

# Target Gene Knockdown by 2',4'-BNA/LNA Antisense Oligonucleotides in Zebrafish

Motoyuki Itoh,<sup>1</sup> Mizuki Nakaura,<sup>1</sup> Takeshi Imanishi,<sup>2</sup> and Satoshi Obika<sup>2</sup>

Gene knockdowns using oligonucleotide-based approaches are useful for studying gene function in both *in vitro* cell culture systems and *in vivo* animal models. We evaluated the efficacy of 2',4'-bridged nucleic acids (BNA)-modified antisense oligonucleotides (AONs) for gene knockdown in zebrafish. We used the *tcf7l1a* gene as a model for testing the knockdown efficacy of 2',4'-BNA AONs and examined how the target sites/affinity and RNase H induction activity of 2',4'-BNA AONs affect knockdown efficacy. We found that *tcf7l1a* gene function was knocked down by 2',4'-BNA AONs that target the start codon and induce RNase H activity. Although nonspecific p53-mediated developmental defects were observed at higher doses, the effective dose of the 2',4'-BNA AONs for *tcf7l1a* is much lower than that of morpholino oligonucleotides. Our data thus show a potential application for 2',4'-BNA AONs in the downregulation of specific genes in zebrafish.

## Introduction

OLIGONUCLEOTIDE-BASED METHODS are useful tools for studying the genetic mechanisms that control development and homeostasis. In vertebrate embryos, including zebrafish, *Xenopus*, and sea urchins, morpholino antisense oligonucleotides (MOs, Fig. 1A) are widely used for this purpose (Nasevicius and Ekker, 2000; Corey and Abrams, 2001). MOs are synthetic DNA analogues composed of morpholine rings joined by phosphoramidate bonds (Summerton, 1999). MOs function solely by a steric block mechanism and in an RNase H-independent manner (Summerton, 1999; Summerton, 2007). It has been reported that MOs have provably low off-target effects because they lack any charge on the backbone and thus cannot interact electrostatically with proteins (Summerton, 2007). Other artificial oligonucleotide analogues that have been used to knock down gene expression in zebrafish embryos include hydroxypropyl-phosphono peptide nucleic acids (HypNA-pPNAs, Fig. 1B) (Urtishak et al., 2003). HypNA-pPNAs also function in an RNase H-independent manner and have a higher hybridization affinity toward RNA and a more stringent mismatch discrimination than MOs, such that 18-mers possess comparable potency to 25-mer MOs in zebrafish (Urtishak et al., 2003).

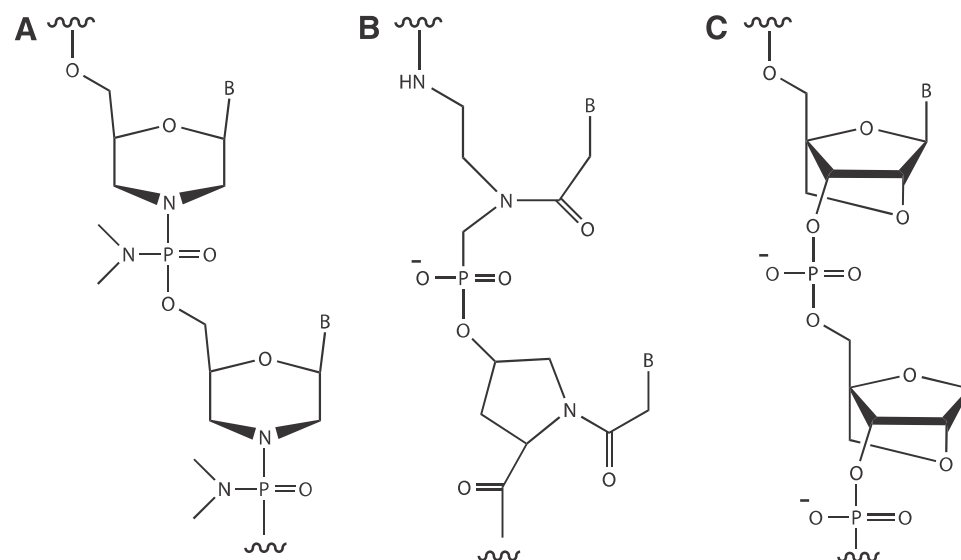
RNase H-dependent oligonucleotides, which can induce mRNA degradation, comprise another class of antisense oligonucleotides (AONs). RNase H specifically recognizes RNA:DNA heteroduplexes composed of the A-form RNA

strand and the B-form DNA and subsequently hydrolyzes the RNA component. We previously developed a 2',4'-bridged nucleic acid (2',4'-BNA, Fig. 1C), also called locked nucleic acid (LNA), that includes a methylene bridge between the 2' and 4' positions in the ribose ring (Obika et al., 1997; Obika et al., 1998; Singh et al., 1998). This modification confers resistance to nucleases. Further, 2',4'-BNA has a high affinity for RNA. 2',4'-BNA:RNA duplexes structurally resemble A-form RNA:RNA duplexes; thus, 2',4'-BNA does not activate RNase H on its own (Singh et al., 1998). However, 2',4'-BNA/DNA chimeric oligomers with a DNA stretch of at least six nucleotides can activate RNase H (Kurreck et al., 2002). Therefore, the 2',4'-BNA/DNA chimeric oligomer can act through both steric blocking and RNase H-dependent mechanisms to efficiently knock down endogenous gene expression (Obika et al., 2001). Recently, modified BNA AONs have been developed as one of the most promising antisense oligonucleotide analogues for clinical applications (Rayburn and Zhang, 2008; Seth et al., 2009; Prakash et al., 2010; Yamamoto et al., 2011; Yamamoto et al., 2012).

Zebrafish is a useful *in vivo* model to understand gene function in both developmental and physiological contexts. Antisense agents, such as morpholino oligos, have been widely used for loss of gene function studies in zebrafish (Summerton, 1999; Nasevicius and Ekker, 2000; Urtishak et al., 2003). However, the efficacy of 2',4'-BNA AONs in zebrafish has not been well studied. Here, we examine whether 2',4'-BNA/DNA chimeric oligomers are able to knock down gene function by screening various target

<sup>1</sup>Graduate School of Pharmaceutical Sciences, Chiba University, Chuo-ku, Chiba, Japan.<sup>2</sup>Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan.

**FIG. 1.** Antisense oligonucleotides. (A) MO, morpholino oligonucleotide. (B) HypNA-pPNA, hydroxypropyl-phosphono peptide nucleic acids. (C) 2',4'-BNA, 2',4'-bridged nucleic acid.



regions and numbers and positions of 2',4'-BNA in the oligonucleotide sequence using zebrafish *tcf7l1a* as a model gene. Although the dose for nonspecific toxicity is close to the effective dose, select 2',4'-BNA AONs can knock down *tcf7l1a* function in zebrafish.

## Materials and Methods

### Fish maintenance

The zebrafish AB strain was raised and maintained under standard conditions approved by the Institutional Animal Care and Use Committee at Chiba University.

### Antisense oligonucleotides

2',4'-BNA/DNA- and 2',4'-BNA/RNA-chimeric oligomers were synthesized by Gene Design, Inc.. The RNA melting temperature ( $T_m$ ) of the BNA/DNA oligomers was estimated using the Exiqon  $T_m$  prediction program ([www.exiqon.com/ls/Pages/ExiqonTMPredictionTool.aspx](http://www.exiqon.com/ls/Pages/ExiqonTMPredictionTool.aspx)). All morpholino oligonucleotides were purchased from Gene tools, LLC. The morpholino oligonucleotides were previously described as follows: *tcf7l1a* MO (Dorsky et al., 2003) and p53 MO (Robu et al., 2007).

### Zebrafish embryo injections

Fertilized eggs were collected, and 0.2–1 nL of oligonucleotides was injected into one- to two-cell-stage embryos. After injection, the embryos were incubated at 28.5°C and raised until 24 hours post fertilization (hpf). Bright-field images were taken using a Zeiss Axiocam HR mounted on a Leica stereoscopic microscope.

### RNA isolation and quantitative real-time polymerase chain reaction

Embryos were injected with 2.4 fmol of 2',4'-BNA oligos. At tail-bud stage (around 10 hpf), 30–50 embryos were collected and total RNA was purified using standard TRIzol and propan-2-ol precipitation. Complementary DNA syn-

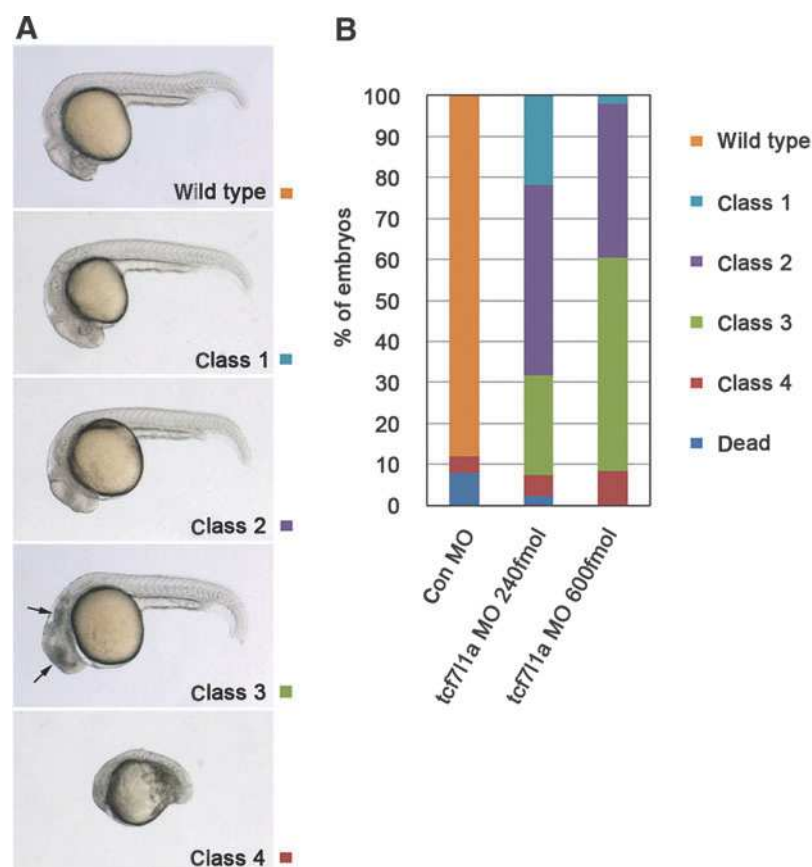
thesis was performed with the ReverTra Ace. Transcript levels of *tcf7l1a* and *Rpl13α* were quantified by real-time polymerase chain reaction (PCR) with Power SYBR Mix (Applied Biosystems) on a 7300 Real-Time PCR detection system (Applied Biosystems). Results are expressed relative to the level of the housekeeping gene *Rpl13α* (Tang et al., 2007). Primer sequences are as follows: *tcf7l1a* (up), 5'-CAAGGCCAGCATATGTACTCC-3'; *tcf7l1a* (low), 5'-AACGGCTGGAGACCAGACTA-3'; *Rpl13α* (up), 5'-TCTGGAGGACTGTAAGAGGTATGC-3'; and *Rpl13α* (low), 5'-AGACGCACAATCTTGAGAGCAG-3'.

## Results and Discussion

### Injection of an antisense morpholino against *tcf7l1a* causes the loss of the anterior head in zebrafish

Antisense MOs have been used to knock down endogenous genes in zebrafish (Nasevicius and Ekker, 2000). To analyze the efficacy of gene knockdown by 2',4'-BNA antisense oligonucleotides (AON), we used the zebrafish *tcf7l1a* gene as a case study. As previously published, *tcf7l1a* functions as a repressor of Wnt signaling (Kim et al., 2000). Wnt signaling plays an important role in anterior–posterior neural patterning, and exaggerated Wnt signaling leads to the loss of the rostral brain region and the expansion of the more caudal brain region (Ciani and Salinas, 2005). The headless (hdl) mutant, in which the *tcf7l1a* gene is inactivated and thus has excessive Wnt signaling, lacks eyes and a forebrain region but exhibits a rostral expansion of the midbrain–hindbrain boundary (Kim et al., 2000). It has been shown that injection of an antisense MO against *tcf7l1a* causes similar head defects to those of *hdl* mutants (Dorsky et al., 2003; Thorpe and Moon, 2004). To confirm this finding, we injected 240 or 600 fmol of the *tcf7l1a* MO into fertilized zebrafish eggs. Injected embryos showed head defects at 24 hpf (70%–90% of the embryos had no eyes, Fig. 2A, B). The morphological head defect phenotype was enhanced by injection of a higher dose of the *tcf7l1a* MO, but this treatment also caused additional neuronal death, possibly due to off-target effects of the MO (Robu et al., 2007).

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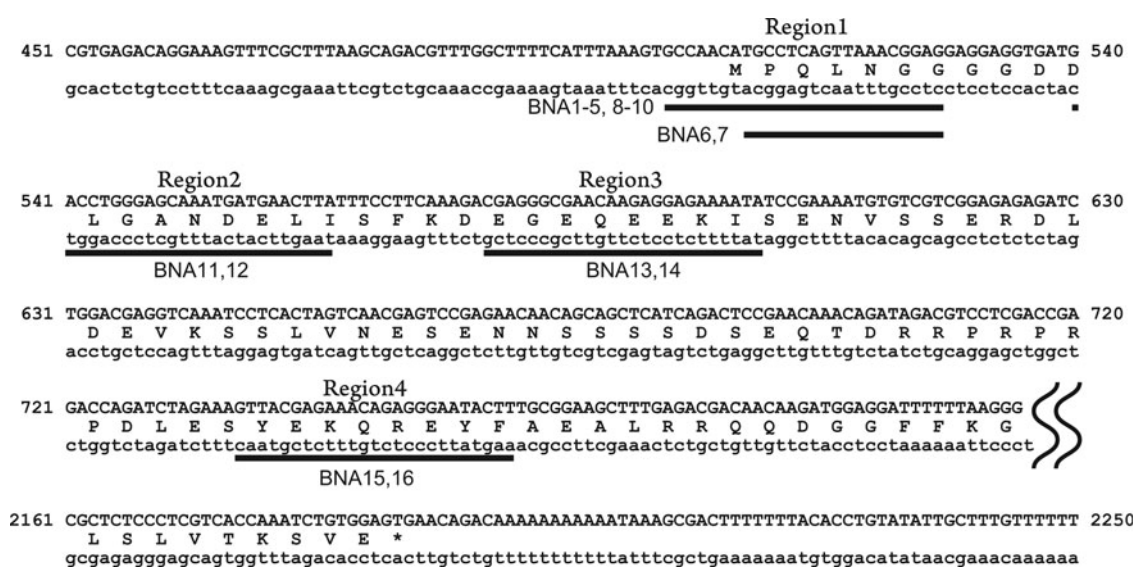


**FIG. 2.** Representative phenotypes obtained after injection of control or *tcf7l1a* MO. At 24 hours post fertilization (hpf), phenotypes were assessed by morphological criteria, according to severity. **(A)** Wild type embryos appear normal, class 1 embryos have small eyes, class 2 embryos lack eyes completely, class 3 embryos have no eyes and exhibit neuronal death (arrows indicate neuronal cell death), and class 4 embryos are severely malformed. **(B)** Distribution of phenotypes in embryos injected with control or *tcf7l1a* MO. Control MO,  $n=25$ ; *tcf7l1a* MO 240 fmol,  $n=41$ ; *tcf7l1a* MO 600 fmol,  $n=48$ .

### 2',4'-BNA/DNA chimeric oligomers that activate RNase H against *tcf7l1a* affect anterior head formation

The *tcf7lla* MO is a 25-bp sequence of oligonucleotides that targets a region that includes the start codon (Region 1, Fig. 3). We first chose the same region (Region 1 in Fig. 3, Table 1) to study the effects of 2',4'-BNA in zebrafish em-

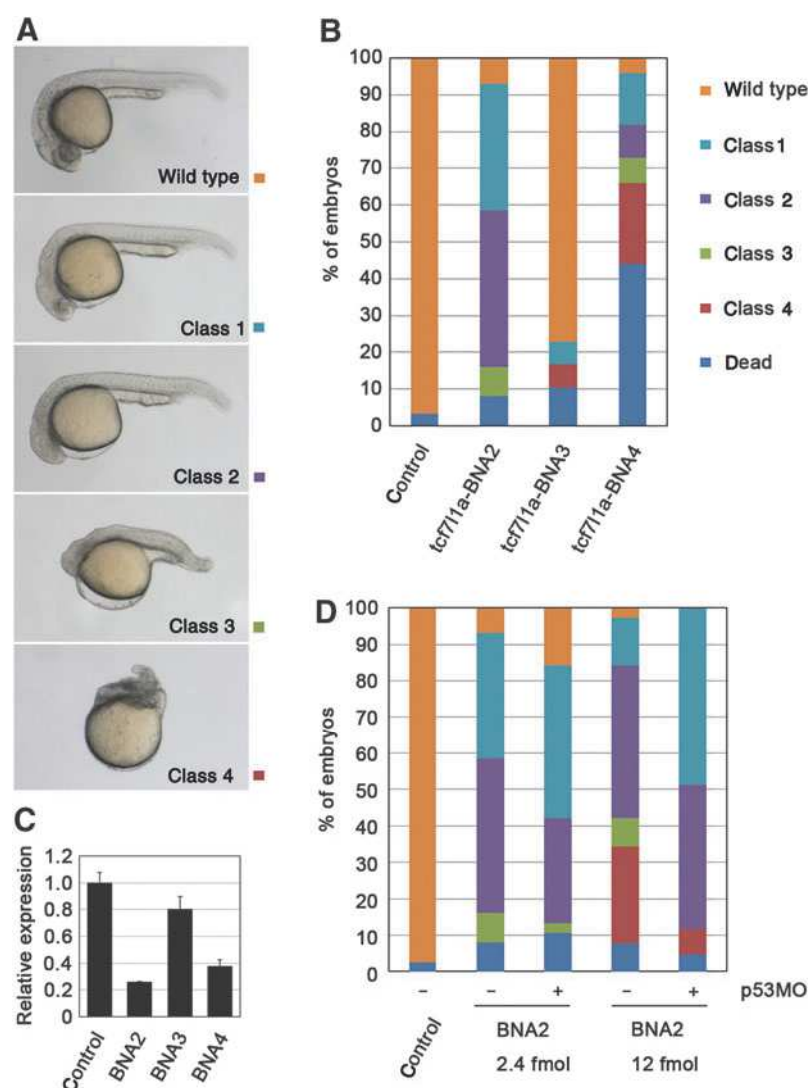
bryos. MOs function solely by a steric block mechanism to inhibit translation and RNA splicing (Summerton, 1999; Summerton, 2007). In contrast, 2',4'-BNA/DNA-mixed AONs either exploit RNase H to cleave their target RNAs and/or interfere sterically with RNA to block its function. Previous studies have shown that a DNA stretch of six nucleotides in the 2',4'-BNA/DNA chimeric oligomer is



**FIG. 3.** Target positions of 2',4'-BNA/DNA chimeric oligomers in *tcf7l1a*. Uppercase and lowercase letters in the sequences represent the sense and antisense strands, respectively. The numbers at the left and right refer to the nucleotide positions in the *tcf7l1a* gene target sequences of the 2',4'-BNA/DNA AONs are underlined.







**FIG. 4.** 2',4'-BNA/DNA chimeric oligomers that activate RNase H against *tcf7l1a* affect anterior head formation, and off-target effects are mediated by p53 activation. (A) Representative phenotypes obtained after injection of *tcf7l1a*-BNA/DNA chimeric oligomers. At 24 hpf, phenotypes were assessed by morphological criteria, according to severity. Wild type embryos appear normal, class 1 embryos have small eyes, class 2 embryos lack eyes completely, class 3 embryos have no eyes and short body axes, and class 4 embryos are severely malformed. (B) Distribution of phenotypes in embryos injected with *tcf7l1a*-BNA/DNA chimeric oligomers. Control, *n* = 33; *tcf7l1a*-BNA2, *n* = 87; *tcf7l1a*-BNA3, *n* = 48; *tcf7l1a*-BNA4, *n* = 100. (C) Quantitative real-time polymerase chain reaction for *tcf7l1a* was performed on 10 hpf embryos injected with *tcf7l1a*-BNA/DNA chimeric oligomers. Results are shown as the relative expression to *Rpl13α* (mean  $\pm$  standard deviation). (D) p53 is involved in 2',4'-BNA/DNA-induced toxicity in zebrafish. Phenotypic classes as defined in (A). The percentage of embryos with short body axes or malformed bodies was reduced and that of embryos with small eyes was reciprocally increased by p53 knockdown.

gene. Six 2',4'-BNA/DNA chimeric oligomers targeting three regions downstream of the start codon were examined (*tcf7l1a*-BNA11-16, Fig. 3). Because their target regions are at a distance from the ATG codon, they cannot function to inhibit translation. As a result, none of the *tcf7l1a*-BNA11-16 AONs showed any head defects at doses of 3 or 10 fmol per embryo (data not shown). Taken together, both protein translation blocking and RNase H-mediated mRNA degradation may play important roles for effective gene knockdown by 2',4'-BNA/DNA chimeric oligomers in zebrafish.

#### Nonspecific toxicity of 2',4'-BNA/DNA chimeric oligomers in zebrafish embryos is mediated by p53 activation

We found that *tcf7l1a*-BNA2 was highly efficient at blocking *tcf7l1a* function but also that some embryos showed nonspecific abnormalities and cell death, which were also observed with the other 2',4'-BNA/DNA chimeric oligomers. Some morpholino AONs and small interfering RNAs have been reported to induce p53-mediated apoptosis nonspecifically in zebrafish and in cultured cells (Scacheri et al. 2004; Robu et al. 2007). Therefore, we examined whether 2',4'-BNA/DNA AON-induced off-target toxicity is mediated by

p53. Embryos were initially injected with a control MO or p53 MO, and then *tcf7l1a*-BNA2 was injected at 2.4 or 12 fmol per embryo. The percentage of embryos with short body axes was reduced by p53 knockdown at both dose levels (class 3, Fig. 4D). Furthermore, when coinjected with 12 fmol of *tcf7l1a*-BNA2, the p53 MO reduced the percentage of malformed embryos (class 4) and reciprocally increased the percentage of embryos with small eyes (class 1, Fig. 4D). Thus, p53 knockdown significantly alleviated the off-target developmental abnormalities induced by 2',4'-BNA/DNA AONs in zebrafish. These data suggest that p53 activation is a major cause of toxicity by 2',4'-BNA/DNA AONs in zebrafish.

#### Conclusions

In conclusion, we have shown that 2',4'-BNA/DNA chimeric oligomers are able to knock down gene function in zebrafish embryos. One advantage of 2',4'-BNA/DNA chimeric oligomers is that the effective dose is much lower ( $\sim$ one-hundredth) than that of morpholino oligos. However, the effective dose is close to the toxic dose that triggers nonspecific developmental defects. Thus, the fine adjustment of target sequences, the number and position of the 2',4'-BNA, and the RNase H induction activity are required to

effectively achieve knockdown using 2',4'-BNA/DNA AONs. Future studies are required to further improve the effectiveness of 2',4'-BNA/DNA AONs in zebrafish.

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#### AU2 ► Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to: ◀AU4  
Prof. Motoyuki Itoh ◀AU5

Graduate School of Pharmaceutical Sciences  
Chiba University  
Research Building1, Room 308  
1-8-1, Inohana, Chuo-ku  
Chiba, 260-8675  
Japan

E-mail: mito@chiba-u.jp

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