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REVIEW ARTICLE

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U7 snRNA: A tool for gene therapy

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Abstract

Most U-rich small nuclear ribonucleoproteins (snRNPs) are complexes that mediate the splicing of pre-mRNAs. U7 snRNP is an exception in that it is not involved in splicing but is a key factor in the unique 3′ end processing of replication-dependent histone mRNAs. However, by introducing controlled changes in the U7 snRNA histone binding sequence and in the Sm motif, it can be used as an effective tool for gene therapy. The modified U7 snRNP (U7 Sm OPT) is thus not involved in the processing of replication-dependent histone pre-mRNA but targets splicing by inducing efficient skipping or inclusion of selected exons. U7 Sm OPT is of therapeutic importance in diseases that are an outcome of splicing defects, such as myotonic dystrophy, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, β-thalassemia, HIV-1 infection and spinal muscular atrophy. The benefits of using U7 Sm OPT for gene therapy are its compact size, ability to accumulate in the nucleus without causing any toxic effects in the cells, and no immunoreactivity. The risk of transgene misregulation by using U7 Sm OPT is also low because it is involved in correcting the expression of an endogenous gene controlled by its own regulatory elements. Altogether, using U7 Sm OPT as a tool in gene therapy can ensure lifelong treatment, whereas an oligonucleotide or other drug/compound would require repeated administration. It would thus be strategic to harness these unique properties of U7 snRNP and deploy it as a tool in gene therapy.

KEYWORDS
adenoassociated virus, gene-editing, gene-therapy, HIV, muscular dystrophy, neurodegenerative disease, RNA-technologies, stem/progenitor cell research

1 | INTRODUCTION

Small nuclear ribonucleoproteins (snRNPs) are complexes composed of small nuclear RNA (snRNA) and proteins in specific structures. Initially, the term snRNA was introduced by Weinberg and Penman in 1968.[1] It was further observed that some of these snRNAs were uridine rich compared to ribosomal or messenger RNAs and thus were described as U snRNAs. These U snRNAs were further numbered (e.g. U1 snRNA) by their order of discovery, and not by size, location or abundance. Valuable research was published by the group led by Joan Steitz in 1980, where they initially reported the role of snRNPs in splicing.[2] The spliceosomes, comprising large complexes that catalyse splicing, are divided into major and minor spliceosomes: U1, U2, U4, U5, U6 and U11, U12, U4atac, U5atac and U6atac snRNP, respectively. Subsequently, our knowledge about U snRNPs that interact with pre-messenger RNA (pre-mRNA) and other proteins forming the spliceosome has expanded.

In simple terms, splicing is the removal of introns and joining of exons from pre-mRNA. Splicing is divided into constitutive and alternative splicing (Figure 1) with alternative splicing considered responsible for protein diversity.[3–6] The interaction between *cis*-acting elements and *trans*-acting factors during alternative splicing define the exons that would be in the mature mRNA.[3] Exonic and intronic splicing enhancers are *cis*-acting elements bound by positive *trans*-active factors such as the nuclear phosphoprotein family (serine/arginine-rich). By contrast, exonic and intronic splicing silencers are bound by negatively acting factors such as heterogeneous ribonucleoproteins (hnRNP).[3] In constitutive splicing, the enhancing elements are dominant, whereas the silencers play a prominent role in controlling alternative splicing. Collaboration between these elements results in the promotion or inhibition of spliceosome assembly at the weak splice sites.[3,7,8]

Exceptionally, one of the U snRNPs, U7 snRNP, is not involved in splicing but is a key factor in the unique 3′ end processing of replication-dependent histone (RDH) pre-mRNAs. Moreover, recent studies showed U7 snRNA as an important tool in therapeutic studies, with reports based on modified U7 snRNP (U7 Sm OPT) targeting splicing to induce efficient skipping or inclusion of selected exons. U7 Sm OPT is designed by changing the histone binding sequence at the 5′ region of U7 snRNA to the complementary sequence of the gene to be modified. Further modifications include changing of U7 snRNP specific proteins, Lsm10 and Lsm11, to the consensus protein ring of spliceosomal snRNPs.

In U7 Sm OPT-based therapy, an antisense oligonucleotide is incorporated into the U7 snRNA. Antisense oligonucleotide and its importance was published for the first time by Stephenson Zamecnik in 1978.[9] Antisense oligonucleotides are short single-stranded nucleotide sequences binding the mRNA to alter gene expression by degradation of the transcript or by inhibition of translation.[10] They do so by various mechanisms, which include ribonuclease H mediated decay of pre-mRNA or steric hindrance. An alternative way of using antisense oligonucleotides is splicing modulation in which these short nucleotides can bind to pre-mRNA splicing elements and disrupt the recognition of splicing regulators.[11] For this purpose, as a tool of manipulation of pre-mRNA splicing, antisense oligonucleotides are used in the U7 Sm OPT therapeutic strategy. Although directly using antisense oligonucleotide has certain benefits, it has some limitations because it is sensitive to degradation, may cause immunoreactivity and usually needs repeated dosage. Thus, incorporating antisense

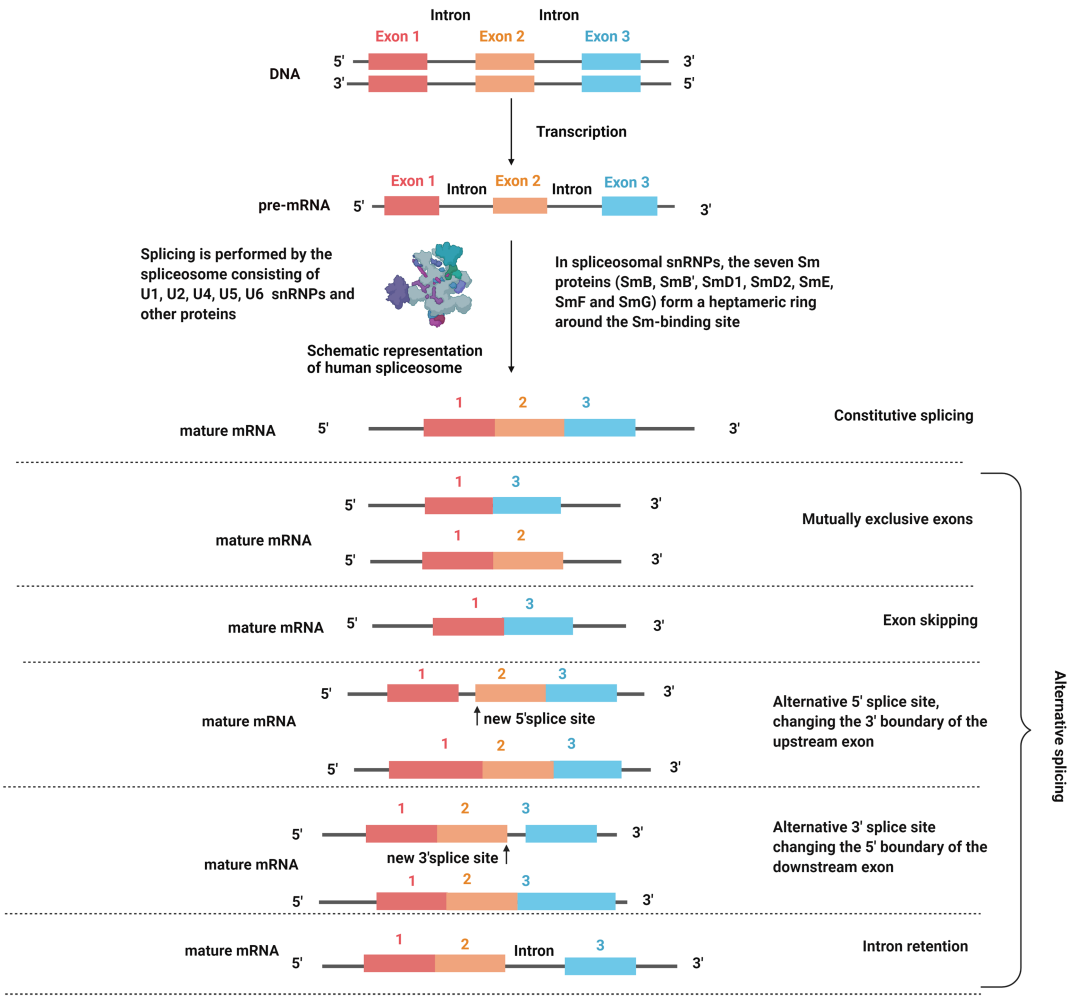

FIGURE 1 Constitutive and alternative splicing

oligonucleotides into U7 snRNP and delivering it using viral vectors overcomes many limitations. U7 Sm OPT snRNP is compact in size, accumulates in the nucleus, is non-toxic to the cells and overcomes the issue of repeated administration of oligonucleotides. Therefore, using U7 snRNA as a tool in gene therapy enables the desired splicing correction to be achieved along with stable antisense oligonucleotide levels. All of these factors are the basis of gene therapy and U7 snRNP clearly appears to have the upper hand compared to other candidates.

The use of U7 snRNP as a tool for gene therapy has shown promise in pre-clinical studies and in human clinical trials. The representative diseases include myotonic dystrophy, Duchenne muscular dystrophy (DMD), amyotrophic lateral sclerosis (ALS), β-thalassemia, HIV-1 infection, and spinal muscular atrophy (SMA). The aim of this review is to discuss the role of U7 snRNA as a tool for gene therapy.

2 | HISTONE GENES AND THEIR PROCESSING

Histone proteins comprise two major classes: canonical RDHs and proteins called histone variants. The genes of RDH are in clusters and are intronless. Interestingly, RDH transcripts are the only mRNAs in metazoan cells that are not polyadenylated.[12,13] By contrast, the genes encoding histone variants contain introns, are not arranged in clusters and produce polyadenylated mRNAs.[13,14] The expression of RDH genes is activated at the S phase of the cell cycle.[15] During the 3′ end processing, RDH pre-mRNAs undergo a single endonucleolytic cleavage after a specific stem-loop structure, located at the 3′ end and recognized by the stem-loop binding protein (SLBP).[16,17] Downstream of the stem-loop structure is a purine-rich conserved sequence, known as the histone downstream element (HDE). HDE is recognized by the 5′ end of U7 snRNA.[18–20] The stem-loop structure, HDE, SLBP and U7 snRNP are respective *cis* and *trans* elements in the post-transcription regulation of RDH mRNAs. These elements determine the cell-cycle regulated expression of canonical histone genes.[15,21,22] The binding of U7 snRNP to HDE aids in the recruitment of other factors involved in processing, known as the histone cleavage complex. Cleavage occurs between the 3′ stem-loop and the HDE, and is catalysed by endonuclease CPSF73[22–27] (Figure 2). It remains unclear which of the mechanisms, polyadenylation or RDH 3′ end processing, developed first in evolution, although it is agreed that U7 snRNP based RDH 3′ end processing was lost in protozoa, plants and fungi, thus making it unique to metazoan cells.[13]

3 | U7 SNRNP

In humans, U7 snRNP comprises 63 nucleotides of U7 snRNA and a protein core consisting of five common Sm proteins, SmB/B′, SmD3, SmE, SmF and SmG, as well as two U7 snRNP-specific Sm-like proteins, Lsm10 and Lsm11.[28] Lsm10 and Lsm11 replace SmD1 and SmD2, respectively, which are found in other U snRNPs.[28–30]

The 5′ part of the U7 snRNA is complementary to the HDE motif of the 3′ UTR of RDH pre-mRNA, and the 3′ region is occupied by a non-canonical Sm binding site (recognized by the Sm/Lsm protein core), followed by a conserved stem-loop secondary structure required for its stability[31] (Figure 2). It has been reported in a

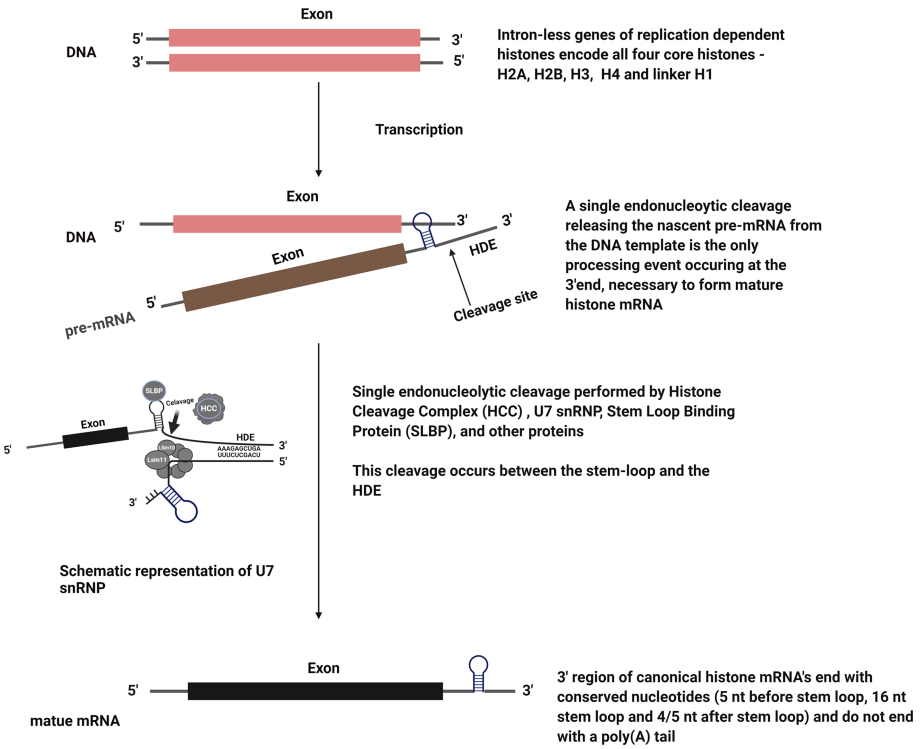

FIGURE 2 Formation of mature RDH transcripts

Drosophila model that blocking the 5′ end of the U7 snRNA with a complementary oligonucleotide specifically blocks the processing of RDH pre-mRNA.[32] This is one such primary finding that is further implemented with respect to using U7 snRNA as a tool for gene therapy.

U7 snRNP follows the same maturation pathway as spliceosomal U snRNPs. During biogenesis, newly transcribed U7 snRNA is exported to the cytoplasm, where it is assembled with core proteins already present in the cytoplasm (SmB/B′, SmD3, SmE, SmF, SmG and Lsm10 and Lsm11) in a process mediated by the survival motor neuron (SMN) complex and protein arginine methyltransferase-5 (PRMT5) complex.[33,34] After assembly, the U7 snRNP is imported into the nucleus and localizes in histone locus bodies.[28,33,35]

In comparison with major spliceosomal snRNPs, the level of U7 snRNP in the cell is very low, reaching approximately 500 molecules.[36] The non-canonical Sm binding site and Lsm10 and Lsm11 are assumed to be critical factors of U7 snRNP, and replacement of the U7 Sm binding site with the consensus sequence leads to a higher production of U7 Sm OPT, reaching a level comparable to that of other U snRNPs.[28,36]

4 | U7 SNRNA IN GENE THERAPY

Factors that make U7 snRNA such an interesting choice as a tool for treating genetic disorders are its small size, good stability and ability to accumulate in the nucleus. However, to further improve its activity, the wild-type U7 snRNA needs to be modified.

Schuemperli and colleagues have shown that the non-canonical Sm binding site of U7 snRNA (AAUUUGUCUAG; U7 Sm WT) can be converted into the consensus sequence derived from major spliceosomal U snRNPs (AAUUUUUGGAG; U7 Sm OPT), leading to the formation of a spliceosomal-type heptameric protein core wrapped around U7 Sm OPT. This change results in the augmented expression of U7 Sm OPT particles, which efficiently accumulate in the nucleus and can be redirected to the sites of pre-mRNA splicing. Moreover, because U7 Sm OPT is unable to bind U7-specific proteins, Lsm10 and Lsm11, U7 Sm OPT particles are non-functional in RDH mRNA processing and thus do not affect this process.[28,36,37] Next, modification of the sequence motif of U7 snRNA, which is complementary to HDE within RDH pre-mRNA, can make the snRNP particle hybridize to almost any RNA sequence within the nucleoplasm. U7 Sm OPT, even when expressed permanently, does not elicit immunological reactions or toxic effects in the cells. This modification enables U7 snRNA, with its original 3′ elements, to be used in gene therapy (Figure 3).

The diseases targeted for U7 snRNA gene therapy are predominantly an outcome of splicing defects. Mutations disrupt splice sites, leading to the creation of cryptic acceptor or donor sites, and aberrant splicing leads to the synthesis of premature terminated or truncated proteins. One disease can be caused by different mutations, thus requiring different therapeutic approaches. Therefore, using antisense oligonucleotides incorporated into U7 Sm OPT proves to be advantageous because it has varied therapeutic applications, such as exon skipping to restore expression, exon skipping to introduce a stop codon, and exon inclusion or displacement of mRNA binding factors (Figure 4).

In modifications made to U7 snRNP for blocking splicing, the binding sites to U1 and U2 snRNP are known to comprise the most effective targets.[38] A stable stem-loop structure as short as 7 bp in an RNA transcript has been shown to abolish enhancer activity, and an exonic splicing enhancer was found to act as intronic splicing enhancer when located in the intron instead of the exon.[39–41] It is further shown that a looping out pre-mRNA leads to exonic sequestration from the rest of pre-mRNA transcript.[3,42]

An initial report based on data obtained from experiments conducted on cell lines mentioned the use of double-target antisense U7 Sm OPT in the treatment of β-thalassemia. This treatment was

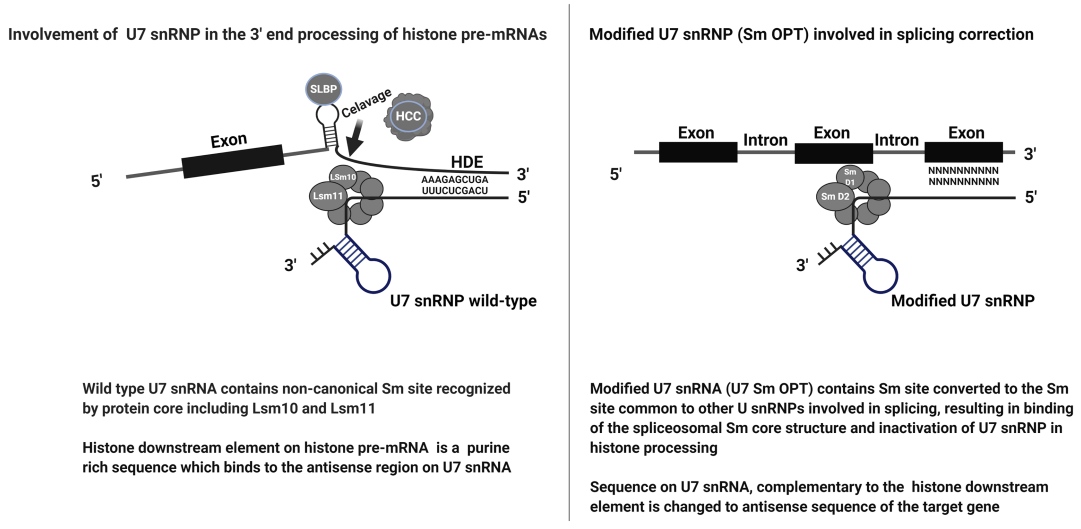

FIGURE 3 Modifications made to U7 snRNP to be used as a tool in gene therapy

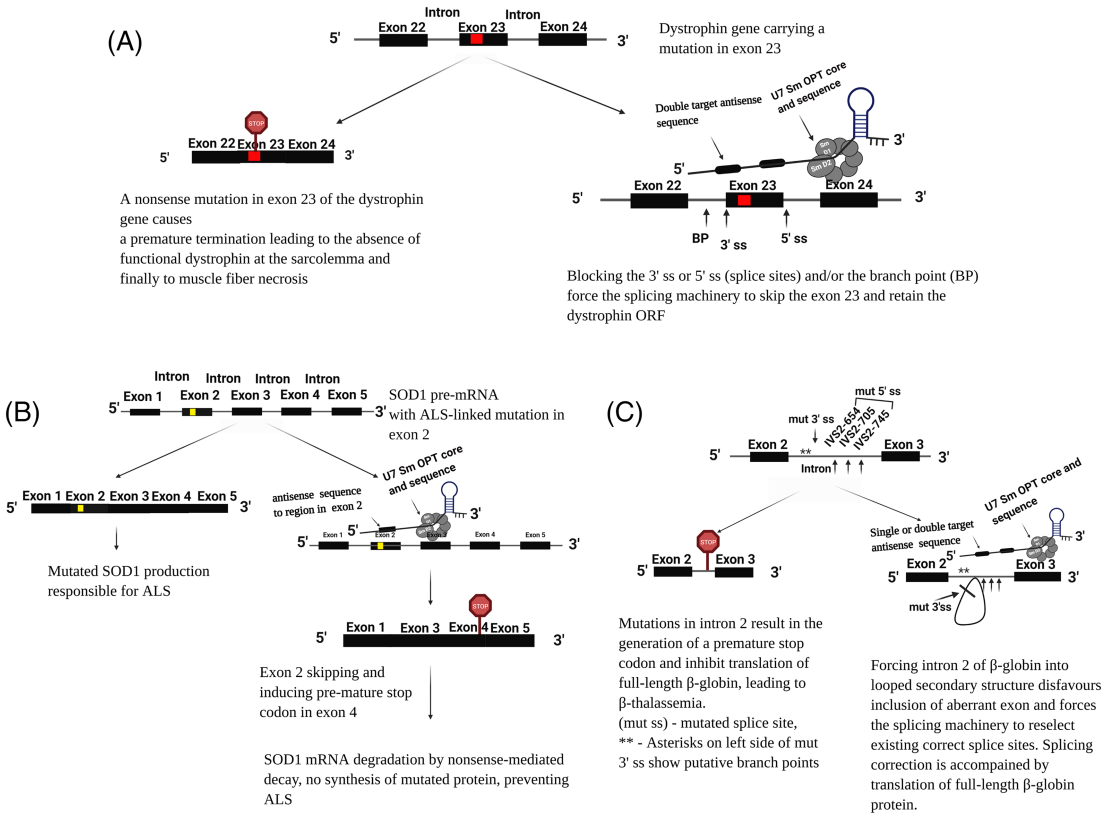

FIGURE 4 Examples of U7 snRNA-based splicing modulation for therapy of (a) DMD, (B) ALS and (C) β-thalassemia

achieved by forcing the aberrant exon into a looped secondary structure and strongly promoting its exclusion from the mRNA.[43] This approach is the fundamental observation reported by the Schuemperli group. Research was conducted on myoblasts obtained from DMD patients,[44] as well as fibroblasts from an SMA patient,[45] and extended to a SMA mouse model.[46] The efficiency of U7 Sm OPT was increased by adding sequences acting as splicing silencers or enhancers.[44–46]

The delivery of engineered constructs is an important aspect in gene therapy and is rapidly evolving subsequent to the study of viral vectors. Adeno-associated viruses (AAV) are non-enveloped, single-stranded DNA, replication defective viruses from the *Parvoviridae* family.[47–49] AAV are currently not known to cause any disease and thus adeno-associated viral vectors can be used to deliver U7 Sm OPT to the nervous system, as well as other targets organs, as a result of the availability of numerous serotypes with different tissue tropism.[50,51] The delivery of U7 Sm OPT by AAV is now broadly used because it ensures high efficiency gene transfer and relatively stable expression.

5 | DISEASES TARGETED BY U7 SNRNA GENE THERAPY

U7 Sm OPT is of therapeutic importance in diseases that are an outcome of splicing defects, such as myotonic dystrophy, DMD, ALS, β-thalassemia, HIV-1 infection and SMA, as described in detail below. Apart from this, using U7 Sm OPT for correction of aberrant splicing of three genes, *PTCH1*, *BRCA1* and *CYP11A*, where mutations cause nevoid basal cell carcinoma syndrome, breast cancer and congenital adrenal insufficiency, respectively, is also reported.[52] A brief summary of the diseases targeted by U7 snRNA gene therapy is provided in Table 1.

5.1 | U7 snRNA therapy for treating myotonic dystrophy

Myotonic dystrophy type 1 caused by expanded CTG repeats in the 3′ UTR of *DMPK* (myotonic dystrophy protein kinase) gene is the most prevalent form of adult muscular dystrophy.[56] Research was conducted on skeletal muscle cells isolated from individuals with myotonic dystrophy 1 using U7 Sm OPT containing 15 CAG repeats. The construct targeted the expanded CUG repeats of mutant *DMPK* transcripts in myotonic dystrophy.[60] This resulted in the subsequent release of mRNA binding factors and MBNL1 from the foci, improving splicing abnormalities and differentiation defects. Thus, permanent targeted degradation of pathogenic *DMPK* mRNA was ensured without affecting the products of wild-type *DMPK* allele over subsequent cell divisions.

5.2 | U7 snRNA therapy for treating DMD

DMD is an X-linked recessive muscle-wasting severe muscular disorder caused by mutations in the dystrophin gene, leading to

TABLE 1 Summary of different research studying the use of U7 snRNP in gene therapy

Condition	Description
Nevoid basal cell carcinoma syndrome[52]	**Cause** – mutation c.584G>A in the *PCTH1* gene at the 3′ end of exon 3 leading to insertion of a 37 bp intronic sequence between exon 3 and exon 4 and premature termination of PCTH1 protein **Study**[52] – used HeLa cell line. U7 Sm OPT contains a sequence complementary to the cryptic donor splice site of *PCTH1* intron 3. U7 Sm OPT was transfected along with a minigene containing the sequence for exon 3, intron 3 and exon 4. Authentic splicing restored
Breast cancer[52]	**Cause** – mutation IVS16+6 T>C, located in intron 16 of *BRCA1* gene leading to a 65 bp insertion of the 5′ end of intron 16 and premature termination of BRCA1 protein **Study**[52] – used HeLa cell line. U7 Sm OPT contains a complementary sequence encompassing activated cryptic splice site in intron 16. Transfected along with a minigene containing the sequence of exon 16, intron 16 and exon 17. Observed partial correction of splicing and premature termination.
Congenital adrenal insufficiency[52]	**Cause** – mutation c.566C>T in the exon 3 of *CYP11A* gene leading to a 61-bp deletion of exon 3 **Study**[52] – used HeLa cell line. U7 Sm OPT contains anti-CYP11A sequence targeting the cryptic donor splice site in exon 3. Transfected along with minigenes containing exon 3. Targeted exon deletion to various degrees rather than restoring splicing was observed
Myotonic dystrophy 1 (DM1)[57]	**Cause** – expanded CTG repeats in the 3′ UTR of the *DMPK* gene **Study**[57] – used skeletal muscle cells isolated from individuals with DM1, containing various CTG expansions, and myoblasts. U7 Sm OPT contains antisense oligonucleotide with 15 CAG repeats. Long-lasting selective destruction of deleterious CUG^{exp} RNAs in DM1 cells and in transduced wild-type myoblasts in a dose-dependent manner was observed
DMD[44,59,60,64,65,70,71]	**Cause(s) –** mutations in the *dystrophin* gene resulting in a premature stop codon leading to the absence of functional dystrophin at the sarcolemma and finally to muscle fibre necrosis. Mutations include, for example: (i) deletion or duplication of exon 2[53]; (ii) nonsense mutation in exon 23 in *mdx* mice[53] (iii) C7360A mutation in exon 51[53]; and (iv) single base change (A to G) in the 3′ splice site of intron 6 of the dystrophin gene, which cause skipping of exon 7 and termination of the ORF within N-terminus of exon 8[62] **Study**[64] – used HEK 293 cells, HSMM cells (wild-type or with exon 2 duplication), fibroblast cell lines, myoblasts and DMD mouse model (Dup2). U7 Sm OPT targeted exon 2 of the *dystrophin* gene contains antisense oligonucleotides targeting splice donor and splice acceptor sites. Exon 2 skipping resulted in generation of a truncated reading frame upstream of the IRES, which led to the synthesis of a functional N-truncated isoform in both human subject–derived cell lines (HEK293, HSMM) and in Dup2 model. Expression of the truncated isoform protected the muscle from contraction-induced injury and corrected muscle force to the same level as that observed in control mice **Study**[59] – used dystrophin deficient mouse models, *mdx* mice. Double target U7 Sm OPT (AAV-U7-SD23/BP22)[59] contains: (i) a 24-nucleotide sequence located across the splicing branching point in intron 22 (BP22) and (ii) a 20-nucleotide sequence in intron 23 that corresponds to the U1 binding region at the donor site (SD23). Reported significant stability of exon 23 skipping in comparison to other U7 snRNA-based constructs in myoblasts or oligonucleotides injected *in vivo* **Study**[65] – used myoblasts from H-2Kb-tsA58 *mdx* mice and C2C12 cells. Double target U7 Sm OPT targeted to block the 5′ splice site and the branch point upstream of exon 23. Using a double target molecule yielded a maximum effect but not an additive effect as in the case of β-thalassemia[65] **Study**[60] – used *mdx* mice and dystrophin/utrophin double-knockout mice. Used the construct as described by Le *et al*.[60] for treatment and showed that arrest of dystrophin process is crucial for maintaining viral genomes. Further reported that nontherapeutic U7 Sm OPT resulted in the loss of AAV genome within three weeks which correlates with dystrophin loss but except in the heart **Study**[44] – bifunctional U7 Sm OPT containing antisense sequences to exon 51, targeting either the acceptor or donor splice site, was transduced into immortalized myoblasts from healthy individuals and from DMD patient carrying a deletion of exons 49 and 50 to restore ORF and dystrophin expression. The second construct carried a 20-nucleotide sequence complementary to exon 51 and a free tail harboring high-affinity binding site for hnRNPA1.[59]The study suggests that either the exonic splicing enhancer, proper internal secondary RNA structure, or combination of two play an important role in correct splicing of exon 51. Remarkably, the hnRNPA1-tailed U7 Sm OPT induced complete exon 51 skipping in patient cells with restoration of dystrophin expression to almost wild-type level **Study**[70] – used double knockout utrophin/dystrophin mice, single dose injected intravenously. The construct described in reference[59] was used. Restored near-normal levels of dystrophin in all muscles, including heart; the treated muscles showed dystrophin detection even 1 year after injection **Study**[71] – Golden retriever muscular dystrophy dog (GRMD), injected intramuscularly with proximal muscles as a test and with contralateral muscles as control. Three-week olf GRMD puppies were injected intramuscularly in their whole left cranial leg muscle compartment. Two GRMD dogs were given high pressure intravenous injection in one of the forelimbs by locoregional delivery. Used constructs AAV1-U7E6 and AAV1-U7E8ref.[71] The constructs contained 2′ O-methylated 22 bp and 24 bp antisense

TABLE 1 (Continued)

Condition	Description
	oligonucleotides against exon-splicing enhancers of exon 6 and exon 8, respectively. Partially functional truncated dystrophin was restored
ALS[80]	**Cause** – single base substitutions such as H48Q,[77] insertion mutation such as 132insTT,[77] CA dinucleotide repeat (D21S223) in exon 2,[78] C to A substitution at codon 41 in exon 2[79] in human *SOD1* gene **Study**[80] – used ALS mouse model B6SJLTg (SOD1*G93A)1Gur/J. U7 Sm OPT contains two steric blocking RNA-based antisense oligonucleotides masking the splicing acceptor site in intron 1 or the exon splicing enhancer in exon 2 to promote efficient skipping of exon 2. U7 Sm OPT was administered using AAV vector by intravenous and intracerebroventricular routes combined. Therapy at birth or at 50 days of age delayed disease onset, prevented weight loss, prevented the decline of neuromuscular junction, and increased life expectancy by 92% and 58%, respectively
β-Thalassemia/HbE disorder HbE; $\alpha_2\beta_2^{26Glu\ to\ Lys83}$	**Cause** – G to A mutation (glutamic acid is changed to lysine) at codon 26 in exon 1 of the human *β-globin* gene, leading to the activation of the cryptic 5′ splice site at codon 25 and generation of aberrantly spliced β^E-*globin* mRNA with a premature termination codon at position 55, leading to the reduction of β^E-globin chains **Study**[83] – used HeLa β^E-cell model and erythroid progenitor cells from β-thalassemia/HbE patients. The U7 Sm OPT (U7 βE4+1) (UCCACUUGCACCUACUUCAACCACC) targeted 102–127 nucleotides of exon 1. Observed near complete splicing correction for 5 months in HeLa cells. Furthermore, observed improved erythroid cell pathology
β-Thalassemia[43,85,86,88,91]	**Cause** – mutation at position 654 (C to T), or 705 (T to G), or 745 (C to G) in intron 2 of *β-globin* gene creates an aberrant 5′ splice site at different positions but a common cryptic 3′ splice site at the nucleotide 579 in the *β-globin* intron 2 **Study**[43] – used HeLa cell line stably expressing plasmids carrying *β-globin* gene with each of the three mutations. Efficient and permanent correction of aberrant splicing and production of β-globin levels similar to cells expressing wild-type gene could be obtained by stable expression of a double target construct U7-BP+5′654. U7–3′[c] and U7–3′/24[c] are other potent candidates **Study**[88] – used HeLa cell line carrying the thalassemic IVS2–705 human *β-globin* gene and cell lines stably expressing U7 Sm OPT, containing a sequence antisense to either the 5′ splice site created by the 705 mutation (U7.5) or to the cryptic 3′ splice site activated in the aberrant splicing pathway (U7.3 and its derivatives). The approach was effective at restoring correct splicing. 65% and 55% correction in cell lines **Study**[86] – used iPSCs derived from mesenchymal stromal cells from a patient with IVS2–654 β-thalassemia mutation. U7 Sm OPT (U7.623) (reference[91]) carrying antisense oligonucleotide (UGUUAUUCUUUAGAAUGGUGCAAAG) targeted the 623 position of intron 2 of the IVS2–654 β-globin pre-mRNA. Erythroblasts generated from these iPSCs expressed ∼80% restoration in splicing compared to healthy cells. Initial report of combined use of U7 Sm OPT with patient-specific iPSCs together to treat patients **Study**[91] – used HeLa cells expressing IVS2–654, IVS2–705 and IVS2–745 human thalassemic *β -globin* genes. U7 Sm OPTU7.623 targeted the 623 position of intron 2 of *β-globin* mRNA and U7.324 targeted cryptic 3′ splice site activated by IVS2–654 mutation in the *β* -globin gene. It increased the levels of correctly spliced *β -globin* mRNA and protein by for at least 6 months. It showed therapeutic potential in haematopoietic stem cells and erythroid progenitor cells from a patient with IVS2–745/IVS2–1 thalassemia. 25-fold correction in patient cells after 12 days of transduction was observed **Study**[85] – used HeLa IVS2–654 cells and erythroid progenitor cells from patients carrying $\beta^{IVS2-654}$ thalassemia mutation. U7. BP+623 targeted the cryptic branch point site and the exonic splicing enhancer in intron 2 of *β-globin* pre-mRNA. Therapeutic potential was shown in both cell models
HIV-1 infection/AIDS[93–95]	**Cause** – HIV type 1 **Study**[93] – used HEK 293 T, HeLa and human T-cell lines CEM-SS or CEM. U7 Sm OPT constructs contain antisense sequences targeting flanking internal HIV-1 exons to reduce tat and rev expression. A merged construct with an additional exonic splicing enhancer and upstream splice donor, named as ESE/SD4, proved 40–50% effectiveness in the context of 'real' HIV-1 replication in human T cells of CEM-SS line **Study**[94] – used HEK 293 T, HeLa, CEM-SS, CEM, P4.2 and $CD4^+$ T cells. Triple combination therapy was used: (i) shRNA targeting nucleotides 330–348 of human *cyclophilin A* mRNA; (ii) shRNA targeting nucleotides 423–443 and 479–498 of the *vif* ORF of HIV; and (iii) U7 Sm OPT ESE/SD4 as in reference.[93] Complete inhibition of viral multiplication in semi-permissive CEM T cells was observed **Study**[95] – used cell lines HEK 293 T, HeLa and human T-cell lines Jurkat and CEM-SS. U7 Sm OPT double target constructs targeted 5′ and 3′ splice site of exon 3 or exon 4 of *cyclophilin A* gene to eliminate either exon or both and inhibit the interaction of cyclophilin A with the capsid protein of the virus. In addition, siRNAs targeting the region between nucleotides 265 to 283 and 330 to 348 of *cyclophilin A* mRNA were used. Treated CEM-SS cell line showed delayed and reduced HIV-1 multiplication
SMA[45,46,104,107,108]	**Cause** – homozygous exon 7 deletion or inactivation of *SMN1* gene. Furthermore, C to T transition in *SMN2* gene located at position 6 of exon 7 causes exon 7 deletion in mRNA, leading to the synthesis of truncated protein

(Continues)

TABLE 1 (Continued)

Condition	Description
	Study[104] – used HeLa cells. U7 Sm OPT contains antisense nucleotide complementary to 36 nucleotides upstream and 34 nucleotides downstream of the intron7/exon 8 junction to target the 3′ splice site of exon 8. Anti-SMN U7 Sm OPT G as the most potent along with anti-SMN U7 Sm OPTs targeting intronic silencer or exon 7 was found. These potent molecules contained ∼20 nucleotides hybridization regions with high G/C content. The length of the most potent anti-SMN RNAs was between 18 to 22 nucleotides. In addition to length and G/C content, competing secondary structures of anti-SMN U7 snRNAs are important. Inclusion of exon 7 is present as long as the expression cassette is retained in the cell **Study** [45] – used HEK 293 T cells, HeLa cells and immortalized human fibroblasts from SMA type 1 patient and a healthy individual. bifunctional U7 Sm OPT (U7-ESE-B) (sequence B – GUGCUCACAUUCCUUAAAU)[54,55] carries an antisense sequence to exon 7 of SMN2 gene together with an exon splicing enhancer or serine arginine repeat. The approach prolonged SMN protein restoration ensuring its localization in gems **Study**[46] – used SMA mice strain FVB.Cg-Tg (SMN2)89Ahmb smn1tm1Msd/J, stock number: 005024 from Jackson laboratories. Used U7-ESE-B construct from reference.[45] Introducing therapeutic U7 snRNA by germline transgenesis resulted in efficient rescue of exon 7 in the most severe SMA mouse model **Study**[105] – used severe SMA mouse strain (Burghes severe model) with stillborn or death by postnatal 4–6 days, stock number: 005204 from Jackson laboratories. Neuromuscular junctions of the diaphragm and soleus muscles having the discrete function in breathing and locomotion were selected for study. Used U7-ESE-B construct from reference.[45] Neuromuscular junction in treated mice showed correct SMN2 splicing, with delayed or no SMA symptoms **Study**[107] – used HeLa cells and SMA1-patient-derived fibroblast. Five U7 Sm OPT constructs were designed to target exon 8 of *SMN* gene. Upregulation of SMN levels similar to control cells was observed **Study**[108] – used HeLa S2 and HeLa cells stably transformed with human SMN2 minigene and SMN Δ7 mice, stock number: 00525 from Jackson laboratories. Used U7-ESE-B construct from reference,[45] delivered by intracerebroventricular injection. Introduction into motoneurons significantly increased life span and improved muscle function. Therapeutic U7 snRNA was also observed to be expressed in the heart and liver

aberrant or reduced levels of the dystrophin protein. The majority of mutations causing DMD are deletion mutations, which disrupt the open reading frame (ORF) and lead to the synthesis of prematurely terminated proteins.[58] Approximately 70% of the mutations lead to the absence of dystrophin protein and thus to severe DMD phenotype.[59] The presence of qualitatively and quantitively altered dystrophin protein results in Becker muscular dystrophy, a mild form of DMD. In Becker muscular dystrophy, by preserving the reading frame, a truncated, partially functional dystrophin protein is produced that contains deletions in the rod domain with an intact N- and C-terminus. For treatment, research is being carried out that aims to use antisense oligonucleotides to induce specific exon skipping at the dystrophin pre-mRNA level. The target for therapy is the central rod-domain because it is known to tolerate large internal deletions. The aim is to convert the "out-of-frame" mutation into an "in frame" mutation, giving rise to internally deleted but still functional dystrophin.[60,61] Moreover, deletion or duplication of exon 2, nonsense mutation in exon 23, C7360A mutation in exon 51, and A to G mutation in the 3′ splice site of intron 6 of dystrophin gene leading to exon 7 skipping are some of the examples of the cause of DMD.[62]

Patients with exon 2 deletions are either asymptomatic or show mild symptoms as a result of the expression of N-truncated isoform of dystrophin.[63] For treatment, two copies each of antisense oligonucleotides targeting splice donor and splice acceptor were introduced into U7 Sm OPT and delivered using AAV. Research conducted on patient fibroblasts, myoblasts and mice using this construct showed efficient skipping of exon 2 resulting in alternative translation initiation in exon 6 (via an internal ribosome entry sequence), leading to the expression of a functional N-truncated protein.[64] Based on these studies, a recent clinical trial "AAV9 U7 snRNA gene therapy to treat boys with DMD exon 2 duplications" is now underway, starting from January 2020, registered at NIH with the clinical trials.gov identifier NCT04240314. This clinical trial focuses on skipping of exon 2 leading to either mRNA containing only one copy of exon 2 mRNA (error-free) or no copy of exon 2, thus giving rise to a highly functional isoform.

One of the earliest research studies on the rescue of DMD via U7 snRNA-mediated exon skipping reported using a 'double-target' U7 Sm OPT to skip exon 23 of *mdx* dystrophin mRNA[59] (Figure 4A). In this study, Goyenvalle and colleagues used U7 Sm OPT equipped with two sequences: (i) a 24-nucleotide sequence complementary to the splicing branch point within intron 22 and (ii) a 20-nucleotide sequence complementary to U1 snRNA binding region within intron 23.[65,66] The engineered U7 snRNA was introduced into skeletal muscles of *mdx* mouse using AAV vector.[67] The observed level and stability of exon skipping were significantly higher than those obtained using other U7 snRNA-based constructs in myoblast cultures or using oligonucleotides injected *in vivo*. Histology performed after treatment showed healthy morphology in corrected muscles.[59] In another study performed on *mdx* mice, AAV vector was encoded with U7 Sm OPT as reported previously by Goyenvalle *et al*.[59] This construct allowed efficient exon 23 skipping, therefore rescuing dystrophin. However, it was observed that, over the course of time, loss of AAV vector genome from muscle fibres led to a decrease in exon-skipping therapeutic effect.[60]

Next, Goyenvalle and colleagues reported bifunctional U7 Sm OPT as a promising tool for DMD therapy.[44,68] The bifunctional

U7 Sm OPT was constructed as follows: first, the U7 Sm OPT was equipped with a splicing silencer sequence complementary to the exonic splicing enhancer located within exon 51 of the human *dystrophin* gene, aiming to promote efficient skipping of exon 51. Furthermore, the U7 Sm OPT carrying splicing silencer was extended by a tail harbouring canonical binding sites for the heterogeneous ribonucleoprotein A1 (hnRNPA1), which naturally contributes to exon skipping. Enhanced exon 51 skipping, induced by this bifunctional U7 Sm OPT, was observed to restore a near wild-type level of dystrophin expression in myoblasts derived from DMD patients. The efficacy of the U7 Sm OPT construct was further confirmed by the observation of positive results after subsequent injection into the tibialis anterior muscle of a mouse model transgenic for the entire human dystrophin locus.[44,69] Based on this strategy, gene therapy employing bifunctional U7 Sm OPT showed positive results with respect to treating muscular dystrophy in both *mdx* mice and golden retriever muscular dystrophy dogs.[61,70]

A study performed on a golden retriever muscular dystrophy dog reported sustained correction of the dystrophic phenotype with U7 Sm OPT carrying antisense oligonucleotides designed for exon skipping therapy. In golden retriever muscular dystrophy dogs, a single base mutation changes the 3′ end of intron 6, leading to skipping of exon 7 and termination within the N-terminal domain of exon 8.[71,72] Using antisense sequence to skip exons 6 and 8, it is possible to restore partially functional truncated dystrophin. The researchers constructed two U7 Sm OPT cassettes for targeting exon 6 or exon 8 skipping. These two different constructs were delivered using AAV vector to treat the forelimb of a golden retriever muscular dystrophy dog by locoregional delivery. No immune rejection was observed after a 5-year follow-up.[71]

The research performed on DMD is very successful and medication such as eteplirsen (brand name Exondys 51) has been approved in the USA to treat DMD.[73] Ataluren is another drug that has been approved for use in certain cases.[74] Another study sheds light on using the *utrophin* gene as therapy for the treatment of DMD in animal models.[75] In this case, miniaturized utrophin, a highly functional and non-immunogenic substitute for dystrophin, is used, thus preventing the most deleterious histological and physiological aspects of DMD in small and large animal models.[75]

5.3 | U7 snRNA therapy for treating ALS

ALS is a neurodegenerative disease involving mostly mutations in *SOD1* (superoxide dismutase 1) gene. The human *SOD1* gene is located on chromosome 21 and encodes the superoxide dismutase 1 enzyme responsible for destroying potentially toxic free superoxide radicals in the body. Over 180 mutations in the *SOD1* gene are described as being involved in ALS.[76] Some of the mutations are single base substitutions such as H48Q,[77] insertion mutation such as 132insTT,[77] CA dinucleotide repeat (D21S223) in exon 2[78] and C to A substitution at codon 41 in exon 2.[79]

One study mentions using U7 Sm OPT to restore the function of SOD1 and prolong the survival of mice in an ALS mouse model.[80] For this, high copy $SOD1^{G93A}$ mice (Jackson SN 2726) were used.[80] U7 Sm OPT containing an antisense sequence targeting exon 2 of the human *SOD1* pre-mRNA was embedded in AAV vectors and administered by intravenous and intracerebroventricular routes combined.[80] It was designed to mask the splicing acceptor site in intron 1 or the exonic splicing enhancer in exon 2 to promote exon 2 skipping. This led to the production of mRNA with a premature stop codon, subjecting it to a RNA degradation pathway and thus reducing the level of toxic protein[80,81] (Figure 4B). This treatment, when initiated at birth or at 50 days of age, lead to a delay of disease onset and an increase in life expectancy of 92% and 58%, respectively, thus indicating the effectiveness of an exon-skipping approach in SOD1-ALS mice. Therefore, in ALS studies, exon 2 is targeted with the aim of producing mature mRNA containing a premature stop codon to inhibit protein synthesis and thus ultimately reduce the level of toxic proteins. This is in contrast to the discussed therapy for DMD (related to exon 2), in which exon 2 carrying the mutation was skipped to produce a wild-type protein or its isoform.

5.4 | U7 snRNA therapy for treating β-thalassemia

β-thalassemia is an autosomal recessive hereditary disease caused by defects in the β- chain, which affect hemoglobin (Hb) production. The hemoglobin E (HbE) $\alpha_2\beta_2 26$ variant, where glutamic acid is changed to lysine as a result of G to A mutation at codon 26 in exon 1 of the human *β-globin* (*HBB*) gene, is a widespread HbE variant found among the Southeast Asian population.[82,83] This substitution activates the cryptic 5′ splice site at codon 25, which generates an aberrantly-spliced β^E*-globin* mRNA containing a premature termination codon at position 55, leading to 16-nucleotide deletion of the 3′ end in exon 1 of *β-globin* pre-mRNA. In a study that focused on the treatment of β-thalassemia patients using engineered U7 snRNA, antisense oligonucleotides, spanning nucleotides from 102 to 130 of *β-globin* mRNA exon 1, were designed to target the 5′ cryptic splice site created by HbE mutation.[83] These oligonucleotides were incorporated into U7 Sm OPT and transiently introduced into cells to test the optimal target site for U7 Sm OPT to attain maximum splicing correction of *β-globin* mRNA. From the series of vectors tested, one of the most active engineered U7 Sm OPT vectors, U7 βE4+1 snRNA, was selected. It was then transduced into the HeLa-β^E model cell line and effectively restored the correctly spliced β^E*-globin* mRNA for at least 5 months. The U7 βE4+1 snRNA was further used to correctly splice β^E*-globin* mRNA in erythroid progenitor cells from β-thalassemia patients, resulting in phenotypic improvements of β-thalassemic erythroid cells.[83]

Aberrant splicing is also associated with mutations in intron 2 of the *β-globin* gene at nucleotide positions 654, 705 or 745. These mutations are not the same (in mutation IVS2–654, C is substituted to T; in mutation IVS2–705, T is substituted to G; in mutation IVS2–745, C is substituted to G) and generate aberrant 5′ splice sites at different positions while activating the same cryptic 3′ splice site at the

579 position, leading to the inclusion of intronic sequences in *β-globin* pre-mRNA.[84–86] Mutations in intron 2 result in the generation of a premature stop codon and inhibit translation into full-length β-globin, leading to β-thalassemia.[84] Antisense oligonucleotides targeting either 5′ or 3′ splice site were shown to repair β-globin mRNA in mammalian cells.[87] Schuemperli and colleagues first reported the use of the U7 Sm OPT-mediated approach in splicing correction of *β-globin* genes carrying mutations IVS2–654, IVS2–705[88] and IVS2–745, expressed from exogenous plasmids in HeLa cells.[43] Some key points from this foundational research were (i) an optimum sequence of approximately 24 nucleotides influenced the efficiency of antisense sequence and (ii) combining two antisense sequences directed against different target sites in intron 2 significantly enhanced the efficiency of splicing correction[43] (Figure 4C).

Mutation IVS2–654 creates an intron inclusion [nucleotides 580–652][89,90] and patients with this IVS2–654 mutation produce a minor amount of correctly spliced *β-globin* mRNAs with severe symptoms of β-thalassemia. An initial report mentioned combining U7 Sm OPT and induced pluripotent stem cells (iPSCs), leading to successful aberrant splicing reduction of the *β-globin* gene in IVS2–654 β-thalassemia disease.[86] In this study, the U7 Sm OPT, called U7.623 snRNA, was constructed as reported by Vacek *et al*.[91] It contained a 25-bp antisense oligonucleotide targeting the 623 position of intron 2 of *β-globin* mRNA[91] to restore correct splicing and protein levels. This U7 Sm OPT was transduced into iPSCs derived from mesenchymal stromal cells from a patient with the IVS2–654 mutation using a lentivirus system. The U7 Sm OPT stably integrated into the genome and maintained splicing correction over many passages. Erythroblasts were further generated from these transduced iPSCs, which expressed high levels of correctly spliced *β-globin* mRNA. These erythroblasts were further differentiated into haematopoietic stem cells and transplanted back into the same patient with a reduced chance of rejection.[86]

Another study reported the restoration of correct $\beta^{IVS2-654}$*-globin* mRNA splicing and hemoglobin production using U7 Sm OPT (U7.BP +623).[85] U7 BP+623 targeted the cryptic branch point and an exonic splicing enhancer. Therapy was performed using thalassemia patient erythroid progenitor cells, as well as HeLa cells carrying the $\beta^{IVS2-654}$ thalassemic mutation. Altogether, this approach showed a positive response by restoring correctly spliced *β-globin* mRNA and correcting thalassemic erythroid cell pathology.[85]

5.5 | U7 snRNA therapy for treating HIV infection

HIV is a single-stranded, positive sense, enveloped RNA retrovirus.[49] HIV cellular tropism is immune cells of the body, with $CD4^+$ T cells being the preferred target.[49,92] HIV-1 exploits alternative splicing, which makes it stand out from other retroviruses. In addition to the standard retroviral genes *gag*, *pol* and *env*, HIV-1 also codes for *tat*, *rev* and *nef*, expressed at an early stage from the integrated provirus genome along with some other proteins. Nef attunes the physiological status of the host cell, whereas Tat and Rev are RNA-binding proteins important for the synthesis of full-length genomic RNA, the regulation of expression of other viral genes and the production of progeny virions.[49] HIV-1 uses alternative splicing in which Tat strongly activates transcription and Rev channels unspliced and partially spliced RNAs into a nucleo-cytoplasmic export.[49]

In a study on HIV-1 inhibition, Schuemperli and colleagues employed U7 Sm OPT equipped with two antisense sequences directed against the *tat* and *rev* pre-mRNA internal exons.[93] Targeting the transcripts leads to exon skipping within *tat* and *rev* ORFs, inhibition of expression of both proteins and thus inhibition of transition into the late phase of the viral replication cycle.[43,93] This U7 snRNA-based approach resulted in the suppression of HIV-1 multiplication by up to 50% in human T cells. It was further suggested that the use of other antivirals or siRNA-based methods in combination with the U7 snRNA-based approach will lead to increased effects, ensuring a robust response and preventing the evolution of viral escape mutants.[93]

The inhibitory effect of the U7 Sm OPT was observed to be stronger for a viral infectivity factor (Vif)-deficient virus. Therefore, a new approach combining RNAi and U7 Sm OPT strategies was developed.[94] Three different cassettes containing antiviral RNAs were expressed from one triple lentiviral vector: shRNA against the host factor cyclophilin A (CyPA), U7 Sm OPT to modulate exon skipping of *tat* and *rev* viral pre-mRNA, and shRNA targeting the *Vif* ORF. This approach dramatically affects HIV-1 infection and allows for a strong inhibition of HIV-1 multiplication in human T cell lines. Moreover, all three therapeutic RNAs exhibit antiviral effects at early stages of the viral replication cycle. Using these strategies, no changes in cell proliferation or morphology have been observed, suggesting a low toxicity to cells.[94]

Inhibition of HIV-1 replication by targeting the cellular protein cyclophilin A is another strategy based on U7 Sm OPT.[95] Cyclophilin A is known to engage Gag polyprotein in HIV-1 replication and is essential for HIV-1 infectivity.[96] Importantly, this protein is neither crucial for early development, nor essential for cell survival.[97] In this research, U7 Sm OPT was exploited as an alternative splicer by inserting appropriate antisense sequences directed against the 3′ and 5′ splice sites of exons 3 and 4 of *cyclophilin A* pre-mRNA, respectively. Using this approach, efficient skipping of these exons was achieved, which in turn disturbed the protein ORF. As a consequence, a significantly reduced level of cyclophilin A protein and delayed multiplication of HIV-1 in T cells were observed.[95] The ability of the cells to sustain HIV-1 replication was impaired. In the same study, RNAi was used in addition to U7 Sm OPT to reduce cyclophilin A. Adding RNAi along with U7 Sm OPT further improved the survival of T cells. It was further hypothesized that using the aforesaid strategy to modify haematopoietic stem cells instead of T cells would be beneficial to achieve a long-lasting effect.[95]

5.6 | U7 snRNA therapy for treating SMA

SMA is an autosomal recessive neuromuscular disorder characterized by the degeneration of anterior horn cells of the spinal cord, leading

to symmetrical muscle weakness and atrophy.[98] SMA is linked to a genetic mutation in the *SMN1* gene,[99] which is unable to correctly code for the SMN protein, either as a result of a deletion at exon 7 or other point mutations.[100] Simultaneously, a *SMN1* paralogous gene, the *SMN2* gene, has identical coding potential but, because of single point mutation in exon 7, an exonic splicing enhancer is disrupted or an exon silencer element is created. This favours alternative splicing at the junction of intron 6 to exon 8, resulting in truncated protein (SMNΔ7).[101,102] It is thus hypothesized that correcting the splicing of exon 7 would help in the treatment of SMA. Because exclusion of exon 7 is a major reason for the pathogenesis of SMA and individuals with SMA usually lack the correct *SMN1* gene and have a functional *SMN2* gene, modulating the inclusion of exon 7 in the human *SMN2* gene represents an attractive therapeutic outlook.[103]

Exchange of the anti-histone region of U7 snRNA with a region complementary to the intron7/exon 8 junction of *SMN2* pre-mRNA increases the inclusion of exon 7.[104] Indeed, the administration of such anti-SMN U7 Sm OPT to a HeLa cell line expressing the *SMN2* minigene favoured exon 7 inclusion in *SMN2* mRNA and resulted in a higher concentration of full-length SMN protein.[104]

Next, a more permanent strategy using bifunctional U7 snRNA therapy to correct *SMN2* pre-mRNA splicing was developed.[45] In this approach, the U7 Sm OPT was modified both with an antisense sequence binding to exon 7 of *SMN2* pre-mRNA and with the splicing enhancer sequence, improving the recognition of the targeted exon. Addition of the splicing enhancer sequence to the U7 Sm OPT strongly stimulated *SMN2* exon 7 recognition and boosted exon inclusion, performing an almost complete re-inclusion of *SMN2* exon 7 in all systems tested. In fibroblasts from a type I SMA patient, this bifunctional approach induced prolonged expression of SMN protein, which was correctly localized within the cell.[45]

Schuemperli and colleagues confirmed the hypothesis that promoting *SMN2* exon 7 inclusion might help to prevent or benefit treatment of SMA.[46] They used a U7 snRNA-mediated splicing modulation approach to rescue a severe mouse model of SMA. The U7 Sm OPT construct was developed to target the 3′ end of exon 7 and carried an exon splicing enhancer to attract stimulatory splicing factors. When introduced into the mouse by germline transgenesis, this U7 Sm OPT construct proved efficient in rescuing a severe SMA mouse model. This process reversed the pathology to milder forms and, in some cases, restored neuromuscular functionality and life expectancy.[46]

U7 Sm OPT construct capable of stimulating the inclusion of *SMN2* exon 7 in severe SMA mouse models was also designed by Voigt *et al*.[105] They checked the effectiveness of the therapy using a severe mouse model where the mice contained two copies of the human *SMN2* transgene and one copy of the mouse *Smn* gene.[107] For this study, the diaphragm and soleus muscle, which have discrete functions in breathing and locomotion, respectively, were selected. It was observed that the diaphragm displayed prominent adverse morphological alterations in a severe mouse model. No significant changes were observed in either muscle of SMA mice undergoing treatment. Research was further extended by investigating neuromuscular junctions (NMJs). Unfortunately, the development of NMJs was incomplete in a severe SMA mouse model, as well as in treated individuals.[105]

To restore full-length SMN, Geib and Hertel[107] modified the histone binding region of the U7 snRNA to a sequence complementary to the 3′ splice site of *SMN* exon 8 to inhibit its recognition and induce exon 7 inclusion. The experiments were carried out in both HeLa cells and fibroblasts derived from SMA-1 patients. AAV vector delivered U7 Sm OPT therapy resulted in a higher rate of exon 7 inclusion, as well as an increase in the level of full-length SMN protein from 33% to 60%. Moreover, the percentage of gems significantly increased after AAV/U7 Sm OPT therapy, ensuring proper SMN nuclear localization.[107]

Recently, Schuemperli and colleagues performed somatic therapy on an SMNΔ7 mouse model (SMN1−/−, SMN2+/+, and SMNΔ7+/+).[108] The mice that would normally survive for only 14–17 days survived for a median of 150 days when treated with the U7 Sm OPT construct, prepared as described previously.[45,46] The SMNΔ7 mice, when injected with U7 Sm OPT, showed therapeutic U7 Sm OPT expressed in both the heart and liver. The mice grew smaller in size compared to wild-type mice and showed mild SMA symptoms, although they were able to feed and drink without any assistance. Additionally, the neuromuscular junctions were less affected compared to SMA models. In conclusion, somatic gene therapy using U7 Sm OPT is feasible for correcting the skipped exon and significantly improving the SMA phenotype.[108]

The research examples described above demonstrate that splicing correction of *SMN* mRNA by the U7 Sm OPT construct works for both transgenic and somatic gene therapy. Nusinersen (SPINRAZA™), an antisense oligonucleotide that acts as a modulator for alternative splicing of the *SMN2* gene and converts its functionality to the *SMN* gene, has been approved against SMA.[109–112] In May 2019, the US Food and Drug Administration approved Zolgensma, which represents the first gene therapy that has been approved to treat children aged less than 2 years who are diagnosed with SMA.[113,114]

5.7 | Limitations and benefits

U7 snRNP is not a spliceosomal U snRNP and thus requires additional modifications to function as a splicing modifier. For example, in U7 Sm OPT, the binding of unique Lsm proteins is changed to canonical Sm proteins. However, it can generate errors in the 3′ end processing of U7 snRNA and lead to the production of a truncated (20 nucleotides shorter) version of U7 snRNA that is unlikely to mediate correct pre-mRNA splicing. Because the higher level of truncated U7 snRNA was observed in mice compared to humans, it has been suggested that the processing of U7 Sm OPT differs in these two species and thus should be taken into consideration.[115,116] It must also be considered that the snRNA processing capacity of the cell is limited, and that going past this limit may lead to additional U7 snRNA by-products, which comprise both 3′ processed and unprocessed species. Understanding the formation of these potentially inactive by-products is necessary to improve the potency of U7 snRNP-based gene

therapy.[116] A limitation for using U7 snRNP in gene therapy is also the amount of vector that can be administered. Administering modified snRNA using the AAV vector requires very high doses that are potentially toxic.[116] Moreover, a study performed on *mdx* mice emphasizes how the immune response against AAV leads to the production of neutralizing antibodies and thus forbids reinjection, indicating it as a major drawback of the AAV vector approach.[60] Successful delivery is not only a hurdle for administering U7 Sm OPT but for all antisense therapies in general.[117,118] Even though all these difficulties exist, viral vectors including AAV vectors and bioreducible lipid nanoparticles comprise useful candidates for delivery, creating a fertile ground for antisense oligonucleotide incorporated U7 snRNA-based therapeutics.[50,119–121]

Some questions that remain to be answered are, for example, how to decide a time point to start the therapy? In the case of diseases that develop in a later stage of life, would individual screening for genetic diseases be practised? Nowadays, screening for breast cancer is performed for individuals with genetic background, and maybe this approach can be expanded to other diseases as well. The economic constraint is a hurdle that needs to be overcome. A further consideration is the number of U7 snRNA copies required to achieve the desired correction. High dosage would ensure improved correction but can pose a threat of potential side effects if the dosage increases beyond the cellular processing capacity. Low dosage treatment can have no or minimum side effects but, at the same time, it may not provide maximum correction. It can thus lead to repeated visits to the therapy centre. These decisions are very complicated because they involve physical, economical, psychological and, importantly, emotional factors concerning the affected individual and its family.

Neurodegenerative diseases still remain to be fully understood, with a number of different hypotheses describing the process and cause of neurodegeneration. SMN deficiency, found in SMA and ALS, leads to lower levels and defects in U snRNP assembly, including U7 snRNP. Therefore, the biogenesis and function of U7 snRNP can paradoxically be disrupted in diseases, and thus used as a potential tool in gene therapy.

RDH pre-mRNA 3′ end processing is undoubtedly the fundamental role of U7 snRNP but, by introducing controlled changes at the histone binding region and the binding site of Sm/Lsm proteins, it can be used as an effective tool for gene therapy. A modification such as converting U7 snRNA into U7 Sm OPT facilitates the accumulation of U7 snRNP in the nucleus and, at the same time, the particle is no longer functional for the processing of *RDH* pre-mRNAs, making it an advantage.[28,36] The benefits of using U7 snRNP in gene therapy include its compact size and the ability to accumulate in the nucleus without causing any toxic effects to the cells. U7 cassettes will fit into smaller vectors and do not carry the risk of transgene misregulation because the therapeutic RNA only corrects the expression of an endogenous gene that is controlled by its own regulatory elements. The benefit of U7 snRNA-based gene therapy is also that a single treatment can ensure a lifelong therapeutic effect, while an oligonucleotide or other drug/compound would require repeated administration. U7 snRNP is a unique resource available exclusively to metazoan cells and it would be strategic to use our resources to the fullest.

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AUTHOR CONTRIBUTIONS

Ankur Gadgil prepared the main text of the manuscript, as well as Figures 1 to 4 and Table 1. Katarzyna Dorota Raczynska revised the manuscript.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article because no datasets were generated or analysed during the current study.

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