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Perspective

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RNA-Guided Adenosine Deaminases: Advances and Challenges for Therapeutic RNA Editing

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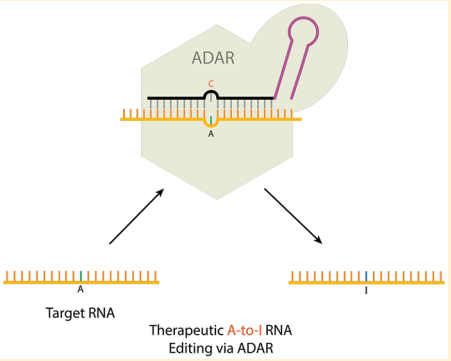
ABSTRACT: Targeted transcriptome engineering, in contrast to genome engineering, offers a complementary and potentially tunable and reversible strategy for cellular engineering. In this regard, adenosine to inosine (A-to-I) RNA base editing was recently engineered to make programmable base conversions on target RNAs. Similar to the DNA base editing technology, A-to-I RNA editing may offer an attractive alternative in a therapeutic setting, especially for the correction of point mutations. This Perspective introduces five currently characterized RNA editing systems and serves as a reader's guide for implementing an appropriate RNA editing strategy for applications in research or therapeutics.



Human genetic diseases are caused by point mutations, insertions/deletions, and chromosomal translocations or copy number variations, with point mutations accounting for ∼58% of all genomic variants causing disease.[1] In this regard, programmable nucleases such as meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas are enabling powerful capabilities to engineer genomes for repairing aberrant function and for deciphering function and programming novel function.[2−7] However, their use for the correction of point mutations *in vivo* poses several challenges. First, the efficiency of homologous recombination (HR) versus nonhomologous end joining (NHEJ) is typically low, particularly in postmitotic cells that comprise the vast majority of the adult body.[8,9] The development of DNA base editors has helped solve in part the problem of reliance on HR to correct point mutations.[10−13] However, these approaches still pose the threat of introducing permanent off-target mutations into the genome, thus presenting formidable challenges in both engineering exquisite targeting specificity without compromising activity and requiring tight regulation of their dose and duration in target cells.[14,15] Finally, several effector systems, such as the CRISPR-Cas systems, are of prokaryotic origin, raising a significant risk of immunogenicity for *in vivo* therapeutic applications.[16,17] To avoid these limitations of DNA nucleases, approaches that instead directly target RNA would be highly desirable, as these would enable tunability and reversibility and importantly no off-target mutations would be permanent. Additionally, RNA, unlike DNA, can be targeted via simple RNA−nucleic acid hybridization.[18] Thus, RNA base editing via RNA-guided adenosine deaminases of human origin could be an attractive approach for *in vivo* correction of disease-causing point mutations. In this Perspective, we provide an overview of the recent advances in the field of RNA base editing while highlighting the challenges that need to be overcome before these sets of tools can be widely used for gene therapy. We also discuss approaches for *in vivo* delivery of RNA editing tools.

ADARs and RNA Editing. Adenosine to inosine (A-to-I) editing is a common post-transcriptional modification in RNA that occurs in a large variety of organisms, including humans. Inosine, being structurally similar to guanosine, functions as a guanosine in the cellular processes of translation and splicing. Adenosine deaminases acting on RNA (ADARs) are enzymes that catalyze the conversion of adenosine to inosine (A-to-I).[19,20] The ADAR family of enzymes is highly conserved among members of the animal kingdom. Three ADAR genes have been identified in vertebrates, ADAR1−3, each of which has one or more double-stranded RNA binding domains (dsRBDs) and a C-terminal deaminase domain. While ADAR1 is ubiquitously expressed across several tissues, ADAR2 is strongly expressed in the cerebellum, lung, and urinary bladder. Both ADAR1 and ADAR2 are known to create thousands of A-to-I edits in the transcriptome.[21] Naturally edited substrates of ADARs include Alu repeat elements, several miRNAs, and mRNA.[22] ADARs are known to play important roles in brain development and defense mechanisms against viruses and other human diseases, including cancers. Complete knockouts of either ADAR1 or -2 enzymes have been shown to be deleterious in mice.[23−25]

ADAR2 Structure and Site Selectivity. Crystallization of the deaminase domain of human ADAR2 bound to its natural substrates, Bdf2 and GLI1 mRNA, has provided unique insights into the catalytic mechanism of ADAR2-mediated A-to-I RNA editing.[26] ADAR2 utilizes a base-flipping mechanism

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Table 1. Comparison of RNA Editing Systems

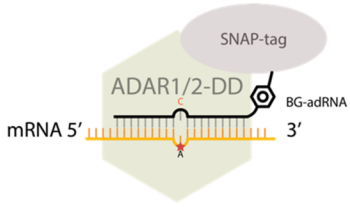
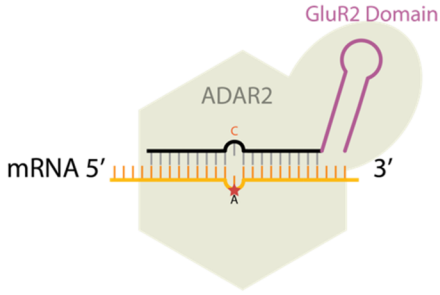
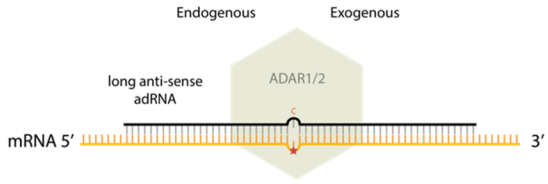
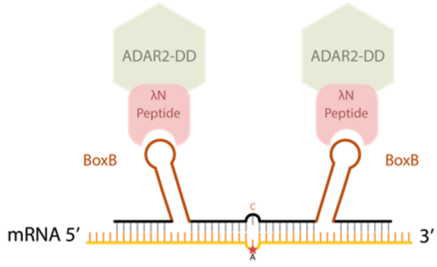
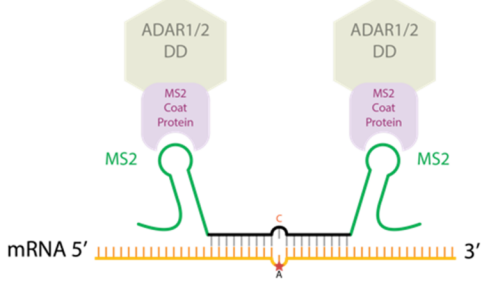
RNA editing system		adRNA sequence (5′ to 3′)	Anti-sense (length, mismatch position)	Deaminase sequence
SNAP-ADAR (36, 39)		O6-benzylguanine-NNNNNNNNNCNNNNNNNNNN	20bp, central	MDKDCEMKRTTLDSPLGKLELSGCEQG LHRIIFLGKGTSAADAVEVPAPAAVLG GPEPLMQATAWLNAYFHQPEAIEEFPV PALHHPVFQQESFTRQVLWKLLKVVKF GEVISYSHLAAALAGNPAATAAVKTALS GNPVPILIPCHRVVQGDLDVGGYEGGL AVKEWLLAHEGHRLGKPGLGPAGGGAP GSGGGSKKLAKARAAQSALAAIFNLHL DQTPSRQPIPSEGLQLHLPQVLADAVS RLVLGKFGDLTDNFSSPHARRKVLAGV VMTTGTDVKDAKVISVSTGTKCINGEY MSDRGLALNDCHAEIISRRSLLRFLYT QLELYLNNKDDQKRSIFQKSERGGFRL KENVQFHLYISTSPCGDARIFSPHEPI LEEPADRHPNRKARGQLRTKIESGEGT IPVRSNASIQTWDGVLQGERLLTMSCS DKIARWNVVGIQGSLLSIFVEPIYFSS IILGSLYHGDHLSRAMYQRISNIEDLP PLYTLNKPLLSGISNAEARQPGKAPNF SVNWTVGDSAIEVINATTGKDELGRAS RLCKHALYCRWMRVHGKVPSHLLRSKI TKPNVYHESKLAAKEYQAAKARLFTAF IKAGLGAWVEKPTEQDQFSLTPSR
ADAR-GluR2 (29, 30, 32, 34)		Transcribed: GUGGAUAUAUAACAA UAUGCUAAAUGUUGU UAUAUAUCCACNNNN NNCNNNNNNNNNNNN NN ASOs: N: rNT n: 2′-OMe rNT n: 2′-OMe rNT and phosphorothioate GGUGUCGAGAAGAGG AGAACAAUAUGCUAA AUGUUGUUCUCGUCU CCUCGACACCnnnnn nNCNnnnnnnnnn	~20bp, ~6bp from 5′	MDIEDEENMSSSSTDVKENNNLDNVSP KDGSTPGPGEGSQLSNGGGGGPGRKRP LEEGSNGHSKYRLKKRRKTPGPVLPKN ALMQLNEIKPGLQYTLLSQTGPVHAPL FVMSVEVNGQVFEGSGPTKKKAKLHAA EKALRSFVQFPNASEAHLAMGRTLSVN TDFTSDQADFPDTLFNGFETPDKAEPP FYVGSNGDDSFSSSGDLSLSASPVPAS LAQPPLPVLPPFPPPSGKNPVMILNEL RPGLKYDFLSESGESHAKSFVMSVVVD GQFFEGSGRNKKLAKARAAQSALAAIF NLHLDQTPSRQPIPSEGLQLHLPQVLA DAVSRLVLGKFGDLTDNFSSPHARRKV LAGVVMTTGTDVKDAKVISVSTGTKCI NGEYMSDRGLALNDCHAEIISRRSLLR FLYTQLELYLNNKDDQKRSIFQKSERG GFRLKENVQFHLYISTSPCGDARIFSP HEPILEEPADRHPNRKARGQLRTKIES GEGTIPVRSNASIQTWDGVLQGERLLT MSCSDKIARWNVVGIQGSLLSIFVEPI YFSSIILGSLYHGDHLSRAMYQRISNI EDLPPLYTLNKPLLSGISNAEARQPGK APNFSVNWTVGDSAIEVINATTGKDEL GRASRLCKHALYCRWMRVHGKVPSHLL RSKITKPNVYHESKLAAKEYQAAKARL FTAFIKAGLGAWVEKPTEQDQFSLTP
Long anti-sense adRNA (32)		...NNNNNNNNNCNNNNNNNNN...	>50bp, central	
λN-ADAR (41–44)		NNNNNNNNNGGCCCT GAAAAAGGGCC NNNNNNNNNNNNNNN NNNCNNNNNNNNNN GGCCCTGAAAAAGGG CCNNNNNNNNN	~40bp, central	NARTRRRERRAEKQAQWKAANGGGGSG GGGSGGGGSNARTRRRERRAEKQAQWK AANLHLDQTPSRQPIPSEGLQLHLPQV LADAVSRLVLGKFGDLTDNFSSPHARR KVLAGVVMTTGTDVKDAKVISVSTGTK CINGEYMSDRGLALNDCHAEIISRRSL LRFLYTQLELYLNNKDDQKRSIFQKSE RGGFRLKENVQFHLYISTSPCGDARIF SPHEPILEEPADRHPNRKARGQLRTKI ESGEGTIPVRSNASIQTWDGVLQGERL LTMSCSDKIARWNVVGIQGSLLSIFVE PIYFSSIILGSLYHGDHLSRAMYQRIS NIEDLPPLYTLNKPLLSGISNAEARQP GKAPNFSVNWTVGDSAIEVINATTGKD ELGRASRLCKHALYCRWMRVHGKVPSH LLRSKITKPNVYHESKLAAKEYQAAKA RLFTAFIKAGLGAWVEKPTEQDQFSLT PLV
MCP-ADAR (32, 46, 47)		AACATGAGGATCACC CATGTCNNNNNCNNN NNNNNNNNNNAACA TGAGGATCACCCATG TC	~40bp, central	MASNFTQFVLVDNGGTGDVTVAPSNFA NGIAEWISSNSRSQAYKVTCSVRQSSA QNRKYTIKVEVPKGAWRSYLNMELTIP IFATNSDCELIVKAMQGLLKDGNPIPS AIAANSGIYGGSGSGAGSGSPAGGGAP GSGGGSKAERMGFTEVTPVTGASLRRT MLLLSRSPEAQPKTLPLTGSTFHDQIA MLSHRCFNTLTNSFQPSLLGRKILAAI IMKKDSEDMGVVVSLGTGNRCVKGDSL SLKGETVNDCHAEIISRRGFIRFLYSE LMKYNSQTAKDSIFEPAKGGEKLQIKK TVSFHLYISTAPCGDGALFDKSCSDRA MESTESRHYPVFENPKQGKLRTKVENG EGTIPVESSDIVPTWDGIRLGERLRTM SCSDKILRWNVLGLQGALLTHFLQPIY LKSVTLGYLFSQGHLTRAICCRVTRDG SAFEDGLRHPFIVNHPKVGRVSIYDSK RQSGKTKETSVNWCLADGYDLEILDGT RGTVDGPRNELSRVSKKNIFLLFKKLC SFRYRRDLLRLSYGEAKKAARDYETAK NYFKKGLKDMGYGNWISKPQEEKNFYL CPV

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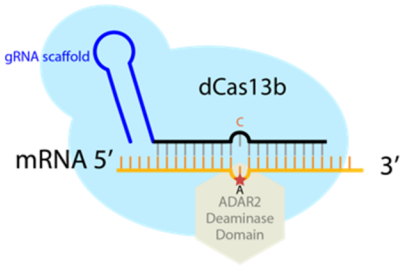
Table 1. continued

RNA editing system		adRNA sequence (5′ to 3′)	Anti-sense (length, mismatch position)	Deaminase sequence
dCas13b -ADAR (REPAIR) (48)		N…NNNNNNNNNCNNN NNNNNN…NGUUGUGG AAGGUCCAGUUUUGA GGGGCUAUUACAAC	~50bp, central	MNIPALVENQKKYFGTYSVMAMLNAQT VLDHIQKVADIEGEQNENNENLWFHPV MSHLYNAKNGYDKQPEKTMFIIERLQS YFPFLKIMAENQREYSNGKYKQNRVEV NSNDIFEVLKRAFGVLKMYRDLTNAYK TYEEKLNDGCEFLTSTEQPLSGMINNY YTVALRNMNERYGYKTEDLAFIQDKRF KFVKDAYGKKKSQVNTGFFLSLQDYNG DTQKKLHLSGVGIALLICLFLDKQYIN IFLSRLPIFSSYNAQSEERRIIIRSFG INSIKLPKDRIHSEKSNKSVAMDMLNE VKRCPDELFTTLSAEKQSRFRIISDDH NEVLMKRSSDRFVPLLLQYIDYGKLFD HIRFHVNMGKLRYLLKADKTCIDGQTR VRVIEQPLNGFGRLEEAETMRKQENGT FGNSGIRIRDFENMKRDDANPANYPYI VDTYTHYILENNKVEMFINDKEDSAPL LPVIEDDRYVVKTIPSCRMSTLEIPAM AFHMFLFGSKKTEKLIVDVHNRYKRLF QAMQKEEVTAENIASFGIAESDLPQKI LDLISGNAHGKDVDAFIRLTVDDMLTD TERRIKRFKDDRKSIRSADNKMGKRGF KQISTGKLADFLAKDIVLFQPSVNDGE NKITGLNYRIMQSAIAVYDSGDDYEAK QQFKLMFEKARLIGKGTTEPHPFLYKV FARSIPANAVEFYERYLIERKFYLTGL SNEIKKGNRVDVPFIRRDQNKWKTPAM KTLGRIYSEDLPVELPRQMFDNEIKSH LKSLPQMEGIDFNNANVTYLIAEYMKR VLDDDFQTFYQWNRNYRYMDMLKGEYD RKGSLQHCFTSVEEREGLWKERASRTE RYRKQASNKIRSNRQMRNASSEEIETI LDKRLSNSRNEYQKSEKVIRRYRVQDA LLFLLAKKTLTELADFDGERFKLKEIM PDAEKGILSEIMPMSFTFEKGGKKYTI TSEGMKLKNYGDFFVLASDKRIGNLLE LVGSDIVSKEDIMEEFNKYDQCRPEIS SIVFNLEKWAFDTYPELSARVDREEKV DFKSILKILLNNKNINKEQSDILRKIR NAFDANNYPDKGVVEIKALPEIAMSIK KAFGEYAIMK GSLQLPPLERLTLGS QLHLPQVLADAVSRLVLGKFGDLTDNF SSPHARRKVLAGVVMTTGTDVKDAKVI SVSTGTKCINGEYMSDRGLALNDCHAE IISRRSLLRFLYTQLELYLNNKDDQKR SIFQKSERGGFRLKENVQFHLYISTSP CGDARIFSPHEPILEEPADRHPNRKAR GQLRTKIESGQGTIPVRSNASIQTWDG VLQGERLLTMSCSDKIARWNVVGIQGS LLSIFVEPIYFSSIILGSLYHGDHLSR AMYQRISNIEDLPPLYTLNKPLLSGIS NAEARQPGKAPNFSVNWTVGDSAIEVI NATTGKDELGRASRLCKHALYCRWMRV HGKVPSHLLRSKITKPNVYHESKLAAK EYQAAKARLFTAFIKAGLGAWVEKPTE QDQFSLT

by which it penetrates the double-stranded RNA (dsRNA) helix from the minor groove next to the target adenosine and flips it out of the duplex, which makes the target adenosine susceptible to deamination. This flipped conformation is stabilized by the E488 residue taking the space previously occupied by the target adenosine and hydrogen bonding with the opposite base. The protonation of the amino group in the glutamine side chain makes it a better hydrogen bond donor to the opposite cytidine base, which explains the hyperactivity of the E488Q mutant. The fact that both G and A as the opposite base would clash with E488 being in this position explains ADAR2's preference for an A-C mismatch or A-U pair at the target site. It has previously been determined that ADAR2 prefers a U or A immediately 5′ to the target A and a G immediately 3′ to the target A. The 5′ base pair preference can again be explained by the clashing of 2-amino groups present in G-C or C-G pairs at the 5′ position. The 3′ G donates a hydrogen bond to S486, serving as a stabilizing interaction. Because all other bases lack the 2-amino group to donate the hydrogen bond at this position, the editing efficiency decreases. Taken together, the structural and mechanistic understanding of ADAR2-mediated RNA editing forms the basis for the design and engineering of guide RNAs used in programmable RNA editing systems. For a more detailed analysis of the ADAR structure and reaction mechanism, see refs 26 and 27.

PROGRAMMABLE RNA EDITING

The idea of programmable RNA editing for gene therapy was first put forth by Woolf and co-workers in 1995.[28] In a pioneering study that outlined the potential of RNA editing, they delivered into single-cell *Xenopus* embryos a luciferase reporter mRNA with a premature stop codon or the reporter mRNA hybridized with a 52-nucleotide RNA oligomer. They observed a significant increase in luciferase activity in embryos injected with the reporter−oligomer hybrids as compared those injected with only the mutant luciferase mRNA. This was attributed to the high levels of ADARs seen in the *Xenopus* embryos and their ability to edit dsRNA. They also went on to propose the idea of recruiting endogenous ADARs for therapeutic RNA editing in humans.

The ADAR-based RNA editing platform has since been engineered to catalyze site-specific RNA by several groups. These approaches rely on an engineered ADAR-associated RNA (adRNA) bearing an ADAR recruiting domain and antisense domain complementary to the target. The following primary approaches have been developed (Table 1).

Recruitment of ADARs via GluR2-adRNA. *Exogenous ADARs.* Fukuda and co-workers and Wettengel and co-workers engineered an adRNA from the GluR2 mRNA, which is a naturally occurring ADAR2 substrate, to enable programmable RNA editing via recruitment of full length ADAR2.[29,30] To achieve this, they fused the *cis*-acting R/G motif from the GluR2 mRNA to an antisense domain complementary to the

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target. The double-stranded RNA binding domain (dsRBD) of ADAR2 recognizes the GluR2 hairpin and thus is recruited to the target RNA. Within the target RNA, a C mismatch is carefully positioned opposite the A to be edited and this enables site-specific A-to-I editing. After systematic characterization of the system against fluorescence or luciferase reporters, it was observed that an antisense domain length of 16−20 nucleotides with the editing site carefully positioned 6−8 nucleotides from the R/G motif yielded the highest editing efficiencies. These studies were carried out in the presence of exogenous ADAR2 overexpression. The use of multiple copies of the adRNA yielded improved RNA editing efficiencies. This system was also validated across multiple endogenous transcripts with 10−40% editing seen across all loci. In addition, the GluR2−adRNA could also achieve significant albeit lower editing efficiencies with overexpression of both ADAR1 isoforms, p110 and p150.[31] Further optimization of the GluR2−adRNA was carried out by replacing several A-U base pairs with G-C base pairs to reduce the level of autoediting of the adRNA.[31] Katrekar and co-workers engineered and optimized the GluR2−adRNA approach for application to two independent mouse models of human disease: the *mdx* mouse model of Duchenne muscular dystrophy (DMD) and the *spf*^{ash} mouse model of ornithine transcarbamylase (OTC) deficiency.[32] ADAR2 or its hyperactive mutant, ADAR2 (E488Q), being only 2.1 kb in length, was readily packaged into an AAV along with two copies of an adRNA with an antisense domain of length 20 with a mismatch located at position 6 and delivered to mice. Upon treatment, RNA editing efficiencies of 0.8% and 3−21% were observed in the two mouse models, respectively, with ADAR2 (E488Q) yielding editing efficiencies significantly higher than those of ADAR2 in the *spf*^{ash} mice. Western blots confirmed partial restoration of protein expression in both mouse models. However, the authors noted that significant toxicity was seen upon delivery of ADAR2 (E488Q) in mice injected via systemic injections, possibly arising due to off-target editing. Although this establishes the utility of RNA editing for *in vivo* gene therapy, it also highlights that further efforts need to be made to address the issue of off-target editing arising due to the overexpression of the ADAR enzymes that could have deleterious effects.[33]

Endogenous ADARs. To improve the specificity of RNA editing, Merkle and co-workers developed chemically synthesized antisense oligonucleotides (ASOs) bearing GluR2 domains to recruit endogenous ADARs.[34] This approach did not require overexpression of exogenous ADARs. By introducing phosphorothioate modifications on four terminal residues at the 3′ end of the ASO and 2′-OMe modifications at all but three residues opposite the nucleotide triplet being targeted, they developed ASOs that enabled 5−35% editing in the 3′ untranslated region of GAPDH across a variety of cell lines. Addition of IFN-α led to an increase in ADAR1-p150 levels, which in turn boosted the editing efficiency by 1.5−2-fold. However, they observed that the use of short antisense domains was not sufficient to effect RNA editing in the open reading frame of the GAPDH transcript in HeLa and A549 cells. The authors overcame this problem by increasing the length of the antisense domain to 40 nucleotides and also included locked nucleic acid modifications in the antisense domain. This approach was used to correct the PiZZ mutation, which is the cause of α1-antitrypsin deficiency, in HeLa cells, and editing efficiencies of 10−20% were observed in the absence of IFN-α. It was also used to edit phosphotyrosine 701 in STAT1 of primary cells and to achieve values of 3−20% in the absence of IFN-α.

In an alternative approach, Katrekar and co-workers also achieved significant RNA editing at endogenous loci in HEK 293T cells via expression of genetically encoded long antisense domains bearing centrally positioned mismatches, both with and without the R/G motif.[32] This resulted from the formation of long dsRNA at the target that is recognized by the dsRBD of the ADAR enzymes and confirmed that long dsRNA itself was sufficient for recruitment of endogenous ADARs in human cells. At one of the three loci tested, they observed a significant decrease in the target mRNA level possibly due to an RNAi-like effect of the long antisense domains. In their *in vivo* studies in *spf*^{ash} mice, they also observed low but distinct editing levels of 0.6% via delivery of only an adRNA with a R/G motif and a short 20-nucleotide antisense domain, in the liver tissue that has endogenous ADAR2 expression. These observations suggest that it is possible to correct disease-causing point mutations *in vivo* via the delivery of only adRNAs. Transcriptome-wide RNA-seq analysis revealed that recruitment of endogenous ADARs demonstrated 100-fold reduction in off-target levels as compared to those under conditions that included ADAR overexpression. Recruitment of endogenous ADARs, thus, helps circumvent the issue of off-target editing arising due to enzyme overexpression. In the future, further engineering of the adRNA will be needed to improve the efficiency and prevent off-targets created by the long antisense domains.

Recruitment of SNAP-ADARs via Benzylguanine(BG)-adRNA. The SNAP tag protein labeling system is derived from the human DNA repair protein O6-alkylguanine-DNA alkyltransferase (hAGT). The hAGT recognizes O6-benzylguanine (BG) as a substrate and forms a covalent linkage. SNAP-ADARs were engineered by Stafforst and co-workers, fusing the deaminase domain of human ADAR1 to a SNAP tag (an engineered hAGT), which can covalently link with a customizable O6-benzylguanine (BG)-adRNA.[35] The BG-adRNAs are 17−22 nucleotides in length and typically carry a C mismatch positioned in the middle of the dsRNA duplex. Using this system, they observed 60−90% UAG to UIG conversion in an *in vitro* editing reaction. In addition, they explored SNAP-ADAR2 DD fusions and found that transfection of BG-adRNA into SNAP-ADAR1/2 DD-expressing HEK 293T cells resulted in 40−80% editing across four endogenous transcripts.[36] The use of hyperactive ADAR E>Q mutants improved the editing efficiency to 65−90%.[36] Although the SNAP-ADARs induced editing of an exogenous reporter when transfected in a plasmid format, the editing levels were found to be much lower (indistinguishable from the Sanger trace background) using the standard BG-adRNA.[37] Next, they engineered a variety of chemical modifications in the BG-adRNA to modulate its stability and targeting fidelity and to allow photoinducible activity.[37−39] 2′-Methoxy modifications on nucleotides other than the triplet containing the targeted base and phosphothioate linkages at the 3′ and 5′ termini of the BG-adRNA improved its stability.[37] This BG-antagomir-adRNA improved the editing efficiency in 293T cells upon co-transfection with the SNAP-ADAR. However, even with the modified BG-antagomir-adRNA, the editing rate was only ∼25% in 293T cells,[37] suggesting that transfection of the SNAP-ADAR is not efficient.[37] Notably, off-target editing in the RNA duplex was suppressed by including 2′-methoxy

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modifications at and near the undesired sites.[37] Additionally, light-inducible RNA editing was also engineered through the chemical attachment of a 6-nitropiperonyloxymethyl (Npom) protecting group to the O6-benzylguanine.[38,39] The Npom group is light-sensitive and absorbs in the range of 330−420 nm. Under 365 nm light, the Npom is released from the BG-adRNA and allows the latter to conjugate with SNAP-ADARs and enable RNA editing. This light-inducible RNA editing can also be performed in living cells, with <5% background editing of a reporter transcript in the absence of light and ≤45% editing during a 10 s exposure to 365 nm light. The level of editing achieved by a 10 s exposure is comparable to that achieved by the canonical BG-adRNA without Npom protection.[39]

Transcriptome-wide RNA-seq analysis showed that the use of hyperactive mutants improved on-target editing but also led to an increase in the transcriptome wide off-targets. Notably, the genomically integrated SNAP-ADAR system significantly outperformed the overexpressed λN-ADAR and dCas13b-ADAR systems with regard to specificity, having orders of magnitude fewer global off-target edits.[36] This is potentially because genomic integration of the SNAP-ADARs and chemical modifications on the BG-adRNA limit the intracellular SNAP-ADAR levels as well as its activity to the target mRNA-adRNA duplex.

Taken together, while the SNAP-ADAR system offers high efficiency and specificity, its use for *in vivo* gene therapy might be challenging. Stable genomic integration is not a feasible solution for *in vivo* gene therapy, and it remains to be seen if the efficiency and specificity profiles of this system will hold under conditions of overexpression. In addition, the inability to genetically encode the BG-adRNA might also pose a problem for *in vivo* gene therapy due to the transient nature of RNA editing. Finally, RNA editing by the SNAP-ADAR system has not been clearly demonstrated *in vivo* other than in the setting of *Platynereis dumerilii* embryos.[39] This could be due to the fact that genomic integration of the SNAP-ADAR is challenging *in vivo* for most organisms, including mammals. Nonetheless, development of delivery approaches that enable direct cellular transduction of SNAP-ADAR:BG-adRNA ribonucleoproteins could open the door to the use of this system for therapeutic RNA editing.

Recruitment of λN-ADARs via boxB-adRNA. The λN-boxB system is derived from the naturally occurring λ-phage N protein-boxB RNA interaction that regulates antitermination during transcription of the λ-phage mRNAs.[40] The λN peptide (22 amino acids) binds to its cognate boxB hairpin (17 nucleotides) with nanomolar affinity. Montiel-Gonzalez and co-workers demonstrated the use of the λN-boxB system for the recruitment of ADARs.[41] They utilized this approach for the correction of a CFTR reporter bearing a premature stop codon as seen in a subset of patients with cystic fibrosis. They carried out their studies in *Xenopus* oocytes and observed 20% correction of the nonsense mutation. In addition, they observed not only partial restoration of protein expression but also restoration of functional currents in the treated oocytes. They also explored the roles of addition of multiple λN domains and boxB hairpins in editing efficiency and noted that the addition of 4λN domains and 2boxB hairpins led to a 6.5-fold increase in the level of on-target editing over the system with a single λN domain and boxB hairpin.[42] Additionally, they demonstrated that it was possible to control the off-target editing in the target mRNA by limiting the amount of RNA guide. A comparison of 4λN-ADAR2 DD and 4λN-ADAR2 DD (E488Q) revealed that the hyperactive mutant was indeed more efficient but also more promiscuous. The number of transcriptome-wide off-targets of the system was, however, significantly reduced by the addition of a nuclear localization signal.[43] Overexpression of an adRNA was shown to significantly increase the number of transcriptome-wide off-targets as compared to that under the enzyme only condition. Sinnamon and co-workers further applied this tool set for the correction of a point mutation in primary neurons derived from a mouse model of Rett syndrome.[44] They utilized AAVs to package the λN-ADAR2 DD (E488Q) along with six copies of the boxB-adRNA, each containing two boxB domains on either side of a 30-nucleotide antisense domain with a C mismatch located at position 10. They targeted the mutated MECP2 transcript and achieved 72% on target editing, with a 20% increase in MECP2 protein levels. However, they also noticed several off-target adenosines being edited with levels of ≤50% in the mRNA-adRNA duplex. Thus, although the genetically encodable λN-ADARs along with its boxB-adRNA can effect robust RNA editing via AAV-mediated delivery in primary cells, concerns over the high levels of off-target editing as well as the viral origin of the system need to be overcome before it can be considered for use in *in vivo* gene therapy.

Recruitment of MCP-ADARs via MS2-adRNA. The MS2-MCP tagging system has been derived from the naturally occurring interaction between the MS2 bacteriophage coat protein (MCP) and a stem loop from its genome.[45] The MCP (130 amino acids) binds to the MS2 stem loop (21 nucleotides) with nanomolar affinity. The use of the MS2-MCP system for ADAR recruitment was described by Azad and co-workers, who tested the MCP-ADAR1 DD against a EGFP reporter bearing a premature stop codon.[46] They observed ∼5% RNA editing efficiency. They also found that the MCP-ADAR1 DD was more efficient than the MCP-ADAR2 DD and its hyperactive mutant, the MCP-ADAR2 DD (E488Q).[47] Concurrently, Katrekar and co-workers[32] developed an independent MS2-MCP-based system for ADAR recruitment. Here the MS2-adRNAs were designed with two MS2 hairpins on either side of a 20-nucleotide antisense domain with a C mismatch located at position 6. They noted efficiencies of 10−80% when the samples were tested against eight endogenous transcripts as compared to the 10−40% efficiencies seen in side-by-side ADAR2-based experiments. Systematic RNA-seq analysis of the MCP-ADARs revealed that ADAR1-based constructs, in general, displayed higher on-target activity but were also more promiscuous than the ADAR2-based constructs. In addition, it was observed that the off-targets primarily arose due to the overexpression of the enzyme, independent of the MS2-adRNA. As observed for the λN-ADARs, it was noted that use of hyperactive mutants ADAR1 (E1008Q) and ADAR2 (E488Q) and/or addition of a nuclear export signal showed higher on-target activity but also led to a significant increase in the number of transcriptome-wide off-targets. The best MCP-ADAR variant, the MCP-ADAR2 DD-NES, displayed an on-target editing yield that was 1.2−2-fold higher than that of ADAR2 while yielding a similar number of off-targets. In addition, they tested out the AAV-delivered MCP-ADAR system in the *mdx* mouse model and observed 2.5-fold higher RNA editing efficiencies *in vivo* as compared to that of ADAR2 or ADAR2 (E488Q), along with partial restoration of dystrophin expression. However, although the MCP-ADAR system displays an editing efficiency that is

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Table 2. Delivery Strategies for Therapeutic RNA Editing Components

delivery method	cargo	advantages	disadvantages	refs
adeno-associated virus (AAV)	ssDNA	infects dividing and nondividing cells potential long-term expression (up to years) low pathogenicity and immunogenicity existing serotypes with diverse tissue tropism	small packaging size (4.7 kb) some integration	62, 65−70
lentivirus	RNA	stable long-term expression infects dividing and nondividing cells high transduction efficiency large packaging size (8−10 kb)	random integration pathogenic	68, 71−73
adenovirus	dsDNA	infects dividing and nondividing cells no integration very high transduction efficiency large packaging size (≤36 kb)	highly immunogenic and pathogenic	74−77
liposome	DNA, RNA, RNP	very low immunogenicity transient expression (hours to weeks) easy production, low cost large capacity	low serum stability low *in vivo* efficiency low tissue specificity some cytotoxicity	78−88
exosome	DNA, RNA, RNP	long circulating life intrinsic tissue/cell specificity low toxicity or immunogenicity can cross the blood−brain barrier avoids endosomal pathway and lysosomal degradation	poor purification techniques high production cost heterogeneity of content	89−91
electroporation	DNA, RNA, RNP	very high efficiency transient presence (hours to weeks) suitable for most cell types	poor cell viability limited applicability *in vivo*	92−95

higher than that of ADAR2 *in vivo*, concerns over off-target editing and the viral origin of the MCP need to be addressed before it can be considered for use in *in vivo* gene therapy.

Recruitment of dCas13b-ADARs via crRNA. Cox and co-workers utilized a crRNA to recruit a catalytically inactive PspCas13b (dCas13b) fused to ADAR1 DD (E1008Q) or ADAR2-DD (E488Q).[48] While the dCas13b-ADAR1 DD (E1008Q) required a spacer length of >70 nucleotides for efficient RNA editing, the dCas13b-ADAR2 DD (E488Q) could edit RNA with short 30−50-nucleotide spacers. This system was characterized using the luciferase reporter. Editing in endogenous transcripts was demonstrated on two mRNA sequences, with RNA editing efficiencies of 25−40% seen at these loci. The system also yielded 15−40% editing efficiencies against all 16 codon triplets in a luciferase reporter. To be packaged into an AAV, a truncated protein Δ984−1090 was created, which displayed a similar on-target editing efficiency. The system showed a 30-fold higher off-target editing efficiency compared to that of ADAR2, which was attributed to the presence of the ADAR2 DD (E488Q). Systematic mutagenesis of the ADAR2 DD (E488Q) yielded the T375G mutation with enhanced specificity. This resulting construct showed a number of off-targets that was 900 times lower; however, the mutation also resulted in a 2-fold decrease in the on-target editing efficiency of the luciferase reporter.

Vogel and co-workers tested overexpression of a crRNA with a spacer length of 50 nucleotides along with ADAR2, SNAP-ADAR2 DD (E488Q), and the Cas13b system mentioned above.[36] Interestingly, they observed similar editing efficiencies in all three scenarios. Furthermore, they observed that an antisense domain with a length of 50 itself was sufficient to recruit overexpressed Cas13b-ADAR protein. These data suggest that the large bacterial Cas protein provides a limited advantage for RNA editing. Whether this system can be potentially used for therapeutic RNA editing remains to be determined.

DELIVERY OF RNA-GUIDED ADENOSINE DEAMINASES AND ADRNA FOR THERAPEUTIC RNA EDITING

The delivery of any therapeutic reagents is an important challenge in gene therapy. For any RNA editing system described above to be used in treating human diseases, an appropriate delivery method must be developed. The RNA editing system should be able to correct any mutation in a disease-relevant transcript, and the delivery strategy must be able to efficiently and specifically deliver all components of the system to the targeted tissue or organ. Common viral delivery vehicles include adeno-associated viruses (AAVs), lentiviruses, and adenoviruses. Nonviral delivery methods include lipid-mediated delivery, exosome delivery, and electroporation. We offer a comparison of all viral and nonviral delivery methods in Table 2 and will expand on two relevant methods for therapeutic delivery of engineered RNA editing systems, namely, lipid-mediated delivery and AAV delivery.

Lipid-Mediated Delivery. Cationic lipids can deliver nucleic acids as well as negatively charged proteins to the cell through triggering of endocytosis. Synthetic liposomes have been widely used as transfection agents *in vitro* and achieved commercial success since their invention in the 1980s.[49] Liposomal delivery is the most commonly used strategy for delivering the engineered deaminase to cultured cells in the laboratory setting.

Many studies have demonstrated the use of liposomes or lipid nanoparticles for the delivery of genome engineering tools *in vivo*. One study showed that lipid nanoparticle delivery of Cas9 mRNA along with a chemically modified sgRNA resulted in significant genome editing in the liver and knockdown of protein levels in serum for >12 months.[50] Lipofectamine was

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also shown to deliver anionic proteins and protein complexes *in vivo*. Zuris, Yeh, and their co-workers have shown delivery of Cre recombinase,[51] the Cas9:sgRNA complex,[51] and the BE3 base-editor:sgRNA complex to the inner ear,[52] leading to efficient recombination, genome editing, and base conversion in hair cells. Although no studies have shown lipid-mediated delivery of RNA editing components *in vivo*, it could be a promising delivery strategy.

On the downside, *in vivo* liposomal delivery is hindered by low efficiency in most tissues, low serum stability, and some toxicity. Although some recent studies have shown tissue specificity by surface modifications,[53−56] an efficient lipid formulation remains to be engineered for each tissue type.

AAV Delivery. Viruses have naturally evolved to propagate by injecting their genetic material into host cells whose transcription and translation machinery is hijacked to produce more viral particles. Due to this unique property, viruses have been vastly engineered to serve as delivery vehicles of gene therapy agents, with >2300 viral vector-based clinical trials conducted to date.[57]

In particular, AAVs have been regarded as one of the most suitable for this purpose due to their ability to infect a variety of cell types, their low immunogenicity, and their stable transgene expression. AAVs have multiple variants that exhibit natural tropisms toward certain tissues, which in turn allows efficient delivery to a broad range of organs, especially the liver, muscle, eye, and heart.[58] Previously, numerous studies have demonstrated the use of AAVs as delivery vehicles for genome engineering tools such as CRISPR-Cas9[59,60] and base editors.[61] Notably, AAVs are the only delivery vehicle, to date, to have successfully delivered the ADAR2-GluR2 and MCP-ADAR systems to correct disease-relevant mutations in mouse models, as described in previous sections.

While AAVs present multiple advantages as gene delivery vehicles, their use is limited by issues such as preexisting immunity, immunogenicity, and potential for integration. Recent efforts have broadened the scope of tissue tropism[62−64] and engineered immuno-stealth[65] through viral surface modifications but with only moderate success. Furthermore, although long-term expression of the RNA editing components might be required to lengthen the therapeutic effects, it remains to be determined whether persistent activity of the deaminase may increase off-target effects, which in turn can also have detrimental consequences for the cell. Nonetheless, on the basis of the considerations mentioned above, AAVs seem to be one of the best working delivery strategies for therapeutic RNA editing.

CONCLUSIONS AND OUTLOOK

Clinical Applications. G(C)-to-A(T) point mutations constitute 47% of the 33000 pathogenic SNPs identified in the human genome.[1,11,96] These include missense and nonsense mutations in the coding region as well as mutations in noncoding regions affecting transcript stability, splicing, and translation. These disease-causing mutations can theoretically be corrected by A-to-I editing of relevant transcripts. Compared to DNA editing, editing RNA may present some advantages moving into the clinic. Whereas genomic changes are usually irreversible, RNA edits can be reversed simply by stopping the administration of editing constructs in case any toxicity or unwanted effects of the therapy are observed. Because no permanent genomic changes are made by RNA editing, it might be possible to reach a broader population of patients, because concerns over ethics and safety of genome editing persist. In addition, the A-to-I RNA editing enzymes, namely ADARs, are of human origin and a subset of RNA editing systems, as discussed in previous sections, utilize only human proteins, circumventing concerns about immunogenicity toward the effector systems.[16,17] Furthermore, recruitment of endogenous ADARs with ASOs or long antisense adRNAs for targeted RNA editing has great promise as it is completely non-immunogenic.

The idea of programmable RNA editing for correction of point mutations *in vivo* was put forth by Woolf and co-workers more than 20 years ago.[28] Since then, significant progress has been made toward understanding the biology of RNA editing via ADARs as well as its prevalence in the transcriptome,[22,24,26,97−109] but the use of RNA editing in therapeutics has been limited. However, since 2013, RNA-guided adenosine deaminases have been applied for the correction of premature stop codons in CFTR and PINK1 reporter mRNA, which are responsible for causing cystic fibrosis and Parkinson's disease, respectively.[29,41] Endogenous ADARs have been used to correct the PiZZ mutation, which is the cause of α1-antitrypsin deficiency, in reporter mRNA.[34] AAV-mediated delivery of RNA-guided adenosine deaminases has been shown to efficiently correct a point mutation in the endogenous MECP2 transcript of primary neurons harvested from a mouse model of Rett syndrome.[44] In addition, utilizing this AAV delivery approach, disease-causing premature stop codons and splice site mutations have also been corrected *in vivo* in mouse models of DMD and OTC deficiency.[32] These studies demonstrate the promise of the RNA editing technology for therapeutic correction of point mutations.

Despite great advancements in RNA editing technology, a few problems with safety and efficiency must be addressed before RNA editing technology can be used in therapeutics.

Off-Target Editing. The most pressing problem associated with the safety of therapeutic RNA editing is off-target editing. Editing of nontargeted transcripts will lead to undesired changes in the transcriptome, including changes in codons, splice sites, and transcript stability. These could cause deficiency, overabundance, or misfunction of proteins as well as potential generation of immunogenic epitopes. Currently, robust RNA editing of disease-causing endogenous transcripts has been demonstrated using overexpressed ADAR2, λN-ADARs, and MCP-ADARs. However, all of these approaches result in off-target editing both within the adRNA-target-RNA duplex and across the transcriptome. While the genomically integrated SNAP-ADAR system offers the best specificity profile as compared to those of the other RNA editing approaches, genomic integration of the SNAP-ADAR is not feasible for *in vivo* gene therapy. Even if SNAP-ADAR can offer the best specificity when overexpressed, the BG-adRNA cannot be genetically encoded and will require additional considerations in terms of synthesis and delivery. In the future, limiting the duration of enzyme and adRNA expression, use of wild type deaminase domains, and nuclear sequestration of these RNA editing enzymes may help limit off-target editing. In particular, further improvement in adRNAs for recruiting endogenous ADARs would also be key with respect to therapeutic applications, because the number of off-targets is significantly smaller without ADAR overexpression.

Delivery. Delivery is another issue that affects the efficiency and safety of therapeutic RNA editing. RNA editing is transient, and re-administration of editing constructs is likely

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to be necessary due to the limited lifetime of both the edited transcripts and the RNA-guided adenosine deaminases. AAV delivery can potentially achieve long-term expression of the editing constructs and thereby minimize the frequency of administrations, but immunity acquired against AAVs might prevent efficient subsequent AAV administration. Furthermore, there have been reports of existing immunity against certain AAV serotypes in the population,[110] potentially rendering even the initial therapeutic administration ineffective for a large fraction of the patient population. On the other hand, synthetic liposomes have very low immunogenicity but are generally unstable *in vivo*. Coupled with a low *in vivo* transfection efficiency for many tissues, lipid-mediated delivery may require more frequent and larger doses, which may then magnify the cytotoxicity of the lipids, off-target editing, and targeting of undesired tissues.

Immunogenicity. As mentioned above, immunogenicity could be problematic in terms of both *in vivo* efficiency and safety. Of the five programmable RNA editing tools discussed, only the ADAR2 and SNAP-ADAR constructs are entirely of human origin and likely to be non-immunogenic. MCP, λN, and Cas13b proteins used in other systems are of either bacterial or viral origin. Immunity against these proteins (in addition to any immunogenic delivery vehicle) may develop after the first administration and decrease the effectiveness of subsequent doses. Furthermore, the immune reaction toward foreign proteins can cause serious safety concerns.[111]

In the future, the engineering of adRNAs for recruitment of endogenous ADARs offers great promise for gene therapy. As ADAR1 is ubiquitously expressed, the focus should be optimizing the adRNA design to recruit ADAR1 for efficient and precise editing of disease-causing mutations across most tissues without the requirement for deaminase overexpression. In addition, in tissues such as the cerebellum and lung, where ADAR2 is strongly expressed, delivery of current GluR2-adRNAs could effect efficient RNA editing. Although ASOs have shown great promise in giving rise to efficient editing in multiple cell types, the *in vivo* delivery of these ASOs is a great challenge due to their inability to be genetically encoded. Taken together, we believe that the utilization of engineered genetically encodable adRNAs to recruit endogenous ADARs would provide the safest therapeutic route for RNA editing technology. In addition, several known cytidine deaminases such as APOBECs have natural mRNA substrates.[112] Theoretically, a similar programmable C-to-U RNA editing could be developed utilizing a guide RNA consisting of both a cytidine deaminase recruiting domain and a targeting domain. If successful, this will have the potential to expand the scope of point mutations that can be corrected at the transcriptomic level.

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