Future
Medicinal Chemistry

Advances in therapeutic development for spinal muscular atrophy

Spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality. The disease originates from low levels of SMN protein due to deletion and/or mutations of *SMN1* coupled with the inability of *SMN2* to compensate for the loss of *SMN1*. While *SMN1* and *SMN2* are nearly identical, *SMN2* predominantly generates a truncated protein (SMNΔ7) due to skipping of exon 7, the last coding exon. Several avenues for SMA therapy are being explored, including means to enhance *SMN2* transcription, correct *SMN2* exon 7 splicing, stabilize SMN/SMNΔ7 protein, manipulate SMN-regulated pathways and *SMN1* gene delivery by viral vectors. This review focuses on the aspects of target discovery, validations and outcome measures for a promising therapy of SMA.

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Spinal muscular atrophy: genetics & phenotype

Spinal muscular atrophy (SMA) is a neurodegenerative disease and a leading cause of infant mortality that occurs in approximately 1 in every 10,000 live births [1]. The disease manifests as progressive degeneration of α motor neurons in the spinal cord and the consequent atrophy of muscles. In >95% of cases, SMA is caused by the deletion, mutation or gene conversion of *SMN1*, a gene that encodes the ubiquitously expressed SMN (Figure 1A) [2,3]. *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for the loss of *SMN1* due to a C to T substitution (C6U in the mRNA) at the sixth position of exon 7 and an A to G substitution (A100G) at the 100th position of intron 7. The combination of C6U and A100G mutations triggers massive skipping of *SMN2* exon 7 [4,5]. The exon 7-skipped transcript encodes SMNΔ7, a truncated protein that is partially functional and quickly degraded [6,7].

SMA patients show remarkable variability in phenotypes. The spectrum ranges from fetuses that die *in utero* or soon after birth (type 0), infants born noticeably afflicted who die within 2 years of birth (type I), patients able to sit upright but not walk and who survive into their teens and adulthood (type II), patients able to walk independently with a normal or near normal lifespan (type III) and patients with adult-onset progressive muscle weakness (type IV). Barring a few exceptions [1,10–11], the copy number of *SMN2* appears to correlate at least partially with SMA disease severity [12–14]. Increased *SMN2* copy number may result from increased conversion of *SMN1* to *SMN2* or duplication of *SMN2* [15,16]. *SMN2* does generate low levels of full-length transcript capable of producing enough SMN for the majority of cell types, except motor neurons and muscles, to survive [17]. There is an ongoing search for tissue and cell-type specific regulators that are impacted by low levels of SMN. Several recent reviews describe progress on various fronts towards SMA therapy [10,18]. Based on independent reports of antisense oligonucleotide (ASO)- and gene-therapy-based preclinical studies, it is increasingly evident that postnatal treatment aimed at upregulation of SMN can substantially increase the lifespan of mouse models of severe SMA [18]. In this review, we provide an account of regulatory mechanisms that could be exploited to accelerate the therapeutic development for SMA.

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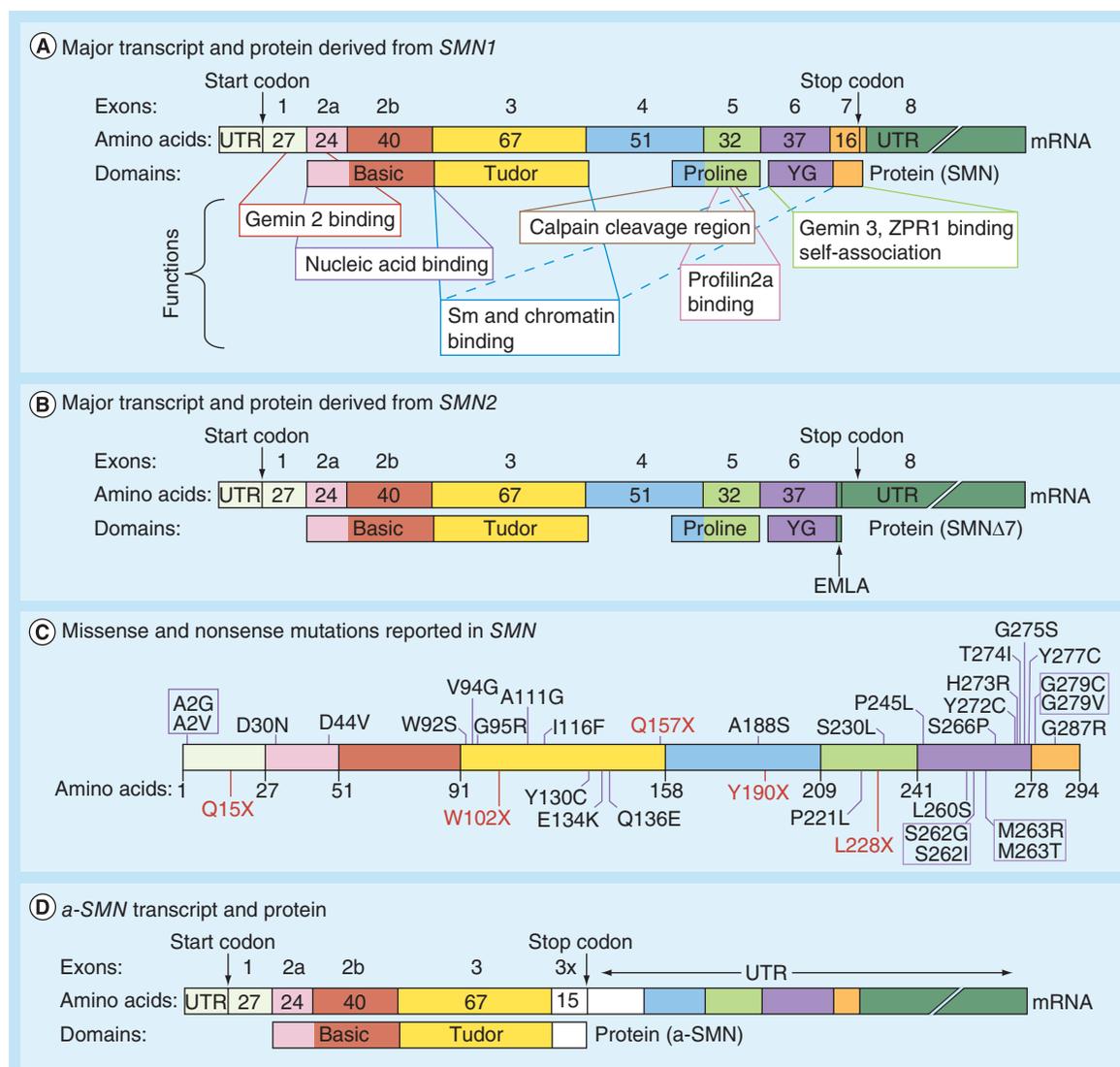
Figure 1. Description of transcripts and proteins encoded SMN. (A) Diagrammatic representation of the major transcript and the full-length protein derived from *SMN1*. Exons are presented as colored boxes with the number of amino acids noted within each box. Arrows indicate locations of the start and stop codons. Corresponding protein domains and their functions are indicated. The presentation is adapted from [8]. The new Tudor domain function of chromatin binding was recently reported [9]. **(B)** Diagrammatic representation of the major transcript and the truncated protein derived from *SMN2*. Arrows indicate locations of the start and stop codons as well as EMLA degron that renders the protein unstable. Other details including color codes are the same as in **(A)**. **(C)** Diagrammatic presentation of SMN protein with the mutations reported to date. Amino acids are represented by their one-letter codes. Position and type of mutations are indicated, with missense mutations shown in blue and nonsense mutations shown in red. Other details including color codes are the same as in **(A)**. **(D)** Diagrammatic representation of a-SMN transcript and protein. Presence of an extra exon due to inclusion of a portion of intron 3 (3x) is shown as white box. Other details including color codes are the same as in **(A)**. UTR: Untranslated region.

Structure–function relationship in SMN

SMN is a multifunctional protein coded by eight exons: 1, 2a, 2b, 3, 4, 5, 6 and 7; exon 8 is not translated (Figure 1A). The SMN protein contains several functional domains including Gemin2-binding, nucleic acid binding, Tudor, self-association and calpain cleavage (Figure 1A) [8,9]. The interaction of SMN with Gemin2 has been suggested to be important for several SMN functions including small nuclear ribonucleoprotein (snRNP) biogenesis [19], signal recognition particle biogenesis [20], DNA recombination [21], motor neuron trafficking of mRNAs [22] and translation regulation [23]. Point mutations across the entire primary structure of the protein have been linked to SMA pathogenesis (Figure 1C; Supplementary Table 1) [1–3,11,24–55]. None of the deletions or missense muta-

tions of SMN produce toxic effects and/or generate protein aggregates. Therefore, loss of SMN function associated with deletions or missense mutations is considered to be the major cause of SMA.

Skipping of exon 7 replaces the sixteen C-terminal amino acid residues of exon 7 with four amino acids (EMLA) coded by exon 8 (Figure 1B). EMLA serves as a degradation signal in SMNΔ7 [7]. Compounds that allow read-through of the first stop codon within exon 8, including a neomycin derivative, were shown to stabilize SMNΔ7 and may potentially provide a therapeutic option for SMA (Figure 2) [56]. Although not tested in SMA patients, the read-through compound ataluren has shown successful results in patients with Duchenne muscular dystrophy [57] and is undergoing Phase III clinical trials.

One of the most well characterized functions of SMN is assembly of snRNPs [63]. Participation of SMN in snRNP assembly is mediated through the SMN complex, which is composed of several proteins including Gemin2-8 and unrip [64]. While snRNP assembly is a cytosolic process, SMN is also localized in nuclear bodies called gems. It remains to be seen if gems have specific functions or whether they are the signatures of an ordered nuclear organization forced by the spatial and temporal arrangement of other macromolecules. Other

> **Key Terms**
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> **Antisense oligonucleotide:** Antisense oligonucleotides (ASOs) are nucleic acid molecules that anneal to specific RNA or DNA sequences. ASOs have been used to block regulatory sequences to modulate alternative pre-mRNA splicing.
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> **Small nuclear ribonucleoprotein:** Small nuclear ribonucleoproteins are the building blocks of spliceosome, which catalyzes the removal of intronic sequences during the essential process of pre-mRNA splicing. SMN is involved in biogenesis of small nuclear ribonucleoproteins.

less studied SMN functions include detection of specific chromatin modifications [9], transcription [65,66], translation [23], signal transduction [67], stress granule formation [68] and macromolecular trafficking [69,70]. Motor neurons are particularly susceptible to the loss of SMN protein, although the reasons underlying this susceptibility are not well understood. One plausible cause of neuronal susceptibility would be loss of SMN interactions with neuron-specific proteins such as HuD, a protein implicated in trafficking of mRNAs [71,72]. For instance, it was shown that interaction of SMN and HuD with mRNA cpg15 rescues motor neuron axonal deficits [71]. A role for SMN in axonal trafficking could be critical for neuromuscular junction (NMJ) formation, neurite outgrowth, myoblast fusion and myofibril integrity [72].
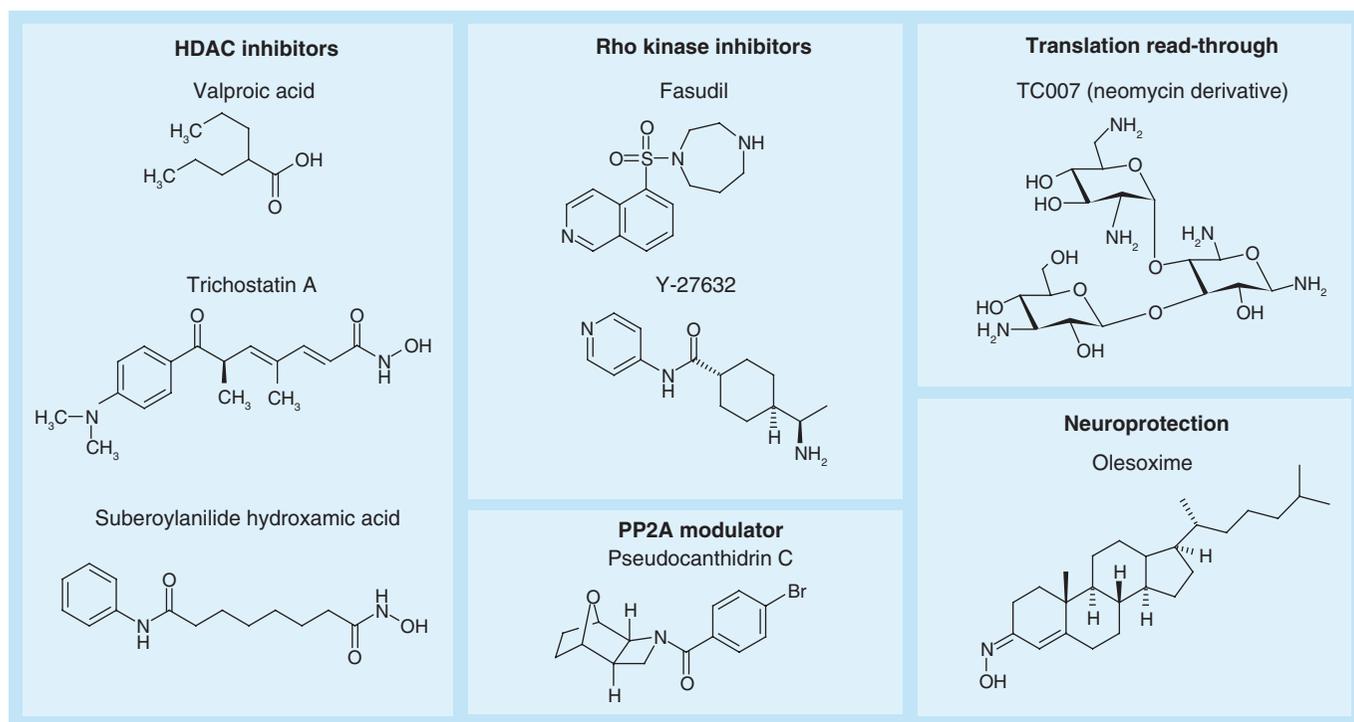
Figure 2. Select small compounds that have been investigated for spinal muscular atrophy therapy. Select HDAC inhibitors that have been tested in mouse models of spinal muscular atrophy (SMA) and in the case of valproic acid in several clinical trials in SMA patients [58]. Rho kinase inhibitors [59,60] and the read-through compound TC007 [56] have also been tested in mouse models of SMA. Pseudocantharidin C modulates the activity of PP2A to increase *SMN2* exon 7 inclusion [61]. The neuroprotective compound olesoxime is currently being tested in a Phase II clinical trial of type II and III SMA patients [62].

The molecular mass of cellular SMN determined by western blot falls between 38 and 42 kDa, a range that is substantially higher than the predicted mass of approximately 32 kDa. This discrepancy of SMN molecular mass could be attributed to post-translational modifications (PTMs). Indeed, phosphorylation of SMN is considered to be important for snRNP assembly [73,74], and phosphorylation of SMN by protein kinase A increases SMN stability [6,75]. Conversely, small molecules that inhibit GSK-3, and presumably prevent GSK-3-mediated phosphorylation of SMN, can increase SMN stability [76]. Ubiquitination of SMN causes its degradation through the ubiquitin proteasome system [6]. The significance of other PTMs, including but not limited to glycosylation, palmitoylation, sumoylation and transamidation, has yet to be explored. A less abundant, shortened isoform of SMN, a-SMN, is generated by alternative splicing in which a portion of *SMN* intron 3 is included in a tissue-specific and developmentally regulated manner (Figure 1D) [77]. The a-SMN isoform is evolutionarily conserved between humans and rodents [78]. Although the physiological role of a-SMN is poorly understood, a-SMN was recently shown to promote axon growth, stimulate cell motility and regulate expression of chemokines (CCL2, CCL7) and IGF-1 [79].

SMN depletion in mice during embryonic development causes a severe phenotype with early postnatal lethality. SMN restoration can ameliorate this phenotype, but only if done soon after birth [80,81]. In *Smn* knockout zebrafish, SMN must be restored relatively soon after motor neuron birth for normal motor axon development [82]. SMN is particularly important in the maturation and repair of NMJs, as early depletion prevents the formation of mature NMJs and leads to early postnatal death [83]. However, mice reach an SMN-refractory period after about 2 weeks of age where SMN depletion does not lead to early postnatal lethal-

ity, but much later in life abnormal NMJs are apparent [83]. While SMN is known to affect motor neurons, muscles and possibly other tissues are also affected independent of motor neuron defects [17,84]. Overall, these studies suggest that treatments for SMA patients should be given as early as possible to increase the SMN level and potentially ameliorate any abnormalities as much as possible.

SMN pre-mRNA splicing regulation

The mechanism of *SMN2* exon 7 splicing has been extensively studied since it is directly relevant to potential SMA therapies. Due to the close proximity of C6U to the 3′ splice site (3′ ss) of exon 7, early studies hypothesized that C6U weakened it. Considering that very small sequence motifs may define the specificity of RNA-protein interactions, it is not uncommon that loss of one motif due to a point mutation could be accompanied by a gain of another motif. Indeed, the inhibitory effects of C6U have been suggested to abrogate an enhancer element associated with SF2/ASF, as well as create a silencer element associated with hnRNP A1 [85,86] (Figure 3A). Furthermore, C6U strengthened a terminal stem loop (TSL1), an inhibitory RNA structure (Figure 3B) [87]. Subsequent studies revealed a negative role of Sam68 and a positive role of hnRNP Q exerted through the 6th position of exon 7 [88]. It is now also known that splicing regulators, such as Tra2-β1, SRp30c, hnRNP G and TDP-43, stimulate exon 7 inclusion by interacting directly or indirectly with exon 7 [88]. Consequently, factors that modulate the phosphorylation of Tra2-β1 and other splicing factors, including the pseudocantharidin compounds, have been shown to affect *SMN2* exon 7 splicing as well (Figure 2) [61].

Available algorithms do not predict the relative significance of exonic positions in splicing regulation due to variable contexts of exons. To circumvent this problem, we developed a novel *in vivo* selection protocol and employed it in the context of the entire exon 7 of *SMN1* [89]. This complex experiment uncovered three regulatory regions: extended inhibitory context (Exinct), conserved tract and 3′-cluster (Figure 3A) [89]. Exinct and 3′-cluster are negative regions located close to the 3′ and 5′ ss of exon 7, respectively. Conserved tract is a positive region that covers most of the middle portion of exon 7 and fully encompasses the previously described GA-rich sequence associated with Tra2-β1 interaction (Figure 3A). The nature of the regulatory regions identified by *in vivo* selection corroborated well with the results of an independent study in which overlapping ASOs were used to probe the significance of regulatory cis-elements within the entire exon 7 in the context of endogenous *SMN2* [93]. The identification

of Exinct, and its further validation by an ASO-based approach, lends additional support to the hypothesis that C6U strengthens a negative cis-element within *SMN2* exon 7.

In recent years, SMA patients with unique missense mutations within exon 7 of *SMN1* and *SMN2* have been reported (Supplementary Table 1). One such example is a mild form of SMA associated with a G to T mutation at the 29th position (G29U in the mRNA) of *SMN1* exon 7 [53]. Although not yet proven, it is expected that G29U would likely trigger *SMN1* exon 7 skipping due to its positioning within the conserved tract that spans from the 16th to 44th positions of exon 7. It is also possible that G29U creates an inhibitory cis-element. Another less severe form of SMA is associated with the loss of *SMN1* coupled with a G to C mutation at the 25th position (G25C) of *SMN2* exon 7 [48,51]. This is one of the rare examples in which deletion of *SMN1* is partially rescued by a single nucleotide mutation within *SMN2* exon 7. G25C moderately stimulated *SMN2* exon 7 inclusion in the context of a minigene. Based on *in vitro* binding assays, it was argued that G25C abrogates a composite regulatory element associated with hnRNP A1 and SF2/ASF [51]. It is still unknown if G25C creates a novel enhancer and/or brings structural changes favorable for *SMN2* exon 7 inclusion. Of note, G25C replaces a hydrophobic residue (glycine) with a charged hydrophilic residue (arginine) with the potential to alter the properties of SMN. However, the contribution of this mutant SMN to modulation of SMA severity requires further examination.

In addition to the defining C6U and A100G mutations of *SMN2*, the weak 5′ ss of exon 7 serves as the limiting factor for *SMN2* exon 7 inclusion. This was first revealed by the results of our *in vivo* selection of the entire exon 7; a non-wild type G residue was overwhelmingly selected at the last position of exon 7 [89]. The presence of a G residue at this exonic position extends the base pairing between the 5′ ss of exon 7 and U1 snRNA, an essential component of U1 snRNP that defines the 5′ ss of an exon. This finding clearly supported the hypothesis that a poor recruitment of U1 snRNP at the 5′ ss is the cause of *SMN2* exon 7 skipping. This hypothesis was validated when a mutant U1 snRNA that increased the base pairing with the 5′ ss of exon 7 robustly stimulated *SMN2* exon 7 inclusion [91]. Subsequent reports revealed a number of inhibitory cis-elements in the vicinity of the 5′ ss of exon 7. These inhibitory elements include TSL2, a 15-nucleotide long intronic splicing silencer N1 (ISS-N1) and an 8-nucleotide long GC-rich sequence (GCRS) that partially overlaps with ISS-N1 (Figure 3A) [91,94–95]. It has been shown that the last 14 residues of ISS-N1

encompass two putative binding sites of hnRNP A1/A2 (Figure 3) [96]. The first residue of ISS-N1 is a cytosine that occupies the 10th position (^{10}C) of *SMN* intron 7 and participates in an inhibitory long-distance interaction (LDI) involving downstream intronic sequences [92,97]. Given the negative role of ISS-N1 and GCRS, it is not surprising that ASO sequestration of these motifs promoted *SMN2* exon 7 inclusion [8,94–95].

TIA1 and TIAR stimulated *SMN2* exon 7 inclusion by binding to intronic U-rich clusters (URC1 and URC2) downstream of ISS-N1 (Figure 3A) [90]. Recently, a missense mutation within TIA1 was linked to Welander distal myopathy, a late onset autosomal dominant disease [98]. Muscle biopsies from Welander distal myopathy patients carrying the TIA1 missense mutation showed elevated levels of *SMN2* exon 7-skipped transcripts [98]. This is the first evidence that demonstrated the critical role of a *SMN* exon 7-splicing-associated factor in the context of a human disease. This finding clearly underscores the likely consequences of aberrant expression of a splicing factor on the severity of SMA. Since TIA1 is a Q-rich domain containing protein, we suspect that other RNA-binding proteins with similar domain organization would affect *SMN2* exon 7 splicing and SMN levels and consequently SMA severity. It should be mentioned that TIA1 is a critical component of stress granules, formation of which is an important cellular process that is negatively impacted by low levels of SMN [68]. Therefore, the impact of TIA1 on the severity of SMA is likely to be compounded by several TIA1-associated functions.

Recently, we interrogated folding of *SMN2* intron 7 and identified several regulatory structures, including TSLs and internal stems formed by LDIs (ISTLs) (Figure 3B & Figure 4) [92]. In particular, we identified ISTL1, a unique structure in which the 5′ and the 3′ strands of a RNA–RNA duplex are separated by more than 250 nucleotides. Interestingly, ^{10}C and half of GCRS are locked in ISTL1 (Figure 3B). Although the significance of ISTL1 is not yet fully understood, it represents an example of a structure generated by a unique folding of the entire *SMN2* intron 7 (Figure 3). The continuous sequence stretch encompassing the 3′ strands of ISTL1, ISTL2 and ISTL3 constitute ISS-N2, an inhibitory region in the middle of intron 7 (Figure 3).

Antisense-based therapy

In recent years, a wide range of research has utilized ASO-based approaches to modulate transcription, splicing and translation [100,101]. A variety of ASO-based strategies have also been adopted to stimulate *SMN2* exon 7 inclusion, albeit with varying degrees of success

Figure 3. Regulators of *SMN* exon 7 splicing (see facing page). (A) Diagrammatic representation of cis-elements and transacting factors that affect splicing of exon 7. Exon 7 sequence is shown in capital letters, while adjacent intronic sequences, including the first 104 nucleotides of intron 7, are shown in lower-case letters. The ss of exon 7 are indicated by arrows; in addition, the 5′ ss is highlighted with a blue arrow. Nucleotide numbering starts from the first position of intron 7. Cis-elements/transacting factors that promote exon 7 inclusion are indicated by (+), while cis-elements/transacting factors that promote exon 7 skipping are indicated by (−). Exinct, the conserved tract and 3′-Cluster were identified by *in vivo* selection of the entire exon 7 [89]. Element 2 and binding sites for hnRNP A1/A2, Sam68, SF2/ASF, hnRNP Q, Tra2-β1, TDP-43, hnRNP G and SRp30c were described previously [88,90]. TIA1 binds to URC1 and URC2 located in intron 7 and promotes exon 7 inclusion [90]. ISS-N1, along with the overlapping GCRS and the ^{10}C, which is involved in LDI, contribute to skipping of exon 7 [90]. TSL2 structure sequesters the 5′ ss of exon 7 and promotes its skipping [91]. The presentation is adapted from [92]. **(B)** Schematic representation of the partial RNA secondary structure formed by the sequences that constitute *SMN2* exon 7, the last 14 nucleotides of intron 6 and the entire intron 7. The structure is based on the results of chemical structure probing performed in [91,92]. Arrows indicate the 5′ and 3′ ss of exon 7. Exon 7 and intron 6 sequences are highlighted in yellow and light blue, respectively. Nucleotide numbering starts from the first position of intron 7. Three TSLs are marked by green boxes. The juxtaposed internal stems formed by LDI (ISTLs) are marked by blue boxes. ISS-N1 and ISS-N2 are highlighted in red. The adjacent 3′-strands of ISTL1, ISTL2 and ISTL3 constitute ISS-N2. Binding sites of TIA1 and hnRNP A1/A2 are indicated. GCRS: GC-rich sequence; ISS-N1: Intronic splicing silencer N1; LDI: Long-distance interaction; ss: Splice site; TSL: Terminal stem-loop; URC: U-rich cluster.

as recently reviewed in [102,103]. Discovery of ISS-N1 in 2006 provided a major breakthrough for ASO-mediated splicing correction in SMA [94]. ISS-N1 earned the name of a 'master checkpoint', since deletion of ISS-N1 or an ASO-mediated sequestration of ISS-N1 fully restored *SMN2* exon 7 inclusion even in the absence of a number of positive regulatory elements [104]. Advantageously, an ASO that anneals to an intronic sequence does not interfere with the translation and transport machinery. Therefore, ISS-N1 serves as an ideal target for the restoration of the full-length protein. Indeed, very low concentrations of an ISS-N1-targeting ASO significantly increased levels of SMN in SMA patient cells [94].

Since the discovery of ISS-N1, several independent studies with ISS-N1-targeting ASOs have been performed, the details of which have been recently reviewed [102]. These studies have employed ASOs with different modifications, including 2′-*O*-methyl coupled with uniform phosphorothioate backbone, 2′-*O*-methoxy-ethyl (MOE) coupled with uniform phosphorothioate backbone and phosphorodiamidate morpholino oligomers (Figure 4B) [102]. Of particular note is a study from the Krainer group that showed approximately 25-fold increase in lifespan of severe type I Taiwanese mice upon early subcutaneous administration of an ISS-N1 18-mer MOE ASO [105]. While this strategy resulted in the longest survival increase of any treatment performed in this model, the effect was less promising when the MOE ASO was delivered through intracerebroventricular (i.c.v.) route, although the i.c.v. dose was substantially lower than subcutaneous administration [105]. On the contrary, independent studies conducted with ISS-N1-targeting phosphorodiamidate morpholino oligomers have shown very promising results for i.c.v. administration in both type I Taiwanese and Δ7 SMA mouse models of SMA [106–108]. These results also underscore the need for the optimization of ASO size, since a difference of a few nucleotides may dramatically impact

the *in vivo* efficacy of an ASO. Furthermore, given that SMA patients exhibit deficits in tissues outside the nervous system [17,84], it appears that treatment should reach the nervous system and peripheral tissues. Currently, the MOE ASO targeted against ISS-N1 (ISIS-SMN$_{Rx}$) is recruiting patients for a Phase II clinical trial [109] after the successful completion of Phase I clinical trial [110].

GCRS and ISS-N2 represent additional antisense targets for ASO-mediated splicing correction in SMA (Figure 4A). Located within *SMN2* intron 7, these unique targets offer different sequence compositions and mechanisms for promoting *SMN2* exon 7 inclusion. The GCRS target in particular provides a rare advantage of utilizing a short ASO to increase *SMN2* exon 7 inclusion. There is a general misconception that a small ASO is bound to be less specific due to preponderance of similar motifs throughout the genome/transcriptome. This misconception is built on an unproven hypothesis that a large ASO does not tolerate mismatched base pairs. Based on the bifunctional ASOs and seed interactions of miRNAs that do not require full complementarity with the target [111,112], large ASOs can function while tolerating more than 50% mismatched base pairs. On the other hand, small ASOs show limited or no tolerance for a single mismatch base pair. Indeed, tiny ASOs targeting seed sequences of microRNAs have shown commendable efficacy *in vivo* [113]. Furthermore, a recent report validated the therapeutic efficacy of a GCRS-targeting short ASO in two severe mouse models of SMA [99]. Therefore, employment of a small ASO as a potential therapy of SMA remains an attractive proposition.

> ## Key Term
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> **ISIS-SMN$_{Rx}$:** ISIS-SMN$_{Rx}$ is the first antisense drug to undergo clinical trial for the treatment of spinal muscular atrophy. Drug is based on proprietary 2′-O-methoxyethyl chemistry of ISIS Pharmaceuticals and targets ISS-N1 sequence within SMN2 intron 7.

A

ASOs used *in vivo*
- PMO
- PMO
- PMO
- MOE (ISIS SMNRx; currently in Phase II clinical trial)
- 2´OMe

GCRS
10

ISS-N1
20

TIA1

URC1 30 40

URC2 50

Element 2 60

70 URC3

GUAAGUCUGCCAGCAUUAUGAAAGUGAAUCUUACUUUUGUAAAACUUUAUGGUUUGUGGGAAAACAAAUGUUUUG

5´ISTL1

hnRNP A1/A2

hnRNP A1/A2

TSL3

5´ISTL2

TSL4

URC3 80 · 90 · 100 · 110 · 120 · 130 · 140 · 150

AACAUUUAAAAAGUUCAGAUGUUAGAAAGUUGAAAGGUUAAUGUAAAACAAUCAAUAUUAAAGAAUUUUGAUGCC

TSL4

hnRNP A1/A2

160 · 170 · 180 · 190 · 200 · 210 · 220

AAAACUAUUAGAUAAAAGGUUAAUCUACAUCCCUACUAGAAUUCUCAUACUUAACUGGUUGGUUGUGUGGAAGAA

5´ISTL3

TSL5

230 · 240 · 250 · 260 · 270 · 280 · 290 · 300

ISS-N2

LS-1

ACAUACUUUCACAAUAAAGAGCUUUAGGAUAUGAUGCCAUUUUUAUAUCACUAGUAGGCAGACCAGCAGACUUUUU

TSL5

5´ISTL4

3´ISTL3

3´ISTL2

3´ISTL1

310 · 320 · 330 · 340 · 350 · 360 · 370

UUUAUUGUGAUAUGGGAUAACCUAGGCAUACUGCACUGUACACUCUGACAUAUGAAGUGCUCUAGUCAAGUUUAA

3´ISTL4

TSL6

TSL7

380 · 390 · 400 · 410 · 420 · 430 · 440

CUGGUGUCCACAGAGGACAUGGUUUAACUGGAAUUCGUCAAGCCUCUGGUUCUAAUUUCUCAUUUGCAG

TSL7

B

Backbone modifications

Phosphodiester

Phosphorothioate

Morpholino

Sugar ring modifications

2'-O-methyl (2'OMe)

Locked nucleic acid (LNA)

Methoxyethyl (MOE)

Terminal modifications

PEG-282

C3 Spacer

Figure 4. Linear representation of the entire *SMN2* intron 7 (see facing page). (A) Colored boxes designate the structural elements and splicing cis-elements. Numbering starts from the first position of intron 7. Double arrow within a given box indicates a TSL, whereas, a double arrow outside of boxes in the corresponding color shows the 5′ and 3′ strands of a particular ISTL. ISS-N1-targeting ASOs used for *in vivo* studies are shown as bars. The linear structure is adapted from [92]. **(B)** Chemical structures for a ASO backbone (unmodified phosphodiester, phosphorothioate and morpholino) and sugar ring modifications (2′-O-methyl, 2′-methoxyethyl and locked nucleic acid). The terminal modifications shown, PEG-282 and C3 spacer, were successfully utilized to increase the *in vivo* stability of a short ASO [99].
ASO: Antisense oligonucleotide; GCRS: GC-rich sequence; ISTL: Internal stem formed by LDI; LDI: Long-distance interaction; TSL: Terminal stem-loop; URC: U-rich cluster.

Transcriptional & epigenetic regulation

Upregulation of *SMN2* transcription could potentially increase SMN without altering the splicing pattern of *SMN2* exon 7. The human *SMN1* and *SMN2* gene promoters are nearly identical in sequence and activity [114,115]. While the principal transcription initiation site (TIS) is located 163 base pairs upstream of the translation initiation site, a second TIS has been mapped 246 base pairs upstream and appears to be used during fetal development [116]. Sequences up to 4.6 kilobases upstream of the TIS have been implicated in transcriptional regulation of *SMN1/ SMN2* and harbor putative bindings sites of transcription activators including CREB and ELK-1 [117,118].

There are very limited studies on the molecular mechanism by which transcription of *SMN2* exon 7 is regulated. Prolactin, a polypeptide hormone and an activator of the JAK2/STAT5 signaling pathway, increased *SMN2* transcription and SMN in both human neuron-committed teratocarcinoma (NT2) cells and the Δ7 SMA mouse model and improved the disease phenotype of Δ7 SMA mice [119]. Inhibition of the MEK/ERK pathway coupled with the activation of the AKT/CREB pathway is another mechanism recently shown to enhance *SMN2* transcription (Figure 5) [118,120]. Oral treatment with selumetinib, a MEK/ERK inhibitor currently in Phase II clinical trials [121], significantly enhanced expression of *SMN2* in spinal cord and increased lifespan by approximately 3.4-fold in a severe SMA mouse model [118]. Similar results were obtained with intrathecal administration of U0126, an inhibitor of the MEK/ERK/Elk-1 signaling pathway [118]. While the MEK/ERK/ELK-1 and the PI3K/AKT/CREB signaling pathways are known to cooperate with beneficial consequence to neurons, it appears that these two pathways have opposing roles in transcriptional regulation of *SMN2* in the spinal cord of type I SMA-like mice. The ERK/ELK-1 pathway becomes constitutively overactive in the spinal cord of SMA-like mice [120], exerting a negative effect on expression (transcription) of *SMN2* [118]. CREB is a positive regulator of *SMN* transcription [122], whereas ELK-1 plays a repressive role on transcription in certain circumstances [123]. In the spinal cord of type I SMA-like mice, inhibition of ELK-1 phosphorylation through MEK/ERK pathway inhibition by U0126 leads to activation of the AKT/CREB pathway. The cross-talk between MEK/ERK and AKT/CREB pathways is facilitated by CaMKII (Figure 5) [118]. Activation of spinal cord NMDA receptor that induces calcium flux and consequently activates AKT/CREB pathway, enhances *SMN2* gene expression in severe SMA mice [120]. Interestingly, massive reprogramming of alternative splicing in response to EGF signaling has been reported [124]. For instance, it has been shown that activation of AKT induces autophosphorylation of SRPKs, an action that enhances SRPK translocation and SR protein phosphorylation [124]. Therefore, it is likely that the increase in *SMN2* transcript levels in response to activation of JAK2/STAT5 and/or AKT/CREB pathway is partly due to an increase in the rate of splicing.

Histone acetylation and deacetylation are important epigenetic mechanisms by which transcription is activated and repressed, respectively. A number of HDAC inhibitors have been found to increase transcription of *SMN2*, including suberoylanilidehydroxamic acid, trichostatin A, and US FDA approved drugs hydroxyurea and valproic acid (Figure 3) [58]. However, clinical trials conducted with valproic acid and hydroxyurea did not show promising results [125–129]. It is possible that HDAC inhibitors would exhibit greatest efficacy if administered immediately to newborns or shortly after birth, although patients this young would be difficult to enroll in clinical trials. Perhaps HDAC inhibitors could be utilized as adjunct therapy, especially for patients with more complicated manifestations of SMA.

RNA decapping

Decapping is a regulated mRNA scavenging process catalyzed by the enzyme DcpS, a member of the HIT family of pyrophosphatases [130]. Quinazolines are a family of DcpS inhibitors that were identified as SMN modulating compounds in a high-throughput screening [131]. In order to improve the delivery across the blood–brain barrier, several quinazoline derivatives were developed, including a promising candidate

Key Term

Quinazolines: Quinazolines are small compounds that stabilize mRNAs by inhibiting the action of decapping enzyme DcpS. RG3039 is a quinazoline derivative that has been shown to extend the lifespan of spinal muscular atrophy mice.
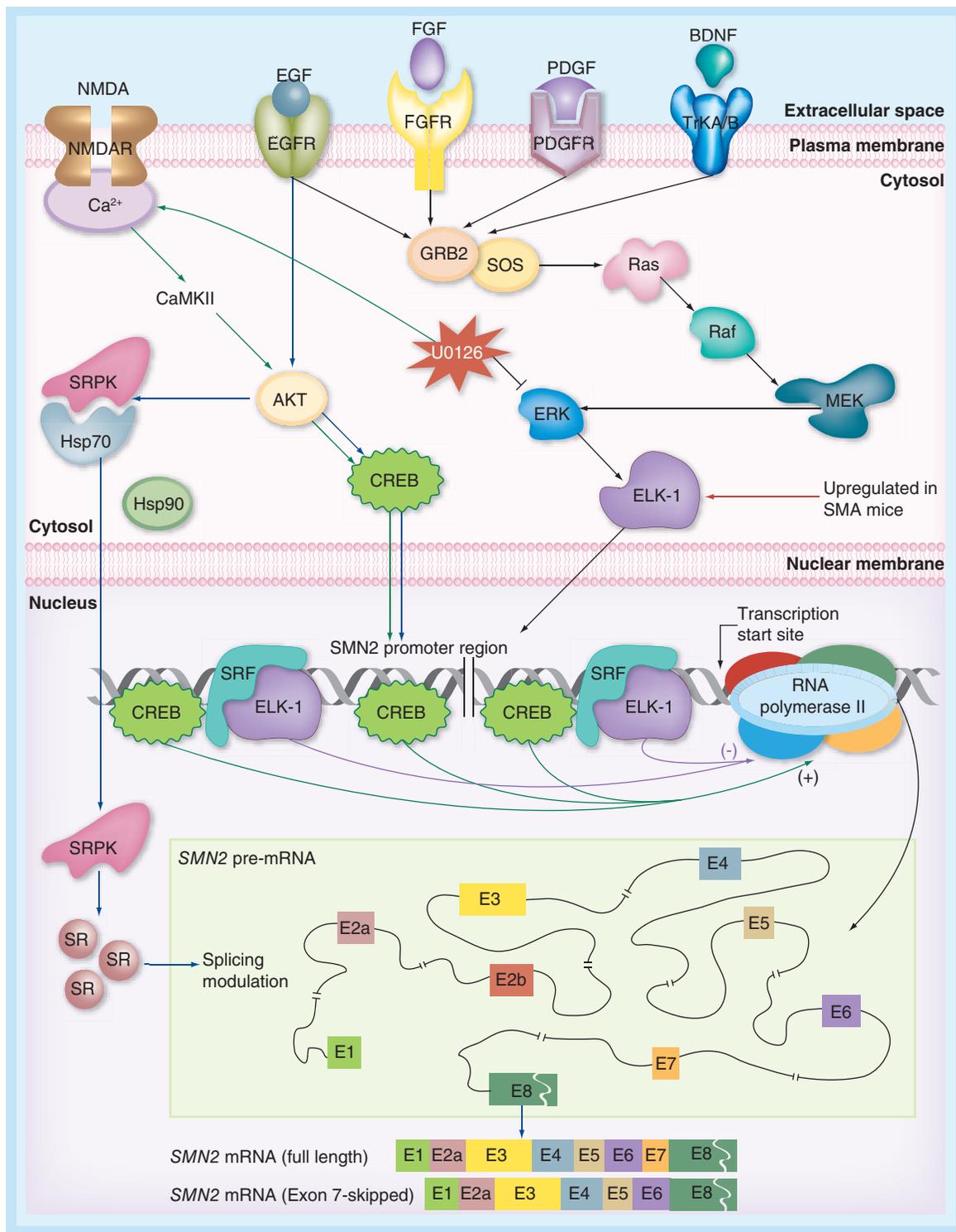
Figure 5. Signaling pathways implicated in regulation of *SMN2* transcription and *SMN2* exon 7 splicing. Simplified version of intracellular MEK/ERK and AKT/CREB signaling pathways that affect *SMN* transcription, including the upstream regulators (NMDA, EGF, FGF, PDGF and BDNF). Activation of spinal cord NMDA receptors or inhibition of ERK by U0126 activates the AKT pathway leading to increased *SMN2* transcription [118,120]. Activation of AKT triggers autophosphorylation of SRPK followed by its nuclear import and consequent phosphorylation of SR proteins that regulate alternative splicing [124]. Binding sites of transcription factors within *SMN2* promoter are shown. In the inset for *SMN2* pre-mRNA, exonic (coding) and intronic (non-coding) regions are indicated by boxes and lines, respectively. Two major *SMN2* splice isoforms are shown at the bottom.
EGFR: EGF receptor; FGFR: FGF receptor; NMDAR: NMDA receptor; PDGFR: PDGF receptor.

known as RG3039. Daily intraperitoneal administration of RG3039 improved NMJ function and modestly extended lifespan of Δ7 SMA mice [132]. Similar results were obtained in Taiwanese type I mice that received daily oral administration of RG3039; RG3039 also significantly extended median lifespan from 18 to 112 days in the intermediate $Smn^{2B/-}$ mouse model [133]. Currently, RG3039 is undergoing Phase I clinical trial.

Gene therapy of SMA

Gene therapy as a treatment for SMA could potentially cure the disease through the delivery of *SMN1* and consequent restoration of SMN. The first gene therapy study reported in 2004 was conducted with a lentiviral vector to deliver *SMN1* gene in the Δ7 SMA mice [134]. The results were modest; treated Δ7 mice survived merely 3 days longer than the usual 14 days, but nevertheless it was a promising result at the time. Over the past several years, adeno-associated vectors (AAV) class 8 and 9 have emerged as potential vehicles for delivering genes to the CNS [135,136] and have been tested in mouse models of SMA. i.c.v. administration at birth of *SMN1* cDNA packaged in AAV8 and scAAV8 vectors markedly increased the median lifespan of the mice to 57 and 157 days, respectively, and both vectors improved neuromuscular architecture and motor function [137]. The majority of gene therapy studies in SMA mouse models, however, have involved scAAV9. Intravenous (iv.) administration of scAAV9 carrying *SMN1* cDNA at postnatal day 1 restored endplate current at neuromuscular junctions and extended lifespan of Δ7 SMA to at least 250 days [138]. The same gene therapy protocol rescued the severe bradycardia and improved electrocardiographic features [139] and the heart structural defects [140] observed in untreated Δ7 SMA mice. Recent studies utilized codon optimized *SMN1* cDNA in scAAV9 with different promoters. Iv. administration of SMNopt*i* under control of the phosphoglycerate kinase promoter spared motor neurons from death, prevented motor function deficits and increased median survival to 199 days (compared with 13 days for untreated mice) [141]. However, iv. administration of codon-optimized *SMN1* under cytomegalovirus control only extended lifespan up to 70 days [142]. Findings from these reports suggest the importance of the promoter in addition to the *SMN1* sequence. However, the efficacy of route of viral administration remains unclear. Although equal efficacies have been reported for iv., i.c.v. and intramuscular deliveries of scAAV9-*SMN1* [143,144], the requirement of high viral titer (up to 10^{14} viral genomes) may limit the clinical utility of peripheral administration. Additional studies in different mouse models of SMA, as well as toxicology studies in non-human primates, are needed for gene therapy

> ### Key Terms
>
> **Adeno-associated vectors:** Adeno-associated vectors have emerged as the powerful tool to deliver coding sequence for making full-length SMN in the CNS. Recently, US FDA has approved a Phase I clinical trial for AAV9 delivery of SMN1 cDNA into spinal muscular atrophy patients.
>
> **Transcriptome-wide analysis:** Transcriptome-wide analysis requires high throughput sequencing of the entire transcripts from a specific cell or tissue type. Recent transcriptome-wide analyses of neuronal tissues from spinal muscular atrophy mice have revealed novel hallmarks of spinal muscular atrophy pathogenesis.

to be an attractive option in SMA. Recently, the FDA approved a Phase I clinical trial for systemic AAV9 delivery of *SMN1* [145].

Disease modifiers of SMA

Several genes, including *NAIP*, *H4N4* and *PLS3* are known to affect SMA severity [146,147]. *NAIP* and *H4N4* genes are located in close proximity to *SMN1* on human chromosome 5. The most severe type I patients are found to have deletions of *NAIP* and/or *H4N4* in addition to deletion of *SMN1*. The *PLS3* gene is located away from the *SMN* locus on human chromosome 10 and encodes the F-actin bundling protein plastin-3 (PLS3). Thus far, PLS3 is the only reported natural disease modifier of SMA because individuals with high PLS3 levels have been found to be protected from the harmful effects of homozygous *SMN1* deletions that cause SMA [146]. Several disease modifiers have been implicated based on studies in cell-based and animal models. For instance, altered phosphorylation of Rho kinase-dependent targets that influence polymerization of actin have been suggested to play an important role in SMA pathogenesis [52]. Consistently, treatment of an intermediate mouse model of SMA ($Smn^{2B/-}$) with Y-27632 or fasudil, both inhibitors of Rho kinase (Figure 2), increased survival and myofiber size [59,60]. Based on a recent transcriptome-wide analysis of motor neurons and surrounding glial cells, multiple genes, including agrin (required for NMJ maintenance), C1q (synapse pruning-promoting complement factor) and Etv1/ER81 (transcription factor for establishing sensory motor circuitry), are likely to be modifiers of SMA [148]. Dysregulation of these genes further underscores the importance of SMN in maturation of NMJs. In addition, zinc-finger protein, ZPR1, which is required for nuclear accumulation of SMN, is downregulated in SMA patients [149]. *In vitro*, overexpression of ZPR1 in SMN-deficient spinal cord neurons from SMA mice stimulated neurite growth and rescued axonal growth deficits [149]. Targeting these modifying factors may provide avenues for the development of

adjunct therapy for SMA. Cellular levels of these factors may also serve as markers of outcome measures for the development of SMA therapy.

Future perspective

The past 15 years have witnessed a remarkable progress in our understanding of SMA pathogenesis, SMN function, *SMN* transcription and *SMN* exon 7 splicing. Several *in vivo* studies have been performed for potential therapeutic compounds identified by available chemical libraries. Similar progress has taken place for the therapeutic development of other genetic diseases. However, genuine progress towards therapy in human patients has been less than impressive due to the lack of reproducibility of most of the *in vivo* studies performed using mouse models. According to a recent report, the minimum cost of testing the efficacy of a single dose of a given compound in a mouse model of amyotrophic later sclerosis (ALS), a disease similar to SMA, comes out to be approximately US$330,000 [150]. This report suggests a minimum time requirement of 9 months to realistically conclude a meaningful preclinical study in ALS. Significantly, the report also provides a clue why most claims based on low-budget preclinical studies in ALS were not reproducible and eventually failed in clinical trials [150]. The findings of this report are instructive for both investigators and funding agencies (public, private and non-profit organizations) in factoring the realistic cost and time of pursuing a preclinical study in an animal model. Ideally, an *in vivo* study is warranted only after rigorous cell-based experiments have validated the efficacy and target specificity of a compound.

SMA is a disease with the potential to be cured by correction of *SMN2* exon 7 splicing. Compounds that correct *SMN2* exon 7 splicing should be rigorously evaluated for the specificity, particularly at higher concentrations. Similarly, the mechanism of compounds that increase SMN levels independent of *SMN2* exon 7 splicing should be stringently evaluated for specificity. Better solubility (in aqueous solutions) and high activity at low concentrations in cell-based studies is a good indicator if a compound is suitable for *in vivo* application. The manner in which ISS-N1 target was discovered and subsequently validated in the cell-based assays led to an overwhelming interest in an ASO-based therapy for SMA. Consequently, ISS-N1 emerged as the natural contender for testing the efficacy of different ASO chemistries in various mouse models of SMA. While several independent reports on ISS-N1-targeting ASOs have shown unprecedented therapeutic benefits *in vivo*, rigorous and independent evaluations of different chemistries with a substantially large number of animals per treatment group over a long period of time

are yet to be conducted. Gender variations should also be factored in the experimental design. Gene therapy is another promising approach for the treatment of SMA, particularly if DNA coding for SMN is safely delivered to all needed tissues at the very early stages of development. Herein, preclinical studies should be conducted with a larger cohort of animals considering variability in administration and tolerability of individual animals.

Progress in the field of ASO-based and gene therapies does not in any way diminish the need for other approaches, particularly small compounds that could serve as major means for therapy at the advanced stages of the disease or as an adjunct therapy to gene-based treatments. The most important lesson to be learnt from the journey of the therapeutic development for SMA is to continue to uncover the mechanism of the disease at the molecular, cellular and organismal level. There are many more therapeutic targets hidden in the sequences and structures of molecules that affect *SMN2* transcription, *SMN2* exon 7 splicing, SMN stability and SMN-dependent processes.

Executive summary

Spinal muscular atrophy: genetics & phenotype

- Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality that occurs in approximately 1 in every 10,000 live births. SMA patients show a broad spectrum of phenotype with type 0 being the most severe and type IV being the mildest.
- Humans carry two near identical copies of *SMN* gene: *SMN1* and *SMN2*. SMA is caused by the loss of *SMN1* coupled with the inability of *SMN2* to compensate for the loss of *SMN1*.
- The major problem with *SMN2* gene is exon 7 skipping that produces SMNΔ7, a truncated SMN that is rapidly degraded.

Structure–function relationship in SMN

- SMN is a multifunctional protein coded by eight exons. The functional domains present within SMN include Gemin2-binding, nucleic acid binding, Tudor, self-association and calpain cleavage. Point mutations across the entire primary structure of the protein have been linked to SMA pathogenesis.
- Interaction of SMN with Gemin2 is important for snRNP biogenesis, signal recognition particle biogenesis, translation regulation, DNA recombination and motor neuron trafficking of mRNAs.

Regulation of *SMN2* exon 7 splicing

- *SMN2*-specific C6U and A100G substitutions cause massive skipping of *SMN2* exon 7. C6U and A100G substitutions create binding motifs for the inhibitory factor hnRNP A1/A2. Depletion of hnRNP A1/A2 promotes *SMN2* exon 7 inclusion.
- *In vivo* selection of the entire *SMN* exon 7 revealed a weak 5′ ss as the limiting factor for *SMN* exon 7 inclusion. The inhibitory cis-elements weakening the 5′ ss of *SMN* exon 7 include terminal stem loop 2, intronic splicing silencer (ISS)-N1, GC-rich sequence (GCRS) and ISS-N2.
- ISS-N2 participates in the formation RNA structures (ISTL1, ISTL2 and ISTL3) facilitated by long-distance interactions. ISTL1 is a rare structure in which two stems of a RNA–RNA duplex is separated by more than 250 nucleotides.
- TIA1 regulates *SMN2* exon 7 splicing by binding to intronic sequences immediately downstream of ISS-N1. Pathogenic TIA1 mutation in Welander distal myopathy has been found to promote *SMN2* exon 7 skipping

Antisense-based therapy

- Antisense oligonucleotides targeting ISS-N1, GCRS and ISS-N2 fully restore *SMN2* exon 7 inclusion and substantially elevate levels of full-length SMN in SMA patient cells. GCRS provides a unique advantage of utilizing a very small ASO for SMA therapy.
- Among various oligonucleotide chemistries tested, ISS-N1-targeting phosphorodiamidate morpholino oligomers produced the best life extension benefits when delivered through the clinically relevant intracerebroventricular route in two independent studies carried with different severe mouse models of SMA.
- The 2′-*O*-methoxyethyl ASO (ISIS-SMN$_{Rx}$) targeted against ISS-N1 has successfully completed the Phase I clinical trial by ISIS Pharmaceuticals (CA, USA). Patients are being recruited for a Phase II clinical trial of ISIS-SMN$_{Rx}$.

Transcriptional & epigenetic regulation

- Upregulation of *SMN2* transcription produces increased levels of SMN despite no apparent change in the splicing pattern of *SMN2* exon 7. Prolactin, an activator of the JAK2/STAT5 pathway, improves the disease phenotype of a severe SMA mouse model by increasing *SMN2* transcription.
- Inhibition of the MEK/ERK pathway coupled with the activation of the AKT/CREB pathway enhances *SMN2* transcription. Oral treatment with selumetinib, a MEK/ERK inhibitor currently in Phase II clinical trials, significantly increased lifespan of a severe SMA mouse model. A number of HDAC inhibitors have been found to increase transcription of *SMN2*. However, an effective HDAC inhibitor for SMA therapy is yet to be found.

RNA decapping

- Quinazolines are RNA-decapping inhibitors shown to upregulate SMN. RG3039, an improved version of quinazoline, is currently undergoing Phase I clinical trial.

Gene therapy

- Gene therapy offers a permanent solution to SMA if DNA coding for SMN is safely delivered to the right tissues at the right time. AAV9-based delivery of DNA coding for SMN has shown the best promise in various preclinical studies in mouse models of SMA.

Disease modifiers

- Genes including NAIP, H4N4 and PLS3 have been known to modify the severity of SMA. Additional SMA modifiers have been suggested based on studies in mouse models of SMA. Targeting these modifying factors may provide avenues for the development of adjunct therapies of SMA.

Executive summary (cont.)

Future perspective

• ASO- and gene-therapy-based approaches have shown the best outcome measures in terms of extension of life span of mouse models of SMA.

• Small compounds continue to be attractive agents of SMA therapy due to their ease of administration.

• To uncover more promising therapeutic targets, research should continue to focus on understanding the mechanism of the disease at the molecular, cellular and organismal level.

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