

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

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**Summary:** TGF- $\beta$ 1 causes cultured trout cardiac fibroblasts to increase collagen synthesis suggesting that this cytokine may play a role in regulating the collagen content of the trout heart during thermal acclimation

## 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 remodeling 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 h and 72 h and an increase in collagen type I protein after 7 d. 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  $\alpha$  I (*colla1*) 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 suggests that this cytokine may play a role in regulating collagen content in the trout heart during thermal acclimation.

**KEYWORDS:** Cardiac fibroblasts, collagen matrix, transforming growth factor beta-1, tissue remodeling

## Introduction

Rainbow trout remain active in waters where temperature can change seasonally between 4 °C and 25 °C. Such change in temperature represents a significant challenge to maintaining normal physiological processes due to 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 hearts capacity to support the oxygen requirements of the animal. For example, (Klaiman et al., 2014) has demonstrated that the magnitude and rate of ventricular pressure generation was greater in hearts from trout acclimated to 4°C compared with 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 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 remodeling 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 is maintained (Weber et al., 1987). In contrast, pathological remodeling, 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; Lee et al., 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 (Hillegass 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 signaling 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 remodeling such as that which occurs after myocardial infarction (Bujak et al., 2007) or with 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 remodeling (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 (*coll1*; 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 if 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 are coded by a separate collagen transcript (*colla1*, *colla2* and *colla3*) (Saito et al., 2001). Expression of the trout *colla3* has been found to be up regulated 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 for *colla1* in response to short-term TGF- $\beta$ 1 signaling (Bauge 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 if 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*. It is predicted that TGF- $\beta$ 1 treatment will cause an increase in collagen production and that this change will 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 (1661±166 g) were obtained from Alma Aquaculture Research Centre (Alma, ON) 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 12:12 cycle. Fish were fed a maintenance diet of Martin Mills (Elmira, ON) floating feed on alternating 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 mM: 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). 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-ethylenediaminetetraacetic acid (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 spent 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), a reliable marker of fibroblastic cells (Sappino et al., 1990). Since 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). This antibody was validated using immunohistology with whole trout muscle tissue preparations (supplemental figure 1). 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 (supplemental figure 2). Of the 27 trout ventricles used to establish cell lines, only 7 lines grew successfully. Cell lines mRTV2, 9, 13, 17, 19, 21 and 22 (male rainbow trut 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 responses detected would be in response to the treatment (Hayes et al., 2005).

### *Transforming growth factor-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  $\sim 8 \times 10^3$  cells per  $\text{cm}^2$ .  $15 \text{ ng} \cdot \text{ml}^{-1}$  human recombinant TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) in a solution of 4 mM HCl + 0.5% BSA was added to fresh medium and neutralized with the addition of an equimolar solution of 4 mM NaOH. Cells were then incubated for 24 h, 48 h, 72 h, or 7 d 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) before pelleting.

### *Cellular proliferation*

Fibroblasts from three separate cell lines (mRTV 17, 21 and 22) were plated into 24-well plates at a density of  $3 \times 10^4$  cells  $\text{ml}^{-1}$  in 500  $\mu\text{l}$  whole medium and allowed to attach overnight at room temperature. After serum starving, half of the cells were treated with  $15 \text{ ng} \cdot \text{ml}^{-1}$  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°C overnight and pelleted at 1710  $g$  and the supernatant was reserved then air-dried. Once dry, 300  $\mu\text{L}$  6N HCl was added to each sample and hydrolyzed at 135 °C for 3 h, followed by air-drying. Samples were reconstituted in 200  $\mu\text{L}$  distilled water, centrifuged at 9 000  $g$ , and then 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 if 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 treatment 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  $\mu\text{g}\cdot\text{ml}^{-1}$  (Lee et al., 1997). 15  $\text{ng}\cdot\text{ml}^{-1}$  TGF- $\beta$ 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 d. The cell culture media used in these experiments was pH buffered and contained a pH sensitive dye. The addition of the L-AA did not alter the color 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% Tween20 (PBS-T) (Fisher, City, ON) 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) was added at a dilution factor of 1:500 in blocking buffer and incubated overnight at 4 °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  $\mu\text{g}\cdot\text{ml}^{-1}$  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 color digital camera (Q-Imaging) using identical acquisition settings.

#### *Measurement of collagen type I in fibroblasts using Western blotting*

Fibroblasts from 3 separate cell lines were grown in 75 cm<sup>2</sup> flasks and treated with 15 ng·ml<sup>-1</sup> TGF- $\beta$ 1, L-AA or solvent solution. 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 d 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  $\mu\text{M}$  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 °C. Lysate was then centrifuged for 20 min at 20 000 g, then supernatant was reserved and measured for protein content with a bicinchoninic acid (BCA) assay (Bio-Rad). 20  $\mu\text{g}$  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 polyvinylidene fluoride membrane with wet transfer at 30 V for 16 h at 4 °C, blocked in 5% skim milk powder in tris-buffered saline with 0.1% Tween20 (TBST) (hereafter referred to as blocking buffer) for 1 h at room temperature then incubated in 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 manufacturer's instructions to induce chemiluminescence, which was subsequently visualized with the ChemiDoc MP. 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 membranes were compared to 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) (supplemental figure 3).

#### *Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

The transcript abundances of genes associated with connective tissue regulation were quantified in cardiac fibroblasts after 24 h, 48 h, and 72 h incubation with TGF- $\beta$ 1 or solvent control. Total RNA was extracted from fibroblasts using Trizol (Life Technologies) according to manufacturer's instructions and quantified using a Nanodrop 8000 (ThermoFisher Scientific, Ottawa, ON). One microgram of total RNA was treated with DNase I (Sigma) and used to synthesize cDNA with the High Capacity cDNA Synthesis Kit (Life Technologies) following 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 abundances were measured in duplicate reactions on a StepOnePlus Real-Time PCR Instrument (Life Technologies) using default cycling conditions and a dissociation cycle. Each 15  $\mu$ l reaction contained 1 x PerfeCta Fast SYBR Green Master Mix (Quanta BioSciences, Gaithersburg,

MD), 200 nM of each gene-specific primer (Table 1) and 1:15 vol:vol cDNA. Custom oligos for *coll1a1*, *timp-2*, *mmp-2*, *tgf $\beta$ r* and *tgf- $\beta$ 1* 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 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, 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 analyzed with a non-parametric test. Differences in hydroxyproline content between control and TGF- $\beta$ 1 treated cells at all times points were analyzed with a two-way ANOVA with a post-hoc Bonferroni correction at 95% significance cutoff. Differences in transcript abundance at each time point were tested with an unpaired student's t-test at 95% cutoff, and data is 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 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 to controls after 48 h 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 (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 d ( $p < 0.06$ ; Fig. 2). In addition, the levels of collagen type 1 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 ascorbic acid and TGF- $\beta$ 1*

There was no evidence of collagen fibril assembly in the ECM of fibroblasts at 24 h and 72 h, regardless of treatment (data not shown); however, by 7 d 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 being 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 time- and gene-specific. After 24 h of TGF- $\beta$ 1 treatment, the transcript abundances of *timp-2* and *mmp-9* were 7.8- and 10.9-fold greater, respectively, than in un-treated 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 *colla1* was 7.4-fold greater compared to 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, including an increase in hydroxyproline content of cells measured in the cells at 48 h and 72 h, and the increase in collagen type I quantified with immunoblotting at 7 d 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 demonstrate 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 demonstrated that L-AA is a co-factor for the extra cellular 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 the co-treatment of the fibroblasts with L-AA and TGF- $\beta$ 1 in the current study is demonstrating the same phenomenon. The treatment of the fibroblasts with L-AA alone did not stimulate collagen production, as demonstrated in figure 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 remodeling. 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 signaling cascade and leads to changes in the expression of transcripts for MMPs and TIMP-2 in cardiac fibroblasts (Husse et al., 2007). While the role 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 role 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 that 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). Such 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. (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 hours 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 *coll1a1* in the current study may indicate that preserving existing ECM collagen is prioritized to synthesizing new fibers. 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; Lee et al., 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, since biological effects were apparent even with this limitation, we are confident that endogenous TGF- $\beta$ 1 plays a role in ECM remodeling 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., (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-signaling 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 regulating the expression of genes involved in cardiac remodeling and regulating collagen deposition (Katsumi et al., 2004). These include collagen type I  $\alpha$ 1 (*colla1*), *timp-2*, *mmp-2*, *mmp-9* and *mmp-13* (Husse et al., 2007; Lee et al., 1999; Lee et al., 2002; Li et al., 2001). This response of the myocardium to stretch is potentially relevant to identifying what triggers cardiac remodeling in the trout heart. Previous authors have suggested that the trigger for cardiac remodeling 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 remodeling (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 remodeling 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, 1991 #120;Keen, 2016 #4365}. 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.

### *Author Contributions*

EFJ and TEG conceived and designed the project. EFJ completed the experiments and analyzed the data. EFJ and TEG wrote the manuscript.

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*Disclosures:* None

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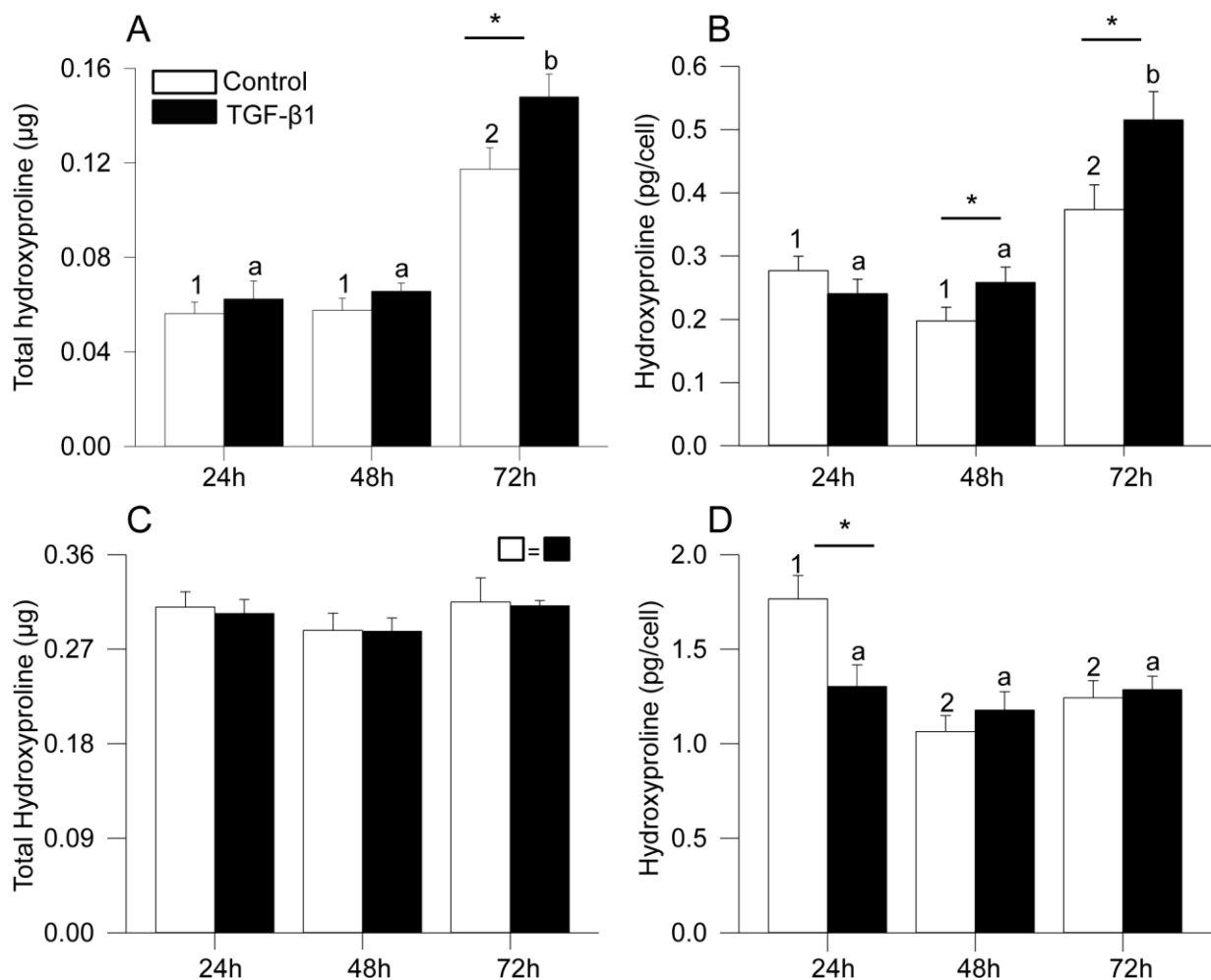
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**Table 1:** Forward and reverse primer sequences used in quantitative real-time PCR to amplify rainbow trout transcripts for matrix metalloproteinase 2 and 9 (*mmp-2* and *mmp-9*), the tissue inhibitor of metalloproteinase (*timp-2*), and collagen type Ia1 (*colla1*). All reactions were run at an annealing temperature of 60 °C.

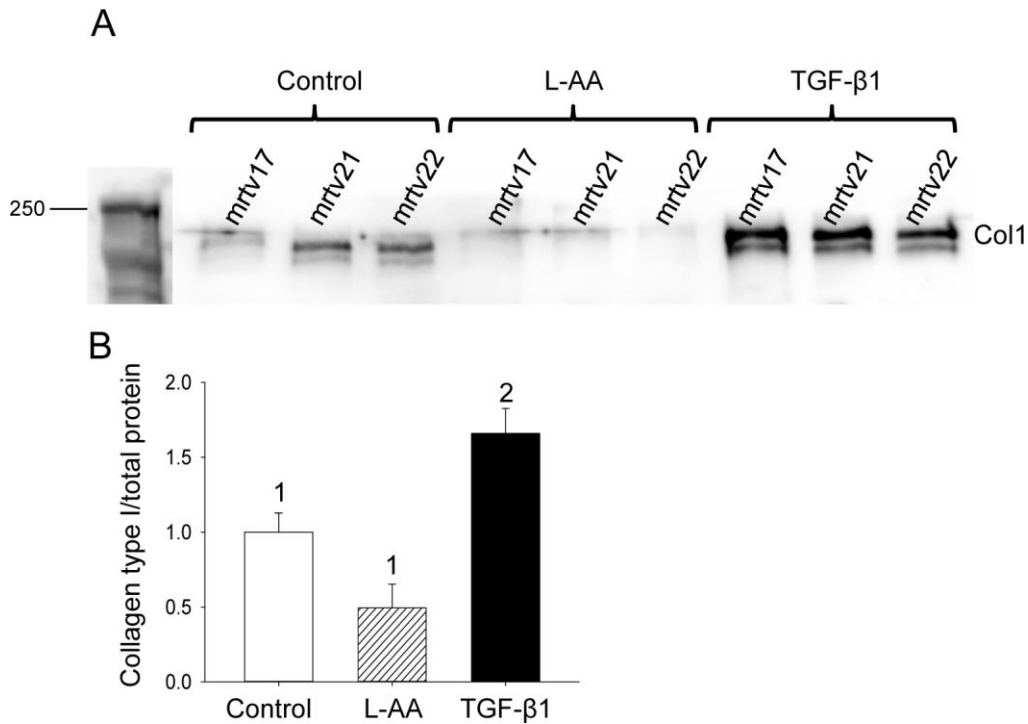
Gene	Sequence (5'-3')	Amplicon (bp)	Accession	Efficiency (%)	R <sup>2</sup>
<i>efla</i>	F: GGGCAAGGGCTCTTTCAAGT R: CGCAATCAGCCTGAGAGGT	188	AF498320	81	0.99
<i>timp-2</i>	F: ACATTTTCCCTCCACGCGAT R: TTCTGCGATGTCTTCCACCC	146	AY606030.1	80	0.99
<i>mmp-2</i>	F: AGACGCATAGACGCTGGCTAA R: GCAGGATAGGCTGGTTGGATAG	78	AB021698.1	84	0.99
<i>mmp-9</i>	F: ACCCCTTCGATGGTAAGGAC R: GGTCCAGTTTTTCGTCATCGT	98	AJ320533.1	82	0.99
<i>colla1</i>	F: CCCGAGCCATGCCAGAT R: CGGATGTGTCCTCGCAGATAA	76	NM_001124177.1	80	0.99

## Figures



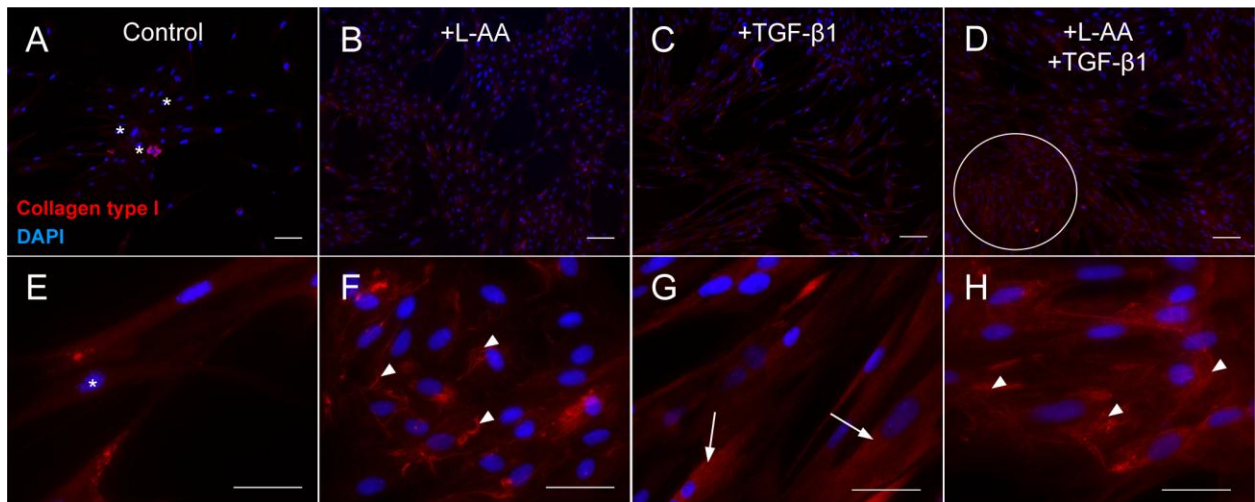
**Figure 1: The effect of TGF-β1 on collagen production and release from cultured trout cardiac fibroblasts.** Panels A and C demonstrate absolute hydroxyproline in (A) cell pellets or (C) culture medium after 24h, 48h and 72h of TGF-β1 treatment. Panels B and D display the average amount of hydroxyproline produced per cell (pg/cell) after 24 h, 48 h and 72 h of TGF-β1 treatment. Numbers indicate a significant effect of time on the amount of hydroxyproline produced within control cells ( $p < 0.05$ ). Letters indicate a significant effect of time on the amount of hydroxyproline produced per cell in the TGF-β1 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 ECM data,  $n = 3$  for cell medium data where each  $n$  represents a separate cell line established from a single heart from different fish, and each  $n$  contains 8-15 technical replicates.

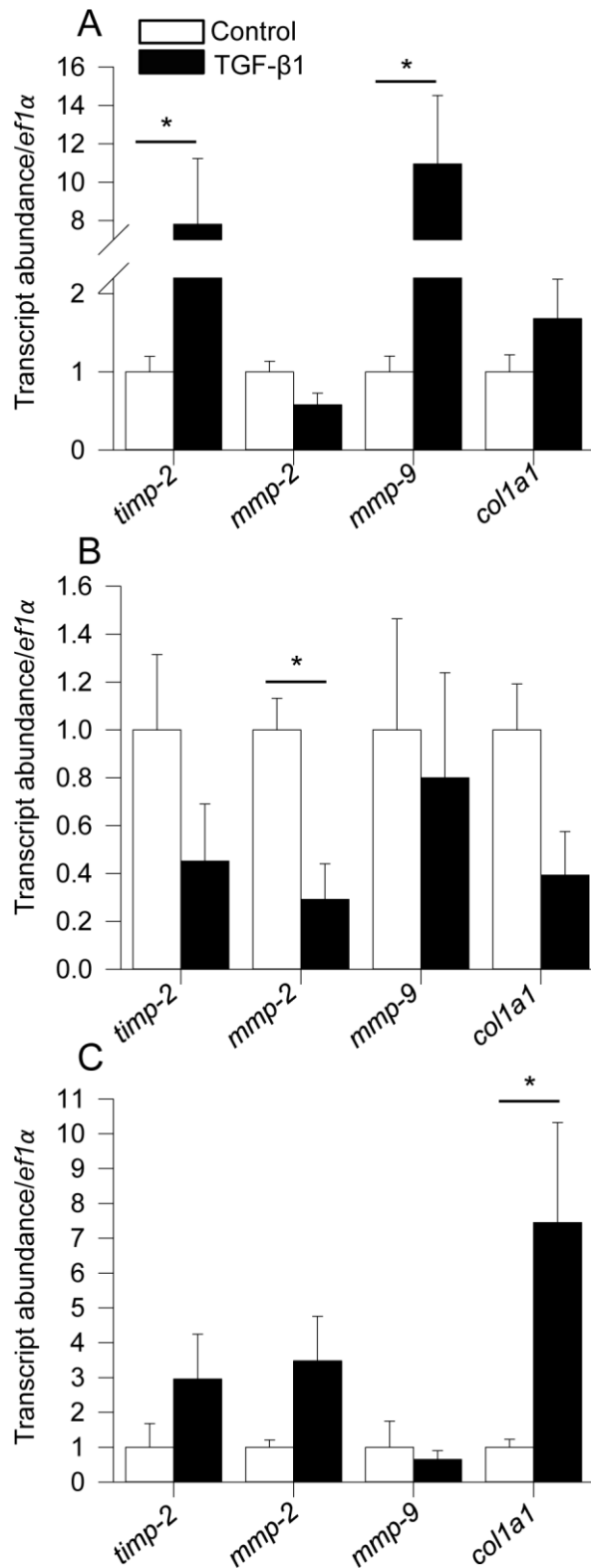


**Figure 2: The effect of TGF-β1 or L-AA treatment on collagen type 1 deposition in cultured cardiac fibroblasts.** Cardiac fibroblast cultures were treated with medium alone (control), or medium containing either L-ascorbic acid or TGF-β1 for 7 d, and collagen type 1 was quantified by Western blot and densitometry. (A) Western blot image for collagen type 1 in fibroblasts from 3 different cell lines, detected using rabbit polyclonal anti-salmon collagen type I. The first lane is the weight ladder imaged at a different wavelength. (B) Mean collagen type 1 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 ( $n = 3$ ,  $p < 0.06$ ).



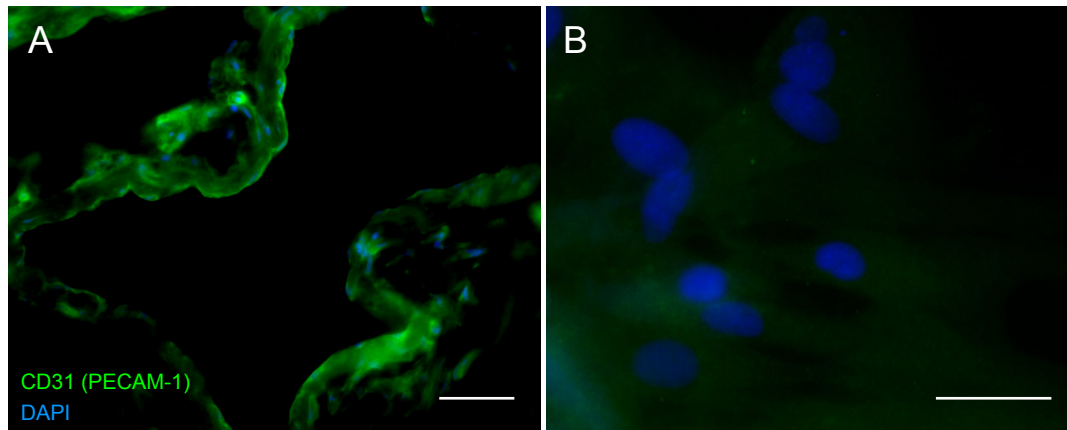


**Figure 3: The effect of ascorbic acid and TGF- $\beta$ 1 on collagen type I production and assembly.** Collagen type I assembly in response to TGF- $\beta$ 1 treatment was assessed by observing the appearance of fibrils after 7d of treatment to TGF- $\beta$ 1 only (C, G), L-AA only (B, F), TGF- $\beta$ 1 and L-AA simultaneously (D, H), as well as a control group (A, D). Cells were stained with polyclonal rabbit anti-salmon collagen type I and counterstained with DAPI. Panels A-D are at 100 x magnification and panels E-H are the same cells as above but at 600 x magnification. 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, full arrows point at areas of high collagen staining in TGF- $\beta$ 1 treated cells that would contribute to the overall increased collagen signal apparent at 100 x magnification, 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 the 100 x images this was 1.5 s for collagen (red) and for the 600 x images, exposure time was 300 ms. Scale bars equal 50  $\mu$ m for 100 x and 20  $\mu$ m for 600 x. n=3.

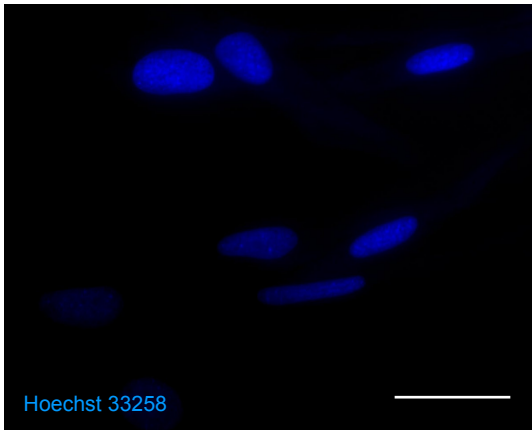


**Figure 4: The effect of TGF- $\beta$ 1 treatment on the expression of genes involved in ECM regulation in cultured trout cardiac fibroblasts.** Panels A, B and C show the transcript abundance for control and TGF- $\beta$ 1 treated cells at 24 h, 48 h and 72 h post-treatment,

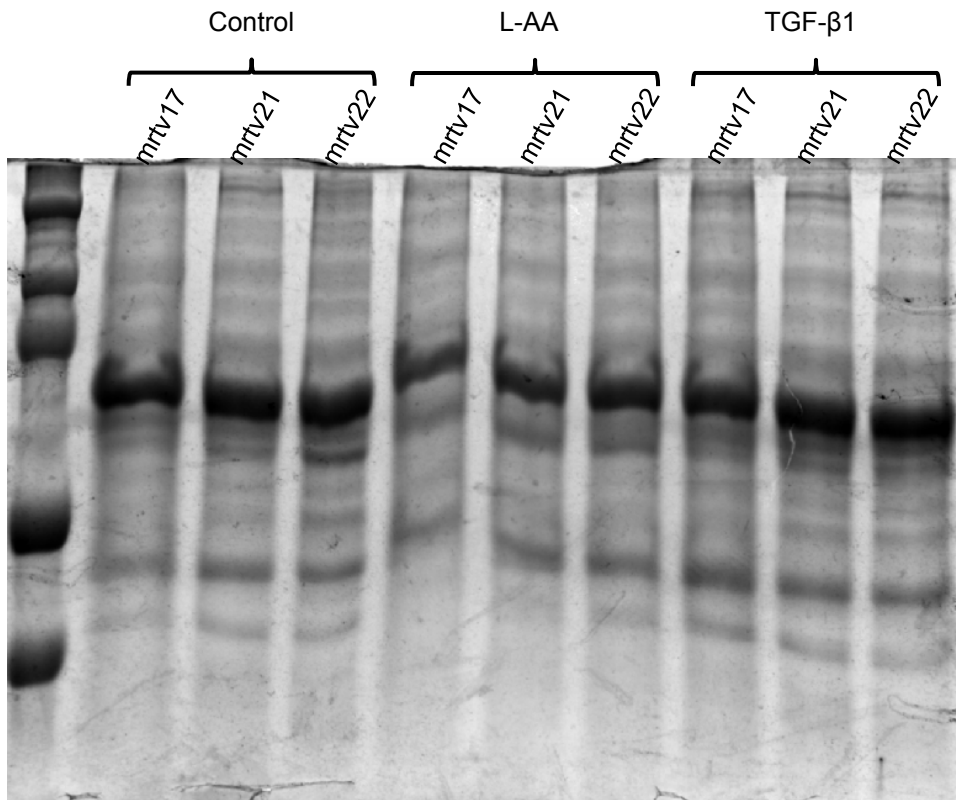
respectively. The amount of transcript in each TGF- $\beta$ 1 group is relative to the control group, which is set to 1 in each panel. mRNA level for each gene was normalized to the transcript abundance of *elongation factor 1 alpha (EF1 $\alpha$ )*. \*, a significant effect of TGF- $\beta$ 1 treatment on gene expression ( $p < 0.05$ ). n = 3-5.



**Supplemental figure 1: Validation of fibroblast cell origin.** Tissue from sectioned rainbow trout muscle tissue (A) and cultured cardiac fibroblasts (B) stained with CD-31 antibody to determine presence or absence of endothelial cells. Muscle tissue is presented at 200 x magnification whereas cells are imaged at 600 x. Cells did not give a distinct CD-31 signal indicating the lack of endothelial cell marker presence. Scale bars = 50  $\mu\text{m}$  (A), 20  $\mu\text{m}$  (B).



**Supplemental figure 2: Mycoplasma test.** Representative image of cultured fibroblasts cells stained with Hoechst 33258 stain for mycoplasma. Image taken at 600 x water immersion. All lines were tested and none were positive for mycoplasma (no nucleated particles surrounding fibroblast nucleus).



**Supplemental figure 3: Total protein stained by coomassie blue used to standardize collagen type I.** Normalizing gel was run at the same time as gel used for Western blotted membrane with identical volumes of protein from the same sample tubes.