



Expression of miR-199a, *lum* and *ets2* in benthic and pelagic Arctic charr morphs at developmental stage 200τ

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Expression of miR-199a, *lum* and *ets2* in benthic and pelagic Arctic charr morphs at developmental stage 200τ.

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Abstract

MiRNAs play an important role in development by regulating gene expression. They are recruited to RISC complexes, which bind to target mRNAs. This interaction causes silencing of the target mRNAs by repressing translation or by initiating degradation. In lake Þingvallavatn there are four morphs of Arctic charr that are very different in morphology and their feeding apparatus. The different morphs of Arctic charr have different levels of miRNA gene expression during their embryonic development. MiR-199a is one of these miRNAs and it and its putative targets, *lum* and *ets2*, were investigated in the large benthic (LB) morph at developmental stage 200 τ . WISH staining was used followed by cryosectioning to gain insight in the cellular expression of miR-199a, *lum*, and *ets2*. Expression of miR-199a and *ets2* was found in the pharyngeal arches outside of the cartilage while the expression of *lum* was found inside of the cartilage. Thus, *ets2* is likely to be a target of miR-199a whereas *lum* is probably not. Expression patterns of miR-199a, *lum*, and *ets2* were also analysed in pelagic planctivorous (PL) charr at the same developmental stage (200 τ). Similar expression was found in PL as in LB. Furthermore, a computational approach was used to find putative targets for miR-206 resulting in 36 possible targets.

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Abbreviations

RNA	Ribonucleic acid
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
mRNA	Messenger RNA
miRNA	micro RNA
UTR	Untranslated region
RISC	RNA induced silencing complex
<i>lum</i>	lumican
<i>ets2</i>	v-ets avian erythroblastosis virus E26 oncogene homolog 2
WISH	Whole mount in situ hybridisation
PFA	Paraformaldehyde
PFAGA	Paraformaldehyde/glutaraldehyde
SSC	Saline-sodium citrate
PBT	PBS with Tween 20
PBS	Phosphate buffered saline
anti-DIG-AP	Anti-digoxigenin antibody linked to alkaline Phosphatase
NBT-BCIP phosphate	Nitro-blue tetrazolium chloride/5-bromo-4chloro-4-indolyl
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
OCT	Optimal cutting temperature compound (10.24% polyvinyl alcohol 4.26% polyethylene glycol 85.5% non-reactive ingredients)
PL	Pelagic planctivorous
PI	Piscivorous

SB	Small benthivorous
LB	Large benthivorous

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Introduction

1.1 Micro RNAs

Micro RNAs (miRNAs) are short, single stranded non-coding RNAs. They play an important role in development by regulating gene expression. The miRNA biogenesis in animals is shown in Figure 1. First DNA coding for miRNA is transcribed and the transcribed single stranded RNA then folds into a hairpin loop to create a primary miRNA (pri-miRNA) structure (Bartel D. P., 2004). The pri-miRNA is then cut by the enzyme Drosha to generate pre-miRNA of ~70 nucleotides long ((Carthew & Sontheimer, 2009) as per Kim 2005), which is then transported from the nucleus to the cytoplasm. There, the pre-miRNA is cut by Dicer to create a mature miRNA/miRNA* duplex of ~20 nucleotides (Carthew & Sontheimer, 2009 as per Kim 2005). These are then assembled into miRISC (Figure 1).

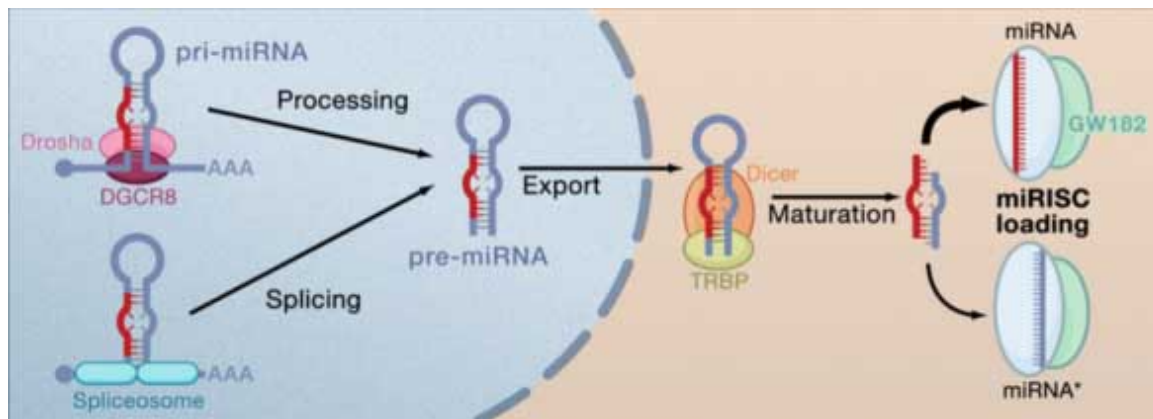


Figure 1 - Adapted from (Carthew & Sontheimer, 2009) **Biogenesis of miRNAs** Pri-miRNAs are processed by Drosha to make pre-miRNA, which is transported to the cytoplasm, then matured with the help of the Dicer enzyme to miRNA/miRNA* duplex and build in miRISC.

MiRISC regulates gene expression by binding to target mRNA. This interaction causes silencing of the target mRNA by repressing translation or by initiating degradation (Yi, Qin, Macara, & Cullen, 2003). Usually, the miRISC with the miRNA strand is more favoured than the complex with the miRNA* strand (Carthew & Sontheimer, 2009). Most miRNAs are partial base complementary with their target. Because of this partial base pairing, each miRNA can potentially bind to many targets (Pasquinelli, 2012). Targets can also have multiple binding sites for different miRNAs (Pasquinelli, 2012). Thus miRNA - target mRNA networks are usually complex.

For miRNAs and their targets to bind, a 7 nucleotide match in the miRNA 'seed' region is essential (Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). This 'seed' region is located at nucleotides 2-8 from the 5' end of the miRNA: Figure 2A. After the binding of miRNA to its target, different repression mechanisms take place; inhibition of translation, post-initiation expression and mRNA deadenylation and degradation: Figure 2B (Liu, Fortin, & Mourelatos, 2008).

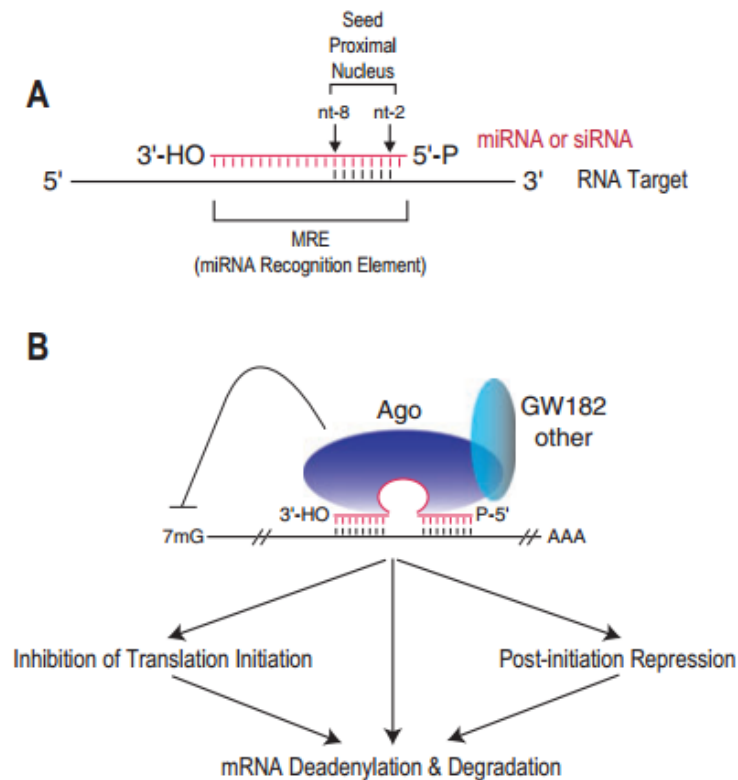


Figure 2 – MiRNA binding to target RNA. (Liu et al., 2008) *A) 7 nucleotide match in the seed region. B) Ago protein is present during binding after which different repression mechanisms take place.*

When using computational approaches to find miRNA targets, the most important part is the pairing of the mRNA targets to the miRNA ‘seed’ region. Other features that improve the target site efficiency are: positioning within the 3’UTR at least 15 nucleotides from the stop codon, positioning away from the centre of long UTRs, site accessibility such as an AU-rich nucleotide composition near the site, and proximity to sites for co-expressed miRNAs (Bartel D. P., 2009).

1.2 Arctic charr

In this project expression and potential targets of miRNA miR-199a were examined in LB and PL Arctic charr morphs from lake Þingvallavatn. In this lake, four different morphs of Arctic charr are present: planktivorous (PL), piscivorous (PI), large benthivorous (LB) and small benthivorous (SB) (S.S.Snorrrason, et al., 1994). They are very different in morphology (Figure 3). They live in different parts of the lake and thus feed on different things; PL on zooplankton, PI on threespine sticklebacks, and SB and LB on benthos and snails (S.S.Snorrrason, et al., 1994). This had led to different adaptations in their feeding apparatus: PL and PI have terminal mouths and evenly protruding jaws whereas LB and SB have sub terminal mouth and shorter lower jaw (Snorrason et al., 1989). These differences are thought to arise during early development. Moreover the different forms of Arctic charr have different expression levels of various genes involved craniofacial development (Ahi, et al., 2014). Because of all these differences in the morphs that are evolutionary so close to each other, the

morphs are good candidates to find candidate genes and miRNAs, which play a role in craniofacial development and evolution of phenotypic diversity.

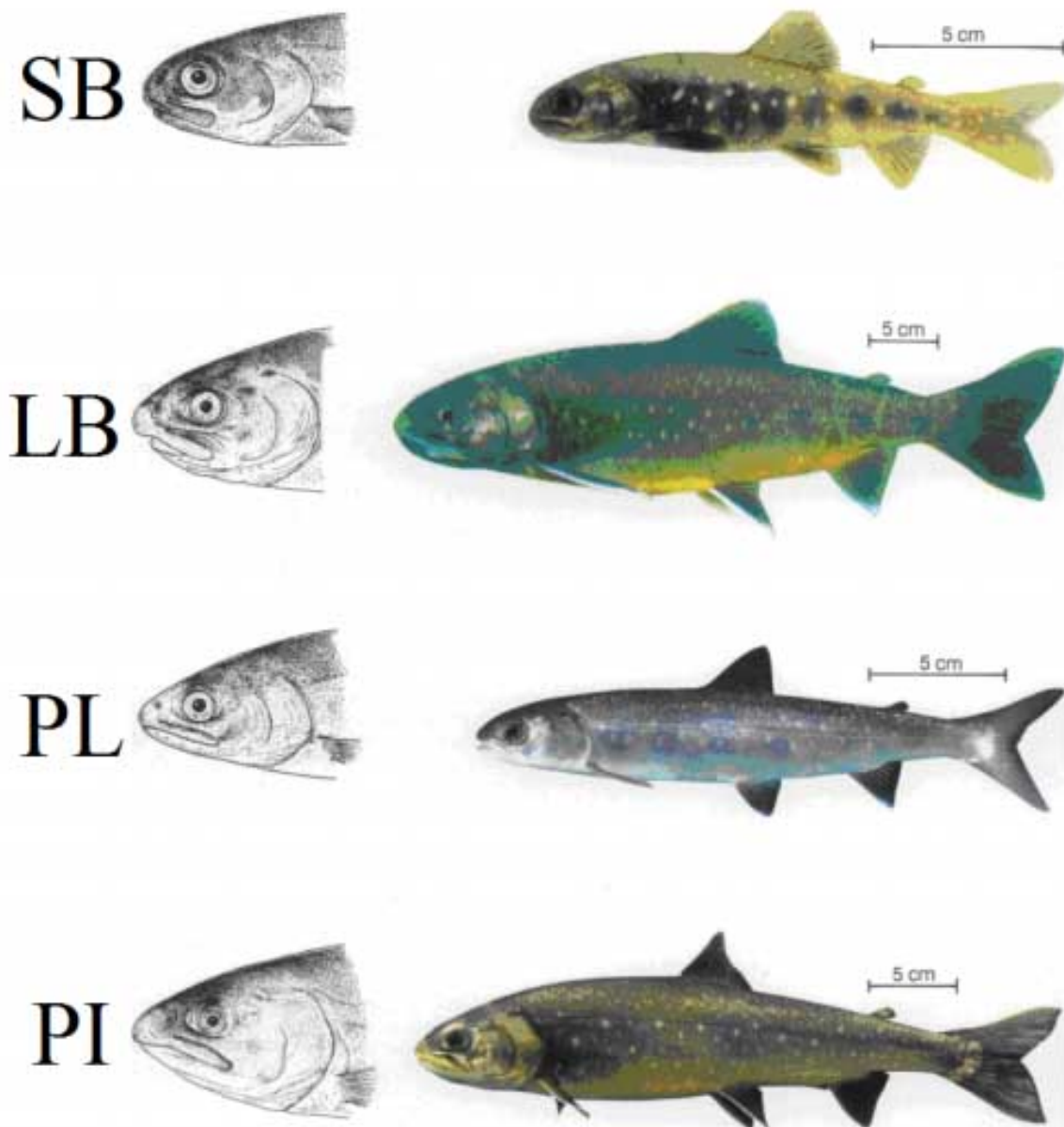


Figure 3 - From Sandlund et al. (1992), morphological and size difference of Arctic charr. SB is small benthivorous, LB is large benthivorous, PL is planktivorous and PI is piscivorous. Differences between morphs are shown, as can be seen in size as well as facial structure.

1.3 Research

In this project the expression of miR-199a and its putative targets were studied in Arctic charr embryos. This project is a continuation of the work of Kapralova et al. 2014 and Ástrós

Skúladóttir 2014. First Kapralova et al. 2014 found 53 conserved miRNAs to be differentially expressed during the development of two contrasting Arctic charr morphs a benthic and a limnetic. One of these miRNAs (miR-199a) was selected for further studies. In her BSc research project Ástrós Skúladóttirs studied the spatial expression pattern of miR-199a in Arctic charr embryos of different morphs at different developmental stages. Ástrós Skúladóttir used 2 computational approaches to search for potential targets for miR-199a. Two interesting candidate targets were selected and their spatial expression studied with WISH (Whole Mount In situ Hybridization). Expression patterns of *lum* and *ets2* were found to have a similar expression pattern to the one seen for miR-199a.

The present work aimed to study in further detail the expression patterns of miR-199a and its putative targets *lum* and *ets2*. To find out whether *lum* and *ets2* are targets for miR-199a, it first has to be shown that they are present in the same tissue. To this end whole mount in situ hybridisation (WISH) was performed in Arctic charr embryos at the same developmental stage (200 τ) for miR-199a and its putative targets *lum* and *ets2*. The WISH was followed by cryosectioning of the embryos to obtain a more detailed idea of their tissue specific expression patterns.

Materials and methods

First the expression pattern of miR-199a, *lum* and *ets2* was assessed with WISH, after which photos were taken of the whole mounts. Next, the whole mounts were subjected to cryosectioning and photographed. Figure 4 shows an overview of the process and a detailed description of all the steps is provided in the following sections.

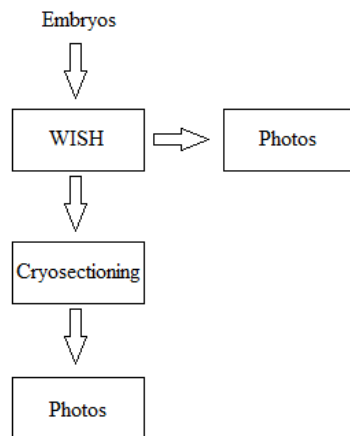


Figure 4 - Overview of the methods used in this project

6.1 Whole mount in situ hybridisation (WISH) protocol

6.1.1 Embryo pre-fixation

Embryos were fixed in 4% PFA for 30 minutes. Small punctures were made in the chorion and embryos were incubated overnight at 4°C in 4% PFA/PBT. After that, embryos were washed in PBT, followed by dehydration in 25% Methanol/PBT, 50% Methanol/PBT, 75% Methanol/PBT and 100% methanol, respectively. After this embryos were stored at -20°C. The embryo pre-fixation steps were performed by someone else.

6.1.2 Probe hybridisation

Embryos were rehydrated in 75% Methanol/PBT, 50%Methanol/PBT, 25%Methanol/PBT and pure PBT. Then for each embryo the chorion and yolk sack were removed. Embryos were incubated in Proteinase K for 30 minutes for a tissue and cell permeabilization. After washing with PBT, PFAGA was used to fix the embryos. Embryos were washed with PBT again and then incubated in pre-heated hybridization buffer for two hours (70°C for *ets2* and *lum* and 64°C for mir-199a and mir-206). Then embryos were incubated in pre-heated hybridization

buffer with the WISH probes overnight (70°C for *ets2* and *lum* and 64°C for mir-199a and mir-206).

6.1.3 Washing, blocking and antibody reaction

Embryos were washed with first 2xSSC, then 2xSSC/0,1% Tween and last 0.2xSSC/0,1% Tween to wash away unspecifically bound RNAs. After PBT washes, embryos were incubated in the blocking solution (4ml BSA (50mg/ml), 5ml Sheep serum, 1ml DMSO in 100ml PBT) for 2 hours to reduce non-specific binding of the anti-DIG-AP. Then, embryos were incubated overnight at 4°C in blocking solution containing 1:2000 anti-DIG-AP.

6.1.4 Colour reaction

The antibody was removed by washing the embryos with PBT. Then embryos were washed with a colouration buffer (10 ml 1M MgCl₂, 20ml 1M Tris pH 9.5, 20ml 1M NaCl, 200µl Levamisole, 20ml 10%Tween in 200ml). This was followed by incubation with colouration buffer containing 20µl NBT-BCIP per ml, in the dark at 4°C, until the staining was visible. Afterwards embryos were washed in PBT and fixed in 4%PFA/PBT. Finally embryos were stored in the dark in PBT.

6.2 Sections

6.2.1 OCT embedding and freezing

After WISH, embryos were incubated in a 15%, followed by a 30% sucrose/PBT solution. This prevents tissue damage resulting from ice crystal formation during freezing. Then, samples were transferred to OCT and incubated for an hour at room temperature. Subsequently the samples were transferred into moulds with fresh OCT and frozen at -80°C.

6.2.2 Sectioning

To section, a Leica CM3050S cryosectioning machine was used. Samples were “glued” on a slicing block by adding OCT in between them and fast freezing the whole together with a freezing spray. The slicing block was mounted on the machine and the foot pedal was used to move the whole up and down over the blade to cut fine sections. Machine settings were: one step on the pedal is one section. Sections were 12,0µm thick and the sectioning speed was set to 70. Slices were guided off the blade and straightened out with a brush. After that sections were retrieved by pressing a slide from Starfrost onto the block where the slice is laying. Samples were then air-dried overnight before taking photos.

6.3 Photos

6.3.1 Whole mounts

Photos of whole mount samples were taken using Leica application suite (LAS), Leica MZ10F microscope and Leica DFC310 FX camera. Samples were pinned down in a petri dish containing 50 ml of 1% agarose gel. A 50% glycerol solution was added to reduce light reflection. The photographs were taken at different magnifications; 2.0x, 3.2x, and 4.0x.

6.3.2 Sections

Oil was used on the Starfrost slides with sections and a cover glass was added on top of that. Photos of the sections were taken using Leica application suite (LAS) and Leica microscope. The photographs were taken at two different magnifications; 5x and 20x.

6.4 Computational approaches for target identification

6.4.1 Conservation approach using Target Scan and zebrafish

To find putative targets for miR-206, the website www.targetscan.org was used, with dre-mir-206 as a search question. The following criteria were applied to narrow down the search:

- 1) If they were representative for both miR-1 and miR-206 they were removed from the list.
- 2) If they were theoretical target genes (unknown genes) they were removed from the list.
- 3) If they did not have early development expression or muscle/bone expression they were removed from the list.
- 4) If there were a lot of target sites for a lot different miRNAs on the target gene, it was removed from the list.
- 5) Literature was used to find out if the targets were already known.

Results

7.1 Intro

7.1.1 WISH expression

To understand the WISH expression, it is necessary to know where the main craniofacial elements are located in Arctic charr. Figure 5A shows an Alcian blue cartilage staining of Arctic charr craniofacial elements at 200τ. Figure 5B shows the expression pattern of miR-199a, WISH staining of the same developmental time point. Staining in this embryo can be seen in the arches, upper- and lower jaw and in the nasal cavity.

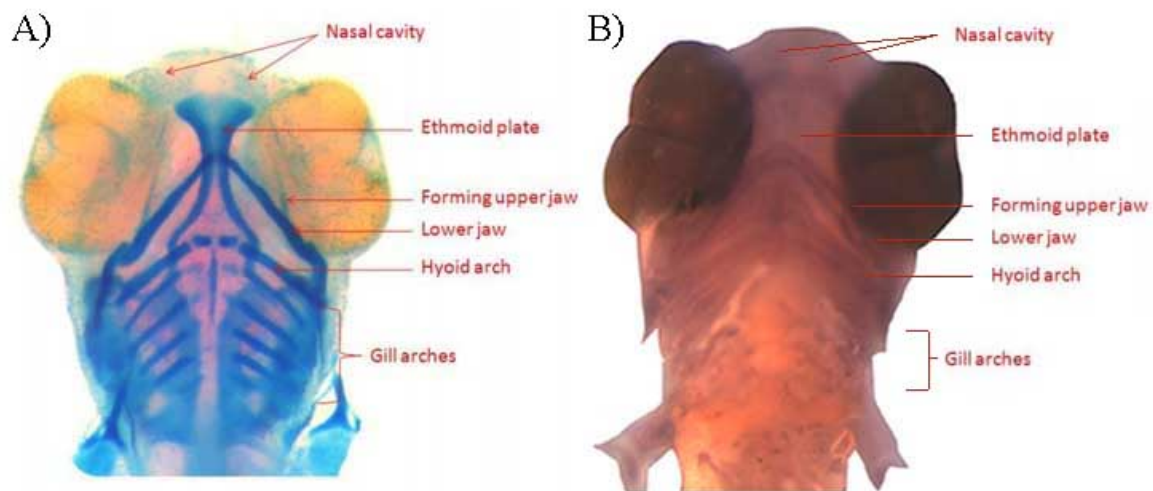


Figure 5 – Craniofacial elements in Arctic charr. On the left; morphological features adapted from Ástrós Skúladóttirs paper. On the right; LB200 embryo, WISH stained for miR -199a. The photos were taken at 4.0x magnification.

7.1.2 Expression patterns are the same in both projects

This project is a continuation of Ástrós Skúladóttir's BSc research project; she studied the expression pattern of miR-199a in the head of LB and PL embryos at different developmental stages. She also looked at the expression patterns of candidate targets for miR-199a, *lum* and *ets2*. She found similar expression patterns for miR-199a, *lum* and *ets2*. To compare the results from this project to what was observed by Ástrós Skúladóttir, photos from samples subjected to WISH for miR-199a, *ets2* and *lum* from both projects were compared.

The expression patterns in the corresponding embryos from Figure 6A and B are similar. In both Figure 6A and B, miR-199a shows staining in the nasal cavity, upper and lower jaw, hyoid and the gill arches. In both *lum* samples, staining can be seen in the nasal cavity, upper and lower jaw, hyoid arch and gill arches. For *ets2*, expression in Figure 6A is hard to distinguish, because the staining is overall very dark. In Figure 6B the staining in *ets2* is

visible in the nasal cavity, upper and lower jaw, hyoid arch and gill arches, which is the same pattern as miR-199a shows.

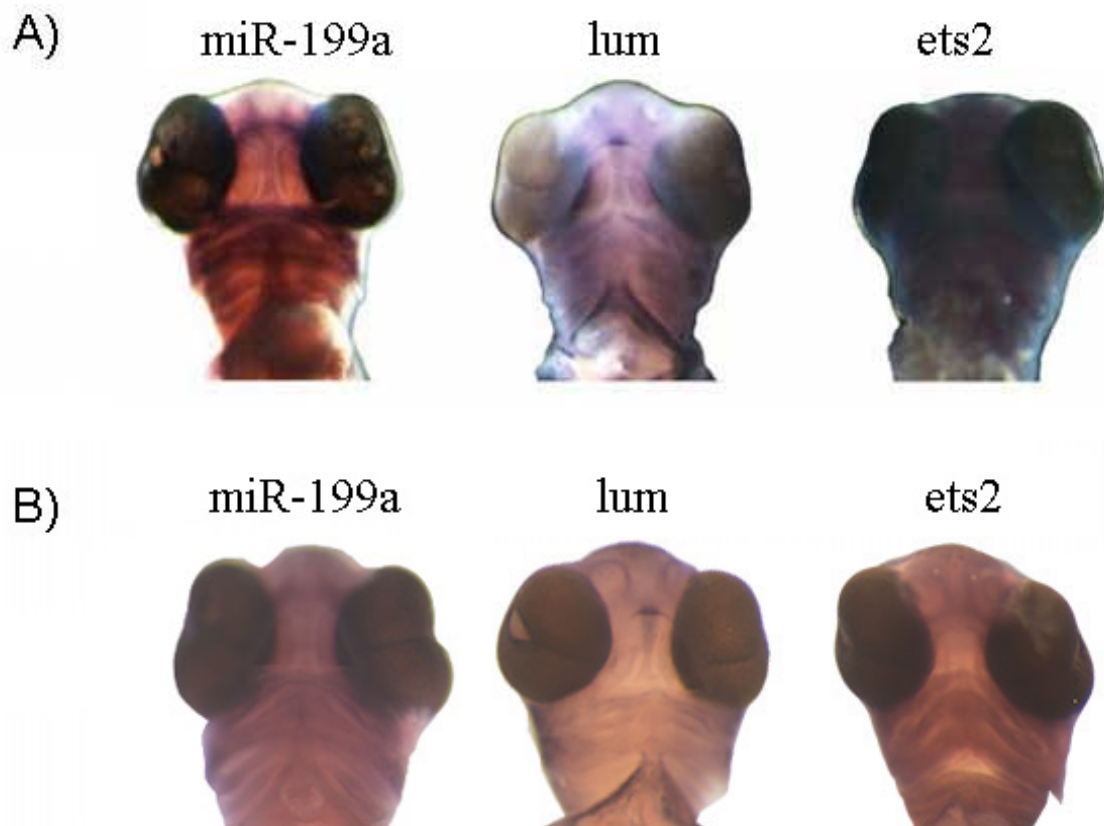


Figure 6 - Expression patterns of miR-199a, lum and ets2 for LB 200 embryos with WISH for miR-199a, lum and ets2. A) Adapted from Ástrós' report and B) The current project. Photos were taken with magnification 4.0x.

The expression patterns between the embryos from the two projects are very similar (reproducible). Now, the starting point is confirmed to be the similar to what was seen by Ástrós Skúladóttir. In the project presented here the tissue specific expression patterns of miR199a, lum and ets2 are studied by using WISH followed by cryosectioning.

7.2 Cryosectioning

7.2.1 miR-199a shows expression in the pharyngeal arches outside of the cartilage cells

In Figure 7A an embryo is shown with WISH staining for miR-199a. Staining can be seen, inter alia, in the arches in the whole mount (arrow). In the cryosectioned samples, staining can be seen in the arches as well (arrows point at the same feature) (Figure 7B and C). Figure 7B and C show that staining is present in the most outer cell layer of the pharyngeal arches, around the cartilage cells.

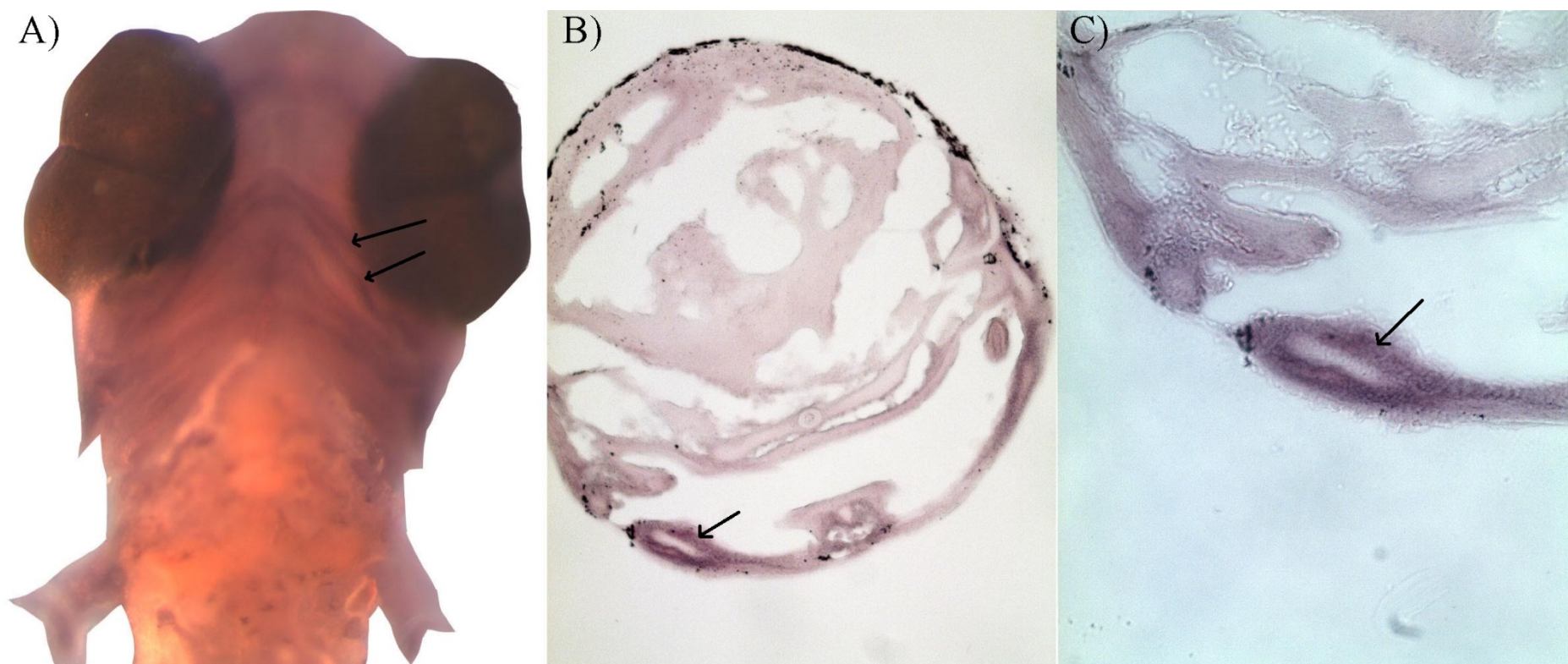


Figure 7 - WISH staining of miR-199a in LB 200. A) Whole mount, photo taken with magnification 4.0x. B) Transversal cryosection, photo is taken with 5x magnification. C) Transversal cryosection, magnification 20x.

7.2.2 *Lum* shows expression in pharyngeal arches inside the cartilage cells

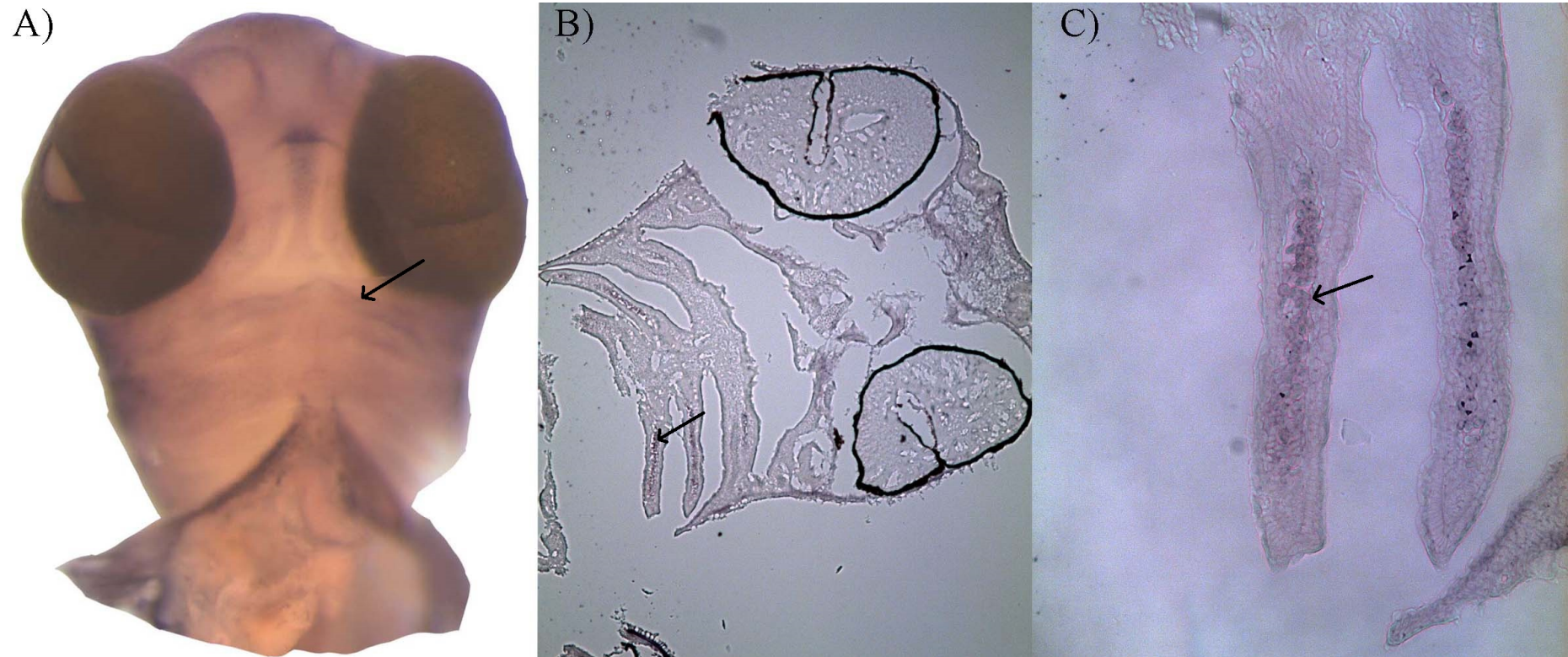


Figure 8 - WISH staining of *lum* in LB 200. A) Whole mount. Photo is taken with magnification 4.0x. B) Longitudinal cryosection, magnification 5x. C) Longitudinal cryosection, magnification 20x.

In Figure 8A staining can be seen, inter alia, in the arches in the whole mount of the LB200 embryo that is WISH stained for *lum* (arrow). In the cryosection staining can be seen in the arches as well (arrows point at the same feature) (Figure 8B). Figure 8B and more clearly C show that staining is present inside the cartilage cells.

7.2.3 *Ets2* shows expression in the pharyngeal arches outside of the cartilage cells

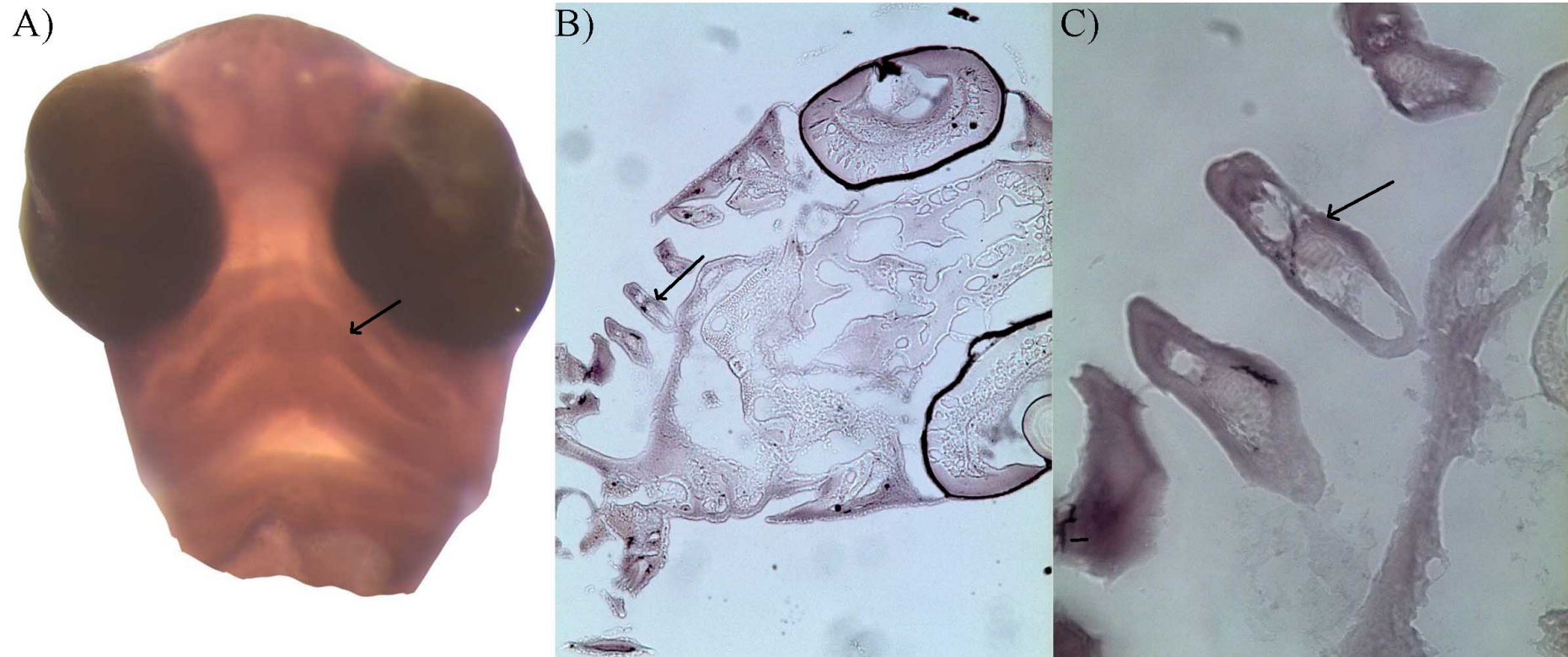


Figure 9 - WISH staining of *ets2* in LB 200. A) Whole mount at magnification 4x. B) Longitudinal cryosection, magnification 5x. C) Longitudinal cryosection magnification 20x.

In Figure 9A the whole mount WISH staining of *ets2* in LB 200 shows expression, inter alia, in the arches. In Figure 9B and C, staining can be observed within the pharyngeal arches, but not inside the cartilage cells. Arrows show the same features.

7.2.4 PL embryos show similar expression patterns as the ones seen in LB

PL embryos at 200 τ were also stained with WISH for *199a*, *ets2* and *lum* and their expression patterns were compared between the two morphs (PL and LB).

Figure 10A and B show that the expression in PL 200 for miR-199a is similar to the one seen for LB 200. Expression is seen in the pharyngeal arches outside of the cartilage cells. Figure 10C and D show the expression patterns of *lum* in PL and LB 200. Staining in PL 200 is very light and seems to be only in the most outer part of the embryo, so no definite conclusion can be drawn from these pictures. Figure 10E and F show that expression in PL 200 for *ets2* is the same as in LB 200. Expression is seen in the pharyngeal arches outside of the cartilage cells.



Figure 10 - WISH stained samples subjected to cryosectioning, 20x magnification. A) LB 200, staining for miR-199a, transversal cryosection. B) PL 200, staining for miR-199a, transversal cryosection. C) LB 200, staining for lum, longitudinal cryosection D) PL 200, staining for lum, longitudinal cryosection. E) LB 200, staining for ets2, longitudinal cryosection. F) PL 200, staining for ets2, longitudinal cryosection.

The cryosections from miR-199a and *ets2* look very similar in both morphs. Expression is found in the arches but not inside the cartilage for both WISH stainings. The samples for *lum*, show expression inside the cartilage for LB200. Staining was too weak to be seen in PL200 samples.

7.3 Troubleshooting cryosectioning

The cryosectioning of samples has not been done in our lab before, so some troubleshooting needed to be performed.

7.3.1 Orientation

Samples were often rotated either sideways or lifted by the body. This gave barely recognisable samples where only main features, such as one of the eyes could be recognized (Figure 11). The heads were cut off of the body before freezing. This helped to get rid of the lifting, but samples were still often rotated. To counter that, samples were put upside down in the moulds. This gave more space for trimming and rotating during sectioning. However the upside down samples were not successful. This is because although there is a lot of room to trim and rotate during sectioning, samples cannot be seen through the white OCT until they are actually reached. This gives approximately the same space to trim as one would have when the samples are the other way around, which is very little.

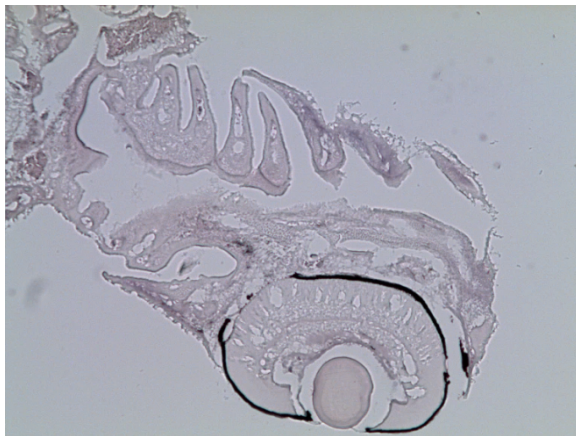


Figure 11 - LB200 WISH miR-199a 5x magnification, longitudinal section.

Because of the rotation issues, a different approach was taken. Samples were sectioned transversally instead of longitudinally. This worked very well, the sections are symmetrical, which means the orientation is perfectly straight (Figure 12).

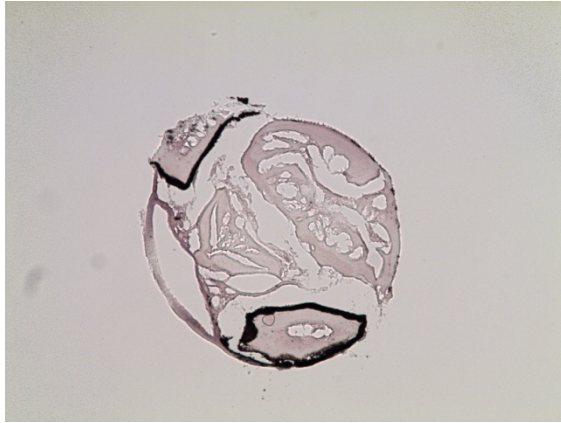


Figure 12- LB200 WISH miR-199a 5x magnification, transversal section through the eyes.

7.3.2 Tissue damage during cryosectioning

Some samples contained holes reflecting damaged tissue (Figure 13A). To counter this, the OCT incubation time had to be increased to give the OCT time to replace the sucrose and prevent tissue damage through crystallisation formed during freezing. This worked well and tissue looked less damaged in later samples. In Figure 13B muscle tissue is shown that is fully intact.

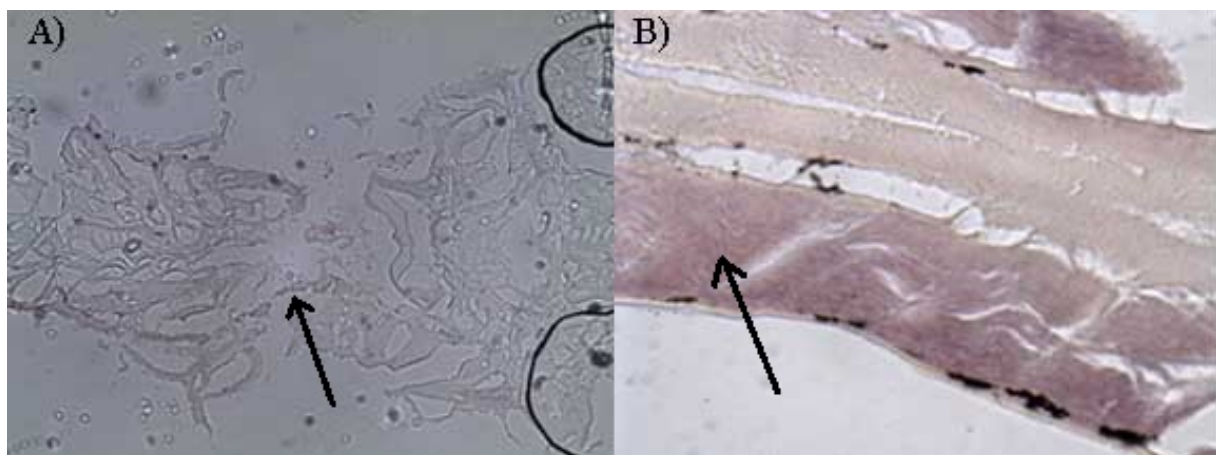


Figure 13 - LB200 WISH embryo cryosection. A) magnification 5x B) magnification 20x

7.4 Putative targets found for miR-206

When searching for dre-mir-206 was used as a “search string” in targetsan.org, 3720 genes were found. After the first step (removing representative miRNA miR-1) 558 genes were left. After the second step (removing unknown target genes) 499 genes were left. After the third step (removing genes with no early development expression) 451 genes were left. After the fourth step (removing everything multiple different miRNA sites on the target gene) 36 targets were left. Targets with a high number of total sites, but also with multiple miR-206 sites were kept in the list. See table 1.

Target gene	Representative 3' UTR	3' UTR expression profile	total sites	miR-206 specific sites
cnn3a	ENSDARG00000008359.1	24h,72h,Adult,Brain,Ovary,Testis	15	5
PFAS	ENSDARG00000003750.1	24h,72h,Adult,Brain,Testis	21	3
tagln2	ENSDARG000000033466.1	24h,72h,Adult,Brain,Ovary,Testis	14	3
ywhaba	ENSDARG000000013078.1	24h,72h,Adult,Brain,Ovary,Testis	14	3
ATXN3L	ENSDARG000000009264.1	24h,72h,Adult,Brain,Ovary,Testis	9	1
numbl	ENSDARG000000024043.1	24h,72h,Adult,Brain,Ovary,Testis	8	1
WDR1	ENSDARG000000077991.1	24h,72h,Adult,Brain,Ovary,Testis	8	1
arfrp1	ENSDARG000000057857.1	24h,72h,Adult,Brain,Ovary,Testis	8	1
ncapg2	ENSDARG000000060023.1	24h,72h,Adult,Brain,Ovary,Testis	8	1
cd63	ENSDARG000000025147.1	24h,72h,Adult,Brain,Ovary,Testis	7	1
nf2a	ENSDARG000000020204.1	24h,72h,Adult,Brain,Ovary,Testis	7	1
dusp22b	ENSDARG000000039850.1	24h,72h,Adult,Brain,Ovary,Testis	7	1
rlbp1b	ENSDARG000000045808.1	24h,72h,Adult,Brain,Ovary,Testis	7	1
slc7a6os	ENSDARG000000010596.1	72h,Brain,Testis	7	1
cad	ENSDARG000000041895.1	24h,72h,Ovary,Testis	6	1
ddost	ENSDARG000000037318.1	24h,72h	6	1
aga	ENSDARG000000004889.1	24h,72h,Adult,Brain,Ovary,Testis	6	1
vps45	ENSDARG000000061180.1	72h,Brain,Testis	6	1
nudcd1	ENSDARG000000036158.1	24h,72h,Adult,Brain,Ovary,Testis	6	1
cacybp	ENSDARG000000014731.1	24h,72h,Adult,Brain,Ovary,Testis	6	1
ttc35	ENSDARG000000003308.1	24h,72h,Adult,Brain,Ovary,Testis	6	1
ARFGAP1	ENSDARG000000067809.1	24h,72h,Adult,Brain,Ovary,Testis	6	1
ccnk	ENSDARG000000034146.1	24h,72h,Adult,Brain,Ovary,Testis	5	1
grna	ENSDARG000000004954.1	24h,72h,Adult,Brain,Ovary,Testis	5	1

tjp3	ENSDARG00000002909.1	24h	5	1
ARL8A	ENSDARG00000078686.1	72h,Adult,Brain,Testis	5	1
bhmt	ENSDARG00000013430.1	24h,72h,Adult,Brain,Ovary,Testis	5	1
ppapdc1a	ENSDARG00000067798.1	72h,Ovary,Testis	4	1
fam92a1	ENSDARG00000004436.1	24h,72h,Adult,Brain,Ovary,Testis	4	1
umps	ENSDARG00000012215.1	24h,72h,Adult,Brain,Ovary,Testis	4	1
timmm9	ENSDARG00000043865.1	24h,72h,Adult,Brain,Ovary,Testis	3	1
safb	ENSDARG00000020467.1	24h,72h,Adult,Brain,Ovary,Testis	2	1
atp5ia	ENSDARG00000078113.1	24h,72h,Adult,Brain,Testis	2	1
prmt3	ENSDARG00000019791.1	72h,Adult,Ovary,Testis	2	1
EIF3C	ENSDARG00000016443.1	24h,72h,Adult,Brain,Ovary,Testis	2	1
CERKL	ENSDARG00000079397.1	72h,Brain,Ovary,Testis	1	1

Table 1 – putative targets for miR-206

Discussion

8.1 Staining

In the PL samples that are WISH stained for *lum* the staining in the whole mounts seems a bit superficial. When looking at the sections from these samples it becomes clear that staining is indeed mostly present in the outer layer of the sample. This could mean that the samples have not been stained for long enough, or the probe just doesn't penetrate deep enough. This gives results that are not very reliable, because the difference between the staining and the background is so small. For further studies it would be interesting to look into this, because better and deeper staining would give clearer results and make them more reliable. One way to improve the signal to noise ratio could be to use a whole mount EDC fixation (Lagendijk, Moulton, & Bakkers, 2012). This will improve the ratio by crosslinking the 5' end of miRNA with amino groups in the protein matrix (Pena, Sohn-Lee, & Rouhanifard, 2009).

8.2 WISH followed by cryosectioning

WISH followed by cryosectioning shows promising results. Expression was visible in slices up to cellular detail. Another way to study tissue expression is to do cryosectioning of the 4% PFA fixed embryos followed by in situ hybridisation of the sections. This will most likely improve the staining/background problem and increase the visibility of the staining. The downside is that large amounts of probe are needed for this approach and miRNA LNA probes for insitu hybridisation are very expensive.

8.3 WISH followed by cryosectioning is a good step towards miRNA target validation

The method presented here, WISH followed by cryosectioning, can be used to study whether a miRNA and its putative targets are located in the same tissue at the same time in development. The co-localisation of a given miRNA and its computationally predicted mRNA targets in the same tissue is an indication of the interaction between the two.

8.3.1 MiR-199a and *ets2* show similar spatial expression

As can be seen in Figure 7, miR-199a shows expression in the pharyngeal arches in cells outside the cartilage. Similarly *ets2* is expressed in the pharyngeal arches in cells outside the cartilage (Figure 9), showing that *ets2* and miR-199a have similar spatial expression patterns and are present in the same cell types. Given the fact that *ets2* has a target site for miR-199a in its 3'UTR (Skúladóttir, 2014) and the miRNA and its putative target are present in the same place at the same time, it is likely that *ets2* is a target for miR-199a. Further analysis is needed to confirm that *ets2* is actually a target for miR-199a. For example a direct interaction between the 3'UTR of *ets2* and miR-199a can be shown by using a luciferase assay, where luciferase would be fused to the *ets2* 3' UTR. Then it can be shown that miR-199 can knock down the expression.

8.3.2 *Lum* shows expression in pharyngeal arches inside the cartilage cells

As can be seen in Figure 8 *lum* shows expression in pharyngeal arches inside the cartilage cells showing that *lum* is not expressed in the same cell types as miR-199a. These results show that miR-199a and its putative target *lum* are not present in the same space at the same time and even though the 3'UTR contains a target site for miR-199a (Skúladóttir, 2014) it is unlikely that *lum* is targeted by miR-199a in LB at the studied developmental time-point.

8.3.3 PL and LB embryos show similar expression patterns

As can be seen in Figure 10, expression for miR-199a and *ets2* in PL and LB embryos at 200 τ is similar, indicating that the differences detected between morphs are not due to different expression patterns and differences between morphs most likely stem from differences in the level of expression of miR-199a and *ets2* (Ahi et al., 2013; Kapralova, et al. 2014). This project shows the importance of studying the expression pattern of a miRNA and its putative targets in detail. PL and LB showed similar expression in whole mounts when WISH-stained for *lum*, miR-199a and *ets2* in Ástrós Skúladóttir's project. However when WISH samples were subjected to cryosectioning it became clear that only *ets2* and miR-199a have similar spatial expression patterns, whereas *lum* appears to be expressed in other tissues.

8.4 Computational target search for miR-206

The putative targets for miR-206 described in in Table 1 have not been verified. Before using them for further research, known expression patterns of the candidates in model species such as zebrafish should be investigated, by database searches. Then candidates with overlapping expression patterns to the one seen for miR-206 will be selected and further studied using the methods described in this project.

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