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Review article

The Yin and Yang of nucleic acid-based therapy in the brain☆

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ABSTRACT

The post-genomic era has unveiled the existence of a large repertory of non-coding RNAs and repetitive elements that play a fundamental role in cellular homeostasis and dysfunction. These may represent unprecedented opportunities to modify gene expression at the right time in the correct space *in vivo*, providing an almost unlimited reservoir of new potential pharmacological agents. Hijacking their mode of actions, the druggable genome can be extended to regulatory RNAs and DNA elements in a scalable fashion.

Here, we discuss the state-of-the–art of nucleic acid-based drugs to treat neurodegenerative diseases. Beneficial effects can be obtained by inhibiting (Yin) and increasing (Yang) gene expression, depending on the disease and the drug target. Together with the description of the current use of inhibitory RNAs (small inhibitory RNAs and antisense oligonucleotides) in animal models and clinical trials, we discuss the molecular basis and applications of new classes of activatory RNAs at transcriptional (RNAa) and translational (SINEUP) levels.

Contents

Abbreviations: 3C, chromosomal conformation capture; AAV, adeno-associated virus; AD, Alzheimer's disease; Ago2, argonaute2; ALS, amyotrophic lateral sclerosis; AntagoNATs, antagonist of natural antisense transcript; APP, amyloid precursor protein; ASOs, antisense oligonucleotides; Aub, aubergine; BACE1, β-amyloid precursor protein cleaving enzyme 1; BBB, blood brain barrier; BD, binding domain; BDNF, brain-derived neurotrophic factor; Cas9, CRISPR-associated protein 9; CCPG1, cell cycle progression 1; CCR4, chemokine (C-C motif) receptor 4; COX2, cyclooxygenase 2; CRISPR, clustered regularly interspaced short palindromic repeats; CSDC2, cold shock domain containing C2, RNA binding; CSF, cerebrospinal fluid; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; ED, effector domain; Emx2, empty spiracles homeobox 2; eRNA, enhancer RNA; Foxg1, forkhead box G1; FRDA, friedreich's ataxia; FTD, frontotemporal dementia; FXN, Frataxin; GFP, green fluorescent protein; GOI, gene-of-interest; HD, Huntington's disease; HTT, huntingtin gene; IGF2, insulin-like growth factor 2; IL24, interleukin24; IL32, interleukin32; iPSC, induced pluripotent stem cells; LDLR, low density lipoprotein receptor; lincRNAs, long intergenic non-coding RNAs; LINE, long interspersed nuclear element; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; NAT, natural antisense transcript; NMHVs, nuclear localization signal-MS2 coat protein RNA interacting domain-; HA epitope, (3x)VP16 transactivating domain; PABP, poly (A)-binding protein; PD, Parkinson's disease; piRNAs, piwi-interacting RNAs; bPLA2G4A, phospholipase A2; polyA, polyadenylated; PR, progesterone receptor; PSEN1, presenilin 1; RAN, repeat-associated non-ATG; RAPGEF3, rap guanine nucleotide exchange factor (GEF) 3; RIP, RNA immunoprecipitation; RISCs, RNA-instructed silencing complexes; RNAa, RNA activation; RNAi, RNA interference; S/AS, sense/antisense pairs; saRNA, small activator RNA; SHANK2, SH3 and multiple ankyrin repeat domains 2; shRNA, small hairpin RNA; SINE, short interspersed nuclear element; SINEUP, SINEB2 sequence to UP-regulate translation; siRNAs, small interfering RNAs; SLC39A, solute carrier family 39; SMA, spinal muscular atrophy; SMN, survival of motor neuron; SOD1, superoxide dismutase 1; SYNGAP1, synaptic ras GTPase activating protein 1; TEs, transposable elements; TFs, transcription factors; TGS, transcriptional gene silencing; TSS, transcription start site; UBE3A, ubiquitin protein ligase E3A; Uchl1, ubiquitin carboxyl-terminal esterase L1; UTR, untranslated region; VEGF, vascular endothelial growth factor; wt, wild type.

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1. The post-genomic era of molecular therapy

In the last years, our understanding of the functional output of the mammalian genome has enormously increased. This has led to profound changes in the current view of how a cell works and how evolution has shaped biological complexity. These discoveries provide new opportunities for translational research with a potentially great impact on molecular medicine.

The classic approach to drug discovery has stemmed from the concept that genomes contain the information to encode for proteins and these are the building blocks of organisms. Proteome complexity is the way to structure cells and tissues with different shapes and functions. Drugs are therefore modifiers of protein activities inhibiting or activating specific signaling pathways.

Despite a long list of success stories that have positively affected the well being of patients, this approach has posed tremendous challenges to drug discovery, resulting in staggering costs, poor specificities and difficulties in addressing major complex diseases. Importantly, it has negatively impacted the ability to address rare diseases that present highly heterogeneous molecular profiles in spite of the small number of patients. Many proteins remain difficult to be targeted, limiting the repertory of therapeutics.

The sequence of the human genome has confirmed that less than 5% is involved in encoding proteins, leaving the remaining sequences, the so-called Junk DNA, with no function and therefore no role in pharmacology.

A pioneering work previously showed that Junk DNA might indeed be transcribed unveiling the existence of small RNAs and the expression of repetitive elements (Klein et al., 1974). However, the first evidence that these unconventional transcripts may have an important functional role comes from the discovery of RNA interference in the late 1990s (Fire et al., 1998). Immediately, it was evident that these RNAs may provide a new class of nucleic acid-based drugs to inhibit gene expression *in vivo*. As for the discovery of recombinant molecular antibodies, the initial excitement and the consequent large investments crashed against technical difficulties that seemed unsolvable. Two decades later, we are now witnessing a renaissance of interest in small inhibitory RNAs due to the refinement of RNA targeting and the optimization of *in vivo* delivery. More than 50 RNA molecules and their derivatives are currently formulated as drugs and being tested in clinical trials for a number of disorders across different organs. Overcoming initial failures, we have now entered a second phase of RNA therapeutics, where we can optimistically expect to witness the first real impact of RNA medicine on patients and society.

Meantime, large genomic projects as those from the ENCODE (Derrien et al., 2012) and FANTOM Consortia (Forrest et al., 2014) have increased enormously the description of the molecular constituents of cells and have contributed to hamper the classical view of gene expression regulation. In addition to a previously underestimated number of alternative variants for protein-coding genes, pervasive transcription of the mammalian genome gives rise to a large repertory of non-coding transcripts, whose expression is tightly regulated in space and time. These include long non-coding RNAs (lncRNAs), small non-coding RNAs and transcripts derived from Transposable Elements (TEs), such as SINE (short interspersed nuclear element) and LINE (long interspersed nuclear element) (Faulkner et al., 2009; Fort et al., 2014; Kapranov et al., 2010; Katayama et al., 2005). Interestingly, the vast majority of genomic *loci* are extensively transcribed from both strands where two genes are located in opposite orientation, giving rise to Sense/Antisense pairs (S/AS) (Derrien et al., 2012; Katayama et al., 2005).

In this context, it is becoming evident that the major transcriptional output of mammalian cells is represented by lncRNAs. While functional annotation of the genome has previously revealed almost 15,000 independent lncRNA genes (Derrien et al., 2012), the most recent version of LNCpedia database contains more than 90,000 annotated human lncRNAs (Volders et al., 2015). By definition, lncRNAs are transcripts longer than 250 nucleotides, with features similar to those of protein-coding genes but without a functional open reading frame (ORF). lncRNAs

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contain a CAP structure and can be polyadenylated, they are typically composed by 2–3 exons and may have introns, which are removed during processing (Derrien et al., 2012). The majority of lncRNAs are enriched in the nucleus, albeit they can exert their functions both in nuclear and cytoplasmic compartments. They can be classified according to their genomic position relative to nearby genes. When lncRNAs are distant from other protein-coding or non-coding genes, they are referred to as long intergenic non-coding RNAs (or lincRNAs). When they entirely or partially overlap with other genes, they are defined as genic lncRNAs. In most cases, the overlap is between a protein-coding gene on one filament and an antisense lncRNA on the opposite strand. This genomic architecture gives rise to natural AS lncRNAs, which are estimated to cover almost all protein-coding genes, potentially regulating their expression (Derrien et al., 2012; Katayama et al., 2005).

An increasing number of studies are unveiling lncRNA functions. As representative examples, in the nucleus lncRNAs can have enhancer-like activity (Ørom et al., 2010), they may control the epigenetic status of the chromatin by recruiting Polycomb Repressor Complex (Rinn et al., 2007) and they can act as decoy for inhibiting splicing and mRNA maturation (Tripathi et al., 2010). In the cytoplasm, lncRNAs were described to activate mRNA decay *via* Alu elements (Gong and Maquat, 2011), to act as "sponges" for miRNA binding (Cesana et al., 2011; Tay et al., 2011) or to enhance translation under stress conditions (Carrieri et al., 2012). Irrespective of the specific lncRNA and its function, it is becoming evident that lncRNAs are organized into independent "domains" that are required to bind protein complexes. RNA folding is now believed to provide functional cues to lncRNA domains, as their primary sequences are poorly conserved. In addition to domain architecture, lncRNAs can take advantage of the exquisite feature of nucleic acid base pairing to specifically select and modulate target RNA and/or DNA molecules.

Therefore, lncRNAs may work as flexible modular scaffolds that combine domains engaged for protein-binding with those targeted to RNA and/or DNA sequences (Guttman and Rinn, 2012).

With this extraordinary amount of data in hand, a large effort is being devoted to understand the grammar of lncRNAs structure/function relationship and how their activities integrate into the biological scenarios the scientific community has been unveiling in the last half of the century.

Importantly, lncRNAs and small RNAs present unprecedented opportunities to modify gene expression at the right time in the correct space *in vivo*, providing an almost unlimited reservoir of new potential pharmacological agents.

RNA-based drugs have the advantage of extending the druggable genome with high specificity to all the protein coding genes as well as to regulatory regions. By taking advantage of common delivery systems to specific target organs, nucleic acid-based therapy may be scalable at a fairly low cost with relatively common pharmacodynamics and pharmacokinetics properties.

2. Challenges for the molecular therapy of neurodegenerative diseases

It is fair saying that despite an enormous amount of knowledge accumulated along the years, the large majority of neurodegenerative diseases remain uncurable. A general problem is that the site of degeneration is not accessible for direct study during life. This is especially relevant during clinical trials for new therapeutic treatments since objective measurements of degeneration are scarce. Importantly, neurodegenerative diseases are characterized by a long pre−symptomatic phase during which degeneration is occurring but no clinical symptoms are evident. Therefore, potential pharmacological treatments may act only on surviving cells that cannot provide a sufficient substrate for disease reversal. Furthermore, there is still no objective way to make a diagnosis in pre−symptomatic phases as well as to monitor disease progression. In summary, to date no drugs seem to block or even slow the neurodegenerative process in any of the most socially challenging neurodegenerative disorders as Alzheimer's Disease (AD), Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis (ALS). We still do not know the initial triggering events of the majority of these sporadic diseases, limiting our ability to identify a crucial drug target.

In the case of familial neurodegenerative diseases, genetic testing can provide the identification of patients in the pre-symptomatic phase. Importantly, it names the triggering cause of the disease and therefore the main validated drug target. It is therefore particularly depressing that no treatment is available even for those diseases such as Huntington's Disease (HD), where the cause is precisely defined.

An increasing number of neurodevelopmental disorders with relatively high prevalence in the general population, including autism spectrum disorders (1%), schizophrenia (1%), epilepsy (0.8%) and intellectual disability (2%), have been recently associated to DNA copy number variations and – in select cases – to haploinsufficiency for specific genes. Polypeptides encoded by these genes are extremely heterogenous including molecules linked to synaptic functions (*e.g.*, *NEUREXIN1A*, *SHANK2*, *SYNGAP1*) as well as effectors falling in other ontology groups (*e.g.*, enzymes involved in chromatin dynamics) (Coe et al., 2012; Devlin and Scherer, 2012; Merikangas et al., 2009; Tam et al., 2009)). Remarkably, animal models often support the etiological link between reduced allele dosage and neuro-cognitive symptoms inferred from human genetics (Etherton et al., 2009; Jiang and Ehlers, 2013; Nuytens et al., 2013). Gene therapy of neuropathogenic haploinsufficiencies is a formidable task. In some cases, genes can be therapeutically expressed at non-physiological levels without toxic consequences, *e.g. SMN1* in clinical trials for spinal muscular atrophy (https://clinicaltrials.gov/ct2/show/NCT02122952) and *hamartin* in mouse models of tuberous sclerosis type 1 (Prabhakar et al., 2015). In the majority of cases, an accurate tuning of gene expression, close to physiological levels, is strictly required. This is the case of *FOXG1*, whose hemideletion and duplication lead to Rett-like and West syndromes, respectively (Florian et al., 2012). Given the complex control of gene expression, the somatic delivery of an extra-copy of the disease gene with the full repertory of regulatory sequences hardly looks a scalable therapeutic option. On the other hand, the homologous recombination-based, CRISPR/Cas9-nuclease-assisted correction of gene deletion *in vivo* would be equally difficult to implement for three concurrent key issues: (1) huge size of Cas9-cDNA; (2) length of the editor DNA, often in the range of megabases; and (3) strong functional prevalence of non-homologous-end-joining (NHEJ) machinery over homologous recombination (HR) in neuronal cells (Orii et al., 2006; Yang et al., 2013). To overcome these limitations, smaller CRISPR enzymes, deliverable by biosafe AAV vectors, have been recently discovered (Kleinstiver et al., 2015; Ran et al., 2015). Furthermore, undesired indels evoked by CRISPR nucleases could be prevented by transient inhibition of the NHEJ machinery (Chu et al., 2015; Maruyama et al., 2015). However, the editor DNA issue is still unfixed. Therefore, although CRISPR enzymes are promising tools for accurate rescue of subtle gene lesions, the vast majority of neurodevelopmental disorders linked to *whole-gene* hemideletions remains at present uncurable.

For all neurological diseases requiring genome engineering and/or correction of gene expression levels, the central nervous system poses special challenges for therapeutic intervention. First, it is isolated from the circulatory system by the Blood Brain Barrier (BBB). While essential nutrients can pass, this vascular gate-controlling system prevents most molecules from entering the

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nervous system from blood circulation. To avoid this limitation, nucleic acid- and protein-based drugs can be delivered directly into the brain by intratechal injection into the cerebrospinal fluid (CSF) or by intranasal administration. Interestingly, it has been shown that properly functionalized exosomes (Alvarez-Erviti et al., 2011) and – more recently – selected variants of recombinant AAV vectors (Deverman et al., 2016) can pass the BBB and effectively support pan-neural nucleic acid delivery upon intravenous administration. Most importantly, the brain is composed by a staggering complexity in neuronal cell types and connectivity that pose currently insormontable obstacles in delivering the drug specifically into the appropriate cell type and in the correct subcellular or synaptic location.

3. The Yin and Yang of molecular therapy for neurodegenerative diseases

Manipulating RNA expression *in vivo* represents a new strategy for the molecular therapy of neurodegenerative diseases. In this context, a first class of therapeutic drugs should promote down-regulation of gene expression, in conditions of dominant neurodegenerative diseases caused by the expression of a pathological, toxic target gene. Therapeutic molecules have been developed using technologies based on small antisense oligonucleotides (ASOs) (Gogtay and Sridharan, 2016; Havens and Hastings, 2016) and small interfering RNAs (siRNAs) (Bobbin and Rossi, 2016; Lorenzer et al., 2015).

A second equally challenging class of drugs should be based on RNA molecules that can increase gene expression *in vivo*. Augmented levels and/or activity of modifiers of pathogenic pathways may contribute to reestablish a homeostatic cellular environment. Hijacking the neuroprotective function of neurothrophins may represent an attractive strategy to preserve cell viability and function in diseased brain. Most importantly, for patients with haploinsufficiencies, a therapeutic approach based on RNA drugs to rescue the physiological amounts of the target protein would in principle be curative. Unfortunately, no molecules of this type have been found to date leaving these diseases untreatable (van Bokhoven, 2011).

This review aims at sharing the excitement over the recent advancements in the field of genomics and on their potential application in developing new nucleic acid-based therapies for brain diseases. For the first time scientists can use a plethora of molecular tools to manipulate gene expression *in vivo* both to inhibit (Yin) and increase (Yang) the expression of validated drug target genes. Since the ability to decrease expression has been within reach for many years, we will briefly describe inhibitory technologies while focusing on specific examples in animal models of neurodegenerative diseases as well as on initial clinical trials data in humans. Importantly, new technologies are now developed to increase the expression of selected genes without permanently modifying host genomes. Here we will focus our attention on the molecular basis of gene-specific activation of transcription (RNAa) and translation (SINEUP). Since these molecular tools are just at their infancy, no clinical data are available.

We are concious that an efficient delivery of exogenous nucleic acids is at the core of drug development. For this matter, we refer the readers to reviews specifically addressing this important topic (some representative reviews cited here) (Gherardini et al., 2014; Gomes et al., 2015; Kanasty et al., 2013; Liu et al., 2013b; Lorenzer et al., 2015; Magen and Hornstein, 2014; McConnell et al., 2014; Reissmann, 2014; Southwell et al., 2012; Zhang et al., 2013).

4. The Yin side of nucleic acid-based therapy: technologies

4.1. Antisense oligonucleotides (ASOs)

Antisense oligonucleotides or ASOs are short, single-stranded synthetic nucleic acids, tipically 8–50 nucleotides long, that bind target RNA molecules by standard Watson–Crick base pairing (Lundin et al., 2015). Upon binding, ASOs modulate target RNA function. Chemical modifications are required to achieve ASOs stability in biological fluids and to increase their potency in binding their mRNA target. Several variants of ASO chemistry have been developed aiming at improving nuclease resistance and overall pharmacokinetic properties (Deleavey and Damha, 2012). In all cases, primary nucleotide sequence is enriched with a series of chemical modifications that overall change nucleic acid biochemical properties. Chemical modifications can target the phosphodiester bond along the nucleic acid backbone (as in phosphorothioate or PS ASOs, thiophosphoramidate and morpholino ASOs) or the sugar mojety at the 2′-position (as in 2′-*O*-methyl or 2OMe and 2′-*O*-methoxy-ethyl or MOE) (Eckstein, 2014; Yamamoto et al., 2011). In some cases, modifications at the sugar mojety are applied to a portion of the ASO sequence, which is then combined with an internal unmodified sequence (as in gapmer ASOs). Additional variants of ASO chemistry include locked nucleic acid molecules, or LNO, where the sugar 2′-hydroxyl group is tethered with the 4′-carbon atom and tricycle-DNA oligonucleotides (Geny et al., 2016), or tc-DNA, where a conformational constrain is inserted around C3′-C4′ and C4′-C5′ bonds (Goyenvalle et al., 2015; Renneberg et al., 2002). Most recently, additional nucleic acid modifications have been introduced to combine chemical adducts at the backbone and at the sugars. One class of such ASOs is provided by peptide nucleic acids, or PNAs, in which polyamide linkages fully substitute the sugar phosphate backbone (Wancewicz et al., 2010). PNAs are resistant to degradation but retain base-pairing capabilities showing enhanced affinity. The choice of chemical modification for ASO design has an impact on their mechanism of action and specific ASO chemistry is selected based on the therapeutic target (Geary et al., 2015).

Therapeutic ASOs work mainly through two mechanisms: 1) RNAse H-mediated degradation of target RNA; 2) non-degradative steric block of target RNA (Bennett and Swayze, 2010; Evers et al., 2015).

ASOs with unmodified sugars can effectively trigger RNAse H-mediated degradation of base-paired target mRNAs, thus resulting in gene silencing. RNAse H specifically cleaves the RNA mojety when bound to ASOs in mRNA-DNA heteroduplex. Single-stranded DNA ASOs are then released from the duplex and made available for catalyzing degradation of additional mRNA molecules for a more efficient silencing. This class of ASOs is currently the most widely used for therapeutic applications. In particular, MOE ASO gapmers targeting mutant SOD1 are the first example of ASOs tested in human clinical trial to treat patients with ALS (Miller et al., 2013).

Chemical modifications at 2′ position of sugars render ASOs and paired RNAs resistant to RNAse H-mediated degradation. While hampering degradation mechanism, 2′ modifications provide enhanced stability, better pharmacokinetic properties and, most importantly, increased binding to target RNA. Non-degradative ASOs act by creating a steric hindrance that blocks target RNA from further processing and therefore inhibits its function. In most cases, non-degradative ASOs negatively regulate mRNA translation, blocking the recognition of target mRNA by the ribosomes and ultimately down-regulating gene expression.

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4.2. RNA interference (RNAi)

It collectively refers to a large array of natural regulatory processes, conserved from yeast to humans, where endogenous molecular machineries, programmable by small-sized RNAs, negatively regulate gene expression (Bobbin and Rossi, 2016).

Three types of small RNAs are involved in RNAi, siRNAs, microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs). siRNAs derive from Dicer-dependent processing of dsRNAs, in turn originating from convergent transcription, bidirectional transposon readthrough or unidirectional transcription of inverted repeats (Elbashir et al., 2001; Fire et al., 1998). miRNAs are generated by processing of long primary transcripts (pri-miRNAs), along the classical Drosha/Dicer pathway or the splicing-machinery-mediated mirtron pathway (Ha and Kim, 2014; Lee et al., 1993). piRNA, still poorly characterized in mammals, are produced in Drosophila *via* Zucchini-mediated parsing of long primary transcripts and their subsequent trimming, and may be amplified thanks to a ping-pong cycle catalized by Vasa, Piwi/Aub and Ago3 (Brennecke et al., 2007; Vagin et al., 2006).

miRNAs and siRNAs are loaded onto RNA-instructed silencing complexes (RISCs), which prevalently act at post-transcriptional level (Agrawal et al., 2003). RISC-mediated silencing may be achieved by straight target-RNA cleavage. This usually occurs upon perfect matching between the siRNA guide included in the RISC complex and its target, and requires the endonucleolytic activity of the Ago2 RISC subunit. Conversely, prevalently in case of miRNA-programmed RISCs, Agos recruit GW182, which in turn conveys to RISC other silencing machineries. The interaction of GW182 with poly(A)-binding protein (PABP) may displace PABP, leading to destabilization of the target mRNA and decrease of its translation. Furthermore, GW182 may recruit deadenylases and the decapping complex *via* a CCR4–NOT complex bridge, further destabilizing the target mRNA and jeopardizing its translation. As for piRNAs, they are loaded onto different RISCs, which are imported into the nucleus and act co-transcriptionally. In these cases, target recognition recruits silencing factors and chromatin modifiers increasing local levels of H3K9me3 and leading to transcriptional silencing (Iwasaki et al., 2015).

Endogenous molecular machineries supporting siRNA- and miRNA-RNAi have been extensively exploited to achieve artificial RNAi for therapeutic applications (Bobbin and Rossi, 2016; Lam et al., 2015). While different types of exogenous RNAs have been employed, immature RNA precursors have been often shown to outperform their small RNA derivatives. Among the best performing effectors there are 29-bp long ds-siRNA precursors as well as hairpin RNA precursors (small hairpin RNAs or shRNAs), based on backbones of endogenous pri-miRNAs. These effectors may be delivered to target cells as pre-made molecules, with appropriate chemical modifications to stabilize them and promote their processing (Deleavey and Damha, 2012; Lam et al., 2015). Alternatively they may be delivered as DNApolII- or DNApolIII-dependent, cDNA-encoding transgenes to achieve a more persistent effect (Lorenzer et al., 2015). Lentiviral and adeno-associated viral vectors are the most commonly used to deliver shRNAs and miRNAs into the brain, as they provide stable expression while being poorly immunogenic, replication incompetent and safe (Keiser et al., 2016). Recently, great therapeutic potentials are held by engineered versions of adeno-associated vectors that are capable of crossing the blood brain barrier and efficiently deliver transgenes into the brain even after a single intravenous injection (Deverman et al., 2016).

4.3. Anti-miRNA oligonucleotides (or antagoMIR or blockmir)

miRNA expression and function has been proved altered in a number of human disorders, such as cancer, autoimmune diseases and neurodegeneration. As a consequence, there has been an increasing interest in developing therapeutic strategies to inhibit miRNAs in disease conditions. Moreover, therapeutic miRNA targeting has the advantage that a single miRNA-drug can affect many target genes simoultaneuosly, ultimately affecting the whole disease pathway. Three main strategies have been adopted to achieve down-regulation of miRNA activities: expression of miRNA sponges, small molecule inhibitors of miRNAs and anti-miRNA ASOs. In the first case, expression of long coding or non-coding transcripts containing multiple copies of miRNA binding sites can act as sponges that compete with natural targets and reduce the actual concentration of active miRNAs. This strategy has been based on recent discoveries that lncRNAs (lincRNAs and pseudogenes) can act as natural miRNA sponges (Cesana et al., 2011; Tay et al., 2011). Currently, the use of miRNA sponges has been limited to *in vitro* applications and some pre-clinical mouse models (Ebert and Sharp, 2010). Circular RNAs, referred to as circRNAs, are a novel class of abundant, nuclease-resistant lncRNAs that are generated by covalent link of 5′ and 3′ termini (Memczak et al., 2013; Salzman et al., 2012). circRNAs can compete with protein-coding mRNAs and lncRNAs and act as sponges for miRNAs (Hansen et al., 2013). As an example, CiRS-7 has been shown to adsorb miR-7, thus releasing its target mRNAs, including Parkinson's disease-associated α-synuclein, from inhibition (Hansen et al., 2013; Lukiw, 2013; Salzman, 2016). Furthermore, from compound libraries screenings small molecules have been identified for their inhibition of the expression of specific miRNAs (Young et al., 2010). However, their therapeutic use is yet limited because of high IC_{50} and poor specificity. Finally, wider therapeutic application is coming from the use of ASOs to specifically target selected miRNAs thus blocking their function. Anti-miRNA oligonucleotides, or antagoMIR or blockmir, are single-stranded ASOs that tightly bind miRNAs and inhibit their activity on the target mRNA(s). Many antagoMIRs targeting different miRNAs are currently being developed for hepatitis C virus infection, liver cancer, heart failure and metabolic disorders of the heart (Li and Rana, 2014). Some of them have already reached the clinic for phase I/II testing. For neurodegenerative disorders, antagoMIRs have been used in mouse models of AD and ALS (see below).

5. The Yin side of nucleic acid-based therapy: applications to neurodegenerative diseases

5.1. Alzheimer's disease

Alzheimer's Disease (AD) is the most common neurodegenerative disorder, affecting nearly 44 million people worldwide and accounting for 60–80% of all forms of dementias. AD neuropathology is characterized by the deposition of insoluble aggregates of beta-amyloid (Aβ) and tau in extracellular plaques and intracellular neurofibrillary tangles, resulting in progressive neuronal loss and cortical atrophy. AD patients suffer from progressive cognitive impairment, gradual loss of memory and learning capabilities, ultimately leading to degeneration of the overall quality of life (Scheltens et al., 2016). Although the precise mechanisms that lead to AD are not fully understood, several studies indicate that processing of amyloid precursor protein (APP) has a key role in the disease. β-amyloid precursor protein cleaving enzyme 1 (BACE1) is involved in APP processing giving rise to Aβ fragments. Increased BACE1 activity has been observed in AD and associated to neurodegeneration and accumulation of Aβ aggregates. Thus, inactivation of BACE1 is considered a potential therapeutic strategy to treat AD. To this purpose, lentiviral vectors were used to deliver BACE1-targeting siRNAs into hippocampus of APP transgenic mice. Upon injection, siRNAs lowered levels of BACE1, reduced amyloid production and rescued neurodegeneration and behavioural

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deficits in APP mice (Singer et al., 2005). In a more recent study, BACE1-specific siRNAs were delivered to the mouse brain by systemic injection of modified exosomes, containing neuron-specific RVG peptide fused to Lamp2b exosomal protein (Alvarez-Erviti et al., 2011). BACE1-siRNA loaded exososomes induced a strong down-regulation of BACE1 mRNA (60%) and protein (62%) in wt mice, thus demonstrating their efficacy as brain delivery system. Synthetic miRNAs were also used to decrease BACE1 levels and rescue disease phenotype in AD mouse model upon surgical delivery through Adeno-associated viral particles (Piedrahita et al., 2015).

BACE1 mRNA is positively regulated by an antisense lncRNA, named BACE1-AS (Faghihi et al., 2008). Interestingly, BACE1-AS is upregulated in brain samples from AD patients thus becaming itself a target for siRNA strategies for AD. Indeed, knockdown of either one of BACE1 or BACE1-AS transcripts by intra-ventricular infusion of modified siRNAs caused concordant downregulation of both transcripts in APP transgenic mice, ultimately leading to reduced Aβ production and plaque deposition (Modarresi et al., 2011).

In addition to siRNA-based strategies, ASOs are also employed to reduce expression of proteins involved in AD pathogenesis. ASOs targeting acetylcholinesterase, mutated APP as well as PSEN1 had shown promising effects in transgenic AD models (Chauhan and Siegel, 2007; Farr et al., 2014; Fiorini et al., 2013).

Finally, antagoMIR strategies are also under study as potential therapeutics for AD. Expression of several miRNAs is dysregulated in AD brains, resulting in alteration of molecular targets and pathways known to be associated to AD pathology (Di Meco and Pratico, 2016). BACE1 mRNA, whose quantity is elevated in AD, is a direct target of miR-188-3p, that conversely is down-regulated in the brain of AD mice. Overexpression of miR-188-3p by intracranial viral delivery reduced BACE1 levels, decreased amyloid inclusions and improved synaptic activity and cognitive functions (Zhang et al., 2014).

5.2. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective loss of motor neurons in the spinal cord and brain. ALS patients suffer from muscle stiffness and severe muscle weakness. They ultimately die by respiratory failure. The progression of ALS is rapid and most patients survive only 3–5 years after diagnosis. The most advanced antisense approach to treat ALS is based on ASOs targeting Superoxide Dismutase 1 (SOD1), a protein mutated in familial cases of the disease. Selection of SOD1-specific ASOs, optimization of delivery and efficacy of treatment were evaluated in a rat model expressing human mutated SOD1 (SOD^{G93A}). SOD1-ASOs were injected intro cerebral ventricles and proved to reach the spinal cord circulating through the cerebrospinal fluid. ASO-treated mice showed decrease levels of SOD1 protein and mRNA throughout the brain and spinal cord. When ASO infusion was performed before disease onset, transgenic rats displayed slower disease progression and increased survival (+37%) (Smith et al., 2006).

Following encouraging pre-clinical data, the first phase-1 clinical trial was funded to test safety, tolerability and pharmacokinetics of ASO treatment targeting SOD1 upon intrathecal administration in patients with SOD1 mutation (patients with different types of SOD1 mutation were enrolled in this study). Cerebrospinal fluid samples taken immediately after infusion indicated the presence of the drug. Analysis of spinal cord samples taken from one participant who later died from ALS indicated: i) higher ASO levels at the site of injection as compared to distant sites; ii) proper estimate of drug persistence in the body (peak concentration at 12 h after infusion and lowered to basal level at 24 h). Overall, a 15% variation in SOD1 levels in the cerebrospinal fluid was observed, predicting that higher doses of the drugs are needed to achieve effective down-regulation (Miller et al., 2013).

Additional antisense strategies are also being developed in SOD^{G93A} animal models using siRNAs. Several studies have demonstrated effectiveness of SOD1 silencing approach in mouse models of ALS, proving reduced SOD1 expression, improved motor neuron survival, and delayed onset of ALS symptoms in treated animals (Nizzardo et al., 2012). In most cases, silencing RNAs are delivered as shRNA precursors using viral vectors.

Most recently, it has been shown that miR-155 is increased in SOD^{G93A} mice as well as in the spinal cord of ALS patients, thus providing a new therapeutic target. Indeed, antisense oligonucleotide therapy devised to inhibit miR-155 (antagoMIR) was developed and tested in mice. After continuous intra-ventricular administration, treated mice showed extended survival and prolongued disease duration (38%) (Koval et al., 2013). Therapeutic potential of antagoMIR for miR-155 in ALS was further validated in a more recent study, where genetic ablation of miR-155 in SOD^{G93A} transgenic mice restored molecular signatures in the periphery (monocyte signature) and in the central nervous system (microglia signature), as determined by proteomics and transcriptomics (Butovsky et al., 2015). Finally, intra-ventricular delivery of LNA formulation of anti-miR-155 molecule delayed disease onset and extended survival in SOD1 mice (Butovsky et al., 2015). The exact molecular targets of anti-miR-155 therapy have yet to be identified.

5.3. Amyotrophic lateral Sclerosis/Frontotemporal dementia

Expanded hexanucleotide repeats in the first intron of C9orf72 gene were recently identified as the most common genetic cause of ALS, FrontoTemporal Dementia (FTD) or concomitant ALS/FTD. Although the exact mechanisms by which repeated elements cause neurodegeneration are currently unknown, recent studies suggest that neurodegeneration by C9orf72 is mediated by RNA toxicity, loss of C9orf72 gene functions or, possibly, a combination of both. As support to this hypothesis, RNA foci are present in ALS/FTD brains. Expanded RNA is involved in repeat-associated non-ATG (RAN) translation that produces aberrant peptides or dipeptides polymers. Recently, three independent studies reported the use of antisense oligonucleotides technology to modulate C9orf72 expression and RNA foci (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013). ASOs were designed to target the sequences upstream or downstream the toxic repeat expansion. In all cases, a more pronounced down-regulation of C9orf72 was obtained with down-stream targeting ASOs. ASOs were tested in patients' fibroblast, patients-derived iPS cells and iPS-differentiated to motor neurons. ASOs mediated therapy was able to efficiently target C9orf72 transcript suppressing RNA foci formation and reversing gene expression alterations observed in patients cells. The positive effect on RNA foci could be obtained with ASOs that down-regulate overall C9orf72 levels, targeting intronic regions, as well as with those that specifically hybridize to transcripts containing the exanucleotide repeat expansion. The use of sense-specific ASOs could not correct RNA signature in patients' fibroblasts indicating that antisense-specific ASOs were probably required for therapeutic effects. Interestingly, siRNA molecules targeting C9orf72 were also tested and surprisingly proved uneffective.

Finally, in Lagier-Tourenne et al. (Lagier-Tourenne et al., 2013), a proof of principle study was performed *in vivo* by delivering C9orf72-specific ASOs in the mouse brain with intracerebroventricular stereotaxic injection. Prolonged down-regulation of C9orf72 was observed. Microarray analysis revealed tolerability of ASO injection *in vivo*, with small changes in overall expression

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profile, long-term reduction of C9orf72 mRNA (30–40% less) and no neuropathological or behavioural defects.

Several studies are ongoing to better define the genetic features of c9orf72-related pathology in patients in order to prepare for future clinical trials targeting this gene.

5.4. Tauopaties

In AD, progressive supranuclear palsy and in a number of other neurodegenerative diseases collectively known as tauopathies, the macrotubule-associated protein tau becomes hyperphosphorylated and aggregates to form intracellular neurofibrillary tangles (Iqbal et al., 2016; Wang and Mandelkow, 2016). Antisense oligonucleotide strategies have been developed to down-regulate tau expression and reduce tau-associated seizures in mice. In a first study, injection of tau-specific ASOs directly into the cerebrospinal fluid reduced levels of endogenous murine tau throughout the entire central nervous system and cerebrospinal fluid. Down-regulation of tau had no effect on baseline motor or cognitive behavior, but was associated to reduced seizures in chemically-induced seizures model (DeVos et al., 2013). More recently, additional ASOs were designed to target human tau and tested in human neuroblastoma cell lines and in transgenic mice expressing human tau. The strongest effect was achieved with ASOs targeting splice sites, thus mediating exon skipping and inducing frame-shifts and premature stop codon, with subsequent mRNA degradation (Sud et al., 2014). siRNAs were also employed to silence Tau levels and rescue disease phenotype *in vivo* in transgenic mice expressing mutated human tau (Xu et al., 2014). Recently, expression profiling studies have revealed altered quantities of miRNAs in AD and age-related tauopathies (Qiu et al., 2015), implying their role in pathogenesis. As a consequence, therapeutic modulation of miRNAs is expected to have an impact on pathways implicated in the disease. miR-129 was identified to be downregulated in brain tissues from patients with AD and taupathies (Santa-Maria et al., 2015). By *in vitro* experiments, miR-129 was found to bind directly the 3′UTR of tau mRNA. *In vivo*, overexpression of miR-129 silenced tau protein and partially abrogated tau toxic effect in a *Drosophila* model producing human tau. In another study, intra-brain delivery of miR-132 mimics *via* osmotic pumps resulted in the reduction of tau and amelioration of long-term memory in an AD mouse model (Smith et al., 2015).

5.5. Parkinson's disease

Several genetic evidences indicate that α-synuclein expression affects PD onset, severity and risk. Increased expression of α-synuclein provokes a series of events altering neuronal homeostasis and ultimately cell death. While α-synuclein is prone to aggregation as the crucial component of Lewy Bodies, the gene has been found mutated in rare familial cases. Importantly, α-synuclein gene triplication (Farrer et al., 2004; Sekine et al., 2010; Singleton et al., 2003) and duplication (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Nishioka et al., 2009) promote early-onset PD, proving that a higher level of wild type α-synuclein can lead to pathogenesis (Devine et al., 2011). In this context, genetic variants in its promoter region are associated to sporadic PD cases with lower expression being beneficial (Chiba-Falek and Nussbaum, 2001). Recent reports support the hypothesis that α-synuclein also plays a critical role in propagating the neurodegenerative process *via* prion-like mechanisms. Therefore, therapeutic strategies aiming at inhibiting its cellular function and expression are under intense scrutiny to achieve disease amelioration.

A large effort is undergoing in optimizing siRNA- and ASO-based drugs to target α-synuclein mRNA. Widespread delivery into the brain was achieved by fusion of rabies virus glycoprotein peptide (RGV) to extra-exosomal N-terminus of Lamp2b protein (Cooper et al., 2014). In normal mice, systemic administration of siRNA-loaded RGV-exosomes decreased α-synuclein mRNA and protein to 40–50% of its endogenous levels in Substantia Nigra, Striatum and cortex. Most importantly, in transgenic mice expressing human phospho-mimic S129D α-synuclein, treatment with RSV-exosome-siRNAs reduced aggregated and insoluble proteins in midbrain dopaminergic neurons.

More recently, down-regulation of α-synuclein expression was obtained using recombinant adeno-associated virus to deliver α-synuclein-specific shRNA in the Substantia Nigra in normal rats (Zharikov et al., 2015). Under physiological conditions, a 35% reduction of mRNA and protein levels did not cause any motor deficit or neuronal degeneration. However, when rats were subjected to chemical mitochondria intoxication, mimicking PD-like symptoms, AAV-shRNA targeting α-synuclein proved to be neuroprotective, safeguarding dopaminergic neurons in the Substantia Nigra and dopaminergic terminals in the Striatum while rescuing motor functions.

5.6. Huntington's disease

Huntington disease (HD) is an inherited, autosomal dominant, fatal neurodegenerative disorder caused by a CAG repeat expansion in the first exon of the gene encoding for huntingtin (HTT) protein. Pathogenic expansion of CAG sequence is translated into an elongated stretch of polyglutamines (polyQ) at the N-terminal of HTT protein, resulting in the production of a mutant protein. The mutant protein is toxic and causes neuronal dysfunction and degeneration leading to motor symptoms, cognitive decline and psychiatric disturbances (Andrew et al., 1993; The Huntington's Disease Collaborative Research Group, 1993). Silencing mutant HTT using nucleic acids would eliminate the basis of HD pathology. However, these approaches have raised some concerns about possible side effects due to the diminishment of HTT physiological functions. Indeed, constitutive loss of wt allele is lethal in mouse embryos and reduced expression leads to neurodegenerative-like phenotypes (Dragatsis et al., 2000; Duyao et al., 1995), while recent data seem to indicate that ablation of HTT in adult neurons is nondeleterious (Wang et al., 2016). Extensive body of research has demonstrated that wt HTT is neuroprotective and plays fundamental roles in several cellular pathways (Saudou and Humbert, 2016). As a consequence, in addition to nonallele-selective silencing approaches, novel strategies are being developed aimed at allele-specific suppression of mutant HTT (Beaudet and Meng, 2016; Keiser et al., 2016). Nucleic acid-based therapeutic strategies, using ASOs and RNAi, hold great promise. Indeed, several preclinical studies have demonstrated the utility of such strategies to improve HD neuropathology and symptoms. Initial studies in patients-derived cells lines indicated selective inhibition of HTT protein expression by double-stranded and single-stranded ASOs (Hu et al., 2009). In mouse models, a sustained therapeutic reversal of HD pathology was obtained by transient repression of HTT synthesis using HTT-targeting ASOs (Kordasiewicz et al., 2012). Transient infusion of HTT-ASOs into the cerebrospinal fluid of symptomatic HD mouse models effectively suppressed HTT mRNA and protein levels and mutant HTT accumulation, causing delayed disease progression and reversal of disease phenotype. Similar ASO infusion into nonhuman primates showed effective reduction of HTT protein in several brain regions (Kordasiewicz et al., 2012). Additional studies described selective down-regulation of mutant HTT by allele-specific and single-nucleotide polymorphism-specific ASOs (Carroll et al., 2011; Kay et al., 2015; Ostergaard et al., 2013; Southwell et al., 2014).

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An alternative approach for silencing HTT expression at post-transcriptional level is the use of siRNAs to trigger the RNAi pathway. Initial studies using RNAi as a therapeutic strategy enabled the reduction of HTT mRNA and protein *in vitro* in cellular models of HD (Chen et al., 2005). Subsequently, RNAi-based approach was applied *in vivo* in preclinical trial for HD (Harper et al., 2005). Single bilateral injections of AAV delivering anti-HTT shRNA were administered into the striatum of HD transgenic mice. Significant reductions in mutant HTT mRNA levels (approximately 55%) were observed, accompanied by a reduction of HTT inclusions and recovery of motor deficits. Several other preclinical studies followed in the most recent years, mainly focusing on siRNA delivery systems using nanoparticles or cholesterol-conjugated molecules and adeno-associated vectors (Aronin and DiFiglia, 2014; Keiser et al., 2016). Nonallele-specific silencing by RNAi proved to be well-tolerated resulting in long-lasting amelioration of neuropathological deficits in mice (Boudreau et al., 2009; Drouet et al., 2009; Dufour et al., 2014) and non-human primates (Grondin et al., 2015; McBride et al., 2011). Selective silencing of mutant HTT was achieved with siRNAs (Pfister et al., 2009) and artifical miRNAs (Monteys et al., 2015) targeting allele-specific polymorphisms. Allele-selective silencing was also demonstrated in patients' cells and in mouse models with single-stranded antisense oligonucleotides (ss-siRNAs), that combine simplicity of ASO technology with the efficiency of RNAi (Yu et al., 2012). Finally, the development of new-generation of brain-targeting adeno-associated viral vectors represents a promising platform for silencing therapy in neurodegenerative disorders (Choudhury et al., 2016; Deverman et al., 2016). In mice, a single intravenous injection of modified AAV encoding an artificial miRNA was sufficient to induce effective silencing of HTT in the brain (Choudhury et al., 2016).

Nonallele-specific HTT silencing approach for HD using ASO technology has recently entered the clinic with a phase1/2 trial (source ClinicalTrial.gov, 2015). Together with safety, its aim will include measuring the level of mutant huntingtin protein in the cerebrospinal fluid using a newly developed assay (Southwell et al., 2015; Wild et al., 2015).

5.7. Transthyretin familial amyloid polyneuropathy

Transthyretin familial amyloid polyneuropathy (TTR-FAP) is an autosomal dominant inherited disease, characterized by progressive neuropathy and cardiomyopathy. Like other forms of TTR amyloidosis, TTR-FAP is caused by mutations in the gene encoding for transthyretin (TTR). Val30Met is the most common mutation in TTR-FAP patients and accounts for 50% of TTR-FAP cases worldwide (Gertz et al., 2015; Parman et al., 2016). Mutated TTR has the propensity to misfold into insoluble and pathologic amyloid fibrils. These form extracellular deposits that accumulate in the peripheral and central nervous system as well as in the heart, giving rise to the clinical manifestations (Sipe et al., 2014). As such, reducing the quantities of TTR deposits is crucial for therapeutic treatment of TTR-FAP. A siRNA-based drug targeting TTR and delivered by means of lipid nanoparticles is in clinical trial for TTR-FAP. Data from phase II study demonstrate a robust knockdown of serum TTR protein with >80% reduction after two doses. Multiple doses were generally safe and well tolerated (Suhr et al., 2015). A randomized, placebo-controlled, double-blind phase III study (APOLLO, NCT01960348) is currently ongoing for TTR-FAP patients with Val30Met to evaluate the safety and efficacy of TTR-targeted siRNA therapy. Additional siRNA sequences and delivery methods are still under development. Preclinical testing indicate that RNAi-mediated knockdown of TTR can efficiently reduce toxic deposition across a broad range of affected tissues in a mouse model of TTR-FAP (Butler et al., 2016).

6. The Yang side of nucleic acid-based therapy: technologies

6.1. Modified mRNA technology

Modified mRNA technology has recently emerged as a novel option to gene therapy approach. The use of DNA and mRNA as genetic source of therapeutic proteins has started decades ago, but only the advent of novel chemistry to stabilize mRNA molecules has prompted the use of mRNA-based therapy to pre-clinical and clinical settings (Sahin et al., 2014). By definition, mRNA has the potential to produce proteins and peptides of therapeutic interest with easy scalability in the number of targets. mRNA-based therapeutics display several advantages over other nucleic acid strategies: i) delivered mRNA does not need to enter the nucleus to be functional; ii) mRNA does not integrate into the genome; iii) production is relatively simple and inexpensive. However, since recent years, there have been two major problems associated to mRNA therapeutics: i) mRNA is highly unstable in cells and *in vivo* to achieve sufficient protein expression and ii) mRNA induces strong immune responses in the host organism, thus hampering repeated administrations. A variety of formulations have been developed to protect nucleic acids from extracellular degradation by RNAses, to facilitate cellular uptake and enable endosomal escape and easy release into the cytoplasm. Nanosized particles are typically employed with different biochemical and biophysical properties: positively charged lipids, cationic polypetides, polymers and micelles (Cheng and Lee, 2016; Li et al., 2016a; Reichmuth et al., 2016; Uchida et al., 2013). Overall, lipid nanoparticles and polyplex nanomicelles are the most widely used for therapeutic mRNA delivery *in vivo* and in clinical trials (Reichmuth et al., 2016; Sahin et al., 2014).

Chemically-modified mRNA synthesized *in vitro* and combined with naturally occurring modified ribonucleotides has been employed to overcome the aforementioned obstacles and has shown a great promise as therapeutic tool for gene therapy. Expression of recombinant proteins after delivery of chemically modified mRNA has been shown to be effective in mice and non-human primates in models of anemia, congenital lung disease and myocardial infarction (Kariko et al., 2012; Kormann et al., 2011; Zangi et al., 2013). Modified mRNA is currently in clinical trails as cancer immunotherapy and vaccine (Kallen and Thess, 2014). Alternatively, unmodified mRNA has been sequence-engineered to display higher stability and translatability when introduced *in vivo* in complexed nanoparticles for vaccines (Kubler et al., 2015; Petsch et al., 2012; Schnee et al., 2016) and therapy in the brain (Lin et al., 2016; Nabhan et al., 2016). Recently, unmodified mRNA encoding for erythropoietin has been proven effective in large animals (Thess et al., 2015).

6.2. AntagoNATs

Inhibition of natural AS lncRNA transcripts (or natural antisense transcripts or NAT) can be used as an indirect mean to modulate endogenous sense mRNA levels. To specifically inhibit NATs, Wahlested and collaborators took advantage of the antisense oligonucleotide chemistry to develop AntagoNATs. AntagoNATs are ASOs that inhibit a regulatory antisense lncRNA, thus indirectly modulating sense gene expression. AntagoNATs are designed to target S/AS overlapping region, thus blocking AS function mainly through RNAse H-mediated degradation (Wahlestedt, 2013). Depending on NAT function, AntagoNATs can be used for down-regulating gene expression, as for BACE1-AS (Faghihi et al., 2008; Modarresi et al., 2011), or for up-regulation, as for BDNF-AS (Modarresi et al., 2012) and SCNA1 (Hsiao et al., 2016).

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6.3. Non-degradative ASOs

The binding capabilities of non-degradative ASOs have also been employed for up-regulating gene expression. Mechanistically this can be obtained by blocking mRNA degradation or by increasing mRNA transcription. ASOs designed to bind miRNAs (referred to as antagoMIR or blockmir) increase mRNA stability interfering with miRNA-mediated mRNA degradation pathway and ultimately leading to enhanced accumulation of protein products (Li and Rana, 2014). Up-regulation of gene expression can also be obtained by ASOs designed to bind AS lncRNAs involved in chromatin remodeling. In this context, a class of AS lncRNAs is required for suppressing gene expression by recruiting components of the Polycomb Repressor Complex 2 (PRC2) to the gene locus from which they are transcribed. ASO binding to lncRNA can therefore block PRC2 complex assembly allowing transcription to restart, thus inducing up-regulation of sense protein-coding mRNA present at the same locus (Zhao et al., 2010).

Alternatively, ASOs that do not degrade target mRNAs can be designed to modulate their processing. When targeting pre-mRNAs, ASOs can modulate splicing and are referred to as exon-skipping ASOs or splice-switching oligonucleotides (SSOs) (Havens and Hastings, 2016). SSOs are designed to interfere with RNA sequences required for the splicing machinery thus inhibiting pre-mRNA interaction with the spliceosome including small nuclear RNAs and splicing factors. As a result, SSOs can repair defective splicing, promote exon-skipping or induce exon-retention, thus restoring the production of a functionally corrected protein. SSOs are usually synthesized using LNA or PNA chemistry to avoid target RNA degradation and achieve potent binding. SSOs have been used in the clinic for the treatment of Duchenne Muscolar Dystrophy (Hodgkinson et al., 2016; Kole and Krieg, 2015; Mendell et al., 2016). In the context of neurodegenerative disorders, therapeutic SSOs have been designed to target ATM in Ataxia Telangectasia (Du et al., 2011, 2007), SMN2 in Spinal Muscolar Atrophy (see below) and microtubule associated protein Tau in Frontotemporal Dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (Peacey et al., 2012). A state-of-the-art description of the use of ASOs as splicing modifiers can be found elsewhere (Havens and Hastings, 2016; Kole et al., 2012; Rigo et al., 2014; Siva et al., 2014).

6.4. RNA activation (RNAa)

6.4.1. Historical and operational features

The first report of transcriptional stimulation by small RNAs, briefly RNA activation (RNAa), dates back to 2006, when Li et al. (Li et al., 2006) showed that siRNA-like molecules targeting the promoters of three genes of oncological interest, *Cadherin E*, *p21*$^{WAF1/CIP1}$ and *VEGF*, were able to elicit a specific and prolonged stimulation of their transcription in human cells. Since then a plethora of similar reports corroborated these early findings, pointing to RNAa as a pervasive phenomenon (Faghihi et al., 2010), that is potentially implicated in endogenous regulation of transcription and of great interest for therapy. Currently we still have limited understanding of its molecular mechanisms. Despite their likely mechanistic heterogeneity, RNAa processes share a well-defined set of phenomenological traits.

First, different types of small activating RNAs (saRNAs) may trigger RNAa. They include siRNA-precursor-like 21nt-dsRNAs (Li et al., 2006), pre-miRNA-like shRNAs (Turunen et al., 2009) and pri-miRNA-like molecules (Diodato et al., 2013). Albeit not necessary, specific covalent modifications may enhance their activity (Kang et al., 2012; Matsui et al., 2010; Place et al., 2010, 2012). Effective saRNAs are usually directed against Transcription Start Site (TSS) (Li et al., 2006; Schwartz et al., 2008) or polyA-site sequences surroundings (Yue et al., 2010), conserved *cis*-active modules regulating gene-of-interest (*GOI*) transcription (Diodato et al., 2013) and the transcribed region of the gene (Liu et al., 2013a). They may be both sense- and antisense-oriented (Diodato et al., 2013). Importantly, fine location of the saRNA-target often is a key issue since shifting it by only a few bases can dramatically affect its outcome. Mismatches between the saRNA and its target may be tolerated, however only if far from saRNA 5′ end (Li et al., 2006).

Gene responsivity to small RNAs depends in complex ways on the differentiative state of cells and on the functional state of the target gene. In some cases, low basal expression of this gene may predispose to transactivation (Yue et al., 2010). In other cases, RNAa is restricted to cells and tissues expressing the target gene (Diodato et al., 2013; Li et al., 2006). In these latter cases, *generalized* delivery of aRNAs might be employed to stimulate selective expression of defective genes within their expression domain with no risks of ectopic activation.

When compared to RNAi, RNAa often displays a later onset, usually with a delay of 24–48 h (Place et al., 2010), and it lasts quite longer since one unique saRNA delivery may exert an effect prolonged over >7 days (Li et al., 2006; Place et al., 2010).

Finally, mRNA-upregulation achieved *via* RNAa is usually within physiological range (often <5-folds) This is important since subtle changes of target mRNA levels may deeply impact the functional state of the cell, giving rise to predictable and overt biological effects (Diodato et al., 2013; Janowski et al., 2007; Voutila et al., 2012).

Until now, many genes of oncological and cardio-vascular interest have been upregulated by RNAa for purposes of experimental therapy. In this respect, RNAa-mediated gene stimulation is of obvious therapeutic interest for dozens of different hemizygous gene deletions at the basis of a wide spectrum of neuropathological conditions, including epilepsy, mental retardation, autism, schizophrenia and neurodegeneration (Lal et al., 2015; Lee et al., 2015). Furthermore, upregulation of specific endogenous genes may help counteracting consequences of trauma and neurodegeneration (Modarresi et al., 2012).

6.4.2. Molecular mechanisms

We are still far from full understanding of RNAa dynamics. Experimental data suggest that RNAa is a heterogeneous process and at least two classes of molecular mechanisms may underlie it. RNAa may take place *via* post-transcriptional/co-transcriptional downregulation of AS-transcripts in charge of limiting S-transcript expression. Alternatively, saRNAs may convey supramolecular complexes stimulating transcription to target chromatin.

6.4.3. Downregulating AS transcripts in charge of inhibiting sense transcript expression

As described previously, the large majority of sense mRNAs presents antisense transcription (Katayama et al., 2005). In some cases, antisense RNAs can inhibit transcription of its sense gene and saRNAs can then function by downregulating the anticorrelated, antisense companion of the transcript they promote. This phenomenon has been documented in a number of examples *in vitro*, (Katayama et al., 2005; Faghihi et al., 2010; Morris et al., 2008; Modarresi et al., 2012). In these cases, saRNAs might act by preventing the repressive regulatory effects exerted by the AS transcript on sense transcription (Fig. 1A). Consistently with this inference, siRNA-mediated, Ago2-dependent knock-down of the convergent *p21* AS partner, *Bx332409*, was followed by the detachment of the repressive epigenetic mark H3K27me3 from the surroundings of the *p21*-TSS and by subsequent upregulation of *p21*-mRNA (Morris et al., 2008).

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6.4.4. Conveying transcription-promoting complexes to chromatin

saRNAs may also act by helping to convey macromolecular complexes catalyzing transcription to the gene of interest. Interstingly, saRNA may directly interact with its target DNA (Fig. 1B) (Hu et al., 2012) or *via* nascent ncRNA molecules, still attached to chromosomal DNA (Fig. 1C) (Schwartz et al., 2008; Matsui et al., 2013).

Among potential supporters of RNAa, Ago1 and Ago2 interact with miRNAs and siRNA and are both detectable in the nucleus (Huang et al., 2012). While the former has a scattered distribution in the nucleoplasm, the latter is confined to the peripheral region adjacent the nuclear envelope (Huang et al., 2013). Ago1 binds pervasively to chromatin, preferentially to TSS surroundings, interacts with RNApolII and is involved in regulation of transcription. As such, it was supposed to be specifically implicated in RNAa. Unexpectedly, however, Ago1 is required for transcriptional gene silencing (TGS) but not for RNAa (Chu et al., 2010; Li et al., 2006). The same applies to its paralogs Ago3 and Ago4 (Chu et al., 2010). Conversely, in a number of cases including those of *p21* and *E-Cadherin* (Li et al., 2006), *PR* (Chu et al., 2010), *LDLR* (Matsui et al., 2010) and *COX2* (Matsui et al., 2013), Ago2 resulted to be linked to target gene chromatin (or ncRNAs stemming from it) in a saRNA-dependent manner and required for RNAa activity. In the case of *COX2*-RNAa, Ago2 and the saRNA were associated to GW182, which was needed for RNAa as well (Matsui et al., 2013). The reason of selective Ago2 requirement for RNAa remains obscure.

Beyond Ago2 and GW182, concomitantly with RNAa, other key players are recruited to target gene chromatin or to RNAs stemming from it. An enrichment of RNA polymerase II (RNApolII) at the 5′ of genes upregulated *via* RNAa was detected by ChIP or RIP (Huang et al., 2012; Place et al., 2008; Matsui et al., 2010; Chu et al., 2010; Yue et al., 2010; Majid et al., 2010). This suggests that saRNAs often act by easing the recruitment of RNApolII to the target gene promoter. Alternatively, miR-483 upregulated *IGF2*-mRNA, by conveying the RNA helicase DHX9 to the CG-rich 5′UTR of this mRNA (Liu et al., 2013a). This suggests that saRNAs might also act co-transcriptionally, possibly by destabilizing secondary structures peculiar to nascent pre-mRNAs and thus promoting their elongation.

Remarkably, saRNAs delivery also leads to extensive and complex changes of the covalent epigenetic profile of chromatin. Depending on the gene of interest, epigenetic changes triggered by saRNAs included an increase of H3K4me2 and/or H3K4me3, H3ac and H4ac, as well as a decrease of H3K9me2 and/or H3K9me3, H3K27me3, H3K9ac and H3K14ac (Majid et al., 2010; Turunen et al., 2009; Matsui et al., 2013; Huang et al., 2012; Janowski et al., 2007; Modarresi et al., 2012; Li et al., 2006). Conversely, no change in CpG methylation was reported at all.

In addition to covalent epigenetic changes, the functional link between RNAa and chromosomal DNA looping is particularly intriguing. As found by Chromosomal Conformation Capture (3C), the 5′ and the 3′ ends of the *PR* gene physically interact in MCF7 cells. While not affecting the degree of this intragenic looping, saRNAs captured ncRNAs stemming from the other end in RIP assays when directed against the two different gene ends (Yue et al., 2010). More remarkably, in A549 cells, targeting of the *COX2* promoter by saRNA12 induced looping of the DNA interposed between *COX2* and *Phospholipase A2* (*PLA2G4A*), whose protein product is required for the synthesis of arachidonic acid, the COX2 substrate. With this mechanism, *COX2* and *PLA2G4A* promoters, which lie on the same chromosome almost 150 kb far from each other in divergent orientation, got into close tridimensional vicinity, leading to coordinated transactivation of both genes (Matsui et al., 2013).

In conclusion, a wealth of information about “basic” molecular events triggered by saRNAs is available nowadays. However, we do not know the general causal order, if any, linking one another. In particular, we presently ignore if the epigenetic events exemplified above are propedeutic to the transcription arousal occurring in RNAa, or are a consequence of it. Further in depth studies will be required to solve this issue.

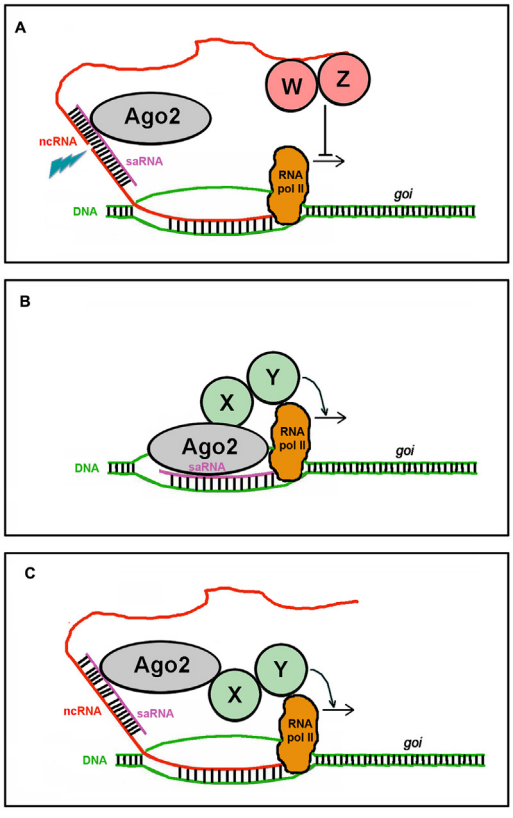

Fig. 1. Putative mechanisms of saRNAs. (**A**) saRNAs may drive Ago2-dependent degradation of nascent, noncoding RNAs, in charge of recruiting W and Z polypeptidic *trans*-repressors to the gene of interest (goi). (**B,C**) Alternatively, saRNAs may drive Ago2-dependent recruitment of X and Y polypeptidic transactivators to the goi. The saRNA may directly interact with locally denatured goi-DNA (**B**) or with a nascent ncRNA, stemming from goi chromatin (**C**).

6.5. RNA-programmable NMHV transcription factors

To expand the repertoire of molecular tools available for therapeutic stimulation of gene transcription, one of our groups recently developed a novel class of artificial transactivators, termed NMHVs (an acronym standing for Nuclear localization signal – MS2 coat protein RNA interacting domain – HA epitope – (3x)VP16 transactivating domain) (Fig. 2) (Fimiani et al., 2015). These are RNA-programmable enzymes like CRISPR-TFs, however they differ from CRISPR-TFs in several aspects. First, they require longer RNA baits for effective and specific target recognition (60 bases vs 20 bases). Moreover, compared to CRISPR, NMHVs (1) are >7-folds smaller, (2) elicit a normalized transcriptional gain around twofold, and (3) selectively stimulate transcription of active genes, while not affecting silent ones. These traits are very different from the corresponding features of more popular, RNA-programmable CRISPR-TFs, which are very large, can switch on *silent* genes, possibly because of their intrinsic helicase activity (Cheng et al., 2013; Kearns et al., 2014), and easily achieve very high expression gains, even >500-folds (Perez-Pinera et al., 2013). For these reasons, CRISPR-transactivators would be hardly employable for scalable and finely tuned correction of haploinsufficiencies. Conversely, *broad* in vivo delivery of NMHV-TFs seem to be better

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suitable for *clean and scalable* transcriptional rescue of these insufficiencies. This prediction, as well as a more thorough assessment of NMHV off-targeting effects, currently waits for experimental validation.

6.6. SINEUPs

The use of long non-coding RNAs as biotechnology and therapeutic tools is still at infancy. Some of us have recently shown that AS Uchl1, a lncRNA antisense to the mouse ortologue of the human Uchl1/Park5 gene, increases UchL1 protein synthesis at post-transcriptional level (Carrieri et al., 2012). AS Uchl1 is a 5′ head-to-head divergent antisense lncRNA, that partially overlaps with Uchl1 mRNA covering initiating AUG. When overexpressed in murine dopaminergic cell line, AS Uchl1 is able to increase UchL1 protein product without affecting its mRNA levels. Under physiological conditions, AS Uchl1 is retained in the nucleus. Upon rapamycin treatment, AS Uchl1 shuttles from the nucleus to the cytoplasm with an unknown mechanism. Once in the cytoplasm, AS Uchl1 induces Uchl1 mRNA association to heavy polysomes, thus increasing its translation. We could demonstrate that AS Uchl1 activity depends on the combination of two RNA elements: at the 5′ end, the overlapping region is indicated as the Binding Domain (BD); while the 3′ non-overlapping region contains an embedded inverted SINEB2 element, which represents the Effector Domain (ED) (Fig. 3). The ED is required for protein up-regulation function of AS Uchl1 and the BD dictates its specificity towards Uchl1 mRNA. By swapping BD sequences, a synthetic lncRNA can be designed in which AS Uchl1 activity is re-directed to target exogenous transcripts, as those encoding for Green Fluorescent Protein (GFP) (Carrieri et al., 2012; Zucchelli et al., 2015b). Therefore, AS Uchl1 can be considered the representative member of a new functional class of AS lncRNAs that utilize embedded inverted SINEB2 elements to increase translation of partially overlapping protein-coding genes acting on their endogenously expressed mRNAs (Carrieri et al., 2012; Zucchelli et al., 2015b). These natural and synthetic molecules were named SINEUPs since their function requires the activity of an embedded inverted SINEB2 sequence to UP-regulate translation (Zucchelli et al., 2015a, 2015b). SINEUPs are thus the first example of gene-specific inducers of protein synthesis. SINEUP modular structure can be employed to artificially engineer their BD and design synthetic SINEUPs to specifically enhance translation of virtually any target gene of interest. Synthetic SINEUPs so far have been proven effective with a number of targets, including GFP (Carrieri et al., 2012), FLAG-tagged proteins (Zucchelli et al., 2015b), secreted recombinant antibodies and cytokines (Patrucco et al., 2015), thus showing SINEUP technology is scalable, once provided the required target recognition with BDs. Most importantly,

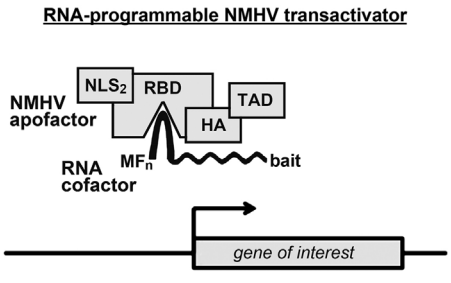

Fig. 2. Schematics of RNA-programmable NMHV transactivators. NMHV transactivator harbors a polypeptidic NMHV apofactor and an RNA cofactor. The former includes an NLS_2 (nuclear localization signal, 2x), an MS2-like RNA binding domain (RBD), an HA hemaggulitinin epitope and a $VP16_3$ TransActivating Domain (TAD). The latter results from a multimerized MS2-interacting finger (MF_n) and a 60–180 base long bait, for the recognition of transcription start site sequences.

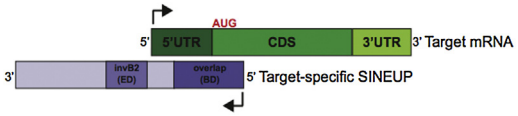

Fig. 3. Schematic representation of target mRNA and target-specific SINEUP. 5′ to 3′ orientation of sense mRNA and antisense SINEUP is indicated. Black arrows represent transcription initiation and orientation. Structural elements of target protein-coding mRNA (green): 5′ untranslated region (5′UTR), coding sequence (CDS) and 3′ untranslated region (3′UTR). Initiating AUG is shown in red. Structural elements of target-specific SINEUP (purple): Binding Domain (BD), SINEUP sequence that overlaps, in antisense orientation, to the sense protein-coding mRNA; Effector Domain (ED), inverted SINEB2 element (invB2) that confers activation of protein synthesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synthetic SINEUPs can act on endogenous mRNAs *in vitro* and *in vivo*, as demonstrated by specific SINEUPs designed to target genes associated to neurodegeneration (PARK7/DJ-1 for PD) (Zucchelli et al., 2015b) and brain developmental disorders (Indrieri et al., 2016).

Over competing technologies aimed to increase quantities of a protein of interest, SINEUPs present three major advantages: 1) Induce a 2-to-5-fold up-regulation thus limiting side effects due to exaggerated overexpression in gene therapy approach; 2) Act on endogenous mRNAs *in situ*, restricting translation enhancement to the time and space of endogenous gene expression, 3) Do not trigger hereditable genome editing. Similarly to other nucleic acid-based therapies for brain disorders, the final outcome of SINEUPs-based therapy will depend on reaching their target mRNAs in the brain. The current renaissance in nucleic acid-based drugs will provide the correct molecular tools for their delivery.

7. The Yang side of nucleic acid-based therapy: applications to neurodegenerative diseases

7.1. Angelman syndrome

UBE3A is an imprinted gene encoding an E3 ubiquitin ligase. Maternal deficiency of UBE3A is the primary cause of Angelman syndrome, a disorder characterized by severe intellectual disability and developmental delay, with behavioural deficits and severe speech impairment, associated to seizures and ataxia. Patients with Angelman syndrome carry one intact copy of paternal UBE3A gene, which is inactivated by an antisense nuclear-retained lncRNA (UBE3A-AS). ASO treatment was developed to target UBE3A-AS (as carried out in AntagoNAT-based therapy) to correct for silenced UBE3A expression in Angelman Syndrom mouse model (Meng et al., 2015). Intra-cerebroventricular injection of single-dose ASO was well tolerated. After ASO treatment (4 weeks), UBE3A-AS expression was reduced by 60–70% and ABE3A mRNA increased 2–5 fold in brain and spinal cord. Correction of phenotypes was evident but not complete.

7.2. Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disorder, caused by mutations in the SMN1 gene that result in a deficiency of SMN protein that ultimately cause neuromuscular defects. The severity of SMA is dictated in part by the copy number of the related duplicated gene SMN2, which has a critical mutation in the exonic splicing site of exon 7. Therapeutic strategies are aimed at replacing insufficient gene dosage by AAV delivery of SMN1 cDNA. An alternative strategy is to use ASOs to re-direct splicing of paralogue SMN2 RNA to boost expression of functional SMN protein. Intra-cerebral ventricle injection into a mouse model of severe SMA resulted in increased SMN protein levels, increased

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numbers of motor neurons in the spinal cord, which in turn led to improvements in muscle physiology, motor function and survival (Passini et al., 2011). In the same study, the SMN-specific ASO was also injected intrathecally into non-human primates (cynomolgous monkeys) delivering therapeutic levels of the nucleotide drug to all regions of the spinal cord.

Phase1/2 clinical trials have recently been completed to assess safety, tolerability and the range of appropriate single intrathecal dose of ASOs targeting SMN2 in patients with SMA (Chiriboga et al., 2016; Hache et al., 2016). Phase 3 trials are currently ongoing for infants. Additional trials were also initiated this year and are currently enrolling a small subset of patients with infantile or childhood-onset SMA (Faravelli et al., 2015) (and www.clinicaltrial.gov, 2016).

7.3. Neurothrophic factor therapy

Neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), belong to a class of secreted neurotrophic factors that are essentials for neuronal growth, differentiation and maintainance. BDNF is also crucial for neuronal plasticity and has been involved in learning and memory. Reduced levels of BDNF are found in neurodegenerative diseases and are associated to psychiatric and neurodevelopmental disorders. Therefore, upregulation of BDNF is believed to have beneficial effects on several brain dysfunctions. Nucleic acid-based therapy has been employed to increase BDNF quantities in the brain. Intranasal administration of BDNF-encoding mRNA proved to be effective in repairing olfactory epithelium architecture and rescuing olfaction defects in a mouse model of olfactory dysfunction (Baba et al., 2015).

BDNF-AS is a negative regulator of BDNF levels, controlling BDNF expression though recruitment of proteins belonging to polycomb repressor complex. Inhibition of BDNF-AS using antagoNATs increased BDNF mRNA and protein levels, ultimately leading to neuronal outgrowth and differentiation *in vitro* and *in vivo* (Modarresi et al., 2012).

Similarly, three distinct siRNAs, targeting convergent antisense transcripts associated to *Bdnf,* elicited an early downregulation of these transcripts, followed by a delayed and transient upregulation of *Bdnf*-mRNA. Such *Bdnf* upregulation was not due to post-transcriptional stabilization of the corresponding mRNA, but reflects a specific reduction of the repressive epigenetic mark H3K27me3 linked to the *Bdnf*-TSS. Consistently, two LNA-DNA gapmers (aka antago-NATs, *i.e.* antagonists of natural antisense transcripts), driving RNAse H-mediated degradation of *Bdnf-AS*, fully recapitulated the effects of siRNA-like *Bdnf* activators (Modarresi et al., 2012). Remarkably, by a similar approach, the same authors also up-regulated *Gdnf* (Modarresi et al., 2012), a neurotrophic factor regulating neurite branching and synaptic plasticity (Airaksinen and Saarma, 2002), specifically down-regulated in substantia nigra and putamen of PD patients (Bäckman et al., 2006; Chauhan et al., 2001; Hunot et al., 1996), as well as in cerebro-spinal fluid and middle temporal gyrus of AD patients (Airavaara et al., 2011; Straten et al., 2009).

Endogenous quantity of neurotrophic factors can be regulated at the post-transcriptional level by networks of endogenous miRNAs, whose expression is altered in brain of patients and in animal models (Di Meco and Pratico, 2016; Zovoilis et al., 2011). Therapeutic strategies aim at increasing neuroprotective factors by inhibiting their physiological negative regulators. From miRNA prediction programs, BDNF mRNA contains several hundreds of miRNA target sites. Of these, a small number has been experimentally validated and only miR-206 has been tested for therapeutic intervention *in vivo* (Varendi et al., 2015). While upregulated in AD brains, miR-206 has been shown to inhibit BDNF by direct targeting its 3′UTR, albeit contrasting results exists on the exact region of miRNA-mRNA interaction (Lee et al., 2012; Miura et al., 2012; Tapocik et al., 2014). Viral-mediated overexpression of miR-206 into the brain inhibited expression of BDNF *in vivo* in the rat brain (Tapocik et al., 2014). Anti-miR-206 could restore levels of BDNF, hippocampal synaptic density and neurogenesis while improving memory in an AD mouse model (Lee et al., 2012).

7.4. Friedreich ataxia

Friedreich's ataxia (FRDA) is a fatal untreatable neurodegenerative disease, mostly caused by the homozygous expansion of GAA repeats, which leads to an insufficient amount of frataxin protein. Increasing quantities of frataxin protein in the brain and in the heart is in principle curative. Delivery of corrected human frataxin gene by adeno-associated viral vectors was sufficient to reverse cardiomyopathy (Perdomini et al., 2014) and improve symtoms (Gerard et al., 2014) in mouse models of the disease. In addition to these viral vector-mediated gene replacement strategies, recent studies indicate the feasibility of mRNA-based and ASO-based approaches for FRDA. Intratechal delivery of unmodified mRNA encapsulated in lipid nanoparticles led to robust expression of exogenous frataxin protein in the CNS and in dorsa root granglia, a major site of pathology (Nabhan et al., 2016). Alternatively, the use of single-stranded ASO targeting GAA repeat region rescued frataxin protein levels in patient-derived fibroblasts (Li et al., 2016b).

Finally, in this respect, it may be of therapeutic interest that one of our groups recently selected two artificial miRNAs targeting the *FXN* promoter, eliciting a normalized *FXN*-mRNA gain up to 2.7 x in immortalized lymphoblasts originating from homozygous FRDA patients (Fimiani and Mallamaci, unpublished results). By this gain, these cells expressed *FXN*-mRNA levels associated to healthy heterozygous carriers (Pianese et al., 2004).

8. Conclusions

The recent years have been characterized by a new renaissance in the field of nucleic acid-based therapeutics and RNA therapeutics in particular. Nowadays, it is possible to envision innovative ways to treat neurodegenerative diseases. By exploiting nucleic acid-based drugs we may hijack or interphere with regulatory pathways modulating RNA and protein levels in time and space *in vivo*. While inhibitory RNAs (Yin) are reaching clinical testing at an unprecedented pace, recent advances in the field of activatory RNAs (Yang) are ready to pass the first phase of discovery and validation providing unprecedented tools in increasing therapeutic protein levels *in vivo*. Whatever the RNA molecule to be delivered (inhibitory siRNA or miRNA, activatory small or long non-coding RNAs or sequence-corrected mRNAs) advances in the chemistry of nucleic acids themselves or in their carrier nanoparticles will allow a more efficient and more precise targeting of brain tissues. Similarly, the advent of new-generation adeno-viral vectors with strongly selected brain tropism will eventually lead to new efficient strategies to solve one of the major issues for gene therapy of neurodegenerative diseases.

The list of potential RNA-based intervention approaches to treat neurodegenerative disorders cannot exclude guide RNA-assisted CRISPR/Cas9 technology. Initially, CRISPR/Cas9 has been employed to generate cellular and animal models of various diseases, including those affecting the brain (Yang et al., 2016a). More recently, RNA-guided endonuclease-based approaches have been used for therapy. During this year, three groups have independently reported that CRISPR/Cas9 could be used to remove the affected allele in mouse dystrophin gene and revert disease phenotype in mouse models of Duchenne Muscular Dystrophy (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016).

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Other two studies demonstrated the efficacy of CRISPR/Cas9 in mouse models of human hereditary liver diseases (Yang et al., 2016b; Yin et al., 2016). For neurodegenerative disorders, provided the appropriate delivery, CRISPR/Cas9 could represent a prowerful tool to eliminate the expression of inherited autosomal dominant mutated genes, as in Huntington's disease or in some forms of Parkinson's disease. However, as for any genome-editing tool, permament genome modification represents a major drawback with ethical concerns that will need to be specifically addressed in the near future.

Finally, nucleic acid-based drugs, for their intrinsic scalability, have the potentials to treat many neglected rare brain developmental disorders that collectively affect an increasingly large number of individuals.

9. Competing financial interests

SG and SZ declare competing financial interests as co-founders and members of TransSINE Technologies (www.transsine.com). SG and SZ are named inventors in a patent issued in the US Patent and Trademark Office on SINEUPs and licensed to TransSINE Technologies.

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