, the oocyte is easily accessible for genetic manipulation. Spermatogonial stem cells seem to be a better target for male germ line(Vassena et al., 2016).

Recently, the CRISPR-Cas9 system was successfully applied in spermatogonial stem cells (SSCs) to efficiently correct genetic diseases in mouse through pre-selection of SSC lines carrying the desired genotype without off-target mutations as shown by whole genome sequencing from a father carrying a homozygous genetic defect(Wu et al., 2014). The mouse SSCs based gene therapy using CRISPR-Cas9 would be of great interest to investigate whether similar gene editing strategies could be used for mutation correction in human SSCs in a setting related to paternal genetic diseases in the future through germ line editing. The system of SSC-based gene therapy could also be applied to treat the following genetic conditions: (1) male infertility induced by genetic defects, (2) father-carrying dominant disease alleles, and (3) sex chromosome-linked dominant genetic diseases (Wu et al., 2014).

The other possible approach to prevent a genetic disorder in the progeny is genomic editing to correct the disorder in induced pluripotent stem cells (iPSC) or Somatic Cell Nuclear Transfer-human Embryonic Stem Cells (SCNT-hESC) obtained from a diseased patient. Pluripotent stem cells would need to be differentiated towards oocytes or sperm containing the genetically corrected information. The possibility of creating stem cell-derived gametes was shown in mice and research is nowadays being performed to attain similar progression in human to overcome certain types of infertility (Vassena et al., 2016).

A research group in China recently employed CRISPR-Cas9 technology to make changes in human germ line cells, changes that could in principle both be expressed in a human being and then later be passed on via sperm or egg cells to subsequent generations by using nonviable embryos or tripronuclear zygotes (Liang et.al. 2015). Furthermore, Tang *et.al*., (2017) demonstrated efficient homologous recombination-mediated correction of point mutations in *HBB* and *G6PD* by injection of Cas9 protein complexed with the appropriate sgRNAs and homology donors into normal one-cell human embryos. However, the long term effects of modifying the germ

line with CRISPR-Cas9 mediated gene editing on human generations is unknown(Lundberg and Novak, 2015).Therefore it seems better to use CRISPR-Cas9 gene editing to cure serious diseases, treat the patient but not the germ line. Most genetic diseases will occur, and in principle can be corrected, in specific somatic cells. Given the existence of alternative non-germ line approaches to treat most Mendelian genetic disorders and the uncertainty of the impact of the technology on the germ line, , no modern regulatory system would authorize clinical use of germ line gene editing at the present time (Lundberg and Novak, 2015).

Even if parents and their physicians agree that avoiding heritable genetic disease in the current and future generations is desirable, there are already several ways to achieve this outcome. Couples can undertake genetic testing prior to pregnancy, or employ in vitro fertilization and preimplantation genetic diagnosis to implant only those embryos without risk for a specific disease (Lundberg and Novak, 2015).

However, some have argued for the use of CRISPR/Cas9 in human germ line editing in more exceptional circumstances such as in the cases when one parent is homozygous for a dominant disorder or both members of a couple affected with the same monogenic disease who wish to have a healthy child with their own genetic material. As an example, the life expectancy of CF patients has increased tremendously in the past decades, with intensive and early symptomatic treatment, with possible transplantation of the lungs and heart, and most recently with drugs that open CFTR channels for ion transport that have also been made available. Healthy pregnancies have been described in women with CF, and intracytoplasmic sperm injection (ICSI) with testicular biopsy has long since been introduced and used for males with congenital absence of the vas deferens, many of whom harbor CF mutation. In those cases, it would suffice to correct the affected genes in the germ line of one of the prospective parents, and all children of this couple would be healthy carriers. Another example of possible candidates for germ line editing is constituted by patients who are homozygous for an autosomal dominant disease such as Huntington's disease. In the case of Huntington's disease, patients who carry two affected alleles are more severely affected than those who carry only one allele, but appear to have the same age of onset. Autosomal dominant polycystic kidney disease also displays childhood or earlier onset when one of both alleles is milder, and the second one leads to more severe disease. Homozygotes for two alleles leading to severe disease are probably lost in utero. Many other autosomal dominant diseases, such as achondroplasia and Marfan syndrome, are severe or lethal in the homozygous form. CRISPR/Cas9 technology could also be envisaged as a mean to correct chromosomal aberrations such as Robertsonian translocation that leads to trisomy 21. A possible approach would be to use CRISPR/Cas9 to separate the two chromosomes and to restore both the centromere and the missing p-arms. However, even when taking into consideration the extremely rapid evolution of the CRISPR/Cas9 toolkit, this particular application may be quite far from being applied(Vassena et al., 2016).

Other candidates for correction would be genes related to infertility. Only a handful of genes are known to cause infertility, but the most common and best described genetic causes of infertility are chromosomal in nature. Although modern technology such as whole genome sequencing will help to identify new genes involved in infertility, the main hurdle here is the identification of patients. Even if genes or other genetic variants causing infertility are identified, there is limited choice of germ cells to manipulate. Finally, it could be envisaged to correct mutations in mitochondrial DNA (mtDNA) that are present in the oocyte. Mitochondrial diseases are caused by a mutation in a proportion of the mtDNA molecules present in the patient's cells, a phenomenon called heteroplasmy. Mutations in the mtDNA are transmitted exclusively via the oocyte, which can carry between 10, 000 and 100, 000 mtDNA copies (Tuppen et al., 2010; Vassena et al., 2016). Mutated mtDNA molecules could be eliminated from the oocyte or the zygote using CRISPR/Cas9 (Wang et al., 2015).

Challenges and Prospects of CISPR Cas 9 Technology in Gene Therapy

Most genetic diseases in humans are caused by point mutations, single base errors in the DNA sequence. However, current genome-editing methods cannot efficiently correct these mutations in cells, and often cause random nucleotide insertions or deletions (indels) as a byproduct making it impractical for therapeutic correction of point mutations in man(Komor *et al*., 2016). A matter of concern when working with SSNs are so called off-targets effects or unspecific activity towards other locations in the genome that share sequence homology with the target(Kueh and Herold1, 2016; Plosky, 2016).

Bioinformatics resources can help decreasing the likelihood of selecting a highly repetitive sequence and thus the chance of unwanted off-target activity. Importantly, CRISPR/Cas9 modified versions exist that keep similar target efficiencies as the wild type CRISPR/Cas9 while reducing off-target activity. In the context of germ line manipulation, it is expected that nickases or CRISPR/Cas9 protein modified versions, Cas9 nickase, although somewhat less active than wild type CRISPR/Cas9, would be preferred in order to minimize undesired off-target effects(Kueh and Herold1, 2016). According to Ran *et al*. (2013), double nicking by rna-guided crispr cas9 for enhanced genome editing specificity. Donor templates used in combination with SSNs require much shorter stretches of homology (1–2 kb) compared with classic targeting construct configurations (5–8 kb), and should be co-delivered with Case 9 /sgRNA (Kueh and Herold1, 2016). Also, correction can be pursued through homology dependent repair using exogenously supplied oligonucleotides, avoiding the need to clone

donor plasmids. Thus, SSNs facilitate the generation of targeting constructs to the point that single stranded oligonucleotides can be used to repair mutations(Kueh and Herold1, 2016).

Two studies addressing a potential alternative to Cas9: the Cpf1 enzyme (Fonfara et al., 2016). CRISPR/Cpf1 creates "sticky ends" overhangs in cleaved DNA that leave unpaired bases either side of the break rather than the blunt ends made by Cas9's double-strand DNA cleavage. Sticky ends are more efficient for homologous repair than blunt ends for DNA repair in cells (Watkins-Chow *et. al.* 2017).

In addition to these , researchers have modified CRISPR/Cas9 technology to get around these problems, creating a new "base editor," which permanently and efficiently converts cytosine (C) to uracil (U) bases with low error in human and mouse cell lines by tethering inactivated Cas9 to the rat cytidine deaminase enzyme APOBEC1 that directly catalyzes conversion of C to U (essentially an equivalent of thymine, T), without DNA cleavage**.** This creates mismatch pair at the target that trigger cellular repair mechanism to remove the mismatch base, "G" and replace with the complement to the remaining one. Thus mismatch repair produces the desired the G to an A conversion (Fig.3) (Komor et al., 2016).

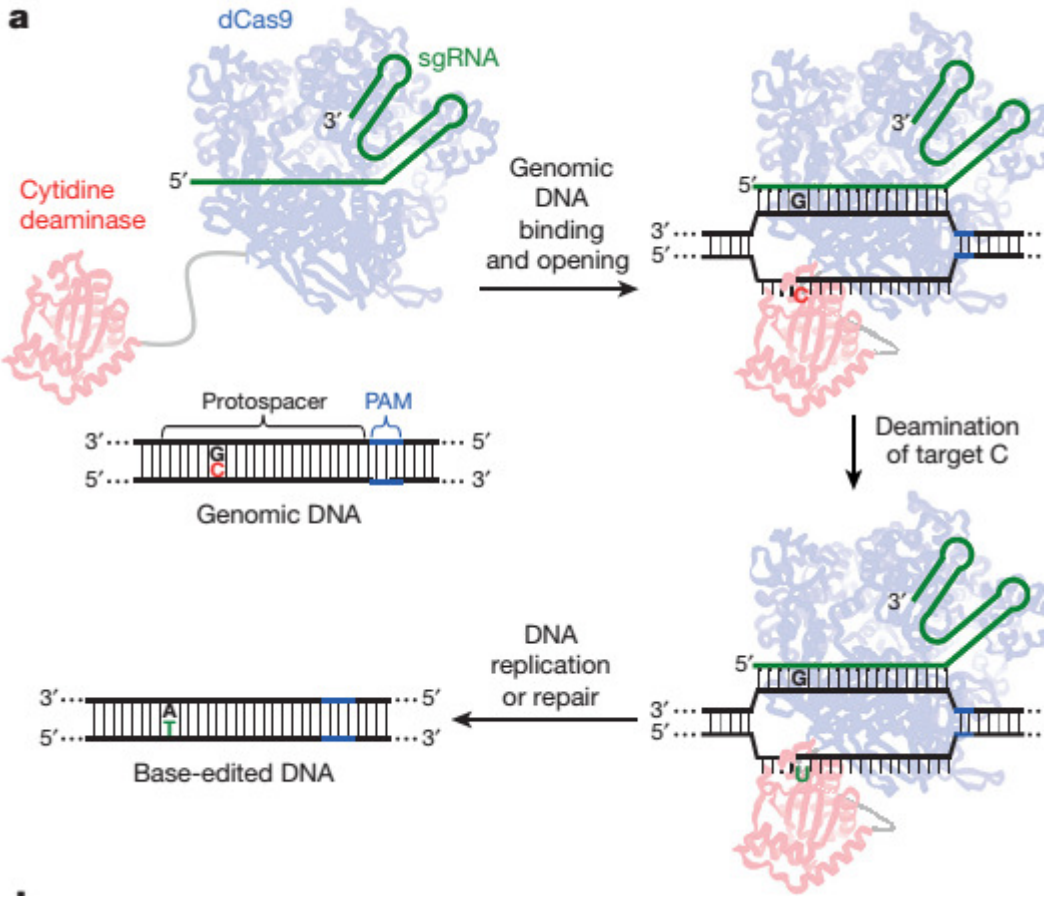

Fig.3 First generation base editor (BE1) mediates specific, guide RNA-programmed C →U conversion *in vitro*

Conclusions

The human genome project revealed that there are 25,000 to 30,000 genes in man that govern all life processes. These genes are not always stable, but, undergo changes. These changes (mutations) have resulted in thousands of human genetic disorders identified by different methods. In the past, there were methods to correct these genetic disorders. However, they involve inactivation or replacement of defective gene by random integration which is prone to other effects. The programmable nucleases with sequence specific nuclease activity (ZFNs, TALENs and CRISPR-Cas9 are now in use for therapeutic gene editing of different animals. Among these, the CRISPR-Cas9 technology, an efficient, inexpensive, fast-to-design, and easy-to-use genomic editing tool, has been rapidly applied in many fields, ranging from basic biology to translational medicine. It has been in genome editing mediated gene therapy in different species of animals. More recently, modified versions of CRISPR-Cas9 that do not need DSB or template has been developed. Therefore, there is a hope to use this technology with some improvement to treat human patients suffering from hereditary diseases at clinical level in the future(Kueh and Herold1, 2016).

Competing Interests
The authors declare that they have no competing interests.

Acknowledgments
The authors are thankful to library and ICT office of Addis Ababa University for providing the facilities to prepare this review paper.

Reference
Benjamin, D., Cox, T., Platt, R. J., & Zhang, F. (2015). Therapeutic genome editing : prospects and challenges. *Nature Medicine*, *21*(2), 121–131. http://doi.org/10.1038/nm.3793
Cooper, T. A., Wan, L., & Dreyfuss, G. (2009). Review RNA and Disease. *Cell*, *136*, 777–793. http://doi.org/10.1016/j.cell.2009.02.011
Daneshvar, K. (2015). CRISPR-Cas9 in Gene Therapy : Much Control On Breaking , Little Control On Repairing. *Peer J Preprint*, *4*, 10–13.
Dayal, M. B. (2015). Preimplantation genetic diagnosis. *The Lancet*, *363*(9421), 1633–1641. http://doi.org/10.1016/S0140-6736(04)16209-0
Dinget.al. (2013). Letter Enhanced Efficiency of Human Pluripotent Stem Cell Genome Editing through Replacing TALENs with CRISPRs. *Cell*, *12*, 393–394. http://doi.org/10.1016/j.stem.2013.03.006
Doudna, J. A., & Gersbach, C. A. (2015). Genome editing : the end of the beginning. *Genome Biology*, *16*(292), 15–17. http://doi.org/10.1186/s13059-015-0860-5
Flynn, R., Grundmann, A., Renz, P., Walther, H., James, W. S., Cowley, S. A., & Moore, M. D. (2015). CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. *Experimental Hematology*, *43*, 838–848. http://doi.org/10.1016/j.exphem.2015.06.002
Friedmann and Schneider. (2006). The Scientific Basis for Gene Therapy : A New Concept in Medicine. *Advances in Genetics*, *51*(6), 1–10. http://doi.org/10.1016/S0065-2660(06)51002-
Gady S. Cojocaru1, G. R. and N. K. (2001). Molecular Biology in Medicine The Use of Microarrays in Medicine. *IMAJ*, *3*, 292–296.
Genetics Home Reference. (2016). Genetic Testing. Retrieved from http://ghr.nlm.nih.gov/handbook/testing?show=all
Greber, B., Tandara, H., Lehrach, H., & Himmelbauer, H. (2005). Comparison of PCR-Based Mutation Detection Methods and Application for Identification of Mouse Sult1a1 Mutant Embryonic Stem Cell Clones Using Pooled Templates. *Human Genetic Mutation*, *490*, 483–490. http://doi.org/10.1002/humu.20168
Hao, W., Fan, L., Chen, Q., Chen, X., & Zhang, S. (2015). Modified Proofreading PCR for Detection of Point Mutations , Insertions and Deletions Using a ddNTP-Blocked Primer. *PLoS ONE*, *10*(4), 1–17. http://doi.org/10.1371/journal.pone.0123468
Hoopes, L. (2008). Genetic Diagnosis : DNA Microarrays and Cancer. *Nature*, *1*(1), 1–3.
Kamps, R., Brandão, R. D., Bosch, B. J. Van Den, Paulussen, A. D. C., Xanthoulea, S., Blok, M. J., & Romano, A. (2017). Next-Generation Sequencing in Oncology : Genetic Diagnosis , Risk Prediction and Cancer Classification. *International Journal of Molecular Sciences*, *18*(308). http://doi.org/10.3390/ijms18020308
Keener, Kevin, T. H., & Balasubramanian, R. (2016). *BIOTECHNOLOGY and its APPLICATIONS*.
Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420–424. http://doi.org/10.1038/nature17946
Kueh, AJ, and Herold, MJ W. and E. (2016). The new editor — targeted genome engineering in the absence of homology-directed repair. *Nature*, *2*(42), 16042–16O43. http://doi.org/10.1038/cddiscovery.2016.42
Liang, P., Xu, Y., Zhang, X. et al. (2015). CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes, Protein & Cell, 6 (5), 363–372.
Lockye, E. J. (2016). The potential of CRISPR-Cas9 for treating genetic disorders *Bioscience*.**9**,1-10
Lundberg, A. S., & Novak. (2015). CRISPR-Cas Gene Editing to Cure Serious Diseases : Treat the Patient , Not the Germ Line CRISPR-Cas Gene Editing to Cure Serious Diseases : Treat the Patient , Not the Germ Line. *The American Journal of Bioethics*, *15*(12), 18–21. http://doi.org/10.1080/15265161.2015.1103817
Mahdieh, N., & Rabbani, B. (2013). An Overview of Mutation Detection Methods in Genetic Disorders. *Iran J Pediatr*, *23*(4), 375–388.
Mann, J. R., & Mattiske, D. M. (2012). REVIEW / SYNTHÈSE RNA interference in mammalian DNA methylation 1. *Biochem. Cell Biol.*, *90*, 70–77. http://doi.org/10.1139/O11-050
Mccammon, M. T., Gillette, J. S., Thomas, D. P., Ramaswamy, S. V, Rosas, I. I., Graviss, E. A., … Quitugua, T. N. (2005). Detection by Denaturing Gradient Gel Electrophoresis of pncA Mutations Associated with Pyrazinamide Resistance in Mycobacterium tuberculosis Isolates from the United States-Mexico Border

Region. *American Society for Microbiology*, *49*(6), 2210–2217. http://doi.org/10.1128/AAC.49.6.2210

Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C, Huang J (2015) CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell* 6:363–372

Natasa Savic, and G. S. (2016). Advances in therapeutic CRISPR/Cas9 genome editing. *Translational Research*, *168*, 15–21. http://doi.org/10.1016/j.trsl.2015.09.008

Nollau, P., & Wagener, C. (1997). Methods for detection of point mutations : performance and quality assessment. *Clinical Chemistry*, *1128*, 1114–1128.

Ohbayashi, F., Balamotis, M. A., Kishimoto, A., Aizawa, E., Diaz, A., Hasty, P., … Mitani, K. (2005). Correction of chromosomal mutation and random integration in embryonic stem cells with helper-dependent adenoviral vectors. *PNAS*, *102*(38), 13628–13633.

Pankowicz FP, Jarrett KE, Lagor WR, et al. (2017).CRISPR/Cas9: at the cutting edge of hepatology.*Gut,***66**,1329-1340

Pastinen, T., Kurg, A., Metspalu, A., Peltonen, L., & Syva, A. (2016). Minisequencing : A Specific Tool for DNA Analysis and Diagnostics on Oligonucleotide Arrays. *Genome Research*, *7*, 606–614.

Plosky, B. S. (2016). Technology Preview CRISPR-Mediated Base Editing. *Molecular Cell*, *62*(4), 477–478. http://doi.org/10.1016/j.molcel.2016.05.006

PRAJNYA, R, C REHDER, S. P. A. D B(2011) Prenatal Diagnosis of Pompe Disease – Enzyme Assay or Molecular Testing? *Indian Pediatrics.* **48,**901-902

Ran,FA,Patrick D. Hsu, Chie-Yu Lin, et.al.,(2013). Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. *Cell* ,**154**, 1380–1389.

Schneider, F. and. (2006). Early Gene Transfer Experiments : Problems and Eventual Success. *Advances in Genetics*, *51*(6). http://doi.org/10.1016/S0065-2660(06)51003-X

Schwank, G., Koo, B., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., … Clevers, H. (2013). Brief Report Functional Repair of CFTR by CRISPR / Cas9 in Intestinal Stem Cell Organoids of Cystic Fibrosis Patients. *Stem Cell*, *13*(6), 653–658. http://doi.org/10.1016/j.stem.2013.11.002

Shuo-Ting Yena, Min Zhang, Jian Min Deng, Shireen J. Usman, Chad N. Smithd, J., & Parker-Thornburg, Paul G. Swintone, James F. Martin, and R. R. B. (2014). Somatic mosaicism and allele complexity induced by CRISPR/ Cas9 RNA injections in mouse zygotes. *DEV Bio*, *393*(1), 3–9. http://doi.org/10.1016/j.ydbio.2014.06.017.Somatic

Singh, L. C. Y., Arora, C. D., Kochar, B. S. P. S., Koopmans, C. M., Bijlenga, D., & Groen, H. Prenatal Diagnosis of Gaucher Disease (2010).

Tang, L, Zeng,Y, Du,H, Gong,M, Peng, J, Zhang, B, Lei, M Zhao, F, Wang,W, Li, X, Liu, J, (2017)CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein Mol Genet Genomics DOI 10.1007/s00438-017-1299-z

Tu, Z., Yang, W., Yan, S., Guo, X., & Li, X. (2015). CRISPR / Cas9 : a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. *Molecular Neurodegeneration*, *1o*(35), 1–8. http://doi.org/10.1186/s13024-015-0031-x

Tuppen, H. A. L., Blakely, E. L., Turnbull, D. M., & Taylor, R. W. (2010). Biochimica et Biophysica Acta Mitochondrial DNA mutations and human disease. *Biochimica et Biophysica Acta*, *1797*(2), 113–128. http://doi.org/10.1016/j.bbabio.2009.09.005

Uppanqala, N. (2010). Types of Genetic Disorders. *Genetics*, *20*, 2.

Vassena, R., Heindryckx, B., Peco, R., Pennings, G., Raya, A., Sermon, K., & Veiga, A. (2016). Genome engineering through CRISPR / Cas9 technology in the human germline and pluripotent stem cells. *Human Reproduction Update*, *0*(0), 1–9. http://doi.org/10.1093/humupd/dmw005

Wang,H. Russa,M.La,and Qi,L.S, (2016)CRISPR/Cas9 in Genome Editing and Beyond .*Annual Review of Biochemistry*.**85**:22.1–22.38

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., & Zhang, F. (2013). Resource One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR / Cas-Mediated Genome Engineering. *Cell*, *153*(4), 910–918. http://doi.org/10.1016/j.cell.2013.04.025

Watkins-Chow,DE, Varshney,GK *et. al.* (2017) highly efficient Cpf1-mediated gene targeting in mice following high concentration pronuclear injection.*Genetics*,**7**,719-723

Weterings, E., & Chen, D. J. (2008). The endless tale of non-homologous end-joining. *Cell Research*, *18*(1), 114–124. http://doi.org/10.1038/cr.2008.3

Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., … Zhu, P. (2014). Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Research*, *25*(1), 67–79. http://doi.org/10.1038/cr.2014.160

Xie F, Ye L, Chang JC, et al.,(2014) Seamless gene correction of b-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Research*;**24**:1526–33

Yang, X. (2015). Applications of CRISPR-Cas9 mediated genome engineering. *Military Medical Research*, *2*(11), 4–9. http://doi.org/10.1186/s40779-015-0038-1

Zahid K, AH, A. Q, Sarwar K, Shakoor S, et al. (2016) Role of Biotechnology in Improving Human Health. J Mol Biomark Diagn 8: 309. doi: 10.4172/2155-9929.1000309