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Abstract	The microRNA (miRNA) pathway and the phenomenon of RNA interference (RNAi), which have both been shown to involve targeting of mRNAs by small RNA molecules, are interconnected and depend partly on the same cellular machinery. RNAi in vertebrates was first reported in zebrafish (*Danio rerio*) 10 years ago. However, reliable RNAi-based gene silencing techniques, based on injection of siRNAs into zygotes, have not been established for this important vertebrate model because of unspecific developmental defects. We have recently shown that these side effects can be attributed to inhibition of the miRNA pathway by siRNAs at early embryonic stages. This review highlights these findings and the function of microRNAs in zebrafish development.

Keywords (separated by '-')	RNAi - siRNA - microRNA - miR-430 - zebrafish

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Chapter 15

Inhibition of the microRNA Pathway in Zebrafish by siRNA

Anders Fjose and Xiao-Feng Zhao

Abstract

The microRNA (miRNA) pathway and the phenomenon of RNA interference (RNAi), which have both been shown to involve targeting of mRNAs by small RNA molecules, are interconnected and depend partly on the same cellular machinery. RNAi in vertebrates was first reported in zebrafish (*Danio rerio*) 10 years ago. However, reliable RNAi-based gene silencing techniques, based on injection of siRNAs into zygotes, have not been established for this important vertebrate model because of unspecific developmental defects. We have recently shown that these side effects can be attributed to inhibition of the miRNA pathway by siRNAs at early embryonic stages. This review highlights these findings and the function of microRNAs in zebrafish development.

Key words: RNAi, siRNA, microRNA, miR-430, zebrafish.

1. Introduction: Connections Between the RNAi and miRNA Pathways

The discovery of RNA interference (RNAi) demonstrated that exogenously introduced double-stranded RNA (dsRNA) molecules could trigger degradation of cognate messenger RNA (mRNA) sequences in the nematode *Caenorhabditis elegans* (1). This RNA silencing process was subsequently shown to involve generation of small interfering RNA (siRNA) molecules of ~22 nucleotides (nt), which act as guides to target particular mRNAs for cleavage (2). Research on developmental regulatory genes in *C. elegans* also led to the discovery of a new category of small endogenous RNA molecules, microRNAs (miRNAs), generated by processing of transcripts containing certain stem-loop structures (3–6). These miRNAs have approximately the same size as siRNAs, and their generation and function depend to a large

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extent on the same cellular machinery (7–9, *see* **Chapters 14** and **16**).

During the past 10 years, rapid progress in the research on these two RNA-mediated silencing mechanisms has revealed that they are widespread in eukaryotic organisms, where they are involved in cellular control of gene expression or protection against parasitic and pathogenic invaders such as transposons and viruses (8, 10). In addition, various studies have uncovered that they have essential roles in developmental processes and can be associated with diseases (11, 12). Importantly, these small-RNA-mediated pathways can also be utilized experimentally or therapeutically to knock down expression of specific genes (13, 14).

Generation of siRNAs and miRNAs from dsRNA precursors that are produced in or introduced into cells depend on the ribonuclease III (RNase III) enzyme known as Dicer (8, 15). In the case of miRNAs, the dsRNA precursors (pre-miRNAs) are ~65 nt stem-loop structures, which are excised from longer primary transcripts (pri-miRNAs) in the nucleus by the RNAse III enzyme Drosha and exported to the cytoplasm by nucleocytoplasmic transport factors (8, 9, 16). In the cytoplasm, one of the strands from the duplexes of miRNAs or siRNAs, which has the weakest base pairing at its 5′ terminus, is preferably incorporated into the RNA-induced silencing complexes (RISCs) miRISC or siRISC, respectively (8). The loaded single-stranded RNA (ssRNA), sometimes called the guide strand, targets RISCs to mRNAs that contain homologous sequences.

Whereas siRNAs, in general, and the miRNAs in plants are perfectly complementary to their targets, animal miRNAs usually base pair with imperfect complementarity to target sequences located within the 3'UTRs of mRNAs. The first 2–8 nucleotides at the 5′ end of miRNAs, commonly referred to as the "seed" region, is most important for target recognition and silencing (17, 18). However, the presence of secondary structures and other features of the 3'UTR sequence surrounding the target site may also influence how miRNAs and mRNAs interact.

Each RISC contains an Argonaute (AGO) protein as its core component and several additional protein factors that may vary in different types of complexes (8). Importantly, different members of the AGO family, which contain the highly conserved RNA-binding domains PAZ and PIWI, are core components in siRISCs and miRISCs. AGO proteins in siRISCs silence targeted mRNAs by catalyzing their endonucleolytic cleavage. In the animal miRNA pathway, AGO proteins are known to inhibit the expression of mRNAs by various mechanisms, including translational inhibition, mRNA degradation, and rapid degradation of nascent peptides (19). In some cases, animal miRNAs may also have sufficient complementarity to sequences

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in the targeted mRNAs to cause endonucleolytic cleavage, indicating that there is no clear distinction between miRNAs and siRNAs (20).

The miRNA gene number in vertebrate species is currently estimated to exceed 500 genes, which can be grouped into >40 families based on seed sequence identity (21–24). Furthermore, various computational predictions and experimental studies indicate that single miRNAs can target >100 different mRNAs (25, 26). Hence, at least 30% of human genes are probably regulated by miRNAs (17). Considering these estimates, it can be assumed that miRNAs have global impact on gene-expression profiles, and may in some cases function as developmental switches. Several recent studies in mice and zebrafish have provided experimental evidence in support of these ideas (11, 25). In particular, the family of miR-430 miRNAs in zebrafish has been shown to be important for the removal of a large group of maternal mRNAs after the initiation of zygotic transcription at the mid-blastula stage, which is critical for normal development (25, 27). Furthermore, establishment of new techniques to manipulate and assay the activity of specific miRNAs in zebrafish, which are based on microinjection of reporter mRNAs and inhibitors of processing and target binding, have led to important contributions to our understanding of miRNA functions in vertebrates (25, 28–30).

RNAi is a powerful tool to knock down expression of specific genes, and it has been employed as a research tool in a variety of species, including animal models such as *C. elegans*, *Drosophila melanogaster*, and mice (31–33). In addition, RNAi methods have been used extensively to study gene functions in mammalian cell culture systems (34). Furthermore, strong efforts have been made to develop RNAi-based therapeutics in humans (35, 36).

Although the first observations of RNAi in vertebrates were reported for zebrafish (37), RNAi has not been established as a reliable tool for analysis of gene functions in this important animal model due to frequent occurrence of unspecific developmental aberrations (28, 38). The factors causing this problem have remained unknown until recently, when it was demonstrated that siRNA treatment led to inhibition of the miRNA pathway in zebrafish embryos (39). In particular, siRNA injections were shown to reduce the level of mature miR-430 miRNAs, and in this way affect the maternal to zygotic transition (25, 39). This finding may help us to develop new RNAi techniques for zebrafish that can circumvent the problem leading to unspecific developmental defects. The observations in zebrafish also suggest a need for investigating whether similar inhibition of the miRNA pathway may occur in mammals under some experimental or therapeutic conditions.

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2. Functions of miRNAs in Zebrafish Development

2.1. Zebrafish miRNAs and Analytical Tools

Identification and characterization of miRNAs in zebrafish have provided important information regarding the conservation of miRNA genes in vertebrates. Zebrafish have also proven to be a particularly valuable system to study miRNA functions in developmental processes. In the initial investigations, small-RNA cDNA libraries from different developmental stages of zebrafish were used for large-scale sequencing to obtain a comprehensive picture of the total number of miRNAs (40–42). An additional approach has been to use prediction algorithms to search in the zebrafish genome database to identify conserved hairpin structures of 60–100 nucleotides (22). These analyses have led to identification of 415 zebrafish miRNAs, and based on seed sequence identity it has been possible to group these into 44 families (22). However, since zebrafish genome sequencing and annotation is not yet complete, the exact number of miRNAs remains unknown.

Synchronous populations of several hundred fertilized zebrafish eggs (zygotes) are easily available for microinjection of reporter constructs, RNA molecules or chemically modified nucleic acids, which can facilitate investigations of miRNA functions in developmental processes. For gain-of-function studies, mature miRNA duplexes, which have been chemically synthesized, can be injected into zygotes to analyze how overexpression of specific miRNAs may affect development (28). Mature miRNA duplexes can also be coinjected with reporter mRNAs to test whether predicted miRNA-binding sites can mediate silencing of the presumptive target mRNA. This type of assay is based on coinjections of specific miRNA duplexes with reporter mRNAs containing a green fluorescent protein (GFP) coding sequence and a 3'UTR region with the predicted miRNA-binding site (25).

Antisense morpholino phosphorodiamidate oligonucleotides (MOs) have been used extensively to knock down gene functions in zebrafish embryos and larvae by inhibition of splicing and/or translation of specific mRNAs (43, 44). Due to the chemical modifications, these oligonucleotides are not susceptible to enzymatic degradation and can be applied to inhibit gene functions in zebrafish larvae several days after microinjection at the zygote stage. MOs have also proved to be very useful as tools to inhibit specific miRNAs, and can be designed to block processing of the pre-miRNA or the activity of the mature miRNA (29, 45). In addition, MOs designed to be complementary to specific miRNA-binding sites in target mRNAs have been shown to efficiently protect the target mRNA from translational inhibition or degradation (30). The efficiency of "target protectors" to block the interaction of miRNAs with a particular binding site in a target

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mRNA can be investigated by coinjections with reporter mRNAs containing a GFP coding sequence and a 3'UTR region with the miRNA-binding site (30). Subsequently, a tested target protector can be used to study whether protection of a specific target mRNA from silencing has any biological effect.

A key step toward understanding the function of specific miRNAs is to determine the spatiotemporal expression pattern during development. However, detection of miRNAs by in situ hybridization has been technically difficult because of their small size. This problem was solved when it was discovered that locked nucleic acid (LNA) modified DNA oligonucleotide probes can provide increased sensitivity for detection of miRNAs by Northern blots (46), and can be applied for whole-mount in situ detection of specific miRNAs in zebrafish embryos and larvae (40, 47).

The temporal and spatial expression patterns of many zebrafish miRNAs have been investigated by in situ hybridization, microarray, and/or Northern analyses of different developmental stages (40, 41, 47). Studies of the temporal profiles revealed that only a few are expressed before the end of gastrulation. The majority of the zebrafish miRNAs also display tissue-specific expression patterns, suggesting functional roles in differentiation or maintenance of tissue identity (47). Similar spatiotemporal features have been observed in other vertebrates, and generally miRNA homologues are expressed in the same type of tissue (12, 48).

2.2. Functional Roles of Specific miRNAs

Studies of abnormalities caused by *dicer* mutations in mice and zebrafish provided the first evidence that generation of mature miRNAs is essential for normal development, but alternative explanations involving failure to produce endogenous siRNAs could not be excluded (49, 50). In zebrafish, homozygous *dicer* mutants were shown to produce mature miRNAs during the first few days, due to the presence of maternally contributed *dicer* (49). As a consequence these zygotic *dicer* (Z*dicer*) mutants have no visible defects other than a developmental arrest around day 10 postfertilization (49).

The problem of maternal *dicer* activity could be circumvented when a germ line replacement technique was used to generate embryos that were maternal–zygotic mutant for *dicer* (MZ*dicer*). These MZ*dicer* embryos did not generate mature miRNAs and displayed severe morphogenesis defects during gastrulation, brain formation, somitogenesis, and heart development (51). Further analyses revealed a causal connection between some of these abnormalities and members of the miR-430 family of miRNAs, which are the only abundant miRNAs at blastula and gastrula stages (41, 51). These miRNAs are expressed ubiquitously from multiple gene copies, mainly located in two large clusters on chromosome 4 (22, 41).

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Experimental evidence that normal development depends on the miR-430 family was obtained from microinjections of preprocessed duplexes of these miRNAs into MZ*dicer* zygotes, which resulted in almost complete rescue of the brain and gastrulation defects (51). In particular, these injections were shown to rescue the formation of normal brain ventricles and brain constrictions, including the characteristic fold of the midbrain–hindbrain boundary (MHB), revealing essential roles for the miR-430 miRNAs during brain morphogenesis. Hence, it could also be concluded that this particular phenotype depended on the loss of a specific mature miRNA and was not due to the inability of MZ*dicer* mutants to generate endogenous siRNAs. However, since miR-430 injections failed to rescue some of the abnormalities (e.g., heart defects) in MZ*dicer* embryos, it remains a possibility that endogenous siRNAs are required for normal development. Alternatively, these additional defects may be associated with the function of other miRNAs.

To elucidate how the lack of mature miR-430 miRNAs could lead to morphogenesis defects, it was important to consider the temporal expression profile and how it related to processes known to occur at the same embryonic stages. Interestingly, this showed that expression of the miR-430 family members begins at the moment of transition from maternal to zygotic expression, and their mature miRNAs rapidly accumulate to high levels (41, 51). This maternal–zygotic transition, which is a universal process in animal development, is also known to involve elimination of many maternal mRNAs (52). In zebrafish, this transition occurs at the mid-blastula stage (~2.5 hpf [hours postfertilization]) around 2 h before initiation of gastrulation (25).

Comparison of mRNA levels showed that >700 genes were expressed at significantly higher levels in MZ*dicer* mutant embryos than in wild-type embryos (25). Interestingly, target predictions indicated an enrichment of putative miR-430 target sites in the upregulated mRNAs, and several of these 3'UTR targets were validated in injection experiments with reporter mRNAs (25). On the basis of these results, it was estimated that the miR-430 family of miRNAs directly regulate several hundred maternal mRNAs (25). Further studies investigated the mechanism by which miR-430 promoted removal of the maternal target mRNAs. These investigations provided evidence that binding of miR-430 to the 3'UTR of maternal mRNAs leads to deadenylation, thereby triggering degradation (25). Hence, miR-430 seems to have a major role in facilitating clearance of a large group of maternal mRNAs at the transition to zygotic expression. In MZ*dicer* mutants, the absence of mature miR-430 leads to delayed degradation of the maternal target mRNAs, and consequently an extended period of mixed maternal and zygotic gene expression. This mixed state probably interferes with processes

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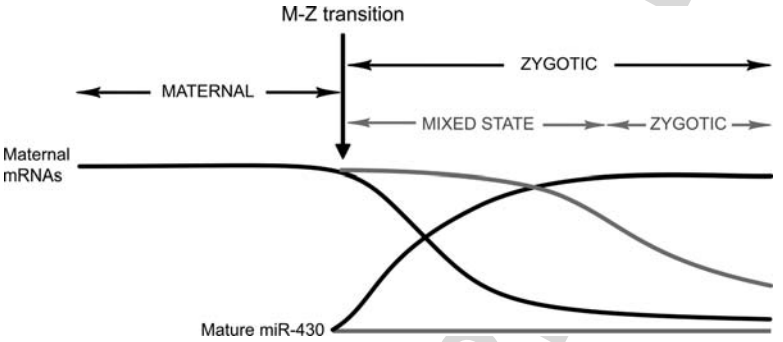


Fig. 15.1. Removal of maternal mRNAs at the maternal–zygotic (M–Z) transition in early zebrafish embryos is mediated by miRNAs from the miR-430 family. In MZ*dicer* embryos (*greyish lines* and characters), the mature forms of miR-430 miRNAs are not generated and as a result the removal of maternal mRNAs is delayed. The delayed removal leads to a mixed state of zygotic and maternal expression.

responsible for gastrulation and other types of morphogenesis (**Fig. 15.1**).

Similar miRNA-based mechanisms are likely to operate at the maternal–zygotic transition in other species of fish. In support of this assumption, conserved and clustered miR-430 miRNAs have been found in other fish genomes, including *Fugu rubripes* and *Tetradon nigroviridis* (51). This miRNA family is also conserved in amphibians, and studies on *Xenopus* have detected high levels of miR-430 during the blastula period (53). However, investigations on mammals and *Drosophila* have shown that unrelated types of miRNAs and/or endogenous siRNAs are involved in targeting of maternal mRNAs for degradation during the transition to zygotic expression (54, 55).

Although the MZ*dicer* mutants displayed morphogenesis defects, such as lack of brain ventricles and brain constrictions, detailed morphological analysis revealed that the body axis and all major subregions and cell types were not affected by the lack of mature miRNAs (51). Hence, mature miRNAs apparently do not have widespread essential roles in cell fate specification or signaling during early zebrafish development. However, several recent studies have demonstrated important roles of particular miRNAs in specific processes during zebrafish development, and similar results have been reported for various miRNAs mammals (11, 12, 21, 28). One important example of conservation of miRNA functions in vertebrates has been revealed for the Hox clusters, which encode transcription factors that are required for correct development of different tissues along the anterior–posterior axis (56). In the Hox clusters of different vertebrates, two conserved miRNAs, miR-10 and miR-196, are located in the same positions (56–58). Furthermore, studies in both zebrafish and mice have shown that these miRNAs target the mRNAs of nearby Hox genes and

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contribute to define their expression domains along the body axis (56, 57, 59).

In addition to an involvement in patterning of the anterior–posterior axis, several zebrafish miRNAs have been shown to participate in regulating the differentiation of highly specialized cell types. By using MOs to block the maturation of miR-375, it was revealed that this miRNA is essential for the formation of the insulin-secreting pancreatic islet (29). Similarly it was demonstrated that zebrafish miR-214 contributes to precise specification of muscle cell types by modulating a component of the hedgehog signal transduction pathway (45). Inhibition of miRNA maturation has also been applied in several other investigations where specific zebrafish miRNAs have been shown to have roles in erythroid maturation, fin regeneration, and cardiac patterning (60–62).

3. RNAi in Zebrafish

Following the discovery of RNAi in *C. elegans* (1), the first study of RNAi in vertebrates was reported for zebrafish by our laboratory (37). Using dsRNA microinjection in zygotes to target three genetically characterized genes, *ntl*, *flh*, and *pax2a*, we demonstrated that the mRNA levels were reduced and the embryonic defects were similar to the known mutant phenotypes of these loci (37). However, the efficiency to induce phenocopies of the mutants was quite low (<30%) and treatment with increasing concentrations of dsRNA was in some cases shown to cause a higher frequency of unspecific defects (37). Despite these limitations, it was assumed that RNAi-based techniques could be established as important tools for loss-of-function studies of specific zebrafish genes. Further attempts were made by several laboratories to explore the potential of dsRNA to knock down expression of specific genes, but these investigations showed conflicting results. Whereas some research groups claimed to generate specific effects with high efficiency (63, 64), other scientists reported that dsRNA injections resulted only in unspecific effects (65, 66).

At the same time as these investigations were made, it was discovered that antisense MOs, designed to inhibit translation and/or splicing, could be used as efficient tools to knock down expression of specific genes in zebrafish (67–69). However, the phenotypes induced by MO injections were also shown to include variable side effects that made interpretations difficult (67). An additional disadvantage of MOs was the high costs required for synthesis of these chemically modified oligonucleotides. Hence, it was still of interest to explore the possibilities to establish efficient

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RNAi-based techniques in zebrafish, and in this connection it was important to consider the potential causes for the high frequencies of unspecific abnormalities in dsRNA-injected embryos. One likely explanation was that these side effects could reflect a global antiviral/interferon response to long dsRNA (70). This issue was also discussed in relation to the application of similar methods in mammals, and using siRNAs to trigger RNAi in mammalian cells, the global responses associated with longer dsRNAs could be avoided (71).

Following the first demonstration of siRNA-mediated gene silencing in mammalian cells, several laboratories reported that siRNA injections could be used to induce gene-specific effects in zebrafish (72–74). In studies on three different zebrafish cell lines, highly efficient gene-specific knock down has also been demonstrated in experiments where several siRNAs were introduced by microinjection and transfection (38). However, when the same siRNAs were used for microinjection into zebrafish zygotes, most of the embryos at later stages displayed severe unspecific defects, including truncated tails, loss of brain structures, and enlarged heart cavities (38). The conflicting results reported by different laboratories are difficult to explain, but they may partially reflect differences in the injection procedures (e.g., amounts of siRNA injected) and variations in target-binding efficiencies of the siRNAs. It has also been important to find an explanation to the observation that siRNAs, which mediated specific gene silencing in zebrafish cell lines, were only able to cause unspecific abnormalities in embryos (38).

4. Inhibition of the miRNA Pathway by siRNA

Remarkably, the MZ*dicer* mutant embryos displayed several defects similar to those caused by siRNAs, and the defects in mutants were partially rescued by injection of preprocessed miR-430 miRNAs (38, 51). Based on these observations, and considering the common factors in the RNAi and miRNA pathways, we have investigated whether the function of miR-430 could be associated with the unspecific defects caused by siRNA treatment (39). Following injection of siRNAs corresponding to sequences in two endogenous genes (*eri1* and *six3a*) and the exogenous reporter gene GFP, we found by Northern analysis of RNA extracted from early embryos (12 hpf) that the levels of processed miRNAs were significantly reduced for the four miRNAs tested, including the miR-430 family member miR-430b (39) (**Fig. 15.2**). Importantly, these siRNA injections also caused brain morphogenesis defects and tail truncations similar to the MZ*dicer*

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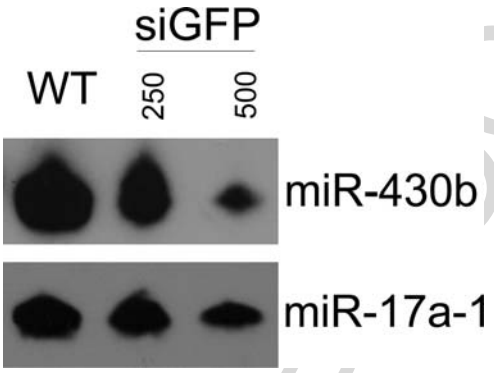


Fig. 15.2. Injection of siRNA affects the endogenous level of miRNAs. Following injection of different amounts (250 and 500 ng) of siRNA containing a GFP sequence (siGFP), Northern analysis of the endogenous levels of the processed forms of miR-430b and miR-17a-1 showed significant reductions as compared to noninjected embryos (WT) at the same developmental stage (12 hpf). Reproduced from (39) with permission from Blackwell Publishing Ltd.

phenotype (39) (**Fig. 15.3**). By contrast, injections of other types of RNA molecules such as single-stranded and double-stranded RNAs without 3′ overhangs did not affect embryonic development or the levels of mature miRNAs (39). Hence, these results suggested that the characteristic features of siRNAs were critical for reducing the levels of processed miRNAs and causing the unspecific defects in zebrafish embryos. Interestingly, we also observed strongest reduction (~70%) in the level of mature miR-430b (39) (**Fig. 15.2**).

To investigate a possible involvement of miR-430, we checked whether coinjection of preprocessed miR-430b duplexes could rescue the effects of siRNA treatment (39) (**Fig. 15.3**). Consistent with the previously reported rescue of MZ*dicer* mutants by miR-430 (51), we observed that a control injection of miR-430b alone did not induce any embryonic defects. However, in additional control experiments with the mature forms of miR-206, and a mutated version of miR-430b (miR-430b-mis), which lacks the power to rescue MZ*dicer* embryos (51), the embryos were affected in a similar way as when injected with siRNA (39). These results indicated that treatment with miRNA duplexes generally leads to the same kind of unspecific abnormalities as in the case of siRNAs, and this assumption was supported by observations of reduced levels of mature miR-430b following miR-206 injection (39). By contrast, coinjections of the three different siRNAs with preprocessed miR-430b duplexes resulted in efficient rescue of the unspecific defects, which were observed when these siRNAs were injected alone (39) (**Fig. 15.3**). Such rescue was not observed in coinjections with miR-430b-mis, which cannot bind target sites because of two-point substitutions in the 5′

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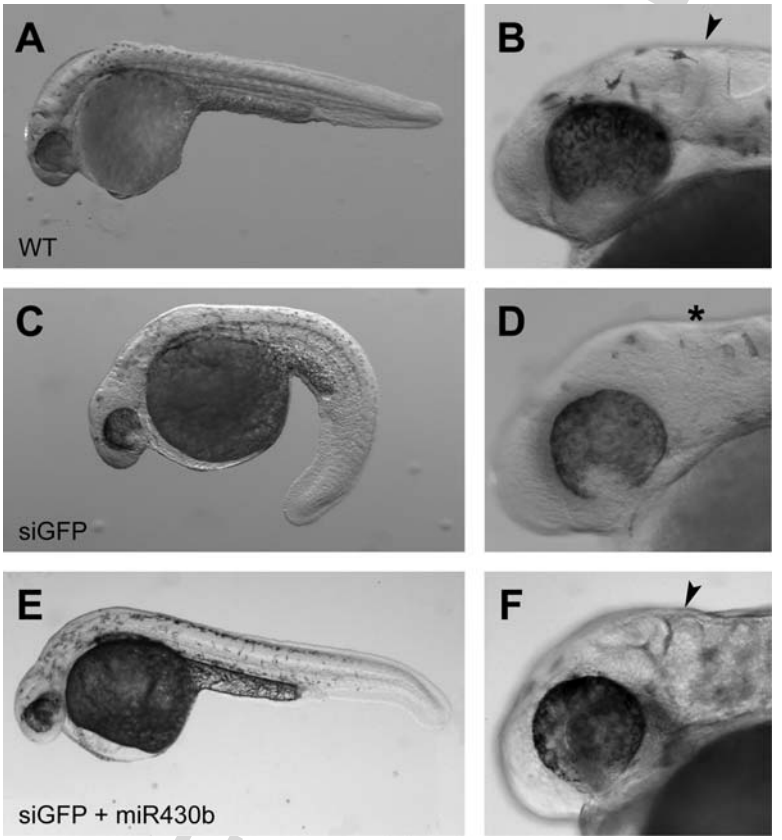


Fig. 15.3. Coinjection of mature miRNAs from the miR-430 family can rescue siRNA-induced abnormalities in zebrafish embryos. (**a**, **b**) Normal embryos have a straight tail and a characteristic fold at the MHB (*arrowhead*). (**c**, **d**) Injection of siRNA containing a GFP sequence (siGFP) caused tail defects and abnormalities at the MHB (*star* in **d**). The unspecific brain and tail defects caused by siRNA injection were rescued by coinjection of preprocessed miR-430b duplexes (**e, f**). Images in **c**–**f** are reproduced from (39) with permission from Blackwell Publishing Ltd.

seed region (51). These results clearly showed that the unspecific brain and tail defects induced by siRNAs, which correlated with a strong reduction of the endogenous level of mature miR-430b, could be prevented by coinjection of this particular miRNA (39).

Taken together, these observations strongly suggest a direct or indirect inhibitory effect of injected siRNA (and miRNA) duplexes on the processing of endogenous miRNAs, particularly miR-430, which is the most abundant miRNA at early embryonic stages. Such inhibition probably results from a competition between pre-miRNAs and injected siRNAs for binding to essential factors. This idea is based on the knowledge that siRNAs can associate with Dicer and other factors required for the generation of siRNAs and mature miRNAs from dsRNAs and pre-miRNAs, respectively (75–77). Accordingly, the injection of large amounts

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of siRNAs would affect the availability of these factors for processing of pre-miRNAs.

The relatively strong reduction of processed miR-430 caused by siRNA injection would be at least partially equivalent to the conditions in MZ*dicer* embryos where there is no production of mature miRNAs (51). Furthermore, the similarities of the unspecific defects relative to the MZ*dicer* phenotype, and the ability to rescue some of these by injection of preprocessed miR-430 duplexes, were also consistent with the role of miR-430 in promoting clearance of maternal mRNAs at the maternal–zygotic transition (25). Hence, our results suggest that siRNA treatment generally will induce these kinds of abnormalities in zebrafish embryos.

Notably, we also observed relatively high frequencies of unspecific defects after injecting smaller amounts of siRNAs, when the endogenous level of mature miR-430 was less affected (39). This could reflect an additional block of miRNA function involving the effector complex miRISC, due to competition with miR-430 (and other miRNAs) for binding to Argonaute proteins and/or other factors (**Fig. 15.4**). It seems likely that this type of inhibition was partially prevented by the coinjection of preprocessed miR-430b duplexes as well. Based on these considerations, it may seem possible to avoid the problem of unspecific effects in siRNA knockdown experiments simply by coinjecting mature miR-430 duplexes. However, with this strategy the efficiency of inducing specific phenotypes would probably be reduced because

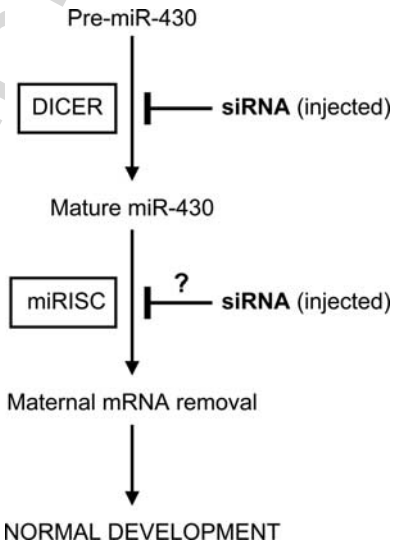


Fig. 15.4. A model proposed to explain how injection of siRNAs may inhibit the function of miR-430, which is essential for normal development. Injection of siRNA has been shown to reduce the level of mature miR-430 (39), but competition for critical factors may also affect miRISC function.

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of the competition between siRNAs and miR-430 for binding to essential components. Furthermore, the inhibition of other miRNAs expressed at early stages may cause some additional defects that cannot be rescued by coinjected miR-430.

Another approach to avoid inhibition of the miRNA pathway in early embryos may be to use chemically modified siRNAs ("caged" siRNAs) that can be activated by photoexposure at later stages during zebrafish development (78). Alternatively, it may be possible to establish transgenic RNAi techniques in zebrafish that are similar to the techniques currently being used in *Drosophila* and mice (79, 80). Vectors developed for inducible or tissue–specific expression of short-hairpin RNAs (shRNAs) should make it possible to circumvent the problems associated with maternal mRNA clearance during the maternal–zygotic transition (81–83). However, it remains possible that the general inhibitory effect of siRNAs (or shRNAs) on the miRNA pathway at later developmental stages could lead to some unspecific defects as well. This general inhibition may also be a problem in studies on mammals, and it will be particularly important to investigate these aspects in relation to the therapeutic use of RNAi in humans.

It cannot be excluded that siRNA treatment can cause some additional side effects in zebrafish. In the case of MO knockdown experiments, about 15–20% of the MOs used in zebrafish show off-targeting effects, and these have been shown to result from induction of a p53-dependent cell death pathway (84). Although the mechanism of MO-induced p53 activation is not known, it appears to depend on the MO sequences used (84). Studies on mammalian cells have also revealed that some siRNA sequences can cause a significant increase in the protein level of p53 (85). However, it is still unclear whether siRNA treatment can result in similar side effects in zebrafish.

5. Conclusions

The discovery of RNAi has provided us with powerful new tools to investigate specific gene functions in several animal models. Similarly, the identification of miRNAs has revealed novel cellular mechanisms that control gene expression. However, the rapid progress in our understanding of these two pathways has shown that they are quite strongly interconnected, and use of RNAi to knock down gene expression may in some cases influence the miRNA pathway and cause side effects. Our work on zebrafish has uncovered a general inhibitory effect of siRNAs on the miRNA pathway, which particularly affects the global role of miR-430 in the removal of maternal mRNAs at the point of transition

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from maternal to zygotic expression. This finding may help us to establish reliable siRNA-mediated gene silencing techniques in the zebrafish model. It also emphasizes the need to investigate how experimental and therapeutic use of RNAi may affect miRNA functions and cause unwanted side effects.

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References

1. Fire, A., Xu, S., Montgomery, M.K. et al. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature*, **391**, 806–811.
2. Bernstein, E., Caudy, A.A., Hammond, S.M. et al. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, **409**, 363–366.
3. Lau, N.C., Lim, L.P., Weinstein, E.G. et al. (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans. Science*, **294**, 858–862.
4. Reinhart, B.J., Slack, F.J., Basson, M. et al. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans. Nature*, **403**, 901–906.
5. Wightman, B., Ha, I., and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans. Cell*, **75**, 855–862.
6. Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843–854.
7. Zeng, Y., Yi, R., and Cullen, B.R. (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci USA*, **100**, 9779–9784.
8. Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol*, **8**, 23–36.
9. Kim, D.H., Behlke, M.A., Rose, S.D. et al. (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol*, **23**, 222–226.
10. Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.
11. Stefani, G. and Slack, F.J. (2008) Small noncoding RNAs in animal development. *Nat Rev Mol Cell Biol*, **9**, 219–230.
12. Kloosterman, W.P. and Plasterk, R.H. (2006) The diverse functions of microRNAs in animal development and disease. *Dev Cell*, **11**, 441–450.
13. Dillon, C.P., Sandy, P., Nencioni, A. et al. (2005) RNAi as an experimental and therapeutic tool to study and regulate physiological and disease processes. *Annu Rev Physiol*, **67**, 147–173.
14. Beal, J. (2005) Silence is golden: can RNA interference therapeutics deliver? *Drug Discov Today*, **10**, 169–172.
15. Hammond, S.M. (2005) Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett*, **579**, 5822–5829.
16. Yi, R., Qin, Y., Macara, I.G. et al. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, **17**, 3011–3016.
17. Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
18. Doench, J.G. and Sharp, P.A. (2004) Specificity of microRNA target selection in translational repression. *Genes Dev*, **18**, 504–511.
19. Pillai, R.S., Bhattacharyya, S.N., and Filipowicz, W. (2007) Repression of protein

UNCORRECTED PROOF

673 synthesis by miRNAs: how many mechanisms? *Trends Cell Biol*, **17**, 118–126.
674 20. Yekta, S., Shih, I.H., and Bartel, D.P. (2004) MicroRNA-directed cleavage of HOXB8 mRNA. *Science*, **304**, 594–596.
675 21. Williams, A.E. (2008) Functional aspects of animal microRNAs. *Cell Mol Life Sci*, **65**, 545–562.
676 22. Thatcher, E.J., Bond, J., Paydar, I. et al. (2008) Genomic organization of zebrafish microRNAs. *BMC Genomics*, **9**, 253.
677 23. Landgraf, P., Rusu, M., Sheridan, R. et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, **129**, 1401–1414.
678 24. Berezikov, E., Guryev, V., van de Belt, J. et al. (2005) Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*, **120**, 21–24.
679 25. Giraldez, A.J., Mishima, Y., Rihel, J. et al. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, **312**, 75–79.
680 26. Lim, L.P., Lau, N.C., Garrett-Engele, P. et al. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769–773.
681 27. Cohen, S.M. and Brennecke, J. (2006) Developmental biology. Mixed messages in early development. *Science*, **312**, 65–66.
682 28. Begemann, G. (2008) MicroRNAs and RNA interference in zebrafish development. *Zebrafish*, **5**, 111–119.
683 29. Kloosterman, W.P., Lagendijk, A.K., Ketting, R.F. et al. (2007) Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. *PLoS Biol*, **5**, e203.
684 30. Choi, W.Y., Giraldez, A.J., and Schier, A.F. (2007) Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science*, **318**, 271–274.
685 31. Lents, N.H. and Baldassare, J.J. (2006) RNA interference takes flight: a new RNAi screen reveals cell cycle regulators in *Drosophila* cells. *Trends Endocrinol Metab*, **17**, 173–174.
686 32. Coumoul, X. and Deng, C.X. (2006) RNAi in mice: a promising approach to decipher gene functions in vivo. *Biochimie*, **88**, 637–643.
687 33. Cullen, L.M. and Arndt, G.M. (2005) Genome-wide screening for gene function using RNAi in mammalian cells. *Immunol Cell Biol*, **83**, 217–223.
688 34. Paddison, P.J. (2008) RNA interference in mammalian cell systems. *Curr Top Microbiol Immunol*, **320**, 1–19.
689 35. Nguyen, T., Menocal, E.M., Harborth, J. et al. (2008) RNAi therapeutics: an update on delivery. *Curr Opin Mol Ther*, **10**, 158–167.
690 36. Durcan, N., Murphy, C., and Cryan, S.A. (2008) Inhalable siRNA: potential as a therapeutic agent in the lungs. *Mol Pharm*, **5**, 559–566.
691 37. Wargelius, A., Ellingsen, S., and Fjose, A. (1999) Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem Biophys Res Commun*, **263**, 156–161.
692 38. Gruber, J., Manninga, H., Tuschl, T. et al. (2005) Specific RNAi mediated gene knockdown in zebrafish cell lines. *RNA Biol*, **2**, 101–105.
693 39. Zhao, X.F., Fjose, A., Larsen, N. et al. (2008) Treatment with small interfering RNA affects the microRNA pathway and causes unspecific defects in zebrafish embryos. *FEBS J*, **275**, 2177–2184.
694 40. Kloosterman, W.P., Steiner, F.A., Berezikov, E. et al. (2006) Cloning and expression of new microRNAs from zebrafish. *Nucleic Acids Res*, **34**, 2558–2569.
695 41. Chen, P.Y., Manninga, H., Slanchev, K. et al. (2005) The developmental miRNA profiles of zebrafish as determined by small RNA cloning. *Genes Dev*, **19**, 1288–1293.
696 42. Lim, L.P., Lau, N.C., Weinstein, E.G. et al. (2003) The microRNAs of *Caenorhabditis elegans*. *Genes Dev*, **17**, 991–1008.
697 43. Eisen, J.S. and Smith, J.C. (2008) Controlling morpholino experiments: don't stop making antisense. *Development*, **135**, 1735–1743.
698 44. Chen, E. and Ekker, S.C. (2004) Zebrafish as a genomics research model. *Curr Pharm Biotechnol*, **5**, 409–413.
699 45. Flynt, A.S., Li, N., Thatcher, E.J. et al. (2007) Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. *Nat Genet*, **39**, 259–263.
700 46. Valoczi, A., Hornyik, C., Varga, N. et al. (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res*, **32**, e175.
701 47. Wienholds, E., Kloosterman, W.P., Miska, E. et al. (2005) MicroRNA expression in zebrafish embryonic development. *Science*, **309**, 310–311.
702 48. Ason, B., Darnell, D.K., Wittbrodt, B. et al. (2006) Differences in vertebrate microRNA expression. *Proc Natl Acad Sci USA*, **103**, 14385–14389.
703 49. Wienholds, E., Koudijs, M.J., van Eeden, F.J. et al. (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet*, **35**, 217–218.
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UNCORRECTED PROOF

50. Bernstein, E., Kim, S.Y., Carmell, M.A. et al. (2003) Dicer is essential for mouse development. *Nat Genet*, **35**, 215–217.
51. Giraldez, A.J., Cinalli, R.M., Glasner, M.E. et al. (2005) MicroRNAs regulate brain morphogenesis in zebrafish. *Science*, **308**, 833–838.
52. Newport, J. and Kirschner, M. (1982) A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell*, **30**, 687–696.
53. Watanabe, T., Takeda, A., Mise, K. et al. (2005) Stage-specific expression of microRNAs during *Xenopus* development. *FEBS Lett*, **579**, 318–324.
54. Lykke-Andersen, K., Gilchrist, M.J., Grabarek, J.B. et al. (2008) Maternal Argonaute 2 is essential for early mouse development at the maternal–zygotic transition. *Mol Biol Cell*, **19**, 4383–4392.
55. Bushati, N., Stark, A., Brennecke, J. et al. (2008) Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in *Drosophila*. *Curr Biol*, **18**, 501–506.
56. Pearson, J.C., Lemons, D., and McGinnis, W. (2005) Modulating Hox gene functions during animal body patterning. *Nat Rev Genet*, **6**, 893–904.
57. Woltering, J.M. and Durston, A.J. (2008) MiR-10 represses HoxB1a and HoxB3a in zebrafish. *PLoS ONE*, **3**, e1396.
58. Tanzer, A., Amemiya, C.T., Kim, C.B. et al. (2005) Evolution of microRNAs located within Hox gene clusters. *J Exp Zoolog B Mol Dev Evol*, **304**, 75–85.
59. Mansfield, J.H., Harfe, B.D., Nissen, R. et al. (2004) MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nat Genet*, **36**, 1079–1083.
60. Pase, L., Layton, J.E., Kloosterman, W.P. et al. (2009) miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2. *Blood*, **113**, 1794–1804.
61. Yin, V.P., Thomson, J.M., Thummel, R. et al. (2008) Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish. *Genes Dev*, **22**, 728–733.
62. Morton, S.U., Scherz, P.J., Cordes, K.R. et al. (2008) microRNA-138 modulates cardiac patterning during embryonic development. *Proc Natl Acad Sci USA*, **105**, 17830–17835.
63. Li, Y.X., Farrell, M.J., Liu, R. et al. (2000) Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev Biol*, **217**, 394–405.
64. Hsieh, D.J. and Liao, C.F. (2002) Zebrafish M2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *Br J Pharmacol*, **137**, 782–792.
65. Zhao, Z., Cao, Y., Li, M. et al. (2001) Double-stranded RNA injection produces nonspecific defects in zebrafish. *Dev Biol*, **229**, 215–223.
66. Oates, A.C., Bruce, A.E., and Ho, R.K. (2000) Too much interference: injection of double-stranded RNA has nonspecific effects in the zebrafish embryo. *Dev Biol*, **224**, 20–28.
67. Heasman, J. (2002) Morpholino oligos: making sense of antisense? *Dev Biol*, **243**, 209–214.
68. Ekker, S.C. and Larson, J.D. (2001) Morphant technology in model developmental systems. *Genesis*, **30**, 89–93.
69. Nasevicius, A. and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. *Nat Genet*, **26**, 216–220.
70. Stark, G.R., Kerr, I.M., Williams, B.R. et al. (1998) How cells respond to interferons. *Annu Rev Biochem*, **67**, 227–264.
71. Elbashir, S.M., Harborth, J., Lendeckel, W. et al. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494–498.
72. Kanungo, J., Li, B.S., Zheng, Y. et al. (2006) Cyclin-dependent kinase 5 influences Rohon–Beard neuron survival in zebrafish. *J Neurochem*, **99**, 251–259.
73. Liu, W.Y., Wang, Y., Sun, Y.H. et al. (2005) Efficient RNA interference in zebrafish embryos using siRNA synthesized with SP6 RNA polymerase. *Dev Growth Differ*, **47**, 323–331.
74. Dodd, A., Chambers, S.P., and Love, D.R. (2004) Short interfering RNA-mediated gene targeting in the zebrafish. *FEBS Lett*, **561**, 89–93.
75. Kok, K.H., Ng, M.H., Ching, Y.P. et al. (2007) Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA. *J Biol Chem*, **282**, 17649–17657.
76. Haase, A.D., Jaskiewicz, L., Zhang, H. et al. (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep*, **6**, 961–967.
77. Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E. et al. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, **436**, 740–744.

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78. Blidner, R.A., Svoboda, K.R., Hammer, R.P. et al. (2008) Photoinduced RNA interference using DMNPE-caged 2′-deoxy-2′-fluoro substituted nucleic acids in vitro and in vivo. *Mol Biosyst*, **4**, 431–440.
79. Hitz, C., Steuber-Buchberger, P., Delic, S. et al. (2009) Generation of shRNA transgenic mice. *Methods Mol Biol*, **530**, 1–29.
80. Dietzl, G., Chen, D., Schnorrer, F. et al. (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*, **448**, 151–156.
81. Emelyanov, A. and Parinov, S. (2008) Mifepristone-inducible LexPR system to drive and control gene expression in transgenic zebrafish. *Dev Biol*, **320**, 113–121.
82. Asakawa, K., Suster, M.L., Mizusawa, K. et al. (2008) Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc Natl Acad Sci USA*, **105**, 1255–1260.
83. Davison, J.M., Akitake, C.M., Goll, M.G. et al. (2007) Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. *Dev Biol*, **304**, 811–824.
84. Robu, M.E., Larson, J.D., Nasevicius, A. et al. (2007) p53 activation by knockdown technologies. *PLoS Genet*, **3**, e78.
85. Scacheri, P.C., Rozenblatt-Rosen, O., Caplen, N.J. et al. (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci USA*, **101**, 1892–1897.

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