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Morpholinos: Antisense and Sensibility

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For over 15 years, antisense morpholino oligonucleotides (MOs) have allowed developmental biologists to make key discoveries regarding developmental mechanisms in numerous model organisms. Recently, serious concerns have been raised as to the specificity of MO effects, and it has been recommended to discontinue their usage, despite the long experience of the scientific community with the MO tool in thousands of studies. Reviewing the many advantages afforded by MOs, we conclude that adequately controlled MOs should continue to be accepted as generic loss-of-function approach, as otherwise progress in developmental biology will greatly suffer.

Introduction

The introduction of antisense morpholino oligonucleotides (MOs) (Heasman et al., 2000) has changed the way developmental biologists study the mechanics and underlying molecular mechanisms of embryonic development. MOs have for the first time allowed the study of gene function directly in non-genetic model organisms, in particular in the frog *Xenopus* but also in chicken, in sea urchin, and in emerging model organisms such as the sea anemone *Nematostella*. MOs bind to and thereby block the translational start site of mRNA molecules; they can also be designed to interfere with mRNA splicing or to protect mRNAs from microRNA (miRNA)-mediated turnover. As with all sequence-based intervention tools, MOs are in principle prone to off-target effects: any given short nucleotide sequence may occur more than once, in addition to some tolerance of mismatches between MO and target site. The scientific community has been aware of these potential pitfalls, guidelines have been published (Eisen and Smith, 2008) (Box 1), and journal referees and editors in general have made sure that agreed-upon standards were adhered to. Work in zebrafish, however, has recently raised serious concerns as to the specificity of MO effects. A comparison of morphant and mutant phenotypes reported a lack of concordance in about 70% of cases. The generally more severe morphant phenotypes were largely ascribed to off-target effects (Kok et al., 2015). The authors of an accompanying Forum article in *Developmental Cell* concluded—at least for the zebrafish community—that for the time being, MO-mediated phenotypes should be verified in genetic null backgrounds or by gene editing approaches (Stainier et al., 2015). Discussing the same issue, Stainier and colleagues concluded that the description of a MO phenotype for the first time without the ability to compare with a genetic mutant should in the future be viewed very critically. The issue has now transcended the zebrafish community and has begun to affect grant approvals and paper reviews in communities studying other developmental model organisms, where genetics is less readily available or undeveloped. These include cnidarians, molluscs, sea urchins, ascidians, lamprey, amphioxus, and amphibia, where MOs are often the only established targeted loss-of-function approach. In these organisms, as well as in zebrafish, MOs have allowed enormous progress in the molecular understanding of developmental mechanisms. While dominant-negative constructs have been useful, they are limited to certain protein classes. For these communities, the sweeping recommendations made based on the Kok et al. paper are an existential threat.

Here, we argue that Kok et al. should not be taken as an argument to ban the first-time use of MOs. Instead, adequately controlled MOs should continue to be accepted as generic loss-of-function approach in the absence of genetic evidence, if progress in developmental biology is not to suffer.

Reassessing Kok et al.

Kok et al. (2015) set out to study vascular development and generated 32 mutant lines in 24 genes, using ZFN, TALEN, and, in one case, CRISPR/Cas9. With one exception, small deletions and insertions of 2–77 nucleotides were induced near the N terminus of the encoded proteins, aiming at non-functional truncated proteins and nonsense-mediated decay of mRNA. Specific abnormalities were recorded in only three mutant lines, in contrast to corresponding morphants in which abnormalities were analyzed and reported for 14 of the 24 genes. The authors extended their analysis to an additional 24 genes, selected from the 98 ZMP (zebrafish mutation project) mutants for which morphant phenotypes have been published. They found matching phenotypes in only five cases to conclude, based on both datasets, that morphants phenocopy mutants in less than 30% of cases. Kok et al. (2015) argue that the reasons for these discrepancies were likely MO off-target effects. There is but one example (*megamind*^{um209}) where MO injection gives a phenotype even in fish in which the MO-targeted part of the gene has been genome edited to prevent MO binding. The general interpretation, however, seems problematic for a number of reasons.

Close scrutiny reveals that in only a minority of cases (4 out of 48), it has been rigorously demonstrated that the mutant is a null, with reduced RNA levels recorded for an additional four lines (Table S1). Thus, the stronger phenotype in morphants may

Box 1. Currently Used MO Controls, Advantages, and Limitations

In 2008, Eisen and Smith published the most comprehensive guidelines on the use of MOs for gene knockdown experiments (Eisen and Smith, 2008). Some journals also provide recommendations concerning MO use in their guidelines for authors (http://www.elsevier.com/journals/developmental-biology/0012-1606/guide-for-authors). Ideally, absence of protein in the targeted tissues should be demonstrated by antibody staining or other assays. Antibodies, which work on whole embryos or tissue sections, however, are not always available, and sufficient amounts to detect proteins on western blots can often not be prepared from embryonic samples. Rescue of phenotypes by co-injection of mRNAs not targeted by the MO should be a standard for MO studies, provided that the mRNA injection alone does not produce a phenotype by itself. More than one MO should be used, including ideally a splice-inhibiting MO, the effect of which is to be demonstrated by RT-PCR. Simultaneous application of two non-overlapping MOs at doses below threshold can further be used to prove MO specificity. Experiments should always include the use of control MOs, for example mismatch MOs which differ in five positions. Control MOs should not affect development when used at the same dose as the specific MO under investigation. Knockdown experiments should be performed in at least three, ideally five, independent experiments, and variation of efficacy within and between experiments should be reported, along with statistical evaluation of results. Of course, comparison with existing mutants may provide a validation of the observed phenotype, even across species in case of non-genetic model organisms such as *Xenopus* (cf. Table S2). Limitations include cases of genetic compensation and processes, which differ between the vertebrates, such as, for example, neural crest development (Barriga et al., 2015).

In zebrafish, it has been reported that MOs non-specifically induce p53 expression even if the target gene is not involved in cell survival (Robu et al., 2007). Knockdown of p53 is sometimes used to minimize non-specific effects of MOs in zebrafish (Kok et al., 2015). The before-mentioned Forum article in *Developmental Cell* proposed to provide dose-response curves for p53 induction to determine tolerable MO doses (Stainier et al., 2015). In the frog *Xenopus*, however, p53 knockdown was never used to prevent unspecific MO effects, indicating a better tolerance. Induction of apoptosis, however, was occasionally investigated, particularly when studying pathways in which a role of apoptosis had been suggested, such as in neural crest cell specification. We are not aware of any case of unspecific MO-induced apoptosis. On the contrary, a MO to the actin-binding protein Calponin-2, which affected directed neural crest cell migration, did not affect apoptosis (Ulmer et al., 2013), nor did control MOs analyzed in that context (Bonano et al., 2008; Honoré et al., 2003). However, species differences might exist, as knockdown agents have been shown to activate p53 in mammalian cells as well, justifying perhaps a necessity for differing standards in differing species.

reflect the fact that a number of the reported mutants are hypomorphs, as acknowledged by Kok et al. (2015) for one of their lines. In addition, rigorous proof of the specificity of morphant phenotypes was only provided in about half the cases (Table S1), questioning the significance of the statement that phenotypes match in less than 30% of cases. An additional general complication for the comparison of mutant and morphant phenotypes in zebrafish arises from extensive genetic diversity among strains. The authors of a study that analyzed copy number variants in three commonly used laboratory zebrafish strains and one native population recommend considering all forms of genetic variation in biological and medical research using zebrafish, as phenotypes might differ greatly between commonly used laboratory strains (Brown et al., 2012). While the relevant information could not be retrieved in the majority cases, backgrounds matched in three and differed in five examples (Table S1).

Importantly, as the authors acknowledge, the discrepancies between morphants and mutants may also be caused by the fact that mutant embryos from heterozygote crossings lack only zygotic gene function, while MO targeting additionally inhibits maternal mRNA translation. Kok et al. claimed to have excluded in their analyses cases where genes have maternal expression. However, scrutiny of e.g., *angiomotin* (*amot*) from their analysis reveals that there is substantial *amot* expression at two-cell stage (Harvey et al., 2013; Table S2). In fact, Harvey et al. report that 61% of zebrafish genes show maternal and zygotic expression, which may pose a challenge for mutant studies in general. Moreover, the authors reporting the *amot* morphant (Aase et al., 2007) not only demonstrated the same angiogenesis phenotype with two independent MOs but also fully rescued the morphant with mRNA injection. Strikingly, they showed that *amot* mutant mice also display a corresponding angiogenesis defect to the fish morphants. This suggests very strongly that the failure of Kok et al. to detect vascular defects in their *amot* fish mutants is due to incomplete reduction of Amot protein activity.

In this context it is also interesting and highly relevant that the group of Didier Stainier recently published a carefully controlled comparative study where he analyzed the genes *EGF-like domain, multiple 7* (*egfl7*) and *vascular endothelial growth factor Aa* (*vegfaa*). Again, the mutants revealed milder phenotype while morphants were severely affected. To investigate possible off-target effects, MOs were injected into the *egfl7* mutant, which was much less sensitive than wild-type embryos, suggesting compensatory mechanisms that were activated in the mutant. *emilin3a*, an extracellular matrix gene like *egfl7*, was identified as a compensating gene, which remarkably was upregulated in the mutant, but not in morphants or in specimens, in which *egfl7* transcript elongation was inhibited by CRISPR interference. In *vegfaa* mutants, the paralogous gene *vegfab* was upregulated, which was again not observed in morphants or upon applying a dominant-negative approach to impair *vegfaa* function (Rossi et al., 2015). The lack of the anticipated mutant phenotypes, which Kok et al. (2015) noted for 21 of their 24 vasculogenesis genes, appears in a new light should compensation of gene loss be more widespread. MO approaches, in contrast, might get to the function of a given gene more directly by avoiding compensatory mechanisms.

Given these limitations in the interpretation of the reported discrepancies, we believe it is unfounded to conclude that MOs are

an inferior tool to study gene loss of function. Analyses of the first available mutant lines in *Xenopus*, which were generated by CRISPR/Cas9, further indicate concordance of phenotypes with MO data in a majority of cases, for example, for five out of six genes studied by Khokha and colleagues (M. Khokha, personal communication). In addition, numerous cases have been documented in which morphant phenotypes in *Xenopus* match mouse and/or zebrafish mutants (Table S2).

Unique Applications of MOs

The use of MOs is very helpful in model organisms with poor experimental genetics. An excellent example of the power of antisense MOs in the analysis of sea anemone development has been published recently (Genikhovich et al., 2015). MOs are also useful in human clinical trials to induce exon skipping in Duchenne muscular dystrophy (Cirak et al., 2011). In addition, MOs have a number of advantages, which make them invaluable even in model organisms in which genetics is in principle an option.

Precise Spatial Targeting

It is frequent that genes are expressed in many tissues during development, and therefore, it is desirable to perform loss-of-function experiments of those genes in a tissue-specific manner, which can in many cases overcome or delay embryonic lethality. Although this can be accomplished by genetic tools, it is much easier to achieve by targeted injection or electroporation of MOs in suitable species (e.g., *Xenopus* or chicken; Schweickert et al., 2010; Ulmer et al., 2013; Ohkawara et al., 2011), if carefully controlled by co-application of lineage tracer and, ideally, the absence of protein from the targeted tissue (cf. Box 1).

Targeting Multiple Gene Products

One of the greatest advantages of MO use is the ability to easily target multiple gene products, which is invaluable for epistatic analysis and to unravel functional gene redundancy; these are much more difficult to analyze genetically or by gene editing.

A great advantage is that one can combine several MOs to simultaneously knock down multiple genes with similar function. Studying the role of BMP antagonists in *Xenopus*, Harland and colleagues used MOs to generate a triple knockdown of follistatin, chordin, and noggin (Khokha et al., 2005). These embryos showed a catastrophic loss of dorsal structures and neural tissue. By contrast, when only one or two of the antagonists were blocked, no or only mild effects were observed. In an elegant control experiment, they showed that knockdown of BMP4 rescued the effect of the triple knockdown. These data clearly indicated a requirement for BMP antagonists in early axis formation. Similarly, only quadruple knockdown of ADMP and BMP2/4/7 embryos eliminated organizer self-regulation in *Xenopus*, causing the induction of neural tissue over the entire ectoderm (Reversade and De Robertis, 2005). These insights into the nature of embryonic induction would have been extremely difficult if not impossible to carry out using genetic tools.

Multiple knockdowns have also proven crucial to overcome functional redundancy between duplicated genes, as they occur in many species. For example, mutation of sonic hedgehog (*shh*) in mouse and humans leads to anterior midline signaling defects. In contrast, zebrafish *shh* mutants have normal anterior development because the gene is functionally redundant with *twhh*, as revealed by double MO knockdown of *shh* and *twhh*-MO, which yields a new phenotype, cyclopia (Nasevicius and Ekker, 2000).

Genetic analysis of epistatic interactions is very challenging. Combinatorial MO injections have proven a powerful tool to dissect the epistatic relationship of interacting genes and proteins. For example, the basic sensing mechanism of leftward flow during left-right symmetry breakage was revealed using double and triple knockdowns in *Xenopus*. Impairing ciliary motility through knockdown of the axonemal dynein motor protein *Danh9* prevented induction of the asymmetric gene cascade in the left lateral plate mesoderm, which was rescued by parallel injection of a MO targeting the Nodal inhibitor *Dand5* at the left-right organizer. The additional (triple) knockdown of *Xnr1* specifically in the flow-sensing cells reversed the rescue, which demonstrated that (1) Dand5 acted downstream of leftward flow, and (2) Xnr1 was the target of Dand5 inhibition (Schweickert et al., 2010). This scenario was later confirmed in the mouse using a combination of genetic mutants and experimental manipulations of embryos in culture (Nakamura et al., 2012). In addition, these experiments were performed by sided injections, assigning left-specific functions to Dnah9 and Nodal and a right-specific one to Dand5, while injections of the very same MOs at the very same concentrations on the respective contralateral sides produced no phenotypes whatsoever. Similarly, combinatorial MO injections helped reveal the functional hierarchy of proteins involved in Bardet-Biedl syndrome (Badano et al., 2006).

Dose-Dependent Effects

The ability to titrate MOs allows graded knockdown of gene function. Allelic series can be generated in tractable organisms such as *Drosophila*, but it is by no means simple to obtain these collections. Graded phenotypes can reveal subtle or intermediary defects, which may be missed in complete loss-of-function mutants. For example, dose-response studies with different MO doses against activin B revealed its role as a morphogen (Piepenburg et al., 2004). Such tunable gene inactivation is difficult to achieve genetically.

Spatiotemporal Gene Regulation In Vivo Using Photoactivatable MOs

Photoactivatable MOs that can be uncaged by light to initiate gene inhibition are available. This tunable reagent has been used to analyze the spatiotemporal regulation of *ntl* in mosaic zebrafish embryos (Shestopalov et al., 2007). Early induction showed the same phenotype as *ntl* mutants, while later light-induced activation revealed differential requirement for chordamesoderm and tail development, respectively, highlighting the power of this timed inactivation approach. The opposite approach, photo-inducible gene inactivation, is also possible with photo-cleavable MOs. If injected as double-stranded MOs as a sense partner blocking an antisense reagent, UV light will cleave the sense and allow activation of the antisense strand at any particular stage of development and region of the embryo. This approach has been used to demonstrate the sufficiency of *ntl* at tail bud stage to drive notochord cell fate (Tallafuss et al., 2012). Photoactivatable MOs can be combined with studying the effects of miRNAs on target mRNAs in vivo. Designing MOs complementary to the miRNA binding sites in the 3′ untranslated region of target RNAs protects mRNAs from miRNA inhibition (Choi et al., 2007), and photoactivation and inhibition could provide formidable ways of controlling this

spatiotemporally. To achieve comparable flexibility in spatiotemporal control of gene expression by genetic means would be very challenging.

Analyzing Maternal Gene Products

Most eggs carry stockpiles of maternal mRNAs and proteins, which can support gene function into early organogenesis. Zygotic transcription in many species only starts hours after fertilization, and early cleavage stages are driven by maternal factors. Not only is it difficult to study maternal gene products genetically, maternal components provided in the egg can also mask the requirement of zygotic gene products after the onset of transcription. MOs target both maternal and zygotic mRNAs simultaneously, providing an advantageous situation for the study of early development.

The study of phenotypes associated with defects in the maternal RNA supplies requires inhibition of gene products as they are synthesized during oogenesis. Zygotic gene editing of eggs or early embryos does not interfere with maternal mRNA (and in the next generation can result in oogenesis defects and sterility). In *Xenopus*, it is possible to inhibit maternally stored mRNAs in oocytes using antisense oligonucleotides. While MOs will not inhibit already translated maternal protein, maternal mRNAs and proteins produced during late oogenesis and early embryogenesis from maternal mRNA stores can be interfered with. This has permitted a powerful molecular dissection of maternal Wnt signaling. In this host transfer assay, antisense MO-injected oocytes are implanted back into the body cavity of a host female to render them competent for fertilization. Using this technique, Heasman and colleagues systematically dissected the role of individual maternal Wnt pathway components (Mir and Heasman, 2008). Genetic or gene editing analysis of Wnt maternal effect mutants would require gene expression or targeting during oogenesis. This is cost, labor, and time intensive, and consequently such studies have yet to be reported.

Target Protector Morpholinos to Study miRNAs

In the analysis of miRNA function, the interpretation of phenotypes resulting from genetic inactivation of individual miRNAs can be challenging. This is because many miRNAs are members of large miRNA families present in multiple copies in the genome. Moreover, miRNA deletion typically causes upregulation of all the transcripts it regulates not just a particular miRNA-mRNA pair of interest. To overcome these problems, miRNA function of specific miRNA-mRNA pairs has been studied by using target protector MOs, which have been developed in zebrafish (cf. Staton and Giraldez, 2011 and references therein).

Throwing Out the Baby with the Bath Water: The Case of Antisense and Interfering RNAs

Previous experience shows that not using the full armamentarium available to interrogate biological systems due to prevailing fashion can be costly. When in 1985 Herbert Jäckle and colleagues showed that one could use antisense RNA injection into *Drosophila* embryos to identify the *Krüppel* gene, there was great excitement in the scientific community (Rosenberg et al., 1985). Experiments in a range of organisms gave mixed results, and this method was gradually felt to be unreliable. However, the late Herbert Steinbeisser found that an antisense RNA, directed against BMP4, was very effective in *Xenopus* (Steinbeisser et al., 1995). This was probably because BMP4 is required relatively late in development. Although antisense BMP4 RNA was used to demonstrate that BMP4 prevented neural differentiation in *Xenopus* (Sasai et al., 1995), prevailing fashion made this useful reagent fall into disuse. When Andy Fire and Craig Mello discovered that double-stranded RNA interfered with gene expression (Fire et al., 1998), it became evident that the results described above may have been due to the formation of RNAi in the microinjected embryos. However, shortly thereafter, a study in zebrafish by Robert Ho came out showing that microinjection of double-stranded RNA had non-specific effects in the zebrafish embryo, probably by triggering an antiviral reaction when injected in large amounts (Oates et al., 2000). Unfortunately, the exploration of antisense or RNAi in *Xenopus* or zebrafish ceased immediately, as the field adopted the newly introduced antisense morpholinos (Heasman et al., 2000). We should avoid the mistakes of the past as new techniques emerge. As the saying goes, make new friends, but keep the old, one is silver and the other gold.

Conclusions

In summary, 15 years of MO use in a variety of developmental model organisms have transformed functional developmental biology for many organisms. Criteria for adequate controls have been established, which by and large have worked well. Artifacts and erroneous interpretations have occurred with MOs (e.g., Ross et al. 2001; Little and Mullins, 2004), as they have occurred in genetics and will occur with the emerging genome modification technologies. Gene editing nucleases promise to revolutionize developmental biology but, at least for the time being, cannot replace MOs in many applications. An ideal scenario involves, where possible, the use of MOs in conjunction with other genetic tools (mutants, CRISPRi, dominant-negative reagents, etc.). Obtaining the same outcome using two or more different approaches is far better for science than any one approach, no matter how well conducted. Scrutiny of the cases in Kok et al. (2015), however, does not support the conclusion that MOs are an unreliable tool, which should be abandoned in the absence of genetic data, because of the incomplete characterization of most mutants cited. MOs have many unique advantages that should not be given up lightly, given the wealth of insight, which has been and will be generated with them. Banning the use of MOs by reviewers and editors would cause great harm to developmental biology, not least to the zebrafish research community, where many of the novel MO techniques were pioneered.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.09.017.

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