CELL & GENE THERAPY INSIGHTS

TRANSLATION & APPLICATION OF GENE EDITING

SPOTLIGHT

REVIEW

The promise of therapeutic genome engineering

Andrew Bassett

Recent advances in genome engineering have revolutionized our ability to specifically and delicately manipulate the genomes of essentially any organism including human cells, and have already led to more accurate cellular and animal models of disease. Such techniques also have the potential to permanently repair genetic mutations associated with human disease, many of which are currently difficult or impossible to treat by traditional means. This review discusses the technologies currently available for genome engineering, the strategies for their application in patients and current progress towards applying such techniques to specific diseases. I highlight the exciting avenues for such therapeutic genome engineering in the future and challenges to its successful application.

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Sequencing of the human genome has provided us with the means to perform detailed analysis of the genetic variants underlying many diseases, and to date nearly 3,500 genes have been linked to specific pathologies (www.omim.org/statistics/geneMap). Certain genetic variants have been shown to be causative in disease through familial inheritance studies. Such well-defined monogenic disorders, including cystic fibrosis, hemophilia B, severe combined immunodeficiency (SCID), and sickle cell anemia provide good candidates for therapeutic genetic intervention. Since they are largely caused by mutation within a single gene, correction of this aberration would therefore be expected to completely revert the pathological symptoms.

Whilst the underlying genetics of many diseases have been well studied, our ability to intervene in this process has, until recently, been limited. Techniques such as developing small molecules to predicted target genes, oligonucleotide-based approaches to interfere with gene function, expression of transgenes to restore dysfunctional gene expression or knockdown of gene expression post-transcriptionally by RNA interference (RNAi) have been applied (Reviewed in [1,2]). However, these techniques have certain limitations, since they need to be constantly supplied to

the tissue of interest, and often require integration of foreign DNA into the genome, which can result in undesirable mutations [3]. The recent explosion in genome engineering techniques such as zinc finger nucleases (ZFNs [4]), transcription activator-like effector nucleases (TALENs [5]) and clustered regularly interspaced short palindromic repeat associated endonucleases (CRISPR/Cas9 [6]) have allowed delicate and specific manipulation of the DNA sequence. This has not only enabled us to more rapidly generate cellular and animal models of genetic variants, but also opened up the possibility of genetic therapies to permanently correct disease-causing aberrations both somatically and more controversially in the germline [7,8].

As well as treatment for heritable genetic diseases, genome engineering may also provide an attractive alternative to treat infectious diseases. The exquisite sequence-specific binding of these reagents can be used to target viral and bacterial genomes, to prevent the spread of specific subtypes of pathogen such as hepatitis B virus [9–14] and bacteria such as antibiotic-resistant *Staphylococcus aureus* [15,16]. Interestingly, such sequence-specific reagents can also be applied to cure latent viral infections, where the virus has integrated into the genome of the host cell, such as in the case of HIV [17–19].

These recent developments in the identification of genetic variants associated with disease and the genome engineering technologies necessary for their correction may therefore realize our ambition of being able to correct genetic aberrations that cause a variety of debilitating diseases.

OVERVIEW OF SYSTEMS

Zinc fingers

The concept of genome engineering, that is the use of specific DNA-binding factors to manipulate DNA sequence, was born over 20 years ago with the discovery that ZFN DNA-binding transcription factors were modular in their architecture [20,21]. This led to the proposal that they could be reprogrammed to bind to essentially any sequence, and carry with them DNA-modifying enzymes to introduce mutations or other modifications to the underlying DNA [20,21]. Each ZFN monomer was shown to bind to three consecutive bases in the DNA, but frustratingly the DNA sequence bound by a single monomer differs depending on its context within the polypeptide [22,23]. This makes prediction of their binding sites challenging, and it is therefore necessary to invoke elaborate selection strategies from libraries of ZFN multimers to identify those that bind to the desired sequence, hindering general application of this technique [24]. Certain sequences, notably those deficient in guanine bases, are also inherently more difficult to target simply due to the absence of monomers that are able to bind to these sequences.

These DNA-binding domains are often fused to a non-specific endonuclease domain (e.g., Fok I [25]), and used to introduce mutagenic double strand breaks (DSBs) in the genome (Figure 1). In order to increase specificity and avoid unwanted DSBs, typically two ZFN DNA-binding domains are produced, each of which carries half of a homo- or hetero-dimeric Fok I nuclease [26,27]. The nuclease

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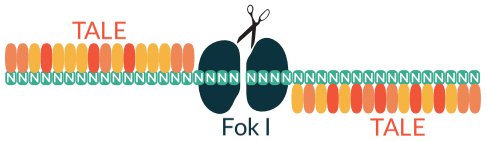
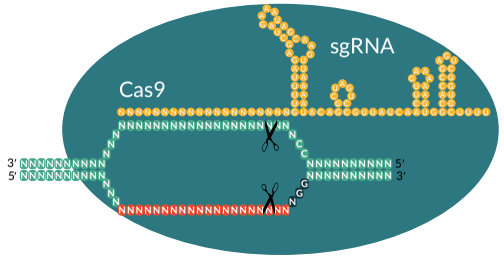
FIGURE 1

Comparison of genome engineering technologies.

	ZFNs	TALEN	CRISPR
			
DNA recognition	2 × 9–18 nt	2 × 14–20 nt	22 nt (2× for paired strategies)
Target site constraints	Some sequences difficult to target (especially non-G-rich)	First base of each TALE monomer is T	PAM (NGG) must follow 20 nt target site
Off-targeting	Some mismatches tolerated	Some mismatches tolerated	Up to 3 mismatches tolerated, position dependent
Simplicity of assembly	Complex, requires selection from library	Relatively simple, but complex cloning protocols	Simple, using synthesized oligonucleotides
***In vivo* delivery**	Easy. Small size allows simple packaging into any viral vector. Also likely to be less immunogenic, since of human origin	Difficult. TALEN pair is too large to be packaged into AAV vector, and highly repetitive nature can result in recombination	Relatively simple. *S. pyogenes* Cas9 too large to be packaged in AAV vector, but *S. aureus* can be
Multiplexing	Possible	Possible	Simple – supply multiple guide RNAs

Schematics of different DNA-binding factors are indicated, along with information about DNA recognition site, sequence constraints on-target site choice, off-targeting, ease of assembly, *in vivo* delivery and multiplexing. AAV: adeno-associated virus; PAM: protospacer adjacent motif.

therefore only becomes functional upon binding of two ZFNs in the correct orientation and spacing on the DNA, improving specificity considerably, but also further limiting the potential target sites within the genome.

TALEs

A second class of highly modular DNA-binding factors, the transcription activator like effector (TALE) proteins, were discovered through studies of the plant pathogen *Xanthomonas*, which uses them to modulate host gene transcription [28–30]. TALEs are made up of repeating 34-amino-acid modules, each of which binds to a single base in the DNA, the identity of which is determined by two amino acids, the repeat variable diresidue (RVD), in each monomer [28,30]. Since each monomer acts independently of its location within the polypeptide, it is possible to predict the sequence of monomers necessary to bind to essentially any DNA sequence [31,32]. In order to achieve specificity within a large genome, polypeptides with up to 20 monomers are necessary, which along with their inherently repetitive nature, makes assembly and manipulation of these proteins somewhat challenging. Although elegant systems have been developed that allow the production of such polypeptides [31,33,34], this process is still somewhat laborious, making assembly of large numbers of constructs time consuming.

Similar to ZFNs, these DNA-binding domains are often designed in pairs, and fused to homo- or hetero-dimeric endonuclease domains to generate TALE nucleases or TALENs [31,32,35]. Unlike ZFNs, they can be targeted to any DNA sequence so long as the first base bound by each TALE is a thymine, and lengths of multimers can be adjusted to provide sufficient specificity (Figure 1).

CRISPR/Cas9

Recent studies on bacterial immunity to viral infection led to the exciting discovery of a class of RNA-guided endonucleases present in many different bacterial species [36–39]. Endogenously, viral fragments are captured into arrays of CRISPR, that are subsequently transcribed into CRISPR RNAs (crRNAs) which bind to the CRISPR-associated endonuclease (Cas9) protein and guide it to specific sites in the viral genome, where it generates mutagenic DSBs. The realization that the sequence specificity was driven by Watson–Crick base pairing of the first 20 nt of the guide RNA molecule with the DNA target site made it possible to reprogram the endonuclease to essentially any target site in a highly predictable manner simply by altering this 20 nt sequence (Figure 1) [40–42]. The most commonly used system derives from *Streptococcus pyogenes*, and can be recapitulated by simply expressing two components: an ~100 nt synthetic guide RNA (sgRNA) and the Cas9 protein. This has led to the demonstration of its activity in many other organisms, including human cells [43–45] and cynomolgus monkeys [46], and its rapid exploitation in model and non-model organisms [47–49]. The simplicity of the system allows the extremely rapid assembly of large numbers of constructs, and combining this with techniques used to generate oligonucleotide arrays has allowed genome-wide libraries of up to

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200,000 guides to be produced, targeting every annotated gene in the human genome [50,51].

The only limitation to the sequences that can be targeted is the necessity for a protospacer adjacent motif (PAM) adjacent to the guide RNA, that is recognized by the Cas9 protein [52]. For the *S. pyogenes* system this is NGG, which should occur on average every eight bases in the DNA. However, CRISPR systems from other bacteria have different PAM requirements [53–55], and there has been success in engineering Cas proteins to utilize different PAM sequences [56], making it likely that in the future it will be possible to target essentially any sequence.

Specificity of Cas9 binding is potentially a problem, especially in large genomes such as in humans, since it is determined by a 20 nt sequence and the requirement for the PAM (NGG). As with all of the genome engineering systems described, there is some tolerance for mismatches, making the situation worse. However, in the case of CRISPR/Cas9, this is somewhat more problematic, since the effect of mismatches is position dependent, and 2–3 mismatches at the 5′ end of the sgRNA can be tolerated [57,58] (Figure 1). Whilst the exact nature and extent of off-targeting is currently under debate [57,59–65], and likely depends on cell type and delivery system, there are several systems that have been developed to address this problem. A single point mutation in Cas9 (D10A) results in inactivation of one of the two endonuclease domains, preventing cleavage of one DNA strand and turning the enzyme into a "nickase", only able to make single strand DNA (ssDNA) breaks, which are rapidly and efficiently repaired by the cell [58,66]. However, supplying two guides in an appropriate orientation and spacing allows a staggered DSB to be made, that results in similar mutagenic rates to the wild-type protein. Similarly, a catalytically dead Cas9 protein (D10AH840A) fused to the dimeric Fok I nuclease allows one to employ an analogous system to that used for the ZFNs and TALENs, with a pair of guide RNAs driving specificity [67]. Somewhat unexpectedly, specificity can also be improved by reducing the length of the guide RNA without significantly reducing catalytic activity at least in some cases [68,69]. Whilst these techniques already vastly improve specificity, no doubt additional improvements will continue to develop through engineering of sgRNA sequence, Cas9 protein, chemical modifications and delivery systems. The recent description of Cas9 structures in complex with sgRNA and target DNA will no doubt facilitate such efforts [70,71].

APPROACHES TO USE

Predominantly, genome engineering systems are used to introduce genetic mutations at desired sites in the genome. This has already enabled more rapid genetic analysis in model organisms [47–49] and allowed genetic modifications to be performed in systems that would otherwise not be amenable to such manipulations [43–46,48].

Such genetic changes can be highly beneficial for therapeutic applications (Table 1), since it is possible to effect a permanent repair to the DNA, that persists for

TABLE 1

Current applications of therapeutic genome engineering.

Hereditary disease	Target gene	Nuclease	Strategy	*in vivo*/ *ex vivo*	Refs
Cystic fibrosis	*CFTR*	CRISPR	HR-based repair of *CFTR* gene in intestinal organoids	*ex vivo*	[113]
Hemophilia B	*Factor IX*	ZFN	HR-based repair of *Factor IX* gene in liver cells	*in vivo*	[97]
SCID	*IL2RG*	ZFN	HR-based repair of *IL2RG* gene in HSC	*ex vivo*	[109]
DMD	*Dystrophin*	CRISPR and TALEN	NHEJ-mediated removal of stop codon, splice site or frameshifted exon. HR-based repair of *Dystrophin* gene	*in vivo*	[81,87–89]
α-hemo-globinopathies	*BCL11A*	TALEN	NHEJ-based deletion of erythroid-specific enhancer in *BCL11A* gene	*ex vivo*	[85]
	β-*globin*	CRISPR and TALEN	HR-based repair of sickle cell anemia mutation in iPSCs and differentiate to erythrocytes	*ex vivo*	[117, 118]
α-tyrosinemia	*Fah*	CRISPR	HR-based repair of *Fah* gene in liver cells	*in vivo*	[132]
Hyper-cholesterolemia	*PCSK9*	CRISPR	NHEJ-based deletion of *PCSK9* gene in liver cells	*in vivo*	[53,133]
Infectious disease					
Hepatitis B virus	Viral	CRISPR and TALEN	NHEJ-induced frame-shifts in essential HBV genes	*in vivo*	[9–14]
HIV	*CCR5*	ZFN and CRISPR	NHEJ-induced frame-shifts in *CCR5* gene in $CD4^+$ T-cells to prevent viral spread	*ex vivo*	[74–78]
	Viral LTR	CRISPR	NHEJ-based deletion of integrated virus	*ex vivo*	[17–19]
Staphylococcus aureus	Bacterial	CRISPR	NHEJ-induced frame-shifts in essential *S. aureus* genes or antibiotic resistance genes	*in vivo*	[15]
Galleria mellonella	Bacterial	CRISPR	NHEJ-induced frame-shifts in essential *G. mellonella* genes	*in vivo*	[16]

the lifetime of the cell, and does not require continued transgene expression after the repair has been completed. This is achieved by targeting DSBs to desired sites in the DNA, and exploiting the endogenous DNA repair mechanisms of non-homologous end joining (NHEJ) or homologous recombination (HR), to generate the desired changes in the DNA [72,73].

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Non-homologous end joining

Repair of a DSB by NHEJ essentially involves re-ligation of the DNA ends, but is somewhat error prone and occasionally small insertions or deletions (indels) of a few bases can occur at the cut site. These indels are typically used to generate frameshift mutations in protein coding sequences resulting in loss of protein function (Figure 2). This has been applied to bacterial pathogens such as *Galleria mellonella* or antibiotic resistant *S. aureus*, where CRISPR nucleases targeting the bacterial genome or antibiotic resistance genes can block bacterial spread, and enable loss of antibiotic resistance [15,16]. Additionally, CRISPR/Cas9 or TALENs targeting key open reading frames in the genome of hepatitis B virus have also been shown to be effective in blocking viral protein expression and replication [9–14].

NHEJ-based loss of function mutations can also be used to introduce protective alleles that have been identified to confer resistance to particular diseases. For example, it was noted that individuals homozygous mutant for the HIV coreceptor gene, *CCR5,* were highly resistant to infection with HIV and yet otherwise healthy [74]. This has resulted in phase I trials using ZFNs to knockout the *CCR5* gene in $CD4^+$ T cells as a therapy for HIV infection [75–78].

However, indels generated by inefficient NHEJ can also be used to mutate other small functional regions of the genome (Figure 2). These may include splice sites to alter splicing patterns, micro RNA (miRNA) target sites or miRNA genes themselves to alter post-transcriptional regulatory networks [79], or transcription factor binding sites to alter gene expression profiles of specific genes [80]. For instance, restoration of Dystrophin function, the causative lesion in Duchenne's muscular dystrophy (DMD), can be achieved by skipping of exons that contain frameshifting mutations. CRISPR/Cas9 reagents have been targeted to these splice site regions and been shown to restore gene function [81]. Interestingly, most disease-linked single nucleotide polymorphisms (SNPs) lie outside of protein-coding sequence (93%, Encyclopaedia of DNA elements [ENCODE] [82]), and many of them fall within gene regulatory elements as defined by DNAse hypersensitivity (perhaps more than 50% [83]) suggesting that this set of targets may be able to revert disease phenotypes [83,84]. Importantly, these regulatory sequences can have tissue-specific or developmental stage dependent effects on gene expression, making them good candidates for therapeutic intervention, such as those near the *BCL11A* gene involved in β hemoglobinopathies [80,85]. Mutation of these regulatory elements specifically reduces gene expression in erythroid but not B-lymphoid cells, allowing specific reactivation of fetal hemoglobin (HbF) in erythroid lineages, but leaving the non-erythroid functions of BCL11A unaffected [80,85].

Interestingly, pairs of DSBs can also result in deletion and inversion of the intervening sequence, or even translocations between chromosomes, making it possible to induce larger chromosomal deletions or rearrangements (Figure 2). This has applications in deleting genes or repeat expansions present in for example Friedreichs Ataxia [86], removal of exons in DMD [81,87–89], or reverting chromosomal translocations observed in certain leukemias

FIGURE 2

Mechanisms of therapeutic genome engineering.

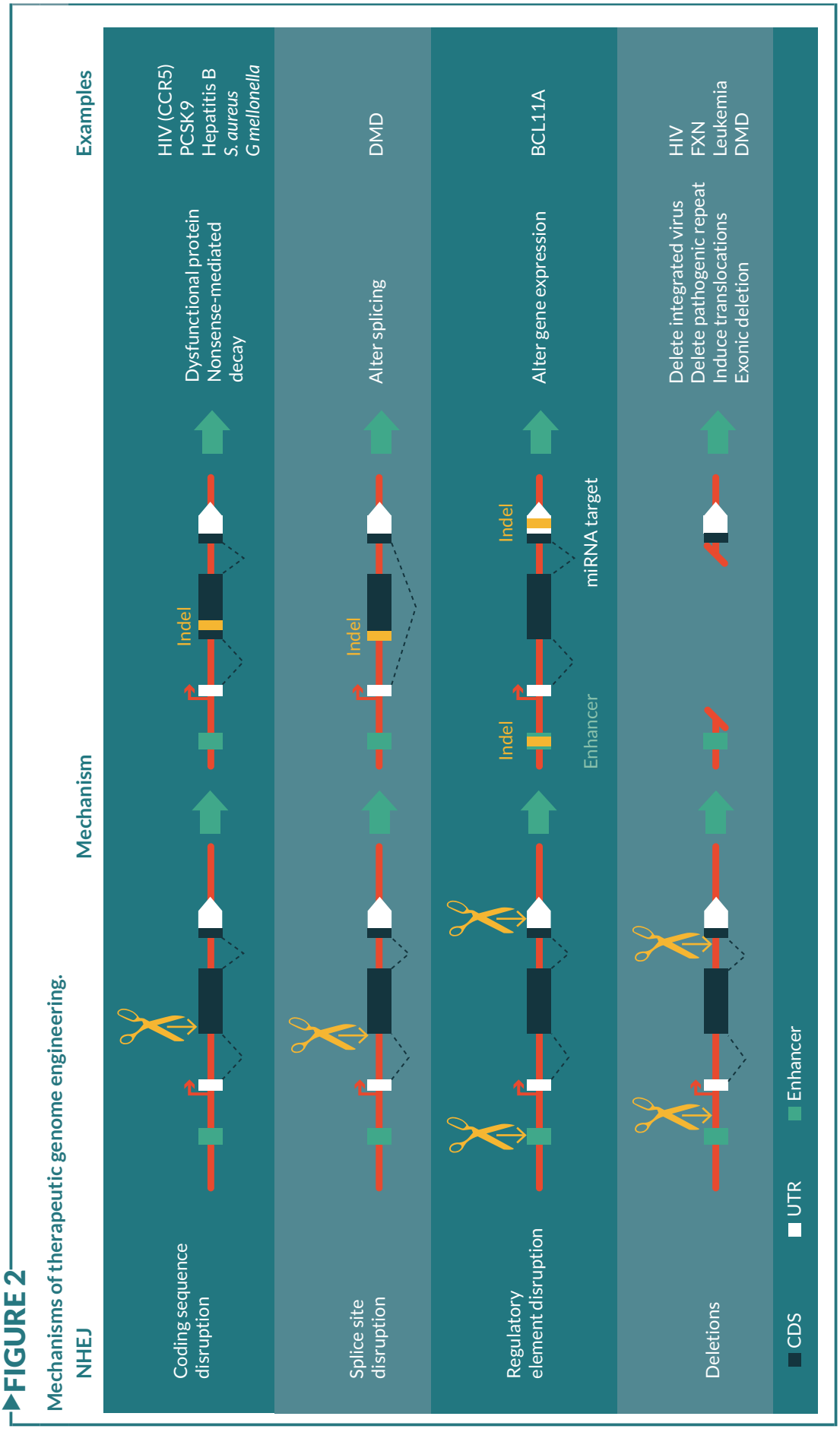

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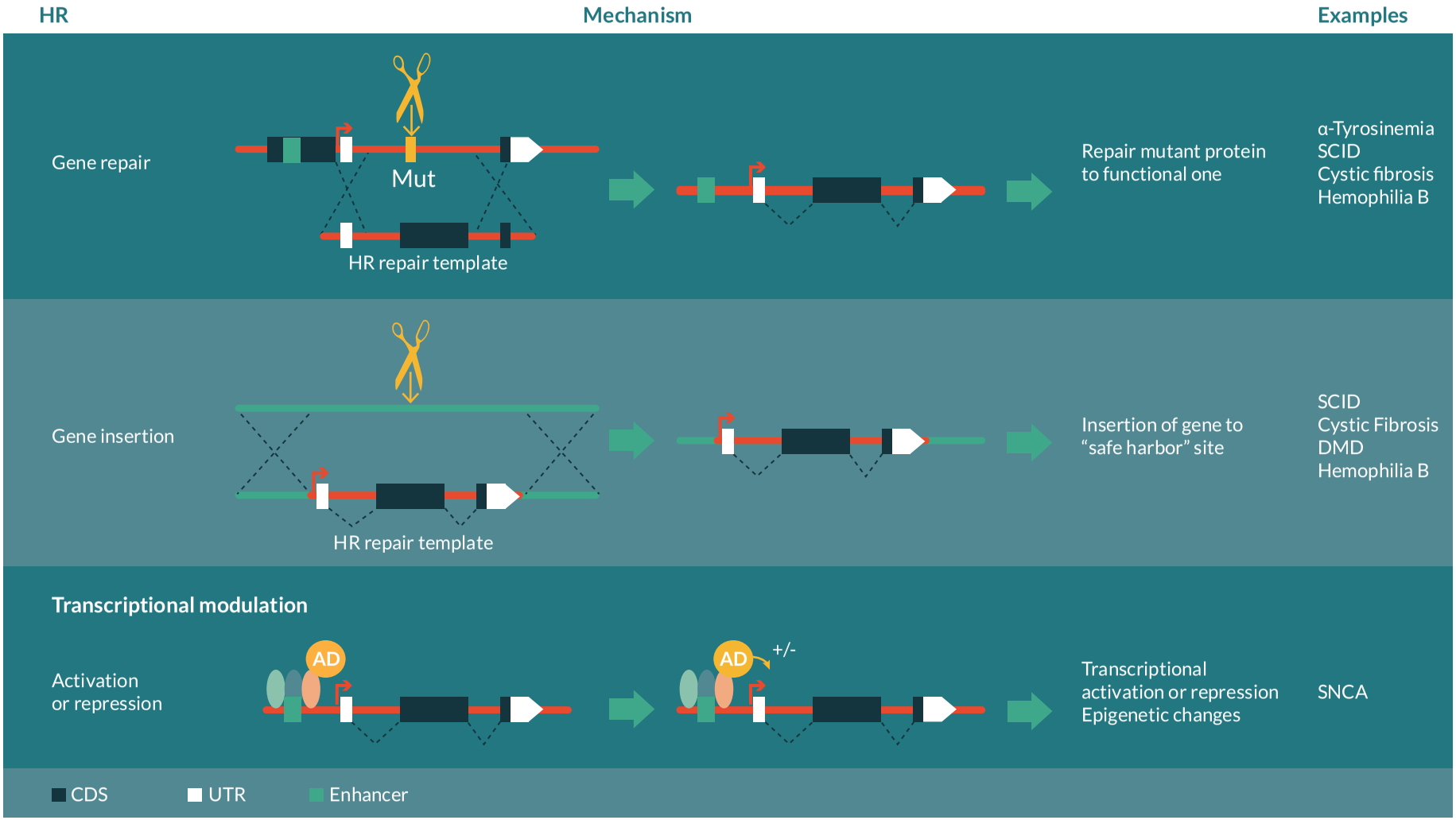
FIGURE 2 CONT.



The choice of strategy depends on the nature of the disease, and may include non-homologous end joining (NHEJ)-based mutations, homologous recombination (HR)-based repair or transcriptional modulation by activation or repression domain fusions. NHEJ-based mutations may be used to generate coding sequence disruptions to inactivate genes, splice site disruption to alter splicing patterns, enhancer or other regulatory element disruption to alter gene expression or deletions between two cuts to remove genomic regions. HR-based repair can be used to accurately repair mutations in genes, or insert transgenes into a defined "safe harbor" sites to avoid problems with random insertion. Transcriptional modulation may include activation or repression of transcription, or epigenetic modifications to cause semi-permanent changes to gene expression. CDS: coding sequence; UTR: untranslated region;

[90–92]. Interestingly, such large deletions can be used to target latent viral infections such as HIV that are currently difficult or impossible to treat. CRISPR/Cas9 nucleases targeted to the long terminal repeats (LTR) of the virus are able to recognize cells with a viral integration, excising the viral genome and generating cells free of integrated virus [17–19] (Table 1).

Homologous recombination

HR-based repair systems normally use the homologous region of the sister chromatid as a template for high fidelity repair, and therefore only occur after DNA replication, in late S and G_2 phases of the cell cycle. By supplying an excess of a desired repair template, this mechanism can be exploited to enable precise, directed changes to be achieved such as the repair of pathogenic mutations, deletion of defined regions of the DNA or integration of transgenes to a specific genomic location [73] (Figure 2, Table 1). Expression of transgenes from a defined "safe harbor" site can prevent problems with undesired mutations generated by random insertions, and ensure uniform expression levels. Recently, ZFNs have been used to integrate Factor VIII and IX transgenes into the albumin locus to restore functional gene expression in mouse models of hemophilia [93], providing an example of such a strategy.

It is highly beneficial in many therapeutic contexts to make very precise changes to the DNA sequence, and indeed essential in certain cases. Many genetic diseases such as cystic fibrosis, SCID, hemophilia B and α-tyrosinemia require the repair of mutant alleles in a precise manner to reverse point mutations that are causative in disease. However, HR-based repair is currently difficult to implement at high efficiency and requires simultaneous delivery of an appropriate repair template. Importantly, NHEJ repair vastly predominates over HR in most cell types, especially those which have withdrawn from the cell cycle, such as neurons, where the latter repair process is absent. Significant steps have been made to improve the rates of HR by inhibiting NHEJ pathways both chemically and with transient knockdown by RNAi [94,95], manipulating the timing of genome engineering relative to the cell cycle [96], use of ssDNA templates for homology-directed repair [97] or chemical enhancement of HR pathways [98]. There is little doubt that the rates will be improved still further with additional developments of these systems. Interestingly, a third type of DNA repair, microhomology-mediated end joining has emerged as a useful, if less precise alternative to HR that occurs at different phases of the cell cycle, does not require the HR repair machinery and therefore could be applied to different cell types [99].

Transcriptional modulation

Although introduction of changes in the DNA sequence are beneficial in many ways, such changes are permanent, which brings additional risks should there be undesired effects, or off-targeting. With this in mind, it is also possible to recruit other functional protein domains to desired sites in the genome using these site-specific DNA-binding factors. This can elicit transient up- or down-regulation of the transcriptional activity of specific genes or even complex alterations to transcriptional profiles [58,100–105].

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This strategy could be used to restore gene expression patterns for diseases where these are perturbed, such as reverting the overexpression of the *α-synuclein* (*SNCA*) gene that often occurs in Parkinson's disease [106]. It is also possible to recruit chromatin-modifying factors to specific sites to modulate the epigenetic status of the cell, a feature which is often perturbed in disease [107,108]. Interestingly, once certain epigenetic states are altered, they are able to persist after removal of the initiating factor, thus providing a semi-permanent change to the cell without altering DNA sequence.

DELIVERY SYSTEMS

Application of therapeutic genome engineering requires delivery of the reagents to cells, which can be achieved either directly *in vivo*, or to patient-derived cells cultured *ex vivo*, that are subsequently re-implanted. Each of these has its own advantages, and the choice of system will depend on the specific disease, the accessibility of tissue and availability of *in vitro* culture systems (Table 1). Also, certain diseases are inherently easier to target, since either they only require a small number of repaired cells in order to rescue the pathological symptoms, or the repaired cells obtain a growth advantage compared to the diseased cells, and therefore will eventually outcompete them when grafted *in vivo*.

Ex vivo

Certain tissues, notably within the hematopoietic system, can be removed from patients, cultured *in vitro* and re-implanted after genetic manipulation. However, the number of situations amenable to such manipulation is limited, often due to inaccessibility of the tissue or lack of appropriate *in vitro* culture conditions. However, when this is possible, it provides significant advantages for genome engineering, namely in the delivery of constructs, and the selection of correctly repaired cells prior to implantation. This is particularly relevant when the efficiency of modification is low, such as for HR-based repair strategies, since those cells with the desired modifications can be clonally selected from a small proportion of modified cells and fully analyzed prior to re-implantation.

This can be a tremendously useful strategy for diseases of the hematopoietic system such as SCID [109] and HIV [75], where cells can be removed, cultured, manipulated and transplanted into patients. In the case of HIV treatment, there is also a fitness disadvantage of HIV infection in $CD4^+$ T-cells, which is highly beneficial during re-implantation of modified cells. Deletion of the *CCR5* co-receptor gene not only prevents viral infection, but these modified cells will outcompete the remaining HIV-infected cells in the patient [75–78]. Gene correction has also been shown to be possible at the *IL2RG* locus, where hematopoietic stem cells (HSCs) from a patient with SCID-X1 were repaired by an HR-based mechanism [109]. Given the corrected cells have a selective advantage over the mutant cells, these HSCs can be produced in sufficient quantities for re-implantation. HSCs were able to be autologously transplanted into mice and gave rise to essentially all cell types in the hematopoietic lineage [109], making this strategy extremely valuable for the treatment of many other hematological disorders [110].

Certain other adult stem cells such as intestinal organoids [111] can be cultured *in vitro*, manipulated genetically and effectively grafted into the colon of mice carrying chemically induced mucosal lesions [112]. A recent study of organoids derived from cystic fibrosis patients has demonstrated correction of the causative mutation in the *CFTR* gene [113], and reverts the cellular phenotypes caused by this mutation. This therefore provides an opportunity to treat the intestinal phenotypes associated with this disease.

These studies provide important paradigms for further development in this area. Indeed, the advent of induced pluripotent stem cells (iPSCs) [114,115] and their ability to be differentiated into essentially any cell type vastly increases the general applicability of such strategies [116]. A recent report has shown that it is possible to use CRISPR- or TALEN-directed HR in iPSCs to correct the mutation in the β-globin gene that causes sickle cell anemia, and differentiate the resulting cells into disease-free erythrocytes [117,118]. However, *ex vivo* genetic engineering is still limited by the inefficient differentiation into certain adult cell types, and problems with graft rejection upon re-implantation [116,119]. Equally, certain tissues such as the brain are relatively inaccessible and there are inherent difficulties in re-implanting new cells due to the complex tissue architectures involved, limiting its use in treating certain diseases.

In vivo

In vivo delivery of genome editing components is an attractive alternative that circumvents the problems of tissue accessibility, ability to culture cells *in vitro* and issues with re-implantation. However, delivery of the components necessary for genome engineering is fraught with the same problems as other gene therapy strategies, predominantly in terms of the efficiency of cellular uptake, and immune responses to such interventions [120–122]. Importantly, the genetic changes made by genome engineering are inherently heterogeneous in nature, introducing cell-to-cell variability in genotype, and are delivered to many different cell types throughout the body. This can be an advantage in some situations where the pathology results from multiple tissues, but may also create additional complications through the generation of genetic changes in otherwise healthy tissues.

A major advantage of genome engineering for gene therapy applications is that the genetic changes that are created are permanent, so they only need to be generated once per cell. Indeed this could be achieved by multiple sequential treatments that could be administered over a period of time, each of which only targets a small proportion of cells. Additionally, if the genetic changes can be made in stem cells, these will repopulate the tissue with the repaired genome, and therefore offer a permanent solution.

Genome engineering reagents can be supplied in a number of different ways–as DNA plasmids, incorporation into viral vectors, or provided as *in vitro*-transcribed mRNAs/sgRNAs or recombinant proteins. Each strategy is being developed, and has advantages for gene therapy in certain situations, or in delivery to certain tissues [121–124].

Viral vectors are perhaps the most advanced of these, and efficient

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systems of delivery of lentiviral (LV) [123] and adenovirus-associated virus (AAV) [124] vectors to a variety of different tissues have been developed and successfully used to deliver ZFNs *in vivo* [97]. However, both TALEN and CRISPR/Cas9 systems require expression of relatively large proteins. For example, the *S. pyogenes* Cas9 protein is too large to be packaged into a single AAV vector with its cognate sgRNA and it is not possible to package a TALEN pair in a single AAV vector (Figure 1). Development of such systems is ongoing, and recent advances include use of dual AAV vectors for CRISPR/Cas9 delivery [125], and development of an AAV vector for the smaller *S. aureus* Cas9 protein [53]. This is able to be packaged into a single AAV along with its respective sgRNA, and has been shown to be highly effective *in vivo* in mice [53]. Other smaller Cas9 variants such as the Cpf1 protein from *Francisella novicida* provide opportunities for other analogous systems [55]. Many current viral vectors will integrate randomly into the genome, and this has caused problems in previous gene therapy trials [3]. The fact that genome engineering reagents only need to be transiently expressed to cause permanent effects on the genome raises the possibility to use integration-deficient forms of the viral vectors removing this caveat [109].

Equally, systems for direct delivery of DNA, RNA and protein are becoming available, including liposome-, nanoparticle- and peptide-mediated delivery systems that are highly effective in certain tissues [121,122,126]. These can be complexed with the appropriate biological molecule non-covalently or covalently [127–129], and significant progress is being made in the stability of such complexes in serum, and their delivery into the cell cytoplasm [121]. Similarly, exosomal vesicles have shown promise as delivery systems for many tissues, and can be targeted to specific cell types by decoration with particular cell surface markers [130]. These delivery systems have the advantage that they will only express transiently, reducing the risks of off-target effects. In the case of mRNA and protein they are also unable to integrate non-specifically into the genome, eliminating such unintended side-effects.

Such *in vivo* delivery is especially applicable when the disease only requires a small proportion of cells to be repaired in order to relieve symptoms, such as in the case of hemophilia B or α-tyrosinemia. Hemophilia B results from a defect in Factor IX activity, that results in defects in blood clotting. Restoration of its activity to even a few percent of normal levels in the liver can transform the pathological symptoms of the patient [131]. A study has used AAV vectors to deliver ZFNs and an homologous repair template, resulting in correction of up to 7% of alleles in mouse liver, and an amelioration in the associated phenotypes [97]. Similarly, a mouse model of hereditary α-tyrosinemia resulting from a defect in splicing of the *Fah* gene can be corrected by hydrodynamic delivery of CRISPR/Cas9 nucleases and an oligonucleotide repair template [132]. Although the initial correction is only in 0.4% of liver cells, these gain a selective advantage and expand to form around 33% of liver tissue after 30 days, resulting in correction of the pathogenic phenotypes [132].

High efficiencies of mutagenesis of up to 50% have been achieved in murine liver cells *in vivo* using vectors to deliver CRISPR/Cas9 to generate NHEJ-mediated loss of function mutations in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene [133]. This results in a 35–40% reduction in blood cholesterol levels, and may have therapeutic potential for prevention of cardiovascular disease.

CONCLUSIONS & FUTURE CHALLENGES

The application of genome engineering approaches to human genetic disease is an exciting prospect, with the potential to correct disease-causing aberrations and result in a permanent cure. The ease with which this can be achieved and the optimal strategies involved depends strongly on the nature of the disease. Diseases where a partial restoration of function is sufficient to rescue the pathology, such as cystic fibrosis, or hemophilia B, or where wild-type cells have a selective advantage *in vivo* such as SCID-X1, HIV or α-tyrosinemia will initially provide the most attractive candidates for treatment. Equally, although considerable advances have been made in understanding the genetic basis of complex heritable polygenic disorders through analyses such as genome-wide association studies, few examples show large effects of a single allelic variant [134]. Therefore at least initially, monogenic disorders will provide the best chances for therapeutic genetic intervention.

Loss of function mutations generated by NHEJ repair are the most efficient and easiest to introduce but there are a limited number of diseases that can be treated in this manner, such as bacterial or viral infections, those where protective null alleles have been identified, or where splicing, transcription or miRNAs can be modulated. Therefore, an important future challenge will be to increase the rates of HR-based repair to a level where they are useful *in vivo*. Although significant progress has been made along these lines, further improvements will be necessary before this is generally applicable, other than to those diseases where repair in a small proportion of cells is sufficient. Similarly, the efficiency of delivery systems and their temporal and tissue specificity also needs to be addressed in order for such *in vivo* therapies to become a reality.

Equally important for *in vivo* gene therapies is the identification and elimination of off-target effects, which may cause unintended and permanent changes elsewhere in the genome. Whilst these problems have been addressed to a large extent in the research environment, this is particularly relevant when therapies are applied to the entire body, with approximately 3.7×10^{13} cells [135]. In this context, even an extremely small proportion of off-target mutagenesis could become relevant.

Another promising avenue that removes some of the problems of efficiency and off targeting is *ex vivo* manipulation of patient-derived cells to obtain the correct genetic changes. The ability to characterize both the desired and off-target changes in the genome would allow delicate and precise manipulations to be performed, and reduce the chances of undesirable effects. Further work to improve culture systems for adult stem cells, and techniques for their efficient re-implantation will therefore be important if this strategy is to be more generally employed.

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Perhaps the most extreme and controversial example of *ex vivo* genome engineering is the modification of the germline, resulting in every cell in the body containing the desired changes. This has been extremely successful in the case of mice [49] and even monkeys [46], and recent work has suggested that this is possible in humans [136]. However, there are many ethical implications to such modifications, not least of which being that any effects, beneficial or detrimental, will be passed on to subsequent generations, therefore irreversibly affecting the course of human evolution [7,8].

The use of genome engineering for therapeutic applications is a stimulating, fast moving field and perhaps one of the most exciting aspects of such therapies is their general applicability. Once the systems for modification of the genome, and delivery of the reagents and are established, it can be applied with relatively minor modifications to essentially any genetic disease. The developments in this area in the coming years will no doubt be extremely interesting, and may signify the beginnings of a cure for many genetic diseases.

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AFFILIATION

Andrew Bassett
University of Oxford, Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE, UK
+44 (0)1865 285489
andrew.bassett@path.ox.ac.uk

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