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A new era of gene editing for the treatment of human diseases

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Summary

The treatment of human diseases using gene-editing technology has been envisioned for several decades with the realisation that so many were associated with mutations in DNA. The Human Genome Project opened new doors for identifying the genetic bases for human suffering. Research on gene editing has been active since the 1970s, but the technology has seen substantial growth and application just within the past decade. Simply stated, CRISPR technology has become a phenomenon in both biomedical and therapeutics research. Concurrently, cell therapies and pluripotent stem cell research have also been refined and now interfaced with CRISPR technology to enhance and maximise their potential in modelling as well as treatment of human diseases. In this review, we discuss the novel and revolutionary modality of gene editing, as this marks a new era in research and medicine. We also discuss gene-modifying technologies leading to CRISPR, as they are still being used for a wide variety of genomic applications. The modes and challenges for delivery of gene editing components are also discussed. Lastly, we review examples of human diseases that are not only amenable to gene editing techniques, but also show true promise of cure in the early 21st century of genetic correction and gene repair.

Keywords: *gene editing, CRISPR, human genetic diseases*

Introduction

The past decade in medicine has seen significant advances in the field of gene editing, many with applications to biomedical research and therapeutic medicine. These novel techniques have not only provided new tools for genetic surgery, but have also heralded a new era of medicine, with possibilities never imagined even half a century ago. As we continue to study the myriad diseases, it is imperative that we also have tools to model them *in vivo* as well as *ex vivo*. With new advances, the scientific community is working tirelessly to apply these tools in various disease models to find the ultimate answer for the cure of many diseases.

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Genetic basis of human diseases

The human genome consists of ~3 billion base pairs of DNA organised in 22 pairs of autosomal chromosomes and one pair of sex chromosomes [1]. An average chromosome has about 120 million base pairs of DNA; a gene has anywhere from 2000 to 200,000 base pairs. Only about 3% of our genomic DNA sequence encodes for genes, of which ~20,000 are protein encoding. Included in this gene pool are those that are also coding for RNA with various functional properties, such as microRNAs, small interfering RNAs, and circular RNAs, which do not require translation into proteins. The human genome has been sequenced,

ABBREVIATIONS:

AAT	alpha-1 antitrypsin
AAV	adeno-associated virus
CAR-T cells	chimeric antigen receptor-T cells
Cas	CRISPR-associated
cccDNA	covalently closed circular DNA
CRISPR	clustered regularly interspaced short palindromic repeat
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
dCas9	dead Cas9
DSB	double-stranded break
ESC	embryonic stem cell
HDR	homology directed repair
HBV	hepatitis B virus
HIV	human immunodeficiency virus
iPSCs	induced PSCs
LTR	long terminal repeat
NHEJ	nonhomologous end joining
precrRNA	precursor CRISPR RNA
PAM	protospacer adjacent motif
PSCs	pluripotent stem cells
saCas9	Cas9 from *Staphylococcus aureus*
sgRNA	single guide RNA
spCas9	Cas9 from *Streptococcus pyogenes*
TALENs	transcription activator like effector nucleases
tracrRNA	transactivating CRISPR RNA
ZFNs	zinc finger nucleases

and the vast functional nature of the noncoding regions is also being slowly realised. The role of gene mutations in human disease is not a new concept; however, with access to information about the human genome, more diseases are being recognised for their genetic basis. These disorders can arise from mutation (or mutations) in one gene (monogenic disorder) [2]; mutations in multiple genes (polygenic inheritance disorder) [3]; or by damage to the entire chromosome(s) [4]. We now realise that it is not only the gene sequence that causes disease, but also epigenetic changes representing a dynamic process reflecting a complex interaction between an organism and the environment. This can occur via at least three different mechanisms, including DNA methylation, histone modification, and microRNAs [5].

The prevalence of monogenic disease is rare overall; however, millions of people are affected worldwide. Diseases such as cystic fibrosis, sickle cell disease, haemophilia, thalassaemia and Huntington disease cause significant morbidity in millions of patients. Additionally, disorders such as chronic myeloid leukaemia (due to chromosomal translocation), Down syndrome (trisomy of chromosome 21) and Turner syndrome (monosomy of X chromosome) are a few examples of diseases caused by chromosomal abnormalities [4]. We now realise that many cancers, autoimmune diseases and neurodegenerative diseases are caused by epigenetic changes [6, 7]. We also have many challenging infectious diseases that affect us at the genetic level or utilise our own genetic mechanisms to cause these disorders [8]. The wide variety of diseases that are caused by genetic or epigenetic changes, or even infectious process, presents a unique opportunity to treat the disease at its core via gene editing.

History of gene editing

The initial concepts of gene therapy arose during the 1960s and early 1970s when restriction enzymes were used to manipulate DNA [9] and, more specifically, to create specified cuts in DNA. In 1972, Friedmann and Roblin envisioned the potential of gene editing after these initial studies were published [10]. In the following decade, Capecchi and Smithies demonstrated that mammalian cells can incorporate an exogenous copy of DNA into their own genome [11, 12]. This process, called homologous recombination, typically involves the accurate repair of mutated or damaged DNA. This is accomplished by transferring the modified gene of interest into cells so that it is integrated into its homologous location at the target site in the genome. Using homologous recombination alone for genetic modification posed many challenges and limitations included inefficient incorporation of exogenous DNA (10^{-6}–10^{-9}), and random integration in undesired genomic location. For these undesired limitations, a breakthrough came in the late 1980s and early 1990s, when it was demonstrated that efficient and accurate genetic modification at the chromosomal level could be accomplished via the induction of a double-stranded break (DSB) at a specific genomic target [13, 14]. These studies were done in eukaryotic cells, which opened doors to establishing more accurate and targeted mechanisms to create these breaks, which are discussed below.

Gene editing

Efficient and accurate induction of a DSB at a specific target sequence is the foundational concept in modern gene editing techniques (fig. 1). Once a DSB is introduced, there are two known cellular mechanisms for repair: nonhomologous end joining (NHEJ) and homology directed repair (HDR). NHEJ involves directly ligating the two ends of DNA without a template [16, 17]; whereas in HDR, the broken strand relies on a repair in a template directed manner [18]. It has been demonstrated that introduction of a DSB at a targeted site can be utilised to improve efficiency of gene targeting through homologous recombination [17, 18]. Since then, gene-editing technologies have focused on the development and implementation of various endonuclease-based mechanisms to introduce DSBs with high precision. This would then utilise the robust, endogenous cellular mechanism to repair this break in order to achieve desired changes in the DNA. Simply stated, gene editing involves the deliberate and targeted insertion, deletion or replacement of DNA in the genome of a living cell. Potential goals of gene editing include replacement of missing gene(s), overexpression of a normal gene, interference with gene expression, disruption of an offending gene, or repair of mutated gene.

DSB-based genome editing platforms: targeted nucleases

Meganucleases

Meganucleases are endoribonucleases that have a large recognition site for DNA sequences (>14 base pairs). Also known as homing nucleases, these are a group of naturally occurring nucleases, which recognise DNA cleavage sites and are associated with parasitic DNA elements, such as group 1 self-splicing introns or inteins (protein introns). They include enzymes such as I-Sce I and I-Cre I, which can be used to target genes in mammalian cells [19–22]. The advantages of this technology are that meganucleases have high specificity to target DNA due to large recognition sites, are relatively small in size (ease in delivery), and produce a 3′ overhang after DNA cleavage (more recombinogenic for HDR). Although this is a promising technology, it is difficult to separate the DNA-binding and -cleavage domains of meganucleases, which poses a challenge in its engineering. Even though there are hundreds of discovered meganucleases, the probability of finding an enzyme that targets a desired locus is low. The presence of already induced DSBs is presented to the error-prone NHEJ DNA repair mechanism. In essence, the process is prone to inserting DNA templates in random, undesired locations. Attempts were made [23, 24] to improve this mechanism; however, the specificity continues to be significantly low.

Zinc finger nucleases

The discovery of zinc finger nucleases (ZFNs) resolved some of the issues that arose from meganucleases [25]. ZNFs are a class of artificial, engineered endonuclease generated by fusing zinc finger DNA binding protein (from a eukaryotic transcription factor) to a DNA-cleavage domain (from FokI restriction endonuclease). Zinc finger domains can be engineered to target specific DNA sequences, and together with nucleases, can create DSBs, making it

a powerful tool in gene editing. The DNA-binding motif recognises and binds to a specific genomic locus, after which the ZFN dimerizes and creates a DSB of the target DNA. As a results of the need for dimerization, one could create two separate ZNF modules to target two proximal sites, which then homodimerizes and creates a DSB, thus increasing the efficiency and improving targeting [26]. As example, ZNF recognised a 3 bp DNA code, and with combinations of 67 ZNFs, a region of 1821 bp genomic sequence was successfully targeted [27].

TALENs (transcription activator like effector nucleases)

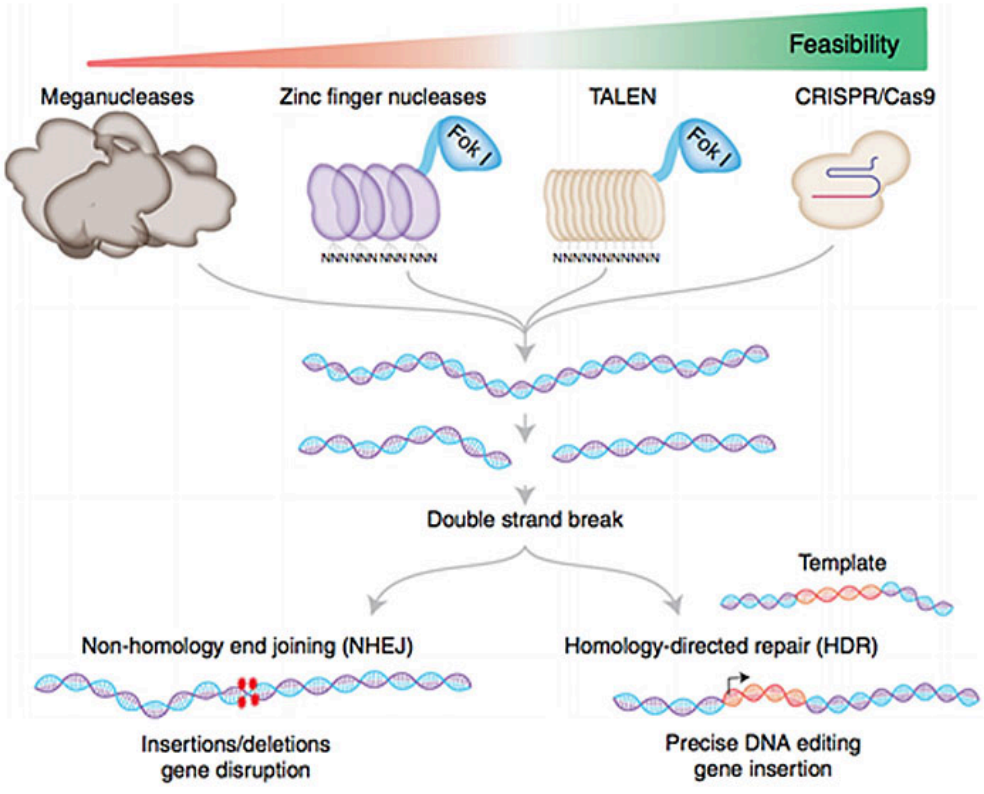
TALENs had advantages over ZFNs in that they could recognise a single nucleotide, as compared with 3 bp with ZFNs [28]. TALENs are naturally occurring bacterial proteins secreted by pathogenic bacteria of the *Xanthomonas* genus to modulate gene transcription in host plants. Similar to ZNFs, TALENs are engineered restriction enzymes that were fused to the catalytic domains of the FokI endonuclease and shown to function as dimers to cleave DNA target site [29]. This system has been shown to efficiently introduce DSBs in both somatic cells [30] and pluripotent stem cells (PSCs) [31]. The drawback of TALENs is their large size (impeding delivery), and studies have shown epigenetic modification in host chromosome leading to DNA accessibility issues.

CRISPR/Cas9-type enzymes

CRISPR stands for clustered regularly interspaced short palindromic repeat DNA sequences. The name "CRISPR" was coined much later, but they were initially discovered in the 1980s when these elements were identified in the 3′ end of the region of the alkaline phosphatase (iap) gene in *Escherichia coli*, which contained a set of 29 nucleotide repeats and 32 nucleotide spacers. Their utility was essentially unknown at that time [32] and it was only because of the Human Genome Project that many organism DNAs were sequenced, and computational analysis showed that CRISPRs were found in the more than 40% of sequenced bacterial and 90% of archaea [33].

Although the system is not naturally found in eukaryotic cells, it is analogous to the adaptive immune system in mammalian cells. The CRISPR system constitutes an adaptive immune defence system that protects bacteria and archaea from invading phages, viruses and plasmids through an RNA-guided DNA cleavage mechanism [34–36]. Instead of recognition of epitopes of foreign proteins, the system is designed to keep record of prior exposures to invasive DNA material from phages or plasmids. In bacteria, the DNA material from an invader (usually a virus) is harnessed and incorporated into its own bacterial genome within the CRISPR locus. CRISPR loci consist of several non-contiguous, highly conserved repeats that are separated by stretches of variable sequences, called spac-

Figure 1: The basic working principle of major genome-editing technologies. Meganucleases are engineered restriction enzymes that recognise long stretches of DNA sequences. Each zinc finger nuclease recognises triple DNA code whereas each TALE recognises an individual base. Unlike protein-DNA recognition in ZFNs and TALENs, simple RNA-DNA base pairing and the PAM sequence determine CRISPR targeting specificity. All these tools result in DNA double-strand breaks, which are repaired either by error-prone non-homology end joining or homology-directed repair. While NHEJ results in random indels and gene disruption at the target site, HDR can be harnessed to insert a specific DNA template (single stranded or double stranded) at the target site for precise gene editing.Reproduced from Adli M. The CRISPR tool kit for genome editing and beyond. Nat Comm 2018; 9(1):1911. doi: 10.1038/s41467-018-04252-2, with permission; © Nature Publishing Group [15].



ers. The sequences of these spacers are always of extra-chromosomal origin [37] and correspond to the viral and plasmid DNA sequences that have been harnessed by the bacteria as an adaptive mechanism.

Adjacent to the CRISPR locus, is a group of genes that encode a family of proteins known as the CRISPR-associated (Cas) genes. Cas proteins coded by these genes carry functional domains similar to endonucleases, helicases, polymerases and nucleotide binding proteins. The Cas protein system and CRISPR sequences generate a complex that can recognise and destroy viral DNA sequences during infections. In the CRISPR/Cas system, immunity occurs in three phases, which comprise adaptation, expression and interference [38] (fig. 2A). When foreign genetic material, such as from phage, is injected into a bacterial cell, the adaptation system selects proto-spacers from the foreign DNA and incorporates them into the CRISPR array. In the second phase, this locus is transcribed as a single precursor CRISPR RNA (precrRNA) containing the full set of CRISPR repeats as well as the foreign DNA material, which then matures into multiple crRNA containing a single spacer. In the final phase, a transactivating crRNA (tracrRNA) binds to the repeat sequences of the precrRNA to form a duplex; and the crRNA guides the Cas nucleases to precisely identify and cleave the foreign nucleic acid.

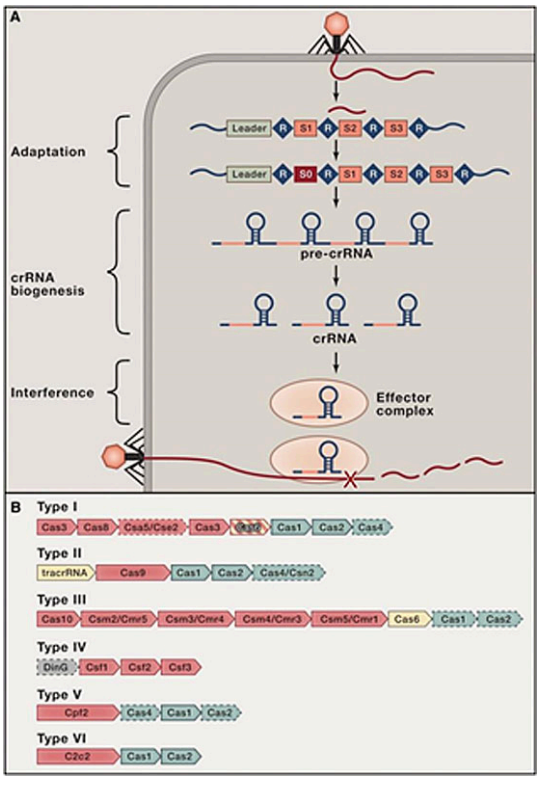
Over the evolutionary time line, the CRISPR/Cas systems have been exposed to, and thus adapted to, a wide range of invaders. There is variation in the repeat sequence of different CRISPRs, and also variations in the endonuclease activity of the Cas protein. There are six main types (I–VI) of Cas grouped into two classes [1, 2] (fig. 2B). Class 1 has multiple RNA guided mechanisms (types I, III and IV), and class 2 includes single large proteins (types II, V and VI) [39]. Types III–VI have all been recently described in detail [40]. Types I and III are found in both bacteria and archaea, whereas type II is unique to bacteria. The bacterial type II is the most studied and best known for the Cas9 proteins, a critical component when it comes to gene editing.

A critical feature of the Cas9 system is the protospacer adjacent motif (PAM), which flanks the 3' end of the DNA target site and dictates the DNA target search mechanism of Cas9. Commonly used Cas9 systems obtained from *Streptococcus pyogenes* (SpCas9) recognises a short "NGG" PAM sequence that occurs frequently in eukaryotic chromosome, whereas the Cas9 from *Staphylococcus aureus* (SaCas9) recognises a longer, less common PAM "NNGRRT". Through engineering of the SaCas9, the target range has increased two- to fourfold, making it more precise in targeting. Cas9 is a unique enzyme because it has a helicase that can unwind the DNA at the site of the RNA-DNA match sequence [35]. It is fascinating that this is an energy independent process [41]. Also, Cas9 is the only one among many Cas proteins with catalytic activity in *Streptococcus thermophilus*.

As discussed above, the endogenous system uses two separate, short RNAs: the mature crRNA (with guiding sequence) and tracrRNA (base pairs with crRNA), both of which are required to form Cas9 protein-RNA complexes. A chimeric RNA, called single guide RNA (sgRNA) was developed by fusion of crRNA and tracrRNA [42, 43] for the purposes of gene editing. The utility of CRISPR/Cas9 was further realised when it was noted that it can be transferred across distinct bacterial genera to confer heterologous resistance to invasive nucleic acid [44]. Furthermore, its potential became ground-breaking when the Cas9 system was used *in vivo* in genome editing in eukaryotic cells [45–47]. It was also demonstrated that Cas9 enzymes can be reprogrammed to target a desired DNA sequence [48].

To summarise, CRISPR/Cas9 typically contains a minimum of two components: customisable sgRNA and a programmable endonuclease (e.g., Cas9). Upon binding to PAM, the Cas9-sgRNA complex detects DNA complementary to the guide RNA and creates a site-specific DSB to generate a blunt end, usually at a position 3 base pairs away from the PAM sequence. This DSB can then be repaired either by NHEJ or HDR of the host cell. This simple yet elegant programmable system opened many doors and has revolutionised the field of gene editing [49].

Figure 2: Function and organisation of CRISPR systems. (A) CRISPR immunity occurs in three stages. Upon introduction of foreign DNA, the adaptation machinery selects protospacers and inserts them into the leader end of the CRISPR locus. During crRNA biogenesis, the CRISPR locus is transcribed and sequence elements in the repeats direct processing of the precrRNA into crRNAs each with a single spacer. The crRNA then assembles with Cas proteins to form the effector complex, which acts in the interference stage to recognise foreign nucleic acid upon subsequent infection and degrade it. (B) CRISPR systems are extremely diverse but can largely be classified into six major types. Representative operons for each type are shown here. Genes only present in some subtypes are shown with dashed outlines. Genes involved in interference are coloured red, those involved in crRNA biogenesis are coloured yellow, and those involved in adaptation are coloured blue. Type IV systems are notable for their frequent occurrence in the absence of CRISPR loci.Reproduced with permission from Wright AV, Nuñez JK, Doudna JA. Biology and applications of CRISPR systems: harnessing Nature's toolbox for genome engineering. Cell. 2016;164(1–2):29–44. doi: 10.1016/j.cell.2015.12.035 [38].



The utility of CRISPR/Cas9, however, has been extended beyond site-specific gene editing with DSBs. With development of the catalytically dead Cas9 enzyme (dCas9), the system was able to recognise and bind to a DNA sequence of choice without cleaving it. This, therefore, prevented polymerase or other transcription factors from binding DNA and hence repressed expression of the gene (fig. 3). The system is now appropriately referred to as CRISPR interference (CRISPRi) [51]. Subsequent studies demonstrated activation of genes (CRISPRa) using modified dCas9 [52]. A recent study also showed utility of dCas9 for precise base editing [53] utilising transfer RNA adenosine deaminase attached to dCas9. Numerous studies have now demonstrated conversion of A/T base pairs to G/C base pairs in a targeted fashion, without creating a DSB. Similar to CRISRi and CRISPRa, this system was utilised for precise targeting rather than for its endonuclease activity. These systems could be used effectively in the future for repairing single base pair mutations in various monogenic diseases.

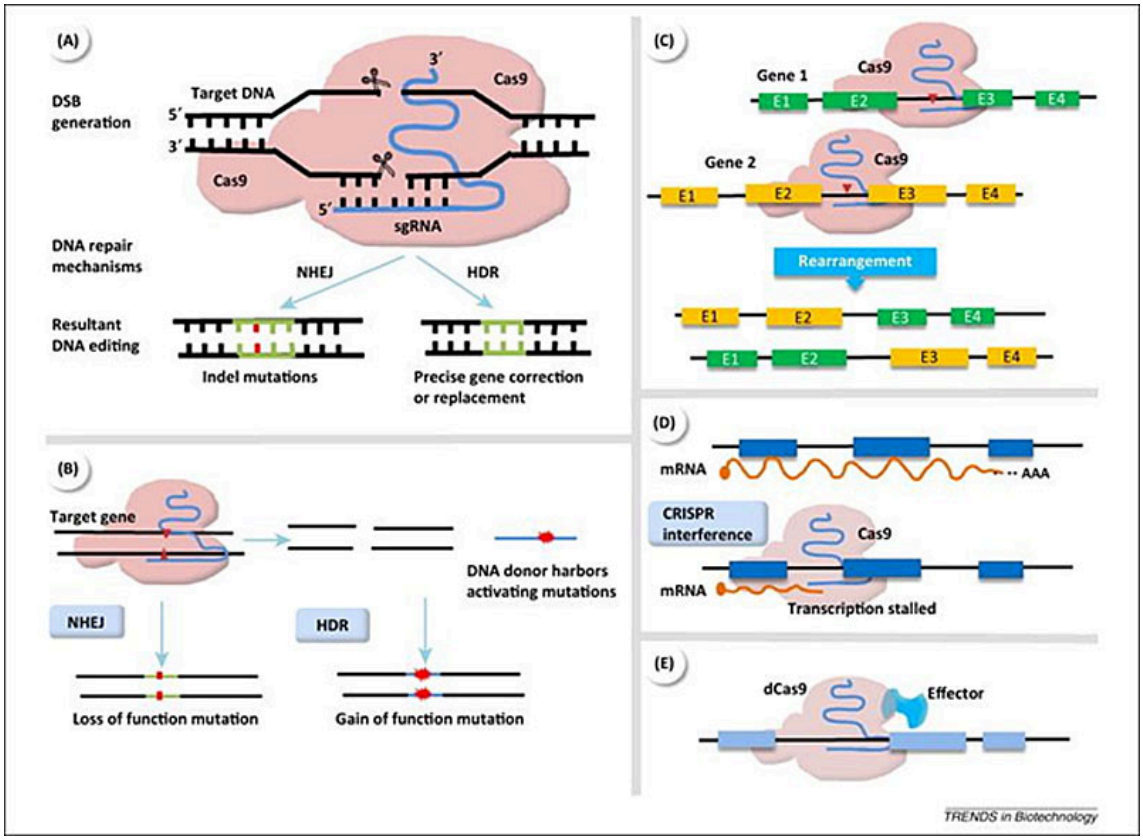
The CRISPR system has also been applied for targeted manipulation and study of epigenetic markers. It allows one to precisely control phenotype or interrogate the relationship between the epigenome and transcriptional control. A study using the fusion protein dCas9-acetyltransferase was used to catalyse the acetylation of histone H3 lysine 27 at a target site, leading to robust transcriptional activation of the target genes from promoters and enhancers [54]. In a separate study, dCas9 directed methylation of a promoter region of targeted loci to decrease gene expression [55]. More recently, the Cas9 system has been shown *in vivo* to activate endogenous target genes through trans-epigenetic remodelling [56], and used effectively in mouse models of diabetes, muscular dystrophy and acute kidney disease.

The CRISPR/Cas9 system has the potential to change the way we think about medicine in the next decade. We will further discuss several examples of how this system is being used to model and treat human diseases.

The challenge: delivery of gene editing tools

Much of the enthusiasm about CRISPR and other gene editing tools revolves around their potential to treat human diseases. However, delivery of these tools into a cell of in-

Figure 3: Mechanisms and applications of CRISPR-Cas9 in cancer modelling. (A) Mechanisms of CRISPR-Cas9-mediated genome editing. Cas9 locates and cuts the target sequence directed by the base-pairing between the sgRNA and the target sequence in the presence of a proto-spacer-adjacent motif (PAM) on the opposite strand. The resulting DSBs stimulate DNA repair processes, including error-prone NHEJ that induces indel mutations, and HDR that leads to precise gene repair or replacement. The yellow line indicates the PAM. Green lines indicate repaired DNA sequence, while the short red lines indicate indel mutations generated by NHEJ. (B) Schematic of CRISPR-Cas9 induced loss or gain-of-functions mutations. Left: NHEJ mediates loss-of-function indel mutations. Right: In the presence of a donor template, activating mutations in the template are introduced into the target gene via homologous recombination. (C) CRISPR-Cas9 mediated chromosomal rearrangement. (D) CRISPR interference: Cas9 binding to DNA sequence per se exerts transcriptional suppressive effects. (E) dCas9 is repurposed as a site-specific DNA-binding domain that transports various effectors to the target loci. Abbreviations: Cas9, CRISPR-associated 9; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, dead Cas9 (catalytically inactivated version of Cas9); DSB, double-strand break; E, exon; HDR, homology-directed repair; NHEJ, non-homologous end-joining; sgRNA, single guide RNA. Reprinted with permission from Lu XJ, Qi X, Zheng DH, Ji LJ. Modeling cancer processes with CRISPR-Cas9. Trends Biotechnol. 2015;33 [6]:317–9. doi: 10.1016/j.tibtech.2015.03.007 [50].



terest poses a major challenge. The components of gene editing have to be delivered to the cell/nucleus of interest, and these could potentially be *in vivo*, *ex vivo* or *in vitro*. Considerations for delivery include physical barriers (cell membranes, nuclear membranes) and degradation by proteases or nucleases of the host, as well as immune system of the host if the components are being delivered *in vivo*.

Delivery of gene editing tools includes two main categories of cargo and vehicle [57]. For CRISPR/Cas9, these consist of (i) a DNA plasmid encoding both the Cas9 protein and the guide RNA; (ii) mRNA for Cas9 translation alongside a separate guide RNA; and (iii) the Cas9 protein with guide RNA (ribonucleoprotein complex). The type of cargo chosen is based on the vehicle of choice, as well as whether the system is *ex vivo*, *in vivo* or *in vitro*. In general, the vehicle can be divided into three general categories of physical delivery, viral vectors and non-viral agents.

Physical delivery

The physical methods to deliver the CRISPR components include microinjection, electroporation, hydrodynamic delivery and new technology in the form of mechanical deformability. Microinjection is considered the gold standard since the efficiency approaches 100% [58]. Either plasmid DNA encoding both the Cas9 protein and the sgRNA, mRNA encoding Cas9 and sgRNA, or Cas9 protein with sgRNA can be directly injected into individual cells using a needle under visualisation with a microscope. The advantage of this method is its high efficiency and less restriction on size of the cargo. However, it can be used only for *in vitro* or *ex vivo* delivery.

Electroporation a method in which pulsed high voltage electrical currents transiently open nanometre-sized pores in the cell membrane, allowing the components of the gene editing tool (typically available in the buffer outside the cell) to flow into the cells. Newer methods are being developed that directly deliver the cargo into the nucleus, including the now established method of nucleofection [59]. This process does not require breakdown of the nuclear envelope. Studies have shown use of electroporation in conferring resistance to human immunodeficiency virus (HIV) infection by adding the natural CCR5Δ32 mutation to human cells [60], and cure of latent herpesvirus infection [61], as well as *in vivo* use to edit genes in a mouse model to introduce Sonic hedgehog medulloblastoma via *in utero* electroporation [62]. Because it is widely available and has great utility in delivery of new CRISPR/Cas9 systems, this technique continues to be refined.

The hydrodynamic technique involves rapidly injecting a bolus containing gene editing cargo into the bloodstream, which results in an increase of the hydrodynamic pressures in the cells of multiple organs (significantly more in liver) [63], temporarily increasing permeability of the membranes and thus influxing the cargo into cells [57]. This technique has been effective in animal models of liver diseases, but its use in clinical application is a challenge because of the risks incurred by using large volume boluses, which can have severe haemodynamic and physiological effects that could result in significant morbidity and mortality.

Novel mechanical techniques are also being developed. A chip containing micro constriction causing mechanical deformation leading to transient holes in the cell membranes allowing the cargo to flow into the cell has been developed recently [64]. Technologies such as microfluidics have become a significant area of research in multidisciplinary science that deals with precise control and manipulation of submillimetre sized fluid droplets.

Non-viral agents

Non-viral delivery methods typically involve a range of technologies that includes lipid nanoparticles/liposomes, lipoplexes/polyplexes, cell penetrating peptides, the unique DNA nanoclew, gold nanoparticles and iTOP (induced transduction by osmocytosis and propanebetaine). There are many other delivery systems that are suitable for CRISPR/Cas9, such as streptolysin O, multifunctional envelope-type nanodevices (MENDs), lipid-coated mesoporous silica particles, and other inorganic nanoparticles, but simply have not been reported in the literature (reviewed in [55]). In addition, many non-viral vector systems appear to lend themselves to the application of gene editing.

Viral vectors

Recombinant and replication-defective viral vectors were the first modalities that enabled the transfer of genetic material into human somatic cells and continue to be used to deliver the tools for gene editing. There are primarily three classes of vectors that have been used to deliver cargo into the host target: retroviruses, adenoviruses and adeno-associated viral vectors [65]. Retroviral vectors are widely used viral vectors that transmit the transgene into the transduced cell progeny by integrating their complementary DNA into the host genome using reverse transcriptase [66]. Lentiviral vectors are a subtype of retroviral vectors derived from HIV that can transduce nondividing cells [67]. Adenoviral vectors deliver double-stranded DNA to the cell nucleus, allowing transient expression of the mRNA and subsequently the protein of interest. Adeno-associated viruses (rAAV) are engineered from a nonpathogenic, nonenveloped Dependovirus genus and Parvoviridae family that are naturally replication-defective. These small, single stranded DNA (~4.7 kilobases) recombinant viruses have been extensively utilised for gene therapy after demonstration that viral particle transduction of single-stranded rAAV donor DNA yields more than 1000-fold higher frequencies of gene repair compared with transfection by donor plasmids [68]. A few years after this finding, it was also noted that rAAV-based gene targeting can be enhanced by 100-fold if a DSB is generated at a predefined target [69], making it an ideal tool for the most recently developed endonuclease approaches to gene editing. Besides a mild immune response, AAV is not known to cause or relate with any diseases in humans, making it an excellent tool for *in vivo* use in gene therapy [70]. They have the ability to deliver only about 5 kilobases of double-stranded DNA into the host cells, which is a limiting factor. Viral vectors, in general, have been found to be highly favourable, and perhaps the most common CRISPR/Cas9 *in vivo* delivery systems [71].

Use of gene editing in the treatment of human diseases

The discussion thus far primarily focused on the tools that have been developed for genetic modification of the cells. The penultimate goal of research in gene editing has been finding advanced cures for human diseases. There has been success in both *in vivo* as well as *ex vivo* use of gene therapy in human diseases. *In vivo* use of gene therapy involves tissue-specific targeting, local delivery, or target cell specific gene expression, whereas *ex vivo* therapies target the cells outside the body, and these modified cells are used either for disease modelling or as therapeutic agents.

There are numerous human diseases that have been targeted for gene therapy. As discussed earlier, the diseases are either monogenic, complex diseases with multiple mutations and, more recently, gene therapies that have targeted infectious diseases as well as epigenetic-based diseases. We will discuss a few promising studies as well as principles of new therapies such as CAR-T cells (chimeric antigen receptor-T cells) and iPSCs (induced pluripotent stem cells).

Haemophilia

Haemophilia B (factor IX deficiency) and haemophilia A (factor VIII deficiency) are X linked disorders resulting from mutations in the F9 and F8 coding genes, respectively. They are both fairly heterogeneous diseases with variable levels of factor proteins in the plasma, resulting in variable clinical presentation; extreme cases have frequent spontaneous haemarthroses as well as spontaneous bleeding in the tissues or central nervous system causing morbidity and mortality. Current standard care is to infuse exogenous factors multiple times per week, which has its own drawbacks including frequent infusions and breakthrough bleeding despite receiving infusions. Gene therapy therefore has been major area of study for correction of the underlying protein deficiency. Initial studies involved rAAV expression of factor IX in haemophilia patients, resulting in expression of therapeutic levels of the factor that slowly declined after a few weeks owing to destruction of the transduced hepatocytes by the host immune system [72]. Later studies showed improved duration of persistence of factor IX when short-term immunosuppression was introduced [73, 74]. With use of a natural variant of factor IX with high specific activity, called factor IX Padua, sustained factor IX activity for an extended period of time was achieved. The transgene was delivered by an optimised AAV capsid and liver-specific expression cassette, which required significantly fewer rAAV particles [75]. CRISPR/Cas9 also has been used to correct factor IX deficiency. Hydrodynamic administration of naked Cas9sgRNA and donor DNA into germline cells *ex vivo* as well as in adult haemophiliac mice [76], and a different study showing AAV vector expressing SaCas9 and sgRNA delivered to adult and neonatal haemophilia mice, were able to restore adequate levels of factor IX [77].

Similarly, for factor VIII deficiency, a single intravenous dose of a codon-optimised AAV5 vector encoding a B domain-deleted human factor VIII (AAV5hFVIIISQ) with a liver-specific promoter was infused in a small cohort of patients, with sustained normalisation of factor VIII activity in the majority of patients who received a high-dose infusion [78]. A recent study showed use of CRISPR/Cas9 in induced pluripotent stem cells (iPSCs) from patients with haemophilia A to correct their chromosomal inversions. The endothelial cells differentiated from these corrected iPSCs were able to express F8 gene functionality and were able to correct the deficiency in an otherwise lethal knockout mouse model [79].

Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin (AAT) deficiency is a genetic disorder that involves both the liver and the lungs. The mutation in SERPINA1 gene results in the accumulation of defective AAT protein in liver leading to damage; its deficiency in the serum can lead to emphysema of the lungs as a result of unchecked neutrophil proteases. For years, this disease has been investigated as a potential target for gene therapy [80]. Lately, CRISPR/Cas9 has been utilised to incorporate A1AT gene into the ROSA26 “safe harbour” locus in murine livers, and this “knock-in” method achieved gene expression in an *in vivo* model. This study also highlighted that use of knock-in into a safe harbour is a promising strategy for long-term correction of inherited diseases [81]. In another recent study, CRISPR was used for the systemic delivery of AAV8CRISPR to exon 2 of hSERPINA1, leading to reduced aggregates in hepatocytes. A second AAV providing a donor template to correct Z mutation using CRISPR also achieved modest correct of this mutation in this study. This study demonstrated the potential of gene editing-based therapeutics to simultaneously correct both the liver and lung disease symptoms associated with AAT deficiency [82].

Cystic fibrosis

Cystic fibrosis is another example of a monogenic disease that has multiorgan involvement. Average life expectancy with cystic fibrosis is about 40 years and, because of its monogenic nature, it has become a key target of research in gene therapy. A recent study showed that 12.5% of ongoing trials of gene therapy were aimed towards cystic fibrosis [83]. Caused by mutation in the cystic fibrosis transmembrane regulator gene leading to dysfunction of the chloride transporter, it causes severe lung disease, chronic endocrine and exocrine pancreatic insufficiency and infertility in men, among many other manifestations. Using CRISPR/Cas9 systems, functional gene corrections have been achieved in iPSCs generated from patients with cystic fibrosis [84] as well as in intestinal stem cell organoids of cystic fibrosis patients [85]. Preclinical research in progress is evaluating methods of delivery into lung tissue that utilise lentiviral vectors [86] as well as AAVs *in utero* [87], and much has been accomplished culminating in first-in-man lentivirus trials for cystic fibrosis [88].

Viral infections

Viral diseases have also garnered interest in gene therapy. With the rise of the CRISPR/Cas9 system, more studies are tackling some key viruses such as hepatitis B virus (HBV), herpesviruses, human papillomaviruses and HIV. In one study, when HIV1 LTR expression-dormant and inducible T cells were transfected with CRISPR/Cas9 targeting the HIV LTR, a significant loss of LTR-driven expression was noted, and further analysis also showed that the editing system was able to remove internal viral genes

from the chromosome [89]. The presence of the conserved transactivation response (TAR) sequence in HIV makes it a preferred site for CRISPR Cas9 system [90]. In later studies, viral DNA genome spanning between the LTR and gag regions were successfully removed in HIV transgenic mice by means of gRNAs and Cas9 transfected with AAVs; the excision was confirmed in examined tissues from kidneys, lungs and heart as well as circulating lymphocytes [91]. This was one of the first *in vivo* studies in HIV. A separate study addressing viral escape and mutation showed that two strong gRNAs targeting two different regions of the HIV genome can completely stop viral replication [92].

Hepatitis B is the most common chronic viral disease worldwide, which leads to liver disease including hepatocellular carcinoma. Because the virus stays dormant in the liver with covalently closed circular DNA (cccDNA), antivirals have not been successful in eliminating the virus. cccDNA acts as a template for transcription for chronic infection and reactivations, making it a target for novel gene therapies. In the past decade, ZFNs and TALENs have been used to target HBV *in vitro* [93] and *in vivo* [94]. CRISPR/Cas9 was used to reduce the production of HBV core and surface antigens in Huh7 hepatocyte-derived carcinoma cells transfected with an HBV expression vector. *In vivo* mouse models showed that this system could cleave intrahepatic HBV genome containing plasmid and facilitate its clearance *in vivo*, resulting in a reduction of serum surface antigen levels [95, 96]. In a similar study, HepG2 cells expressing HBV receptor sodium taurocholate cotransporting polypeptide were used to show that HBV infection could be reduced up to eightfold by HBV targeted gRNA. This was achieved by multiple deletions and mutations in the cccDNA caused by the Cas9 cleavage, which were subsequently repaired by the host's NHEJ mechanism [97]. Further work will be needed on this promising field, as we still search for a cure for hepatitis B.

Cancer immunotherapy

Cancer therapeutics is a wide area of current research. Most recent advances include chimeric antigen receptor (CAR) T cells, where T cells, which are typically autologous, are manipulated *ex vivo* to express the antigen-binding domain from a B-cell receptor, which is fused to the intracellular domain of a CD3 T-cell receptor (CD3ζ). When these T cells are infused back into the patient, they recognise specific cell surface antigens (as in a cancer cell) that activate T cell response independent of MHC recognition. These cells are milestones in treatment of haematological malignancies, and their use was recently approved by FDA for the treatment certain types of lymphoma. In these malignancies, the CAR-T cells target CD19, a pan B cell antigen, which has been highly successful in treatment of acute lymphoblastic leukaemia. To increase the efficiency of the CAR-T cells, CRISPR/Cas9 has been used to increase their antitumor efficiency by disrupting programmed death protein 1 [98]. Further studies are being done to expand CAR-T therapy to target solid tumours [99].

Disease modelling and stem cell therapies

Another frontier in biomedical research is to generate cell lines that can act as disease models. Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs), and induced pluripotent cells (iPSCs) can both be modified using CRISPR technology for disease modelling, gene correction therapy, antiviral therapy and antitumor therapies [100]. For example, one strategy is to knock out disease-relevant genes in human PSCs using CRISPR/Cas9 in order to explore the pathogenic mechanism in the derived cells. These cells then could be used as disease models for drug therapies. This has been shown by developing retinoblastoma1 null human ESCs generated using CRISPR/Cas9 and testing the utility of various chemotherapeutic agents to identify their sensitivities [101]. Another frontier in this technology is the generation of organoids, which are three-dimensional cultures derived from PSCs, spermatogonial stem cells, or primary tissues [100]. The utility of organoids in modelling cystic fibrosis was discussed earlier [85]. Similar studies have generated kidney disease model organoids by knockout podocalyxin, PDK1 or PKD2 genes of human ESCs using CRISPR/Cas9 [102]. In one study, injection of CRISPR/Cas9 components (including the plasmid of the missing gene) into the zygotes of cataract mice led to generation of healthy mice [103]. Regeneration of functionally recovered stem cells using CRISPR/cas9 technology has also been studies in β-thalassaemia, muscular dystrophy and Huntington disease [104–106].

Epigenetic diseases

Whereas somatic cells all have essentially the same genomic sequence, there is a wide range of phenotypic expression that is mostly attributable to epigenetic changes. These modifications are heritable during cell division, and play significant roles in cellular development, tissue differentiation and responsiveness [107]. The cellular response is reproducible in various cell types to intrinsic processes and external stimuli [108], suggesting that environment plays a key role in gene expression and regulation. There are three main forms of epigenetic information, namely DNA methylation, histone modifications and non-coding RNA-mediated processes (such as microRNAs, long non-coding (lnc) RNAs, and enhancer RNAs) [5]. DNA methylation is the best understood and the clearest example of epigenetic processing and information. For example, during DNA replication, the enzyme DNA methyltransferase 1 recognises hemi-methylated CpG and methylates the daughter strand at appropriate complimentary CpG sites [107]. Depending on the methylation state, there is differential binding of transcriptional factors and enhancers, leading to variability in gene expression.

Histone modifications are characterised by ATP-independent processes involving acetylation, methylation, phosphorylation, ubiquitylation, citrullination and/or sumoylation of nucleosomal histones, which act as spools around which DNA winds. Each of these modifications plays a role in gene activity, silencing or even isolating both active and inactive genes [107]. Higher-order organisation of chromatin structure, which is an assemblage of nucleosomes that assumes a three-dimensional, reproducible conformation, is also considered a form of epigenetics [109, 110]. Non-coding RNAs engage in various steps in post-transcriptional gene regulation and their utility in regulation of epigenetic processes is now well established [111].

The dysregulation of epigenetic modifications has been implicated in various human diseases. Some notable exam-

ples include Rett syndrome and Werner syndrome, which are caused by loss of function due to epigenetic mutations [112, 113]. Diseases such as Fragile X syndrome, Fredrich's ataxia, Prader-Willi syndrome, Angelman syndrome are caused by mutations in gene regulatory regions that are involved in both genetic and epigenetic regulation of methylation [114–116]. Alzheimer's disease, Parkinson's disease, and various types of cancer are believed to result from random changes in epigenetic modification due to age-related changes and environmental stressors [117–119].

The CRISPR/Cas9 system with its myriad of applications has created an entirely new approach to the study and treatment of diseases with aberrant epigenetic mechanisms. The clinical application of epigenetic modifications is in its infancy, but there are some promising studies. For example, a dCas9 complex was recently used to reactivate, both transcriptionally and translationally, a heavily methylated tumour suppressor gene "Maspin" in lung cancer cells and reduce cancer cell growth [120]. In another study, the CRISPR system restored tumour suppressor BRCA1 functional activity in cervical and breast cancers by targeting and decreasing DNA methylation of its promoter region [121]. The Cas9 system has been shown *in vivo* to activate endogenous target genes through trans-epigenetic remodelling [56], and has been used effectively in mouse models of diabetes, muscular dystrophy and acute kidney disease. In addition, CRISPR-Cas9 was used *in vivo* in mice to demonstrate transcriptional repression and prevent vision loss in a mouse model of retinitis pigmentosa, by reprogramming rode to cone-like cell fate via inactivation of *Nrl* (a master regulator gene of rod photoreceptor determination) [122]. Epigenetic-based therapies are appealing because they do not change or mutate a DNA sequence, and target the functional aspect of genes. They are at the forefront of research in the field of gene editing, and more specifically as a genomic target of the CRISPR/Cas9 system.

Conclusion

Gene editing has made significant advances in the past 10 years. From the conceptual outlook in the 1970s until now, we have made even more significant headway since 2013 with development of the CRISPR/Cas9 systems. They are feasible to use, efficacious and are now becoming more economical than even five years ago. Their utility in not only gene editing but also gene expression, epigenetic studies and modifications, and in iPSCs has revolutionised the field of biomedical research. Ultimately, patients will undoubtedly benefit from this technology once it becomes more widely available. There are hosts of human diseases that are being investigated currently worldwide (table 1), a few examples of which we have discussed in this review article. These technologies certainly will be further refined and one day may be used as mainstream therapeutics for many diseases.

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Table 1: Clinical conditions for which human gene transfer trials have been approved.

Monogenic disorders
Adrenoleukodystrophy
Alpha-1 antitrypsin deficiency
Aromatic L-amino acid deficiency
Batten disease
Becker muscular dystrophy
Beta thalassaemia
Canavan disease
Chronic granulomatous disease
Crigler–Najjar syndrome
Cystic fibrosis
Duchenne muscular dystrophy
Fabry disease
Familial adenomatous polyposis
Familial hypercholesterolaemia
Familial lecithin-cholesterol acyltransferase deficiency
Fanconi anaemia
Galactosialidosis
Gaucher's disease
Gyrate atrophy
Haemophilia A and B
Hurler syndrome (mucopolysaccharidosis type I)
Hunter syndrome (mucopolysaccharidosis type II)
Huntington's chorea
Junctional epidermolysis bullosa
Late infantile neuronal ceroid lipofuscinosis
Leukocyte adherence deficiency
Limb girdle muscular dystrophy
Lipoprotein lipase deficiency
Metachromatic leukodystrophy
Sly syndrome (mucopolysaccharidosis type VII)
Netherton syndrome
Ornithine transcarbamylase deficiency
Pompe disease
Purine nucleoside phosphorylase deficiency
Recessive dystrophic epidermolysis bullosa
Sanfilippo A (mucopolysaccharidosis type IIIA)
Sanfilippo B (mucopolysaccharidosis type IIIB)
Sickle cell disease
Severe combined immunodeficiency
Spinal muscular atrophy
Tay Sachs disease
Wiskott–Aldrich syndrome
von Gierke disease (glycogen storage disease type Ia)
X-linked myotubular myopathy
Cardiovascular disease
Anaemia of end stage renal disease
Angina pectoris (stable, unstable, refractory)
Coronary artery stenosis
Critical limb ischaemia
Heart failure
Intermittent claudication
Myocardial ischaemia
Peripheral vascular disease
Pulmonary hypertension
Venous ulcers
Infectious disease
Adenovirus infection
Cytomegalovirus infection
Epstein–Barr virus
Hepatitis B and C

HIV/AIDS
Influenza
Japanese encephalitis
Malaria
Paediatric respiratory disease
Respiratory syncytial virus
Tetanus
Tuberculosis
Cancer
Gynaecological: breast, ovary, cervix, vulva
Nervous system: glioblastoma, leptomeningeal carcinomatosis, glioma, astrocytoma, neuroblastoma, retinoblastoma
Gastrointestinal: colon, colorectal, liver metastases, post-hepatitis liver cancer, pancreas, gall bladder, hepatocellular carcinoma
Genitourinary: prostate, renal, bladder, ano-genital neoplasia
Skin: melanoma (malignant/metastatic)
Head and neck: nasopharyngeal carcinoma, squamous cell carcinoma, oesophaegeal cancer
Lung: adenocarcinoma, small cell/non-small cell, mesothelioma
Haematological: leukaemia, lymphoma, multiple myeloma
Sarcoma
Germ cell
Li-Fraumeni syndrome
Thyroid
Neurological diseases
Alzheimer's disease
Amyotrophic lateral sclerosis
Carpal tunnel syndrome
Chronic traumatic brain injury
Cubital tunnel syndrome
Diabetic neuropathy
Epilepsy
Giant axonal neuropathy
Late infantile neuronal ceroid lipofuscinosis
Multiple sclerosis
Myasthenia gravis
Pain
Parkinson disease
Peripheral neuropathy
Spinal muscular atrophy type 2
Ocular diseases
Achromatopsia
Age-related macular degeneration
Choroideraemia
Diabetic macular oedema
Glaucoma
Leber congenital amaurosis
Macular telangiectasia type 2
Retinitis pigmentosa
Superficial corneal opacity
X-linked retinoschisis
Inflammatory diseases
Arthritis (rheumatoid, inflammatory, degenerative)
Degenerative joint disease
Severe inflammatory disease of the rectum
Ulcerative colitis
Other diseases
Chronic renal disease
Diabetic ulcer/foot ulcer
Detrusor overactivity
Erectile dysfunction
Fractures
Hearing loss
Hereditary inclusion body myopathy
Graft versus host disease / transplant patients
Oral mucositis
Parotid salivary hypofunction
Systemic scleroderma
Type I diabetes
Wound healing
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