Chapter 13
Deep Intronic *NF1* Mutations and Possible Therapeutic Interventions

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13.1 Deep Intronic Mutations: A Specific Type of Splicing Mutation

Splicing is a complex and fine-tuned cellular mechanism consisting of the removal of introns from pre-mRNA transcripts to generate mature messenger RNAs. This essential process is completed by the spliceosome in different reactions that require the participation of several *cis* elements and *trans*-acting factors (reviewed in Hammond and Wood 2011). The recognition of intron–exon boundaries is crucial for correct splicing, so they are marked by highly conserved, almost invariant dinucleotides of the donor or 5′ intronic splice site (GT, in genomic DNA) and the intronic acceptor or 3′ splice site (AG, in genomic DNA). Other important genomic sequences for splice site identification are the branch site and the polypyrimidine tract, both of which are located upstream of the intronic acceptor site. Other relevant *cis* elements include nucleotides motifs representing splice site enhancers (SE) and silencers (SS) that are located within both intronic and exonic regions and participate in the recruitment of enhancer or silencer *trans*-acting factors to the splicing machinery. Genomic variants in the DNA sequences of these elements can alter the correct recognition of a bona fide exon or, alternatively, create a new cryptic splice site that can be identified by the splicing machinery. Hence, alterations in these DNA sequences produced by mutations can lead to errors in the splicing process, leading to exon skipping, partial exon deletion, or

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incorporation of an intronic region into the mature RNA. In all of these cases, either *in-frame* or *out-of-frame* abnormal transcripts may be produced by the mutated allele.

Classically, it was thought that splicing mutations were restricted to DNA changes that disrupt the invariant canonical donor and acceptor splice sites, so only these mutations were reported as splicing mutations in mutation databases (currently fewer than 10 % of all point mutations reported in the *Human Gene Mutation Database* (HGMD Professional 2011.4; http://www.hgmd.org). However, over the last decade, a large number of studies have identified other types of mutation that also affect the correct splicing of a gene, particularly in genetic analyses that have been focused on large genes, using RNA-based approaches for constitutive mutation detection (Teraoka et al. 1999; Ars et al. 2000). These findings have served to extend the group of disease-causing mutations that impact splicing to a range of different mutation types (nonsense, missense, frameshift, etc.) affecting any sequence that is important for splice site recognition and mRNA processing. For example, the largest unbiased comprehensive study of >2,000 unrelated NF1 patients found that approximately 30 % of *NF1* point mutations affected correct mRNA splicing (L Messiaen, personal communication). A small subset of these mutations (about 2 %) (Messiaen and Wimmer 2008; Pros et al. 2008; Wimmer et al. 2007) comprises single-nucleotide changes in sequences residing deep within introns, which create novel donor or acceptor sites that, in conjunction with a nearby cryptic splice counterpart, define a new cryptic exon that the spliceosome then incorporates into mature messenger RNA. Most of these mutations are missed by conventional DNA-based mutation detection techniques because they are located deep within intronic sequences, distant from intron–exon boundaries, and hence are not scanned when this type of mutation detection approach is employed. Moreover, a high proportion of these mutations create mRNAs with premature termination codons (PTC), rendering the transcripts susceptible to degradation by the nonsense-mediated mRNA decay (NMD) machinery. It is therefore desirable to use an NMD inhibitor prior to RNA extraction to be able to detect the mis-splicing effects characteristic of deep intronic mutations (Messiaen et al. 2000). Together, these observations suggest that the frequency of this type of mutation has probably been underestimated for most studied genes.

13.2   Use of Antisense Oligonucleotides to Modulate Aberrant Splicing

In recent years, antisense oligonucleotide (AON)-mediated therapies have been increasingly used to restore gene function by modulating aberrant splicing in different scenarios created by disease-causing mutations. Correction of altered RNA splicing can be achieved by masking a splice motif with AONs complementary to a specific sequence of the pre-mRNA of interest, thereby inhibiting by steric

hindrance the recognition of this region by the splicing machinery. The first indication that AONs could be used as therapeutic agents for genetic diseases was obtained from studies in β-thalassemia, in which abnormal splicing of the beta-globin (*HBB*) gene due to activation of intronic cryptic sites was corrected using an AON approach (Dominski and Kole 1993). Different applications of AON-dependent splicing modulation have been described in the literature, including forcing the skipping of one or more exons flanking a frameshift in order to restore the open reading frame of a gene (Aartsma-Rus et al. 2003, 2004), forcing the selection of an alternative splice site in order to prevent the synthesis of pathogenic transcripts (Mercantante and Kole 2002), preventing the inclusion of an aberrant cryptic exon inserted into the mRNA due to a deep intronic mutation (Du et al. 2007; Rincon et al. 2007; Pros et al. 2009; Rodriguez-Pascau et al. 2009; Vega et al. 2009), and inducing the elimination of in-frame exons that contain a pathogenic mutation (Aartsma-Rus et al. 2003, 2004; reviewed in Perez et al. 2010).

Deep intronic mutations are ideal targets for AON function because they are located within intronic regions, leaving bona fide splice sites intact. Steric blockage by AONs of a newly created splice site prevents the splicing machinery from recognizing the cryptic exon and promotes normal splicing.

13.2.1   Types of AON and Delivery Vehicles

The development of AON technology as a successful means of applying antisense therapy had to overcome a range of obstacles and continues to face several technical challenges, some related to AON stability and cellular delivery.

To avoid degradation by cellular nucleases, different analogs have been used for AON design, including phosphorodiamidate morpholino oligomers (PMO) (Summerton 1999), locked nucleic acids (LNA) (Koshkin and Wengel 1998), peptide nucleic acids (PNA) (Larsen et al. 1999), and 2′-*O*-methyl phosphor-othioate (2′OMe) (Manoharan 1999). All have shown stability against degradation, high target affinity, and good biological activity (reviewed in (Kurreck 2003). Antisense phosphorodiamidate morpholino oligomers (PMOs) have been used successfully for splicing modulation in neurofibromatosis type 1 (Pros et al. 2009; Fernandez-Rodriguez et al. 2011). PMOs are analogs of oligonucleotides with a six-membered morpholinoring, replacing the ribose or deoxyribose backbone, and uncharged phosphorodiamidate intersubunit linkages. In addition to high binding specificity, stability, and resistance to nucleases, PMOs exhibit highly durable activity (Summerton and Weller 1997). Due to these advantageous properties, PMOs have been used for therapeutic purposes in several disorders, including β-thalassemia (Lacerra et al. 2000; Suwanmanee et al. 2002), Duchenne muscular dystrophy (McClorey et al. 2006), Hutchinson–Gilford progeria syndrome (Scaffidi and Misteli 2005), ataxia-telangiectasia (Du et al. 2007), and propionic and methylmalonic acidemias (Rincon et al. 2007).

Another important aspect in AON technology is the delivery of the antisense oligomers into target cells. Initially, different techniques were developed to transfect in vitro cell cultures, including electroporation, liposomes, cationic polymers, and other endosomal escape reagents (Thierry et al. 2003; Merdan et al. 2002). However, for AON delivery in vivo, these procedures were characterized by low efficiency and inadequate levels of toxicity. In this context, modification of AON ends to facilitate cellular delivery has been found to be a more effective solution than the use of unmodified forms. Two promising examples are Morpholino oligos linked to cell-penetrating peptides (PPMOs) and Vivo-Morpholinos (Moulton and Jiang 2009). PPMOs are typically an arginine-rich cell-penetrating peptide linked to a Morpholino oligo. Cell-penetrating peptides offer two advantages over unmodified Morpholinos: enhanced uptake into endosomes and enhanced endosomal escape (Abes et al. 2008). Vivo-Morpholinos are eight guanidinium groups on a dendrimeric scaffold linked to a Morpholino oligo (Li and Morcos 2008) that facilitates efficient delivery into most mouse tissues (Morcos et al. 2008) and has been used successfully as a therapeutic agent in different animal models (e.g., Osorio et al. 2011).

13.3 Use of Antisense Oligonucleotides to Reverse the Effect of Deep Intronic *NF1* Mutations

As mentioned previously, approximately 2 % of germline *NF1* mutations are deep intronic nucleotide changes that activate or create novel splice sites, causing the pathogenic inclusion of cryptic exons in mRNA (Messiaen and Wimmer 2008; Pros et al. 2008) (summarized in Table 13.1). These mutations are an ideal target for antisense therapies since the bona fide splice sites remain intact, conserving their potential for normal splicing. Next, a summary of the results obtained to date on the use of AONs to reverse the effects of *NF1* deep intronic mutations is presented (Pros et al. 2009; Fernandez-Rodriguez et al. 2011), focusing on the differences between distinct mutations and cell-type specificities, the required doses and duration of AON effects (in our case, PMOs), the mode of action, and the impact on neurofibromin function.

13.3.1 Effects of PMOs Are Mutation-Dependent and Variable Between Cell Types

To test the efficiency of PMO treatment in restoring normal splicing for this type of mutation, samples were obtained from seven patients with four independent *NF1* germline deep intronic mutations (Table 13.1, Fig. 13.1). Primary lymphocyte and fibroblast cell lines were derived from these patients, and specific PMOs were

Table 13.1 Details of the *NF1* deep intronic mutations reported in the literature

Mutation	Intron	mRNA effect	# Patient present study	References
c.288 +2025T>G[a]	3	r.288_289ins288+1917_288 +2024	1	Pros et al. (2008)
c.889 -942G>T	6	r.888_889ins889-931_889- 873	–	Pros et al. (2008)
c.1393-592A>G	10a	r.1392_1393ins1393- 673_1393-597	–	Pros et al. (2008)
c.1527 +1159C>T	10b	r.1527_1528ins1527 +1103_1527+1157	–	Spits et al. (2005), Wimmer et al. (2007), and Pros et al. (2008)
c.1642-449A>G	10c	Not described	–	Jeong et al. (2006)
c.3198- 314G>A[a]	19a	r.3197-3198ins3198-214- 3198-312, r.3197- 3198ins3198-245-3198- 312	7	Fernandez-Rodriguez et al. (2011)
c.5749 +332A>G[a]	30	r.5749_5750ins5749 +155_5749+331	2, 3	Perrin et al. (1996), Ars et al. (2000), and Wimmer et al. (2007)
c.5750- 2792A>G	30	r.5749_5750ins5750- 278_5750-108	–	Raponi et al. (2006)
c.7908-321C>G[a]	45	r.7907_7908ins7908- 322_7908-391	4[b], 5[b], 6	Pros et al. (2008)

Sequence changes are described at the cDNA level (indicated by "c.")

The +1 nucleotide corresponds to the A of the ATG translation initiation codon in the reference sequence NM_000267.2

[a]Mutations in which PMO treatment has been performed.

[b]Patients from the same family.

designed to block the effects of mutations causing the inclusion of cryptic exons in the *NF1* gene. Three of the studied mutations generated a cryptic donor splice site (c.288+2025T>G, c.5749+332A>G and c.7908-321C>G), whereas the fourth generated a cryptic acceptor site (c.3198-314G>A). All mutations used a wild-type counterpart to insert a cryptic exon into the mature mRNA (Fig. 13.1), which either led to the generation of out-of-frame transcripts susceptible to degradation by the NMD machinery or produced a truncated form of neurofibromin. All PMOs were designed, synthesized, and purified by Gene Tools (Philomath, OR). For one of the mutations (c.3198-314G>A), PMOs were also designed to block the recognition of the two wild-type counterpart sequences used as donor sites for cryptic exon inclusion (Fig. 13.1). Optimal conditions for PMO treatment were established using fibroblasts, and, when possible, PMO treatment was subsequently studied using EBV-transformed lymphocytes.

To determine the effect of PMO concentration on aberrant splicing correction, a dose–response experiment was performed for different mutations, in which
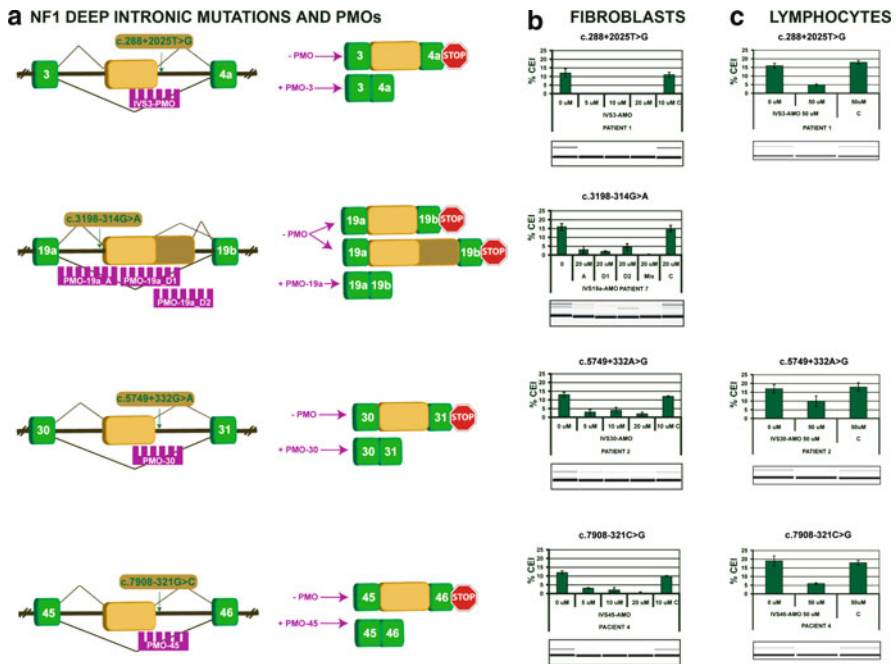
Fig. 13.1 Panel (**a**): Schematic representation of the four mutations examined in the present study and the antisense morpholino designed to treat them. Panel (**b**): Correction of aberrant splicing by PMO treatment of fibroblast cultures. RT-PCR analysis of total RNA was performed using specific primers to analyze both transcripts (wild-type and cryptic exon-containing transcripts). The *y*-axes of each graph show the proportions of cryptic exon-containing transcripts vs. the total. The data are represented by a bar consisting of the mean ± SD for at least three independent experiments. The corresponding Agilent electrophoresis gel is shown below each graph. For mutations in introns 3, 30, and 45, a dose response after 24 h of treatment using different PMO concentrations (5, 10, and 20 μM) is shown. Controls for PMO specificity (C) were as follows: IVS30-PMO, IVS45-PMO, and IVS3-PMO for c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G mutations, respectively. For mutation c.3198-314G>A (intron 19a), PMO treatment is shown using three different antisense oligonucleotides and the sum of all of three. Correction is observed in all cases and is complete when a mixture of the three is used. *CEI* cryptic exon inclusion, *WT* wild-type. Panel (**c**): PMO treatment in EBV-transformed lymphocytes cell lines for mutations c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G. EBV-immortalized lymphocytes were treated at 72 h with 50 μM PMO. Controls for PMO specificity (C) were: IVS30-PMO, IVS45-PMO, and IVS3-PMO for c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G mutations, respectively. *CEI* cryptic exon inclusion, *WT* wild-type

fibroblasts were treated with three different PMO concentrations (5, 10 and 20 μM) for 24 h (Fig. 13.1). In the absence of PMO, the percentage of aberrantly spliced transcripts (containing the cryptic exon) was approximately 10–15 % of the total *NF1* mRNA (wild-type + aberrantly spliced transcripts). As envisaged, the low percentage of cryptic exon-containing *NF1* mRNAs was the result of partial degradation by the NMD machinery and the production of wild-type transcripts from the mutated allele, due to partial recognition of bona fide intron–exon

boundaries by the splicing machinery. When cells were treated with the specifically designed PMO, either complete correction or a dose-dependent correction of aberrant splicing was observed, depending upon the mutation. 20 µM was found to be the optimal concentration of PMO for most mutations tested and was therefore used for the remaining mutations and experiments (Fig. 13.1). The response to PMO treatment was clearly mutation-dependent. By contrast, cells from different patients carrying the same *NF1* mutation (e.g., c.5749+332A>G and c.7908-321C>G) exhibited similar results upon addition of PMO (Pros et al. 2009).

To evaluate the effect of time on mutation correction after Morpholino treatment, a time course was performed for three of the mutations using fibroblast cell lines (Pros et al. 2009). In general, the efficiency of *NF1* splicing correction in fibroblasts after 24 h of treatment ranged from 87 to 100 % for the different mutations.

Several factors could account for the variance in PMO activity between the mutations studied, for example, differences in the strength of the cryptic acceptor/ donor splice sites or variations in the extent to which PMO accesses the pre-mRNA secondary structures. Different strategies could be used to enhance the PMO-dependent restoration of normal splicing for these mutations, including the following: designing a different PMO for blocking the mutation site; using a combination of Morpholinos, one directed at the specific mutation site and the other blocking the wild-type cryptic site used as a counterpart; using a Morpholino to target only the latter; designing Morpholinos to target exon splicing enhancer (ESE) elements. Reports have indicated that the use of PMOs may, in some cases, be more efficient for one of the cryptic splice sites than the other (Du et al. 2007). In this sense, our results with mutation c.3198-314G>A, in which three PMOs were designed to block all of the cryptic splice sites used, indicated that although each PMO was able to reduce the levels of mutant transcripts, complete correction was only observed when a combination of the three PMOs was used (Fig. 13.1), as has also been described for other genetic disorders (Gurvich et al. 2008).

To gain insight into the cell-type specificities of Morpholino treatment, in addition to fibroblasts, transformed lymphocytes derived from the same patients were also analyzed. The highest degree of normal splicing restoration (although not complete) in EBV-transformed lymphocyte cell lines was observed at 72 h, following treatment with 50 µM PMO (Fig. 13.1). Lower concentrations of Morpholino were also tested but were found to be less effective. In general, a lower degree of aberrant splicing correction was observed in transformed lymphocyte cell lines (30–70 % depending upon the mutation), and a higher concentration of Morpholino, together with a longer exposure time, was needed to produce similar effects to those observed in fibroblasts. The differences between the effects observed in fibroblasts and lymphocytes could be explained by the inherent difficulty of transfecting lymphocyte cell lines (Galletti et al. 2007; Seiffert et al. 2007). The authors of another comparative study, using electroporation to deliver Morpholino into cells, achieved similar transfection efficiencies in dermal fibroblasts and B-lymphocyte cell lines. However, different electroporation protocols were required for the two cell types. It was shown that a higher number of electroporation cycles and a higher

Morpholino concentration were needed to achieve the same efficiency in lymphocytes as in fibroblasts (Scaffidi and Misteli 2005). The different conditions required for the two cell types were consistent with findings presented by other groups using the same delivery systems in lymphocytes (Du et al. 2007) and fibroblasts (Rincon et al. 2007).

13.3.2 Effects of PMOs Are Durable, Specific, and Impact on the Function of Neurofibromin

The parallel use of a different control PMO in all of the assays revealed the specificity of the mutation-specific PMOs (Fig. 13.1). Only mutation-specific PMOs had a corrective effect on aberrant splicing in all cases, indicating that the effect of PMO treatment was sequence-specific. The stability and duration of the effect of PMO treatment on aberrant splicing correction were assessed in primary fibroblast cell cultures. Although differences were observed for distinct mutations, near-complete correction was maintained for 20 days after PMO administration in all cases (Pros et al. 2009).

The mode of action of Morpholinos on *NF1* mutations was also investigated. To rule out the possibility that the decreased aberrant transcript levels after PMO treatment was caused by an enhancement of the NMD pathway, fibroblasts from different patients were treated with puromycin (an NMD inhibitor) in the presence or absence of PMO. A total or considerable correction of aberrant splicing after PMO treatment (depending on the mutation) was observed in the presence of puromycin, which indicated that the PMOs acted directly on *NF1* splicing, independently of the NMD mechanism (Pros et al. 2009). Moreover, an increase in wild-type transcript levels (relative to control genes) was observed after PMO treatment, which confirmed that PMO induced correct splicing by blocking the recognition of the new splice site created by the mutation (Pros et al. 2009). Taken together, these results indicate that PMO acts in a sequence-specific manner by preventing the splicing machinery from recognizing the newly created aberrant splice sites in the *NF1* gene.

Finally, since neurofibromin function cannot be assessed directly, an indirect functional analysis was performed to confirm that correction of aberrant splicing by PMO treatment was also attained at the functional level. Since neurofibromin is a well-characterized negative regulator of Ras, Ras-GTP levels in patient-derived primary fibroblast cultures were measured. Fibroblast cultures carrying four different deep intronic mutations were evaluated by comparing Ras-GTP levels before and after specific PMO treatment. Cell lysates were prepared, and a Ras activation assay was performed (Fig. 13.2). Levels of active Ras-GTP in untreated fibroblast cultures from all patients were higher than the levels found in the wild-type control fibroblasts, which was consistent with the observation of lower neurofibromin activity in mutant fibroblasts. However, Ras-GTP levels of mutant fibroblasts
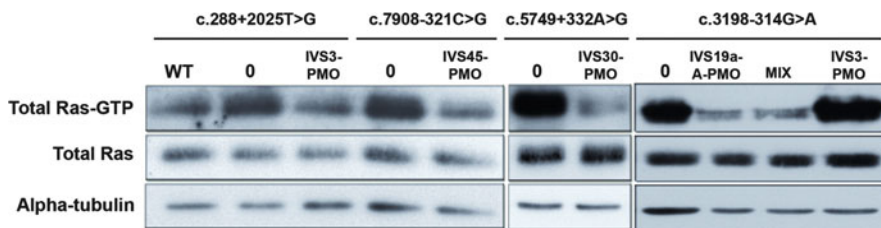
Fig. 13.2 Reduction of Ras-GTP levels after PMO treatment of patient fibroblasts. Neurofibromin GTPase activity was indirectly assessed by quantifying Ras-GTP levels in cell lysates. In all cases, mutation-specific PMO treatment decreased active Ras to levels comparable to those of WT control fibroblasts, presumably by restoration of neurofibromin function. The use of a nonspecific PMO for mutation g.3198-314G>A (IVS45-PMO 20 μM) showed no effect on the levels of Ras-GTP

decreased significantly with PMO treatment, reaching levels comparable to those of wild-type control fibroblasts. This result not only suggested that PMO treatment corrected the aberrant splicing but also indicated that this correction led to the restoration of WT neurofibromin, increasing its overall GAP activity toward Ras proteins.

13.4   AON Therapeutics: From Bench to Bedside

No definitive conclusions have been reached about the in vivo applicability of AON technology for the treatment of NF1 patients with deep intronic mutations; to date, no preclinical animal model or a clinical trial has been performed using this antisense technology for NF1. However, the success of recent clinical trials using AON-directed exon 51 skipping in patients with Duchenne muscular dystrophy (DMD) has created reasonable expectations of eventual success (trials are currently being extended to other exons) (reviewed in Muntoni and Wood 2011; van Putten and Aartsma-Rus 2011). The path from bench to bedside using AON technology is subject to the step-by-step fulfillment of certain requirements, as is the case for any therapeutic agent to treat human disease. The steps comprise in vitro proof of concept followed by the successive phases of clinical trials. The best example of AON technology as a therapeutic strategy is DMD. First, a proof of principle was obtained using cultures of healthy and patient-derived primary human myoblasts (Aartsma-Rus et al. 2002, 2003, 2004). A mouse model of DMD, *mdx* mice, was used for in vivo preclinical trials using different AON chemistries (Goyenvalle et al. 2010; Heemskerk et al. 2009; Wu et al. 2008; Yin et al. 2009). Two exploratory clinical trials using AONs were performed, with the aim of correcting the reading frame of mRNAs encoding a truncated form of dystrophin and producing transcripts similar to those found in patients with Becker muscular dystrophy (a milder form of the disease). The approaches in both trials consisted in forcing the

skipping of exon 51 of the *DMD* gene. One used 2′OMePS (PRO051) (van Deutekom et al. 2007) and the other morpholino oligomers (AVI-4658) (Kinali et al. 2009). In both cases, the direct injection of AON into the muscle corrected significant levels of dystrophin expression, proving the feasibility of the technology without generating clinically important side effects. In a recent phase, I–IIa clinical trial, systemic administration by subcutaneous injection of PRO051 was found to correct dystrophin expression, with no serious adverse events and a modest improvement in the walking capacity of treated patients, although with considerable variance between patients (Goemans et al. 2011). The first systemic clinical trial with PMOs also showed satisfactory levels of dystrophin restoration after treatment (Cirak et al. 2011). More clinical trials for DMD are currently in progress, including phase III trials and some studies involving other exons of the *DMD* gene (Muntoni 2010; van Putten and Aartsma-Rus 2011). For other muscular dystrophies and other diseases, AON therapy is at different points on the path from bench to bedside (Muntoni 2010; van Putten and Aartsma-Rus 2011).

Certain challenges remain in the development of AON therapies (Goyenvalle et al. 2010), such as ensuring efficient AON delivery to all affected tissues or even directing tissue-specific targeting. The first AON chemistries used in clinical trials exhibited poor cellular uptake and a relatively rapid clearance from the circulation, requiring repeated administration. However, recent developments using cell-penetrating peptides conjugated to PMOs (PPMO) or Vivo-Morpholinos have addressed most of these delivery issues, representing a major step forward and revealing what could prove to be an effective strategy, although with room for improvement (Moulton and Jiang 2009; Muntoni 2010).

There are certain limitations to the development of AON therapies, mainly as a result of regulatory constraints and safety considerations. Data collected from the first DMD clinical trials are encouraging, but the number of patients involved has been low in number, the final dosing regimens need to be properly established, and data for treatments longer than 1 year must be provided. It has not yet been confirmed that long-term AON treatment is safe and improves the DMD patient's quality of life, so a cautious view should be taken for now. Given that each AON is mutation-specific (or exon-specific, in the case of forcing skipping), one of the *caveats* of clinical trials using this technology is the low number of patients that could be recruited, since a personalized AON approach will be required for most diseases. Under current regulations, each AON is considered a new drug, so each AON targeting a different exon or mutation would have to undergo the many different steps of clinical development, with all the costs that this process entails (Muntoni 2010). To overcome this hurdle, and since AONs of a certain chemical class generally show more similarities than differences, the same type of chemically identical AONs should be treated as a single drug class, irrespective of the mutation or exon involved, as this would enable sufficient clinical data to be accumulated to develop the technology as a valid therapy for deep intronic mutations and other RNA mis-splicing diseases.

Much work remains to be done in the case of NF1, starting with preclinical animal models. If successful, the foundations provided by DMD AON therapeutics

may facilitate clinical trials with NF1 patients, potentially leading to reasonable treatment opportunities for NF1 patients with deep intronic alterations causing missplicing and possibly also for patients with other AON-treatable *NF1* mutations.

13.5   Conclusions

Some NF1-causing mutations are located well inside intronic regions of the *NF1* gene. These deep intronic mutations lead to the inclusion of a cryptic exon in the *NF1* mRNA, eventually generating an aberrant neurofibromin. We reviewed a body of results that prove the effectiveness of PMOs in correcting abnormal splicing produced by these mutations in the *NF1* gene. Lymphocyte and fibroblast primary cultures derived from seven NF1 patients, bearing four independent *NF1* mutations, were tested. In all cases, PMOs restored the correct splicing of the gene and neurofibromin function, indirectly evaluated by the capacity to downregulate Ras-GTPase. Overall, these results represent a proof of concept for the in vitro capacity of PMOs to correct the effect of the NF1-causing mutations examined. The success of current clinical trials using AON technology to treat DMD patients should spur the development of NF1 preclinical animal models. Deep intronic mutations account for approximately 2 % of all *NF1* constitutional mutations reported to date. AON technology could be applied to patients carrying this type of mutation and perhaps also other types of mutation that generate aberrant *NF1* transcripts.

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