

## RESEARCH ARTICLE

# Transforming growth factor beta-1 (TGF- $\beta$ 1) stimulates collagen synthesis in cultured rainbow trout cardiac fibroblasts

Elizabeth F. Johnston and Todd E. Gillis\*

**ABSTRACT**

Cold acclimation of rainbow trout, *Oncorhynchus mykiss*, causes collagen to increase within the extracellular matrix (ECM) of the myocardium, while warm acclimation has the opposite effect. The mechanism responsible for this remodelling response is not known. In mammals, transforming growth factor beta-1 (TGF- $\beta$ 1) stimulates collagen deposition within the myocardial ECM. Therefore, we hypothesized that TGF- $\beta$ 1 regulates trout myocardial ECM turnover and predicted that TGF- $\beta$ 1 would induce collagen deposition in cultured rainbow trout cardiac fibroblasts. We found that treatment of trout cardiac fibroblasts with 15 ng ml<sup>-1</sup> human recombinant TGF- $\beta$ 1 caused an increase in total collagen at 48 and 72 h and an increase in collagen type I protein after 7 days. We also found that TGF- $\beta$ 1 treatment caused an increase in the transcript abundance of tissue inhibitor of metalloproteinase 2 (*timp-2*) and matrix metalloproteinase 9 (*mmp-9*) at 24 h. Cells treated with TGF- $\beta$ 1 also had lower levels of the gene transcript for *mmp-2* after 48 h and higher levels of the gene transcript for collagen type I  $\alpha$ 1 (*col1a1*) after 72 h. These changes in gene expression suggest that the increase in collagen deposition is due to a decrease in the activity of matrix metalloproteinases and an increase in collagen synthesis. Together, these results indicate that TGF- $\beta$ 1 is a regulator of ECM composition in cultured trout cardiac fibroblasts and suggest that this cytokine may play a role in regulating collagen content in the trout heart during thermal acclimation.

**KEY WORDS:** Myocardium, Collagen matrix, Cytokine, Tissue remodelling

**INTRODUCTION**

Rainbow trout remain active in waters where temperature can change seasonally between 4 and 25°C. Such a change in temperature represents a significant challenge to maintaining normal physiological processes because of the effects of temperature on biochemical and biophysical processes. One critical organ required for maintaining aerobic function is the heart, and many studies have demonstrated that thermal acclimation of trout causes significant changes to its morphology, composition and function (Bailey and Driedzic, 1996; Bailey et al., 1990; Farrell et al., 1991; Graham and Farrell, 1989; Hassinen et al., 2007; Keen et al., 2016; Klaiman et al., 2011; Lurman et al., 2012; Vornanen et al., 2005). It is thought that these changes help to compensate for the effects of temperature on the heart's capacity to support the oxygen requirements of the animal. For example, Klaiman et al.

(2014) demonstrated that the magnitude and rate of ventricular pressure generation was greater in hearts from trout acclimated to 4°C than in those from animals acclimated to 11 or 17°C. In addition, cold acclimation has been demonstrated to cause cardiac hypertrophy and to increase cardiac connective tissue, while warm acclimation causes the opposite response (Keen et al., 2017; Klaiman et al., 2011). These reversible changes in tissue composition are thought to help maintain cardiac function through the ~20°C seasonal change in ambient temperature (Keen et al., 2017; Klaiman et al., 2011). For example, passive stiffness of the heart increases when trout are cold acclimated, and this reduced compliance is thought to help support the increased muscle mass with cold-induced hypertrophy (Keen et al., 2017; Klaiman et al., 2011).

The extracellular matrix (ECM) of the vertebrate heart plays an important role in regulating cardiac function by acting as a dynamic scaffold that maintains the structural integrity of the myocardium. Changing the amount or type of connective tissue within the ECM alters the physical properties of the heart, resulting in changes in myocardial compliance (Carver et al., 1991). In mammals, physiological remodelling of the heart with aerobic exercise training causes a proportional increase in both muscle mass and connective tissue so that the biomechanical properties of the myocardium are maintained (Weber et al., 1987). In contrast, pathological remodelling, or cardiac fibrosis, that occurs with cardiac disease or injury leads to irregular myocardial stiffening (Burchfield et al., 2013; Zeisberg et al., 2007), and this irreversible change, caused by a permanent increase in collagen, reduces contractile function (Shih et al., 2011).

Under physiological conditions, collagen is constantly turned over in the myocardium; therefore, rates of synthesis and degradation are manipulated to maintain biomechanical properties (Siwik et al., 2001). In the mammalian heart, the degradation of collagen in the ECM is mediated by the activity of a family of zinc-dependent endopeptidases known as matrix metalloproteinases (MMPs) (Visse and Nagase, 2003) and their inhibitors, called tissue inhibitors of MMP 2 (TIMP2) (Husse et al., 2007; Lee et al., 2002, 1997). In fish, MMP-13 degrades collagen into gelatin, which is then digested by MMP-2 and MMP-9 into waste products that are removed from the body (Hillegeass et al., 2007; Kubota et al., 2003; Li et al., 2002). An increase in collagen production by the fibroblasts can also lead to an increase in deposition into the ECM (Li et al., 2001). These processes have been demonstrated to be regulated in the mammalian heart, in part, by the cytokine transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (Border and Noble, 1994). TGF- $\beta$ 1 is ubiquitously expressed and binds to most cell types to initiate intracellular signalling cascades involved in cellular proliferation, differentiation and migration (Overall et al., 1991; Van Obberghen-Schilling et al., 1988). In addition, TGF- $\beta$ 1 production and release increases during pathological remodelling such as that which occurs after myocardial infarction (Bujak et al., 2007) or with

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Received 27 March 2017; Accepted 8 May 2017

the onset of hypertension (Lijnen et al., 2003). These pathologies are associated with an increase in blood pressure, which results in greater levels of stretch and/or shear stress on the endocardium and triggers TGF- $\beta$ 1 production to initiate pathological remodelling (Katsumi et al., 2004; MacKenna et al., 1998). Once released, TGF- $\beta$ 1 stimulates cardiac fibroblasts to increase the transcription of *timp-2* (Visse and Nagase, 2003) and collagen type I (*coll*; Kolosova et al., 2011; Reed et al., 1994), resulting in an increase in connective tissue in the ECM. Collagen type I is also a main constituent of the ECM in other vertebrate hearts, including that of rainbow trout (Eghbali and Weber, 1990); however, it is not known whether the role TGF- $\beta$ 1 in regulating collagen deposition is conserved. The collagen protein is composed of three amino acid chains that assemble to form a fibril. In fish, each of the three chains is coded by a separate collagen transcript (*colla1*, *colla2* and *colla3*) (Saito et al., 2001). Expression of trout *colla3* has been found to be upregulated in response to chronic cold acclimation (Keen et al., 2016). In mammalian models, it is well established that there are TGF- $\beta$ 1-responsive sequences on the promoter for *colla1* (Verrecchia and Mauviel, 2007) and *colla2* (Inagaki et al., 1994). Many mammalian studies have focused on the transcript abundance of *colla1* in response to short-term TGF- $\beta$ 1 signalling (Baugé et al., 2011; Pan et al., 2013; Verrecchia et al., 2001). Thus, we studied *colla1* as a representative gene for trout collagen type I deposition and also because it is known to be responsive to TGF- $\beta$ 1 stimulation.

The purpose of the present study was to determine whether TGF- $\beta$ 1 regulates ECM collagen content in the rainbow trout heart. To do this, we characterized how exposure of cultured trout cardiac fibroblasts to TGF- $\beta$ 1 influenced collagen production and assembly, as well as the expression of gene transcripts associated with collagen regulation. The specific gene transcripts examined included *colla1*, *mmp-2*, *mmp-9* and *timp-2*. We predicted that TGF- $\beta$ 1 treatment would cause an increase in collagen production and that this change would be accompanied by an increase in the expression of *colla1* and *timp-2* as well as a decrease in the expression of *mmp-2* and *mmp-9*. Such changes in gene expression would support an increase in collagen synthesis and a decrease in the activity of MMPs.

## MATERIALS AND METHODS

### Animal husbandry

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (1661±166 g), were obtained from Alma Aquaculture Research Centre (Alma, ON, Canada) and kept in 2000 l flow-through tanks at the Hagen Aqualab (University of Guelph, ON, Canada). Water temperature was 12.0±1.0°C and photoperiod was maintained on a 12 h:12 h cycle. Fish were fed a maintenance diet of Martin Mills (Elmira, ON, Canada) floating feed on alternate days. The University of Guelph Animal Care Committee approved all experiments in this study under the auspices of the Canadian Council on Animal Care (CCAC).

### Establishing primary cultures of trout fibroblasts

Methods to establish primary cultures of trout cardiac fibroblasts were adapted from Fryer et al. (1965). Adult rainbow trout were killed by a blow to the head and then the skin on the ventral surface above the heart was scraped of mucous and rinsed with 70% ethanol. The heart was removed using a sterile scalpel, rinsed and then placed in ice-cold Hank's balanced salt solution (in mmol l<sup>-1</sup>: 1.26 CaCl<sub>2</sub>, 0.49 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.4 MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.3 KCl, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 4.16 NaHCO<sub>3</sub>, 137.9 NaCl, 0.33 Na<sub>2</sub>HPO<sub>4</sub> and 5.55 dextrose) containing 1% penicillin-streptomycin (P/S) (Hyclone,

Little Chalfont, UK) and 1% amphotericin B (AmB) (Life Technologies, Carlsbad, CA, USA). All remaining work was conducted within a class II biosafety cabinet at room temperature (21°C). The ventricle was isolated and then minced into small (~1 mm) pieces in Leibovitz's L-15 medium (Life Technologies) before being subjected to protein hydrolysis in a 0.16% trypsin-EDTA solution (HyClone) with constant stirring for 15 min at room temperature. Dissociated cells and tissue were split into three 25 cm<sup>2</sup> flasks in L-15, supplemented with 10% fetal bovine serum (FBS), 1% P/S and 1% AmB (hereafter referred to as whole medium) and allowed to attach to the flask bottom overnight at room temperature. The next day, the exhausted medium was replaced with whole medium containing 15 ng ml<sup>-1</sup> basic fibroblast growth factor (bFGF; Life Technologies) and 30 ng ml<sup>-1</sup> insulin-like growth factor 1 (Life Technologies) to facilitate fibroblast proliferation (Kohei et al., 1997). When cells were 60–70% confluent, populations were sub-cultured with 0.16% trypsin-EDTA. Cells at passages 4–6 were cryobanked in liquid nitrogen in L-15 with 10% dimethyl sulfoxide (DMSO). To confirm that fibroblast populations had been established, cells were cultured on acid-etched coverslips and stained with monoclonal mouse anti-vimentin (Sigma Aldrich, St Louis, MO, USA), a reliable marker of fibroblastic cells (Sappino et al., 1990). As vimentin also stains endothelial cells, cultures were incubated with anti-mouse cluster of differentiation 31 (PECAM-1) antibody (2H8-s, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA). This antibody was validated using immunohistology with whole trout muscle tissue preparations (Fig. S1). Cultured cells did not demonstrate fluorescence of the CD31 protein, indicating that these cells were not endothelial in nature. All lines also tested negative for mycoplasma as demonstrated using a Hoechst 33258 (Sigma) stain (Fig. S2). Of the 27 trout ventricles used to establish cell lines, only seven lines grew successfully. Cell lines mRTV2, 9, 13, 17, 19, 21 and 22 (male rainbow trout ventricle) were used between passages 7–9 (hydroxyproline assay and gene expression) or 11–13 (ascorbic acid and western blot) in the experiments below. For cell culture experiments, the *n*-value refers to a separate cell line at various passages. Cells were maintained at 21°C at all times without CO<sub>2</sub>. Cells were serum-starved for 24 h before all experiments to arrest growth prior to experimentation so that any cellular changes detected would be a response to the treatment (Hayes et al., 2005).

### TGF- $\beta$ 1 treatment

Cryobanked cells were thawed into 5 ml whole medium and then centrifuged at 500 g for 5 min to remove cryofreezing medium. Cells were plated into 6-well plates at a density of ~8×10<sup>3</sup> cells cm<sup>-2</sup>. Human recombinant TGF- $\beta$ 1 (15 ng ml<sup>-1</sup>; R&D Systems, Minneapolis, MN, USA) in a solution of 4 mmol l<sup>-1</sup> HCl+0.5% BSA was added to fresh medium and neutralized with the addition of an equimolar solution of 4 mmol l<sup>-1</sup> NaOH. Cells were then incubated for 24 h, 48 h, 72 h or 7 days at room temperature. Control cells were exposed to the same volume of the cytokine solvent. At each sampling point, medium was reserved and cells were dissociated with trypsin then counted in a TC20 automated cell counter (Bio-Rad, Hercules, CA, USA) before pelleting.

### Cellular proliferation

Fibroblasts from three separate cell lines (mRTV17, 21 and 22) were plated into 24-well plates at a density of 3×10<sup>4</sup> cells ml<sup>-1</sup> in 500  $\mu$ l whole medium and allowed to attach overnight at room temperature.

After serum starving, half of the cells were treated with  $15 \text{ ng ml}^{-1}$  TGF- $\beta$ 1 and the other half were treated with only the solvent solution. Cells from two wells were counted in duplicate, daily, over the next 5 days. Cells were dissociated with 0.16% trypsin for 5 min and then  $50 \mu\text{l}$  FBS was added to the dissociated cells to inhibit trypsin. Cells were then counted with 4% Trypan Blue vital exclusion dye.

### Hydroxyproline assay

Hydroxyproline is an amino acid that occurs regularly (typically every third residue) in the helices of the collagen chains and can thus be used as an indirect measure of total collagen (Kafienah and Sims, 2004). The concentration of hydroxyproline in cultured cells, which includes the ECM, as well as in conditioned medium, was measured according to Kafienah and Sims (2004). In brief,  $400 \mu\text{l}$  absolute ethanol was added to the cell pellets and to  $200 \mu\text{l}$  of conditioned medium. These were then incubated at  $4^\circ\text{C}$  overnight and pelleted at  $1710 \text{ g}$  and the supernatant was reserved then air-dried. Once dry,  $300 \mu\text{l}$  of  $6 \text{ mol l}^{-1}$  HCl was added to each sample and hydrolysed at  $135^\circ\text{C}$  for 3 h, followed by air-drying. Samples were reconstituted in  $200 \mu\text{l}$  distilled water, centrifuged at  $9000 \text{ g}$ , and the hydroxyproline content in the supernatant was assayed as previously described (Kafienah and Sims, 2004).

### Ascorbic acid treatment and immunocytochemistry for collagen type I

L-Ascorbic acid (L-AA) induces mammalian cultured fibroblasts to synthesize and secrete collagen type I into the ECM, and aids in the formation of structured triple-helix filaments (Murad et al., 1981). To determine the combined effects of L-AA and TGF- $\beta$ 1 on the accumulation and excretion of collagen in the ECM, cultured cells, grown on coverslips, were exposed to TGF- $\beta$ 1 in the presence or absence of L-AA. This was done to determine whether TGF- $\beta$ 1 had an effect on collagen production as well as assembly. L-AA was added daily for 7 days to control fibroblasts and to those that had been treated with TGF- $\beta$ 1. Treatment groups included L-AA only, TGF- $\beta$ 1 only and TGF- $\beta$ 1 plus L-AA. The control group was not treated with either TGF- $\beta$ 1 or L-AA. L-AA (Sigma), dissolved in L-15, was added to cells to a concentration of  $50 \mu\text{g ml}^{-1}$  (Lee et al., 1997). TGF- $\beta$ 1 at a concentration of  $15 \text{ ng ml}^{-1}$  was added once on day 0 and again after 72 h when the whole medium was replaced. Cells were sampled after 24 h, 72 h and 7 days. The cell culture media used in these experiments was pH buffered and contained a pH-sensitive dye. The addition of L-AA did not alter the colour of the media, indicating that the pH was not altered. At each sampling point, cells were fixed in 4% paraformaldehyde for 10 min followed by washing with phosphate-buffered saline (PBS), permeabilized with PBS containing 1% Tween 20 (PBS-T) (ThermoFisher Scientific, Ottawa, ON, Canada) for 10 min then blocked with 1% normal goat serum (Invitrogen) in PBS-T for 1 h. Anti-salmon collagen type I (Cedarlane, Burlington, ON, Canada) was added at a dilution factor of 1:500 in blocking buffer and incubated overnight at  $4^\circ\text{C}$ . The next day, cells were rinsed with PBS-T and incubated in 1:500 goat anti-rabbit Alexa Fluor 568 (Life Technologies) in blocking buffer for 1 h at room temperature. Cells were then counterstained with  $1 \mu\text{g ml}^{-1}$  4',6-diamidino-2-phenylindole (DAPI) for 5 min and mounted on glass slides with ProLong Gold (Invitrogen) and left to dry overnight at room temperature. Cells were imaged on a Nikon Eclipse 90i microscope equipped with a 12-bit colour digital camera (Q-Imaging) using identical acquisition settings.

### Measurement of collagen type I in fibroblasts using western blotting

Fibroblasts from three separate cell lines were grown in  $75 \text{ cm}^2$  flasks and treated with  $15 \text{ ng ml}^{-1}$  TGF- $\beta$ 1, L-AA or solvent control. The ascorbic acid treatment group was added to determine whether it also acts to increase collagen production as well as fibril assembly. Whole medium was replaced after 72 h and TGF- $\beta$ 1 was re-added at this time. Cells were grown for 7 days in total and then sampled. Conditioned medium from cultures was removed and reserved. Cells were washed with 5 ml PBS, then  $500 \mu\text{l}$  radioimmunoprecipitation assay (RIPA) buffer with  $1 \mu\text{mol l}^{-1}$  phenylmethane sulfonyl fluoride (PMSF; Sigma) was added to cells on ice and incubated for 5 min. Cells were removed from flasks by scraping and then transferred to an Eppendorf tube and mixed constantly for 30 min at  $4^\circ\text{C}$ . Lysate was then centrifuged for 20 min at  $20,000 \text{ g}$ , and supernatant was reserved and measured for protein content with a bicinchoninic acid (BCA) assay (Bio-Rad). A  $20 \mu\text{g}$  sample of protein was loaded into each lane of a 6% polyacrylamide gel and run at 160 V for 1.5 h. Proteins were electro-blotted onto a polyvinylidene fluoride membrane with wet transfer at 30 V for 16 h at  $4^\circ\text{C}$ , blocked in 5% skim 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 rabbit anti-salmon collagen type I antibody (Cedarlane) in blocking buffer plus 0.05% sodium azide overnight at  $4^\circ\text{C}$ . The next day, blots were incubated in goat anti-rabbit IgG-HRP antibody at a dilution of 1:1000 in blocking buffer (Santa Cruz Biotechnology, Dallas, TX, USA). An Amersham ECL Plus detection kit (GE Healthcare, Chicago, IL, USA) was used as per the manufacturer's instructions to induce chemiluminescence, which was subsequently visualized with the ChemiDoc MP imaging system (Bio-Rad). To control for loading volumes, membranes were incubated in 1:1000 mouse anti-actin cy3-conjugated antibodies (Sigma) in TBS+1% BSA for 1 h at room temperature and visualized with ChemiDoc MP. It was found that treatment of fibroblasts with L-AA induced an increase in actin; thus, data were compared with whole protein content by running a parallel gel of identical protein and loading volumes and staining with Coomassie Blue (BioRad) to quantify total protein of samples (Eaton et al., 2013) (Fig. S3).

### Reverse transcription-quantitative PCR (RT-qPCR)

The transcript abundance of genes associated with connective tissue regulation were quantified in cardiac fibroblasts after 24, 48 and 72 h incubation with TGF- $\beta$ 1 or solvent control. Total RNA was extracted from fibroblasts using Trizol (Life Technologies) according to the manufacturer's instructions and quantified using a Nanodrop 8000 (ThermoFisher Scientific). A  $1 \mu\text{g}$  sample of total RNA was treated with DNase I (Sigma) and used to synthesize cDNA with the High Capacity cDNA Synthesis Kit (Life Technologies) following the manufacturers' instructions. Duplicate cDNA reactions in which the Multiscribe RT enzyme was omitted were included for 10% of total samples, chosen randomly, to verify the efficacy of the DNase treatment. Transcript abundance was measured in duplicate reactions on a StepOnePlus Real-Time PCR Instrument (Life Technologies) using default cycling conditions and a dissociation cycle. Each  $15 \mu\text{l}$  reaction contained  $1 \times$  PerfeCta Fast SYBR Green Master Mix (Quanta BioSciences, Gaithersburg, MD, USA),  $200 \text{ nmol l}^{-1}$  of each gene-specific primer (Table 1) and 1:15 v:v cDNA. Custom oligos for *coll1a1*, *timp-2* and *mmp-2* were designed using Primer Express. The sequence of the gene-specific primers for *mmp-9* was that used by Keen et al. (2016). All reactions generated a single-peaked



**Table 1. Forward and reverse primer sequences used in quantitative real-time PCR to amplify rainbow trout transcripts for matrix metalloproteinase 2 and 9 (*mmp-2* and *mmp-9*), the tissue inhibitor of metalloproteinase (*timp-2*), and collagen type I  $\alpha 1$  (*col1a1*)**

Gene	Sequence (5'-3')	Amplicon (bp)	Accession no.	Efficiency (%)	R <sup>2</sup>
<i>ef1<math>\alpha</math></i>	F: GGGCAAGGCTCTTTCAAGT R: CGCAATCAGCCTGAGAGGT	188	AF498320	81	0.99
<i>timp-2</i>	F: ACATTTTCCCTCCACGCGAT R: TTCTGCGATGTCTTCCACCC	146	AY606030.1	80	0.99
<i>mmp-2</i>	F: AGACGCATAGACGCTGGCTAA R: GCAGGATAGGCTGGTTGGATAG	78	AB021698.1	84	0.99
<i>mmp-9</i>	F: ACCCCTTCGATGGTAAGGAC R: GGTCCAGTTTTGTCATCGT	98	AJ320533.1	82	0.99
<i>col1a1</i>	F: CCCGAGCCATGCCAGAT R: CGGATGTCTCCTCGAGATAA	76	NM_001124177.1	80	0.99

All reactions were run at an annealing temperature of 60°C. Elongation factor 1 $\alpha$  (*ef1 $\alpha$* ) was used as a control.

dissociation curve at the predicted amplicon melting temperature. The mRNA abundance of each gene was quantified by fitting the threshold cycle to the antilog of standard curves prepared from serially diluted cDNA. Isoform transcript abundance was normalized to the mRNA abundance of *elongation factor 1 $\alpha$*  (*ef1 $\alpha$* ). The transcript level of this housekeeping gene did not change in response to TGF- $\beta$ 1 treatment. All primers used had an efficiency of 80–110%. All non-reverse transcribed control samples failed to amplify.

### Statistical analyses

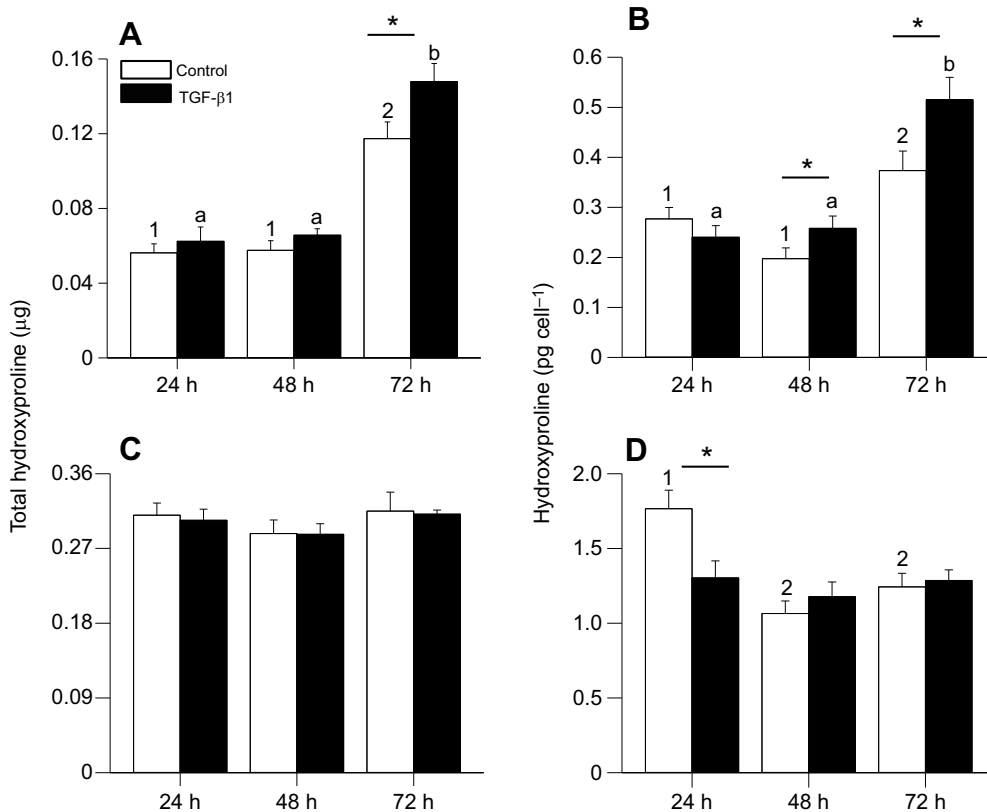
Data for hydroxyproline concentration, collagen protein and gene expression were first tested for normality using a Shapiro–Wilk test. Non-normal data were log-transformed prior to analysis and tested again. Data that were non-normal after log transformation were analysed with a non-parametric test. Differences in hydroxyproline content between control and TGF- $\beta$ 1-treated cells at all time points were analysed with a two-way ANOVA with a *post hoc* Bonferroni

correction at 95% significance cut-off. Differences in transcript abundance at each time point were tested with an unpaired Student's *t*-test at 95% cut-off, and data are expressed as fold-change from control, which was set to 1. Differences in collagen protein production by the control, L-AA-treated and TGF- $\beta$ 1-treated groups were tested with a one-way ANOVA and *post hoc* Bonferroni correction. Statistical analyses were completed using SigmaPlot v.12.

## RESULTS

### Collagen deposition in cultured fibroblast preparations measured by hydroxyproline and western blot protocols

Total hydroxyproline was quantified as an initial measurement of collagen chain production in the cell pellet and in the exhausted medium. Total hydroxyproline in the cell pellet of TGF- $\beta$ 1-treated cells was greater than that in untreated cells after 72 h ( $P < 0.05$ ; Fig. 1A). When standardized to cell number, hydroxyproline levels were 23% and 27% greater in TGF- $\beta$ 1-treated cells compared with



**Fig. 1. The effect of TGF- $\beta$ 1 on collagen production and release from cultured trout cardiac fibroblasts.** (A,C) Absolute amount of hydroxyproline in cell pellets (A) or culture medium (C) after 24, 48 and 72 h of TGF- $\beta$ 1 treatment. (B,D) Average amount of hydroxyproline produced per cell after 24, 48 and 72 h of TGF- $\beta$ 1 treatment in cell pellets (B) or culture medium (D). Different numbers indicate a significant effect of time on the amount of hydroxyproline produced within control cells ( $P < 0.05$ ). Different letters indicate a significant effect of time on the amount of hydroxyproline produced per cell in the TGF- $\beta$ 1-treated group ( $P < 0.05$ ). \*Significant effect of TGF- $\beta$ 1 on hydroxyproline concentration between control and TGF- $\beta$ 1-treated cells ( $P < 0.05$ ).  $n = 5$  for extracellular matrix (ECM) data,  $n = 3$  for cell medium data, where each  $n$  represents a separate cell line established from a single heart from a different fish, and each  $n$  contains 8–15 technical replicates.

controls after 48 and 72 h, respectively ( $P < 0.05$ ; Fig. 1B). The amount of hydroxyproline did not change in the exhausted medium at any time point, whether expressed as absolute values (Fig. 1C) or when standardized to cell number (Fig. 1D). Western blot analysis determined that the level of collagen type I protein was 1.7-fold higher in the cells treated with TGF- $\beta$ 1 than in the untreated control cells at 7 days ( $P < 0.06$ ; Fig. 2). In addition, the levels of collagen type I were 3.4-fold higher in cells treated with TGF- $\beta$ 1 than in cells treated with just L-AA ( $P < 0.05$ ; Fig. 2). The average amount of collagen type I between control and L-AA-treated fibroblasts did not differ significantly.

### Structural collagen deposition in cells treated with L-AA and TGF- $\beta$ 1

There was no evidence of collagen fibril assembly in the ECM of fibroblasts at 24 and 72 h, regardless of treatment (data not shown); however, by 7 days there were differences in both staining intensity and localization between the treatment groups (Fig. 3). Collagen immunostaining of the control cells resulted in weak immunofluorescence and no fibrils (Fig. 3A,E). The signal from immunostained collagen in fibroblasts treated with L-AA alone was comparatively weak, with dense fibrils evident in the ECM (Fig. 3B,F). Conversely, immunostained collagen in fibroblasts treated with TGF- $\beta$ 1 alone was more intense, but was contained entirely around the fibroblasts, and distinct fibrils were absent (Fig. 3C,G). Finally, fibroblasts treated with both TGF- $\beta$ 1 and L-AA resulted in intensely stained collagen both within the cells and secreted into the ECM, with long and densely stained fibrils evident throughout the ECM (Fig. 3D,H).

### The influence of TGF- $\beta$ 1 on gene expression in cultured cardiac fibroblasts

The effects of TGF- $\beta$ 1 treatment on fibroblast gene expression were time and gene specific. After 24 h of TGF- $\beta$ 1 treatment, the

transcript abundance of *timp-2* and *mmp-9* was 7.8- and 10.9-fold greater, respectively, than that in untreated control cells ( $P < 0.05$ ; Fig. 4A). After 48 h of TGF- $\beta$ 1 exposure, the transcript abundance of *mmp-2* was 3.3-fold lower than that in the controls ( $P < 0.05$ ; Fig. 4B), and after 72 h of TGF- $\beta$ 1 exposure, the transcript level of *coll1a1* was 7.4-fold greater than that in the control cells ( $P < 0.05$ ; Fig. 4C).

### Cellular proliferation

The number of cells in the TGF- $\beta$ 1-treated and control cultures increased with time but there was no difference in cell numbers between fibroblast cultures at any sampling time (data not shown).

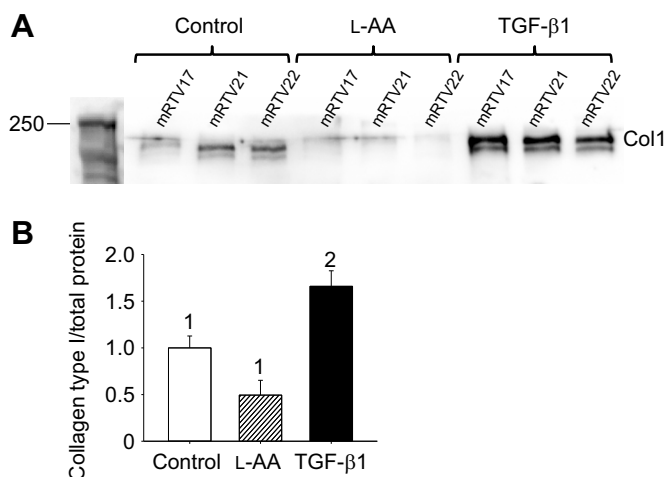
### DISCUSSION

This is the first study to characterize the response of fish cardiac fibroblasts to TGF- $\beta$ 1 and highlights the conserved cellular pathways involved in regulating ECM composition in the vertebrate heart. Cultured cardiac fibroblasts responded to physiologically relevant concentrations of TGF- $\beta$ 1 by increasing collagen production and secretion, and this response was associated with transcriptional-level changes consistent with increased collagen synthesis and decreased collagen degradation.

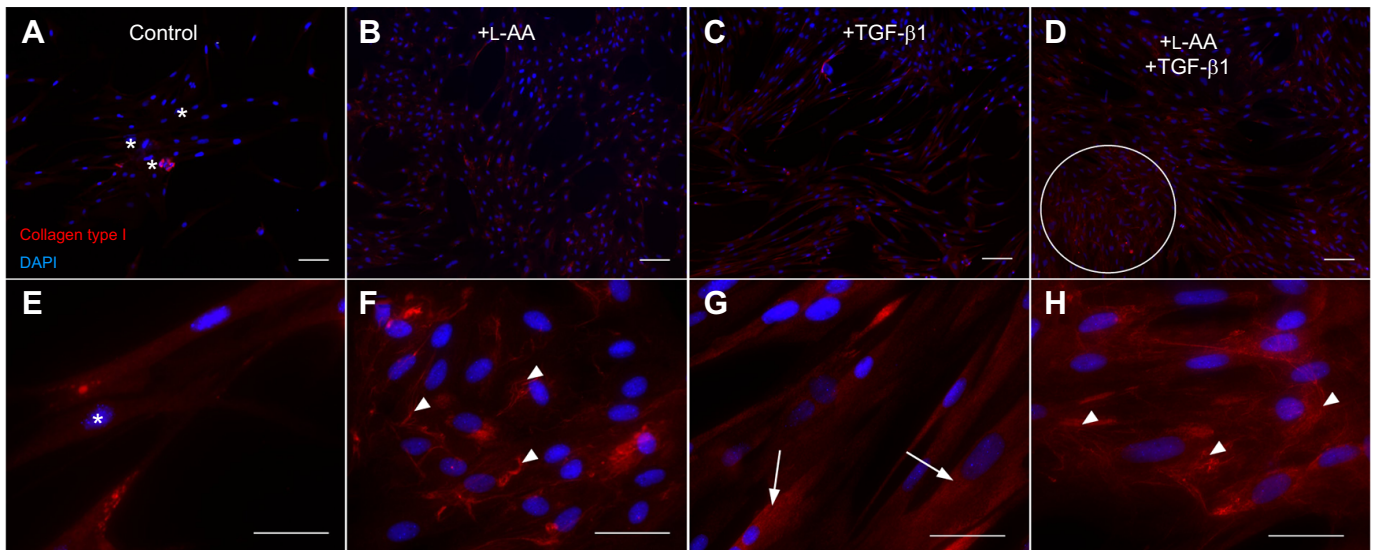
### TGF- $\beta$ 1 regulates collagen production in trout cardiac fibroblasts

Treatment of the trout cardiac fibroblasts with TGF- $\beta$ 1 stimulated collagen production, as indicated by an increase in the hydroxyproline content of cells measured at 48 and 72 h, and an increase in collagen type I quantified with immunoblotting at 7 days post-TGF- $\beta$ 1 treatment. The collagen produced by the cells following TGF- $\beta$ 1 treatment was retained by the cells as there was no difference in collagen content of the culture medium from the control and treated fibroblasts. In addition, the immunocytochemistry data show that structural collagen was not transported into the ECM in response to TGF- $\beta$ 1 treatment. This response is not surprising as previous work with fibroblasts derived from goldfish skin, rat heart and human skin has revealed that L-AA is a co-factor for the extracellular assembly of collagen (Hata and Senoo, 1989; Ju et al., 1998; Lee et al., 1997; Murad et al., 1983; Tamamori et al., 1997). L-AA facilitates the hydroxylation of proline within the collagen amino acid chain, which results in the synthesis of a stable mature collagen polypeptide (Boyera et al., 1998; Peterkofsky, 1972). Deficiencies in L-AA concentration lead to unstructured amorphous collagen and can result in fibrotic tissue pathologies (i.e. scurvy) (Asboe-Hansen, 1963; Thomas et al., 1995). The assembly of collagen fibrils following co-treatment of the fibroblasts with L-AA and TGF- $\beta$ 1 in the current study demonstrates the same phenomenon. Treatment of the fibroblasts with L-AA alone did not stimulate collagen production, as shown in Fig. 2, indicating that it is TGF- $\beta$ 1 that stimulates synthesis in this system. While these separate effects of TGF- $\beta$ 1 and L-AA on collagen synthesis and deposition are well established in mammalian cardiac systems (Eghbali et al., 1991), this is the first description of a cellular response to the combination of these factors in cardiac fibroblasts from any fish species.

One common role of TGF- $\beta$ 1 in vertebrate tissues is to initiate cellular pathways involved in tissue growth and remodelling. In the mammalian heart, TGF- $\beta$ 1 is produced in response to increased physical stress on the myocardium and exerts its effects through an integral membrane receptor (TGF- $\beta$ R1) that initiates a signalling cascade and leads to changes in the expression of transcripts for MMPs and TIMP-2 in cardiac fibroblasts (Husse et al., 2007).



**Fig. 2. The effect of TGF- $\beta$ 1 or L-ascorbic acid (L-AA) treatment on collagen type I deposition in cultured cardiac fibroblasts.** Cardiac fibroblast cultures were treated with medium alone (control), or medium containing either L-AA or TGF- $\beta$ 1 for 7 days, and collagen type I was quantified by western blot and densitometry. (A) Western blot image for collagen type I in fibroblasts from three different cell lines, detected using rabbit polyclonal anti-salmon collagen type I antibody. The first lane is the weight ladder imaged at a different wavelength. (B) Mean collagen type I levels measured by densitometry and standardized to the total amount of protein in the same lane. Differences in collagen abundance were determined with a one-way ANOVA and *post hoc* Bonferroni test ( $n = 3$ ,  $P < 0.06$ ).



**Fig. 3. The effect of L-AA and TGF- $\beta$ 1 on collagen type I production and assembly.** Collagen type I assembly in response to TGF- $\beta$ 1 treatment was assessed by observing the appearance of fibrils after 7 days of treatment with TGF- $\beta$ 1 only (C,G), L-AA only (B,F), and TGF- $\beta$ 1 and L-AA simultaneously (D,H), compared with that of a control group (A,E). Cells were stained with polyclonal rabbit anti-salmon collagen type I antibody and counterstained with DAPI. Images in A–D are shown at higher magnification in E–H, respectively. While collagen type I signal is high in TGF- $\beta$ 1-treated cells (C,G), fibril assembly is apparent only in groups treated with L-AA (B,F,D,H). Asterisks indicate individual nuclei, arrowheads indicate assembled collagen fibrils, arrows point at areas of high collagen staining in TGF- $\beta$ 1-treated cells that would contribute to the overall increased collagen signal apparent in A–D, and the circle denotes an area of dense ECM in a replicate that was treated with both TGF- $\beta$ 1 and L-AA. Micrographs are representative images of one cell line that was used for each of the treatments and imaged using the same exposure time for each magnification. For images in A–D, exposure time was 1.5 s for collagen (red), and for images in E–H, exposure time was 300 ms. Scale bars: 50  $\mu$ m for A–D and 20  $\mu$ m for E–H.  $n=3$ .

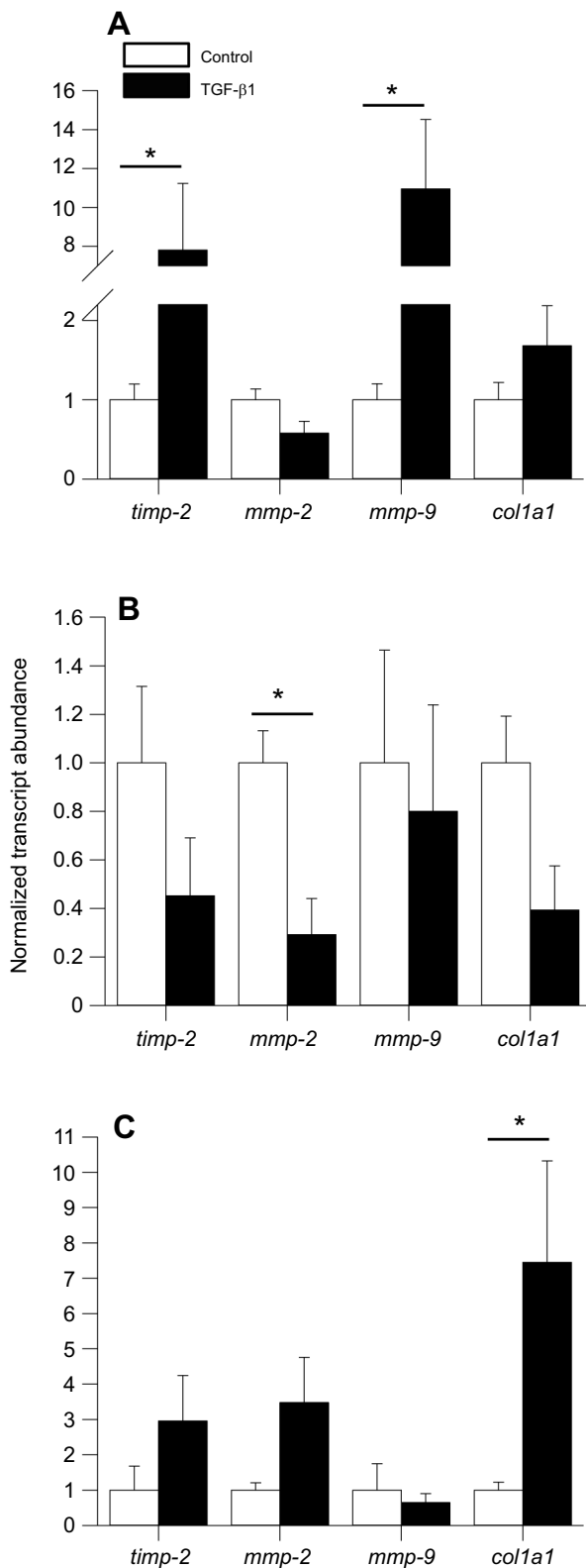
While the effect of TGF- $\beta$ 1 in the heart of fish species has not before been documented, there is some evidence of its importance in regulating other teleost cellular processes. This is due to the broad general effect of TGF- $\beta$ 1, which is ubiquitously expressed and therefore plays a number of important roles in other systems. Recent work with zebrafish explant cultures demonstrates that TGF- $\beta$ 1 promotes keratocyte migration, indicating its conserved role in cell motility across vertebrate species (Tan et al., 2011). In the current study, we found that TGF- $\beta$ 1 stimulated temporal changes in the expression of *mmp-9*, *mmp-2*, *timp-2* and *colla1* in trout cardiac fibroblasts, suggesting that collagen is dynamically regulated by TGF- $\beta$ 1 in fish hearts as well. This is supported by our previous work which demonstrated that the decrease in collagen content in the zebrafish heart with cold acclimation is accompanied by an increase in *mmp-2*, *mmp-9* and *timp-2* (Johnson et al., 2014). These parallel changes in the expression of transcripts for MMPs and TIMP may help provide control over changes in collagen deposition. Such control is important as Li et al. (2002) have suggested that an increase in the production of MMPs, without a counterbalancing increase in TIMPs, leads to functional defects. In the current study, the increase in *mmp-9* at 24 h was accompanied by an increase in *timp-2*, which if translated into protein would prevent collagen degradation by blocking the enzymatic activity of *mmp-9*. The changes in *mmp* and *timp-2* gene expression observed prior to an increase in *colla1* in the current study may indicate that preserving existing ECM collagen is prioritized over synthesizing new fibres. Indeed, an increase in collagen was first detected at 72 h after TGF- $\beta$ 1 exposure, supporting a delay in collagen synthesis. However, even without an increase in collagen production, a decrease in the activity of MMPs caused by reduced levels of the protein, or an increase in TIMP-2 production, can result in an increase in collagen deposition (Husse et al., 2007; Lee et al., 1999, 2002; Li et al., 2001). For

example, overexpression of TGF- $\beta$ 1 in a transgenic mouse leads to an increase in TIMP-2 protein in the heart and this causes an increase in interstitial collagen as well as a decrease in MMP activity (Seeland et al., 2002).

The concentration of human TGF- $\beta$ 1 used in the present study is within the range measured in an infarcted rat heart (Sun et al., 1998); however, it is not known how effective the human cytokine is at interacting with trout TGF- $\beta$ R1. The amino acid sequence of human TGF- $\beta$ 1 shares 48% identity with trout TGF- $\beta$ 1, and the amino acid sequence of trout TGF- $\beta$ R1 shares 81% identity to the human isoform. These differences in sequences may translate into differences in binding affinity; however, as biological effects were apparent even with this limitation, we are confident that endogenous TGF- $\beta$ 1 plays a role in ECM remodelling in the trout heart.

#### Activation of cardiac fibrosis

The change in gene expression characterized in the cultured cardiac fibroblasts in response to TGF- $\beta$ 1 is quite similar to that quantified in the trout heart with cold acclimation. Keen et al. (2016) demonstrated that cold acclimation of trout caused an increase in the expression of *timp-2* and *colla3*, as well as a decrease in the expression of *mmp-2* and *mmp-13* in the heart. These changes in transcript abundance would support the increase in collagen content characterized in this same study by increasing the synthesis of collagen and decreasing the activity of MMPs (Keen et al., 2016). One critical trigger of TGF- $\beta$ 1 production and collagen deposition in the mammalian heart is an increase in blood pressure. An increase in blood pressure causes the endothelial cells to stretch, stimulating TGF- $\beta$ 1 production as well as the activation of a cell-signalling pathway involving p38 MAPK, c-Jun N-terminal kinase (JNK1/2) and extracellular regulated-kinase 1/2 [ERK1/2 (p42/44)] (Katsumi et al., 2004; MacKenna et al., 1998). The end result of both of these stretch-triggered responses is the activation of transcription factors



**Fig. 4. The effect of TGF- $\beta$ 1 treatment on the expression of genes involved in ECM regulation in cultured trout cardiac fibroblasts.** Transcript abundance for control and TGF- $\beta$ 1-treated cells at 24 h (A), 48 h (B) and 72 h (C) post-treatment, respectively. The amount of transcript in each TGF- $\beta$ 1 group is given relative to the control group, which is set to 1 in each panel. mRNA level for each gene was normalized to the transcript abundance of *elongation factor 1 alpha (EF1 $\alpha$ )*. \*Significant effect of TGF- $\beta$ 1 treatment on gene expression ( $P < 0.05$ ).  $n = 3$ –5.

regulating the expression of genes involved in cardiac remodelling and regulating collagen deposition (Katsumi et al., 2004). These include *col1a1*, *timp-2*, *mmp-2*, *mmp-9* and *mmp-13* (Husse et al., 2007; Lee et al., 1999, 2002; Li et al., 2001). This response of the myocardium to stretch is potentially relevant to identifying what triggers cardiac remodelling in the trout heart. Previous authors have suggested that the trigger for cardiac remodelling during cold acclimation is an increase in blood viscosity resulting from a temperature-induced decrease in erythrocyte membrane fluidity (Graham and Farrell, 1989; Keen et al., 2017; Klaiman et al., 2011). This would potentially lead to an increased load on the heart and stretching of the myocardium (Graham and Farrell, 1989; Keen et al., 2017; Klaiman et al., 2011). Given that these same mechanical forces trigger TGF- $\beta$ 1 release in the mammalian heart during pathological remodelling (Takahashi et al., 1994) and that TGF- $\beta$ 1 induces similar cellular responses in cultured trout fibroblasts (present study), we propose that the reversible ECM remodelling of trout hearts during thermal acclimation is mediated by TGF- $\beta$ 1.

### Significance and perspectives

This study demonstrates for the first time that TGF- $\beta$ 1 is involved in stimulating collagen production by trout cardiac fibroblasts. Furthermore, the transcriptional changes in key regulators of ECM collagen content in response to TGF- $\beta$ 1 treatment are quite similar to those previously reported in studies of mammalian cardiac fibroblasts and in the trout heart during cold acclimation (Eghbali et al., 1991; Keen et al., 2016). These results therefore suggest that the cellular pathway responsible for cardiac fibrosis is conserved between fish and mammals. This work also indicates that trout fibroblasts are a good model for examining the regulation of cardiac connective tissue. The next step, using this model, is to identify the cellular mechanisms that enable cardiac connective tissue to be removed from the trout heart, as occurs during warm acclimation. Knowledge gained from such studies has the potential to be translated into novel treatment modalities for cardiac fibrosis in humans.

### Acknowledgements

The authors would like to thank Dr N. Vo (Waterloo) and Dr L. Lee (University of the Fraser Valley) for technical advice on this project, as well as Dr S. L. Alderman for comments on an earlier draft.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: E.F.J., T.E.G.; Methodology: E.F.J., T.E.G.; Formal analysis: E.F.J., T.E.G.; Investigation: E.F.J., T.E.G.; Resources: T.E.G.; Data curation: E.F.J.; Writing - original draft: E.F.J., T.E.G.; Writing - review & editing: E.F.J., T.E.G.; Supervision: T.E.G.; Project administration: T.E.G.; Funding acquisition: T.E.G.

### Funding

This work was supported by an Ontario Graduate Scholarship to E.F.J. and a Discovery Grant from the National Sciences and Engineering Research Council of Canada to T.E.G.

### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.160093.supplemental>

### References

- Asboe-Hansen, G. (1963). Connective tissue. *Annu. Rev. Physiol.* **25**, 41–60.  
 Bailey, J. R. and Driedzic, W. R. (1996). Decreased total ventricular and mitochondrial protein synthesis during extended anoxia in turtle heart. *Am. J. Physiol.* **271**, R1660–R1667.



- Bailey, J. R., Sephton, D. H. and Driedzic, W. R. (1990). Oxygen uptake by isolated perfused fish hearts with differing myoglobin concentrations under hypoxic conditions. *J. Mol. Cell. Cardiol.* **22**, 1125–1134.
- Baugé, C., Cauvard, O., Leclercq, S., Galéra, P. and Boumédiène, K. (2011). Modulation of transforming growth factor beta signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of Sp1 in both early and late response cells to transforming growth factor beta. *Arth res ther* **13**, R23.
- Border, W. A. and Noble, N. A. (1994). Transforming growth factor  $\beta$  in tissue fibrosis. *N. Engl. J. Med.* **331**, 1286–1292.
- Boyera, N., Galey, I. and Bernard, B. A. (1998). Effect of vitamin C and its derivatives on collagen synthesis and cross-linking by normal human fibroblasts. *Int. J. Cosmet. Sci.* **20**, 151–158.
- Bujak, M., Ren, G., Kweon, H. J., Dobaczewski, M., Reddy, A., Taffet, G., Wang, X.-F. and Frangogiannis, N. G. (2007). Essential role of Smad3 in infarct healing and in the pathogenesis of cardiac remodeling. *Circulation* **116**, 2127–2138.
- Burchfield, J. S., Xie, M. and Hill, J. A. (2013). Pathological ventricular remodeling: mechanisms: part 1 of 2. *Circulation* **128**, 388–400.
- Carver, W., Nagpal, M. L., Borg, T. K. and Terracio, L. (1991). Collagen expression in mechanically stimulated fibroblasts. *Circ. Res.* **69**, 116–122.
- Eaton, S. L., Roche, S. L., Llavero Hurtado, M., Oldknow, K. J., Farquharson, C., Gillingwater, T. H. and Wishart, T. M. (2013). Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. *PLoS One* **8**, e72457.
- Eghbali, M. and Weber, K. T. (1990). Collagen and the myocardium: fibrillar structure, biosynthesis and degradation in relation to hypertrophy and its regression. *Mol. Cell. Biochem.* **96**, 1–14.
- Eghbali, M., Tomek, R., Sukhatme, V. P., Woods, C. and Bhambi, B. (1991). Differential effects of transforming growth factor-beta 1 and phorbol myristate acetate on cardiac fibroblasts. Regulation of fibrillar collagen mRNAs and expression of early transcription factors. *Circ. Res.* **69**, 483–490.
- Farrell, A. P., Johansen, J. A. and Suarez, R. K. (1991). Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **9**, 303–312.
- Fryer, J. L., Yusha, A. and Pilcher, K. S. (1965). The *in vitro* cultivation of tissue and cells of pacific salmon and steelhead trout. *Ann. N. Y. Acad. Sci.* **126**, 566–586.
- Graham, M. S. and Farrell, A. P. (1989). The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38–61.
- Hassinen, M., Paajanen, V., Haverinen, J., Eronen, H. and Vornanen, M. (2007). Cloning and expression of cardiac Kir2.1 and Kir2.2 channels in thermally acclimated rainbow trout. *Am. J. Physiol.* **292**, R2328–R2339.
- Hata, R.-I. and Senoo, H. (1989). L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. *J. Cell. Physiol.* **138**, 8–16.
- Hayes, O., Ramos, B., Rodriguez, L. L., Aguilar, A., Badia, T. and Castro, F. O. (2005). Cell confluency is as efficient as serum starvation for inducing arrest in the G0/G1 phase of the cell cycle in granulosa and fibroblast cells of cattle. *Anim. Reprod. Sci.* **87**, 181–192.
- Hillegass, J. M., Villano, C. M., Cooper, K. R. and White, L. A. (2007). Matrix metalloproteinase-13 is required for zebra fish (*Danio rerio*) development and is a target for glucocorticoids. *Toxicol. Sci.* **100**, 168–179.
- Husse, B., Briest, W., Homagk, L., Isenberg, G. and Gekle, M. (2007). Cyclical mechanical stretch modulates expression of collagen I and collagen III by PKC and tyrosine kinase in cardiac fibroblasts. *Am. J. Physiol.* **293**, R1898–R1907.
- Inagaki, Y., Truter, S. and Ramirez, F. (1994). Transforming growth factor-beta stimulates alpha 2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. *J. Biol. Chem.* **269**, 14828–14834.
- Johnson, A. C., Turko, A. J., Klaiman, J. M., Johnston, E. F. and Gillis, T. E. (2014). Cold acclimation alters the connective tissue content of the zebrafish (*Danio rerio*) heart. *J. Exp. Biol.* **217**, 1868–1875.
- Ju, H., Hao, J., Zhao, S. and Dixon, I. M. (1998). Antiproliferative and antifibrotic effects of mimosine on adult cardiac fibroblasts. *Biochim. Biophys. Acta* **1448**, 51–60.
- Kafienah, W. and Sims, T. J. (2004). Biochemical methods for the analysis of tissue-engineered cartilage. *Methods Mol. Biol.* **238**, 217–230.
- Katsumi, A., Orr, A. W., Tzima, E. and Schwartz, M. A. (2004). Integrins in mechanotransduction. *J. Biol. Chem.* **279**, 12001–12004.
- Keen, A. N., Fenna, A. J., McConnell, J. C., Sherratt, M. J., Gardner, P. and Shiels, H. A. (2016). The dynamic nature of hypertrophic and fibrotic remodeling of the fish ventricle. *Front. Physiol.* **6**, 427.
- Keen, A. N., Klaiman, J. M., Shiels, H. A. and Gillis, T. E. (2017). Temperature-induced cardiac remodelling in fish. *J. Exp. Biol.* **220**, 147–160.
- Klaiman, J. M., Fenna, A. J., Shiels, H. A., Macri, J. and Gillis, T. E. (2011). Cardiac remodeling in fish: strategies to maintain heart function during temperature change. *PLoS One* **6**, e24464.
- Klaiman, J. M., Pyle, W. G. and Gillis, T. E. (2014). Cold acclimation increases cardiac myofilament function and ventricular pressure generation in trout. *J. Exp. Biol.* **217**, 4132–4140.
- Kohei, K., Yamazaki, M., Honda, T., Goto, K.-I., Moriya, H. and Fujimura, S. (1997). Effect of insulin-like growth factor 1 and basic fibroblast growth factor on DNA synthesis and collagen production in cultured anterior cruciate ligament cells. *J. Orthop. Sci.* **2**, 349–356.
- Kolosova, I., Nethery, D. and Kern, J. A. (2011). Role of Smad2/3 and p38 MAP kinase in TGF-beta1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J. Cell Physiol.* **226**, 1248–1254.
- Kubota, M., Kinoshita, M., Takeuchi, K., Kubota, S., Toyohara, H. and Sakaguchi, M. (2003). Solubilization of type I collagen from fish muscle connective tissue by matrix metalloproteinase-9 at chilled temperature. *Fish. Sci.* **69**, 1053–1059.
- Lee, L. E., Caldwell, S. J. and Gibbons, J. (1997). Development of a cell line from skin of goldfish, *Carassius auratus*, and effects of ascorbic acid on collagen deposition. *Histochem. J.* **29**, 31–43.
- Lee, A. A., Delhaas, T., McCulloch, A. D. and Villarreal, F. J. (1999). Differential responses of adult cardiac fibroblasts to *in vitro* biaxial strain patterns. *J. Mol. Cell. Cardiol.* **31**, 1833–1843.
- Lee, K.-S., Hong, S.-H. and Bae, S.-C. (2002). Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-beta and bone morphogenetic protein. *Oncogene* **21**, 7156–7163.
- Li, Y. Y., Feng, Y., McTiernan, C. F., Pei, W., Moravec, C. S., Wang, P., Rosenblum, W., Kormos, R. L. and Feldman, A. M. (2001). Downregulation of matrix metalloproteinases and reduction in collagen damage in the failing human heart after support with left ventricular assist devices. *Circulation* **104**, 1147–1152.
- Li, J., Schwimmbbeck, P. L., Tschope, C., Leschka, S., Husmann, L., Rutschow, S., Reichenbach, F., Noutsias, M., Kobalz, U., Poller, W. et al. (2002). Collagen degradation in a murine myocarditis model: relevance of matrix metalloproteinase in association with inflammatory induction. *Cardiovasc. Res.* **56**, 235–247.
- Lijnen, P. J., Petrov, V. V., Fagard, R. H. (2003). Association between transforming growth factor- $\beta$  and hypertension. *Am. J. Hypertens.* **16**, 604–611.
- Lurman, G. J., Petersen, L. H. and Gamperl, A. K. (2012). *In situ* cardiac performance of Atlantic cod (*Gadus morhua*) at cold temperatures: long-term acclimation, acute thermal challenge and the role of adrenaline. *J. Exp. Biol.* **215**, 4006–4014.
- Mackenna, D. A., Dolfi, F., Vuori, K. and Ruoslahti, E. (1998). Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J. Clin. Invest.* **101**, 301–310.
- Murad, S., Grove, D., Lindberg, K. A., Reynolds, G., Sivarajah, A. and Pinnell, S. R. (1981). Regulation of collagen synthesis by ascorbic acid. *Proc. Natl. Acad. Sci. USA* **78**, 2879–2882.
- Murad, S., Tajima, S., Johnson, G. R., Sivarajah, S. and Pinnell, S. R. (1983). Collagen synthesis in cultured human skin fibroblasts: effect of ascorbic acid and its analogs. *J. Invest. Dermatol.* **81**, 158–162.
- Overall, C. M., Wrana, J. L. and Sodek, J. (1991). Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *Biol. Chem.* **266**, 14064–14071.
- Pan, X., Chen, Z., Huang, R., Yao, Y. and Ma, G. (2013). Transforming growth factor beta1 induces the expression of collagen type I by DNA methylation in cardiac fibroblasts. *PLoS One* **8**, e60335.
- Peterkofsky, B. (1972). The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* **152**, 318–328.
- Reed, M. J., Vernon, R. B., Abrass, I. B. and Sage, E. H. (1994). TGF-beta 1 induces the expression of type I collagen and SPARC, and enhances contraction of collagen gels, by fibroblasts from young and aged donors. *J. Cell Physiol.* **158**, 169–179.
- Saito, M., Takenouchi, Y., Kunisaki, N. and Kimura, S. (2001). Complete primary structure of rainbow trout type I collagen consisting of alpha1(I)alpha2(I)alpha3(I) heterotrimers. *Eur. J. Biochem.* **268**, 2817–2827.
- Sappino, A. P., Schürch, W. and Gabbiani, G. (1990). Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.* **63**, 144–161.
- Seelard, U., Haeuselner, C., Hinrichs, R., Rosenkranz, S., Pfitzner, T., Scharffetter-Kochanek, K. and Bohm, M. (2002). Myocardial fibrosis in transforming growth factor-beta(1) (TGF-beta(1)) transgenic mice is associated with inhibition of interstitial collagenase. *Eur. J. Clin. Invest.* **32**, 295–303.
- Shih, H., Lee, B., Lee, R. J. and Boyle, A. J. (2011). The aging heart and post-infarction left ventricular remodeling. *J. Am. Coll. Cardiol.* **57**, 9–17.
- Siwik, D. A., Pagano, P. J. and Colucci, W. S. (2001). Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. *Am. J. Physiol.* **280**, 53–60.
- Sun, Y., Zhang, J. Q., Zhang, J. and Ramires, F. J. A. (1998). Angiotensin II, transforming growth factor- $\beta$ 1 and repair in the infarcted heart. *J. Mol. Cell. Cardiol.* **30**, 1559–1569.



- Takahashi, N., Calderone, A., Izzo, N. J., Jr, Mäki, T. M., Marsh, J. D. and Colucci, W. S.** (1994). Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. *J. Clin. Invest.* **94**, 1470-1476.
- Tamamori, M., Ito, H., Hiroe, M., Marumo, F. and Hata, R. I.** (1997). Stimulation of collagen synthesis in rat cardiac fibroblasts by exposure to hypoxic culture conditions and suppression of the effect by natriuretic peptides. *Cell Biol. Int.* **21**, 175-180.
- Tan, B., Pascual, A., de Beus, A., Cooper, K. and Hull, E.** (2011). TGFbeta (transforming growth factor beta) and keratocyte motility in 24 h zebrafish explant cultures. *Cell Biol. Int.* **35**, 1131-1139.
- Thomas, P. J., Qu, B.-H. and Pedersen, P. L.** (1995). Defective protein folding as a basis of human disease. *Trends Biochem. Sci.* **20**, 456-459.
- Van Obberghen-Schilling, E., Roche, N. S., Flanders, K. C., Sporn, M. B. and Roberts, A. B.** (1988). Transforming growth factor  $\beta$ 1 positive regulates its own expression in normal and transformed cells. *Biol. Chem.* **263**, 7741-7746.
- Verrecchia, F. and Mauviel, A.** (2007). Transforming growth factor-beta and fibrosis. *World J. Gastroenterol.* **13**, 3056-3062.
- Verrecchia, F., Chu, M.-L. and Mauviel, A.** (2001). Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J. Biol. Chem.* **276**, 17058-17062.
- Visse, R. and Nagase, H.** (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* **92**, 827-839.
- Vornanen, M., Hassinen, M., Koskinen, H. and Krasnov, A.** (2005). Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. *Am. J. Physiol.* **289**, R1177-R1184.
- Weber, K. T., Clark, W. A., Janicki, J. S. and Shroff, S. G.** (1987). Physiologic versus pathologic hypertrophy and the pressure-overloaded myocardium. *J. Cardiovasc. Pharmacol.* **10** Suppl. 6, S37-S50.
- Zeisberg, E. M., Tarnavski, O., Zeisberg, M., Dorfman, A. L., McMullen, J. R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W. T., Roberts, A. B. et al.** (2007). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat. Med.* **13**, 952-961.