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Chapter 9

Discovering the Role of MicroRNAs in Microcystin-Induced Toxicity in Fish

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1. Introduction

MicroRNAs (miRNAs) form a class of endogenously expressed small, non-coding RNAs, that play key roles in the regulation of gene expression of a broad spectrum of biological processes. However, in the field of toxinology, a science of naturally occurring toxins, the relationship between toxicity and microRNA expression is poorly understood. Microcystins (MCs) are potent cyclic peptide hepatotoxins produced by cyanobacteria, which pose a serious threat to aquatic organisms and may also affect human health through the consumption of contaminated waters or food. Although a number of cell physiologic pathways, potential targets for miRNA regulation, are implicated in the response to MCs in animals, no research so far investigated the role for miRNA genes in the mechanism of microcystin (MC)-induced toxicity in fish. The chapter aims to summarize recent achievements of our team in the field, focusing on expression profiling *in vivo* of liver microRNA levels of whitefish (*Coregonus lavaretus*) following MC-LR exposure.

2. Body

2.1. MicroRNAs in fish cells

MicroRNAs (miRNAs) form a class of endogenously expressed small, non-coding RNAs, that play key roles in the regulation of gene expression of a broad spectrum of biological processes. Figure 1 summarizes crucial steps in microRNA processing. MiRNAs are transcribed by RNA polymerases II or III as primary transcripts (pri-miRNAs), which are fur-

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ther processed by the nuclear RNase III enzyme Drosha to stem-loop-structured miRNA precursor molecules (pre-miRNAs). The pre-miRNAs are subsequently transported to the cytoplasm where the RNase III enzyme Dicer cleaves off the double stranded (ds) portion of the hairpin and generates a short-lived dsRNA of about 19–23 nucleotides (nt) in size. The duplex is subsequently unwound and only one strand gives rise to the mature miRNA, which is incorporated into miRNA-protein complexes (miRNPs) [1-2]. The mature miRNAs binds to partially complementary recognition sequences located in the 3'-untranslated regions (3'-UTRs) of mRNAs and target them for degradation or translational repression (reviewed in [3]).

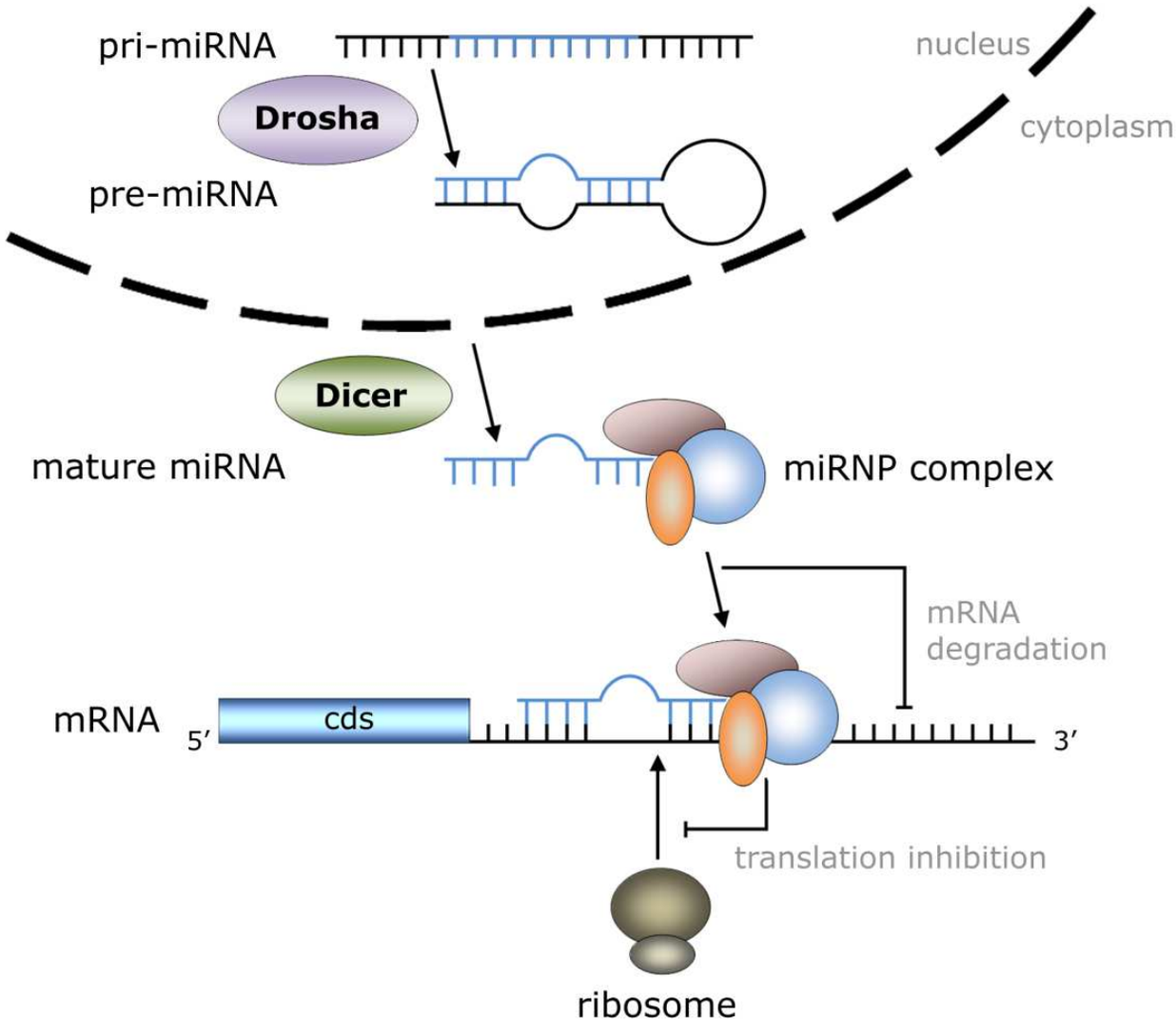

Figure 1. miRNA processing and target recognition. The pri-miRNA is processed by the Drosha enzyme to a stem-loop-structured miRNA precursor molecule (pre-miRNA). The pre-miRNAs is transported to the cytoplasm where the Dicer enzyme cleaves off the double stranded (ds) portion of the hairpin and generates the mature miRNA, which is incorporated into miRNA-protein complexes (miRNPs). The mature miRNA binds to partially complementary recognition sequences on 3'-UTRs of mRNAs and targets them for decay or translational repression.

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In metazoans miRNA complementarity to their targets is far from perfect, so one miRNA can bind up to 200 targets, and each mRNA could have recognition sites for more than one miRNA. It is estimated that about 30% of the human protein-coding genes are negatively regulated by miRNA, which suggests that miRNAs are very important regulators of gene expression process [3]. Although specific functions and target mRNAs have been assigned to only a few dozen of miRNAs, much experimental evidence suggests that miRNAs participate in the regulation of a vast spectrum of biological processes. miRNAs control diverse cellular processes including animal development and growth, cell differentiation, signal transduction, cancer, neuronal disease, virus-induced immune defense, programmed cell death, insulin secretion, and metabolism (see [4] and references therein). Understanding of RNA interference (RNA*i*) has been made possible through a variety of experimental and bioinformatics approaches using different model organisms, including fish [5-6].

To discover aberrantly expressed miRNAs in fish and to determine how altered miRNA function contributes to a disease, new RNA*i* technologies may be applied (Figure 2). In toxicological studies attention is focused on the relationship between exposure to a chemical and adverse effects it produces in cells, tissues or organisms. So, when a treatment study is carried out small RNA may be collected from a tissue to generate miRNA libraries, from either control or exposed fish. That is the first important step to establish the full repertoire of miRNAs that are differentially regulated in treated fish. Then the miRNA libraries are subjected to massively parallel sequencing, a next generation sequencing technique, which is a combination of emulsion PCR and pyrosequencing [7]. In comparison to microarray analyses, this approach is not limited to previously identified miRNAs and is expected to have superior sensitivity at high sequencing depth. Such approaches have expanded the catalogue of differentially expressed miRNA genes in various fish tissues [6]. The genome-wide screen for regulated miRNAs should yield candidate miRNAs for further profiling (Real Time qPCR) and functional analyses (e.g. Renilla luciferase reporter assay).

As miRNAs regulate many different pathways and orchestrate integrated responses in cells and tissues, it is reasonable to think that they also play key roles in coordinating networks in the poisoned organs. Indeed, there are reports concluding that miRNAs may be key molecules involved in aberrant gene expression in liver cells exposed to hepatotoxic agents, other than MC-LR. For example, Fukushima and co-workers [8] have shown that two well known hepatotoxicants which induce hepatocellular injuries and necrosis, acetaminophen or carbon tetrachloride, were capable of modulating expression of two miRNAs (miR-298 and miR-370) in rats, and that those effects were accompanied by impaired liver metabolism. The observation that miRNAs levels in rat livers were changed by hepatotoxic compounds prompted our team to investigate the role of fish microRNAs in the context of liver-specific MC-LR toxicity.

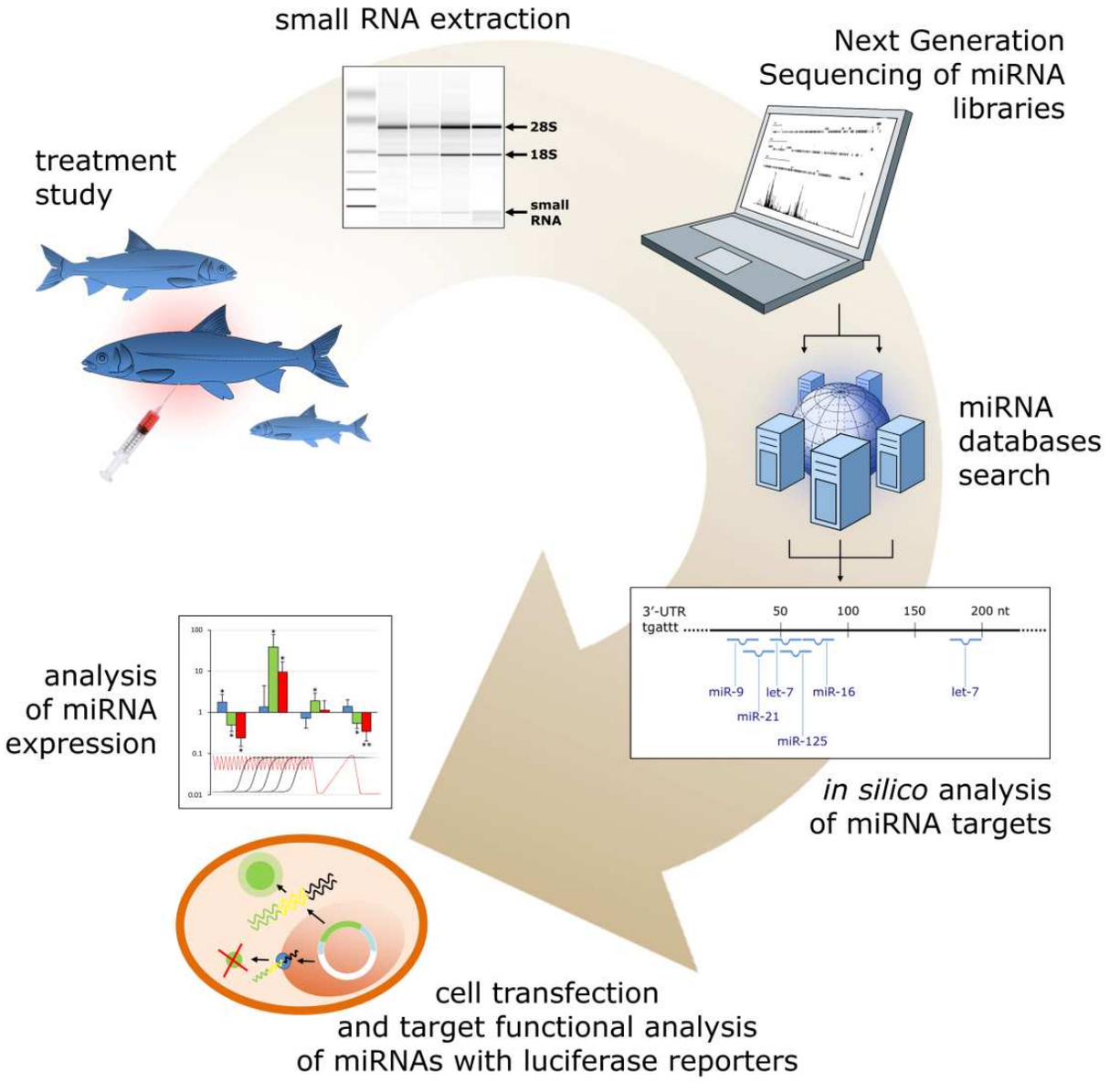

Figure 2. Studying fish miRNAs. MicroRNA discovery has been recently revolutionized by next-generation sequencing. Following ligation of specific linkers to small RNAs (which comprise miRNAs), cDNAs can be produced, which are ideally suited to sequencing using short-read platforms. Databases now offer online catalogues of known microRNAs, which may further be examined for their pathways and functions through a variety of approaches, such as target functional analysis of candidate miRNAs using luciferase reporter assays and miRNA profiling with Real Time PCR.

2.2. Microcystins as potent cyanobacterial toxins

Microcystins (MCs) are potent hepatotoxins produced by cyanobacteria of the genera *Planktothrix*, *Microcystis*, *Aphanizomenon*, *Nostoc*, or *Anabaena*, which have received worldwide concern in recent decades. Mass growths of cyanobacteria, leading to production of blooms, scums and mats, can occur in nutrient-enriched waterbodies (particularly with phosphorus and nitrogen), enhanced by higher temperature and pH values. MCs can be found in lakes,

ponds and rivers used for recreational activities as well as in sources for drinking water preparation [9]. In surface waters, concentrations of total MCs (cell-bound and dissolved) measured with ELISA may reach high levels, of up to 1300 μg/l [9], and thus the toxins may pose a threat to aquatic organisms and humans [10]; the World Health Organization recommends 1 μg/l as the maximum acceptable level for microcystin-LR (MC-LR) in drinking water [11]. So far, more than 100 different structural analogues of MCs have been identified, among which MC-LR is one of the most common and abundant [12].

MCs have strong affinity to serine/threonine specific protein phosphatases (PP1 and PP2A), thereby acting as inhibitors of the enzymes [13]. The acute toxicity of MC can be explained by the PP inhibition, which leads to an excessive phosphorylation of cell proteins, to alterations in the cytoskeleton, and a loss of cell shape [14]. Another biochemical feature of MC toxicity is the production of reactive oxygen species (ROS). MC-related ROS generation has been reported using both *in vitro* approaches with different cell lines of fish and mammals [15-16], as well as in a number of *in vivo* studies in rodent liver, heart and reproductive system [17-19]. This process is related to mitochondrial metabolism and it may lead to cell death and to genotoxicity [20]. Oxidative stress caused by MC exposure is believed to be involved in a series of heart, liver and kidney pathologies [19, 21], neurodegenerative effects [22] and embryotoxicity [23].

In recent years, new insights on the key molecules involved in the signal-transduction and toxicity have been reported [24], which highlighted the complexity of the interaction of these toxins with animal cells (Figure 3). Key proteins involved in MC up-take, biotransformation and excretion have been identified, demonstrating the ability of aquatic animals to metabolize and excrete the toxin. After having caused damage to intestinal (or gill) cells these toxins penetrate liver cell membranes through a bile acid carrier. In liver cells MCs inhibit serine/threonine-specific protein phosphatases, PP1 and PP2A, through the binding to them, thus perturbing signaling pathway controlled by the enzymes. The consequences are induction of mitochondrial permeability and loss of mitochondrial membrane potential leading to dysfunction of the mitochondria, induction of reactive oxygen species (ROS), DNA damage (through lowered expression of DNA-PK), and cell apoptosis (through increase Ca2+ levels, CaMKII). MC activity leads to the differential expression/activity of transcriptional factors (e.g. c-myc, p53) and protein kinases (NeK2) involved in the pathways of cellular differentiation, proliferation, tumor promotion activity, and metastasis [25].

2.3. Likely silencing targets in MC-exposed fish cells

In the field of toxinology, a science of naturally occurring toxins, the relationship between toxicity and microRNA expression is poorly understood. However, based on current knowledge about genes involved in the animal cell response on the exposure to environmental stressors, putative targets for miRNA regulation emerge. Genes of transcription factors, *p53* and mapk (mitogen activated protein kinases) regulated proto-oncogenes e.g. *c-myc*, that are involved in MC-LR toxicity (Figure 3), are good candidates for tight and robust regulation by microRNAs. The nuclear phosphoprotein p53 is induced in response to cellular stress. It plays a role as a transcriptional trans-activator in DNA repair, apoptosis and tumor suppres-

sion pathways. Interestingly, the protein is a substrate of PP2A [26] and therefore its activity is likely to be regulated, in part, by MC-LR. Furthermore, p53 is a regulator of the expression of the anti- and pro-apoptotic genes including members of the BCL-2 family such as *BCL-2* and *BAX*, as well as *CDKN1A*, encoding $p21^{Cip1}$, which is a cyclin dependent kinase inhibitor (CDKI), an important effector that acts by inhibiting CDK activity in p53-mediated cell cycle arrest in response to various agents. Indeed, we have shown previously that intraperitoneal injection of whitefish, *Coregonus lavaretus*, with MC-LR at subacute dose of 100 μg/kg body weight induced mRNA expression of tumor suppressor p53 and cyclin dependent kinase inhibitor 1 (cdkn1a) in the liver of exposed fish [27]. Interestingly, it was proven in human cell lines that p53 is a transcription factor for some miRNAs, such as miR-34a [7]. miR-34a mediates some of the well-known effects of p53, i.e. cell cycle arrest or apoptosis, and reduced miR-34a levels can serve as a biomarker for any dysfunction along the p53 axis [28]. Yet, its role in controlling miRNA network in fish awaits investigation (Figure 3).

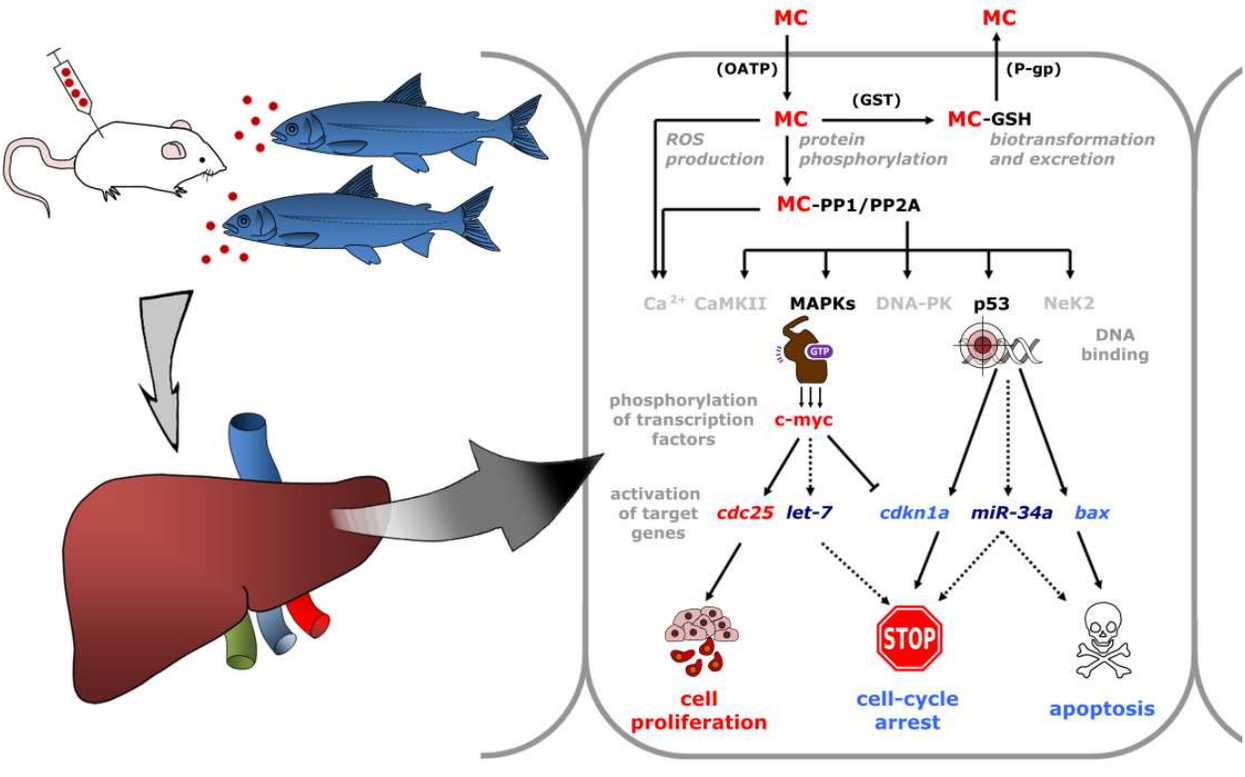

Figure 3. Suggested pathways of MC up-take, toxicity, biotransformation and excretion in vertebrates. Based on the current knowledge, microRNAs (e.g. let-7 or miR-34a) may play roles in MC-LR dependent cell proliferation, cell-cycle arrest or apoptosis.

In the other pathway (Figure 3), mitogen-activated protein kinases (MAPKs) regulate the expression of proto-oncogenes which on the other hand regulate the transcription of genes involved in the growth and differentiation [29]. Expression of MAPKs is mediated by PP2A and are likely to be regulated by MC. The expression of three proto-oncogenes c-fos, c-jun and c-myc were reported to increase in liver, kidney and testis of Wistar rats injected intra-

venously with MC-LR, with higher levels registered in liver [30]. Expression of these genes suggest that a possible mechanism for the tumor-promoting activity of the toxin could be controlled by MAPKs. Importantly, c-MYC controls expression of let-7 miRNA members by binding to their promoters. The levels of let-7 have been reported to decrease in models of MYC-mediated tumorigenesis, and to increase when MYC is inhibited by chemicals [31]. It is also found that MYC can repress $p21^{Cip1}$ transcription (Figure 1), thereby overriding a p21-mediated cell cycle checkpoint [32].

2.4. miRNA expression in whitefish exposed to MC-LR

In 2008, we began a study of MC-LR induced transcriptional changes in European whitefish, *Coregonus lavaretus* L., a sentinel organism frequently used for pollution monitoring in aquatic systems [27]. To obtain necessary information for the study, full-length cDNA of p53 or cdkn1a of whitefish were determined, using molecular cloning and rapid amplification of cDNA ends (RACE). The *short term* treatment study showed that MC-LR at a dose of 100 μg/kg body weight induced hepatocyte cell DNA fragmentation and up-regulated mRNA expression of p53 and cdkn1a genes in whitefish liver. Interestingly, the elevated transcript levels of both genes were observed only from 48 through the 72 h of exposure, and were accompanied by pathological signs of severe injury of the liver and loss of normal organ functions (elevated levels of blood AspAT AlaAT, and hepatosomatic index; [27]).

Whereas, the above study confirms that MC-LR exposure underlies various acute and chronic effects in fish, it is still little known about aberrant gene expression profiles and molecular pathways involved in the liver of MC-LR challenged organisms. Therefore, to improve our knowledge about adverse effects of MC-LR on hepatocyte cell responses in fish, we performed an initial microRNA study to examine the abundance of 9 selected miRNAs (omy-miR-21, omy-miR-21t, omy-miR-122, omy-miR-125a, omy-miR-125b, omy-miR-125t, omy-miR-199-5a, omy-miR-295, omy-let-7a), in liver samples of whitefish exposed for 24 or 48h to MC-LR at a dose of 100μg/kg body weight [4]. Interestingly, the study showed that MC-LR treatment affected expression levels of two miRNAs, omy-miR-125a (up-regulation) and omy-let-7a (down-regulation) [4].

Following the early demonstration that MC-LR modulates expression of let-7a and miR-125a, in our most recent work [33] we aimed at profiling expression of other 6 miRNAs and 8 mRNAs (Table 1) in the liver of challenged whitefish during the first 48 h after single intraperitoneal injection. From studies on mammals we chose miRNAs which play regulatory roles in pathways of signal transduction (let-7c, [34]; miR-9b, [35]), apoptosis and cell cycle (miR-16a, [36]; miR-21a, [37]; miR-34a, [7]) and fatty-acid metabolism (miR-122, which is a liver specific miRNA, [38]). The selection of mRNA targets (Table 1) was based on their reported aberrant tissue expression on exposure to environmental stressors, and included mRNAs involved in apoptosis and cell cycle (bax, [20]; cas6, cdkn1a, p53, [27]), signal transduction (p-ras, [39]), cellular iron homeostasis (frih, [40]), gene silencing by miRNAs (dcr, [41]), and nucleosome assembly (h2a, [4]). Together with the RNA expression, we analyzed levels of tumor suppressor protein p53 to assess its potential contribution in molecular mechanisms of liver toxicity induced by MCs in fish.

miRNA	Putative biological process*
omy-let-7c	signal transduction
omy-miR-9b	signal transduction
omy-miR-16a	apoptosis, cell cycle
omy-miR-21a	apoptosis, cell cycle
dre-miR-34**	cell cycle, signal transduction
omy-miR-122	fatty-acid metabolism, maintenance of adult liver phenotype
mRNA [gene abbreviation]	Biological process***
bcl2-associated X protein (bax)	apoptosis
caspase 6 (cas6)	apoptosis
cyclin-dependent kinase inhibitor 1a (cdkn1a)	cell cycle
dicer (dcr)	gene silencing by miRNA
ferritin heavy chain (frih)	cellular iron ion homeostasis
histone 2A (h2a)	nucleosome assembly
tumor protein 53 (p53)	apoptosis, cell cycle, signal transduction
HNK Ras –like protein (p-ras)	signal transduction

* based on literature review; see text for details.

** putative *miR-34* gene is present in *Salmo salar* genome; Contig_142190, whole genome shotgun sequence, GenBank ACC. No. AGKD01142167.1, nucleotides from 5978 through 6053.

*** in terms of Gene Ontology Annotation (http://www.ebi.ac.uk/QuickGO).

Table 1. miRNA and mRNA targets selected under study.

Quantifying miRNAs in different tissues is an important initial step in investigating their biological functions. To this end, we determined the expression levels of 6 selected miRNAs in adult whitefish liver using Real-Time qPCR. Prominent expression of miR-122 in the liver of whitefish was observed which is consistent with other data from fish [4,6] and mammals [42]. Variable expression levels of other miRNAs studied in the liver of whitefish corroborated results of previous work on normal human tissues [43], and they are also in agreement with available data on the fish miRNome isolated from rainbow trout [6] and zebrafish [44] miRNA libraries. While the actual expression values of miRNAs can vary by orders of magnitude between whitefish and humans [43], their relative abundance in a particular tissue should tend to be more conserved in

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evolution. Indeed, the order of individual miRNA abundances in human liver (miR-122 > let-7c ≈ miR-21 ≈ miR16 > miR-34a > miR-9; [43]) held in whitefish as well [33].

Our treatment study [33] identified miRNAs whose expression levels rose (from 2.7-fold for miR-122 to 6.8-fold for let-7c) in MC-LR treated fish, compared to the respective levels in control fish (Figure 4). The increase, which was most apparent at 24 h of the experiment, was correlated with a reduction in the expression of mRNAs: ferritin H (frih) and HNK Ras –like protein (p-ras) and an overexpression of bcl2-associated X protein (bax), cyclin dependent kinase inhibitor 1a (cdkn1a), dicer (dcr), histone 2A (h2a) and p53. Expression of the remaining caspase 6 (cas6) mRNA did not change over 48 h of the treatment. Moreover, exposure to MC-LR did not alter whitefish p53 protein levels [33].

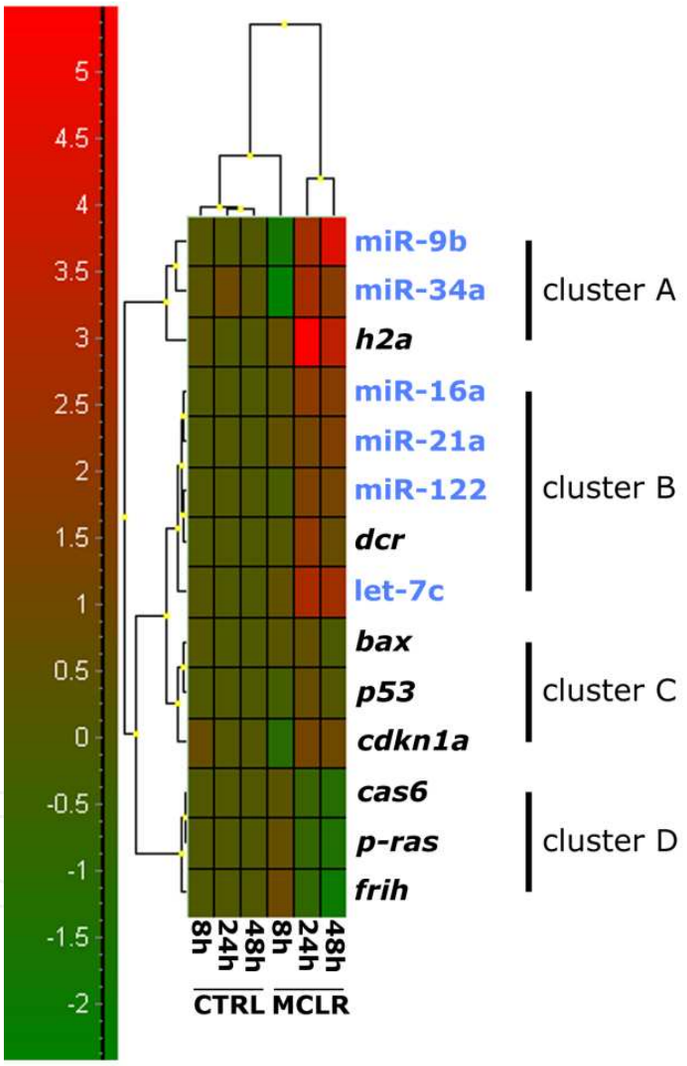

Figure 4. Heat map and hierarchical clustering of differentially expressed genes and miRNAs in MC-LR treated whitefish. Each row represents one gene/miRNA and each column represents a mean of 5 replicates/duration of exposure. Colors represent expression levels of each individual gene/miRNA: red, up-regulation; green, down-regulation. Four distinct clusters (A through D) based on the observed expression profiles could be identified by the analysis. The analysis and visualization were performed using GenEx 5 software (MultiD Analyses AB; Sweden), based on raw expression data from our recent study [33].

The experiment allows one to determine which miRNAs change expression as a group or as a cluster. Genes that function together may define regulatory networks and regulate a common set of regulated genes. Using clustering software, we divided the significantly regulated miRNAs into different groups. In Figure 4 there were four different types of expression profiles among the miRNAs and genes. Some groups showed transient changes in the expression profile (clusters B and C) while others stably increase (cluster A) or decrease (cluster D) during the treatment with MC-LR. Bearing in mind a variety of likely silencing targets for, and the onset of, the aberrant miRNAs expression (Table 2; [33]) it may be concluded that they are involved in diverse molecular pathways, such as liver cell metabolism, cell cycle regulation and apoptosis, and may contribute to the early phase of MC-LR induced hepatotoxicity. Whereas, this argues that at least some of miRNAs listed in Table 2 are good candidates to pursue in future studies, a key to further elucidation of the miRNA role in the toxicity mechanism is the generation of more complete lists of their numbers and expression changes in healthy and challenged fish.

MicroRNA*	Fold change	Reported silencing targets	Reference
let-7c	6.8	Rat sarcoma viral oncogene, RAS	[34]
		Myelocytomatosis viral related oncogene, c-MYC	[45]
miR-9b	4.4	Caudal related homebox protein, CDX2	[35]
miR-34a	4.0	B-cell lymphoma 2, BCL2	[46]
		Myelocytomatosis viral related oncogene, neuroblastoma derived (avian), MYCN	[47]
miR-16a	3.6	B-cell lymphoma 2, BCL2	[36]
miR-122	2.7	Cationic amino acid transporter, CAT-1	[48]

*Only miRNAs which were significantly up-regulated ($p<0.05$) are included in the column.

Table 2. Reported mammalian silencing targets for differentially expressed miRNAs in MC-LR treated whitefish (100µg/kg body weight) after 24 h of the challenge [33].

On the other hand, the lack of p53 stabilization observed in our study infers the presence of alternate checkpoint mechanisms for deregulated growth signals and/or DNA damage in whitefish cells and may suggest post-transcriptional regulation of *p53*. Indeed, recent work by Liu and coworkers [49] suggest that two checkpoint kinases, ATM and ATR, which act upstream of p53, are promising candidates for the role. Further studies should also reveal if the lack of p53 induction in fish liver following exposure to many compounds known to cause DNA damage and DNA replication defects [49-50], is controlled by the miRNA network, a role it is known to fulfill in other organisms. For example, miR-125b has been previously confirmed to be a negative regulator of p53 in both zebrafish and humans [51].

3. Conclusions

We are only beginning to understand the complexities of miRNA-mediated gene regulatory networks in fish cells. It should be expected that environmental contaminants that have the potential to induce oxidative stress and hypoxia in animal cells, like MCs, will also be agents deregulating miRNA expression. In our initial studies [4, 33] we observed rapid changes in liver microRNA levels of whitefish following MC-LR exposure. Bearing in mind a variety of likely silencing targets for and the onset of the aberrant miRNAs expression observed in the study, one may conclude that they are involved in various molecular pathways and may contribute to the early phase of MC-hepatotoxicity. This argues that studied miRNAs are good candidates to pursue in future studies, however, a key to further elucidation of the miRNA role in the toxicity mechanism will be the generation of more complete lists of their numbers and expression changes in healthy and challenged fish, using next generation sequencing methods (Figure 2). As miRNA field continues to evolve, the new markers should help elucidating a variety of issues intrinsic to MC toxicity. As more profiling studies are performed after MC-LR treatment, and on different model organisms, it might be possible to obtain a miRNA snapshot map, the "core of the MC-LR toxicity connectivity grid". Finally, the revealed miRNA pathways underlying hepatotoxic effects of MC-LR may provide therapeutic targets for a variety of liver diseases.

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