



Patterns of MiRNA Expression in Arctic Charr Development

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Abstract

Micro-RNAs (miRNAs) are now recognized as a major class of developmental regulators. Sequences of many miRNAs are highly conserved, yet they often exhibit temporal and spatial heterogeneity in expression among species and have been proposed as an important reservoir for adaptive evolution and divergence. With this in mind we studied miRNA expression during embryonic development of offspring from two contrasting morphs of the highly polymorphic salmonid Arctic charr (*Salvelinus alpinus*), a small benthic morph from Lake Thingvallavatn (SB) and an aquaculture stock (AC). These morphs differ extensively in morphology and adult body size. We established offspring groups of the two morphs and sampled at several time points during development. Four time points (3 embryonic and one just before first feeding) were selected for high-throughput small-RNA sequencing. We identified a total of 326 conserved and 427 novel miRNA candidates in Arctic charr, of which 51 conserved and 6 novel miRNA candidates were differentially expressed among developmental stages. Furthermore, 53 known and 19 novel miRNAs showed significantly different levels of expression in the two contrasting morphs. Hierarchical clustering of the 53 conserved miRNAs revealed that the expression differences are confined to the embryonic stages, where miRNAs such as sal-miR-130, 30, 451, 133, 26 and 199a were highly expressed in AC, whereas sal-miR-146, 183, 206 and 196a were highly expressed in SB embryos. The majority of these miRNAs have previously been found to be involved in key developmental processes in other species such as development of brain and sensory epithelia, skeletogenesis and myogenesis. Four of the novel miRNA candidates were only detected in either AC or SB. miRNA candidates identified in this study will be combined with available mRNA expression data to identify potential targets and involvement in developmental regulation.

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Introduction

Since the initial discoveries of *lin-4* and *let-7* miRNAs have emerged as key regulators of animal development (reviewed in [1,2]). These small (~22 nt) non coding RNAs, regulate gene expression by inducing mRNA degradation or translational repression, making for a specific and “fine-tunable” response (reviewed in [3]). miRNAs originate from different parts of the genome (intergenic regions, exons or intronic sequences [4] and are transcribed either as independent transcriptional units or as clusters of several miRNAs (reviewed in [5]). A common feature of all miRNA genes, regardless of their genome location, is the folding of their primary transcript into a stem-loop structure. This hairpin structure is recognized and converted into a miRNA-miRNA* duplex by the miRNA processing machinery (see [5]). One of the strands, dubbed the “mature” miRNA, is then loaded into the miRISC complex while the complementary “star” sequence is often degraded [6]. In most cases of miRNA mediated gene regulation the target repertoire is determined by the “seed” region (nt 2-8 located at the 5’ end of the mature miRNA) of the miRNA [7].

In general miRNAs are highly conserved among taxa [5]. Comparative studies show how new miRNAs have continuously been emerging during the evolution of metazoan genomes [8,9] through various mechanisms including gene duplications of preexisting miRNAs followed by changes in their sequences, or *de novo* appearance from random hairpins [10]. However, once they become integrated into the regulatory network, their primary sequence and particularly their seed region, becomes subject to strict selective constraints [5,11]. Variation in timing and expression patterns among species suggests that these molecules may play an important role in shaping physiological differences. For example comparison of two fish species (medaka and zebrafish) showed that heterochrony in miRNAs expression is associated with neuromast and craniofacial development [12]. This was suggested to reflect the differences in formation of the head and sensory epithelia observed between medaka and zebrafish. Morphological differences arising in development can potentially drive evolutionary change, adaptive divergence and speciation (discussed in [1]). More specifically, it has been suggested that miRNAs may generally cover more restricted regulatory niches than transcription factors and thus frequently be

more important in terminal differentiation programs [13]. It has also been proposed that miRNAs are involved in enhancing species evolvability by stabilizing gene expression and signaling cascades leading to the increased distinctness of developmental phenotypes, thereby increasing heritability of traits and facilitating natural selection ([14] and discussion in [15]).

Arctic charr as a model species to study adaptive divergence

The high level of phenotypic polymorphism present in Northern freshwater systems offers an excellent opportunity to study adaptive divergence [16]. These watersheds, with their rivers and lakes, were formed after the last glacial epoch 10 000–16 000 years ago. The short evolutionary history characterized by physical variability and topographic dynamics sets a stage where the early steps of divergence may be playing out in multiple locations and species. Studies of whitefish (*Coregonus clupeaformis*), threespine stickleback (*Gasterosteus aculeatus*) and Arctic charr (*Salvelinus alpinus*) have shown that fish inhabiting these systems exhibit an extremely high level of inter-population variation in phenotype with many populations diversifying along a benthic to limnetic habitat axis [17–20]. Although Arctic charr in Iceland originates from a single Atlantic lineage [21], this species shows an extremely high level of variation in phenotype between populations and many examples of polymorphism (i.e. sympatric morphs) have been documented [17,22–24]. The Arctic charr morphs of Lake Thingvallavatn constitute an extreme example of local phenotypic diversity. Four morphs grouped into two morphotypes have been described in the lake: a limnetic morphotype represented by planktivorous (PL) and piscivorous (PI) charr, with pointed snout and evenly protruding jaws, and a derived, benthic morphotype represented by small (SB) and large benthivorous (LB) charr, blunt snout, short lower jaw and relatively large pectoral fins [25]. These morphs also differ extensively in life history characteristics (size and age at maturity) and embryology [26–29]. The morphs also exhibit strikingly clear differentiation in ecology as reflected in different habitat use, diet and endoparasite fauna [27,28,30]. Several common garden experiments have shown that some key morph specific traits have a definite genetic basis [31,32]. A recent study, using neutral microsatellite markers, revealed significant but subtle genetic differentiation between the three most common morphs in Lake Thingvallavatn, which is consistent with a scenario of early evolution of reproductive isolation, followed by slow divergence by drift with restrictive gene flow [33]. Notably, a study of immune system genes revealed more pronounced genetic differentiation among the morphs in the lake, consistent with a scenario where parts of the immune systems have diverged substantially among Arctic charr morphs from Lake Thingvallavatn [34]. The adaptive nature of the trophic morphology and feeding behavior of the Thingvallavatn morphs has been demonstrated in a series of laboratory rearing experiments [29,31,35]. Moreover the role of developmental heterochrony in the evolution of the Thingvallavatn Arctic charr morphs was demonstrated in a study showing that some skeletal elements of the head start ossifying earlier and/or faster in small benthivorous embryos than in embryos derived from the planktivorous morph [35].

Some of the key differences in functional traits that define the charr morphs are without doubt rooted in differences in the expression of developmental genes. We hypothesize that miRNAs may, through their potentially stabilizing effect of phenotypes [14], play a fundamental role in the divergence of developmental processes that induce differential cranial morphologies in Arctic charr morphs. As a first step of addressing this hypothesis we

utilized high-throughput sequencing techniques to identify and annotate Arctic charr miRNAs and to study their expression during the development of two contrasting Arctic charr morphologies. To this end we used a common garden set up to generate embryonic series of two contrasting Arctic charr morphotypes, a benthic morphotype, represented by the SB-charr from Thingvallavatn and a limnetic morphotype represented by fish from the Hólar aquaculture stock (AC). These two morphs differ greatly in adult size, color and head morphology (Figure 1): SB are small, dark and have a sub-terminal mouth and rounded snout whereas AC are large, silvery and have a pointed snout and a longer lower jaw. We sampled AC and SB embryos at four developmental time-points reflecting important events in Arctic charr craniofacial development and used high-throughput sequencing to quantify differences in miRNA expression between the morphs. More specifically we identified and annotated Arctic charr miRNAs using homology to known miRNAs in other species. Furthermore, we identified a large set of novel miRNA candidates by aligning reads to genomic sequences from the closely related Atlantic salmon, *Salmo salar*. Expression levels for both known and novel miRNAs were compared between AC and SB.

Materials and Methods

Sampling and methodology

All sampling from the wild and rearing in aquaculture was performed according to Icelandic law and with proper permissions. Fish from Lake Thingvallavatn were caught by the authors for the purpose of this study with fishing permissions obtained from the Thingvellir National Park Commission and the owner of the Mjóanes farm. SSS and ZOJ hold special permits for sampling fish from nature for scientific purposes according to Icelandic law (clause 26 of law 61/2006 on salmonid fishing). Control fish from Hólar aquaculture stock were obtained from a national breeding programme, and were not specifically bred for the purpose of this project. These fish are held at the arctic charr breeding station, a quarantined rearing and holding facility, at Hólar University College. After stripping for gametes, parent fish were killed by a sharp blow to the head and checked for absence of breathing when placed in water. Setting up crosses and the subsequent killing of parents was performed by the authors. Ethics committee approval is not needed for regular or scientific fishing in Iceland (The Icelandic law on animal protection, Law 15/1994, last updated with Law 157/2012). The rearing of embryos was performed according to Icelandic regulations (licence granted to Hólar University College aquaculture and experimental facilities) in Verið, Sauðárkrúkur, Iceland. Sampling of embryos was performed by University College Aquaculture Research Station (HUC-ARC) personnel. HUC-ARC has an operational license according to Icelandic law on aquaculture (Law 71/2008), that includes clauses of best practices for animal care and experiments. For this study the last gestation age at which embryos were sacrificed was 434 (τ_s) units. For RNA extraction, samples were flash frozen in RNA later. Prior to freezing eggs were permeabilized by puncture with a needle. Samples for staining (not described in this study) were treated with an overdose of phenoxyethanol before fixing.

For this study we used developmental time-series from pure crosses of two Arctic charr morphs, Hólar aquaculture charr (AC) and small benthic charr (SB) from Lake Thingvallavatn. These strains were selected mainly for their pronounced differences in body size, coloration and head morphology (Figure 1). As stated above, the AC crosses were made with parents from the Hólar breeding programme [36]. Fish from the small benthic morph (SB)



Figure 1. Two contrasting Arctic charr morphs differing in size, coloration and head morphology. Top: Arctic charr from aquaculture stock (AC) is large, silvery and has a pointed snout and long lower jaw, Bottom: Small benthic charr from Thingvallavatn (SB) is small, dark and has a sub-terminal mouth and rounded snout.
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were caught in Lake Thingvallavatn using gill-nets. Eggs from several females were pooled and fertilized using milt from several males from the same group. Eggs were reared at approximately 5°C in a hatching tray (EWOS, Norway) under constant water flow and in complete darkness at the Holar University College experimental facilities in Verið, Sauðárkrókur. Exact water temperature was recorded twice daily to estimate the relative age of the embryos using tau-somite (τ_s) units defined as the time it takes for one somite pair to form at a given temperature [37]. Embryos were collected throughout development and either fixed in 4% PFA or stored in RNAlater (Ambion) at -80°C . Based on embryos sampled at different developmental stages and stained with alcian blue (cartilage) and alizarin red (bone), four time-points (141, 161, 200 and 434 τ_s) were selected to represent important stages of bone and cartilage development. Stages 141, 161 and 200 are embryonic whereas stage 434 is a fry stage and for simplicity these stages will be referred to as stages 1, 2, 3 and 4, respectively. Two independent samplings were performed: one was used for high-throughput small-RNA-sequencing (miRNA-seq) and the other one for qRT-PCR.

Small RNA sequencing

Total RNA from each stage of each morph was isolated from a pool of 6 whole embryos and enriched for small RNAs using the mirVana kit (Ambion). The purity and amount of small RNA was verified on a BioAnalyzer (Agilent Technologies). The samples were prepared for sequencing following the small RNA v1.5 sample preparation protocol from Illumina. Briefly, 3' and 5' RNA adapters were ligated to small RNAs, which were subsequently, reverse transcribed into DNA and PCR amplified. The samples were then run on polyacrylamide gels and the DNA eluted from bands corresponding to 20–30 nucleotide RNA fragments. miRNA and transcriptome sequencing (mRNA-seq) was performed at deCODE Genetics (Reykjavik, Iceland) using the TruSeq smallRNA (v1.5) kit (Illumina) on an Illumina GAII_X instrument. Raw reads were submitted to NCBI Sequence Read Archive (SRA) under accession number SRP039492.

miRNA-seq data processing

Raw reads were processed with cutadapt [38] as follows: First, adaptor sequences were removed and only reads with adaptors were kept. Next, we used the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) (script available on request) and the quality scores associated with the reads to remove bases with a Phred based quality score [39] of less than 20 from read ends. Sequences retaining less than 15 nucleotides after filtering were discarded. Reads where 10% or more of the bases had a Phred quality score lower than 20 were also discarded. Finally, identical reads were reduced to one copy with the redundancy noted in the read name. The sequence filtering and collapsing was repeated for each sample.

Annotation of ncRNAs

To annotate sequences using known RNAs we used Rfam version 10.1 [40] and miRBase [41–44] version 20 databases. The Rfam database was searched with HMMER (version 3.0; <http://hmmer.janelia.org/>) with an e-value cutoff of 0.01. For the miRBase the search command from the fasta package version 36.3.6d [45] was used to detect homology between the mature miRNAs and the collapsed sequences (e-value cutoff 0.01).

On a quest for novel miRNAs

To identify novel miRNAs we used a probabilistic model of miRNA biosynthesis implemented in miRDeep 2 [46]. As a sequenced Arctic charr genome is not currently available, we used the genome sequences from the closely related Atlantic salmon [47] for reference. Collapsed reads were aligned to the Atlantic salmon genome with the mirDeep 2 mapping program (10 minimum reads per miRNA) for both morphs with the time-points combined. To facilitate mapping, collapsed sequences with strictly lower read count than 4 were omitted from the detection. The miRDeep2 algorithm then uses the reference regions bracketing the aligned reads to compute a hairpin structure and estimates the probability of each sequence being a true miRNA precursor based

on the position of the reads, their frequency, the energetic stability of their secondary structure and conservation of the 5' ends.

Sequences with log score greater or equal to 2 were considered as potential miRNAs, the predicted hairpins were searched against hairpins from mirBase (version 20) with blastall (version 2.2.26, -W 7) [48]. Candidates were annotated as known miRNAs if the alignment length was greater or equal to 60 nucleotides and expected value for the match was lower than 0.01 ($-e$ 0.01) otherwise the hairpins were classified as novel.

PCR amplification and sequencing of miRNA clusters

To assess the degree of sequence conservation for genomic clusters containing known and novel miRNAs between Arctic charr and Atlantic salmon we selected 4 clusters containing known miRNAs (miR-19c, 18b* and 20b; miR-133a and miR-133b and miR-143-3p and miR-143-5p; miR-219-3p and miR-219-5p) and 3 clusters containing novel miRNA candidates (sal-nov-235, sal-nov-242 and sal-nov-343) and PCR amplified their genomic regions from the Arctic charr genome. Primers were designed with Primer3 (<http://primer3.wi.mit.edu/>) (Table S1). The same PCR program was used for all primer pairs: an initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 45 seconds; 45 seconds at a 53°C; 1 min at 72°C, then a final step of 10 min at 72°C. PCR products were treated with ExoSap and sequenced on an Applied Biosystems 3500xL Genetic Analyzer using BigDye chemistry. Raw sequencing data was base-called by Sequencing Analysis Software v5.4 with KBTMBasercaller v1.41 (Applied Biosystems), and run through Phred and Phrap, prior to trimming primer sequences, visual editing of ambiguous bases and putative polymorphisms in Consed [49]. Fasta files were exported and aligned with ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and manually inspected for alignment errors in Genedoc (www.psc.edu/biomed/genedoc). All sequences were deposited in Genbank under the accession numbers [KJ573796-KJ573802]. These sequences were then searched using blast against the salmon database. The conservation between Arctic charr and salmon ranged between 91–94% for the known miRNAs and 92–98% for the novel miRNA candidates. Mismatches were always located outside of the miRNA mature-star sequence.

Differential expression analysis

The R package edgeR [50] was used to study the differential expression of conserved and novel miRNA candidates between morphs and among developmental time-points in a generalized linear model, where the additive covariates (no-interaction) corresponded to developmental time-point and different morphs. The normalization factors were calculated for each sample using the function calcNormFactors. As there are no replicates in any of the experimental conditions, the options `method = "deviance"`, `robust = TRUE` and `subset = NULL` were used for estimating the common dispersion (function estimateGLMCommonDisp) parameters as recommended by the edgeR user manual. The trended and tagwise dispersion were also estimated (function estimateGLMTrendedDisp and estimateGLMTagwiseDisp) with default options. The statistical significance of the terms was assessed by comparing likelihood difference to a reduced model without the time or the morph terms, with the function glmLRT in edgeR. The first 20 bases of each annotated sequence (novel and previously described miRNAs) were used as an identifier and the counts were aggregated for sequences that share the first 20 bases. This allowed us to work at the sequence level without lumping together isoforms (isomiRs). An entry (first 20 bases) was only considered for the statistical testing if the counts per million reads

were strictly greater than 3 in at least two experimental points resulting in 1862 tags. We adjusted for multiple testing using the Benjamini-Hochberg false discovery rate [51]. The R script used for this analysis is available in File S1.

Descriptive analysis

Cluster analysis was performed using the heatmap function and plotted using the gplots package in R (<http://www.r-project.org/>). Prior to the clustering analysis expression levels for each miRNA were normalized across samples using a Variance Stabilizing Transformation.

Real-time quantitative PCR analysis

In order to verify the observed differential expression between morphs in our miRNA-seq data, we selected 9 miRNAs (sal-miR-17, sal-miR-26a, sal-miR-30b, sal-miR-122, sal-miR-140, sal-miR-181a*, sal-miR-196a, sal-miR-199a and sal-miR-206) for qPCR analysis. We concentrated on the 3 embryonic stages, as in both our analyses (for morph or developmental effect) the expression profiles between the samples of the last stage appeared to be very similar (see results). For the qPCR analysis two separate RNA extractions (biological replicates) were used for each data point. RNA was extracted from pools of 6 whole embryos/fry using a standard TRI Reagent (Sigma) protocol and treated with DNaseI (New England Biolabs) in order to limit genomic DNA contamination. All samples were from the same sampling effort and were extracted and processed simultaneously. cDNA was synthesized using the Exiqon universal cDNA Synthesis Kit II. The consistency of the cDNA synthesis among samples was verified using a spike in template along with a Control primer set (Exiqon). For the qPCRs we used SYBR Green master mix (Exiqon) and LNA primers (Exiqon). All qPCRs were done in duplicates (technical replicates) in a 10 µl reaction volume in 96 well-PCR plates on an ABI 7500 real-time PCR System (Applied Biosystems) following manufacturer instructions (Exiqon). The same PCR program was used for all miRNA primer pairs: starting with a 2 min hold at 50°C followed by a 10 min initial denaturation at 95°C and 45 cycles of 10 sec denaturation at 95°C and 1 min annealing/extension at 60°C. A melting curve analysis was performed at the end of each PCR to verify the specificity of the amplification. U2 spliceosomal snRNA (Primer sequence: GGTACTGCAATACCGGGG) was initially selected as a reference gene. However, the use of non-miRNA genes as reference has been shown to be problematic and the use of mean expression is often more appropriate [52]. We therefore opted to use the geometric mean for the expression values of the miRNAs under study as a reference. Relative expression (fold change) for each miRNA compared to stage 1 in AC was calculated in R using a script provided in File S2.

Results

Small RNA sequencing descriptive statistics

In order to identify miRNAs involved in Arctic charr development and morph differences, we made 8 small RNA libraries from four developmental time-points of two contrasting morphs of Arctic charr. The sequencing depth ranged from 29.1 to 33.9 million reads with a mean depth of 32.4 million reads per sample. After removing the adapters using cutadapt [38] and filtering out low quality reads using the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), we obtained on average 24.8 million reads per sample (Table 1). The size distribution of the collapsed reads of all 8 libraries accounting for redundancy is shown in Figure S1. The majority of the reads were 21–23

nucleotides, corresponding to the typical miRNA size range. Details of the size distribution for both unique and collapsed reads for all 8 libraries are shown in Figure S2. All 8 libraries showed similar distribution with a peak at 21–23 nt. Furthermore, annotation of the collapsed reads using the Rfam database confirmed that our small RNA libraries were highly enriched with miRNAs (Table 2).

A total of 326 conserved and 427 novel miRNA candidates were found in the data

All collapsed reads were compared to the mature miRNA sequences available in miRBase (release 19) using ssearch [45] and 326 candidates (Table S2) were identified with high confidence (e -value <0.001). The 10 most abundant miRNAs account for 65% of the total conserved miRNAs (Figure 2) with sal-miR-206 and sal-miR-1 alone accounting for 36% of the total miRNAs. We identified 427 novel miRNA candidates (Table S3) of which 37% were represented by the 10 highest expressed putative miRNAs. We sequenced the genomic regions of three novel miRNAs (sal-nov-235, sal-nov-242 and sal-nov-334). They were all highly conserved between Arctic charr and Salmon (Table S1). Furthermore their mature and star sequences are located in highly conserved blocks in medaka, fugu, tetraodon and stickleback. Several of the conserved miRNAs were present in two or three isoforms (isomiRs). For example sal-miR-451 exists in 3 isoforms (Table S2). Two of these (sal-miR-451_1 and sal-miR-451_3) are highly conserved among vertebrates, whereas the third (sal-miR-451_2) has not previously been described in other species. This derived isoform differs in one base (G->U substitution) located at the 3' end of the mature sequence and is the predominant isoform of sal-miR-451 in our data (Table S2). Another interesting example is sal-miR-152, where 4 isoforms are found in our data (Table S2) with the most abundant being the ancestor sequence. The three other isoforms are one mutation away from the ancestral form. Interestingly, these mutations (T->A, C, or G) are located at the same site (position 5) for all 3 derived isoforms (Table S2).

51 known miRNAs and 6 novel miRNA candidates are differentially expressed among developmental stages

We found 51 known miRNAs to be differentially expressed among developmental time-points. Hierarchical clustering (Figure 3, see also Table S4 for background data) of miRNA expression showed that the 8 samples grouped into four clusters according to developmental time: one major division separates the

three embryonic stages (1, 2 and 3) from the last post-hatching stage (4) and three divisions for each of the three embryonic stages (Figure 3). This major division between embryonic and post hatching stages indicates a clear shift in miRNAs expression between these developmental phases. The second division separates stage 3 from stages 1 and 2 and the third division separates stages 1 and 2 (Figure 3). Interestingly, there are two major divisions in the miRNA expression pattern clustering: node one depicts miRNAs that are highly expressed during the embryonic stages and their expression decreases in the last stage while the second node includes miRNAs with high expression in stage 4 and low expression in the embryonic stages. For example members of the 430 family (miR-430 a, b, c and d) are highly expressed in the embryonic stages and their expression decreases markedly in late development. In addition other miRNAs, such as sal-miR-219 a and b and miR-181c, show higher expression in the embryonic stages. On the other hand, miRNAs such as sal-miR-22a, 140, 182, 183, 192, 215 and different members of the let-7 family show increasing expression over time. Of the novel candidates, 6 putative miRNAs were found to be differentially expressed among developmental points (Table 3). Three of them sal-nov-1, sal-nov-5 and sal-nov-18 are also differentially expressed between morphs.

53 known miRNAs and 19 novel miRNA candidates are differentially expressed between AC and SB embryos

We tested for differential expression between morphs using a Generalised Linear Model and adjusted for multiple testing using the Benjamin-Hochberg false discovery rate as described in methods. We found 53 miRNAs to be differentially expressed between AC and SB. These miRNAs cluster by morph during the embryonic stages (stages 1-3) (Figure 4, see also Table S4 for background data). During these 3 stages miRNAs such as sal-miR-130, 133, 153, 17, 30, 451, 219, 26, 199a and 145 are highly expressed in AC, whereas sal-miR-206, 133, 122, 181a, 192, 196a and 223 are highly expressed in SB. The expression of some of these “morph specific” miRNAs for example sal-miR-130, 153, 17, 30b and 30c in AC and sal-miR-196a, 206, 192 and 122 in SB observed in the embryonic stages decreases markedly in the last stage. During the last stage the observed miRNA expression differences between the two morphs disappear (Figure 4). Of the novel miRNA candidates, 19 putative miRNAs were found to be differentially expressed between AC and SB (Table 3). Two of them, sal-nov-4 and 5 were only expressed in AC and at most/all stages whereas expression of another putative novel miRNA, sal-

Table 1. Summary of read numbers from small RNA sequencing.

| Sample | Number of reads (NR) | NR after adapter trimming | NR after collapsing |
|--------|----------------------|---------------------------|---------------------|
| SB 1 | 33.4 M | 23.1 M | 2.4 M |
| SB 2 | 32.8 M | 28.2 M | 0.9 M |
| SB 3 | 33.9 M | 30.5 M | 0.7 M |
| SB 4 | 29.1 M | 21.6 M | 0.7 M |
| AC 1 | 32.8 M | 24.2 M | 1.7 M |
| AC 2 | 32.5 M | 21.1 M | 2.0 M |
| AC 3 | 31.3 M | 23.2 M | 0.7 M |
| AC 4 | 33.6 M | 26.2 M | 1.0 M |

Number of reads (NR, in millions of reads) in high-throughput data from small RNA libraries of four developmental points and two morphs of Arctic charr. AC = Aquaculture charr, SB = Small Benthic charr.

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Table 2. High-throughput reads annotated using the Rfam database.

| snc RNA | Number of reads |
|---------|-----------------|
| miRNA | 50841311 |
| rRNA | 49033 |
| mRNA | 5755 |
| tRNA | 24451 |
| SNORD | 151004 |
| U | 208259 |
| sno | 25857 |

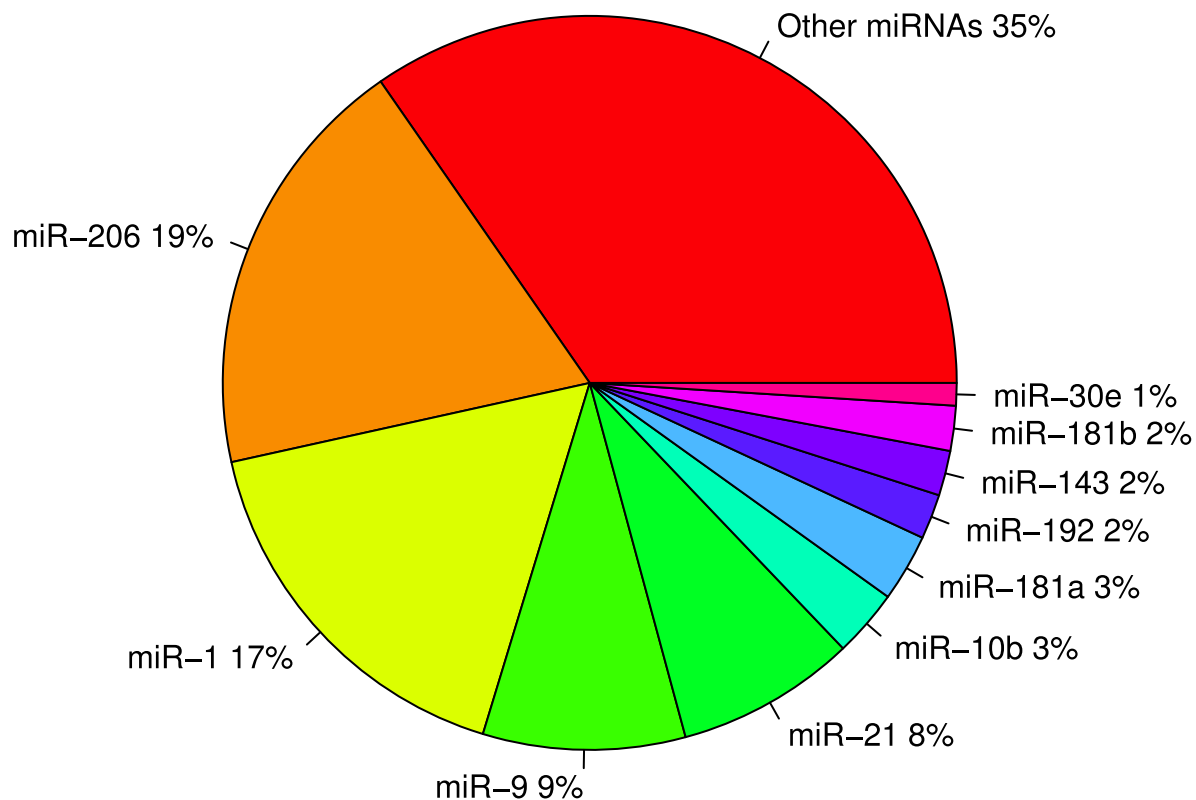
doi:10.1371/journal.pone.0106084.t002

nov-3, was only detected in SB offspring and at all four developmental points. With three exceptions (sal-nov-4, 5 and 16) all of the differentially expressed putative miRNAs showed very low expression levels (Table 3). We selected 9 miRNAs and further studied their expression by qPCR using independent biological replicates. The selection was based on the dynamics and degree of differential expression between morphs and/or among developmental points seen in the sequencing data. We concentrated on the three embryonic stages, as in both our analyses (for morph or developmental effect), the expression profiles between the samples of stage 4 appeared to be very similar. Eight of these miRNAs (miR-17, miR-26a, miR-30b, miR-140, miR-181a*, miR-196a, miR-199a and miR-206) amplified well (Figure 5), whereas miR-

122 showed double peaks in melting curve analysis and was discarded from further analysis. Five (miR 17, 26a, 30b, 140 and 206) out of eight miRNAs tested with qPCR showed similar expression patterns to what was expected from the high-throughput sequencing (Figure 5, A–E). Three miRNAs (miR-196a and miR-199a and miR-181a) exhibited similar expression patterns in one or two of the three stages under study, (Figure 5, F–H).

Discussion

The molecular mechanisms underlying the development of Arctic charr morphologies are likely to have parallels in other vertebrate species and studying them is of interest in both developmental and evolutionary contexts. Given the recent divergence in northern populations of Arctic charr, it is likely that the observed phenotypic polymorphism rooted in development in this species arose mostly by differences in gene regulation as opposed to changes in protein coding sequences. In a recent study [53] two genes involved in matrix remodeling in bone formation (*sparc* and *mmp2*) showed consistent differences in expression during the development of embryos derived from benthic and limnetic morphs of Arctic charr, suggesting that these genes might be involved in the development of these distinct Arctic charr morphologies. Little is known about what controls such differences in expression. While numerous studies demonstrate the involvement of transcription factors and other regulatory elements in the phenotypic evolution of birds [54] and fish [55–60], there are few known examples of miRNAs playing a role in morphological variation. In a recent study Arif *et al.* [61] experimentally demonstrated that differences in the “naked valley” phenotype

**Figure 2. Relative abundance of known miRNAs in all samples combined.** Together the 10 most abundant known miRNAs constitute 65% of all known miRNAs.

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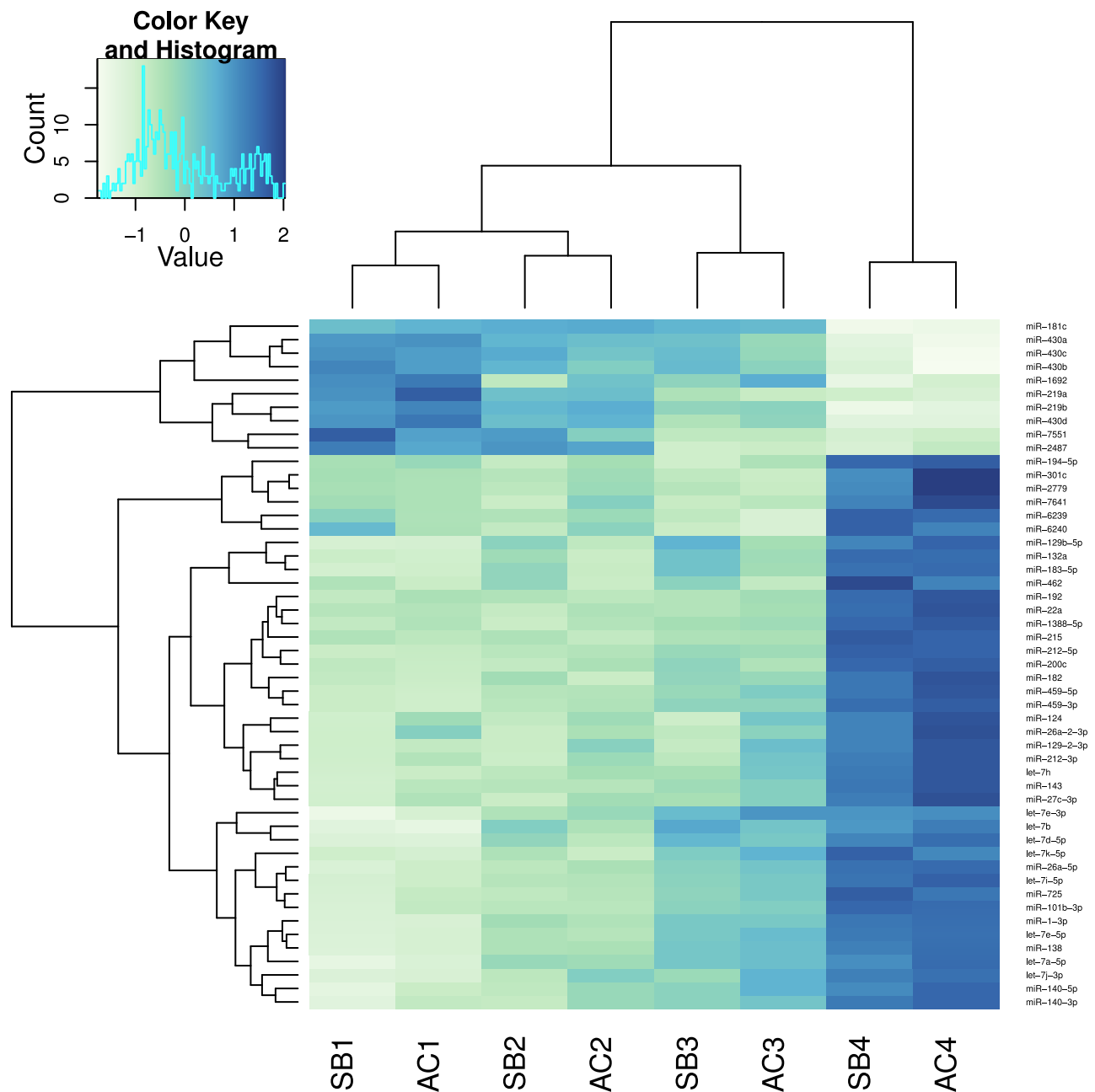


Figure 3. Heat-map showing relative expression of the 51 miRNAs significantly differentially expressed among developmental stages. Expression levels for each miRNA were normalized across samples using variance stabilizing transformation. Blue denotes high and white low relative expression. AC stands for Aquaculture charr and SB stands for Small benthic charr. Numbers 1, 2, 3 and 4 depict the four developmental time-points.

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observed among natural populations of *D. melanogaster* were caused by variation of miR-92a expression. Our study is the first phase of assessing the involvement of miRNAs in the development of key morphological traits and their potential role in the morphological evolution of the highly polymorphic Arctic charr. In so doing we also hope to shed light on some of the developmental circuitry operating at these levels of development.

Using small RNA-seq we found 326 known and 427 novel miRNA candidates in Arctic charr. A few of the candidates, termed novel (Tables 3 and S2) are absent from miRbase but have been previously identified in other salmonid species [62–64].

When only the 326 known miRNAs are considered, the 10 most abundant ones account for 65% of reads (Figure 2). These miRNAs are highly conserved among taxa and have been shown to have important functions during development. The two most abundant miRNAs in our data, miR-206 and miR-1, together account for 36% of the total known miRNAs (Figure 2). Their role in skeletogenesis and myogenesis has been studied in some detail, for example miR-206 has been found to induce myogenic differentiation [65–67] while inhibiting osteoblast differentiation [68], and miR-1 has been found to regulate skeletal muscle and cardiac development [69,70]. These miRNAs are highly conserved in

Table 3. Novel miRNA candidates differentially represented between morphs and/or developmental stages in the small-RNA-seq data.

| Name | Expressed | SB1 | SB2 | SB3 | SB4 | AC1 | AC2 | AC3 | AC4 |
|------------|------------|------|------|-------|-------|-------|------|-------|-------|
| sal-nov_1 | Morph/Time | 7 | 4 | 1 | 1 | 17 | 31 | 25 | 10 |
| sal-nov_2 | Morph | 8 | 13 | 16 | 9 | 104 | 89 | 151 | 88 |
| sal-nov_3 | Morph | 34 | 78 | 63 | 17 | 0 | 0 | 0 | 0 |
| sal-nov_4 | Morph | 0 | 0 | 0 | 0 | 13076 | 5397 | 5755 | 560 |
| sal-nov_5 | Morph/Time | 0 | 0 | 0 | 0 | 1800 | 563 | 343 | 0 |
| sal-nov_6 | Morph | 47 | 9 | 14 | 18 | 73 | 58 | 23 | 28 |
| sal-nov_7 | Morph | 25 | 5 | 1 | 9 | 48 | 45 | 14 | 5 |
| sal-nov_8 | Morph | 207 | 168 | 20 | 7 | 256 | 117 | 47 | 71 |
| sal-nov_9 | Morph | 130 | 59 | 13 | 54 | 268 | 361 | 173 | 70 |
| sal-nov_10 | Morph | 16 | 6 | 12 | 10 | 45 | 53 | 52 | 25 |
| sal-nov_11 | Morph | 8 | 13 | 16 | 9 | 104 | 89 | 151 | 88 |
| sal-nov_12 | Morph | 8 | 12 | 13 | 6 | 14 | 17 | 15 | 6 |
| sal-nov_13 | Morph | 21 | 44 | 112 | 133 | 113 | 110 | 236 | 308 |
| sal-nov_14 | Morph | 2 | 32 | 31 | 53 | 27 | 25 | 96 | 103 |
| sal-nov_15 | Morph | 3 | 22 | 32 | 31 | 6 | 7 | 8 | 10 |
| sal-nov_16 | Morph | 644 | 1472 | 1725 | 983 | 966 | 648 | 944 | 1011 |
| sal-nov_17 | Morph | 24 | 30 | 27 | 2 | 65 | 41 | 69 | 50 |
| sal-nov_18 | Morph/Time | 13 | 89 | 121 | 372 | 3 | 11 | 17 | 219 |
| sal-nov_19 | Morph | 8 | 4 | 9 | 0 | 207 | 63 | 107 | 15 |
| sal-nov_20 | Time | 70 | 274 | 967 | 5358 | 128 | 213 | 988 | 6078 |
| sal-nov_21 | Time | 3472 | 7090 | 12625 | 26369 | 5005 | 4127 | 10188 | 20766 |
| sal-nov_22 | Time | 121 | 107 | 56 | 1 | 206 | 71 | 26 | 0 |

Included are miRNA names, differential expression and number of raw reads per stage per morph.
doi:10.1371/journal.pone.0106084.t003

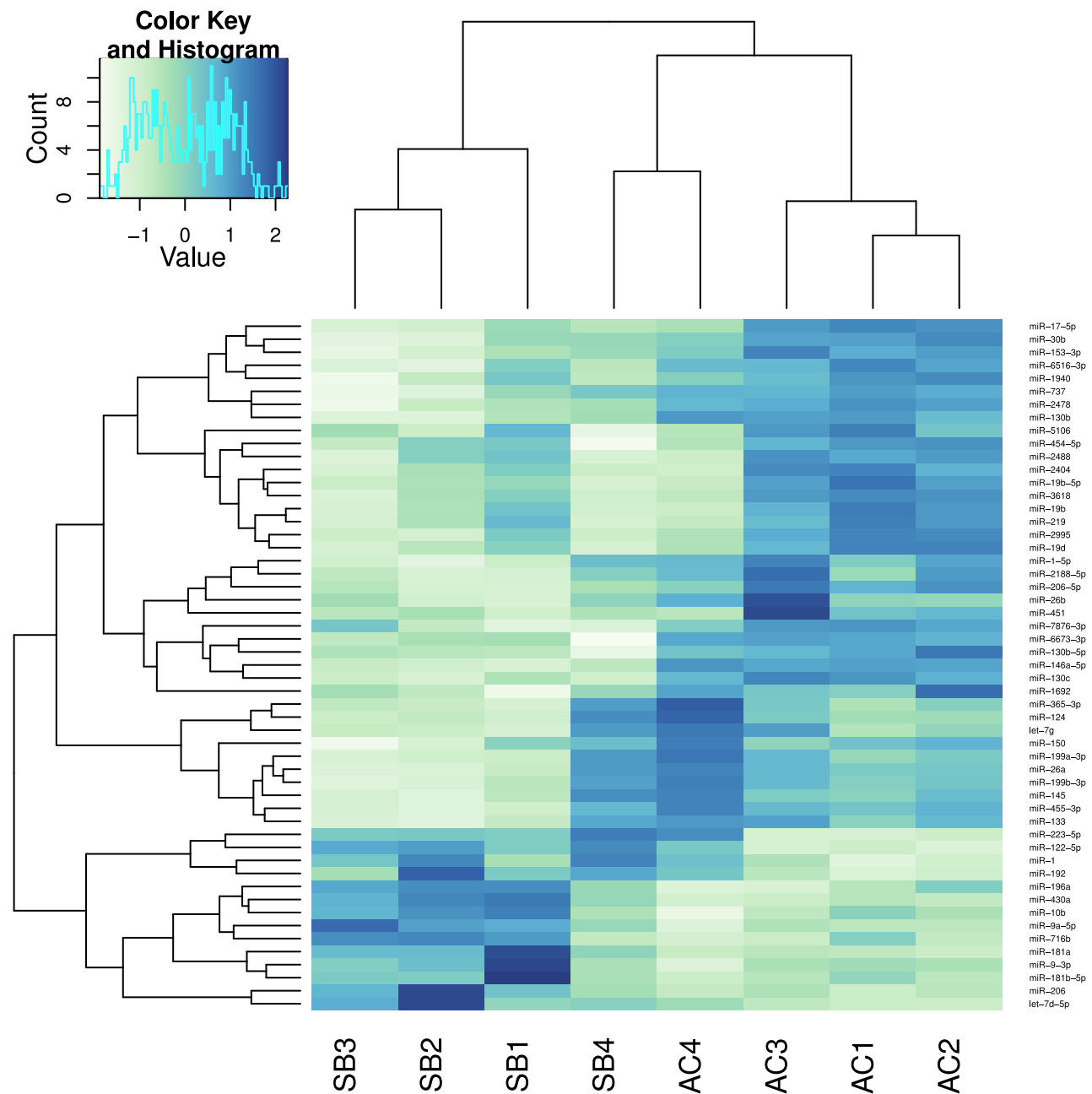


Figure 4. Heat-map showing relative expression of the 53 miRNAs significantly differentially expressed between AC and SB morphs. Expression levels for each miRNA were normalized across samples using variance stabilizing transformation. Blue denotes high and white low relative expression. AC stands for Aquaculture charr and SB stands for Small benthic charr. Numbers 1, 2, 3 and 4 depict the four developmental points.
doi:10.1371/journal.pone.0106084.g004

animal evolution [8] with miR-1 retaining its muscle-specific expression from *C. elegans* to human [71]. Other highly expressed miRNAs are involved in cardiogenesis (miR-21), neurogenesis (miR-9), gut and gall bladder development (miR-143 and miR-192) [72]. One of the oldest miRNAs in the animal kingdom (miR-10) [8] is also among the 10 most highly expressed miRNAs in our data. Encoded in the intron of *hoxB4*, this miRNA is suggested to play a role in anterior posterior patterning.

A few miRNAs exist in multiple forms in our data. Among these the most interesting examples are miR-152 and miR-451. In the case of miR-152 the polymorphism is located in the seed region,

which suggests functional divergence. In the case of miR-451 the ancestral and derived forms differ in one base (G->U substitution) located in the 3' end of the mature sequence. Interestingly the derived form of miR-451 is also the most abundant one. Although not as essential as the seed region, 3' miRNA-target pairing has a role in defining target specificity within miRNA families [73]. The derived form of miR-451 might have evolved following the whole genome duplication Salmonids underwent 25–100 million years ago or as a result of gene duplication. Other possibilities for the presence of this miRNA in our data include post-transcriptional editing of the ancestral form. However, without a sequenced

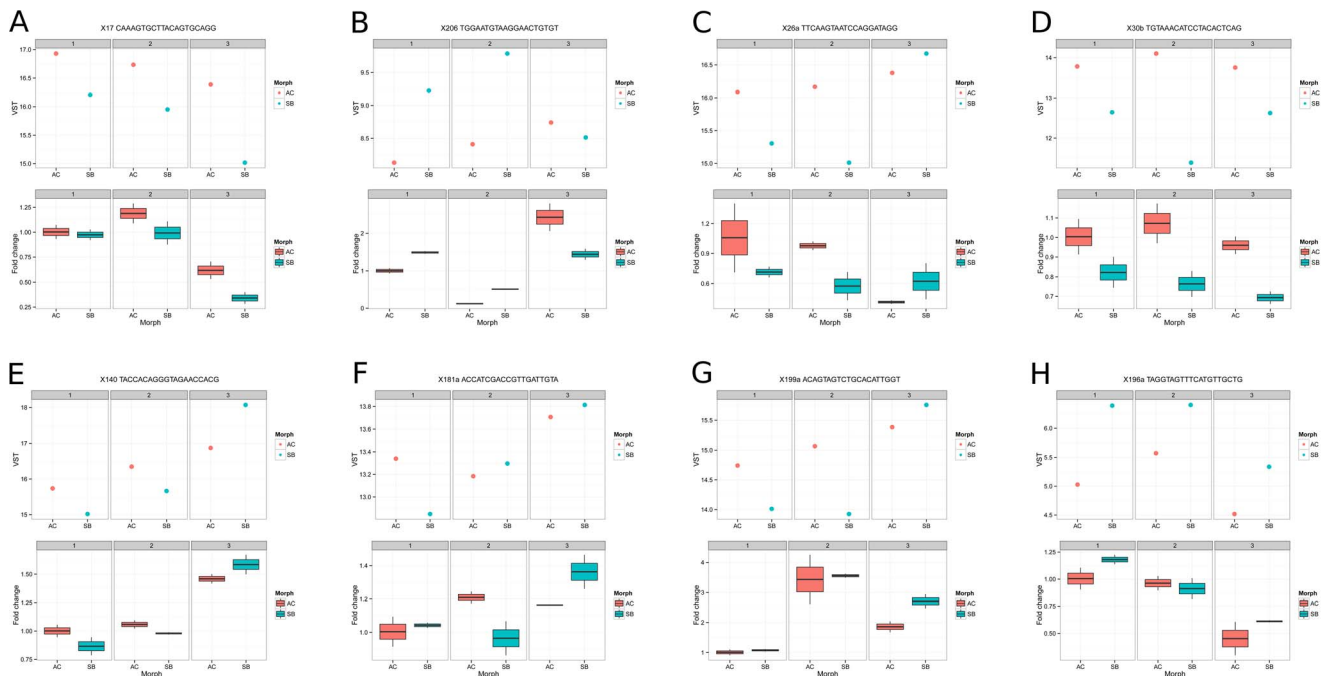


Figure 5. Comparison of expression of 8 selected miRNAs (miR-17, 26a, 30b, 140, 181a, 196a and 199a) at three developmental time-points for two contrasting Arctic charr morphs (AC and SB) quantified by small RNA-seq (upper panel) or qPCR (lower panel). doi:10.1371/journal.pone.0106084.g005

Arctic charr genome, distinguishing between evolutionary scenarios represents a challenge. The derived form of mir-451 might be specific to Arctic charr as it is not present in the salmon genome and has not been reported in rainbow trout. As miRNAs often co-evolve with their targets [5] further phylogenetic analysis will help shedding light on the evolution of miR-152 and miR-451 and their targets in Arctic charr.

51 previously annotated miRNAs were found to be differentially expressed among developmental points. The cluster analysis of these miRNAs showed a clear shift in miRNA expression between the embryonic stages and the post-hatching stage, visible from both dendrograms. Although samples grouped by developmental stage, the major division was between stages 1, 2, 3 and stage 4. These findings were further confirmed by the existence of two major clades of miRNA expression: one containing miRNAs highly expressed during the embryonic stages and one the last stage. Among the miRNAs showing high expression in early development are the members of the 430 family (miR-430 a, b, c and d). In zebrafish these miRNAs are involved in the maternal to zygotic transition by deadenylation and clearing of maternal transcripts [74]. The majority of the miRNAs showed higher expression in the last developmental stage. Examples include miR-1, members of the let-7 family, miR-22a, miR-140, miR-182, miR-183, miR-192 and miR-215. The evolutionarily ancient and highly conserved let-7 family is involved in the regulation of the timing of developmental events in *C. elegans*, in particular the transition from larval stage 4 (L4) to adult [75]. In vertebrate development let-7 is temporally regulated and it is thought to play a role in late temporal transitions during development [75]. Other miRNAs found to be highly expressed in the last developmental stage are also involved in major developmental processes such as muscle differentiation (miR-1), endochondrial bone development (miR-140) and neuromast differentiation (miR-182 and 183) [12,82]. Overall 72 miRNAs (19 novel miRNA candidates and 53 conserved miRNAs) were found to be differentially expressed

between AC and SB at the developmental points under study. Of those sal-miR-196a, sal-miR-206, 122, 192, 196a, 223 and 181a were more highly expressed in SB whereas sal-miR-26a, 30b, 17-5p, 153-3p, 130b and c, 199a were more highly expressed in AC (Figure 4). All of the conserved miRNAs showing variation in expression between the Arctic charr morphs have been found to play an important role in development. For example, miR-196a, which is encoded in a Hox cluster, has been found to be involved in axial and appendicular patterning in chicken [76] and zebrafish [77]. Another muscle specific miRNA, miR-206, shows large expression differences between SB and AC especially at stage 3, where there is a 2.5 fold difference between the two morphs. This miRNA is involved in muscle differentiation and its expression is up-regulated by MyoD in differentiating muscle fibers. Loss of function of MyoD leads to down-regulation of miR-206 and severe deformities in the craniofacial elements [78]. miR-206 has also been shown to directly affect osteoblast differentiation and its overexpression in the osteoblasts of transgenic mice leads to bone abnormalities [68].

Other conserved miRNAs, miR-130b and c, miR-133, miR-199a-3p, miR-26a and miR-451 were highly expressed in AC throughout early development compared to SB. Some of these miRNAs are involved in myogenesis and skeletogenesis, for example, miR-199a-3p is important for normal skeletal development. In mouse a knockdown of Dnm3os (the primary precursor of a miR-214-miR-199a cluster) leads to skeletal abnormalities such as craniofacial hypoplasia [79]. miR-26 contributes to neurogenesis and myogenesis [80] and is involved in rainbow trout embryonic development [62] whereas miR-451 has been found to be involved in erythroid maturation in zebrafish [81]. Nineteen novel miRNA candidates were found to be differentially expressed between the two Arctic charr morphs in this study. Of those, two were only expressed in AC while one putative miRNA was only expressed in SB. These novel miRNA candidates were detected in most/all stages in one morph and not detectable in any

of the stages in the other morph (Table 3), therefore it is unlikely that they represent a sequencing or technical error. As we used the Salmon genome to detect novel miRNAs, none of the novel miRNAs is likely to be morph or even Arctic charr specific, although expression differences can be expected. Several scenarios exist as to why these miRNAs are not expressed in both morphs, for example they might have been lost, their sequence might have been modified leading to the instability of the miRNA secondary structure or their expression repressed.

miRNAs are understudied in fishes and at present represent just a fraction of the miRNAs described in mammals. In the latest release of miRBase (version 20) from June 2013 there are only 255 mature miRNA sequences available for zebrafish, whereas 2578 mature sequences have been described in humans. Here we find 427 novel miRNA candidates, which are not Arctic charr specific. Some of these miRNAs are in highly conserved blocks (sal-nov235, 242, 334) among fishes, indicating that these miRNAs might have arisen early in the evolutionary history of fishes.

Concluding Remarks and Future Directions

The theoretical underpinnings of our study are based on the general proposition that differences in the level, timing and pattern of miRNA expression or acquisition of new miRNAs can influence variation in developmental circuits, so as to generate diverse and possibly discrete morphological phenotypes, thereby creating substrate for natural selection to act upon. We use a system of two contrasting morphs of Arctic charr and as a first step we surveyed miRNA expression at four developmental stages thereby homing in on the miRNA genes that may have a bearing on the morphological and functional differences between the morphs. Differences in expression levels were detected in 72 miRNAs. Interestingly, the majority of these miRNAs (53/72) are evolutionarily stable and have been previously described as part of important developmental processes such as neurogenesis, erythropoiesis, skeleto- and myogenesis, specifically in craniofacial elements. Some miRNAs (e.g. the let-7 and miR430 families) show indications of differences in timing of expression. Other miRNAs (sal-miR-152 and sal-miR-451) exhibit sequence divergence. We are currently working on follow up experiments e.g. looking for the putative targets of the interesting miRNA candidates found in this study and defining their expression pattern using *in situ* hybridization in embryos derived from additional morphs and populations.

Supporting Information

Figure S1 Length distribution of reads in the small-RNA-seq data for all samples combined. A major peak is observed at 22 nt, corresponding to the typical miRNA size. (TIF)

Figure S2 Length distribution of reads in individual miRNA-seq samples. A–D: Small benthic (SB) stages 1–4; E–

H: Aquaculture (AC) stages 1–4. Left panel: redundant reads, Right panel: unique reads. (PPTX)

Table S1 Background information and primers used for amplification of selected miRNA clusters. The names of *S. salar* contigs and miRNAs in clusters used for primer design, sequence identity (%) between Arctic charr and Salmon, and forward and reverse primer sequences are shown. (XLS)

Table S2 Conserved Arctic charr miRNAs. 326 known miRNAs were identified in the small-RNA-seq data. Included are miRNA names, miRNA sequences, number of raw reads per sample and names of miRNA orthologs. (XLS)

Table S3 Putative novel arctic charr miRNAs. 427 novel miRNA candidates. Included are novel miRNA candidate IDs, miRDeep2 score (the log-odds score assigned to the hairpin by miRDeep2), significant ($p < 0.005$) randfold p-value of the putative miRNA to form a hairpin structure, consensus mature, consensus star, consensus precursor sequences and genomic coordinates in Salmon. (XLS)

Table S4 Background data for figures 3 and 4. Normalized number of reads in all 8 samples for miRNAs that showed differential expression between morphs and/or developmental time-points. (CSV)

File S1 R code used for differential expression analysis of miRNA-seq data. (R)

File S2 R code used to calculate relative expression (fold change) for each miRNA compared to stage 1 in AC. (R)

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Author Contributions

Conceived and designed the experiments: SRF ZOJ SSS KHK. Performed the experiments: KHK SRF ZOJ. Analyzed the data: HJ KHK SRF. Contributed reagents/materials/analysis tools: HJ KHK SRF SSS ZOJ. Wrote the paper: KHK ZOJ SRF HJ SSS.

References

- Plasterk RHA (2006) Micro RNAs in animal development. *Cell* 124: 877–881.
- Mishima Y (2012) Widespread roles of microRNAs during zebrafish development and beyond. *Dev Growth Differ* 54: 55–65. doi:10.1111/j.1440-169X.2011.01306.x
- Li J, Zhang Z (2012) miRNA regulatory variation in human evolution. *Trends Genet* 1–9. doi:10.1016/j.tig.2012.10.008
- Zhao Y, Srivastava D (2007) A developmental view of microRNA function. *Trends Biochem Sci* 32: 189–197. doi:10.1016/j.tibs.2007.02.006
- Berezikov E (2011) Evolution of microRNA diversity and regulation in animals. *Nat Rev Genet* 12: 846–860. doi:10.1038/nrg3079
- Bartel DP, Lee R, Feinbaum R (2004) MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Genomics: The miRNA Genes*. 116: 281–297.
- Lewis BP, Shih I, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of Mammalian MicroRNA Targets. *Cell* 115: 787–798.
- Heimberg AM, Sempere LF, Moy VN, Donoghue PCJ, Peterson KJ (2008) MicroRNAs and the advent of vertebrate morphological complexity. *Proc Natl Acad Sci U S A* 105: 2946–2950. doi:10.1073/pnas.0712259105
- Tarver JE, Sperling EA, Nailor A, Heimberg AM, Robinson JM, et al. (2013) miRNAs: Small Genes with Big Potential in Metazoan Phylogenetics. *Mol Biol Evol* 30: 2369–2382.

10. Liu N, Okamura K, Tyler DM, Phillips MD, Chung W-J, et al. (2008) The evolution and functional diversification of animal microRNA genes. *Cell Res* 18: 985–996. doi:10.1038/cr.2008.278
11. Saunders MA, Liang H, Li W-H (2007) Human polymorphism at microRNAs and microRNA target sites. *Proc Natl Acad Sci U S A* 104: 3300–3305.
12. Ason B, Darnell DK, Wittbrodt B, Berezikov E, Kloosterman WP, et al. (2006) Differences in vertebrate microRNA expression. *Proc Natl Acad Sci U S A* 103: 14385–14389. doi:10.1073/pnas.0603529103
13. Hobert O (2008) Gene regulation by transcription factors and microRNAs. *Science* (80-) 319: 1785–1786. doi:10.1126/science.1151651
14. Peterson KJ, Dietrich MR, McPeck MA (2009) MicroRNAs and metazoan macroevolution: insights into canalization, complexity, and the Cambrian explosion. *Bioessays* 31: 736–747.
15. Ebert MS, Sharp P (2012) Roles for microRNAs in conferring robustness to biological processes. *Cell* 149: 515–524. doi:10.1016/j.cell.2012.04.005
16. Wilson AJ, Gíslason D, Skúlason S, Snorrason SS, Adams CE, et al. (2004) Population genetic structure of Arctic charr, *Salvelinus alpinus* from northwest Europe on large and small spatial scales. *Mol Ecol* 13: 1129–1142. doi:10.1111/j.1365-294X.2004.02149.x
17. Snorrason SS, Skúlason S (2004) Adaptive speciation in northern fresh water fishes — patterns and processes. In: Dieckmann U, Metz H, Doebeli M, Tautz D, editors. *Adaptive speciation*. Cambridge University Press, Cambridge. pp.210–228.
18. Robinson BW, Schluter D (2000) Natural selection and the evolution of adaptive genetic variation in northern freshwater fishes. *Adaptive genetic variation in the wild*. New York: Oxford University Press. pp.65–94.
19. Schluter D (1993) Adaptive Radiation in Sticklebacks: Size, Shape, and Habitat Use Efficiency. *Ecology* 3: 699–709.
20. Schluter D, McPhail JD (1993) Character displacement and replicate adaptive radiation. *Trends Ecol Evol* 8: 197–200.
21. Brunner PC, Douglas MR, Osinow A, Wilson CC, Bernatchez L (2001) Holarctic phylogeography of Arctic charr (*Salvelinus alpinus* L.) inferred from mitochondrial DNA sequences. *Evolution* 55: 573–586.
22. Gíslason D, M Ferguson M, Skúlason S, Snorrason S, Ferguson MM, et al. (1999) Rapid and coupled phenotypic and genetic divergence in Icelandic Arctic char (*Salvelinus alpinus*). *Can J Fish Aquat Sci* 56: 2229–2234.
23. Cresko WA, Amores A, Wilson C, Murphy J, Currey M, et al. (2004) Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. *Proc Natl Acad Sci U S A* 101: 6050–6055. doi:10.1073/pnas.0308479101
24. Woods PJ, Skulason S, Snorrason SS, Kristjánsson BK, Malmquist HJ, et al. (2012) Intraspecific diversity in Arctic charr, *Salvelinus alpinus*, in Iceland: I. Detection using mixture models.
25. Snorrason S, Skúlason S, Sandlund O, Malmquist H, Jonsson B, et al. (1989) Shape polymorphism in Arctic charr, *Salvelinus alpinus*, in Thingvallavatn, Iceland. *Physiological Ecol Japan* 1: 393–404.
26. Jonsson B, Skúlason S, Snorrason SS, Sandlund OT, Malmquist HJ, et al. (1988) Life History Variation of Polymorphic Arctic Charr (*Salvelinus alpinus*) in Thingvallavatn, Iceland. *Can J Fish Aquat Sci* 45: 1537–1547.
27. Malmquist HJ, Snorrason SS, Skulason S, Jonsson B, Sandlund OT, et al. (1992) Diet differentiation in polymorphic Arctic charr in Thingvallavatn, Iceland. *J Anim Ecol* 61: 21–35. doi:10.2307/5505
28. Sandlund OT, Gunnarson K, Jonasson PM, Jonsson B, Lindem T, et al. (1992) The Arctic charr *Salvelinus alpinus* in Thingvallavatn. *Oikos* 64: 305–351.
29. Skulason S, Noakes DLG, Snorrason SS (1989) Ontogeny of trophic morphology in four sympatric morphs of arctic charr *Salvelinus alpinus* in Thingvallavatn, Iceland. *Biol J Linn Soc* 38: 281–301. doi:10.1111/j.1095-8312.1989.tb01579.x
30. Frandsen F, Malmquist HJ, Snorrason SS (1989) Ecological parasitology of polymorphic Arctic charr, *Salvelinus alpinus* (L.), in Thingvallavatn, Iceland. *J Fish Biol* 34: 281–297.
31. Skúlason S, Snorrason SS, Ota D, Noakes DLG (1993) Genetically based differences in foraging behaviour among sympatric morphs of arctic charr (Pisces: Salmonidae). *Anim Behav* 45: 1179–1192.
32. Skúlason S, Snorrason SS, Noakes DLG, Ferguson MM (1996) Genetic basis of life history variations among sympatric morphs of Arctic char, *Salvelinus alpinus*. *Can J Fish Aquat Sci* 53: 1807–1813.
33. Kapralova KH, Morrissey MB, Kristjánsson BK, Ólafsdóttir GA, Snorrason SS, et al. (2011) Evolution of adaptive diversity and genetic connectivity in Arctic charr (*Salvelinus alpinus*) in Iceland. *Heredity* (Edinb) 106: 472–487.
34. Kapralova KH, Gudbrandsson J, Reynisdóttir S, Santos CB, Baltanás VC, et al. (2013) Differentiation at the MHCII α and Cath2 Loci in Sympatric *Salvelinus alpinus* Resource Morphs in Lake Thingvallavatn. *PLoS One* 8: e69402.
35. Eiríksón GM, Skulason S, Snorrason SS (1999) Heterochrony in skeletal development and body size in progeny of two morphs of Arctic charr from Thingvallavatn, Iceland. *J Fish Biol* 55: 175–185.
36. Svavarsón E (2007) *Árangur í kynbótum á bleikju og næstu skref [reference in icelandic]*. Fræðingur landbúnaðarinnar (conference proceedings) 4: 121–125.
37. Gorodilov YN (1996) Description of the early ontogeny of the Atlantic salmon, *Salmo salar*, with a novel system of interval (stage) identification. *Environ Biol Fishes* 47: 109–127. doi:10.1007/BF00005034
38. Martín M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* 17: 10–12.
39. Ewing B, Green P (1998) Base-Calling of Automated Sequencer Traces Using Phred. II. Error Probabilities. *Genome Res* 8: 186–194.
40. Burge SW, Daub J, Eberhardt R, Tate J, Barquist L, et al. (2013) Rfam 11.0: 10 years of RNA families. *Nucleic Acids Res* 41: D226–32.
41. Griffiths-Jones S (2004) The microRNA Registry. *Nucleic Acids Res* 32: D109–11. doi:10.1093/nar/gkh023
42. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 34: D140–4.
43. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res* 36: D154–8.
44. Kozomara A, Griffiths-Jones S (2011) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* 39: D152–7.
45. Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. *Proc Natl Acad Sci* 85: 2444–2448.
46. Friedländer MR, Chen W, Adamidi C, Maaskola J, Einspanier R, et al. (2008) Discovering microRNAs from deep sequencing data using miRDeep. *Nat Biotechnol* 26: 407–415. doi:10.1038/nbt1394
47. Di Génova A, Aravena A, Zapata L, González M, Maass A, et al. (2011) SalmonDB: a bioinformatics resource for Salmo salar and *Oncorhynchus mykiss*. *Database* (Oxford) 2011: bar050. doi:10.1093/database/bar050
48. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410.
49. Gordon D, Abajian C, Green P (1998) ConSeq: A Graphical Tool for Sequence Finishing. *Genome Res* 8: 195–202. doi:10.1101/gr.8.3.195
50. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.
51. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B* 57: 289–300.
52. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, et al. (2009) A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 10: R64.
53. Ahi EP, Guðbrandsson J, Kapralova KH, Franzdóttir SR, Snorrason SS, et al. (2013) Validation of reference genes for expression studies during craniofacial development in arctic charr. *PLoS One* 8: e66389.
54. Mallarino R, Campàs O, Fritz JA, Burns KJ, Weeks OG, et al. (2012) Closely related bird species demonstrate flexibility between beak morphology and underlying developmental programs. *Proc Natl Acad Sci U S A* 109: 16222–16227.
55. Sylvester JB, Rich CA, Loh Y-HE, van Staaden MJ, Fraser GJ, et al. (2010) Brain diversity evolves via differences in patterning. *Proc Natl Acad Sci U S A* 107: 9718–9723.
56. Roberts RB, Hu Y, Albertson RC, Kocher TD (2011) Craniofacial divergence and ongoing adaptation via the hedgehog pathway. *Proc Natl Acad Sci U S A* 108: 13194–13199.
57. Manousaki T, Hull PM, Kusche H, Machado-Schiaffino G, Franchini P, et al. (2013) Parsing parallel evolution: ecological divergence and differential gene expression in the adaptive radiations of thick-lipped Midas cichlid fishes from Nicaragua. *Mol Ecol* 22: 650–669. doi:10.1111/mec.12034
58. Henning F, Jones JC, Franchini P, Meyer A (2013) Transcriptomics of morphological color change in polychromatic Midas cichlids. *BMC Genomics* 14: 171.
59. Filteau M, Pavey SA, St-Cyr J, Bernatchez L (2013) Gene coexpression networks reveal key drivers of phenotypic divergence in lake whitefish. *Mol Biol Evol* 30: 1384–1396.
60. Yamamoto Y, Byerly MS, Jackman WR, Jeffery WR (2009) Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution. *Dev Biol* 330: 200–211.
61. Arif S, Murat S, Almudi I, Nunes MDS, Bortolamiol-Becet D, et al. (2013) Evolution of mir-92a Underlies Natural Morphological Variation in *Drosophila melanogaster*.
62. Ramachandra RK, Salem M, Gahr S, Rexroad CE, Yao J (2008) Cloning and characterization of microRNAs from rainbow trout (*Oncorhynchus mykiss*): their expression during early embryonic development. *BMC Dev Biol* 8: 41. doi:10.1186/1471-213X-8-41
63. Ma H, Hostuttler M, Wei H, Rexroad CE, Yao J (2012) Characterization of the rainbow trout egg microRNA transcriptome. *PLoS One* 7: e39649.
64. Bekaert M, Lowe NR, Bishop SC, Bron JE, Taggart JB, et al. (2013) Sequencing and Characterisation of an Extensive Atlantic Salmon (*Salmo salar* L.) MicroRNA Repertoire. *PLoS One* 8: e70136.
65. Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol* 174: 677–687.
66. Sweetman D, Goljanek K, Rathjen T, Oustanina S, Braun T, et al. (2008) Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133. *Dev Biol* 321: 491–499.
67. Dey BK, Gagan J, Dutta A (2011) miR-206 and -486 induce myoblast differentiation by downregulating Pax7. *Mol Cell Biol* 31: 203–214. doi:10.1128/MCB.01009-10
68. Inose H, Ochi H, Kimura A, Fujita K, Xu R, et al. (2009) A microRNA regulatory mechanism of osteoblast differentiation. *Proc Natl Acad Sci U S A* 106: 20794–20799. doi:10.1073/pnas.0909311106

69. Chen J-F, Mandel EM, Thomson JM, Wu Q, Callis TE, et al. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 38: 228–233.
70. Liu N, Williams AH, Kim Y, McAnally J, Bezprozvannaya S, et al. (2007) An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. *Proc Natl Acad Sci U S A* 104: 20844–20849.
71. Niwa R, Slack FJ (2007) The evolution of animal microRNA function. *Curr Opin Genet Dev* 17: 145–150. doi:10.1016/j.gde.2007.02.004
72. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, et al. (2005) MicroRNA expression in zebrafish embryonic development. *Science* 309: 310–311.
73. Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of microRNA-target recognition. *PLoS Biol* 3: e85.
74. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, et al. (2006) Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* 312: 75–79. doi:10.1126/science.1122689
75. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, et al. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408: 86–89.
76. McGlenn E, Yekta S, Mansfield JH, Soutschek J, Bartel DP, et al. (2009) In ovo application of antagomiRs indicates a role for miR-196 in patterning the chick axial skeleton through Hox gene regulation. *Proc Natl Acad Sci U S A* 106: 18610–18615.
77. He X, Yan Y-L, Eberhart JK, Herpin A, Wagner TU, et al. (2011) miR-196 regulates axial patterning and pectoral appendage initiation. *Dev Biol* 357: 463–477. doi:10.1016/j.ydbio.2011.07.014
78. Hinitz Y, Williams VC, Sweetman D, Donn TM, Ma TP, et al. (2011) Defective cranial skeletal development, larval lethality and haploinsufficiency in Myod mutant zebrafish. *Dev Biol* 358: 102–112.
79. Watanabe T, Sato T, Amano T, Kawamura Y, Kawamura N, et al. (2008) Dnm3os, a non-coding RNA, is required for normal growth and skeletal development in mice. *Dev Dyn* 237: 3738–3748. doi:10.1002/dvdy.21787
80. Wong CF, Tellam RL (2008) MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. *J Biol Chem* 283: 9836–9843.
81. Pase L, Layton JE, Kloosterman WP, Carradice D, Waterhouse PM, et al. (2008) miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2. *Blood* 113: 1794–1804. doi:10.1182/blood-2008-05-155812
82. Papaioannou G, Inloes JB, Nakamura Y, Paltrinieri E, Kobayashi T (2013) let-7 and miR-140 microRNAs coordinately regulate skeletal development. *Proc Natl Acad Sci U S A* 110: E3291–300.