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Controlling morpholino experiments: don't stop making antisense

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One of the most significant problems facing developmental biologists who do not work on an organism with well-developed genetics – and even for some who do – is how to inhibit the action of a gene of interest during development so as to learn about its normal biological function. A widely adopted approach is to use antisense technologies, and especially morpholino antisense oligonucleotides. In this article, we review the use of such reagents and present examples of how they have provided insights into developmental mechanisms. We also discuss how the use of morpholinos can lead to misleading results, including off-target effects, and we suggest controls that will allow researchers to interpret morpholino experiments correctly.

Introduction

To understand the molecular basis of early development, developmental biologists have long wished for techniques that allow the experimenter specifically to inhibit the functions of particular genes in particular cells at particular developmental stages. That goal has not yet been achieved, although researchers working on mouse embryos are closest to this aim, with their use of targeted mutations and Cre recombinase. Even here, however, there are some difficulties: attempts to interfere with the function of one gene may lead to unwanted side-effects on another (Carvajal et al., 2001), and users of Cre recombinase need to be aware of the potential toxicity of Cre expression (Schmidt-Supprian and Rajewsky, 2007).

But what of other species? There is no doubt that research on other vertebrates and on invertebrates has led to fundamental insights into early development, and that the use of such species can have significant practical advantages over the use of mammalian embryos, in terms of accessibility, cost and time, in addition to their intrinsic interest. In none of these species is it yet possible routinely to carry out specific gene targeting, and although conventional genetic screens are of inestimable value in understanding a particular process, they cannot be guaranteed to target the desired gene nor can they be guaranteed to produce a null mutation. Thus, researchers need a way to inhibit the functions of genes. Dominant-negative approaches have their place, but the best hope, certainly in terms of specificity, lies in antisense RNA technologies. Antisense RNA strategies can be applied not only to 'genetic' invertebrate and vertebrate organisms, but also to 'non-genetic' organisms and to tissue culture cells, and they may be of increasing use in searches for new drugs.

Both RNA interference (RNAi) and morpholino (MO) techniques have had a huge influence on our understanding of gene function in the early embryo, and will continue to do so. But are these techniques too good to be true? All antisense techniques, with the possible exception of those employed by the animal itself, suffer from some off-target effects. These effects can make it difficult to discern how much of an antisense-mediated phenotype results from the knockdown of the gene of interest, and how much results from the knockdown of other genes. Clarifying this issue requires the establishment of stringent controls that are applied across the board. Here, we discuss appropriate controls for antisense technologies for the study of animal development, focussing on controls for MO experiments, and we make recommendations about the use of these controls in zebrafish and frog embryos. These issues have been discussed elsewhere for RNAi experiments in the fruit fly *Drosophila melanogaster* (Dasgupta et al., 2007; Ma et al., 2006) and in mammalian cells (Svoboda, 2007). Some of the controls we recommend are also likely to be important for establishing the use of MOs for other species and for therapeutic applications.

Antisense techniques

The idea of introducing antisense RNA into a cell to inhibit the translation, processing or stability of its endogenous mRNA complement was developed over 20 years ago (Izant and Weintraub, 1984; Izant and Weintraub, 1985). The technique proved successful in inhibiting the translation of exogenous RNA injected into oocytes of the frog *Xenopus laevis* (Harland and Weintraub, 1985; Melton, 1985), as well as in inhibiting the translation of endogenous mRNA (Izant and Weintraub, 1985). And the use of antisense oligonucleotides in the hands of Heasman and Wylie (Wylie and Heasman, 1997) has allowed researchers to study the functions of maternally inherited transcripts in frog oocytes, including those encoded by genes such as *Vg1* and *VegT* (Birsoy et al., 2006; Zhang et al., 1998). In these cases, the antisense reagent interfered with gene function by hybridising to endogenous RNAs and by mediating their degradation via RNaseH (Summerton, 1999). Further studies in the nematode worm *Caenorhabditis elegans* revealed, surprisingly, that double-stranded (ds) RNAs also interfere with endogenous gene function (Fire et al., 1998). This work led to the discovery in animals of families of small, non-coding RNAs that regulate many, if not all, aspects of gene expression (Farazi et al., 2008; Mendes Soares and Valcarcel, 2006), and to a Nobel prize for the discoverers of dsRNA-mediated interference (Couzin, 2006).

At least in the frog, however, with just a few exceptions (Li and Rohrer, 2006; Lombardo and Slack, 1997; Nakano et al., 2000; Steinbeisser et al., 1995), the use of conventional antisense RNA, antisense oligonucleotides, or RNAi has not met with success in understanding the roles of zygotically expressed genes (Heasman, 2002). Similarly, in the zebrafish *Danio rerio*, antisense RNA has been shown to have widespread effects that are sequence independent, preventing the practical application of this approach to the study of specific gene functions during development (Oates et al., 2000). It remains possible that new approaches will make techniques such as RNAi feasible in these species, but for now they do not seem to be practicable.

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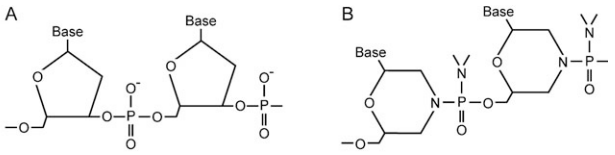

Fig. 1. Structures of conventional DNA and morpholino oligonucleotides. (**A**) Conventional DNA oligonucleotide. (**B**) Morpholino oligonucleotide. Note the six-membered morpholino ring in B and the non-ionic phosphorodiamidate link between the two rings.

These problems in testing gene function delayed the field until early this century, when it was shown, with some fanfare [Editorial, *Nature Genetics* 26(2), 129-130], that MOs could be targeted to knockdown specific gene expression both in frog (Heasman et al., 2000) and in zebrafish (Ekker, 2000; Nasevicius and Ekker, 2000). The power of MOs to test gene function was quickly recognised and applied to other organisms, including another species of frog, *X. tropicalis* (Nutt et al., 2001), the chick *Gallus gallus* (Kos et al., 2001), an ascidian, *Ciona savignyi* (Satou et al., 2001), oocytes of the mouse *Mus musculus* (Coonrod et al., 2001), and the sea urchin,*Strongylocentrotus purpuratus* (Coffman et al., 2004). The importance of MO technology in advancing the field was heralded by an entire issue of the journal *Genesis* [30(3), July 2001], devoted to gene targeting studies employing MOs in a variety of organisms.

MOs are synthetic oligonucleotides composed of chains of about 25 subunits that are similar to DNA and RNA oligonucleotides, except that they have a morpholine ring rather than a ribose ring (Fig. 1). This feature still allows MOs to undergo Watson-Crick base pairing, but it offers significant advantages over conventional oligonucleotides (Corey and Abrams, 2001; Heasman, 2002; Heasman et al., 2000). In particular, MOs are resistant to nucleases and are therefore remarkably stable (see below), and the fact that they do not carry a negatively charged backbone means that they are less likely to interact non-specifically with other components of the cell and may be less toxic as a result.

MOs do not act through an RNaseH mechanism but instead can be designed to inhibit translation (Summerton, 1999) (Fig. 2A), or, as more recent experiments have shown, to prevent the proper splicing of RNA (Draper et al., 2001) (Fig. 2B-E). As we discuss in this article, MOs are now used in a wide variety of applications. For example, a recent study shows that it is possible in principle to use MOs to do the same types of genome-wide screens in vertebrate embryos, such as *X. tropicalis* (Rana et al., 2006), that have been done successfully using RNAi in *C. elegans* embryos (Gonczy et al., 2000; Kamath et al., 2003; Sonnichsen et al., 2005) and in tissue culture cells in *D. melanogaster* (Agaisse et al., 2005; Boutros et al., 2004; Friedman and Perrimon, 2006). This study (Rana et al., 2006) also showed that fluorescent MOs can be used to monitor the distribution of MOs within the embryo. More recently, photoactivatable MOs have been used to provide spatiotemporal control over gene knockdown, raising the possibility of targeting gene perturbations to specific regions of the embryo, perhaps even in individual cells, at particularly relevant stages of development (Shestopalov et al., 2007) (Fig. 2F). Finally, MOs can also be used to block the functions of microRNAs, one of the non-coding RNA families in animals identified as a result of the original dsRNA study. This can be achieved by targeting either the mature microRNA or the microRNA precursor (Kloosterman et al., 2007).

Use of morpholinos in frogs, zebrafish and other organisms

The first descriptions of the use of MOs in developmental biology research were made eight years ago (Ekker, 2000; Heasman et al., 2000; Nasevicius and Ekker, 2000), and since then the research community has adopted them with enthusiasm. The ease of use of MOs, especially in zebrafish and frogs, and the exciting results that have been obtained, have meant that any paper describing a new gene cannot afford to neglect experiments using an MO to knockdown the gene and thus to learn about its normal function. However, an unfortunate side effect of the enthusiasm for MOs is that because proper standards for controls have not been adopted, there are undoubtedly publications in which at least some of the supposedly specific results are due to off-target effects, rather than to the specific knockdown of only the gene(s) of interest. One purpose of this review is to discuss this issue and to suggest a series of standard controls; our suggestions complement and supplement those put forward by Corey and Abrams (Corey and Abrams, 2001).

Translation-blocking MOs

Many investigators use MOs directed against the initiation codon of the target mRNA. This approach is particularly attractive in those (now rather few) model species whose genome sequence is unknown, because one does not need to know the intron-exon structure of the target gene to inhibit its function. The design of MOs intended to inhibit translation has been extensively studied, and the rules for success prove to be relatively simple (see Box 1).

There are so many examples of the use of translation-blocking MOs that it is difficult to select just a few to describe. Here, we mention two examples, one from each of our labs. Both cases were very informative about the function of specific proteins during development. The first example describes a straightforward case, whereas the second example describes a case in which detective work was necessary to solve a mystery.

In the first example, MOs were used to explore the role of Notch signalling in zebrafish neurogenesis. In the absence of Notch signalling, cells that would normally become trunk neural crest develop as supernumerary Rohon-Beard sensory neurons (Cornell and Eisen, 2000), suggesting that Notch signalling is required for the neural crest fate. However, the use of MOs to knockdown Neurogenin 1 (Neurog1), a proneural protein, revealed that Notch

Box 1. Designing and storing translation-blocking MOs
(see also www.gene-tools.com)
- The MO should normally be about 25 bases in length, with a GC content of about 50%, and little or no secondary structure.
- The MO should be designed to be complementary to sequence between the 5′ cap and about 25 bases 3′ of the AUG translation start site. There is a sharp decrease in the efficacy of MOs that are positioned any more 3′ of the translation start site than this.
- It is wise to carry out a BLAST search using the proposed sequence to confirm that it, or a similar sequence, is not present elsewhere in the genome of the species under study.
- It is also wise to re-sequence the MO target in the particular strain under study, to avoid being misled by SNPs or sequencing errors.
- MOs should be stored in aliquots at –20°C. They can be heated to 65°C and then put on ice prior to injection. The concentration of a dissolved MO can be calculated by measuring its absorbance at 265 nm in 0.1 N HCl and dividing by ε, the molar absorbance provided on the MO product information sheet (see www.gene-tools.com).

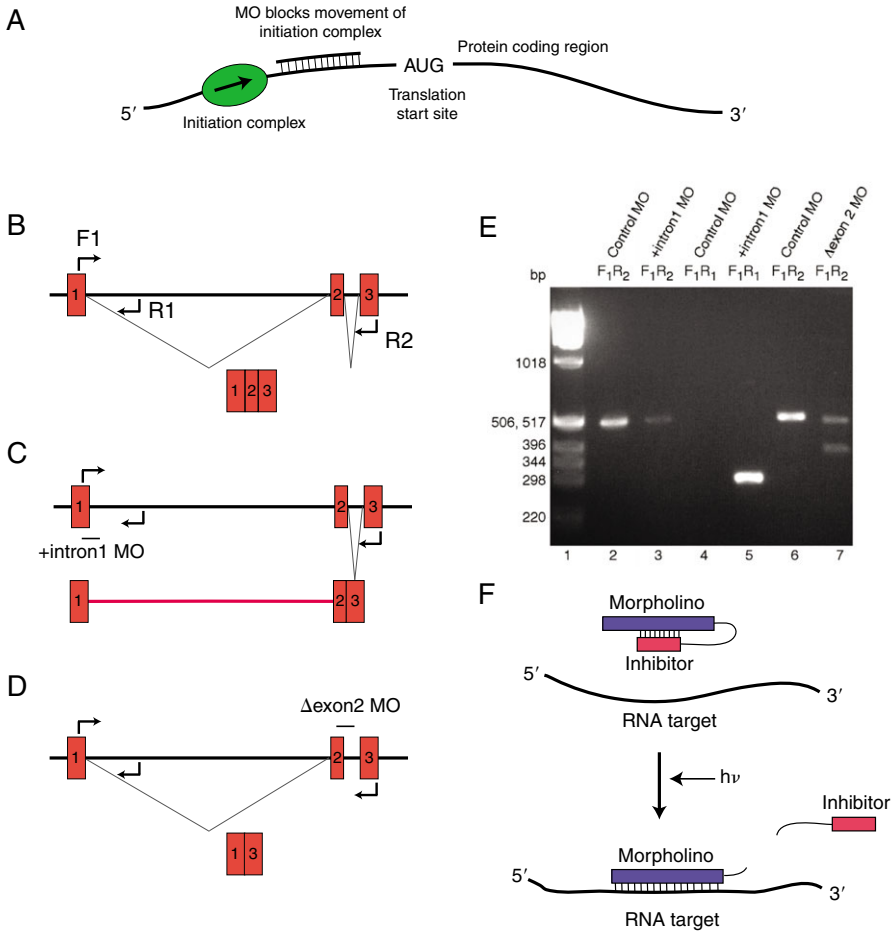

Fig. 2. Different uses of antisense morpholino oligonucleotides. (**A**) Inhibition of translation by an antisense morpholino oligonucleotide. The MO is targeted to sequence 5′ of the translation start site and inhibits progression of the initiation complex. (**B-E**) Use of MOs to prevent correct splicing of *X. tropicalis NRH1*. (B) The first three exons of *NRH1*. Arrows indicate the positions of the forward (F_1) and reverse (R_1, R_2) PCR primers used to test the efficacy of the splicing MOs. Exon 1 is 326 bp, intron 1 is 9013 bp, exon 2 is 134 bp, intron 2 is 740 bp, and exon 3 is 363 bp. If the mRNA is correctly spliced, the combination of F_1 and R_1 should yield no PCR product and F_1 and R_2 should give a product of 490 bp. (C) The +intron1 MO is designed to cause intron 1 to be retained. This would result in the first 15 amino acids of NRH1 being followed by 67 missense amino acids before a stop codon is reached. If intron 1 is retained in this way, the combination of F_1 and R_1 should give a PCR product of 280 bp, and F_1 and R_2 should yield a product in excess of 9.0 kb. (D) The Δexon2 MO is designed to exclude exon 2 from the mature RNA. In this case, exon 3 would no longer be in frame with exon 1, and a truncated protein consisting of 15 correct amino acids followed by 25 missense residues would be formed before a stop codon is encountered. Deletion of exon 2 in this way should cause the combination of F_1 and R_1 to yield no PCR product, and F_1 and R_2 should yield a product of 360 bp. (E) Verification of the efficacy of MOs +intron1 and Δexon2. Comparisons of lane 2 with lane 3, and lane 6 with lane 7, indicate that both MOs reduce levels of correctly spliced transcript by ⩾50%. Note that the predicted 9.5 kb band in lane 4 is absent, probably because the PCR conditions do not efficiently amplify products of this size. (**F**) Activation of a caged morpholino. An inhibitory oligonucleotide is coupled to a specific MO through a photocleavable linker. Irradiation [10 seconds of 360 nm light (hν)] cleaves the linker and allows the MO to exert its effects. B-D are redrawn, and E is reproduced, with permission, from Knapp et al. (Knapp et al., 2006). F is redrawn, with permission, from Shestopalov et al. (Shestopalov et al., 2007).

signalling is required only to prevent neurogenesis, because the absence of both Notch signalling and Neurog1 restored normal trunk neural crest formation (Cornell and Eisen, 2002).

In the second example, MOs were used to investigate the function of activin in the early *X. laevis* embryo. Activin had long been known to have powerful mesoderm-inducing activity in amphibian embryos (Albano et al., 1990; Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990), but its role in normal development was unclear until MOs were used to inhibit its effects (Piepenburg et al., 2004). In the course of these experiments, a MO designed to inhibit translation of activin B caused defects in development, as well as the dose-dependent downregulation of several mesodermal markers. A control MO with four evenly spaced nucleotide changes had no such effect, suggesting that the phenotype was specific. But, to the authors' surprise, no phenotype was observed with a second MO, positioned 5′ of the first, which was predicted to be equally effective in inhibiting activin B translation. Eventually, it was found that the 5′ untranslated region of activin B derived from the *X. laevis* colony maintained in the authors' laboratory differed from that of the published sequence, such that two nucleotides in the second MO did not match the target. When a perfectly matched MO was injected into *X. laevis* embryos, it recapitulated the original phenotype, indicating (but not proving) that the effect is specific. 'Rescue' experiments (see below) also indicated that the MOs used exerted a specific effect, and the authors went on in later experiments to demonstrate that the loss of activin function causes the misregulation of genes involved in cell-cycle regulation (Ramis et al., 2007). In addition to providing new insights into the role of activin B, these experiments make the point that it is important to re-sequence the MO target sequence in the strain of animal under study, to avoid being misled by single-nucleotide polymorphisms (SNPs) or errors in published sequences.

Splice-inhibiting MOs

The difficulty of ensuring that MOs designed to inhibit translation do actually knockdown protein levels and thus have their intended effect (see below) caused researchers to investigate the possibility of using MOs to inhibit pre-mRNA splicing or even to cause exon skipping (Draper et al., 2001; Gebski et al., 2003). Splice-inhibiting MOs have the advantage that one can quantify the efficacy of the MO (Draper et al., 2001). In addition, because such MOs do not affect spliced maternal transcripts they will normally allow genetic mutations to be phenocopied when the gene in question also has a maternal function (Bennett et al., 2007; Gore et al., 2007; Gore et al., 2005). MOs designed to inhibit splicing have often been used to corroborate data obtained with MOs that block translation. As with

translation-blocking MOs, there are now many examples of the use of splice-inhibiting MOs. Thus, we have chosen two examples, one from each of our labs. In the first example, the Eisen laboratory showed that the knockdown of zebrafish Islet1 protein with two different splice-inhibiting MOs, either individually or in combination, results in the same phenotype. In this case, motoneurons in MO-injected embryos develop interneuron-like characteristics, providing evidence that Islet1 functions both to promote motoneuron differentiation and to inhibit the differentiation of some ventral interneurons (Hutchinson and Eisen, 2006).

In the second example, the Smith group investigated the function of neurotrophin receptor homologue (NRH1) proteins in mesoderm formation in frog embryos (Knapp et al., 2006). MOs targeted against the translation start sites of two related *X. laevis* proteins indicated that NRH1 activity is required for expression of *Xbra* and *Chordin* in the early embryo, but that *Goosecoid* expression is less dependent on signalling through this receptor. The downregulation of *Xbra* and *Chordin* was also observed in *X. tropicalis* (a diploid species whose genome has been sequenced), using one MO designed to inhibit the translation of NRH1, and two MOs that prevented its correct splicing. The fact that similar results were obtained in two species, and using both translation-blocking and splice-inhibiting MOs, suggests that the observed effects are specific.

There has been considerable work on the best design for splice-inhibiting MOs (Morcos, 2007). In contrast to MOs designed to block translation, it is not possible to apply general guidelines to the design of splice-inhibiting MOs, because genes differ in their genomic structures and proteins in their functional domains. For example, a truncated version of a protein may act as a dominant-negative or even as a constitutively active form of the wild-type protein, or retention of an intron might, if the mRNA remains in frame, not affect the function of a protein. It is also possible that the use of splice-inhibiting MOs will reveal cryptic splice donor or acceptor sites, resulting in the retention of a short stretch of intron or the deletion of a short stretch of exon. This might cause in-frame insertions or deletions in the protein of interest. The important point, therefore, is to determine the sequence of the modified mRNA, to ask what protein is encoded by it, and, if necessary, to test the function of that protein.

microRNA-blocking MOs

MicroRNAs are short (about 22 nucleotides), non-coding RNA molecules that regulate gene expression by both blocking the translation and promoting the decay of their target mRNAs (Alvarez-Garcia and Miska, 2005; Bushati and Cohen, 2007; Farazi et al., 2008). Various approaches have been used to block the functions of individual microRNAs by means of antisense technologies. The most direct has been to use antisense oligonucleotides that are complementary to the target microRNA, and for these oligonucleotides to be modified in some way to increase their stability. These modifications include the introduction of 2′-*O*-Me-modified nucleotides and phosphorothioate linkages; and, to assist their delivery into liver cells of living mice, such 23-mer RNAs have been covalently linked to cholesterol molecules (Krutzfeldt et al., 2006; Krutzfeldt et al., 2005). Recently, MOs have also been used in such an approach. For example, in zebrafish, MOs complementary to *microRNA-214* (*miR-214*) have revealed that this microRNA regulates expression of *su(fu)*, which encodes a modulator of Hedgehog signalling (Flynt et al., 2007), and MOs complementary to *miR-140* have revealed that this microRNA regulates Pdgf signalling during palate formation (Eberhart et al., 2008). In *X. laevis*, the activities of *miR-15* and *miR-16* have been inhibited by the use of 2′-*O*-methylantisense oligonucleotides and of MOs to reveal that these microRNAs target the type II Nodal receptor Acvr2a (Martello et al., 2007).

In an intriguing alternative approach, Schier and his colleagues have 'protected' the two zebrafish *miR-430* targets *ndr1* (also known as *squint*; this gene encodes a Nodal-related signalling molecule) and *lefty* (a Nodal antagonist) by introducing 'target protectors', MOs that are complementary to the microRNA binding sites. Protection of the *ndr1 miR-430* binding site resulted in an elevation of Nodal signalling, and protection of the *lefty* site caused a diminution (Choi et al., 2007). This result illustrates both the utility of MOs in endeavours of this sort, as well as the roles of microRNAs in modulating levels of Nodal signalling.

Problems with MOs

For all these successes, there are difficulties with the MO technique, and there have undoubtedly been some misleading results. The main difficulties in interpreting experiments fall into three classes (see Box 2). First, it is important to know how effective the knock-down has been, and, second, the possibility of 'off-target' effects has to be addressed; that is, the possibility that the MO inhibits the function of an irrelevant gene instead of, or in addition to, the intended gene. As discussed below, in addressing these problems, it is very helpful if the genome of the species under investigation has been sequenced and well annotated. The third problem is that it can be difficult, especially in small embryos, to inject precise and reproducible volumes of MO.

It is also important in interpreting experiments that make use of antisense oligonucleotides to consider the mechanisms by which the reagent of choice acts. For example, RNAi leads to the destruction of mRNA by RNase H activity, whereas MOs interfere only with translation or with pre-mRNA splicing. In all cases, if the experiment is designed properly, the protein that is encoded by the mRNA should be knocked down by the antisense reagent. However, the mRNA may or may not persist, depending on the antisense strategy that is used, and, in some cases, mRNA levels may even increase, as the embryo attempts to compensate for loss of the protein. In many cases, this may be of no importance, but if the RNA has functions instead of, or in addition to, its role as mRNA, changes in its level may be very significant indeed. For example, the knockdown of *VegT* mRNA causes a decrease in VegT protein, but it also causes the release of *Vg1* mRNA from the vegetal cortex of *X. laevis* embryos and a decrease in the amount of Vg1 protein (Heasman et al., 2001). *Bicaudal C* and *Wnt11* mRNAs also become dispersed from the vegetal cortex. This phenomenon is observed only if *VegT* mRNA is destroyed; MOs designed to inhibit VegT translation but not to degrade *VegT* RNA have no effect on RNA localisation (Heasman et al., 2001). In this particular case, 'rescue' experiments, in which *VegT* mRNA is injected into the oocyte in an effort to replace the depleted transcripts, suggest that the release of *Vg1*, *Bicaudal C* and *Wnt11* mRNAs from their vegetal position

Box 2. Three potential problems with the use of MOs

- It can be very difficult to estimate the efficacy of MOs without a good antibody.
- It is very difficult to rule out the possibility that the MO inhibits the function of an irrelevant gene, as well as, or instead of, its intended target.
- Especially in smaller embryos, it can be very difficult to inject precise and reproducible volumes of MOs.

does not compromise development significantly, because such embryos still form all three germ layers. In other cases, however, the phenotype of embryos injected with a particular antisense RNA or MO may indeed depend on whether or not the target mRNA persists.

Effectiveness of gene knockdown

As discussed above, many investigators use MOs targeted against the initiation codon of the target mRNA. Simple as this approach might be, it suffers from the fact that it is not possible to determine, without a suitable way to test for the protein, how effectively the protein has been knocked down. If an antibody is available that works well in vivo, it can provide a high-resolution view of protein expression, even at the single-cell level, and such antibodies can reliably report the downregulation of protein in cells of MO-injected embryos (Hutchinson and Eisen, 2006). However, the antibody might not work well in vivo, in which case it might be necessary to use western blotting. Unfortunately, a significant drawback of this technique is that the sensitivity might be such that one can determine effectiveness only on a rather large population of animals. Similarly, although it might be possible to test for the presence of a protein using an enzyme assay or a protein gel, these approaches are likely to require a large number of animals and may not give the resolution required for the experiment.

The determination of knockdown efficiency is clearly an essential step. A common alternative is to express a form of the target protein that is tagged, for example with the influenza haemagglutinin (HA) epitope or green fluorescent protein (GFP), and to show that the MO can knockdown expression of the tag in question (Collart et al., 2005). Such an experiment is better than nothing – it demonstrates at least that the MO can recognise its target mRNA in the cytoplasmic milieu – but it cannot confirm that the endogenous protein is targeted, nor can it predict the extent to which expression of the endogenous protein is inhibited. It is therefore of the greatest importance to obtain a high-affinity specific antibody for the gene product under study. This might prove to be difficult and expensive, but there is no doubt that such an antibody will be an essential research tool.

MOs designed to inhibit splicing have the great advantage that one can determine, by PCR (polymerase chain reaction), how effective the reagent has been, although it is important to note that a 50% reduction in mRNA levels does not necessarily lead to a 50% reduction in protein levels! However, incorrectly spliced pre-mRNAs often remain in the nucleus and their nuclear localisation can be visualised using RNA in situ hybridisation (Hutchinson and Eisen, 2006), providing another measure of MO efficacy.

In the case of MOs designed to knockdown particular microRNAs, their efficiency can be estimated by northern blots or by RNA in situ hybridisation. For example, Plasterk and colleagues have investigated the function of *miR-375* in zebrafish and shown that this microRNA is necessary for normal formation of the pancreatic islet (Kloosterman et al., 2007). In the course of control experiments, an MO complementary to mature *miR-206* was injected into zebrafish embryos and, at intervals thereafter, embryos were subjected to RNA in situ hybridisation and northern blotting. Both techniques revealed a significant reduction in levels of *miR-206* that was specific in the sense that levels of another microRNA, *miR-124*, were unaffected by the *miR-206* MO. Additional experiments indicated that this decrease in *miR-206* levels did not occur because the MO interferes with the RNA isolation procedure, nor because MOs affect microRNA stability. Rather, the MOs inhibit microRNA maturation. Consistent with this suggestion, MOs targeting the cleavage sites of the Drosha and Dicer enzymes that process precursor microRNAs also caused a loss of *miR-206* (Kloosterman et al., 2007).

Although MOs are remarkably stable (Nutt et al., 2001; Summerton, 2007), their levels may nevertheless fall slightly as development proceeds, and, although zebrafish and frog embryos do not increase in volume during development, numbers of nuclei increase dramatically, so that the intranuclear concentration of MOs designed to inhibit splicing may decrease significantly. Thus, it cannot be assumed that a MO that is effective for the first few hours or days following injection will remain so over a longer period of time. It is therefore essential to assay the efficacy of MOs over the entire time course of an experiment. If the efficacy of a particular MO is inadequate at later stages of development, it may be possible to use iontophoresis (McWhorter et al., 2003) or electroporation (Thummel et al., 2006) into individual or small groups of cells to supplement MO levels.

Off-target effects of MOs

A more insidious problem with the use of MOs, including those targeted to the initiation codon and those designed to inhibit splicing, is that they may have 'off-target' effects. In other words, they might affect the production of a completely irrelevant gene product, and the observed phenotype may be only partially the result of, or in the worst case have nothing to do with, the gene under study. Two published examples of such off-target effects come from work on sea urchins and zebrafish. Unfortunately, there are likely to be many other cases, many yet to be recognised, in which phenotypes caused by MOs are non-specific.

In the sea urchin example, embryos injected with either of two MOs directed against the *S. purpuratus Runt* gene, *SpRunt*, caused early developmental arrest, whereas injection of either of two other MOs produced gastrula stage defects (Coffman et al., 2004). The first two MOs were the more effective at reducing levels of SpRunt protein, but, remarkably, they also targeted two different histones, thus causing the early phenotype. One MO, designated m1 and designed to inhibit the translation of SpRunt, inhibited the synthesis of histone H3, whereas another, designated m4 and designed to interfere with the splicing of *SpRunt* mRNA, inhibited the translation of histone H4. It is to the credit of the authors that they carried out meticulous controls in their experiments (see below), and it is sobering to realise that in an MO of 25 nucleotides, just 18 (in the case of m1) or 20 (in the case of m4) need match a target sequence for inhibition of protein synthesis to occur. As the authors point out, *S. purpuratus* embryos develop at 15°C, so the 'stringency' of hybridisation is lower in sea urchins than in some other species, but the point is clear nevertheless. We note that *X. laevis* embryos can be cultured between 14°C and 24°C. Thus, until someone carries out an experiment to directly test the effectiveness of the same MO in embryos raised at different temperatures, to circumvent the potential problem of the relationship between 'stringency' and temperature, it may be sensible for embryos injected with MOs to be incubated closer to the higher temperature.

In the zebrafish example, researchers in Julian Lewis's laboratory were studying the interaction between the membrane-associated scaffolding protein Magi1 and the Notch ligand DeltaD (Wright et al., 2004). Two MOs, one designed to interfere with splicing of the *magi1* mRNA and one directed against its translation start site, caused a narrowing of the hindbrain and midbrain of the embryo. This phenotype appeared specific, because it was not observed when the researchers used a version of the translation-blocking MO that had five nucleotide changes. However, this phenotype had been observed previously as a non-specific effect of MOs (Ekker and Larson, 2001), and 'spurred on by the doubts of a [*Development*]

Box 3. A summary of our recommendations

Comparison with existing mutant. Should a mutation in the gene of interest be available, a careful comparison should be made between the phenotype of the mutant and that of morpholino-injected embryos, so as to reveal any off-target morpholino effects.

Loss of protein. Whenever possible, the effect of each morpholino on protein levels should be verified by antibody staining or other assays.

Incorrectly spliced pre-mRNA. A splice-inhibiting morpholino should be used for every target gene that has more than one exon. The effect of splice-inhibiting morpholinos should be verified by RT-PCR; the altered splice products should be verified by sequencing, and where necessary the function of the modified protein thus encoded should be investigated. In addition, RNA in situ hybridisation should be used to determine whether unspliced pre-mRNA is retained in the nucleus.

Minimisation of off-target effects. At least two morpholinos should be designed against each target gene; it is best to have both a translation-blocker and a splice-inhibitor. These morpholinos should be tested independently to ensure that they give similar phenotypes, and also simultaneously to test for synergism. The latter will involve titrating these morpholinos down to a level at which they yield no phenotype, co-injecting them into embryos, and showing that together they yield the same phenotype as they yield independently. It is important to ensure that injection volumes are consistent in such experiments.

RNA rescue. Co-injection of a form of the targeted RNA that is not recognised by the morpholino should be used to show that the effects of the morpholino are specific. In the case of morpholinos designed to interfere with splicing, the rescuing RNA can be the wild-type version; in the case of morpholinos designed to interfere with translation, the rescuing RNA should be made 'immune' to the morpholino in question, either by removing the appropriate regions of the 5′ untranslated region or by creating silent mutations in the coding sequence.

Control morpholino. A control morpholino should always be used. This could be the standard control morpholino, a morpholino that affects a gene that is not expressed in the cells of interest, or a mismatch, scrambled or invert morpholino. We favour a five-base mismatch morpholino because this is most similar to the experimental morpholino, but one or more of the other control morpholinos may be more appropriate under some conditions.

referee', the authors went on to demonstrate that *deltaD* mutant embryos did not show the narrowed-ventricle phenotype, but that it did occur when these embryos were injected with the Magi1 MOs described above. Thus, it appears that the Magi1 MOs cause a phenotype that is robust and consistent, but nevertheless non-specific.

The non-specific neural defects observed in the above experiments, and in 15-20% of all MO experiments carried out in zebrafish, are likely, as explained below, to derive from the activation of p53 and consequent p53-induced apoptosis (Robu et al., 2007). The mechanism by which some MOs activate p53 is unknown, and it is also a mystery that MOs activate predominantly a truncated form of p53 (the D113 isoform), whereas it is the full-length version that is required for the induction of cell death. Nevertheless, a MO that targets the translation start site of p53 is effective at relieving non-specific neural cell death in response to injection of some MOs (Robu et al., 2007), and this is likely to prove a powerful adjunct to MO experiments in the zebrafish embryo. The caveat here is that it may be difficult to use MOs to study developmental processes that involve p53-mediated cell death.

It is also worth noting that not all cell death in experiments using MOs is non-specific. For example, a reduction in Cytochrome C oxidase activity caused by MO injection results in cell death in the hindbrain and neural tube, but this cannot be reduced by co-injection of a p53 MO (Baden et al., 2007).

These examples should suffice to illustrate the potential hazards of using MOs in the study of early development. To what extent can use of the right controls allow researchers to distinguish between bona fide and spurious results?

Appropriate controls

Given the potential problems described above, how can investigators ensure that the phenotype they observe is specific, that it is the genuine consequence of the loss of function of the gene under investigation? We suggest several approaches and we recommend that they become standard controls for the use of MOs (see Box 3). We recognise that some of the controls we recommend will not be appropriate or will not work in every case. However, we suggest that researchers use as many controls as possible, so as to provide the strongest evidence for MO specificity.

The right control construct

The most obvious strategy to control MO experiments is to create control animals that are identical to the MO-injected experimental animals in every respect except that the MO injected into the controls should not interfere with the function of the gene being tested. One type of control is a MO that targets an exogenous gene that is not present in the species being tested. For example, the company Gene Tools provides a 'Standard Control' MO directed against human β-globin pre-mRNA (www.gene-tools.com/node/23#standardcontrols). Although this reagent controls for the use of MOs in general, it does not necessarily control for the use of any specific MO, and in particular it does not control for the activation of p53 (see above). Therefore, for some experiments (for example, those where it is not possible to carry out a 'rescue'), it might be preferable to select as a control a MO that more closely resembles the experimental MO. This control MO might be a randomised version of the specific MO, or its invert, or it might differ in a particular number of nucleotides. Unfortunately, there is no consensus on how many nucleotides should differ in the control MO, and the number may not be the same for different target sequences (depending, for example, on how GC-rich they are, on the species under study, or on the culture conditions). As discussed above, it is possible that MOs introduced into species that develop at a lower temperature might recognise their targets even if several nucleotides have been changed.

Our experience has shown that a 25-mer MO that differs in four nucleotides from the experimental MO can have an effect similar to the experimental MO when injected at high concentration into zebrafish embryos, but no effect when injected at a lower concentration (Cornell and Eisen, 2002). Similarly, in *X. tropicalis*, 25-mer MOs that differ in five nucleotides from the target sequence have little effect on translation (Rana et al., 2006). Thus, based on this rather limited information, if the Gene Tools standard control MO is inappropriate, we recommend using five-nucleotide mismatch MOs as a control for the injection process itself, for the introduction of significant amounts of exogenous oligonucleotides, and for the specificity of a particular MO. To our knowledge, no one has published a systematic comparison of the optimal number of nucleotide mismatches under various conditions, and although such a study would be expensive to undertake, it would provide important baseline information for the design of MO controls. In the meantime,

in designing MOs, it would be wise to conduct a BLAST search using the proposed sequence, and to reject the sequence if it differs by fewer than five nucleotides from a sequence near the translation start site of any unrelated gene or indeed near a splice junction of an unrelated gene.

Use of mismatch MOs

We described above that MOs that differ by five out of 25 nucleotides from their target sequence appear not to interfere with translation of the target mRNA, whereas at least some MOs that differ from the target sequence by four nucleotides do interfere, at least when injected at high concentration. It is instructive, and sobering, to ask how likely it is that a 25-mer might recognise a target that differs from the intended one. Theoretical calculations based on the *X. tropicalis* genome sequence indicate that there is a probability of about 0.3 that a particular translation-blocking MO will match another target sequence near a translation start site with four or fewer mismatches (Rana et al., 2006). And if one takes into account the possibility that the MO might instead interfere with mRNA splicing, the chance of off-target effects increases to 0.5. The strength of a phenotype obtained using a MO with three or four mismatches is likely to be relatively weak, but clearly, even if a 'control' MO causes no phenotype, it is still possible that the phenotype observed with the 'specific' MO is an artefact. Therefore, although using a five-nucleotide mismatch MO control is wise, by itself this control is insufficient, and we recommend using additional controls, as described below. We note that, although these calculations were made for *X. tropicalis*, the conclusions will be similar for vertebrate species of comparable genome sizes.

Use of different MOs targeting the same gene

The pessimistic calculations outlined above suggest that for every two MOs introduced into a developing embryo, one will elicit non-specific effects. At first sight this represents an untenable situation: how can such experiments be interpreted? But in practice things are not so bad. The strengths of the off-target phenotypes are likely to be weak, and concerns can be addressed by designing a second, non-overlapping MO against the gene of interest. The chance of both MOs yielding an off-target effect may be 0.25, but the chance of the two off-target effects being the same is considerably lower. Thus, if the phenotypes caused by the two reagents are similar, one can be more confident that they really shed light on the function of the intended target gene. Even more confidence can be gained if the same phenotype is observed when two MOs are injected together at low levels that do not elicit phenotypes on their own. This can be achieved by generating a 'dose-response' curve for each MO, by titrating it down to a level at which it does not elicit a phenotype, and then injecting the two MOs together at these low concentrations (Maves et al., 2002). In carrying out such experiments, it is essential to take great care in controlling injection volumes and to repeat the experiment on at least three independent occasions. These precautions are crucial because a two- or threefold variation in injection volumes in quantitative experiments of this sort may lead to misleading results.

In practice, as shown by the *SpRunt* experiments described above, it might be sensible to use three or even four MOs targeted against the gene of interest. Two of these might be designed to block translation of the protein and two designed to inhibit correct pre-mRNA splicing. The interpretation of such experiments must take into account that different MOs might have different 'penetrances', such that a high dose of one MO yields the same phenotype as a low dose of another. Thus, the experimenter will need to construct a dose-response curve and inject a range of concentrations of each MO to make valid comparisons.

Finally, as an alternative to using different MOs, it may be possible to use closely related species to verify the phenotype produced by a MO. For example, the function of genes such as NRH1 can be tested in both *X. laevis* and *X. tropicalis* (Knapp et al., 2006). Such an approach requires caution, however, and it may be dangerous to make comparisons across different genera. For example, *Fgf8* is necessary for heart development in ray-fin fish and in tetrapods, but in zebrafish it is *fgf8a* that plays this role and in the stickleback *Gasterosteus aculeatus* it is *fgf8b* (Jovelin et al., 2007). This observation suggests that the heart function of the two *fgf8* duplicates was conserved in the common ancestor of zebrafish and stickleback after genome duplication in the ray-fin fish lineage, but that the expression of the two paralogues subsequently diverged. It will be interesting in the future to design MOs to target the different paralogues of the tetraploid species *X. laevis*, to investigate whether they have different functions, and to compare the resulting phenotypes with those observed in the diploid species *X. tropicalis*.

Together, these considerations lead us to suggest that, for any gene, at least two MOs should be designed, with one, if possible, designed to inhibit correct pre-mRNA splicing. Even if antibodies are unavailable, this will allow some quantification of the effect of the MO. We suggest that for each MO a dose-response curve is prepared, and that co-injection of two MOs at below threshold levels should also be carried out in an effort to search for synergy (see Box 3).

Rescue

None of the controls described above is perfect. It is always possible that a MO will have off-target effects, and it will not always be feasible to alleviate these by injection, for example, of a MO targeted against p53. The most reliable control, therefore, is to attempt to 'rescue' the phenotype by introducing the gene product of interest in a form that is immune to the MO, most commonly by injecting RNA at the one-cell stage. For MOs targeted against the translation start site, this might be achieved by removing the 5′ untranslated region of the mRNA or by introducing silent mutations into the coding region. Such a difficulty does not arise for MOs designed to inhibit pre-mRNA splicing. As mentioned above, different MOs may have different penetrances, and this also holds true for different mRNAs. Thus, when doing rescue experiments, it is important to achieve appropriate levels of injected MO and mRNA. We therefore recommend paying particular attention to injection volumes, injecting a variety of concentrations and also comparing embryos from the same batch that have been injected with MO alone or with MO plus mRNA (Little and Mullins, 2004).

For those genes that are expressed ubiquitously or that have no overexpression phenotype, rescue experiments of this sort might be straightforward. However, for genes that are expressed in a restricted manner, or that have a strong overexpression phenotype, obtaining a true rescue may be difficult or impossible. In particular, it might become necessary to drive expression of the gene in its correct spatial pattern at the correct levels. This may not be straightforward or even possible in some cases, but it might be possible to devise molecular assays that suffice. For example, perfect rescue of the loss of a mesoderm-inducing factor may not be possible because the inducing factor has to be expressed in a precise spatiotemporal manner (Piepenburg et al., 2004). It might, however, be feasible to assay for the expression of one or more of its target genes.

Conclusions

It should be apparent from this article that morpholino technologies provide powerful approaches for the study of early development. Indeed, in many cases, if one wants to know the function of a gene, currently there is no alternative. But it should be equally apparent that interpretation of the results of morpholino experiments, as for experiments in general, depends entirely on appropriate controls. These controls allow the experimenter to rule out off-target effects, and provide a high level of confidence in the specificity of the MO and the validity of the results. As enterprising researchers design more and more sophisticated uses for MOs, it behoves us all to ensure that they are matched by the sophistication of the controls. Therefore, our strongest recommendation is to take these controls seriously, both as experimenters and as reviewers.

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