The use of RNAi technologies for gene knockdown in zebrafish

Amanda Kelly and Adam F. Hurlstone

Advance Access publication date 27 April 2011

Abstract
Despite being a popular and versatile model organism in which to study development and model disease, the use of zebrafish has been hampered by the lack of a reliable, stable and cost-effective method of gene knockdown. It is therefore not surprising that the discovery of RNAi as an exploitable method of post-transcriptional gene regulation has created a lot of excitement within the zebrafish research community. However, despite concerted efforts in the field, progress in the use of RNAi technologies in zebrafish has been extremely slow and a reliable means of RNAi-mediated gene knockdown remains elusive. The following reviews progress in the field to date, highlights the major pitfalls identified and suggests possible future directions.

Keywords: RNA interference; zebrafish; siRNA; dsRNA; vector-mediated RNAi; Argonaute 2

INTRODUCTION
The ability to knockdown or knockout genes has proved invaluable in the elucidation of gene function and for the development of disease models in a variety of model systems. At present the most widely used method of gene knockdown in zebrafish is morpholinos, antisense oligonucleotides which either block translation of mRNAs or interfere with correct splicing of mRNA [1]. Although they have proved a powerful tool, their short-lived nature and numerous reports of ‘off-target’ effects has limited their use [2]. The discovery of RNAi, an evolutionarily conserved, innate and potent method of sequence-specific gene knockdown, has therefore opened up a new potential avenue of exploration for targeted gene knockdown in zebrafish.

The RNAi pathway: a brief overview
RNAi is a process of post-transcriptional gene regulation in which small RNA effector molecules sequence-specifically regulate the expression of cognate mRNAs by either causing their destruction or repressing their translation. The RNA effector molecules can either be endogenously expressed microRNAs (miRNAs) or exogenous small interfering RNAs (siRNAs). Endogenous miRNAs are transcribed in the nucleus either as part of a host gene or as a gene in its own right. Primary miRNA transcripts, consisting of a double-stranded stem, a terminal loop and flanking single-stranded (ss)RNA, are cleaved by the RNaseIII-like enzyme Drosha into ~70 nt long stem–loop precursor miRNAs [3–6], which are then transported out of the nucleus by the nuclear transport receptor complex Exportin 5-RanGTP [7–9]. The loop and flanking ssRNA are then removed by Dicer, giving rise to ~22 nt duplex miRNAs with asymmetric 3′-ends [10–12]. The mature miRNA duplex is then unwound and one strand preferentially loaded into RNA-induced silencing complex (RISC) [13], the major component of which is a member the Argonaute (Ago) family of proteins [14]. RISC complexes loaded with miRNAs then bind to mRNAs with which they have complementary sequence. In the case of endogenous miRNAs the majority bind through a seed region (usually nucleotides 2–8) to the 3′-UTR of their target mRNA and either repress translation or cause the destabilization of the mRNA. Some mRNAs however bind with complete complementarity to the target and result in the cleavage of the mRNA (Figure 1). Concurrent with the discovery of miRNAs and the miRNA pathway was

Corresponding author. Adam F. Hurlstone, Faculty of Life Sciences, Michael Smith Building, Oxford Road, The University of Manchester, Manchester, M13 9PT. E-mail: adam.hurlstone@manchester.ac.uk

Amanda Kelly is a former postgraduate student of the Hurlstone Laboratory, the University of Manchester.

Adam F. Hurlstone is a lecturer and member of the Molecular Cancer Studies group, the University of Manchester.

© The Author 2011. Published by Oxford University Press. All rights reserved. For permissions, please email: journals.permissions@oup.com
Figure I: The RNAi Pathway. Endogenous primary miRNAs (pri-miRNAs) are transcribed in the nucleus, processed into ~70 nt precursor miRNAs (pre-miRNAs) by Drosha, transported out of the nucleus by Exportin 5 and further processed into mature miRNAs by Dicer. Dicer also processes long dsRNA molecules into small interfering (si)RNA duplexes. One strand of the miRNA or siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by binding through a seed region in the 3'-UTR and causing translational repression or mRNA destabilization or, by binding with complete complementarity and cleaving the mRNA. The major component of RISC is a member of the Argonaute (Ago) family of proteins of which there are four. Agos 1–4 are all capable of causing translational repression and mRNA destabilization. However, only Ago2 has endonuclease activity and can result in cleavage of the target mRNA.
the discovery in Caenorhabditis elegans that exogenous double-stranded (ds) RNA could also down-regulate mRNAs with which it shares sequence homology [15, 16]. The dsRNAs are ‘diced’ by Dicer into ~22 nt ds siRNAs which resemble endogenous mature miRNAs and follow down the same pathway as described above [17]. However, unlike miRNAs the majority of siRNAs bind to their target with complete complementarity and result in cleavage of the target mRNA (Figure 1).

This discovery has led to an explosion of the use of RNAi as a method to knockdown genes both in vitro and in vivo. The small interfering effector molecules can either be delivered as dsRNAs, chemically synthesized siRNAs or through the use of DNA vector-based systems leading to stable expression of customized siRNAs.

The importance of the RNAi pathway in zebrafish
To date, >300 zebrafish miRNA sequences have been deposited in miRBase [18–20] and the total predicted number of miRNAs stands at >400 [21]. They have been shown to be involved in numerous biological pathways and are expressed throughout development and across tissues [22]. Furthermore, the zebrafish RNAi pathway has been shown to be essential for zebrafish development, as disruption of the dicer gene results in developmental arrest at 10 dpf [23]. The disruption of dicer gene should prevent the biogenesis of mature miRNA, however, due to maternal contribution of dicer mRNA and protein, mature miRNAs do accumulate in these embryos for the first few days. The use of a germ line replacement technique to produce maternal-zygotic dicer (MZdicer) mutants which lack any maternally contributed dicer, results in a complete loss of mature miRNA biogenesis and the effects on development are much more severe. Although these mutant embryos are able to undergo normal axis formation and differentiation of numerous cell types, severe morphogenetic abnormalities occur during gastrulation, brain development, somitogenesis and heart development [24]. Thus miRNAs and functional RNAi machinery play an essential role in zebrafish development. In particular, the miRNA miR–430 is extremely important during zebrafish development, as injection of dicer processed miR–430 into MZdicer mutants rescued many of the brain and gastrulation defects associated with loss of mature miRNAs [24]. The miR–430 was later shown to be essential for the deadenylation and clearance of maternally contributed mRNAs after the initiation of zygotic transcription [25].

Targeted gene knockdown in zebrafish: dsRNA and siRNA
The first attempts at targeted gene knockdown in zebrafish by RNAi focussed on the injection of dsRNA. Wargelius et al. [26] and Li et al. [27] both reported highly efficient gene knockdown using this technique; however, Wargelius et al. [26] also reported high levels of ‘off-target’ effects that were present in all injected fish regardless of the dsRNA sequence injected. However, Oates et al. [28], Zhao et al. [29] and Mangos et al. [30] reported no evidence of targeted gene knockdown, but instead a more global knockdown of mRNA, which they speculated was most likely attributable to an interferon response as a result of injection of dsRNA. As a result of this, dsRNA has not been widely used for gene knockdown experiments in zebrafish.

The discovery by Caplen et al. [31] and Elbashir et al. [32] that chemically synthesized siRNAs do not elicit such an immune response and can effectively knockdown targets for which they were completely complementary in both invertebrate and vertebrate systems, provided new hope that the zebrafish RNAi pathway might be exploitable. The siRNAs were first used in zebrafish by Dodd et al. [33], who reported efficient and specific silencing of the dystrophin gene. Since then, there has been a few publications reporting specific and effective knockdown [34–36]. However, in contrast to these reports, Gruber et al. [37] suggested that injection of siRNAs into embryos results in a host of ‘off-target’ effects causing morphological defects, abnormal development and early death, similar to phenotypes seen in dicer null embryos. Zhao et al. [38] were later able to show that the ‘off-target’ effects associated with siRNA were caused by saturation of the RNAi machinery which prevented the biogenesis and activity of essential endogenous miRNAs. Furthermore, they were able to show that co-injection of pre-processed miR–430 duplexes with siRNAs significantly rescued the developmental defects associated with siRNA injection. As with dsRNA, due to these concerns of non-specific effects, the use of siRNAs as a means for gene knockdown in zebrafish has not been widely taken up by the zebrafish research community.
Targeted gene knockdown in zebrafish: vector-based approaches

As well as concerns over ‘off-target’ and toxic effects of injection of large doses of siRNAs in zebrafish embryos, a major limitation to the use of siRNAs in zebrafish embryos is the brief temporal manner in which they could be used. To this end, focus has shifted from the use of siRNAs towards the employment of DNA vector-based systems, in the hope that such systems would avoid the need for the injection of high doses of siRNA, while at the same time, when combined with methods for zebrafish transgenesis, provide a means of stable and heritable gene knockdown. Furthermore, through the use of different promoter elements knockdown could be controlled in both a spatial and temporal manner. However, despite the massive potential of such systems, at present there have been very few publications in this area.

Of the RNAi vector systems that have so far been described, the simplest uses two, in tandem, RNA polymerase (Pol) III-responsive promoters to drive expression of the sense strand and antisense strand of the siRNA, respectively, leading to the direct in vitro/in vivo production of siRNA (Figure 2a). Such vectors have been shown to be highly efficient in a variety of human cells [39–42]. However, in zebrafish although U6 promoter-driven expression of siRNA was reported to give rise to some ‘on target’ gene-specific knockdown, a variety of non-specific effects were also reported, similar to those seen with siRNA injection [43]. These non-specific effects could either have been caused directly, through interaction of the siRNA with other targets, or indirectly through the saturation of the RNAi machinery. Other vectors commonly used in gene knockdown studies in vitro and in other organisms drive expression of transcripts which enter the RNAi pathway further upstream and so the amount of siRNA production is regulated by the availability of RNAi components in the cell. These vectors fall broadly into two main categories: the RNA Pol III-responsive vectors which drive ‘ubiquitous’ expression of short hairpin (sh)RNAs, that

![Figure 2: Vectors used for gene knockdown studies in zebrafish. (a) RNA polymerase (Pol) III-responsive promoter-driven expression of siRNA. Two in tandem Pol III-responsive promoters drive expression of the sense and antisense strand of siRNA, respectively, leading to production of double-stranded siRNA. (b) RNA Pol III-responsive promoter-driven expression of short hairpin (sh)RNA. This vector gives rise to an RNA transcript which resembles Drosha-processed precursor miRNA. (c) RNA Pol II-responsive promoter-driven expression of a customized primary miRNA and reporter gene. The primary miRNA sequence with customized targeting sequence is embedded with an intronic region of a host gene. The host gene can be a reporter gene, such as green fluorescent protein, to allow for easy visualization of cells expressing customized miRNA.](https://academic.oup.com/bfg/article-abstract/10/4/189/209773)
resemble drosha-processed precursor miRNAs [44] (Figure 2b) and vectors which use RNA Pol II-responsive promoters to drive expression of longer transcripts based on endogenous primary miRNAs, such as human miR-30 as described by Zeng et al. [45, 46]. In the case of the latter, the primary miRNA sequence with customized targeting sequence is embedded within an intronic region of a host gene. The host gene is usually a reporter gene which allows for easy visualization of transgene expression (Figure 2c). The use of different Pol II-responsive promoters also allows for knockdown to be controlled in a tissue-specific/conditional manner. However, despite extensive use of these types of vectors in mammalian and other systems, there have been very few and contradictory reports of their use in zebrafish.

Wang et al. [47] and Su et al. [48] used in vivo T7 promoter-driven shRNAs and Cytomegalovirus (CMV) promoter-driven shRNAs respectively and reported significant (~70% as analysed by qPCR) and sequence-specific knockdown of GFP and the zebrafish gene no tail (ntl). However, work in our own laboratory using both the zebrafish RNA Pol III-responsive promoter, H1, to drive expression of shRNA and the zebrafish RNA Pol II-responsive promoter, H2Az/f, to drive expression of customized miRNAs targeted against GFP has yielded little evidence of targeted gene knockdown either transiently in injected embryos or in stable transgenic lines. Furthermore, we were also unable to find evidence of knockdown in zebrafish cell culture using a variety of different cell types including AB.9, PAC.2 and ZFL cells. This is despite the same vectors being able to knockdown GFP in HEK 293 cells by >90% (unpublished data). In agreement with these findings, Dong et al. [49] also found no evidence of GFP knockdown in zebrafish embryos using a similar RNA Pol II-responsive system under the control of the CMV promoter when targeting the open-reading frame (ORF). Again, this was despite demonstration of efficiency of the customized miRNAs in HEK 293 cells and the detection of abundant customized GFP miRNAs in zebrafish embryos by northern blot. Surprisingly, however (or perhaps not if you consider how the majority of endogenous metazoan miRNAs function), the same group was able to show that by targeting the 3′-UTR of GFP or the erythroid-specific gene, gata1, specific and heritable knockdown could be achieved, with efficiency of GFP knockdown ranging from ~45% at 24 hpf to 62% at 72 hpf. Furthermore, they showed that knockdown could be driven by an ubiquitous promoter such as CMV or tissue specifically with the erythroid-specific gata1 promoter or the hemangioblast-specific Imo2 promoter. Whilst the demonstration of vector-mediated RNAi when directed against 3′-UTRs represents a major advancement in the use of RNAi for gene knockdown in zebrafish, it is important to note that the levels of knockdown so far observed are only modest, and use of such an approach may at best only be used to model the effects of haplo-insufficiency of a gene and not total knockdown. In addition, targeting the 3′-UTR had previously been avoided due to the increased likelihood of ‘off-target’ effects as binding of the siRNA/miRNA to the target is dependent on a seed region (usually bases 2–8) only, whereas targeting of the ORF requires complete complementarity between siRNA/miRNA and the target. Therefore, caution should be taken if using this approach and the use of several distinct siRNA/miRNA sequences for each target gene is to be advised to ensure that phenotypes observed are due specifically to knockdown of the target gene. The mechanism of gene knockdown, however, is not only governed by the position and amount of sequence similarity, but also by the relative abundance of different silencing complexes. The major component of the silencing complex is a member of the Ago family, of which there are four. Although all four Ago proteins are able to bind to the 3′-UTR of their cognate genes and repress translation, only Ago2 has intrinsic endonuclease activity and is able to directly cleave message where there is complete complementarity between the siRNA/miRNA and the target (Figure 1) [50, 51]. That targeting of the 3′-UTR and not the ORF leads to gene knockdown in zebrafish embryos suggests that levels of zebrafish Ago2 may be limiting. This further suggests that even when targeting the ORF, it is likely that siRNAs/miRNAs may actually act upon other targets through a seed region in the 3′-UTR and so may also give rise to a variety of ‘off-target’ effects. Indeed, work by Wu et al. [52] has demonstrated that decreasing relative abundance of Ago2 compared to the other Ago proteins correlates with increasing ‘off-target’ effects of siRNAs and vice versa in a variety of human cell lines.

In an attempt to induce cleavage-mediated RNAi directed against the ORF, we investigated the effect
of over-expression of human Ago2 in zebrafish cells and embryos. In agreement with work by Diederichs et al. [53] in human cells and by Chen et al. [54] in the Xenopus central nervous system (CNS), where vector-mediated RNAi against the ORF had been shown to be ineffective, over-expression of Ago2 did lead to induction of RNAi-mediated knockdown in two independent zebrafish lines (PAC.2 and ZFL cells) and the level of knockdown was similar to that seen when targeting the 3′-UTR (~50%). Transient over-expression of Ago2 in zebrafish embryos by means of mRNA injection, however, failed to result in knockdown (unpublished data). This may simply be due to insufficient levels of Ago2 expression being achieved through mRNA injection and work has already begun on the creation of a stable transgenic Ago2 expressing line to test this theory. Equally however, it may suggest that other factors are also limiting RNAi in zebrafish embryos; this could be other components of the RNAi machinery or levels of transgene expression.

**Future directions**

Future work should look at whether vector-mediated RNAi in zebrafish might be improved through increasing transgene expression or increasing expression of components of the RNAi machinery. It is also important to note that most attempts at RNAi in zebrafish using siRNAs or vector-mediated approaches have concentrated on being able to detect global knockdown of genes at the whole organism level. However, given that there is likely to be variability in the RNAi potential of different cell types/tissues, it is plausible that different lineages may be more susceptible than others to RNAi-mediated knockdown. Indeed, although RNAi vectors have been successfully used to knockdown genes in many mouse tissues [55], some cell types such as differentiating chondrocytes seem to be resistant to RNAi-mediated gene knockdown [56]. Equally, despite reports of successful vector-mediated RNAi in Xenopus embryos [57], Chen et al. [54] have reported inefficient RNAi-mediated gene knockdown in the CNS of Xenopus tadpoles. Therefore, full analysis of the efficacy of different zebrafish cell types to perform RNAi would be appropriate. It should also be noted that experiments so far have only looked at knockdown in zebrafish embryos in the first few days post-fertilization. With the use of DNA-based vector strategies it is now possible to assess knockdown throughout development and in adults. Indeed, preliminary results from our laboratory have suggested that adult zebrafish brains may be amenable to RNAi, as a 50% knockdown of the Parkinson’s disease associated gene, PINK1, has been obtained in this tissue. Interestingly, this knockdown coincided with high level Ago2 expression and high levels of customized miRNA expression compared to other tissues (unpublished data).

**SUMMARY**

Despite the enthusiasm for the use of RNAi technologies for gene knockdown in zebrafish, the promise of efficient, stable and targeted gene knockdown has not yet been achieved. Initial studies using dsRNA resulted in global mRNA knockdown brought about by the induction of interferon response, while the injection of high doses of siRNAs in zebrafish embryos is not feasible as saturation of the RNAi pathway prevents the biogenesis and activity of essential endogenous miRNAs. It was hoped, therefore, that a move towards DNA vector-mediated delivery of RNAi would avoid the problems associated with injection of high doses of siRNAs and at the same time, enable stable and heritable knockdown of target genes. Despite the successful use of such vector-based RNAi systems in other model organisms such as the mouse [55] and chick [58] progress in zebrafish has been extremely slow with very few publications in this area. Those results which are published are also contradictory with some suggesting modest knockdown can be achieved in this manner while others finding no evidence of knockdown. Interestingly the report by Dong et al. [49] suggests that while targeting the ORF is ineffective, targeting the 3′-UTR does lead to knockdown. These results, however, are yet to be confirmed by others and so how robust this approach is as a general method of gene knockdown in zebrafish is yet to be determined. Furthermore, this approach should be used with caution as exact sequence match between miRNA/siRNA and the target mRNA is not required. Apparent resistance of zebrafish embryos to ORF-targeted RNAi despite being able to detect transgene-mediated production of customized miRNAs, may suggest that some component of the RNAi machinery or the level of transgene expression may be limiting in zebrafish. Interestingly, resistance to RNAi technologies has also been reported in mouse chondrocytes [56] and in the Xenopus CNS [54], suggesting that this is not
only a zebrafish-specific problem, but may be applicable to other organisms/cell types. Further work to see whether RNAi can be induced in zebrafish either through increasing levels of expression of miRNAs/siRNAs or through over-expression of components of the RNAi machinery will therefore be of benefit not only to the zebrafish research community but may have broader implications for the use of RNAi technologies in other organisms.

Although there are still avenues that need to be explored, it seems that the complexity of the RNAi pathway and potential for ‘off-target’ effects of customized siRNAs/miRNAs may mean that an easy, reliable, ‘one strategy fits all’ solution to RNAi targeted gene knockdown in zebrafish may not be feasible. As such, morpholinos remain the most effective method of gene knockdown in zebrafish and a stably inheritable system in which expression of genes can be spatially and temporally controlled remains elusive.

Key Points

- Use of zebrafish is hampered by the lack of an efficient, reliable, cost-effective and stable method of gene knockdown.
- Attempts to knockdown genes through delivery of dsRNA and siRNAs into zebrafish embryos have resulted in a host of non-specific effects.
- Reports of vector-mediated RNAi against ORFs in zebrafish embryos are limited and contradictory, but appear to suggest this is not a reliable approach for targeted gene knockdown.
- Targeting the 3’-UTR has been suggested as a more effective alternative to targeting the ORF for gene knockdown in zebrafish. However, the robustness of this system requires further validation and caution should be taken when using this approach due to the potential for ‘off-target’ effects.
- Despite the limitations of morpholinos, they remain the most effective method of gene knockdown in zebrafish.

FUNDING

BBSRC and Summit Plc. (formally DanioLabs) (to A.K.); Cancer Research UK (to A.F.H.).

References


