REVIEW

Deoxyribozymes and bioinformatics: complementary tools to investigate axon regeneration

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Abstract For over 100 years, scientists have tried to understand the mechanisms that lead to the axonal growth seen during development or the lack thereof during regeneration failure after spinal cord injury (SCI). Deoxyribozyme technology as a potential therapeutic to treat SCIs or other insults to the brain, combined with a bioinformatics approach to comprehend the complex protein-protein interactions that occur after such trauma, is the focus of this review. The reader will be provided with information on the selection process of deoxyribozymes and their catalytic sequences, on the mechanism of target digestion, on modifications, on cellular uptake and on therapeutic applications and deoxyribozymes are compared with ribozymes, siRNAs and antisense technology. This gives the reader the necessary knowledge to decide which technology is adequate for the problem at hand and to design a relevant agent. Bioinformatics helps to identify not only key players in the complex processes that occur after SCI but also novel or less-well investigated molecules against which new knockdown agents can be generated. These two tools used synergistically should facilitate the pursuit of a treatment for insults to the central nervous system.

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Introduction

The first true-to-detail drawings of struggling axons at injury sites in the central or peripheral nerves system (CNS or PNS) were prepared from 1887 to 1903 by Santiago Ramon y Cajal (1852–1934). Since then, a plethora of publications have been generated in an attempt to comprehend the events that occur after CNS or PNS injury. Over time, a large number of genes, proteins and substances have been discovered and collected, all of which are involved in and are able to influence these processes. Although considerable improvements can be observed with regard to most of the promising candidates, satisfying results in treating insults to the CNS have not been achieved. Early responses to prevent complications and accelerate recovery are necessary during the medication of CNS trauma. Hence, the availability of safe and easy to administer therapeutics is long-awaited and the pursuit of a treatment or even a cure for paralysis continues.

Knockdown agents such as deoxyribozymes might be relevant in this regard. They have the ability to act as enzymes after binding to their target mRNA via sequence-specific binding-arms and cleaving the target via a catalytic loop structure. After releasing the digested fragments, the deoxyribozyme is able to bind and cleave further mRNA molecules of the same gene. Deoxyribozymes have been overlooked during the last few years but harbor great therapeutic potential. This review summarizes the selection process of deoxyribozyme development, the digestion mechanism, requirements for modifications of the length of the sequence-specific binding arms, existing knowledge

about the uptake mechanism of nucleic-acid-based molecules into cells and therapeutic applications. Furthermore, to comprehend the multifactotrial and complex mechanisms that occur after CNS trauma, the review also summarizes results obtained via bioinformatics approaches.

Deoxyribozymes

Selection of deoxyribozyme sequences

Deoxyribozymes are single-stranded DNA molecules, which are also called DNA enzymes or DNAzyme. They were developed by Breaker and Joyce (1994) and Santoro and Joyce (1997) by using systematic evolution of ligands by exponential enrichment (SELEX; Fig. 1). The idea of developing catalytic DNA molecules arose because RNA in

the form of ribozymes is able to function as an enzyme. However, the simple translation of a ribozyme sequence into DNA (replacing U with T) did not show satisfying results. No detectable cleavage of the target mRNA occurred. As a consequence, a random search for DNA sequences was undertaken by using SELEX. The first attempt resulted in Pb^{2+}-dependent deoxyribozymes, one being called E2 (Fig. 2a, b). They cleaved at a phosphodiester immediately 3′ to a single ribonucleotide embedded within a DNA substrate (Fig. 1a, b) but were either unable to cleave when extended RNA of the same sequence replaced the original DNA substrate (Breaker and Joyce 1994, 1995) or did cleave but with significantly lowered rates (Roth and Breaker 1998).

The next attempt was carried out by another group, Faulhammer and Famulok (1996), who were able to identify a Ca^{2+}-dependent deoxyribozyme, Mg5, in spite of Ca^{2+} never being part of the selection process. However, this

Fig. 1 Systematic evolution of ligands by exponential enrichment (SELEX) strategies to identify RNA-cleaving deoxyribozymes (red RNA, green potential deoxyribozyme). a General four-step selection strategy to enrich functional deoxyribozymes. b First approach used to identify the E2 deoxyribozyme. c Type of approach that Santoro and Joyce (1997) employed to isolate the 10–23 and 8–17 deoxyribozyme (modified after Silverman 2005)
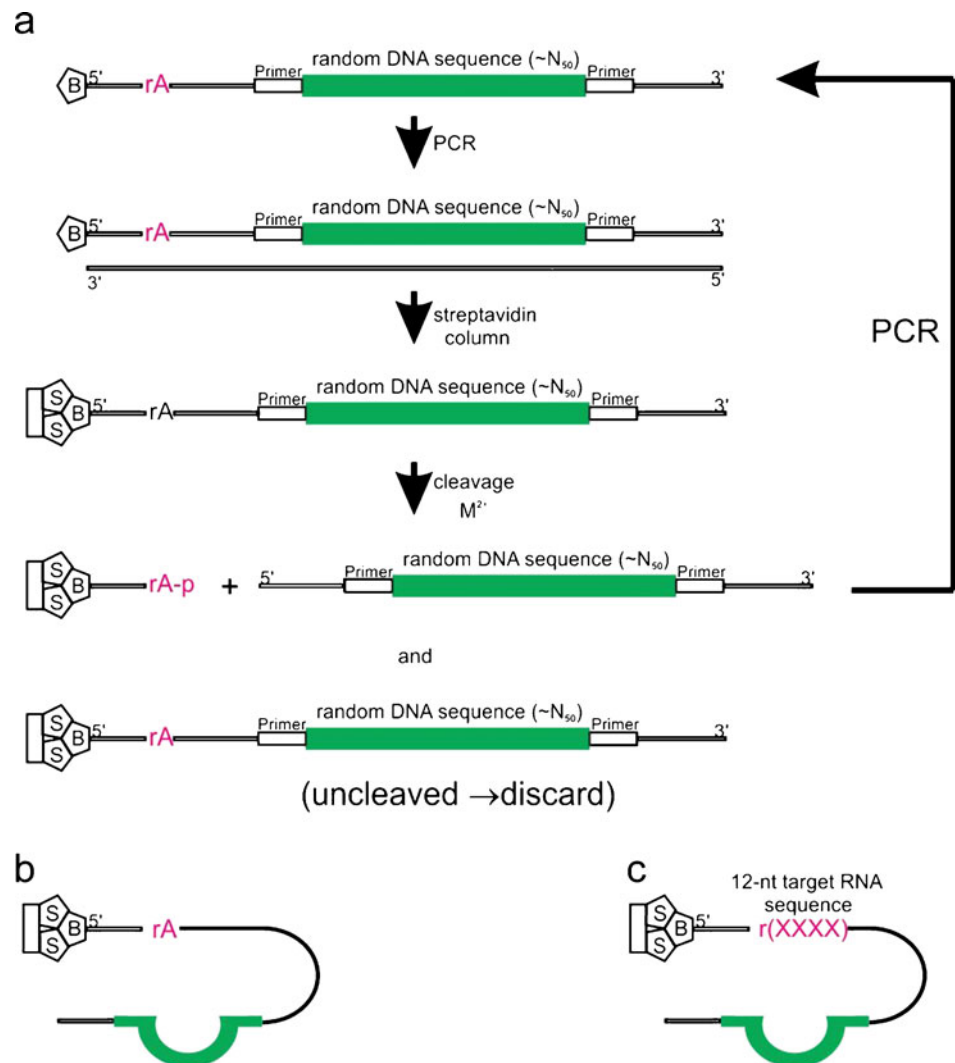
Fig. 2 Examples of various RNA-cleaving deoxyribozymes, which perform in trans (red RNA, green DNA, arrows cleavage sites). a, b Two of the first generation of Pb^{2+}-dependent deoxyribozymes (Breaker and Joyce 1994, 1995). c, d The most frequently used deoxyribozymes 10–23 and 8–17 (Santoro and Joyce 1997). e, f Later developed deoxyribozymes, of which f does not require divalent cations (Feldman and Sen 2001; Roth and Breaker 1998). Note that the respective names and the relevant references are given below each deoxyribozyme (modified after Baum and Silverman 2008)
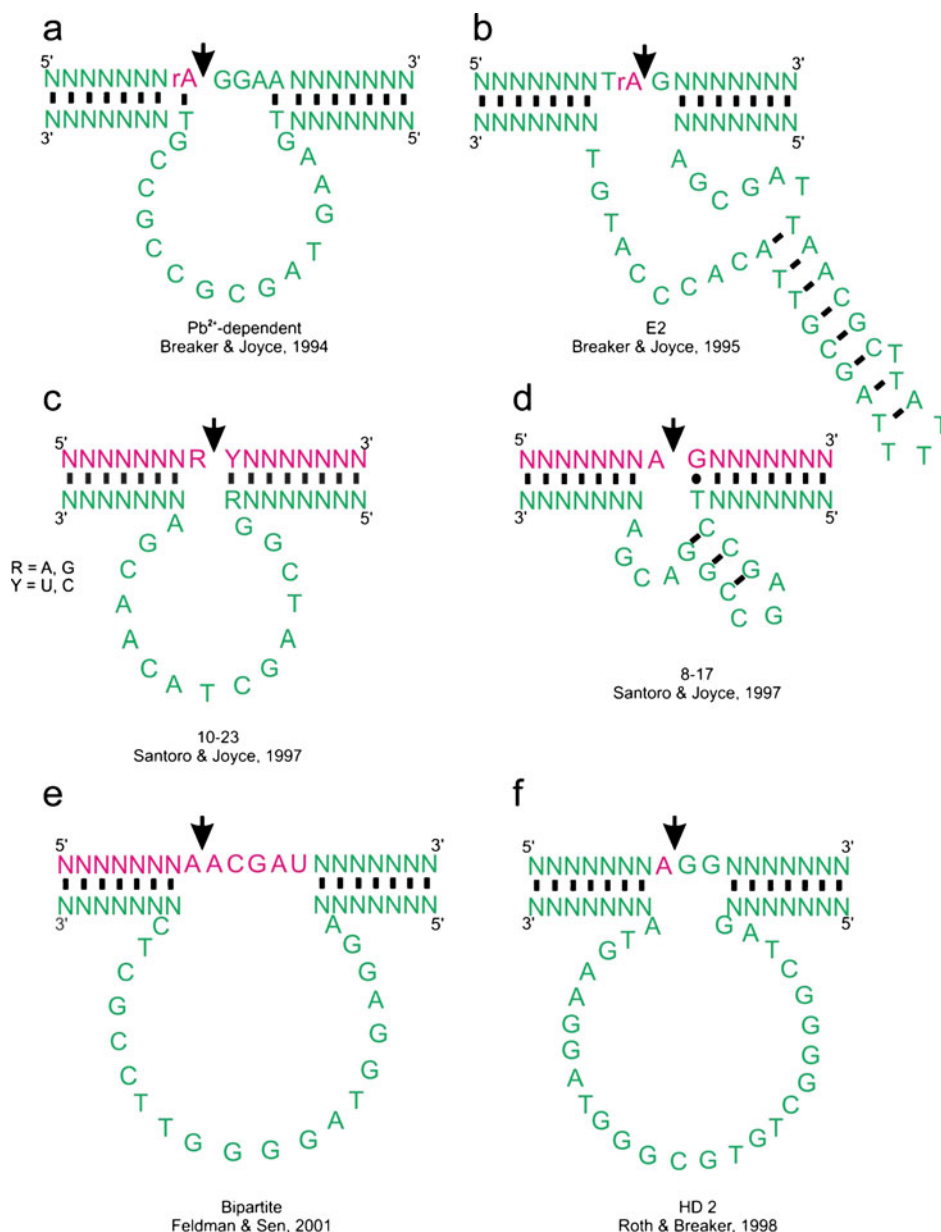
deoxyribozyme too was unable to cleave within an extended RNA sequence.

To tackle this problem, Santoro and Joyce (1997) generated a library of approximately 10^{14} single-stranded DNA molecules, in which each contained a 5′ biotin moiety followed (in a 5′→3′ direction) by a short oligodeoxynucleotide spacer, 12 target ribonucleotides (RNA sequence to be cleaved) and 50 random deoxynucleotides (potential DNA enzymes). The random region was surrounded by fixed-sequence deoxynucleotides as sites for primer hybridization (Fig. 1a, c). These molecules were applied to a streptavidin-coated solid support and eluted with a solution containing 10 mM MgCl$_2$ at pH 7.5 and 37°C. Molecules that cleaved an RNA phosphoester within a small fraction of the bound molecule released the 3′ cleavage product into the eluate.

The released material was recovered and amplified by the polymerase chain reaction (PCR). The PCR pool was then subject to generate a progeny population of DNA molecules, which were enriched for the desired population. Two sides in the target RNA sequence emerged to be hotspots for cleavage, one during amplification round 6–8 and the other during round 9–10. After the eighth and tenth rounds, the enriched sequences were cloned and analyzed in detail. From a total of 62 clones, two motifs (8–17 and 10–23, Fig. 2c, d) showed extensive Watson-Crick pairing to the target RNA sequence located upstream and downstream of the cleavage site. The names of two deoxyribozymes were coined based on the 17th or 23rd clone of the 8th or 10th cycle of the in vitro selection process. The catalytic core of the 8–17 and 10–23 enzymes was located

between the two substrate-binding arms that contained 13 and 15 deoxynucleotides, respectively (Santoro and Joyce 1997). Both deoxyribozymes required divalent metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, or Cu^{2+} to cleave the target mRNA. The 8–17 DNA enzyme contained a short internal stem-loop (3 base pairs, at least two being G-C) followed by an unpaired region of 4–5 nucleotides. In contrast, the 10–23 DNA enzyme contained an unpaired loop structure where the eighth nucleotide position of the catalytic core was either a T, C, or A. The highest level of activity was provided with a T at this position. Apart from this, the entire loop sequence was intolerant to any changes (Sugimoto et al. 1999; Li et al. 2000).

Around the same time, Geyer and Sen (1997) identified an RNA-cleaving deoxyribozyme, Na8 that worked independently of divalent metal ions but it was able to increase RNA cleavage 108-fold. Monovalent ions such as Na^+ were the effective cofactors. From the same laboratory, Feldman and Sen (2001) published a deoxyribozyme that preferred several unpaired ribonucleotides at the cleavage site. This newly formed bipartite I deoxyribozyme (12–17, Fig. 2e) had, however, an absolute requirement for Mg^{2+} or certain other divalent metal salts such as Mn^{2+}, Zn^{2+} and Cu^{2+} for cleavage.

Roth and Breaker (1998) presented several histidine-dependent deoxyribozymes (HD2, Fig. 2f). To ensure the absence of divalent ions during the selection process, EDTA was added. HD2 required, in addition, K^+ and the imidazole group from the histidine. This imidazole group most likely accelerated the cleavage reaction by facilitating deprotonation of the target site, the 2′ hydroxyl group.

The latest identification of a deoxyribozyme with broad pH tolerance by in vitro selection was performed by Xiao et al. (2010). The 9NL27 deoxyribozyme is one of several 10MD5 deoxyribozyme variants that enhance the hydrolysis of an uncatalyzed P-O bond at a rate of 10^{12}. The deoxyribozymes 9NL27 and 10MD5 have comparable activities in the presence of a mixture of 1 mM Zn^{2+} and 20 mM Mn^{2+} at pH 7.5, whereas 10MD5 is essentially inactive when only 1 mM Zn^{2+} is used. In contrast, 9NL27 shows only a three-fold reduction under these conditions.

Digestion mechanism

One factor that influences the dissociation rate of deoxyribozymes to its substrate is the length of the binding arms, which form Watson-Crick base pairs with the target mRNA upstream and downstream of the cleavage site. Early studies by Sun and colleagues (1999) showed that a deoxyribozyme with a sequence length of 10 nucleotides of each arm (arm I, left site and arm II, right side of the deoxyribozyme, Fig. 3) performed best using a target sequence to human papilloma virus (HPV 16 E7), whereas deoxyribozymes with an arm I
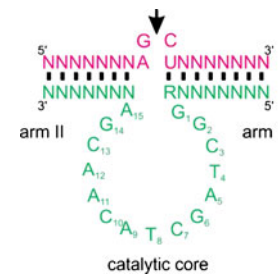

Fig. 3 Detailed labeling of the 10–23 deoxyribozyme after binding to a RNA substrate (*red* RNA, *green* DNA, *arrow* cleavage site). The 15-base sequence of the conserved catalytic core is numbered in the 5′→3′ orientation from 1 to 15. The binding domains on the 5′ and 3′ sides of the catalytic domain are designated *arm I* and *arm II*, respectively (modified after Cairns et al. 2000)

and arm II length combination of 8/10 or 6/10 performed better with a target sequence to c-myc. When the arm length of a deoxyribozyme to HPV 16 E7 was reduced to 6/10, a strong reduction was observed, whereas changes to 10/4 or 4/10 led either to an almost undetectable cleavage activity to HPV 16 E7 or strong reduction to c-myc (Cairns et al. 2000). Notably, when both binding arms are too long, dissociation of the target mRNA does not occur. Thus, only an antisense effect can be observed. When designing a deoxyribozyme, the length of the hybridization arms should be between 7 and 10 nucleotides on each side (Santoro and Joyce 1997) to secure cleavage and release as shown by Choi et al. (2010) for an arm I/armII length ratio of 10/10 and by Yang et al. (2009) for a ratio of 7/9.

Digestion by the 10–23 deoxyribozyme requires a dinucleotide combination of G-C, G-U, A-C, or A-U in the target mRNA sequence, whereas for digestion by the 8–17 deoxyribozyme, solely A-G is sufficient (Fig. 2c, d). Hence, the 10–23 deoxyribozyme has a broader target range with a substrate requirement of an unpaired purine (A or G) and paired pyrimidine (C or U). This is ideal, because the more sites tested in long-folded mRNA, the greater the likelihood of finding highly accessible and cleavable sites (Cairns et al. 1999). The best efficiency of substrate cleavage was observed when the target site consisted in an A and U for the 10–23 deoxyribozyme (Santoro and Joyce 1997) and, in descending order, followed rAU=rGU≥rGrC>>rArC. To increase the activity, in particular, to the last two dinucleotide combinations (GC and AC), a substitution of dG with deoxyinosine (dI) is efficient. Thus, the three hydrogen bonds between rC-dG are converted to two hydrogen bonds between rC-dI, which improves the biological activity of the deoxyribozymes (Cairns et al. 2003).

Finally, cleavage occurs at the 3′ side of a single unpaired ribonucleotide, preferably the purine that is followed by the paired pyrimidine within an all-RNA substrate (Santoro and Joyce 1997). Hereby, the deoxyribozyme bends the substrate away from the cleavage point, exposing the reactive

site and buckling its own catalytic core (Kenward and Dorfman 2009). Then, RNA cleavage is catalyzed through transesterification when the oxygen of a MO⁻ (M =metal ion) or OH⁻ group serves as a nucleophile to attack the phosphorus center of the adjacent phosphoester linkage in an S_N2-like reaction. In other words, the divalent cations serve as a Lewis base or metal hydroxide, generating an end-product of 2′,3′-cyclic phosphate and 5′-hydroxyl RNA termini (Fig. 4). Afterwards, the catalytic core of the deoxyribozyme unwinds and the complex extends.

Modifications of deoxyribozymes

The origin of modifications for knockdown agents, which are prone to nucleolytic degradation in body fluids, started during antisense research. Thus, chemical modifications not only enhance bioactivity but also lower toxicity and increase target affinity (Kurreck 2003). To prevent deoxyribozyme digestion through serum nucleases and, at the same time, to increase the efficiency of the enzyme, the following modifications are the most frequently used: (1) phosphorothioate linkage, (2) 2′-O-methyl nucleotides, (3) 3′-terminal nucleotide inversions such as thymidine at the 3′ end and (4) locked nucleic acids (Fig. 5).
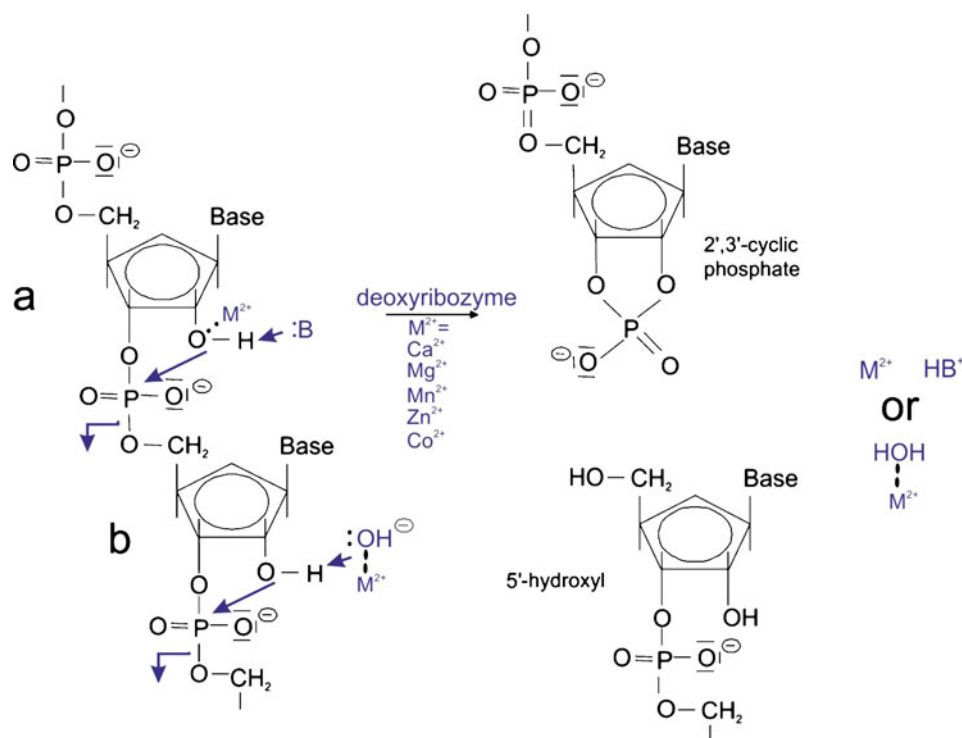
Phosphorothioate oligonucleotides Here, one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulfur. Introduction of a small number of

phosphorothioate oligonucleotides (PTOs), e.g., three at the 3′ end, rendered greater stability to the deoxyribozyme without influencing the efficacy (Sioud and Leirdal 2000). In a study by Sun et al. (1999), the introduction of phosphorothioate (PS) bonds into the hybridization arms and into the pyrimidine residues of the catalytic core selectively inhibited human glioma cell growth by up to 90%. However, Schubert et al. (2003) observed a deceleration of the cleavage rate to one-fifth when PS linkages were introduced throughout the entire binding arms; this might be attributable to a decrease in target affinity. In such cases, potential toxic side effects might also arise (for a review, see Levin 1999).

2′-O-methyl nucleotide The introduction of methylphosphonates into deoxyribozyme backbones increases the relative hydrophobicity and eases diffusion across membranes (Shoji et al. 1991). After the introduction of two 2′-O-methyl at the 5′ and 3′ end or four 2′-O-methyl RNA monomers as end-blocks into a deoxyribozyme, a two-fold or six-fold increase, respectively, of the original cleavage rate was obtained (Warashina et al. 1999; Schubert et al. 2003).

Locked nucleic acids These are ribonucleotides containing a methylene bridge that connects the 2′-oxygen of the ribose with the 4′-carbon (for a review, see Orum and Wengel 2001). Incorporation of locked nucleic acids (LNAs) into the binding arms improves the hybridization potential of

Fig. 4 Representation of two hypothetical mechanisms for the catalysis of RNA cleavage by the 10–23 deoxyribozyme with metal cations (M⁺) such as Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺ and Co²⁺. **a** Mechanism involving a divalent metal hydroxide that functions as a general base (*B*). This base deprotonates the 2′-OH group. **b** Mechanism involving a divalent metal cation that functions as a Lewis acid. A Lewis acid is an electron-pair acceptor that polarizes the 2′-OH bond and accelerates deprotonation. In both cases, the RNA is cleaved to a 2′-3′ cyclic phosphate and 5′-hydroxyl termini with a metal cation and oxidized base or with a metal cation associated with H₂O, respectively (modified after Santoro and Joyce 1998)
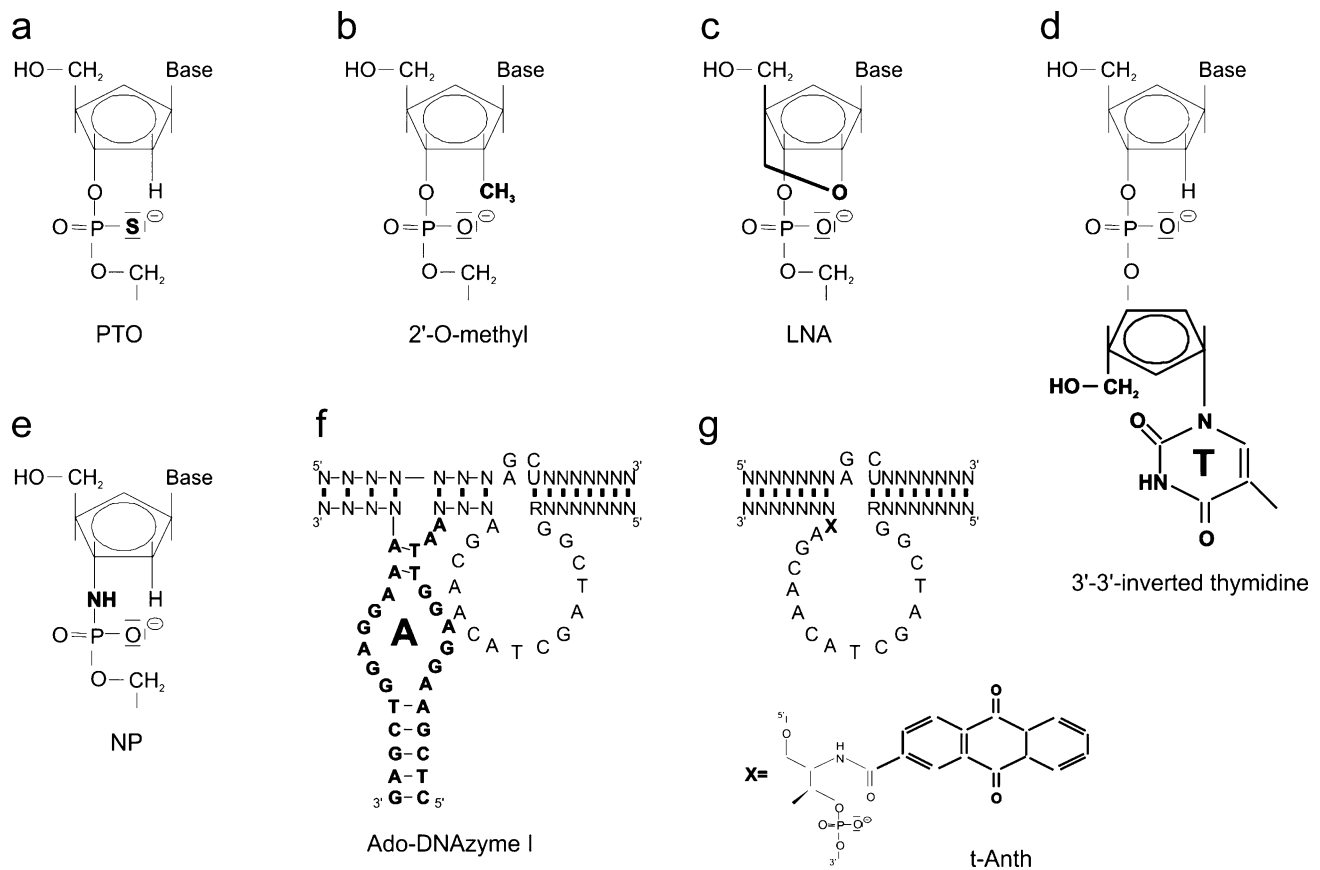
Fig. 5 Modifications to increase stability and/or efficacy of deoxyribozymes (*bold typeface* denotes the modification). **a** Phosphorothioate oligonucleotides (*PTO*) replaces an oxygen atom with a sulfur atom. **b** A 2′-O-methyl group substitutes the hydrogen at the 2′-deoxyribose position. **c** Locked nucleic acids (*LNA*) are RNA-based nucleotides connecting the 2′-oxygen with the 4′-carbon. **d** The 3′-3′-inverted thymidine (*T*) increases the stability of the deoxyribozyme against nucleases. **e** N3′-P5′ phosphoramidate (*NP*) enhances the affinity to the single-stranded RNA substrate. **f** Ado-DNAzyme I contains a regulatory sequence that is able to bind adenosine (*A*). **g** Anthraquinone (*t-Anth*) is tethered between A15 and the binding arm II (summarized data from Takahashi et al. 2000; Wang et al. 2002; Schubert and Kurreck 2004, Asanuma et al. 2006)

several DNA enzymes, enhances the folding of the catalytic loop (Vester et al. 2006) and induces a conformational change toward the A-type helix (Bondensgaard et al. 2000).

Inverted nucleotide Sioud and Leirdal 2000 introduced a 3′-3′-inverted thymidine into a deoxyribozyme and achieved a six-fold increase in stability with sustained cleavage activity, whereas others (Sun et al. 1999) have observed a ten-fold enhancement in serum stability. In combination with the arm length of the deoxyribozymes, Sun et al. (1999) have shown that an inverted thymidine influences the cleavage rate, ranging from a slight loss of efficiency to a four-fold increase of activity. In contrast, Schubert et al. (2003) has not been able to confirm this observation.

Further modifications of deoxyribozymes (Fig. 5), which increase the catalytic activity but are less common, are listed below:

- *N3′-P5′ phosphoramidate* The N3′-P5′ phosphoramidate (NP) modification showed an increase of about two-fold

in the relative efficiency of a deoxyribozyme to the translation initiation region of influenza A virus (Takahashi et al. 2000)

- *Adenosine binding* To regulate the binding of a deoxyribozyme to its target mRNA, (1) a regulator oligonucleotide and adenosine (Ado-DNAzyme I) or (2) adenosine alone (Ado-DNAzyme II) is required (Fig. 5). By adding adenosine to Ado-DNAzyme I, the catalysis of the 10–23 and 8–17 deoxyribozyme can be increased by up to 35-fold (Wang et al. 2002)

- *Intercalator tethering* When the intercalator, anthraquinone (t-anth), is tethered via D-threoninol directly through an amide bond between position A_{15} and the binding arm II, an eight-fold increase in activation is obtained (Asanuma et al. 2006).

Other modifications are focusing on the catalytic core of the 10–23 deoxyribozyme; the core is sensitive to any changes and should be preserved. In particular, positions G_1, G_2, T_4, G_6 and G_{14} (Fig. 3) are highly conserved and

the substitution with any other base leads to the complete loss of catalytic activity. Known exceptions are: a nucleotide replacement at position 8, the least conserved base, from a T→N (N=C, G, A) and another at positions 7–12 by any naturally occurring nucleotides. Further substitutions are possible at position 14 from a G→A and at position 15 from an A→G, without severe effects (Joyce 2001; Zaborowska et al. 2002). From these results, one can conclude that nucleotides at positions 1–6 and 13–14 are directly involved in forming the catalytic center. Curiously, Schubert et al. (2003) have demonstrated that modifications with 2′-O-methyl groups at positions 2, 7, 8, 11, 14 and 15 do not interfere with the correct folding of the catalytic centre and even double the original value of its catalytic rate.

Finally, alteration of the deoxyribozyme size (17 to 33 nucleotides long) and chemistry do not affect the degree of uptake into cells in culture (Dass et al. 2002). For internalization, deoxyribozymes do not need support through electroporation, transfection, or infection.

Cellular uptake

In spite of the uptake of modified single-stranded DNA molecules having been widely investigated, some mechanistic aspects refuse to give up their secrets. DNA molecules that are associated with the cell membrane are internalized into the cytoplasm by cell surface receptors such as CD4 (Yakubov et al. 1993) and gp 120 (Stein et al. 1993) with the involvement of protein kinase C (PKC), which are unspecifically used as free-riders via receptor-mediated endocytosis. Alternatively, pinocytosis and absorptive endocytosis might also play an important role in oligonucleotide (ODN) uptake (Patil et al. 2004). Which of these mechanisms is used depends on factors such as ODN chemistry, length, conformation and concentration, cell type, stage of cell cycle, degree of cell differentiation and intracellular conditions or cell environment (e.g., pH and cation concentration; Akhtar et al. 2000). Hence, no universal mechanism that conclusively explains the internalization process has been identified. After uptake, when labeled, the DNA molecules appear in a characteristic punctated cytoplasmic distribution (Loke et al. 1989; Grimpe and Silver 2004). The fate of the DNA molecule depends on its localization into either endosomes or lysosomes. Lysosome membranes contain proton (hydrogen) pumps, which change the inside pH to 4.5-5. Such conditions not only promote acidic hydrolysis but also activate lysosomal enzymes that rapidly degrade the ODNs. In contrast, endosomes keep a neutral pH in their lumen so that no degradation of the ODNs occurs and hence, the ODNs are ready to work. The way in which the DNA molecule escapes the lysosomes or endosomes is, once again, unclear. A likely mechanism for penetrating these compartments is the conformational change of membrane proteins to destabilize the membrane, as occurs with bacterial toxins (Farahbahksch et al. 1987) and viruses (White et al. 1983). After being released into the cytoplasm, the DNA molecules are reported to become localized (1) predominantly in the cytoplasm or (2) within the nucleus (Zamecnik and Stephenson 1978; Cerruzi et al. 1990; Chin et al. 1990; Leonetti et al. 1991).

In conclusion, mention should be made of the special care that has to be taken when selecting cells for investigations. An often-observed problem is encountered on the utilization of cloned cell lines when using deoxyribozymes or other knockdown agents: frequently, these cell lines show limited or no uptake of the agent, whereas primary cells show good to excellent internalization. Concerning the CNS, the author has successfully used hippocampal slice cultures (Grimpe and Silver 2004), primary astrocytes (Grimpe and Silver 2004) and primary retinal ganglion cells (Ries et al. 2007) with high efficiency of the internalization of deoxyribozymes. Schwann cells from the PNS also exhibit high efficiency (Grimpe et al. 2005), whereas dorsal root ganglion cells fail to internalize a deoxyribozyme (data not shown). For cloned cell lines, the aid of transfection reagents, electroporation, or infection is necessary for internalization, whereas primary cells do not need them. When such enhancing techniques are used on primary cells, the uptake of the ODNs, however, is increased. Laboratories that are newly interested in this research field will probably make the mistake of initially selecting the wrong cell type and will experience a lack of effect from their agents. In a further observation, the simple administration of chemically stabilized deoxyribozymes in a saline solution led to efficient uptake and biological effects in vivo. This finding points to unknown uptake mechanisms that occur inside organisms and that are not encountered in cell culture as yet (Schubert and Kurreck 2004). To circumvent these problems, Li et al. (2010) have used a single-stranded DNA expression vector that generates a 10–23 deoxyribozyme under the control of two separated promoters.

Therapeutic approaches

Nucleic-acid-based knockdown technologies such as deoxyribozymes, ribozymes, short interfering RNA (siRNA) and antisense ODNs depend on the accessibility of the target site. To identify suitable sites along the mRNA, theoretical approaches such as the MFOLD program (http://unafold.math.rpi.edu/) have been developed. Although the MFOLD program predicts RNA secondary structure based on free-energy calculations (Walter et al. 1994; Amarzguioui et al. 2000), interactions with proteins such as single-stranded proteins, to which every mRNA is attached, are excluded from analysis. Therefore, mRNA targeting is still a relatively random affair in which several agents are tested until an accessible site is identified.

The above-mentioned knockdown technologies have widespread applications in the fields of human immunodeficiency virus (HIV), cancer, influenza, hepatitis, asthma and spinal cord injury (SCI) research but the mechanism between these various technologies is quite different. Deoxyribozymes are non-naturally occurring, whereas ribozymes, in contrast, are single-stranded natural RNA catalysts. Both require mono- or divalent ions. siRNA molecules are small double-stranded RNA molecules with a 3′ overhang of one nucleotide on each side, whereas antisense ODNs are small single-stranded DNA molecules. The last two utilize the cellular machinery for the sequence-specific silencing process. siRNA molecules are incorporated into the RNA-induced silencing complex (RISC), which binds to the mRNA of interest and stimulates an mRNA degradation mechanism, such as nuclease activity, which leads to the silencing of the particular mRNA (Bertrand et al. 2002; McManus and Sharp 2002; Scherr et al. 2003; Kim and Rossi 2007). Antisense ODNs, however, bind stoichiometricly to the target mRNA and interfere with gene expression or other mRNA-dependent cellular mechanisms. In the presence of RNAse H, they induce an enzymatic degradation of the target mRNA (S.T. Crooke et al. 1995). The therapeutic applications of these four knockdown agents will be discussed below.

Deoxyribozymes Based on its catalytic efficiency of $3.2\times10^8 M^{-1} min^{-1}$, which is comparable to those of naturally occurring ribozymes, only the 10–23 deoxyribozyme has been applied in vivo to inactivate target mRNA molecules (Santoro and Joyce 1997). This efficiency is near-catalytic "perfection", consistent with the Albery and Knowles definition, in which the reaction rate is limited only by the rate of diffusional encounter between the enzyme and substrate (Albery and Knowles 1976). Therapeutic use of DNA-based over RNA-based technologies is predicated on the absence of a 2′-OH group at each phosphodiester linkage, which makes DNA ~100.000-fold more stable to hydrolysis than RNA under physiological conditions (Li and Breaker 1999). In other words, the half-life of an unmodified deoxyribozyme in human serum has been measured to be about 2 h compared with<1 min for hammerhead ribozymes (Schubert and Kurreck 2004). Further support for DNA-based technology comes from the finding that a DNA:RNA duplex tends to dissociate more easily than an RNA:RNA duplex. This means that mismatches in the hybridization arms, which might occur during binding to nontarget mRNAs, inhibit cleavage activity and increase dissociation from the target molecule (for a review, see Shaw and Arya 2008). Furthermore, the cleavage rates with long RNA molecules are several orders of magnitude lower than those with short substrate molecules (Thomson et al. 1996). Hence, when dealing with long-sequence targets, as likely in biological systems, a deoxyribozyme should be more

active and potentially more selective than a ribozyme. As Table 1 shows, deoxyribozymes have been investigated with a variety of molecules. Their application to neurological disorders, however, is limited. The two companies that have been founded to date, namely the US-based CytoGenix (Tan et al. 2004) and the Germany-based Sterna Biologicals (Sel et al. 2008), test deoxyribozymes of the 10–23 type in preclinical trials of cancer, viral infections diseases such as influenza, small pox and HIV and asthma research. Whereas CytoGenix have generated a mammalian single-stranded DNA expression vector from which a 10–23 deoxyribozymes is expressed (called SynDNA), Sterna Biologicals administers intranasally a 10–23 deoxyriboyzme modified by an inverted 3′-3′-thymidine (Fig. 5d).

Ribozymes The most frequently described and investigated ribozyme with therapeutic potential is the small hammerhead ribozyme (Birikh et al. 1997) followed by the hairpin ribozyme (Fig. 6). Further ribozymes or self-cleaving RNAs are the rRNA intervening sequence from *Tetrahymena thermophila* (Kruger et al. 1982), RNase P (Guerrier-Takada et al. 1983), hepatitis delta virus (Wu et al. 1989), transcript from a *Neurospora crassa* mitochondrial DNA plasmid (Saville and Collins 1990) and plant pathogenic RNAs (viroids, satellite RNAs, virusoids; Diener 1989). The hammerhead ribozyme requires a recognition sequence of NUH for cleavage of the target mRNA, where N is any nucleotide and H equals A, C, or U. Cleavage occurs between U and H (James and Turner 1997). In contrast, the hairpin ribozyme has a sequence requirement of BN/GUC, where "/" is the site of cleavage and B equals G, U, or C (Hampel 1998). For clinical use, efforts have been made to reduce the number of nucleotides in the binding arms and within the catalytic loop structure without compromising the cleavage ability or specificity of the hammerhead ribozyme. For most efficient cleavage, the 5′-arm requires 5–6 nucleotides, whereas the 3′-arm, which is the most crucial in this respect, has no limitation in the number of nucleotides, as long as it also contains a minimum of 5 or 6 bases. A reduction in the catalytic loop and the insertion of DNA, which leads to chimeric miniribozymes or minizymes (Fig. 6), results in faster cleavage of longer RNA substrates, at least in vitro. Here, the binding arms of miniribozymes or minizymes should rather have a length of around 10 nucleotides (Hendry et al. 1998). The actual cleavage reaction of ribozymes occurs on a susceptible 3′,5′-phosphodiester linkage leading to the formation of a 2′,3′-cyclic phosphodiester and 5′ hydroxyl group (Cech 1987), which is similar to the mechanism used by deoxyribozymes. During this process, the ribozyme requires the presence of Mg^{2+}. Crystallization experiments have revealed that Mg^{2+} not only assists in RNA folding but also participates directly in the cleavage mechanism (Dahm and Uhlenbeck 1991; Pley et al. 1993).

**Table 1** Representative in vivo targets of deoxyribozymes (modified from Baum and Silverman 2008)

Target gene/molecule	Target site/function	Reference
Akt1		Yang et al. 2009
Egr1	Zinc finger transcription factor	Santiago et al. 1999; Fahmy and Khachigian 2004; Mitchell et al. 2004; Bhindi et al. 2006; Wu et al. 2010
Epstein-Barr virus	Latent membrane protein	Lu et al. 2005
Hepatitis B virus	X protein	Hou et al. 2006
Hepatitis B virus	HBs and HBe antigens	Wo et al. 2005
Hepatitis B virus	Core protein	Trepanier et al. 2006
HIV-1	Gag	Sriram and Banerjea 2000; Dash and Banerjea 2004; Sood et al. 2007a
HIV-1	Tat protein	Umwalla et al. 2006; Sood et al. 2007b
HIV-1	5′-untranslated region	Jakobsen et al. 2007
Human telomerase reverse transcriptase		Yuan et al. 2007
Influenza virus A		Takahashi et al. 2004
Isocitrate lyase from *Mycobacterium tuberculosis*		Li et al. 2005
c-Jun	Leucine zipper transcription factor	Fahmy et al. 2006; Zhang et al. 2004
Beta-catenin	Colon polyps and cancer	Choi et al. 2010
b-Lactamase		Chen et al. 2004; Hou et al. 2007a
12-Lipoxygenase		Liu et al. 2001
c-Myc	Proto-oncogene	Sun et al. 1999; Dass et al. 2002; Pun et al. 2004
Ornithine decarboxylase		Ackermann et al. 2005
Pencillin-binding protein		Hou et al. 2007b
PML/RARa fusion gene	Acute promyelocytic leukemia	Kabuli et al. 2004
Respiratory syncytial virus (RSV)	Nucleocapsid protein	Zhou et al. 2007
SARS	Associated coronavirus 5′-untranslated region	Wu et al. 2007
Survivin		Liang et al. 2005
TGF-β1		Isaka et al. 2004
Twist	Helix-loop-helix transcription factor (apoptosis)	Hjiantoniou et al. 2003
Vascular endothelial growth factor receptor 2 (VEGFR)		Zhang et al. 2002
Xylosyltransferase-1	Glycosaminoglycan chain synthesis	Grimpe and Silver 2004; Hurtado et al. 2008

siRNA This knockdown technology has been the most frequently used during the last two decades, with high expectations for therapeutic application. A plethora of publications has focused on the mechanism of siRNA gene silencing, the delivery of the siRNA agent, its therapeutic utilization and any unwanted side-effects. New online tools provide effective design algorithms, which are based on a combination of mRNA target sequence, secondary structures and siRNA duplex end-stabilities and aim to minimize potential sequence-dependent off-target effects. Most of the proposed clinical applications of RNA interference (RNAi) incorporate chemically synthesized 21-nt siRNA duplexes that have two-nucleotide 3′ overhangs. When longer siRNAs (27 nucleotides) are used, a lower concentration is required for silencing the target. These agents then contain an asymmetrical 3′ overhang with two nucleotides and one blunt end. Here, the 3′overhang is recognized by Dicer for processing (Amarzguioui et al. 2005). Off-target effects seem to be avoidable when the complementary region of the siRNA is localized within the open reading frame evading the 3′ untranslated region (for a review, see Jackson and Linsley 2010). To elude a type I interferon response of double-stranded RNA agents, which are longer than 30 base pairs, or of double-stranded and single-stranded RNA agents via Toll-like receptors, the following immunostimulatory sequence motifs should be avoided: 5′-GUCCUUCAA-3′ and 5′-UGUGU-3′ (Hornung et al. 2005; Judge et al. 2005).

Various delivery strategies to guide the therapeutic siRNAs to their specific location have been utilized. For example, they have been coupled to antibody fragments (Song et

Fig. 6 Ribozyme structure. **a** RNA-based hammerhead ribozyme. **b** RNA-based hairpin ribozyme. **c** RNA-based miniribozyme. **d** DNA-RNA based chimeric minizyme (incorporation of deoxythymidine [*T*]; modified after James and Turner 1997; Berzal-Herranz and Burke 1997; Hendry et al. 1998)
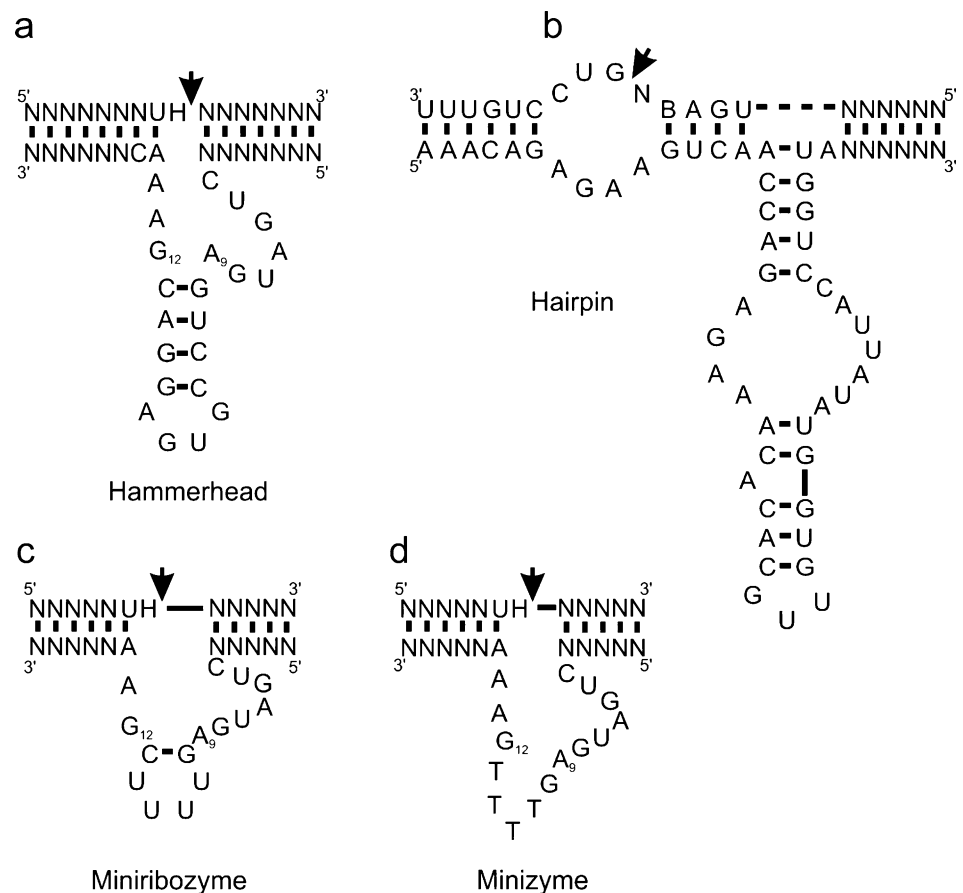
al. 2005), aptamers (McNamara et al. 2006), or packaged into nanoparticles coated with receptor-targeting ligands (Hu-Lieskovan et al. 2005), whereby the siRNA is incorporated through endocytosis. Modifications with the 2′-O-methyl group not only avoid interferon induction (Morrissey et al. 2005) but also, when staggered between the two siRNA strands, seem to provide an optimal balance between retention of RNAi potency and protection against degradation (Czauderna et al. 2003). A summary on the therapeutic targets of siRNA agents is provided by Koutsillieri et al. (2007) and Lares et al. (2010).

In light of all the success achieved with siRNA and the investments made by big pharma, the latest decisions of the pharmaceutical industry are surprising. Roche closed its three R&D facilities working on siRNA in December 2010 (after a total of $500 million had been invested during the previous three years) and Pfizer and Abbott announced that it had no further interest in this technology. Novartis also showed reduced enthusiasm by ending a 5-year partnership with Alnylam at the end of 2010. However, Novartis will finish its program including 31 siRNAs, on which they are working internally (Pollak 2011; Schmidt 2011).

Antisense ODNs The trailblazer of all knockdown agents is antisense technology. Our long-time experience with this technique provides the necessary foundation for the design of efficacious and specific ODNs. In spite of the size of the ODN being important and leaving little leeway, a hard-and-fast rule of a right-sized ODN does not exist; hence, it must be determined by empirical means. Some ODNs as short as 12 nucleotides have been found to provide a good discrimination of the target gene expression, whereas in other cases, a 16-base long ODN shows no selectivity. If an ODN is too short, it will not be selective at all but if it is too long, it will not be accessible to the tissue and will be difficult to work with. Control ODNs are essential for every experiment utilizing this technique. Hence, "mismatch ODNs", which are scrambled antisense sequences, with the potential of keeping the same GC contents as their specifically working counterpart, are often used. Another control is the utilization of "mixed base ODNs", which are sense sequences that contain 4 to 5 scrambled bases on their 3′ and 5′ ends. Pure sense controls are not to be recommended, because antisense mechanisms are involved in naturally occurring gene regulation such as for fibroblast growth factor-2 (FGF-2). Here, sense and antisense transcripts form double-stranded RNA duplexes in vivo. With the administration of a sense ODN to the antisense transcript, one generates a control that has biological activity and produces physiological effects that create confusing or even misleading results. Other

common types of control involved "randomized" and "reverse ODNs", in which the first is obtained by mixing up the bases of the antisense ODN and the latter by reversing the antisense sequence with respect to its 5′-3′ orientation. Moreover, the latter is unable to hybridize to the antisense mRNA target (Leslie et al. 1999). Needless to say, it is essential to analyze all antisense and control sequences for their specificity through a BLAST search.

For the clinical utilization of antisense ODNs, S.T. Crooke (1995), the founder, chairman and CEO of Isis Pharmaceuticals, the company that generated the first Food and Drink Administration (FDA)-approved an antisense drug in 1998, namely Vitravene, targeting cytomegalovirus retinitis in HIV-positive patients, eloquently summarized the pharmacokinetics of antisense PTOs. He states that antisense PTOs are readily absorbed from a variety of administration routes such as intramuscular, subcutaneous, intradermal, or intraperitoneal applications. These PTOs reach their maximum plasma concentration within 15–30 min. Distribution occurs rapidly and broadly to the peripheral tissue with accumulation of most of the drug in the liver and kidney without crossing the blood–brain barrier. Clearance of the antisense PTOs after being internalized into the cell takes place slowly (elimination half-life: 40–50 h) by slow metabolism (half-life: ~24 h) in all tested organs. S.T. Crooke and colleagues (R.M. Crooke et al. 1995; Cossum et al. 1993, 1994; Sands et al. 1994; Agrawal et al. 1991) have observed minimal differences between species and between different sequences from the above-described statements. Thus, the in vivo delivery of antisense PTOs to peripheral tissues has proved to be relatively facile. In conclusion, Crook writes, "In animals and humans, perhaps to the surprise of some, antisense PTOs have displayed excellent parenteral pharmacokinetic properties concentrating in a number of peripheral tissues, including the liver, kidney, bone marrow, skeletal muscle and skin. Thus, specialized drug delivery systems have not been required to show potent in vivo activities." Today, he adds, "Despite the early success, all the necessary elements were not in place at that time to make multiple products. Due to the worldwide shrinkage of the drug market in 2004, when big pharma experienced losses in the billions, Vitravene was discontinued because its patient group was too small." Raymond Warrell, chairman and CEO of Genta adds, "If you're going to have an effect with a single antisense agent, which attacks only a single gene product, you have to be sure that your target has an absolutely central role in the complex biology of the disease you're trying to treat." An example is the Isis anti-PKCα drug, which can be given at high doses as its side-effects are modest compared with those of other anticancer agents. "But it failed because PKCα was a bad choice of target", says S.T. Crooke. "It's just not very significant in the malignancies that we looked at" (Jones 2011). The revival of antisense drugs can be observed based on the number of licenses that the big pharmaceutical companies have distributed in the last two years (Pollak 2011). Not only target specificity but also safe treatment requires the investigation of the liver and kidney function through blood analyses, such as a complete blood count (CBC) and chemistry. Furthermore, the slow administration of antisense PTOs, in particular when using the systemic route, is essential in a safe treatment paradigm. In summary, Table 2 compares the advantages and disadvantages of the deoxyribozyme technologies with the other above-mentioned knockdown techniques.

Table 2 Comparison of deoxyribozymes with other knockdown technologies (*siRNA* short interfering RNA, *qPCR* quantitative polymerase chain reaction; modified after Baum and Silverman 2008)

Knockdown technologies	Advantage of deoxyribozyme over other knockdown technologies	Disadvantage of deoxyribozyme over other knockdown technologies
Ribozyme	• More stable chemically and biologically • Less expensive • Easier to synthesize • Recognizes dinucleotide base pairs and not triplets as ribozymes • Use as ATP-dependent ligase and kinase	• Narrower range of reactions
siRNA	• Catalytically independent of cellular machinery • Does not require transfection, infection, or electroporation for uptake into cells	• Difficult to engineer for activity under physiological conditions (e.g., low divalent metal ion concentrations) • Sequence dependency whereas siRNA can digest any sequence
Antisense oligonucleotide	• Allows direct catalytic digestion of target mRNA • Positive digestion can be analyzed/visualized by using qPCR	• More complex design because of sequence, structure, or cofactor requirements beyond base pairing

Presently, 347 clinical studies exist for SCI and 348 for traumatic brain injury, whereas 15 recruitments to studies with siRNA (completed or active), two trials with ribozymes (completed or active) and 88 clinical trials (completed or active) based on antisense technology can be found on the FDA database (www.clinicaltrials.gov). The lack of deoxyribozymes trials demonstrates that this technology is still in its infancy. A combination of SCI or traumatic brain injury with one of these knockdown technologies has not been initiated.

Utilization of deoxyribozymes in axon regeneration

After trauma to the CNS, e.g., spinal cord injury, immediate early genes such as c-jun and also c-fos, nor-1 (Landry et al. 2006) and Zif268 (Densmore et al. 2010) are up- or down-regulated. If the blood-spinal cord-barrier opens after such an injury, a lesion scar forms, which, among other factors, prevents re-growth of the majority of severed axons. The scar consists in extracellular matrix molecules (ECMs), e.g., proteoglycans (PGs), laminins, fibronectin and collagen. Deoxyribozymes have been designed to some of these molecules and show a successful reduction of the specific mRNA in the CNS. These deoxyribozymes will now be discussed in more detail.

c-Jun This basic region leucine-zipper transcription factor is activated through double phosphorylation by the c-Jun N-terminal kinase (JNK) on Ser-63 and Ser-73 within its transcriptional activation domain. JNK 3, one of three genes, is predominantly found in CNS neurons such as pyramidal neurons in the CA1, CA4 and the subiculum region of the hippocampus, whereas lower levels have been identified in the heart and testis. Maximum activation of JNK requires the phosphorylation of Thr and Tyr residues in the activation loop. If only the Thr residue via mitogen-activated protein kinase kinase 7 (MKK7) is phosphorylated, a significant increase of JNK3 can still be observed. In contrast, phosphorylation by MKK4 alone induces only low levels of JNK3. JNK activates, in addition to c-Jun, anti-activating transcription factor-2 (ATF-2), Jun B and Jun D (Ip and Davis 1998). Because JNK is involved in neuronal differentiation, apoptosis, neuronal diseases such as Alzheimer's disease and Parkinson's diseases and stroke, it is being pursued by several companies for CNS drug indications (Resnick and Fennell 2004).

The first study utilizing a deoxyribozyme to c-jun (Dz13) demonstrated that it blocked endothelial cell proliferation and migration. After injection of Dz13 into tumor cells, vascular endothelial growth factor (VEGF)-induced neovascularization in the rat cornea and B16 melanoma growth was inhibited (Zhang et al. 2004). The latest discoveries show that Dz13 is a potent inducer of caspase-2 (Dass et al. 2010a) and of the transcription factor, E2F1 (Dass et al. 2010b), based on off-target effects. Both molecules are involved in apoptotic processes, whereas the E2F class is additionally involved in mitosis, DNA replication, DNA damage checkpoints, DNA repair, development and differentiation. Further analysis has shown that ERK, Akt and p38 are also up-regulated after Dz13 administration (Dass et al. 2010b). The same group (Dass and Choong 2010) have found that systemic application of Dz13 represents no toxic effects for blood or solid tissues in adult or fetal mice, with only a slight hepatotoxicity being noticed by means of histology.

Laminin Unlike most regions of the adult brain and spinal cord, the hippocampus possesses a remarkable capacity for continued axon growth and reformation of precise synaptic connections within the CNS. Indeed, new functional granule neurons with lengthy axons are generated in the hippocampus from a resident population of stem/progenitor cells throughout the adulthood of many species, including humans. Grimpe et al. 2002 describe a narrow sickle-shaped laminin γ1 chain pathway beginning in the hilus and extending along the CA3 pyramidal cell body layer (stratum pyramidal). Staining is also present in and around the portion of the apical dendrites closest to the cell body (stratum lucidum) but is lacking entirely in the basal and distal-most portions of the apical dendrites of the young CA3 pyramidal neurons. Thus, laminin staining is confined strictly within the stratum pyramidal and stratum lucidum, providing the newly generated granule neurons with a growth-promoting guidance highway for their mossy axons. O'Keefe and Nadel 1978 and Amaral and Dent 1981 have identified the identical terrain as a mossy fiber pathway.

In hippocampal slice cultures, the mossy fibers show the ability to regenerate after being lesioned at the hilus/CA3 region. However, when a deoxyribozyme to the laminin γ1 chain is administered, the laminin in this pathway is reduced and the mossy fibers lose their ability to regenerate. Instead, they are much shorter and take a meandering course. When the deoxyribozyme is washed out, laminin immunoreactivity returns and mossy fiber regeneration resumes (Grimpe et al. 2002).

Xylosyltransferase-1 In the last decade, PGs have been identified as ECM molecules that participate in the growth inhibitory environment that is formed after an injury in the spinal cord. Whether their glycosaminoglycan (GAG)-chains or their protein cores prevent the regeneration of severed axons is still controversial. However, prevention of the initiation of these GAG-chains, which begin primarily

in the Golgi apparatus (Hoffmann et al. 1984), increases axon growth as demonstrated by Moon et al. (2001) and Bradbury et al. (2002). In the Golgi apparatus, the core proteins of PGs receive the long unbranched GAG-side chains. Chondroitin sulfate (CS)-, dermatan sulfate (DS)- and heparan sulfate (HS)-PGs all have a special link tetrasaccharide, composed of xylose, galactose, galactose and glucuronic acid. This link-molecule is attached to a serine hydroxyl side chain, embedded in a specific consensus peptide sequence at the core protein, to serve as a primer for polysaccharide growth. The first carbohydrate of the GAG-chains, namely xylose from UDP-xylose, is catalyzed by the chain-initiation enzyme UDP-D-xylosyltransfease (XT-1; Götting et al. 2000). Furthermore, sugars such as D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) are added by specific glycosyl transferases as disaccharide units individually to the link tetrasaccharide. While still in the Golgi compartment, many of the polymerized sugars are covalently modified by a sequential and coordinated series of reactions, such as sulfatation at the C2 position of GlcA and/or the C4 or C6 position of GalNAc residues. In principle, PGs have the potential for almost limitless heterogeneity regarding the number and types of GAG chains attached to even one single type of core protein.

In Grimpe and Silver (2004), the local administration of a deoxyribozyme to XT-1 reduced the presence of GAG chains, which normally create an inhibitory penumbra around the epicenter of spinal cord lesions. The reduction of fully glycosylated PGs allowed for the regeneration of microtransplanted adult sensory axons around and beyond the central core of the lesion. This result was confirmed in Hurtado et al. (2008), in which endogenous severed sensory axons were able to enter and grow into spinal cord tissue rostral of a peripheral nerve graft after deoxyribozyme to XT-1 treatment. In an untreated spinal cord, this entry and growth was stalled based on enhanced GAG-chain synthesis.

These examples demonstrate that deoxyribozymes are successful in reducing the mRNA of proteins. In addition, the number of deoxyribozymes provided in Table 1 demonstrates that their application is flourishing. Hopefully, more neuroscientists will recognize this technology as a fruitful and rewarding alternative compared with established techniques.

Bioinformatics

At the beginning of the 21st century, microarray experiments were introduced to the neurobiology field, in particular for the study of the pathophysiology of the injured spinal cord. With this approach, bioinformatics and statistical software have to a large extent found their way into the SCI community.

DNA-microarrays

Three phases after SCI are typically recognized: (1) the acute phase, (2) secondary tissue loss and (3) the chronic phase (Tator 1996, 1998). After immediate mechanical damage to the spinal nervous tissue in the acute phase (minutes to days), cell death (necrosis), a barrage of action potentials (spinal shock), hemorrhages and swelling occurs, leading to motor functional restrictions or loss. During the secondary phase (minutes to weeks), ischemic cell death, electrolytic shifts and edema continue and, in addition, lipid peroxidation, free-radical production, apoptosis and reactive gliosis occur. Within 24 h, neutrophils invade the spinal parenchyma, followed by lymphocytes, resulting in an increase in cytokines and chemokines, which contribute to the loss of tissue. At this time, inhibitory factors such as PGs are expressed and the scar gradually matures at the peri-lesion site. In the chronic phase (days to years), apoptosis continues, additional demyelination causes more conduction deficits and, in about 25% of all patients, tethering of the spinal cord occurs. In approximately 20% of all SCI patients, a cyst forms at the injury site, which usually continues to enlarge (syringomyelia; Hulsebosch 2002).

Starting with Farlow et al. (2000), several scientific groups began to analyze the changes in gene expression throughout these three phases in various PNS- and CNS-trauma and regeneration models. Up to the present day, a wide range of different approaches have been performed, some with rather specialized questions, such as those investigating two temporally distinct microglia-related gene clusters (Byrnes et al. 2006) or those analyzing the effect of N-methyl-D-aspartate receptor inhibition (Nesic et al. 2002) and others with broader questions as summarized in Bareyre and Schwab 2003. In spite of providing important new insights into the pathophysiology of the injured spinal cord, all these approaches have in common that only a relative small number of genes have been selected for further investigations. The sheer volume of data creates additional challenges. Dedicated software and database systems to store and organize the microarray data and images are required. A description of the experimental parameters (annotation) via the MIAME standard, normalization of the data and clustering techniques have proven valuable but require a new standard in statistical analysis. For the first time, data and text-mining methods, k-nearest neighbor classifications, support vector machines, machine learning algorithms and neural nets have found applications in the field. Kaiser and Nisenbaum (2003) have combined published data sets from five different microarray studies to examine global changes in nerve tissue gene expression. They have been able to extend the biological conclusions derived from each study.
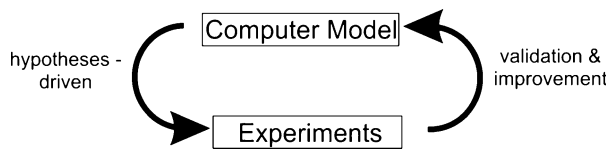
Fig. 7 Representaion of the course of action in the author's laboratory utilizing a bioinformatics (applied systems biology) approach (created by B. Grimpe)

Novel approach

The next logical step is to build upon all existing data to comprehend the complex process of events and, at the same time, to identify suitable target molecules for the design of new deoxyribozymes to prevent regeneration failure. Therefore, it will be essential to collect all existing information on genes and proteins found in publications and databases, to connect these data utilizing bioinformatics and to validate the computer-based results through hypothesis-driven experiments (Fig. 7). To provide such an overall picture of SCI, its underlying biochemical pathways and potential protein interactions and to lay hands on the sheer number of applied substances, Ries et al. (2007) have written a preliminary computer program suite, called the regeneration data mining project (RDMP), to build an in-silico protein interaction model, drawing both from natural language sources and from various databases available on-line. A large MySQL database provides the sequence, interaction, literature and other data records plus query capability. RDMP is able to collect, identify and generate a list of proteins involved in processes of axonal growth from abstracts that have been collected in PubMed and classified with the medical subject heading (MeSH) term "nerve regeneration" (Ries et al. 2007). MeSH is a controlled vocabulary maintained by the National Medical Library, which covers subjects in the medical domain. The RDMP recognizes protein names by a dictionary/rule-based method similar to that of Egorov et al. (2004). The dictionary has been extracted from the DEF field of the universal protein resource UniProt

database (Emmert et al. 1994). A list of English stop words and the most frequent words used in natural language texts were obtained from MySQL v4.1, to which the letters from the alphabet were added. An example in Table 3 illustrates the method's main steps. In this approach, publications build the foundation of the analysis, instead of microarray databases. The latter contain a high number of false-positive and false-negative results, which have to be identified and filtered out, whereas the review process by journals for manuscripts should assure the quality of the data.

With the prototypic program suite, Grimpe and colleagues were able to identify 942 proteins, which were being evaluated in the area of nerve regeneration at that time. When classified by using gene ontology terms, proteins from every field of biochemistry were found in our list (Ries et al. 2007). This confirmed the original hypotheses that SCI is based on complex events. It further demonstrates the challenges that one is facing in modern science. Within the 942 proteins, we indentified osteopontin. Further analysis determined a novel function for osteopontin and its receptor CD44 and for the laminin-CD44 interaction in neurite outgrowth of retinal ganglion cells, an event that remained elusive at that time (Ries et al. 2007). When continuing to use the 942 proteins as a seed to search the Biomolecular Interaction Network Database (BIND), the authors were able to generate a protein-protein (P-P) interactions map. By analyzing these P-P interactions, which were visualized by Cytoscape (Fig. 8), three molecules new to and hence uninvestigated in the field of SCI were identified. These proteins were:

(1) **Protein inhibitor of activated STAT (PIASγ)**: *Function*: Specific inhibitor of transcription factors. *Interactions*: with nuclear hormone receptors as a co-regulator (Moilanen et al. 1999; Kotaja et al. 2000), with a specific SUMO ligase to p53, c-Jun (Schmidt and Muller 2002; for a review, see Shuai and Liu 2005) and Smad7 (Imoto et al. 2003) and as a suppressor to regulate transforming growth factor (TGF)-β-mediated Smad3 activation (Imoto et al. 2003).

Table 3 Course of action of the RDMP software to identify proteins from abstracts of publications that are collected under the medical subject heading (MeSH)-term "nerve regeneration" (from Ries et al. 2007)

Text	Action
"…detected ankyrin G, which was…"	Obtain raw text instance
Keep "detected", "ankyrin"; discard "G", "which", "was"	Split into words, trim non-alphanumeric characters, discard stop words and single letters
Discard "detected", keep "ankyrin"	Match as substring to name dictionary: 118 hits for "ankyrin"; no hit for "detected"
…ankyrin-3|ankyrin G…	Use rules to generate spelling alternatives
…ankyrin 3|ankyrin-3|ankyrin G|ankyrin-G…	Match each alternative in full length to the raw text
"…detected **ankyrin G**, which was…"	"ankyrin G" matches
"ankyrin G", Q12955, 12507143	Recognized protein name, primary accession number and PubMed identifier

Text sample from Kretschmer et al. (2002)

Fig. 8 Small part of the protein interaction network generated by the regeneration data mining project and rendered by Cytoscape v2.5.0 (*pink dots* proteins, *blue lines* protein interaction). In the *circle* at the *bottom* of the image and magnified in the *insert*, several proteins are presented that have so far not been investigated after SCI, such as PIASγ (*A*) and SH3-domain-GRB-2-like 3 (*B*), whereas glial fibrillary acidic protein (*C*) is one of our 964 seed list proteins (created by B. Grimpe)
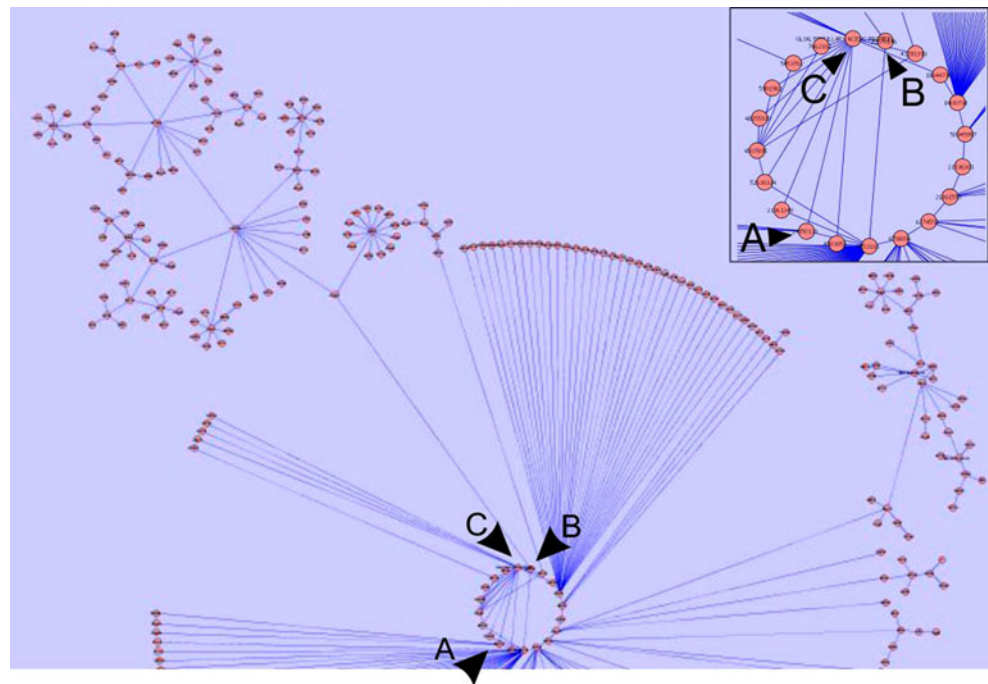
(2) **Son of sevenless (Sos)**: Interacts directly with mitogen-activated protein kinase, a protein from our seed list. This is a guanine nucleotide exchange factor (Boguski and McCormick 1993; Simon et al. 1991). _Function_: Regulates Ras activity (Downward 1992); Sos1 knockout results in embryonic lethality in mice, indicating its essential function for mammalian development (Wang et al. 1997). _Localization_: Human cells contain two SOS (hSos) genes that encode closely

related ubiquitously expressed proteins (Chardin and Mattei 1994); they are normally localized in the cell cytosol but, in response to growth factor stimulation, they are recruited to the plasma membrane (Bar-Sagi

Fig. 9 Real time PCR was performed on contused spinal cords (*n*=3) and sham (*n*=2) operated animals in triplicate. RNA was isolated 1, 3, 6, 24 and 48 h after lesion. The normalized expression values of the genes (*PIAS* protein inhibitor of activated STAT, *SOS* son of sevenless, *SH3* Src-homology-3) are presented on a logarithmic scale (created by B. Grimpe)

Table 4 Twenty of the most frequently found protein names in abstracts of publications (from supplemental table 1 in Ries et al. 2007)

Number	Protein name/abbreviation	Number of publications
1	Nerve growth factor/NGF	775
2	Collagen	304
3	Calcitonin gene-related peptide/CGRP	289
4	Glial fibrillary acidic protein/GFAP	251
5	Brain-derived neurotrophic factor/BDNF	246
6	Laminin	224
7	Acetylcholinesterase/AChE	208
8	Neurotrophin-3/NT-3	148
9	Insulin-like growth factor/IGF	138
10	Ciliary neurotrophic factor/CNTF	129
11	α- or β-Tubulin	127
12	Fibroblast growth factor (FGF-1/-2)	118
13	α-, β-, γ-Actin	90
14	Substance P/SP	86
15	Fibronectin	76
16	Acetyltransferase	63
17	p75	59
18	Myelin-associated glycoprotein/MAG	54
19	Glial-cell-line-derived neurotrophic factor/GDNF	51
20	Nitric oxide synthase/NOS	50

1994). Only one publication (Marciano et al. 2004), which describes the investigation of, among others, SOS during hippocampal apoptosis after traumatic brain injury, has surfaced so far.

(3) **SH3-GRB2**: *Src*-homology-3 (SH3) domain, folding of SH3-domain-carrying proteins into a conserved secondary structure appears critical for binding to its ligand rather than for its primary structure (Feng et al. 1994; Yu et al. 1994). *Functions*: Signal transduction (for a review, see Pawson 1995), cell polarization (Chenevert et al. 1992), enzymatic activation (Leto et al. 1990; Volpp et al. 1989), transcriptional regulation (Bustelo et al. 1992; Giachino et al. 1997) and limb morphogenesis (Chan et al. 1996).

Real time quantitative PCR of injured spinal cords (*n*=3) normalized to β-actin revealed that PIASγ and SOS have their expression peak at 3 h after contusion, whereas the SH3-GRB2 peak occurs after 6 h (Fig. 9). None of these proteins have been investigated regarding their potential function after SCI, in spite of their importance being demonstrated during, for example, neuronal development.

Furthermore, the prototypic program suite has enabled the authors to rank the proteins based on their number of publications (Table 4). The generated list projects the political importance of the respective proteins and the direction that is favored by the regeneration community in order for it to comprehend the processes after trauma rather than the scientific importance. Nevertheless, the scientific information received about these selected and extensively studied proteins will still be important for fitting together the pieces of the puzzle of regeneration failure.

Concluding remarks

Deoxyribozymes have great potential to serve as knockdown agents for a variety of neurobiological problems and for therapeutic purposes because of their easy administration, reliability, specificity and low production costs. When used thoughtfully and within its limits, this technology is especially suitable for in vivo approaches to manipulate the environment of the injured spinal cord or brain tissue. In combination with a bioinformatics approach to indentify suitable but less-well-studied target molecules, the way is paved for a better comprehension of the mechanisms that lead to regeneration failure of axons after CNS trauma and, ultimately, to finding a treatment for paralysis.

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