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RESEARCH ARTICLE

microRNA-29b knocks down collagen type I production in cultured rainbow trout (*Oncorhynchus mykiss*) cardiac fibroblasts

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ABSTRACT

Warm acclimation of rainbow trout can cause a decrease in the collagen content of the heart. This ability to remove cardiac collagen is particularly interesting considering that collagen deposition in the mammalian heart, following an injury, is permanent. We hypothesized that collagen removal can be facilitated by microRNA-29b (miR-29b), a highly conserved, small, non-coding RNA, as a reduction in this microRNA has been reported during the development of fibrosis in the mammalian heart. We also used a bioinformatics approach to investigate the binding potential of miR-29b to the seed sequences of vertebrate collagen isoforms. Cultured trout cardiac fibroblasts were transfected with zebrafish mature miR-29b mimic for 7 days with re-transfection occurring after 3 days. Transfection induced a 17.8-fold increase in miR-29b transcript abundance (P<0.05) as well as a 54% decrease in the transcript levels of the *col1a3* collagen isoform, compared with non-transfected controls (P<0.05). Western blotting demonstrated that the level of collagen type I protein was 85% lower in cells transfected with miR-29b than in control cells (P<0.05). Finally, bioinformatic analysis suggested that the predicted 3′-UTR of rainbow trout *col1a3* has a comparatively higher binding affinity for miR-29b than the 3′-UTR of *col1a1*. Together, these results suggest that miR-29b is a highly conserved regulator of collagen type I protein in vertebrates and that this microRNA decreases collagen in the trout heart by targeting *col1a3*.

KEY WORDS: Cardiac remodelling, Heart, Cultured fibroblasts, Extracellular matrix, MicroRNA, Transcriptional regulation

INTRODUCTION

Thermal acclimation of rainbow trout causes remodelling of the active (contractile) and passive (structural) components of the myocardium (Keen et al., 2017). These changes are thought to be responsible, as least in part, for the capacity of the trout heart to maintain function over a range of seasonal temperatures (Keen et al., 2017). Recent work has demonstrated that cold acclimation causes cardiac hypertrophy and an increase in cardiac connective tissue, while warm acclimation causes a decrease in the relative size of the heart and a reduction in cardiac connective tissue (Keen et al., 2016; Klaiman et al., 2011). The ability of trout to remove connective tissue from the heart in response to a physiological stressor is quite novel amongst vertebrates (Klaiman et al., 2011). In humans, for example, pathological collagen deposition within the heart is considered permanent, and contributes to declining cardiac function (Wynn, 2007).

Connective tissue in the vertebrate heart is primarily composed of collagen type I and is produced by cardiac fibroblasts. We have recently demonstrated that the synthesis of collagen type I is induced in rainbow trout cardiac fibroblasts in response to physiologically relevant levels of human recombinant TGF-β1 (Johnston and Gillis, 2017). A similar response is well documented in mammalian cardiac fibroblasts (Leask and Abraham, 2004; Verrecchia and Mauviel, 2007). Together, these results suggest that the major cellular pathways involved in the regulation of extracellular matrix (ECM) deposition in the heart are probably conserved amongst vertebrates, including fish. However, virtually nothing is known about the cellular processes involved in the removal of collagen from the fish heart.

Previous experiments, completed using mammalian models, indicate that microRNAs (miRs) play an important role in regulating the expression of collagen gene transcripts in the heart (van Rooij et al., 2008). miRs are a class of small (~22 nucleotide) non-coding RNAs that bind with the 3′-untranslated region (UTR) of mRNA to promote degradation of the mRNA or inhibit its binding to the ribosomes (Bartel, 2004; Griffiths-Jones et al., 2006). Such targeting would lead to a decrease in the expression of the gene transcript and, as a result, in the synthesis of the associated protein. In mammalian cardiac fibroblasts, one miR implicated in regulating the expression of collagen gene transcripts and consequently collagen protein is miR-29b (van Rooij et al., 2008). Abonnenc et al., (2013) demonstrated that miR-29b transfected into mouse cardiac fibroblasts targets gene transcripts involved in the fibrotic response including several collagen isoforms and matrix metalloproteinases (MMPs) (Abonnenc et al., 2013). In addition, van Rooij et al. (2008) found that the levels of miR-29b are significantly decreased at the site of an infarction in hearts from mice and humans, and reduced in response to TGF-β1 treatment. Furthermore, experiments involving fibroblasts from non-cardiac tissues have shown that the miR-29 family, including miR-29b, is required for the prevention of fibrosis (Maurer et al., 2010; Qin et al., 2011; Roderburg et al., 2011). Inhibition of miR-29b activity would contribute to an increase in collagen synthesis as there would be an increase in the copy number of collagen gene transcripts available for translation. It is not known whether miR-29b is able to regulate collagen in non-mammalian species; however, the sequence of miR-29b is highly conserved among vertebrate species, and this may reflect its importance as a regulator of collagen in fibroblasts. For example, the sequences of zebrafish and rainbow trout miR-29b are 100% identical to that of human miR-29b (alignment calculated). In mammalian cells, miR-29b binds to the mRNA of the COL1A1 isoform, and as a result inhibits collagen protein synthesis (Kriegel et al., 2012; Li et al., 2012; Mayer et al., 2017). In teleost fish, the *col1a3* isoform is more similar to the human COL1A1 isoform than to human COL1A2

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(Saito et al., 2001). This suggests that *col1a3* may be the target of miR-29b in the trout heart.

We hypothesized that miR-29b has the capacity to knock down collagen type I transcripts in rainbow trout cardiac fibroblasts. Using cultured trout cardiac fibroblasts, we tested the prediction that transfection of multiple trout cell lines with miR-29b would decrease collagen type I gene transcript and protein levels. To increase confidence that any effects that we detected experimentally were the result of the miR-29b transfection, we also characterized the binding potential of miR-29b to the *col1a1* and *col1a3* mRNA 3′-UTRs in trout using a predictive bioinformatics approach. Finally, we examined the transcript abundance of other important ECM-related genes including vimentin, matrix metalloproteinase-2 (MMP-2) and β′-actin, to determine whether miR-29b transfection affects the expression of these related genes.

MATERIALS AND METHODS

Sequence alignment

miR-29b sequences were obtained from miRBase (www.mirbase.org) with the exception of rainbow trout miR-29b, which was obtained from microTrout (http://www.mennigen-lab.com/microtrout.html: miR-29b-3p) and converted to RNA to maintain consistency with miRBase sequences. Sequences were aligned in ClustalOmega (www.ebi.ac.uk/Tools/msa/clustalo/) to determine the percentage conservation.

Cell culture

Cell lines were established from male rainbow trout ventricles as previously described (Johnston and Gillis, 2017) and used between passages 19 and 21. Cells were routinely kept at 21°C in Leibovitz's L-15 medium with 10% fetal bovine serum, 1% penicillin–streptomycin, 1% amphotericin B (hereafter referred to as whole medium) and split 1:2 during passaging.

Transfection with miR-29b

Cells were plated at ∼0.5×10^6 cells per well of 6-well plates and allowed to attach overnight. The next day, whole medium was replaced and cells were again allowed to grow overnight so that fibroblast confluency was ∼40% at the beginning of the experiment. Zebrafish miR-29b mature mimic (Life Technologies, Carlsbad, CA, USA) was diluted to a working concentration of 100 nmol l^{-1} and combined with Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions. Mature miR-29b mimic/lipofectamine complex was added to cells at a final concentration of 10 nmol l^{-1} in Opti-MEM (Life Technologies) in serum- and antibiotic-free L-15; 24 h after addition of miR-29b, transfection medium was replaced with whole medium. This was done to mitigate cytotoxic effects of the liposome vector and to ensure cell growth and survival. Cells were incubated for a total of 7 days, with re-transfection as described above occurring after 3 days. This time point was chosen based on previous experiments utilizing rainbow trout cardiac fibroblast cultures (Johnston and Gillis, 2017). Cells in the control group were given medium+lipofectamine, without the miR mimic, to ensure that any effects of transfection on protein and gene expression were the result of the miR mimic. A second control group was transfected with nonsense small interfering RNA (siRNA) (Thermo Fisher Scientific product no. 4464060), to test the influence of small RNA transfection on collagen deposition in cultured cardiac fibroblasts. These control cells were transfected using the same methods as for miR-29b.

RNA extraction and miRNA reverse transcription

Acid–phenol:chloroform RNA extraction was performed with the mirVana miRNA extraction kit (Life Technologies) according to manufacturer's instructions. Briefly, DNase I (Sigma-Aldrich) diluted 1:100 in 300 µl lysis buffer was added to cells on ice and incubated for 8 min. Cells were collected by scraping, homogenized with a sterile needle and syringe and incubated for 10 min on ice. A *Caenorhabditis elegans* mature miR-39 spike-in control was added at a concentration of 5.6×10^8 copies μl^{-1} (Qiagen, Hilden, Germany) immediately after lysis to serve as a normalization control between samples. After washing, total RNA was collected with a glass filter and collection tube, eluted with 100 µl 95°C RNase-free water, then re-precipitated overnight with 1 ml isopropanol and 1 µl 20 mg ml^{-1} glycogen. RNA was pelleted at 12,000 ***g*** for 1 h then the pellet was washed twice with cold 75% ethanol and reconstituted in 10 µl RNase-free water. Reconstituted RNA was quantified with a Nandrop2000 (Thermo Fisher Scientific). A 1 µg sample of total RNA was reversed transcribed with a miScript II kit (Qiagen) according to the manufacturer's instructions, using HiFlex buffer to reverse transcribe all small non-coding RNA, which would include transfected miR-29b, the miR-39 spike-in, as well as endogenously produced mRNA. The reverse transcription enzyme was omitted from 10% of samples to serve as a genomic contamination control.

Quantitative real-time PCR using SYBR green assay

Quantitative real-time PCR (qPCR) was completed using the Qiagen miScript SYBR Green PCR kit in 10 µl reaction volume with the following concentrations: 1× SYBR Green Supermix, 500 nmol l^{-1} forward primer (reverse complement of target, Table 1), 500 nmol l^{-1} Universal miScript primer, 2 µl water and 1 µl cDNA. The Qiagen Universal primer sequence is proprietary,

Table 1. Forward primer sequences used in quantitative real-time PCR (qPCR)

Gene	Sequence (5′–3′)	Accession no.	Efficiency (%)	R^2
cel-39-3p	TCACCGGGTGTAAATCAGCTTG	MIMAT0020306	103	0.99
miR-29b	TAGCACCATTTGAAATCAGTGTT	MIMAT0048668	102	0.99
col1a1	CCCGAGCCATGCCAGAT	NM_001124177.1	94	0.99
col1a2	CAGGCTACCCAGAACATCACATA	NM_001124207.1	106	0.99
col1a3	GCGACAGCGGAACCGTTAT	NC_035096.1	105	0.99
col4a4	TTACCTGGGCCTAAAGGTGA	XM_021587195.1	107	0.99
mmp2	AGACGCATAGACGCTGGCTAA	AB021698.1	85	0.99
β-actin	ACACCCGACTACCACTTCAG	AF157514.1	83	0.99
vim	AAGCTGCATGATGAGGAGGTTGC	NM_001124729.1	92	1

Primers were used to amplify rainbow trout transcripts for *Caenorhabditis elegans* microRNA-39b (*cel-39*), microRNA-29b (*miR-29b*), collagen type I alpha I (*col1α1*), collagen type I alpha 2 (*col1α2*), collagen type I alpha 3 (*col1α3*), collagen type IV alpha 4 (*col4a4*), matrix metalloproteinase 2 (*mmp2*), *β-actin* and vimentin (*vim*). The Qiagen Universal primer was used as the reverse primer for all genes examined to maintain consistency across reactions. Use of the proprietary reverse primer is also why amplicon sizes are not reported, as the exact resulting nucleotide length is not known.

thus the exact amplicon size is unknown. Reactions were run in a 96-well plate in a CFX96 Real-Time System (Bio-Rad) under the following conditions: 95°C for 15 min, 94°C for 15 s, 40×55°C for 30 s and 70°C for 30 s.

Western blot for collagen type I

Cells transfected for 7 days in 6-well plates were collected by scraping in radioimmunoprecipitation assay (RIPA) buffer containing 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and homogenized with a sterile needle and syringe on ice. Lysate was centrifuged at 20,000 ***g*** for 20 min and supernatant was reserved. Samples were diluted in 5× Laemmli buffer and boiled at 95°C for 5 min prior to SDS-PAGE; 20 µg protein was loaded into each lane of a 6% polyacrylamide gel and run at 160 V for ∼1 h. Proteins were electro-blotted onto polyvinylidene fluoride membrane with wet transfer at 30 V for 16 h at 4°C, blocked in 5% skimmed milk powder in Tris-buffered saline with 0.1% Tween 20 (TBST; hereafter referred to as blocking buffer) for 1 h at room temperature then incubated in 1:1000 rabbit anti-salmon collagen type I (Cedarlane) in blocking buffer plus 0.05% sodium azide overnight at 4°C. The next day, blots were incubated in goat anti-rabbit IgG-HRP at a dilution of 1:1000 in blocking buffer (Santa Cruz Biotechnology). An Amersham ECL Plus detection kit (GE Healthcare) was used as per the manufacturer's instructions to induce chemiluminescence, which was subsequently visualized with the ChemiDoc MP. Antibodies were removed by washing in mild stripping buffer (200 mmol l^{-1} glycine, 0.1% SDS, 1% Tween 20, pH 2.2) for 10 min at room temperature, then dried overnight. To control for loading volume, membranes were wetted in 7% acetic acid–10% methanol, then incubated in SYPRO ruby blot stain (Bio-Rad) (Fig. S1). Collagen type I and total protein levels were quantified in ImageLab 5.2.1. (Bio-Rad) using densitometry analysis. Collagen type I was normalized to total protein below 100 kDa to avoid using altered collagen protein levels above 100 kDa, and collagen levels in the nonsense siRNA- and miR-29b-treated cells were standardized to control, which was set to 1.

Validating siRNA entry into cells with fluorescent control

To the best of our knowledge, no fibroblast cell lines from rainbow trout cardiac tissue have been used in experiments involving mRNA knockdown via siRNA transfection. Thus, a fluorescent siRNA control was used to ensure that the combination of Lipofectamine RNAiMax and miR/siRNA was able to permeate fibroblast cells. Cells were plated into 24-well plates at a density of $\sim 0.4\times10^5$ cells per well and left for 24 h, then incubated with 10 nmol l^{-1} MISSION Cy5 Universal Fluorescent Control (Sigma-Aldrich) in Lipofectamine RNAiMax for 24, 48 and 72 h, and 7 days. Medium was replaced after 24 h (as above). Before imaging, cells were incubated in 5 µg ml^{-1} Hoechst 33258 (Sigma-Aldrich) for nuclear visualization. Three groups of cells were re-transfected after 3 days, and incubated with the fluorescent siRNA for a total of 7 days, as in miR experiments, while three groups did not receive additional fluorescent siRNA.

Statistical analysis of treated cells

All data were treated with a Shapiro–Wilk test for normality. Any data that did not meet normality were log-transformed prior to analysis. Average normalized transcript abundance levels between control and transfected groups were analysed with an unpaired *t*-test at a confidence interval of 95%. For each treatment, n=3, where n represents a cell culture established from the ventricle of a different individual rainbow trout. Average normalized collagen protein levels between control, nonsense siRNA- and miR-29b-transfected groups were analysed with a one-way ANOVA at a confidence interval of 95%. For each treatment, n=3, where n represents a cell culture established from the same ventricle of a male rainbow trout, as previously described (Johnston and Gillis, 2018).

Bioinformatics analysis

Methods were in alignment with previously published techniques by Kuc et al. (2017). An alignment of the collagen transcript (type 1 alpha 1 in most species and type 1 alpha 3 in rainbow trout) was generated to understand the evolutionary conservation of miR-29b binding sites. *Oncorhynchus mykiss* collagen type 1 alpha 1 (*col1a1*; NM_001124177.1) or collagen type 1 alpha 3 (*col1a3*; NM_001124206.1) was used as query in a BLASTN v2.8.0 (Zhang et al., 2000) search against the *Euteleostomi* (taxid:117571) database to determine orthologues of the rainbow trout-specific collagen. Orthologues chosen were annotated as belonging to collagen type 1 alpha 1 and *E*-value <0.01. Annotated 3′-UTRs were acquired from the following species and genome assembly identifiers: *Danio rerio*: GRCz11 (GCF_000002035.6); *Salmo salar*: ICSASG_v2 (GCF_000233375.1); *Salvelinus alpinus*: ASM291031v2 (GCF_002910315.2); *Xenopus laevis*: Xenopus_laevis_v2 (GCF_001663975.1); *Homo sapiens*: GRCh38.p12 (GCF_000001405.38); and *Mus musculus*: GRCm38.p4 (GCF_000001635.24). The predicted 3′-UTRs for *col1a1* and *col1a3* in *Oncorhynchus mykiss* were extracted directly from the genome (Berthelot et al., 2014); 1360 bases for *col1a1* and 1134 bases for *col1a3* downstream from the end of the gene were extracted based on the GAZE gene prediction for a longer 3′-UTR within the Genoscope genome browser (https://www.genoscope.cns.fr/trout/; Howe et al., 2002). UTRs were aligned in Seaview and coloured in Jalview v2.10.5 using the 'Nucleotide' colour scheme at 85% identity. miR-29b binding sites were predicted using TargetScanFish v6.2 in zebrafish and manually inspected for site conservation between species (Lewis et al., 2005).

To determine how miR-29b could be influencing other aspects of ECM remodelling, a gene ontology (GO) analysis was carried out using all predicted miR-29b targets in zebrafish. Ensembl gene IDs for miR-29b targets were downloaded from TargetScanFish v6.2 and run through the DAVID bioinformatics pipeline (Huang da et al., 2009a,b; Lewis et al., 2005). Gene IDs classified under cellular composition and GO term 0031012 (extracellular matrix) were downloaded and TargetScanFish total context+ scores recorded to show the comparative strength of the predicted binding.

RESULTS

Conservation of miR-29b

All miR-29b-3p sequences investigated were 100% conserved between vertebrate species, with the exception of Atlantic salmon, which was 86% conserved compared with all other species (Fig. 1).

Effect of miR transfection on miR overexpression and collagen type I targets

Transfection of trout cardiac fibroblasts with mature zebrafish miR-29b mimic resulted in a 17.8-fold increase in miR-29b compared with untransfected cells (P<0.05, Fig. 2). There was a significant effect of miR-29b transfection on collagen isoform expression, where the relative transcript abundance of *col1a3* was reduced by 54% (P<0.05, Fig. 2). The average transcript abundance of *col1a1*, *col1a2*, *β-actin*, *mmp2*, *vim* and *col4a4* isoforms did not change with miR-29b transfection and overexpression (P>0.05, Fig. 2).

Effect of miR transfection on collagen type I protein

Transfection of trout cardiac fibroblasts with mature zebrafish miR-29b mimic resulted in an 85% decrease of collagen type

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	Seed region	
Rainbow trout	UAGCACCAUUUGAAAUCAGUGUU	23
Zebrafish	.....-	22
Atlantic salmon	.....G...A-	22
Carp	.....	23
Xenopus	.....	23
Human	.....	23
Rhesus macaque	.....	23
Mouse	.....	23
Rat	.....	23
Cow	.....	23
Platypus	.....-	22
Chicken	.....	23
King cobra	.....	23

Fig. 1. miR-29b sequence conservation in several vertebrate species. The miR-29b nucleotide sequence is 100% conserved between mammals and there is also complete conservation between zebrafish and human miR-29b. The rainbow trout miR-29b sequence was obtained from microTrout (see Materials and Methods). The miR-29b sequence is 86% conserved between humans and Atlantic salmon. Dots indicate conserved nucleotides, dashes indicate a nucleotide deletion. The seed region is composed of nucleotides ~2–7.

I protein after 7 days with re-transfection, compared with the control liposome group (P<0.05) (Fig. 3). Transfection of trout cardiac fibroblasts with the nonsense siRNA had no effect on collagen expression (Fig. 3).

Validation of siRNA entry into cells

Fluorescently labelled siRNA was incorporated into cells after 24 h of incubation (Fig. 4). siRNA remained in cells after 48 and 72 h incubation even after medium replacement after 24 h.

Seed sequences

The seed sequence of a miR is composed of ~8 nucleotides within the miRNA sequence and is the main part of the miRNA that is

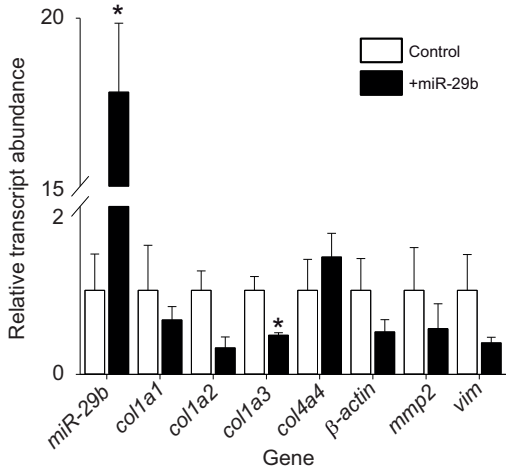

Fig. 2. The effect of miR-29b transfection on its own gene expression and that of hypothesized collagen mRNA targets. Transcript abundance of cells transfected with 100 nmol l^{-1} mature zebrafish miR-29b mimic or liposome vector control. The amount of transcript (*miR-29b*, *col1a1*, *col1a2*, *col1a3*, *col4a4*, *β-actin*, *mmp2*, *vim*) is given relative to the control group, which was set to 1 for each gene. mRNA level for each gene was normalized to the transcript abundance of *Caenorhabditis elegans* miR-39 spike-in control. Asterisks denote a significant effect of miR-29b transfection on transcript abundance at 95% confidence. n=3, where each n represents a population of fibroblasts cultured from a ventricle of a different individual male rainbow trout.

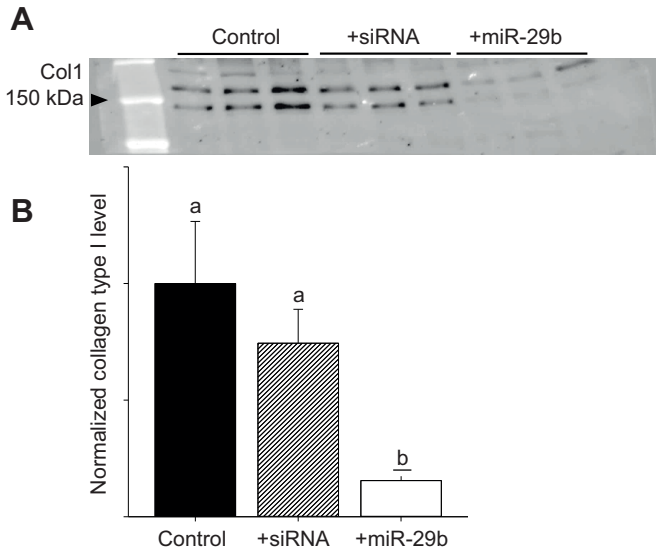

Fig. 3. The effect of miR-29b transfection on collagen type I protein levels. Cardiac fibroblast cultures were transfected with 10 nmol l^{-1} mature zebrafish miR-29b mimic or a nonsense small interfering RNA (siRNA), and re-transfected after 3 days, with sampling occurring at 7 days. (A) Representative western blot image for collagen type I acquired from three different cell lines. (B) Mean collagen type I levels measured by densitometry and standardized to total protein on the same membrane, which was cut in half and blotted separately. Differences were detected using a one-way ANOVA and letters represent a significant change relative to control (P<0.05). n=3 for each experiment, with each n being a protein sample extracted from cultured cells derived from the same trout ventricle maintained in separate passages and cryovials, and thawed on different days, as previously described (Johnston and Gillis, 2017).

responsible for targeting mRNA (Kehl et al., 2017). To confirm that miR-29b was able to target *col1a1* and *col1a3*, TargetScanFish-predicted binding sites were manually searched for in the generated alignment. The conserved binding site shown in Fig. 5A is a predicted 8mer seed match in zebrafish *col1a1a* (total context+ score −0.26) while the conserved binding site in Fig. 5B is a predicted 8mer seed match in zebrafish *col1a1b* (total context+ score −0.39) based on the TargetScan algorithm. Please note that the total context+ score indicates the likelihood of binding, with a lower a score indicating a higher likelihood.

The GAZE software used to find putative longer 3′-UTRs makes use of the express sequence tag (EST) cDNA database to generate predictions on gene products (Howe et al., 2002). However, to confirm that these putative UTRs could be expressed, the GAZE predictions were searched against the EST database to find specific matches that contained the miRNA binding sites of interest. For both GAZE predicted UTRs, one cDNA sequence (*col1a1* – accession no. BX072793.3; *col1a3* – accession no. CX148961) was found that contained the binding site of interest and a polyadenylation signal as expected for a 3′-UTR.

Other targets of miR-29b

The results were filtered to only include other ECM targets (GO term 0031012) of miR-29b (Table 2). Collagen type IV demonstrated the strongest possible candidate for miR-29b binding (total context+ score −1.0), followed by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMT) 10 (total context+ score −0.9). Other notable targets included other ECM proteins (laminin, collagens), MMPs and ADAMT 6 (total context+ scores >−0.5).

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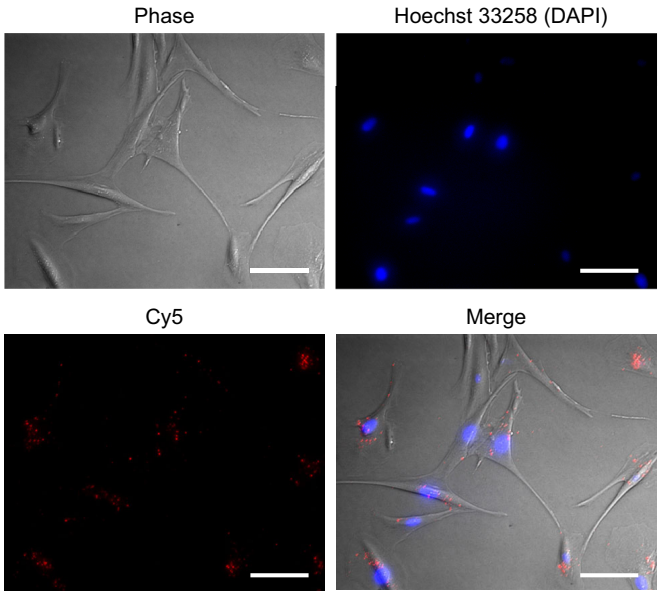

Fig. 4. Representative image of intracellular fluorescently labelled non-functional siRNA in rainbow trout cardiac fibroblasts. Cardiac fibroblasts from trout were incubated for 24, 48 and 72 h after transfection with MISSION Cy5-labelled siRNA, then visualized at each time point. Cells in the images are fibroblasts from cell line mRTV 22 (as per Johnston and Gillis, 2017) after 24 h of incubation. Phase, Cy5 and DAPI image settings were adjusted for the merged image to allow for better visualization of nuclei and siRNA. Images were taken at 400× magnification. Scale bars: 50 μm. Other time points can be found in Fig. S2.

col1a1b (total context+ score −0.7) is included in this table because it is of interest for this study but was not classified into GO:0031012.

DISCUSSION

This is the first known study to demonstrate a functional connection between the overexpression of miR-29b and subsequent decrease in collagen type I protein via *col1a3* mRNA knockdown in a fish cultured cell line. These results establish that miR-29b is an effective regulator of collagen in the fish myocardium and, along with the high conservation of this protein, suggest that this miR plays a role during ECM remodelling in the trout heart.

Knockdown of collagen type I transcript

In mammals, mRNA destabilization is the dominant process by which translational repression occurs (Eichhorn et al., 2014). In this study, the reduction of the *col1a3* isoform transcript abundance positively correlated with the decrease in abundance of the collagen type I protein, indicating that the endogenous complexes, including Argonaute, required for either translational repression or mRNA destabilization, are functional in a teleost species. As the miR that was transfected into the fibroblast cells was already in a mature form, it is not known how the function of cleaving enzymes, namely Drosha and Dicer, may perform in fish compared with mammals. For example, it has been established that the structure of the miR terminal loop is critical for allowing Drosha and Dicer to cleave and thus mature the miR (Zhang and Zeng, 2010). It would be of interest to examine the genome sequence as well as function of fish miR cleavage enzymes in particular, as the teleost genome duplication event may be associated with differential enzyme conformation (Shaffer and Gillis, 2010). The genome duplication may have also resulted in many more functional miRs in trout, or isoforms of known miRs.

The results of our overexpression experiments establish that miR-29b is a regulator of collagen in fish; however, the levels of this miR in the trout heart under physiological conditions are not known. Based on the concentration of the mature mimic measured in the current study and the number of cells exposed, it is estimated that each cell would have been treated with 10 fmol l^{-1} of the mature mimic. As demonstrated by the results, this concentration did have a significant effect on collagen concentration in the cells, but whether this concentration is applicable to an *in vivo* system is unknown. Unfortunately, little is known about absolute concentrations of miRs in living tissue. Furthermore, a previous study demonstrated that miR turnover is high and variable (minutes to hours), indicating that *in vivo* concentrations are in flux (Rüegger and Grosshans, 2012). Future work should attempt to quantify the physiological levels of mature miR-29b in the trout heart in order to fine-tune more complex *in vitro* experiments that utilize multiple miRs. However, the concentration chosen for experiments in this study (10 nmol l^{-1}) was similar to that used in previous studies, and within a safe range for reducing the possibility of non-specific silencing (Jafarinejad-Farsangi et al., 2015; Jin et al., 2015; Mizuno et al., 2016).

miR-29b regulation of collagen type I protein

In fish, collagen type I is composed of three monomers, transcribed from three different transcripts (*col1a1*, *col1a2* and *col1a3*), with *col1a3* being unique to fish (Keen et al., 2016). This is different from collagen expression in mammals, where the collagen molecule is composed of two strands translated from the *col1a1* gene and one from the *col1a2* gene (Pöschl et al., 1988). In this study, *col1a3* appeared to be the mRNA target for knockdown, as the transcript abundance of this isoform was significantly reduced after miR-29b transfection. This therefore suggests that the observed decrease in collagen type I protein was caused by miR-29b knocking down *col1a3* expression. In mammals, *col1a1* is the target of miR-29b (Kriegel et al., 2012; Li et al., 2012; Mayer et al., 2017); therefore, unless the transcript of this gene is completely decreased, there is still the likelihood of intact collagen being present in the heart. This suggests that the same concentration of miR-29b may have a more profound effect on the collagen content of the fish heart, where the translational inhibition of a single peptide would inhibit the formation of the intact triple helix.

Seed sequences

The most effective sites of translational repression have been mapped to the mRNA 3′-UTR sequence, where the seed region of miR (5′–3′, nucleotides 2–7) forms a perfect complementary pair with the mRNA (Eichhorn et al., 2014). Once this is achieved, inhibition of mRNA translation can be accomplished through a number of mechanisms including translational repression, endonuclease cleavage or degradation initiated by 5′ de-capping (Valencia-Sanchez et al., 2006). In this study, the seed sequences in the trout *col1a1* and *col1a3* UTR that demonstrated the best possible position for miR-29b binding were 7 and 8 nucleotides in length. These sites were mapped to the same nucleotide sequence in the miR-29b sequence, as determined by TargetScan. The 7mer-A1 sequence was mapped to *col1a1*, and the 8mer sequence to *col1a3*. Previous studies have suggested that the size of the seed sequence on the target mRNA affects the binding potential of the miR (Bartel, 2009). An 8mer sequence may therefore have more affinity than a 7mer-A1 sequence. This suggests that the *col1a3* isoform may be a stronger target for miR-29b than *col1a1* and this may help explain the lack of downregulation of *col1a1*. Furthermore, the 7mer

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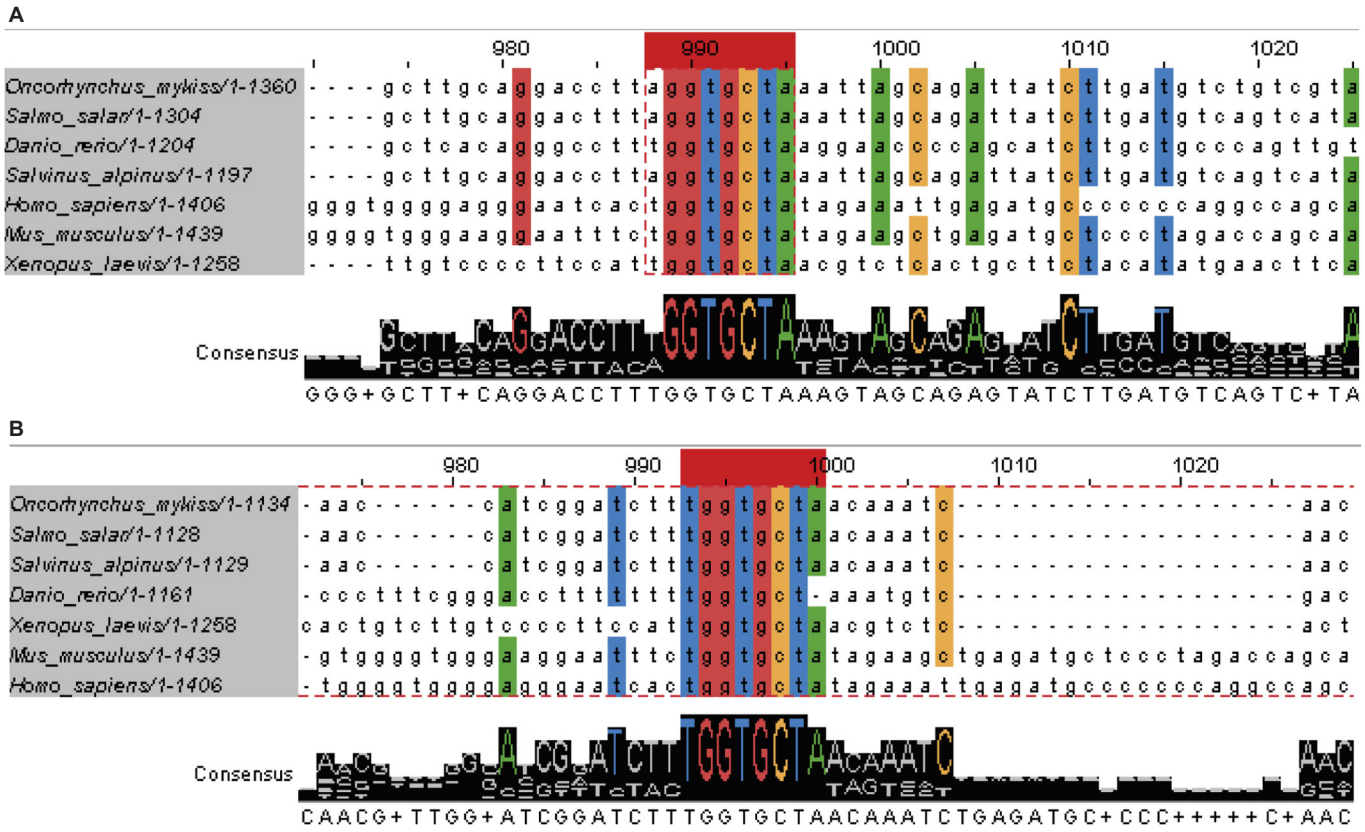

Fig. 5. Alignment of miR-29b binding sites in the *col1a1* and *col1a3* 3′-UTRs. miR-29b binding site conservation between teleosts, *X. laevis*, *M. musculus* and *H. sapiens*. *col1a1* (A) and *col1a3* (B) binding sites were predicted using the TargetScanFish algorithm, which uses zebrafish transcripts, and manually searched within the alignment. Nucleotides are colour coded, and a larger letter within the consensus sequence indicates greater conservation of that specific nucleotide. The 3′-UTR miR-29b binding region is highlighted in red.

sequence on *col1a1* was found in all vertebrate species analysed, indicating that this is likely to be a highly conserved seed sequence.

Other targets of miR-29b

Other possible ECM targets of miR-29b were investigated through GO analysis, and demonstrated similar or greater potential binding affinity. The total context+ scores suggest the binding affinity for all binding sites within the UTR for that miRNA, where lower scores indicate a higher probability of binding (Bartel, 2009). In the cultured fibroblasts, the conserved 8mer binding site within the *col1a3* UTR had a total context+ score of −0.39 in zebrafish *col1a1b*, and we found a decrease in the levels of this transcript. Therefore, a binding site with an even lower score may have strong regulatory potential. One such target that we identified was collagen type IV, which is a basement membrane protein that acts as an intercellular anchor for myocytes, and assists in maintaining tissue integrity during contraction (Bruggink et al., 2007). Therefore, if miR-29b levels are altered in response to thermal acclimation, collagen IV may also be affected. However, the role of collagen IV in the trout myocardium is unknown. We did not find an effect of miR-29b overexpression on the transcript abundance of rainbow trout collagen type IV alpha 4; however, this may be due to the fact that *col4a4* is the only annotated rainbow trout collagen IV gene. This miRNA could have an impact on other collagen IV transcripts

Table 2. Extracellular matrix (ECM)-related targets of miR-29b

Ensemble ID	Gene name	Total context+ score
ENSDARG00000002831	collagen, type IV, alpha 4 (*col4a4*)	−1.00
ENSDARG00000075188	ADAM metallopeptidase with thrombospondin type 1 motif, 10 (*adamts10*)	−0.90
ENSDARG00000036279	laminin, gamma 1 (*lamc1*)	−0.46
ENSDARG00000012405	collagen, type I, alpha 1a (*col1a1a*)	−0.39
ENSDARG00000077084	collagen, type XXVIII, alpha 1a (*col28a1a*)	−0.30
ENSDARG00000051962	matrix metallopeptidase 15a (*mmp15a*)	−0.26
ENSDARG00000041982	ADAM metallopeptidase with thrombospondin type 1 motif, 6 (*adamts6*)	−0.20
ENSDARG00000069473	Fras1-related extracellular matrix 1a (*frem1a*)	−0.14
ENSDARG00000008388	matrix metallopeptidase 14b (membrane-inserted) (*mmp14b*)	−0.11
ENSDARG00000073711	multimerin 2b (*mmm2b*)	−0.09

In silico-predicted miR-29b targets based on TargetScanFish v6.2 and classified as ECM components based on DAVID bioinformatics pipeline (GO:0031012). Total context+ score is calculated by TargetScanFish to potential strength of binding, with lower context scores having a higher chance of repression by the miRNA.

that are currently not annotated. Another reason for this lack of *col4a4* change could be the prediction algorithm used. ECM targets for miR-29b were determined using TargetScanFish which uses zebrafish 3′-UTRs to find potential binding sites (Lewis et al., 2005). However, salmonids underwent a subsequent genome duplication event 80 MYA that separates them from other teleosts such as zebrafish, which could have resulted in altered regulation (Lien et al., 2016; Mennigen and Zhang, 2016). It is possible that miR-29b could still be impacting protein levels of *col4a4*, but further study is necessary to confirm this relationship.

Conclusions and perspectives

This study demonstrates for the first time that miR-29b can cause a reduction in collagen protein in rainbow trout cardiac fibroblasts. Furthermore, the bioinformatics analysis together with the results of the qPCR measurements suggest that this is accomplished by miR-29b targeting, and decreasing, *col1a3* translation. As the protein product of *col1a3* is one of three collagen monomers in intact fish col1, this represents a potentially effective strategy to reduce deposition of the polymer. This suggests that miR-29b can play a role in the decrease in collagen content of the trout heart observed to occur during warm acclimation. Finally, while other studies have used bioinformatics analyses to characterize the conservation of regulatory processes across vertebrate lineages, this is the first known study that has coupled such analysis with functional assays to provide support for the predicted, conserved effects of the same miR across vertebrate lineages.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.F.J., P.M.C., T.E.G.; Methodology: E.F.J., I.G.C., T.E.G.; Formal analysis: E.F.J., I.G.C.; Investigation: E.F.J.; Writing - original draft: E.F.J., I.G.C., T.E.G.; Writing - review & editing: E.F.J., I.G.C., P.M.C., T.E.G.; Supervision: P.C., T.E.G.; Project administration: T.E.G.; Funding acquisition: T.E.G.

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Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.202788.supplemental

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