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Transcriptome profiling of infectious diseases and cancer in zebrafish

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MicroRNA expression during bacterial infections in zebrafish

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Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play important roles in controlling mRNA stability and translation. Several miRNAs have been implicated in development and function of the immune system and their aberrant expression has been linked to pathogenesis of human diseases. Recently, zebrafish infection models have been developed to study the vertebrate immune system. Signaling pathways of the immune response were found to be conserved between human and zebrafish, but the interaction between miRNAs and the zebrafish immune system has not yet been addressed. Here, we used a custom-designed microarray platform to evaluate miRNA expression in zebrafish infection models. By infection studies with two pathogens, *Mycobacterium marinum* and *Salmonella typhimurium*, modeling human tuberculosis and salmonellosis, respectively, we identified a set of common infection-responsive miRNAs, including members of the miR-21, miR-146 and miR-181 families, previously linked to immunity and cancer in human and mammalian models. The context of innate immunity was sufficient for the infection response of these miRNAs, as their differential expression occurred not only in adult fish but also in embryos prior to development of adaptive immunity. We also determined that predicted target genes of the strongly infection-inducible miR-146 family are conserved between human and zebrafish. These include key signaling intermediates of the TLR pathway that is pivotal for pathogen recognition and activation of the innate immune response, suggesting a miR-146-mediated feed back mechanism to properly control the inflammatory response. Together, our results uncovered overlap in human and zebrafish immune-related miRNAs as well as miRNA target genes, indicating the usefulness of zebrafish infection models to increase our knowledge of miRNA functions in the vertebrate immune system.

Introduction

The discovery of microRNAs (miRNA) has added a new level of complexity to how biological processes are controlled. MicroRNAs are small non-coding single stranded RNAs with a length of 19-25/21-25 nucleotides (nt) that control gene expression by regulating mRNA stability and translation in a unique tissue-specific, developmental stage-specific and disease-specific manner (Schmidt et al., 2009). They are involved in the regulation of variety of biological processes, including cell cycle, differentiation, development, metabolism and disease pathogenesis (Sonkoly and Pivarcsi, 2009). MiRNA genes are evolutionarily conserved and located in introns and exons of protein-coding genes and non-coding regions of the genome. They are transcribed mostly by RNA polymerase II into several kilobases long, double-stranded primary transcripts (pri-miRNA). Pri-miRNA is subsequently cleaved in the nucleus by the Drosha /DGCR8 complex into one or more ~ 70 nt-long precursor miRNA strands (pre-miRNA). Pre-miRNA is exported to the cytoplasm by

exportin-5, where it is bound to RNase Dicer and to the RNA-induced silencing complex (RISC) and processed into a mature miRNA molecule. Only the active or mature strand is retained in the RISC complex, the passenger strand is removed and degraded. However, in some cases both strands of the miRNA duplex (the primary miRNA and its star sequence) are incorporated in the RISC complex with similar efficiency. In animals, the mature miRNAs usually bind to complementary sequences in the 3' untranslated region of target mRNAs, particularly with their seed sequences (nucleotide 2-8 from the 5' end). This results in repression of gene expression by translational inhibition and mRNA degradation. Under certain conditions miRNAs can also induce the translation of target mRNAs (Vasudevan et al., 2007) or transcriptionally silence gene expression (Kim et al., 2008). The physiological impact of this is still unknown. MiRNA biogenesis is controlled by extensive transcriptional and post-transcriptional regulation.

Proteins in miRNA biogenesis as well as several miRNAs have been implicated in the immune system, both in the process of haematopoiesis and in the function of haematopoietic cell lineages. The miR-17~92 cluster has been shown to be a ubiquitous regulator of B-cell, T-cell and monocyte development. MiR-142 was shown to be a regulator of T cell development, miR-150 has been implicated in B cell differentiation, miR-155 was shown to be involved in T- and B-cell maturation, miR-181a in B-cell differentiation and CD4⁺ T-cell selection, activation and sensitivity, and miR-223 is specifically expressed cells of the granulocytic lineage (Baltimore et al., 2008; Carissimi et al., 2009; Lindsay, 2008; Lu and Liston, 2009; Sonkoly et al., 2008; Taganov et al., 2006; Xiao et al., 2007).

MiRNAs have not only been linked to development of immune cells but also to the process of infection or inflammation. There is increasing evidence that they have important roles in regulating innate immune responses, the first line of defence to bacteria, viruses, and other pathogens. As part of the first immune response, monocytes and macrophages recognize microbial ligands with their pattern recognition receptors, of which the Toll-like receptors (TLRs) form one of the major families. Due to activation of the downstream signaling pathways hundreds of genes are induced to defeat the pathogen. Several miRNAs are induced or repressed during bacterial and viral infections. After exposure of (human or mouse) monocytes to bacterial lipopolysaccharide (LPS) miR-132, miR-146, and miR-155 showed significant up-regulation (O'Connell et al., 2007; Taganov et al., 2006; Tili et al., 2007). In addition, miR-146 is also induced by proinflammatory stimuli, including interleukin-1 (IL-1) and tumor necrosis factor (TNF), in a NF- κ B-dependent manner, and miR-155 was found to be stimulated by interferon (IFN) or TNF (O'Connell et al., 2007; Taganov et al., 2006). MiR-146 and miR-155 might be components of negative feedback loops attenuating TLR signaling pathways, where miR-146 limits IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) expression, and miR-155 suppresses FADD, RIP and IKK (Pedersen and David, 2008). In contrast, miR-125b, which targets *TNF* mRNA, is down-regulated following LPS-induced TLR

signaling, thereby stimulating the production of TNF (Pedersen and David, 2008; Tili et al., 2007). There is also evidence that miRNAs play role in viral infections either by contributing to the antiviral defence or facilitating the replication of the pathogen.

As miRNAs regulate the normal development and function of the immune system, their aberrant expression have been suggested to be involved in pathogenesis of diseases. A large number of recent studies demonstrated deregulated miRNAs in immune-related disorders like inflammatory or autoimmune diseases. In inflammatory diseases such as psoriasis and atopic eczema, miR-146 and miR-125b were found to be deregulated (Carissimi et al., 2009). In rheumatoid arthritis miR-146 and miR-155 are commonly up-regulated. (Nakasa et al., 2008; Stanczyk et al., 2008). Many other miRNAs (miR-17~92, miR-21, miR-129, miR-203, miR-451 etc) have been detected in other types of immune-related disorders supporting the fact that these miRNAs play critical role in inflammation (Carissimi et al., 2009; Sonkoly and Pivarcsi, 2009). Since the immune system is tightly connected with cancer, not surprisingly the expression of many of the immunity-related miRNAs is also changed in a variety of tumors (Bhaumik et al., 2008; Costinean et al., 2006; Esquela-Kerscher and Slack, 2006; Garzon et al., 2009; Hurst et al., 2009; Lu et al., 2005; Medina and Slack, 2008).

Although many recent studies have contributed to the discovery of novel miRNAs and have aimed to shed light on their role in gene expression, only a limited number of the mRNA targets of miRNAs have been experimentally validated and the contribution of miRNA regulation to the pathogenesis of human diseases is far from understood. As miRNAs are highly evolutionary conserved across vertebrates, studies using different model organisms can provide valuable information about their functions in development of human diseases. In the last decade, zebrafish, which has an innate and adaptive immune system similar to that of mammals, has been commonly applied as model system for immunological research (Traver et al., 2003; van der Sar et al., 2004). Zebrafish models have been developed for different infections, including *Mycobacterium marinum* infection as a model for tuberculosis and *Salmonella typhimurium* infection as a model for food poisoning (Davis et al., 2002; Meijer et al., 2004; Meijer et al., 2005; Stockhammer et al., 2009; van der Sar et al., 2003; van der Sar et al., 2009). Zebrafish is also a convenient model to study miRNA function and its miRNA family has been well described (Davis et al., 2002; Kloosterman et al., 2007; Kloosterman et al., 2006; Schier and Giraldez, 2006; Wienholds et al., 2005). However, the functions of miRNAs in the immune system of zebrafish have not yet been addressed.

Here, we have used a custom-designed microarray platform to evaluate miRNA expression in adult zebrafish and zebrafish larvae infected with different bacterial pathogens. Common microRNA induction profiles from different bacterial infections are assessed and compared to human miRNA expression profiles in the literature. We have focused on miR-146 family and their predicted target genes in the

TLR pathway, and have compared microRNA expression with previously published expression data of predicted target genes (Stockhammer et al., 2009; van der Sar et al., 2009). Our results show that there are infection-responsive miRNAs highly conserved between human and zebrafish and also that there is considerable overlap in miRNA target genes, indicating that the zebrafish can be a useful model to dissect functions of miRNAs in the vertebrate immune system.

Results

Microarray analysis of microRNA profiles in response to infection

In order to study the effects of bacterial infection on microRNA (miRNA) expression profiles we used RNA samples from different infection experiments in adult and embryonal zebrafish that we had previously analyzed for gene expression differences (Stockhammer et al., 2009; van der Sar et al., 2009). Bacteria tested included *Salmonella typhimurium*, which causes a lethal infection in 1-day-old zebrafish embryos (van der Sar et al., 2003) and *Mycobacterium marinum* strains Mma20 and E11, which respectively cause acute disease or chronic granulomatous tuberculosis in zebrafish adults (van der Sar et al., 2004) and also induce granuloma formation in zebrafish larvae (Davis et al., 2002). We analyzed samples from embryos injected into the blood at 27 hpf with *S. typhimurium* bacteria (Stockhammer et al., 2009). In addition, embryos at 48 hpf were infected with *M. marinum* E11 using a novel infection system, in which bacterial suspensions with polyvinylpyrrolidone (PVP) as a carrier are injected into the yolk sac (Spaink et al., unpublished results). For comparison a non-pathogenic bacterium *Lactobacillus casei* Shirota was used in the yolk infection system and the effect of PVP carrier was determined as a control. In adult infection experiments, *M. marinum* strains Mma20 and E11 were injected into the intraperitoneal cavity. Details of the experimental set-up of the embryonic and adult infection studies are shown in Fig. 1.

To quantify microRNA gene expression profiles we used a custom-designed 8x15k Agilent zebrafish array. In the infection experiments with embryos, the total number of probes that showed more than 1.5-fold induction or repression of the expression level ($P < 1.00E-4$) was highest for the *M. marinum* E11 yolk-infected embryos (Fig. 2A). The number of differentially expressed probes was 8 times lower when similar doses of non-pathogenic *L. casei* Shirota were injected into the yolk and 18 times lower in control injections with PVP carrier. Similarly, the number of differentially expressed probes was 10 times lower in *S. typhimurium* caudal vein infections than in *M. marinum* E11 yolk-infected embryos. The numbers of differentially expressed probes in adult infection experiments with *M. marinum* were in a similar range as in the *M. marinum* E11 yolk-infected embryos (Fig. 2A). Comparing the two *M. marinum* strains, a higher number of probes were differently expressed in infections with the Mma20 strain that causes acute disease than in the case of the E11 strain that causes chronic disease. For both strains, at 1 dpi 1.5 to 2-fold more probes were up-

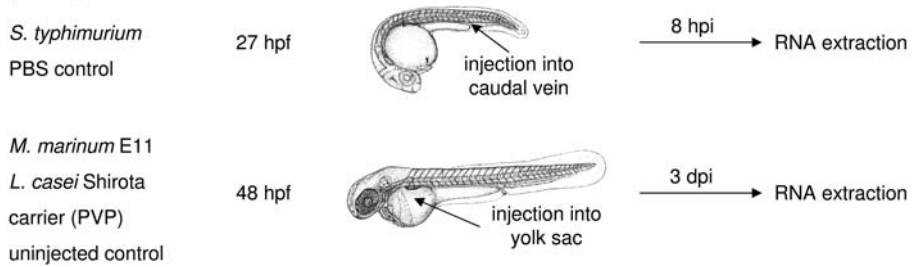
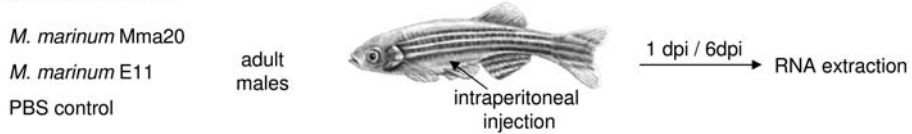
A) Embryonal infections**B) Adult infections**

Figure 1. Overview of the infection experiments. (A) For infection of embryos with *S. typhimurium*, bacteria were microinjected into the caudal vein close to the urogenital opening after the onset of blood circulation (27 hpf). An equal volume of PBS was likewise injected for the control group (Stockhammer et al., 2009). RNA samples for transcriptome analysis were collected at 8 hours post infection (hpi). For yolk infection experiments, embryos were staged at 48 hpf and *M. marinum* E11 bacteria were injected in PVP suspension. For comparison a non-pathogenic bacterium *Lactobacillus casei* Shirota was injected in PVP suspension at similar concentrations as used for *M. marinum*. As controls PVP-injected and uninjected embryos were employed. For gene expression analysis we extracted RNA samples at 3 days post infection (dpi). (B) In adult infection experiments, *M. marinum* strains Mma20 and E11 were injected into the intraperitoneal cavity of male zebrafish and PBS-injected males were used as a control. For microarray analysis we collected RNA samples from two time points, 1 and 6 days post infection (dpi).

regulated than down-regulated, while at 6dpi experiments 3 to 4 times more probes were down-regulated than up-regulated.

Annotation of differentially expressed microRNAs

The custom-designed microarray platform contained probes for both known and predicted miRNAs, the latter classified into three categories: C1 - novel confident miRNAs for which there is compelling experimental evidence, C2 - candidate miRNAs with clear hairpin structure but less experimental evidence, and C3 - additional hairpins identified in the zebrafish genome sequence that are less likely to be miRNAs. The distribution of differentially expressed probes over the known and predicted miRNA categories in the different embryonal and adult infection experiments is shown in Fig. 2B. The total number of known miRNAs regulated during infection was highest for the *M. marinum* yolk infections in embryos (181 out of a total of 496 probes), much lower for the other embryonal infections (13-20 probes),

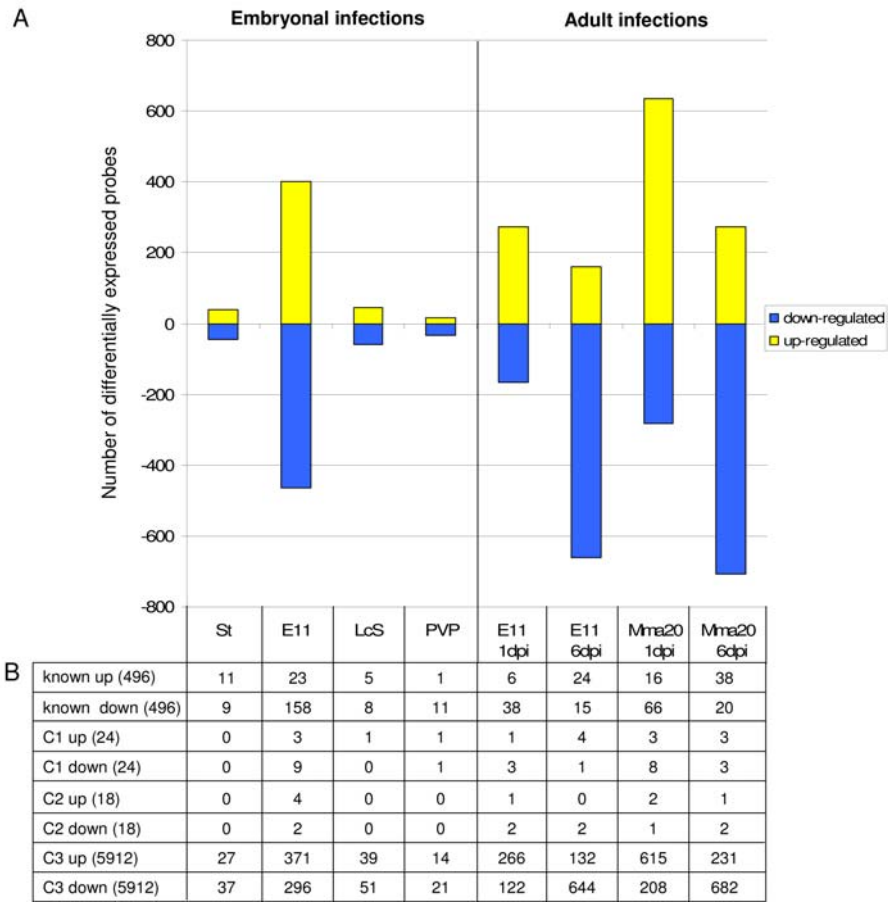


Figure 2. Differentially expressed miRNA probes in adult and embryonal infections. (A) Total number of up-regulated and down-regulated probes. (B) Number of up-regulated and down-regulated probes in four annotation categories: known miRNAs, and predicted miRNAs, C1, C2, and C3, with decreasing level of confidence as further detailed in the text. The total number of probes on the array per category is: 546 probes in known, 62 probes in C1, 54 probes in C2 and 6942 probes in C3 category. Embryonal infections were performed by injection of *S. typhimurium* (St) into the caudal vein at 27 hpf or by yolk injection of *M. marinum* (E11) or *L. casei* Shirota (LcS) at 48 hpf. PVP is the carrier control for yolk injections. Adult infections were performed with *M. marinum* strains E11 (E11 1dpi, E11 6dpi) and Mma20 (Mma20 1dpi, Mma20 6dpi). For further details of the experimental set-up of the infection studies see Figure1.

and intermediate for the adult *M. marinum* infections (39-82 probes). Furthermore, in the *M. marinum* yolk-infected embryos a particularly high number of known miRNAs fell into the down-regulated category (158) compared with the other infections. In comparison with the known miRNAs, the number of predicted miRNAs

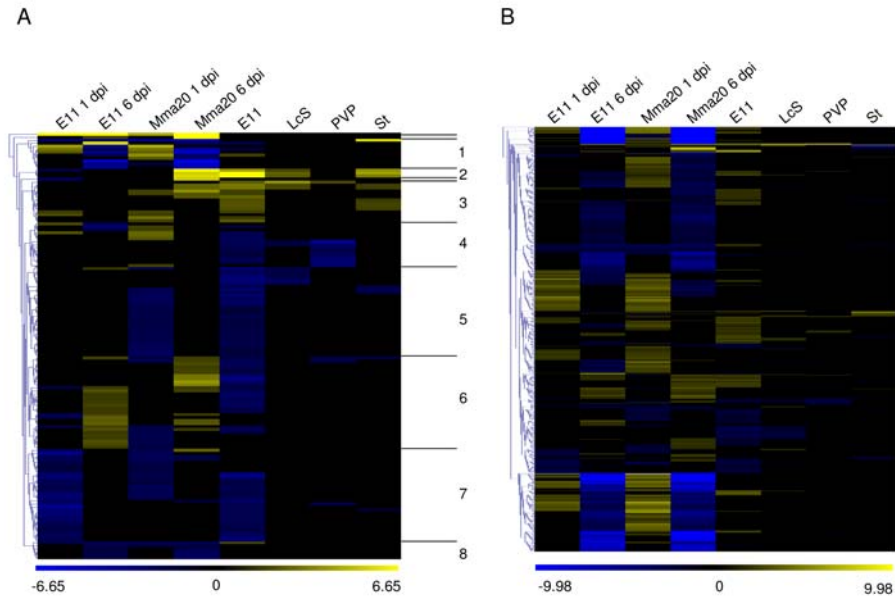


Figure 3. Hierarchical cluster analysis of differentially expressed miRNA probes in adult and embryonic infection experiments. A) The set of significantly up- and down-regulated known miRNAs, C1, and C2 category probes were analyzed together by one-dimensional hierarchical cluster analysis using MultiExperiment Viewer with settings for average linkage method with Euclidean distance. Induced genes are indicated by increasingly brighter shades of yellow, and down-regulated genes are indicated by increasingly brighter shades of blue. Eight clusters were defined in this set of probes named 1-8. Clusters containing less than 3 miRNAs were excluded. B) Cluster analysis of significantly up- and down-regulated C3 category probes.

(total of the C1-C3 categories) that showed differential expression in the infection experiments was between 3 and 20-fold higher. In particular, in all *M. marinum* embryo or adult infections the number of differentially expressed probes in the C3 category was very high (ranging between 388 and 913 out of a total of 5912 probes). In the much smaller C1 and C2 categories (consisting of 24 and 18 probes, respectively), the highest number of changes was observed in the *M. marinum* embryo infection (C1 12 probes, C2 6 probes). In the adult infection experiments with the *M. marinum* E11 and Mma20 strains, C3 probes were mostly up-regulated at 1dpi, and down-regulated at 6dpi. In contrast, the known miRNAs were more frequently down-regulated at 1dpi and up-regulated at 6dpi.

Common microRNA induction profiles between different bacterial infections

To further analyze the common response to different bacterial infections, we performed a two-dimensional hierarchical cluster analysis. To limit the complexity of

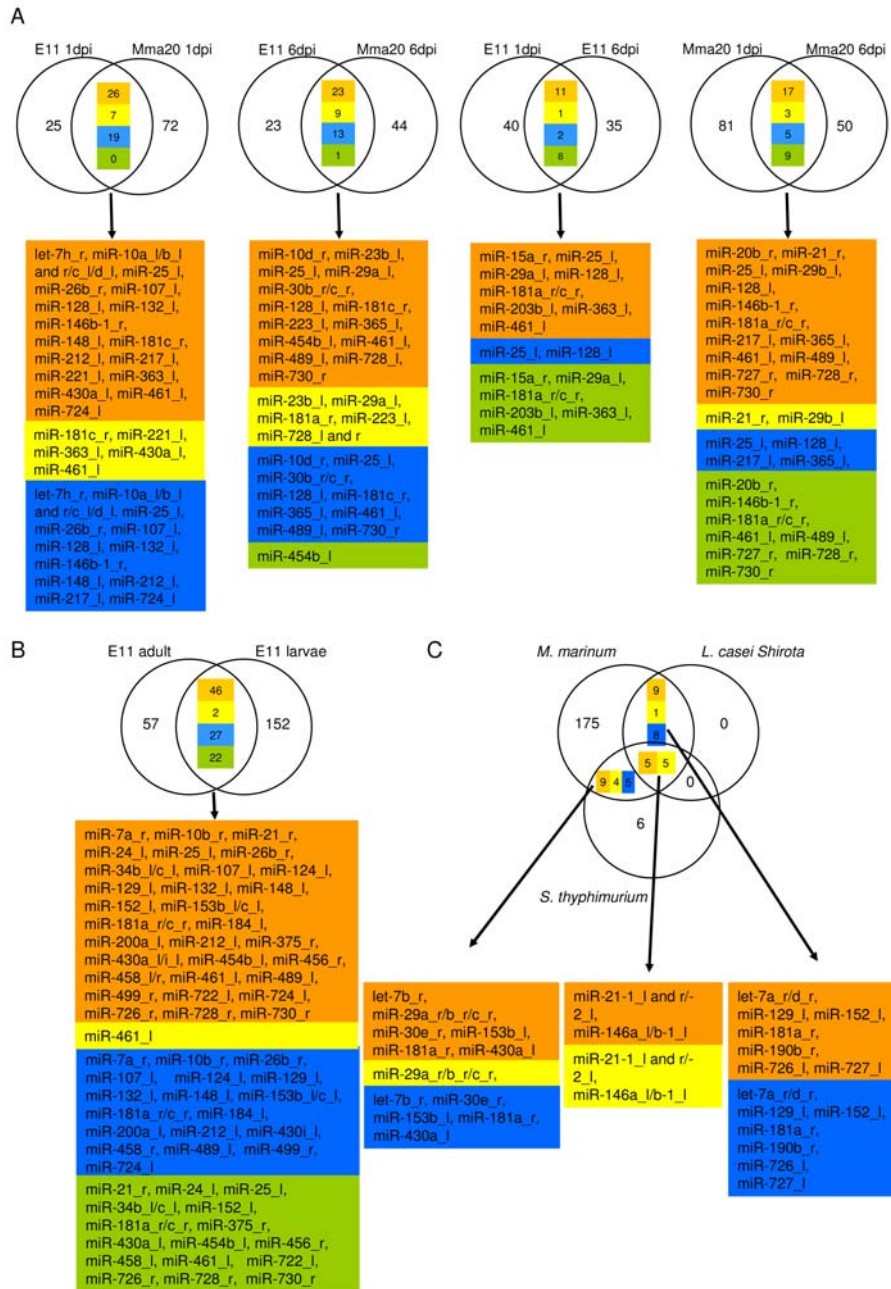


Figure 4. Venn diagrams showing the overlap between differentially expressed miRNA probes in adult and embryonic infection experiments. Orange - total number of overlapping probes; yellow - overlapping probes showing up-regulation in both groups; blue - overlapping probes showing down-regulation in both groups; green - overlapping probes showing up-regulation in one group and down-regulation in the other.

probes showing down-regulation in both groups; green - overlapping probes showing regulation in opposite directions. Only the probes in the category of known miRNAs are denominated in the colored boxes below the Venn diagrams. Probes correspond to the left (l) or right (r) arms of the pre-miRNA hairpin structure. A) Comparison of differentially expressed known miRNAs, C1, and C2 category probes in adult infections with the Mma20 and E11 strains. The Venn diagrams show the overlap in responsive miRNAs between the strains and at the two time points of analysis (1 and 6 dpi). B) Comparison of known miRNA, C1, and C2 category probe expression patterns in adult (1dpi and 6 dpi combined) and embryonal *M. marinum* E11 infection experiments. Numbers in yellow/blue/green boxes do not add up to 46 due to the fact that miRNAs can be changed in different directions at the 1dpi and 6 dpi time points. C) Comparison of known miRNAs, C1, and C2 category probes in embryonal infections with *M. marinum* E11, *S. typhimurium* and *L. casei* Shirota.

the cluster analysis we included only those probes that were differentially expressed in two or more infection experiments. We divided the probes into two main groups for which we performed separate cluster analysis (Fig. 3A,B). In the first set we combined the probes for known miRNAs and probes for miRNAs in the small C1 and C2 categories that include candidate miRNAs supported by experimental evidence. In the second set only C3 category probes that are less likely to represent miRNAs were evaluated. The cluster analysis of the C3 probes showed that in adult infection with the *M. marinum* E11 and Mma20 strains at 1 dpi a largely overlapping set of probes was up-regulated (Fig. 3B). In addition, the large numbers of down-regulated C3 probes observed with both strains at 6 dpi was also largely overlapping. For further analysis we concentrated on the set with known miRNAs and C1 and C2 predicted miRNAs. The clustering pattern of this probe set was notably different from that of the C3 probe set, with much less overlap between the *M. marinum* adult infection samples. We could distinguish eight major clusters of probes showing different trends in expression over the embryonal and adult infection experiments (Fig. 3A). MiRNAs in cluster 1 exhibited significant changes in several adult infection samples but not in embryonal infections. In contrast, miRNAs in clusters 2 and 3 were commonly induced in several samples of both adult and embryonal infections. Cluster 4 includes a non-specifically regulated group of miRNAs that was down-regulated in embryonic yolk injections with *M. marinum* but also by the PVP carrier used for these infections. In cluster 5, miRNAs are commonly down-regulated in *M. marinum* Mma20 infection of adults (1 dpi) and in *M. marinum* E11 embryonal yolk infection. Cluster 6 miRNAs were up-regulated at 6 dpi in adult *M. marinum* infection (Mma20 or E11 strains or both), but not in the embryonal *M. marinum* E11 infection. In cluster 7 miRNAs were commonly decreased in adult (1 dpi) and embryonal *M. marinum* infections, while cluster 8 miRNAs were commonly down-regulated in adult *M. marinum* infections at 6 dpi but not in embryonal infection.

In addition to the cluster analysis, we used Venn diagrams to show the common responsive miRNAs in different infection experiments. As shown in Fig.4A, in the *M. marinum* adult infections several tens of regulated miRNAs were overlapping

Table 1. Comparison of miRNA regulation between zebrafish and human or mammalian models.

Infection experiment										
Embryonal						Adult				
Zebrafish miRNA		Human miRNA	5'	E11	L6	PVP	E11 1dpi	E11 6dpi	Mma20 1dpi	Mma20 6dpi
dre-miR-20b* (miR-20b_r)		hsa-miR-20b*	-	-2.2	-	-2	-	-	-2.2	1.7
dre-miR-21 (miR-21-1_l)		hsa-miR-21	1.7	3.2	1.9	-	-	-	-	2.7
dre-miR-21* (miR-21-1_r)		hsa-miR-21*	3.8	4.2	1.8	-	-1.9	-1.4	-	8.3
dre-miR-21 (miR-21-2_l)		hsa-miR-21	1.6	3.1	1.9	-	-	-	-	2.6
dre-miR-21* (miR-21-2_r)		hsa-miR-21*	-	1.7	1.4	-	-	-	1.8	4.3
dre-miR-128* (miR-128-2_l)		hsa-miR-128*	5.9	-	-	-	-3	-3.2	-4.1	-4.3
up-regulation inhibits <i>Reelin</i> and <i>DCX</i> expression and reduces neuroblastoma cell motility and invasiveness; Targeting of the <i>Bmi-1</i> oncogene/stem cell renewal factor inhibits glioma proliferation and self-renewal; inhibits glioma cell proliferation by targeting transcription factor <i>E2F3a</i> ; Inhibition of <i>SNAP25</i> expression by HIV-1 Tat; significantly overexpressed in acute lymphoblastic leukemia										
									<i>Reelin</i> , <i>DCX</i> , <i>Bmi-1</i> , <i>E2F3a</i> , <i>UBE2W</i>	Evangelisti C et al 2009 Godlewski J et al 2008 Zhang Y et al 2009 Mi S et al 2007 Fulci V et al 2009

		Infection experiment										Function/associated diseases in human or mammalian animal models	Demonstrated target genes	Reference
		Embryonal					Adult							
Zebrafish miRNA	Human miRNA	g	E11	LCS	PVP	E11 1dpi	E11 6dpi	Mma20 1dpi	Mma20 6dpi					
dre-miR-181a* (miR-181a-1_r)	hsa-miR-181a*	-1.7	-1.9	-	-1.7	-	1.7	-1.6	1.6	specifically expressed and dynamically regulated in hematopoietic cells; expression in hematopoietic stem cells associated with increased B-cell development; decreased in HTLV-I, which dampens TCR signaling and T-cell activation to persist in the host; Down-regulation contributes to an aggressive leukemia phenotype through mechanisms associated with the activation of innate immunity pathways mediated by toll-like receptors and interleukin-1beta; miR-181a promotes early CD4 and CD8 double-positive T cell development; positively regulated by protein kinase Akt1; critical player in EpCAM-positive hepatic cancer stem cells	AKT3, CDX2, GATA6, NLK	Chen CZ et al 2004 Bellon M et al 2009 Debernardi S et al 2007 Larson RA. 2009 Fulci V et al 2009 Liu G et al 2008 Androulidaki A et al 2009 Ji J et al 2009		
dre-miR-181a-2* (miR-181a-2_r)	hsa-miR-181a-2*	-	-1.8	-1.3	-	-1.8	1.6	-	1.5					
dre-miR-181c* (miR-181c_r)	hsa-miR-181c*	-	-1.8	-	-	3	-2	3.1	-4.9					
dre-miR-461* (miR-461_r)	not in human	-	1.6	-	-	2.7	-3.9	2.5	-5.4	not in human and other mammals				
dre-miR-728 (miR-728_r)	not in human	-	-2.1	-	-	-	2.1	-1.9	1.5	not in human and other mammals				
dre-miR-730* (miR-730_r)	not in human	-	1.7	-	-	-	-2.2	3.8	-2.5	not in human and other mammals				
(block1581404_C1_r)		-	-1.7	-1.3	-	-1.8	1.8	-1.9	1.4					
(sblock96274_C1_r)		-	-			10	17	4.6	18.4					

The miRNAs that showed more than 1.5-fold differential expression ($P < 10^{-4}$) in four or more experimental samples from the infection studies are listed in the table. miRNA names were derived from miRBase and star sequences (not in all cases present in miRBase) were named accordingly. Probe names on the custom microarray are indicated in brackets behind the zebrafish (dre) miRNA names, and the corresponding human (hsa) miRNA names are given. Data on function, association to diseases in human and in mammalian animal models were collected from the literature. The literature data are for primary miRNAs, not for the star sequences, and literature data for miR-181a and miR-181c are combined. Experimentally demonstrated target genes for the primary miRNAs are also reported in the table (human gene symbols are in uppercase letters, mouse gene symbols begin with an uppercase letter followed by lowercase letters). In summary, these miRNAs are involved in several immune response related mechanisms (immune cell development, regulation of innate immunity, cytokine regulation, inflammation), and implicated in multiple cancer related mechanisms in a number of types of cancer. Yellow indicates up-regulation, blue indicates down-regulation. Light yellow and blue indicate significant fold changes ($P < 10^{-4}$) but below the 1.5-fold threshold. The numbers indicate the fold change values for *S. typhimurium* (St), *M. marinum* (E11), *L. casei* Shirota (LcS), and PVP carrier control injections of embryos and *M. marinum* (E11 and Mma20) adult infections, with experimental set-up as indicated in Fig. 1.

between the E11 and Mma20 strains and between the 1dpi and 6dpi time points. However, regulation of other miRNAs appeared to be strain or time point dependent. For example, miR-15a and miR-363 were up-regulated by E11 at 1 dpi and down-regulated by E11 at 6dpi, but were not regulated by Mma20 at both time-points. Similarly, miR-203b and miR-29a were not regulated by Mma20, but down-regulated by E11 at 1dpi and up-regulated at 6 dpi. On the other hand, miR-146b* (146b-1-r), miR-20b, miR-21 (miR-21-2-r), miR-217, miR-29b, miR-365, miR-489, miR-727, miR-728, and miR-730 were regulated by Mma20 at both time points but not by E11 at both time points. We also found miRNAs that changed in the opposite direction at 1 and 6 dpi time points in infections with both the E11 and Mma20 strains, such as miR-181c (down at 1 dpi, up at 6 dpi) and miR-461 (up at 1 dpi, down at 6 dpi). In addition, we identified that 46 known or C1/C2 category miRNAs were commonly regulated in adult and embryonal *M. marinum* E11 infections, of which 2 were up-regulated in the same direction, and 27 down-regulated in the same direction (Fig.4B). Comparison of all embryonal infection experiments showed that miR-21, miR-146a, and miR-146b were commonly up-regulated by the pathogenic *M. marinum* and *S. typhimurium* bacteria as well as by the non-pathogenic *L. casei* Shiota bacteria (Fig.4C). In conclusion, we observed common aspects in the response of miRNAs to different bacterial infections as well as differences related to the bacterial species or strains, the time points of analysis, and the adult or embryonal stage of the zebrafish host.

Functions and disease associations of commonly infection responsive miRNAs

To further narrow down the list of miRNAs that are commonly responsive to bacterial infections in zebrafish we took those miRNAs that occurred in more than four infection experiments. These miRNAs include miR-20b, miR-21, miR-128, miR-146a, miR-146b, miR-152, miR-181a, miR-181c, miR-461, miR-728, miR-730 and two predicted miRNAs in the C1 category (Table 1). We searched the literature for data on the function of the known miRNAs and their association to diseases in human and in mammalian animal models (Table 1). Reviewing the literature revealed that these miRNAs are involved in several processes related to the immune response, such as immune cell development, regulation of innate immunity, cytokine regulation, and inflammation. The miR-146 family, whose members miR-146a and miR-146b we find commonly up-regulated in infections of zebrafish, has previously been shown to affect the TLR signaling pathway that is involved in the innate immune response to bacterial infection (Taganov et al., 2006; Williams et al., 2008). Down-regulation of miR-181 family members, which we observed in four infection experiments, has been shown to contribute to an aggressive leukemia phenotype through mechanisms associated with the activation of innate immunity pathways mediated by TLRs and interleukin-1beta. The involvement of miR-181 in T-cell development has also been demonstrated (Chen et al., 2004; Larson, 2009). The interleukin-12 p35 subunit gene is a demonstrated target of the miR-21 family (Lu et al., 2009), whose members were

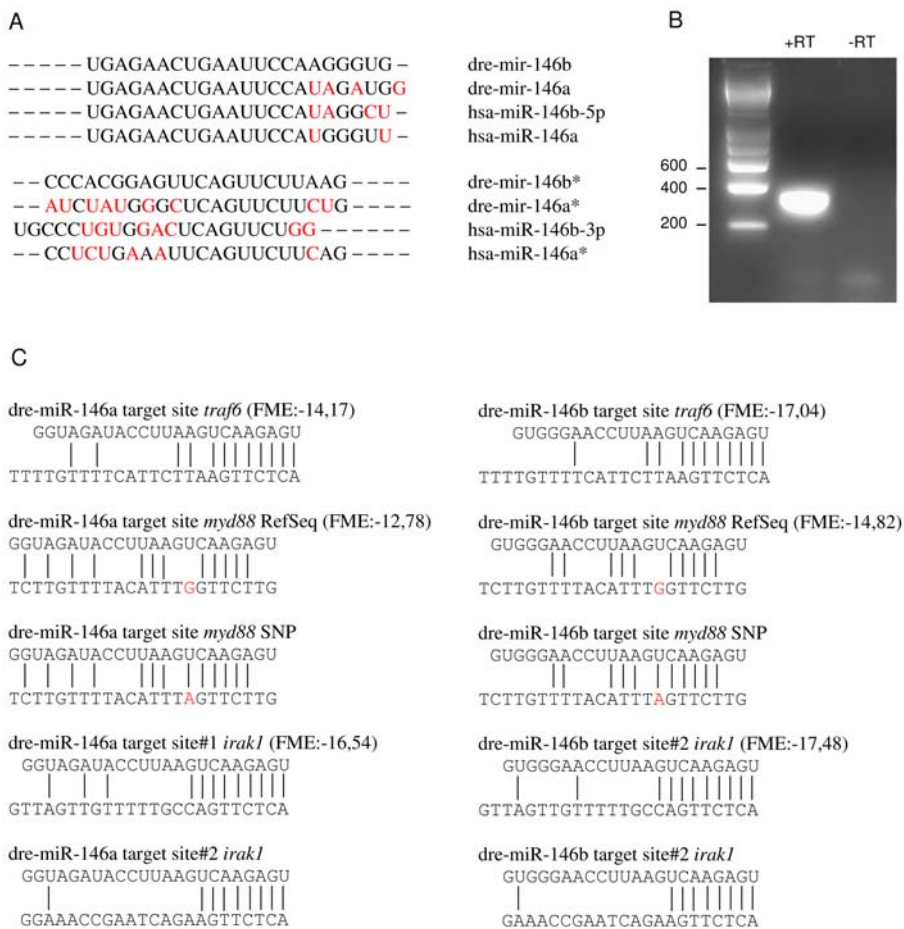


Figure 5. Members of the miR-146 family in human and zebrafish and their target sites in zebrafish TLR and IL-1R pathway genes. A) Sequence alignment of the members of the human (hsa) and zebrafish (dre) miR-146 family. Nucleotide differences with the dre-miR-146b/b* sequences are indicated in red. B) Confirmation of the presence of miR-146a/b target sites in the 3'UTR of the zebrafish *irak1* gene. A target site for miR-146a and miR-146b is located in the Zv8 genomic sequence between nucleotides 255-276 downstream of the stop codon of the *irak1* transcript. To check if this target site is included the 3'UTR of the *irak1* transcript RT-PCR was performed on RNA from *S. typhimurium*-infected embryos. RT-PCR reactions were performed with (+RT) and without (-RT) the reverse transcription step to verify that genomic DNA contamination was removed. With inclusion of the RT step a 316 bp fragment was amplified that includes the miR-146a/b target site. C) Alignments of predicted target sites of zebrafish miR-146a and b in TLR and IL-1R pathway genes, *traf6* (NM_199821), *myd88* (NM_212814) and *irak1* (Zv8 genome). We compared target sites of *myd88* transcript NM_212814 from the RefSeq database and the *myd88* transcript from the Ensembl database (ENS DART0000001143) where we detected a SNP (G > A polymorphism, indicated in red)

that increases the complementarity with the miR-146a/b sequences. In all alignments the miR sequences are shown on top in the 3' to 5' direction. Free minimum energy values (FME) for the interactions are indicated next to the alignments.

up-regulated in four zebrafish infections and down-regulated in one. MiR-152 has also been implicated in the immune system (Hiroki et al., 2009; Kannan et al., 2009; Lehmann et al., 2008). Furthermore, in previous studies most of the commonly infection responsive miRNAs (miR-20b, miR-21, miR-128, miR-146b, miR-152, miR-181a, miR-181c) have been implicated in various processes related to the development of cancer, such as apoptosis, proliferation, invasion, metastasis, and cell motility. Aberrant expression of these miRNAs has been detected in different types of cancer, including breast, liver, lung, and colon cancer, and leukemia (Table 1). Considering the common expression characteristics in adult and embryonal zebrafish infection experiments and the clear link with the immune response in human and other animal models, we focused our further analyses on family members of miR-146.

Targeting of the TLR and IL-1R pathways by the miR-146 family in human and zebrafish

MiR-146 was one of the most consistently induced miRNAs in our infection studies in zebrafish and has previously been linked to innate immune response in mammalian systems (Hou et al., 2009; Perry et al., 2009; Taganov et al., 2006). In human, the miR-146 family members are derived from two genes, *MIR146A* and *MIR146B*, located on chromosomes 5 and 10, respectively. The miRNAs processed from the left arm of the stem loop precursor miRNAs, miR-146a and miR-146b-5p, differ in their mature sequence only by two nucleotides at the 3' end (Taganov et al., 2007). In addition, mature miRNAs, miR-146a* and miR-146b-3p, are also processed from the right arm of the precursor miRNAs. Recently, it has been reported that there is a rapid increase in miR-146a and miR-146b expression in immune cells following activation of members of the TLR and IL-1 receptor families, activated by microbial ligands and pro-inflammatory cytokines, respectively. The induction of most TLR pathways and IL-1 signaling is initiated by the recruitment of adaptor protein MyD88 to the receptor. MyD88 transmits the signal to IL-1 receptor associated kinase 1 (IRAK1) that then recruits TNF receptor-associated factor 6 (TRAF6) into the complex. This activates IKB kinase and JNK and, in turn, the downstream NF- κ B and AP-1 transcription factors and results in up-regulation of several hundreds of immune-responsive genes. IRAK1 and TRAF6 have been shown to represent potential targets of the miR-146 family, and overexpression of miR-146a and b resulted in down-regulation of IRAK1 and/or TRAF6 (Taganov et al., 2006). It has also been shown that miR-146a expression is regulated via NF- κ B and JNK-1/2, whilst miR-146b expression was mediated via MEK-1/2 and JNK-1/2 (Perry et al., 2009). Together, these data suggest a role for the miR-146 family in negative feed back regulation of the innate immune response.

According to the data in miRBase (www.mirbase.org/) the zebrafish miR-146 fam-

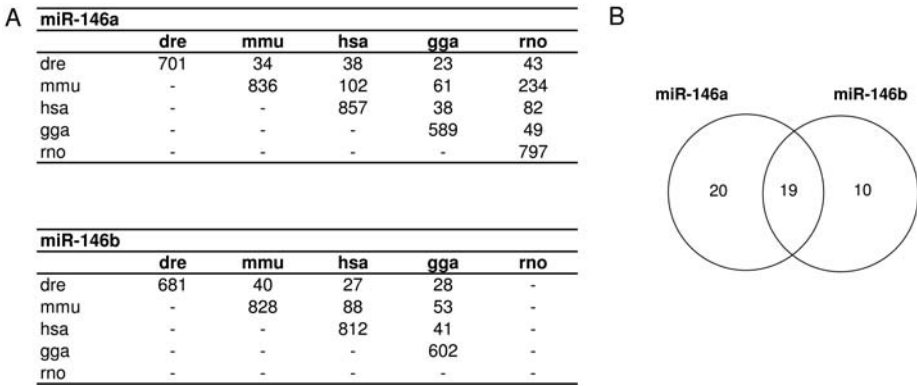


Figure 6. Conservation of predicted targets of the miR-146 family. Target genes of miR-146 family members in zebrafish (dre), human (hsa), mouse (mmu), rat (rno) and chicken (gga) were predicted using MicroCosm Targets. The predicted targets from zebrafish, mouse, rat, and chicken, were converted to human Ensembl Gene ID homologues and were compared across different species. If the predicted targets had the same human homologues, then these targets were considered to be potentially conserved across these species. The numbers of overlapping targets between species are indicated in the table. B) Comparison of miR-146a and miR-146b predicted targets. The Venn diagram shows the overlap between the targets of miR-146a and miR-146b that were commonly predicted for human and zebrafish.

ily consists of two members, dre-miR-146a and dre-miR-146b, derived from genes located on chromosomes 13 and 21, respectively. Experimental evidence is indicated in miRBase only for mature miRNAs derived from the left stem loop precursor arms. However, in our microarray analysis we also detected differential expression of probes for miRNAs derived from the right stem loop precursor arms, which we named dre-miR-146a* and dre-miR-146b*. The alignment of mature sequences of human and zebrafish miR-146 family members is shown in Fig. 5A.

To determine if the specificity of miR-146 members for the *irak1* and *traf6* genes in the TLR/IL-1R pathways might be conserved in zebrafish we used different target prediction programs. MiRanda prediction identified *traf6* as a putative target of both miR-146a and b from zebrafish, however *irak1* was not identified. We checked *irak1* mRNAs/ESTs in the database, but only few sequences were present, of which only two ESTs contained a very short 3'UTR. Upon inspection of the *irak1* genomic sequence we identified two perfect miR-146 target sites present at nucleotide locations 293-316 and 903-924 downstream of the *irak1* stop codon. By reverse transcriptase PCR on RNA from *S. typhimurium*-infected zebrafish embryos we confirmed that the *irak1* transcript contains a longer 3'UTR containing at least the first target site (Fig. 5B). MiRanda analysis predicted *myd88* as a third gene in the TLR pathway that could be a potential target of zebrafish miR-146 a and b. However, it was not predicted for human miR-146. Interestingly, zebrafish *myd88* is polymorphic for the pre-

Table 2. Ranking of predicted miR-146 target sites in the TLR pathway genes *myd88*, *irak1* and *traf6*.

	miRanda ranking				RNAhybrid ranking				TargetScan ranking				Integration algorithm			
	<i>myd88</i>	<i>irak1</i>	<i>traf6</i>		<i>myd88</i>	<i>irak1</i>	<i>traf6</i>		<i>myd88</i>	<i>irak1</i>	<i>traf6</i>		<i>myd88</i>	<i>irak1</i>	<i>traf6</i>	
dre-miR-146a	10	16	1	13	44	7	-	6	3	14	2	2	-	-	-	-
dre-miR-146a*	-	-	-	190	65	2	-	5	2	102	93	-	-	-	-	-
dre-miR-146b	12	14	3	28	35	3	-	4	1	13	1	1	-	-	-	-
dre-miR-146b*	-	-	-	24	-	-	-	-	-	20	-	-	-	-	-	-
hsa-miR-146a	-	17	2	567	189	25	-	8	9	570	8	1	-	-	-	-
hsa-miR-146a*	-	130	26	-	451	153	-	-	60	-	159	23	-	-	-	-
hsa-miR-146b-5p	-	33	5	592	352	82	-	7	11	575	23	2	-	-	-	-
hsa-miR-146b-3p	61	-	-	143	37	-	57	74	-	31	158	-	-	-	-	-

Three different target site prediction methods, MiRanda, RNAhybrid and TargetScan, were used to identify putative miR-146 family target genes in transcript databases downloaded from the MicroCosm Targets database (formerly known as miRBase Targets). As the zebrafish *irak1* transcript in this database contains an incomplete 3'UTR we replaced this sequence with the longer sequence derived from the Zv8 genome. The predicted target genes were ranked by calculating a weight for the prediction based on different parameters used by the target site prediction methods. In miRanda the weight is calculated from local alignment score, thermodynamic stability, and the number of predicted target sites in the transcript. In RNAhybrid the weight is based on *P*-value for the significance of increasing free energy and the number of predicted target sites. In TargetScan the weight is based on site type (matching to 5' seed region and contribution of 3' pairing) and the number of predicted target sites. The integration algorithm calculates a final weight based on the three prediction methods. Numbers in the table show the position of the gene in the weight ranking of all predicted target genes.

dicted target site, which contains a SNP (single nucleotide polymorphism) in the 5' seed region that is most important for the miRNA interaction (Fig. 5C). Alignments of miR-146 a and b with their predicted target sites in all three TLR pathway genes, *traf6*, *irak1*, and *myd88* are shown in Fig. 5C.

To further investigate the conservation of miR-146 target sites in the *myd88*, *irak1* and *traf6* genes we employed two target site prediction methods, RNAhybrid and TargetScan, in addition to miRanda, and also used an integration algorithm to rank these predictions (Table 2). This showed that miR-146 target sites in *traf6* are in the top 2 ranking for miR-146a and b from both human and zebrafish. MiR-146a and b target sites in zebrafish *irak1* (containing the longer 3'UTR from genomic prediction) ranked in the top 2 and for human *IRAK1* ranked on 8th and 23rd position using the integration algorithm. MiR-146 target sites in zebrafish *myd88* were in the top 20, but for human *MYD88* were predicted only by the RNAhybrid method and with low ranking. Target sites in the TLR pathway genes are also predicted for the miR-146 star/3p sequences but with lower ranking.

Other predicted targets of the human and zebrafish miR-146 family

To predict further conserved targets we downloaded miRanda-predicted target gene lists of zebrafish (dre-miR-146a, -146b), human (hsa-miR-146a, -146a*, -146b-5p, -146b-3p), mouse (mmu-miR-146a, -146b, -146b*), rat (rno-miR-146a) and chicken (gga-miR-146a, -146b, -146b*) miR-146 family members from MicroCosm Targets (formerly known as miRBase Targets). To compare targets across different species, we converted the predicted targets from zebrafish, mouse, rat and chicken to the homologous human Ensembl Gene IDs and HGNC gene symbol (Fig. 6A). Among 701 predicted target genes for miR-146a in zebrafish, we found 38 overlapping between zebrafish and human, of which 7 were also conserved in at least one other species (Fig. 6 and Table 3). For miR-146b, 27 out of 681 predicted target genes in zebrafish were overlapping with human, with 6 conserved in at least one other species (Fig. 6 and Table 3). Next, we compared the results for miR-146a and b showing that 19 genes were predicted to be commonly targeted by miR-146a and b in both human and zebrafish, 19 genes only targeted by miR-146a, and 10 genes only targeted by miR-146b (Fig. 6B). To this list of 48 genes we added those predicted targets that overlapped between zebrafish and at least two other species but not human (Table 3). This list of in total 64 genes was used for functional annotation and analysis of biological pathways (Table 3). These analyses revealed that most of the predicted targets in the list have immune related functions and were previously shown to be associated with immunological diseases or cancer. Genes were mostly classified into signaling in the immune system (*ARHGAP17*, *COMMD7*, *HSPA1A*, *IRAK1*, *IRF5*, *LCK*, *PIAS2*, *TRAF6*, *TRPC1*), haemostasis (*FGB*, *MMP11*), apoptosis (*ARD1*, *ARSG*, *CTSB*, *DAXX*, *FAS*), cell cycle signaling (*CHEK1*, *PPP2R4*, *ASPM*), gene expression (*ARID3B*, *BCOR*, *FBXO8*, *IARS*, *POLR2A*, *PUM2*, *SMYD1*, *SNRPC*, *TGIF*, *YEATS4*, *ZNF143*, *ZNF326*), and metabolic processes (*AMARC*, *B3ALT2*, *GNE*, *NUDT4*, *PHKA1*, *POLQ*, *PTGES2*,

Table 3. Pathway annotation of predicted target genes of the miR-146 family.

Ensembl Gene ID	Gene symbol	# species conserved	dre	hsa	mmu	rno	gga	Biological pathways	Description	Reference
ENSG000002042110	AMACR	3	a/b	-	-	a	a/b	Steroid metabolism	Alpha-methylacyl-CoA racemase	Mubiru JN et al 2005 Marx A et al 2006
ENSG00000102030	ARD1A	2	a/b	a/b	-	-	-	Apoptosis-Akt-HIF1 signaling, Metabolism: amino acid	N-terminal acetyltransferase complex ARD1	Li W et al 2006 Amatizurra A et al 2008 Lim JH et al 2008
ENSG00000140750	ARHGAP17	3	a/b	-	a/b	a	-	Signaling by Rho GTPases, by EGFR	subunit homolog A	
ENSG00000179361	ARID3B	2	a	a/b	-	-	-	Regulation of transcription	AT rich interactive domain 3B (BRIGHT-like)	Cowden Dahl KD et al 2009 Kim D et al 2007 Kobayashi K et al 2006 Takebayashi A et al 2006
ENSG00000141337	ARSG	2	a/b	a/b	-	-	-	Apoptosis-Sphingolipid Metabolism	Arylsulfatase G Precursor	
ENSG00000066279	ASPM	4	a/b	b	a	a	-	Negative regulation of asymmetric cell division	Asp (abnormal spindle) homolog, microcephaly associated	Bord J et al 2002 Hagemann C et al 2008 Lin SY et al 2008 Wu SC et al 2008
ENSG00000162630	B3GALT2	2	a	a/b	-	-	-	Sphingolipid metabolism	Beta-1,3-galactosyltransferase 2	Felner KM et al 1998 Arrip JH et al 1999 Vall M et al 1998
ENSG00000183337	BCOR	2	a/b	a/b	-	-	-	Negative regulation of transcription, DNA-dependent, chromatin modification	BCL6 co-repressor	Hyunh KD et al 2000 Wansada JA et al 2008 Srinivasan RS et al 2003
ENSG00000105583	C19ORF56	2	a/b	a	-	-	-	-	UPF0139 membrane protein C19orf56	Aoki H et al 2000
ENSG00000165698	C9ORF9	2	a/b	b	-	-	-	-	Uncharacterized protein C9orf9	Boelens MC 2009 Amira N et al 2004
ENSG00000140743	CDR2	2	a/b	a/b	-	-	-	-	Cerebellar degeneration-related protein 2 (Paraneoplastic cerebellar degeneration-associated antigen)	Albert ML et al 1998
ENSG00000149554	CHEK1	2	a/b	a/b	-	-	-	Cell Cycle Checkpoints	Serine/threonine-protein kinase Chk1	Jamil S et al 2008 Matsura K et al 2008 Barre B, Perkins ND 2007
ENSG00000184908	CLCNKB	2	a/b	a/b	-	-	-	Chloride transport	Chloride channel protein ClC-Kb (Chloride channel Kb)	Embark HM et al 2004
ENSG00000149600	COMMD7	2	b	a/b	-	-	-	Negative regulation of NF-kappaB transcription factor activity	COMM domain-containing protein 7	Maine GN, Burstein E 2007
ENSG00000212710	CTAGE1	3	a/b	-	a/b	a	-	-	Cutaneous T-cell lymphoma-associated antigen 1	de Ble P et al 2006 Usener D et al 2003 Eichmüller S et al 2001
ENSG00000225932	CTAGE4	3	a/b	-	a/b	a	-	-		
ENSG00000150527	CTAGE5	3	a/b	-	a/b	a	-	-		
ENSG00000244693	CTAGE6	3	a/b	-	a/b	a	-	-		
ENSG00000164733	CTSB	3	a/b	-	a/b	a	-	Regulation of apoptosis	Cathepsin B	Johnson EM et al 2009 Rose PP et al 2007 Yan S, Sloane BF 2003 Li-Korokyi HS et al 2004
ENSG00000102390	CXORF26	2	a	a	-	-	-	-		
ENSG00000204209	DAXX	2	a	a/b	-	-	-	Apoptosis (JNK, MAPK, SUMO signaling)	Death domain-associated protein 6	Yang X et al 1997 Tavali N et al 2008 Kiamura T et al 2009

Ensembl Gene ID	Gene symbol	# species conserved	dre	hsa	mmu	rno	gga	Biological pathways	Description	Reference
ENSG00000165487	<i>EFHA1</i>	3	a b	-	a b	a	-	Calcium ion binding	EF-hand domain family, member A1	Winston JT et al 1999
ENSG000000026103	<i>FAS</i>	3	a b	a b	a	-	-	Apoptosis	Tumor necrosis factor receptor superfamily member 6 Precursor (FASLG receptor)/Apoptosis-mediating surface antigen FAS(Apo-1 antigen)(CD95 antigen)	Xie FJ et al 2009
ENSG00000164117	<i>FBXO8</i>	2	a	a b	-	-	-	Regulation of ARF protein signal transduction	F-box only protein 8	Giordanengo V et al 2004
ENSG00000171564	<i>FBG</i>	2	a b	a	-	-	-	Hemostasis, Formation of Platelet plug	Fibrinogen beta chain Precursor	Merkley MA et al 2009
ENSG00000159821	<i>GNE</i>	3	a b	-	-	a	a b	Carbohydrate metabolic process, glycolysis, gluconeogenesis	Glucosaminase (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	Wheeler DS et al 2009
ENSG00000204389	<i>HSPA1A</i>	2	a b	a b	-	-	-	Signaling in Immune system, Antigen processing and presentation, Proteolysis	Heat shock 70kDa protein 1A	Wang X et al 2008
ENSG00000196305	<i>IARS</i>	2	a	a b	-	-	-	Gene Expression, Cytosolic tRNA aminoacylation	Isoleucyl-tRNA synthetase, cytoplasmic, isoleucine tRNA synthetase	Figueredo C et al 2008
ENSG00000184216	<i>IRAK1</i>	4	a b	a b	a b	a	-	Signaling in Immune system, TCR signaling	Interleukin-1 receptor-associated kinase 1	Gabal VL et al 2008
ENSG00000126604	<i>IRF5</i>	2	a b	a	-	-	-	Toll-like receptor signaling pathway	Interferon regulatory factor 5	Lee SW et al 2004
ENSG00000152315	<i>KCNK13</i>	3	a b	b	-	a	-	Potassium transporters: inward current	Potassium channel, subfamily K, member 13	Bannerman DD et al 2002
ENSG00000111731	<i>KIAA0528</i>	2	b	b	-	-	-	-	-	Altmann R et al 2007
ENSG00000108244	<i>KRT23</i>	2	a b	a	-	-	-	-	Hypothetical protein LOC9847	Mizobe T et al 2007
ENSG00000182866	<i>LCK</i>	2	b	a b	-	-	-	Signaling in Immune system, TCR signaling, hemostasis, HIV infection	Keratin 23 (histone deacetylase inducible)	Liu G et al 2008
ENSG00000198934	<i>MAGEE1</i>	4	a b	a b	a b	a	-	-	Melanoma-associated antigen E1 (MAGE-E1 antigen)(Hepatocellular carcinoma-associated protein 1)	Yang L et al 2009
ENSG000000099953	<i>MMP11</i>	2	a b	a	-	-	-	Hemostasis: Leukocyte Extravasation, Bladder Cancer Signaling	Matrix metalloproteinase-11	Pandey AK et al 2009
ENSG00000236761	-	3	a b	-	a b	a	-	-	-	Hu G and Barnes BJ 2009
ENSG00000242426	-	3	a b	-	a b	a	-	-	-	Balkhi MY et al 2009
ENSG00000173598	<i>NUDT4</i>	3	a	-	-	a	a	Cyclic nucleotide metabolic process, regulation of RNA export from nucleus	Nudix (nucleoside diphosphate linked moiety X)-type motif 4	Yanai H et al 2007

Ensembl Gene ID	Gene symbol	# species conserved	dre	hsa	mmu	rno	gga	Biological pathways	Description	Reference
ENSG00000173598	<i>NUDT4P1</i>	3	a	-	-	a	a	-	Nudix (nucleoside diphosphate linked moiety X)-type motif 4 pseudogene 1	Li Y et al 2005
ENSG00000015781	<i>PANK4</i>	2	a	a	-	-	-	Pantothenate and CoA biosynthesis	Pantothenate kinase 4	Xiang RL et al 2007
ENSG000000104883	<i>PEX1G</i>	2	a/b	a/b	-	-	-	Peroxisome fission	Peroxisomal membrane protein 11C (Peroxin-11C)/PEX1 (gamma)	Wilfred BR et al 2007
ENSG000000067177	<i>PHKA1</i>	2	b	a/b	-	-	-	Glucose metabolism	Phosphorylase kinase, alpha 1 (muscle)	Tanaka A. et al 2003
ENSG000000078043	<i>PIAS2</i>	2	a/b	a	-	-	-	Immune response IL-12 signaling pathway, P53 signaling pathway, SUMO-1 pathway, Proteolysis, Cancer, Jak-STAT signaling pathway	E3 SUMO-protein ligase PIAS2 (Protein inhibitor of activated STAT2)(DAB2-interacting protein)(DIP1)(Androgen receptor-interacting protein 3)(ARIP3)	Thoms S, Erdmann R 2005
ENSG000000051341	<i>POLQ</i>	4	b	-	b	a	b	Metabolism of nucleotides	Polymerase (DNA directed), theta	Dharancy et al 2009
ENSG000000181222	<i>POLR2A</i>	2	a	a/b	-	-	-	Gene Expression, MicroRNA (miRNA) Biogenesis, viral infection	DNA-directed RNA polymerase II subunit RPB1	Prakash S et al 2005
ENSG000000119383	<i>PPP2R4</i>	2	a/b	a	-	-	-	Cell cycle Regulation of G1/S transition (part 1), Small cell lung cancer	Protein phosphatase 2A activator, regulatory subunit 4	Wuyts W et al 2005
ENSG000000148334	<i>PTGES2</i>	3	a	-	a/b	a	-	Lipid metabolism	Prostaglandin E synthase 2	McDonnell N et al 2000
ENSG000000055917	<i>PLM2</i>	3	a/b	-	a/b	a	-	Regulation of translation	Pumilio homolog 2 (Drosophila)	Saito M et al 2009
ENSG000000075826	<i>SEC31B</i>	2	a/b	a	-	-	-	Claithrin-dependent protein traffic	Protein transport protein Sec31B (SEC31-related protein B)	Harold S et al 2008
ENSG000000139574	<i>SLC38A6</i>	2	a	a/b	-	-	-	Amino acid transport	Probable sodium-coupled neutral amino acid transporter 6	van den Akker E et al 2005
ENSG000000115393	<i>SMYD1</i>	3	a/b	-	a/b	a	-	Negative regulation of transcription	SET and MYND domain containing 1 (BOP)	Liu ST et al 2006
ENSG000000124562	<i>SNRPC</i>	3	a/b	a/b	-	-	-	Spliceosomal snRNP assembly	U1 small nuclear ribonucleoprotein C (U1 snRNP protein C)(U1C protein)	Kim J et al 2005
ENSG000000227524	<i>SNRPB1</i>	2	b	a/b	-	-	-	-	Small nuclear ribonucleoprotein polypeptide E-like 1	Kawamura K et al 2004

Ensembl Gene ID	Gene symbol	# species conserved	dre	hsa	mmu	rno	gga	Biological pathways	Description	Reference
ENSG00000177426	TGIF1	3	a/b	a/b	-	a	-	Negative regulation of transcription from RNA polymerase II promoter	Homeobox protein Tgif1 (5'-TG-3'-interacting factor 1)	Chen F et al 2003
ENSG00000168286	THAP11	2	a/b	a/b	-	-	-	Regulation of transcription	THAP domain-containing protein 11	Zhu CY et al 2009
ENSG00000133872	TIME166	3	b	a/b	a/b	-	-	-	Transmembrane protein 66 Precursor (Protein FOAP-7)(hepatitis B virus-HBV X-transactivated gene 3 protein)	Romanuk TL et al 2009 Mannherz O et al 2006
ENSG00000175104	TRAF6	4	a/b	a/b	a/b	a	-	TLR, TNFR and TCR signaling	TNF receptor-associated factor 6 (Interleukin-1 signal transducer)(RING finger protein 85)	Fujii Y et al 1999
ENSG00000144935	TRPC1	2	a/b	a/b	-	-	-	Signaling in Immune system: CREB pathway, MEK2 in T lymphocytes	Short transient receptor potential channel 1	Takahashi Y et al 2007 Pani B et al 2006 Marasa BS et al 2006 Beck B et al 2008 Bair AM et al 2009 Yeh TS et al 2004
ENSG00000137267	TUBB2A	2	a/b	a/b	-	-	-	Metabolism of proteins, Protein/Tubulin folding	Tubulin beta-2A chain	Mhalimeh S et al 2004
ENSG00000135763	URE2	2	a/b	a/b	-	-	-	-	Unhealthy ribosome biogenesis protein 2 homolog	Park JH, Roeder RG 2006 Italiano A 2008
ENSG00000127337	YEATS4	4	a/b	a/b	a/b	a	-	Positive regulation of transcription, DNA-dependent	YEATS domain-containing protein 4 (Glioma-amplified sequence 41)(Gas41)(NuMA-binding protein 1)(NuB1-1)	
ENSG00000166478	ZNF143	2	a/b	a	-	-	-	Transcription: RNA Polymerase III Transcription	Zinc finger protein 143 (Selenocysteine tRNA gene transcription-activating factor)(ISlat)(SPH-binding factor)	Mach CM et al 2002 Schuster C et al 1995 Grossman CE et al 2004 Mylinski E et al 2007 Gérard MA et al 2007 Mylinski E et al 2006
ENSG00000162664	ZNF326	2	a/b	a	-	-	-	Regulation of transcription	Zinc finger protein 326, ZAN75	Lee JY et al 2007, 1998

The table includes the most reliable predicted targets based on conservation among multiple species and all targets that were overlapping between zebrafish and human. Analysis of biological pathways was performed using the Reactome pathway database, GeneAlicart (www.genecards.org) and AmiGO pathways (amigo.geneontology.org/cgi-bin/amigo/go.cgi). Literature references are given that link these genes to immune functions. Species analyzed included zebrafish (dre), human (hsa), mouse (mmu), rat (rno) and chicken (gga). a - miR-146a predicted target gene, b - miR-146b predicted target gene, a/b - predicted target gene of both miR-146a and b. An extended version of this table with details on immune functions reported in the literature is included as a supplementary file.

TUBB2A), all pathways previously linked to immune functions.

Comparison between miRNA expression and the expression of the predicted target genes

Next we compared miR-146 expression with expression of predicted target genes from Table 3 under infection conditions. For comparison we took previously published data from van der Sar et al. 2009, where the zebrafish host transcriptome response to *M. marinum* Mma20 and E11 was determined using the same samples as for miRNA analysis in the present study. In addition, we used data from Stockhammer et al. 2009, where a time-course transcriptome profiling of the zebrafish embryonic innate immune response to salmonella infection was performed. We found that approximately half (35) of the predicted target genes in Table 3 showed significant ($P < 10^{-4}$) expression changes (up- or down-regulation) in either of these infection studies (Table 4). It is possible that the number of infection-responsive genes might still be higher since not all predicted target genes (13) were present on the microarray platform used in the expression studies. Responsiveness to both *M. marinum* and *S. typhimurium* infection was observed for 10 predicted target genes, 17 were responsive only to *M. marinum* infection, 8 only to *S. typhimurium* infection, and 13 predicted target genes represented on the microarray were not responsive to both infections. *Irak1*, one of the genes of the TLR and IL-1R pathways that are experimentally validated targets in human, was not present on the microarray. *Traf6* the other experimentally validated target in TLR and IL-1R signaling pathways was up-regulated at the end stage of *S. typhimurium* infection in embryos (24hpi). The interferon-responsive factor 5 (*IRF5*) gene is another key immune response gene that has been experimentally validated in mammals and for which a miR-146 a and b target sites are also predicted in zebrafish. Expression of *irf5* was up-regulated in all samples of the *M. marinum* infection study. In conclusion, many of the predicted miR-146 target genes that are conserved between human and zebrafish can be linked to bacterial infection processes by expression changes.

Discussion

To increase knowledge of miRNAs involved in the vertebrate host response to bacterial infections we have analyzed miRNA expression profiles of zebrafish infected with two model pathogens, *S. typhimurium* and *M. marinum* (Stockhammer et al., 2009; van der Sar et al., 2009). While adult zebrafish have complex innate and adaptive immunity similar to the human immune system, zebrafish embryos rely solely on the function of their innate immune response. Therefore, by using both embryos and adult zebrafish as hosts for bacterial infection, we could link miRNA expression profiles to the innate and adaptive components of the immune response. We identified a set of commonly responsive miRNAs in embryonic and adult infection experiments that includes highly conserved miRNAs with strong associations to

Table 4. Responsiveness of predicted target genes of miR-146 to different infections.

Human gene symbol	Unigene Code	Zebrafish gene symbol	E11 1 dpi	Mma20 1 dpi	E11 6 dpi	Mma20 6 dpi	St-5h	St 8h	St 24h
AMACR	Dr.77673	c1qtnf3				-1.64948			
ASPM	Dr.96472	aspm	1.47073	1.34309	1.78499	2.45658			
B3GALT2	Dr.79277	b3galt2							-1.41804
C19ORF56	Dr.117007	zgc:73111			-1.34411				
CHEK1	Dr.83316	chk1	1.38266	-1.51612	-1.42253				
CLCNKB	Dr.78662	zgc:64141				-2.01377	-1.25287	-1.64969	-1.58357
CTAGE1	Dr.105440	ctage5				1.28439			
CTAGE5	Dr.105440	ctage5				1.28439			
CTSB	Dr.3374	ctsb				2.82477			3.31843
CXORF26	Dr.78321	zgc:92611			-1.33198				
DAXX	Dr.73516	daxx				1.5038			
EFHA1	Dr.77336	zgc:113196		1.3113					
FAS	Dr.82180	fas			-2.1401	1.5653		3.6617	12.54386
FBXO8	Dr.83230	fbxo8		-1.41323					2.19152
FBG	Dr.8505	fbg		1.37155		3.47284		1.3093	
GNE	Dr.132714	gne		-1.16012		-1.15201			
HSPA1A	Dr.115994	hsp70l							15.85009
IARS	Dr.5185	iars							3.64018
IRF5	Dr.7758	irf5	1.4546	1.34987	1.45303	2.67239			
LCK	Dr.91511	lck				-1.52648			
-	Dr.105440	ctage5				1.28439			
NUDT4P1	Dr.134107	zgc:101062							-2.15537
PANK4	Dr.132622	zgc:66285				-1.3341			
PIAS2	Dr.25643	pias2						-1.1846	
PPP2R4	Dr.9399	ppp2r4						1.34357	
PTGES2	Dr.16120	ptgesl		-1.27843		-1.98551			-1.70173
SLC38A6	Dr.45587	slc38a6	1.43176			2.09576			
SMYD1	Dr.21307	smyd1a				-2.47098			
SNRPC	Dr.18318	snrpc		-1.39496	-1.58672				-1.65606
SNRPE1	Dr.117027	snrpe	-1.33143	-1.44041	-1.69165	-1.14326			-1.32162
TGIF1	Dr.139	tgif1							1.42374
THAP11	Dr.75932	zgc:65871		-1.15521	-1.28304				-1.45774
TMEM66	Dr.11537	zgc:153286		1.30393		1.33354			7.13477
TRAF6	Dr.74618	traf6							2.27878
TUBB2A	Dr.46855	zgc:112335	-1.1894			-2.91869			

Predicted miR-146 target genes from Table 3 are shown that showed infection-dependent expression changes in previously reported data sets (Stockhammer et al., 2009; van der Sar et al., 2009). Zebrafish UniGene IDs are from build #105. Significant fold change values ($P < 10^{-4}$) are indicated for infections of adult zebrafish with *M. marinum* E11 and Mma20 strains at 1 and 6 dpi (van der Sar et al., 2009) and for infection of zebrafish embryos with *S. typhimurium* at 5, 8 and 24 hpi (Stockhammer et al., 2009). Yellow - up-regulation, blue - down-regulation.

the immune system and development of cancer in human and mammalian models. We also determined that predicted target genes of the strongly infection-inducible miR-146 family are conserved between human and zebrafish, including key signaling intermediates of the TLR pathway that is pivotal for the innate immune response to bacterial infections (Hou et al., 2009; Perry et al., 2009; Taganov et al., 2006).

The set of commonly infection-responsive miRNAs included the highly conserved miRNAs, miR-20b, miR-21, miR-128, miR-146a/b, miR-152, and miR-181a/c. The mammalian counterparts of these miRNAs are differentially expressed in many types of cancer and have been implicated in tumor growth, invasion and metastasis (Debernardi et al., 2007; Evangelisti et al., 2009; Hiroki et al., 2009; Lei et al., 2009; Perry et al., 2009; Shi et al., 2008; Zhu et al., 2008). The mammalian counterparts of miR-21, miR-146 and miR-181 have also been linked to the immune system. Our study is the first that connects these miRNAs with mycobacterium and salmonella

infections. It is well known that the signaling pathways involved in the immune response and inflammation are strongly overlapping with those involved in the development of cancer, and it has previously been suggested that changes in expression of miRNAs might link these processes (Williams et al., 2008). In agreement, deregulated expression of miRNAs, for example miR-146a, has been observed in several inflammatory diseases, such as psoriasis, rheumatoid arthritis and osteoarthritis, as well as in different types of cancer such as papillary thyroid carcinoma, cervical cancer, ovarian cancer, breast cancer, pancreatic cancer and prostate cancer (Nakasa et al., 2008; Perry et al., 2009; Xia et al., 2009). The infection data reported here further support the notion that many miRNAs implicated in cancer processes are also connected to regulation of the immune response.

In connection to the immune response, the miR-146 family is one of the most interesting. We found that expression of miR-146a and b was commonly induced by infection in adult zebrafish as well as in embryos, indicating that the context of innate immunity is sufficient for the induction of the miR-146 miRNAs. This is consistent with results in human and animal models that have implicated the miR-146 family in regulation of innate immunity signaling pathways (Hou et al., 2009; Perry et al., 2009; Taganov et al., 2006). Induction of miR-146a and b was observed in human immune cells stimulated with microbial components (LPS) and proinflammatory mediators IL-1 β , TNF α (Taganov et al., 2006). Furthermore, viral infections and *Helicobacter pylori* infections also increased expression of miR-146 (Belair et al., 2009; Hou et al., 2009; Motsch et al., 2007). NF- κ B, the central transcription factor of the immune response, was shown to control miR-146 transcription (Taganov et al., 2006). Furthermore, two key intermediates of the common TLR and IL-1R signaling pathway, *TRAF6* and *IRAK1*, were shown to be potential molecular targets of the miR-146 family members (Taganov et al., 2006). Based on these results, the miR-146 miRNAs were proposed to fine-tune the innate immune response by negative feedback regulation of the TLR and IL-1R signaling pathways (Taganov et al., 2006). Consistent with this hypothesis, miR-146a expression directly or indirectly down-regulated the IL-1 β -induced release of chemokines, IL-8 and RANTES (Williams et al., 2008) and the LPS-induced production of IFN- γ and nitric oxide (Dai et al., 2008). In addition to a role in fine-tuning TLR and IL-1R signaling, miR-146a was also proposed as a negative regulator of the RIG-I-dependent antiviral pathway by targeting *TRAF6*, *IRAK1*, and *IRAK2* (Hou et al., 2009). In contrast to these data, miR-146b was found to be down-regulated in an *in vivo* model of acute inflammation triggered by LPS infusion of healthy human volunteers, indicating that this miRNA might respond differently in leukocytes *in vivo* than in *in vitro* studies or that miR-146b levels might be oscillating during inflammation owing to a possible sensing mechanism (Schmidt et al., 2009). In our *in vivo* infection studies, miR-146a and b were consistently induced, but during early stages of mycobacterium infection in adult zebrafish we observed down-regulation of the miR-146 star sequences that currently have unknown function. Thus, it will be of interest to study the dynamic

pattern of miR-146 family members in greater detail using *in vivo* models. Besides its role in innate immunity, miR-146a was recently suggested to also modulate adaptive immunity (Schmidt et al., 2009). The zebrafish model, in which innate and adaptive immunity can be studied in separation, can therefore contribute to dissecting the diverse functions of the miR-146 family.

Similar as for the miR-146 family, we also observed increased expression of miR-21 primary miRNAs and star sequences during several conditions of infection in zebrafish embryos and adults. MiR-21 was also found to be up-regulated in cultured human gastric epithelial cells upon *H. pylori* infection, which is a major risk factor for gastric cancer (Zhang et al., 2008). Significantly increased levels of miR-21 were observed in several human gastric cancer tissues and cell lines as well, and the over-expression of miR-21 was shown to promote cell proliferation and migration and inhibit apoptosis in a gastric cancer cell line (Zhang et al., 2008). The up-regulation of miR-21 expression during *H. pylori* infection was found to be mediated by AP-1 and STAT3, which in turn are activated by NF- κ B activation and IL-6 secretion in the gastric mucosa (Belair et al., 2009; Loffler et al., 2007). In addition, the miR-21 gene was found to be transactivated by the NF- κ B p65 subunit following infection with *Cryptosporidium parvum*, a protozoan parasite that elicits strong innate immune responses of the gastrointestinal epithelium (Zhou et al., 2009). MiR-21 also showed elevated expression during Epstein-Barr virus infection (Cameron et al., 2008) and was shown to regulate interleukin-12 p35 expression during allergic airway inflammation. Thus, like for miR-146, the infection-responsiveness of miR-21 in zebrafish indicates an evolutionary conserved role for this miRNA family in the vertebrate immune response.

Another interesting miRNA family that we found to be regulated in zebrafish infection experiments is the miR-181 family that has an important role in normal haematopoiesis, B-cell differentiation and T-cell development in mammals and has been associated with aggressive leukemia (Chen and Lodish, 2005; Li et al., 2007; Pekarsky et al., 2006). Little is known of the role of miR-181 in bacterial infections, but LPS stimulation of macrophages positively regulated miRNA-181c expression in a protein kinase Akt1-dependent manner (Androulidaki et al., 2009). Like miR-146a/b, the miR-181 family may function in negative regulation of the innate immune response, as expression levels of its members were inversely correlated with expression levels of predicted targets in TLR and IL-1R signaling (Marcucci et al., 2008). In our experiments miR-181a and c family members were down-regulated during bacterial infections of zebrafish, but showed opposite patterns of regulation in adult zebrafish. While miR-181a miRNAs were down-regulated at early stages and up-regulated at later stages of mycobacterium infection with two different strains, miR-181c expression showed the exact inverse pattern. In further studies the zebrafish-mycobacterium model may prove useful to dissect the functions of these members of the miR-181 family in the immune response.

Besides several hundreds of known miRNAs or predicted miRNAs with experi-

ment support (C1/C2 categories), our custom microarray used in the zebrafish infection studies contained probes for several thousands of additional hairpin sequences identified in the zebrafish genome sequence (C3 category). Many of these probes were infection responsive. Interestingly, there was a striking common regulation of C3 probes during mycobacterium infections of adult fish with the *M. marinum* E11 and Mma20 strains. We examined the response to these stains at 1 and 6 days after infection and observed a strongly overlapping pattern at both stages, despite the fact that there is a major difference in disease phenotypes elicited by the two strains. While E11-infected fish develop a chronic infection and show no symptoms of disease, Mma20-infected fish develop acute infection and are close to the end point of the disease at 6 dpi. The common regulation of C3 probes is in sharp contrast with what we have seen for gene expression where in a 2D cluster analysis of the data set the strains rather than the time points clustered together (van der Sar et al., 2009). In addition, the clustering pattern for the C3 category (Fig.3B) was very different from the clustering pattern of the known and C1/C2 category miRNAs (Fig. 3A) that showed much less overlap between the strains and time points. These observations warrant further study, as it is currently unknown if the C3 probes represent miRNAs or might correspond to other types of non-coding or coding RNAs.

The knowledge of target genes is crucial for the understanding of miRNA function. We focused on the infection-inducible miR-146 family to investigate possible conservation of target genes between zebrafish and mammals. As discussed above, mammalian miR-146a and b are thought to fine-tune TLR and IL-1R signaling by targeting *IRAK1* and *TRAF6*. Here we show the presence of conserved miR-146 target sites in the 3'UTRs of zebrafish *irak1* and *traf6*. Additionally, we found a miR-146a/b target site in the 3'UTR of MyD88, which functions upstream of *irak1* and *traf6* as the common adaptor of several TLRs and IL-1R. However, targeting of MyD88 by miR-146 in zebrafish awaits experimental confirmation and appears not to be conserved in human or rodents. We have previously shown that several TLR pathway genes and downstream effector genes are induced during infections in zebrafish (Stockhammer et al., 2009; van der Sar et al., 2009). In addition, we observed that many negative regulatory genes of the TLR pathway, for example *irak3*, are induced during salmonella infection (Stockhammer et al., 2009). The induction of miR-146, as a putative negative regulator of several genes in TLR pathway, is in line with these findings. Together these gene expression and miRNA expression data suggest the importance of feed back mechanisms to properly control the inflammatory response that might in some cases be more damaging than the infection itself.

We attempted to predict other targets than the TLR pathway genes for miR-146. The available target prediction programs generate lists of hundreds to thousands of candidate target genes probably containing many false positive hits. We reasoned that predicted targets common between multiple species or distant species like human and zebrafish could be the most likely candidates for being true *in vivo* targets. We found 64 targets that appeared conserved between zebrafish and human, of which 27

were conserved also in mouse, rat and/or chicken. The majority of these genes were previously linked to immune response processes with gene ontology or pathway annotations related to apoptosis, regulation of NF- κ B transcription factor activity, haemostasis, infections, T-cell development and function, and TLR signaling (Table 3). In addition, many of these genes are linked to tumor progression, consistent with the previously proposed link between miR-146 function in cancer and the immune response (Williams et al., 2008). One of the predicted targets conserved between human and zebrafish is interferon response factor 5 (*IRF5*), which is a critical transcription factor in the immune response downstream of TLR signaling and also has been linked to tumor suppression (Balkhi et al.; Hu and Barnes, 2009; Pandey et al., 2009; Yang et al., 2009). Targeting of human *IRF5* by miR-146 is supported by experimental data (Tang et al., 2009), consistent with the possibility that other predicted targets conserved between zebrafish and human may also be bona fide target genes. We also examined how the expression of the predicted miR-146 target genes responded to infection in zebrafish. In general, it cannot be predicted if and how the induction of miR-146 might affect transcript levels of its target genes. MiR-146 induction might affect only translation of its targets, or it might down-regulate their transcription or dampen their induction. However, it was notable that many of the predicted targets could be linked by expression changes to the infection process. This, together with the fact that most of the predicted targets have functional annotations linked to immunity, suggests that our list of conserved predicted targets may be a good starting point for experimental validation studies.

In conclusion, we have shown here that infection-responsive miRNAs are highly conserved between human and zebrafish. It is likely, as we have shown for the miR-146 family, that there is also considerable overlap in miRNA target genes, indicating that the zebrafish can be a useful model to dissect functions of miRNAs in the vertebrate immune system. Several miRNAs are not only responsive to infection in zebrafish adults, but also in embryos prior to the onset of adaptive immunity. In the embryo model, miRNAs can be easily over-expressed by micro-injection of synthetic miRNA duplexes, or knocked down using morpholinos (Schier and Giraldez, 2006). In addition, target protecting morpholinos can be used to experimentally validate predicted target genes *in vivo* (Choi et al., 2007). In future studies these strategies will be applied to analyze the function of infection-responsive miRNAs in the zebrafish embryo model.

Materials and methods

Bacterial strains and growth conditions

M. marinum strains E11 and Mma20 have been described before (van der Sar et al., 2004). Bacteria were grown at 30 °C in Middlebrook 7H9 medium supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (BD Biosciences) and 0.05% Tween-80. *S. typhimurium* wild type strain SL1027 bacteria containing

DsRed expression vector pGMDs3 were used for the infection of zebrafish embryos (Stockhammer et al., 2009; van der Sar et al., 2003). Bacteria were freshly grown at 37 °C overnight on Luria-Bertani agar plates supplemented with 100 µg/ml carbenicillin. *M. marinum* and *S. typhimurium* bacterial numbers were determined by measuring the optical density at 600 nm and by plating and CFU determination. *L. casei* Shirota bacteria were taken from Yakult fermented milk drink (Yakult Europe B.V., Almere) specified to contain 10⁸ bacteria/ml. Bacteria were collected by centrifugation and washed extensively with PBS.

Zebrafish husbandry and infection experiments

Zebrafish (*Danio rerio*) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (<http://ZFIN.org>). The infection experiment of adult male zebrafish with *M. marinum* E11 and Mma20 strains has been previously described (van der Sar et al., 2009). The analysis of miRNA expression profiles was done using powdered tissue from the same fish as used for mRNA expression analysis in the previous study (van der Sar et al., 2009). As before, three fish per strain and time point (1 and 6 days post infection) were used and PBS-injected fish were used as a control. Infection experiments at the embryonic stage were performed using mixed egg clutches from different pairs of wild type zebrafish. Embryos were grown at 28.5–30 °C in egg water (60 µg/ml Instant Ocean sea salts) and staged by morphological criteria (Kimmel et al., 1995). For the duration of bacterial injections embryos were kept under anaesthesia in egg water containing 0.02% buffered 3-aminobenzoic acid ethyl ester (tricaine; Sigma-Aldrich). For infection with *M. marinum* E11 embryos were staged at 48 h post fertilization (hpf). Bacteria were resuspended at desired concentrations in a 2% suspension of polyvinylpyrrolidone (PVP, average mol wt 40000, Calbiochem, San Diego) in PBS and approximately 1 nl of this mixture was injected into the yolk sac of 48 hpf embryos. PVP was chosen as the vehicle for yolk injections as it is commonly used in clinical medicine and experimental animals. The amount of injected *M. marinum* per embryo totalled 2,000 or 20,000 CFU. As we observed only a minor concentration effect on miRNA expression, these samples were treated as biological duplicates in the microarray analysis. *L. casei* Shirota was used as a non-pathogenic bacterium for comparison and injected in PVP suspension at similar concentrations as used for *M. marinum*. PVP-injected and uninjected embryos were employed as controls. Pools of 10–30 embryos from each group were taken at 72 h post infection (hpi). For the *S. typhimurium* infection study embryos were staged at 27 hpf and approximately 250 CFU of *S. typhimurium* bacteria in PBS were injected into the caudal vein close to the urogenital opening. As a control an equal volume of PBS was likewise injected. Triplicate pools of 20–40 embryos were collected at 8hpi. In all infection experiments, bacterial injections were controlled using a Leica MZ Fluo 3 stereomicroscope with epifluorescence attachment, a Femtojet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary pipettes.

RT-PCR analysis of *irak1*

RNA from *S. typhimurium*-infected 1-day-old embryos at 24 hpi (Stockhammer et al. 2009) was used to amplify the 3' UTR of *irak1*. Traces of genomic DNA were removed by incubation with DNA-free solution (Ambion). RT-PCR analysis was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) using forward (5'AGAAAGAAACGCACAACAGATATGTTG3') and reverse (5'GTGCTGTTTAACAAGGCCGTAATC3') primers, located respectively at nucleotides 1-27 and 293-316 downstream of the *irak1* stop codon. To confirm the identity of the amplified sequence, the PCR product was cloned in pCRII-TOPO vector (Invitrogen) and sequenced using the sequencing service of ServiceXS (Leiden, The Netherlands).

Microarray design

Custom-designed 8x15k microarray slides were ordered from Agilent Technologies. The 15k custom design was obtained from Edwin Cuppen and Eugene Berezikov, Hubrecht Institute, Utrecht, The Netherlands and will be submitted into the Gene Expression Omnibus database. The 15k design contained a duplicate of 7604 probes of 60-oligonucleotide length. The probes consisted of 2x22 nucleotide sequences antisense to mature miRNAs separated by a spacer of 8 nucleotides (CGATCTTT) and with a second spacer with the same sequence at the end. From 7604 probes 546 were designed for left (5') and right (3') arms of the hairpins of zebrafish miRNAs that are currently known in miRBase. The remainder 7058 probes were classified into 3 categories: 62 probes into C1 category (novel confident miRNAs for which there is compelling experimental evidence), 54 probes into C2 category (candidate miRNAs with clear hairpin structure but less experimental evidence), and 6942 into C3 category (additional hairpins identified in the zebrafish genome sequence that are less likely to be miRNAs).

RNA isolation, labeling and hybridization

Adult fish and embryos for RNA isolation were snap frozen in liquid nitrogen and subsequently stored at -80 °C. Adult fish were homogenized in liquid nitrogen as described (van der Sar et al., 2009) and portions of 50–100 µg of powdered tissue were used for RNA extraction. Total RNA from adult or embryo sample was isolated using the miRNeasy Mini kit to preserve small RNA species. RNA labeling was carried out with miRCURY™ LNA microRNA, Hy3™/Hy5™ Power Labeling kit (Exiqon) using 1 µg of total RNA according to the manufacturer's instructions. RNA samples from mycobacterium-infected adult fish were labeled with Hy3 and hybridized against Hy5-labeled RNA samples from PBS-injected controls. For the yolk infection study of 2-day-old embryos, the RNA samples from mycobacterium-injected, *L. casei* Shirota injected, and PVP-injected embryos were labeled with Hy3 and hybridized against Hy5-labeled samples from uninjected controls. RNA samples from salmonella-infected 1-day-old embryos were labeled with Hy3 and hybridized

against Hy5-labeled RNA samples from PBS-injected controls. The dual colour hybridization of the microarray chips was performed according to Agilent protocol GE2-v5_95_Feb07 and GE2_105_Jan09 (www.Agilent.com) for two-color microarray-based gene expression analysis except that hybridization and washing was performed at 37 °C.

Data analysis

Microarray data were processed from raw data image files with Feature Extraction Software 10.1.1 and 10.5.1 (Agilent Technologies). Processed data were subsequently imported into Rosetta Resolver 7.2 (Rosetta Biosoftware) and subjected to default ratio error modeling. Ratio results from control vs. infected replicates were combined using the default ratio experiment builder. Significance cut-offs for the ratios of infected versus control were set at 1.5-fold change at $P \leq 10^{-4}$. Two-dimensional hierarchical cluster analyses were performed with MultiExperiment Viewer version 4.4.1 (TM4 Software Development Team) settings for average linkage method with Euclidean distance.

Target prediction

To predict miR-146 family member target sites in TLR pathway genes *traf6*, *irak1* and *myd88* and rank these predictions we employed three different miRNA target site prediction methods: miRanda (www.microrna.org/) (John et al., 2004), RNAhybrid (bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (Rehmsmeier et al., 2004), and TargetScan (www.targetscan.org/) (Lewis et al., 2003), and an integration algorithm for these three methods developed by Yanju Zhang and Fons Verbeek, Leiden Institute for Advanced Computer Science. To predict further conserved targets of miR-146 family members in zebrafish, human, mouse, rat and chicken we used MicroCosm Targets (formerly known as miRBase Targets) (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>). The predicted targets from zebrafish, mouse, rat and chicken were converted to human Ensembl Gene ID homologues using the Biomart program of the Ensembl database. Targets which appeared across zebrafish and human or multiple other species were selected to study their involvement in biological pathways using the Reactome pathway database (www.reactome.org/), GeneALaCart (www.genecards.org) and AmiGO pathways (amigo.geneontology.org/cgi-bin/amigo/go.cgi).

Supplementary data

Supplementary data associated with this chapter can be found at http://apo.szbk.u-szeged.hu/transfer/A_ORDAS/.

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