The expression and function of microRNAs in vertebrate embryonic development

Wigard Kloosterman

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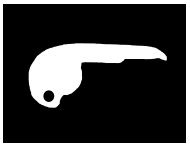
Cover: Front, mosaic of the expression of *miR-194*, *miR-1* and *miR-124* in 3 day old zebrafish embryos. Back, stereogram of a 24 hour old zebrafish embryo (see picture below). The texture is built from 3 day old embryos stained for *miR-194*, *miR-1* and *miR-124*.



The expression and function of microRNAs in vertebrate embryonic development

De expressie en functie van microRNA's tijdens de ontwikkeling van het vertebrate embryo

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 5 september 2007 des middags te 4.15 uur

door

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Contents

CHAPTER 1

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General introduction

microRNAs and RNA silencing

The first microRNA (miRNA), *lin-4*, was found in 1993 in the worm *C. elegans*. This small RNA of 22 nucleotides (nt) regulates the *lin-14* mRNA posttranscriptionally by binding to its 3' untranslated region (UTR) and is essential for the temporal control of postembryonic developmental events. Several years later, the *let-7* small RNA was also found to be important for the timing of developmental events in *C. elegans*. It turned out that *let-7* is fully conserved in many other animal species and human. Consequently, several labs started to search for more small RNAs, which resulted in the discovery of hundreds of miRNAs targeting thousands of mRNAs.

In parallel with the discovery of miRNAs, a related RNA silencing phenomenon termed RNA interference (RNAi), was discovered. In *C. elegans*, RNAi is triggered by long double-stranded RNA, which is processed into small interfering RNAs (siRNAs) of similar size as miRNAs. siRNAs can induce the degradation of complementary mRNAs by binding to perfectly complementary regions. Nowadays, siRNA-mediated gene-silencing is widely used to knockdown genes in cell lines and model organisms.

The miRNA and the RNAi pathway have many protein factors in common, such as those for processing of double stranded RNA or the protein complexes for executing the silencing response. Although all miRNAs are encoded on the genome, siRNAs can be derived from exogenous sources such as viruses.

Over the past few years it has become clear that gene regulation by miRNAs is essential for development of multicellular organisms. The following sections describe the details of miRNA discovery, action, biogenesis and function.

miRNA biogenesis

miRNA genes are transcribed as capped and polyadenylated transcripts termed primary miRNA (pri-miRNA) (Figure 1). Probably, most animal miRNAs are transcribed by RNA polymerase II (1, 2). However, some miRNAs on human chromosome 19 are interspersed with Alu repeats and transcribed by RNA polymerase III (3). miRNA genes reside in both intergenic regions and in exons annotated to non-coding RNAs, but the majority of miRNAs map to intronic regions (4, 5). Processing of miRNAs starts in the nucleus with recognition of the pri-miRNA by the Drosha/DGCR8 complex (6-10). DGCR8, a double stranded-RNA binding protein, interacts with the single-stranded (ss)/double-stranded (ds) RNA junction at the beginning of the miRNA hairpin (11). Subsequently, the RNAse III enzyme Drosha cleaves the stem at ~11 bp away from the ss/ds-RNA junction to release a precursor miRNA

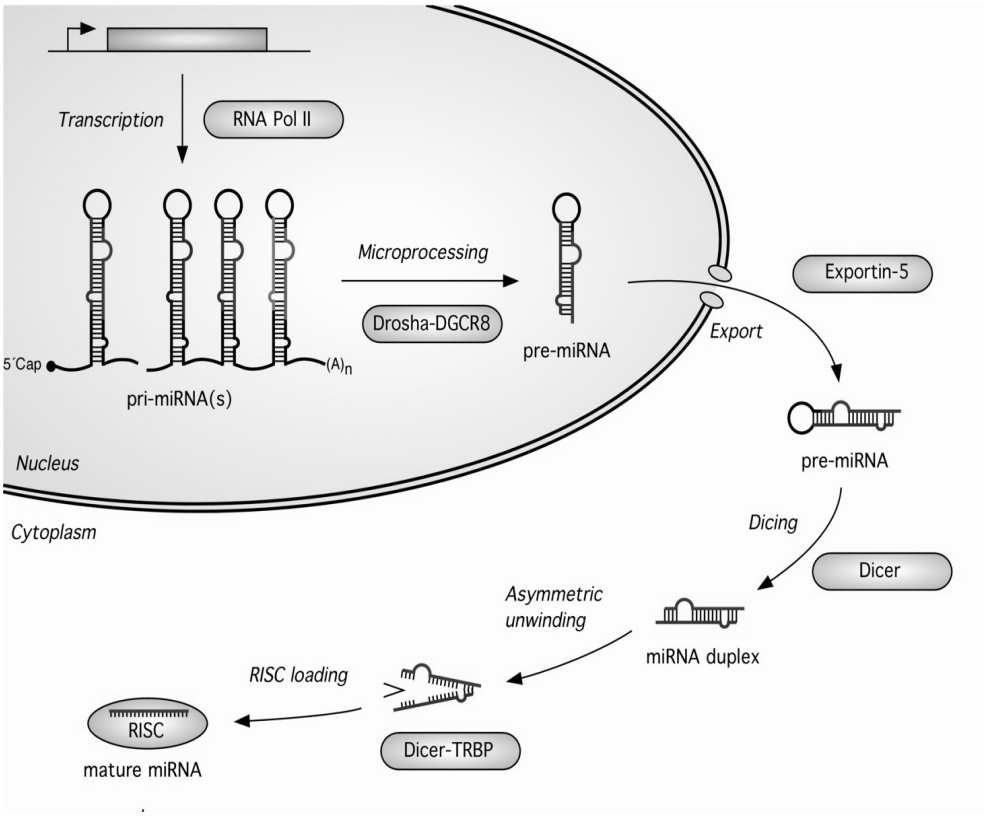

Figure 1. Biogenesis and mechanism of action of animal miRNAs. Adapted from Wienholds and Plasterk, FEBS Lett 579, 2005.

(pre-miRNA) (10, 11). Intronic miRNAs can be processed prior to splicing, although it is unclear if miRNAs located in spliced introns may also be processed (5).
The pre-miRNA is transported to the cytoplasm by the Exportin-5 protein (Figure 1) (12, 13). There, the final miRNA processing step is accomplished by the RNAse III enzyme Dicer, which cleaves the loop from the stem, releasing a mature miRNA duplex (14-17). In human cells, Dicer cleavage requires association with the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) and an Argonaute (Ago) (18). One strand of the mature miRNA duplex, the guide strand, which has its 5' end at the most flexible end of the duplex, is subsequently loaded into an RNA-induced-silencing-complex (RISC) (19, 20). The other strand (passenger strand) is degraded (21).

miRNA mechanism of action

The RISC complex that is involved in miRNA silencing (miRISC) contains at least

an Argonaute family protein. In human, there are four Argonaute proteins (22) and all four can bind miRNAs suggesting that they may be part of miRISC (23, 24). Argonautes have two characteristic domains: the PAZ domain, which recognizes the miRNA 3' end and the PIWI domain, which belongs to the RNase H family of enzymes (25). It has been shown that Ago2 can cleave complementary mRNAs when loaded with an siRNA (23, 24).

Once miRNAs are loaded into a RISC complex, they target partially complementary mRNAs and repress translation (26). In exceptional cases, miRNAs have near perfect complementarity to their target and induce mRNA cleavage (27). For animal miRNAs, especially the miRNA seed (nucleotide 2-8 from the 5' end of the miRNA) should fully basepair to the target mRNA to ensure regulation (28-30). Nevertheless, binding of the miRNA 3' end may also contribute to regulation and even compensate for improper complementarity of the seed region (28, 29, 31).

miRNA target sites are usually located in the 3'UTR of mRNAs, although target sites are functional when placed in the coding sequence or 5'UTR (30). The 3'UTR context of a miRNA target site may also influence regulation, e.g. the target site for the *lsy-6* miRNA in the *cog-1* 3'UTR is only functional in its natural UTR context (31).

One miRNA may have several target sites in the 3'UTR of a single mRNA. The distance between such target sites is important for regulation, with the strongest cooperative effect for sites separated by 13 to 35 nt (32).

Based on the interaction between the *lin-4* miRNA and the *lin-14* mRNA, it was proposed that miRNAs translationally regulate gene expression (33-35). Since repressed mRNA are associated with polysomes, miRNAs seem to repress translation after the initiation step (34, 36, 37). Indeed, reporter assays have shown that translational repression is a cap-independent process and that repression is caused by ribosome drop off during elongation of translation (38). However, investigation of *let-7* mediated repression in human cell lines, showed that repression is cap-dependent, suggesting inhibition of translation initiation (39, 40). The overall consensus is that repressed mRNAs are localized to P-bodies, which are cytoplasmic sites of mRNA degradation (39, 41, 42). This observation is consistent with a moderate drop of mRNA expression that is often associated with miRNA repression (43-48). The reduction of mRNA abundance is a consequence of accelerated deadenylation of targeted transcripts and this process seems independent of translational inhibition, indicating that miRNAs repress mRNAs by independent mechanisms (45, 48).

miRNA target predictions

Many computational algorithms have been employed to predict miRNA targets in animals (49). The initial approaches used both complementarity between the target and the miRNA and the predicted free energy of the miRNA/target duplex as impor-

tant features of miRNA target prediction (50). Using conservation as a criterion, it was soon recognized that short sequences of 6-8 nt, which are complementary to the miRNA 5' end, are most important for miRNA regulation (51-54). With the availability of more sequenced genomes, animal miRNA targets are nowadays predicted with strong emphasis on seed conservation (52, 55, 56). These studies show that individual miRNAs may target several hundred transcripts and that about 30% of the genes in vertebrates are subject to regulation by miRNAs (52, 55, 56). Another pattern based approach for miRNA target prediction estimates that some miRNAs may have thousands of target genes (57).
The importance of human miRNA target sites for fitness is demonstrated by the lower SNP density in conserved miRNA target sites as compared to conserved control sites (58). Furthermore, in some instances SNPs that create or destroy miRNA target sites might cause phenotypic variation (59, 60).

miRNA identification

The first miRNA, *lin-4*, was found in 1993 by genetic means in *C. elegans* (33, 61). The small RNA nature of *lin-4* was regarded as an odd phenomenon specific to *C. elegans*. The significance of this discovery was only realized several years later when another small RNA, *let-7*, was discovered in *C. elegans* (62). This finding triggered a hunt for new miRNAs and many distinct miRNAs were cloned from *C. elegans* and vertebrates (63-66). Strikingly, the *let-7* miRNA is perfectly conserved in many animal species, but not in plants, yeast and bacteria (67). Taking into account miRNA conservation and secondary structure, several computational algorithms were developed to identify new miRNAs (68-71). Together with experimental validation such as Northern blotting and PCR-based strategies, computational predictions of miRNAs is a strong approach especially for identifying rare miRNAs.
To reach a description of the complete set of miRNAs in animal species, miRNA cloning remained a continuing effort and several new miRNAs have been found by tissue- or stage-specific cloning (66, 72-75). Interestingly, miRNAs have also been found in the genomes of several viruses (76-78).
To extend beyond the set of conserved and highly expressed miRNAs, new strategies have been developed that predicted and confirmed the existence of many more vertebrate miRNAs (79-81). These approaches use computational predictions or extensive cloning together with micro-array based platforms for miRNA detection. Although, these studies suggest that there are many more miRNAs to be discovered, most of the new miRNAs are expressed at much lower levels (or are restricted to only a few cells) and are less deeply conserved (75).
Using recently developed massively parallel sequencing technologies (82), many new small RNAs have been discovered (83-85). However, it remains an issue as to whether all newly cloned small RNA sequences represent miRNAs or other small

RNAs, such as siRNAs, tiny non-coding RNAs (tncRNAs), 21-U RNAs, piwi-interacting RNAs (piRNAs) or novel types of small RNAs, which are also often found in small RNA libraries (85-87). Currently, all miRNAs are deposited in a miRNA database, miRBase, from which features such as sequence, genomic location, miRNA targets and conservation can be easily extracted (88) (Table 1).

miRNA detection

Although miRNAs are highly conserved and abundantly expressed, researchers have overlooked them for many years. Because of their small size they cannot be detected by conventional methods for RNA detection. Nowadays, several optimized protocols exist for miRNA expression analysis.

Northern blotting with radioactively labeled DNA probes is the most widely used method for miRNA detection (61-63, 65). Although this method is technically straightforward and allows quantification of miRNA levels and determination of miRNA size, it might not always be sensitive enough for detection of very low abundant miRNAs. Several technical improvements have been developed to enhance miRNA detection by Northern blotting. Instead of UV-cross linking of the RNA to a membrane, chemical carbodiimide-mediated cross linking can provide a 25-50-fold increase in sensitivity of small RNA detection probably due to much better access of the RNA by a complementary probe (89). Another improvement has been made through the use of Locked Nucleic Acid (LNA)-modified DNA probes (90). LNA-modified probes exhibit improved hybridization properties and increase the sensitivity of miRNA detection by tenfold or more (90). The optimal LNA count in LNA-modified probes is one LNA-base per every three bases in the probe (90).

Table 1. Number of miRNAs in various organisms

Species	**Number of miRNAs**
Drosophila melanogaster	78
Caenorhabditis elegans	132
Xenopus tropicalis	177
Gallus gallus	149
Homo sapiens	474
Mus musculus	377
Danio rerio	337
Bos taurus	98
Epstein Barr virus	23

Alternatively, miRNAs can be detected in a highly sensitive and quantitative manner using RT-PCR. These methods consist of a reverse transcriptase step with linear or stem-loop primers followed by real-time PCR and detection with a fluorescently-labeled probe (91, 92). RT-PCR based methods for miRNA detection can be multiplexed to enable simultaneous detection of hundreds of miRNAs from a single cell or tissue (93, 94). However, high-throughput miRNA profiling is most commonly done using miRNA microarrays. Many labs have developed their own microarray platform, differing in nature of the spotted probes and sample preparation (95-97).
Analysis of miRNA expression by Northern blotting, RT-PCR or microarrays gives only a crude estimation of the spatial specificity of miRNAs. *In situ* detection of miRNAs has been challenging. In worms mouse and zebrafish, miRNA-responsive sensors have been used to visualize miRNA expression indirectly (98-100). The advantage of this method is that the activity of the miRNA is monitored and not only its presence. However, for this technique construction of miRNA-responsive reporters and transgenic animals is required. *In situ* hybridization is a much better way to monitor miRNA expression in an animal. This is preferentially done by detecting the mature miRNA. Using high affinity DIG-labeled LNA-modified DNA probes miRNAs can be efficiently detected in fish, mouse and *Xenopus* embryos (101, 102). In *Drosophila*, usually long riboprobes are used to detect the primary miRNA transcript, because LNA-modified probes do not work as good as in zebrafish (103, 104).

miRNA expression

In our lab we have performed an *in situ* hybridization screen using LNA-modified DNA probes targeting 115 conserved vertebrate miRNAs (102). We could detect most miRNAs only during segmentation and later stages of development, but not in early development. In zebrafish, the few miRNAs expressed in early development primarily belong to the *miR-430* family of miRNAs (105). Similarly, in mouse most miRNAs are not expressed in the early stages up to 8.5 days post fertilization (dpf) (101, 106). However, the cluster of miRNAs, *miR-17-92*, which is related to zebrafish *miR-430* is also expressed in the early mouse embryo.
Most conserved miRNAs have a very tissue-specific expression pattern, suggesting that they play a role in maintenance of tissue identity (102). About half of all conserved zebrafish miRNAs are expressed in specific regions of the central nervous system (102). It is unclear whether miRNAs that are less conserved also exhibit these diverse and tissue-specific expression patterns (75). In zebrafish, miRNAs that are less conserved generally have lower expression levels, although the significance of this phenomenon is unknown (75). Some miRNAs reside in introns of coding genes and these intronic miRNAs are usually expressed together with their host gene mRNA, e.g. *miR-126* and *EGFL7* in endothelial cells (107). Also miRNAs that are located in genomic clusters are usually processed from a common transcript (102,

107). When comparing the expression patterns of some deeply conserved miRNAs, such as *miR-124*, *miR-10a* or *miR-1*, it is intriguing to see that also the expression is largely conserved from fly to human (103), suggesting that they have ancient roles in animal development. Comparing a large set of expression patterns for conserved miRNAs between zebrafish, medaka, chicken and mouse showed that, although expression is largely conserved, some differences occur and these might be related to differences in physiology (108, 109).

miRNA inhibition

Because of the widespread role of miRNAs in diverse cellular and developmental processes, tools to selectively inhibit miRNAs may be therapeutically attractive. In addition, inhibiting individual miRNAs is essential for their functional characterization. miRNAs can be efficiently inhibited in cell lines using antisense approaches with LNA/DNA mixmers or 2'-O-methyl oligonucleotides (110-113). Both types of chemistry confer a high affinity towards complementary RNA and both methods decrease the cellular concentration of a targeted miRNA (113, 114). At least for 2'-O-methyl oligonucleotides it has been demonstrated that loss of the targeted miRNA is due to degradation and not blocking of upstream processing events (115). *In vivo*, 2'-O-methyl oligonucleotides have been used to inhibit miRNA function in the *Drosophila* embryo and in adult mouse (114, 116). The intravenous administration of cholesterol conjugated 2'-O-methyl oligonucleotides resulted in long-lasting down-regulation of the targeted miRNA in mouse (114). Nevertheless, the phenotypic results of antisense inhibition should be taken with caution, because the knockdown phenotype for *miR-1* in Drosophila differed substantially from the corresponding knockout phenotype (104, 116).

In zebrafish, 2'-O-methyl oligonucleotides can also be used to inhibit the effects of miRNA misexpression (30). However, the higher concentrations needed to target endogenous miRNAs are toxic to the early embryo (see Chapter 6). Instead, morpholino oligonucleotides are generally micro-injected in the zebrafish embryo to knockdown gene expression. Morpholinos are not very toxic to the zebrafish embryo and they can thus be used to target miRNAs (30, 117). Morpholinos can deplete the embryos of mature miRNAs by disrupting miRNA processing (see Chapter 6).

miRNA function

Loss of all miRNAs

From their conservation and expression patterns it is clear that miRNAs might play important roles in development and indeed, animals without miRNAs cannot live or reproduce (17, 118, 119). Mice deficient in *dicer* die at embryonic day 7.5 and lack multipotent stem cells (118). In addition, conditional inactivation of *dicer* in embryonic stem cell lines compromises proliferation and differentiation although

these effects might also be attributed to changes in centromeric silencing rather than compromised miRNA production (120, 121). Also, *Drosophila* mutants lacking *dicer-1* in the germ line stem cells show that the miRNA pathway is essential for stem cell division and to bypass the G1/S checkpoint of the cell cycle (122). However in zebrafish, *dicer* deficient primordial germ cells were transferred to wild-type embryos and these could grow into fertile zebrafish including a germ line that lacks *dicer* and miRNAs (105). Together, these data show that not all organisms employ the same mechanisms to proliferate and differentiate pluripotent stem cells and that at least in zebrafish, *dicer* and miRNAs are not required for germ line stem cell development. Although the zebrafish zygotic *dicer* mutant lives for almost two weeks due to maternally contributed Dicer (119), analysis of the maternal zygotic *dicer* mutant embryos, showed that zebrafish lacking miRNAs can differentiate multiple cell types in early development, but morphogenetic processes are severely affected (105). However, disruption of *dicer* in growing oocytes in mouse caused failure to progress through the first cell division, suggesting that maternal miRNAs might be essential for mouse zygotic development (123). Several conditional approaches to knockout *dicer* in the mouse have been taken to circumvent the embryonic lethality of *dicer* null mutants. These studies have indicated that Dicer and probably also miRNAs are essential for morphogenesis of the skin (124, 125), lung epithelium (126) and the vertebrate limb (127).

miRNAs as regulators of developmental timing

The first miRNA, *lin-4*, was discovered in *C. elegans* as a small RNA species with antisense complementarity to the heterochronic gene *lin-14* (33, 61). Negative post-transcriptional regulation of *lin-14* by *lin-4* is essential for formation of a temporal gradient of the LIN-14 protein, ensuring proper transition between *C. elegans* larval stages. Besides *lin-14*, also the heterochronic gene *lin-28* is a target of *lin-4*. *Lin-28* homologues also exist in animals, including mouse and human. The *lin-28* 3'UTR harbors target sites for *miR-125a* and *let-7b* (homologs of *C. elegans lin-4* and *let-7*) and is expressed and downregulated during development (128).

The *let-7* miRNA regulates heterochronic genes in a similar manner as *lin-4* and the complementary elements conferring regulation by *let-7* are in some cases evolutionarily conserved, e.g. *lin-41* (67). Together with the finding that *let-7* regulates the *C. elegans hunchback* homolog *hbl-1* and several other transcription factors during the larval to adult transition, this indicates that *let-7* acts as a master switch controlling temporal patterning (129-131). However, the *let-7* family miRNAs *miR-48*, *miR-84* and *miR-241* also play a role in the *C. elegans* heterochronic pathway to control cell fate transitions (132).

miRNAs involved in signaling pathways

Although a role in developmental timing appeared to be the critical function for the *lin-4* miRNA, recently a role was proposed for *lin-4* in regulating life span, possibly through the insulin/insulin-like growth factor-1 pathway (133). Overexpression of *lin-4* resulted in prolonged life span and *lin-4* loss of function mutations caused premature death. These data suggest a dual function for *lin-4* in controlling developmental timing during embryonic development and affecting life span through a major signaling pathway.

The Notch signaling pathway is essential for patterning and development and studies in *Drosophila* have shown that Notch targeted genes are regulated by miRNAs via conserved motifs (134). Ectopic expression of some of these miRNAs induces phenotypes that are reminiscent of Notch pathway loss of function. This repression of Notch induced transcripts might be necessary to prevent their overexpression.

Several positive and negative feedback loops exist which incorporate miRNAs. The *C. elegans* miRNA *miR-61* is a direct transcriptional target of LIN-12/Notch and functions in a positive feedback loop promoting a secondary vulval cell fate, thus implicating that *miR-61* plays a major role in specifying cell fate (135).

Photoreceptor differentiation in the *Drosophila* eye is mediated by *miR-7* (Figure 2). This miRNA functions in a reciprocal negative feedback loop to inhibit the expression of the YAN protein, which acts as a transcriptional repressor of *miR-7*. The expression of *miR-7* is initially triggered by EGF signaling, which results in phosphorylation and inactivation of YAN, generating a stable change in gene expression (136). Thus, major signaling pathways may induce changes in gene expression in the first place, but use miRNAs to stabilize the resulting expression program. It is yet unclear through which target genes outside the feedback loop *miR-7* promotes photoreceptor differentiation.

The Hedgehog pathway is another major signaling route in development. Morpholino-mediated inhibition of *miR-214* in zebrafish, yielded embryos with U-shaped somites, reminiscent of defects in Hedgehog signaling (117). *miR-214* seems to fine-tune the expression of suppressor of fused, a negative regulator of Hedgehog signaling (117).

Although not directly related to signaling, miRNAs more often reside in feedback loops to control their own expression. Granulocytic differentiation is enhanced by *miR-223* expression (137). Before the differentiation process, the transcription factor NFI-A allows weak expression of *miR-223*. Upon stimulation with retinoic acid NFI-A is replaced by the transcription factor C/EBPα, which induces high expression of *miR-223*, which in turn represses the expression of NFI-A post-transcriptionally, similar to *miR-7* and YAN.

The expression of *miR-7* in flies and *miR-61* in worms is induced by EGF and Notch signaling respectively. However, miRNAs might also function upstream of signaling

pathways. Hoxb8 is a transcription factor that mediates retinoic acid induced expression of Sonic hedgehog, a signaling molecule that regulates anterioposterior patterning in limb buds. *miR-196* functions as a secondary regulator of *hoxb8* expression in the vertebrate hindlimb, where the primary level of *hoxb8* regulation appears to be transcriptional (138).

miRNAs in apoptosis and metabolism

Forward genetic screens in flies have led to the discovery of miRNAs involved in programmed cell death. The *bantam* miRNA accelerates proliferation and prevents apoptosis by regulating the pro-apoptotic gene *hid* (139). Similarly, *miR-14* functions as a cell death suppressor, although its cellular target is unknown (140). However, fly *miR-14* mutants display another phenotype, they are obese and have elevated levels of triacylglycerol, showing that *miR-14* plays a role in fat metabolism. In a similar gain-of-function screen for genes affecting tissue growth, the *miR-278* locus was identified (141). *miR-278* mutants are lean and have elevated insulin production, implicating this miRNA in energy homeostasis, possibly by interacting with the expanded transcript, which is known to be involved in growth control. In vertebrates, *miR-375* is expressed in the pancreatic islet and suppresses glucose induced insulin secretion (142). The *myotrophin* (*mtpn*) gene was validated as a target of *miR-375* and siRNA mediated knockdown of *mtpn* mimicked the effect of *miR-375* on insulin secretion.

miRNAs involved in myogenesis and cardiogenesis

The sequence of *miR-1* is conserved from worms to mammals. It is highly expressed in the muscles of flies and the muscles and heart of mice (103, 143). Interestingly, a genetic knockout of *Drosophila miR-1* does not affect formation and physiological function of the larval musculature, but malformation of the muscles and death are only triggered upon feeding and growth (104). So, some miRNAs might not be essential for establishing a tissue type, but they are required for subsequent growth and maintenance of the tissue. In mouse, *miR-1* expression is directed by muscle differentiation regulators, such as serum response factor, MyoD and Mef2 in the similar cells as one of its targets, Hand2 (143). Overexpression of *miR-1* results in developmental arrest, thin walled ventricles and heart failure due to premature differentiation and proliferation defects of myocytes. There are two *miR-1* genes in mouse, human and zebrafish. Both copies are expressed in the embryonic heart (144). Genomic disruption of only one of the copies (*miR-1-2*) causes ventricular septal defects, abnormalities in cardiac conduction and an increased number of cardiomyocytes (144). Apparently, the *miR-1-1* copy does not fully compensate for loss of *miR-1-2*, indicating that the intracellular miRNA pressure is of essential importance to ensure the correct degree of regulation of *miR-1* target mRNAs.

In cell culture, *miR-1* promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle differentiation (145). HDAC4 is also a possible target of *miR-140* during osteoblast differentiation and skeletogenesis, showing that two different miRNAs can target the same gene during differentiation (146). *miR-1* localizes in a genomic cluster together with *miR-133*, but these two miRNAs differ in their seed sequence and they have distinct functions (145). In contrast to *miR-1*, *miR-133* inhibits muscle differentiation and promotes proliferation by repressing serum response factor.
Although *miR-181* is hardly detectable in skeletal muscle, this miRNA is strongly upregulated during myoblast differentiation and inhibits the expression of Hox-A11, which is a repressor of differentiation (147). *miR-181* might be involved in establishing a muscle phenotype, while *miR-1* and *miR-133* are involved in muscle maintenance. In addition, *miR-181* can induce the differentiation of hematopoietic stem cells to B-lineage cells, demonstrating a dual role for a single miRNA in different cell types (148).
Not all miRNAs are directly influencing developmental processes. Mice lacking *miR-208*, which is expressed in the heart, do not display obvious defects in size, shape or structure of the heart. However, *miR-208* is essential for cardiac hypertrophy in response to stress (149).

miRNAs in the brain

The brain represents a complex tissue, with multiple different cell types. Many miRNAs are expressed in specific brain regions or neurons in vertebrates, suggesting their importance in brain functioning (102). Some miRNAs have been implicated

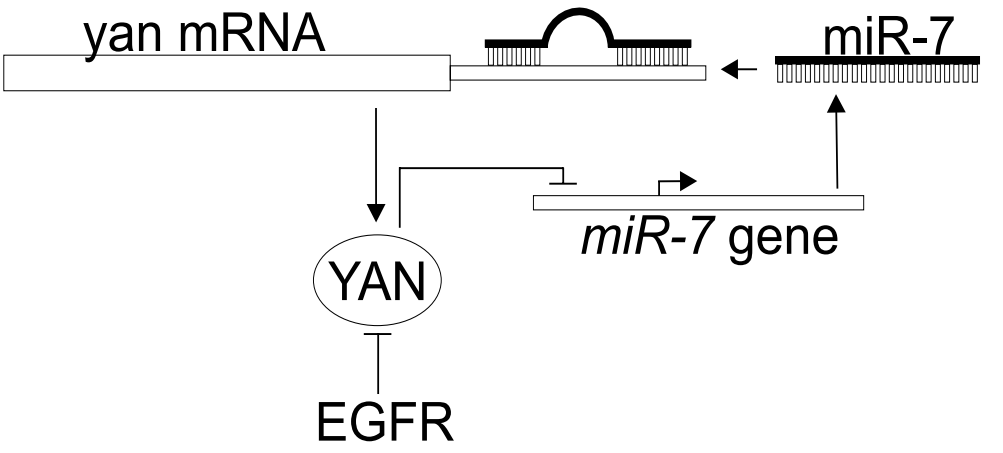

Figure 2. Model for the role of *miR-7* and YAN in photoreceptor differentiation in *Drosophila*. Adapted from Li and Carthew, Cell 2005 Dec 29;123(7):1267-77.

in specifying asymmetric gene expression in chemosensory neurons in *C. elegans* (98, 150). The *lsy-6* miRNA is expressed in left neurons and *miR-273* is expressed in right neurons and this reciprocal expression is essential for neuronal asymmetry.
In the mammalian brain, *miR-134* was found to localize to synaptic sites in rat hippocampal neurons (151). *miR-134* can inhibit the expression of the protein kinase Limk1, which controls the development of dendritic spines. The silencing can be released upon extracellular stimuli resulting in spine growth.
Another miRNA, *miR-132*, is expressed in cortical neurons and a target of the transcription factor cAMP-response element binding protein (CREB) (152). Inhibition of *miR-132* attenuates neuronal outgrowth and the effects on neuronal morphogenesis might be mediated by the GTPase-activating protein p250GAP.
Although these two examples propose roles for miRNAs in neuronal outgrowth and plasticity, *miR-124a* is involved in the differentiation of neural progenitors into mature neurons by degradation of nonneural transcripts (153). *miR-124a*, together with *miR-9* and *miR-132*, is targeted by the transcriptional repressor REST in nonneural cells. However, antisense inhibition of *miR-124* in the developing neural tube did not affect neuronal differentiation, but decreases the expression of some neuronal markers (154, 155). *miR-124* rather ensures that expression of neuronal progenitor genes is inhibited in mature neurons (154).
The *miR-430* family of miRNAs, which is the major miRNA family expressed during early zebrafish development, performs a more general role in zebrafish brain morphogenesis (105). In addition, this miRNA clears the embryo of maternal mRNAs to promote the maternal to zygote transition (45).

The expression of miRNAs and their targets

In principal, there are two ways that miRNAs could regulate their target mRNA (156). Fine tuning targets contain just one site for one miRNA and confer only weak regulation. This is the case for the majority of targets (157). In these instances, weak regulation of a target might become significant when the target is already expressed at very low levels in the cell. The second class comprises switch targets, where multiple target sites are present in the 3' UTR resulting in strong repression (156, 157). These could be target sites for just a single miRNA or target sites for different miRNAs, resulting in cooperative regulation provided that the miRNAs are expressed in the same cells.
Intuitively one would expect that miRNAs and their targets are expressed in the same cells, because only then is a real physical interaction possible. However, the studies of *miR-7* and YAN in *Drosophila*, *miR-196* and Hoxb8a in mouse and *miR-223* and NFI-A during granulopoiesis indicate that miRNAs and their targets might have reciprocal expression patterns, i.e if the miRNA is expressed at a high level, the target is lowly expressed. This trend towards mutually exclusive expression of

miRNAs and their targets has been shown on a larger scale by independent studies in mammals (158, 159) and *Drosophila* (157). There are 5 important results from these studies. First, mRNAs are in general expressed at higher levels prior to miRNA expression. A very nice biological example of this is the clearance of maternal mRNAs by the *miR-430* family (45). Second, non-conserved target sites are preferentially found in genes that are not expressed in the tissue where the miRNA is expressed. These might thus be mRNAs that randomly acquired target sites during evolution, which could persist because there is no selection against them. Third, conserved targets are expressed in the tissue where the miRNA is expressed, but usually at lower levels compared to the surrounding tissue. Fourth, mRNAs that are preferentially co-expressed with a miRNA specifically avoid target sites for that miRNA and these are referred to as anti-targets. Anti-targets could cooperate with miRNAs to establish the correct biological environment in a particular cell lineage or be involved in basic processes common to all cells. Fifth, house keeping genes have shorter UTRs than other messengers and the target site density is lower compared to genes with longer 3'UTRs, probably to avoid targeting by miRNAs.

In conclusion, miRNAs might repress transcripts that are already expressed at low levels in the cells where the miRNA is expressed. As such, they could provide robustness to developmental expression programs that are primarily regulated at the transcriptional level.

Outline of this thesis

In **Chapter two** we describe the effects of wild type and mutant *let-7* miRNAs on the developing zebrafish embryo and on a GFP reporter with the *let-7* complementary sites from the *lin-41* 3'UTR. We conclude that the 5' part of *let-7* is most important for its function.

It has been challenging to detect miRNA expression by *in situ* hybridization. In **Chapter three** we describe a technique to visualize miRNA expression in embryos based on LNA-modified DNA probes. We describe the experimental conditions for *in situ* hybridization of miRNAs and we report some miRNA expression patterns in the mouse.

In **Chapter four** we show the results of an *in situ* hybridization screen for 115 conserved vertebrate miRNAs in zebrafish embryonic development. Our expression data demonstrate the enormous variation and tissue-specificity of miRNA expression in a vertebrate organism.

Computational predictions and cloning efforts have indicated that there might be up to a thousand miRNAs in vertebrate genomes. To extend the miRNA repertoire of the zebrafish, we have cloned many novel miRNAs from embryos and adult brains. **Chapter five** describes the cloning and expression of new miRNAs from zebrafish. This study demonstrates that many poorly conserved miRNAs are expressed at low levels.

The functions of miRNAs in vertebrate developmental are only beginning to be understood. **Chapter six** describes the use of antisense morpholinos to knockdown miRNAs in the first days of zebrafish development. We show that knockdown of *miR-375* results in defects in pancreatic islet development.

Chapter seven provides a general discussion of the work in this thesis.

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CHAPTER 2

Substrate requirements for *let-7* function in the developing zebrafish embryo

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Abstract

MicroRNAs (miRNAs) are involved in the regulation of gene expression at the post-transcriptional level by base pairing to the 3'-UTR (untranslated region) of mRNAs. The *let-7* miRNA was first discovered in *Caenorhabditis elegans* and is evolutionarily conserved. We used zebrafish embryos as a vertebrate *in vivo* system to study substrate requirements for function of *let-7*. Injection of a double-stranded *let-7* miRNA into the zygotes of zebrafish and frogs causes specific phenotypic defects. Only the antisense strand of the *let-7* duplex has biological activity. In addition, co-injected mRNA of *gfp* fused to the 3'-UTR of a zebrafish *lin-41* ortholog (a presumed target of *let-7*) is silenced by *let-7*. Point mutant studies revealed that the two *let-7* target sites in the *lin-41* 3'-UTR are both essential and sufficient for silencing. *let-7* and *miR-221* together, but not either of them alone, can silence a construct with one of the *let-7* target sites replaced by a target site for *miR-221*, showing that two different miRNAs can provide the required cooperative effect. *let-7* target sites can be moved around: they are also functional when positioned in the coding sequence or even in the 5'-UTR of *gfp*. We took advantage of reporter and phenotypic assays to analyze the activity of all possible point mutant derivatives of *let-7* and found that only the 5' region is critical for function of *let-7*.

Introduction

Hundreds of microRNAs (miRNAs) have been discovered in eukaryotes (1-11) and they form an abundant class of posttranscriptional regulators [for reviews see (12, 13) and many references therein]. miRNAs are initially transcribed as longer precursors and subsequently processed into 21–23 nt double-stranded RNAs with 2 nt 3' overhangs by the RNAse III-like endoribonucleases Drosha (14) and Dicer (15-18), respectively. miRNAs regulate the gene expression by incorporating into a RISC (RNA-induced silencing complex) complex that binds to miRNA complementary elements in the 3'-UTR (untranslated region) of target genes. Targets have been predicted computationally for many miRNAs, based on the conservation of miRNA targets and known miRNA-target interactions (19-23). Despite the numerous targets predicted for many miRNAs, only few studies have addressed a role for distinct miRNAs in animals: In flies, the *bantam* miRNA was shown to be involved in the control of cell proliferation (24) and *miR-14* suppresses apoptosis and is required for fat metabolism (25). Some miRNAs from mouse are implicated in the modulation of hematopoietic lineage differentiation (26). The *Caenorhabditis elegans lsy-6* and *mir-273* miRNAs regulate chemosensory laterality (27, 28). *lin-4* is the founding member of the miRNA class of genes. It acts on *C. elegans* by binding to complementary sites in the 3'-UTR of the heterochronic genes *lin-41* and *lin-28* (29, 30). The *let-7* miRNA also regulates developmental timing in *C. elegans* by inhibiting the expression of heterochronic genes, among which *lin-41* (31, 32). At least two out of six *let-7* target sites in the *C. elegans lin-41* gene, together with the 27 bp sequence in between, were shown to be necessary for *let-7*-mediated gene silencing (33). Both *let-7* and *lin-41* are conserved in evolution and *let-7* target sites are also present in the *lin-41* orthologs of *Drosophila* and zebrafish (31).

Here, we demonstrate that injection of a synthetic *let-7* miRNA (in double-stranded form) causes specific defects in the vertebrate embryo. Furthermore, we employ the zebrafish embryo to show that two *let-7* target sites from the zebrafish *lin-41* gene mediate silencing. Both target sites are essential for silencing; an mRNA with one *let-7* target site replaced by a *miR-221* target site can be silenced by both miRNAs together. Target sites for *let-7* are also functional when placed, in the coding sequence or in the 5'-UTR of a reporter gene.

No study systematically determined the importance of every position of a miRNA. The *let-7* mutant allele (*n2853*) in *C. elegans* harbors a mutation at position 5 from the 5' end of the miRNA (32). This single point mutation abolishes the function of *let-7* in *C. elegans*. Since the *let-7* miRNA is strongly conserved, we took this miRNA to derive a complete mutational spectrum using zebrafish as an *in vivo* vertebrate system.

Results and Discussion

Effects of injected let-7 on development

During the first 48 h of zebrafish development, no endogenous *let-7* miRNA is expressed (31, 34). We observed a specific phenotype upon injection of a double-stranded *let-7* miRNA in one-cell stage zebrafish embryos (Figure 1A). At 26 h post-fertilization (hpf), embryos were generally retarded in development. More pronounced characteristics were a lack of proper eye development and reduced tail and yolk sac extension. The embryos died after 2 days. This phenotype was only induced by a double-stranded version of *let-7*. Injection of either the sense or the antisense strand alone did not affect development, although these species remain stable *in vivo* for at least 48 h (data not shown). A control miRNA bearing five mutations (mm*let-7*) failed to induce the phenotype, indicating the specificity of the observed phenotype (Figure 1A). Furthermore, an injected pre-*let-7* hairpin is processed *in vivo* and induces a phenotype similar to that of a mature *let-7* duplex (Figure 1B).
Because of its perfect conservation, we also investigated the effects of *let-7* microinjection on *X. tropicalis* embryos. Strikingly, this resulted in similar defects as for zebrafish, i.e. embryos were retarded in development and exhibited a reduced eye size and tail length (Figure 1A). Development was virtually normal in embryos injected with the mm*let-7* duplex. The phenotypic effects caused by *let-7* misexpression in zebrafish could be specifically inhibited by co-injection with a 2'-O-methyl oligonucleotide complementary to the *let-7* miRNA, but not by a control 2'-O-methyl oligonucleotide unrelated in sequence to *let-7* (Figure 3B) (35). Injection of the oligonucleotide alone did not cause any developmental abnormalities. Similarly, a morpholino directed against *let-7* inhibited the misexpression phenotype, but did not induce a phenotype when injected alone (data not shown). The results presented here show that the *let-7* miRNA is active in the zebrafish embryo and severely affects normal development upon misexpression. Although the biological significance of the observed effects remains unclear, it seems likely that ectopic expression of *let-7* causes precocious downregulation of one or more endogenous *let-7* targets. Misexpression studies can be important for defining miRNA function, especially in cases where knockout is difficult because of redundancy.

The antisense strand of the let-7 duplex is biologically active and an asymmetric duplex exhibits enhanced activity

We next investigated which strand of the *let-7* duplex exerts a biological effect by examining the activity of heteroduplexes (Figure 2). A heteroduplex containing two mutations at the 5' end of the antisense strand of *let-7* (heteroduplex 1) fails to induce a phenotype, while a heteroduplex with two mutations at the 3' end of the sense strand (heteroduplex 2) functions extremely well. This indicates that only the antisense strand of the *let-7* duplex exerts a biological effect. The phenotype induced

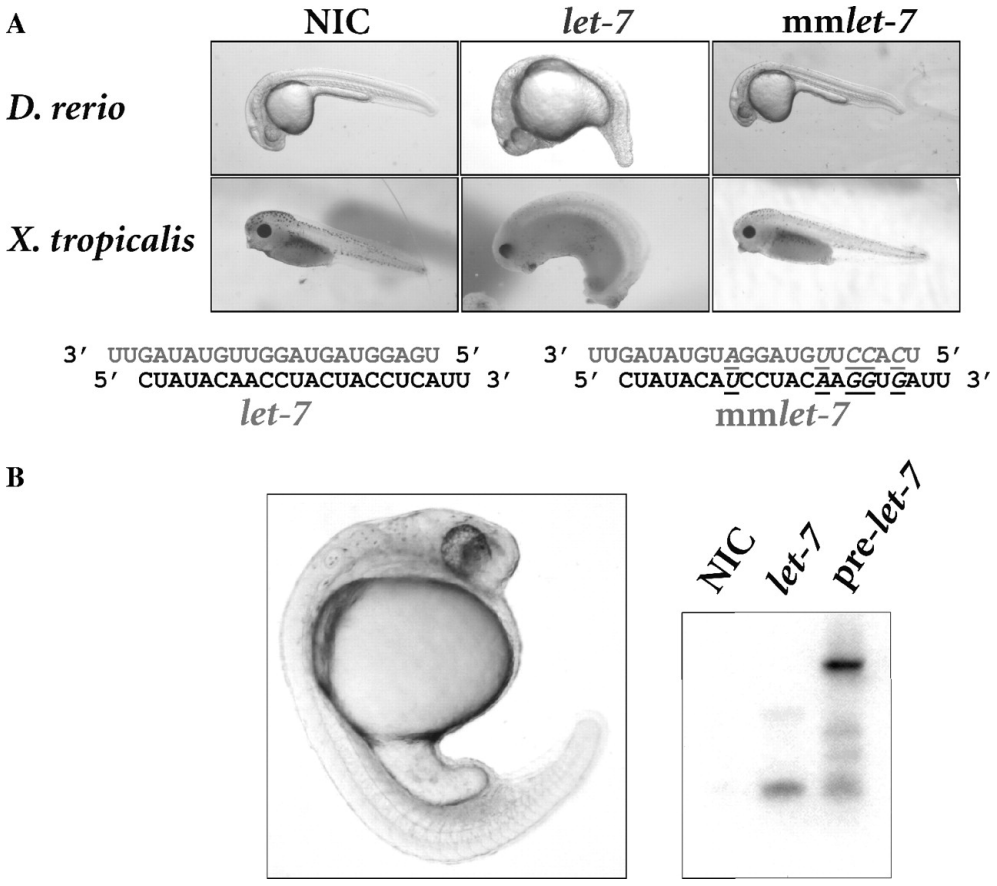

Figure 1. Misexpression of *let-7* in vertebrate embryos. (A) Lateral views of *D. rerio* and *X. tropicalis* embryos injected with *let-7* and a mutant version containing five mismatches (underlined). (B) Phenotype induced by injection of a pre-*let-7* hairpin. The northern blot shows processing of pre-*let-7* in zebrafish embryos. Pictures were taken at 28 h post-fertilization (hpf) and 2 days post-fertilization (dpf) for *D. rerio* and *X. tropicalis*, respectively. NIC, not injected control.

by heteroduplex 2 is even stronger than that induced by the *let-7* homoduplex. In contrast, injection of a heteroduplex containing two mismatches at the 5' end of the sense strand (heteroduplex 3) resulted in a reduced phenotype. These data support recent work, which shows that both the absolute and the relative stabilities at the 5' ends of siRNA or miRNA duplexes are critical determinants for incorporation of one or the other strand into RISC (36-38). Our experiments demonstrate that the *in vivo* activity of a miRNA is subject to the same criteria.

Silencing of a gfp::lin-41 3'-UTR reporter by let-7

The *let-7/lin-41* miRNA-target couple was experimentally verified in *C. elegans* (32), but conserved sequences are found in *Drosophila* and zebrafish (31). To show that injected *let-7* acts as a miRNA on the zebrafish *lin-41* 3'-UTR, we fused a 379 bp fragment comprising part of the zebrafish *lin-41* 3'-UTR and containing two *let-7* target sites (Figure 3A) to *gfp*. Co-injection of *let-7* with mRNA derived from this construct resulted in the translational repression of GFP expression (Figure 3B). The *gfp::lin-41* 3'-UTR mRNA levels remained unaffected, whereas GFP protein levels were dramatically reduced upon *let-7* overexpression (Figure 3C). Silencing of *gfp* is dependent on the *lin-41* 3'-UTR, since *gfp* mRNA without the *lin-41* 3'-UTR was not silenced by *let-7* (Figure 3B). Furthermore, silencing of *gfp::lin-41* 3'-UTR could be blocked by the 2'-O-methyl oligonucleotide complementary to *let-7*, and the mm*let-7* version did not silence *gfp::lin-41* 3'-UTR. We did not observe downregulation of the *gfp::lin-41* 3'-UTR reporter by endogenous *let-7* as shown by a recent study using mouse embryos transiently expressing *lacZ* miRNA sensor constructs (39). This is likely due to the fact that there are no detectable amounts of *let-7* expressed during these stages of development.

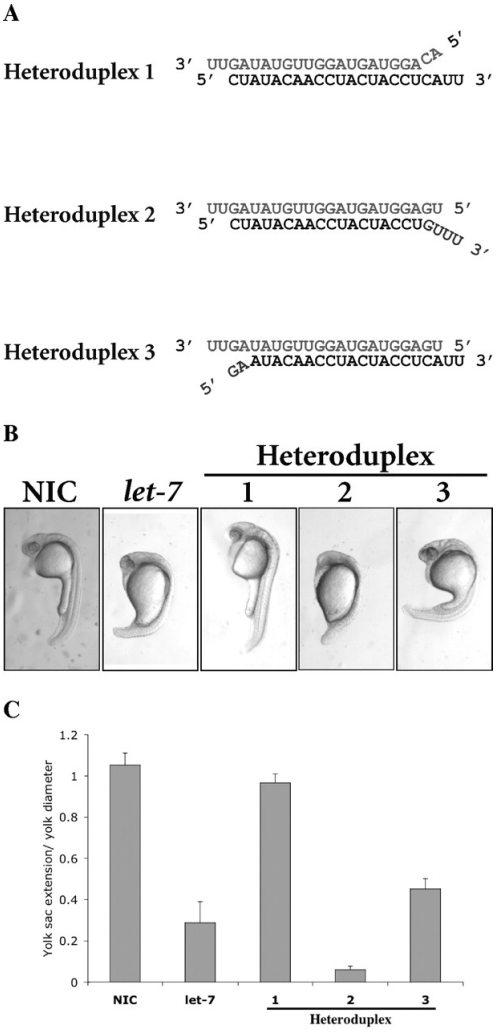

Figure 2. Phenotypic effects of injection of 3 *let-7* heteroduplexes. (A) Sequences of heteroduplexes. The upper strand is the antisense strand of the *let-7* duplex. (B) Phenotypes of 28 h zebrafish embryos injected with heteroduplexes. (C) Bar diagram showing relative length of the yolk sac extension of fish injected with *let-7* heteroduplexes.

To investigate the interaction between *let-7* and its target sites in more detail, *gfp* was fused to a 100 bp fragment of the *lin-41* 3'-UTR containing only the two *let-7* target sites including inter-

vening sequence (p*gfp::lin-41* 3'-UTR-1). In addition, a similar construct was made that contains the same fragment, but with a point mutation in both *let-7* target sites (*gfpl::lin-41* 3'-UTR-2) (Figures 3A and 4A). mRNA derived from *gfp::lin-41* 3'-UTR-1 could only be silenced by wild-type *let-7*, while expression of *gfp::lin-41* 3'-UTR-2 was inhibited exclusively by a *let-7* duplex with a compensatory mutation (*let-7*mm5, Figure 3D). In addition, duplex *let-7*mm5 does not induce a phenotype in fish embryos. Taken together, our data show that injection of a mature *let-7* miRNA duplex can specifically induce gene silencing via the *lin-41* 3'-UTR in the developing zebrafish embryo.

We next asked whether the sequence intervening the *let-7* target sites is an essential

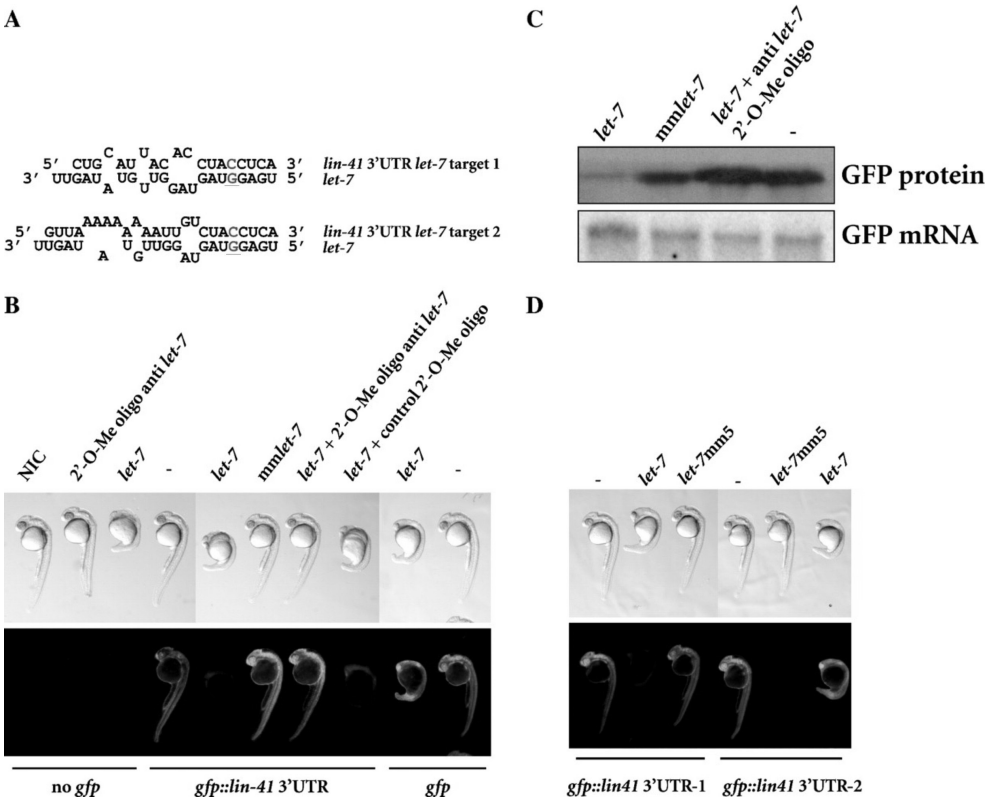

Figure 3. Effects of *let-7* injection on *gfp::lin-41* fusions and zebrafish development. (A) Structure of *let-7* bound to its target sites in the zebrafish *lin-41* 3'-UTR. The position of the point mutation in the target and *let-7* is underlined. At these positions the C in the target is changed to a T and the G in *let-7* is changed to an A. (B) Lateral views of 28 h zebrafish embryos injected with *gfp* with and without the *lin-41* 3'-UTR. The upper panel shows phenotypes and the lower panel GFP fluorescence. (C) Western and northern analysis of GFP in 28 h zebrafish embryos co-injected with *gfp:lin-41* 3'-UTR. (D) Phenotypes and GFP expression in 28 h embryos injected with mutant (*gfp::lin-41* 3'-UTR-2, *let-7*mm5) and wild-type (*gfp::lin-41* 3'-UTR-1, *let-7*) *let-7* and *gfp::lin-41* 3'-UTR fusions.

component for silencing. In *C. elegans*, it was shown that silencing of a *lacZ* reporter fused to the *C. elegans lin-41* 3'-UTR is dependent on two specific *let-7* target sites and the 27 bp sequence between the target sites (33). In contrast, we found that a *gfp* reporter containing both *let-7* target sites from the zebrafish *lin-41* gene separated by a 5 bp sequence is still silenced by co-injected *let-7* (Figure 4A, p*gfp::let-7*tar1+2), while in the natural situation both *let-7* target sites are separated by a 57 bp sequence. Similar constructs containing either two copies of target site 1 or two copies of target site 2 fused to *gfp* were also silenced by *let-7* (Figure 4A, p*gfp::let-7*tar1-2x and p*gfp::let-7*tar2-2x), showing that both target sites are equally potent in silencing. However, reporter constructs with a point mutation (Figure 3A) in either *let-7* target site 1 or 2 could not be silenced by *let-7*, demonstrating that both target sites act together to mediate a silencing response (Figure 4A, p*gfp::lin-41* 3'-UTR-1 mut1 and p*gfp::lin-41* 3'-UTR-1 mut2). GFP expression from p*gfp::lin-41* 3'-UTR-1 mut1 and p*gfp::lin-41* 3'-UTR-1 mut2 cannot be downregulated by co-injection of a mixture of *let-7* and *let-7*mm5. This is probably due to a competition effect (i.e. *let-7* binding to the mutant target site and *let-7*mm5 binding to the wild-type target site), since a mixture of *let-7* and *miR-221* could silence a reporter construct with *let-7* target site 2 replaced by an artificially designed *miR-221* complementary site (Figure 4A, p*gfp::let-7*tar1+*miR-221*tar), whereas *let-7* and *miR-221* alone did not affect the GFP expression from this construct. These data are in agreement with previous studies in cultured mammalian cells (40), showing that miRNAs can act cooperatively to repress gene expression, although our data differ from these studies because we used a combination of two copies of one target site instead of two copies of two target sites acting together.

Taken together, we demonstrate that injected *let-7* can act on the zebrafish homolog of the *lin-41* gene, but we have no way of knowing whether endogenous *let-7* silences *lin-41 in vivo* or whether the phenotype we observe is the result of injected *let-7* acting on *lin-41*.

Silencing of reporters containing let-7 targets in the coding region or 5'-UTR

To investigate whether silencing of the GFP reporter is restricted to the presence of *let-7* target sites specifically in the 3'-UTR, constructs were made with the *let-7* target unit comprising of both target sites with a 5 bp spacer inserted in the 5'-UTR (*gfp::let-7*tar 5'-UTR) and 12 bp before the stop codon in the *gfp* coding sequence (*gfp::let-7*tar cds) (Figure 4A). Surprisingly, both constructs could be specifically silenced by co-injected *let-7*, but not by *let-7*mm5 (Figure 4B). These data indicate that GFP is specifically downregulated due to *let-7* sites in the coding region or the 5'-UTR. In the latter case, it cannot be ruled out that translation initiation is inhibited due to steric hindrance of a *let-7* loaded RISC complex just upstream of the start codon.

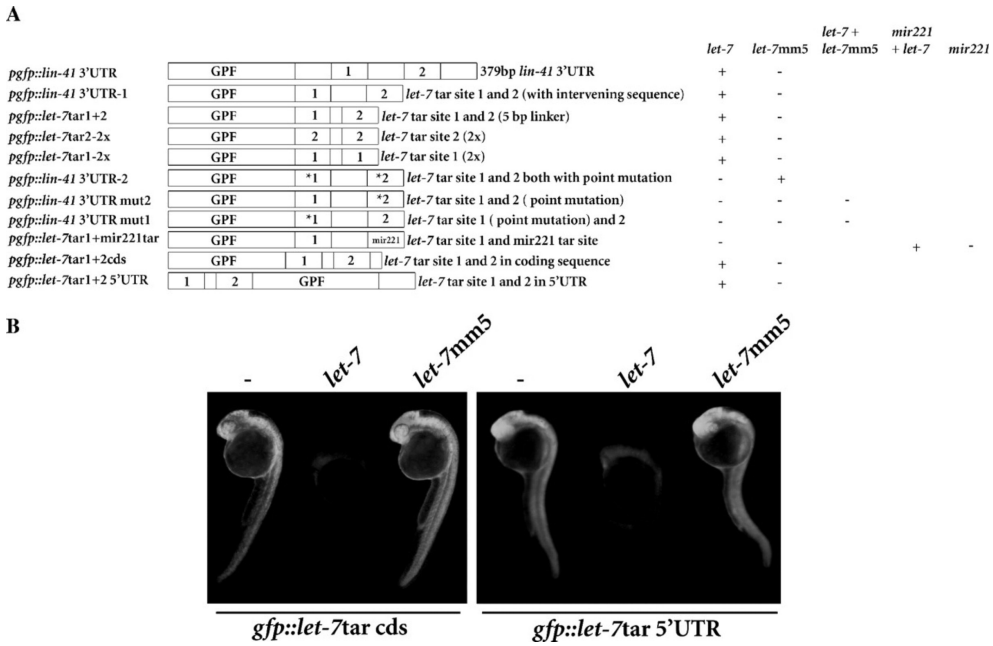

Figure 4. *let-7* target site 1 and 2 are sufficient for silencing. (A) Overview of constructs and miRNAs analyzed in this study. + indicates strong silencing, – indicates no silencing. (B) GFP fluorescence in 24 h zebrafish embryos injected with *let-7* or *let-7*mm5 and *gfp::let-7*tar 5'-UTR or *gfp::let-7*tar cds.

In plants, it is known that miRNA targets are also present in the coding sequence of the mRNA, but plant miRNAs often target the mRNA for degradation via single complementary sites (41). No natural examples are known yet of animal miRNAs regulating a target gene via complementary sites in the coding sequence or 5'-UTR, although it was shown that siRNA off-target effects can be mediated through translational repression of mRNAs due to imperfect base pairing of the siRNA with the coding sequence (42).

In our zebrafish system, we find that miRNA-target sites do not necessary need to be located in the 3'-UTR, since *let-7* targets both in the 5'-UTR or in the coding sequence could induce a specific silencing response. This is important information for studies aimed at predicting miRNA targets, which are currently only focusing on 3'-UTR sequences. As also suggested by others (12, 13, 20), these should include 5'-UTR and coding sequences as well.

The 7 bases at the 5' end of let-7 are most important for its activity

To define the most important positions for functionality of the *let-7* miRNA, we performed a mutational scan by introducing point mutations at each of the 22 positions of *let-7*. For all 66 (3 x 22) mutants, we determined phenotypic defects and the influence on *gfp::lin-41* 3'-UTR mRNA translation (Figure 5A and B) as these assays are good indicators for the activity of *let-7*. Similar data were obtained by both the analysis of GFP protein level and the examination of phenotypic defects: mutations induced in the region spanning positions 1–7 from the 5' end of the antisense strand of the *let-7* duplex eliminate the function of the miRNA (Figure 5). However, small growth defects were observed for *let-7* duplexes with mutations at position 1. Versions of *let-7* with a mutation at position 8 showed a strong phenotype but only a low reduction in the GFP protein level. Remarkably, the change of an A to a G at positions 3 and 7, enabling the formation of a G-U instead of an A-U base pair with the *lin-41* reporter construct, did not inhibit protein expression or affect normal development.

Most prominently at positions 10 and 13, GFP silencing and phenotypic effects were different for the 3 mutant *let-7* derivatives. These differences probably reflect changes in the *let-7* duplex free energy, which is relatively low for positions 9–14 in natural miRNA duplexes (36, 37). For example, at position 10 the change of the original A-U base pair in the *let-7* duplex into an U-A base pair still causes strong silencing. However, the change to a G-C or C-G base pair increases stability of the duplex and decreases its activity. Similar although less pronounced effects were observed for mutations at position 9. Thus, mutations at certain positions in the miRNA duplex can affect its activity, not because of an altered interaction with the target RNA, but because of a change in the stability of the miRNA duplex itself.

Recently, the specificity of miRNA-target interaction was analyzed in cell culture using luciferase reporter assays (40). In this study, the miRNA target instead of the miRNA itself was analyzed for critical positions and, similar to our observations, these reporter studies indicated that base pairing of the first 8 nt in the 5' region of the miRNA is most important for activity, although the binding of the 3' end of a miRNA also appeared to contribute to gene silencing. These observations were made with reporter constructs containing multiple miRNA-target sites similar to our *gfp::lin-41* reporter. Investigation of rules governing the interaction of a miRNA with a single target site showed that interaction of the proximal (i.e. 5') part of a miRNA with its target is indeed the most susceptible to mutations (43). Only a target:miRNA mismatch in the 5' part flanked by 4 bp on each side resulted in silencing of the reporter. Similarly, mutational analysis of the plant *miR-165/166* complementary site of PHABULOSA revealed that disrupting miRNA pairing near the 5' region causes stronger developmental consequences and reduced miRNA-directed cleavage *in vitro* (44).

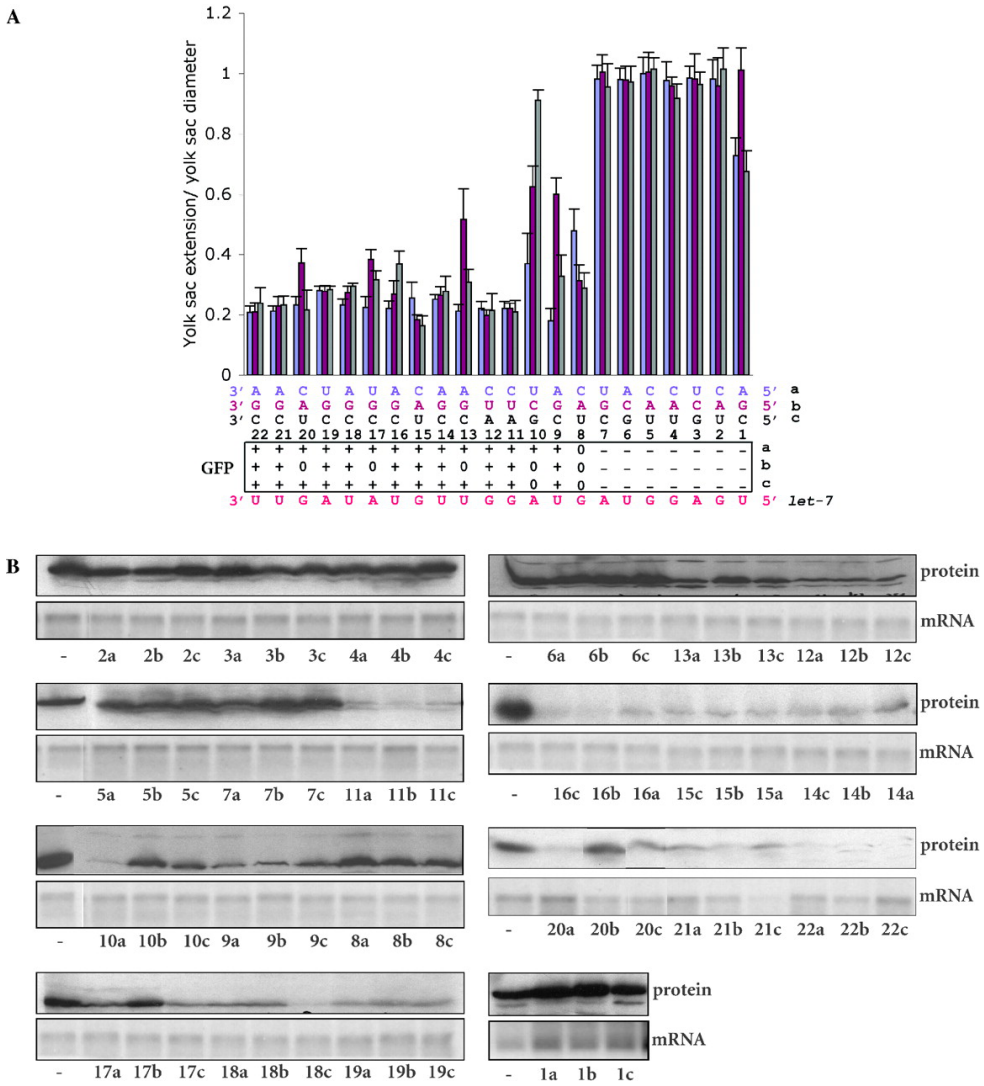

Figure 5. Mutational analysis of the *let-7* miRNA. (A) Phenotypes were scored by measuring the length of the yolk sac extension relative to the diameter of the yolk (bar diagram). For each data point, five individuals were measured. Approximate GFP levels, as assessed from western blots (B), are indicated in the table. – indicates no decline of GFP protein; + indicates a strong decline of GFP protein; 0 indicates a moderate decline of GFP protein. At each position, the three types of possible mutations are shown in the sequences beneath the bars. The first series of mutants (purple bars and sequence a) has an equal number of hydrogen bonds compared with the wild-type *let-7* duplex. Both other series (dark red and gray bars and sequences b and c) have a different number of hydrogen bonds, depending on the nature of the mutation, i.e. U/A to C/G or vice versa. The *let-7* sequence is depicted in red. (B) Western blot analysis of GFP protein level for 3 x 22 mutant derivatives of *let-7*. As an injection control, the level of *gfp::lin-41* 3'-UTR mRNA was determined by northern blotting. For each sample, 3 µg of RNA, obtained from 10 individuals, was loaded. Numbering is from the 5' end of *let-7* and corresponds to that in (A).

For our phenotypic assay, it is unclear whether the affected target mRNA(s) that cause(s) the phenotype contain one or multiple *let-7* targets. Our data add to the previous studies that irrespective of the targets, the major sequence determinants for *in vivo* function of *let-7* in zebrafish lie in the 5' 1–7 residues.

This study represents the first complete mutational analysis of a miRNA in a vertebrate model organism. It is striking and unexplained that the *let-7* miRNA is perfectly conserved throughout evolution, while only mutations in the first 7 residues affect its activity. It could be that the sensitivity of both assays described here is too low, since they make use of a rather artificial over expression setup. Another explanation might be that the mutational spectrum was derived by injection of mature *let-7* miRNA sequences. The processing of the precursor into its mature form might also be affected by mutations in (the 3' part of) *let-7*, which was not addressed by our screen.

Materials and Methods

Construction of gfp reporters

A 379 bp fragment containing two putative *let-7* target sites in the zebrafish *lin-41* 3'-UTR (Al794385) was amplified from genomic DNA using primers: *lin-41* 3'-UTR F, GGCATTGAATTCATAAGACTGCTGCAAGCTGAGAG (EcoRI restriction site is underlined) and *lin-41* 3'-UTR R, GGCATTTCTAGATCAGGGATATAACTTGCGTTC (XbaI restriction site is underlined). This fragment was cloned into pCS2 (Clontech), containing a *gfp* cDNA sequence cloned between restriction sites BamHI and ClaI (p*gfp*) resulting in p*gfp::lin-41* 3'-UTR. p*gfp::lin-41* 3'-UTR-1, -2, -1 mut1, -1 mut2, p*gfp::let-7*tar1+2, p*gfp::let-7*tar1-2x, p*gfp::let-7*tar2-2x, p*gfp::let-7*tar1+*miR-221*tar and g*fp::let-7*tar cds were made by cloning of double-stranded oligonucleotides *lin-41* 3'-UTR-1 (5'-gaattcataatcttcctgcattacacctacctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctcatctaga-3'), *lin-41* 3'-UTR-2 (5'-gaattcataatcttcctgcattacacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctcatctaga-3'), *lin-41* 3'-UTR-1 mut-1 (5'-gaattcataatcttcctgcattacacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctcatctaga-3'), *lin-41* 3'-UTR-1 mut2 (5'-gaattcataatcttcctgcattacacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctatctcatctaga-3'), *let-7*tar1+2 (5'-gaattcataatcttcctgcattacacctacctcatctaggttaaaaaaaaaattgtctacctcatctaga-3'), *let-7*tar1-2x (5'-gaattcataatctctcctgcattacacctacctcatctagctgcattacacctacctcatctaga-3'), let-7tar2-2x (5'-gaattcataatcttcgttaaaaaaaaaattgtctacctcatctaggttaaaaaaaaaattgtctacctcatctaga-3'), *let-7*tar1+*miR-221*tar (5'-gaattcataatcttcctgcattacacctacctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttaacccagcagtgtatgtagcttctaga-3') and *let-7* tar cds (5'-gaattctcttcctgcattacacctacctcatctaggttaaaaaaaaaattgtctacctcactaagtctaga-3') in the EcoRI and XbaI sites of p*gfp*. Construct *gfp::let-7*tar 5'-UTR was made by cloning of oligonucleotide *let-7*tar 5'-UTR (ggatcctcttcctgcattacacctacctcatctaggttaaaaaaaaaattgtctacctcactaagccatgg) in the NcoI and BamHI sites of p*gfp*. *let-7* and *miR-221* target sites are underlined, and the mutations are shown in bold.

Microinjections

Wild-type and mutant *let-7* deprotected and desalted RNA oligonucleotides (Proligo) were dissolved in RNAse free water at a concentration of 100 µM. Pre-*let-7* (UGAGGUAGUAGGUUGUAUAGUUUUAGGGUCACACCCACCACUGGGAGAUAACUAUACAAUCUACUGUCUUUC) was obtained from Biolegio. Oligos were annealed using a 5x buffer containing 30 mM HEPES–KOH, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$ and 50 mM NH_4Ac. 2'-O-methyl oligonucleotides (anti-*let-7*, UCUUAACUAUACAACCUACUACCUCAACCUU and control, UCUUCAGCCUAUCCUGGAUUACUUGAAACCUU; Dharmacon) were dissolved in RNAse free water at a concentration of 500 µM. Anti-*let-7* morpholino (AACTATACAACCTACTACCTCA) was dissolved in water at a concentration of 25 ng/nl and injected at a concentration of 10 ng/nl. mRNA derived from Green fluorescent protein (GFP) reporter constructs was obtained by in vitro transcription using SP6 (Boehringer) and SacII linearized plasmid as a template. Injection mixtures contained 10 µM of a *let-7* duplex and 100 ng/µl *gfp* mRNA and 50 µM 2'-O-methyl oligonucleotide where indicated. This solution was injected into the one-cell stage of wild-type embryos derived from the TL line using 1 nl per embryo. *Xenopus tropicalis* embryos were injected in the two-cell stage using 2 nl per cell.

Northern blot analysis
Total RNA from embryos was isolated using TRIzol Reagent (Invitrogen). *gfp* mRNA was detected using RNA from 10 embryos (3 µg), separated on 1.5% agarose gels according to the standard procedures. A random primed 32P-dCTP radiolabeled probe covering the complete *gfp* cDNA sequence was used for hybridization. *let-7* was detected using RNA isolated from 5 embryos (1.5 µg). RNA was separated on a 15% polyacrylamide gel. A radiolabeled probe complementary to *let-7* was used for hybridization.

Western blot analysis
Protein was isolated by boiling 5 embryos (5 mg) for 10 min in 10 µl loading buffer. Prior to loading lysates were centrifuged for 5 min at 14 000 g. Western blotting was performed according to the standard procedures. GFP was detected using a rabbit polyclonal antibody.

Acknowledgements

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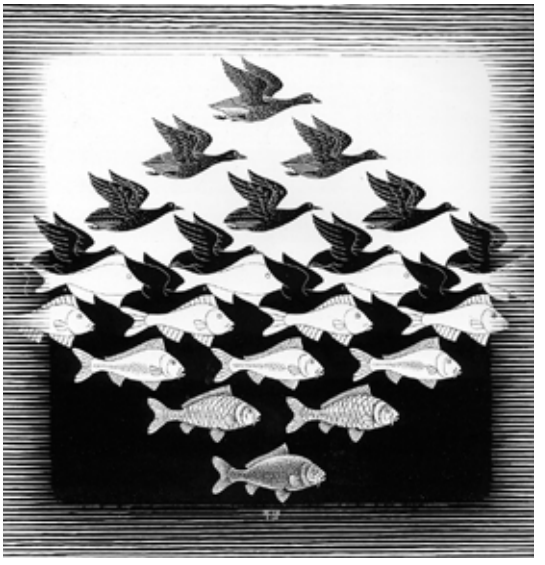
CHAPTER 3



In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes

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Abstract

MicroRNAs (miRNAs) are 20-23 nt RNA molecules that regulate gene expression posttranscriptionally. A key step towards understanding the function of the hundreds of miRNAs identified in animals is to determine their expression during development. Here, we performed a detailed analysis on the conditions for in situ detection of miRNAs in the zebrafish embryo using LNA (locked nucleic acid)-modified DNA probes and we report expression patterns for 15 miRNAs in the mouse embryo.

Results and Discussion

Several hundred different miRNAs have been cloned from various species (1) and recent estimates suggest that vertebrate genomes contain up to a thousand miRNA genes (2, 3). miRNAs function in processes ranging from insulin secretion to hematopoietic lineage differentiation (see (4) and references therein). Recent evidence shows that miRNAs play essential roles in vertebrate development and can regulate brain morphogenesis in zebrafish (5) and cardiogenesis in mice (6). In addition, altered miRNA expression levels have been reported in many human cancers (7).

Detailed spatial expression analysis of miRNAs has been technically challenging because of their small size. We recently reported the expression patterns for 115 conserved vertebrate miRNAs in the developing zebrafish embryo using LNA-modified DNA oligonucleotide probes (8).

LNA comprises a new class of bicyclic high-affinity RNA analogues in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation by the introduction of an O2',C4'-methylene bridge, resulting in unprecedented hybridization affinity towards complementary DNA and RNA molecules (9). The thermal stability and improved mismatch discrimination of short LNA oligonucleotides has made them useful for SNP genotyping assays, antisense-based gene silencing and gene expression profiling (10).

In the present study, we describe the conditions for optimal *in situ* detection of miRNAs using LNA probes (see also Materials and Methods).

First, we compared the ability of LNA-modified DNA probes to detect *miR-206* (muscle specific), *miR-124a* (brain specific) and *miR-122a* (liver specific) in zebrafish embryos with unmodified DNA probes of identical length and sequence. While we obtained expected signals for all three miRNAs when LNA-modified probes were used for hybridization, we observed no such expression patterns with corresponding DNA probes (Supplementary Figure 1A and B).

Next, we determined the minimal LNA probe length required for specific staining. Therefore, we performed *in situ* hybridizations with systematically shortened probes against *miR-124a* and *miR-206*. We could specifically detect *miR-124a* and *miR-206* with shortened versions of the LNA probes complementary to a 12 nt (nucleotide) region at the 5'-end of the miRNA (Figure 1A). *In situ* staining was virtually lost when 10-nt or 8-nt probes were used. Shorter probes would allow the design of T_m -normalized probe sets for large scale *in situ* screens or for microarray profiling. Furthermore, the successful use of very short probes with T_m values in the range of body temperatures suggests that therapeutic applications, like antisense inhibition of miRNAs, may also be possible.

To determine the optimal hybridization temperature for detecting miRNAs in zebrafish using LNA-modified probes, in relation to their T_m (melting temperature)

values (Supplementary Table 1), we tested probes covering the complete mature miRNA for *miR-122a* (T_m = 78 °C), *miR-206* (T_m = 73 °C) and *miR-153* (T_m = 68 °C, brain specific) and 14-mer probes for *miR-124a* (T_m = 70 °C) and *miR-206* (T_m = 55 °C) (Supplementary Figure 2A and B). In most cases the signal decreased when the hybridization temperature became higher than ~20 °C below the T_m value of

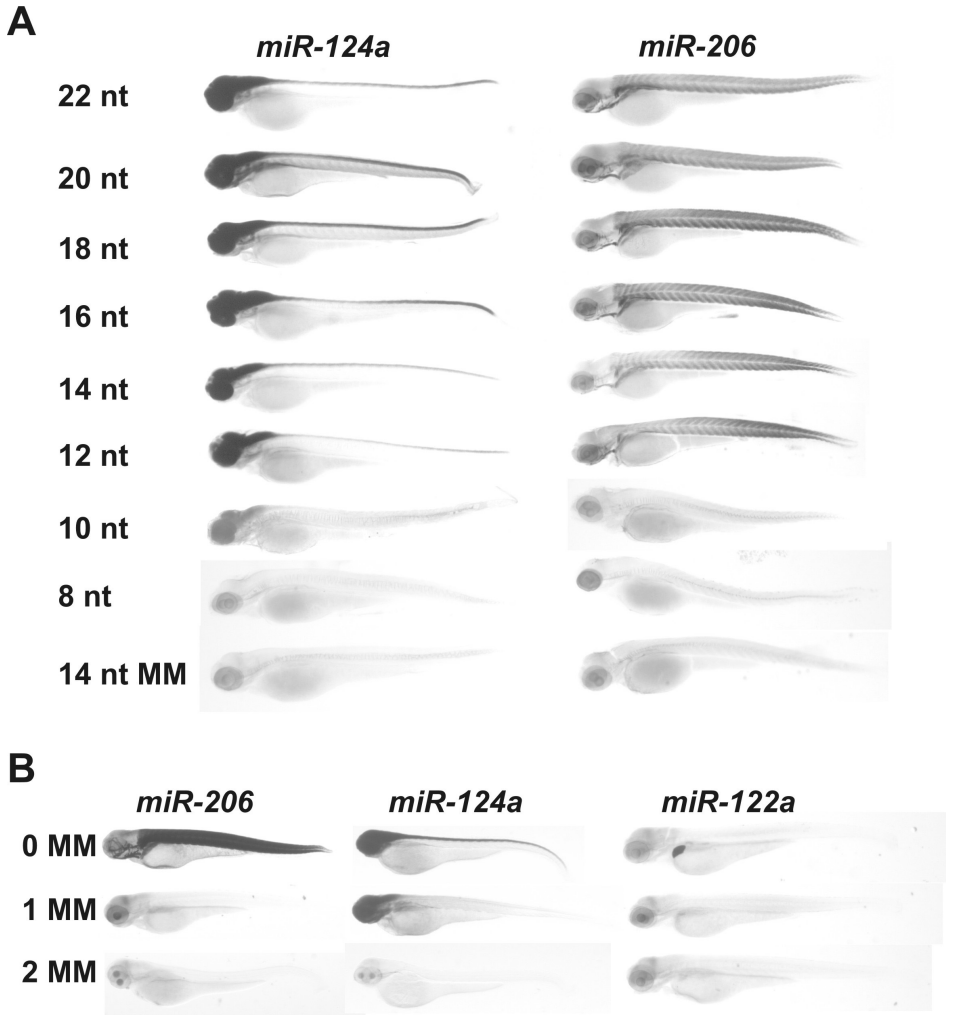

Figure 1. miRNA detection in zebrafish embryos using LNA-modified DNA probes. (A) *In situ* detection of *miR-124a* and *miR-206* using probes that are 2, 4, 6, 8, 10, 12 and 14 nt shorter than the original 22 nt probes. A single central mismatch (MM) in the 14 nt probes for *miR-124a* and *miR-206* prevents hybridization (14 nt MM). (B) Assessment of the specificity of LNA-modified probes using perfectly matched (0 MM) and single (1 MM) and double (2 MM) mismatched probes for the detection of *miR-124a*, *miR-122a* and *miR-206*.

the probe. Background staining increased at hybridization temperatures lower than ~28 °C below the T_m value of the probe. We obtained optimal miRNA detection at hybridization temperatures of 20-25 °C below the T_m value of the probe.

Next, we investigated the optimal hybridization time for LNA-based miRNA *in situ* hybridization (Supplementary Figure 3). We obtained substantial *in situ* staining even after ten minutes of hybridization for *miR-122a* and *miR-206* in 72 hour fish embryos. After one hour, the signal strength was comparable to the staining obtained after an overnight hybridization. This indicates that the hybridization times can be easily shortened for *in situs* using LNA probes, which would shorten the miRNA *in situ* protocol for zebrafish embryos from three to two days.

Some members in a miRNA family differ by one or two bases only and it might be that these do not have identical expression patterns (11). To examine the specificity of LNA-modified probes we performed *in situ* hybridizations with single and double mismatched probes for *miR-124a*, *miR-206* and *miR-122a* (Figure 1B and Supplementary Table 1). For *miR-122a* and *miR-206* specific staining was lost upon introduction of a single central mismatch in the LNA probe. For the *miR-124a* probe two central mismatches were needed for adequate discrimination. We expect that shorter LNA probes would exhibit enhanced mismatch discrimination. Thus, we tested single central mismatch versions of the 14-mer LNA probes for *miR-206* and *miR-124a* and in both cases the hybridization signal was completely lost (Figure 1A). In addition, we performed *in situ* hybridization with probes for *miR-206* and *miR-124a* containing mismatches at either the 3' end or the 5' end (Supplementary Figure 4). Although signals were lost for all *miR-206* mismatch probes, we only observed discrimination for *miR-124a* with a 14-mer probe containing a mismatch at the 5' end.

To investigate if the *in situ* signal is coming only from the mature miRNA or also from its precursor stemloop, we tested probes targeting segments of the precursor stemloop that do not comprise the mature miRNA, i.e. probes complementary to the star (arm of the stem opposite of the mature miRNA) and loop sequences of *miR-183* and *miR-217* precursors (Supplementary Table 1). However, we observed no *in situ* signal with these probes, although on a northern blot we could detect the precursor for *miR-183* with probes targeting star, loop and mature sequences of the precursor stemloop (Supplementary Figure 5A and B).

To explore the usefulness of the LNA probe technology for detection of miRNAs in organisms other than zebrafish, we performed whole mount *in situ* hybridizations on mouse and *Xenopus tropicalis* embryos with probes for *miR-124a* and *miR-1* (Figure 2 and Supplementary Figure 6). *miR-124a* was specific for tissues of the central nervous system in both organisms. *miR-1* was expressed in the body wall muscles and the muscles of the head in *Xenopus*. In mouse, *miR-1* was mainly expressed in the somites and the heart. These data are in agreement with the expression patterns in zebrafish and with expression studies based on dissected tissues from mouse, which

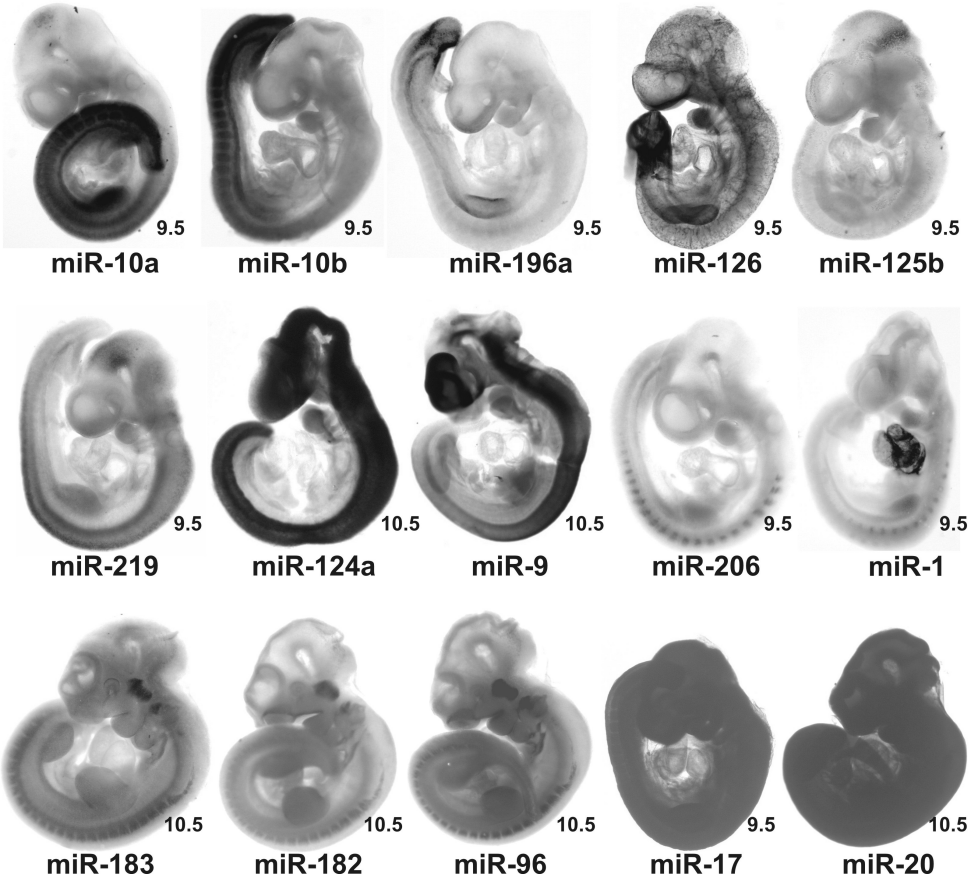

Figure 2. Expression of 15 miRNAs in 9.5 and 10.5 dpc mouse embryos: *miR-10a* and *miR-10b*, posterior trunk; *miR-196a*, tailbud; *miR-126*, blood vessels; *miR-125b*, midbrain-hindbrain boundary; *miR-219*, midbrain, hindbrain and spinal cord; *miR-124a*, central nervous system; *miR-9*, forebrain and the spinal cord; *miR-206*, somites; *miR-1*, heart and somites; *miR-182*, *miR-96* and *miR-183*, cranial and dorsal root ganglia; *miR-17-5p* and *miR-20* are ubiquitously expressed.

show that *miR-124a* is brain specific and *miR-1* is a muscle specific miRNA (12). Next, we determined the whole mount expression patterns for 80 conserved vertebrate miRNAs in mouse embryos (Supplementary Table 2). However, for only a few miRNAs we observed tissue specific expression patterns in 9.5 or 10.5 dpc (days post conception) mouse embryos (Figure 2), which might be due to the low expression levels of many miRNAs during early stages of mouse development (13). For *miR-1*, *miR-206, miR-17, miR-20, miR-124a, miR-9, miR-126, miR-219, miR-196a, miR-10b* and *miR-10a* the patterns were similar to what we previously observed in the zebrafish (14). In addition, *miR-10a* and *miR-196a* were found to be active in the posterior trunk in mouse embryos as visualized by miRNA-responsive

sensors and we also found these miRNAs to be expressed in the same regions (15). For *miR-182, miR-96, miR-183* and *miR-125b* the expression patterns were different compared to zebrafish.

In conclusion, we determined the optimal hybridization conditions for the detection of miRNAs in animal embryos using LNA-modified probes and we described novel miRNA expression patterns in mouse embryos. We expect that the LNA technology will be widely used not only in fundamental studies of miRNAs but also in cancer diagnosis.

Materials and Methods

Experimental material

Experiments with animals were approved by the DEC (Dier Experimenten Commissie) of the KNAW (Koninklijke Nederlandse Academie van Wetenschappen). Zebrafish, mouse and *Xenopus tropicalis* were kept under standard conditions. For all in situ hybridizations on zebrafish we used 3 day or 5 day old homozygous albino embryos. For *Xenopus tropicalis* 3 day old embryos were used and for mouse we used 9.5 dpc or 10.5 dpc embryos.

Design and synthesis of LNA-modified oligonucleotide probes

All LNA-modified DNA oligonucleotide probes (miRCURY detection probes, kindly provided by Exiqon, Denmark) are listed in Supplementary Table 1 and Supplementary Table 2. LNA probes were labeled with digoxigenin-ddUTP using the 3'-end labeling kit (Roche) according to the manufacturers recommendations and purified using sephadex G25 MicroSpin columns (Amersham).

Whole mount in situ hybridizations

All washing and incubation steps were performed in 2 ml eppendorf tubes. Embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS and subsequently transferred through a graded series (25% MeOH in PBST (PBS containing 0.1% Tween-20), 50% MeOH in PBST, 75% MeOH in PBST) to 100% methanol and stored at –20 °C up to several months. At the first day of the *in situ* hybridization embryos were rehydrated by successive incubations for 5 min in 75% MeOH in PBST, 50% MeOH in PBST, 25% MeOH in PBST and 100% PBST (4 x 5 min). Fish, mouse and *Xenopus* embryos were treated with proteinaseK (10 µg/ml in PBST) for 45 min at 37 °C, refixed for 20 min in 4% paraformaldehyde in PBS and washed 3 x 5 min with PBST. After a short wash in water, endogenous alkaline phosphatase activity was blocked by incubation of the embryos in 0.1 M tri-ethanolamine and 2.5% acetic anhydride for 10 min, followed by a short wash in water and 5 x 5 min washing in PBST. The embryos were then transferred to hybridization buffer (50% Formamide, 5x SSC, 0.1% Tween, 9.2 mM citric acid, 50 µg/ml heparin, 500 µg/ml yeast RNA) and prehybridized for 2-3 hour at the hybridization temperature. Hybridization was performed in fresh pre-heated hybridization buffer containing 10 nM of labeled LNA probe. Post-hybridization washes were done at the hybridization temperature by successive incubations for 15 min in HM^- (hybridization buffer without heparin and yeast RNA), 75% HM^-/25% 2x SSCT (SSC containing 0.1% Tween-20), 50% HM^- /50% 2x SSCT, 25% HM^-/75% 2x SSCT, 100% 2x SSCT and 2 x 30 min in 0.2x SSCT. Subsequently, embryos were transferred to PBST through successive incubations for 10 min in 75% 0.2x SSCT/25% PBST, 50% 0.2x SSCT/50% PBST, 25% 0.2x SSCT/75% PBST and 100% PBST. After blocking for 1 hour in blocking buffer (2% sheep serum, 2mg/ml BSA in PBST), the embryos were incubated overnight at 4 °C in blocking buffer containing anti-DIG-AP FAB fragments (Roche, 1/2000). The next day, zebrafish embryos were washed 6 x 15 min in PBST, mouse and *X. tropicalis* embryos were washed 6 x 1 hour in TBST containing 2 mM levamisole and then for 2 days at 4 °C with regular refreshment of the wash buffer. After the post-antibody washes, the embryos were washed 3 x 5 min in staining buffer (100 mM tris HCl pH9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% tween 20). Staining was done in buffer supplied with 4.5 µl/ml NBT (Roche, 50 mg/ml stock) and 3.5 µl/ml BCIP (Roche, 50 mg/ml stock). The reaction was stopped with 1 mM EDTA in PBST and the embryos were stored at 4 °C. The embryos were mounted in Murray's solution (2:1 benzylbenzoate:benzylalcohol) via an increasing methanol series (25% MeOH in PBST, 50% MeOH in PBST, 75% MeOH in PBST, 100% MeOH) prior to imaging.

Northern blotting

Total RNA was isolated from 5 day old embryos using trizol (Invitrogen). For each sample 5 µg RNA was separated

on a 15% polyacrylamide gel and blotted according 3 to standard procedures. Blots were prehybridized for 30 min at 60 °C in hybridization buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, 7% SDS) and hybridized overnight at 60°C in hybridization buffer containing 0.1 nM DIG-labeled LNA probe. After stringent washes (once for 30 min at 50 °C in 2X SSC 0.1% SDS and once for 30 min at 50 °C in 0.5X SSC 0.1% SDS) blots were rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween, pH 7.5) and blocked for 30 min at room temperature in washing buffer containing 5% milk powder. Subsequently, blots were incubated for 1 hour at room temperature with anti-DIG-AP antibody (Roche) in blocking buffer, washed 3 times for 15 min in washing buffer and 2 times for 5 min with AP-buffer (0.1 M Tris-HCl pH9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween). Signal was detected using CDP-star chemiluminescent substrate (Roche).

Acknowledgements

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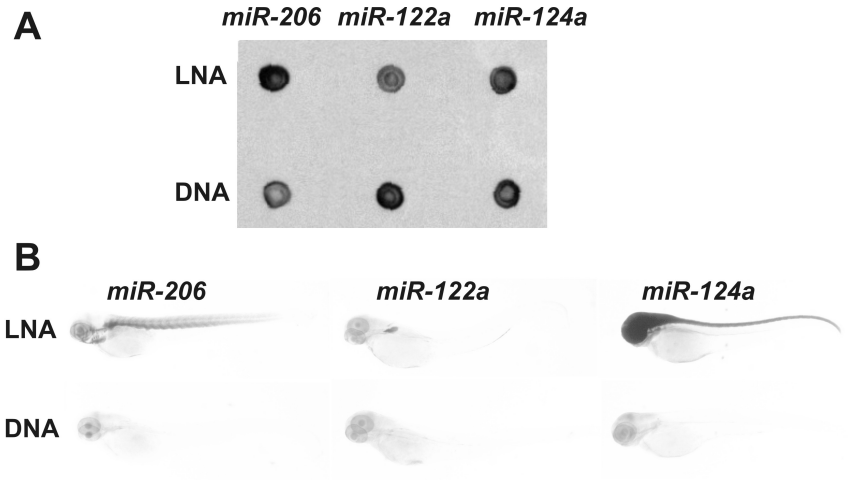
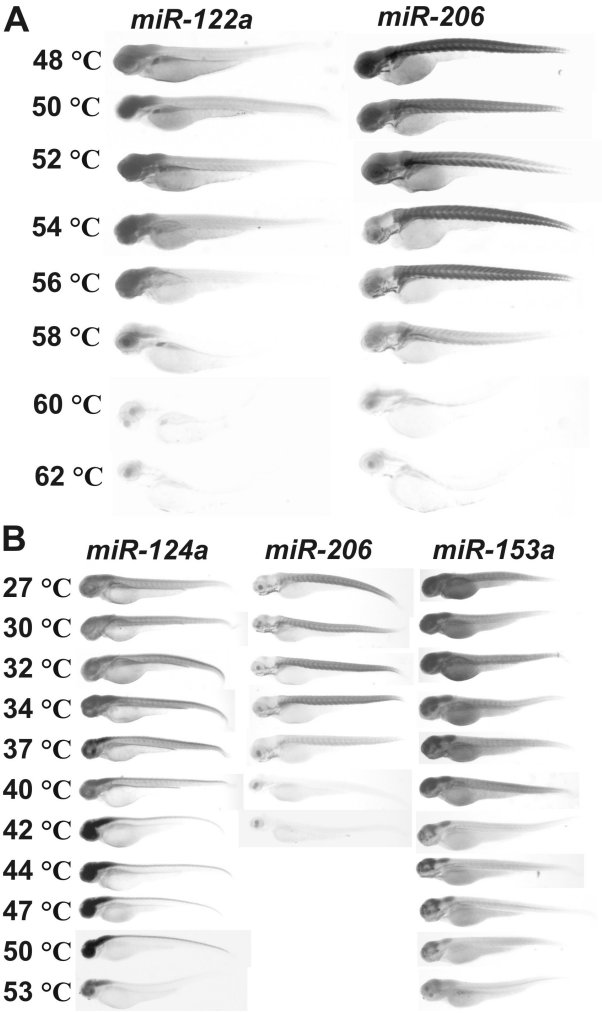
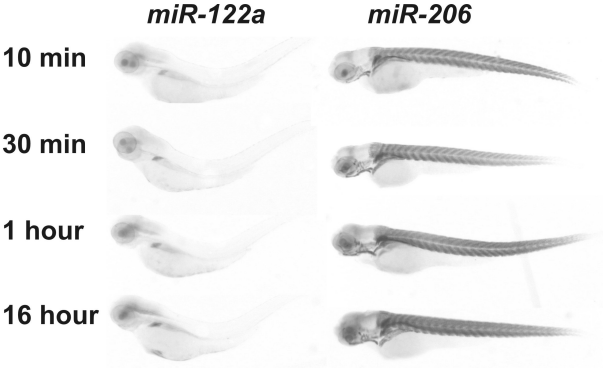
Supplementary data



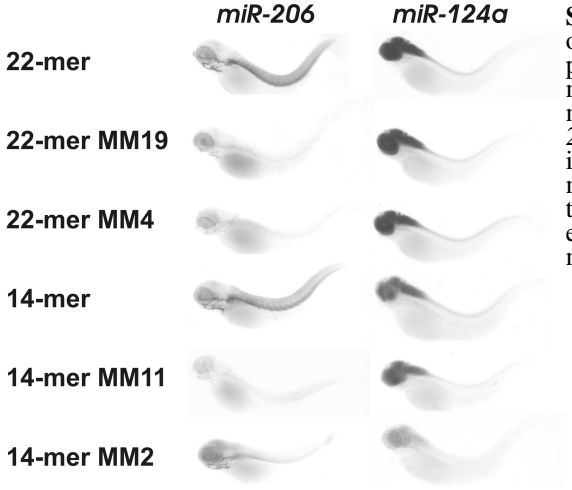
Supplementary Figure 1. Detection of *miR-124a, miR-122a* and *miR-206* with DIG-labeled DNA and LNA probes in 72h zebrafish embryos. (A) Dot-blot of DIG labeled DNA and LNA probes. Per labeled probe, 1 pmol was spotted on a positively charged nylon membrane. Equal labeling was obtained for both LNA-modified and unmodified DNA probes. (B) *In situ* hybridization of *miR-206* (muscle specific), *miR-122a* (liver specific) and *miR-124a* (brain specific) in zebrafish embryos. LNA probes were hybridized at 59 ºC (*miR-122a* and *miR-124a*) and 54 ºC (*miR-206*). DNA probes were hybridized at 45 ºC. Only LNA probes give clear staining. Lowering the hybridization temperature for DNA probes resulted in higher background staining (data not shown). Similar experiments to detect miRNAs in fish embryos using *in vitro* synthesized RNA probes, that carried a concatamer against the mature miRNA, were also unsuccessful (data not shown).



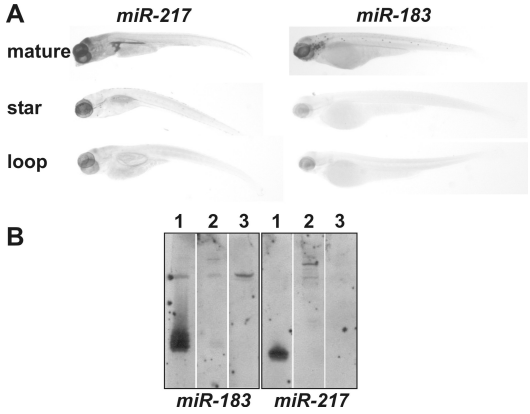
Supplementary Figure 2. Determination of the optimal hybridization temperature for *in situ* detection of miRNAs in the zebrafish embryo. (A) Hybridization temperature series on zebrafish embryos with 22-mer LNA-modified probes for *miR-122a* (T_m = 78 °C) and *miR-206* (T_m = 53 °C). (B) Hybridization temperature series on zebrafish embryos with 14-mer LNA-modified probes for *miR-124a* (T_m=70 °C) and *miR-206* (T_m=55 °C) and a full-length (20-mer) LNA-modified probe for *miR-153* (T_m=68 °C). For most probes the *in situ* signal strength is reduced at hybridization temperatures higher than ~20 °C below the calculated T_m values of the probes. On the other hand, at temperatures lower than ~28 °C below the T_m, the background staining increases. We did not detect any increase in background staining at the lower temperatures tested for the 14-mer probe against *miR-206*. In general, optimal *in situ* detection is obtained at a hybridization temperature around 20-25 °C below the T_m value of the LNA-modified probe.



Supplementary Figure 3. Determination of the optimal hybridization time for *in situ* detection of miRNAs in the zebrafish embryo.



Supplementary Figure 4. Assessment of the specificity of LNA-modified probes using perfectly matched and mismatched 14-mer and 22-mer LNA-modified probes for *miR-124a* and *miR-206*. In contrast to the experiment shown in Figure 1B, where the effect of central mismatches (MM) was assessed, now the effect of mismatches at the 3' or 5' end of the probe was tested (see Supplementary Table 1 for probe sequences).



Supplementary Figure 5. Detection of *miR-183* and *miR-217* using LNA-modified probes designed to target the mature, star and loop sequences of the miRNA precursor. (A) *In situ* detection of *miR-183* and *miR-217*. In contrast to the probes against mature miRNAs, we could not detect any *in situ* expression patterns with the probes complementary to the star and loop sequences of the miRNA precursors (for sequences see Supplementary Table 1), indicating that the LNA-modified probes mainly detect mature miRNAs *in situ*. (B) Detection of *miR-183* and *miR-217* by Northern blot analysis of RNA from 5 day old zebrafish embryos using the same LNA-modified probes as in (A).

Supplementary Figure 6. *In situ* hybridizations of miRNAs in *Xenopus tropicalis* embryos. Expression of *miR-1* is restricted to the muscles in the body and the head and *miR-124a* is expressed throughout the central nervous system. The patterns are similar to the expression of these miRNAs in the zebrafish embryo.

Supplementary Table 1. Short probes, mismatch probes and star and loop probes used in this study

Probe name	Probe sequence (5' to 3')	miRNA sequence (5' to 3')	T_m *
miR124a/LNA	tggcattcaccgcgtgccttaa	uuaaggcacgcgggugaaugcca	80
miR124a/LNA-2	gcattcaccgcgtgccttaa	uuaaggcacgcgggugaaugcca	78
miR124a/LNA-4	attcaccgcgtgccttaa	uuaaggcacgcgggugaaugcca	72
miR124a/LNA-6	tcaccgcgtgccttaa	uuaaggcacgcgggugaaugcca	71
miR124a/LNA-8	accgcgtgccttaa	uuaaggcacgcgggugaaugcca	70
miR124a/LNA-10	cgcgtgccttaa	uuaaggcacgcgggugaaugcca	60
miR124a/LNA-12	cgtgccttaa	uuaaggcacgcgggugaaugcca	45
miR124a/LNA-14	tgccttaa	uuaaggcacgcgggugaaugcca	27
miR124a/LNA/MM4	tggaattcaccgcgtgccttaa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA/MM19	tggcattcaccgcgtgccataa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA/2MM	tggcattcaaagcgtgccttaa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA/1MM	tggcattcaacgcgtgccttaa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA-8/MM2	aacgcgtgccttaa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA-8/MM11	accgcgtgccataa	uuaaggcacgcgggugaaugcca	ND
miR124a/LNA-8/MM	acgcgtaccttaa	uuaaggcacgcgggugaaugcca	ND
miR206/LNA	ccacacacttccttacattcca	uggaauguaaggaagugugugg	73
miR206/LNA-2	acacacttccttacattcca	uggaauguaaggaagugugugg	70
miR206/LNA-4	acacttccttacattcca	uggaauguaaggaagugugugg	64
miR206/LNA-6	acttccttacattcca	uggaauguaaggaagugugugg	58
miR206/LNA-8	ttccttacattcca	uggaauguaaggaagugugugg	55
miR206/LNA-10	ccttacattcca	uggaauguaaggaagugugugg	49
miR206/LNA-12	ttacattcca	uggaauguaaggaagugugugg	35
miR206/LNA-14	acattcca	uggaauguaaggaagugugugg	32
miR206/LNA/MM4	ccaaacacttccttacattcca	uggaauguaaggaagugugugg	ND
miR206/LNA/MM19	ccacacacttccttacatacca	uggaauguaaggaagugugugg	ND
miR206/LNA/2MM	ccacacactatcttacattcca	uggaauguaaggaagugugugg	ND
miR206/LNA/1MM	ccacacactaccttacattcca	uggaauguaaggaagugugugg	ND
miR206/LNA-8/MM2	taccttacattcca	uggaauguaaggaagugugugg	ND
miR206/LNA-8/MM11	ttccttacatacca	uggaauguaaggaagugugugg	ND
miR206/LNA-8/MM	ttccttaaattcca	uggaauguaaggaagugugugg	ND
miR122a/LNA	acaaacaccattgtcacactcca	uggagugugacaaugguuuugu	78
miR122a/LNA/2MM	acaaacaccaaagtcacactcca	uggagugugacaaugguuuugu	ND
miR122a/LNA/1MM	acaaacaccatagtcacactcca	uggagugugacaaugguuuugu	ND
miR183/LNA	cagtgaattctaccagtgccata	tatggcactggtagaattcactg	73
miR-183/LNA STAR	tttatggccctttggtaattcac	gtgaattaccaaagggccataaa	72
miR-183/LNA loop	ctgatagtgtgctttcacagtga	tcactgtgaaagcacactatcag	73
miR217/LNA	atccaatcagttcctgatgcagta	tactgcaggaactgattggat	75
mir-217/LNA STAR	gagtgcatcaggaactgatggctc	gagccatcagttcctgatgcactc	80
mir-217/LNA loop	gatggctcctgaatatcatccaat	attggatgatattcaggagccatc	73
miR153/LNA	tcacttttgtgactatgcaa	ttgcatagtcacaaaagtga	68

* The melting temperatures (T_m) were predicted using a thermodynamic nearest neighbour model based on the determination of T_m values of a large set of LNA oligonucleotide duplexes (>1000) by UV spectroscopy. The LNA T_m prediction tool is accessible at www.exiqon.com.

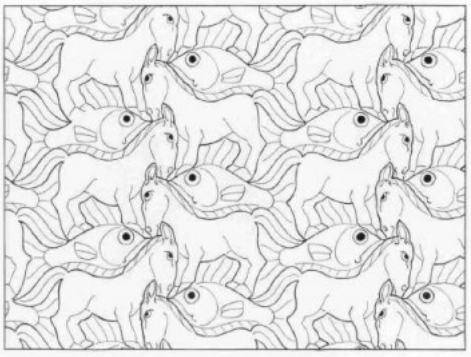
Supplementary Table 2. In situ probes and detection of miRNAs in mouse

miRNA	probe sequence (5' to 3')	miRNA sequence (5' to 3')	9.5 day	10.5 day
let-7a	aactatacaacctactacctca	UGAGGUAGUAGGUUGUAUAGUU	2	2
let-7b	aaccacacaacctactacctca	UGAGGUAGUAGGUUGUGUGGUU	2	3
let-7c	aaccatacaacctactacctca	UGAGGUAGUAGGUUGUAUGGUU	1	2
let-7f	aactatacaatctactacctca	UGAGGUAGUAGAUUGUAUAGUU	2	2
let-7i	agcacaaactactacctca	UGAGGUAGUAGUUUGUGCU	1	1
miR-1	tacatacttctttacattcca	UGGAAUGUAAAGAAGUAUGUA	4	4
miR-100	cacaagttcggatctacgggtt	AACCCGUAGAUCCGAACUUGUG	1	2
miR-101	cttcagttatcacagtactgta	UACAGUACUGUGAUAACUGAAG	2	2
miR-108	aatgcccctaaaaatccttat	AUAAGGAUUUUUAGGGGCAUU	2	3
miR-10a	cacaaattcggatctacagggta	UACCCUGUAGAUCCGAAUUUGUG	4	4
miR-10b	acaaattcggttctacagggta	UACCCUGUAGAACCGAAUUUGU	4	4
miR-122a	acaaacaccattgtcacactcca	UGGAGUGUGACAAUGGUGUUUGU	2	2
miR-124a	tggcattcaccgcgtgccttaa	UUAAGGCACGCGGUGAAUGCCA	4	4
miR-125b	tcacaagttagggtctcaggga	UCCCUGAGACCCUAACUUGUGA	4	4
miR-126	gcattattactcacggtacga	UCGUACCGUGAGUAAUAAUGC	4	4
miR-126*	cgcgtaccaaaagtaataatg	CAUUAUUACUUUUGGUACGCG	1	1
miR-128a	aaaagagaccggttcactgtga	UCACAGUGAACCGGUCUCUUUU	2	2
miR-130a	gccctttaacattgcactg	CAGUGCAAUGUUAAAAGGGC	1	3
miR-132	cgaccatggctgtagactgtta	UAACAGUCUACAGCCAUGGUCG	1	2
miR-135a	tcacataggaataaaaagccata	UAUGGCUUUUUAUUCCUAUGUGA	2	2
miR-137	ctacgcgtattcttaagcaata	UAUUGCUUAAGAAUACGCGUAG	1	1
miR-138	gattcacaacaccagct	AGCUGGUGUUGUGAAUC	2	2
miR-140	ctaccatagggtaaaaccact	AGUGGUUUUACCCUAUGGUAG	1	1
miR-141	gcatcgttaccagacagtgtt	AACACUGUCUGGUAACGAUGC	1	2

miR-142-3p	tccataaagtaggaaacactaca	UGUAGUGUUUCCUACUUUAUGGA	1	2
miR-142-5p	gtagtgctttctactttatg	CAUAAAGUAGAAAGCACUAC	1	2
miR-143	cgagctacagtgcttcatctca	UGAGAUGAAGCACUGUAGCUCG	3	3
miR-144	tagtacatcatctatactgta	UACAGUAUAGAUGAUGUACUA	1	2
miR-145	aagggattcctgggaaaactggac	GUCCAGUUUUCCCAGGAAUCCCUU	2	2
miR-152	aagttctgtcatgcactga	UCAGUGCAUGACAGAACUU	2	3
miR-153	tcacttttgtgactatgcaa	UUGCAUAGUCACAAAAGUGA	1	N.D.
miR-155	ccctatcacgattagcattaa	UUAAUGCUAAUCGUGAUAGGGG	1	1
miR-16	ccaatatttacgtgctgcta	UAGCAGCACGUAAAUAUUGG	1	2
miR-17-5p	actacctgcactgtaagcactttg	CAAAGUGCUUACAGUGCAGGUAGU	3	3
miR-181a	actcaccgacagcgttgaatgtt	AACAUUCAACGCUGUCGGUGAGU	2	3
miR-182	tgtgagttctaccattgccaaa	UUUGGCAAUGGUAGAACUCACA	4	4
miR-182*	tagttggcaagtctagaacca	UGGUUCUAGACUUGCCAACUA	2	2
miR-183	cagtgaattctaccagtgccata	UAUGGCACUGGUAGAAUUCACUG	4	4
miR-184	cccttatcagttctccgtcca	UGGACGGAGAACUGAUAAGGG	2	3
miR-187	ggctgcaacacaagacacga	UCGUGUCUUGUGUUGCAGCC	1	2
miR-190	acctaatatatcaaacatatca	UGAUAUGUUUGAUAUAUUAGGU	1	2
miR-192	ggctgtcaattcataggtca	UGACCUAUGAAUUGACAGCC	1	2
miR-193	ctgggactttgtaggccagtt	AACUGGCCUACAAAGUCCCAG	1	2
miR-194	tccacatggagttgctgttaca	UGUAACAGCAACUCCAUGUGGA	2	2
miR-195	gccaatatttctgtgctgcta	UAGCAGCACAGAAAUAUUGGC	2	3
miR-196a	cccaacaacatgaaactaccta	UAGGUAGUUUCAUGUUGUUGGG	4	4
miR-199a	gaacaggtagtctgaacactggg	CCCAGUGUUCAGACUACCUGUUC	1	2
miR-199a*	aaccaatgtgcagactactgta	UACAGUAGUCUGCACAUUGGUU	1	2
miR-19a	tcagttttgcatagatttgcaca	UGUGCAAAUCUAUGCAAAACUGA	2	3
miR-19b	tcagttttgcatggatttgcaca	UGUGCAAAUCCAUGCAAAACUGA	3	3
miR-20	ctacctgcactataagcacttta	UAAAGUGCUUAUAGUGCAGGUAG	3	3
miR-200a	acatcgttaccagacagtgtta	UAACACUGUCUGGUAACGAUGU	1	2
miR-200b	catcattaccaggcagtattaga	UCUAAUACUGCCUGGUAAUGAUG	1	2
miR-203	agtggtcctaaacatttcac	GUGAAAUGUUUAGGACCACU	1	1
miR-204	caggcataggatgacaaagggaa	UUCCCUUUGUCAUCCUAUGCCUG	2	2
miR-206	ccacacacttccttacattcca	UGGAAUGUAAGGAAGUGUGUGG	4	4
miR-215	ctgtcaattcataggtcat	AUGACCUAUGAAUUGACAG	1	2
miR-216	cacagttgccagctgagatta	UAAUCUCAGCUGGCAACUGUG	1	2
miR-217	atccaatcagttcctgatgcagta	UACUGCAUCAGGAACUGAUUGGAU	1	2
miR-218	acatggttagatcaagcacaa	UUGUGCUUGAUCUAACCAUGU	1	1
miR-219	agaattgcgtttggacaatca	UGAUUGUCCAAACGCAAUUCU	4	4
miR-223	ggggtatttgacaaactgaca	UGUCAGUUUGUCAAAUACCCC	1	2
miR-23a	ggaaatccctggcaatgtgat	AUCACAUUGCCAGGGAUUUCC	1	2
miR-25	tcagaccgagacaagtgcaatg	CAUUGCACUUGUCUCGGUCUGA	2	2
miR-26a	agcctatcctggattacttgaa	UUCAAGUAAUCCAGGAUAGGCU	2	2
miR-27a	gcggaacttagccactgtgaa	UUCACAGUGGCUAAGUUCCGC	1	2
miR-27b	cagaacttagccactgtgaa	UUCACAGUGGCUAAGUUCUG	1	1
miR-29b	acactgatttcaaatggtgcta	UAGCACCAUUUGAAAUCAGUGU	1	1
miR-29c	taaccgatttcaaatggtgcta	UAGCACCAUUUGAAAUCGGUUA	1	1
miR-301	gctttgacaatactattgcactg	CAGUGCAAUAGUAUUGUCAAAGC	1	1
mir-30a-3p	acagcaaacatccaactgaaag	CUUUCAGUUGGAUGUUUGCUGU	1	1
miR-30b	gctgagtgtaggatgtttaca	UGUAAACAUCCUACACUCAGC	1	1
miR-30c	gctgagagtgtaggatgtttaca	UGUAAACAUCCUACACUCUCAGC	1	2
miR-338	caacaaaatcactgatgctgga	UCCAGCAUCAGUGAUUUUGUUG	2	2
miR-7	aacaaaatcactagtcttcca	UGGAAGACUAGUGAUUUUGUU	1	N.D.
miR-7b	aacaaaatcacaagtcttcca	UGGAAGACUUGUGAUUUUGUU	2	2
miR-9	tcatacagctagataaccaaaga	UCUUUGGUUAUCUAGCUGUAUGA	4	4
miR-9*	actttcggttatctagcttta	UAAAGCUAGAUAACCGAAAGU	1	1
miR-96	agcaaaaatgtgctagtgccaaa	UUUGGCACUAGCACAUUUUUGCU	4	4
miR-99a	cacaagatcggatctacgggtt	AACCCGUAGAUCCGAUCUUGUG	1	2

1= no staining, 2= weak ubiquitous staining, 3= strong ubiquitous staining, 4= specific staining,
N.D.= not determined

CHAPTER 4



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MicroRNA expression in zebrafish embryonic development

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 21 nucleotides in length, that can regulate gene expression by base-pairing to partially complementary mRNAs. Regulation by miRNAs can play essential roles in embryonic development. We determined the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebrafish embryos by microarrays and by *in situ* hybridizations, using locked nucleic acid-modified oligonucleotide probes. Most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development, suggesting that their role is not in tissue fate establishment but in differentiation or maintenance of tissue identity.

Current estimates of miRNA gene numbers in vertebrates are as high as 500 (1), of which many are conserved, and miRNAs may regulate up to 30% of genes (2). The miRNA first discovered, *lin-4*, is involved in developmental timing in the nematode *C. elegans* (3). In mammals, miRNAs have been implicated in hematopoietic lineage differentiation (4) and homeobox gene regulation (5). Zebrafish that are defective in miRNA processing arrest in development (6). Recently, miRNAs were shown to be dispensable for cell fate determination, axis formation and cell differentiation, but required for brain morphogenesis in zebrafish embryos (7). Together, these findings indicate that miRNAs can play essential roles in development. However, little is known about the individual roles of most miRNAs. To focus future miRNA studies, we determined the spatial and temporal expression patterns of 115 conserved vertebrate miRNAs (see Material and Methods) in zebrafish embryos.

First we determined the temporal expression of miRNAs during embryonic development by microarray analysis (Figure 1A and Supplementary Figure 1A). Up to segmentation (12 hours postfertilization (hpf)) most miRNAs could not be detected. Most miRNAs became visible at 1 to 2 days post-fertilization and showed strong expression when organogenesis is virtually completed (96 hpf). In adults the majority of miRNAs remained expressed (Figure 1A). In addition we determined the expression of miRNAs in dissected organs of adult fish. For some miRNAs a high degree of tissue specificity was observed (Supplementary Figure 1B and 2).

In situ hybridization of miRNAs had thus far not been possible in animals. Recently LNA (locked nucleic acid)– modified DNA oligonucleotide probes have been shown to increase the sensitivity for the detection of miRNAs by northern blots (8). By northern blots analysis and *in situ* hybridization, using LNA probes, we detected predominantly mature miRNAs, which were reduced in *dicer* knockout zebrafish (Supplementary Figure 3). We used these LNA probes for the wholemount *in situ* detection of the conserved vertebrate miRNAs in zebrafish embryos and made a catalogue of miRNA expression patterns (Supplementary Figure 4).

Most miRNAs (68%) were expressed in a highly tissue specific manner. For example, *miR-140* was specifically expressed in the cartilage of the jaw, head and fins, and its presence was entirely restricted to those regions (Figure 1B). Figure 1C shows representative examples of six miRNAs that were expressed in different organ systems: nervous system, digestive system, muscles, circulatory system, sensory organs and excretory system. Even within organs there is specificity, as exemplified in Figure 1D, where *miR-217* can be seen to be expressed in the exocrine pancreas, and *miR-7* in the endocrine pancreas (Langerhans islets). More than half of the miRNAs (43) were expressed in (specific regions of) the central nervous system (Supplementary Figure 4). Many miRNA genes are clustered in the genome and therefore probably expressed as one primary transcript, and indeed we observed that many such clustered genes showed identical or overlapping expression patterns (Supplementary Figure 4 and 5). We compared the *in situ* data with microarray data for zebrafish and

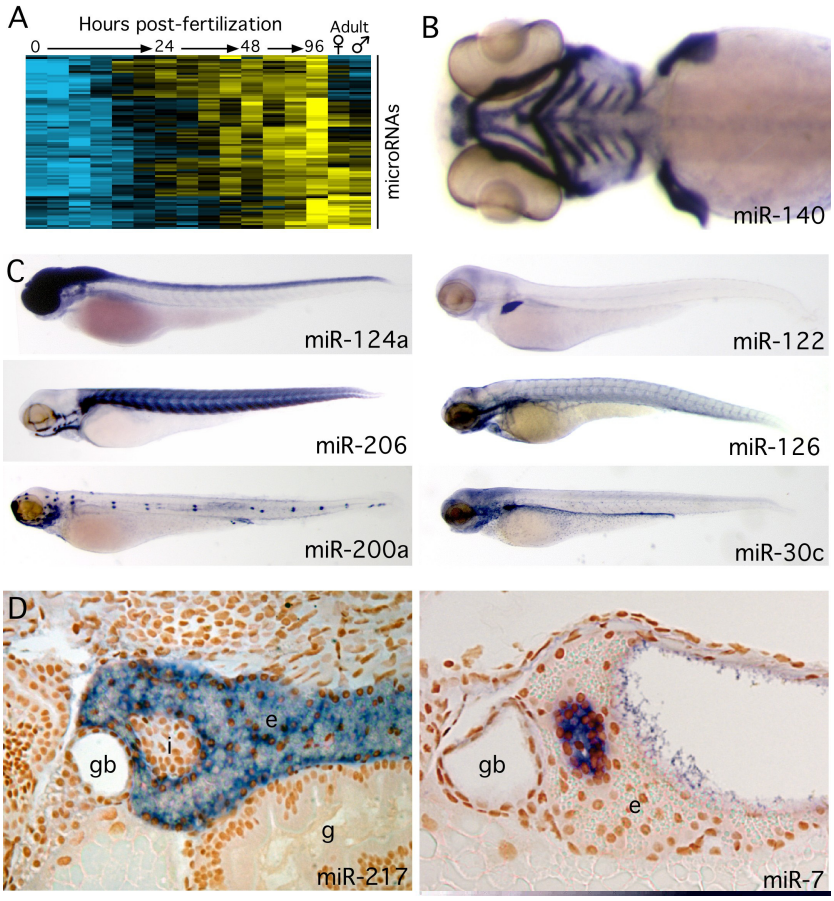

Figure 1. miRNA expression in zebrafish embryonic development. (A) Microarray expression levels of 90 (of the 115) miRNAs during embryonic development. Colors indicate relative and mean-centered expression for each miRNA: blue, low; black, mean; yellow, high. (B) Ventral view of *miR-140* whole-mount *in situ* expression in cartilage of pharyngeal arches, head skeleton and fins at 72 hours postfertilization (hpf). (C) Lateral views of miRNA whole-mount *in situ* expression in different organ systems at 72 hpf: *miR- 124a*, nervous systems; *miR-122a*, liver; *miR-206*, muscles; *miR-126*, blood vessels and heart; *miR-200a*, lateral line system and sensory organs; *miR-30c*, pronephros. (D) Histological analysis of miRNA *in situ* expression in the pancreas at 5 days post-fertilization. Abbreviations: e, exocrine pancreas; i, pancreatic islet; gb, gall bladder; g, gut.

mammals (Supplementary Figure 2). Up to 77% of the *in situ* expression patterns were confirmed by at least one of the microarray data sets. In addition, miRNA *in situ* data showed patterns that cannot easily be detected by microarrays. For example, some miRNAs were expressed in hair cells of sensory epithelia (Supplementary Figure 6).

In conclusion, we here describe the first comprehensive set of miRNA expression patterns in animal development. We found these patterns to be remarkably specific

and diverse, which suggests highly specific and diverse roles for miRNAs. Most miRNAs are expressed in a tissue-specific manner during segmentation and later stages but were not detected during early development. Although we cannot exclude a role for undetectable early miRNAs, this observation indicates that most miRNAs may not be essential for tissue fate establishment but rather play crucial roles in differentiation or the maintenance of tissue identity.

Materials and Methods

Animals

Zebrafish were kept under standard conditions (9). Embryos were staged according to (10). Wild-type zebrafish were used for northern blot analysis and microarray analysis. Homozygous albino embryos and larvae and progeny of a *dicer1 hu715*/+ (11) incross were used for the *in situ* hybridizations.

Identification of zebrafish miRNAs

Since the miRNA registry 5.0 (12) contained sequences for only 26 mature zebrafish miRNAs (corresponding to 30 precursor sequences), we designed a computational approach to identify additional zebrafish orthologs of known mammalian miRNAs. First, all human, mouse and rat precursor miRNA sequences from the miRNA registry 5.0 (12) were searched against the zebrafish genome assembly (Zv4) using the blastn program (13), and hits with lengths of at least 18 bp and identity of at least 16 bp were used as anchors for extracting relevant zebrafish genomic regions corresponding in length and position of the hit to the respective mammalian precursor miRNA sequence. Next, RNAfold software (14) was used to select only those regions that can form hairpin structures. As an additional filter, we used the Randfold program (15), which evaluates stability of the secondary structure relative to random sequences of the same nucleotide content. The regions with Randfold score of 0.005 or less were aligned with respective mammalian miRNA precursor sequences using CLUSTALW program, and average percentage identity was calculated for every alignment. In cases where several zebrafish regions were predicted for a given mammalian miRNA, the region with the highest alignment identity was considered as a true ortholog. Finally, redundancy in predictions (which occurred because of the use in the search of orthologous miRNAs from several species) was removed, and positions of mature miRNA sequences within predicted precursors were mapped by additional blast search. In this way we have identified 142 zebrafish miRNA regions corresponding to 126 unique mature miRNA sequences. 106 miRNAs that had no more than two mismatches were selected for further analyses. Of these, 23 had previously been identified in zebrafish (16), which validates this set. 74/106 (70%) matched perfectly and 100/106 (94%) had no more than one mismatch. In total we investigated the expression of 115 miRNAs. These included the 106 miRNAs we identified from the zebrafish genome, three other previously identified zebrafish miRNAs, four other miRNAs that showed expression when assayed using microarrays, and two recently identified miRNAs.

Microarray analysis

For the microarray analysis we used a microarray that was recently developed for the detection of mammalian miRNAs (17), containing probes for all the mammalian miRNAs currently known (miRNA registry 5.0). Since the microarray does not reliably discriminate between one or a few mismatches (17), we could use this array to study the expression of the zebrafish miRNAs. To perform the hybridizations, total RNA was isolated using trizol (Sigma) and size-selected for small RNAs in the range of mature miRNAs (18-26 nucleotides) by PAGE. Oligonucleotide microarrays were spotted on glass slides and hybridized as described previously (17). Briefly, oligonucleotides with complementary sequence to the miRNAs (miRNA registry 5.1 (12)) were modified with a free amino group linked to the 5' termini through a 6-carbon spacer (IDT) and were printed onto amine-binding slides (CodeLink, Amersham Biosciences). Printing and hybridization were done according to the protocols from the manufacturer of the slides with the following modifications: the oligonucleotide concentration for printing was 20 μM in 150 mM sodium phosphate, pH 8.5. Printing was done on a MicroGrid TAS II arrayer (BioRobotics) at 50% humidity. For each microarray experiment 10 μg of total RNA was used. For the developmental time-course, RNA was labeled using a reverse transcription and amplification protocol as described previously (17). For all other samples the labeling method was modified to a direct labeling procedure as follows: after sizeselection of the RNA, it was ligated to pCU-Cy3 using T4 RNA ligase. Ligation products were diluted 5 times in hybridization buffer and used directly for hybridization. Hybridization was done at 50°C for 10 hrs in 5X SSC, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. The hybridized arrays were scanned using an arrayWoRxe biochip reader (Applied Precision) and primary data were

analyzed using the Digital Genome System suite (Molecularware). Data for the developmental time-course is log transformed, mean centered and normalized. Data for the different tissues are mean centered and normalized. Hierarchical clustering was performed using CLUSTER 3.0/TreeView software (18). Only miRNAs that were detected in at least one experiment (20-fold over background) are shown. Primary microarray data is deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE2625-GSE2628.

LNA-modified DNA oligonucleotides
LNA is a high-affinity RNA analogue with a bicyclic furanose unit locked in an RNA mimicking sugar conformation (19), which results in unprecedented hybridization affinity towards complementary single-stranded RNA molecules. This makes LNA-modified DNA probes ideally suited for RNA targeting. The LNA probes were labeled with digoxigenin (DIG) using a DIG 3'- end labeling kit (Roche) and purified using Sephadex G25 MicroSpin columns (Amersham). For northern blot analysis and *in situ* hybridizations approximately 1-2 pmol of labeled probe was used.

Northern blot analysis
Total RNA was isolated from embryos at 48 hpf, 72 hpf and 5 day old embryos using trizol (Invitrogen). From each sample, 10 µg RNA was separated on 12.5% polyacrylamide gels and was blotted according to standard procedures. Blots were prehybridized in hybridization buffer (0.36M Na_2HPO_4, 0.14M NaH_2PO_4, 1mM EDTA, 7% SDS) for 30 min at 45°C and hybridized overnight in hybridization buffer containing 0.1 nM probe at 45°C. After stringent washes (2 times 30 min at 45°C in 2X SSC 0.1%SDS) blots were rinsed in washing buffer (0.1M maleic acid, 0.15M NaCl, 0.1% Tween, pH 7.5) and blocked in washing buffer containing 5% milk powder for 30 min at room temperature. Subsequently, blots were incubated with anti-DIG-AP antibody (Roche) in blocking buffer for 1 hour at room temperature, washed 3 times for 15 min in washing buffer and 2 times for 5 min with AP-buffer (0.1M Tris-HCl pH9.5, 50 mM $MgCl_2$, 0.1M NaCl, 0.1% Tween). Signal was detected using CDP-star chemiluminescent substrate (Roche).

Whole-mount in situ hybridizations
Whole-mount *in situ* hybridizations were performed essentially as described (21), with the following modifications: Hybridization, washing and incubation steps were done in 2.0 ml eppendorf tubes. All PBS and SSC solutions contained 0.1% Tween (PBST and SSCT). Embryos of 12, 16, 24, 48, 72 and 120 hpf were treated with proteinase K for 2, 5, 10, 30, 45 and 90 min, respectively. After proteinase K treatment and refixation with 4% paraformaldehyde, endogenous alkaline phosphatase activity was blocked by incubation of the embryos in 0.1 M ethanolamine and 2.5% acetic anhydride for 10 min, followed by extensive washing with PBST. Hybridizations were performed in 200 µl of hybridization mix. The temperature of hybridization and subsequent washing steps was adjusted to approximately 22°C below the predicted melting temperatures of the LNAmodified probes. Staining with NBT/BCIP was done overnight at 4°C. After staining, the embryos were fixed overnight in 4% paraformaldehyde. Next, embryos were dehydrated in an increasing methanol series and subsequently placed in a 2:1 mixture of benzyl benzoate and benzyl alcohol. Embryos were mounted on a hollow glass slide and covered with a coverslip.

Plastic sectioning
Embryos and larvae stained by whole-mount *in situ* hybridization were transferred from benzyl benzoate/benzyl alcohol to 100% methanol and incubated for 10 min. Specimens were washed twice with 100% ethanol for 10 min and incubated overnight in 100% Technovit 8100 infiltration solution (Kulzer) at 4ºC. Next, specimens were transferred to a mold and embedded overnight in Technovit 8100 embedding medium (Kulzer) deprived of air at 4ºC. Sections of 7 µm thickness were cut with a microtome (Reichert- Jung 2050), stretched on water and mounted on glass slides. Sections were dried overnight. Counterstaining was done by 0.05% neutral red for 12 sec, followed by extensive washing with water. Sections were preserved with Pertex and mounted under a coverslip.

Image acquisition
Embryos and larvae stained by whole-mount *in situ* hybridization were analyzed with Zeiss Axioplan and Leica MZFLIII microscopes and subsequently photographed with digital cameras. Sections were analyzed with a Nikon Eclipse E600 microscope and photographed with a digital camera (Nikon, DXM1200). A subset of images was adjusted for levels, brightness, contrast, hue and saturation with Adobe Photoshop 7.0 software to optimally visualize the expression patterns.

Acknowledgements

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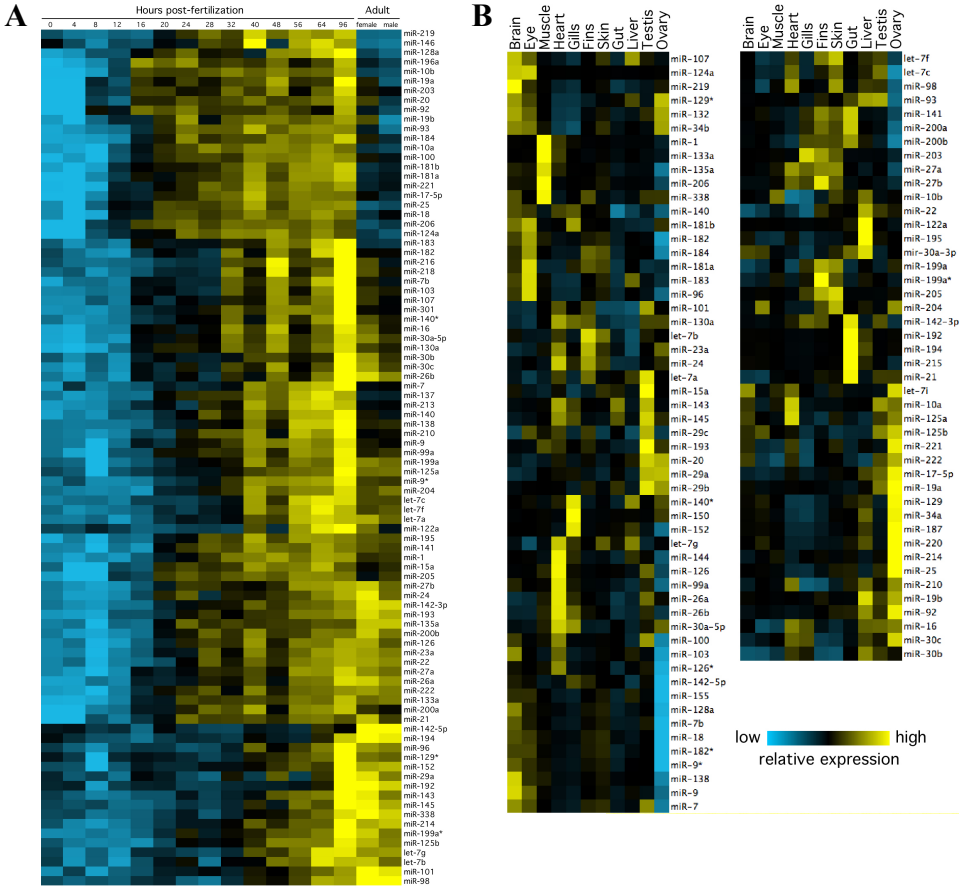
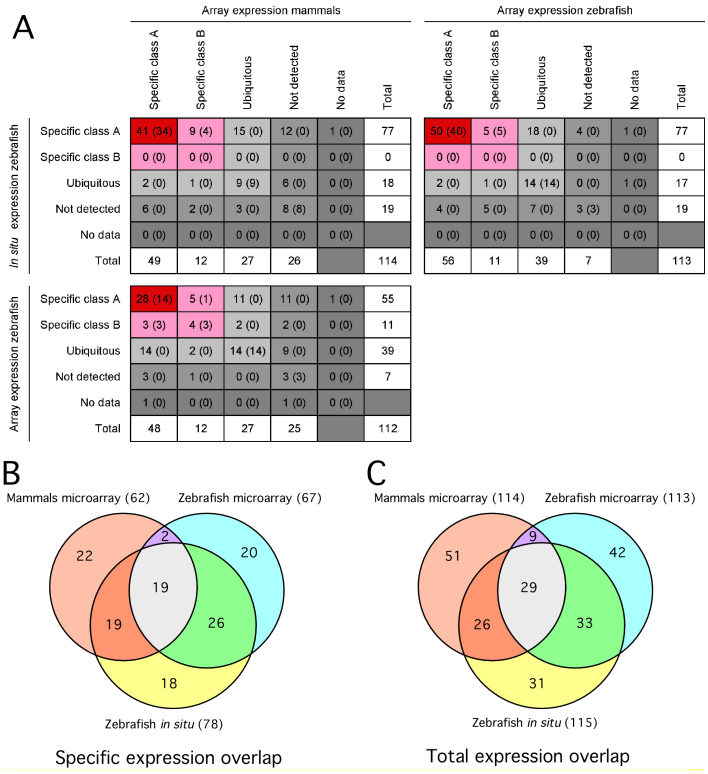
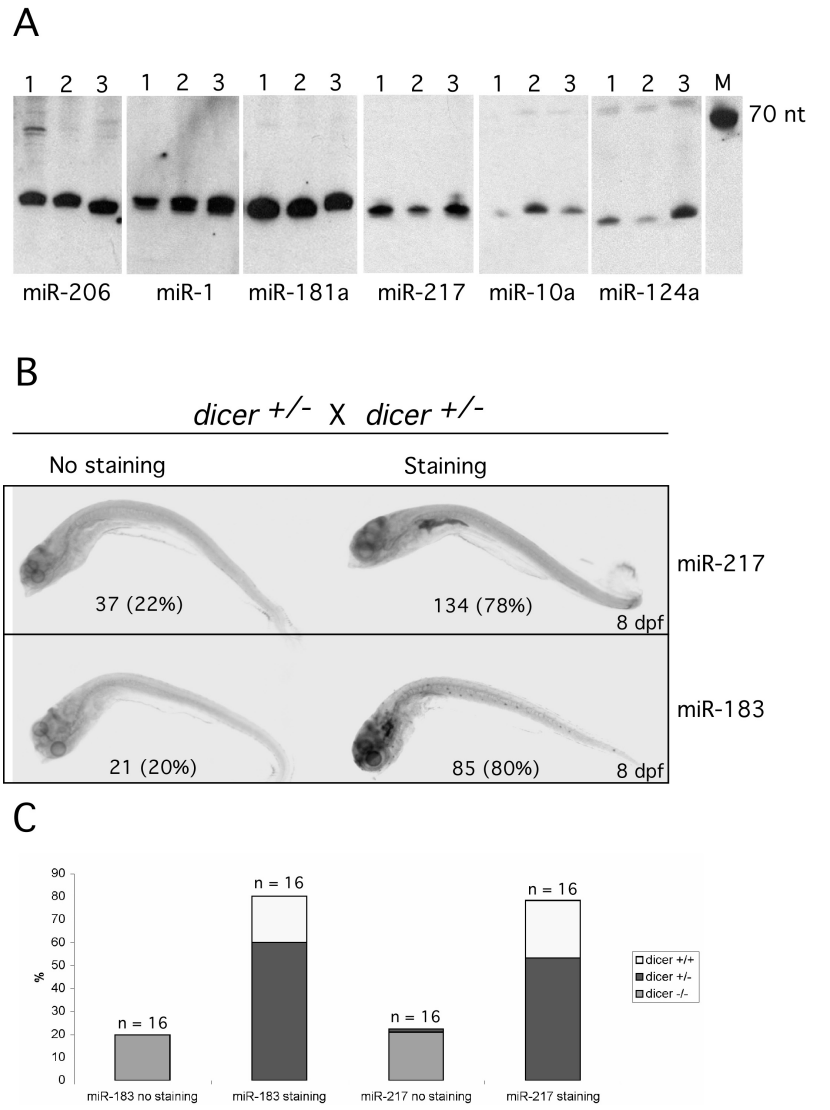
Supplementary data



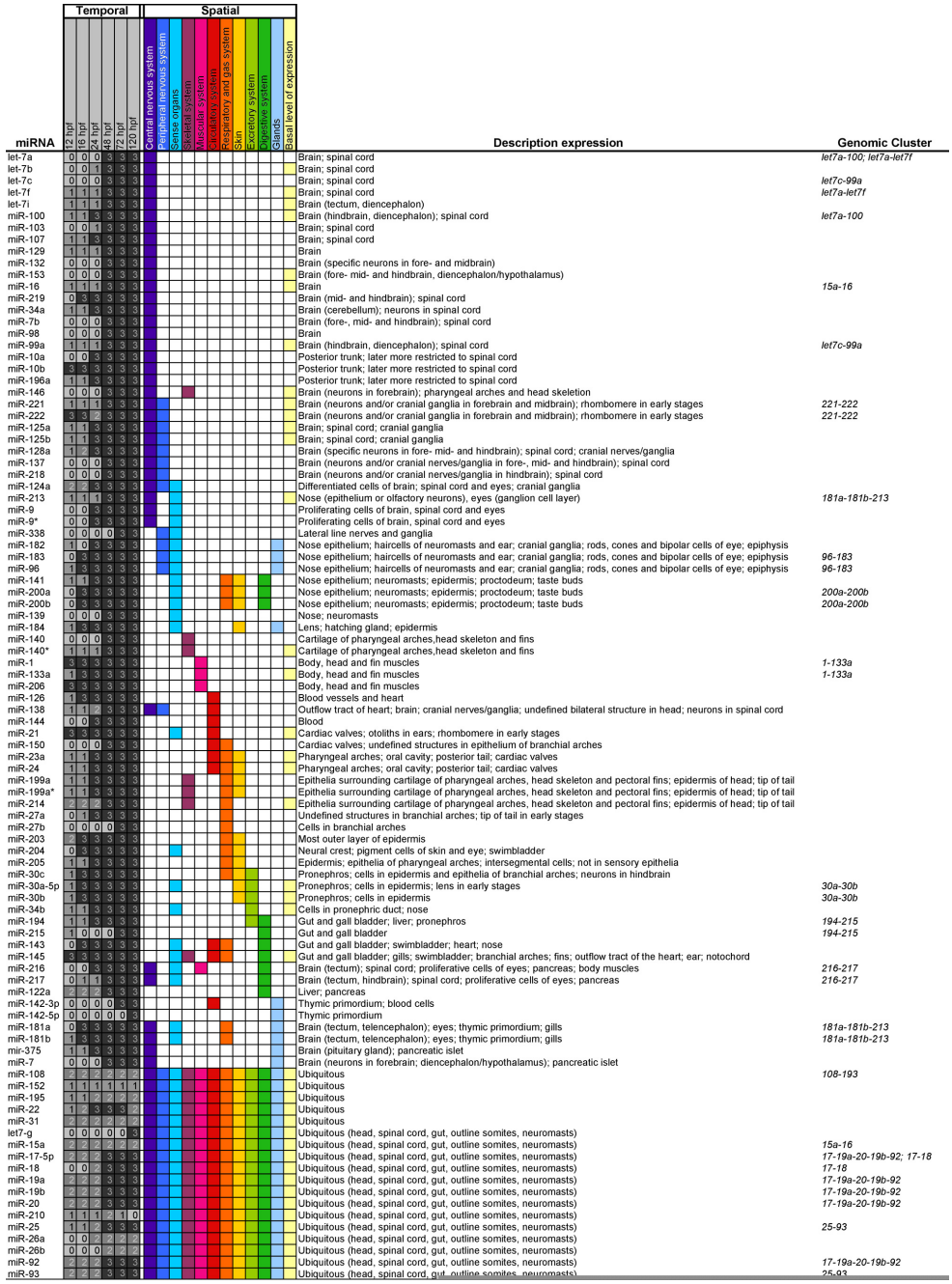
Supplementary Figure 1. Microarray expression profile of conserved miRNAs in zebrafish. (A) Relative expression levels of 90 miRNAs during embryonic development. Lanes 1-14 show levels at 0, 4, 8, 12, 16, 20, 24, 28, 32, 40, 48, 56, 64 and 96 hours post-fertilization and lanes 15 and 16 show levels of adult females and males. (B) Relative miRNA expression levels in different organs from adult zebrafish. Colors indicate relative expression compared to the mean for each miRNA: blue, low; black, mean; yellow, high. These profiles do not reflect the absolute expression levels, but rather represent mean-centered and normalized expression levels for each gene. Only miRNAs that were detected in at least one experiment of each panel (20-fold over background) are shown. Specific miRNA expression was found in several tissues, for example *miR-206* and *miR- 122a* were expressed almost exclusively in muscle and liver, respectively. There was also clustering of miRNA expression in two or more tissues: many miRNAs were expressed in the brain as well as the eyes, or are co-expressed in the gills, fins and skin. In total we found that 67 of 113 miRNAs that we could analyze by microarrays were tissue-specific. We compared 66 of these expression patterns with the predominant expression patterns of the same miRNAs in mammalian tissues reported by several microarray experiments (22-25). 21/66 (32%) of the tissue-specific expression patterns in zebrafish were similar in mammals (Supplementary Figure 2A and B). These included brainspecific expression (*miR-128a*, *miR-138*, *miR-9*), muscle-specific expression (*miR-1*, *miR-133a*, *miR-206*), liver-specific expression (*miR-122a*) and gut-specific expression (*miR-194*). This overlap is an underestimate, because not all the same tissues have been analyzed by microarrays in zebrafish and mammals.



Supplementary Figure 2. Overlap between predominant miRNA expression patterns in mammals and zebrafish. (A) Pair-wise comparisons between miRNA expression data from mammals (microarray and literature data) and miRNA expression data from zebrafish (microarray and *in situ* hybridization data). To facilitate the comparisons, the miRNAs were first grouped according to their expression: specific class A (highly specific expression), red; specific class B (marginal specific- and/or low absolute expression), pink; ubiquitous expression, light gray; no detectable expression, gray; no data, dark gray. In the different data sets, a given miRNA can fall into other expression groups. Therefore we made square matrices, which reflect overlap between miRNAs from the different expression groups within each data set. The numbers of miRNAs belonging to each of these combinations of expression groups are shown. Next, the corresponding miRNA expression patterns were compared. The numbers of miRNAs with overlap in the expression patterns are denoted in brackets. miRNAs for which there was no data in either of the data sets were excluded from the comparison. miRNAs in specific class A and B were both classified as specific and therefore were both used for the calculations of the overlaps in specific expression patterns. (B) Three-way overlap between the specific (class A and B) miRNA expression of microarray data in mammals and zebrafish and *in situ* data in zebrafish. (C) Three-way overlap between the total miRNA expression patterns of microarray data in mammals and zebrafish and *in situ* data in zebrafish. Note that four miRNAs had overlap between *in situ* hybridizations and both microarrays in mammals and zebrafish but did not have overlap between the microarrays. Forty-five miRNAs had overlap in specific expression patterns between the microarray data for zebrafish and the *in situ* hybridization data for zebrafish (A, B). Thus, the *in situ* hybridizations confirmed 67% of the 68 specific microarray expression patterns in zebrafish. The overlap between microarray data for mammals and our *in situ* data for zebrafish was similar. Of the 61 miRNAs with predominant microarray expressions in mammals, 38 (62%) had overlap with the *in situ* hybridization data. Conversely, 45 (58%) and 38 (47%) of the 78 miRNAs with specific *in situ* patterns had overlap with the microarray expression patterns in zebrafish and mammals, respectively. Interestingly, 60 (77%) of the 78 miRNAs with specific *in situ* patterns had overlap with either the microarray data for zebrafish or for mammals (A, B). In addition, overlap between non-specific patterns (ubiquitous and no detectable expression) was also observed (B, C). Thus, in total a large fraction of the *in situ* patterns are consistent with microarray data for mammals or zebrafish.

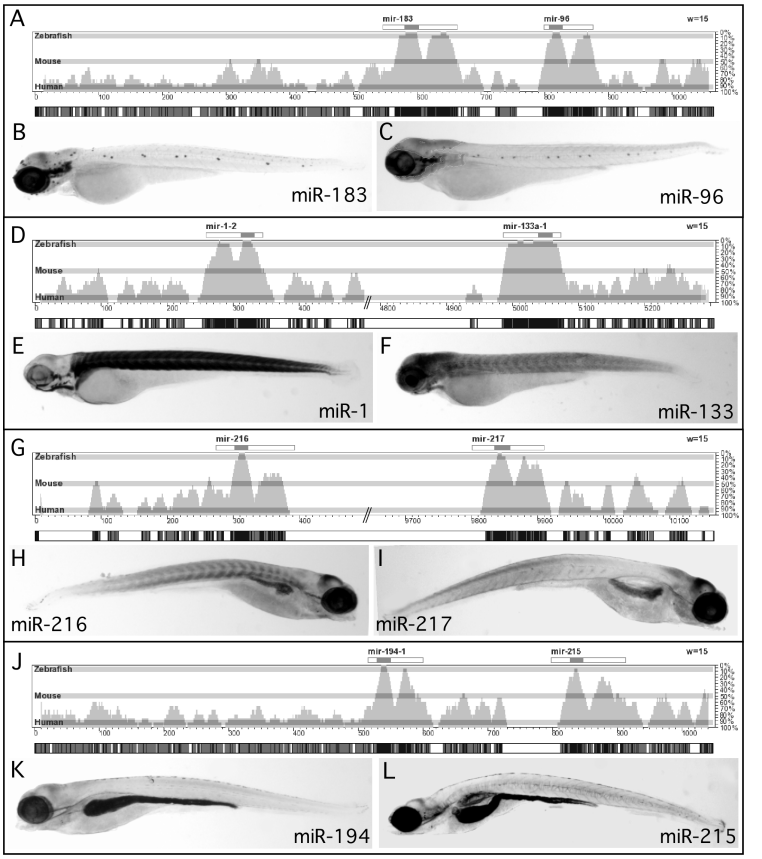


Supplementary Figure 3. miRNA detection in zebrafish using digoxigenin-labeled LNA-modified probes. (A). Northern blot analysis of six different miRNAs at 48 hpf (lane 1), 72 hpf (lane 2) and 5 days post-fertilization (lane 3). M, 70 nt marker. (B) Whole-mount *in situ* hybridizations on progeny of a *dicerhu715*/+ incross at 8 days post-fertilization. Total number of embryos (5 and 8 days post-fertilization) that show staining or no staining for each miRNA is indicated. See Supplementary Figure 4 for description of the expression patterns. (C) Genotypes of embryos in (B). From each class 16 embryos were genotyped. In the 'no staining' classes 31 *dicer*-/- embryos and one *dicer*+/- embryo were detected. In the 'staining' classes only *dicer*+/- embryos or wild-type embryos were detected. This indicates that mature miRNAs cannot be detected in *dicer*-/- embryos and suggest that we specifically detect mature miRNAs in wild-type embryos.

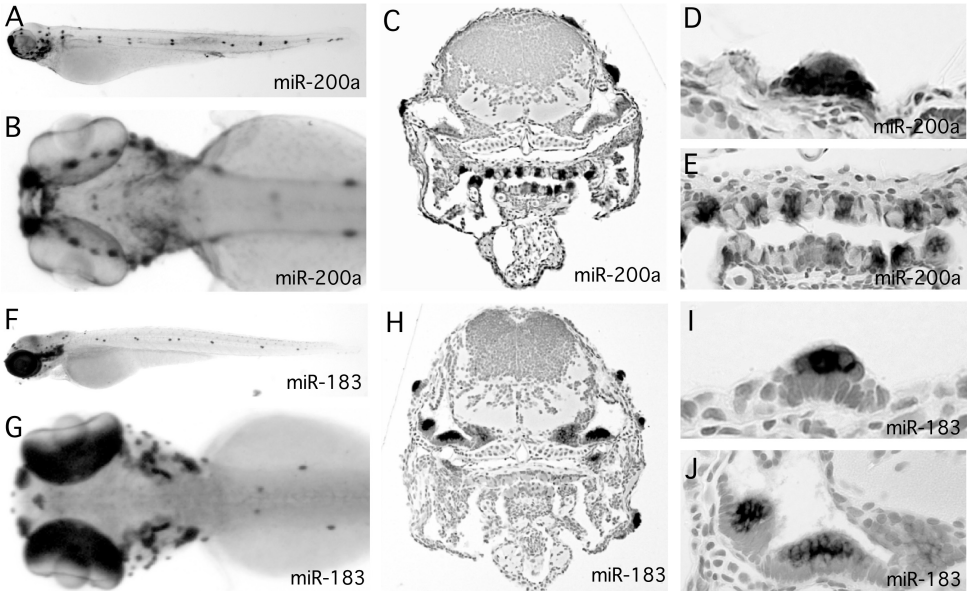


Supplementary Figure 4. Catalogue of miRNA expression patterns in zebrafish embryos. Expression patterns were determined by whole-mount *in situ* hybridizations using LNA-modified probes (20). To determine the miRNA expression patterns more precisely at cellular level, some embryos were sectioned. Expression was determined in embryos at 12, 16, 24,

48 and 72 hours post-fertilization (hpf) and in five-day old larvae (120 hpf). Temporal expression, spatial expression in 11 different organ systems and basal level of expression in all organs is represented graphically. miRNAs that did not show expression were excluded from the figure. Temporal expression numbers indicate: 0, no expression; 1 background staining or weak ubiquitous expression; 2, ubiquitous expression; 3, specific expression in one or more organs systems. Some miRNAs in the latter class were expressed in many organs and therefore these miRNAs were finally annotated as ubiquitously expressed. Genomic miRNA clusters within 10,000 base-pairs are indicated. Note that some miRNAs are encoded by more than one gene, which can be in different clusters. Pictures were mainly taken from embryos at 72 hpf. If additional structures were visible at earlier or later stages, pictures were also taken from these stages.

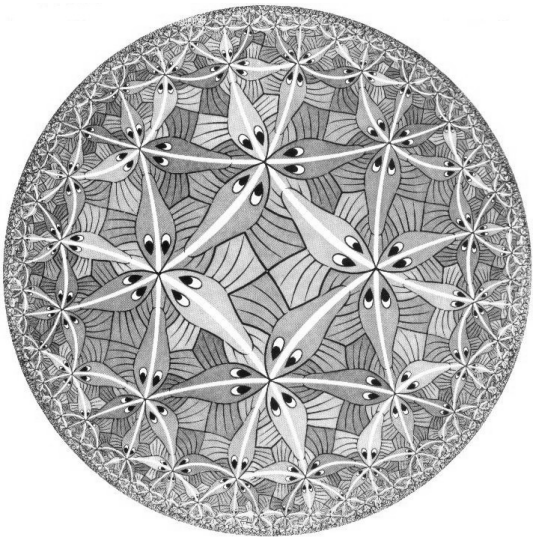


Supplementary Figure 5. Examples of miRNAs in genomic clusters. (A, D, G, J) VISTA-like plots of human/mouse/zebrafish comparisons of the genomic regions containing clustered miRNAs. Pre-miRNAs and mature miRNAs are indicated as open and filled boxes at the top of each figure, respectively. Divergence for each position is calculated in a 15- nucleotide window and plotted graphically. (B, C, E, F) Lateral views of miRNA expression in embryos at 72 hpf. (H, I, K, L) Lateral views of miRNA expression in 5-day old larvae. Many of these clusters consist of miRNAs from the same family, and in some cases we cannot exclude the possibility that the probes cross-hybridized. However, some clusters consist of unrelated miRNAs that nonetheless had similar expression patterns. We also observed differences between clustered miRNAs. For example, *miR-216* and *miR-217* were both expressed in the pancreas, but *miR-216* was also expressed in the muscles. This indicates that *miR-216* expression might be under additional control or that there are additional, yet unidentified, copies of *miR-216* in the genome that are differentially expressed.



Supplementary Figure 6. miRNA expression in sensory epithelia. (A, B) In whole-mount embryos *miR- 200a* was expressed in the nose, neuromasts of the lateral line, epithelium of the lips, mouth cavity and branchial arches. (C, D, E) On sections *miR-200a* was expressed in (C, D) hair- and supporting cells of the neuromasts and in (C, E) taste buds. (F, G) In whole mount embryos *miR-183* was expressed in the nose, neuromasts of the lateral line, ear, eye (rods, cones and bipolar cells), cranial ganglia and epiphysis. (H, I, J) On sections *miR-200a* was expressed in (H, I) hair cells of the neuromasts and in (H, J) sensory epithelia of the ear. (A, F) lateral views of embryos at 72 hpf. (B, G) Dorsal views of embryos shown in (A, F), respectively. (C, H) Cross sections through the head, at the position of the ear, of five-day old larvae. (D, E) Higher magnifications of image shown in (C). (I, J) Higher magnifications of image shown in (H). *miR-200a* apparently was expressed in sensory epithelial structures that can sense chemicals, and *miR-183* was expressed in sensory epithelia that can sense light or vibrations. Thus, all of the sensory epithelial structures present in zebrafish had specific miRNAs expressed.

CHAPTER 5



Adapted from Nucleic Acids Research 34, 2558 (2006)

Cloning and expression of new microRNAs from zebrafish

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Abstract

MicroRNAs (miRNAs) play an important role in development and regulate the expression of many animal genes by post-transcriptional gene silencing. Here we describe the cloning and expression of new miRNAs from zebrafish. By high-throughput sequencing of small-RNA cDNA libraries from 5-day-old zebrafish larvae and adult zebrafish brain we found 139 known miRNAs and 66 new miRNAs. For 65 known miRNAs and for 11 new miRNAs we also cloned the miRNA star sequence. We analyzed the temporal and spatial expression patterns for 35 new miRNAs and for 32 known miRNAs in the zebrafish by whole mount *in situ* hybridization and northern blotting. Overall, 23 of the 35 new miRNAs and 30 of the 32 known miRNAs could be detected. We found that most miRNAs were expressed during later stages of development. Some were expressed ubiquitously, but many of the miRNAs were expressed in a tissue-specific manner. Most newly discovered miRNAs have low expression levels and are less conserved in other vertebrate species. Our cloning and expression analysis indicates that most abundant and conserved miRNAs in zebrafish are now known.

Introduction

Over the past few years it has become clear that the expression of many genes is extensively controlled at the post-transcriptional level by microRNAs (miRNAs) (1, 2). Although miRNAs were initially recognized as an oddity specific to developmental switches in *Caenorhabditis elegans* (3, 4), the cloning and computational prediction of hundreds of miRNAs in both animals and plants uncovered a whole new layer of gene regulation (5). It appears now that a mammalian genome may contain >500 genes encoding miRNAs (6, 7).

miRNAs are transcribed as long RNA polymerase II transcripts (8, 9) which fold into characteristic stem–loop structures. Once cleaved by the nuclear enzyme Drosha (10), a smaller precursor miRNA is transported to the cytoplasm (11, 12), where the Dicer protein mediates maturation of the miRNA into a 20–23 nt species (13-16), a process which is coupled to loading into a miRNP complex. These small-RNA molecules bind to the 3'-untranslated region of mRNAs by partial basepairing, which primarily results in inhibition of mRNA translation (17). mRNAs that are repressed by miRNAs are localized in cytoplasmic foci called P-bodies (18-20).

While plant miRNAs usually bind with perfect complementarity to their target mRNA and induce mRNA degradation (21), animal miRNAs in most cases regulate a mRNA containing a sequence complementary to the 7 nt seed of the miRNA (nt 1–7 or 2–8) (22-24). Computational predictions indicate that thousands of genes might be regulated by miRNAs and that the average number of genes that is targeted by a miRNA is 200 (25, 26). Many miRNA target sites are evolutionarily conserved and the mRNAs that bear conserved target sites are expressed at lower levels in the tissue where the miRNA is expressed compared with the tissues where the miRNA is not expressed (27, 28). In addition, the mRNAs with conserved target sites are often expressed in developmental stages prior to miRNA expression (27).

The important role of miRNAs in animal development has been shown by several approaches. Removal of all miRNAs results in developmental arrest in mouse and fish (29-31). Several other studies have revealed the details of processes where miRNAs act. For example, the *miR-1* knockout in *Drosophila* results in aberrant muscle growth (32), while in mouse, *miR-1* regulates the transcription factor Hand2 during heart development (33). In addition, miRNAs may regulate major signaling pathways like the notch signaling pathway in *Drosophila* (34). In mouse and chick, *miR-196* expression regulates the expression of Sonic hedgehog through targeting the transcription factor Hoxb8 (35).

In zebrafish there are currently 369 miRNA genes expressing 168 different miRNAs (5). Many of the conserved miRNAs have a striking organ-specific expression pattern in zebrafish and are mostly expressed at later stages of development (36). Also in *Drosophila*, miRNAs exhibit diverse spatial expression patterns during embryonic development as indicated by the analysis of the expression of primary

miRNA transcripts (37). Strikingly, the expression patterns of some highly conserved miRNAs like *miR-1* and *miR-124* are similar in flies, fish and mouse, suggesting ancient roles in tissue development (37, 38).
Encouraged by recent studies which indicate that there are many more miRNAs than currently known (6, 7), we attempted to find new miRNAs in the zebrafish by sequencing small-RNA cDNA libraries made from 5-day-old zebrafish larvae and adult zebrafish brain. We found 139 known miRNAs and 66 new miRNAs in the zebrafish. In addition, we studied the temporal and spatial expression of miRNAs with unknown expression from three sources: 32 miRNAs predicted or cloned previously from zebrafish (7, 39), 34 miRNAs cloned in this study and one miRNA cloned from human (E. Berezikov, R. H. A. Plasterk and E. Cuppen, unpublished data). We used locked nucleic acid (LNA) probes to detect these 67 miRNAs *in situ* in the zebrafish embryo and on northern blots with total RNA from different developmental stages and adult tissues. In contrast to our previous *in situ* hybridization screen for conserved vertebrate miRNAs (36), we could only detect miRNA expression *in situ* for a subset of 28 miRNAs. For 53 miRNAs we could detect a 22 nt species on northern blots. The remainder of 14 miRNAs could not be detected by *in situ* hybridization or northern blotting, although 13 of these were cloned in this study and passed our computational analysis. These might thus represent low abundant miRNAs or miRNAs expressed only in a few cells.
Our data show that there are many more miRNAs in zebrafish than so far described. They also demonstrate that next to the highly abundant and tissue-specific miRNAs, most of which are conserved (36), there is a large set of miRNAs expressed at much lower levels, many of which are less conserved.

Results

Cloning of new miRNAs from zebrafish

Recently, miRNAs were profiled in zebrafish by cloning small-RNAs from different developmental stages and zebrafish cell lines (39). In addition to the miRNAs already known from other organisms, several new miRNAs were found. The total number of zebrafish miRNA genes in the miRNA registry is currently 369, corresponding to 168 different miRNAs (5). Computational identification, verification and cloning of miRNAs by our group and others has shown that the number of miRNAs in humans might be much higher and extend towards a thousand (E. Berezikov, R. H. A. Plasterk and E. Cuppen, unpublished data) (6, 7).
In order to find more miRNAs in the zebrafish we performed sequencing of two newly generated small-RNA libraries derived from 5-day-old zebrafish larvae (zf-larvae) and adult zebrafish brain (zf-brain). These two sample types were chosen based on our previous *in situ* expression analysis of conserved vertebrate miRNAs in the zebrafish embryo, which revealed that strongest expression for most miRNAs

is observed in later stages of development and that one-third of this set of miRNAs was found to be expressed in the zebrafish embryonic brain (36).
For each library 12 288 individual clones were sequenced. Of these 12 288 sequence reads 2182 from the zf-larvae library and 1231 reads from the zf-brain library were too short to be analyzed. The remaining sequence reads were selected based on the presence of a 3' poly(A)-tail and a 5' adapter sequence, both of which resulted from the cloning process. Clones containing inserts shorter than 18 bp were also removed from the dataset. The clones that fulfilled these criteria were analyzed using a computational pipeline that includes RNA secondary structure. Based on this analysis, 13 094 sequences could be annotated as miRNAs, representing 205 distinct miRNAs. From these small-RNA sequences, 3,150 (zf-larvae) and 9,607 (zf-brain) could be annotated as known miRNAs from zebrafish. In total, we found 139 out of 168 known zebrafish miRNAs and 126 of these were found in both the zf-brain library and the zf-larvae library (Supplementary Figure S1 and Supplementary Table 1). For 65 of the known miRNAs, we sequenced clones from both the 3p- and 5p-arm of the miRNA hairpin, i.e. both the miRNAand the miRNA star sequence were found. All of the known miRNAs that we found were represented by multiple clones in the library (Figure 1). In addition, 2.6% of the clones represent 66 novel miRNAs (zf-larvae, 121 clones and zf-brain, 216 clones). These clones could be assigned to 116 different hairpins in the zebrafish genome, thus representing 116 potential new miRNA genes. For 11 of the new miRNAs, clones were sequenced from both the 5p- and the 3p-arm. Together, this limits the total number of unique miRNA hairpins to 66. Of the new miRNAs 37 were found only once in one of both libraries. The overlap between the zf-larvae and zf-brain libraries for new miRNAs was 19 (Supplementary Figure S1). Although the majority of the known miRNAs have a clear homolog in other vertebrate species, many of the newly identified miRNAs are less conserved (Figure 1). The first nucleotide of the newly identified miRNAs is most often a U (50%), although this bias was less strong than for known miRNAs (72%) (25).
In total, we found 66 novel miRNAs from zebrafish and these represent a new set of less conserved miRNAs.

In situ hybridization analysis of the spatial expression of known and new miRNAs in the zebrafish

To determine the spatial and temporal expression of known and new miRNAs during zebrafish development, we performed *in situ* hybridization and northern blotting using LNA probes (Tables 1 and 2). In total we analyzed the expression of 67 miRNAs, derived from three sources: 34 miRNAs cloned from the zf-larvae and the zf-brain libraries, one miRNA cloned from human (E. Berezikov, R. H. A. Plasterk and E. Cuppen. unpublished results) and 32 miRNAs that were found previously (7, 39). Of

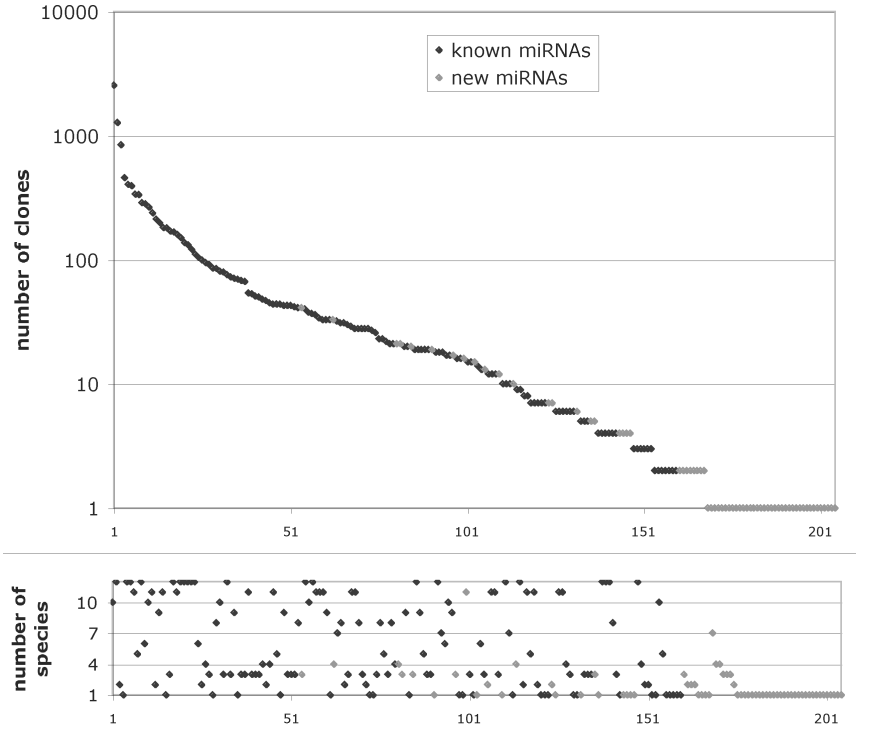

Figure 1. Cloning frequency and conservation for all miRNAs cloned from zebrafish small-RNA cDNA libraries. The upper panel depicts the cloning frequency for all small RNAs that were found in the two libraries and that passed our computational pipeline. All 139 known miRNAs (black data points) were cloned more than once, while 37 out of the 66 new miRNAs (grey dots) were represented by a single sequenced clone. The lower panel shows a scatter plot of the conservation of known (black dots) and new (grey data dots) miRNAs in 12 vertebrate species (zebrafish, fugu, tetraodon, mouse, rat, human, dog, macaca, opossum, chicken, chimpanzee, cow). Forty-four of the new miRNAs were only found in zebrafish, while most of the known miRNAs were found in several species according to our conservation criteria.

the latter set, six were already in our previous *in situ* screen (36) (*miR-130a, miR-187, miR-101b miR-135, miR-193b, miR-301b*), but the sequences of these probes turned out to contain some mismatches compared with the miRNA sequences cloned by Chen *et al*. (39), so that we decided to test new and correct probes.

First, we analyzed the expression of all 67 miRNAs by *in situ* hybridization on different zebrafish embryonic stages. Only a subset of 28 miRNAs could be detected *in situ* (Figure 2, Tables 1 and 2). For many of the miRNAs, the expression was restricted to specific tissues or cell types, although overall we observed less tissue-restricted expression as in our previous study for the conserved vertebrate miRNA set (Figure 2, Tables 1 and 2). Several miRNAs were expressed in (parts of) the brain (e.g. *miR-92b, miR-500a/b* and *miR-135*) and these have unique patterns as

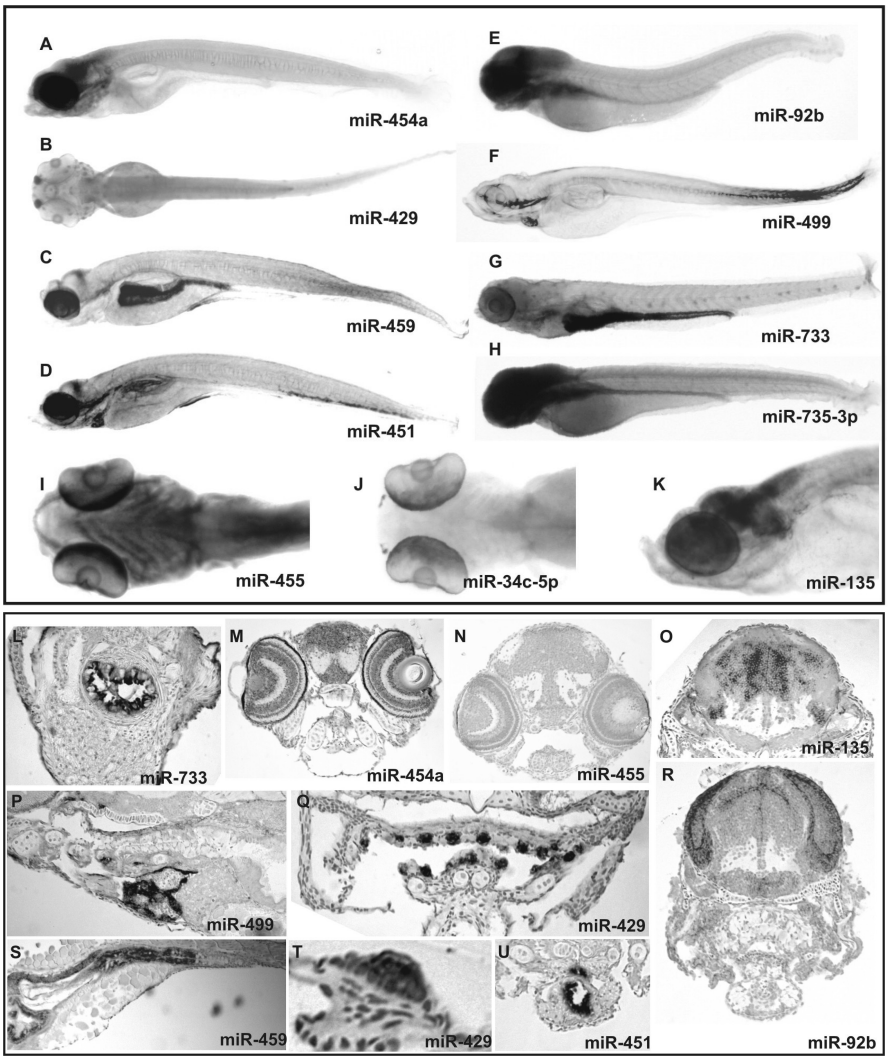

Figure 2. Examples of expression patterns of miRNAs in the zebrafish embryo as revealed by whole mount *in situ* hybridization. Upper panel, whole mount pictures of 72-h-old and 5-day-old larvae. Lower panel, pictures of sections from 5-day-old larvae. (A and M) *miR-454a* is expressed in the brain, the pharyngeal arches and the jaw and the eye; (B, Q and T) *miR-429* is expressed in the hair and supporting cells of the lateral line organ (T), the taste buds (Q), the nose and the epithelium of the lips; (C and S) *miR-459* is expressed in the anterior gut; (D and U) *miR-451* is expressed in the blood cells; (E and R) *miR-92b* is expressed in the proliferating zones of the preoptic region, optic tectum, tegmentum, telencephalon and octaval area; (F and P) *miR-499* is expressed in the ventricle and atrium, the muscles of the head and the somitic muscles; (G and L) *miR-733* is expressed ubiquitously but primarily in the intestine; (H) *miR-735-3p* is expressed ubiquitously, but primarily in the central nervous system; (I and N) *miR-455* is expressed in the cartilage of the pharyngeal arches and head skeleton; (J) *miR-34c-5p* is expressed in the nose; (K and O) *miR-135* is expressed in the pallium, optic ganglion, optic tectum, ventral telencephalon and at the beginning of the medulla oblongata.

revealed by sectioning of the embryos (Figure 2 lower panel). Other examples of tissue-specific expression are *miR-451*, which is expressed in the blood cells, *miR-455*, which is expressed in the cartilage of the pharyngeal arches and head skeleton and *miR-459*, which is only expressed in the anterior part of the gut.
The expression of several miRNAs was ubiquitous in the later stages of development (*miR-130a/b/c* and *miR-301b*), whereas all members from the *miR-430* family of miRNAs were expressed ubiquitously only in the earlier stages up to 48 h of development, as described previously (30).
Of the 35 new miRNAs analyzed in this study, we could only detect four by *in situ* hybridization, which probably reflects the low abundance of these new miRNA as already indicated by the low cloning frequency. *miR-34c-5p* was expressed in the nose; *miR-499* was expressed in the heart, the somitic muscles and the muscles of the head; *miR-735-3p* was expressed ubiquitously but primarily in the central nervous system; *miR-733* was also expressed ubiquitously but more strongly in the gut and the cells surrounding the yolk.

Northern blot analysis of known miRNAs

As outlined above, we could observe a clear *in situ* expression pattern in the embryo for only 28 of the 67 miRNAs analyzed. We next went on analyzing the expression of all miRNAs by northern blot analysis, since this is a more sensitive method for detecting miRNAs, and it also determines the length of the RNA species, providing further evidence for the existence of a bonafide miRNA. Furthermore, the *in situ* hybridization analysis is only restricted to embryonic stages of development, whereas northern blot analysis enabled us to detect miRNAs in RNA derived from adult zebrafish tissues.
We first analyzed the temporal expression in five developmental stages ranging from 24 h to adult fish for the set of 32 miRNAs that was already cloned or predicted previously (Figure 3A and Table 1). While some miRNAs could mainly be detected in RNA from adult zebrafish (*miR-202, miR-460*), others were expressed at all time points analyzed (*miR-130a/b/c*) and some were also expressed mainly in embryonic stages, but at much lower levels in the adult fish (*miR-363* and *miR-301b*). Again, all members of the *miR-430* miRNA family were expressed abundantly in the early embryonic stages up to 72 h, but were absent in RNA from 5-day-old larvae and adult fish.
Next, we compared the *in situ* patterns of miRNAs in the embryo with the spatial expression in the adult zebrafish (Figure 3B and Table 1). For the majority of miRNAs, the expression in RNA derived from adult tissues is similar to the expression in the embryo. For example, *miR-135* is specific for the embryonic and the adult brain. Similarly, *miR-459* is expressed in the embryonic anterior gut and in the adult fish it is expressed exclusively in the gut. However, for some miRNAs the expression in

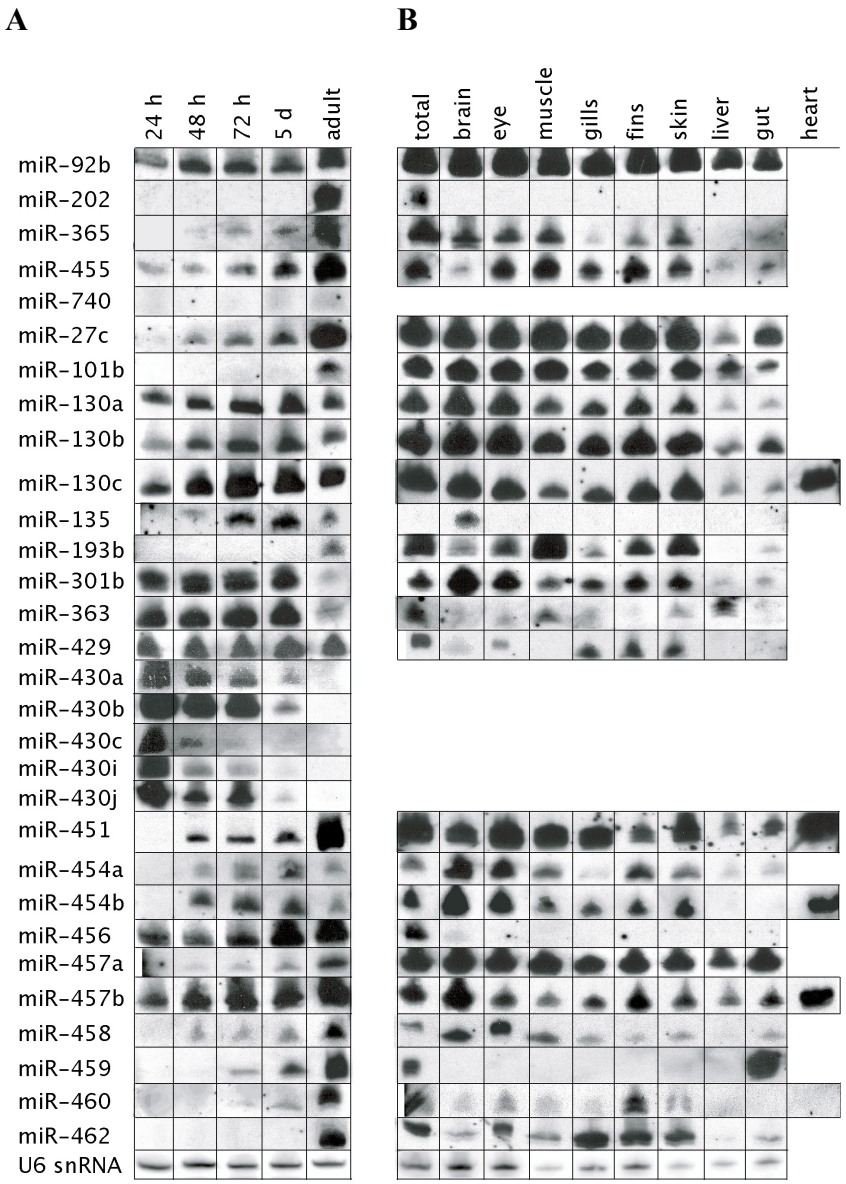

Figure 3. Northern blot analysis of the expression of known miRNAs from zebrafish. (A) Expression of miRNAs in five developmental stages: 24 h, 48 h, 72 h, 5 days and adult zebrafish. (B) Expression of miRNAs in RNA derived from 10 adult zebrafish tissues: total, brain, eye, muscle, gills, fins, skin, liver, gut and heart. For some miRNAs we did not analyze the expression in the heart, because we could not obtain enough heart tissue for analyzing all the miRNAs by northern blotting. U6 snRNA serves as a loading control.

the embryo was different compared with the expression in the adult, e.g. *miR-455* is expressed in the cartilage of the embryo (Figure 2), but the expression in the adult is in many tissues. Overall, on northern blots we could detect 30 out of the 32 known miRNAs analyzed.

Northern blot analysis of newly cloned miRNAs

Although we were able to obtain *in situ* expression patterns for the majority of known miRNAs, we could only detect 4 out of the 35 novel miRNAs that were cloned in this study (Figure 2 and Table 2). We next went on analyzing the expression of these 35 newly identified miRNAs on northern blots using the same LNA probes as for *in situ* hybridization (Figure 4). First, we scanned the whole set of 35 miRNAs for presence in large quantities of total RNA (15 µg) from 24-h-old embryos and adult zebrafish (data not shown). For all miRNAs that gave a positive signal, we performed new northern blots with total RNA from different developmental stages and with total RNA from 10 dissected tissues from adult zebrafish. In total, we could detect 16 miRNAs on time series northern blots (Figure 4A). As for the known miRNAs analyzed in Figure 3A, some miRNAs were expressed throughout development (*miR-15c* and *miR-736*) and others were expressed only in the adult (*miR-731*). Another set of the new miRNAs was most abundant in 5-day-old larvae, while there was a strong drop in expression in the adult zebrafish (*miR-726-3p*, *miR-729*, *miR-728* and *miR-190b*). Except for *miR-726-3p*, these have all been cloned primarily from the 5-day-old larvae library. An additional three miRNAs could be detected on northern blots containing RNA from dissected tissues (Figure 4B). For most miRNAs we could only detect an RNA species corresponding to the length of miRNAs, but for two we saw some additional bands corresponding to larger RNA molecules (*miR-735-3p* and *miR-733*), which is probably background.

There was a wide variety in expression in RNA from dissected tissues. For example, *miR-499* is specific for the heart and the muscles. However, many newly cloned miRNAs were expressed in the adult brain (*miR-739, miR-34c-5p, miR-728, miR-723-3p, miR-737-3p, miR-727-5p*). Encouraged by this finding, we analyzed the expression of the miRNAs, which we did not initially detect in total RNA samples from 24-h-old embryos or adult fish, in RNA from adult fish brain. By doing this, we could detect four more miRNAs that were expressed in the adult brain sample, whereas hardly any signal was detected in the total RNA sample (Figure 4C). Thus, by increasing the amount of RNA on the northern blot and by looking in a specific tissue, we enriched enough to detect some additional new miRNAs. In total, we could detect 23 out of 35 newly cloned miRNAs by northern blotting.

To quantify the differential expression level of known and new miRNAs, we performed parallel northern blots using a dilution series of total adult fish RNA and an adult brain RNA sample. We probed these for two miRNAs that are easily detectable,

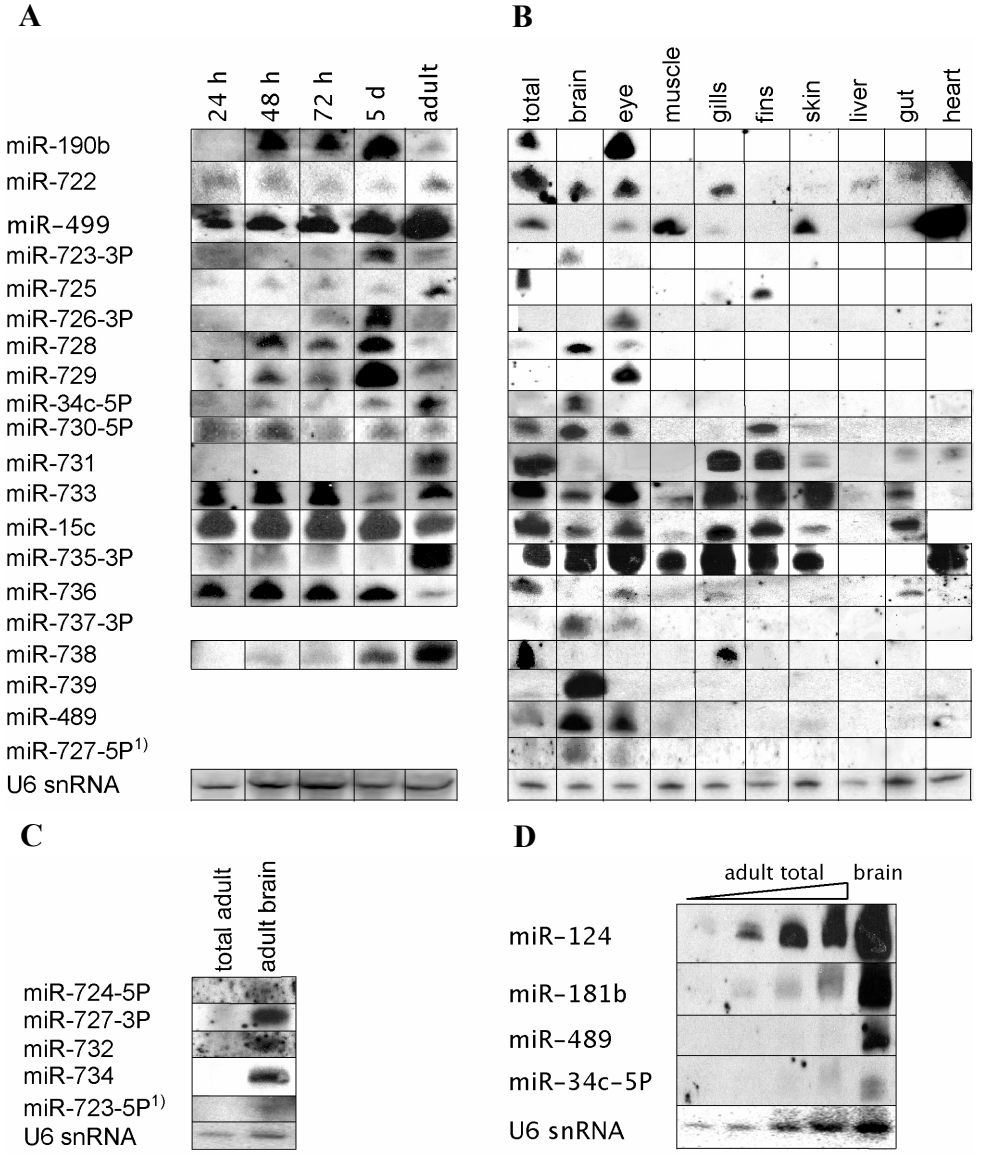

Figure 4. Northern blot analysis of newly cloned miRNAs from zebrafish. (A) Expression of miRNAs detected in five developmental stages: 24 h, 48 h, 72 h, 5 days and adult zebrafish. (B) Expression of miRNAs in 10 different tissues from adult zebrafish: total, brain, eye, muscle, gills, fins, skin, liver, gut and heart. (C) miRNAs detected by specifically probing RNA from adult fish brain. (D) Dilution series of adult fish RNA and one adult brain RNA sample. Blots were probed for two abundant and brain-specific miRNAs (*miR-124* and *miR-181b*) and two new and brain specific miRNAs cloned in this study (*miR-489* and *miR-34c-5p*). U6 snRNA serves as a loading control. 1), regarded as the miRNA star sequence.

Table 1. Overview of expression data for known miRNAs

miRNA	expression time[1]	expression adult tissue[1]	expression in situ	temporal expression in situ					
				12h	16h	24h	48h	72h	5d
dre-mir-101b	adult	ubiquitous	no expression	0	0	0	0	0	0
dre-miR-130a	24h-adult	ubiquitous	head (gills, brain, jaw)	0	0	1	3	3	3
dre-miR-130b	24h-adult	ubiquitous	ubiquitous	0	0	1	3	3	3
dre-miR-130c	24h-adult	ubiquitous	head (gills, brain, jaw)	0	0	1	3	3	3
dre-miR-135	48h-adult	brain	structures in brain	0	0	0	3	3	3
dre-miR-187	not expressed	N.D.	no expression	0	0	0	0	0	0
dre-mir-193b	adult	stronger in muscle, fins, skin	brain, jaw	0	0	0	0	3	3
dre-mir-202	24h-adult	not in any of the analyzed tissues	no expression	0	0	0	0	0	0
dre-mir-27c	48h-adult	ubiquitous but not in liver	weak ubiquitous, pharyngeal arches, jaw	0	0	0	1	3	3
dre-mir-301b	48h-adult	ubiquitous but strongest in brain and eye	ubiquitous	0	0	0	2	2	2
dre-mir-363	24h-adult	strongest in muscle, liver, skin	no expression	0	0	0	0	1	1
dre-mir-365	48h-adult	brain, eye, muscle, fins, skin	weak brain	0	0	0	0	3	3
dre-mir-429	24h-adult	gills, fins, skin	nose, neuromasts (hair and supporting cells), taste buds, proctodeum	0	0	3	3	3	3
dre-mir-430a	24h-5d	N.D.	ubiquitous in early stages	2	2	2	2	1	1
dre-mir-430b	24h-5d	N.D.	ubiquitous in early stages	2	2	2	2	1	0
dre-mir-430c	24h-48h	N.D.	ubiquitous in early stages	2	2	2	2	1	0
dre-mir-430i	24h-72h	N.D.	ubiquitous in early stages	2	2	2	2	1	1
dre-mir-430j	24h-5d	N.D.	ubiquitous in early stages	2	2	2	2	1	1
dre-mir-451	48h-adult	ubiquitous	blood	0	0	3	3	3	3
dre-mir-454a	48h-adult	ubiquitous, but stronger in brain and eye	brain and pharyngeal arches	0	0	0	3	3	3
dre-mir-454b	48h-adult	ubiquitous, but stronger in brain and eye	brain and pharyngeal arches	0	0	0	3	3	3
dre-mir-455	72h-5d	eye, muscle, fins, skin	cartilage in head	0	0	0	3	3	3
dre-mir-456	24h-adult	weak brain	weak brain	0	0	1	1	3	3
dre-mir-457a	24h-adult	ubiquitous	brain	0	0	0	3	3	3
dre-mir-457b	24h-adult	ubiquitous	brain	0	0	0	3	3	3
dre-mir-458	48h-adult	stronger in brain and eye	no expression	0	0	0	1	1	1
dre-mir-459	72h-adult	gut	gut	0	0	0	0	3	3
dre-mir-460	72h-adult	fins	no expression	0	0	0	0	0	0
dre-mir-461	not expressed	N.D.	no expression	0	0	0	0	0	0
dre-mir-462	adult	gills, fins, skin	liver and head	0	0	0	0	3	3
dre-mir-92b	24h-adult	ubiquitous	outline of tectum and telencephalon	0	0	0	3	3	3
dre-miR-740	weak 5d-adult	N.D.	no expression	0	0	0	0	0	0

N.D.=not determined. 0=no expression, 1=weak ubiquitous or background expression, 2=strong ubiquitous expression, 3=specific expression, [1]determined by northern blot analysis

also *in situ*, and that are expressed in the brain (*miR-124* and *miR-181b*), with two new miRNAs that we found to be expressed at least in the adult fish brain (*miR-489* and *miR-34c-5p*) (Figure 4D). Although *miR-124* and *miR-181b* were easily detected also in the more diluted total RNA samples, *miR-489* and *miR-34c-5p* could only be detected clearly in the adult brain samples. The expression level of *miR-34c-5p* in the undiluted total RNA sample is comparable to the expression level of *miR-124* in the most diluted (27x) total RNA sample, showing that there is a 30 fold difference in abundance between these two miRNAs. These examples indicate that indeed many of the miRNAs that we cloned in addition to the already known miRNAs are much less abundant and therefore more difficult to detect.

Table 2. Overview of expression data for new miRNAs				temporal expression in situ					
new ID	expression time[a]	expression adult tissue[a]	expression in situ	12h	16h	24h	48h	72h	5d
dre-miR-489[b]	no expression	brain, eye	no expression	0	0	0	0	0	0
ZF_nl_11	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-724-3p[c]	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-724-5p	no expression	weak brain[d]	no expression	0	0	0	0	0	0
dre-miR-725	48h-adult	gills, fins	no expression	0	0	0	0	0	0
dre-miR-726-3p	72h-adult	eye	no expression	0	0	0	0	0	0
dre-miR-726-5p[c]	no expression	no expression	no expression	0	0	0	0	0	0
ZF_nl_139	no expression	no expression	no expression	0	0	0	0	0	0
ZF_nl_149	no expression	no expression	no expression	0	0	0	0	0	0
ZF_nl_157	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-727-3p	no expression	brain[d]	no expression	0	0	0	0	0	0
dre-miR-727-5p[c]	no expression	brain, eye	no expression	0	0	0	0	0	0
dre-miR-728	48h-adult	brain, eye	no expression	0	0	0	0	0	0
dre-miR-729	48h-adult	eye	no expression	0	0	0	0	0	0
ZF_nl_21	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-190b	48h-adult	eye	no expression	0	0	0	0	0	0
ZF_nl_236	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-34c-3p[c]	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-34c-5p	48h-adult	brain	nose	0	0	0	0	3	3
dre-miR-722	24h-adult	brain, eye, gills, skin, liver	no expression	0	0	0	0	0	0
dre-miR-730-3p[c]	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-730-5p	24h-adult	brain, eye, fins, skin	no expression	0	0	0	0	0	0
ZF_nl_263	no expression	no expression	no expression	0	0	0	0	0	0
ZF_nl_264	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-731	adult	gills, fins, skin, gut, heart	no expression	0	0	0	0	0	0
ZF_nl_286	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-732	no expression	weak brain[d]	no expression	0	0	0	0	0	0
dre-miR-733	24h-adult	eye, brain, muscle gills, fins, skin, gut	ubiquitous, but more in yolk and gut	2	2	3	3	3	3
dre-miR-15c	24h-adult	ubiquitous, not in liver	no expression	0	0	0	0	0	0
ZF_nl_33	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-734	no expression	braind	no expression	0	0	0	0	0	0
dre-miR-735-3p	24h-adult	ubiquitous but not in liver and gut	ubiquitous (brain, neural tube, outline neuromasts)	2	2	2	3	3	3
ZF_nl_384	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-736	24h-adult	eye, gut	no expression	0	0	0	0	0	0
dre-miR-737-3p	no expression	brain, eye	no expression	0	0	0	0	0	0
dre-miR-738	48h-adult	gills	no expression	0	0	0	0	0	0
dre-miR-739	no expression	brain	no expression	0	0	0	0	0	0
ZF_nl_51	no expression	no expression	no expression	0	0	0	0	0	0
dre-miR-499	24h-adult	heart and muscles and fins	heart and muscles in head	0	0	0	3	3	3
dre-miR-723-3p	5d-adult	brain	no expression	0	0	0	0	0	0
dre-miR-723-5p[c]	no expression	weak brain[d]	no expression	0	0	0	0	0	0

0=no expression, 1=weak ubiquitous or background expression, 2=strong ubiquitous expression, 3=specific expression

[a]determined by northern blot analysis, [b]predicted based on verified mammalian sequence

[c]regarded as miRNA star sequence, [d]only checked for expression in brain

Cloning and expression of miRNA star sequences

For 65 known and 11 new miRNAs we also cloned the other arm of the hairpin (Supplementary Table 1). The 65 star sequences of known miRNAs were represented by 621 clones and the 11 star sequences of new miRNAs were represented by 45 clones. In most cases, the miRNA was cloned more often than the star sequence.

In our expression analysis, we included six star sequences of new miRNAs. We were unable to detect any of these by *in situ* hybridization. However, we detected *miR-723-5p* and *miR-727-5p* by northern blot analysis (Figure 4). As expected, the expression of these two miRNA star sequences overlaps in both cases with the expression of the miRNA.

Discussion

Regulation of gene expression by miRNAs is an important process for development of multicellular organisms, and organisms without miRNAs cannot live (29-31). This is strengthened further by recent insight into the abundance and conservation of miRNAs and the high number of miRNA targets found in animals (5, 25, 26).

Here we describe the cloning and expression of new miRNAs from the zebrafish. By deep sequencing two small-RNA cDNA libraries we found 66 new miRNAs and 11 star sequences corresponding to 116 potential miRNA hairpins in the zebrafish genome. The majority (97.4%) of small-RNAs that were found in the libraries corresponded to known miRNAs and 56% of the new miRNAs were represented by a single sequenced clone. However, only 13% of the new miRNAs that we detected on northern blots were cloned only once, indicating that these single cloned miRNAs are more difficult to detect and also that miRNA cloning frequency reflects miRNA abundance. In addition, 67% of the newly cloned miRNAs are, out of 12 different species, conserved only in zebrafish according to our conservation criteria (>90% identity for the mature miRNA and >70% identity for the precursor). Thus, our cloning and expression data show that although many miRNAs are abundantly expressed, there are also miRNAs that have much lower expression levels or that are expressed in only a few cells. For example, *miR-34c-5p* has a very restricted expression pattern in the nose of the embryo. Of the new miRNAs 37 were picked up only once, indicating that our sequencing did not reach saturation yet. Furthermore, we screened only two libraries derived from a limited amount of tissues. If some miRNAs are only expressed in specific adult tissues, and some of them are (Figures 3 and 4), these will be missed by our libraries, which do not contain all adult tissues. In order to determine the complete set of miRNAs that governs gene expression in an organism, one should perform saturated sequencing of small-RNA cDNA libraries from several tissues.

Current estimates, which are based on cloning and computational predictions of miRNAs from human, suggest that there might be up to a thousand miRNAs (6, 7). Our data show that there are more miRNAs to be found, but predict that these will be lowly expressed and less conserved. The set of abundantly expressed miRNAs in zebrafish is, based on this study and previous work (36, 39), limited to 150 different miRNAs and mostly contains conserved miRNAs.

Analysis of the spatial and temporal expression of miRNAs may shed light on their

role in tissue specific processes. Many of the new miRNAs analyzed in this study have a cell type or tissue specific expression, providing a basis for understanding specific aspects of development that are under miRNA control. The differences in temporal expression of many miRNAs suggests that some play a role in early development during gastrulation and segmentation, whereas others may be required for organ morphogenesis in later stages of embryonic development and a few miRNAs are exclusively expressed in the adult fish. For some miRNAs there is a difference in expression in the embryo compared to the adult, suggesting differences in function. For example, *miR-92b* is expressed in the embryonic brain, but it is detected in many tissues in the adult fish.

In many cases, miRNA expression patterns correlate with miRNA function in the *Drosophila* embryo, where, for example, knockdown of miRNAs expressed in the peripheral or central nervous system induced nervous system defects (37, 44). Careful examination of the phenotypes caused by depletion of specific miRNAs in the vertebrate embryo together with computational prediction of miRNA targets may further help in understanding their role in development.

Materials and Methods

Small-RNA library construction

Two small-RNA cDNA libraries were prepared by Vertis Biotechnologie AG (Freising-Weihenstephan, Germany). RNAs smaller than 200 bases were isolated from 5-day-old zebrafish larvae and dissected adult zebrafish brain using the mirVana miRNA isolation kit (Ambion). Subsequently, the population of small-RNAs ranging in size from 15 to 30 bp were purified from 12.5% polyacrylamide gel. This small-RNA fraction was poly(A)-tailed followed by ligation of an RNA linker to the 5' end of the RNA. First strand cDNA synthesis was performed using oligo(dT)-linker primers and M-MLV-RNase H– reverse transcriptase. The resulting cDNA was then PCR-amplified in 15 (larvae library) or 23 (brain library) cycles. After limited exonuclease treatment to generate 5' overhangs, the gel purified fraction of the cDNA in the range of 95–110 bp was directionally ligated in the EcoRI and BamHI sites of pBSII SK+. Ligations were electroporated into T1 Phage resistant TransforMaxTM EC100TM electrocompetent cells (Epicentre) resulting in 1.25 (larvae library) and 1.3 (brain library) x 10^6 recombinant clones.

Sequencing of small-RNA cDNA libraries

Both libraries were plated on Luria–Bertani (LB) amp plates and 12 288 individual colonies were automatically picked and put into 384-well plates (Genetix QPix2; New Milton Hampshire, UK) containing 75 µl LB-Amp and grown overnight at 37°C with continuous shaking. All following pipetting steps were performed using liquid handling robots (Tecan Genesis RSP200 with integrated TeMo96 and Velocity11 Vprep with BenchCell 4x). 5 µl of culture were transferred to a 384-well PCR plate (Greiner) containing 20 µl water. Cells were lysed by heating for 15 min at 95°C in a PCR machine. Lysate (1 µl) was transferred to a fresh 384-well plate containing 4 µl PCR mix (final concentrations: 0.2 µM M13forward, TGTAAAACGACGGCCAGT; 0.2 µM M13reverse, AGGAAACAGCTATGACCAT, 400 µM of each dNTP, 25 mM Tricine, 7.0% Glycerol (w/v), 1.6% dimethyl sulfoxide (w/v), 2 mM $MgCl_2$, 85 mM Ammonium acetate, pH 8.7 and 0.2 U Taq Polymerase in a total volume of 10 µl) and the insert was amplified by 35 cycles of 20 min at 94°C, 10 min at 58°C, 30 min at 72°C. After adding 30 µl water, 1 µl of PCR product was directly used for dideoxy sequencing by transferring to a new 384-well PCR plate containing 4 µl sequencing mix (0.027 µl BigDye terminator mix v3.1 (Applied Biosystems, Foster City, CA), 1.96 µl of 2.5x dilution buffer (Applied Biosystems), 0.01 µl sequencing oligo (100 µM stock T7, GTAATACGACTCACTATAGGGC) and 2 µl water). Thermocycling was performed for 35 cycles of 10 min at 94°C, 10 min at 50°C, 20 min at 60°C and final products were purified by ethanol precipitation in 384-well plates as recommended by the manufacturer (Applied Biosystems) and analyzed on ABI3730XL sequencers with a modified protocol for generating 100 nt sequencing reads.

Sequence analysis

Base calling and quality trimming of sequence chromatograms was done by phred software (40). After masking of vector and adapter sequences and removing redundancy, inserts of length 18 bases and longer were mapped to the zebrafish genome using megablast software (ftp://ftp.ncbi.nlm.nih.gov/blast/). Not all inserts matched perfectly to a genome, and detailed analysis of non-matching sequences indicated that many of them represented known miRNAs with several additional nucleotides added to one of the ends. These non-genomic sequences may be artifacts of the cloning procedure or a result of non-templated modification of mature miRNAs (41). Such sequences were corrected according to the best blast hit to a genome. Next, for every genomic locus matching to an insert, repeat annotations were retrieved from the Ensembl database (http://www.ensembl.org) and repetitive regions were discarded from further analysis, with the exception of the following repeats: MIR, MER, L2, MARNA, MON, Arthur and trf, since these repeat annotations overlap with some known miRNAs. Genomic regions containing inserts with 100 nt flanks were retrieved from Ensembl and a sliding window of 100 nt was used to calculate RNA secondary structures by RNAfold (42). Only regions that folded into hairpins and contained an insert in one of the hairpin arms were used in further analysis. Since every non-redundant insert produced independent hits at this stage, hairpins with overlapping genomic coordinates were merged into one region, tracing locations of matching inserts. In cases when several inserts overlapped, the whole region covered by overlapping inserts was used in downstream calculations as a mature sequence. Next, gene and repeat annotations for hairpin genomic regions were retrieved from Ensembl, and repetitive regions (with above mentioned exceptions) as well as ribosomal RNAs, tRNAs and snoRNAs were discarded.

To find homologous hairpins in other genomes, mature regions were blasted against human, macaca, chimpanzee, mouse, rat, dog, cow, opossum, chicken, tetraodon, zebrafish and fugu genomes. Hits with length of at least 20 nt and identity of at least 70% were extracted from genomes along with flanking sequences of length similar to that observed in original hairpins to which a certain mature query sequence belonged. Extracted sequences were checked for hairpin structures using RNAfold, and positive hairpins were aligned with the original hairpin using clustalw (43). Only homologs with at least 70% overall identity and 90% identity within the mature sequence were considered. In cases where several homologous hairpins in a species were identified, the best clustalw-scoring hairpin was retained. Next, homologs from different organisms were aligned with the original hairpin by clustalw to produce a final multiple alignment of the hairpin region. Chromosomal location of homologous sequences were used to retrieve gene and repeat annotations from respective species Ensembl databases. Hairpins that contained repeat/RNA annotations in one of the species, as well as hairpins containing mature regions longer that 25 nt or with GC-content higher than 85% were discarded. For remaining hairpins, randfold values were calculated for every sequence in an alignment using mononucleotide shuffling and 1000 iterations. The cut-off of 0.01 was used for randfold and only regions that contained a hairpin below this cut-off for at least one species in an alignment, were considered as miRNA genes. Finally, positive hairpins were split into known and novel miRNAs according to annotations. To facilitate these annotations and also to track performance of the pipeline, mature sequences of known miRNAs from miRBase (5) were included into the analysis from the very beginning.

In situ hybridization

Albino zebrafish embryos and larvae of 12, 16, 24, 48, 72 and 120 hpf were fixed in 4% PFA in phosphate-buffered saline (PBS) overnight at 4°C. Proteinase K treatment was done for 2, 5, 10, 30, 45 and 90 min, respectively. In situ hybridization was performed as previously described (36, 38). LNA-modified DNA probes (LNA probes) were designed and synthesized by Exiqon (Denmark). The LNA probes were labeled with digoxigenin (DIG) using the DIG 3' end labeling kit (Roche) and purified using Sephadex G25 MicroSpin columns (Amersham).

Plastic sectioning

Embryos and larvae stained by whole-mount *in situ* hybridization were transferred from benzyl benzoate/benzyl alcohol to 100% methanol and incubated for 10 min. Specimens were washed twice with 100% ethanol for 10 min and incubated overnight in 100% Technovit 8100 infiltration solution (Kulzer) at 4°C. Next, embryos were transferred to a mold and embedded overnight in Technovit 8100 embedding medium (Kulzer) deprived of air at 4°C. Sections of 7 µm thickness were cut with a microtome (Reichert-Jung 2050), stretched on water and mounted on glass slides. Sections were dried overnight. Counterstaining was done with 0.05% neutral red for 12 s, followed by extensive washing with water. Sections were preserved with Pertex and mounted under a coverslip.

Image acquisition

Embryos, larvae and sections were analyzed with Zeiss Axioplan and Leica MZFLIII microscopes and subsequently photographed with digital cameras. Images were adjusted with Adobe Photoshop 7.0 software.

Northern blotting

Total RNA was isolated using trizol (Invitrogen). For each sample 15 µg RNA was separated on 15% denaturing polyacrylamide gels and blotted according to standard procedures. Blots were prehybridized for 30 min at 60°C in hybridization buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA and 7% SDS) and hybridized overnight at 60°C in hybridization buffer containing 0.1 nM probe. After stringency washes (once for 30 min at 50°C in 2x SSC/0.1% SDS and once for 30 min at 50°C in 0.5x SSC and 0.1% SDS) blots were rinsed in PBST (PBS with 0.1% Tween-20) and blocked for 30 min at room temperature in PBST with 5% milk powder. Subsequently, blots were incubated for 1 h at room temperature with anti-DIG-AP antibody (Roche) in blocking buffer, washed six times for 15 min in PBST and twice for 5 min with AP-buffer (0.1 M Tris–HCl, pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl and 0.1% Tween). Signal was detected by using CDP-star chemiluminescent substrate (Roche) and exposing the blots to X-ray films. Films were scanned and pictures were processed using Adobe photoshop 7.0 software. To control for equal loading, blots were hybridized for 2 h at 37°C with a radio-labeled probe against U6 snRNA. After washing twice in 2x SSC/0.2% SDS, blots were exposed to phosphor-imager screens.

Acknowlegdements

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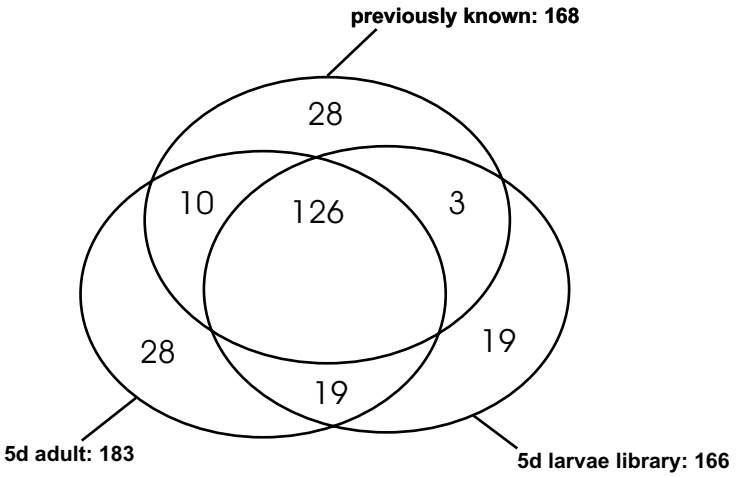
Supplementary data

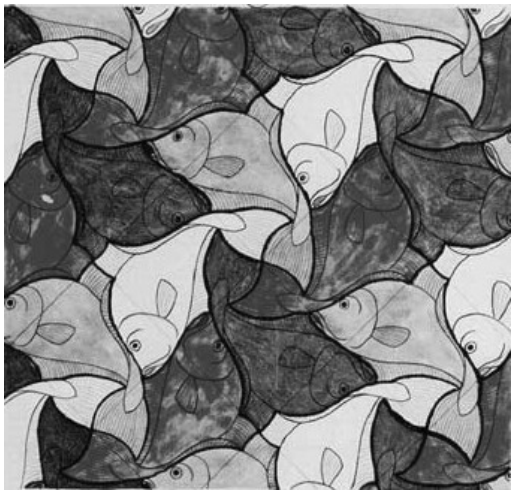


Supplementary Figure 1. Venn-diagram of known zebrafish miRNAs from the miRNA registry, miRNAs cloned from the zf-brain library and miRNAs cloned from the zf-embryo library.

Table S1. Overview of miRNA cloning and expression

	known	new	
miRNAs in registry	168		
miRNA genes in registry	369		
miRNAs cloned	139	66	
star sequences cloned	65	11	
miRNA genes	255	116	
analyzed miRNAs	32	35	
analyzed star sequences	0	6	
miRNAs detected in situ	24	4	
star sequences detected in situ	0	0	
miRNAs detected on Northern	30	23	
star sequences detected on Northern	0	2	
	brain	larvae	total
known miRNAs cloned	136	129	139
of which found in both libraries			126
new miRNAs cloned	47	38	66
of which found in both libraries			19

CHAPTER 6



Targeted inhibition of miRNA maturation with morpholinos reveals a role for *miR-375* in pancreatic islet development

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Abstract

Several vertebrate microRNAs (miRNAs) have been implicated in cellular processes such as muscle differentiation, synapse function, and insulin secretion. In addition, analysis of *dicer* null mutants has shown that miRNAs play a role in tissue morphogenesis. Nonetheless, only a few loss-of-function phenotypes for individual miRNAs have been described to date. Here, we introduce a quick and versatile method to interfere with miRNA function during zebrafish embryonic development. Morpholino oligonucleotides targeting the mature miRNA or the miRNA precursor specifically and temporally knock down miRNAs. Morpholinos can block processing of the primary miRNA (pri-miRNA) or the pre-miRNA, and they can inhibit the activity of the mature miRNA. We used this strategy to knock down 13 miRNAs conserved between zebrafish and mammals. For most miRNAs, this does not result in visible defects, but knockdown of *miR-375* causes defects in the morphology of the pancreatic islet. Although the islet is still intact at 24 hours postfertilization (hpf), in later stages the islet cells become scattered. This phenotype can be recapitulated by independent control morpholinos targeting other sequences in the *miR-375* precursor, excluding off-target effects as cause of the phenotype. The aberrant formation of the endocrine pancreas, caused by *miR-375* knockdown, is one of the first loss-of-function phenotypes for an individual miRNA in vertebrate development. The miRNA knockdown strategy presented here will be widely used to unravel miRNA function in zebrafish.

Introduction

MicroRNAs (miRNAs) have a profound impact on the development of multicellular organisms. Animals lacking the Dicer enzyme, which is responsible for the processing of the precursor miRNA into the mature form, cannot live (1-3). miRNA mutants have been described only for *Caenorhabditis elegans* and *Drosophila*, reviewed in (4). From these studies, it is clear that invertebrate miRNAs are involved in a variety of cellular processes, such as developmental timing (5, 6), apoptosis (7, 8), and muscle growth (9). Analysis of conditional *dicer* null alleles in mouse has indicated a general role for miRNAs in morphogenesis of the limb, skin, lung epithelium, and hair follicles (10-13). Overexpression studies in mouse have implicated specific vertebrate miRNAs in cardiogenesis and limb development (14, 15). In zebrafish, embryos lacking both maternal and zygotic contribution of Dicer have severe brain defects (2). Strikingly, the brain phenotype of maternal-zygotic *dicer* mutant zebrafish can be restored by injection of *miR-430*, the most abundant miRNA in early zebrafish development. Despite all these studies describing functions for miRNAs in development, no vertebrate miRNA mutant has been described to date. Genetically, it is challenging to obtain mutant miRNA alleles in zebrafish, because their small size makes them less prone to mutations by mutagens, and for many miRNAs, there are multiple alleles in the genome or they reside in families of related sequence.

Temporal inhibition of miRNAs by antisense molecules provides another strategy to study miRNA function. 2′-O-methyl oligonucleotides have been successfully used *in vitro* and *in vivo* to knock down miRNAs (16-18). Morpholinos are widely applied to knock down genes in zebrafish development (19) and have recently been used to target mature *miR-214* in zebrafish (20). However, off-target phenotypes are often associated with the use of antisense inhibitors.

Here, we show that morpholinos targeting the miRNA precursor can knock down miRNAs in the zebrafish embryo. Several independent morpholinos can knock down the same miRNA, and these serve as positive controls to filter out off-target effects. Morpholinos can block miRNA maturation at the step of Drosha or Dicer cleavage, and they can inhibit the activity of the mature miRNA. We show that inhibition of *miR-375*, which is expressed in the pancreatic islet and pituitary gland of the embryo (21), results in dispersed islet cells in later stages of embryonic development, whereas no effects were observed in the pituitary gland. The morpholino-mediated miRNA knockdown strategy presented here, is an extremely fast and well-controlled method to study miRNA function in development.

Results

Morpholinos targeting the mature miRNA deplete the embryo of specific miRNAs

Since it is difficult to obtain a genetic mutant for a miRNA in zebrafish, we looked for alternative strategies to deplete the embryo of specific miRNAs. Antisense mol-

ecules such as 2′-O-methyl and locked nucleic acid (LNA) oligonucleotides have been used to inhibit miRNAs in cell lines (16, 18, 22), *Drosophila* embryos (23), and adult mice (17). We tried to use these molecules to inhibit the function of endogenous miRNAs in the zebrafish embryo. Although they can be used to suppress the effects of miRNA overexpression (24), injection of higher concentrations required to obtain good knockdown of endogenous miRNAs resulted in toxic effects, when injecting 1 nl solution at a concentration of approximately 10 μM and approximately 50 μM for LNA and 2′-O-methyl oligonucleotides respectively (unpublished data). Therefore, we switched to morpholinos because these are widely used to inhibit mRNA translation and splicing in zebrafish embryos (19) and have also been shown to target miRNAs in the embryo (2, 20, 24). We injected 1 nl of 600 μM morpholino solution with a morpholino complementary to the mature *miR-206* in one- or two-cell–stage embryos. Subsequently, embryos were harvested at 24, 48, 72, and 96 hours postfertilization (hpf) and subjected to *in situ* hybridization and Northern blotting (Figure 1A and 1B). This analysis showed that the mature miRNA signal is suppressed up to 4 d after injection of the morpholino. The knockdown effect was specific for this miRNA; parallel *in situ* analysis of the same embryos with a probe for *miR-124* did not show any effects on expression of this miRNA (Figure 1B). Thus, miRNA detection can be specifically and efficiently suppressed during embryonic and early larval stages of zebrafish development using morpholinos antisense to the mature miRNA.

The zebrafish embryo can be used to monitor the effect of miRNAs on green fluorescent protein (GFP) reporters fused to miRNA target sites (24). To determine the effect of a morpholino in this assay system, we constructed a GFP reporter for *miR-30c* and tested it in the presence and absence of a mature *miR-30c* duplex. Injected *miR-30c* silences this GFP reporter, which is in line with previous reports using similar strategies in the embryo (Figure 1C) (2, 20, 24). Co-injection of the *miR-30c* duplex and a morpholino targeting mature *miR-30c* rescues the reporter signal, whereas injection of a control morpholino did not reverse the silencing by *miR-30c*. These data indicate that a morpholino can block the activity of a mature miRNA duplex in a functional assay .

There are three possible explanations for the observed reduction in the detection signal for a miRNA that is targeted by a morpholino. First, the hybridization of a morpholino could disturb isolation of the miRNA. Second, the morpholino could destabilize the miRNA. Third, the morpholino could inhibit the maturation of the miRNA.

To examine the effect of a morpholino on the isolation of a mature miRNA, we incubated a mature *miR-206* duplex and a control duplex (*miR-205*) with a morpholino against *miR-206 in vitro*. After isolation, samples were analyzed by Northern blotting for the presence of *miR-206* and *miR-205*. We could still detect *miR-206*, indicating that there is no effect of the morpholino on the RNA isolation procedure (Figure 1D).

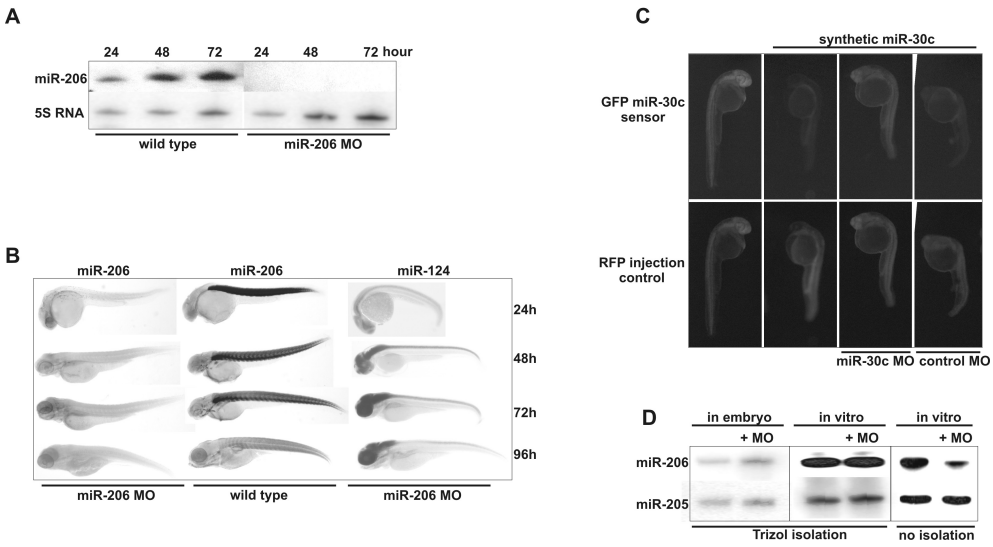

Figure 1. Morpholinos targeting the mature miRNA deplete the zebrafish embryo of specific miRNAs. (A) Northern blot for *miR-206* in wild-type and MO *miR-206*–injected embryos at 24, 48, and 72 hpf. 5S RNA serves as a loading control. (B) *In situ* analysis of *miR-206* and *miR-124* expression in different stage embryos after injection of MO *miR-206*. (C) Effect of a morpholino targeting *miR-30c* on a silencing assay with *miR-30c* and a responsive GFP sensor construct. (D) *In vivo* and in *vitro* effects of a morpholino on the stability and RNA extraction of a synthetic *miR-206* duplex. *miR-205* serves as a loading control.

However, when morpholino and miRNA duplex were incubated together *in vitro* and loaded on a denaturing gel without isolation, we observed a decrease in the signal for *miR-206*, indicating that the morpholino can bind to the miRNA *in vitro* and still does so in the denaturing gel.

Next, we wanted to know whether a morpholino could affect the stability of a mature miRNA *in vivo*. Therefore, we injected a mature *miR-206* and a control duplex (*miR-205*) together with a morpholino against *miR-206* in the embryo. After incubation for 8 h, RNA was isolated and subjected to Northern blot analysis to probe for injected *miR-206* and injected *miR-205*. In contrast to the data obtained for endogenous *miR-206*, there was no decrease observed in the amount of injected *miR-206* in the morpholino-injected embryos (Figure 1D) (endogenous *miR-206* is not yet expressed at this stage).

Since these data show that there is no effect of a morpholino on miRNA isolation or stability, we conclude that morpholinos deplete the embryo of miRNAs by inhibiting miRNA maturation. If this is the case, then we expect morpholinos targeting other regions of the miRNA precursor to act as well as the morpholinos designed against the mature miRNA, and this is indeed what we find (see next section).

Morpholinos targeting the miRNA precursor interfere with primary miRNA processing

Injection of antisense oligos in embryos might result in off-target effects. Thus, phenotypic data retrieved from antisense knockdown experiments should be treated with caution. In *Drosophila*, 2′-O-methyl oligo–mediated knockdown of embryonically expressed miRNAs caused defects that clearly differed from the phenotype of the corresponding knockout fly (9, 23). In sea urchin experiments, off-target effects of morpholino knockdowns are well documented, though low incubation temperatures favor off-target interactions (25).

To filter out off-target effects, we sought a control strategy that would allow us to compare effects of morpholinos with independent sequences targeted to the same miRNA. Because our data on morpholinos targeting the mature miRNA suggested that miRNA biogenesis might be affected, we designed morpholinos targeting the Drosha and Dicer cleavage sites of the precursor miRNA (Figure 2A). We decided to test this strategy on *miR-205*, since it is expressed relatively early and there are only two, but identical, copies in the fish genome. Four different morpholinos were designed to inhibit *miR-205* biogenesis: two targeting the Drosha cleavage site complementary to either the 5′ or 3′ arm of the stem, and two morpholinos similarly targeting the Dicer cleavage site (Supplementary Figure 1). These morpholinos were injected under similar conditions as described for *miR-206* and compared to the morpholino targeting mature *miR-205*. Interestingly, all five morpholinos induced complete or near-complete loss of *miR-205* (Figure 2B).

Many miRNAs are highly expressed during later stages of embryonic development (21). Therefore, we tested how long the effect of the morpholinos would last. Although for this series of morpholinos the knockdown is best at 24 hpf, the effect is still significant up to 72 hpf (Figure 2C).

Next, we tested a similar series of morpholinos against the *miR-30c* precursor and analyzed *miR-30c* expression by Northern blotting (Supplementary Figure 2). However, we only observed knockdown for the morpholino targeting mature *miR-30c*, but not for the other four morpholinos targeting the *miR-30c* precursor. This could be because *miR-30c* resides in a family of closely related species, with more sequence variability in the regions outside of the mature miRNA. The precursors of the family members might not all be targeted by these morpholinos (Supplementary Figure 2). Thus, not all miRNAs are equally prone to knockdown by morpholinos that target the miRNA precursor.

To investigate the effect of morpholinos on exogenously introduced pri-*miR-205*, we injected mRNA derived from a GFP construct with pri-*miR-205* in the 3′ UTR. Again, we could not detect mature *miR-205* derived from this construct after targeting by morpholinos (Figure 2D). Interestingly, the *miR-205* precursor also could not be detected in the embryos co-injected with morpholinos, whereas pre-*miR-205* could

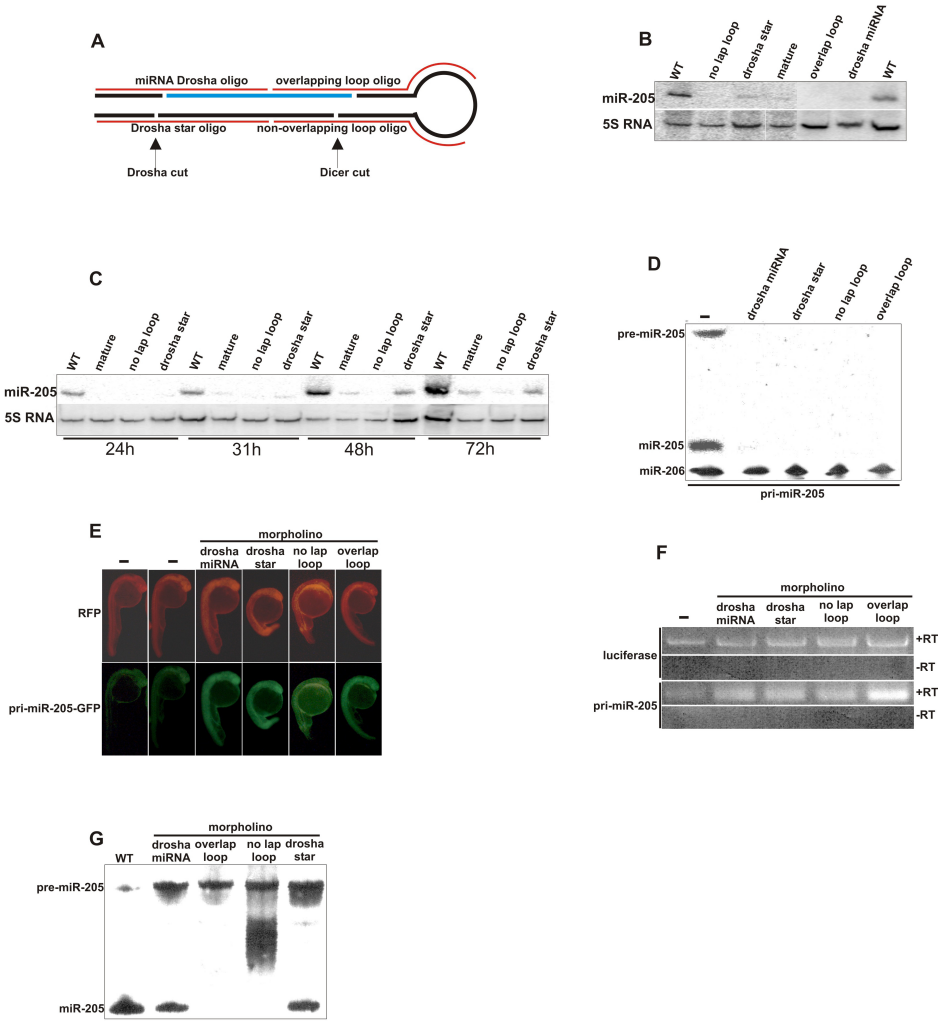

Figure 2. Morpholinos targeting the precursor miRNA interfere with miRNA maturation. (A) Design of morpholinos targeting the precursor miRNA. (B) Northern blot analysis of *miR-205* in 30-h-old embryos injected with different morpholinos against pri-*miR-205*. 5S RNA serves as a loading control. (C) Time series of *miR-205* expression after injection of mature, no lap loop, and drosha star morpholinos against pri-*miR-205*. (D) Northern blot analysis of *miR-205* derived from embryos injected with a GFP-pri-*miR-205* transcript and four different morpholinos targeting pri-*miR-205*. Co-injected *miR-206* serves as an injection and loading control. Embryos were collected 8 h after injection. (E) GFP expression in 24-h embryos injected with morpholinos and a GFP-pri-*miR-205* construct as used in (C). Pri-*miR-205* is positioned just upstream of the polyA signal in the 3′ UTR of the GFP mRNA. Red fluorescent protein (RFP) serves as an injection control. (F) RT-PCR analysis of injected GFP-pri-*miR-205* mRNA with (+) and without (−) co injected morpholinos. Luciferase serves a an injection control. Embryos were collected 8 h after injection. (G) Northern analysis of the effect of morpholinos on an injected *miR-205* precursor. Embryos were collected 8 h after injection. WT, wild type.

be detected in the absence of morpholinos (Figure 2D). Because pri-*miR-205* was cloned in the 3′ UTR of GFP, we monitored GFP fluorescence after injection of this construct. In the presence of a morpholino, GFP fluorescence increased (Figure 2E), suggesting accumulation of the primary miRNA. Therefore, we performed RT-PCR on 8-h-old embryos injected with GFP-pri-*miR-205* and a control mRNA (luciferase) (Figure 2F). In the presence of a morpholino, the GFP-pri-*miR-205* mRNA level is higher compared to control embryos that were not injected with morpholinos. This experiment confirms the GFP data and shows that morpholinos targeting the miRNA precursor inhibit Drosha cleavage.

Next, we tested whether processing of the pre-miRNA might also be inhibited by morpholinos. Therefore, we injected a *miR-205* precursor in the one-cell stage embryo. Northern analysis showed that the precursor was processed into mature miRNA in the embryo (Figure 2G). However, co-injection of the overlap loop and nonoverlapping loop morpholinos blocked processing completely. There was only a little effect of morpholinos targeting the Drosha cleavage site, probably because they only partially overlap the precursor.

A similar analysis was performed for *miR-375*, which is expressed in the pancreatic islet and pituitary gland (21), and which has two copies in the zebrafish genome, that differ in the regions outside the mature miRNA.

Overlap loop and loop morpholinos were designed for both *miR-375-1* and *miR-375-2*, and a morpholino against the miRNA star sequence could be used to target both copies of *miR-375* simultaneously (Figure 3A). The efficacy of all morpholinos was assessed by determining their effect on injected pri-*miR-375-1* or pri-*miR-375-2* transcripts (Figure 3B). As expected, each morpholino targeted the transcript to which it was directed. However, the star *miR-375* morpholino did not knock down *miR-375* completely. In addition, morpholino oligonucleotide (MO) *miR-375* did not interfere with processing of *miR-375* from pri-*miR-375-1*, possibly because this primary transcript forms a more stable hairpin. In all cases, the lack of a signal for mature miR-375 coincided with the absence of pre-*miR-375*, which could be detected in the absence of a complementary morpholino.

Next, all morpholinos were injected separately and in combination, and embryos were subjected to Northern blotting to determine endogenous *miR-375* expression at 24 and 48 hpf (Figure 3C). In contrast to the results obtained by *in situ* hybridization (see last section), the morpholino to mature *miR-375* only slightly decreased the expression of *miR-375*. However, MO *miR-375* could inhibit the activity of a mature *miR-375* duplex in a GFP-*miR-375*-target reporter assay (Figure 3E). The morpholinos targeting only one copy of *miR-375* reduced *miR-375* expression, with the strongest effect for the morpholinos targeting pri-*miR-375-1*. However, simultaneous injection of morpholinos targeting pri-*miR-375-1* and pri-*miR-375-2* completely knocked down mature *miR-375*, indicating that both transcripts are expressed.

To further determine the contribution of each transcript to mature *miR-375* accu-

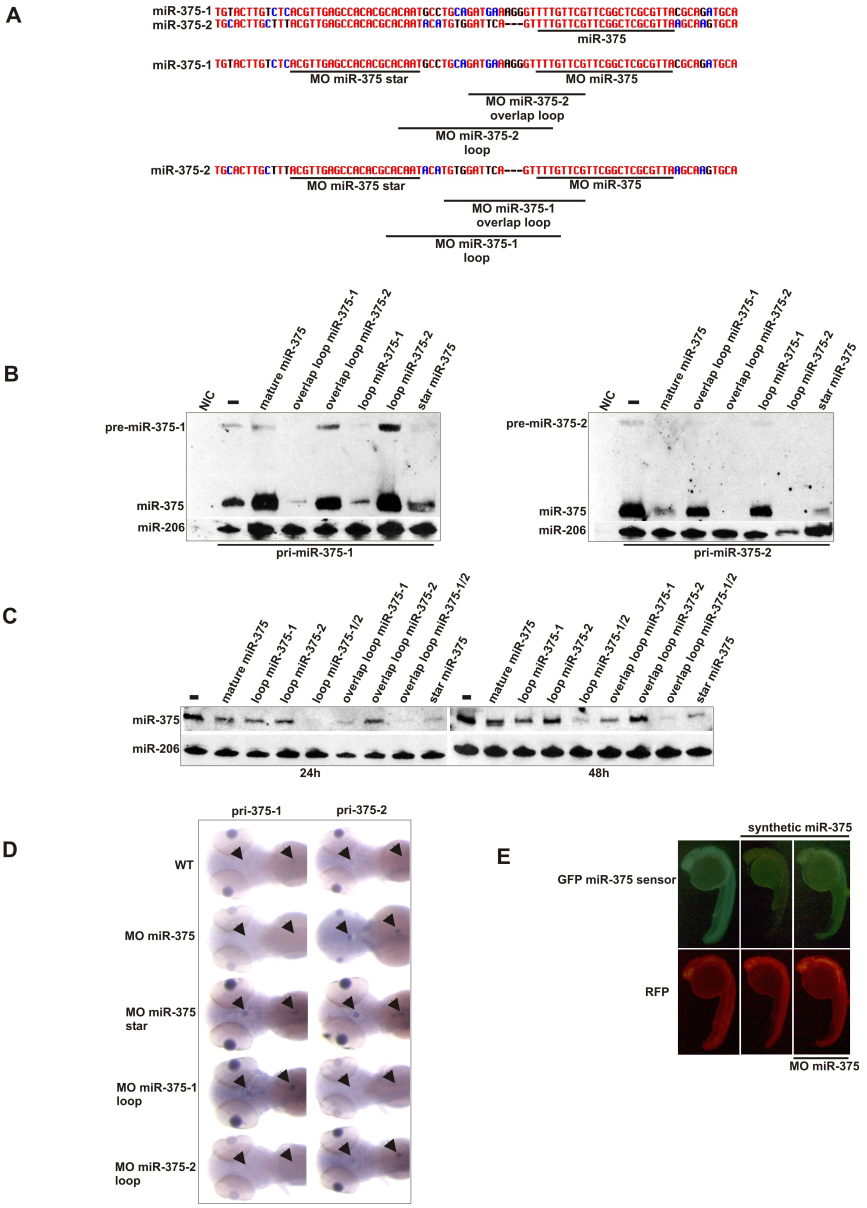

Figure 3. Specific morpholinos deplete the embryo of *miR-375*. (A) Sequence alignment of the two *miR-375* genes from zebrafish and design of morpholinos targeting the dre-*miR-375-1* and dre-*miR-375-2* precursors. (B) Northern blot analysis of the effect of morpholinos on the expression of *miR-375* derived from injected pri-miRNA mRNAs for *miR-375-1* and *miR-375-2*. MO-375-1 overlap loop and loop morpholinos target exclusively the pri-*miR-375-1* construct, and MO-375-2 overlap loop and loop morpholinos target exclusively the pri-*miR-375-2* construct. Co-injected *miR-206* serves as a loading and injection control. Embryos were collected 8 h after injection. (C) Northern blot analysis of the effect of morpholinos on

endogenous *miR-375* expression at 24 hpf and 48 hpf. *miR-206* serves as loading control. (D) *In situ* hybridization for pri-*miR-375-1* and pri-*miR-375-2* on wild-type (WT) and morpholino-injected embryos. Arrowheads indicate the pituitary gland and the pancreatic islet. (E) Analysis of GFP expression in 24-h embryos injected with a *miR-375* GFP sensor construct, a synthetic *miR-375* duplex and MO *miR-375*. Red fluorescent protein (RFP) serves as an injection control. NIC, Noninjected control.

mulation, we performed *in situ* hybridization for pri-*miR-375-1* and pri-*miR-375-2* (Figure 3D). Both transcripts could not be detected in wild-type embryos. However, *pri-miR-375-1* was detected in the pancreatic islet and the pituitary gland in embryos injected with the *miR-375-1* loop morpholino and the morpholino to *miR-375* star. Similarly, pri-*miR-375-2* was only detected in embryos injected with the *miR-375-2* loop morpholino, the morpholino to *miR-375* star and mature *miR-375*. Thus, both transcripts are expressed in the pituitary gland and the pancreatic islet, similar to *miR-1* in the developing mouse heart (15). Together, this indicates that these morpholinos inhibit primary miRNA processing and result in primary miRNA accumulation, as we described for *miR-205*.

Concluding, our data demonstrate that morpholinos targeting the miRNA precursor can interfere with primary miRNA processing at either the Drosha or Dicer cleavage step and that morpholinos targeting the mature miRNA can inhibit their activity in a functional assay. Taken together, our data show that different morpholinos targeting the same miRNA may serve as positive controls for miRNA knockdown phenotypes in the embryo.

Knockdown of many miRNAs does not result in any observed developmental defects

To identify functions for individual miRNAs in zebrafish embryonic development, we knocked down a series of 11 conserved vertebrate miRNAs and analyzed their expression after morpholino knockdown (Figure 4). Injected embryos were monitored phenotypically by microscopic observation until four days postfertilization (dpf). Knockdown of most miRNAs resulted in loss of *in situ* staining for the respective miRNA. However, we could not observe gross morphological malformations after knockdown of these miRNAs (Figure 4A). Therefore, we analyzed embryos injected with morpholinos against either *miR-182, miR-183*, or *miR-140* in more detail, because we could easily stain the tissues that express these miRNAs (Figure 4B). Embryos injected with morpholinos against *miR-182* or *miR-183*, which are expressed in the lateral line neuromasts and hair cells of the inner ear, were treated with DASPEI, which stains hair cells. Embryos injected with a morpholino against *miR-140*, which is expressed in cartilage, were subjected to Alcian Blue staining, a cartilage marker. However, staining of these specific cell types that express the miRNA did not uncover any defects upon knockdown (Figure 4B).

In conclusion, knockdown of many miRNAs does not appear to significantly affect

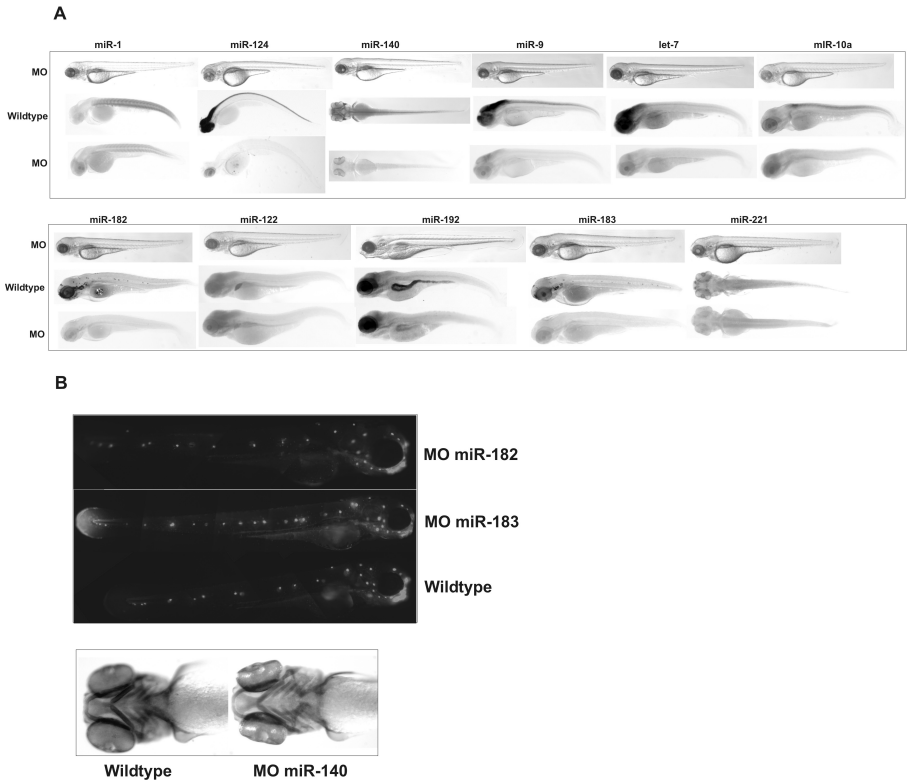

Figure 4. Knockdown of many miRNAs does not affect zebrafish embryonic development. (A) Phenotypes and *in situ* analysis of 3- and 4-d-old embryos after injection of morpholinos against 11 different mature miRNAs. (B) Daspei staining of 72-h-old embryos injected with MO *miR-182* and MO *miR-183* and wild-type control (upper panel). Alcian Blue staining of 72-h-old embryos injected with MO *miR-140* and noninjected control (lower panel).

zebrafish embryonic development, at least not to the extent that can be visualized by the methods used in these examples.

Knockdown of miR-375 affects pancreatic islet morphology

miR-375 is known to be expressed in the pancreatic islet and the pituitary gland, and was first isolated from pancreatic beta cells (21, 26). This miRNA is conserved in vertebrates and may regulate insulin secretion by inhibiting myotrophin (26).

We injected a morpholino against mature *miR-375* into the one-cell–stage embryo. This morpholino effectively knocked down *miR-375* in the first 4 d of development (Figure 5A), and it could also block the activity of an injected *miR-375* duplex, as monitored by its effect on a GFP reporter silenced by *miR-375* (Figure 3E).

During the first 5 dpf, there was no clear developmental defect except for a general delay in development. At around 7 dpf, approximately 80% of the injected embryos

died. Next, we analyzed the development of both the pituitary gland and the pancreatic islet, by *in situ* hybridization with pit1 and insulin markers. This analysis revealed no change in the formation of the pituitary gland (Figure 5B). However, analysis of insulin expression showed a striking malformation of the islet cells in 3-d-old morphant embryos (Figure 5B). Wild-type embryos have a single islet at the right side of the midline, whereas the *miR-375* knockdown embryos have dispersed insulin-positive cells. The effect is sequence specific, because a morpholino complementary to the mature *miR-375* morpholino inhibited the pancreatic islet phenotype (Figure 5E).

The pancreatic islet consists of four cell types, α, β, δ, and PP, expressing glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. Insulin is the first hormone expressed, and somatostatin co-localizes partially with insulin, whereas glucagon-expressing cells are distinct (27). A more detailed analysis using somatostatin and glucagon as marker genes revealed a similar pattern of scattered islet cells in the *miR-375* morphant (Figure 5C).

In zebrafish, insulin is first expressed at the 12-somite stage in a few scattered cells located at the midline, dorsal to the yolk (28). Insulin-positive cells migrate posteriorly and converge medially to form an islet by 24 hpf. To look at the development of the pancreatic islet in time, we collected MO *miR-375* and noninjected control embryos at different stages, and investigated the expression of insulin (Figure 5D). At the 16-somite stage, insulin-positive cells are scattered at the midline in both noninjected and MO *miR-375*–injected embryos, and a presumptive islet is formed by 24 hpf. Subsequently, when the insulin-positive islet is moving to the right side of the embryo in later stages, the islet breaks apart and insulin-positive cells become scattered in morphant embryos (Figure 5D). Also, in later stages, the phenotype persists, although *miR-375* is re-expressed at approximately 5 dpf in morpholino-injected embryos (Figure 5A).

Next, we analyzed the effect of all *miR-375* control morpholinos described in the previous section, by staining for insulin (Figure 6A). Both the dispersion phenotype and the knockdown were striking for embryos injected with MO *miR-375*. Injection of the overlap loop and loop morpholinos targeting pri-*miR-375-1* also resulted in scattered insulin-positive cells at 72 hpf, although the effect was weaker compared to MO *miR-375*. The miR-*375-2* loop and overlap loop morpholinos hardly induced any scattering of insulin-positive cells, whereas the effect was very strong in embryos injected with morpholinos to pri-*miR-375-1* and *-2* simultaneously. The effect of the *miR-375* star morpholino on insulin-positive cells was moderate compared to MO *miR-375*.

To further prove the specificity of the pancreatic islet phenotype, we injected two control morpholinos against *let-7* and *miR-124* and analyzed these for *miR-375* and insulin expression. None of these control morpholinos showed loss of *miR-375* expression or abnormal development of the islet cells (Figure 6A).

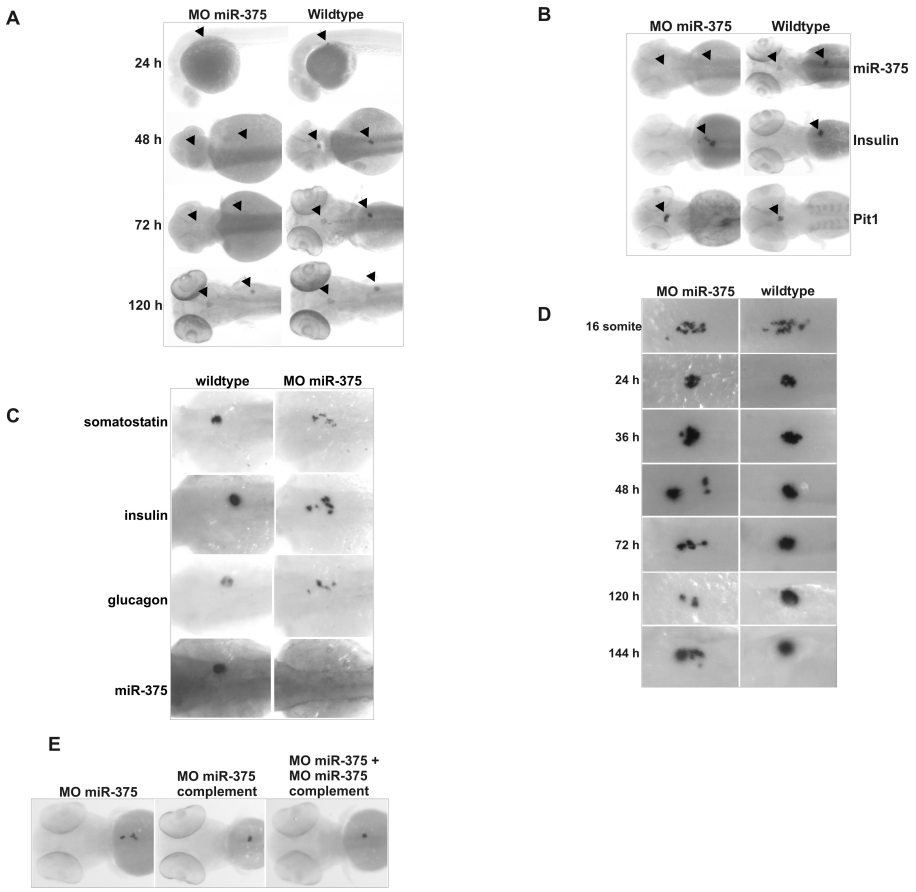

Figure 5. Knockdown of *miR-375* results in aberrant migration of pancreatic islet cells. (A) *In situ* analysis of *miR-375* knockdown in MO *miR-375* injected embryos and noninjected controls at 24, 48, 72, and 120 hpf. Arrowheads indicate the pituitary gland and the pancreatic islet. (B) *In situ* analysis of the pancreatic islet (insulin staining) and the pituitary gland (pit1 staining) in *miR-375* morphants and noninjected controls. Arrowheads indicate the pituitary gland and the pancreatic islet. (C) *In situ* analysis of pancreatic islet development in wild-type and morphant embryos using insulin, somatostatin, and glucagon as markers. (D) Time series of insulin expression in wild-type and morphant embryos injected with MO *miR-375*. (E) Insulin expression in 72-hpf embryos injected with MO *miR-375* and a complementary morpholino.

Next, we analyzed *miR-375* knockdown embryos with markers staining the endocrine or exocrine pancreas (Figure 6B). Similar to insulin staining, islet1 expression showed dispersed islet cells in embryos of 48 hpf and 72 hpf but not 24 hpf. Embryos injected with MO *miR-375* exhibited delayed development of the exocrine pancreas, liver, and gut as shown by ptf1a and foxa2 staining. At 72 hpf, these markers showed a similar pattern in MO *miR-375*–injected embryos as in noninjected embryos at 48

hpf. However, co-injection of *miR-375-1/2* loop morpholinos did not delay development of the exocrine significantly, but these embryos still displayed the scattered insulin-positive cells (Figure 6A). This shows that loss of *miR-375* mainly results in malformation of the endocrine pancreas, whereas surrounding tissues that do not express *miR-375* are not affected.

Discussion

Functional data on miRNAs in vertebrate development have been obtained mainly from overexpression studies and analysis of conditional *dicer* knockouts. For example, the role of *miR-430* in zebrafish brain morphogenesis has become clear from experiments that rescued *dicer* null mutants by injection of an miRNA duplex that mimicked a *miR-430* family member (2).

miRNA expression can be conveniently studied in zebrafish embryos. However, dissecting miRNA function by disrupting miRNA genes is difficult in zebrafish, because the miRNA is too small to efficiently search for mutations by a target-selected mutagenesis approach (29). In addition, it is unclear what such point mutations would do to processing or function of the miRNA.

It has been shown previously that morpholinos can target miRNAs in the zebrafish embryo (20, 24). In a recent study, mature *miR-214* was targeted by a morpholino in zebrafish, and this resulted in a change in somite shape, reminiscent of attenuated hedgehog signaling (20). Although the phenotype could be rescued by simultaneous inhibition of a negative regulator of hedgehog signaling, no positive control morpholinos were reported that could mimic the phenotype. In addition, data were lacking that showed an effect of the morpholino on endogenous *miR-214* levels.

The results in this paper show that morpholinos targeting the miRNA precursor form a reliable and efficient tool to deplete the embryo of miRNAs during the first 4 d of development, when most organ systems are formed and miRNAs are expressed. We have shown that miRNA expression can be inhibited by targeting the mature miRNA, the precursor miRNA or the primary miRNA. Our data show that such morpholinos can inhibit miRNA processing at the Drosha cleavage step or the Dicer cleavage step, probably by steric blocking, although the exact mechanism is unclear. In addition, morpholinos targeting the mature miRNA can inhibit their activity, probably by preventing binding to a target mRNA.

We used morpholinos targeting the mature miRNA for a set of 13 conserved vertebrate miRNAs to identify their developmental functions. By microscopic analysis we could not observe clear defects associated with loss of 11 of these miRNAs during the first 4 d of embryonic development, although *in situ* hybridization revealed specific loss of most knocked-down miRNAs. Because all the targeted miRNAs are expressed in very specific tissues and we did not investigate most morphants in much detail by marker analysis, we may have missed subtle defects. In addition,

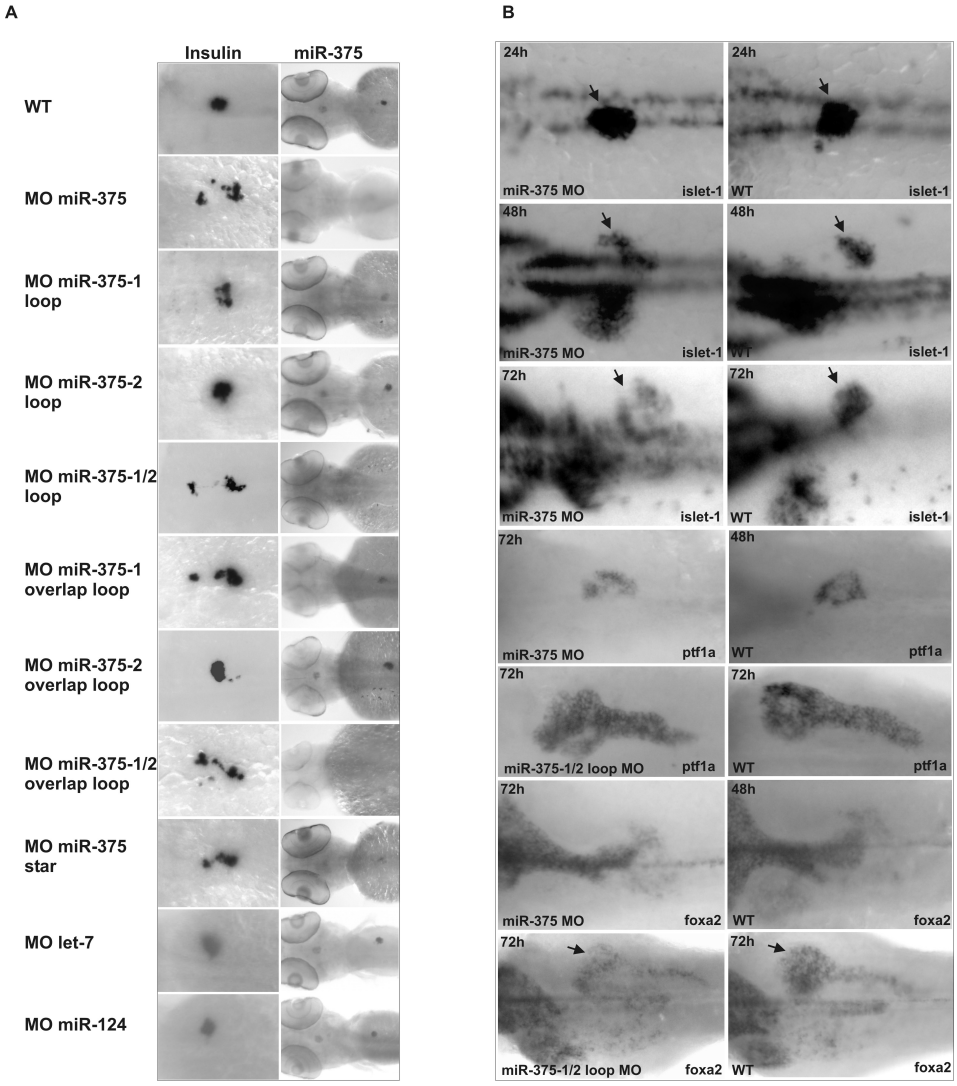

Figure 6. Specific effects of *miR-375* knockdown on the development of the endocrine pancreas. (A) *In situ* analysis of *miR-375* and insulin expression in 72-hpf embryos injected with morpholinos against the *miR-375* precursor and negative control morpholinos for *let-7* and *miR-124*. (B) Expression of islet1, foxa2, and ptf1a in wild-type and *miR-375* knockdown embryos. Arrows indicate the pancreatic islet. WT, wild type

many miRNAs reside in families of related sequence (e.g. *let-7* and *miR-182*), and these should possibly be targeted simultaneously by different morpholinos to obtain a biological effect. Furthermore, in those instances in which miRNAs of unrelated sequence target a similar set of mRNAs when expressed in the same tissue (21), removing only one miRNA might not have a profound impact on transcript levels or expression. Finally, microarray analysis and computational predictions have shown that a single miRNA may regulate hundreds of mRNAs (30, 31), but that some miRNAs act as a backup for mRNAs that are already repressed transcriptionally (32). Thus, knockdown of such miRNAs might not dramatically affect gene expression, but ensure robustness of protein interaction networks as for example *miR-7* in *Drosophila* (33).

In zebrafish, there are two copies of *miR-375*, and in human and mouse only one copy has been identified (34). To verify the *miR-375* knockdown phenotype, we designed control morpholinos targeting both precursors simultaneously (MO *miR-375* star) and separately. Complete knockdown was only observed in those instances in which both *miR-375* copies were targeted simultaneously. This also led to scattered islet cells, proving the specificity of the phenotype. However, knockdown with *miR-375-1/2* loop morpholinos did not delay development as seen in the knockdown with the mature *miR-375* morpholino. This shows the strength of using control morpholinos and excludes the delayed development as a relevant *miR-375* loss-of -function phenotype. A moderate version of the phenotype was also observed in embryos injected with a morpholino specifically targeting *miR-375-1*. Thus, a reduction in the level of *miR-375* already disturbs islet integrity. Similar to mouse *miR-1* (15), two *miR-375* copies survived evolution and are expressed similarly in time and space, probably to ensure the high intracellular concentration of *miR-375* necessary to repress many weakly binding targets.

In a forward genetic screen, several mutants were identified with improper development of the endocrine pancreas (35). These mutants fall into three classes: (1) mutants with severely reduced insulin expression; (2) mutants with reduced insulin expression and abnormal islet morphology; and (3) mutants with normal levels of insulin expression and abnormal islet morphology. However, in all of these mutants, islet cells do not merge into an islet from their first appearance at approximately the 14-somite stage. Our *miR-375* knockdown phenotype differs from this, because in the first instance, an islet is formed at approximately 24 hpf, but in later stages, the islet falls apart into small groups of cells. This rules out a general role for *miR-375* in early endocrine formation as is seen for Wnt5 (36), but rather indicates a role in maintenance of tissue identity, which is assumed to be a general function of miRNAs in development (21). It is as yet unclear which *miR-375* targets are involved in the phenotype. Work in cell lines has implicated *miR-375* in insulin secretion by targeting *myotrophin* (26). The zebrafish homolog of *myotrophin* also contains a 7-nucleotide seed match to *miR-375* (unpublished data), but future studies should

reveal whether this target or many other predicted targets are relevant to the phenotype. The specific expression of *miR-375* in the pancreatic islet and its implication in insulin secretion make it a candidate drug target in diabetes, e.g., to influence insulin levels in the blood. However, our data show that if *miR-375* is used as a drug target, developmental side effects need to be taken into account.

Materials and Methods

Morpholino and miRNA injections.
Morpholinos were obtained from Gene Tools LLC (http://www.gene-tools.com) and dissolved to a concentration of 5 mM in water. Morpholinos were injected into one- or two-cell–stage embryos at concentrations between 200 μM and 1,000 μM, and per embryo, one nl of morpholino solution was injected.
RNA oligos (Table S2) were obtained from Sigma (http://www.sigmaaldrich.com) and dissolved to a concentration of 100 μM in distilled water. Oligos were annealed using a 5x buffer containing 30 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, and 50 mM NH_4Ac. Typically, 1 nl of a 10 μM miRNA duplex solution was injected.

Construction of miR-30c and miR-375 GFP reporters and pri-miRNA constructs.
The *miR-30c* and *miR-375* reporter constructs were made by cloning two annealed oligos containing two perfectly complementary miRNA target sites into pCS2 (Clontech, http://www.clontech.com) containing a *gfp* gene between BamHI and ClaI restriction sites. A construct containing pri-*miR-205* was made by amplifying a genomic region (801 base pairs) containing the *miR-205* precursor (*miR-205*-hairpinF ggcattgaattcataaCCTCTTACCTGCATGACCTG; *miR-205*-hairpinR ggcatttctagaGTGTGTGCGTGTATTCAACC). The resulting PCR fragment was cloned between XbaI and EcoRI restriction sites of PCS2GFP. Pri-*miR-375-1* and pri-*miR-375-2* constructs were made by amplifying genomic regions containing *miR-375-1* and *miR-375-2* precursors (WK*miR-375*-1F-pCS2 gcccgggatccTGTGTCTTGCAGGAAAAGAG; WK*miR-375*-1R-pCS2 attacgaattcTCAAACTCTCCACTGACTGC; and WK*miR-375*-2F-pCS2 gcccgggatccGCCCTCCCATTTGACTC; WK*miR-375*-2R-pCS2 attacgaattcAATGAGTGCACAAAATGTCC), and cloning of the resulting PCR fragments into the BamHI and EcoRI sites of pCS2. mRNA was synthesized using SP6 RNA polymerase. Luciferase mRNA was derived from pCS2 containing luciferase between BamHI and EcoRI sites.

In situ hybridization, Northern blotting, and RT-PCR.
In situ hybridization was performed as described previously (37). LNA probes for miRNA detection were obtained from Exiqon (http://www.exiqon.com) and labeled using terminal transferase and DIG-11-ddUTP. cDNA clones for pri-*miR-375-1*, pri-*miR-375-2*, pit1, insulin, somatostatin, and glucagon were used for antisense DIG-labeled probe synthesis by T7 or Sp6 RNA polymerase.
For Northern blotting, total RNA was isolated from ten embryos per sample using Trizol reagent (Invitrogen, http://www.invitrogen.com). RNA was separated on a 15% denaturing polyacrylamide gel. Radiolabeled DNA probes complementary to miRNAs or 5S RNA (atcggacgagatcgggcgta) were used for hybridization at 37 °C. Stringency washes were done twice for 15 min at 37 °C using 2 × SSC 0.2% SDS. Alternatively, DIG-labeled LNA probes were used for hybridization at 60 °C and stringency washes were performed at 50 °C with 2 × SSC 0.1% SDS for 30 min and 0.5 × SSC 0.1% SDS for 30 min.
For RT-PCR, RNA was isolated with Trizol, treated with DNAse (Promega, http://www.promega.com) and subsequently purified again using Trizol. cDNA was made with a poly dT primer. Primers used for amplification were *miR-205*-hairpinF and *miR-205*-hairpinR, and lucF (ATGGAAGACGCCAAAAACATAAAG) and lucR (ATTACATCGATTTACACGGCGATCTTTCC).

Alcian Blue and Daspei staining.
For Alcian Blue staining, embryos were fixed for 1 h at room temperature in 4% PFA in PBS, rinsed for 5 min in 50% MeOH and stored overnight in 70% MeOH at 4 ºC. Next, embryos were incubated for 5 min in 50% MeOH and for 5 min in 100% EtOH. Embryos were stained at room temperature with Alcian Blue (Sigma) for 90 min with continuous shaking. Subsequently, embryos were rinsed in 80%, 50% and 25% EtOH for 2 min each and two times in water containing 0.2% Triton and neutralized in 100% Borax solution. Finally, embryos were incubated for 60 min in digest solution (60% Borax solution, 1 mg/ml colleganase-free and elastase-free trypsin, 0.2% trypsin) and stored in 70% glycerol.

Staining of the hair cells was done by incubating live embryos for 5 min in a 200 µM solution of Daspei (Sigma) in + chorion. After rinsing twice in + chorion, embryos were anesthetized using MS222 and mounted in methylcellulose.

Acknowledgments

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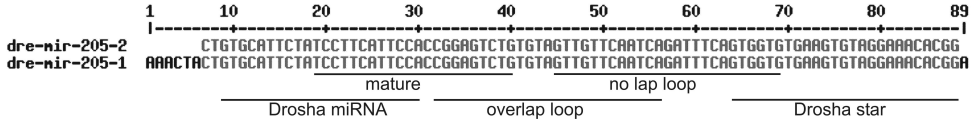
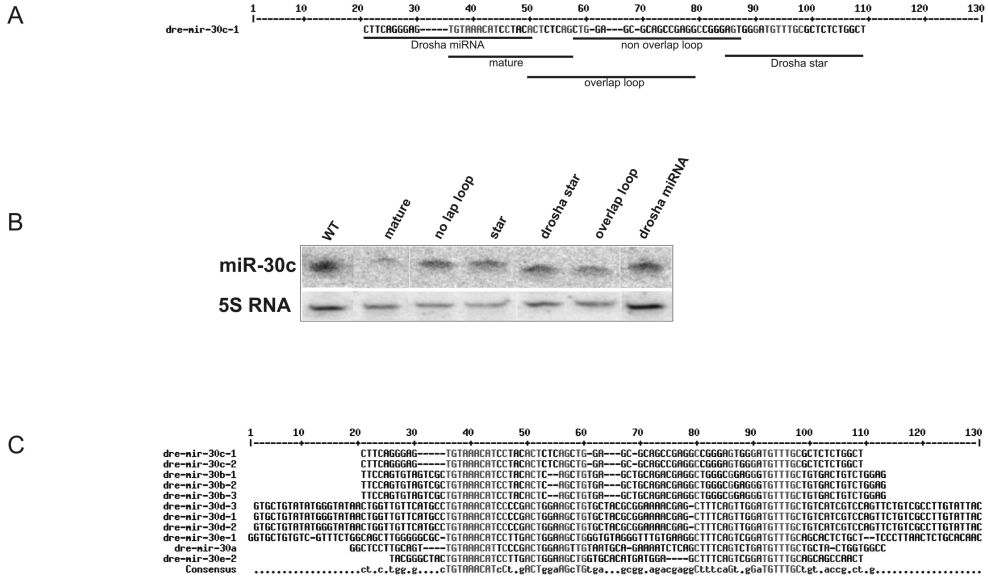
Supplementary data



Supplementary Figure 1. Design of Morpholinos Targeting the *miR-205* Precursor



Supplementary Figure 2. Morpholino-mediated knockdown of *miR-30c.* (A) Design of morpholinos targeting the *miR-30c* precursor. (B) Northern analysis of *miR-30c* expression in 24-h-old embryos injected with different morpholinos targeting the *miR-30c* precursor. (C) Alignment of the precursor of *miR-30* family miRNAs.

CHAPTER 7

General discussion

The importance of miRNAs for development and life of multicellular organisms is clear from several points. First, animals without miRNAs cannot live (1-3). Second, there are hundreds of miRNAs expressed and many of these are conserved and expressed at very high levels, with striking tissue-specific distributions (4, 5). Third, miRNAs target thousands of mRNAs and miRNA target sites are often conserved (6).

When examining the conservation patterns of miRNA genes, one will immediately notice that many mature miRNA sequences are almost perfectly conserved, sometimes from worms up to humans (e.g. *let-7*, *miR-1*). In chapter 2 of this thesis, we determined the sequence requirements for *let-7* mediated silencing and saw that mutations in the first 1-8 bases were most detrimental to *let-7* function. Also one point mutant *let-7* allele in *C. elegans*, that was discovered in a forward genetic screen, has a mutations at position 5 from the 5' end (7). But why have all 22 nucleotides in *let-7* been perfectly maintained in evolution, if only the miRNA seed is required for activity? The most obvious answer is of course that mutations in other parts of the miRNA might affect processing or strand selectivity of the miRNA duplex. We have created a mutant zebrafish with a mutation in mature *miR-222* at nucleotide 20 from the 5' end. The levels of mature *miR-222* from this allele are severely reduced, but instead the miRNA star sequence (the other arm of the stem) is highly expressed (Figure 1). This can be explained by the change in the binding energy of the duplex free ends. The mutation makes the end of the duplex which contains the 5' end of the miRNA star, more flexible and this favors stabilization of the miRNA star sequence (8). Similarly, a SNP at position 8 of human *miR-125a* prevents processing of pri-*miR-125a* to pre-*miR-125a* (9). Possibly this SNP destabilizes the miRNA stem-loop, which disables recognition by the Drosha protein complex. In addition, this *miR-125a* SNP reduces miRNA mediated translational repression (9).

Although this explains at least part of the question why mature miRNA sequences are so strongly conserved, it does not explain, why there are so many miRNAs, which do slightly differ in their mature sequences and which reside in families of related sequence. Intuitively one would expect that these family members are derived from a common ancestor as a consequence of duplication events, rather than independent convergent evolution. Subsequently, these duplicated miRNAs randomly acquired some mutations, which made them differ from their ancestor. However, the ancestor remained unchanged, ensuring that its function was maintained. Apparently, the mutated duplications did not need to act together with their 'parent miRNA', which allowed them to gain mutations. Nevertheless, at a certain point these mutated duplicates got fixed in evolution, probably because they acquired their own function or they started to act together with their parent miRNA. For example, comparing

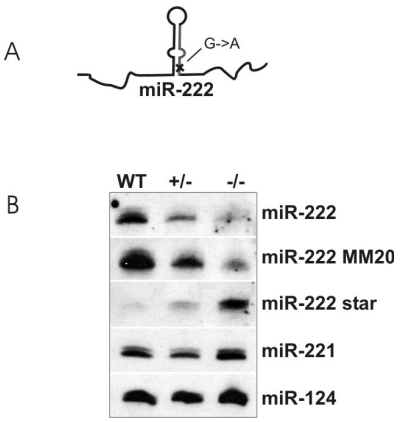


Figure 1. Effects of a single point mutation on *miR-222* expression. (A) Using reverse genetics, a *miR-222* allele was isolated with a G to A transition at position 20 from the *miR-222* 5' end. (B) Northern analysis of *miR-222*, *miR-222* star, *miR-221* and *miR-124* in wildtype, heterozygous and homozygous *miR-222* mutants.

miR-183, *miR-182* and *miR-96*, which are located in a cluster, immediately shows that there are some small variations between them (Table 1). However, each of these variants is perfectly conserved from zebrafish to human. In the first place this could mean that family members are completely redundant. Alternatively, this could mean that it is advantageous to maintain one copy (or in some cases several copies, e.g *miR-124*, *let-7a*, *miR-375*, *miR-9*) without changing it, while allowing mutations in other duplications, to fine-tune target repression combining all variants together. Finally, it could also suggests that the members within a miRNA family have a somewhat different array of target mRNAs and acquired new functions compared to the miRNA they have been originating from, especially in those instances where mutations occurred in the miRNA seed.

Another question, related to redundancy, emerges when thinking about the number of identical copies that are present in the genome for some miRNAs. At least for *miR-1* in mouse and *miR-375* in zebrafish it is known that both copies are expressed similarly in time and space (10) (Chapter 6). Although this suggests that these miRNAs have redundant functions, eliminating *miR-1-2* in mouse has dramatic consequences for the developing heart (10). Thus *miR-1-1* cannot fully compensate for the loss of *miR-1-2*. Similarly, loss of only *miR-375-1* in zebrafish already causes pancreatic islet defects in presence of *miR-375-2* (Chapter 6). Apparently, every miRNA requires its own 'miRNA pressure' to ensure the exact degree of target repression. Since every miRNA has hundreds of target mRNAs and maybe even more target sites, there is not necessarily an excess of miRNA molecules or a pool of free miRNAs. Most likely all miRNA molecules are bound to target sites. Also, the variation in the sequence of miRNA family members might help in fine tuning the expression of target genes. Members of the mixed pool of these related miRNAs could all recognize the same targets by seed pairing, but they could vary a little in the overall degree of complementarity, and together, they could provide the required degree of regulation.

Table 1. Alignment of *miR-96*, *miR-182* and *miR-183* between mouse, zebrafish and human

miRNA	sequence
mmu-miR-182	UuUGGCAaUgGuAgAacUcaca
mmu-miR-96	UuUGGCAcUaGcAcAuuUuugcu
mmu-miR-183	UaUGGCAcUgGuAgAauUcacug
hsa-miR-182	UuUGGCAaUgGuAgAacUcaca
hsa-miR-96	UuUGGCAcUaGcAcAuuUuugcu
hsa-miR-183	UaUGGCAcUgGuAgAauUcacug
dre-miR-182	UuUGGCAaUgGuAgAacUcaca
dre-miR-96	UuUGGCAcUaGcAcAuuUuugcu
dre-miR-183	UaUGGCAcUgGuAgAauUcacug
mmu-miR-96	UUUGGCACUAGCACAUUUUUGCU
hsa-miR-96	UUUGGCACUAGCACAUUUUUGC
dre-miR-96	UUUGGCACUAGCACAUUUUUGCU
mmu-miR-182	UUUGGCAAUGGUAGAACUCACA
hsa-miR-182	UUUGGCAAUGGUAGAACUCACA
dre-miR-182	UUUGGCAAUGGUAGAACUCACA
mmu-miR-183	UAUGGCACUGGUAGAAUUCACUG
hsa-miR-183	UAUGGCACUGGUAGAAUUCACUG
dre-miR-183	UAUGGCACUGGUAGAAUUCACUG

The first wave of miRNA discovery and target prediction is now followed by a second flood of information concerning the elucidation of miRNA functions. The overall consensus is that miRNAs dampen the expression of genes that do not need to be expressed in a certain cell type (11, 12). It has been proposed that miRNAs might confer robustness to gene expression networks by buffering stochastic changes (12, 13). In many cases, there is a large number of target genes, which are all moderately regulated by the miRNA via a single target site (14). However, there are exceptions to this. For example, *let-7* regulates *lin-41* in *C. elegans* via multiple target sites in the *lin-41* 3'UTR and the repressive effect is very strong (7, 15, 16). This single interaction is very important for developmental transitions in the worm, since mutations in *let-7* can be rescued by mutations in *lin-41* (16). That miRNAs do not only function as a fail-safe mechanism to repress leaky transcripts that are supposed to be mutually exclusively expressed with the miRNA and primarily regulated at the transcriptional level, is clear from the severe developmental consequences of *miR-1-2* disruption in mouse (10).

To really understand the complete network of miRNA mediated regulation we need to develop methods to disrupt the interaction between a miRNA and a single target. This relies strongly on a recurrent issue in the miRNA field, which is the reliable prediction of miRNA targets. Conserved seed pairing is a general reliable predictor of miRNA regulation (6). However, identical sites behave differently within differ-

ent genomic contexts (17). Probably, a stable environment with a low ΔG makes the target site less accessible to the miRNA, compared to an unstable environment (10, 18). Thus, incorporating structural characteristics of the miRNA target will improve target identification (19). In addition to secondary structure of the target site context, there are also conditional requirements for the susceptibility of a target site. For example, expression of the zebrafish *nanos* gene is only silenced in the soma, but not in germ cells (20). Another study has shown that stress conditions can relieve the *CAT-1* mRNA from miR-122 mediated repression (21). The relieve is accompanied by release of the mRNA from P-bodies and requires binding of an AU-rich element binding protein.

Genomic disruption of miRNA target sites would be a laborious but powerful approach to analyze the significance of single miRNA-target interactions. In zebrafish, this is still practically impossible. As an alternative, we managed to block miRNA target sites by injection of morpholinos complementary to the complete target site or part of the target site. The disadvantage of morpholinos is that they only provide a short time window of about 3 days in which the effect should be visible. However, for some early expressed miRNAs, such as *miR-430*, this will be a very quick and strong approach to investigate the individual contributions of miRNA-target interactions in the miRNA-target interaction network.

Although the consequences of miRNA disruption are now slowly accumulating (Chapter 1), changing a single miRNA target site may also have huge phenotypic effects (22, 23). For example, Texel sheep have exceptional muscularity and they contain a G to A substitution in the 3'UTR of the *myostatin* gene, which is associated with this phenotypic trait (23). Loss of *myostatin* causes muscle doubling in mice and human. The substitution creates a target site for *miR-1* and *miR-206*, which are both strongly expressed in muscle, which is also the primary site of myostatin expression. Consequently, *myostatin* expression is reduced in Texel sheep, demonstrating that gain of a single miRNA target site may have profound phenotypic consequences. That this is not a sheep specific phenomenon is indicated by the finding of a G to A substitution in the 3'UTR of the human *slitrk1* gene that is associated with Tourette's syndrome (22). This polymorphism located within the predicted binding site for *miR-189*, replaces a G:U wobble base pair with an A:U pairing, which confers stronger regulation of *slitrk1*.

Probably there are many more polymorphisms associated with miRNA target sites. Examining a large panel of 92.967 human SNPs located in human 3 'UTRs, revealed that 8.354 SNPs (8.9%) were affecting the miRNA target site content of 7.406 genes, either creating or destroying a target site (24). Comparing these true SNPs with a set of randomly simulated SNPs, showed that there are much less true SNPs in conserved target sites compared to random simulated SNPs. Also SNP density is much lower in regions matching to the miRNA seed compared to the rest of the site (25). This indicates that there is negative selection against these SNPs in conserved sites,

due to phenotypic consequences that affect individual fitness.
Differences in gene expression patterns and levels between individuals within a species and between species are thought to be causal to phenotypic variation (26). There are several major mechanisms that an organism uses to regulate gene expression (26). The classical way involves transcription factors, which have been studied extensively for many decades. At the posttranscriptional level, miRNAs are controlling translational and mRNA stability in multicellular organisms. Although miRNAs themselves are strongly conserved, miRNA target seem to have evolved much more rapidly (27). This is reasonable since each miRNA may regulate hundreds of targets and changing the miRNA would have a much bigger impact compared to a mutation in a single target site (27).
In conclusion, variations in miRNA target sites might be a source of phenotypic variation. Unraveling the complete miRNA regulatory network including combinatorial regulation will be a tremendous task. Refined prediction programs, careful expression analysis and high throughput tools for miRNA inhibition, will help us to built a complete picture of miRNA-mediated gene regulation also in relation to transcriptional control. This will also contribute to our understanding of the genetic changes in miRNA targets that underlie disease.

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Samenvatting

Erfelijk materiaal

DNA, het erfelijk materiaal, is opgebouwd uit vier bouwstenen: moleculen die worden aangeduid met de letters A (adenine), T (thymine), C (cytosine) en G (guanine). De lettervolgorde en structuur van DNA bepalen voor het overgrote deel de zichtbare en niet zichtbare kenmerken van plant, dier en mens, maar uiterlijke omstandigheden hebben ook grote invloed op de ontwikkeling van een organisme.
Een gen is een stukje DNA dat wordt gekenmerkt door een specifieke volgorde van deze bouwstenen. De mens heeft er ongeveer 20.000, evenals de muis, de zebravis en de worm. Genen kunnen worden aan- en uitgeschakeld, ze kunnen zelfs in verschillende gradaties actief zijn net als een dimmer op de lichtschakelaar. Actief betekent in dit geval dat er een boodschapperRNA (mRNA) wordt gemaakt met dezelfde lettervolgorde als het gen. Het mRNA brengt een boodschap van het DNA in de celkern naar buiten. Buiten de celkern wordt het mRNA vertaald naar een eiwit. Eiwitten zijn actief bij allerlei processen, van wondherstel en celdeling tot het afbreken van suiker. De meeste genen zijn alleen actief in specifieke weefsels of celtypen. Gedurende de embryonale ontwikkeling worden dus voortdurend verschillende genen actief in verschillende cellen.

Het besturen van de activiteit van genen

Een gen is alleen actief als de cel dat ook toestaat. Het aan- of uitschakelen van een gen kan worden geregeld door in meer of mindere mate de vorming van het mRNA toe te laten. Het kopiëren van de informatie van een gen naar een mRNA wordt dan gereguleerd. Een jaar of 7 geleden is er een ander proces ontdekt, dat de activiteit van genen reguleert. Hierbij wordt de vertaling van een mRNA naar een eiwit aan banden gelegd door kleine RNA moleculen (microRNA's), die zelf niet in eiwit vertaald kunnen worden.

microRNA's

De eerste microRNA werd pas in 1993 ontdekt in het rondworpje *Caenorhabditis elegans*. Tot dan toe waren ze altijd over het hoofd gezien, omdat ze zo klein zijn. In 2000 werd er nog een microRNA, met de naam *let-7*, ontdekt in *C. elegans*. Het bijzondere is nu dat precies dezelfde *let-7* microRNA niet uniek is voor wormen maar ook voorkomt bij de mens en de meeste andere dieren, zoals bijvoorbeeld de fruitvlieg of de zebravis. Door gericht te zoeken naar kleine RNA moleculen werden er nog honderden microRNA's gevonden. Velen daarvan zijn bewaard gebleven gedurende de evolutie van worm tot mens. Dat geeft aan dat ze belangrijk zijn. Het is inmiddels bekend dat microRNA's zich kunnen hechten aan het mRNA en op die

manier de vertaling van het mRNA naar een eiwit kunnen verstoren. Een microRNA werkt dus als een soort rem op het mRNA.

Werkzaamheid van microRNA's

In hoofdstuk 2 worden experimenten beschreven die aantonen welk deel van de microRNA het belangrijkst is voor zijn activiteit. Deze experimenten zijn verricht door gebruik te maken van een *in vivo* systeem, namelijk het zebravissen embryo. In de pas bevruchte eicel kan een beetje GFP (groen fluorescerend eiwit)-mRNA worden gespoten, waar een microRNA aan vast kan hechten. In afwezigheid van de microRNA is het GFP-mRNA actief en wordt het embryo knalgroen. In aanwezigheid van de microRNA is het GFP-mRNA niet actief, omdat de microRNA eraan vast hecht, en dan is er geen groene fluorescentie. Vervolgens is elk van de 22 bouwstenen van de microRNA veranderd in een andere willekeurige bouwsteen en is er gekeken of de veranderde microRNA nog steeds het GFP-mRNA kan remmen. Hieruit blijkt dat een microRNA niet meer werkzaam is na een verandering in één of meer van zijn eerste 8 bouwstenen. Deze eerste 8 bouwstenen van een microRNA wordt de "seed" genoemd. In het algemeen kan worden gesteld dat de "seed" het belangrijkste gedeelte is van de microRNA voor het hechten aan de mRNA.

Expressie van microRNA's

Wat doen die microRNAs nu precies en bij welke processen zijn ze betrokken? Om daar achter te komen is het allereerst nodig vast te stellen in welke celtypen elke individuele microRNA zich bevindt. In hoofdstuk 3 wordt een methode beschreven om microRNAs zichtbaar te maken in een embryo. Daarbij is gebruik gemaakt van LNA's (Locked Nucleic Acid). LNA lijkt op DNA en het kan heel goed en specifiek hechten aan een complementair RNA, dus ook een microRNA. Net zoals een puzzelstukje maar op één plaats in een puzzel past, kan elke LNA maar aan één enkele microRNA plakken, waarbij de bouwsteen A plakt aan T en C aan G. Door de LNA te voorzien van een gekleurd labeltje, wordt de microRNA indirect zichtbaar wanneer de LNA zich eraan vasthecht.
Van deze methode is gebruik gemaakt in hoofdstuk 4. Voor ongeveer 115 microRNA's van zebravissen, die allemaal ook bij de mens voorkomen, is bepaald waar ze zich in het embryo bevinden. Het *let-7* microRNA zit bijvoorbeeld in de hersenen, *miR-206* in de spieren en *miR-122* in de lever van het embryo. Elke microRNA heeft zijn eigen specifieke expressie patroon en dezelfde microRNA's komen ook bij ons in dezelfde weefsels voor.

Nieuwe microRNA's

Hoofdstuk 5 gaat verder in op het bepalen van de weefselspecifieke patronen van nieuwe microRNA's. Uit embryo's en volwassen vissen zijn kleine RNA moleculen

geïsoleerd en op die manier zijn nieuwe microRNA's opgespoord. Veel van die nieuwe microRNAs zijn niet zo wijdverspreid bewaard gebleven in de evolutie en sommigen komen alleen maar voor in de zebravis. Het lijkt erop dat ze nog niet zo lang geleden zijn ontstaan. Het is ook een stuk moeilijker om die nieuwe microRNA's op te sporen en zichtbaar te maken, omdat ze in kleinere aantallen worden gemaakt.

Functies van microRNA's

Nu precies in kaart is gebracht welke microRNA's er zijn en in welke weefsels ze zich bevinden, weten we van elke microRNA waar het actief is. Tijdens de embryonale ontwikkeling van de lever is *miR-122* belangrijk en bij de ontwikkeling van sterke spieren is *miR-206* nodig. Om aan te tonen dat microRNA's ook daadwerkelijk onmisbaar zijn voor de ontwikkeling van weefsels en organen, moeten ze stuk voor stuk worden uitgeschakeld. In hoofdstuk 6 wordt een methode beschreven om de activiteit van microRNA's plat te leggen met behulp van zogenaamde morpholino's. Voor elke microRNA hebben we een specifieke morpholino gemaakt die de microRNA gericht kan uitschakelen gedurende de eerste paar dagen van de embryonale ontwikkeling. Voor *miR-375* kon een heel duidelijk effect op de ontwikkeling van de eilandjes van Langerhans worden waargenomen. Dat zijn de cellen die insuline uitscheiden. Als *miR-375* uitgeschakeld wordt met een morpholino vormt zich eerst nog een eilandje, maar dat valt uit elkaar met het vorderen van de ontwikkeling. Blijkbaar remt *miR-375* specifieke mRNA's in de eilandjes van Langerhans, zodat er een mooi compact groepje insuline uitscheidende cellen wordt gevormd. Dit proces raakt verstoort als *miR-375* uit wordt geschakeld. Natuurlijk is dit nog maar een tipje van de sluier. Het is nog niet eens bekend welke mRNA's door *miR-375* worden geremd in hun activiteit. Bovendien zijn er nog honderden andere microRNA's die stuk voor stuk bestudeerd moeten worden om hun functie te achterhalen.

Dankwoord

De Groningse volkszanger Ede Staal zong ooit: "'t het nog nooit, nog nooit zo donker west, of 't wer altied wel weer licht". Inderdaad, als je nog midden in je promotie onderzoek zit en er mislukt weer een experiment na weken van voorbereiding, dan zakt de moed je wel eens in de schoenen. Maar achteraf gezien was het eigenlijk best een relaxte tijd. Gewoon van maandag tot vrijdag proefjes doen, soms ook in het weekend. Verder regelmatig een borreltje op het werk of een congresje in het buitenland; lekker skiën in de Rocky Mountains in de baas zijn tijd, wat wil een OIO nog meer? Aan de andere kant houdt het onderzoek je wel bezig, ook buiten het werk om. Elke keer als ik een groen stoplicht zie denk ik aan GFP (Green Fluorescent Protein)-vissen en bij een rood stoplicht aan RFP. Ik heb de Matthäus Passion gezongen en steevast als ik de frase "Wenn ich einmal soll scheiden, so scheide nicht von **mir**" zong, moest ik weer aan microRNA's, afgekort miR, denken. Ook als ik in mijn orkestpartij bijvoorbeeld het maatcijfer 124 zie staan, krijg ik onmiddellijk het beeld voor mijn ogen van het expressie-patroon van *miR-124* (komt tot expressie in hersenen en ruggenmerg).

Dankzij mijn vader ben ik vier jaar geleden op het Hubrecht lab terecht gekomen. Ik was toen bijna klaar met mijn studie en pa zei: "Ga eens een kijkje nemen bij Ronald Plasterk, een uitstekend columnist en volgens mij ook moleculair geneticus". Ik voegde daad bij deze raad, hoewel ik nog nooit van alleskunner Ronald had gehoord. Met drie emailtjes en een kort bezoek aan het Hubrecht Instituut was mijn aanstelling een feit.

Het was een hele overgang van het zorgeloze en overzichtelijke studentenleven in het Noorden naar de harde werkelijkheid van het OIO-bestaan in Utrecht. Heit en mem zorgden gelukkig altijd voor een veilige thuishaven als ik de drukke stad even wilde ontvluchten.

Ronald, gedurende drie-en half jaar ben jij, naast al je drukke maatschappelijke en wetenschappelijke activiteiten, mijn begeleider geweest. Vooral je snelheid van handelen heb ik erg gewaardeerd. De eerste meeting in Keystone, waar jij me mee naar toe nam, was een hele belevenis, ook al heb ik toen mijn duim zo ernstig gekneusd tijdens het skiën dat ik een half jaar lang met links heb moeten pipetteren. Ik vind het jammer dat je mijn promotor niet kunt zijn.

Na een wat stroeve start - de eerste vier maanden nog geen Science paper - verliep het onderzoek best goed. René en Titia gaven me tijdens mijn eerste schreden op het lab vaak hele nuttige tips om de proefjes geslaagd te laten verlopen.

Maar vooral dankzij de niet aflatende inspanningen van Ewart stroomden de resultaten binnen. De hele wereld stond versteld van de mooie expressie-patronen die Erno, jij en ik produceerden. Samenwerken met jou was erg gezellig, vooral op vrijdagmiddag dechorioneren onder invloed van Hooghoudt Dubbele Graan Jenever. Zelfs met een stramme pet klaarde jij het klusje nog sneller dan ik.

Florian en ik hebben dat *in situ* werk later nog eens dunnetjes overgedaan. Het was misschien een beetje een mosterd-na-de-maaltijd-project, maar het heeft toch geresulteerd in een mooi verhaal, waar we trots op kunnen zijn. Verder zijn jouw snowboardkunsten ongeëvenaard. Ik krijg nog steeds pijnlijke huiveringen bij de gedachte aan die ene afdaling tijdens de meeting in Breckenridge, waarbij mijn linkerski linksom de boom wilde en mijn rechterski rechtsom.

Ook mijn studenten Anne, Hsin-Yi en Laurens hebben allemaal een steentje bijgedragen aan het onderzoek, dank daarvoor.

Mijn laatste serieuze proeven heb ik samen met Sam uitgevoerd. Wij hebben in de pauzes ook geëxperimenteerd met het beleggen van boterhammen en wel op zodanige wijze, dat het haast onmogelijk is dat onze aderen niet zijn dichtgeslibd met het vet van kilo's roomboter en reuzachtige stukken kaas. Ik hoop dat er nog een leuk verhaal komt uit ons microRNA-target-blokkerende-morpholino's-projectje!

Naast het proeven doen werd er op woensdagmiddag vaak gevoetbald ter ontspanning. Het waren echt geen vriendschappelijke wedstrijdjes. We wilden elkaar altijd in de pan hakken. Ondanks alle bloeddorstige aankondigingen heeft gelukkig niemand ooit gebruik gemaakt van schoenen met stalen noppen of scherpe punten. Bas en de andere voetballers, bedankt voor alle voetbalmiddagen!

Ook het racefietsen was een bron van ontspanning. Kobus, de door jou in het wielrenners-jargon geintroduceerde termen 'zakrot' en 'zwanus' zal ik nooit vergeten.

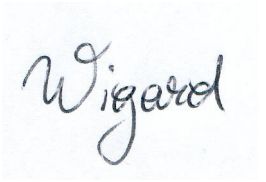
Maar echt hard fietsen deed ik eigenlijk alleen samen met mijn dierbare broers Tomas en Menno. De supersonische snelheden die wij haalden met ons Cipollini-treintje of afdalend van 'La Madeleine' doen, wanneer ik eraan terugdenk, mijn hart nog steeds op hol slaan.

Tenslotte was er nog een andere activiteit om de pijn van mislukte experimenten te verzachten. Wat dat betreft was het wel een cultuurschok voor mij om te beginnen op het Hubrecht Insitituut. Ik was tijdens mijn studententijd weliswaar al een notoire zuiplap, maar de drinkmentaliteit die ik op het Hubrecht aantrof sloeg werkelijk

alles. Elke mogelijkheid voor een borrel werd benut. Ik was nog maar nauwelijks een jaar in dienst of het drankspektakel bereikte een hoogtepunt met de augurkje-wodkaatje-borrel. Daar maak ik verder geen woorden meer aan vuil...

Waar je als OIO in het eerste jaar nog volledig in beslag wordt genomen door pipetteren, raak je mettertijd steeds verder verwijderd van het lab. Er moeten papers worden geschreven, studenten worden begeleid en presentaties worden gemaakt. Waar het administratieve zaken betrof, was Ira onmisbaar. Voor het corrigeren van de grammaticale missers in enkele van de manuscripten heb ik hulp gehad van oom Gerrit.

Bijna aan het eind van mijn promotie tijd diende er zich op de valreep nog een groots ontwikkelingsbiologisch experiment aan. Ik weet zeker dat deze proef een fenomenaal resultaat zal opleveren. Agnes, mijn liefste, we krijgen samen een kleintje, dat is zeer bijzonder, ik kijk er naar uit!



Curriculum vitae

Wigard Kloosterman is geboren op 29 mei 1979 te Emmen. Hij behaalde in 1997 zijn VWO diploma aan de Ubbo Emmius Scholengemeenschap te Stadskanaal. Na een jaartje werken als productie medewerker, begon hij in 1998 met de studie biologie aan de Rijksuniversiteit Groningen. Tijdens de afstudeerfase liep hij stage bij de vakgroep Microbiële Fysiologie (Prof. Dr. L. Dijkhuizen) en Tumor Immunologie (Prof. Dr. W. Helfrich). In 2003 studeerde Wigard *cum laude* af met als afstudeerrichting Biotechnologie. In augustus van dat jaar begon hij met zijn promotie onderzoek in de groep van Ronald Plasterk aan het Hubrecht Laboratorium te Utrecht. De resultaten daarvan staan beschreven in dit proefschrift. Vanaf augustus 2007 is hij werkzaam binnen de Research & Development afdeling Genetics van de DSM Anti-Infectives Business Group in Delft.

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