

THEMATIC REVIEW

65 YEARS OF THE DOUBLE HELIX

The advancements of gene editing and potential application to hereditary cancer

Zi Ying Tan^{1,2}, Taosheng Huang³ and Joanne Ngeow^{1,2,4,5}¹Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore²Institute of Molecular and Cell Biology, Singapore³Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA⁴Cancer Genetics Service, Division of Medical Oncology, National Cancer Centre Singapore, Singapore⁵Oncology Academic Clinical Program, Duke-NUS Medical School Singapore, SingaporeCorrespondence should be addressed to J Ngeow: Joanne.Ngeow.Y.Y@singhealth.com.sg

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Abstract

Hereditary cancer predisposition syndromes are associated with germline mutations that lead to increased vulnerability for an individual to develop cancers. Such germline mutations in tumour suppressor genes, oncogenes and genes encoding for proteins essential in DNA repair pathways and cell cycle control can cause overall chromosomal instability in the genome and increase risk in developing cancers. Gene correction of these germline mutations to restore normal protein functions is anticipated as a new therapeutic option. This can be achieved through disruption of gain-of-function pathogenic mutation, restoration of loss-of-function mutation, addition of a transgene essential for cell function and single nucleotide changes. Genome editing tools are applicable to precise gene correction. Development of genome editing tools comes in two waves. The first wave focuses on improving targeting specificity and editing efficiency of nucleases, and the second wave of gene editing draws on innovative engineering of fusion proteins combining deactivated nucleases and other enzymes that are able to create limitless functional molecular tools. This gene editing advancement is going to impact medicine, particularly in hereditary cancers. In this review, we discuss the application of gene editing as an early intervention and possible treatment for hereditary cancers, by highlighting a selection of highly penetrant cancer syndromes as examples of how this may be achieved in clinical practice.

Key Words

- ▶ CRISPR-Cas
- ▶ gene correction
- ▶ germline mutation
- ▶ human genetics
- ▶ precision medicine

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Introduction

Hereditary cancers are due to inheritance of cancer predisposition genes in which germline mutations are associated with increased cancer risks (Garber & Offit 2005). Mutations can be in the form of (i) large genomic deletions encompassing all or most of protein function,

(ii) frameshift mutations and (iii) point mutations which introduce stop codon (nonsense) or amino acid substitution (missense). Pathogenic germline mutations can be broadly classified as loss-of-function or gain-of-function mutations which are disease-causing or

disease-associated, in adherence to American College of Medical Genetics standards and guidelines (Richards *et al.* 2015). Decades' worth of clinical, epidemiological and functional studies have established genotype–phenotype association of cancer predisposition genes and the key pathways they control (Vogelstein & Kinzler 2004, Hodgson 2008, Rahman 2014). Such key pathways are signalling cascade-controlling cell proliferation, DNA damage and DNA-mismatch repair pathway, cell cycle checkpoint, apoptosis and cell survival. For patients who have been diagnosed with hereditary cancers, current treatment options include surgery, radiation, chemotherapy and gene therapy. Despite the strides made and successes achieved in cancer treatments, patients with hereditary cancers and other genetic disorders are looking primarily for measures that can mitigate their increased risk and prevent cancer initiation. This is an area whereby there has been limited success, and emerging genome editing strategies may provide the possibility of precision medicine based on targeted gene correction.

Genome editing toolbox

The fundamental concept of the gene editing field is that targeted DNA double-strand breaks (DSBs) in mammalian cells could be used to stimulate endogenous DNA repair machinery. Two major repair pathways are either homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Capecchi 1989, Takata *et al.* 1998). HDR is dependent on a repair template with ends homologous to the break site, whereas NHEJ functions to repair without a template, and is therefore error prone and often ends up with insertions and/or deletions (indels) at the break site. HDR is useful in targeted gene editing, whereas NHEJ is used in targeted mutagenesis to generate functional knockout. The molecular scissors that recognize and cut DNA are categorized into zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system, which are able to recognize specific DNA/RNA sequences via protein–DNA or RNA–DNA interactions and cleave DNA. The detailed comparison of ZFNs, TALENs and CRISPR-Cas has been reviewed extensively (Doudna 2014, Chira *et al.* 2017, Guha *et al.* 2017).

ZFNs and TALENs

ZFNs and TALENs are hybrid proteins, which contain DNA-binding domains tethered to DNA cleavage restriction

enzyme *FokI* (Li *et al.* 2011) (Fig. 1A and B). Both classes of protein are dimeric in nature, thus requiring design of two independent DNA-binding domains to target a single sequence. Specificity of ZFNs depends on forward and reverse strand sequences conferred by left and right zinc finger DNA-binding modules, respectively, plus a linker between these two DNA-binding arrays and *FokI* catalytic domain that can induce HDR (Kim *et al.* 1996, Miller *et al.* 2007). ZFNickase is engineered via inactivation of one monomer in a ZFN dimer, thus improving HDR by reducing unwanted mutagenesis from NHEJ (Ramirez *et al.* 2012). In contrast, TALENs work in a more sophisticated way – for each 33–39 amino acid repeat positions 12 and 13, determine the target specificity and follow the rules. The rules are: HD binds to C, NK or NN binds to G, NI binds to A and NG binds to T (Boch *et al.* 2009, Miller *et al.* 2011).

CRISPR-Cas

Compared to the ZFN and TALEN systems, the CRISPR-Cas gene editing platform is less time consuming, yet highly affordable. Since the initial identification of bacterial CRISPR array in *Escherichia coli* (Ishino *et al.* 1987), Cas9 isolated from *Streptococcus pyogenes* was later discovered to be able to cleave DNA when guided by RNA (Jinek *et al.* 2012). The application of class 2 type II CRISPR-Cas9 (Makarova *et al.* 2017) has evolved exponentially from bacterial immune system surveillance to mammalian genome editing toolbox especially within the last 5 years (Hsu *et al.* 2014, Lander 2016). The principle of CRISPR lies in two components: a Cas9 protein and a ~20bp single-guide RNA (sgRNA), which refers to a synthetic fusion of naturally occurring bacterial CRISPR RNA (crRNA) and trans-activating crRNA (Jinek *et al.* 2012). sgRNA directs the Cas9 protein to its target, with the latter introducing DSBs. Target sites must reside next to and 5' of a protospacer adjacent motif (PAM) sequence that matches 5'-NGG-3'. Therefore, target site recognition can be programmed by designing the 5' end of the sgRNA. Crystal structure of Cas9–sgRNA–DNA ternary complex showed that Cas9 consists of two lobes, a recognition (REC) lobe and a nuclease (NUC) lobe, which create a positively charged groove at their interface, to accommodate sgRNA:DNA heteroduplex (Nishimasu *et al.* 2014). The formation of a DNA–RNA heteroduplex based on base-pairing interaction at a specific recognition site necessitates the complementary binding by the REC lobe and cleavage by Cas9–sgRNA complex by the NUC lobe. Upon conformational change, the target DNA strand

is cleaved by one of the Cas9 nuclease domains HNH, whereas the non-target strand is cut by the RuVC domain, resulting in blunt-end DSBs (Fig. 1C) (Sternberg *et al.* 2015, Jiang & Doudna 2017).

Instead of DSBs, Cas9 nuclease can also be transformed to single-strand DNA Cas9 nickase (Cas9n) via inactivation of one of the two nuclease domains from the NUC lobe. D10A mutation on RuVC domain, or H840A or N863A mutation on HNH domain, renders Cas9n cuts either complementary or non-complementary DNA target strands, thereby improving specificity and increasing HDR repair (Fig. 1D) (Jinek *et al.* 2012, Gasiunas *et al.* 2012, Cong *et al.* 2013, Nishimasu *et al.* 2014). Co-localization of paired Cas9n can be targeted to sites on opposite strands (guided by distinct sgRNA–Cas9 complexes) separated by 4–100bp, so that both indel mutations via the NHEJ mechanism and HDR repair can be generated through double nicking with improved specificity (Mali *et al.* 2013, Ran *et al.* 2013). However, being an active monomer and the addition of a second sgRNA, paired Cas9n system may able to cleave off-target genomic sites and introduce new point mutations (Mali *et al.* 2013, Ran *et al.* 2013). To circumvent this limitation, fusion of an active non-specific FokI endonuclease cleaving domain to a catalytically dead Cas9 (dCas9) domain – which contains both D10A and H840A mutations in its nuclease – would ensure that the fusion protein cleaves only when two simultaneous distinct Cas9–sgRNA complexes bind to adjacent sites (Fig. 1E) (Guilinger *et al.* 2014, Tsai *et al.* 2014). This provides an increased specificity (~4-fold higher), since the chance of dimers causing off-target binding event is lower than the chance of Cas9n monomer off-target binding events (Guilinger *et al.* 2014).

As hereditary cancers can be associated with more than one genetic mutation known to increase cancer risk, targeting and editing multiple genomic sites in one step may be more desirable in these cases in order to save time and cost. This requires stacking of multiple sgRNA cassettes into one plasmid or delivery of multiple constructs, which could be tedious (Cong *et al.* 2013, Xie *et al.* 2015). The latest reports show that multiplex gene editing for up to four genes in mammalian cells and three genes in mouse brain is feasible by using a single-customized CRISPR–Cpf1 crRNA array (Zetsche *et al.* 2016). This is because Cpf1 (also known as Cas12a) is smaller than Cas9 and target recognition relies on only a single but shorter crRNA strand (Zetsche *et al.* 2015).

In addition to various Cas9 protein modifications, altering the length of the DNA:sgRNA length interface seems to influence on-target specificity of CRISPR–Cas9.

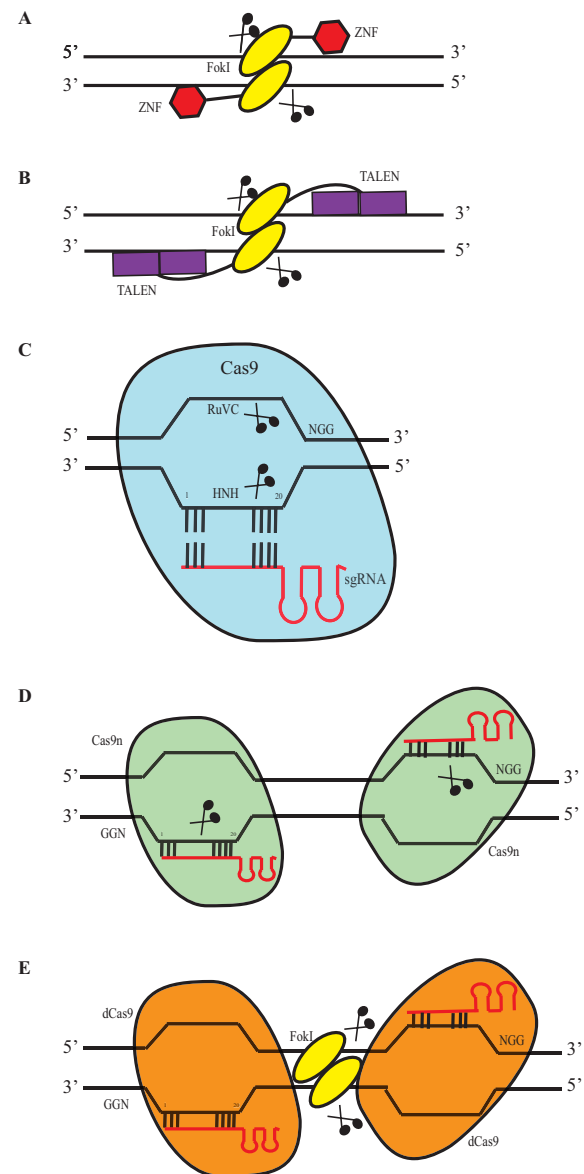


Figure 1

Genome editing toolbox. (A) ZFNs and (B) TALENs are customized DNA-binding domains fused to non-specific FokI endonuclease, which must dimerize to cut the target DNA. (C) CRISPR–Cas9 is an RNA-guided endonuclease that uses sgRNA:DNA base pairing to mediate binding of Cas9 to target sequence. Cas9 cleaves complementary strand with its HNH domain, and cleaves non-complementary strand via RuVC domain, generating a DSB. DNA recognition relies on a PAM sequence (NGG) immediately next to target region complementary to sgRNA. (D) A Cas9n is generated by inactivating nuclease catalytic site in either HNH or RuVC domain, thereby causing single-strand break ('nick'). A paired Cas9n can be targeted by two distinct sgRNAs to improve target specificity. (E) Catalytically dCas9 is another engineered variant, which is defective in DNA cleavage, but retains target recognition and binding ability. It can be fused to non-specific FokI endonuclease so that target site cleavage can only occur when FokI is dimerized, hence increasing target specificity. Cas9n, Cas9 nickase; CRISPR, clustered regularly interspaced short palindromic repeat; dCas9, dead Cas9; DSBs, double-strand breaks; PAM, protospacer adjacent motif; sgRNA, single-guide RNA; TALENs, transcription activator-like effector nucleases; ZFNs, zinc finger nucleases.

Truncated gRNA (<20bp) is shown to decrease unwanted mutagenesis at off-target sites by as much as 5000-fold or more via abrogation of indel formation, with not abrogation of 'functional binding' (Fu *et al.* 2014, Dahlman *et al.* 2015). Coupled with double Cas9n, truncated gRNA can lead to further reduction of off-target effects. This is in contrast to the target effect from lengthening the 5' end of the complementary region, which results in reduction of on-target editing efficiency (Ran *et al.* 2013, Cho *et al.* 2014).

Apart from DNA as the substrate of Cas9, nuclear-localized RNA-targeting CRISPR-Cas9 (RCas9) enabled recognition of endogenous mRNA (O'Connell *et al.* 2014, Nelles *et al.* 2015, 2016). This is based on a mismatch PAM sequence in the PAMmer/RNA hybrid with single-strand RNA (ssRNA) that allows exclusive cleavage of ssRNA in the presence of non-PAM target double-strand DNA (O'Connell *et al.* 2014, Nelles *et al.* 2015). Further development of RCas9 showed that it can support specific targeting and eliminate microsatellite repetitive RNAs such as CUG and CCUG, CAG and GGGGCC, which are toxic RNA species accumulated in nuclear foci responsible for inherited neurological disorders Huntington's disease, myotonic dystrophy and amyotrophic lateral sclerosis (Batra *et al.* 2017). Notably, RCas9 targeting reversed disease phenotypes such as reduced polyglutamine protein, relocated repeat-bound proteins similar to healthy cells, eliminated RNA loci and efficiently reversed myotrophic-related splicing in patient myotubes, suggesting the potential of RCas9 in human therapeutic applications (Batra *et al.* 2017).

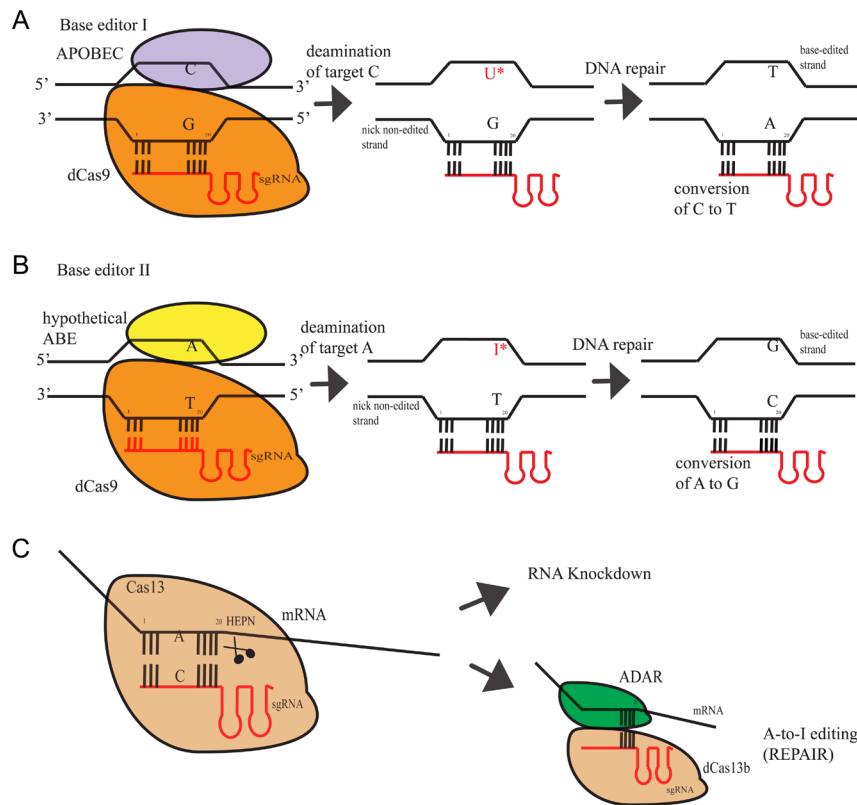
CRISPR 2.0

Repurposing CRISPR-Cas9 has continuously expanded the repertoire of the genome editing toolbox. This is largely based on the exploitation of a dCas9. Given its precise target recognition and binding without DSBs, dCas9 can be fused to various enzymes for different purposes. For example, fusing dCas9 with nucleoside deaminases for direct irreversible nucleotide modification (Komor *et al.* 2017a), fusing dCas9 with fluorescent reporter for tracking and imaging (Nelles *et al.* 2016), and fusing dCas9 with transcriptional activators to mediate gene expression (Liao *et al.* 2017, Zhou *et al.* 2018) and even nuclear reprogramming (Liu *et al.* 2018).

Unlike programmable nucleases which introduce small fragment of transgene or indels by NHEJ at the target site, programmable nucleoside deaminases enable nucleotide substitution or by converting C to T (or G to

A to a lesser extent) without DSBs (Komor *et al.* 2017b). The technique, called base editing (BE), can be achieved through fusion of dCas9 to APOBEC, a cytidine deaminase able to induce cytosine to uracil conversion, without DNA cleavage (Fig. 2A) (Komor *et al.* 2016, Nishida *et al.* 2016, Yang *et al.* 2016). This technique was tested in somatic cell nuclear transfer cloned human embryo – derived from immature oocytes and beta thalassemia patients' skin and blood cells – and was able to correct *HBB*-28 (A>G) point mutation at over 20% repairing efficiency (Liang *et al.* 2017). The fourth generation of BE (BE4) is now being tested to increase efficiency of C:G to A:T BE by approximately 50% while halving the frequency of off-target compared to BE3 (Komor *et al.* 2017a). Interestingly, Cas9-hypothetical adenine base editor (ABE) has been reported to mediate conversion of A:T to C:G base pairing in genomic DNA without DSBs (Gaudelli *et al.* 2017) (Fig. 2B). In this massive effort to evolve tRNA adenine deaminase (TadA) for seven generations in bacteria, subclasses of a novel TadA protein variant (ABE1–ABE7) fused to CRISPR-Cas9 are found to be capable of editing all four transitions (C to T, A to G, T to C and G to A) (Gaudelli *et al.* 2017). This exciting discovery expedites the goal to edit human genetic diseases due to point mutation or single nucleotide polymorphism, as demonstrated by the successful conversion of point mutation that causes hereditary hemochromatosis in lymphoblastic cell line (Gaudelli *et al.* 2017).

Distinct from RNA-guided DNA-targeting CRISPR effectors such as Cas9 and Cpf1, a new variant type VI CRISPR-associated RNA-guided RNA-targeting effector Cas13 (previously known as C2c2) has been discovered to recognize RNA and is specialized to the RNA interference (Abudayyeh *et al.* 2016, East-Seletsky *et al.* 2016, Shmakov *et al.* 2017). In contrast to Cas9 which cleaves DNA, Cas13a enzyme lacks a DNase domain and cleaves RNA with its higher eukaryotes and prokaryotes nucleotide-binding (HEPN) endoRNase domain, which is commonly associated with ribonucleases (Fig. 2C) (Liu *et al.* 2017). The crRNA-target RNA duplex binds in a positively charged central channel of the nuclease lobe, leading to conformational change of the Cas13a protein. This in turn triggers activation of the HEPN catalytic site of Cas13a and subsequently cleavage of both the ssRNA target and collateral RNAs (Liu *et al.* 2017). Interestingly, it has been shown that two orthogonal Cas13a subfamilies LbuCas13a and LbaCas13a cleave ssRNA preferentially at uridines and adenosines, respectively (East-Seletsky *et al.* 2017). Importantly, Cas13a from *Leptotrichia wadei* (LwaCas13a) has been shown to knockdown RNA as effectively as RNA

**Figure 2**

CRISPR 2.0. Base editor I is a fusion protein of dCas9 and APOBEC (A), whereas II is a hypothetical ABE evolved from tRNA adenosine deaminase (B) that catalyses conversion of C>T or A>G, respectively, without generating DSBs. (C) Cas13 is a RNA-guided RNase that cleaves target single-strand RNA with HEPN domain, resulting in RNA knockdown. Fusion of dCas13b to ADAR enzyme catalyses A-to-I RNA editing (REPAIR). ABE, adenine base editor; CRISPR, clustered regularly interspaced short palindromic repeat; DSBs, double-strand breaks; HEPN, higher eukaryotes and prokaryotes nucleotide-binding domain.

interference, but with improved specificity (Abudayyeh *et al.* 2017). This establishes CRISPR–Cas13a as a flexible platform for RNA manipulation – viral RNA detector ‘SHERLOCK’ (Gootenberg *et al.* 2017), transcript tracking and knockdown, and makes it an attractive starting route to expand tools for RNA perturbations and binding.

Like Cas13a, subtype Cas13b proteins – isolated from *Prevotella sp.* – have collateral RNase activity that is activated by target recognition (Shmakov *et al.* 2017, Smargon *et al.* 2017). Analogous to Cas9 BE principle, catalytic inactive dCas13b – mutated at two histidine to alanine sites on HEPN domain H133A/H1058A – can revert two disease-associated G-to-A mutations when it is fused with a catalytic domain of A-to-I editing ADAR2 (Fig. 2C) (Cox *et al.* 2017). The proof-of-concept studies achieved 35% G-to-A correction of 878G>A (*AVPR2* W293X) in X-linked nephrogenic diabetes insipidus and 23% correction of 1517G>A (*FANCC* W506X) in fanconi anaemia (FA) (Cox *et al.* 2017). This dCas13b–ADAR2 system is referred to as RNA editing for programmable A-to-I replacement (REPAIR), which is engineered to edit full-length transcripts containing pathogenic mutations (Cox *et al.* 2017). Use of CRISPR–Cas in RNA-target knockdown or RNA editing is especially beneficial in fixing genetic mutations without causing permanent

DNA changes to the genome, as compared to CRISPR–Cas9 counterpart. More importantly, it bypasses ethical issues as it is considered ‘safer’ for genetic manipulation in living cells without leaving a trace. It is also suitable for transient but rapid alteration to transcript in less actively dividing cells such as neurons and muscle cells.

These data demonstrated that collective power from rationalized bioinformatics/phylogenetic classification, biochemical/structural studies, molecular engineering and functional experiments of CRISPR proteins has revealed more functionally useful proteins and guided scientists in extending innovative applications of CRISPR–Cas genome editing toolbox (Nakade *et al.* 2017, Shmakov *et al.* 2017).

Towards realization of gene editing as potential application in hereditary cancers in the clinic

CRISPR–Cas9 has been rapidly implicated in numerous studies to modify target genes in a variety of human diseases, both in mouse model and human cell lines, as has been reviewed extensively (Sander & Joung 2014, Cai *et al.* 2016, Chira *et al.* 2017). Depending on the nature of disease-causing mutations that predispose carriers to cancer development, there are various ways to perform

specific types of gene editing (Fig. 3). For a gain-of-function germline mutation, the pathogenic function of this protein can be inactivated either via indel formations by NHEJ-mediated gene editing or RNA knockdown by RNA-targeting Cas13 protein (Fig. 3A), so that the constitutively activated mutant oncogene can be silenced. Nonsense mutations that lead to loss-of-function protein can be rescued through HDR-mediated repair, thus restoring normal function (Fig. 3B). Missense mutation of a single base pair can be corrected via the BE mechanism through the addition of four possible nucleotide transitions (Fig. 3C). Precise correction of nonsense and missense mutation via targeted gene editing can restore the normal protein level, activity and function of mutated tumour

suppressor and oncogenes, subsequently maintaining normal cell proliferation through downstream signalling pathways. At the RNA level, REPAIR can be used to correct G>A mutation (Fig. 3D) without genome modification (Cox *et al.* 2017).

Mechanisms behind hereditary cancer predisposition

Hereditary cancers are due to inheritance of cancer predisposition in which germline mutations confer increased risks of cancer (Garber & Offit 2005, Hodgson 2008, Rahman 2014). Averagely 10% of all cancers are caused by inherited mutations, as opposed to *de novo* mutations. The germline mutations cause defects in

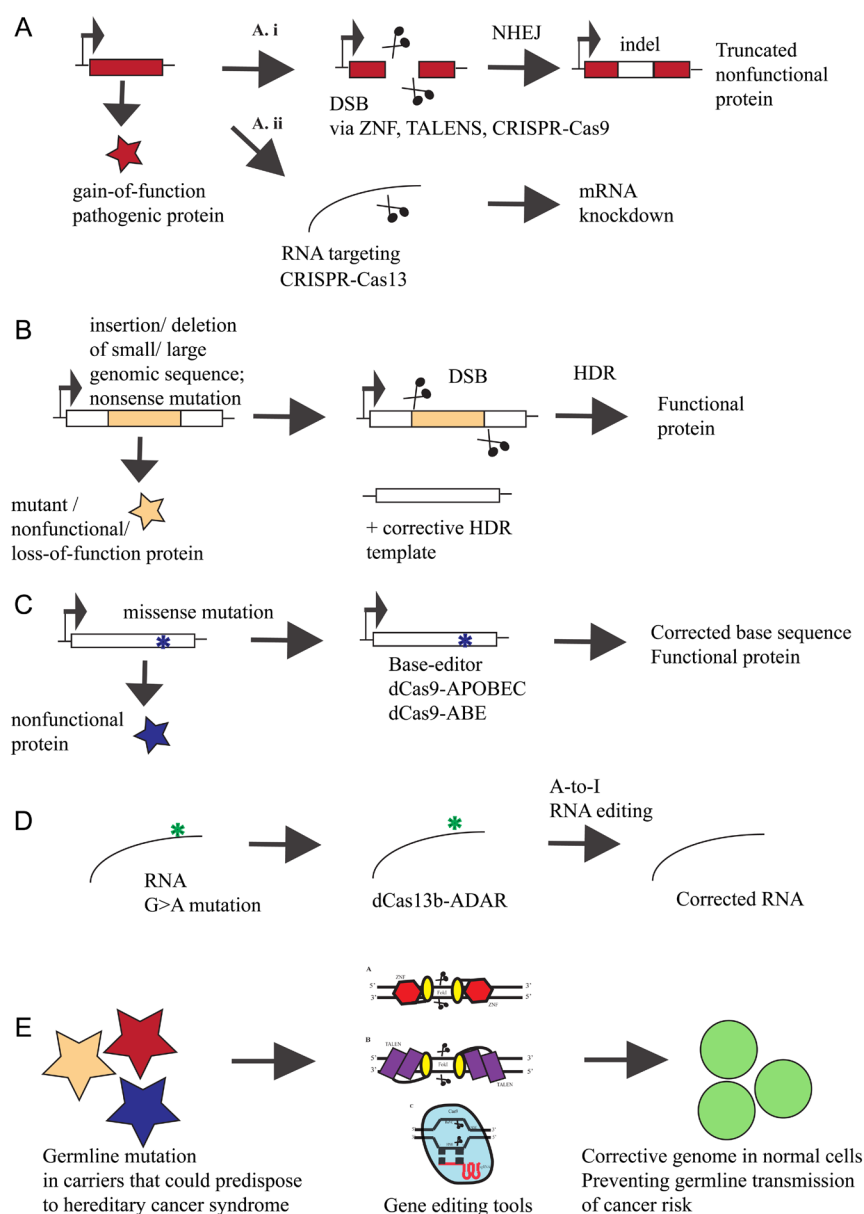


Figure 3

Towards therapeutic gene editing applications in inherited cancers. (A) Inactivation of pathogenic mutation can be achieved either by (i) NHEJ-mediated indel formation on the targeted locus or (ii) RNA targeting by CRISPR-Cas13 to knockdown RNA. (B) Loss-of-function mutation caused by indel and nonsense mutations can be repaired through HDR-mediated pathway after DSB is generated. (C) Missense mutations can be corrected by base editor methods dCas9-APOBEC and dCas9-ABE, in which all four transitions (C to T, A to G, T to C and G to A) are possible. (D) REPAIR by dCas13b-ADAR2 can edit G>A mutation post-transcriptionally without genome modification. (E) Pathogenic germline mutations can be corrected by various gene editing tools in order to stop germline transmission of disease-causing mutations and eliminate risks of developing inherited cancers. ABE, adenine base editor; CRISPR, clustered regularly interspaced short palindromic repeat; DSBs, double-strand breaks; HDR, homology-directed repair; NHEJ, non-homologous end joining; REPAIR, RNA editing for programmable A-to-I replacement; TALENs, transcription activator-like effector nucleases; ZFNs, zinc finger nucleases.

particular biochemical or molecular pathways that lead to genome instability and subsequently uncontrolled cell growth which promote tumour development (Table 1). Hereditary cancers can be broadly categorized into groups according to the key pathways, which are disrupted due to heritable germline mutations. Mutations in genes encoding for proteins involved in DNA repair damage pathway are accounted for numerous familial clustering of cancers, such as *BRCA1/2* in hereditary breast and ovarian cancer, FA-complementation group in FA, mismatch repair genes in Lynch syndrome, and so on. Loss of DNA repair fidelity in patients harbouring this group of germline mutations leads to increased mutational burden and subsequently higher risk of cancer growth at young age. Genes controlling cell cycle is another major pathway contributing to hereditary cancers. For example, tumour suppressors *TP53*, *PTEN*, *NF1* and *APC* are major players in P53, PI3K/Akt/mTOR, Ras/MAPK and β -catenin/WNT signalling pathways, respectively. Abnormal cell division occurs due to uncontrolled signalling pathways caused by loss of function of these mutated proteins. Contrary to these, gain-of-function oncogenes, such as *RET* and *MET* in multiple endocrine neoplasia type 2 (MEN2) and hereditary papillary renal carcinoma, result in constitutive activated tyrosine kinase pathway, causing cancerous growth. Correcting mutations in these target genes can rebuild functional proteins and the molecular pathways affecting genome stability and cell cycle, thus restoring cellular homeostasis.

Gene editing of cancer predisposition genes

Most of the patients inheriting pathogenic variants of cancer predisposition genes develop early-onset cancers, often at young age. Ideally, gene editing should prevent or reduce risk of early-onset cancers in children with germline mutation inheritance, rather than being offered as cancer treatment option when tumours have already been developed. Hence, the earlier the patients receive gene editing therapeutics, the better prognosis outlook in lifetime cancer risks. Time points of early intervention via gene editing to prevent hereditary cancer initiation can be broadly categorized into three stages: embryo, prenatal, children and young adults (Fig. 4).

Osteogenesis imperfecta patients transplanted with allogenic foetal mesenchymal stem cells (fMSCs) prenatally and postnatally did not suffer from new skeletal damage for the remainder of pregnancy and during infancy, as compared to a patient with similar *COL1A2* genetic defect who was not transplanted and did not die at 5 months

of age (Gotherstrom *et al.* 2014). Based on this clinical case report, prenatal cellular therapy via *in utero* fMSCs transplantation has potential in treating congenital disease before birth, without targeting *COL1A2*-mutated cells (Sagar *et al.* 2018). Moreover, transgenic epidermal stem cell cultures could generate an entire functional epidermis in a child with junctional epidermolysis bullosa, which is caused by inherited mutated laminin gene and often lethal due to chronic wounds and eventually skin cancer (Hirsch *et al.* 2017). These suggest that gene-corrected stem cells could repopulate in the young patients, infants and even in prenatal stage who are carrying inherited mutation, without necessarily targeting cancer cells. Successes from prenatal fMSCs transplantation and gene therapy to treat congenital diseases imply clinical feasibility in using gene-edited stem cells, particularly induced pluripotent stem cells (iPSCs) from patients, as source for cellular therapy or engraftment for long-term repopulation. This would potentially treat hereditary cancers at individual level, but not for their next-generation offspring because it does not cause permanent genome modification.

Gene editing without heritable genome modification

It may not be that straightforward to carry out gene editing in one target cell types especially when one germline mutation increases risks in multiple types of cancers, for instance Li-Fraumeni and Lynch syndromes, as compared to hereditary gastric diffuse cancer when specific mutated *CDH1* is known to affect stomach only. Delivery of gene editing vehicles into patients locally must be efficient to reach a certain threshold unit sufficient enough to transform target cells *in vivo*, which later repopulate in the body. Likewise, transplantation of gene-edited cells *ex vivo* must achieve substantial level of editing and harbour regeneration ability in order to engraft. Alternative approach is to engineer an inducible CRISPR-Cas9 cassette or inducible gRNA into adeno-associated viruses (AAVs) locus in zygotic genome, so that it can be activated for precise editing in a spatiotemporal manner through chemical induction and sgRNAs in later growth phase, in various context-dependent disease scenarios (Davis *et al.* 2015, de Solis *et al.* 2016, Liu *et al.* 2016).

For haematological oncology such as FA, current treatments include administration of androgen, hematopoietic growth factors and bone marrow transplant (Shukla *et al.* 2012, Ebens *et al.* 2017). Early study reports disease-corrected hematopoietic progenitors can be generated from FA-induced iPSCs using lentiviral transgenes gene therapy method (Raya *et al.* 2009).

Table 1 Various key pathways affected by germline mutations in inherited cancers.

Key proteins/pathways function	Cancer predisposition genes	Inherited cancers	Inheritance	Increased tumour risk
DNA damage repair hereditary syndrome Double-strand break repair: homologous recombination	<i>BRCA1/2</i> FA genes* <i>RECQL3</i> <i>ATM</i>	Hereditary breast-ovarian cancers Fanconi anaemia Bloom syndrome Ataxia-telangiectasia	AD AR AR AR/AD	Breast, ovarian, prostate, pancreas Bone marrow failure, head and neck cancers, leukaemia Leukaemia, lymphoma, breast, colon, skin Breast, leukaemia, lymphoma
Double-strand break: homologous recombination and non-homologous end joining	<i>MRE11</i> <i>WRN</i>	Nijmegen syndrome Werner syndrome	AR/AD AR	Lymphoid, breast Soft tissue sarcoma, colon, thyroid, skin, pancreas
Mismatch repair genes	<i>MSH2</i> <i>MSH6</i> <i>MLH1</i> <i>PMS2</i>	Lynch syndrome	AD	Colon, endometrial, ovarian, prostate, gastric
Base excision repair Nucleotide excision repair	<i>MUTYH</i> XP genes: <i>XPA</i> , <i>XPC</i> , <i>DDB2</i> , <i>ERCCT-5</i> , <i>POLH</i>	Polypsis Xeroderma pigmentosum	AR AR	Colon, duodenum, gastric Melanoma
Tumour suppressor p53 signalling pathway Hypoxia-induced factor signalling pathway	<i>TP53</i> <i>VHL</i>	Li-Fraumeni Von Hippel–Lindau syndrome	AD AD	Sarcoma, breast, brain, adrenocortical carcinoma Retinal angioma, cerebellar, spinal, brain stem hemangioblastomas, renal cell carcinoma, pheochromocytoma Paraganglioma–pheochromocytoma, gastrointestinal stromal tumour
β -Catenin/WNT signalling pathway Ras/MAPK	<i>SDHA</i> <i>SDHB</i> <i>SDHD</i> <i>APC</i> <i>CDH1</i> <i>NF1</i> <i>PTPN11</i>	Hereditary paraganglioma–pheochromocytoma Familial adenomatous polyposis Hereditary diffuse gastric cancer RASopathies Noonan syndrome	AD AD AD AD AD	Polyposis, colon, small bowel, stomach, pancreas, thyroid Gastric cancer Neurofibrosarcoma, optic glioma, leukaemia Juvenile myelomonocytic leukaemia, neuroblastoma, breast and colon cancer Rhabdomyosarcoma, neuroblastoma, bladder cancer, fibrosarcoma
Retinoblastoma pathway	<i>HRAS</i> <i>Rb1</i> <i>CDKN2A</i>	Costello syndrome Hereditary retinoblastoma Hereditary melanoma pancreatic syndrome	AD AD AD	Retinoblastoma, osteosarcoma Melanoma, glioblastoma, pancreatic cancer
PI3K/AKT/mTOR signalling pathway	<i>PTEN</i> <i>MEN1</i>	Cowden syndrome, PHTS Multiple endocrine neoplasia 1	AD AD	Hamartoma, breast, thyroid Pancreatic neuroendocrine tumours, pituitary adenoma, parathyroid hyperplasia
TGF- β signalling pathway Proto-oncogene Tyrosine kinase	<i>BMPRIA</i> , <i>SMAD4</i> <i>RET</i> <i>MET</i>	Juvenile polyposis syndrome Multiple endocrine neoplasia 2 Hereditary papillary renal carcinoma	AD AD AD	Colon, stomach, small bowel Pheochromocytoma, medullary thyroid carcinoma Type 1 papillary renal cell cancer, bilateral multifocal type 1 papillary renal cell cancer
Telomere biology disorder Telomere maintenance	<i>DKC1</i>	Dyskeratosis congenita	AD	Bone marrow failure, oral leukoplakia, nail dysplasia, head and neck cancer

Cancer predisposition genes are genes that can be targeted in gene editing so that mutations can be corrected and affected pathways can be restored. AD, autosomal dominant; AR, autosomal recessive; PHTS, PTEN hamartoma tumours syndrome.

To date, two *FANCA* lentiviral gene transfer clinical trials are ongoing, one at Fred Hutchinson Cancer Research Center (NCT01331018), another one at Vall d'Hebron Research Institute (NCT02931071). Reports from clinical trial show that while safe integration of gene transfer cassette was well tolerated, the rapidly diminished number of transduced cells in the periphery remained the most challenging task (Adair *et al.* 2016a,b). To overcome this limitation, gene-corrected patient iPSCs-derived hematopoietic stem cells (HSCs) can be transplanted to FA patients (Navarro *et al.* 2016). The critical differences between allogenic HSCs and gene-corrected iPSCs are that the former method requires matched donors and immunosuppressant drugs. iPSCs are the ideal cell type for gene editing therapeutics due to their self-renewal property, differentiation potential, less immunogenicity and less aggressive than current treatment options such as chemotherapy and radiation. Numerous studies demonstrate successful editing of mutated FA genes such as *FANCA*, *FANCC*, *FANCI*, *FANCD1* and *FANCF* by ZNF-mediated, helper-dependent adenoviral vector, lipid-cationic-mediated CRISPR-Cas9 BE and RNA-edit

platforms (Liu *et al.* 2014a, Rio *et al.* 2014, Osborn *et al.* 2015, 2016, Cox *et al.* 2017, Komor *et al.* 2017a, Skvarova Kramarzova *et al.* 2017) (Table 2). Colony-forming unit potential of gene-corrected FA iPSCs is reasonably high at approximately 12% of CD34+CD43+ (Rio *et al.* 2014), suggesting reasonable fitness in HSC repopulation. Gene-corrected FA iPSCs are proven to display phenotypic reversions such as restoration of normal *FANCA* protein expression level, *FANCD2* monoubiquitination and being able to form *FANCD2* nuclear foci, rescue of mitomycin C sensitivity and chromosomal instability (Liu *et al.* 2014a). However, another study reported that gene-corrected FA primary cells may be outcompeted by diseased cancerous cells or normal untreated cells, rendering the benefit of treatment low (Osborn *et al.* 2015). Clonal derivation of FA primary cells is not possible (Osborn *et al.* 2016). Therefore, long-term repopulating HSCs and progenitor cells differentiated from gene-corrected iPSCs (Sugimura *et al.* 2017) are the ideal cell types for transplantation in FA patients.

In glioblastoma and neuroblastoma, affected cells are heterogenous postmitotic cells, it is extremely

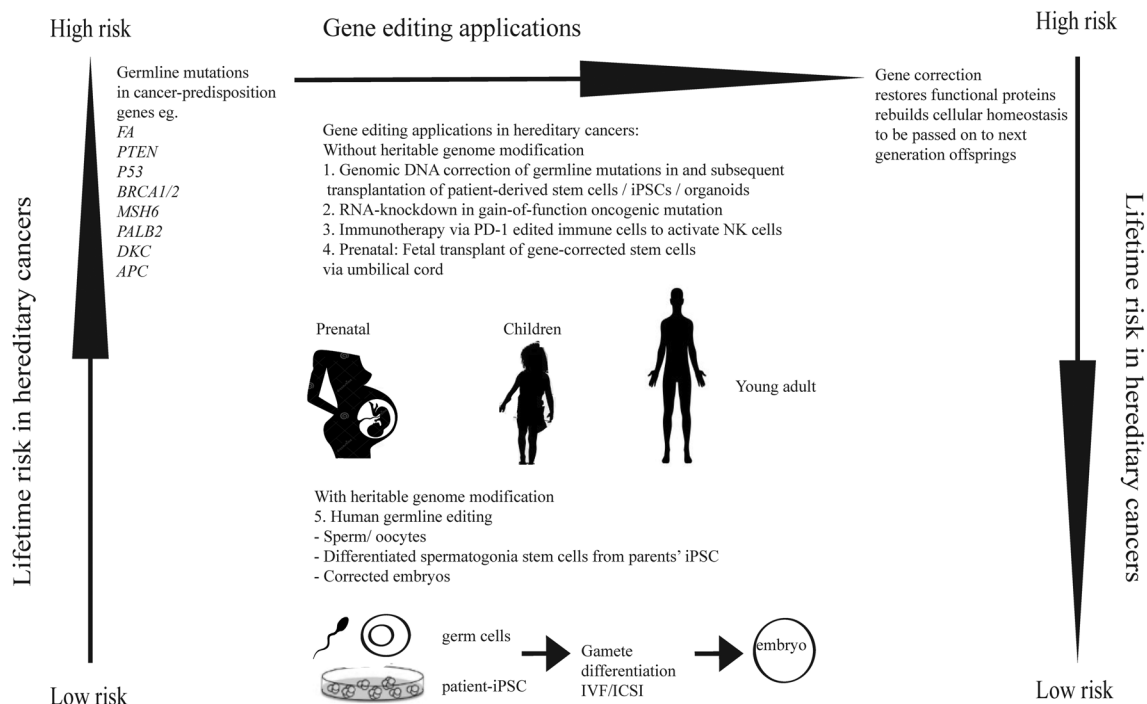


Figure 4

Gene editing applications show great promises in correcting hereditary germline mutations as a medical intervention to prevent early development of hereditary cancers in patients. Stages where gene editing intervention can be introduced are (i) embryo/human germline; (ii) prenatal stage; (iii) children and young adults in order to lower lifetime hereditary cancer risk. Germline mutations can be corrected through multiple ways of gene editing with or without heritable genome modification in order to restore normal protein functions, either through human germline or gene-corrected patient-derived iPSCs and stem cells, knockdown RNA in gain-of-function oncogene expression, and immunotherapy via PD-1 to increase activated NK cells. iPSCs, induced pluripotent stem cells; NK, natural killer.

Table 2 Examples of gene editing in various germline mutations associated with human inherited diseases.

Inherited cancers	Mutations	Edited cells	Delivery method/agent	Editing method	Reference
Cowden syndrome	<i>PTEN</i> c.164+1_164+2insG (42MGBA); c.125T>G L42R (T98G)	Glioblastoma cell lines 42MGBA and T98G	AAV-mediated	HDR	Hill <i>et al.</i> (2017)
Fanconi anaemia	<i>FANCA</i> c.710-5T>C and c.3558insG	Patient iPSC	Lentiviral, AAVS1 locus	ZFN-mediated targeted insertion; HDR	Rio <i>et al.</i> (2014)
	<i>FANCC</i> c.456+4A>T	Patient fibroblast	Lipofectamine	CRISPR HDR	Osborn <i>et al.</i> (2015)
	<i>FANCI</i> c.1461T>A and c.3058+4A>G	Patient fibroblast and iPSC	AAV-puromycin plasmid	CRISPR HDR	Osborn <i>et al.</i> (2016)
	<i>FANCC</i> c.1517 G>A W506X	HEK293FT	AAV	RNA-edit by ADAR	Cox <i>et al.</i> (2017)
	<i>FANCD1</i> (886delGT and 6162insT)	Patient fibroblast and iPSC	Lipofectamine-plasmid	CRISPR HDR	Skvarova Kramarzova <i>et al.</i> (2017)
	<i>FANCF</i>	HEK293T cells	Lipofectamine-plasmid	Base editing by APOBEC	Komor <i>et al.</i> (2017a)
Tumour suppressor	<i>p53</i> C>T (Tyr163Cys)	Human breast cancer HCC1954	Nucleofection	Base editing by APOBEC	Komor <i>et al.</i> (2016)
Hypertrophic cardiomyopathy (HCM)	<i>MYBPC3</i> 4 bp GAGT deletion	Sperm; human zygote	AAV	HDR	Ma <i>et al.</i> (2017)
β-thalassemia	<i>HBB</i> -28 A>G	SCNT cloned human	Lentiviral plasmid	Base editing by APOBEC	Liang <i>et al.</i> (2017)

AAVs, adeno-associated viruses; CRISPR, clustered regularly interspaced short palindromic repeat; HDR, homology-directed repair; iPSCs, induced pluripotent stem cells; SCNT, somatic cell nuclear transfer; ZFN, zinc finger nucleases.

challenging to edit non-dividing cells as CRISPR editing pathway requires DNA replication and cell division. As compared to current megatherapy offered to children with neuroblastoma, genetically corrected neural iPSCs may show great promise in treating inherited brain tumours. For example, *PTEN* correction in two glioblastoma multiforme (GBM) cell lines by AAV-mediated gene editing showed attenuated cellular proliferation ([Hill *et al.* \(2017\)](#)), which was a desired outcome after restoration from hyperactivated PI3K/Akt/mTOR cell cycle pathway due to a mutated tumour suppressor gene *PTEN*. This implies that gene-corrected patient-derived glioma stem cells could potentially be beneficial in brain cancer treatment.

Moreover, intestinal organoids have been transplanted to repair damaged colon, albeit at low engraftment success ([Yui *et al.* 2012](#), [Huch *et al.* 2013](#)). Organoids are self-organizing stem cells in a diverse and complex multicellular tissue system. A proof-of-concept study showed gene-corrected organoids cultured from cystic fibrosis patients' intestinal stem cells can be achieved and expanded ([Schwank *et al.* 2013](#)). This combination of gene-edited stem cells 3D organoids derived from patients' adult stem cells is envisioned as autologous stem

cell therapy for currently incurable hereditary diseases ([Yin *et al.* 2016](#)). This could be relevant to treating young adults with hereditary cancers such as Lynch syndromes, by incorporating gene-corrected intestinal organoids into young patients' colon after polyp removal to repair damage or intervene earlier to displace the polyp growth due to germline mutations.

In contrast, constitutively activated mutant oncogene can be inactivated either by indels by NHEJ-mediated gene editing or RNA knockdown. Hereditary lung cancer syndrome target never smokers with an estimated 31% risk with germline *EGFR* T790M mutation ([Gazdar *et al.* 2014](#)). Analysis of tumour DNA for *EGFR* exons 18–21 revealed an additional L858R mutation in exon 21 ([Gazdar *et al.* 2014](#)). L858R is a classical activating oncogene mutation accounting for ~40% of all *EGFR* mutation. Targeted disruption of allele specific oncogene L858R by the CRISPR-Cas9 via adenovirus (Ad) has led to tumour growth inhibition in mouse lung cancer xenograft model ([Koo *et al.* 2017](#)). As highlighted, RNA knockdown by CRISPR can be applied routinely or daily course of targeted gene silencing; this may be potentially useful in silencing gain-of-function oncogene expression

such as constitutively activated *RET* in MEN2, a dominant inherited cancer that affects neuroendocrine organs. *RET* knockdown by shRNA in medullary thyroid cancer derived cells increased cells' sensitivity to cisplatin-induced apoptosis (Bagheri-Yarmand *et al.* 2015). This can be an alternative choice to achieve long-term silencing of oncogenes without DNA modification in patients who are inheriting these germline mutations.

Gene editing in immune cells to tackle hereditary cancers could potentially alter immune surveillance and influence the course of overall cancer treatment. For example, studies show that among children with FA, dyskeratosis congenita and Cowden syndrome patients, natural killer (NK) cells are significantly lower (Jyonouchi *et al.* 2011, Myers *et al.* 2011, Starink *et al.* 1986). Targeting *PD-1* in mouse model of GBM shows that NK cells are increased and the tumour growth is slower in the treated group (Huang *et al.* 2015). This implies that targeting *PD-1* via CRISPR could be potentially useful in activating NK cells differentiation and cytotoxic potential through T-cell-based therapy in patients with hereditary cancers. However, this application of gene editing does not aim to prevent transmission of germline mutations to offspring.

Gene editing with heritable genome modification

While editing the germline is the easiest and most powerful way to demonstrate gene editing's curative potential in inherited cancers, it is also the most embroiled in controversy (Doudna & Sternberg 2017). Proof-of-concept studies have demonstrated that CRISPR-Cas9 gene editing in sperm and zygote resulted in successful correction of inherited disease-causing mutations. For instance, the *MYBPC3* and *HBB* genes accounted for hypertrophic cardiomyopathy and beta thalassemia, in early human embryonic developmental stage *in vitro* (Liang *et al.* 2017, Ma *et al.* 2017) (Table 2). In this study, they specifically targeted the 4bp GAGT deletion in the *MYBPC3* gene in human embryos with a total targeting efficiency of 72.7%, which is higher than 27.9% in iPSC lines. This suggests a more efficient delivery by zygote microinjection. DSBs in human gametes and zygotes were preferentially resolved using an endogenous HDR mechanism, using WT allele from the oocyte as the repair template, as they did not find any evidence of HDR using exogenous single-strand oligo donor (Ma *et al.* 2017).

Potential clinical routine for the hereditary cancer prevention via gene editing would most likely involve genetic testing, genetic counselling, preimplantation genetic diagnosis and prenatal testing. Options exist for

couple who intend to reproduce when they know both of them are carriers of pathogenic mutation of hereditary cancer predisposition genes. Human germline editing is possible through editing either male or female germ cells, IVF or ICSI-produced embryos and differentiated gametes from patient-derived iPSCs (Fig. 4) (Vassena *et al.* 2016). In addition to common risk of embryo manipulation *in vitro* such as chromosomal instability, there are multiple risks associated with these germline or embryo gene editing steps (Ishii *et al.* 2017a). While there remain numerous uncertainties at the human embryo editing step, it is crucial to achieve balance between the opportunities of rescuing hereditary germline mutations of cancer predisposition genes and the risks of gene correction via CRISPR.

As of this writing, human germline gene editing therapy is limited to the *in vitro* research model (Evitt *et al.* 2015, ACMG Board of Directors 2017, Ormond *et al.* 2017, Howard *et al.* 2018). Clinical use of human germline gene editing is prohibited currently. It should proceed only if (1) safety and efficacy issues are resolved; (2) society has agreed on bounds; (3) appropriate oversight is in place; (4) justice and equity concerns are addressed and (5) it is transparent, as largely recommended by a total of 11 organizations, led by the American Society of Human Genetics (Table 3) (Ormond *et al.* 2017) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Detailed ethical, legal and social issues are being highlighted in Supplementary Fig. 1 for consideration before moving towards realization of a responsible gene editing in humans (Howard *et al.* 2018, de Wert *et al.* 2018). In the future, gene editing may also impact on genetic counsellors when it becomes an available option in the clinics (Capps *et al.* 2017).

Challenges in translating gene editing from bench to bed

The simplicity of short length sgRNA in guiding specificity in the whole genome has made the CRISPR-Cas9 system a popular choice, but at the same time, off-target issues remain as the primary concern in therapeutic application (Fu *et al.* 2013, Pattanayak *et al.* 2013, Cho *et al.* 2014). Numerous online tools are available to assess the optimized sgRNA design based on customized queries, namely CRISPR design tool, CasFinder, E-Crisp, CCTop and ZiFiT targeter. Conversely, there are tools for off-target prediction such as CROP-IT and Cas-OFFinder. For therapeutic applications, off-target analysis must be carried out to assess nuclease dosage and delivery

Table 3 ASHG human germline editing statement.

Arguments	The Hinxton group	NAS, NAM, CAS and UK RSIS	NAS and NAM committee on human gene editing	ASGCT and JSGT	ISSCR	Baltimore <i>et al.</i> (2015)	EGE	Lanphier & Urnov (2015)	ACMG	NIH	HFEA
Basic research should be conducted	X	X	X	X	X	X					X
Preclinical research should be conducted		X	X								
There should be a partial or full moratorium on research							X	X		X	
Diverse stakeholders should be involved in decision-making	X	X	X	X	X	X	X	X	X		
Any public policies regulating this area of science should be flexible	X										
Clinical use should not proceed currently	X	X	X	X	X	X	X	X	X		
Clinical use should proceed only if safety and efficacy issues are resolved	X	X	X	X	X	X	X		X		
Clinical use should proceed only if society has agreed on bounds	X	X	X	X	X		X		X		
Clinical use should proceed only if appropriate oversight is in place	X	X	X				X				
Clinical use should proceed only if justice and equity concerns are addressed	X			X			X				
Clinical use should proceed only if it is transparent			X			X					
Clinical use should be discouraged worldwide						X					

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ACMG, American College of Medical Genetics; ASGCT, American Society for Gene and Cell Therapy; CAS, Chinese Academy of Sciences; EGE, European Group on Ethics in Science and New Technologies; HFEA, UK Human Fertilization and Embryology Authority; ISSCR, International Society for Stem Cell Research; JSGT, Japan Society of Gene Therapy; NAM, US National Academy of Medicine; NAS, US National Academy of Sciences; NIH, National Institutes of Health.

approach to ensure optimal conditions that produce the most favourable genomic surgery with high accuracy (Bolukbasi *et al.* 2015). In fact, it is particularly challenging to decide what is a 'tolerable' off-target activity from a genomic surgery (Tasan & Zhao 2017). Several novel unbiased genome-wide off-target detection platforms have been built in cell-free assays or cell-based assays, as have been reviewed extensively (Zhang *et al.* 2015,

Tsai & Joung 2016, Jung *et al.* 2017, Tasan & Zhao 2017). A combination of both *in vivo* and *in vitro* analysis may be more comprehensive in identification of off-target sites, which can be affected by native chromatin structure and overall cell fitness effects due to certain mutations (Tasan & Zhao 2017). However, it is still not clear that these measurements would be sufficient to prevalidate *in vivo* editing approaches.

In addition, not every germline mutations can be targeted by *S. pyogenes* CRISPR-Cas9 system due to the absence of PAM sequence immediately near the target site. Therefore, more combinations of Cas9 variants from different prokaryotes that exhibit alternative non-canonical PAM sequences are continuously being discovered and engineered for a broader usage.

For a successful editing process, another challenge lies in effective delivery of gene editing tools to the target cell, and ensuring that they have sufficient half-lives for editing to take place before getting diluted out of cells (Yin *et al.* 2017). Delivery methods can be categorized into viral or non-viral. There are currently three types of viral delivery systems: retroviruses (lentiviral vector is the subclass), Ads and AAVs. AAVs are widely used in the gene therapy as they have the advantage of non-pathogenic, high transduction rate and stable expression in dividing and non-dividing cells (Daya & Berns 2008). Compared to other virus vehicles, the limitation of AAVs is their 4.7 kb transgene size limit. Non-viral delivery system in gene transfer includes cell-penetrating particle (CPP) (Liu *et al.* 2016), cationic materials (like lipofectamine) and nanoparticles (Jin *et al.* 2009, Chen *et al.* 2016). In contrast, non-viral vectors are cheaper and easier to prepare, have no issue of insertional mutagenesis, allow for a limitless size for the transgene and are less immunogenic and oncogenic but tend to obtain lower expression of transgene. CPP-mediated gene editing has been demonstrated to disrupt endogenous gene in human cell line via delivery of CRISPR-Cas9 complex (Ramakrishna *et al.* 2014, Suresh *et al.* 2017) and TALENs (Liu *et al.* 2014b). A recent study demonstrated a novel delivery vehicle called CRISPR-Gold – which is composed of gold nanoparticle conjugated to DNA plus cationic endosomal disruptive polymers – could deliver Cas9 ribonucleoprotein complex and donor DNA into a wide variety of cell types, including immune cells, muscle cell progenitors, human iPSCs and human embryonic stem cells (Lee *et al.* 2017). The group also tested CRISPR-Gold in correcting DNA mutation that causes Duchenne muscular dystrophy in mice's legs via local injection, reporting that there is no toxic effect and minimal off-target effect observed (Lee *et al.* 2017). However, gold nanoparticle uptake into immune cells is known to activate pro-inflammatory cytokines, suggesting that this approach does have immunostimulatory properties (Dykman & Khlebtsov 2017). Despite showing efficient delivery at low nanomolar protein concentration, this system ought to be optimized in terms of adjusting physiochemical parameters such as size, shape, electrical

charge and surface functionalization for safety evaluation in preclinical development (Zuris *et al.* 2015).

Most recently, study reports presence of preexisting humoral and cell-mediated adaptive immune system of Cas9 in humans (Charlesworth *et al.* 2018). Abundances of antibody targeting SpCas9 and SaCas9 found in human serum are as high as 65 and 79%, respectively. This suggests that preexisting anti-Cas9 antibodies in human would most probably impede Cas9 nuclease activity. This important factor must be taken into account as the CRISPR-Cas9 genome editing is making a stride into clinical trials.

Conclusion

Inheritance of pathogenic germline mutations predisposes carriers to increased risk of developing cancers. Genome editing shows great promise to correct pathogenic mutations. However, biological risks possibly introduced by genome editing platforms are difficult to assess. These include possible toxicity of nucleases delivered into the human cells, undesirable mutation from irreversible genomic changes, gene repair failure that instead results in obtain off-target mutation causative of other diseases and embryo mosaicism (Cornu *et al.* 2017, Ishii 2017a). Mosaicism in gene-edited embryos is unacceptable in clinical applications, even though studies have shown that mosaic embryos can develop into healthy euploid newborns at significantly lower implantation rates as compared to euploid embryo transfer (Ma *et al.* 2017, Munné & Wells 2017, Munné *et al.* 2017).

The rise of gene editing will be a constant tug-of-war between two forces – private interests/for-profit initiatives and public good – because human germline genome editing is likely to be expensive, limited geographically and might not be covered by all payers and health systems (Ormond *et al.* 2017). If the main government of a country does not cover the cost of many known inherited genetic disorders, the benefit of disease prevention by germline modification will only be restricted to the wealthier social entities who can afford it, potentially creating social disparities based on market economy (Evitt *et al.* 2015, Ishii 2017b). Without public funding to support germline editing research, there is a risk that research will go offshore with less oversight and transparency (Ormond *et al.* 2017).

Notably, the National Institutes of Health just announced \$190 million funding to support Somatic Cell Genome Editing program over 6 years. The focus of this

program is to dramatically accelerate the translation of these technologies to the clinic for treatment of as many genetic diseases as possible. Clinical trial has started in Europe/United States by CRISPR Therapeutics to treat inherited blood disorder beta thalassemia. With this, we anticipate the day that pathogenic variants will be eliminated from the population as a result of gene drives and mechanistic evolution via gene editing techniques (Esvelt *et al.* 2014) (Fig. 3E), offering precision medicine in the best interest of patients with hereditary cancers.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0039>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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