Gene editing and Rett syndrome: Does it make the cut?

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Abstract

Rett syndrome (RTT) is a rare neurogenetic disorder caused by pathogenic variants of the Methyl CpG binding protein 2 (*MECP2*) gene. RTT is characterized by apparent normal early development followed by regression of communicative and fine motor skills. Comorbidities include epilepsy, severe cognitive impairment, and autonomic and motor dysfunction. Despite almost 60 clinical trials and the promise of a gene therapy, no cure has yet emerged with treatment remaining symptomatic. Advances in understanding RTT has provided insight to the complexity and exquisite control of *MECP2* expression, where loss of expression leads to RTT and over expression leads to *MECP2* duplication syndrome. Therapy development requires regulated expression that matches the temporal and spatial endogenous expression of *MECP2* in the brain. Gene editing has revolutionised gene therapy and promises an exciting strategy for many incurable monogenic disorders including RTT by editing the native locus and retaining endogenous gene expression. Here we review the

literature on the currently available editing technologies and discuss their limitations and applicability to the treatment of RTT.

Introduction

Gene replacement therapy is appealing for monogenic disorders as it restores the functional copy of a deficient gene to re-establish expression and has shown promising results in recent clinical trials for monogenic neurological disorders such as spinal muscular atrophy (SMA)[1]. However, for disorders such as Rett syndrome (RTT), where gene expression is dose-dependent, gene editing may prove the only suitable gene therapy solution as it can potentially reinstate endogenously controlled Methyl CpG binding Protein 2 (MeCP2) expression in the brain. Gene editing technologies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein 9 (Cas9), have rapidly changed the gene therapy field by offering an alternative form of therapy from conventional gene replacement therapies by altering the genome *in situ*. Applications of genomic editing technologies have created opportunities in many sectors, ranging from basic research to applied biotechnology as well as in the clinic with novel therapies such as EDIT-101 for Leber Congenital Amaurosis 10 entering clinical trials[2]. Editing technologies are continuously advancing and can now target and modify DNA with high specificity, resulting in precise and permanent editing events. However, site-specific targeting of gene editing is not perfect due to off-target editing events. Alternative editing technologies, including transient approaches such as RNA editing, are being pursued concurrently and provide a potentially safer option to DNA editing strategies. RNA editing creates temporary edits where off-target editing events are short lived. For monogenic disorders, both DNA and RNA editing may offer therapeutic options that maintain native regulation of gene expression. The development of gene editing technologies that reactivate the silenced wild type X chromosome are also underway and may be of therapeutic utility to address the mosaic expression of *MECP2* in RTT. Here we outline the reported editing tools designed for the central nervous system (CNS) and discuss whether RTT falls within their technological reach and if they overcome the therapeutic challenges posed by this complex disorder.

Rett syndrome and gene editing opportunities

Rett syndrome is a rare neurodevelopmental X-linked disorder arising from loss of function pathogenic variants in the *MECP2* gene and predominantly affects females[3]. RTT occurs in 1 in

10,000 live female births with more than 800 different pathogenic variants identified with over 95% of disease-causing mutations arising *de novo*[4]. *MECP2* plays a significant role in regulating transcription, especially in neurodevelopment, as it is highly expressed in neurons[3, 5-7]. Onset of RTT typically occurs around 6-18 months of age after a period of normal development followed by developmental stagnation and regression along with the onset of stereotypic midline hand movements and severe impairment of communication and apparently also of cognition[8-10].

Murine studies have demonstrated the reversal of some key RTT phenotypes in mouse models by reintroducing *MECP2* after symptomatic onset[11-13]. Although these studies are not clinically translatable to humans as they involve genetic manipulation of the genome using Cre-recombinase, they provide evidence of phenotype rescue and restoration of functional *MECP2* following symptomatic presentation; they therefore encourage and motivate the search for gene-based "cures" in human patients. Currently, therapies for RTT are limited to symptomatic treatments that target associated comorbidities. Of almost 60 clinical trials conducted, there has been a significant focus on symptomatic treatment and enhancing quality of life. As phenotypic reversal has been demonstrated in murine models, clinical trials focusing on curative therapies, such as gene replacement therapy, have naturally attracted the attention of major pharmaceutical companies.

The pathology of RTT predominantly lies in the brain with neurons, astrocytes and microglia being most affected[5, 14]. Despite the allure of canonical gene-addition therapy, gene replacement therapy for RTT is vexed with many challenges including access through the blood-brain barrier (BBB) and the complexity of *MECP2* expression in the human brain. The BBB comprises a network of endothelial cells, astrocytes and pericytes and acts as an interface between circulating blood and the brain; it remains a substantial obstacle to therapies targeting neural cells by oral or systemic administration. Successful delivery by alternate delivery modalities such as via the CSF may be achieved however this mode of delivery is also limited by the penetration capacity. For gene therapies to successfully target the affected cells, the therapeutic payload must first be delivered across the BBB, unless the treatment is administered via the intrathecal route, as employed when adeno-associated virus (AAV) vectors are used. The current preferred and only clinically validated vector for CNS-targeted AAV-based gene therapy, following systemic administration, is AAV9.

MECP2 expression in the human brain is complex as the gene is located on the X chromosome and is subject to X chromosome inactivation (XCI), along with around 90% of gene loci on the X chromosome, which means that each cell is functionally monosomic for *MECP2* and most other loci on the X in both males and females. Girls with RTT, therefore, express both healthy and mutant copies of *MECP2* in a mosaic expression pattern. Skewed XCI has been reported in RTT where an

imbalance of each allele expression results in differences in disease severity, despite their harbouring the same mutation[15].

The spatial and temporal expression of *MECP2* in the brain is tightly regulated by endogenous elements (5' promoter and 3' untranslated region), which control expression levels throughout life[6, 16, 17]. This stringent regulation of expression can be referred to as the "Goldilocks" principle, whereby too little and too much expression both lead to a disease phenotype. Over-expression of *MECP2* leads to *MECP2*-duplication syndrome, with effects most marked in males[18], while under-expression results in a severe encephalopathy in males (affecting every cell) and in females (where under-expression is mosaic) it leads to RTT. Conventional gene replacement therapy is challenged by the complex nature of *MECP2* expression, as introducing additional working copies of the gene into a cell that is already expressing a healthy copy of the gene would be expected to result in over-expression-related phenotype. Gene editing, on the other hand, where mutations are corrected at the native locus, provides an appealing potentially safer alternative, providing a therapeutic opportunity that could restore endogenous gene expression. However, the technology is not without its limitations. Here we review the literature on editing technologies and their delivery to the CNS and consider whether RTT falls within their technological reach (Figure 1).
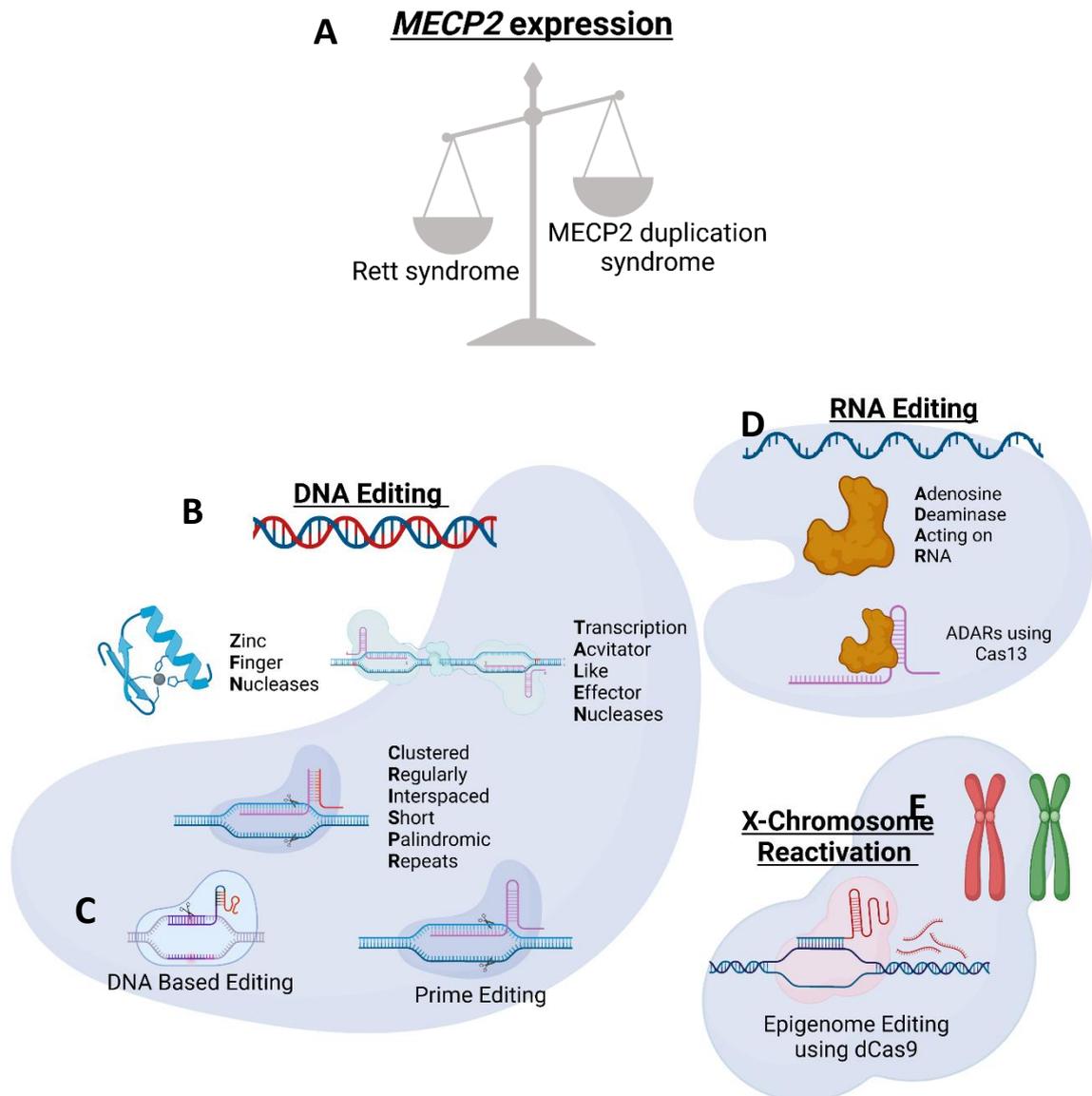
Figure 1. Overview of editing technologies reviewed to overcome the balancing act of MECP2 expression within the 'Goldilocks' principle. A) Under expression of *MECP2* leads to RTT as detailed by the lower scale, however, overexpression leads to MECP2 duplication syndrome detailed by the higher scale. **B)** Editing strategies include the use of Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). **C)** Specifically for DNA editing, ZFNs, TALENs and CRISPR editing is used but so is DNA base editing using nicking Cas enzymes and also Prime Editing using CRISPR technology. **D)** RNA editing involves the use of the naturally occurring adenosine deaminases acting on RNA (ADARs) but can also be specifically guided by adopting CRISPR technology and using Cas13 enzymes. **E)** Reactivation of the inactive wild type X-Chromosome to reintroduce *MECP2* expression is a possible therapy by editing the epigenome of the inactive X-Chromosome using dead Cas9 (dCas9).

DNA Editing strategies:

The ability to alter an organism's DNA at the native locus using genomic editing techniques provides the potential to correct pathogenic variants to both prevent and treat disease. Site-specific breaks in the DNA are required to achieve this and currently, three distinct classes of nucleases have been discovered and bioengineered for laboratory and clinical use. These are zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9[19-21]. Each of these technologies involves a nuclease for DNA cleavage and editing occurs as the cell undergoes repair following a break in the DNA.

Zinc Finger Nucleases

ZFNs were the first reported programmable nucleases to cleave DNA at specific target sites using an adapted *FokI* endonuclease[20, 22-24]. While ZFNs have been tested in human gene therapy clinical trials for induced mutagenesis and knockdown of the HIV co-receptor CCR5 for prevention of HIV infection[25] and other disorders, such as HPV and blood disorders, this technology has not yet expanded to the CNS and neuronal disorders. Interestingly, zinc finger-based artificial transcription factors have been shown to cross the BBB in a Marfan's syndrome mouse model demonstrating their potential utility in editing genes in disorders of the CNS[26].

Transcription Activator-Like Effector Nucleases

TALENs emerged from ZNFs, where the *FokI* endonuclease is combined with modular DNA binding domains of transcription activator-like effectors (TALEs)[27]. The multiple scaffold designs use different amino- and carboxyl-terminal truncations, various *FokI* nuclease linkers and several nuclear localisation sequences, which enables a more versatile system to that of ZNFs[28]. TALENs are currently being tested in clinical trials for diseases such as human papillomavirus (HPV) related cervical cancer and relapsed and refractory B-cell acute lymphoblastic leukaemia[29], but again have yet to be tested in CNS disorders. TALENs are limited by their ability to only edit simple mutations.

Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR Associated Protein

CRISPR-Cas has revolutionised the gene editing field by creating a more user-friendly editing tool that can cut DNA with high specificity[30]. These cuts are introduced by a Cas enzyme directed by a single guide RNA (sgRNA) to precise recognition sites known as protospacer adjacent motifs (PAM), where a break in the DNA is introduced[31, 32]. Various different Cas enzymes exist and create an array of cuts at targeted sites, for example Cas9 creates a double stranded blunt cut while Cas12 results in staggered ended cuts[33]. Utilisation of the most common system, CRISPR-Cas9, in mammalian cells requires a nuclear localisation signal for mammalian expression, a fused CRISPR-RNA (crRNA) with trans activating crRNA (tracrRNA) transcript and the sgRNA appended to the PAM sequence

appropriate to the CRIPSR-Cas9 species[31, 32]. Currently, there are more than 20 clinical trials testing CRISPR-Cas9 gene editing strategies for the treatment of a range of diseases ranging from sporadic cancers to inherited genetic diseases[29]. Excitingly, the phase I/II clinical trial targeting the CEP290 gene for Leber congenital amaurosis type 10 in photoreceptor cells is the first *in vivo* clinical trial (NCT03872479) using CRISPR-Cas9[2].

Each of these editing systems has its own strengths and shortcomings, many of which are common across all editing technologies, such as off-target cutting, which can result in unpredictable side effects. In addition, in the context of X-linked disorders like RTT and dominant genetic disorders, editing poses the threat of damaging the wild type allele, which is essential for maintaining the base level of expression of the gene of interest. ZFNs have advanced the field of programmable site-specific gene editing technology, however, their utility is limited as they lack specificity and require a high workload in generating the ZFN constructs, which can only interact with sequences composed of triplets[34]. While TALENs offer more specific targeted binding to longer sequences and are more flexible in that they recognise individual nucleotide bases which can be designed for specific recognition sites within the gene of interest, their use entails a complex workload. CRISPR-Cas9, on the other hand, is a more versatile gene editing toolkit for both laboratory and clinical use, and for complex disorders such as RTT where mutations span the full length of the *MECP2* gene.

CRISPR technology, however, is not without its own limitations. While the commonly used SpCas9 has a small and therefore more versatile PAM consensus sequence (NGG) which is frequently found throughout the genome, enabling more guide flexibility, the enzyme coding region is very large (4101bp) resulting is limitations to downstream transduction and transfection applications. SaCas9 (3156bp), on the other hand has a more restrictive PAM site (NNGRRT) reducing guide design flexibility, however this results in less off-target cutting. Clinical concerns such as the uncontrolled expression of the Cas enzymes have been documented where Cas9 has been shown to trigger an immune response and result in uncontrolled off-target cutting, causing accidental permanent changes in the genome. Specifically, off-target cutting has also been shown to create large deletions and rearrangements at target sites[35]. In an attempt to provide a "kill switch" for Cas9 and reduce these off-target effects, phage derived anti-CRISPRs are being developed[36]. However, this technology is still in its infancy and requires further refinement to achieve therapeutic delivery. Another concern surrounding the use of CRISPR-Cas9 mediated therapies is the cell-mediated immune response to non-self-proteins, as anti-Cas9 responses have been observed in healthy human adults[37]. This pre-existing adaptive immunity suggests Cas9 therapies may be impeded by natural neutralising antibodies[37].

Novel Integration Strategies for Non-dividing Cells

A fundamental impediment to editing cells of the CNS is bringing about changes to the genome in non-dividing cells. Non-dividing cells, such as neurons, commonly use the error-prone Non-Homologous End Joining (NHEJ) repair strategy to repair breaks in DNA. Neurons are the major target cells in RTT and are post-mitotic, rendering homology directed repair (HDR)-mediated gene editing ineffective. Novel research strategies that utilise the aforementioned DNA editing methods to introduce double stranded breaks in post-mitotic cells for gene editing are being continuously developed which include vSLENDR, HITI, SATI and HMEJ and are discussed below.

Virus-mediated Single-cell Labelling of Endogenous Proteins via HDR (vSLENDR)

vSLENDR is a CRISPR-Cas9 viral-mediated genome editing strategy with demonstrated success in mitotic and post mitotic cells in *in vivo* and *in vitro* models[38]. This strategy hijacks the HDR repair pathway which occurs in post mitotic cells such as neurons[39] and uses AAV-mediated delivery of a repair template[38]. As neurons are the primarily affected cells in RTT this makes vSLENDR an applicable tool for editing the *MECP2* gene. vSLENDR experiments have been performed in an array of post-mitotic murine cells using dual vector delivery systems with one vector containing the Cas9 machinery and the other containing the repair template. While these experiments were extensive and a good proof of principle concept, use of dual vector delivery systems is far from being accepted into clinical trials. Studies have also highlighted concerns surrounding the use of AAV delivery for Cas9, which can occasionally result in cleavage of the AAV capsid-encoding DNA and integration into the genome[40]. Another consideration of the vSLENDR knock-in strategy, is its inability to circumvent the deleterious consequences of the existing expression from the mutant allele.

Homology Independent Targeted Integration (HITI)

The CRISPR-Cas9 HITI system essentially hijacks the NHEJ repair machinery in cells to knock-in genes at targeted sites assisted by a repair construct[41]. The HITI strategy utilizes the same sgRNA target site on the target genome to that of the repair construct allowing both the native locus and the template to be simultaneously cut. This method has been tested in both mitotic and post mitotic cells and shows effective knock-in in non-dividing neurons *in vitro* and *in vivo*[41]. Despite the promising applicability to edit post mitotic cells, the technology has its limitations such that it can only insert DNA at precise locations and cannot be used to repair point or frameshift mutations[42] which make up the majority of mutations in RTT. Additionally, while the data indicates a high template insertion frequency, demonstrating a high chance of the uni-directional insertion of the repair construct, there is still the possibility that the repair construct could be inserted in the incorrect orientation, or not integrated at all[41].

Homology Mediated End Joining (HMEJ)

Similarly, HMEJ also utilises CRISPR-Cas9 mediated targeted integration whereby both the transgene donor vector containing gRNA target sites with ~800 bp homology arms and the targeted genome region are cleaved and either homologous recombination or NHEJ can result in transgene integration[43]. The strategy is used for knock-in of relatively small genes and achieves effective yields in mitotic and post-mitotic cells including neurons[43]. This knock-in strategy could be applied to *MECP2* deletions but would require personalised treatment specific for each patient, which incurs greater costs. However, HMEJ cannot be applied to insertion mutations or single base pair mutations. Despite showing efficacy *in vivo,* only invasive methods of injection directly into the visual cortex have been tested[43] demonstrating proof of principle but not providing a clear and safe translational path. Ideally, less invasive delivery methods such as systemic injections can be used to treat the young girls with RTT.

Single homology Arm donor mediated intro Targeting Integration (SATI)

Due to the limitations of HITI, SATI was designed with the aim of expanding the capabilities of HITI gene knock-in. SATI performs gene knock-in by utilising a donor vector possessing a single homology arm guided by a uniquely designed intron-targeted cleavage site[44]. This strategy has been demonstrated in post-mitotic cell populations of neurons *in vivo* and *in vitro,* providing sufficient demonstration to be considered for use in treatments for RTT[44]. Validation of this strategy in humanised models will aid in the clinical translatability for patients. The use of a single homology arm has shown greater flexibility in this system compared to systems such as HMEJ, which require two homology arms and reduce the size of the insert gene which can be packaged. However, as with HITI, SATI can only be used for gene knock-in and will not be able to target the range of mutations observed in RTT.

Other methods such as PITCh (Precise Integration into Targeted Chromosome)[45] have been demonstrated using TALENs and CRISPR editing machinery. Although these systems have been demonstrated *in vitro* and *in vivo* in cell lines, no reported cases of use in non-dividing cells have been demonstrated. The utility of PITCh in non-dividing cells is yet to be determined.

DNA Base Editing

Many pathogenic variants in the human genome are point mutations involving changes in a single nucleotide[46]. Base editing is a strategy used to target and correct point mutations without causing a double stranded break in the DNA and thus without incurring the associated side effects. There are twelve possible base edits consisting of four transitions and eight transversions. Currently, DNA base editors can only perform the four transition edits. The first class of DNA base editor was the cytosine

base editor (CBE), APOBEC1, which facilitated C to A base changes by converting cytosine to uracil and then adenosine[47]. There are several classes of CBEs, each advancing on the efficiency of the previous class[47, 48]. The third-generation base editors (BE3) are a class of CBE's that utilise a Cas9 nickase (nCas9) to introduce a single stranded break as well as the cytidine deaminase and uracil glycosylase inhibitor (UGI) for conversion of the base pairs[47]. Another class of base editors are the adenine base editors (ABEs) which facilitate adenine to inosine (I) conversions where I is read as G by polymerases, by fusion of dCas9 with a mutant tRNA adenosine deaminase enzyme (TadA) from *E. coli*[49].

Editing the specific region of DNA where the variant lies is favourable, however, the vast majority of RTT causing variants are C to T transition mutations[50] which are not yet within the capabilities of DNA base editing technology. The delivery of base editing machinery to patients is therapeutically challenging as they require two trans-RNA splicing AAV vectors, each encoding half of the therapeutic construct as it is too large to package into a single AAV vector. Furthermore, the efficiency of these trans-splicing vector-mediated gene expression models is low and dual vector delivery is far from clinical translation, which limits the likely clinical use of this therapy for RTT[51]. Base editing requires personalised therapies specific to each patient, which incurs greater costs.

CRISPR Prime

CRISPR prime editing is a novel method for genome editing designed to target insertions, deletions and all twelve possible base edits without causing double stranded breaks or requiring a donor template[52]. Prime editing involves the use of a novel hybrid prime editor (PE) enzyme consisting of an RNA-programmable nickase Cas9 (nCas9) fused to a reverse transcriptase (RT) to correct mutations at the specified DNA target site[52]. The specified DNA target site is directed by the prime editing guide RNA (pegRNA) which includes extra nucleotides that can be used as a template to synthesise new DNA sequences[52]. Unlike base editing, CRISPR prime can be used to correct mutations including insertions and deletions which account for a large majority of RTT mutations. The prime editing system is limited in packaging by its size (6.2Kb) which does not fit into commonly used vectors, such as AAVs which have a packaging capacity of ~4.7Kb. Studies using prime editing have only been demonstrated using dual lentiviral vectors to accommodate the large DNA constructs, leaving concerns surrounding the use of lentiviruses and their potential for integration into the human genome.

Despite their promise, there are many concerns faced by DNA editing. One of the most obvious is off-target cutting of the Cas9 enzyme, targeting regions other than those specified by the guides,

which can have unsought and, indeed, unknown effects and can be assessed by single-cell whole genome sequencing in preclinical studies. Although off-target editing aims to be circumvented by selecting guides through *in silico* programs which predict the target sites, the efficiency of these cannot be confirmed until the guides are experimentally tested. Another concern is the potential for integration of AAV genomic DNA such as the inverted terminal repeat (ITR) sequences (at the cut sites).

RNA editing strategies:

For disorders such as RTT, where gene replacement therapy is complicated by the potential toxicity of over-expression, RNA editing is an alternative strategy that permits temporary changes to the expression of message RNA (mRNA). The transient nature of mRNA allows for temporary treatment, reducing concerns of permanent off-target damage. Additionally, adenosine deaminases acting on RNA (ADARs) are naturally expressed in the human body, thus reducing adverse immune-related responses. However, RNA editing enzymes are of limited application in that they are highly base-pair specific, thus restricting the number of mutations they can correct. Currently, there are two main methods use in RNA base editing which consist of ADARs and CRISPR-associated base editing.

Base Editing using ADARs

The single stranded nature of RNA makes it easier to edit compared to double stranded DNA by using the naturally occurring ADAR enzymes which act to catalyse the chemical conversion of adenosine (A) to inosine (I) which mimics guanosine (G) in dsRNA substrates[53]. In contrast to DNA base editing, RNA base editing provides a more translational strategy to gene editing therapy as there are fewer sequence constraints due to the available machinery and the single stranded nature of RNA, allowing for more mutations to be targeted. *MECP2* is well suited to test the efficacy of programmable RNA editing in vivo as 36% of mutations are caused by G>A or C>T mutations that create opal stop codons. Indeed, recent studies testing programmable RNA editing using AAV-mediated ADAR2 in a *MECP2$^{317G>A}$* (R106Q) mouse model showed a 50% editing efficiency of *Mecp2* RNA and a restoration of MeCP2 protein localization to heterochromatin in neurons[54]. While high levels of off-target editing were reported, this study demonstrates a proof of principle concept that programmable RNA editing can be utilized to repair mutations in mouse models of RTT.

As *MECP2* message transcripts have a half-life of ~5.2 hours, both incorrect (off-target) and on-target edits in individual transcripts are short lived and will not persist in the cell. However, a major limitation of this approach is single base pair editing, resulting in a personalised treatment approach specific for each mutation. Additionally, this strategy cannot be applied to deletion mutations as it only corrects single nucleotide polymorphisms.

Base Editing using CRISPR

With the discovery of CRISPR-Cas systems, RNA base editing has become a more programmable and precise editing tool as coupling ADARs with CRISPR enables specific targeting of RNA regions[55]. The RNA Editing for Programmable A to I Replacement (REPAIR) strategy makes use of the catalytically inactive type VI CRISPR-associated RNA guided ribonuclease Cas13 (dCas13) to direct the ADAR2 enzyme to mRNA transcripts[56]. The programmable nature of Cas13 enzymes as well as the freedom they possess, not being constrained by PAM sequences, allows them to target and bind specifically within full length transcripts that harbour mutations[56]. REPAIR is a highly effective strategy and has low off-target editing effects. An engineered version is able to fit within the 4.7kb packaging limit of AAV vectors, permitting a more clinically translational therapy[56]. Despite this, the REPAIR system has currently only been tested *in vitro* and it is yet to be determined whether it can effectively edit *in vivo*. Following on from REPAIR, RNA Editing for Specific C-to-U Exchange (RESCUE) has been developed to increase the editing potential of ADAR2[57]. RESCUE has only been demonstrated in cellular models and has not been packaged into delivery systems for therapies[57]. Although both REPAIR and RESCUE only target a single site and demonstrate high on-target editing, off-target editing events have been reported[56, 57].

X Chromosome Reactivation

Reactivating the inactive wild type X chromosome offers an altogether different strategy for RTT as, unlike gene addition therapy, it preserves the endogenous regulatory elements, thus maintaining natural gene expression. The challenge of this concept lies in selectively reactivating the *MECP2* gene alone without activating any of the other 900-1400 genes on the X chromosome. Several strategies can be adopted to reactivate genes such as epigenome editing using dead Cas9 (dCas9), but different strategies are also being investigated which use non-editing strategies such as antisense oligonucleotides.

Reactivation epigenome editing strategies:

Dead Cas9 (dCas9) lacks endonucleolytic activity and, when fused to a transcriptional repressor or activator domain, acts as an epigenetic effector (epi-effector)[58]. This approach has been demonstrated, whereby fusion of dCas9 to the catalytic domain of TET1 targets the *CDKL5* gene on the X-chromosome to cause demethylation of the CpG island at the promoter, resulting in reactivation of gene expression on the inactive allele[59]. The demethylation is thought to be involved in loss of the repressive H3K27me3 histone mark in the promoter of *CDKL5* on the inactive chromosome[59]. The role of epigenetic editing to increase expression of *MECP2* has recently been studied in the context of autism spectrum disorders (ASD), as increased methylation at the *MECP2* promoter and decreased *MECP2* expression has been observed in the brains of ASD patients[60]. These

studies demonstrate the potential for reactivation of the wild type *MECP2* allele as a possible option for RTT using a similar approach.

Conclusion/limitations/future directions

In theory, gene editing strategies offer an appealing therapeutic solution for RTT as they can circumnavigate the issues of *MECP2* dosage. However, these strategies require further refinement in pre-clinical studies before their utility can be evaluated for translation into the clinic. As promising as these strategies are, it is critical that their strengths and limitations are identified, such that these can be harnessed and improved upon to meet the requirements for safe and effective RTT therapies.

Overarching challenges common to all RTT therapies include the ability to traverse the BBB and target the affected cells, which are primarily non-dividing neurons but also astrocytes and glial cells[6]. Whilst AAV9 is currently the gold standard of virus-mediated delivery for targeting the CNS by systemic injection, the efficiency of transduction of neural cells is suboptimal and delivery mechanisms still need further advancement. Additionally, concerns surrounding viral DNA integration after AAV-mediated delivery of CRISPR machinery need to be addressed[40]. To avoid this, other possible systemic delivery options include delivery either by lentivirus or by colloidal drug carriers such as nanoparticles; however, these strategies also require refinement[61].

While the current editing technologies such as ZFN, TALENs and CRISPR-Cas generate targeted and specific breaks in the genome, they vary in their targeting specificities and delivery pay-loads. However, strategies harnessing these technologies to edit non-dividing cells such as neurons, which are the main target sought in RTT therapies, still pose a risk from off-target editing that could impact the subsequent expression of *MECP2* (particularly if the wild type allele is mutated) or other loci.

DNA editing most commonly occurs as the result of creating double stranded breaks in DNA. However, the implications of initiating the repair system outside of homeostatic behaviour is unknown. Studies have shown that creating a double-stranded break in DNA can trigger the dysregulation of a cascade of repair pathways. Two studies have shown dysregulation of the p53 pathway, which has the potential to lead to cancer development, however this was demonstrated in human pluripotent stem cells and immortalised human retinal pigment epithelial cells[62, 63]. Although these cell lines are not relevant to RTT, dysregulation of such pathways should be assessed using transcriptome analysis to ensure that there are not severe off-target effects from CRISPR therapies.

RNA editing is an appealing alternative for gene editing due to the temporary nature of RNA and proof of principle studies demonstrating this strategy are underway[54] This single-base RNA editing approach, where an adenine base (A) is converted to a guanine base (G) would address some known

RTT causing pathogenic variants. However, the reported off-target cutting events are notable and remain to be addressed.

Once a successful mechanism has been set up to achieve the molecular reversion of the disease-causing *MECP2* variants in patients with RTT, other challenges will arise. We mention four.

(i) How to establish the diagnosis so that treatment can be commenced when it will be maximally effective, perhaps before the onset of symptoms? Newborn genome screening could detect many cases of RTT before the onset of symptoms, although it would also raise complex practical and ethical questions of managing large volumes of personal data.

(ii) How to ensure that the new genome-based treatment is available in an equitable fashion? This is a general problem for all health services but is perhaps especially acute in the context of new, gene-based, high-tech therapies. If such treatments are made available only to communities that are already well-served then access to genomics will become another mechanism through which inequities in healthcare, and more generally in life chances, become further entrenched[64].

(iii) If treatment is begun after the onset of symptoms, then what complications or hazards might be expected to arise during therapy or afterwards, as a direct consequence? Such potential hazards include autonomic instability, raised intracranial pressure and the experience of psychiatric symptoms such as confusion and distress[65]. The potential physiological hazards of *MECP2* gene editing can be monitored and managed, although the monitoring might be intensive and invasive. These warnings are not intended as reasons for not attempting to develop such treatments but in the belief that one must not do so 'lightly' but with a recognition of the potential hazards, and therefore tentatively and with caution. It will be imperative for professionals, patients and the public all to embark upon such endeavours with their eyes fully open.

Finally, (iv) ethical consideration surrounding the inheritance of edited cells (i.e. editing of the germline) is central to many ethical debates in the field of gene editing. Although neither the intent nor the target, a therapy intended to edit somatic cells, such as neurons, could result in modifications to germline cells at any point in development. This would be most improbable with the intrathecal injection of a therapeutic viral vector but would be less improbable with a treatment administered systemically. Germline alterations, whether corrections of the *MECP2* variant or completely off-target changes, might then be passed from the modified individual to their offspring. The issue here is that such off-target effects of the therapy could not only affect the individuals receiving the treatment but their children and further descendants too.

The field of gene editing is rapidly expanding, which provides promise of rapid advances in these technologies that can improve the shortcomings of each technology and move therapeutic options for RTT closer to the clinic. Concurrently, the field of delivery has, and is, continuing to improve with a strong focus on CNS delivery. However, while many of these strategies have shown proof of principle in pre-clinical studies, further refinement of technologies and more sophisticated experimental approaches are required before these strategies can enter clinical assessment.

References

1.   Al-Zaidy SA, Mendell JR. From Clinical Trials to Clinical Practice: Practical Considerations for Gene Replacement Therapy in SMA Type 1. Pediatr Neurol. 2019;100:3-11.

2.   Maeder ML, Stefanidakis M, Wilson CJ, Baral R, Barrera LA, Bounoutas GS, et al. Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. Nat Med. 2019;25(2):229-33.

3.   Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. 1999;23(2):185-8.

4.   Trappe R, Laccone F, Cobilanschi J, Meins M, Huppke P, Hanefeld F, et al. MECP2 mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. Am J Hum Genet. 2001;68(5):1093-101.

5.   Armstrong DD. Neuropathology of Rett syndrome. J Child Neurol. 2005;20(9):747-53.

6.   Olson CO, Zachariah RM, Ezeonwuka CD, Liyanage VR, Rastegar M. Brain region-specific expression of MeCP2 isoforms correlates with DNA methylation within Mecp2 regulatory elements. PLoS One. 2014;9(3):e90645.

7.   Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet. 1998;19(2):187-91.

8.   Hagberg B, Aicardi J, Dias K, Ramos O. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. Ann Neurol. 1983;14(4):471-9.

9.   Bienvenu T, Souville I, Poirier K, Aquaviva C, Burglen L, Amiel J, et al. Five novel frameshift mutations in exon 3 and 4 of the MECP2 gene identified in Rett patients: Consequences for the molecular diagnosis strategy. Hum Mutat. 2001;18(3):251-2.

10.   Milunsky JM, Lebo RV, Ikuta T, Maher TA, Haverty CE, Milunsky A. Mutation analysis in Rett syndrome. Genet Test. 2001;5(4):321-5.

11.   Giacometti E, Luikenhuis S, Beard C, Jaenisch R. Partial rescue of MeCP2 deficiency by postnatal activation of MeCP2. Proc Natl Acad Sci U S A. 2007;104(6):1931-6.

12.   Guy J, Gan J, Selfridge J, Cobb S, Bird A. Reversal of neurological defects in a mouse model of Rett syndrome. Science. 2007;315(5815):1143-7.

13.   Tropea D, Giacometti E, Wilson NR, Beard C, McCurry C, Fu DD, et al. Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. Proc Natl Acad Sci U S A. 2009;106(6):2029-34.

14.   Lioy DT, Garg SK, Monaghan CE, Raber J, Foust KD, Kaspar BK, et al. A role for glia in the progression of Rett's syndrome. Nature. 2011;475(7357):497-500.

15.   Amir RE, Van den Veyver IB, Schultz R, Malicki DM, Tran CQ, Dahle EJ, et al. Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes. Ann Neurol. 2000;47(5):670-9.

16.   Zoghbi HY. Postnatal neurodevelopmental disorders: meeting at the synapse? Science. 2003;302(5646):826-30.

17. Fukuda T, Itoh M, Ichikawa T, Washiyama K, Goto Y. Delayed maturation of neuronal architecture and synaptogenesis in cerebral cortex of Mecp2-deficient mice. J Neuropathol Exp Neurol. 2005;64(6):537-44.

18. Van Esch H. MECP2 Duplication Syndrome. Mol Syndromol. 2012;2(3-5):128-36.

19. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43(6):1565-75.

20. Li L, Wu LP, Chandrasegaran S. Functional domains in Fok I restriction endonuclease. Proc Natl Acad Sci U S A. 1992;89(10):4275-9.

21. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science. 2009;326(5959):1509-12.

22. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A. 1996;93(3):1156-60.

23. Kim YG, Chandrasegaran S. Chimeric restriction endonuclease. Proc Natl Acad Sci U S A. 1994;91(3):883-7.

24. Kim YG, Smith J, Durgesha M, Chandrasegaran S. Chimeric restriction enzyme: Gal4 fusion to FokI cleavage domain. Biol Chem. 1998;379(4-5):489-95.

25. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med. 2014;370(10):901-10.

26. Bailus BJ, Pyles B, McAlister MM, O'Geen H, Lockwood SH, Adams AN, et al. Protein Delivery of an Artificial Transcription Factor Restores Widespread Ube3a Expression in an Angelman Syndrome Mouse Brain. Mol Ther. 2016;24(3):548-55.

27. Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, et al. Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A. 2012;109(43):17382-7.

28. Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG, 2nd, et al. In vivo genome editing using a high-efficiency TALEN system. Nature. 2012;491(7422):114-8.

29. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduct Target Ther. 2020;5:1.

30. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709-12.

31. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013;8(11):2281-308.

32. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823-6.

33. Banakar R, Schubert M, Collingwood M, Vakulskas C, Eggenberger AL, Wang K. Comparison of CRISPR-Cas9/Cas12a Ribonucleoprotein Complexes for Genome Editing Efficiency in the Rice Phytoene Desaturase (OsPDS) Gene. Rice (N Y). 2020;13(1):4.

34. Ho BX, Loh SJH, Chan WK, Soh BS. In Vivo Genome Editing as a Therapeutic Approach. Int J Mol Sci. 2018;19(9).

35. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat Biotechnol. 2018;36(8):765-71.

36. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. Nature. 2013;493(7432):429-32.

37. Charlesworth CT, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat Med. 2019;25(2):249-54.

38. Nishiyama J, Mikuni T, Yasuda R. Virus-Mediated Genome Editing via Homology-Directed Repair in Mitotic and Postmitotic Cells in Mammalian Brain. Neuron. 2017;96(4):755-68 e5.

39. Cox DB, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. Nat Med. 2015;21(2):121-31.

40. Hanlon KS, Kleinstiver BP, Garcia SP, Zaborowski MP, Volak A, Spirig SE, et al. High levels of AAV vector integration into CRISPR-induced DNA breaks. Nat Commun. 2019;10(1):4439.

41. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. 2016;540(7631):144-9.

42. Suzuki K, Izpisua Belmonte JC. In vivo genome editing via the HITI method as a tool for gene therapy. J Hum Genet. 2018;63(2):157-64.

43. Yao X, Wang X, Hu X, Liu Z, Liu J, Zhou H, et al. Homology-mediated end joining-based targeted integration using CRISPR/Cas9. Cell Res. 2017;27(6):801-14.

44. Suzuki K, Yamamoto M, Hernandez-Benitez R, Li Z, Wei C, Soligalla RD, et al. Precise in vivo genome editing via single homology arm donor mediated intron-targeting gene integration for genetic disease correction. Cell Res. 2019;29(10):804-19.

45. Nakade S, Tsubota T, Sakane Y, Kume S, Sakamoto N, Obara M, et al. Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9. Nat Commun. 2014;5:5560.

46. Suzuki K. Nature of Mutations in Genetic Disorders. Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 1999;6th Edition.

47. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016;533(7603):420-4.

48. Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW, et al. Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. Sci Adv. 2017;3(8):eaao4774.

49. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature. 2017;551(7681):464-71.

50. Ravn K, Roende G, Duno M, Fuglsang K, Eiklid KL, Tumer Z, et al. Two new Rett syndrome families and review of the literature: expanding the knowledge of MECP2 frameshift mutations. Orphanet J Rare Dis. 2011;6:58.

51. Lai Y, Yue Y, Liu M, Ghosh A, Engelhardt JF, Chamberlain JS, et al. Efficient in vivo gene expression by trans-splicing adeno-associated viral vectors. Nat Biotechnol. 2005;23(11):1435-9.

52. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019;576(7785):149-57.

53. Bass BL, Nishikura K, Keller W, Seeburg PH, Emeson RB, O'Connell MA, et al. A standardized nomenclature for adenosine deaminases that act on RNA. RNA. 1997;3(9):947-9.

54. Sinnamon JR, Kim SY, Fisk JR, Song Z, Nakai H, Jeng S, et al. In Vivo Repair of a Protein Underlying a Neurological Disorder by Programmable RNA Editing. Cell Rep. 2020;32(2):107878.

55. Qu L, Yi Z, Zhu S, Wang C, Cao Z, Zhou Z, et al. Programmable RNA editing by recruiting endogenous ADAR using engineered RNAs. Nat Biotechnol. 2019;37(9):1059-69.

56. Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, et al. RNA editing with CRISPR-Cas13. Science. 2017;358(6366):1019-27.

57. Abudayyeh OO, Gootenberg JS, Franklin B, Koob J, Kellner MJ, Ladha A, et al. A cytosine deaminase for programmable single-base RNA editing. Science. 2019;365(6451):382-6.

58. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173-83.

59. Halmai J, Deng P, Gonzalez CE, Coggins NB, Cameron D, Carter JL, et al. Artificial escape from XCI by DNA methylation editing of the CDKL5 gene. Nucleic Acids Res. 2020;48(5):2372-87.

60. Lu Z, Liu Z, Mao W, Wang X, Zheng X, Chen S, et al. Locus-specific DNA methylation of Mecp2 promoter leads to autism-like phenotypes in mice. Cell Death Dis. 2020;11(2):85.

61. Ingusci S, Verlengia G, Soukupova M, Zucchini S, Simonato M. Gene Therapy Tools for Brain Diseases. Front Pharmacol. 2019;10:724.

62.   Haapaniemi E, Botla S, Persson J, Schmierer B, Taipale J. CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. Nat Med. 2018;24(7):927-30.

63.   Ihry RJ, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, et al. p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. Nat Med. 2018;24(7):939-46.

64.   Hart JT. The inverse care law. Lancet. 1971;1(7696):405-12.

65.   Clarke AJ, Abdala Sheikh AP. A perspective on "cure" for Rett syndrome. Orphanet J Rare Dis. 2018;13(1):44.