miR-199a expression and its targets during development in different Arctic charr morphs

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Raunvísindadeild
Háskóli Íslands
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15 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefnafræði

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Háskóli Íslands
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I hereby declare that this thesis is based on my own observations, is written by me and has neither in part nor as whole been submitted, either in part or as a whole, for a higher degree.

Hér með lýsi ég því yfir að þessi ritgerð er samin af mér og hefur hún hvorki að hluta til né í heild verið lögð fram áður til hærri prófgráðu.

Reykjavík, maí 2014

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Útdráttur

Fjórar svipgerðir Atlantshafs bleikjunnar í Þingvallavatni hafa mismunandi formgerð, sérstaklega hvað varðar lögun munnsins. Miðað við þróunarsögu bleikjunnar á Íslandi, telst það líklegt að þau gen, sem eru mikilvæg við myndun andlitsþáttanna, séu tjáð á mismunandi hátt milli mismunandi svipgerða bleikjunnar. miRNA gegna mikilvægu hlutverki í sérhæfingu og þroskun brjósksins. miR-199a er sérstaklega áhugavert í þessu samhengi þar sem það er vel varðveitt meðal hryggdýra, sýnir mismunandi tjáningarmynstur í óbirtum raðgreiningargögnum hópsins og er tjáð í stoðkerfi annarra hryggdýra. Til að fá betri skilning á þeim þroskunarferlum sem miR-199a tekur þátt í, hvaða áhrif það hefur á önnur gen og hvaða genum það stjórnar, er mikilvægt að skilgreina þau mRNA sem hafa bindiset fyrir miR-199a. Með þetta í huga var tjáningarmynstur miR-199a rannsakað í höfðum kuðungableikju og murtu á þremur þroskunarstigum (177 DD, 200 DD og 238 DD) með *in situ* þáttapörun (*in situ hybridization*). miR-199a var tjáð í andlitsþáttum beggja svipgerða bleikjunnar á öllum þroskunarstigum og tjáning þess minnkaði frá fyrsta þroskunarstigi til þess síðasta auk þess að vera mismunandi tjáð milli svipgerða. Borin voru kennsl á fjögur möguleg mRNA, sem hafa bindiset fyrir miR-199a með tölvugreiningu á röðunum. Tvö þeirra (lum og ets2) voru rannsökuð með *in situ* þáttapörun og sýndu svipað tjáningarmynstur og miR-199a í báðum svipgerðum á stigi 200 DD. miQPCR var einnig notað til að skoða breytileika í tjáningu miR-199a milli svipgerða en án árangurs.

Abstract

The four morphs of Arctic charr in Þingvallavatn differ significantly in morphology, especially in their feeding apparatus. Given the evolutionary history of Arctic charr in Iceland it is likely that genes engaged in the formation of the craniofacial structures are expressed differentially between the morphs. miRNAs are known to play an important role in the differentiation and the development of the cartilage. miR-199a is of particular interest because it is highly conserved among vertebrates, exhibits a differential expression pattern in transcriptome data from different Arctic charr morphs and is reported to be specifically expressed in the skeletal system in other vertebrates. To gain a better understanding of the developmental processes miR-199a is involved in, which genes it interacts with and regulates, it is important to identify its mRNA targets. With this in mind the expression of miR-199a was studied during the embryonic development in the head of two Arctic charr morphs, large benthivorous and planktivorous using WISH at three developmental stages (177 DD, 200 DD and 238 DD). miR-199a was expressed in the craniofacial elements at all stages in both morphs and its expression decreased from the earliest to the latest stage as well as showing differences between the morphs. Four candidate mRNA targets were identified with computational approaches. Two of them (lum and ets2) were studied with WISH and showed similar patterns of expression as miR-199a in both morphs at stage 200 DD. miQPCR was also used to determine the expression differences of miR-199a between morphs although without success.

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Abbreviations

AC	Aquaculture
AGO	Argonaute
angptl4	angiopoietin-like 4
Anti-DIG-AP	Anti-Digoxigenin antibody linked to Alkaline Phosphatese
BSA	Bovine serum albumin
CAF1	Chromatin assembly factor 1
casp3b	caspase 3, apoptosis-related cysteine protease b
CCR4	Chemokine (C-C motif) receptor 4
cDNA	Complementary DNA
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF4G	Eukaryotic initiation factor 4G
ets2	v-ets avian erythroblastosis virus E26 oncogene homolog 2
exp5	exportin-5
GW182	Glycine-tryptophan protein of 182 kDA proteins
LAS	Leica Application Suite
LB	Large benthivorous
lum	lumican
miQPCR	miRNA-specific real-time quantitative PCR
miRISC	miRNA-induced silencing complex
miRNA	microRNA
mRNA	Messenger RNA

NBT/BCIP	Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate
ncRNA	Non-coding RNA
NEB	New England BioLabs®
PABP	Poly(A)-binding protein
PBS	Phosphate buffered saline
PBT	PBS with Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PFAGA	Paraformaldehyde/glutaraldehyde
PI	Piscivorious
PL	Pelagic planktivorous
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
RAN-GTP cofactor	RAN-guanosine triphosphate
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Real-time PCR
SB	Small benthivorous
SDN1	Small RNA degrading nuclease 1
SSC	Saline-sodium citrate
tRNA	Transfer RNA
UTR	Untranslated region
WISH	Whole mount in situ hybridization

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I am thankful to my mentor Zophonías O. Jónsson for providing me with the opportunity to learn and work in his laboratory. I am also very grateful to Kalina H. Kapralova because without her extremely good guidance skills, patience and enthusiasm this project would not have been successful. Thanks to Eshan Ahi for providing me with important information and methods.

1 Introduction

1.1 microRNAs

1.1.1 General

microRNAs (miRNAs) represent a class of endogenous, single-stranded, non-coding RNA (ncRNA) molecules that are able to bind to target messenger RNAs (mRNAs) thus silencing their expression (see Plasterk 2006) by inducing mRNA degradation and/or repressing their target translation. miRNAs are able to regulate many cellular processes such as cell proliferation, apoptosis, cell differentiation and development of organisms (Kargul & Laurent 2010). They exemplify the emerging view that ncRNA may rival proteins in regulatory importance (see Pasquinelli 2012). These ~22-24 nucleotide RNAs were discovered about 20 years ago in larval mutants of the nematode *Caenorhabditis elegans* (see Plasterk 2006). *lin-4* was the first small RNA that was found to bind to and silence the expression of a target mRNA. This miRNA was found to interfere with the translation of lin-14 without reducing its mRNA abundance, polyadenylation, or polyribosomal content (Olsen & Ambros 1999). A second small RNA, let-7, was found to inhibit lin-41 and was also found to be highly conserved among worms, flies and humans suggesting that these new gene regulators may be ubiquitous and often well conserved (Reinhart et al. 2000; Pasquinelli et al. 2000). These findings revealed some aspects of the miRNA's unique functionality, such as their role as molecular switches through a precise time regulation of cellular events and via synchronous inhibition of genes that function interdependently (Pasquinelli et al. 2000). Since the initial discovery of miRNAs as essential regulators of development, over 3000 mature miRNAs have been identified in species ranging from plants to humans (see Kargul & Laurent 2010), implying a common evolutionarily ancient mechanism of regulation of gene expression (see Kozomara & Griffiths-Jones 2011). Although many miRNAs and their target binding sites are deeply conserved, an interaction between them often produces only a subtle reduction (<2-fold) in protein level (Farh et al. 2005). Some miRNAs can be deleted with no apparent effect on phenotype (Farh et al. 2005). Early observations of miRNA expression profiles revealed that miRNAs tend to be anticorrelated with their target genes in contiguous developmental stages or tissues (Farh et al. 2005; Stark et al. 2005). The small size of miRNAs provides a limited amount of sequence information for specificity and as partial pairing between a miRNA and its target mRNA is often sufficient, a single miRNA can operate in highly complex regulatory networks (see Pasquinelli 2012). This property not only means that a single miRNA can regulate numerous mRNAs but also that predicting its targets is not always straightforward.

1.1.2 miRNA biogenesis and function

The general miRNA biogenesis pathway involves miRNAs that are processed from precursor molecules, primary miRNA (pri-miRNA), which are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes (see Krol, Loedige, et al. 2010). The pri-miRNAs fold into hairpins, which act as

substrates for two members of the RNase III family, Drosha (and its partner DGCR8/Pasha), and Dicer. DGCR8 binds to Drosha which cleaves the substrate resulting in a ~70 nucleotide long precursor miRNA (pre-miRNA) (see Pasquinelli 2012).The pre-miRNA is exported to the cytoplasm through the nuclear export protein, exportin-5 (exp5), together with Ran-guanosine triphosphate (Ran-GTP cofactor) (Yi et al. 2003). In the cytoplasm the pre-miRNAs are processed by Dicer into a ~20 nucleotide mature miRNA/miRNA* duplex. The importance of Dicer in mRNA maturation has been demonstrated by Wienholds et al. (2003) by showing that zebrafish and mice lacking Dicer cannot synthesize miRNAs. While $Dicer^{-/-}$ mice die during the earliest stages of embryonic development, the zebrafish embryos that are homozygous for the mutant Dicer gene had formed most of their organs by 72 hr of development and were able to swim and eat in the absence of Dicer. The explanation lies in the presence of maternal Dicer transcripts in the developing zebrafish embryos. Finally the miRNA is incorporated into the miRNA-induced silencing complex (miRISC). The function of this complex is to induce the translational repression or deadenylation and degradation of mRNA targets (Yi et al. 2003). The two key factors in the assembly and function of miRISC are Argonaute (AGO) proteins, which directly interact with miRNAs, and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression (see Pasquinelli 2012; Yi et al. 2003; Braun et al. 2011). The formation of the miRISC and its functions involves many additional factors. Another miRNA function is the inhibition of translation initiation. Although the precise mechanisms by which this is achieved are not very well understood, several theories currently exist. The cap and the poly(A) tail structures act in synergy to enhance translation (Humphreys et al. 2005). This has been demonstrated by a model in which eukaryotic initiation factor 4G (eIF4G), a subunit of the 5'cap-binding initiation factor, binds the poly(A)-binding protein (PABP) and promotes circularization of the mRNA molecule, a structure that is translationally superior to the linear form (see Rane 2007). Alternatively, in an intact circular mRNA, the miRISC complex may come into close proximity to the translation initiation complex eIF4F and physically or biochemically hinder its function (see Rane 2007). A more elaborate mechanism became evident with the discovery of the miRNAs properties to induce deadenylation of mRNAs (see Chekulaeva & Filipowicz 2009) leading to their destabilization (see Chekulaeva & Filipowicz 2009). This function is mediated through the interaction of Ago proteins with the chromatin assembly factor 1 (CAF1). CAF1 recruits the CCR4-NOT protein complex, harbouring both a chemokine (C-C motif) receptor (CCR4) and CAF1 deadenylase enzymes (Fabian et al. 2009). This, in addition to GW182's interaction with PABP, induces mRNA deadenylation.

1.1.3 miRNA degradation

In contrast to miRNA biogenesis, limited data of the decay of miRNAs has been documented (Krol, Busskamp, et al. 2010). It is generally thought that miRNAs are highly stable molecules and experimentation using RNA polymerase II inhibitors or depletion of miRNA processing enzymes, have indicated that the half-lives of miRNAs in cell lines or in organs correspond to many hours or even days (Krol, Busskamp, et al. 2010; see Pasquinelli 2012). Such slow turnover is unlikely to be a universal feature of miRNAs as they often play a role in developmental transitions or act as on and off switches, conditions that require a more active metabolism (Chatterjee & Grosshans 2009). Some progress has been made recently in identifying the enzymes involved in

miRNA degradation. In *Arabidopsis thaliana*, degradation of mature miRNAs is mediated by small, RNA degrading nuclease 1 (SDN1), SDN2 and SDN3 (Ramachandran & Chen 2008). Inactivation of these SDN genes, resulting in the stabilization of several miRNAs, is associated with developmental phenotypes (Ramachandran & Chen 2008). In *C. elegans*, an enzyme, XRN-2, catalyses the degradation of mature miRNAs (Chatterjee & Grosshans 2009). The miRNAs have to be released from the miRISC to make the miRNA 5' end accessible to the enzyme so degradation can start. The susceptibility of miRNAs to XRN-2 depends on their mRNA target availability, as the miRISC association with mRNA prevents the miRNA degradation (Chatterjee & Grosshans 2009). In summary, degradation is likely to be an important step in the regulation of miRNA function, in a similar way to that established for mRNAs (see Krol, Loedige, et al. 2010).

1.1.4 Identifying the target mRNAs of a miRNA

The extent of the base pairing between the miRNA and mRNA appears to be an important factor for the fate of the target mRNA (see Berezikov 2011). Most miRNAs imperfectly base-pair with complementary sequences in the 3'UTR of target mRNAs (see Berezikov 2011). There are rare examples of near-perfect complementarity between a miRNA and its target site that enables the cleavage of the mRNA. One of these examples is miR‑196 which binds to the 3'UTR of the *HoxB8* mRNA with an almost perfect complementarity and causes the cleavage of the mRNA within the miRNA-binding site (see Pasquinelli 2012). In the majority of miRNA mediated gene regulation, a 7 nucleotide seed sequence, at position 2 to 8 from the 5'end, appears to be crucial for an efficient mRNA targeting (Doench & Sharp 2004). As previously stated, the binding of the mature miRNA to its target mRNA takes place within the miRISC. miRISC has to contain at least one catalytically active member of the AGO protein family (AGO1–AGO4 in mammals; dAGO1 and dAGO2 in flies; ALG-1and ALG-2 in *C. elegans*) to lead to an endonucleolytic cleavage of the mRNA (see Krol, Loedige, et al. 2010). The binding of the miRISC, which includes GW182 proteins, to the 3′UTR target sequences can result in the recruitment of deadenylation factors that remove the poly(A) tail and make the mRNA susceptible to exonucleolytic degradation (Braun et al. 2011).

1.1.5 Tissue-specific expression of miRNAs

Many miRNAs exhibit striking organ, tissue and/or developmental stage specific expression patterns (see Plasterk 2006). Disruption of the Dicer gene leads to the depletion of all miRNAs in zebrafish (Wienholds et al. 2003). If two heterozygous fish are crossed, their homozygous progeny for the Dicer mutation usually develops normally until the age of one week when the growth stops and they die. During the first few days of embryonic development, zebrafish embryos that lack zygotic Dicer can form new miRNAs due to the activity of maternal Dicer (Wienholds et al. 2003). The temporal pattern of miRNA expression in these zebrafish embryos shows that miRNAs are produced after cells have already differentiated and tissues have been formed (Wienholds et al. 2005). miRNAs are not crucial for early zebrafish development as was emphasized in an experiment (Giraldez et al. 2005) where the maternal expression of Dicer was abrogated by transplanting germ cells from fish embryos with mutant Dicer into wild-type embryos of the same age. The fish were fertile but their germline was

homozygous for the Dicer mutation (Giraldez et al. 2005). While the progeny of these fish were lacking maternal Dicer and they became arrested in early development, they were still able to form several tissues. In summary, miRNAs are required for full embryonic development of zebrafish but they are not required for the initial differentiation of tissues in these animals.

1.2 *Salvelinus alpinus* (Arctic charr)

1.2.1 Arctic charr from Þingvallavatn

Arctic charr in Þingvallavatn is an extreme example of intra-lacustrine diversity (Kapralova et al. 2011). Four morphs grouped into two morphotypes are found to coexist in the lake: two limnetic represented by planktivorous (PL) and piscivorous (PI) and two benthic represented by small (SB) and large benthivorous (LB) (Snorrason et al. 1994). The four morphs differ in many features including morphology (Figure 1), behaviour and diet (Snorrason et al. 1994). PL and PI have fusiform bodies, small pectoral fins, high number of dense gill rakers, terminal mouths, evenly protruding jaws, and pointed snouts (Snorrason et al. 1994). PL feeds mainly on zooplankton and PI on threespine sticklebacks. SB and LB have dark stocky bodies, long pectoral fins, low number of gill rakers, subterminal mouths, short lower jaws, and blunt snouts (Snorrason et al. 1994). SB and LB feed mainly on benthos and snails (Snorrason et al. 1994). The genetic component of the trophic morphology and feeding behaviour of the morphs has been demonstrated in a series of laboratory rearing experiments (Skúlason et al 1989b; Skúlason et al. 1993; Eiríksson et al. 1999). The four morphs spawn in the stony littoral habitat, a habitat zone ranging from 0 to 8 m depth (Skúlason et al. 1989a). LB spawns in late July to early August, SB spawns at variable times ranging from mid-August to the end of October, PI spawns from September to November and PL spawns in the last days of September and the first 10 days of October (Skúlason et al. 1989a). Although possibilities for interbreeding between the morphs exist the gene flow between morphs is rather limited (Kapralova et al. 2011) and morphs were found to be genetically differentiated (Kapralova et al. 2013).

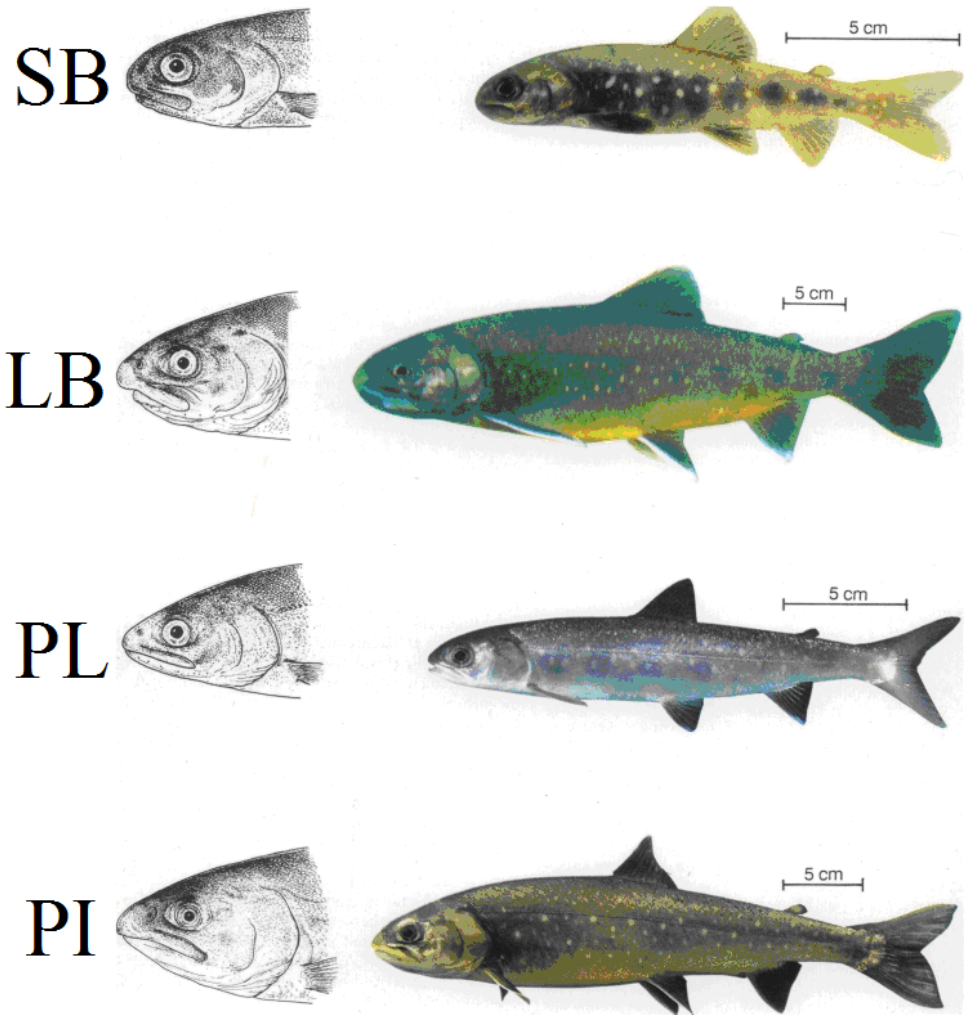

Figure 1 – Morphological and size difference of Arctic charr. *The figure shows the morphological difference of the craniofacial structures and size difference of the four morphs from Þingvallavatn, small benthivorous=SB, large benthivorous=LB, planktivorous=PL and piscivorous=PI. The size scale is 5 cm, from Sandlund et al. (1992).*

1.2.2 Heterochrony

Variation in developmental rate and timing could be responsible for the evolution of different morphs (Parsons et al. 2011). This phenomenon is called heterochrony. A single perturbation of the timing or the rate of developmental event can lead to changes not only in isolated traits of an organism but can affect the whole organism (see Webster & Zelditch 2005). Changes in the patterning of a morphospace (possible form, shape or structure of an organism) during development could also have an effect on adaptation (see Webster & Zelditch 2005). Thus, heterochrony is thought to play an important role in evolution. Developmental heterochrony has been shown to play a role in Arctic charr evolution. In their study Eiríksson et al. (1999) showed that some skeletal elements in SB embryos ossified earlier and/or faster than in PL embryos. The high phenotypic diversity seen in the Arctic charr morphs and their low level of genetic differentiation (Kapralova et al. 2011) suggests that transcription factors and other regulatory elements might be involved in the development of Arctic charr

morphologies. Little is known about the expression of such regulatory elements in Arctic charr. For example Ahi et al. 2013 and Ahi et al. *in prep*, showed that some genes involved in matrix remodelling in bone formation and a transcription factor showed consistent differences in expression during the development of benthic and limnetic Arctic charr. Moreover Kapralova et al. *under revision* showed that some muscle and bone specific miRNAs were differentially expressed between morphs.

1.2.3 Bone formation and the involvement of miRNAs in bone formation

Organ shaping during ontogeny is a complex process (Kimmel et al. 1998). The skeleton is an important building block of many organisms that consists of cartilages and bones. Skeletogenesis in the head is a different process from the one occurring elsewhere in the body. Much of the skull and all of the pharyngeal skeleton, including jaws, hyoid and gill arches have neural crest-derived cartilages and bones, unlike more posterior axial and appendicular skeletons which have mesoderm-derived cartilages and bones (see Helms & Schneider 2003). The cranial neural crest arises from the dorsal margins of the neural folds and Wnt protein is necessary for neural crest induction (García-Castro et al. 2002). If Wnt signalling is not present, neural crest cells cannot be generated. Snail family members are also involved in the generation on neural crest cells see (Helms & Schneider 2003). Neural crest cells display distinct migratory patterns where they pass between neural and facial epithelia, and around paraxial mesoderm until they reach the pharyngeal arches and the frontonasal process (see Helms & Schneider 2003). During their migration to the facial primordial, signals that they encounter can alter their fate. ErbB4, a receptor tyrosine kinase, is expressed in neural ectoderm and is involved in controlling the migration of neural crest cells (Gassmann et al. 1995).

miRNAs are known to play an important role in differentiation and development across a whole range of organisms and tissue types (Lin et al. 2009). Little is known about the precise role of miRNAs in cartilage development and their role in chondrogenic differentiation. Kobayashi et al. (2008) have shown that differential disruption of the Dicer gene in mice resulted in a highly abnormal cartilage development. miR-199a is of a particular interest, because it is highly conserved among vertebrates, is differentially expressed between morphs during development (Kapralova et al. *under revision*) and is reported to be specifically expressed in the skeletal system (Jia et al. 2012). Kobayashi et al. (2008) obtained experimental evidence to present miR-199a as a novel miRNA that is directly implicated in the chondrogenic differentiation process, acting as an inhibitor of early chondrogenesis. They were also able to provide evidence that miR-199a inhibits early chondrogenesis by targeting and suppressing the expression of the Smad protein family 1 (Smad1), a major regulator of bone and cartilage development (see Chen et al. 2004). Thus, it is expected that the expression of miR-199a is highest in the early time points of development and as cartilage and bones accumulate the expression decreases.

To gain better understanding of the nature of the developmental processes miRNAs are involved in and what genes they interact with and regulate, it is essential to identify their mRNA targets. Several computational target prediction tools have been developed, and these tools continue to be modified as more targets are being

experimentally validated (Lewis et al. 2003). In this study two target prediction applications, four mRNA targets of miR-199a were established; angiopoietin-like 4 (angptl4), caspase 3 (casp3b), v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ets2) and lumican (lum).

Different techniques have been applied to detect tissue-specific miRNAs in animals and plants, namely in situ hybridization, northern blotting, real-time PCR, deep sequencing and microarray technologies. The main aim of this study was to detect the expression of miR-199a and its targets mRNAs (angptl4, casp3b, ets2 and lum) using DIG-labelled antisense RNA probes for whole mount in situ hybridization (WISH). First, this technique was used to investigate the spatial expression of miR-199a during Arctic Charr development. Second, the spatial expression of miR-199a was compared between two contrasting morphs of Arctic charr (LB and PL) at three developmental time points. Third, the spatial expression of the putative targets of miR-199a was investigated for LB and PL at stage 200 DD and this pattern was compared to the one seen for miR-199s. Finally a miRNA-specific real-time quantitative PCR (miQPCR) was performed to test whether miR-199a is differentially expressed between the heads of two morphs of Arctic charr (Aqucalture=AC and SB) and whether the expression differs between two developmental time points, 177 DD and 200 DD.

2 Materials and methods

2.1 Materials

2.1.1 Charr embryo pre-fixation and storage

1. The charr embryos were fixed in Verið, Sauðákróki in 4% Paraformaldehyde (PFA) for 30 minutes. The chorion was punctured and the embryos incubated at 4°C overnight in 4% PFA.

2. The embryos were stored in methanol at -20°C.

2.1.2 miQPCR

cDNA synthesis

1. DNase digested RNA

2. T1 mix: miQ Linker and PEG (50%).

3. T2 mix: Neb 10X Buffer (NEB), $MgCl_2$ (25 mM), ddH_2O, RNase inhibitor (40 U/μL) and T4 RNA ligase 2 (NEB, 200 U/μL).

4. cDNA mix 1: dNTPs (10 mM), ddH_2O and miQ RT primer.

5. cDNA mix 2: 5X RT Buffer, DTT (0.1 M) and ddH_2O.

6. Superscript II (Invitrogen)

miQPCR

1. cDNA template made in the previous step.

2. PowerSYBRgreen® PCR Master Mix: ddH2O, forward primer, reverse primer (UNI. Rev) and SYBR Green Mix.

2.1.3 WISH

1. 400 mL Schott bottle was washed in a dishwasher and baked at 180°C overnight. The pour ring and the bottle top were treated with RNase Zap and rinsed with MilliQ water.

2. Phosphate buffered saline (PBS) with Tween 20 (PBT): 1X PBS with 0.1% Tween 20; that is 350 μL added to 350 mL PBS.

3. Rehydration: 75%, 50% and 25% methanol in PBT.

4. Permeabilisation: Proteinase K.

5. Paraformaldehyde/glutaraldehyde (PFAGA): Dilute glutaraldehyde stock solution (70%) to 0.2% in PFA and add 0.1% Tween 20; that is 143 µL glutaraldehyde stock solution and 50 µL Tween 20 into 50 mL PFA.

6. Hybridization Buffer: 2% blocking reagent, 0.1% Triton X (10%), 0.1% CHAPS (10%), 5x 20 X SSC, 50% Formamide, 50 µg/mL tRNA (10 mg/mL), 50 µg/mL heparin (50 mg/mL), 5mM EDTA (2M) and MilliQ water.

7. Salt washes: 2 X SSC, 2 X SSC/0.1% Tween 20 and 0.2 X SSC/0.1% Tween 20.

8. Blocking solution: BSA (50mg/ml), sheep serum, dimethylsulophoxide (DMSO, Sigma) and PBT.

9. Antibody reaction: 1:2000 Anti-DIG-AP to blocking solution.

10. Colouration buffer: $MgCl_2$, NaCl, Tris pH 9.5 (1 M), 10% Tween 20, Levamisole and MilliQ water.

11. 20 µL/mL Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP)

12. Paraformaldehyde fixative (PFA): Dissolve 4% PFA powder in PBS at 65°C in a fume hood. Add several drops of 1M NaOH; that is 20 g PFA powder to 450 mL PBS.

2.2 Methods

2.2.1 Computer search: miR-199a target genes

The targets of miR-199a in Arctic charr were identified by two different approaches: a conservation approach and a candidate gene approach. Two of the most widely utilized engines that rely on base pairing between the seed sequence of the miRNA and the 3'UTR of its targets, in addition to evolutionary conservation of the targeted sequence, were used.

Conservation approach

TargetScan (http://www.targetscan.org/fish_62/), a program which combines thermodynamics-based modelling of RNA:RNA duplex interactions with comparative sequence analysis to predict miRNA targets conserved across multiple genomes (Lewis et al. 2003), was used to define mRNA targets for miR-199 in zebrafish since information about Arctic charr is currently not available. TargetScan applies the following (Lewis et al. 2003; Friedman et al. 2009):

1. An alignment score for W-C base-pairing of the seed sequence, of the miRNA to its target, in addition to base-pairings beyond the seed sequence that can compensate for mismatches in the seed.

2. Recognizing a conserved adenosine at the first position and/or a W-C match at position 8.

3. Context scoring, which evaluates the AU content 30 nucleotide flanking both sides of the targeted site, in addition to the distance of the latter from the end of the 3'UTR

4. Using three different levels to determine the degree of conservation; highly conserved between human, mouse, rat, chicken and dog, conserved between human, mouse, rat, and dog, and poorly conserved among any combination of species.

A table with 3614 putative target genes containing i) the representative 3'UTR, ii) the 3'UTR expression profile, iii) the total number of target sites and iv) the total context+ score was generated when searched for miR-199a. These genes were sorted based on where and when they are expressed. The best candidates were then sorted based on a high total number of target sites and a low context+ score number. The final target genes were further analysed by studying the expression pattern of the top candidates in more detail using The Zebrafish Model Organism Database (http://zfin.org/cgi-bin/webdriver?MIval=aa-xpatselect.apg). Two putative targets (casp3b and ets2) with a craniofacial expression in zebrafish were selected for further studies.

Candidate gene approach

A craniofacial candidate gene list from Ahi et al. *in prep* with 3'UTR sequences from Arctic charr was used and run against the miR-199a sequence using miRanda. The miRanda algorithm (see Betel et al. 2008; John et al. 2004) applies the following:

1. An alignment score between a miRNA and its target; that is "the sum of match and mismatch scores" for base-pairing and gap penalties.

2. The alignment score involves an asymmetric Watson-Crick (W-C) 5' to 3' base-pairing, with more weight assigned to the 5' end (seed sequence), which can be compensated for by stronger complementation between the 3' end of the miRNA and its target, while allowing mismatches and G:U wobbles in the seed sequence.

3. Determining the degree of evolutionary conservation of the targeted sequence and its position in 3'UTRs of human, rat, and mouse genes.

Three putative targets (angptl4, ets2 and lum) were identified for further studies using miRanda.

2.2.2 miQPCR

cDNA synthesis

The first step in this study was the challenging conversion of RNA into complementary DNA (cDNA) by reverse transcription. Extracted and DNase digested RNA (100 ng/μL), for whole body and head, was diluted to 10 ng/μL and reverse transcribed into cDNA using SuperScript® II (Invitrogen) following the manufacturer´s protocol. A randomly chosen sample was prepared without the addition of a reverse transcriptase (-RT) which was used as a control for genomic DNA. The reaction volume (20 μL) was then diluted 5 fold by adding 80 μL RNase-free MilliQ water and stored at -21°C for the following miQPCR.

Target mRNA primer design

The aim was to design qPCR primers overlapping exon boundaries or located in separate exons. Gene exon/intron boundaries are for the most part well conserved between orthologues (Dvoráková et al. 2007) and as a sequenced Arctic charr genome is not currently available, zebrafish was used to locate the exon/intron boundaries prior toprimer design. The primer design was done in CLC Genomics Workbench and primers were designed based on the mRNA sequence information from the Arctic charr transcriptome (Gudbrandsson et al. *in prep*). The resulting sequence was then blasted against the *Salmo salar* genome using SalmonDB (http://salmondb.cmm.uchile.cl/blast/) to generate the primer sequences. Two primer sequences (forward and reverse) of a size ~18-25 bp each were chosen and analysed using OligoAnalyzer 3.1 (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). This online tool performs; a self-dimer analysis on each primer and a hetero-dimer analysis of the two primers together. Two important factors should be considered during the self-dimer analysis: First, the GC content of the sequence and second, the predicted melting temperature (T_m) between the mRNA primer and the target sequence with an optimum T_m of 55-58°C. Finally the forward and reverse primers were tested for the ability to form hetero-dimers using OligoAnalyzer 3.1. It should not be favourable for the primer to dimerise and the ΔG for dimers has to be greater than -6 kcal/mol. The reverse primer was then reversed using Reverse Compliment (http://www.bioinformatics.org/sms/rev_comp.html). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to generate a more detailed primer report containing the length of forward and reverse primer, T_m, GC% and the length of the sequence between the primers (>200 bp) (Table 1).

Table 1 – Primer design. *The name of the primer, the method which it was designed for, the sequence length, T_m and GC% are demonstrated.*

Primer	Method	Length	T_m (°C)	GC%
angpt14_F	WISH	22	57.27	40.91
casp3_F	WISH	22	59.90	50.00
casp3_F	qPCR	22	58.33	45.45
Lum_F	WISH	19	58.33	52.63
angpt14_R	WISH	20	59.46	55.00
casp3_R	WISH	22	59.25	50.00
casp3_R	qPCR	20	58.60	50.00
Lum_R	WISH	22	59.24	54.55

Detection of amplified products using SYBR Green

SYBR Green is a fluorescent DNA binding dye whose fluorescence is monitored as the

amplified product accumulates during the PCR cycles (see Benes & Castoldi 2010). miQPCR was performed using pre-designed miRNA-specific primers by Kalina H. Kapralova and Zophonías O. Jónsson and PowerSYBRgreen® PCR Master Mix as recommended in the Manufacturer´s protocol (Applied Biosystems) with the exception of using 10 µL final reaction volume. Each 10 µL qPCR reaction consisted of 9 µL PowerSYBRgreen® PCR Master Mix and 1 µL previously made and diluted cDNA. The miQPCR was conducted in 96 well-PCR plates using ABI 7500 real-time PCR System (Applied Biosystems). The reactions were run with two biological (i.e. two independent RNA extractions) and two technical replicates (i.e. the qPCR reaction was repeated twice to account for pipetting errors) for each sample. The run started with an initial 2 min hold at 50°C followed by a 10 min hot start at 95°C. The amplification was performed with 50 cycles of 15 sec denaturation at 95°C and 35 sec extension at 60°C. A dissociation step (60°C-95°C) was performed at the end of the amplification phase to identify a specific melting temperature for each miRNA primer. The melting curve was obtained to verify the qPCR product specificity.

Selection of reference genes and data normalization

To date, information on stable reference genes (housekeeping genes) for validation of microRNA expression is lacking (Wessels et al. 2011) but reference gene/-s had to be chosen to minimize experimental error in the miQPCR. Expression levels of two putative developmental miRNA (miR-199a and miR-206) in two charr groups (AC and SB) at three developmental time points (150 DD, 161 DD and 200 DD) were calculated by converting C_T values into raw data and analysed by the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001) using a combination of two reference genes (i.e. the geometric mean of the C_T values of the two selected reference genes) for normalization. Relative quantities and standard deviation were calculated by comparing the expression of miR-199a and miR-206 in the two morphs at three developmental time points. Statistical differences of the expression of the two miRNAs were determined using a Student‘s t-test.

2.2.3 WISH

In this study, WISH was carried out using a standard protocol with DIG-labelled antisense RNA probes for embryos at three developmental time points of LB and PL. However, few specific modifications were made at HÍ by James McEwan and Valerie H. Maier. The WISH procedure is listed below. Each step was performed on a rocking platform except the proteinase K step and the colour reaction.

1. Embryo pre-fixation was performed at Hólar University College where the embryos were fixed in 4% (w/v) PFA overnight 4 °C and dehydrated in 100% methanol at -20 °C. Methanol (meOH) was used as a long-term storage solution for fixed embryo samples.

2. The embryos were rehydrated in the following methanol series: 75% meOH/PBT, 50% meOH/PBT, 25% meOH/PBT and 100% PBT.

3. The embryos were carefully dechorionated using watchmaker‘s forceps under a dissecting microscope. The embryos were removed from the egg and the yolk sac from the jaw and the gill arches so that the proteinase K could penetrate the cartilage of the developing craniofacial elements. During the steps above, it is important to work on ice to prevent RNA degradation.

4. For different developmental time points, the time and concentration of proteinase K varies (Table 2).

5. The embryos were fixed in PFAGA for 20 minutes and

6. pre-hybridized in pre-heated hybridization buffer for 2 hours at different temperature depending on the probe used (ets2, lum=70°C; miR-199a=64°C).

7. The embryos were hybridized in pre-heated hybridization buffer containing 1µg/ml anti-sense DIG labelled probe for a desired gene (ets2, lum and miR-199a) and incubated overnight in a humidified hybridization chamber at an appropriate temperature.

8. The embryos were washed in the following SSC series at hybridization temperature: 2 X SSC, 2 X SSC/0.1% Tween 20 and 0.2 X SSC/0.1% Tween 20.

9. The embryos were added to a blocking solution for 2 hours and

10. Incubated overnight at 4°C with 1:2000 anti-DIG-AP in a blocking solution.

11. The antibody was removed by eight 15 min washes of PBT and

12. Embryos were washed four 5 min washes with the colouration buffer.

13. The DIG signal was visualized using colouration buffer containing 20 µL per mL of NBT-BCIP. The embryos were incubated in the dark at 4°C. Development was allowed to proceed until desired signal was strongest with minimal background.

14. When desired staining was accomplished, the reaction was stopped, the coloration buffer removed by 4 PBT washes and embryos fixed in 4% (w/v) PFA and stored in PBT in the dark at 4°C.

The staining time for miR-199a at stage 200 DD was 22 hours and for 177 DD and 238 DD 12.5 hours. The staining time for lum at stage 200 DD was 2.5 hours and for ets2 it was 4 hours. Photographs of the embryos were taken using Leica Application Suite (LAS), Leica automated microscope and digital camera. The photographs were taken at different magnification for each embryo: 2.0x, 3.2x, 4.0x and 5.0x (6.0x).

Table 2 – Proteinase K. *Temperature, digestion time and concentration of proteinase K diluted in PBT.*

Time point	Time (min)	Concentration (µL/mL)	T (°C)
178 DD	22	2	37
200 DD	30	2	37
238 DD	40	3	37

3 Results

Chen et al. (2004) suggested that the expression of miR-199a is highest in the early time points of development and as cartilage and bones accumulate the expression lowers. In this study, the expression of miR-199a was examined by performing WISH for LB and PL at three developmental time points, 177 DD, 200 DD, and 238 DD representing key stages of the craniofacial cartilage formation.

3.1 miR-199a has a craniofacial expression in the developing Arctic charr

Before the expression pattern of miR-199a is analysed, it is necessary to locate the main cartilage craniofacial elements in Arctic charr. Figure 2 (left panel) shows a ventral view of an Arctic charr head at stage 200 DD, where the cartilage has been stained with alcian blue. All craniofacial elements, such as the Meckel cartilage or lower jaw, the hyoid arch and the five gill arches, have been formed by this stage. The right panel of Figure 2 shows a WISH of mir-199a at the same developmental stage. The WISH staining has a craniofacial expression pattern including the upper and lower jaw, the hyoid and the gill arches. miR-199a expression is also observed in the ethmoid plate and the nasal cavity of the fish.

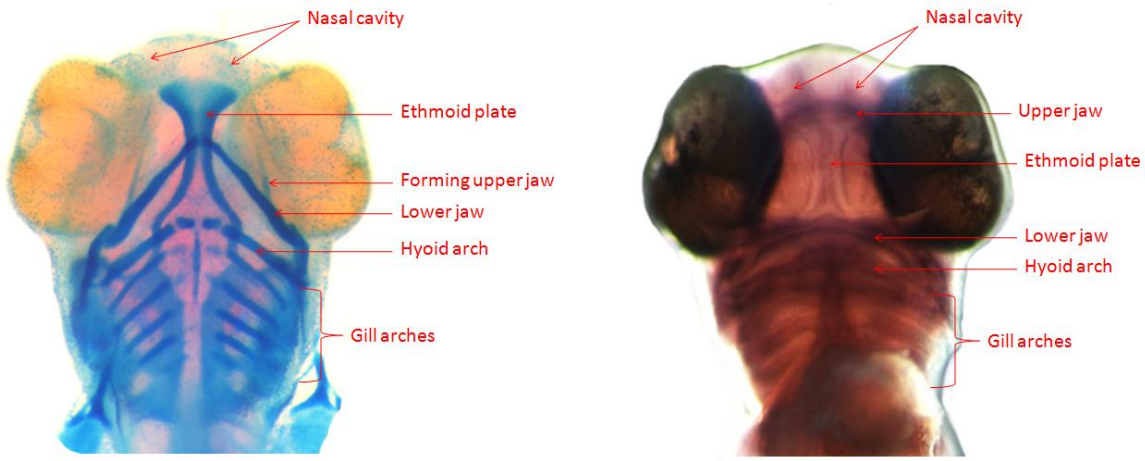

Figure 2 - Comparison of morphological features and staining by WISH. *The embryos are at stage 200 DD. The left picture exhibits the main cartilage/bone forming features. The two photos were taken under a Leica dissecting microscope at 2.0x magnification.*

3.2 Heterochrony of miR-199a expression in two morphs at three developmental stages

The next step was to compare the expression of miR-199a in LB and PL at three developmental time points. At stage 177 DD the staining of the six LB embryos was very strong all over the cartilage although it was most apparent in the outer surface of the cartilage (Figure 3), possibly in the perichondrium (a layer of dense irregular connective tissue that surrounds the cartilage of developing bone). The ethmoid plate was not very strongly stained. The staining was consistent between individuals. The six PL embryos at stage 177 DD were not as strongly stained as LB. The staining was consistent between embryos and most apparent in the lower jaw, hyoid arch and the gill arches. While the staining of the upper jaw appeared similar in intensity to the one seen in LB, the staining in the ethmoid plate was much weaker in PL embryos.

At stage 200 DD the five LB embryos showed consistent staining in the cartilage of the upper jaw, the ethmoid plate and the nasal cavity but only in the outer surface of the cartilage of the lower jaw, the hyoid arch and the gill arches (Figure 3). The staining varied between the six PL embryos at stage 200 DD. All of them had a strong staining in the upper and lower jaw, the nasal cavity and the hyoid arch but the staining was much weaker in the gill arches. Only two out of the six embryos were stained in the ethmoid plate. The staining of both LB and PL at stage 200 DD was weaker than at 177 DD.

For both LB and PL, the staining at stage 238 DD was much weaker than the previous stages (Figure 3). The six LB embryos at stage 238 DD had a consistent staining in the upper jaw, the perichondrium of the hyiod arch and a very weak staining in the gill arches. The ethmoid plate was not stained in any of the embryos. The staining of the six PL embryos at stage 238 was also consistent but it was different from LB. It was much stronger and there was a distinct staining in the hyoid arc and the gill arches. As in LB, there was no staining in the ethmoid plate.

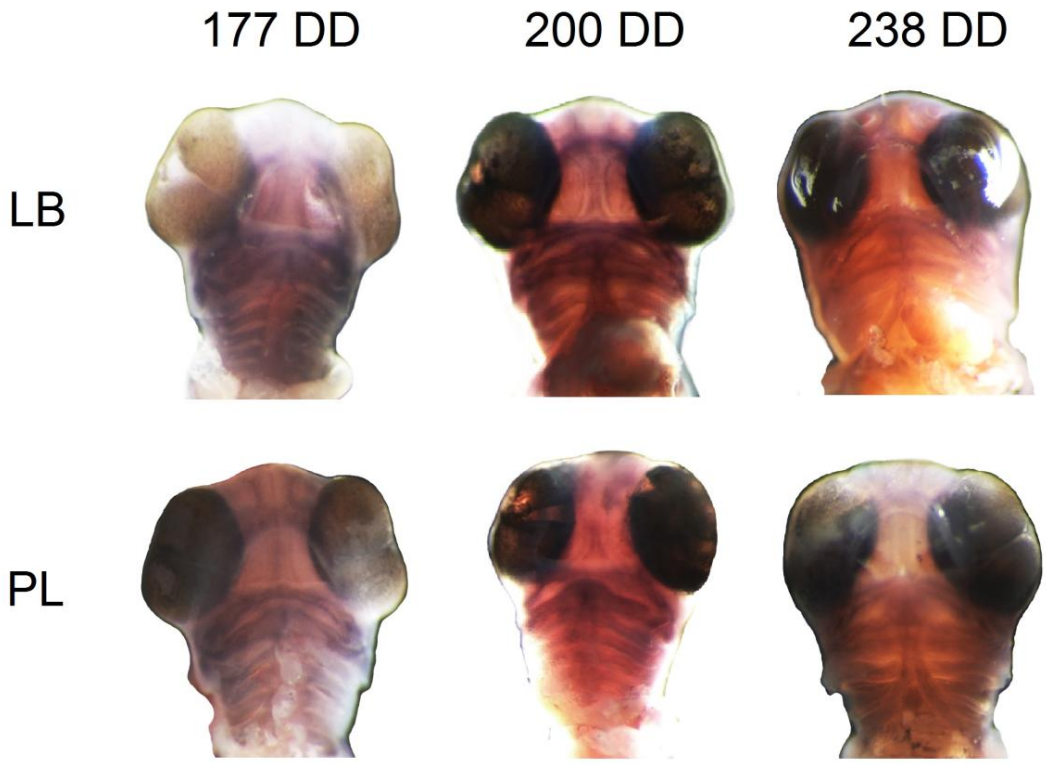

Figure 3 – Heterochronous expression of miR-199a. *WISH stained embryos for miR-199a at three developmental time points, 177 DD, 200 DD and 238 DD for LB and PL. All photos were taken under a Leica dissecting microscope at 3.2x magnification.*

3.3 Overlapping expression of target genes

The targets of miR-199a in Arctic charr were identified using two different approaches; conservation approach and candidate gene approach. According to the conservation approach the most likely targets are ets2 and casp3b. casp3b has 102 putative binding sites for miR-199 in zebrafish and was therefore considered a strong candidate target in Arctic charr. Although ets2 has only one binding site, the fact that it is a transcription factor made it a strong candidate for further studies (Chan et al. 2012). Unfortunately the casp3b probe synthesis was not successful as no PCR product was obtained. Therefore, the expression of casp3b was studied further. The candidate gene approach using miRanda and a list of interesting Arctic charr candidate genes from Ahi et al. *in prep*, yielded three candidate genes containing one or more binding sites for miR-199. Three putative target genes for miR-199a were generated, ets2, angptl4 and lum. Interestingly ets2 was identified as a putative target by both the conservation and the candidate gene approach. Unfortunately the probe synthesis was unsuccessful for angptl4 as no insert was incorporated in the plasmid during the cloning step. Therefore WISH was

performed for LB and PL at stage 200 DD only for lum and ets2. Of the 3 embryos for each morph, one was used as a negative control with sense probe. The two LB embryos stained for lum showed consistent staining in the front of the upper jaw, the ethmoid plate, the lower jaw, the hyoid arch and gill arches. The two PL embryos stained for lum had a different staining between individuals where one had very weak staining except in the front of the upper jaw but the other one stained strongly in the front of the upper jaw, the ethmoid plate, the lower jaw, the hyoid arch and the gill arches. The two LB embryos stained for ets2 were so strongly stained that it was impossible to distinguish between cartilage and other tissues (Figure 4).The two PL embryos showed weaker staining, but little can be interpreted from the photo, although a weak pattern of staining can be seen on the surface of the lower jaw, the hyoid arch and the gill arches. No staining can be seen in the ethmoid plate. By comparing the two morphs stained for lum to the two morphs stained for miR-199a a similar staining pattern can be discerned. Another WISH for ets2 was performed for AC (photos not shown) which had a similar pattern of staining as for ets2 in PL.

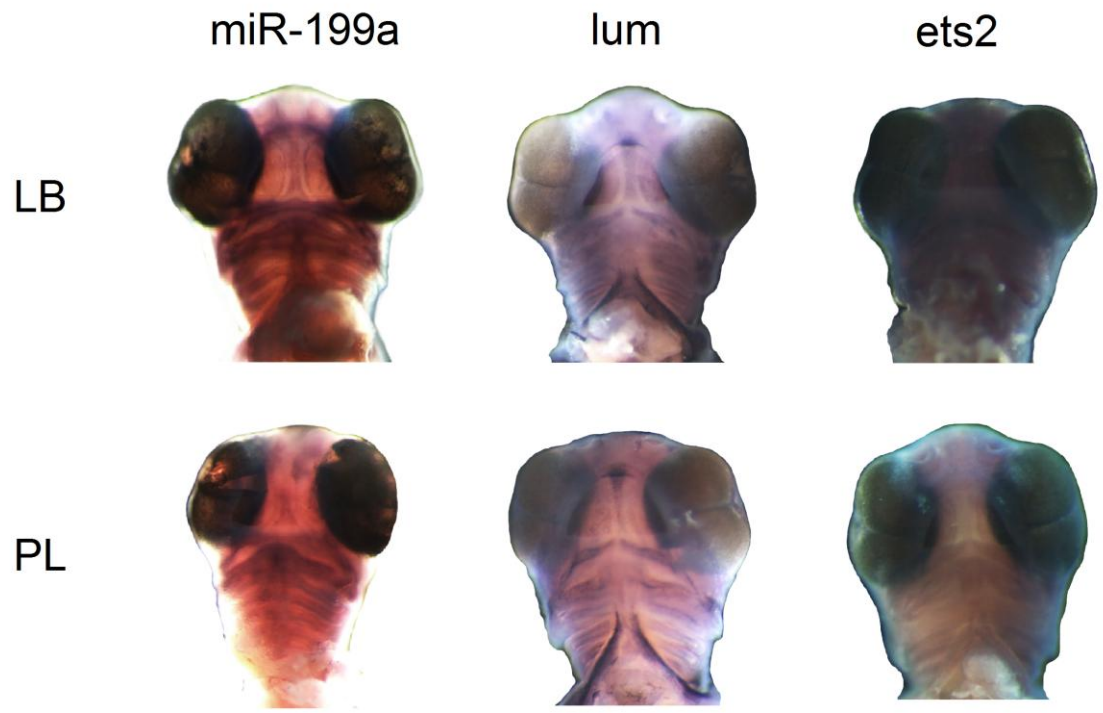

Figure 4 – Overlapping expression of miR-199a, lum and ets2. *WISH stained embryos for miR-199a and its putative targets, lum and ets2 for LB and PL at stage 200 DD. For each target, the embryos of both morphs were stained simultaneously in the same tube. All photos were taken under a Leica dissecting microscope at 3.2x magnification.*

3.4 Expression of miR-199a: miQPCR

miQPCR was performed for miR-199a and miR-206 on cDNA generated from head and whole embryo samples from two charr groups, AC and SB at three developmental time points, 150 DD, 161 DD and 200 DD. The difference in the expression of the two miRNA in each stage for each morph was analysed. Also, the expression of the two miRNA were compared independently for AC and SB at each time point and compared between time points for each morph. The cDNA synthesis of head RNA was not successful for the two stages that were tested (177 DD and 200 DD) and miQPCR for these samples was therefore not successful. A miQPCR for whole embryo was performed for AC and SB at the three developmental time points. To normalize the data, two or more reference genes had to be established. In this study, four reference genes which showed stable expression levels between morphs and developmental time points in Arctic charr (Kapralova et al. *under revision*) were tested. Moreover all these genes are expressed in the head of zebrafish (Wienholds et al. 2005). miQPCR was only successful for miR-21 and miR-24 so they were used to normalize the data for miR-199a and miR-206. The geometric mean of the expression of the two reference genes was used for the normalization. The raw C_T values were transformed to relative expression ratios (relative quantity). The relative quantity of the expression of the two miRNA are illustrated in Figure 5 and 6.

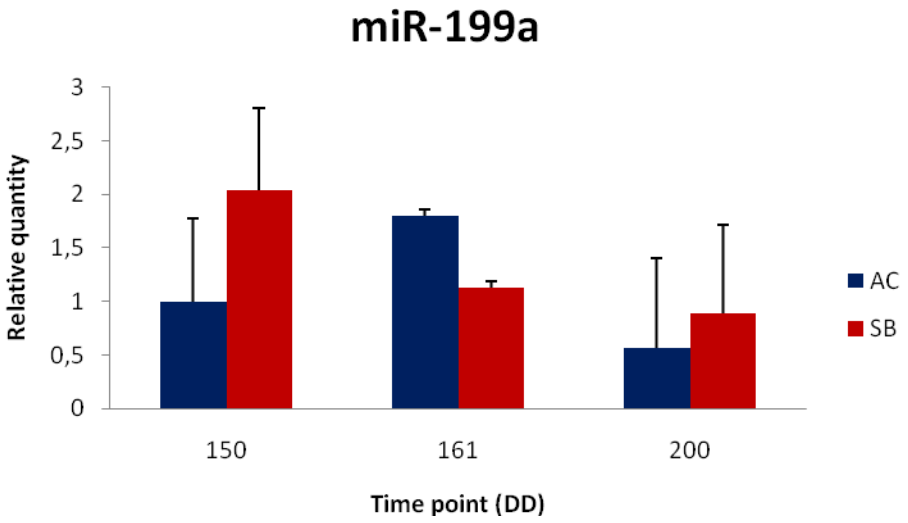

Figure 5 – The relative quantity of miR-199a expression. *The miR-199a expression ratios for AC and SB at 150 DD, 161 DD and 200 DD are illustrated. AC at 150 DD was set as reference with relative quantity equal to 1. The relative quantity for SB is almost twice that for AC at 150 DD but then it decreases as time progresses. The relative quantity for AC increases from stage 150 DD to 161 DD but then decreases roughly twofold at 200 DD. Error bars represent the standard deviation from the mean.*

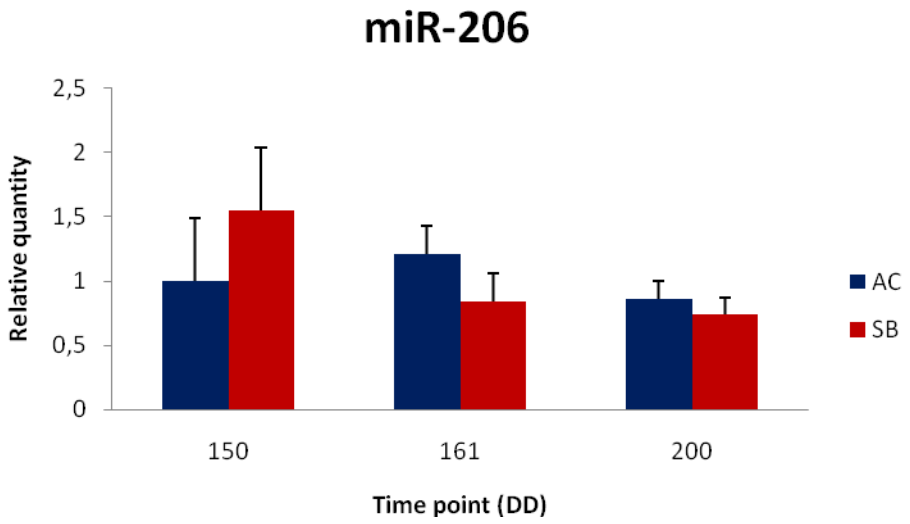

Figure 6 – The relative quantity of miR-206 expression. *The expression ratios of miR-206 for AC and SB at 150 DD, 161 DD and 200 DD are illustrated. AC at 150 DD was set as reference with relative quantity equal to 1.The relative quantity of SB is 0.6x higher than for AC at 150 DD but it decreases as the time progresses like in Figure 5. For AC, the relative quantity increases a bit at 161 DD but decreases at 200 DD like in Figure 5. Error bars represent the standard deviation from the mean.*

The miRNA expression data was further analysed with a Student‘s t-test. Three null hypothesis were tested:

1. The expression between miR-199a and miR-206 does not differ in AC and SB at 150 DD, 161 DD and 200 DD.

2. For each miRNA the expression does not differ between AC and SB at 150 DD, 161 DD and 200 DD.

3. For each miRNA the expression does not differ in AC and SB between pairs of time points.

The tests were carried out for all time-point pairs 150 DD, 161 DD and 200 DD.

H_0: $\mu_1 - \mu_2 = 0$
H_1: $\mu_1 - \mu_2 \neq 0$

The p-values were not significant for any of the tests described above, so the null hypothesis could not be rejected.

4 Discussion

This study yield four interesting result points and each one of them is discussed in a separate sub-chapter bellow.

4.1 miR-199a shows craniofacial expression in the developing Arctic charr

In developing Arctic charr embryos miR-199a is expressed in a tissue-specific manner, mainly in the craniofacial elements such as the Meckel cartilage, the hyoid arch and the gill arches. Expression was also observed in the ethmoid plate and the nasal cavity. miR-199a is highly expressed in the developing skeletal system of a wide range of organisms (Lin et al. 2009). It is expressed in the head, the limbs, and the body mesoderm of chicken (Ason et al. 2006). In zebrafish miR-199a is expressed in the epithelia surrounding the cartilage of the pharyngeal arches, the oral cavity, the pectoral fins and the epidermis of the head (Wienholds et al. 2005). A similar expression pattern in the epithelia surrounding the pharyngeal arches is observed in medaka (Ason et al. 2006). Although the expression of miR-199a is similar in evolutionarily distant vertebrates such as chicken, zebrafish and medaka its timing of expression in specific tissues varies (Ason et al. 2006). The similarity in expression between the two fish species suggests that miR-199a expression is very conserved as zebrafish and medaka diverged ~150–200 million years ago (Furutani-Seiki & Wittbrodt 2004).

In the Arctic charr, miR-199a is expressed in the whole craniofacial structure; the upper and lower jaw, the hyoid and gill arches, the ethmoid plate and the nasal cavity. The expression appears to be in the perichondrium and the epithelia surrounding the cartilage but further studies are needed for more precise tissue and cell localization. The expression of miR-199 is likely to be important in early chondrogenesis as it is expressed in the early development and its expression decreasing from the earliest time point (177 DD) to the latest time point (238 DD) as the cartilage and bones accumulate.

4.2 Heterochrony of miR-199a expression in two morphs at three developmental stages

miR-199a expression studied by WISH showed similar spatial pattern but different intensity between LB and PL at three developmental time points. Overall miR-199a expression decreased with developmental time. Differences in expression were observed between the morphs: expression was higher in LB at 177 DD and 200 DD and higher in PL at 238 DD.

Although WISH is not a quantitative method and cannot detect subtle differences in gene expression, the intensity of miR-199a expression differed noticeably between LB and PL and between developmental stages, suggesting that the observed differences in the expression might be rather large. In some cases, morphs showed a uniform WISH staining whereas in other cases a difference in the intensity of the staining could be detected within morphs. This is probably due to the fact that development is a dynamic process and some

differences may occur between samples.

miR-199 is an evolutionarily old miRNA (Heimberg et al. 2008) and its sequence is well conserved in Arctic charr (Kapralova et al. *under revision*). The high degree of sequence conservation and the similarity in spatial expression seen across taxa suggests that the functions of miR-199a are also evolutionarily stable. Thus it can be speculated that small changes in the miRNA expression can lead to changes in the target mRNA expression and result in the fine tuning of phenotypes.

Ason et al. (2006) compared the expression of many miRNAs between medaka and zebrafish. These two species are separated in evolution by 150-200 million years. Medaka develops more slowly than zebrafish, although the timing for specific tissues varies (Furutani-Seiki & Wittbrodt 2004). Comparable expression of many miRNAs, is observed in later stages for medaka than zebrafish although the eyes, the body wall and the brain develop faster than the jaw, head and sensory epithelia in medaka relative to zebrafish (Ason et al. 2006). Five lateral line-specific miRNAs (miR-96, miR-182, miR-183, miR-200a, and miR-200b) stain the neuromasts along the body. The timing of observed miRNA expression within the lateral line, corresponds to the period of neuromast differentiation although the pro-neuromasts are present much earlier in medaka (Sapede et al. 2002). In zebrafish, the lateral line differentiation and miRNA expression are observed earlier. The location of the neuromast is different in zebrafish and medaka and since migration is coupled to differentiation for neuromast development (Sapede et al. 2002) and miRNA expression is involved in differentiation, the miRNA expression can possibly be associated with the location change of neuromasts along the body of each fish. Delayed expression has also been observed for many miRNAs that are expressed in the craniofacial cartilages and bones. Ason et al. (2006) showed that of nine miRNAs expressed in the pharyngeal arches (f.e. miR-27b, miR-140, miR-145 and miR-199a), seven showed delayed expression.

Although LB and PL have a much shorter evolutionary history and belong to the same species they exhibit different adult phenotypes of the feeding apparatus (Snorrason et al. 1994) and these differences have a genetic component (Skúlason et al. 1989). The observed differences in the timing of expression of miR-199 between LB and PL likely reflects changes in developmental events related to the forming craniofacial elements.

4.3 Overlapping expression of target genes

miR-199a and its putative targets showed similar patterns of expression in both morphs at stage 200 DD. This can be regarded as an indirect indication of co-localization of the miRNA and its target. These results are very interesting although further studies are needed to test for an interaction between the putative targets and miR-199a. This can be done by using for example a luciferase assay as in Fasanaro et al. (2008) where luciferase assays showed that Ephrin-A3 is a direct target of miR-210. When predicting a target of a miRNA a few factors need to be taken in account:

1. Not all predicted targets are genuine, as they may be subject to spatial or temporal restrictions (John et al. 2004). Here the spatial or temporal expression of miR-199a was tested and it was shown that miR-199a expresses at the same time and in a similar pattern as its putative targets.

2. Binding to the target site might be modulated by 3'UTR *cis*-acting sequences or transacting factors and multiple miRNA may target a single 3'UTR (Doench & Sharp

2004). Thus, the level of a mRNA or its product is governed by the combinatorial effect of its targeting miRNAs.

3. The accessibility of the target site should be considered (Kertesz et al. 2007). It was experimentally proven that target sites that are embedded in a closed stem structure do not allow effective inhibition by the targeting miRNA (Kertesz et al. 2007).

4. Nonconserved miRNAs-targeted sites respond equally well to inhibition by miRNAs (see Farh et al. 2005) but these nonconserved sites appear to be present in genes that are not expressed in the same tissue as the targeting miRNAs (Doench & Sharp 2004). On the other hand, mRNAs that preferentially coexist with miRNAs in a specific tissue are thought to have evolved through selective elimination of the targeting sites and are known as "antitargets" (see Farh et al. 2005).

In summary, this shows that the complexity of miRNA regulation is rather complex and it is likely that multiple of miRNAs and other regulatory networks are involved in the building of diverse phenotypes.

Both putative targets, ets2 and lum, have a binding site for miR-199a. ets2 is a conserved proto-oncogene and a transcription factor that has been shown to be highly expressed during development in cartilage, including the craniofacial elements (Maroulakou et al. 1994). Moreover the target site of ets2 for miR-199 is conserved between zebrafish and Arctic charr which might indicate that they are a part of an essential developmental network conserved deep in the evolution tree. lum belongs to a leucine-rich proteoglycan gene family and its expression has been shown to increase in osteoblasts as the matrix matures which suggests that lum might be associated with the regulation of collagen fribril formation in the bone matrix (Raouf & Seth 2002).

As stated by Farh et al. (2005) and Stark et al. (2005) miRNA tend to be anticorrelated with their target genes. Although the data here did not show such a straightforward relationship between miR-199a and ets2 and the expression of miR-199a and ets2 appeared to be in the same direction (i.e. high in LB and lower in PL). Some elements showed an anticorrelated pattern in expression. In the ethmoid plate the expression of miR-199a is quite apparent in PL while the expression of ets2 in this same element was not detectable, suggesting that miR-199a is regulating ets2 in the ethmoid plate. However these results have to be interpreted with caution as the observed pattern can be the result of transcriptional as well as post-transcriptional regulation. Further studies are needed to show the direct involvement of miR-199a in regulating ets2 and lum levels.

4.4 Expression of miR-199a: miQPCR

In this part a more quantitative method (miQPCR) was used to study the miR-199a expression between the heads of five Arctic charr morphs (AC, LB, SB, PI and PL) at two developmental stages, 177 DD and 200 DD.

For this part of the study a new qPCR protocol for miRNAs was used. To test whether the miQPCR protocol works, RNA extractions from a previous miRNA study (Kapralova et al. *under revision*) were used as a control. The miQPCR protocol worked well for the control samples (RNA extracted from whole embryo of AC and SB at 150, 161 and 200 DD), but was unsuccessful for the RNA extracted from heads. Because of the identical steps the two protocols follow after the RNA extraction it is likely that something went wrong with the RNA extraction step for the head samples. The only difference between the

RNA extractions from whole embryos and from heads is the step when the head is removed from the embryo. Thus, the most likely explanation for the unsuccessful miQPCR is RNA degradation which is triggered during this step. Since the samples extracted from heads did not work, the whole embryo samples were used for all subsequent analysis. Four reference genes which showed stable expression levels between morphs and developmental time points in Arctic charr (Kapralova et al. *under revision*) were tested but only two of them, miR-21 and miR-24 amplified successfully. These two genes were used to normalize the data. The results showed that the expression of miR-199a and miR-206 did not differ between AC and SB nor did it differ between developmental time points. These findings are not in accordance with a previous study by Kapralova et al. In review. In their study the authors found and confirmed the differential expression of these same miRNAs using two different methods, high throughput sequencing and qPCR with LNA™-enhanced PCR primers (Exiqon). Apart from using different qPCR methods the present study and the one by Kapralova et al., used different reference genes for data normalization (miR-21 and miR-24 vs U2) which might have also contributed to the conflicting results. It can be concluded that although the miQPCR method was overall successful, good reference genes need to be established before this method can be used to quantify miRNA expression in Arctic charr.

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