Published in final edited form as:

Genesis. 2011 March ; 49(3): 117–123. doi:10.1002/dvg.20689.

Use of fully modified 2'-O-methyl antisense oligos for loss-of-function studies in vertebrate embryos

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Abstract

Antisense oligonucleotides are commonly employed to study the roles of genes in development. Although morpholino phosphorodiamidate oligonucleotides (morpholinos) are widely used to block translation or splicing of target gene products, the usefulness of other modifications in mediating RNase-H independent inhibition of gene activity in embryos has not been investigated. In this study, we investigated the extent that fully modified 2'-O-methyl oligonucleotides (2'-OMe oligos) can function as translation inhibiting reagents in vivo, using *Xenopus* and zebrafish embryos. We find that oligos against *Xenopus* β -catenin, *wnt11* and *bmp4*, and against zebrafish *chordin* (*chd*) can efficiently and specifically generate embryonic loss-of-function phenotypes comparable to morpholino injection and other methods. These results show that fully modified 2'-OMe oligos can function as RNase-H independent antisense reagents in vertebrate embryos and can thus serve as an alternative modification to morpholinos in some cases.

Keywords

antisense oligonucleotides; embryo; 2'-O-methyl RNA; morpholino

Antisense deoxyoligonucleotides (oligos) are used routinely to disrupt gene function in developing embryos. This strategy is particularly important in organisms where gene targeting by homologous recombination is limited or impossible, including *Xenopus*, zebrafish and many non-traditional model organisms. Antisense oligos inhibit gene function through one of two main mechanisms. Oligos composed of DNA bases with phosphodiester or phosphorothioate linkages can bind to and degrade the target mRNA by eliciting RNase H activity (Cazenave *et al.*, 1987; Dash *et al.*, 1987; Shuttleworth and Colman, 1988). Alternatively, oligos composed of various nuclease resistant modified nucleotides and linkages can bind with high affinity to the target mRNA and inhibit translation (Boiziau *et al.*, 1991) or splicing. Chimeric oligos, which incorporate nuclease resistant termini flanking an RNase H competent core, have also been designed to improve oligo longevity and specificity (reviewed in Hulstrand *et al.*, 2010).

Chimeric oligos have been particularly useful in targeting maternal mRNAs in *Xenopus* oocytes, since RNase H-dependent antisense mechanisms are quite effective in these cells. In fertilized embryos however, simple phosphorothioate-modified, RNase H-dependent oligos are highly unstable and exhibit greater toxicity (Dash *et al.*, 1987). Although the use of second and third-generation oligo modifications has somewhat mitigated this limitation (Hukriede *et al.*, 2003; Lennox *et al.*, 2006), RNase H-independent morpholino phosphorodiamidate oligos (morpholinos) have become the predominant reagent used for antisense experiments in *Xenopus* and zebrafish embryos (reviewed in Heasman, 2002;

Eisen and Smith, 2008). Although morpholinos were initially adopted to fill the clear need for a stable and specific antisense reagent in genetically intractable organisms, other types of RNase H-independent oligos have been successfully used for clinical applications (Sazani and Kole, 2003) and may thus be suitable for antisense experiments in embryos.

In this study, we investigated whether fully modified 2'-O-methyl (2'-OMe) RNA antisense oligos could function as effective translational inhibitory reagents in *Xenopus* and zebrafish embryos. 2'-OMe modified oligos have several features that make them attractive antisense reagents, including, rapid hybridization kinetics, thermal stability, and resistance to RNase H and other nucleases. Importantly, 2'-OMe-modified oligos inhibit in vitro translation with a similar efficacy and specificity to morpholinos (Stein *et al.*, 1997) and can inhibit translation and splicing in vivo (reviewed in Manoharan, 1999). In contrast to morpholinos, 2'-OMe modified oligos are readily available from many commercial sources and can be produced in small scales and purified as desired.

To test the activity of 2'-OMe oligos in vertebrate embryos, we obtained a fully modified 2'-OMe RNA antisense 25mer oligo targeted against the start codon of *Xenopus* β -catenin, using a previously published sequence (Heasman *et al.*, 2000). This oligo (*β cat-2ome*) was injected at 3, 6 and 12 ng into one-to-two cell embryos. Injection of 12 ng was lethal, producing a cell-dissociation phenotype in all cases, which was not rescued by RNA injection and is therefore a non-specific effect (data not shown). Embryos injected with the 3 ng (not shown) and 6 ng doses survived and exhibited a high incidence of mild and severe ventralization, respectively (Fig. 1A, B). These embryos displayed all the morphological hallmarks of dorsal axis deficiency, including delayed and symmetrical gastrulation, appearance of animal pole "cysts," failure or delay of neural plate formation and reduced head and dorsal axial structures. Our data are consistent with the results obtained using the isosequential morpholino oligo (Heasman *et al.*, 2000).

To determine the extent that Wnt/ β -catenin target genes were affected by injection of *β cat-2ome*, we analyzed gastrula stage embryos injected with 6 ng oligo by RT-PCR (Fig. 1C). Injected embryos expressed lower levels of *nodal-related 3 (nr3)* and *siamois (sia)*, which are regulated directly by Wnt/ β -catenin signaling. Levels of the Organizer marker *goosecoid (gsc)* were also reduced, whereas a ventrally expressed gene, *sizzled (szl)*, was unaffected at this stage. β -catenin RNA levels were not affected by *β cat-2ome* oligo injection, suggesting that the oligo was not acting by causing RNA degradation. By contrast, analysis of β -catenin protein abundance in late blastula stage embryos showed that *β cat-2ome* oligo was sufficient to reduce total β -catenin abundance by ~33% (Fig. 1D). These reductions in protein levels persisted at least in to the neurula stages, which has also been observed for the corresponding morpholino (Heasman *et al.*, 2000), and is consistent with levels of depletion sufficient to elicit ventralization in *Xenopus* (Heasman *et al.*, 1994; Heasman *et al.*, 2000). Ventralized embryos could also be obtained by injecting 6ng *β cat-2ome* oligo into oocytes and fertilizing them by host-transfer into egg-laying females (data not shown), indicating that, like MOs, 2'-OMe oligos can be used both before and after fertilization in *Xenopus*. Taken together, these data show that injection of an antisense 2'-OMe-modified oligo against β -catenin can reduce β -catenin protein levels without degrading β -catenin RNA, as well as inhibit β -catenin activity and disrupt dorsal axis formation.

We next directly compared the activity of *β cat-2ome* with β -catenin-MO in embryos from the same female. Embryos were injected with 8 ng, 6ng or 4ng of 2'OMe oligo or MO and frozen at the gastrula stage for RT-PCR and immunoblot analysis. The 8 ng and 6ng (not shown) doses of MO and 2'-OMe caused similar degrees of ventralization (Fig. 2A), resulting in predominantly anterior truncations. However, at the 4 ng doses, the β -catenin-MO was unable to ventralize embryos, whereas 4 ng of the *β cat-2ome* oligo yielded >50%

ventralized embryos. In a separate experiment, we found that 3ng of 2ome oligo was sufficient to cause partial axis reduction in about 50% of the embryos as well as cause a reduction in Wnt target gene expression (data not shown). By RT-PCR, we found that the 8 ng and 6 ng doses of both oligo types caused similar reductions in *nr3* expression levels (Fig. 2B) as well as in *sia* and *gsc* (not shown). We also compared beta-catenin protein levels in sibling embryos from the same experiment, frozen at the late blastula stage (Fig. 2C). These results fit well with our RT-PCR data, showing that the higher doses cause similar levels of inhibition, whereas the low MO dose causes very little depletion.

To confirm that *βcat-2ome* oligo was acting specifically by inhibiting β-catenin, we performed rescue experiments by injecting mouse *β-catenin* mRNA. This transcript does not contain a binding site for the oligo and will therefore not compete for binding to the endogenous transcript. We injected embryos with *βcat-2ome* (6 ng) at the two-cell stage, followed by injection of *β-catenin* RNA (50 pg) into one dorsal blastomere at the 4-cell stage. Embryos were then frozen at the gastrula and neurula stages for gene expression analysis or left for phenotype assessment. *β-catenin* was able to rescue normal axis formation (Fig. 3A–C) and RT-PCR analysis showed that dorsal and ventral markers were restored to near-normal levels in 2'-OMe-injected embryos coinjected with *β-catenin* RNA. These data thus demonstrate that the ventralization generated by oligo injection is indeed due to β-catenin depletion and not due to non-specific oligo effects.

We used *β-catenin* as the initial target in these proof-of-principle experiments because the effects of its depletion on dorsoventral patterning are well characterized (Heasman *et al.*, 1994; Heasman *et al.*, 2000). To explore the generality of using 2'-OMe-modified oligos, we next tested a 2'-OMe oligo against *Xenopus wnt11* (*wnt11b*) and compared its activity to the isosequential morpholino (Pandur *et al.*, 2002) in affecting pronephros development (Tételin and Jones, 2009). Injection of *wnt11*-MO into a ventral vegetal blastomere is sufficient to inhibit pronephros development on the injected side. We injected 20ng of *wnt11*-MO or 6ng of *wnt11*-2ome oligo into ventral vegetal blastomeres of 8 cell embryos obtained from the same female. These embryos were raised to the tailbud stage and analyzed by in situ hybridization for *pax8*, a marker of the pronephric anlagen and otic vesicle at this stage. Both reagents caused morphogenetic abnormalities and reduced the pronephric expression of *pax8* to a similar degree (Fig. 4A, B), thus providing a second example of 2'-OMe functionality in embryos.

We also tested a third example in *Xenopus*, targeting *bone morphogenetic protein 4* (*bmp4*) using a sequence isosequential to the published MO sequence (Reversade *et al.*, 2005). Injection of *bmp4-2ome* (3–4 ng/embryo) caused ventral tail and proctodeal defects in over 60% of embryos (Fig. 4D). Molecular analysis of gastrula stage embryos showed reduction in ventroposterior marker expression (*szl*, *evx1*; data not shown), confirming defects in ventral cell fate specification and recapitulating known effects of *bmp4* inhibition.

We further tested the effectiveness of 2'-OMe oligos in zebrafish by targeting *chordin* (*chd*), using an oligo isosequential to the published MO sequence (Nasevicius and Ekker, 2000). We injected *chd-2ome* (3–5 ng) into one-to-two cell zebrafish embryos and raised these to the tailbud stage (Fig. 5). Injection of *chd-2ome* caused reduction of head and axial structures, indicative of ventralization, in over 50% of embryos, mimicking the effects of the morpholino oligo as well as the phenotype of the *chordino* mutant (Nasevicius and Ekker, 2000; Hammerschmidt *et al.*, 1996).

In summary, these data demonstrate that 2'-OMe-modified oligos can be used comparably to MOs for gene knockdown experiments in vertebrate embryos. Although we tested oligos targeted against the start codon to inhibit translation, 2'-OMe-modified oligos would most

likely be effective in blocking splicing as well, since they are used in this context in other experimental settings, including human clinical trials. Antisense 2'-OMe oligos have also been used successfully to inhibit miRNA/siRNA function in vivo (Hutvagner et al., 2004). Also, Lennox et al. (2006) have found that chimeric oligos consisting of 2'-OMe modifications on the 5' and 3' termini with a central phosphodiester region can be effective RNase H-dependent antisense reagents in early embryos. The 2'-OMe modification can be readily used in chimeric oligo synthesis, allowing for some greater flexibility in choice of antisense mechanisms. The fully modified 2'-OMe-modified oligos we tested functioned similarly to the morpholino oligos, although we found non-specific effects beginning at doses of 12 ng for all oligos tested (data not shown), whereas morpholinos are often injected at much higher doses (Heasman, 2002). Ribose methylation occurs naturally during rRNA and tRNA biogenesis (Maden, 1990) and during piRNA/siRNA biogenesis in animals (Horwich et al., 2007) and miRNA biogenesis in plants (Yang et al., 2006). It is therefore possible that oligo breakdown may alter endogenous nucleotide pools or the activity of proteins involved in RNA metabolism. Also, the high affinity of 2'-OMe modified bases for their RNA targets may allow a certain degree of mismatched binding to occur with high doses, resulting in spurious inhibition. In our hands, morpholinos have a wider range of functional doses, whereas 2'-OMe-modified oligos could be used at lower effective doses. In conclusion, fully modified 2'-OMe oligos can provide a useful alternative antisense reagent for investigators performing gene knockdown studies in embryos.

Methods

Xenopus and zebrafish embryos

Eggs were collected from hCG-stimulated females (1000U) and fertilized using sperm macerated in 0.3× MMR (1× MMR: 100mM NaCl, 2mM KCl, 2mM CaCl₂, 1mM MgCl₂, 15 mM HEPES, pH 7.6). Embryos were dejellied in 2% cysteine/0.1x MMR, pH 7.8, extensively washed in 0.1x MMR and placed into Ficoll solution for microinjection (2% Ficoll 400 [GE Healthcare] in 0.5x MMR). Following injection, embryos were maintained in Ficoll overnight at 16–18°C and transferred to 0.1x MMR until the desired stage. For molecular analysis, embryos were placed in microcentrifuge tubes with minimal buffer and frozen on dry ice.

Zebrafish embryos were obtained from natural spawning of adults. Embryos were rinsed with embryo medium (Westerfield, 1995) and staged according to Kimmel et al. (1995). Injections were done into the yolk cell at the one-to-two cell stage.

Antisense oligonucleotides

The antisense oligos used were fully modified 25mers with 2'-O-methyl ribonucleotides and HPLC purified (Integrated DNA Technologies, IDT; Coralville, IA). Oligos were dissolved to 1 mM in deionized water and stored at –80°C. Working solutions of 0.25 mM (~1.0–1.5 µg/µl) were made for injections. The following sequences were synthesized, mN= 2'Ome residue, regions matching the start codon are underlined where applicable:

β-catenin-2ome: 5' mUmUmU mCmAmA mCmCmG mUmUmU mCmCmA
mAmAmG mAmAmC mCmAmG mG 3' (n.t. 260–236, 5'UTR; Heasman et al., 2000).

wnt11-2ome: 5' mCmCmA mGmUmG mAmCmG mGmGmU mCmGmG mAmGmC
mCmAmU mUmGmG mU (*wnt11b*; n.t. 285–309, Pandur et al., 2002)

bmp4-2ome: 5' mCmAmG mCmAmU mUmCmG mGmUmU mAmCmC mAmGmG
mAmAmU mCmAmU mG 3' (n.t. 119–95; Reversade et al., 2005).

(zebrafish) *chordin-2ome*: 5' mAmUmC mCmAmC mAmGmC mAmGmC mCmCmC mUmCmC mAmUmC mAmUmC mC (n.t. 174–150; Nasevicius and Ekker, 2000).

Realtime RT-PCR

Total RNA was isolated by homogenizing in an RNA lysis buffer containing proteinase K (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.5% SDS, 250 µg/ml PK), followed by phenol:chloroform extraction and ethanol precipitation (Kerr et al., 2009). RNAs were treated with DNase I (Turbo DNA-free kit, Ambion) and reverse transcribed using MMLV reverse transcriptase (Invitrogen) primed with 0.25 µg random hexamers. cDNA was diluted 1:10 and 5 µl was used for PCR. Reactions were run on a LightCycler 480 according to the manufacturer's instructions and reagents (Roche Applied Science). Amplified products were detected by SYBR Green or by exonuclease probes (Universal Probe Library probes; Roche Applied Science). The cycling conditions were: 94°C (10 minutes, one cycle), 94°C, 60°C, 72°C, (10 seconds each, for 40 cycles). Quantitation was performed by comparing samples to a standard curve of serially diluted control cDNA, followed by normalization against *ornithine decarboxylase* (*odc*). Individual representative reactions are shown, each of which was repeated at least twice with similar results. The primers and detection probes used are shown in Table 1.

Immunoblotting

Control and oligo-injected embryos were frozen at the late blastula stage and homogenized in 10 µl per embryo cell lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, plus protease and phosphatase inhibitor cocktail [Thermo/Pierce]). Lysates were boiled in SDS-PAGE sample buffer and one embryo equivalent was loaded onto 10% pre-cast gels (Thermo/Pierce) and run in Tris-HEPES-SDS buffer according to the manufacturer's instructions. Proteins were transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked in Sea Block (Thermo/Pierce), incubated with primary antibodies (rabbit anti-β-catenin, Sigma, 1:4000; mouse anti-α-tubulin mAb DM1A, Sigma, 1:10,000) diluted in SeaBlock containing 0.1% Tween-20. Washes were done using PBS+0.1% Tween 20, followed by incubation with secondary antibodies (goat anti-mouse DyLight 800, Thermo/Pierce, 1:3000; goat anti-rabbit DyLight 700, Thermo/Pierce, 1:3000) diluted in PBS+ 0.1% Tween-20 + 0.02% SDS. Blots were washed and scanned on an Odyssey near-infrared imager (Licor), according to the manufacturer's instructions. Band intensities were determined using the Odyssey software and β-catenin levels were normalized against α-tubulin.

In situ hybridization

Whole-mount in situ hybridization was performed essentially as described (Sive et al., 2000; Cuykendall et al., 2009). Antisense RNA probes labeled with digoxigenin-11-UTP (Roche Applied Science) were synthesized using polymerases and reaction buffers from Promega. cDNA for *Xenopus tropicalis pax8* (in pCS107; BC125805) was obtained commercially (imaGenes) and linearized by digestion with *EcoRI*, followed by transcription with T7 RNA polymerase.

Acknowledgments

Grant support: NIH 5R01 GM083999 to DWH

The authors would like to thank Diane Slusarski for contributing zebrafish embryos and for critical reading of the manuscript, and Dan Weeks for critical reading of the manuscript. This work was supported by The University of Iowa and by NIH 5R01 GM083999-02 to D.W.H.

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Fig. 1. 2'-O-methyl oligos against *Xenopus* β -catenin inhibit dorsal axis formation and β -catenin signaling

(A) Phenotypes of uninjected embryos (stage 31/32); 30/30, 100% normal phenotype (two experiments). Anterior is to the left in all cases.

(B) Phenotypes of embryos injected with 6ng β -cat-2ome oligo; 24/27, 89% axis deficiency (two experiments).

(C) Quantitative real-time PCR of dorsal (*gsc*, *sia* and *nr3*) markers, ventral markers (*szl*) and β -catenin (β -cat) in control (Un) and 6ng β -cat-2ome oligo-injected (6ng 2ome) embryos at stage 10.25.

(C') Average (mean) relative expression levels of *nr3* in controls and in 6ng β -cat-2ome oligo-injected embryos from six separate experiments (including those in Fig. 1C and 3D). Error bars indicate standard deviation; p-value by t-test was ~ 0.0001 .

(D) Immunoblotting analysis of β -catenin protein levels (top panel) and α -tubulin (lower level) in uninjected control (Un) and in 6ng β -cat-2ome oligo-injected embryos at late blastula, early gastrula and early neurula stages. The relative abundance of β -catenin compared with tubulin is shown below each lane.

Fig. 2. Comparison of 2'-O-methyl oligo and morpholino oligo activity

(A) Phenotypes of uninjected, β -cat morpholino-injected and β -cat-2ome oligo-injected embryos, 8 ng and 4 ng doses (stage 28). Un, 18/18, 100% normal; 8 ng MO, 8/16, 50% axis deficiency; 4 ng MO, 0/10, 0% axis deficiency; 8 ng 2ome, 14/14, 100% axis deficiency, 4 ng 2ome, 9/17, 53% axis deficiency.

(B) Quantitative real-time PCR of *nr3* in control (Un) and oligo-injected embryos at stage 10.25. Oligo doses are 8 ng, 6 ng, 3 ng.

(C) Immunoblotting analysis of β -catenin protein levels (top panel) and α -tubulin (lower level) in uninjected control (Un) and in β -cat-MO and 2ome oligo-injected embryos at the late blastula stages (8 ng, 6 ng, 3 ng). The relative abundance of β -catenin compared with tubulin is shown below each lane.

Fig. 3. Specificity of 2'-O-methyl oligos against β -catenin

(A) Phenotypes of uninjected embryos (stage 17); 20/20, 100% normal phenotype.

(B) Phenotypes of embryos injected with 6ng β -cat-2ome oligo; 15/15, 100% axis deficiency.

(C) Phenotypes of embryos co-injected with 6ng β -cat-2ome oligo and 50pg β -catenin mRNA (dorsal injection); 3/15, 20% axis deficiency.

(D) Quantitative real-time PCR of dorsal and ventral markers in control (Un) and oligo-injected embryos at stage 10.25.

(E) Quantitative real-time PCR of anterodorsal (*otx2*, *myod*) and ventroposterior (*szl*, *evx1*) markers in control (Un) and oligo-injected embryos at stage 14.

Fig. 4. 2'-O-methyl oligos against *Xenopus wnt11* and *bmp4* cause pronephric and tail fin/proctodeal defects

(A) *pax8* expression in an uninjected control embryo (stage 24/25); 20/20, 100% normal expression (one experiment).

(B) *pax8* expression in embryos injected unilaterally with 20ng of *wnt11-MO*; 5/9, 56% reduced pronephros (one experiment).

(C) *pax8* expression in embryos injected unilaterally with 6ng of *wnt11-2ome*; 6/8, 75% reduced pronephros (one experiment).

(D) Phenotypes of uninjected embryos (stage 36/7); 40/40, 100% normal phenotype (two experiments).

(E) Phenotypes of embryos injected with 4ng *bmp4-2ome* oligo. Arrows indicate ventral tail fin (solid line) and proctodeal (dashed line) abnormalities; 16/28, 57% tail defects (two experiments).

Fig. 5. 2'-O-methyl oligos against zebrafish *chd* cause dorsoventral patterning defects
(A) Phenotypes of uninjected embryos (~ 30 hours post fertilization); 10/10, 100% normal phenotype.
(B) Phenotypes of embryos injected with 4ng *chd-2ome* oligo; 8/15, 53% axis deficiency.

Table 1

Realtime PCR primers and exonuclease detection probes

Target	Detection	Upstream Primer (5'-3')	Downstream Primer (5'-3')	Reference
<i>odc</i>	UPL #69	CAGCTTCAGCAATGACGACT	AGATCAGCAACATAAAAGGCATC	New
<i>gsc</i>	UPL #126	CCAAGAAACCAAGTACCCAGA	GCTCTTCGATTTTGAACCAA	New
<i>nr3</i>	UPL #43	TTTGTGGACTTTCAGAAGATCG	GGGAGCAAACCTCTTAATGTAGGAA	New
<i>szl</i>	UPL #12	GAAGGCCCAGTTGAGTTCAT	GCAATAACATACACTGTGGGTCTG	New
<i>-catenin</i>	SYBR	GGACAAACCCAGGACTACA	ATTGCATCCTGACCATAGCC	New
<i>otx2</i>	SYBR	CGGGATGGATTTGTTGCA	TTGAACCAGACCTGGACT	Heasman et al., 2000
<i>myod</i>	SYBR	AGCTCCAAGTCTCCGACGGCATGAA	AGGAGAGAATCCAGTTGATGGAAACA	Rupp and Weintraub, 1991
<i>evx1</i>	SYBR	ATATGATGAGCCACGCAGCAG	CAGATGCTGCAGCTCTTTGGC	Gawantka et al., 1995