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miRNAs associated with immune response in teleost fish

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ABSTRACT

MicroRNAs (miRNAs) have been identified as important post transcriptional regulators of gene expression. In higher vertebrates, a subset of miRNAs has been identified as important regulators of a number of key genes in immune system gene networks, and this paper review recent studies on miRNAs associated with immune response in teleost fish. Challenge studies conducted in several species have identified differently expressed miRNAs associated with viral or bacterial infection. The results from these studies point out several miRNAs that are likely to have evolutionary conserved functions that are related to immune response in teleost fish. Changed expression levels of mature miRNAs from the five miRNA genes miRNA-462, miRNA-731, miRNA-146, miRNA-181 and miRNA-223 are observed following viral as well as bacterial infection in several teleost fish. Furthermore, significant changes in expression of mature miRNAs from the five genes miRNA-21, miRNA-155, miRNA-1388, miRNA-99 and miRNA-100 are observed in multiple studies of virus infected fish while changes in expression of mature miRNA from the three genes miRNA-122, miRNA-192 and miRNA-451 are observed in several studies of fish with bacterial infections. Interestingly, some of these genes are not present in higher vertebrates. The function of the evolutionary conserved miRNAs responding to infection depends on the target gene(s) they regulate. A few target genes have been identified while a large number of target genes have been predicted by *in silico* analysis. The results suggest that many of the targets are genes from the host's immune response gene networks. We propose a model with expected temporal changes in miRNA expression if they target immune response activators/effector genes or immune response inhibitors, respectively. The best way to understand the function of a miRNA is to identify its target gene(s), but as the amount of genome resources for teleost fish is limited, with less well characterized genomes and transcriptomes, identifying the true target genes of the miRNAs associated with the immune response is a challenge. Identifying such target genes by applying new methods and approaches will likely be the next important step to understand the function of the miRNAs associated with immune response in teleost fish.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that down regulate gene expression at the post-transcriptional level (Bartel, 2004; Chekulaeva and Filipowicz, 2009). The primary miRNA transcripts are processed by endonucleases into two short mature miRNAs originating from the 5′ and the 3′ end of the precursor miRNA (Meister and Tuschl, 2004). The miRNAs originating from the 5′ end of a precursor miRNA are labelled with the suffix -5p and the miRNAs originating from the 3' end with the suffix -3p (Griffiths-Jones et al., 2006; Ambros et al., 2003). These short miRNAs, typically 21–24 nucleotides in length, are assembled into the miRNA-induced silencing complex (miRISC), a ribonucleoprotein complex that consists of Argonaute proteins as well as other effector proteins (Krol et al., 2010). Often only one of the two miRNAs (either -5p or -3p) from the precursor miRNA is incorporated into the miRISC while the other is degraded. In such cases the functionally active miRNA from a given precursor miRNA is often labelled the mature miRNA while the other is called the star miRNA (miRNA*) (Krol et al., 2010). The function of the mature miRNA is to guide the miRISC to the target transcript, the mRNA transcript that is negatively regulated by miRISC (Hausser and Zavolan, 2014).

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Identification of a target transcript depends on partial base-pairing between the miRNA and the target sequence (target site) that usually is located in the 3′ UTR of the target mRNA. The most important part of the miRNAs that contributes to the recognition of target mRNAs are the nucleotides 2–8 in the 5′ end, the miRNA seed sequence (Lewis et al., 2005; Bartel, 2009; Hausser and Zavolan, 2014). Typically in animals, there is a perfect complementarity between the seed sequence and the target site of the mRNA followed by some incomplementarities (bulges) before some additional complementarity between the 3′ end of the miRNA and the target site (Hausser and Zavolan, 2014). Target sites located in the 3′ UTRs of the target transcripts and with perfect seed matches are often referred to as canonical target sites. Computational methods that aim to predict which genes are regulated by a particular miRNA typically identify canonical target sites (Peterson et al., 2014). However, there are examples of target sites in animal mRNAs where there is an absence of perfect seed pairing that is compensated by e.g. extensive pairing of the 3' end of the miRNA to the target sequence (non-canonical target sites) (Pasquinelli, 2012).

Thousands of different miRNA genes have been identified in vertebrates (Kozomara and Griffiths-Jones, 2011). As the short seed sequence is the most important part of the miRNA when it comes to recognizing target transcripts the miRNAs may potentially regulate a very large number of genes. Based on predictions of canonical target sites in the transcriptome as much as 60% of all protein coding genes may be regulated by miRNAs in mammals (Friedman et al., 2009). In vertebrates, miRNAs act as key *trans* acting factors that regulate gene networks controlling fundamental biological processes like growth, immune response, tissue development and maintenance of tissue specific functions (Bushati and Cohen, 2007; Lagos-Quintana et al., 2002; Stefani and Slack, 2008; Friedman et al., 2009; Sonkoly, Stahle, and Pivarcsi, 2008). There are also examples that intronic miRNAs, miRNA genes located in the intron of a protein coding gene (host gene), target the host gene transcript (*cis*-regulation) (Gao et al., 2012). Post-transcriptional regulation by miRNAs results in down regulation of the protein expression from the target mRNAs. This may be caused by increased degradation of the target mRNA or repression of translation (with no change in target mRNA level) (Selbach et al., 2008; Pasquinelli, 2012; Wilczynska and Bushell, 2015).

Most orthologous miRNAs are highly conserved among vertebrates with limited sequence variation particularly in the seed (Bartel, 2009). Also, there are often several very similar paralogs of a miRNA gene in a species. The sequences of the mature miRNAs and precursor miRNAs are used to annotate miRNAs into different families using lettered suffixes (Ambros et al., 2003). As a consequence of having identical seed sequences many orthologous miRNAs are expected to have similar preferences for target sites (Lewis et al., 2005). Although most of the miRNAs are highly conserved there are examples of orthologous miRNA genes that have evolved differently. One such miRNA gene-pair in that has evolved differently is the teleost miRNA genes miRNA-462 and miRNA-731 that share a common ancestry with miRNA genes miRNA-191 and miRNA-425 in humans (Schyth et al., 2015; Andreassen et al., 2013; Li et al., 2010). While these clustered miRNA genes are involved in immune response in Rainbow trout, they do not seem to have such functions in humans where they are involved in cell cycle control. There are also miRNA genes that are specific to teleosts and absent in higher vertebrates, e.g. miRNA-734 and miRNA-7132 (Yang et al., 2015; Yan et al., 2012; Andreassen et al., 2013). A few miRNA genes have been discovered in single species only and are often referred to as species specific miRNAs (Griffiths-Jones et al., 2006; Pasquinelli et al., 2000). Several of the conserved miRNA genes are closely located in the genome (clustered) and such clusters are often conserved in evolution. There are e.g. 26 miRNA gene clusters conserved in species like Atlantic salmon, Atlantic cod, zebrafish and humans (http://www.mirbase.org/) (Chen et al., 2005; Andreassen et al., 2013; Andreassen et al., 2016).

The sequences of the mature miRNAs define which genes they regulate. Therefore, a prerequisite to study miRNA regulation is to have access to the sequences of all the biologically active mature miRNAs in each species. Applying recent advances in sequencing technology and bioinformatics tools, e.g. (Friedlander et al., 2008; Li et al., 2012), the mature miRNAs and their corresponding miRNA genes have been characterized in a large number of commercially important teleost species (e.g. (Xu et al., 2013; Bizuayehu et al., 2013; Andreassen et al., 2013; Ma et al., 2012; Bizuayehu et al., 2015; Andreassen et al., 2016; Fu et al., 2011; Yan et al., 2012; Zhu et al., 2012)). Currently, there are miRNAs from nine teleost fish species in miRBase (v.21) (http://www.mirbase.org/), the main database for miRNAs and miRNA genes. The number of species and the number of miRNA genes discovered in each of the species are low, but the numbers are expected to increase when high throughput methods are applied to search for miRNAs expressed in different tissues and developmental stages, e.g. (Bizuayehu et al., 2015; Andreassen et al., 2016; Juanchich et al., 2016).

miRNA studies may provide knowledge on how gene networks of fundamental biological processes are regulated. Given the important regulatory roles of miRNAs it is not surprising that disruption of miRNA function may cause disease, and the effect miRNAs have on health have been extensively studied in humans as well as in model organisms (Jiang et al., 2009; Esquela-Kerscher and Slack, 2006). In commercially important teleost species research on miRNAs have also included studies on how miRNAs affect economically interesting traits like growth or food conversion. To understand the interaction between miRNAs and genes that affect health or other interesting traits it is necessary to identify the subset of miRNAs that participate as key regulators. In addition to help understand regulation of gene networks, there is a potential for therapeutic use of such key regulator miRNAs to combat infection (Jackson and Linsley, 2010). Knowledge of naturally occurring variation in these miRNA genes or their target sites that affect traits like health and growth may also be used to perform marker assisted selection in breeding programs.

A common approach used in the initial search for miRNAs affecting e.g. health is to identify miRNAs that show differential expression in a challenged group (a group of individuals that have been exposed to a pathogen). This may be the miRNAs that are e.g. important in immune responses (Liang et al., 2007; Guo et al., 2014). Next, the particular mRNA transcripts targeted and gene networks that are regulated need to be identified, and finally, further functional studies must be conducted to reveal the particular role of the miRNAs in a given gene network (Hausser and Zavolan, 2014; Sonkoly, Stahle, and Pivarcsi, 2008; Forster et al., 2015).

Studies of miRNAs in teleost fish have revealed that they participate in regulation of early development, organogenesis, regeneration, tissue differentiation, cellular homeostasis, tissue specific functions, lipid metabolism, growth, reproduction and responses to environmental stimuli (Bizuayehu and Babiak, 2014; Bizuayehu et al., 2015; Mennigen, 2016; Takacs and Giraldez, 2010; Andreassen et al., 2016). In recent years, the role of miRNAs in immune response in teleost fish has also been studied in several species. This review focuses on summarizing and comparing the present knowledge on teleost fish miRNAs associated with immune response and with regulation of genes in immune system gene networks. We also discuss current limitations to research on miRNA regulation in teleost fish. Finally, we discuss recent advances in methods for target gene identification that are likely to be useful in

future studies of miRNAs in teleost fish.

2. miRNAs associated with immune response in teleost fish

The miRNA regulation of immune system gene networks has been extensively studied in higher vertebrates (Forster et al., 2015; Zhou, O'Hara, and Chen, 2011; Li and Shi, 2013). They are much less well studied as regulators of immune responses in teleost fish. Most studies so far have focused on identifying the miRNAs that are differentially expressed between controls and challenged groups. Different virus' and stimulation with poly I:C as well as different pathogenic bacteria and stimulation with LPS have been used in challenge studies in several fish species and cell lines, respectively. The underlying assumption in these studies is that the miRNAs responding to a challenge with a change in their expression are candidate miRNAs that may regulate genes in the immune system gene networks.

2.1. Evolutionary conserved miRNAs differentially expressed following viral challenge

Several studies have identified subsets of miRNAs that are differentially expressed in materials challenged with DNA or RNA virus or treated with poly I:C in teleost fish. Common to these studies is the use of global methods (deep sequencing or microarrays) to identify the miRNAome followed by quantitative analysis to point out those miRNAs that are differentially expressed (DE miRNAs) in the challenge group(s). Sometimes these findings are validated by RT-qPCR, but in most cases only a very limited number of DE miRNAs have been tested by RT-qPCR. In most of these studies *in silico* analysis have also been carried out utilizing the available genome or transcript sequences from the species of interest to predict putative target genes (Zhang et al., 2014; Najib et al., 2016; Han et al., 2016; Guo et al., 2015; Schyth et al., 2015; Andreassen submitted; Wu et al., 2015). A few miRNAs have been further investigated by studying their effect on putative target genes or to reveal their association to e.g. mortality (Bela-ong et al., 2015; Zhang et al., 2016; Andreassen Submitted). The species investigated in these studies are Japanese flounder, Grouper, Miiuy Croaker, Snakehead fish, Common Carp, Rainbow trout and Atlantic salmon. An overview of the species, the viruses used in challenge experiments and references is given in Table 1.

There are differences in the methods applied, number of samples analyzed, the materials (tissue/cell lines) and the time post challenge investigated as well as statistical approaches/thresholds used for significance (adjusted/not adjusted for multiple testing) which results in a huge variety in the number of DE miRNAs identified in each study. Many of the DE miRNAs that show different expression in the challenge group are not necessarily miRNAs that are directly involved in regulation of immune response gene networks. Some are likely to be miRNAs responding to changes in cell homeostasis due to infection while others may be false positives. However, one could assume that if the same miRNAs are detected as DE miRNAs in several distant teleost species challenged with different virus under different conditions they are likely to be evolutionary conserved miRNAs that are involved in regulation of immune gene networks in the hosts. When applying a comparative approach a comparison of the results from these studies shows that there is a small subset of orthologous miRNAs identified as DE miRNAs in several evolutionary distant teleost fish. An overview of the subset of evolutionary conserved DE miRNAs is given in Table 2. The ten miRNAs listed have been discovered as responding to viral challenge (both DNA and RNA virus) in at least three species and in four of the studies in Table 1. In addition to the ten miRNAs listed in Table 2, miRNA-455 was discovered in three different species (Japanese flounder, Grouper and Snakehead fish) and may, thus, also be a miRNA with evolutionary conserved important functions in teleost fish immune responses. The miRNA genes, but not the mature miRNAs, are listed in Table 2 as many of the studies don't specify family (a, b etc) or arm (5p/3p) of the mature miRNAs detected as DE miRNA. Some studies also apply microarrays for detection and these will not necessarily discriminate between miRNAs from the same family. In most cases, however, if a miRNA from a certain miRNA gene is detected in several studies this is likely to be the same mature miRNA as they share the same seed sequence.

Two of the evolutionary conserved DE miRNAs in Table 2 are teleost specific (miRNA-462 and miRNA-731). These two genes are clustered in all teleost fish genomes studied (Schyth et al., 2015; Andreassen et al., 2013; Andreassen et al., 2016). The miRNA gene, miRNA-2188, that was detected as a DE miRNA in Japanese flounder and Atlantic salmon after RNA virus challenge

Table 2
Evolutionary conserved miRNA genes associated with immune response in teleost fish following viral challenge.

miRNA[a]	Species[b]
miRNA-462	JP1, JP2, G, SH, RT, AS,
miRNA-731	JP1, JP2, G, SH, RT, AS,
miRNA-146	JP1, JP2, SH, RT, AS,
miRNA-21	JP1, JP2, SH, RT, AS,
miRNA-181	JP1, JP2, G, SH, RT, AS, MC
miRNA-155	JP1, JP2, RT, AS, MC
miRNA-223	JP1, JP2, RT, AS
miRNA-1388	JP1, JP2, SH, AS
miRNA-100	JP1, JP2, G, SH, RT
miRNA-99	JP2, G, SH, RT

SH: Snakehead fish, RT; Rainbow trout, AS: Atlantic salmon, MC, Miiuy croaker.
[a] miRNA gene with mature miRNAs (5p or 3p) that responded to viral challenge in at least three species (Table 1).
[b] JP1: Japanese flounder challenged with megalocytivirus, JP2; Japanese flounder challenged with VHSV, G: Grouper.

Table 1
Overview of teleost fish studies on miRNAs in viral challenged materials.

Authors/Reference	Species	Viral challenge	materials, time post challenge (poc)
Zhang et al., 2014	Japanese (Olive) flounder	megalocytivirus, dsDNA	spleen, 2–14 days poc
Zhang et al., 2016	Japanese (Olive) flounder	megalocytivirus, dsDNA	spleen, 2–6 days poc
Najib et al., 2016	Japanese (Olive) flounder	viral hemmoragic septicemia virus (VHSV), RNA neg. strand	head kidney, 6–72 h poc
Guo et al., 2015	Grouper	singapore grouper iridovirus (SGIV), dsDNA	spleen, 48 h poc
Liu et al., 2016	Snakehead fish	snakehead fish vesiculovirus (SHVV), RNA neg strand	cell lines, 3 and 24 h poc
Bela-ong et al., 2015	Rainbow trout	viral hemmoragic septicemia virus (VHSV), RNA neg. strand	muscle tissue, 1–21 days poc
Schyth et al., 2015	Rainbow trout	viral hemmoragic septicemia virus (VHSV), RNA neg. strand	Liver, 6 and 7 days poc
Andreassen et al. submitted	Atlantic salmon	salmonide alphavirus (SAV), RNA pos. Strand	cardiac tissue, 2–4 weeks poc
Wu et al., 2015	Common Carp	spring viremia carp virus (SVCV), RNA neg.strand	cell lines, 36 h poc
Han et al., 2016	Miiuy croaker	Poly I:C stimulation	spleen, 24 h poc

(Andreassen Submitted; Najib et al., 2016) is also a miRNA that has not been discovered in mammals. Many studies (Table 1) also reported that there were novel miRNAs, assumed to be species specific miRNAs, that were differentially expressed in a challenge group. Together, this indicates that there are miRNA-related regulatory differences of the immune response between teleost fish and higher vertebrates as these miRNA genes are absent in higher vertebrates. This also suggests that further identification of their target genes could provide details on how teleost immune response regulation differs from higher vertebrates. On the other hand, seven of the DE miRNAs in Table 2 (miRNA-146, miRNA-21, miRNA-181, miRNA-155, miRNA-223, miRNA-100, miRNA-99) that are involved in immune response in teleost fish have also been reported as miRNAs that are associated with immune response in higher vertebrates (Forster et al., 2015; Zhou, O'Hara, and Chen, 2011). This could indicate that for each of these miRNAs the regulation of at least on common target gene that are part of the immune system gene networks has been evolutionary conserved in all vertebrates.

Orthologous miRNAs from different teleost species usually share the same seed and much of their mature miRNA sequence. One would assume that this sequence conservation among distant teleost species is a result of the fact that orthologous miRNAs regulate the same target gene(s). However, a comparison of the putative target genes predicted by *in silico* analysis to be regulated by the evolutionary conserved DE miRNAs in Table 2 does not point to any common evolutionary conserved orthologous genes targeted by the same orthologous miRNA in all of the teleost species studied. There might be method related reasons why we are not able to identify common target transcripts when comparing results from the *in silico* methods applied (see section 3). However, one target of the mature miRNA miR-731-5p have been predicted to target the same transcript in two different teleost species (Japanese flounder and Atlantic salmon) indicating that this target site is evolutionary conserved (Andreassen Submitted; Zhang, Zhou, and Sun, 2016). The target gene, IRF7, was also validated as a target of miR-731-5p in Japanese flounder by use of a dual luciferase assay (Zhang et al., 2016).

2.2. Fish virus encoded miRNAs

Several viruses have been shown to manipulate the host by producing virus encoded miRNAs. Most common are the herpesviruses, but also adenoviruses and polyomviruses have been shown to encode miRNAs (Boss and Renne, 2011). These are DNA viruses that replicate in the nucleus of the host cells and may use the hosts nuclear machinery for their miRNA processing. In accordance with this, the three fish viruses that have been reported as encoding miRNAs are all DNA viruses. Cyprinid herpesvirus encoded miRNAs have been reported in studies of carp (Donohoe et al., 2015) and Singapore grouper iridovirus (SGIV) encoded miRNAs have been reported in studies of Singapore grouper (Yan et al., 2011) while megalocytivirus encoded miRNAs have been reported in a study of Japanese flounder (Zhang et al., 2014). In Japanese flounder these viral miRNAs were predicted to target signal transduction and immune response genes in the host (Zhang et al., 2014). The virus encoded miRNAs are usually assumed to modulate expression of the host cell transcripts, but one of the SGIV encoded miRNAs (SGIV-miR13) seems to regulate the virus transcripts, not the host transcripts (Yan et al., 2015).

Although most host miRNAs responding to pathogene challenge seem to target host genes, there is a possibility that they could be used in anti-viral defense by targeting viral transcripts. There are very few studies that has investigated this possible role for host miRNAs in teleost fish, but there are some examples where the *in silico* predictions of target genes have indicated that some targets are pathogen transcripts (Wu et al., 2015).

2.3. Evolutionary conserved miRNAs differentially expressed following bacterial challenge

Several groups have recently reported miRNAs that are differentially expressed in materials challenged with pathogenic bacteria or LPS. Studies have been carried out in species like snout bream, zebrafish, common carp, half-smooth tongue sole, nile tilapia and miiuy croaker (Yuhong et al., 2016; Ordas et al., 2013; Zhao et al., 2016; Sha et al., 2014; Wang et al., 2016; Xu et al., 2016; Chu and Xu, 2016; Cui et al., 2016; Gong et al., 2015). These studies differ when it comes to the pathogen used in the challenge, which tissue type is studied and the time point post challenge when samples are collected. As in the studies performed on fish challenged with virus, the various conditions used in the challenge as well as the different methods and statistical approaches have led to a large variation in the number of miRNAs detected as differentially expressed (DE miRNAs). An overview of studies of differentially expressed miRNAs in fish challenged with pathogenic bacteria or stimulated with LPS is given in Table 3.

As when we compared studies of virus challenged materials, one could assume that if the same orthologous miRNAs are detected as DE miRNAs in several teleost fish challenged with different bacteria, under different experimental conditions, they are likely to have functions that are related to regulation of evolutionary conserved immune gene networks that are responding to bacterial infection. A comparison of differentially expressed miRNAs identified in recent studies (Table 3) showed that there was a small subset of orthologous miRNAs identified as DE miRNAs in several species. An overview of these evolutionary conserved DE miRNAs is given in Table 4. Totally, there were eight such DE miRNAs responding to bacterial challenge (miRNA-462, miRNA-731, miRNA-181, miRNA-223, miRNA-146, miRNA-122, miRNA-192 and miRNA-451). Five of these (miRNA-462, miRNA-731, miRNA-181, miRNA-223 and miRNA-146) were also identified as evolutionary conserved DE miRNAs that responded to viral infection (Table 2). The three remaining miRNAs, miRNA-122, miRNA-192 and miRNA-451, have all been reported as miRNAs associated with immune response and inflammation in higher vertebrates as well (Sun et al., 2016; Forster et al., 2015; Zhou, O'Hara, and Chen, 2011).

The target genes of the miRNAs discovered as DE miRNAs in these studies (Wang et al., 2016; Yuhong et al., 2016; Zhao et al., 2016; Xu et al., 2016) have been predicted using *in silico* analysis utilizing 3′UTRs derived from various sources as input. Typically, this approach has revealed a very large number of putative target transcripts, e.g. several thousand targets for only 30 DE miRNAs (Zhao et al., 2016). There is, undoubtedly, a large number of false positive predictions when one applies such methods (see section 3) (Witkos et al., 2011).

2.4. Experimental validations of target transcripts

A few studies have reported target transcript validations by using of experimental approaches (Chu and Xu, 2016; Cui et al., 2016; Zhang et al., 2016). In Japanese flounder Zhang et al (Zhang et al., 2016). used dual luciferase assays to show that miR-731-5p target IRF7 and p53 transcripts by binding to the predicted target sites in the 3′UTRs *in vitro*. They also tested the effect of miR-731-5p on translation of target transcripts (western blotting) by manipulating the miRNA level using chemically engineered oligonucleotides (miRNA agomirs and antagomirs). Their results showed that IRF7 and p53 are the direct targets of this miRNA. Also, as the level of miR-731-5p increased early in cytomegalovirus infection, this resulted in an inhibition of the host immune response that could be

Table 3
Overview of teleost fish studies on miRNAs in materials challenged with pathogenic bacteria.

Authors/Reference	Species	Type of bacterial challenge	materials, time post challenge (poc)
Yuhong et al., 2016	Snout bream	LPS	head kidney, spleen and liver pooled, 2–24 h poc
Ordas et al., 2013	Zebrafish	salmonella typhimurum	embryo 8 h poc, adult 6 days poc
Zhao et al., 2016	Common carp	flavobacterium columnare	liver, 40 h poc
Sha et al., 2014	Half-smooth tongue sole	vibrio anguillarum	head kidney, spleen, liver and intestine pooled, 20 h poc
Gong et al., 2015	Half-smooth tongue sole	vibrio anguillarum, polyIC andLPS	head kidney cell culture, 6–24 h poc
Wang et al., 2016	Nile tilapia	streptococcus agalactiae	spleen, 6–72 h poc
Xu et al., 2016	Miiuy croaker	vibrio anguillarum	spleen, 48 h poc
Chu and Xu, 2016	Miiuy croaker	vibrio anguillarum and LPS	cell culture 3–6 h poc, spleen 12–48 h poc
Cui et al., 2016	Miiuy croaker	vibrio anguillarum and LPS	cell culture 3–6 h poc, spleen 12–48 h poc

Table 4
Evolutionary conserved miRNA genes associated with immune response following bacterial challenge.

miRNA[a]	Species[b]
miRNA-462	ZF, NT, HS, SB
miRNA-731	ZF, NT, HS, SB
miRNA-181	ZB, HS, SB
miRNA-223	ZF, NT, HS, SB
miRNA-146	ZF, NT, HS
miRNA 122	HS, MC, SB
miRNA 192	NT, HS, MC, SB
miRNA 451	NT, HS, SB

[a] miRNA gene with mature miRNAs (5p or 3p) that responded to bacterial challenge in at least three species (Table 3).
[b] ZF; Zebra fish, NT; Nile tilapia, HS; Half-smooth tongue sole, MC; Miiuy croaker, SB; Snout bream.

explained as a result of negative regulation of the immune response activator IRF7 by miR-731-5p. A miR-731-5p target site in 3′UTRs of IRF7 from Atlantic salmon has also been reported (Andreassen Submitted) suggesting that this may be a conserved target gene in teleost fish. Studies of miiuy croaker miRNA profiles following bacterial infection revealed a large number of miRNAs with a change in expression, and applying luciferase assays the two miRNAs miR-122 and miR-192 were also reported to target host cell immune response activators (TLR14 and IL-1R1). These miRNAs both showed a reduced expression at the early time points post challenge, but there are some discrepancies in the reported direction of change and the targets of miR-122 (Xu et al., 2016; Chu and Xu, 2016; Cui et al., 2016).

2.5. A model for miRNA function in teleost fish immune homeostasis

A common finding of target validation studies and studies that carried out target gene predictions by *in silico* analysis is that many of the target genes are host genes with roles as immune response activators or immune response inhibitors, e.g. (Zhang et al., 2014; Zhang et al., 2016; Chu and Xu, 2016; Han et al., 2016). We suggest a model where there are temporal changes in expression of the evolutionary conserved miRNAs targeting host genes that are part of immune system gene networks. The temporal changes suggested could contribute to an optimized and immediate initiation of the immune response as well as to help prevent an inappropriate inflammatory response that may be injurious to the host. To serve these functions the expression may change during infection as exemplified in Fig. 1. This example illustrates the changes of a miRNA that target a host immune response activator gene. In a non-infected cell there are immune gene activators (e.g. IRFs) that are constitutively expressed (Honda and Taniguchi, 2006). A constitutive low level expression of the miRNAs targeting the transcripts of immune gene activators may repress their translation and help inhibit that immune responses are triggered (Fig. 1a). As the host is infected, the transcription of the immune response activators is stimulated. This will lead to an increased expression level of activator transcripts that are above the threshold counteracted by host miRNAs. Additionally, if the levels of host miRNAs decrease at this early time point in infection this would lead to less translational repression of immune gene activator transcripts. Such a change of miRNA expression would then contribute to an immediate activation of the immune response as the threshold for triggering the immune response is lowered (Fig. 1b). As the inflammation is potentially harmful to the host the immune response needs to be controlled. This could be accomplished if the level of host miRNA targeting the immune gene activators is increased in the late stage of infection to prevent further increase of activator transcripts (Fig. 1c). At this stage in infection both the activators and the miRNA targeting the activator would show higher expression level than what is observed in normal cellular state (constitutive expression levels). Similar temporal changes could be expected for a miRNA that target an immune response inhibitor gene (Xu et al., 2016). However, in the case that the target is an immune response inhibitor gene it could be expected that the miRNA expression increases at initiation of inflammation (1b) leading to a decreased expression of the inhibitor, thus, lowering the threshold for triggering an immune response. In the late stage of infection, the expression of the miRNA could decrease to help prevent pathological inflammation.

These suggested functions (Fig. 1) of the host miRNAs associated with immune responses in teleost fish are in agreement with findings in higher vertebrates (Liu and Abraham, 2013; Forster et al., 2015; Zhou, O'Hara, and Chen, 2011). The model suggests that there is a balanced expression of miRNA/target transcript levels that change in a controlled manner following infection. Imbalance could potentially be harmful to the host. An early increase of miRNAs targeting activator transcripts e.g. in the initiation of the inflammatory response (Fig. 1b) could result in down-regulation of the immune response. Such imbalance caused by viral manipulation of the host miRNAs was reported to result in less resistance to infection in a study by Zhang et al. (Zhang et al., 2016). On the other hand, if there is no increase of such miRNAs, or not a large enough increase, to balance the effect of the immune system activators (a high expression of activators balanced by a high expression of regulatory miRNAs) this could lead to pathological inflammation and increased mortality.

The particular function of the conserved miRNAs responding to infection depends on what transcript(s) they directly regulate post transcriptionally. However, as illustrated in Fig. 1, their biological effect on the immune response is also depending on the timing and the direction of their change in expression. Thus, the host miRNA genes themselves need to be controlled so that their change in expression is in tune with other activators or inhibitors. Studies in teleost fish have suggested that the expression of some miRNA genes participating in immune gene networks are controlled in a

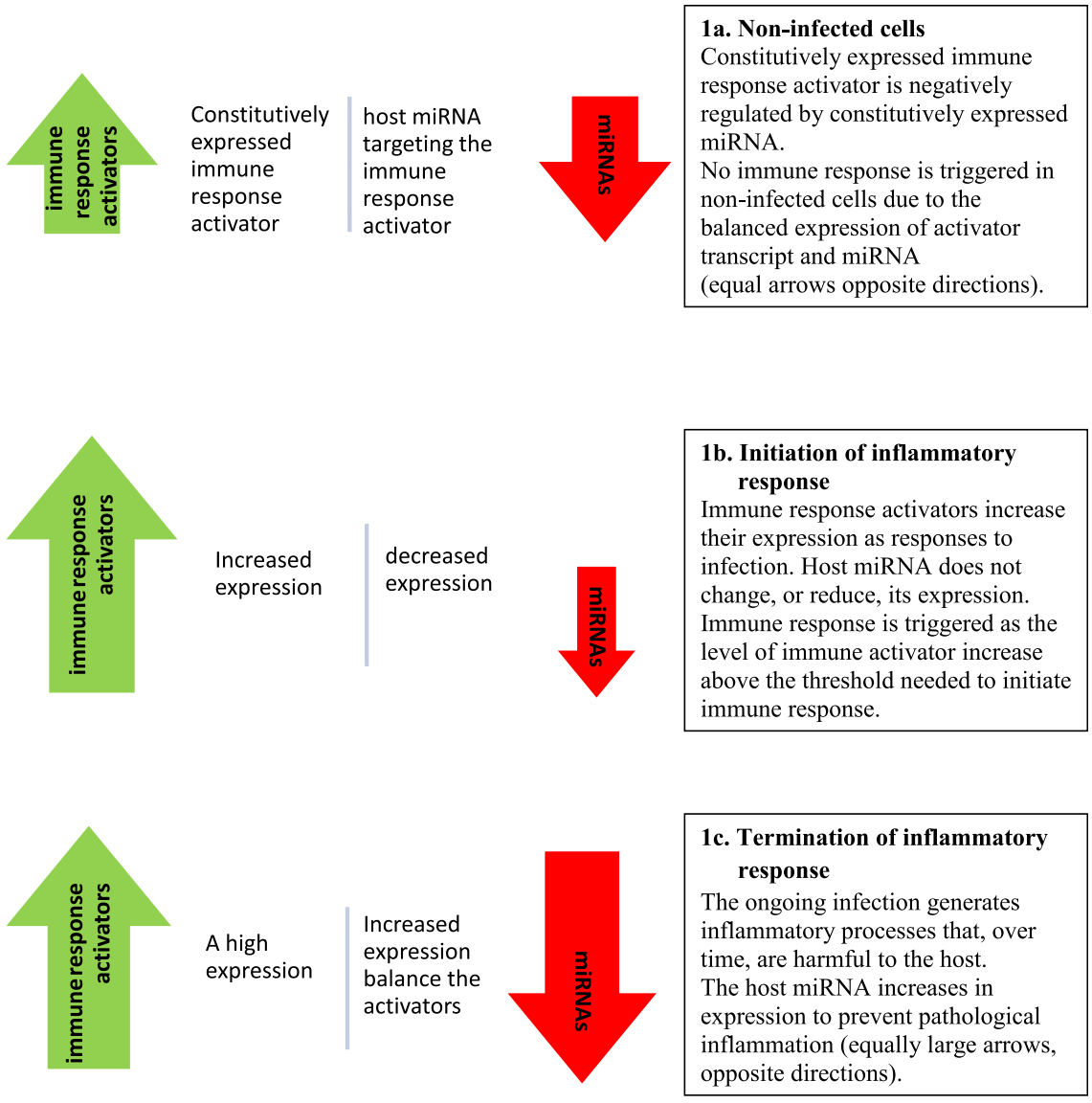

Fig. 1. Shows a schematic illustration of the proposed model with temporal changes of a host miRNA if regulating a host immune response activator.

feedback loop by the same genes that the miRNAs target. The basis for such suggestions is that response elements like ISRE, NFkB and AP1 have been discovered in the upstream genome sequences of several of the DE miRNA genes identified to be associated with immune responses in teleost fish (Tables 2 and 4) (Schyth et al., 2015; Chu and Xu, 2016; Cui et al., 2016; Andreassen Submitted). Such a feedback regulation model could help to explain how the hosts manage to respond with an appropriate change of their miRNA expression to prevent pathological inflammation in the late stage of infection (Fig. 1c). Similar models, where the activators promote expression of their negative miRNA regulators, has also been suggested in higher vertebrates (Forster et al., 2015).

3. Studying miRNA in teleost fish; limitations and challenges

A well characterized and species specific miRNAome is the proper reference to utilize for expression profiling to discover miRNAs responding to infection in teleost fish. Evolutionary conserved orthologous mature miRNAs are, often, but not always, identical in their sequences. Because of this strict sequence conservation, the studies aiming at identifying miRNAs associated with immune responses in one fish species has in some cases been based on miRNA sequences from more well studied teleost fish species. The disadvantage of such an approach is that some orthologous miRNAs do show small sequence variation between species. In addition, species specific miRNAs have been discovered in most teleost fish (http://www.mirbase.org/). The use of a non-species specific miRNAome as a reference would then generate incomplete results as the method applied would only identify differentially expressed miRNAs that match the reference. The fact that many teleost fish miRNAomes are rather incomplete, thus, represents a limitation in the study of miRNAs important in immune homeostasis. The lack of a well characterized miRNAome in a species is often due to an equally poorly characterized genome. A recent study in rainbow trout exemplifies how several hundred new miRNAs may be characterized if the genome sequence is improved and if the discovery is carried out in a large number of tissues and developmental stages (Juanchich et al., 2016).

The characterization of miRNAs and the annotation of miRNAs are based on common criteria agreed on by the scientific

community (Ambros et al., 2003; Kozomara and Griffiths-Jones, 2014) and final annotation is carried out by miRBase (http://www.mirbase.org/). Future miRNA research in teleost fish would benefit if all experimentally identified miRNAs are annotated in agreement with these criteria and submitted to miRBase. This would assure that one would be able to easily identify and compare orthologous miRNAs from different studies and it would facilitate the use of such results in comparative immunology. Uniform annotation would also reduce the number of falsely reported novel and species specific miRNAs. One example is the 26 miRNAs reported as novel in a carp study (Wu et al., 2015). Twelve of these are identical to annotated miRNAs already present in mirBase and observed in several species, some even previously described in carp (Yan et al., 2012) (http://www.mirbase.org/). This illustrates the importance of using common criteria and nomenclature for annotation of miRNAs in teleost fish.

The accuracy and quality of target gene prediction by use of *in silico* methods depends on availability of high quality full-length mRNA transcripts with complete 3′UTR sequences (Andreassen et al., 2009) from putative target genes. The source for putative target transcript sequences has, however, in many cases been incomplete transcript sequences generated from ESTs that are of a rather low sequence quality. If both the 3′UTR sequences are incomplete (not full-length mRNA) and are low quality sequence the actual target genes may not be detected at all. Transcript sequences generated by automatic prediction tools using genome sequence as source are often not accurately predicting the 3′UTRs and these are not a good source for input in target gene prediction analyses. Generation of better characterized, high quality full-length transcript sequences in teleost fish species would, thus, greatly enhance the possibility of predicting the correct target gene as well as greatly benefit any high-throughput study of the transcriptome.

The *in silico* target prediction methods have a precision of only 25–50% depending on algorithm used (defined as number of correctly predicted targets as a proportion of total predicted targets) (Witkos et al., 2011). This means that the *in silico* methods always predict a large number of false target transcripts (false positives). Also, if using a complete transcriptome as input, the results may include several true target transcripts, but they may be targets regulated in other tissues or developmental stages and not relevant to the immune responses studied. One approach to improve the precision could be to include only transcripts from genes of known immune functions and only those transcripts known to change their expression following infection with the virus or bacteria initially used in the challenge study (Xu et al., 2016; Andreassen Submitted). This could possibly limit the predicted targets to a number of transcripts feasible for validation using other experimental approaches with minimal loss of the targets that are relevant to the immune responses studied.

miRNA expression profiling is usually the first step in order to discover particular miRNAs that are differentially expressed in challenged groups. The platform used in teleost fish studies are either microarrays or deep sequencing (high-throughput sequencing). Microarray platforms have the advantage of being less expensive than deep sequencing. However, they have limited specificity and may not properly differ between mature miRNAs from the same family and they also only detect the miRNAs that are included on the microarray. The deep sequencing methods, on the other hand, precisely identify the mature miRNA sequences so that members from the same family may be distinguished. They also have the potential to discover novel miRNAs e.g. those that are only expressed in the challenged group (Pritchard et al., 2012). As the number of reads that match a particular mature miRNA directly reflects the relative expression of this miRNA in a sample the results from the use of deep sequencing methods may easily be used for quantitative measurements (Love et al., 2014). Lately, small RNA deep sequencing has become less expensive, thus, this seems to be the best platform for large scale miRNA profiling.

Validation of differentially expressed miRNAs discovered by large scale miRNA profiling by RT-qPCR is recommended (Pritchard et al., 2012). Robust methods applying conserved stably expressed miRNAs as endogenous controls have been developed for such miRNA expression studies in several teleost fish species (Johansen and Andreassen, 2014; Andreassen et al., 2016; Zhu et al., 2015). Importantly, such validation allows for analysis of a larger number of individual samples than the small number often used in the large scale miRNA profiling. RT-qPCR validations may then reduce the number of false positive DE miRNAs identified if applying large scale profiling only.

For teleost fish it is a challenge that species specific miRNAomes are incomplete. There is also, in general, a lack of high quality reference genomes and high quality full-length transcriptomes. It would greatly benefit the miRNA research in teleost fish if such resources were developed in more species. Microarray platforms are limited to detect only the miRNAs present on the array (not novel miRNAs) and don't discriminate well between closely related mature miRNAs. Large scale miRNA profiling using deep sequencing platforms followed by RT-qPCR particularly developed for measurements of miRNA expression therefore appears as the best approach for discovering DE miRNAs.

4. Future directions for miRNA research in teleost fish

The best way to understand the regulatory role of a miRNA is to identify its target gene(s). Identification of target transcripts using experimental approaches will likely be the next important step in research on miRNAs with a role in immune homeostasis. Most studies on miRNAs in teleost fish (Tables 1 and 3) have, so far, applied *in silico* predictions to suggest target genes. Only a few studies have carried out experimental validations of target genes (Zhang et al., 2016; Chu and Xu, 2016; Cui et al., 2016). The methods applied for validation of target genes in these studies examine the potential negative regulation by one particular miRNA of one particular putative target transcript. One common assay, also applied in these studies, use 293 HEK (human embryo kidney) cell lines that are highly transfectable. The ability of a certain miRNA to inhibit translation by binding to a certain 3′UTR may then be tested using a dual luciferase assay (Zhang et al., 2016; Chu and Xu, 2016; Cui et al., 2016). Recently, a dual luciferase assay that may detect miRNA activity in primary cells has been developed. Applied for validation of teleost fish target transcripts the assay would allow for the study of miRNA/target in their natural cellular context rather than in the mammalian HEK cell line (Beillard et al., 2012).

Newly developed genome wide approaches based on immunoprecipitation identify a large number of miRNAs and their true target transcripts in one experiment. These CLIP (crosslinking and immunoprecipitation) based methods directly capture RNA bound by Argonaut proteins (miRISC) by crosslinking RNA and protein (UV light) followed by immunoprecipitation using Argonaut specific antibodies. The RNAs may then be sequenced to reveal the actual target site sequences in the RNA pool investigated. Compared to *in silico* prediction methods such experimentally based methods detect less false positive target sites. In addition, they may also detect non canonical target sites. Although they define the region of RNA targeted by the miRNAs they do not identify which particular miRNA that is interacting with a particular target sequence (Hausser and Zavolan, 2014; Chi et al., 2009; Broughton and Pasquinelli, 2016). This is a disadvantage as this means the method cannot distinguish between which miRNA, out of several

family members, that are the one actually binding the target sequence (Broughton and Pasquinelli, 2016).

The disadvantages of the CLIP methods may, however, be overcome by the CLASH (crosslinking and sequencing of hybrids) methods (Grosswendt et al., 2014; Broughton and Pasquinelli, 2016). These methods also involve purification of protein-RNA complexes (miRNA and target sequence) as in the CLIP methods. But additional ligation steps promote intermolecular ligation of the RNA molecules that were precipitated together with the same protein. These molecules then form a hybrid consisting of a miRNA ligated to its target sequence. These chimeric sequences may then be sequenced to reveal true miRNA-target sequences (Grosswendt et al., 2014; Broughton and Pasquinelli, 2016).

The use of cross species comparisons to disclose conserved sequences in upstream genome sequences of miRNA genes may represent a powerful tool to identify *cis*-elements that regulate the transcription of miRNAs. This approach have been applied to identify putative response elements in the upstream sequences of some teleost fish miRNA genes associated with immune response. These studies identified cross species conserved *cis*-elements that are known to bind transcription factors that participate in the immune response gene networks. This further indicates that these miRNA genes (miRNA-463/731, miRNA-192 and miRNA-122) have important roles in immune responses (Schyth et al., 2015; Cui et al., 2016; Chu and Xu, 2016; Andreassen Submitted). The development of more genome resources with good quality from more species will facilitate the use of large scale cross species comparisons.

A combination of methods (single target validation and genome wide methods as well as cross species comparisons) could help identify all true target transcripts of the miRNAs associated with immune response in teleost fish. The manipulation (e.g. knock down) of miRNAs responding to infection may further increase the understanding of their particular roles. In economically important teleost fish species such knowledge may eventually be applied in the development of diagnostic markers for e.g. marker assisted breeding or used to develop novel therapeutic tools.

5. Conclusions

The studies of miRNAs associated with immune response in teleost fish have identified several miRNAs responding to infection with changed expression. A comparison of findings in teleost fish points out a smaller subset of evolutionary conserved miRNAs that are likely to have important roles in regulation of the immune system gene networks. We suggest a model where the temporal changes of their expression following infection could be explained by their role as regulators of immune gene activators or inhibitors. There is a large percentage of false positives targets predicted from the *in silico* methods. The identification of the true target transcripts using experimental approaches will, therefore, be the next important step in research on miRNAs associated with immune response in teleost fish. Newly developed experimental methods like dual luciferase assays, CLIP and CLASH may be utilized to reach this goal.

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