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Review

A Primer for Morpholino Use in Zebrafish

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Abstract

Morpholino oligonucleotides are the most common anti-sense "knockdown" technique used in zebrafish (*Danio rerio*). This review discusses common practices for the design, preparation, and deployment of morpholinos in this vertebrate model system. Off-targeting effects of morpholinos are discussed as well as method to minimize this potentially confounding variable via co-injection of a tP53-targeting morpholino. Finally, new uses of morpholinos are summarized and contextualized with respect to the complementary, DNA-based knockout technologies recently developed for zebrafish.

Introduction

Morpholino oligonucleotides (MOs) are the most widely used anti-sense knockdown tools in the zebrafish (*Danio rerio*) community. MOs have been used to accelerate gene discovery through large-scale screening,[1,2] to probe candidate gene function,[3] and to verify mutant phenotypes.[4–6] Additionally, MOs have been used as a tool for reducing both maternal and zygotic gene function in zebrafish.[7] Here we describe current practices in MO applications, injection, experimental design, and data collation. We conclude by discussing several new uses of MOs and their utility, reflecting on their applicability in the context of the exciting new and complementary emerging knockout technologies for the zebrafish.

Background

MOs were first developed by Dr. James Summerton as a way to inhibit the translation of RNA transcripts *in vivo*.[8–11] Their application in basic science is a windfall of this human therapeutic development effort. MOs are typically employed as oligomers of 25 morpholine bases (Fig. 1A) that are targeted via complementary base pairing to the RNA of interest. A neutrally charged phosphorodiamidate backbone results in molecules with high binding affinity for RNA, thereby facilitating steric hindrance of proper transcript processing or translation.[11,12] Two types of MO applications in zebrafish include splice blocking[13] and translational blocking.[12] The mechanism of action for splice blocking MOs is thought to be binding and inhibiting pre-mRNA processing via inhibition of the splicesome components (Fig. 1B–D). RT-PCR can be used to identify the quality and quantity of any new transcripts as well as knockdown of the wild-type mRNA.[13] Translational blocking MOs bind complementary mRNA sequences within the 5′ untranslated region (UTR) near the translational start site hindering ribosome assembly (Fig. 1E, F).[12] If available, the level of knockdown should be assessed using an antibody to the protein of interest.[4] If an antibody is unavailable, a transgenic or co-injected mRNA with 5′ UTR of the gene of interest upstream of hemagglutinin[14] or GFP[15] could be used to assess level of knockdown. In zebrafish, MOs are traditionally introduced into the yolks of 1–8-cell-staged embryos. The cytoplasmic bridges connecting these early embryonic cells allow rapid diffusion of hydrophilic MOs, resulting in ubiquitous delivery. The movement of MOs may be facilitated by their neutrally charged backbone reducing interactions with cellular machinery and masking this molecule from standard nucleic acid binding proteins.[4]

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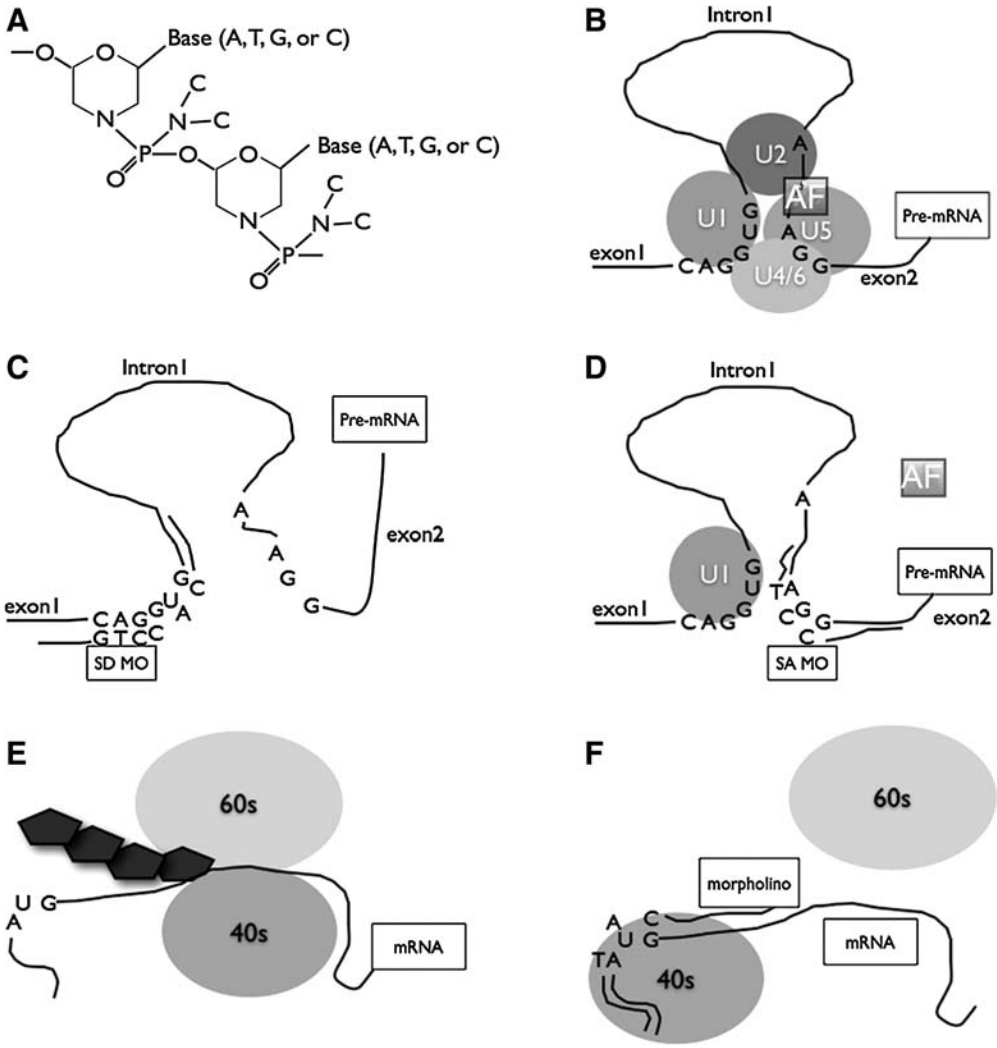

FIG. 1. Morpholino antisense oligonucleotides and mechanisms of gene knockdown. The main antisense chemistry used in zebrafish are morpholino phosphorodiamidate oligonucleotides (morpholinos, MOs).[11] MOs are composed of a phosphorodiamidate backbone with a morpholine ring and the same bases as DNA (**A**).[26,50] Standard use is through steric hindrance of the normal endogenous splicing (**B**) or translation (**E**) mechanisms. MOs targeting the splice donor site inhibit binding of the U1 complex, thus inhibiting lariat formation and incorporation of the intron (**C**). Inclusion of the intron often leads to premature stops and nonsense-mediated decay of the transcript. MOs targeting the splice donor are proposed to function by preventing binding of the U2AF protein (AF) required to recruit the U2 complex, thereby disrupting lariat formation (**D**).[13] Translational blocking MOs bind the AUG or 5′ UTR hindering the scanning of the 40S ribosome and block translational inhibition and elongation by the full ribosomal complex (**F**).[12] Figure adapted from Ekker and Larson.[29]

These modes of MO action have complementary uses in the zebrafish. Splice-site MOs inhibit zygotic transcripts[16] and can thus more closely mimic observed mutations. Moreover, splice blockers can be used to target specific isoforms at a given locus through the specific inhibition of isoform-specific splice sites.[17] In contrast, translational MOs can inhibit both maternal and zygotic transcripts,[4] uncovering phenotypes not observed in standard genetic screens.

MO perdurance is a function of several key variables. MOs are not recognized by any known enzymes and therefore are not easily eliminated from the cell or its progeny.[11] MO efficacy is regulated by binding affinity; therefore, we hypothesize that they are limited by dilution as they are asymmetrically distributed due to differential mitotic activity in different cellular lineages. Most MO phenotypes are identified within the first 3 days of development, but effects have been observed at 5 dpf.[18–20] Protein kinetics and activity may be another mechanism limiting the window of effectiveness for MOs, specifically protein turnover. We know that most MO knockdowns are incomplete and thus result in the formation of small but measurable amounts of protein. We also know that some genes only require a modest percentage of the normal, wild-type amount of protein for effective function. Therefore, long-half-life proteins could potentially reach a functional, no-phenotypic threshold through accumulation of the protein product despite long-lasting effects of MO knockdown on the targeted transcript pool. For an in-depth discussion of MO background, see the recent review by Eisen and Smith.[21]

Morpholino Design

The first key to successful MO use is an accurate RNA target sequence derived from either genomic DNA or cDNA. The zebrafish genome can provide a good starting point, but it contains sequence of variable confidence and quality. First, many protein sequences are based on computer-based algorithms for exon identification, in which the 5′ exons and translational start-site predictions are problematic due to a 3′ bias in expressed sequence tag training sets.[22,23] Second, for many genes, the primary sequence data are obtained from expressed sequence tag work and/or sequence data that can be of variable quality. Third, most vertebrate genes are alternatively spliced, and yet only a subset of such transcriptional complexity can be inferred from genomic sequence and/or are seldom completed due to the extensive work required for such deep transcriptional analysis for a given locus.[22] This can be especially problematic when using MOs as splice blockers. Finally, many of the zebrafish lines are polygenic and will show polymorphism in their genomic sequence. In practice, many zebrafish researchers will clone and sequence the target RNA encoded by their zebrafish strain of interest, a process that addresses many limitations inherent in genome projects while providing experimental confirmation of gene expression.

For splice-site MOs, the choice of exon target is critical. If information suggests an exon when removed can cause a mutant phenotype (i.e., that exon encodes the active site for an enzyme, or the sequence-specific DNA binding domain of a transcription factor), this exon can be targeted using splice-site MOs. For example, the splice-site MO targeting the intron 4–exon 5 splice acceptor site removing the final exon of *h2afza* was able to phenocopy a gene-break transposon insertion in intron 4 that results in a mutant mRNA lacking exon 5 encoding the most C-terminal residues of that histone.[24] Further, co-injection of a MO targeting both the splice junction and splice branch site further increases the efficiency of RNA knockdown.[13] Other factors include removing exons that in their absence lead to a frameshift, removing exons that allow for easy assessment such as RT-PCR, or altering the normal amount of an alternatively spliced transcript. Alternatively, splice-site targeting can result in the inclusion of introns. This method can often lead to frameshift and premature stop codons, and these transcripts are potentially eliminated by nonsense-mediated decay. For a detailed discussion of splice-site MO design, see the study by Morcos.[13] To assess the features of a potential MO, IDT Tools provides a software package (oligo analyzer, homodimer, and hairpin prediction) that is freely available at http://www.idtdna.com/SciTools/SciTools.aspx.

For translational MOs, the 25 bases surrounding the start codon is the most common target. Design of translational blockers against the sequences within the initial coding region avoids the sequence divergence and errors common in UTR sequences. MOs that target mRNA sequence downstream of the start codon and immediate codons rapidly decrease in efficiency as a function of distance, making it critical to determine the 5′ coding exon for a given target gene.[10]

We previously published an online software package, A Morpholino Design software (AMOD),[25] to facilitate translational morpholino design; unfortunately, due to changes in algorithm availability and other long-term upkeep issues, this has recently been removed from the web. Vector NTI (Invitrogen; Carlsbad, CA) is available at http://www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/Vector-NTI/Download.html, and it offers an adequate substitute for AMOD by user customization of the primer design tool. Other primer design programs would also undoubtedly serve as well.

To begin designing a morpholino, the user can input the sequence containing 20 bp of the first coding exon and the 5′ UTR sequence into the Vector NTI software package. Then select the "Analyses → Primer Design → Hybridization Probes" menu selection. In the next window, set the primer to complementary and the maximum number of output options to 20–50 to make sure the MOs span your target region. Then select the "oligo similarity" button to set the similarity threshold to greater than or equal to 95%; this allows an MO to be designed to sequences that may have an ambiguity at one site of the 25 bp. Next, set the key "oligo parameters." In general for MOs, set the length to 25 nucleotides, the %GC to between 40% and 60%, and the Tm between 48 and 55 degrees. To help reduce the selection of MOs that self-complement, set the "Hairpin Loop Stem Length >=" to 3, the "Palindromes <=" to 5, and the "Nucleotide Repeats <=" to 3. Changing the allowed values for ΔG within hairpin loops or for primer–primer complementation can further reduce the self-complementation of the designed MOs. The last submenu, "Oligo Quality Specifics," places weights on each selection for Tm, %GC, Palindromes, Nucleotide Repeats, Hairpin Loops, and Dimers. Essentially, setting a value to 10 forces your selection to be true, and setting at 9 gives precedence but the program will still identify a "primer" if it cannot match all your choices. Therefore, it is important to visually inspect the designed MO by selecting the "Analyses → Oligo Analysis → Thermodynamic Properties." It is crucial to make sure that the MO G/C is maintained between 40% and 60%, and MOs with obvious secondary structure or islands including four or more consecutive G bases are eliminated.

For both splice-site and translational blocking MOs, Gene Tools LLC also offers a free MO design service.

A Practical Guide for Morpholino Use

Morpholino sources

Currently, Gene Tools LLC (Philomath, OR; http://www.gene-tools.com/) is the only supplier of custom MOs for research use. Open Biosystems (http://www.openbiosystems.com/GeneExpression/Non%2DMammalian/Fish/Zebrafish Morpholinos/) sells smaller aliquots for some predesigned MOs previously synthesized by Gene Tools.

Morpholino preparation

MOs are shipped as lyophilized stocks. Resuspending MOs initially in high-grade water, previously vetted for embryo use, retains the ability to independently assess oligomer concentration using standard laboratory spectrophotometric assays (keeping in mind the specific absorbance properties of MO bases). We prefer to store main stocks in evaporation-resistant centrifuge tubes. An extensive discussion on storage of soluble MOs has been previously published.[26] In normal use, we have found only a single method for successful

long-term storage—lyophilization. When in an aqueous solution, some MOs of one sequence will rapidly lose efficacy (in 1–2 weeks), while others have lasted for more than 7 years (Ekker lab, unpublished data). For MOs with a short shelf life, storage temperature (−80°C vs. −20°C vs. 4°C) and concentration (diluted vs. concentrated stock) do not seem to yield a dramatic change in shelf life over time.

For working stocks of MOs that will be injected directly into zebrafish embryos, we have preferred to use near-isotonic solutions such as that developed for cell transplantation work for the zebrafish (i.e., Danieau solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$ 5.0 mM HEPES, pH 7.6]) as described in *The Zebrafish Book*.[27] MOs diluted in straight aqueous, weakly ionic and weakly buffered solutions can work, but at larger volumes (above 6 nL) these solutions can have an adverse effect on embryonic development independent of the MO. The working solution should be spun for 30 s at top speed in a microcentrifuge before each use to remove any small particles in the injection solution. Some laboratories will heat their working solution at 65°C to reduce secondary structure and to dissolve precipitated MO.[21]

For many laboratories, MO dosage is commonly described in mass (i.e., 3 ng/embryo) rather than the seemingly more obvious method of concentration (i.e., molarity). The origin of this resolves around the question of how much exogenous nucleic acid will be tolerated by a zebrafish embryo. Up to 1 ng of single-stranded mRNA (independent of mRNA length) can be readily injected into a zebrafish embryo without any major negative consequences.[28] In contrast, 100 pg of double-stranded RNA or DNA will kill most zebrafish embryos.[28] The first use of MOs employed a mass standard as the description of nucleic acid introduction when using these oligomers in zebrafish.[4] We have maintained this approach because, in practice, most MOs are designed around similar design constraints (such as typically 25 bases in length and 40–60% G/C content), and the relatively subtle differences in effective molarity due to slight differences in molecular weight are small. In addition, the working range (i.e., activity profile) is experimentally determined for each new synthesis of an MO.

How much morpholino to use

Two methods have been used to develop a standard curve of MO effectiveness *in vivo*. One approach has been to phenocopy known zebrafish mutations[4,26] and empirically ascertain when injected animals exhibit a distinctive loss-of-function phenotype as determined using the reference mutant phenotype. A list of MOs that phenocopy 20 different cloned zebrafish mutations has shown that nearly all MOs exhibited the appropriate biologically specific phenotype in greater than 50% of injected embryos at doses of 5 ng or less; injection of 6 ng or more sometimes resulted in embryos displaying defects not noted in mutants and were thus formally considered to be off-target effects.[26,29] This functional data set indicates caution in interpretation of phenotypes observed at higher doses. A corollary of this work is that calibration of delivery in each experiment is critical, as a twofold increase in delivered MO can readily result in a shift into the higher category of MO usage, increasing the risk that any observed effect is due to off-targeting such as binding of that particular MO against a related (but not identical) sequence found in a different RNA target.

The second approach has been to judge MO effectiveness as a quantitative measure of knockdown—for example, adding MOs until a given arbitrary threshold such as greater than 80% reduction of wild-type targeted RNA or protein. Interestingly, this approach has been included since the first described use of MOs in zebrafish[4] and parallels those observations made from phenotyping known mutations. For example, 80% or greater knockdown of GFP was achieved with ~5 ng MO dose, whereas doubling to 9 ng (which achieved greater than 90% quantitative knockdown) also showed off-targeting effects. Proof of efficacy does not demonstrate specificity, a topic extensively discussed in the review article by Eisen and Smith.[21]

Injection setup and calibration

Calibration is an essential step in reproducibility for MO use and to reduce the risk of off-target effects. The standard injection setup we use for MOs is the same as we deploy for injecting mRNA[28] or making transgenic zebrafish.[30] Kwik-fil borosilicate capillary needles (World Precision Instruments, Sarasota, FL; 1B100F-4) are pulled on a Flaming Brown Micropipette Puller Model P87 (Sutter Instruments, Novato, CA) using a trough filament (Heat 410, Pull 190, Velocity 170, Time 170, Pressure 500). One tip is for reproducible needle production and protection of the filament—make sure to ramp the puller temperature to determine the proper setting for heat. The needle is back-loaded with injection solution using a Microloader tip (Eppendorf, Hamburg, Germany) (Fig. 3A), and inserted with a silicon gasket into a three-axis micromanipulator (Narshige, Greenvale, NY) (Fig. 3B, C). The tip of the needle is clipped using a Dumont number 5 forceps under high power on a dissecting microscope (Fig. 3D, E). A PLI-100 micro-injector with foot pedal (Harvard Apparatus, Holliston, MA) is used to reproducibly inject equal amount of the solution into the embryo, and we typically set the instrument to an incoming pressure of 24 psi. To calibrate the amount of solution injected, 10 thirty millisecond pulses are injected into a 1-λ microcapillary (Drummond Scientific, Broomall, PA) (Fig. 3F, G). The amount of solution in the capillary is measured using a millimeter ruler; these capillaries have a 1 μL total capacity and are 33 mm in length; 1 mm thus represents 30 nL of solution. The injection time of the PLI-100 is then adjusted to deliver 1.5 to 6 nL of solution with each pulse, or the needle is rebroken to start with a fresh calibration process (for a demonstration, see Supplemental Video 1, available online at www.liebertonline.com).

Embryo loading

Agarose embryo holding trays for use in injections are produced by allowing a molten 1% agarose solution made with trace amounts of methylene blue in embryo water (0.006% Instant Ocean in MilliQ water) to harden around a polycarbonate plastic plate[27] with six asymmetric raised rows (one side is perpendicular, while the other is a 45° angle to the surface of the plate) inside of a 9 cm Petri plate (Fig. 2A). Embryo water is added to the hardened tray to facilitate the removal of the polycarbonate plate and hydrate the agarose (Fig. 2B). The water and plate are removed (Fig. 2C), and the trays are kept inverted at 4°C until use (Fig. 2D). Trays can be reused up to 2 months. To avoid dehydration of the plate, embryo water is added to each tray before use.

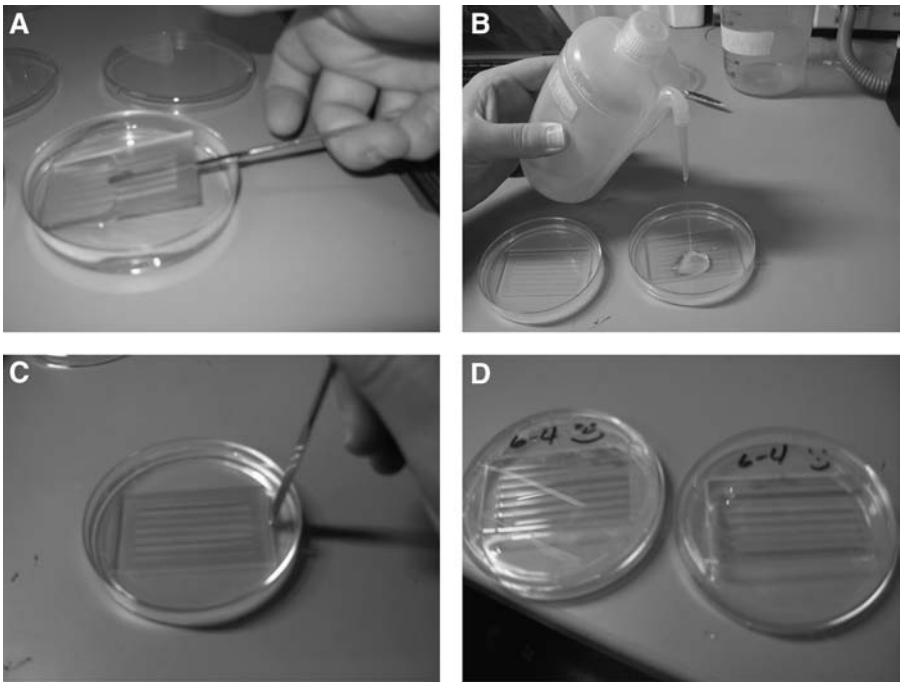

FIG. 2. Zebrafish embryo holding trays for injection—*The Zebrafish Book* design. The process of pouring trays involves the lowering of a plastic mold into molten 1% agarose (**A**). Special caution needs to be taken to reduce the number of air bubbles that are underneath the tray, as this will disrupt the final rows. Embryo water is added to the tray to lubricate the mold for removal as well as hydrate the tray (**B**). If a tray is hydrated properly embryos can be kept on the tray for up to 20 min without harm. If the agarose has spilled over the top of the mold, the spatula is used to slice around the edges before removing the tray (**C**). This leaves an indention in the plate for which to load embryos. Embryo water is left in the tray for a minimum of 5 min to completely hydrate the tray. The final trays (**D**) are kept up to 2 months at 4°C, thus eliminating evaporation. Up to 40 embryos can be loaded into a row for injections.

One- to four-cell embryos are loaded on to the agarose tray. Up to 40 embryos can be loaded per row; higher density loading prevents the embryos from rolling and rotating during injections. To avoid dehydration, either add embryo water to the trays or transfer the embryos within 15–20 min of injection.

Morpholino injections

MOs are traditionally injected into the center of the yolk to reduce the chance of secondary effects due to a mechanical disruption of the early blastomeres (Fig. 3H). MOs can be injected in this fashion between the 1- and 8-cell stage and still give ubiquitous delivery.[4] Later injection time points can be used to target specific structures such as the YSL or Kupffer's vesicle.[31,32] The first step in using a new MO is to determine the optimum delivery dose. For example, MOs can be initially injected at four doses: 1.5, 3, 4.5, and 6 ng. If needed, the dosages are increased or decreased to optimize the phenotype-to-toxicity ratio. Injections of lower than 1.5 nL should be avoided, as the PLI injection systems do not accurately measure these smaller volumes; instead, injection of a diluted stock is recommended. Injection of volumes higher than 12 nL should be avoided as this can disrupt embryo development. Concentrations of the working MO are altered to allow for changes in dosages. Optimally, the best concentration for a particular MO should be injected at ~3 nL, as this is a highly reproducible volume for the microinjector (highest precision). Once the injection process is finished, the embryos should be placed in embryo water in the incubator at 29°C as temperature regulation controls for any differential binding kinetics for the MO.

Nonspecific effects and the tP53 morpholino

In a large-scale screen utilizing translational blocking MOs, one regular class of reproducible phenotypes resembling those previously identified as off-targeting were observed.[29] The most reproducible of these phenotypes is cell death; this can be visualized by a white fuzziness at the borders of the eyes, brain ventricles, and somites using dark field microscopy at 22 h postfertilization.[29] This phenotypic class of off-target effects is due to an ectopic upregulation of the p53 apoptosis pathway (Fig. 4).[33] For example, the *wnt5* mutant phenotype (Fig. 4E, K) was compared to two independent MOs targeting *wnt5* mRNA (Fig. 4B, D). These experiments demonstrate several important points. First, not all MOs cause nonspecific events, as can be seen by comparing the morphology and cell death index between MO2 (Fig. 4D, J) and the *wnt5* mutant (Fig. 4E, K). Second, aberrant morphologies correlate with increased cell death in the MO that appeared to produce an off-targeting phenotype (Fig. 4B, H). With the addition of the *tP53*-targeted MO, this MO-induced cell death returns to normal levels and with it the aberrant phenotype is reduced, resulting in wnt5 MO1 now phenocopying (Fig. 4C, I) the wnt5 mutant (compare to Fig. 4E, K). Finally, the *tP53* MO does not eliminate all cell death, as can be seen by the cell death present in both the *chordin* mutant[34] and *chordin*

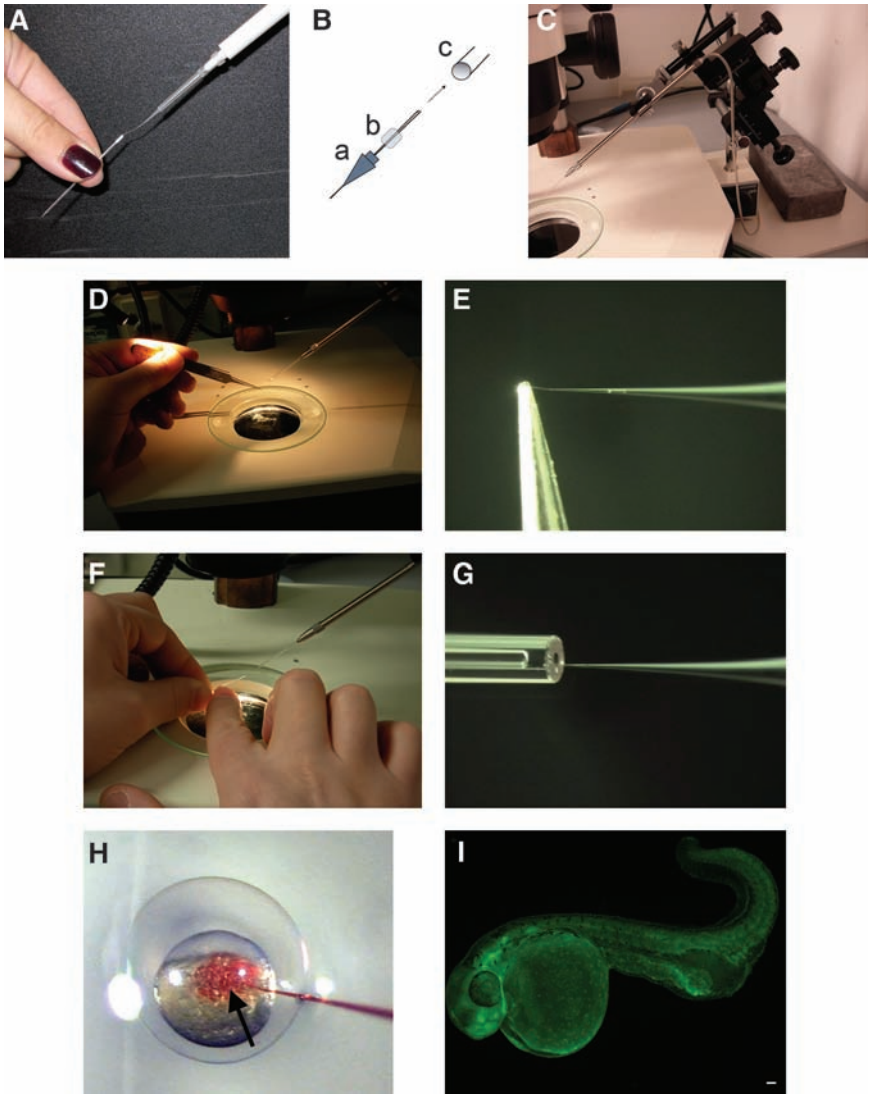

FIG. 3. Microinjector setup and calibration. Quantitation of the amount of solution injected is critical to determining the relevancy of the work. The solution is back-loaded using an Eppendorf microloader pipette tip (**A**). The needle is then loaded into the shaft (c) of the three-axis micromanipulator (**C**) to get a proper seal the silicon gasket (b), and metal cap (a) must be added to the needle before insertion (**B**). To open the tip a number 5 forceps is used to break the tip of the needle (**D**). Imaging under the highest power of the dissecting microscope helps observe the needle during this process (**E**). Pressure from the microinjector should be applied at this stage to determine the amount of solution injected. A 1-λ capillary is moved toward the tip of the needle gently inserting the tip of the needle into the capillary and applying 10–30 ms pumps from the microinjector (**G**). Two hands can help keep the capillary steady during this process (**F**). Embryos are loaded onto the agarose plates. Under high power, move the needle next the embryo with the micromanipulator, quickly insert the needle into the center of the yolk, and inject the proper dosage of solution (**H**). As a training tool, we utilize a *chordin* MO with FITC label, so that positive injections can be scored. The phenotype of this training MO is shown in (**I**). The MO should be distributed ubiquitously throughout the embryo, suggesting a proper delivery and display the *chordin* phenotype of an expanded blood island—the area just posterior to the yolk sac extension (for a demonstration of this process, see Supplemental Video 1).

MO with *tP53* MO (Fig. 4F, L). In practice, the *tP53*-targeted MO (GCGCCATTGCTTTGCAAGAATTG)[35] is co-injected at 1 to 1.5 times the dose of the experimental MO to reduce side effects.[33] Including this MO in all injections can be very helpful and valuable as standard practice for non-P53 pathway-dependent biological work.

Morpholino specificity

When targeting a gene of unknown function, determining a clear link between an observed MO-induced phenotype and the gene target is essential. For p53-independent processes, the field has settled on a set of common controls (reviewed by Eisen and Smith[21]). To avoid sequence-specific off-targeting due to a single MO target sequence, observing the noted phenotype(s) with a second MO of independent sequence (typically nonoverlapping) is required. It is common that the optimal dose will be different between the two MOs. The second experiment is a co-injection experiment using a reduced dosage of both MOs such that the phenotype is only slightly apparent with each MO alone. For most genes, the co-injection of these two MOs should synergize to produce a much stronger effect than if the two individual effects were added together. This is a strong indication that the MO effect is specific to the gene targeted in that experiment. On the other hand, MOs targeting a different target sequence should not yield these same phenotypes. This second test can be addressed in several ways such as generating a specific four or five base mismatch MO or the phenotype can be compared against a battery of MOs developed for other purposes in a zebrafish laboratory. This latter approach is informative so long as the other MOs do not inhibit the development of the structure initially studied. For example, in our large-scale MO screen,[1] we observed a series of phenotypes that occurred at a much higher frequency than expected in a variety of genes. These phenotypes were the same as those characterized as off targeting effects,[29] and therefore are not specific. Alternatively, we identified phenotypes that were unique to a given organ system, thus suggesting with high probability that these morphant phenotypes were specific.

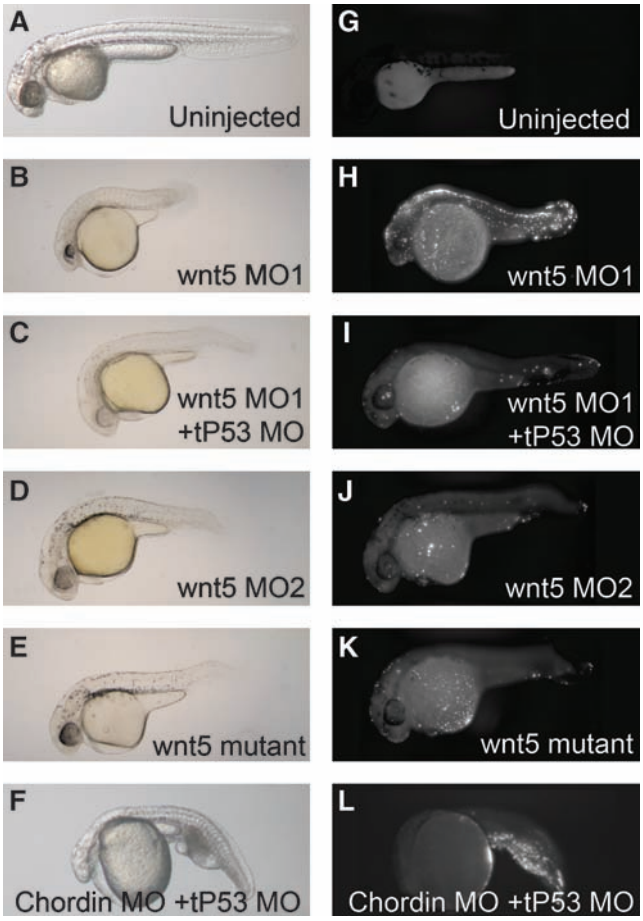

FIG. 4. tP53 knockdown ameliorates nonspecific MO neural death phenotypes. Brightfield images of 1 dpf embryos are shown in panels (**A–F**). Fluorescent images detecting cellular apoptosis after TUNEL detection are shown in panels (**G–L**). (**B, H**) Wnt5 MO1-injected embryos. Note the extensive apoptosis that is ameliorated after co-injection with a *tp53* MO (**C, I**). The resulting embryos show the strong wnt5 mutant phenotype (compare to **E, K**). (**D, J**) Not all wnt5-targeted MOs exhibit the off-targeting neural death phenotype. (**F, L**) The *tp53* MO does not block all cell death in the zebrafish embryo. Note the *chordin* loss of function-induced, tissue-specific apoptosis in *chd*MO/*tp53*MO co-injected embryos. Reprinted from Robu *et al.*[33]

Reversal of morpholino phenotype using RNA rescue

MO specificity can be addressed in a variety of ways.[19] One commonly used approach for phenotypes observed in the first 24–48 h of development is to reverse the noted effects by a strategy called RNA "rescue."

Synthetic mRNA encoding the protein from the targeted locus is injected into the yolk of 1–2-cell embryos.[28] mRNA distribution is a function of an active transport process active in the early zebrafish embryo, porting RNAs from the yolk to the overlying blastomeres[28]; the distribution of injected mRNA is not quite as uniform as small, hydrophilic molecules such as MOs, and must be injected earlier to take advantage of this process underway in the developing early embryo. A key to the RNA rescue experiment is to be sure that the injected synthetic mRNA does not encode the MO target sequence. For translation-blocking MOs against the 5′ UTR sequence, the open reading frame can be simply cloned by PCR into a standard transcription vector.[28] For MOs that target part of the open reading frame, the rescue constructs can be engineered to change the nucleotide sequence without altering the encoded protein through degeneracy of the genetic code.

Conducting these experiments requires careful and simultaneous control of the delivery and concentration of two different reagents. The mRNA is generated *in vitro*, aliquoted, concentration and quality determined, and stored in deep freeze (−80°C). A typical dose range of 50 pg to 1 ng is delivered in 1.5–9 nL solution per embryo. Overexpression phenotypes and LD50 (dose at which 50% embryo lethality occurs) are determined as important references for subsequent rescue studies. To conduct rescue experiments, embryos are divided into several experimental groups—those injected with the targeting MO and with a control mRNA (i.e., GFP-encoding) versus those injected with the targeting MO and with the gene-specific mRNA, as well as mRNA constructs or MO alone.

Database resources for morpholinos

ZFIN[36–40] (www.zfin.org) collates published MO sequences within each gene page and is a valuable resource to the community. ZFIN has requested that researchers include the sequences of all MOs used in publications so that the service can be provided in the most timely and accurate manner possible.

A second database available includes both published and unpublished MOs (MODB).[41] MODB is the combined collection of MOs from several large-scale screens and includes both sequence and phenotypic data from various groups collated via an online database strategy. A MySQL database was designed with a Phenotypic Attribution Trait Ontology–compliant phenotyping system. The benefit of the online database includes utilizing wireless Internet connections in the screening area, making screening data directly available to the consortium in a single, central, and accessible location online.[41]

Exciting new applications of morpholinos

The large number of MOs previously designed and collated within ZFIN and MODB has brought the community to the point where previously validated MOs are available for many members of canonical signaling pathways. This is a powerful resource that can be utilized to rapidly interrogate pathways linking genes that are involved in a particular phenotype. For example, a panel of Notch receptors and ligands was used to investigate the identity of the Notch receptor and ligand involved in development of proper choroid plexus size.[20] We foresee panels developed to interrogate all the major pathways involved in development.

The recent commercialization of photoactivatable MOs will further increase the utility of MOs, as evidenced by the recent temporal interrogation of the *no tail* phenotype.[42] The ability to activate the *no tail* MO in a 100 mm region demonstrated a role for *no tail* in somite formation independent from the early embryonic role in tail development.[42] The further combination of the pathway panels and photoactivation technology

will further expand our knowledge of the general mechanisms of development.

MOs can be targeted to previously inaccessible tissues and time points using electroporation. Recent studies have used this method to deliver MOs to investigate genetic requirements for regeneration of the eye[43] and of the fin.[44,45] Electroporation has been used to deliver DNA to the neural tube of the zebrafish,[46,47] suggesting that this method might also be suitable for delivery of MOs into the brain.

The future of morpholinos

Recently, two papers describing the use of zinc finger nucleases (ZFNs) introduced a new and potentially powerful knockout approach to the zebrafish community.[48,49] While this will replace some current uses of MOs, the two technologies are more complementary than competitive. One of the current shortcomings of the zinc finger technology is its high cost and the multiple generation time for standard diploid genetics necessitates a minimum time lag of about a year before loss of function tissue can be examined. In contrast, MOs provide an inexpensive and rapid means to assess gene function, even for genes with available mutants; initial scientific inquiry is rapid and can provide foci for downstream work once the mutant line is obtained in a given laboratory. MOs can be utilized to screen through a small set of candidate genes or through a gene to look for sensitive exons, followed by the directed knockout of the best candidate. Such a screening approach is currently impractical with ZFN technology. We anticipate one use of ZFNs will be to "hard copy" that subset of genes of high biological interest when screened using MOs. These zinc finger knockouts will facilitate such candidate genes to be fully analyzed using traditional genetic approaches. MOs and ZFNs thus represent a set of complementary and synergistic tools to help in the genetic analysis of the zebrafish, *D. rerio*.

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