Norwegian University of Life Sciences

Department of Animal and Aquacultural Sciences

Master Thesis 2015
60 credits

Optimizing methods for isolation and *in vitro* cultivation of primary cardiomyocytes from Atlantic salmon (*Salmo salar* L.)

A study of cellular responses to hydrogen peroxide, lipo-polysaccharide and docosahexaenoic acid

KEHAN YI

谨以此论文献给我的父母

This thesis I dedicate to my parents

Acknowledgements

Abstract

Atlantic salmon (*Salmo salar* L.) has been the most produced species in Norway. However, production related losses remain significant recent years, and a large proportion of this were due to viral diseases. Among these diseases, PD, HSMI, and CMS, commonly affect the heart of farmed salmon. Therefore, the primary goal of this work was to establish a salmon cardiomyocyte culture system that enabling us to carry out further studies on how salmon cardiomyocytes react to different stimulus.

In the first study, we tested two different isolation methods with two different cell culture media, the combination of collagenase and trypsin was proven to be the better one in cell isolation than trypsin alone. And then, in the time study, heart cells were harvested at day 2, 3, 5, 7, 9, 11, respectively to analyze the expression level of several gene markers, the significantly increased expression of PCNA, CAV3, and GATA4 indicates that primary cultured salmon cardiomyocytes have strong abilities to proliferate and differentiate *in vitro*. Also, we found that the seeding density is important for good proliferation and development of cardiomyocytes in culture. Besides, to learn the morphological changes of cardiomyocytes in culture with time, cells were cultured up to 60 days, spontaneously beating cell structures were observed during this period, and some of these structures even kept contracting over 5 weeks.

Then, we tried to increase cell proliferation by either adding growth factor to the culture media or improving the coating materials. No positive effect was observed on cell proliferation towards supplementation of 125 ng/mL bFGF, but it seems possible to increase cell yield by improving the coatings, since ECL, fibronectin, and ECM coating all performed better than laminin.

Finally, we studied the stress responses of cardiomyocytes to H_2O_2 and the effects of DHA on LPS induced immune response. Cardiomyocytes from three experimental groups were incubated with 100 μM H_2O_2 for 30, 60, and 90 min, respectively. Our

results suggest that cardiomyocytes responded to H_2O_2 by up-regulating SOD1 and GPx-3, and a 30 min incubation may even result in hypertrophic growth in salmon cardiomyocytes. However, no strong immune reaction was observed when we stimulated cardiomyocytes with 100 ng/mL LPS, and salmon cardiomyocytes responded little to DHA as well, only with significantly increased expression of SOD1 among all the gene markers tested.

Table of Contents

Abbreviations

BDM	2,3-Butanedione monoxime
bFGF	Fibroblast growth factor-basic
BSA	Bovine serum albumin
CAT	Catalase
CAV	Caveolin
cDNA	Complementary DNA
CMS	Cardiomyopathy syndrome
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBs	Embryoid bodies
ECL	Entactin-collagen IV-laminin
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EPA	Eicosapentaenoic acid
ESC	Embryonic stem cell
FAs	Fatty acids
FBS	Fetal bovine serum
GATA	GATA binding protein
GPx	Glutathione peroxidase
H	Hepatocyte growth media
HSMI	Heart and skeletal muscle inflammation
HSP70	Heat shock 70 kDa protein
H_2O_2	Hydrogen peroxide
IL	Interleukin
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anaemia

Iso1	Isolation method 1
Iso2	Isolation method 2
LNA	Linolenic acid
LPS	Lipopolysaccharide
M	Muscle cell growth media
MEF	Myocyte enhancer factor
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
MX1	Myxovirus resistance 1, interferon-inducible protein
Nkx2-5	NK2 homeobox 5
Nrf2	Nuclear factor erythroid 2-related factor 2
PCNA	Proliferating cell nuclear antigen
PBS	Phosphate buffered saline
PD	Pancreas disease
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Reverse transcriptase
Stat1	Signal transducer and activator of transcription 1
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
α	Alpha
β	Beta

1 INTRODUCTION

1.1 Atlantic salmon farming

Atlantic salmon (*Salmo salar* L.) farming was established in Norway in the early 1970s and has experienced remarkable growth in the last few decades. In 2013, the first-hand value of Norwegian fish farming reached NOK 40 billion, up 35% from 2012, and the produced quantity was 1.25 million tonnes. Atlantic salmon has been the most produced species, which contributed 93.6% to the total production in 2013, as shown in Fig. 1 (http://www.ssb.no/en/jord-skog-jakt-og-fiskeri).
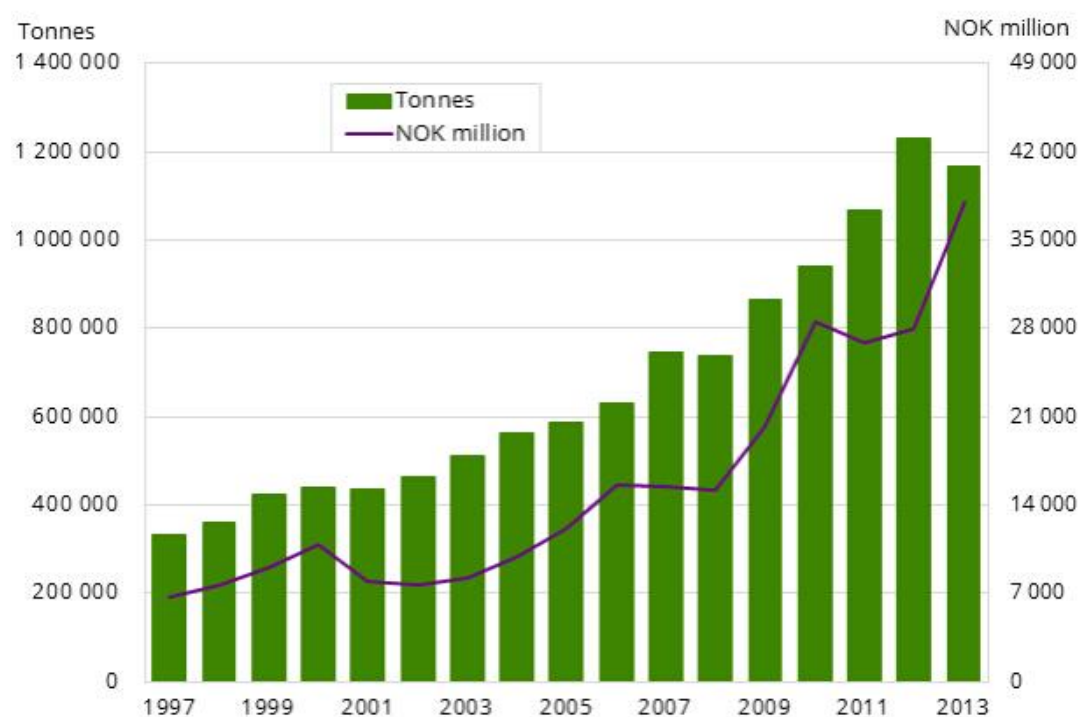
Fig. 1. Sales of salmon quantity and first hand value from 1997 to 2013. Source: Statistics Norway

While the amount of salmon production raises continuously, the production related losses remain significant. The mortality rate fluctuated between 10.6% and 13.2% from 2006 to 2011, with an annual average of 11.8%. In year 2012, a decline in

mortality rate was observed (8.2%), according to Statistics Norway (http://www.ssb.no/204971/fish-farming.stock-and-loss-of-fish-due-to-escape-and-disease.salmon-and-rainbow-trout.1-000-sy-374), this may due to genetically improved brood stock as well as improved hygiene during the hatchery phase (Johansen, 2013). However, disease continues to represent one of the most important challenges to the aquaculture industry and viral diseases continue to dominate the national situation (Johansen, 2013). The most serious viral diseases are infectious salmon anaemia (ISA), pancreas disease (PD), infectious pancreatic necrosis (IPN), heart and skeletal muscle inflammation (HSMI), and cardiomyopathy syndrome (CMS).

1.2   Heart related diseases

Heart is considered as the powerhouse of the cardiovascular system, and the normal function of heart is essential for the function of other organs. But three of these viral diseases, PD, HSMI, and CMS, commonly affect the heart of farmed salmon (Yousaf *et al*, 2013).
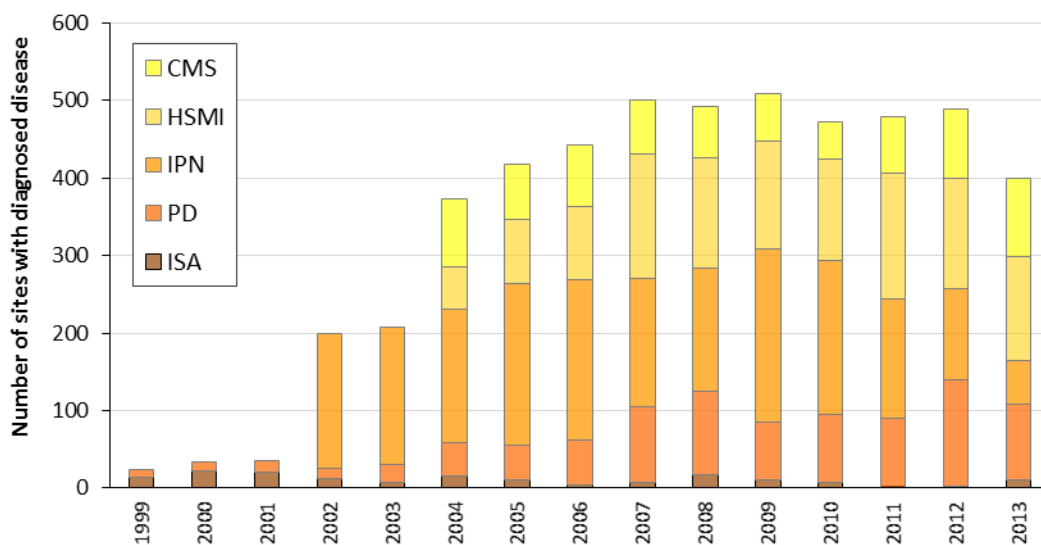
Fig. 2.   Number of diagnosed farm sites with different viral diseases (compiled from Johansen, 2013; Hjeltnes, 2014).

From the annual report published by the Norwegian Veterinary Institute (Fig. 2), we can see that PD is always the most serious disease in Norwegian aquaculture; statistics for HSMI and CMS were included in 2004, and since then, the total number of fish sites that affected with PD, HSMI and CMS kept at a relatively high level, which indicates the heart health may play an important role in protecting against these viral diseases.
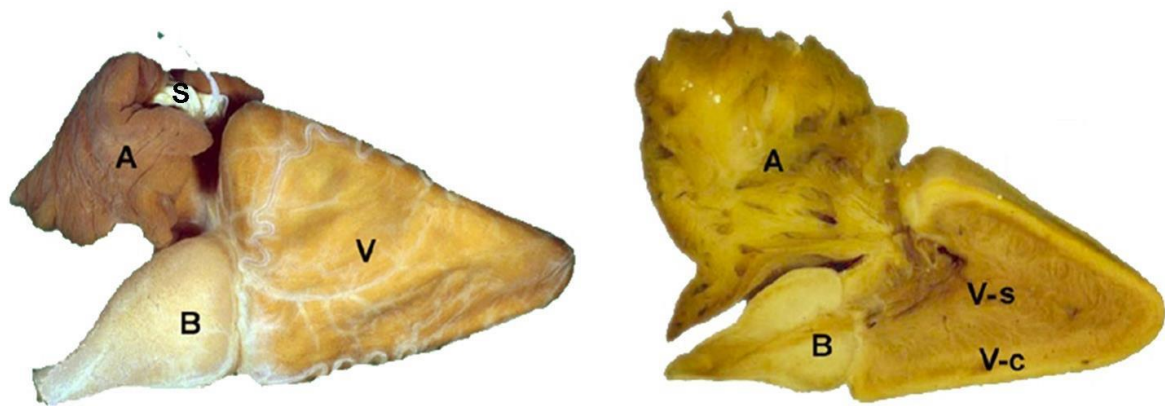
Fig. 3. The Atlantic salmon heart has one atrium and one ventricle. Venous blood flows through the sinus venosus (*S*, a thin walled sac) into the atrium (*A*), and then flows into the ventricle (*V*) and leaves the heart through the bulbus ateriosus (*B*) to the gills and then to the rest of the body. The picture on the right shows the sagittal section of Atlantic salmon heart. The ventricular myocardium of salmonids has two distinct layers. The outer layer, compact myocardium, is marked with "*V-c*", while the inner layer, spongy myocardium, is marked with "*V-s*". Photo: Trygve T. Poppe

In year 2010, a novel virus of *Totiviridae* family, which named piscine myocarditis virus (PMCV), was identified to be the most likely causative agent for CMS (Haugland *et al*., 2011). Fish with CMS have significant inflammatory lesions in the spongy layer of both the atrium and ventricle (Fig. 3), while the compact muscle

layers of the ventricle are, as a rule, normal (Hjeltnes, 2014). HSMI was linked to a reovirus, with a proposed name piscine reovirus (PRV) (Palacios *et al*., 2010). For fish infected with PRV, heart is the primary organ affected, and the main symptoms are epi-, endo-, and myocarditis and myocardial necrosis (Fig. 4), as well as necrosis of red skeletal muscle, indicating that these tissues experience a severe inflammatory process (Martinez-Rubio *et al*., 2013). PD is an extremely infectious disease of farmed Atlantic salmon caused by salmonid alphavirus (SAV). A SAV infection in salmon can lead to severe damage of pancreas, followed by pathology in the cardiac tissue and skeletal muscle. The myopathy of the heart tissue can be similar with HSMI and CMS.
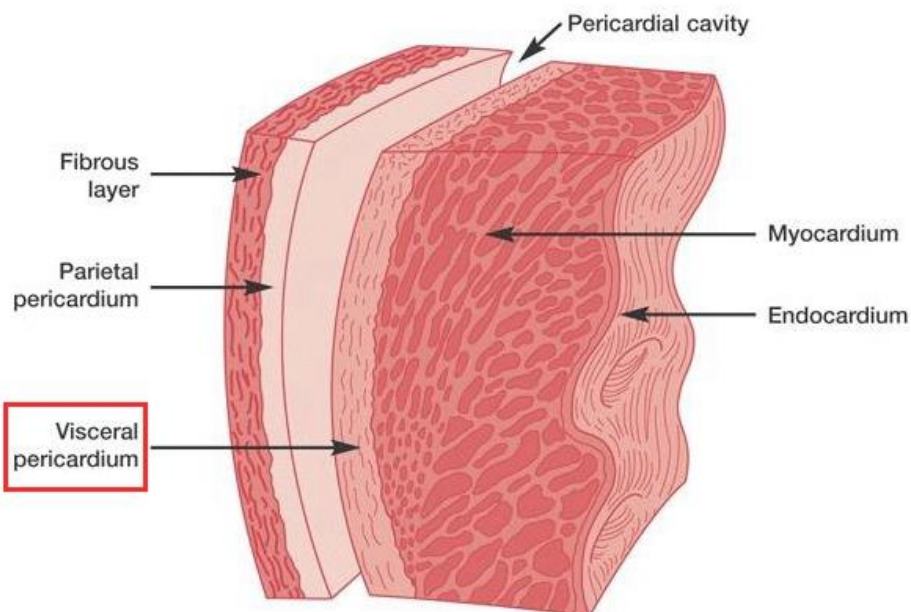
Fig. 4. Structure of the heart wall. The pericardium is a double-walled sac that containing the heart and the root of great vessels. The visceral layer is also known as epicardium. During ventricular contraction, the wave of depolarization moves from the endocardium to the epicardial surface (Picture adapted from: https://drsvenkatesan.wordpress.com/tag/epicardium/).

1.3 Cell culture systems

Cell culture is one of the major tools used in research within cellular and molecular biology, providing excellent model systems for studying the physiology and biochemistry of cells, the effects of different nutrients, drugs and toxic compounds. The term "cell culture" refers to the removal of cells from an organism and their subsequent growth under controlled environment. Cells isolated directly from animal tissues are known as primary cells and as soon as they are subcultured they become a cell line.

Cell lines have at least one passage. With each subsequent subculture, the cell population becomes more homogenous as the faster growing cells predominate. The use of continuous cell lines is cost effective and convenient, however, continuous cell lines may differ from the *in vivo* situation in many important aspects, since cell lines are either derived from tumors and have already lost key features of cell cycle control or they are immortalized by random mutation or deliberate modification.

The preparation of primary cultures is labor intensive, a mixture of different cell types is obtained from the isolation and they can be maintained *in vitro* only for a limited period of time (days to weeks). But primary cells provide the most comprehensive picture of normal cellular physiology and they are superior model of the *in vivo* situation. However, both the primary cultures and cell lines exclude the influence of other organs, and the influence of the circulatory and immune system (Sultan & Haagsman, 2001). Thus, it could be very challenging to extrapolate from the results of *in vitro* work back to the biology of the intact organism.

1.4 Cell types in the heart and growth of cardiomyocyte

Cardiac function is determined by the coordinated and dynamic interaction of several

cell types together with components of the extracellular matrix (ECM) (Banerjee *et al*., 2006).The cellular components of heart consist of cardiomyocytes, fibroblasts, and vascular cells (Borg & Baudino, 2011). Cardiac growth can be given by one or a mix of two mechanisms: cardiomyocyte hypertrophy and cardiomyocyte hyperplasia. The former refers to an enlargement of the cell, while the latter involves cell proliferation. Both of these two mechanisms are found in zebrafish with anemia induced cardiomegaly (Sun *et al*., 2009). In primary cell preparations from larvae of rainbow trout, cells were cultured with Dulbecco's modified eagle medium with 20% fetal calf serum. The formation of autonomously contracting cell aggregates were observed after 7 days and the morphology of the different contracting structures varied from ball shape (1 week after seeding) to tube shape (contracting structures emerged 1 month after subculture) (Grunow *et al*., 2010). Recently, Fuerstenau-Sharp *et al*. (2015) developed a novel protocol which combined the derivation of induced pluripotent stem (iPS) cells from blood-derived T lymphocytes with an optimized directed differentiation to cardiomyocytes. To start the subsequent differentiation, the culturing media was supplemented with certain growth factors and morphogens, including basic fibroblast growth factor (bFGF), activin A and bone morphogenic protein 4 (BMP-4), and different media replenishments were applied daily. Routinely, beating aggregates appeared from day 9 to 12 of differentiation during the cardiac differentiation of iPS cells.

1.5   Cardiac transcription factors

A transcription factor is a protein that binds to specific DNA sequences, they can regulate the expression of other genes in a tissue-specific and quantitative manner (Latchman, 1997; Bruneau, 2002). Cardiac transcription factors are essential transcriptional activators that expressed predominantly in the myocardium and regulating the expression of the cardiac genes encoding structural proteins or

regulatory proteins characteristic of cardiomyocytes (Akazawa & Komuro, 2003). Here is a brief description about five important cardiac transcription factors, GATA binding protein 4 (GATA4), NK2 homeobox 5 (Nkx2-5), myocyte enhancer factor 2C (MEF2C), α-myosin heavy chain (αMHC) and caveolin-3 (CAV3).

	Cardiac Structures	Cardiac Expression of Network Genes				
		NK2	MEF2	GATA	Tbx	Hand
Cnideria Hydra, jellyfish	Striated muscle cells associated with gastrodermis	1	1	?	1	0
Nematodes C. elegans	Contractile pharynx	1	1	2	1	0
Anthropods Drosophila	Tubular heart Open circulatory system	1	1	2	3	1
Urochordates or tunicates Ascidians	Tubular heart, striated Open circulatory system Bidirectional pumping	1	1	2	≥3	1
Cephalochordates Amphioxus	Tubular heart, non-striated Closed circulatory system Unidirectional pumping	1	1	2	?	1
Fish	Single atrium and ventricle	≥3	4	3	≥4	1
Amphibians	Two atria and one ventricle Pulmonary circulation	≥3	4	3	≥5	1
Amniotes Reptiles, birds, mammals	Four-chambered heart	≥2	4	3	≥7	2

Fig. 5. Evolution of the heart and the core cardiac transcription factors (Olson, 2006). The structures of the hearts of representative animals and their evolutionary relationships are shown. The numbers of cardiac regulatory genes, which are known to be expressed in the cardiac structures of each organism, are shown.

Transcription factor GATA4 belongs to a family of zinc finger proteins involved in regulating cell lineage differentiation during vertebrate development and six GATA transcription factors have been identified in vertebrates yet (Morrisey *et al.*, 1997; Simon, 1995). Three members of this family, GATA-4/5/6, are expressed in overlapping but distinct spatial and temporal patterns in the developing heart, and GATA4 and GATA6 continue expression in the adult heart (Peterkin *et al.*, 2005; Suzuki & Evans, 2004; Morrisey *et al.*, 1997).

GATA4 (Fig. 6) contains two distinct zinc finger domains (*Zn*) and a C-terminal nuclear localization sequence (*nls*, amino acid sequence that 'tags' a protein for import into the cell nucleus by nuclear transport) that together constitute the DNA binding and protein-protein interaction domain. GATA4 also contains two transcriptional activation domains (*TAD*) in the N-terminus (the start of a protein or polypeptide terminated by an amino acid with a free amine group) (Molkentin, 2000; Morrisey *et al.*, 1997).
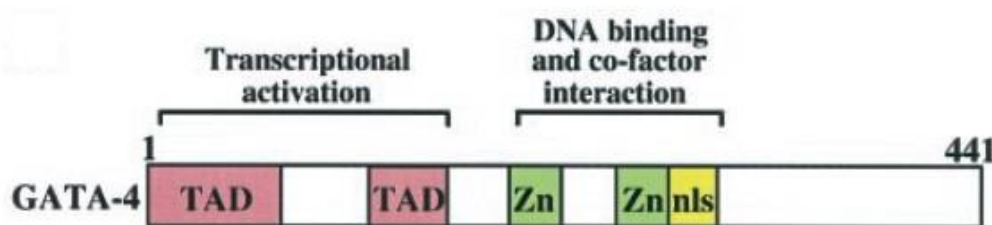
Fig. 6. Protein structure of GATA4 transcription factor (Molkentin, 2000).

GATA4 plays a central role in cardiac development and is critical for survival of the embryo. Homozygous GATA4-deficient mice died during embryonic development due to failure of forming the ventral pericardial cavity and heart tube between 8.0 and 10.5 days post coitum (dpc) (McCulley & Black, 2012; Kuo *et al.*, 1997). Antisense disruption of transcripts for GATA4 inhibits the ability of the pluripotent P19 embryonal carcinoma cells to differentiate into beating cardiomyocytes and interferes with expression of cardiac muscle markers (Grépin *et al.*, 1995). Also, GATA4 is proven to be an important regulator of cardiomyocyte proliferation through direct transcriptional activation of cell cycle regulators in mice (Rojas *et al.*, 2008). And the forced expression of GATA4 is proven to be sufficient to induce a hypertrophic response in the primary cultured neonatal rat cardiomyocytes and transgenic mice (Liang *et al.*, 2001). It is also demonstrated that the GATA4 regulates apoptosis and survival in HL-1 mouse cardiomyocytes and freshly isolated adult rat ventricular myocytes (Kim *et al.*, 2003). And the activation of GATA4 by survival factors in part

serves to protect the heart against oxidative stress (Suzuki & Evans, 2004). GATA4 works in combination with other essential cardiac transcription factors as well, such as Nkx2-5.

Nkx2-5 acts near the top of a large transcriptional cascade controlling multiple cardiac genes (McCulley & Black, 2012). Nkx2-5 expression is first detected in mesodermal cells specified to form heart at embryonic day 7.5 in the mouse and expression is maintained throughout the developing and adult heart (Lien *et al*., 1999). Expression of Nkx2-5 is regulated by GATA4 and SMAD proteins and is also controlled by Nkx2-5 itself in an auto-regulatory loop (McCulley & Black, 2012; Liberatore *et al*., 2002; Lien *et al*., 1999). Nkx2-5 and GATA4 specifically cooperate in activating cardiac-specific atrial natriuretic factor (ANF) and other cardiac promoters, and physically interact both *in vitro* and *in vivo* (Durocher *et al*., 1997). It appears that Nkx2-5 and GATA4 function within a mutually reinforcing transcriptional network to control cardiac gene expression (Lien *et al*., 1999).

In addition to interactions with Nkx2-5, GATA4 also functions as a transcriptional partner or in transcriptional pathways with several other important cardiac transcription factors, including MEF2C (McCulley & Black, 2012). The MEF2 proteins are MADS-box transcription factors which play an important role in myogenesis. In mice, the expression of MEF2C gene is detected in the developing heart at 7.5 dpc, shortly after the expression of the Nkx2-5 and GATA genes (Dodou *et al*., 2004; Morrisey *et al*., 1996; Edmondson *et al*., 1994; Lints *et al*., 1993). GATA4 directly activates MEF2C transcription in the second heart field in combination with Isl1 (McCulley & Black, 2012; Dodou *et al*., 2004).
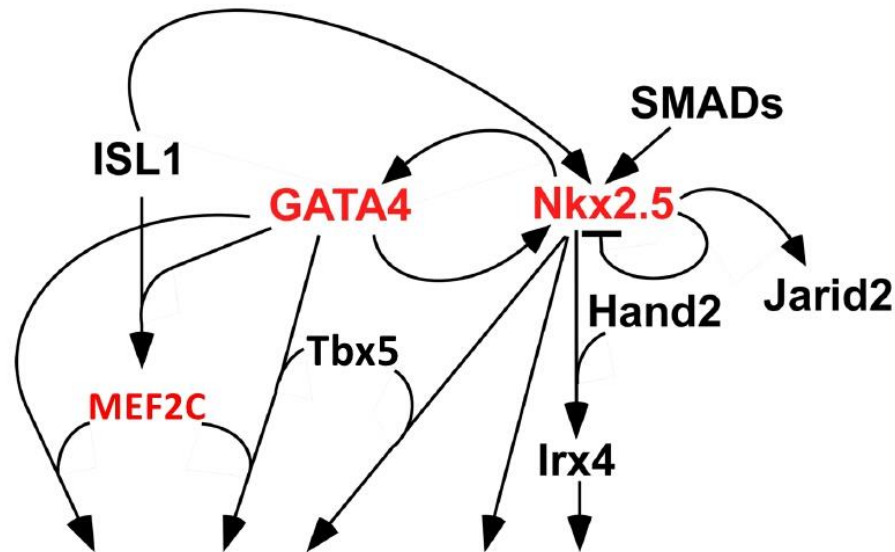
Fig. 7. Part of the transcription factor pathways that involved in myocardial development and heart morphogenesis. Three transcription factors that mentioned above, GATA4, Nkx2-5 and MEF2C, are marked in red. Adapted from McCulley & Black, 2012

Additionally, MEF2-binding sites have been reported in several cardiac promoters and their mutation was shown to decrease promoter activity in cardiomyocytes, including the MEF2 site in αMHC (Morin *et al*., 2000; Molkentin & Markham, 1993). This protein is found in cardiomyocytes, where it forms part of a larger protein called type II myosin. Type II myosin is one of the major component of cell structure called sarcomere. And sarcomere is the basic unit of muscle contraction, which helps generate the mechanical force that is needed for cardiac muscle to contract, allowing the heart to pump blood to the rest of the body (http://ghr.nlm.nih.gov/gene/MYH6). The expression of αMHC is synergistically activated by MEF2 and GATA4 (Morin *et al*., 2000).

In many different cell types, the plasma membrane is heavily decorated with small pits of 60–80 nm in diameter, which constitute a specialized type of microdomain called caveolae (Bastiani & Parton, 2010). CAV3 is a component of the caveolae plasma membrane and Song *et al*. (1996), found that CAV3 protein is selectively expressed only in cardiomyocytes and smooth muscle cells. Caveolae have been

implicated in endocytosis, lipid and cholesterol metabolism, calcium signaling and numerous other cellular processes (Bastiani & Parton, 2010; Kurzchalia & Parton, 1999).

1.6 Lipopolysaccharide (LPS)

LPS, also known as endotoxin, is the major component of the outer membrane of Gram-negative bacteria, which contributing greatly to the stability of membrane structure, and protecting the membrane from certain chemicals. It acts as a potent activator of immune system and stimulates host cells (mainly monocytes and macrophages, but also endothelial cells, smooth muscle cells, and neutrophils) to produce inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) (Schletter *et al*., 1995). The structure of LPS molecule is made up of three parts (Fig. 8):

- O-Antigen, a repeating O-specific oligosaccharide subunit

- Core oligosaccharide

- Lipid A, which is responsible for the toxic properties of the molecule
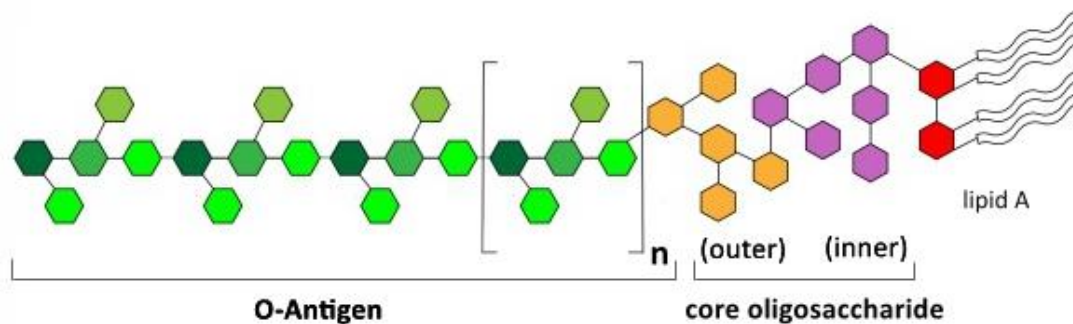

Fig. 8. The structure of LPS molecule (picture compiled from http://archive.constantcontact.com/fs064/1102652087693/archive/1109194888177.html).

It has been known that lower vertebrates, most notably fish and amphibians, are resistant to endotoxic shock when compared to mammals (Iliev *et al*., 2005a; Berczi *et al*., 1966). This might be caused by the differences in the receptor-mediated recognition of LPS between fish and mammals (Iliev *et al*., 2005a). It is reported that primary cultured monocyte–macrophage lineage cells (rtMOCs) responded with lower sensitivity to LPS from *Escherichia coli* and *Pseudomonas aeruginosa*. And the sensitivity of rtMOCs to LPS was not influenced by the presence of serum which suggests that the resistance to endotoxic shock in fish may be due to the lack of serum-borne factors that confer sensitivity to LPS in mammals. Regardless, *in vitro* studies showed that LPS can induce strong immune responses in macrophages from different fish species at μg/mL concentration (Iliev *et al*., 2005b; MacKenzie *et al*., 2003; Pelegrín *et al*., 2001). Upon LPS exposure (100 μg/mL), macrophages differentiated *in vitro* showed significant increased expression of trout TNFα gene over that of freshly isolated monocyte, which suggests that terminally differentiated macrophages play a central and key role in TNF production as a response to infection (MacKenzie *et al*., 2003).

1.7   *Polyunsaturated fatty acid and its immune functions*

In fish, dietary lipids are a major provider of energy, which also serve as important structural components in cell membranes, carriers of lipid-soluble vitamins and minerals (Ruyter *et al*., 2000). And they are also the source of essential fatty acids which are vital for development of tissues and normal growth (Ruyter *et al*., 2000; Tocher, 2010), such as linoleic acid (LA, 18:2n-6), α-linolenic acid (LNA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3).

Cold water fish, like salmon, has the ability to elongate and desaturate dietary 18:2n-6 and 18:3n-3 (Ruyter & Thomassen 1999); however, the rates of synthesis of C$_{20}$ and

C_{22} polyunsaturated fatty acids (PUFA) from 18:3n-3 may be insufficient to meet the Atlantic salmon's requirement for these very long chain PUFA (Ruyter *et al*., 2000). It is reported that in Atlantic salmon fry, increased dietary LA gave increased percentages of LA and its metabolite arachidonic acid (ARA, 20:4n-6) in the liver and blood phospholipids (PL); dietary LNA greater than 0.5% led to increased percentages of both LNA and EPA in liver PL, but there was no significant increase in the percentage of DHA; furthermore, lower doses of EPA and DHA resulted in a better growth and reduced mortality in salmon fry (Ruyter *et al*., 2000).

It is well known that dietary PUFA can regulate the inflammatory responses in fish. Diets containing high inclusion of vegetable oils, particularly n-6 PUFA-rich oils such as soybean (Montero *et al*., 2003) or sunflower (Bell *et al*., 1993), have lower n-3/n-6 PUFA and EPA/ARA ratios, which can lead to increased pro-inflammatory responses in fish. This is caused by the eicosanoids that derived from PUFA, including PGs (prostaglandins), TXs (thromboxanes), and LTs (leukotrienes) etc. Eicosanoids derived from n-6 PUFA, especially ARA, have pro-inflammatory and immunoactive functions, whereas eicosanoids derived from n-3 PUFA, either produce a reduced inflammatory response or actually terminate the inflammatory response. Since EPA competitively interferes with eicosanoid production from ARA catalyzed by cyclooxygenase (COX) and lipoxygenases (LOX), and is itself converted to 3-series PGs and TXs, and 5-series LTs, which are generally much less biologically active than the corresponding 2-series PGs and TXs, and 4-series LTs produced from ARA (Sargent *et al*., 2002). Also, a group of mediators, the E-series resolvins from EPA, and the D-series resolvins, neuroprotectins that derived from DHA, have been identified to be anti-inflammatory (Serhan *et al*., 2000, 2002; Hong *et al*., 2003; Mukherjee *et al*., 2004).

Furthermore, when compared to terrestrial mammals, fish have much higher concentrations of DHA and EPA than ARA in their tissues. Thus, fish have

correspondingly high dietary requirements for n-3 PUFA (Sargent *et al*., 1999), and different dietary n-3/n-6 ratios can affect their resistance to infections (Thompson *et al*., 1996; Martinez-Rubio *et al*., 2012, 2013). It is demonstrated that fish fed diet containing a low ratio (0.3) of n-3/n-6 PUFA are slightly less resistance to *Aeromonas salmonicida* and *Vibrio anguillarum* infections (Thompson *et al*., 1996). Martinez-Rubio *et al*. (2012) demonstrated that functional diets containing 2.45 n-3/n-6 PUFA significantly reduced heart inflammation and pathology caused by piscine reovirus (PRV) when compare to 1.53 in the commercial feed; also, a corresponding lowered expression of inflammatory and immune markers in heart tissue was measured in fish fed with functional feed. However, recent studies from Lopez-Jimena *et al*. (2015) showed that a decreasing n-3/n-6 PUFA ratio (0.87, high omega-6) in diets of rainbow trout is associated with reduced SAV replication when compared to diets with high n-3/n-6 PUFA ratio (3.08, high omega-3), and an increasing in the severity of heart inflammation was observed from fish fed high omega-3.

2   MATERIALS AND METHODS

2.1   Animals

Atlantic salmon (*Salmo salar* L.) were either obtained from the fish lab at the Norwegian University of Life Sciences (NMBU, Ås) or from the Norwegian Institute for Water Research (NIVA, Drøbak). The size of fish varied from 400 to 1200 g, they were fed a commercial feed prior to isolation of heart cells.

2.2   Chemicals and reagents

Ethyl 3-aminobenzoate methane-sulfonate (MS-222) was purchased from Norsk Medisinaldepot AS (Oslo, Norway). Ethanol (96%), as well as D-glucose were purchased from VWR International (Radnor, PA, USA). Sodium hydrogen carbonate ($NaHCO_3$) was from AppliChem GmbH (Ottoweg, Darmstadt, Germany). Sodium chloride (NaCl), calcium chloride dihydrate ($CaCl_2 \cdot H_2O$) and potassium dihydrogen phosphate (KH_2PO_4) were supplied by Merck KGaA (Darmstadt, Germany). Ethylene diamine tetra-acetic acid disodium salt dihydrate (EDTA-Na_2), potassium chloride (KCl), hepes sodium salt, 2,3-Butanedione monoxime (BDM), magnesium sulfate ($MgSO_4$), taurine, trypsin $10\times$ solution, HEPES solution, antibiotic antimycotic solution $100\times$ (10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per mL), penicillin-streptomycin (10,000 units penicillin, 10 mg streptomycin per mL), extracellular matrix (ECM) gel, fibroblast growth factor-basic (bFGF), bovine serum albumin (BSA), cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA, 22:6*n*-3), *Escherichia coli* lipopolysaccharides (*E. coli* LPS) and phosphate buffered saline (PBS) tablet were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagenase (type 1, 315 U/mg) was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Leibovitz's L-15 Medium ($1\times$) with

GlutaMAX^{TM}-I and bovine plasma fibronectin were from Invitrogen Gibco®, Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories GmbH (Pasching, Austria). Heparin was obtained from Biochrom AG (Berlin, Germany). Mouse laminin and entactin-collagen IV-laminin (ECL) cell attachment matrix were obtained from EMD Millipore (Billerica, MA, USA).

2.3 Equipment and kits

Disposable syringe (1 mL) and injection needles were supplied by JØRGEN KRUUSE A/S (Langeskov, Denmark). Blood transfusion set and Vasofix® Braunüle® IV Cannula were purchased from B.Braun (Melsungen, Germany). FALCON® 18 cm cell scrapers were purchased from Corning Science (Reynosa, Tamaulipas, Mexico) and 25 cm cell scrapers from VWR International (PA, USA). Filter paper circles were supplied by Schleicher & Schuell MicroScience GmbH (Dassel, Germany) while 100 μm nylon filter by SEFAR AG (Heiden, Switzerland). 50 mL centrifuge tubes and 10 mL serological pipette were obtained from SARSTEDT AG & Co. (Nümbrecht, Germany). Accu-jet® pro pipette controller was obtained from BrandTech Scientific, Inc. (Essex, CT, USA). 6-well plates and Nunc^{TM} EasYFlasks^{TM} 75 cm^2 were both from Thermo Fisher Scientific (Roskilde, Denmark). RNeasy plus Mini Kit and QIAshredder columns were purchased from QIAgen (Valencia, CA, USA). RNA Clean & Concentrator^{TM}-5 was bought from Zymo Research (Orange County, California, USA). NanoDrop® ND-1000 Spectrophotometer was supplied by NanoDrop Technologies, Inc. (Wilmington, DE, USA). TaqMan® Reverse Transcription kit, Veriti® 96-well Thermal Cycler, MicroAmp^{TM} 8-cap strip and 0.2 mL 8-tube strip, MicroAmp® Optical 96-well reaction plates were all obtained from Applied Biosystems (Foster City, CA, USA). LightCycler® 480 sealing foil, 96-well plates and $2\times$ SYBR Green I Master were all supplied by Roche Diagnostics GmbH (Mannheim, Germany). LightCycler® 480

qPCR instrument was supplied by Roche Instrument Center AG (Rotkreuz, Switzerland). Centrifuge 5415R, Eppendorf Research plus pipette and filter-tips, Multipette® stream and 0.1 mL Combitips Advanced® were both obtained from Eppendorf AG (Hamburg, Germany).

2.4   Quality assurance

As cell cultures request a strict hygienic condition, it is important to work as sterile as possible. Equipment such as Erlenmeyer and volumetric flasks, scissors, forceps, knife handle and funnels were all autoclaved before using. Coating of wells, preparing of buffers and washing of cells were all carried out in sterile bench.

2.5   Isolation and culturing of cardiomyocytes

2.5.1   Cell isolation method 1

Isolation method 1 is based on a protocol for isolation of cardiomyocytes from rainbow trout (Nurmi & Vornanen, 2002).

Fish were anesthetized with MS-222 and stunned by a blow in the head. Then their body surface was washed with 70% ethanol to get rid of the mucus and bacteria. To prevent blood clotting, 0.1 mL heparin (5000 U/mL) was injected into the dorsal vein before the abdomen was opened. The intact heart was carefully excised and quickly transferred to a clean petri dish. An intravenous indwelling cannula was inserted through the bulbus arteriosus leading into the heart. With a pump and infusion set, the buffer could easily flow throughout the heart (ca. 2.5 mL/min). Heart was first perfused with buffer 1 (Ca^{2+}-free buffer: 100 mM NaCl, 10 mM KCl, 1.2 mM

KH$_2$PO$_4$, 20 mM glucose, 10 mM hepes sodium salt, 10 mM BDM, 4 mM MgSO$_4$, and 50 mM taurine at pH 7.4) for 5 min to remove the blood, then followed by a 20 min perfusion with buffer 2 (buffer 1 with 0.75 mg/mL collagenase type 1 and 0.5 mg/mL trypsin) to digest the connective tissues surrounding the heart cells. Thereafter, the digested heart was transferred to ice cold L-15 media in a petri dish and opened with forceps in order to shake loose the cardiomyocytes. The resulting cell suspension was then filtered through a 100 μm nylon filter into a 50 mL centrifuge tube. Heart remnants were washed for 3-4 times until no cells attached. The cell suspension was centrifuged at 300 ×g for 2 min at 4 ℃, precipitate could be seen at the bottom of the tube. Supernatant was removed by pipetting and new L-15 was added to wash the cells. Before centrifugation, cell pellets were re-suspended with pipette. The cells were washed twice before adding growth media.
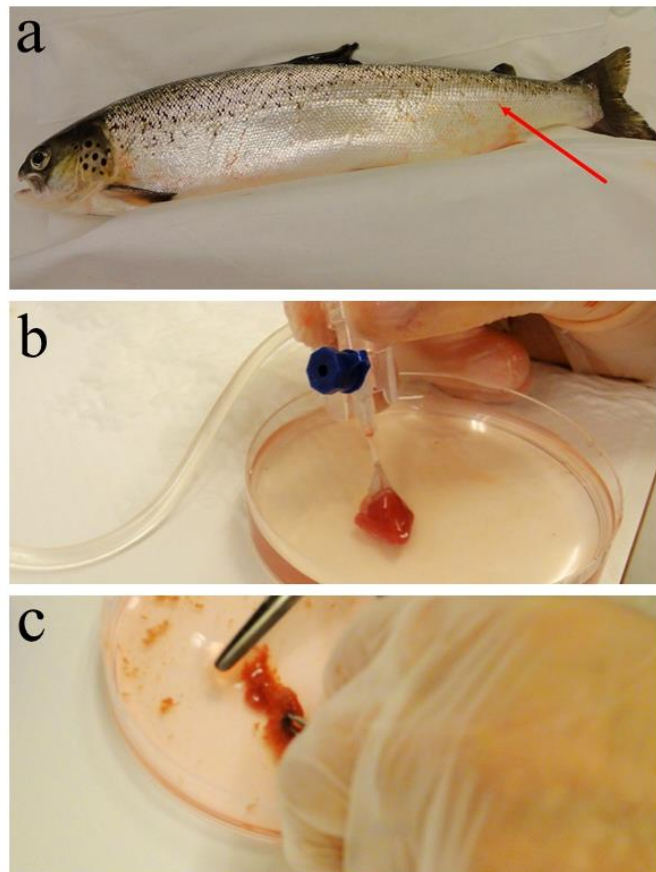

Fig. 9. **(a)** The injection point of heparin. **(b)** Heart that under perfusion. **(c)** After enzymatic digestion, the heart was torn down with forceps to loosen the cells.

2.5.2 Cell isolation method 2

Isolation method 2 is based on a protocol for isolation of hepatocytes from rainbow trout which developed by Seglen (1976) and modified by Dannevig and Berg (1985). The same isolation steps were followed as mentioned in isolation method 1, but different perfusion buffers were used. Firstly, heart was perfused with buffer 3 (buffer with EDTA: 143 mM NaCl, 6.7 mM KCl, 10 mM hepes sodium salt and 20 mM EDTA-Na$_2$) for 5 min to open the tight junctions and then followed with a 20 min perfusion with buffer 4 (buffer containing: 143 mM NaCl, 6.7 mM KCl, 10 mM hepes sodium salt, 1 mM CaCl$_2$ and 1 mg/mL collagenase type 1).

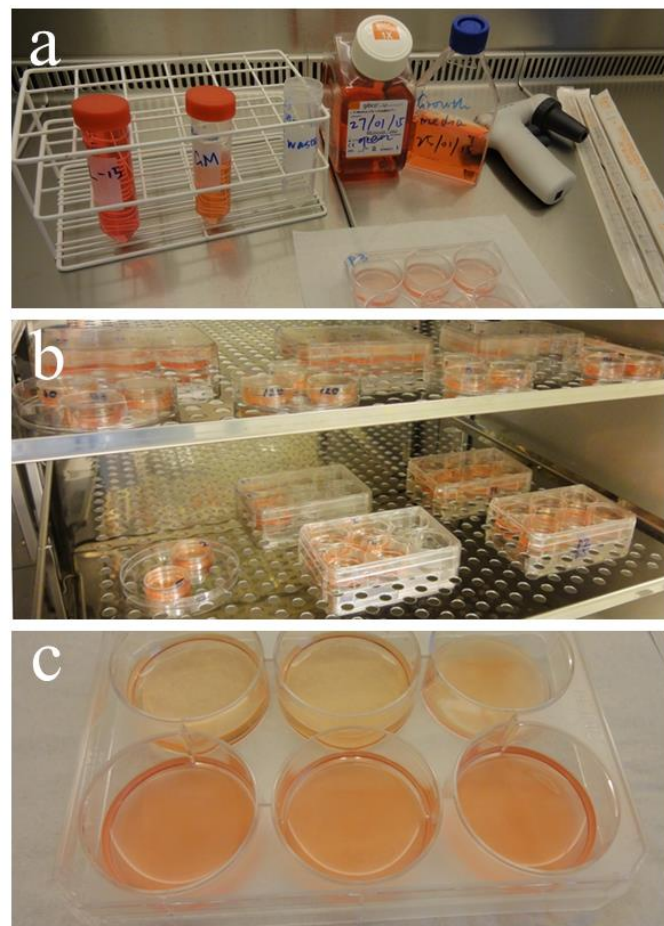***Fig. 10.*** **(a)** Washing of cells was carried out in a sterile bench. **(b)** Cells were cultured in the cell incubator. **(c)** The day after seeding, red color could be seen from the old media due to contaminating blood cells (the second row). After thorough washing with L-15, the majority of the red blood cells loosened and could thereafter be removed by pipetting, cells were added fresh growth media (the first row).

2.5.3 Culturing of cardiomyocytes

Before seeding, the precipitated cardiomyocytes, isolated with either method 1 or method 2, were re-suspended in growth media. And two different growth media, L-15 muscle cell growth media (10% FBS, 10 mM HEPES, 1% antibiotic antimycotic solution) and L-15 hepatocyte growth media (10% FBS, 10 mM HEPES, 1% penicillin-streptomycin, 9 mM $NaHCO_3$), were prepared. Normally, cells were plated on laminin coated 6-well plate (culture area 9.6 cm^2 per well, coated with 16 μL 1 mg/mL laminin), and they were incubated at 13 ℃, CO_2-free environment (Fig. 10). Every day, cells were thoroughly washed with pure L-15 media before given 3 mL fresh growth media.

2.6 Study 1: Evaluation of two different methods for isolation and culturing of cardiomyocytes

The aim of this first study was to test the efficiency of the two different protocols described above for isolation of cardiomyocytes from Atlantic salmon heart, and the two different growth media for culturing of the isolated cells.

In this study, cardiomyocytes were isolated from 4 fish. And in treatment 1 (*Iso1+M*) and treatment 2 (*Iso1+H*), heart cells were isolated according to isolation method 1 and cultivated in either muscle cell growth media (*M*) or hepatocyte growth media (*H*), respectively. Besides, in treatment 3 (*Iso2+M*) and treatment 4 (*Iso2+H*), cells were isolated according to method 2 and they were cultivated in either muscle cell growth media or hepatocyte growth media, respectively. Cardiomyocytes from four different treatments were separately plated on laminin pre-coated 6-well plate. Microscopy was performed every day and images were taken to follow the cell development. At day 9 (the day of seeding is defined as day 0), cells were harvested for isolation of RNA for gene expression analysis of cardiac specific markers.

2.7   Study 2: A time study of cardiomyocytes in culture

The aim of this study was to study the proliferation and development of cardiomyocytes in culture with time. Heart cells were isolated according to isolation method 1 and they were cultured in L-15 muscle cell growth media (isolation and culture conditions with the best outcome in study 1).

Cells were harvested at six different time points, day 2, 3, 5, 7, 9, 11, respectively, with three parallels each. Microscopy was performed every day to monitor differences in cell density and cell morphology between groups. The harvested cells were further used for isolation of RNA for gene expression analysis of some cardiac specific markers.

2.8   Study 3: Influence on cell proliferation and development by supplementation of basic fibroblast growth factor (bFGF) to cell culture media

The aim of this study was to test if supplementation of the growth factor bFGF to the growth media would increase the proliferation degree of cardiomyocytes in culture. Heart cells were isolated according to isolation method 1 and they were cultured in L-15 muscle cell growth media. In total two groups were included in this trial. For treatment group, duplicate wells (*Day2+FGF* & *Day7+FGF*) were added 15 µL of 25 µg/mL bFGF at day 1. And for duplicate wells in control group (*Day2-Control* & *Day7-Control*), only L-15 muscle cell growth media was added for culturing. Cells from both groups were harvested at day 2 and day 7 respectively for isolation of RNA for gene expression analysis. Microscopy was performed every day and images were taken at day 2 and day 6 respectively to monitor differences in development between the groups.

2.9 Study 4: Long term cultivation of cardiomyocytes

The aim of this study was to study the development of cardiomyocytes during long term cultivation. In this trial, heart cells were isolated according to isolation method 1 and they were cultured in L-15 muscle cell growth media up to 60 days. Microcopy pictures were taken every day in order to monitor the morphology of cardiomyocytes at different time points.

2.10 Study 5: Stress response of cardiomyocytes to H_2O_2

The aim of this study was to evaluate the H_2O_2 induced stress response in cardiomyocytes. Heart cells were isolated according to isolation method 2 and they were cultivated in L-15 muscle cell growth media. Four groups were set with three parallels each. Group 1 was the control group. Group 2, 3 and 4 were the experimental groups, and they were incubated with 100 μM H_2O_2 for 30, 60, and 90 min, respectively before harvesting, while group 1 was incubated in normal muscle cell growth media for 30 min. The harvested cells were further used for isolation of RNA for gene expression analysis of heart specific and stress response related gene markers.

2.11 Study 6: Effects of DHA on immune response

The aim of this study was to test the effects of DHA on the expression of antioxidative and inflammatory gene markers in cardiomyocytes. A further aim was to test if salmon cardiomyocyte is able to respond to LPS by influencing genes involved in immunity and inflammation. In this trial, four groups were set with three parallels each and group 1 was the control group.

2.11.1   Solubilization of DHA and making of growth media

To facilitate the uptake of DHA into cardiomyocytes, DHA was complexed to bovine serum albumin (BSA) in the ratio 2.7:1 (unit: mole). Initially, DHA and BSA were dissolved in NaOH and PBS respectively according to the calculation and operation steps below:

✓ Solution 1:   100 mM DHA (MW 328.49) in 100 mM NaOH

25 mg DHA (0.0761 mmol) was mixed with 0.761 mL NaOH at 37 ℃

✓ Solution 2:   9.27 mM BSA (MW 67 KDa) in PBS

0.621 g/mL BSA in PBS

Solution was carefully stirred to avoid foaming

After dissolving, Solution 2 was kept in 37 ℃ water bath

✓ Solution 3:   Solution without DHA

0.8 mL of Solution 2 was mixed with 0.2 mL 100 mM NaOH

✓ Solution 4: water soluble 20 mM DHA solution

3.044 mL of Solution 2 was slowly added into 0.761 mL Solution 1

Volume sol.1/sol.2 = 1:4

pH 7 was measured

✓ Growth media 1: media without DHA

2% FBS, 10 mM HEPES, 1% antibiotic antimycotic solution, 1 ‰ Solution 3

✓ Growth media 2: media with 20 μM DHA

2% FBS, 10 mM HEPES, 1% antibiotic antimycotic solution, 1 ‰ Solution 4

In case that the fatty acids (FAs) in FBS may affect the uptake of DHA, only a small amount (2%) of FBS was used for preparing of growth media, and the fatty acid

composition of growth media 1 and 2 was analyzed (Table 1). The concentration of total EPA and DHA in media 1 is 0.0007 mg/mL, and a much higher concentration 0.0027 mg/mL in media 2.

Table 1

Fatty acid composition of growth media 1 and 2.

Fatty acid[*]	Growth media 1 (media without DHA)	Growth media 2 (media with 20 µM DHA)
12:0	4.7	5.6
14:0	2.4	0.5
16:0	22.1	19.7
16:1 n-7	4.4	0.7
18:0	10.7	10.8
18:1 n-7	4.1	2.0
18:1 n-9	25.6	20.1
18:2 n-6	3.9	4.8
20:1 n-9	3.7	1.0
20:3 n-6	0.5	1.4
20:4 n-6	1.4	4.4
22:1 n-9	5.0	0.0
22:5 n-3	0.8	2.6
22:6 n-3	1.3	25.4
EPA+DHA	1.7	25.4
Sum identified	98.9	99.8

[*] The quantity of each fatty acid is given as the percentage of total FAs. And FAs contributing to less than 1.0%, are not included in the list.

2.11.2   Adding of LPS

Heart cells were isolated according to isolation method 1 and they were cultured in normal L-15 muscle cell growth media for 4 days until confluence achieved. At day 4, heart cells in group 1 (*Control group*) and group 3 (*LPS group*) were washed and added with media without DHA (growth media 1), while cells from group 2 (*DHA*

group) and group 4 (*DHA&LPS group*) were washed and added media supplemented with 20 μM DHA (growth media 2). At day 8, group 3 and group 4 were incubated with 100 ng/mL LPS for 6 hours before cardiomyocytes from all groups were harvested for isolation of RNA.

2.12   Study 7: Test of four different coating materials for culturing of cardiomyocytes

It is an aim to increase the cell yield from each heart. One way to achieve this is to see if it is possible to increase the cell proliferation by optimizing the coating materials of the cell wells. The aim of this study was therefore to test how different well coatings influence cell proliferation. Four different coatings were tested in this trial with one well for each experimental group. Cells were isolated according to isolation method 1 and they were cultured in L-15 muscle cell growth media.

Group 1 was coated 45 μL 1 mg/mL entactin-collagen IV-laminin (ECL) cell attachment matrix (6-well plate, culture area 9.6 cm^2 per well). For group 2, bovine plasma fibronectin (1 mg) was reconstituted by adding 1 mL sterile deionized distilled water and 13 μL fibronectin was used for coating. For group 3, cells were seeded on laminin coated well (as mentioned in 2.5.3). In group 4, extracellular matrix (ECM) gel, which contains laminin as a major component, collagen type IV, heparan sulfate proteoglycan, entactin and other minor components, was used for coating. As a high original protein concentration (8-12 mg/mL), 25 μL ECM gel was diluted with an equal amount of L-15 media and 50 μL of diluted ECM gel was used for coating. Microscopy was performed every day to monitor differences in development between the groups. And to evaluate the proliferation of cardiomyocytes, six images were randomly taken from each group at day 1, 2, 5, and 7, respectively to calculate the average number of cells. At day 7, cells from all groups were harvested for isolation of RNA for gene expression analysis.

2.13   Isolation of RNA

At the day of harvest, cells were thoroughly washed twice with 2 mL of PBS and RNA isolation was carried out by using an RNeasy® Plus Mini Kit.
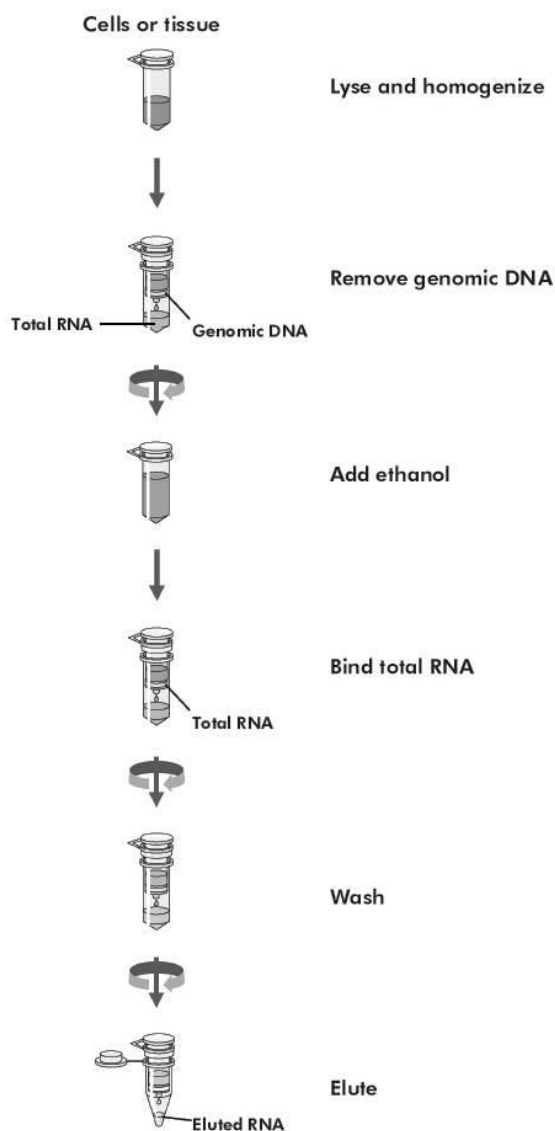

Fig. 11.   A short workflow for RNA purification. All centrifugation steps were performed at 20~25 ℃ room temperature.

Firstly, samples were lysed with 350 μL Buffer RLT Plus (containing 10 μL/mL β-mercaptoethanol, a highly denaturing guanidine-isothiocyanate-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA) before cells

were scraped and collected, then cell lysates were pipetted into a QIAshredder Mini spin column and centrifuged at 16,100 ×g for 2 min. The homogenized cell lysates were stored at -70 ℃ (before moving on to the next step, the frozen lysates were incubated at 37 ℃ in a water bath until completely thawed).

Secondly, homogenized lysates were transferred to a gDNA Eliminator spin column which placed in a 2 mL collection tube and spun at 8,000 ×g for 30 s to remove genomic DNA. The column was discarded and 1 volume (350 μL) of 70% ethanol was added to the flow-through (well mixed by pipetting) to provide appropriate binding conditions for RNA.

Afterwards, up to 700 μL of the sample was applied to an RNeasy spin column (where total RNA binds to the membrane) and centrifuged at 8,000 ×g for 15 s. The flow-through was discarded. To wash away the contaminants, 700 μL Buffer RW1 was added to the RNeasy spin column and centrifuged at 8,000 ×g for 15 s. The flow-through was discarded. Further, 500 μL Buffer RPE was added to the RNeasy spin column and centrifuged at 8,000 ×g for 15 s. The flow-through was discarded and 500 μL of Buffer RPE was added again, to wash the spin column membrane, the column was centrifuged at 8,000 ×g for 2 min.

Finally, the RNeasy spin column was placed in a new 1.5 mL collection tube and 30 μL of RNase-free water was added directly to the spin column membrane, the tube was centrifuged at 8,000 ×g for 1 min to elute the RNA. The eluted RNA was stored at -70 ℃.

2.14   *RNA purification and up-concentration*

Due to a low RNA concentration from the step above, RNA was further purified and up-concentrated by using an RNA Clean & Concentrator™-5 kit.

Firstly, 2 volumes (60 μL) of RNA Binding Buffer was added to each sample and well mixed by pipetting. Then 1 volume (90 μL) of 97% ethanol was added to the mixture before all the mixture was transferred to a Zymo-Spin IC Column in a collection tube. Then the column was centrifuged at 12,000 ×*g* for 1 min and the flow-through was discarded.

Secondly, 400 μL RNA Prep Buffer was added to the column and centrifuged at 12,000 ×*g* for 1 min. The flow-through was discarded. Thirdly, 800 μL RNA Wash Buffer was added to the column and centrifuged at 12,000 ×*g* for 30 s. The flow-through was discarded and this wash step was repeated again with 400 μL RNA Wash Buffer. After that, the Zymo-Spin IC Column was put in a new collection tube and centrifuged at 12,000 ×*g* for 2 min. The spin column was carefully removed from the collection tube and transferred into an RNase-free tube.

Finally, the column matrix was directly added 7 μL RNase-free water and stood for 1 min at room temperature. Then it was centrifuged at 10,000 ×*g* for 30 s. The eluted RNA was stored at -70 ℃.

To evaluate the purity and concentration of eluted RNA, NanoDrop® ND-1000 Spectrophotometer was used to do the absorbance measurements. Since nucleotides, RNA, single-stranded DNA, and double-stranded DNA all absorb at 260 nm, they will contribute to the total absorbance of the sample. The ratio 260/280 is used to assess the purity of RNA and a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.
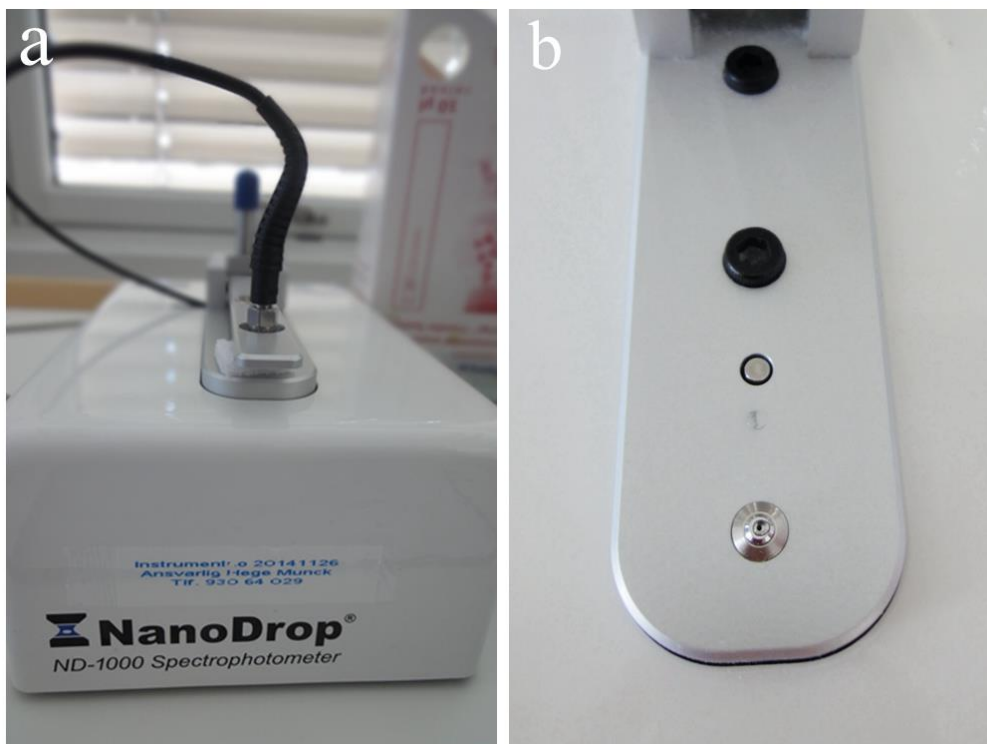
Fig. 12. **(a)** NanoDrop® 1000 Spectrophotometer was used to measure the purity and concentration of eluted RNA. **(b)** 1 µL of RNA sample was pipetted directly to the pedestal.

2.15   Complementary DNA synthesis

All the RNA samples that were used for complementary DNA (cDNA) synthesis had an A260/280 ratio around 2.0. The synthesis of cDNA was completed by using TaqMan® Reverse Transcription Reagents.

A reverse transcriptase (RT) is an enzyme used to generate cDNA from a single-stranded RNA template, a process termed reverse transcription. RT buffer provides a suitable chemical environment for optimum activity and stability of enzyme RT. $MgCl_2$ here is a catalyst, Mg^{2+} and nucleotide complexes are the substrate for RT. dNTP (deoxynucleotide triphosphate) is the building-block from which RT synthesizes a new DNA strand. Oligo $d(T)_{16}$ primer is a short sequence of deoxy-thymine nucleotides which binds to the polyadenylated (poly A+) mRNA and

provides a free 3'-OH end that can be extended by RT to create a cDNA strand. Random hexamers are oligonucleotide sequences of 6 bases [d(N)$_6$] which are synthesized randomly to give a numerous range of sequences that can anneal to random complementary sites on a target RNA. For each sample, the following cDNA-mixture was prepared (Table 2).

Table 2

Reagents used for making cDNA-mixture.

Reagents	Volume (µL)
10×RT buffer	2.0
25 mM MgCl$_2$	4.4
10 mM dNTP mix	4.0
50 µM Oligo d(T)$_{16}$	0.5
50 µM Random hexamers	0.5
RNase inhibitor (20 U/µL)	0.4
MultiScribe™ RT (50 U/µL)	0.5
RNA 200 ng	X
DEPC-H$_2$O to final volume (reaction volume)	20

The cDNA-mixture was held on ice and cDNA synthesis was run on a Veriti® 96-well Thermal Cycler with the following program (heated cover set point 105 ℃):

> ➤ 25 ℃ for 10 min (primer incubation)
>
> ➤ 48 ℃ for 60 min (RT process)
>
> ➤ 95 ℃ for 5 min (RT inactivation)
>
> ➤ 4 ℃ for ∞

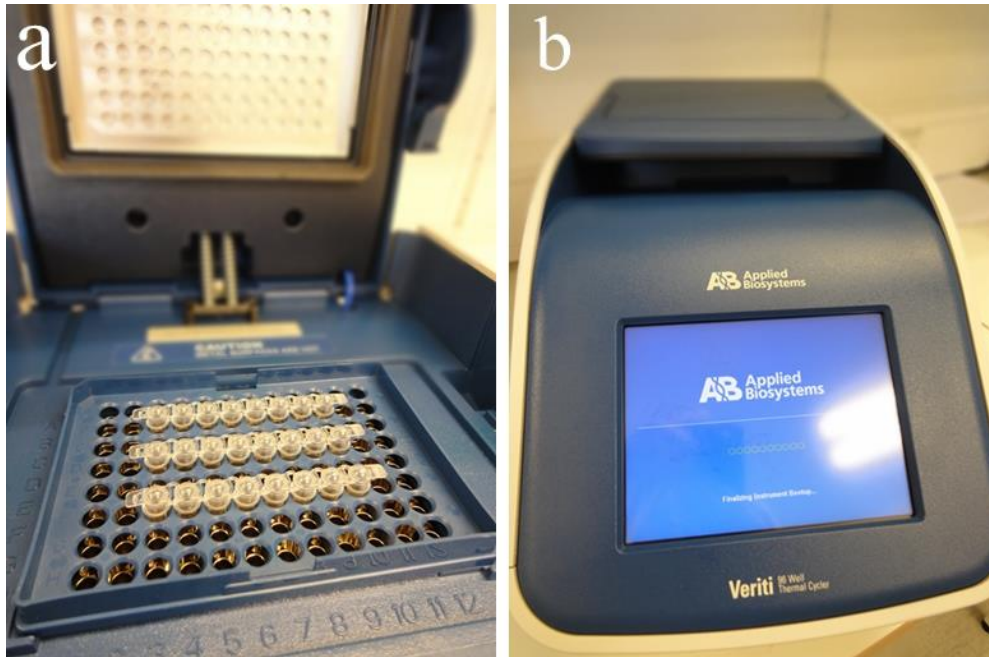Then the reverse transcription products (cDNA) were stored at -20 ℃ for qPCR.

Fig. 13. **(a)** 8-tube strips with cDNA-mixture. **(b)** cDNA synthesis was carrying out by Veriti® 96-well Thermal Cycler.

2.16 Quantitative polymerase chain reaction (qPCR)

In qPCR, the amount of amplified DNA is quantified as it accumulates in the reaction after each amplification cycle by using of fluorescent makers (SYBR Green) that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of amplicons generated in the exponential phase of the reaction, which enables us to determine the initial amount of target with great precision. The reverse transcription products (cDNA) were used as template in qPCR with gene-specific primers and the calculation of relative gene expression levels is based on the formula below:

$$R = E_{target}^{\Delta Ct\ target\ (control-treatment)} / E_{reference}^{\Delta Ct\ reference\ (control-treatment)}$$

The PCR master mix consisted of 5 µL LightCycler® 480 SYBR Green I Master, 1 µL forward and reverse primer (final concentration 0.5 µM) and 4 µL 1:10 H_2O diluted cDNA products. All samples were run in duplicate. Melting curve analysis was run to confirm the presence of a single PCR product and cycle threshold (Ct) values were calculated using second derivative methods (Roche Diagnostics). The running conditions as follow:

➢ 95 ℃ for 5 min (pre-incubation)

➢ Quantification with 45 cycles of
$\begin{cases} 95\ ℃\ \text{for 15 s (denaturation)} \\ 60\ ℃\ \text{for 15 s (annealing)} \\ 72\ ℃\ \text{for 15 s (extension)} \end{cases}$

➢ Melting curve
$\begin{cases} 95\ ℃\ \text{for 5 s} \\ 65\ ℃\ \text{for 1 min} \\ \text{heating til 97 ℃} \end{cases}$

➢ 40 ℃ for cooling

The qPCR primers (Table 3) were designed manually based on Primer-BLAST software from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Invitrogen (CA, USA). The stability of two potential reference genes, eukaryotic translation initiation factor 3 (ETIF3) and RNA polymerase II polypeptide J (RPL2), were tested by RefFinder program (http://leonxie.com/referencegene.php) and the one with the better stability was used as reference gene. Primer efficiencies were calculated in the beginning by making of standard curves.

Fig. 14. LightCycler® 480 qPCR instrument and 96-well plate. By using this system, the change in fluorescence over the process of reaction is measured.

Data from gene expression analysis is presented as mean value $\pm$ SEM (standard error of mean). Differences between the different experimental conditions were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using JMP®, Version 11.2.1. SAS Institute Inc., Cary, NC, 1989-2007.

Table 3   Primer sequences for qPCR.

Target gene	Short name	Accession no.	Primer sequence from 5' to 3'
Proliferating cell nuclear antigen	PCNA	BT045597	F-GGGAGGAGGATCTTGTTGGC
			R-ACGCAATAGCGATGCCATGA
Caveolin-3	CAV3	NM_001141855	F-GCCATCCACACCTTCTGTGA
			R-CCTGTCCCATTCCCATGCTT
Atrial myosin heavy chain	αMHC	CV428926	F-AGCGAAGGAACAAGCCAACT
			R-CGAACACGGAGCTTGTTGAC
GATA binding protein 4	GATA4	HM475152	F-TCTCCATTCGACAGCTCCGT
			R-CATCGCTCCACAGTTCACACA
NK2 homeobox 5	Nkx2-5	DW550500	F-CCCAGTACGTCCACACCCTT
			R-GGAGGTCGGTAAGGCACAGT
Myocyte enhancer factor 2C	MEF2C	GU252207	F-CACCGTAACTCGCCTGGTCT
			R-GCTTGCGGTTGCTGTTCATA
Superoxide dismutase [Cu-Zn]	SOD1	BT057716	F-TTCTGTTGTACGCTGTCCCAAAAGC
			R-GCAGCTTGGTACGCAAAGTGAACA
Glutathione peroxidase 3 (plasma)	GPx-3	CA345853	F-CCTTCCAGTACCTGGAGTTGAATGC
			R-CTCATGATTGTCTCCTGGCTCCTGT
Catalase	CAT	est04a09	F-CCAGATGTGGGCCGCTACAA
			R-TCTGGCGCTCCTCCTCATTC
Heat shock 70 kDa protein	HSP70	BT059361	F-TCATTCATCGGGTTGCGGAG
			R-GTGTGGTCCTGTTACCCTGG
Nuclear factor erythroid 2-related factor 2	Nrf2	BT059007	F-CCGGACTCCTCGCCTTCGGA
			R-GTGGATAGTTGGCTTGTCCCTTCGT
Tumor necrosis factor alpha-1 precursor	TNFα	NM_001123589	F-AGGTTGGCTATGGAGGCTGT
			R- TCTGCTTCAATGTATGGTGGG
Interleukin 1, Beta	IL1b	CA377361	F-GTATCCCATCACCCCATCAC
			R-TTGAGCAGGTCCTTGTCCTT
Signal transducer and activator of transcription 1 isoform alpha	Stat1a	CB513054	F-CGGTGGAGCCCTACACTAAG
			R-GGGATCCTGGGGTAGAGGTA
Myxovirus resistance 1, interferon-inducible protein	MX1	SSU66477	F-TGATCGATAAAGTGACTGCATTCA
			R-TGAGACGAACTCCGCTTTTCA
Jun B proto-oncogene	JunB	NM_001139901	F- CTATCGGAACCAAAGCCTCA
			R- GGATGATCAATCGCTCCAGT
Eukaryotic translation initiation factor 3	ETIF3	DW542195	F-CAGGATGTTGTTGCTGGATGGG
			R-ACCCAACTGGGCAGGTCAAGA
RNA polymerase II polypeptide J	RPL2	CA049789	F-TAACGCCTGCCTCTTCACGTTGA
			R-ATGAGGGACCTTGTAGCCAGCAA

2.17   Microscopy and cell counting

Microscopic observation was performed by Nikon phase contrast 2 microscope (Nikon Corporation, Minato-ku, Tokyo, Japan) with $10\times$ magnification and microscopy images were processed with Leica DC100 digital imaging system (Leica Microsystems Ltd, Wetzlar, Germany).

For the cell counting in study 7, six images were randomly taken from each group at four different time points to estimate the growing trend of cardiomyocytes. All cell types were included for counting, and the average cell number in every six images was used to represent the general growth of cardiomyocytes in the corresponding group and corresponding time point.
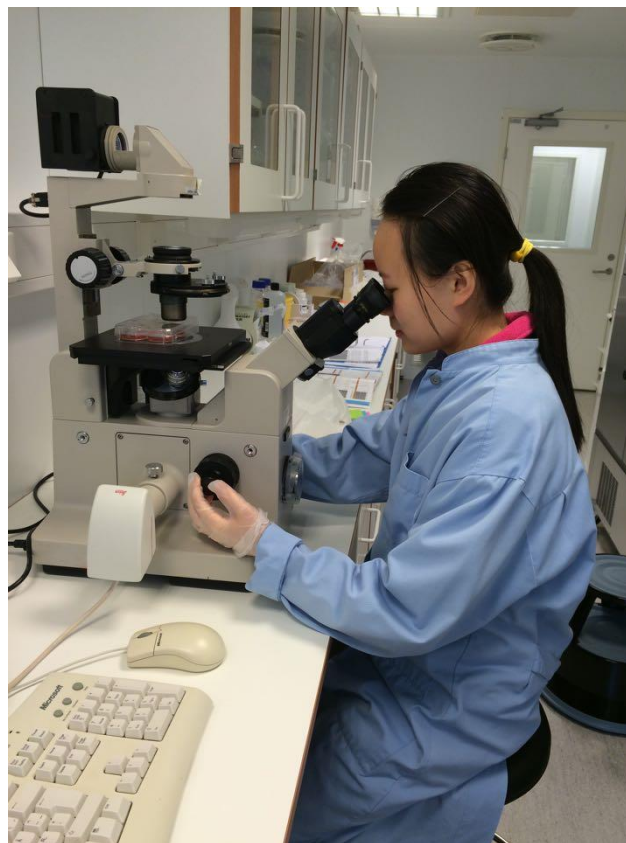

Fig. 15.   Microscopy was carried out in the cell room.

3 RESULTS AND DISCUSSION

3.1 Study 1: Evaluation of two different methods for isolation and culturing of cardiomyocytes
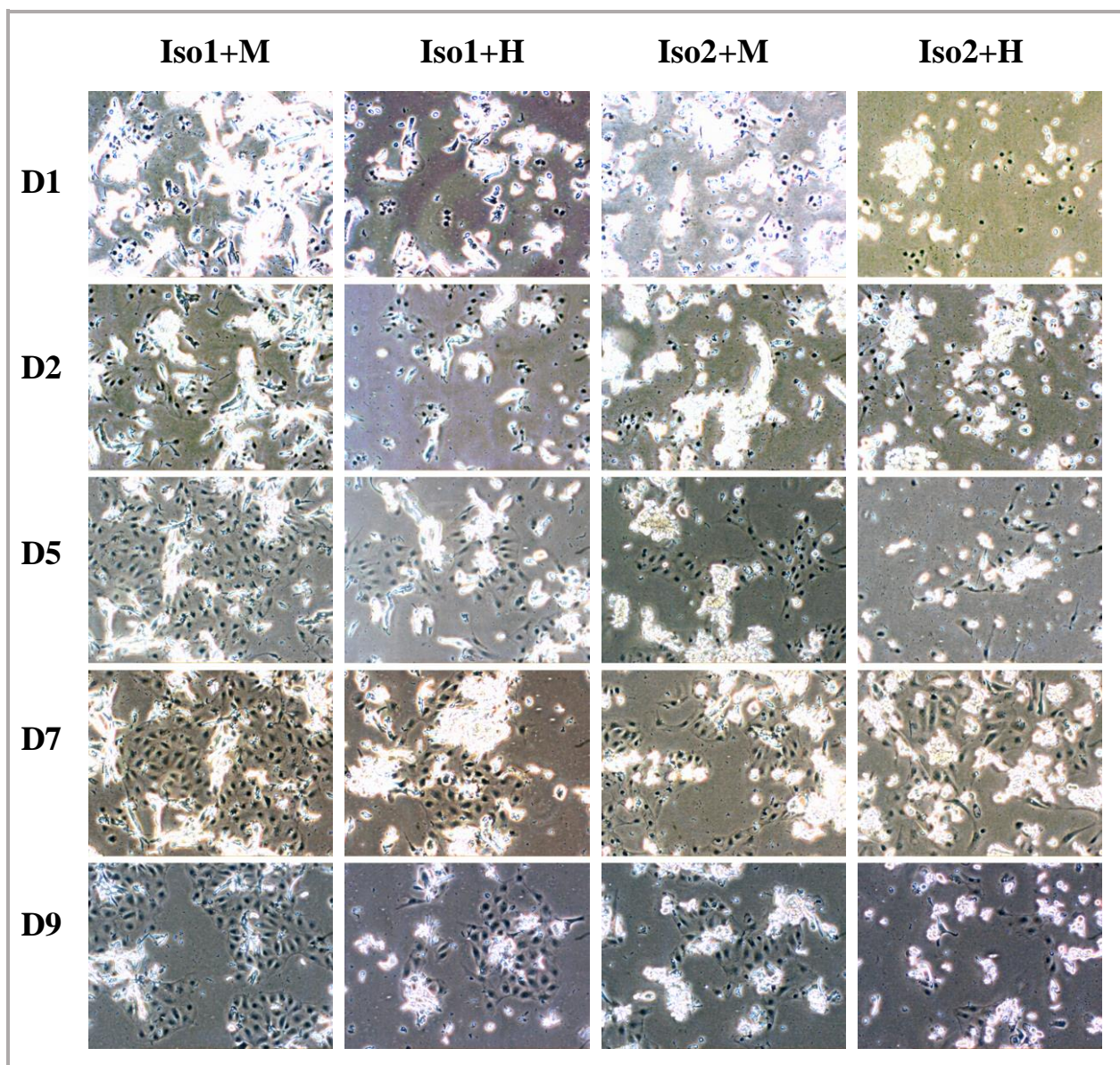

Fig. 16. Phase contrast microscopic images of salmon cardiomyocytes from 4 different treatments at day 1, 2, 5, 7, and 9 respectively (10×magnification). *Iso1* = Isolation method 1, *Iso2* = Isolation method 2, *M* = Muscle cell growth media, *H* = Hepatocyte growth media.

To evaluate the efficiency of two different protocols for isolation of cardiomyocytes, as well as two different growth media for cell proliferation and differentiation in culture, the density changes and morphological developments of cardiomyocytes were observed by microscopy and photographed from day 1 to day 9 after seeding (Fig. 16). Different layers of cells were observed from the images at day 1, and the cells that we expect to be cardiomyocytes appeared with dark nuclei and transparent cytoplasm. They were not in contact with each other in the beginning and spreading out separately on laminin-coated wells. An increase in cell number could be seen when comparing the microscopic images at day 2, 5, 7 with the images at day 1, indicating that cells from all treatments proliferated during this period.

However, when looking at the images at day 1, it seems that a higher number of cells were obtained by using isolation method 1 (with a mixture of two proteases, collagenase and trypsin, for tissue digestion & addition of BDM for cytoprotection) than by using isolation method 2 (where only collagenase was used as the protease & EDTA was used to open the tight junctions). And from day 5 to day 9, the highest number of cells was found in treatment 1 (*Iso1+M*), where cells had been isolated by using both collagenase and trypsin, and cultivated in the muscle cell growth media. The better outcome with treatment 1 (*Iso1+M*) than with treatment 2 (*Iso1+H*) for isolated cells to grow into clusters may be due to a higher density of cardiomyocytes obtained from day 1, and probably not due to differences in growth media composition during the culture period. Considering the fact that the isolation reagents being used in both methods had a relatively similar composition, and the same amount of time that being used for enzymatic digestion, the main differences between these two different isolation methods are the mixture of proteases used for tissue digestion and the addition of BDM.

To isolate intact and not damaged cells, it is essential to optimize the protocol for tissue digestion by proteases. In order to isolate single cells, collagens, the major fibrous component of extracellular connective tissue, have to be broken down. And

collagenases are endopeptidases that break peptide bonds in collagen so that cells can be released. Trypsin is a pancreatic serine protease which cleaves peptide chains mainly on the C-terminal side of lysine or arginine during the hydrolyzation process. Since trypsin is one of the most highly specific proteases known, it shows little selectivity for extracellular proteins, so purified trypsin alone is usually not so effective for tissue dissociation (http://www.worthington-biochem.com/tissuedissociation/trypsin.html). Hence, the combination of trypsin and collagenase is often used for tissue digestion (Brette *et al*., 2008; Goldstein *et al*., 2004; Messina *et al*., 2004). Many protocols, similar with our cell isolation method 1, have been successfully used for isolating fish cardiomyocytes, including adult zebrafish (Brette *et al*., 2008) and sexually matured rainbow trout (Goldstein *et al*., 2004). And in these two protocols, a combination of collagenase and trypsin was applied in enzymatic digestion, with the same concentrations that being used in method 1.

In spite of this, trypsin alone is still widely used for dissociating tissues. It is demonstrated that the highest yield (ca. 70%) of viable hepatocytes was obtained by perfusing the liver with 0.1% trypsin (Miyazaki, 1977). And in the studies from Grunow *et al*. (2010), 0.1% trypsin was used to digest the dissected larvae of trout (*O. mykiss*). It seems that trypsin alone plays some role in cell isolation as well, but even at this optimum concentration, the membrane of isolated cells could still be damaged by enzymatic digestion and mechanical destruction (Miyazaki, 1977). And collagenase, in some ways, can be superior to trypsin as a means to dissociate tissues (Masson-Pévet *et al*., 1976; Miyazaki *et al*., 1984). In the preparations of new-born rat cardiomyocytes, both enzymes, crude collagenase and crude trypsin were tested by Masson-Pévet *et al*. (1976), they found that trypsinized heart cells were severely damaged whereas no evident injury was observed in collagenase-dissociated cardiomyocytes. Besides, in the preparations of adult rat hepatocytes, it was observed that cells prepared with collagenase were morphologically less injured and could be maintained for a longer period in the primary culture than those treated with trypsin

(Miyazaki *et al*., 1984). The trypsin concentration that we used in isolation method 1 ranges from 0.005% to 0.1% due to different trypsin activity and potency, the damage caused by trypsin for cell membrane might be minimized since the concentration that we used is around the optimum range (0.1%) (Miyazaki, 1977), but it is also worth noting that with the addition of BDM, the cytoskeleton and cell vitality were well protected. In the isolation of adult rat cardiomyocytes, it is demonstrated that BDM, at a 15 mM concentration, clearly improved cell yield (Thum & Borlak, 2001). Therefore, it seems that with the protection of 10 mM BDM and a combination of two proteases, trypsin and collagenase, contributed to a higher cell yield in cell isolation method 1.

Probably the differences in proteases and BDM were the most important factors determining the better outcome in isolation method 1. However, other factors that differed between the two different protocols, may also have influenced the outcome. In cell isolation method 2, washing buffer with 20 mM EDTA was used to open the tight junctions in heart tissue and followed by a perfusion with 1 mg/mL collagenase and 1 mM $CaCl_2$. In Thum and Borlak (2001), three different protocols were tested for isolation of adult rat cardiomyocytes, with the protection of BDM, the washing solution with 0.5 mM EDTA and isolation media with 1 mg/mL collagenase, 20 μM $CaCl_2$ got the best cell yield and cell vitality. Also, for calcium repletion, calcium was added to the culture media in eight subsequent steps to increase the final calcium concentration to 1 mM. When compared to this study, the concentration of EDTA (20 mM) in isolation method 2 could be too high for salmon cardiomyocytes and the immediately followed calcium recovering might stressed the cells. Besides, BDM was not added here to protect the cells. Furthermore, when compared to isolation method 1, glucose and taurine were not included in method 2, which may not meet the nutritional requirements of cardiomyocytes during the isolation process, especially under the situation that cells were stressed.

In addition, there is also difference between the two different growth media, although

we expect that the outcome was primarily determined by the isolation protocols that we have used. And it is only the NaHCO$_3$ in L-15 hepatocyte growth media that differed. But in our study, cardiomyocytes were cultured in CO$_2$-free environment and CO$_2$ is not really required for cell culture, since HEPES is added to both growth media to maintain the pH value. Also, L-15 media contains phenol red as a pH indicator, and it seems that NaHCO$_3$ did not really interfere since no color changes was observed during our cell culture.

At day 9, cells were harvested for isolation of RNA for gene expression analysis. From Fig. 17 and 18, we can see that five of these genes, PCNA, CAV3, αMHC, Nkx2-5, and MEF2C, seem to show higher expression levels in cardiomyocytes cultured with hepatocyte growth media when compared to those with muscle cell growth media, and only gene GATA was showing the opposite trend, which may indicate that cells cultured with hepatocyte growth media were to a higher degree of proliferation (Liang *et al*., 2001), while cells cultured with muscle cell growth media were to a higher degree of differentiation and maturation.

This may be because there were fewer cells all days in the hepatocyte growth media when compared to those in muscle cell growth media, but not due to the differences in growth media composition. Cells are usually in a proliferative status until confluence achieved, however, it seems that cardiomyocytes in the hepatocyte growth media would never reach confluence, which may result in a higher expression of gene PCNA in this group. And cells cultured with muscle cell growth media were close to confluence around day 5 to day 7, which probably turned the cultures more into mature cardiomyocytes. However, this cannot be stated with certainty since the trial was only run in duplicate and which do not allow for testing of significant difference between the treatments. Hence, the main aim of this trial was to test the expression of several genes, including cardiac specific genes that was not previously tested in our laboratory.

Besides, other factors may also have influenced the results in the first cell trial. Through microscopic images, decrease in cell number was observed at day 9 when compared to day 7 in group 1 (*Iso1+M*), which may indicate some degree of cell apoptosis. Concerning apoptosis may start at day 9, and the gene expression results may not fully demonstrate the difference in growth and differentiation status of cardiomyocytes cultured with these two growth media, we have decided to draw our conclusion based on the microscopic images at day 7 and not put much emphasis on interpreting the gene expression results in this study.

Therefore, treatment 1 (*Iso1+M*) turned out to be the one with the best culturing outcome, since the largest confluent monolayer was formed in this group at day 7. And it was decided to proceed with the combination of isolation method 1 and muscle cell growth media for isolating and culturing of cardiomyocytes in the later studies, due to the fact that the cells seem to proliferate and differentiate with this combination.
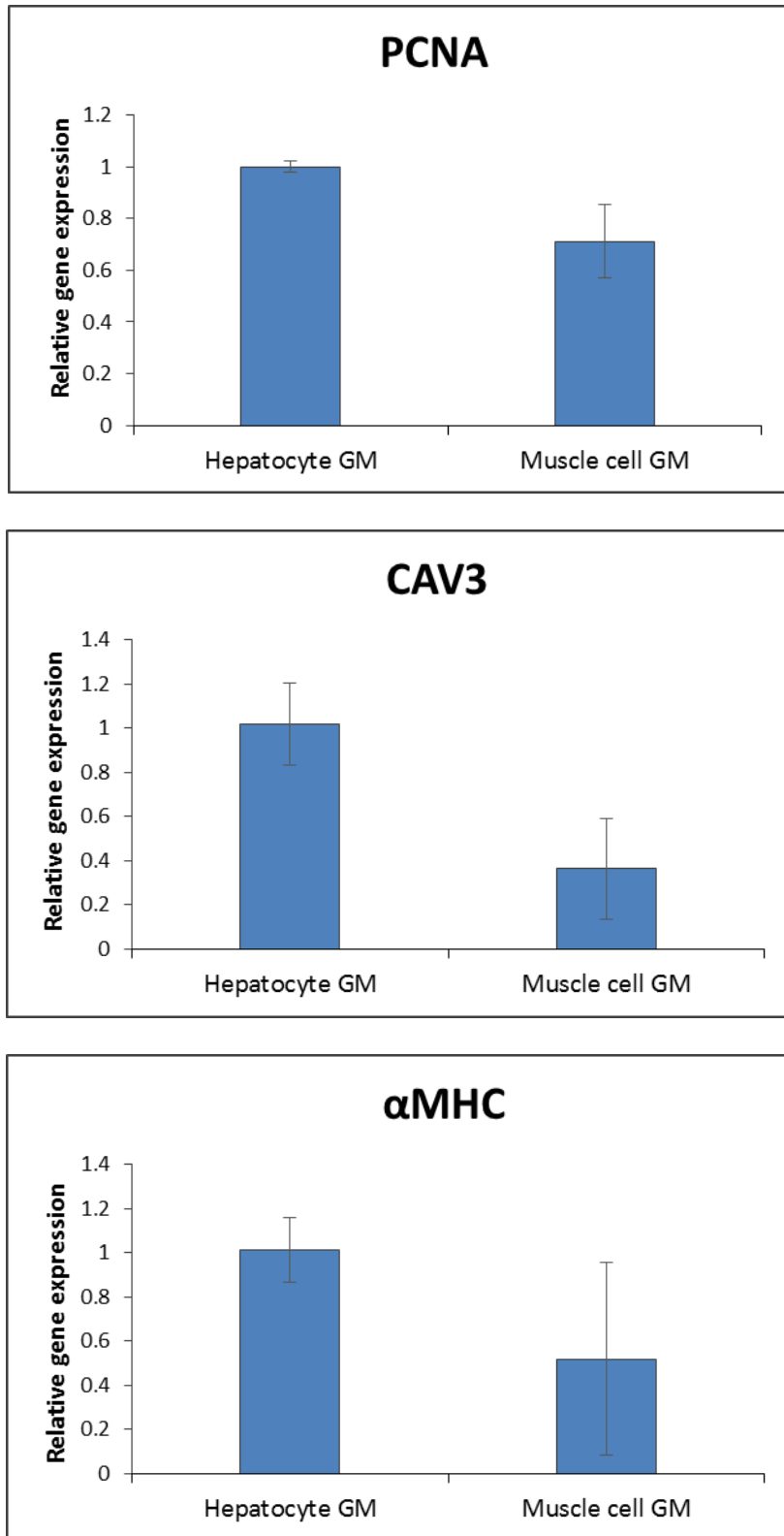
Fig. 17. Relative gene expression of PCNA CAV3, and αMHC in cardiomyocytes cultured with two different growth media. Bars are mean of relative expression from two parallels shown with standard error of mean (*n=2*). Mean of hepatocyte growth media group is set to 1, data from muscle cell group is relative to 1.

Fig. 18. Relative gene expression of GATA4, Nkx2-5, and MEF2C in cardiomyocytes cultured with two different growth media. Bars are mean of relative expression from two parallels shown with standard error of mean (*n=2*). Mean of hepatocyte growth media group is set to 1, data from muscle cell group is relative to 1.
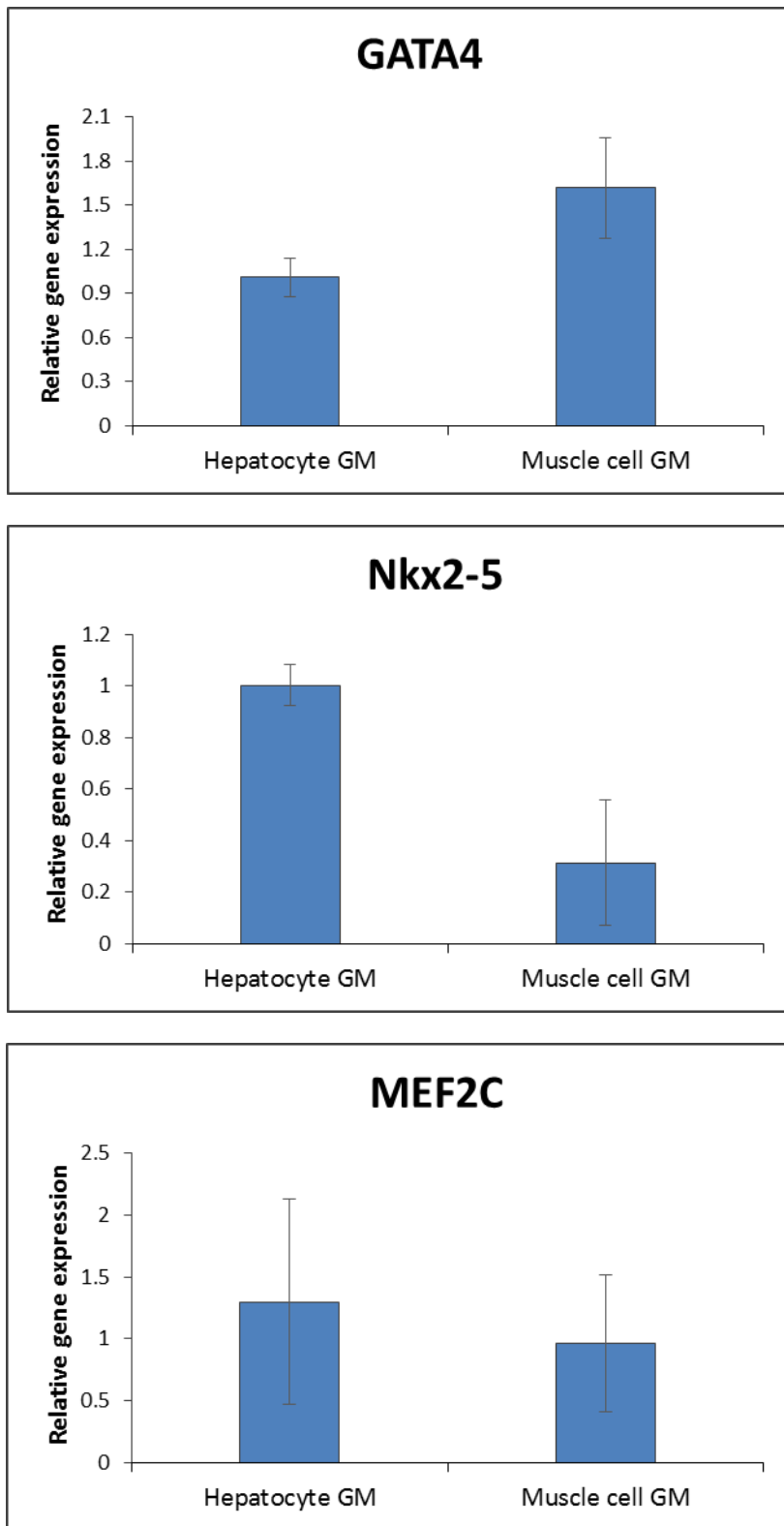
3.2   Study 2: A time study of cardiomyocytes in culture

In order to learn more about how PCNA and cardiac specific genes are regulated at different stages of cardiomyocyte proliferation and differentiation, a time study was performed with cells isolated and cultivated according to treatment 1 (*Iso1+M*) in study 1. In this study, cultured cardiomyocytes were harvested at six different time points, day 2, 3, 5, 7, 9, 11, respectively after seeding. And the harvested cells were further used for gene expression analysis.
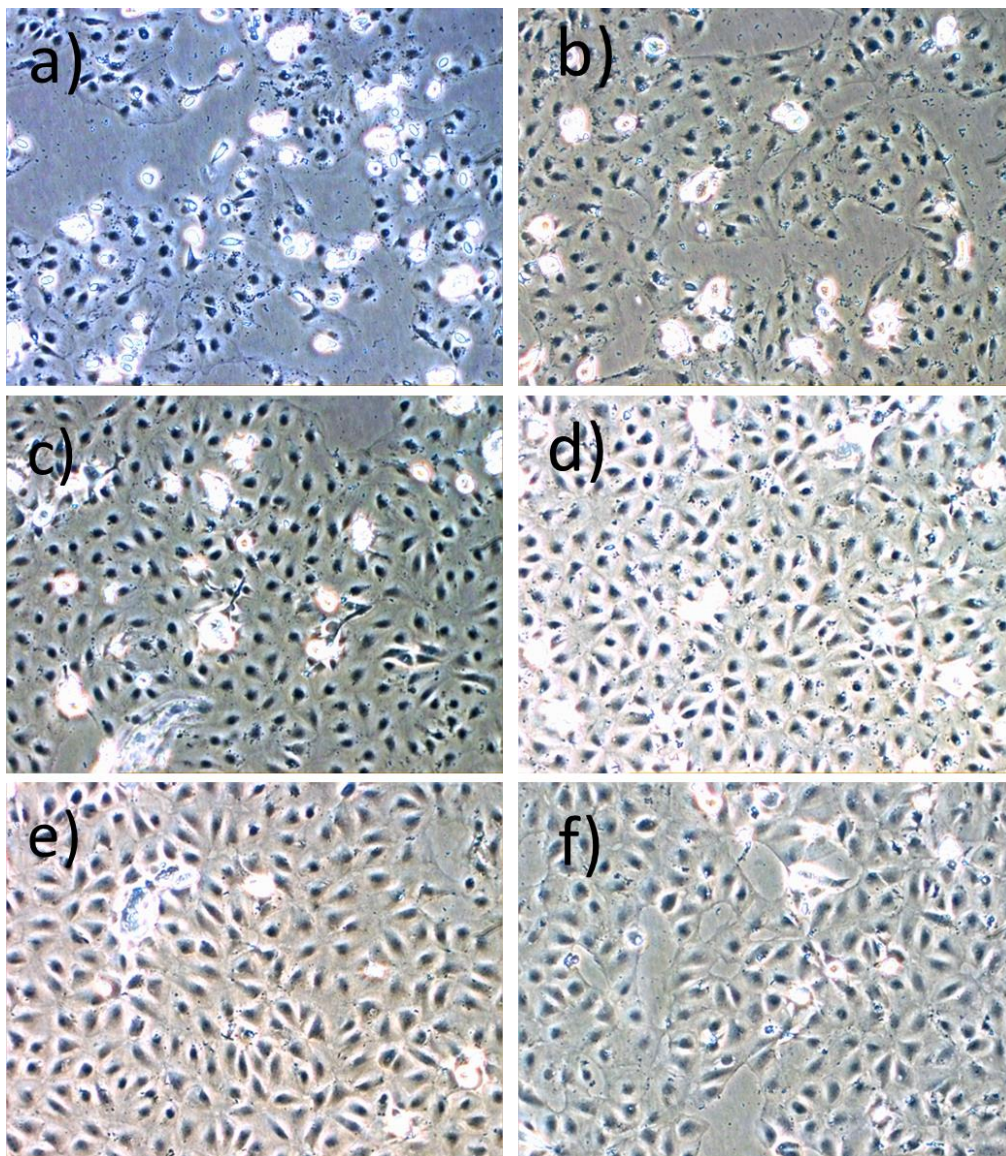

Fig. 19.   Phase contrast microscopic images of salmon cardiomyocytes from one well ($10\times$ magnification) at six different time points: **(a)** Day 1; **(b)** Day 3; **(c)** Day 5; **(d)** Day 7; **(e)** Day 9; and **(f)** Day 11.

From the gene expression results (Fig. 20 and 21), we can see that PCNA, CAV3, GATA4, and MEF2C showed a similar trend of changes from day 2 to day 11. And significant increased expression was observed in PCNA ($P < 0.0001$), CAV3 ($P = 0.0007$), and GATA4 ($P < 0.0001$). Also, the increased expression of MEF2C was close to a significant level ($P = 0.0577$). Besides, a similar trend of expression changes was observed in gene αMHC and Nkx2-5. Both of them showed a significant decreased expression in the mid-term of cell culture and then followed by a significant increase at the end of cell culture. The expression of Nkx2-5 reached its lowest level at day 5, while αMHC reached at day 7, a little bit later than Nkx2-5. It has been known that GATA4, Nkx2-5, MEF2C and αMHC are involved in cardiac transcription network. And αMHC together with CAV3 are related to sarcomeric activity, part of cardiomyocyte development.

PCNA is involved in the control of eukaryotic DNA replication, and the expression level of PCNA reflects the proliferative status of cells. In our study, the significantly increased expression of PCNA indicates that newly isolated salmon cardiomyocytes have the ability to proliferate *in vitro* up to 11 days, it is also consistent with our microscopic images, since increase in cell number could be seen.

GATA4 and MEF2C are known to play key central roles in the up-regulated networks in cardiomyocyte-specific differentiation of murine embryonic stem cells (ESCs) (Gan *et al*., 2014). And the expression of GATA4 can promote the expression of its downstream gene MEF2C (McCulley & Black, 2012; Dodou *et al*., 2004). This is in concordance with our gene expression results, since both GATA4 and MEF2C showed a similar trend of increased expression from day 2 to day 11, and that may indicate the newly isolated salmon cardiomyocytes can differentiate *in vitro*. Also, the slightly non-significant up-regulation of GATA4 after day 5 indicates that the differentiation and maturation process of cardiomyocytes was almost completed at this time.

Furthermore, it is reported that the forced expression of GATA4 can induce a

hypertrophic response in the primary cultured neonatal rat cardiomyocytes and transgenic mice (Liang *et al*., 2001). Combined with the significant increased expression of PCNA during cell culture, it seems that not only can cultured salmon cardiomyocytes proliferate and differentiate *in vitro*, but also develop hypertrophic growth. This could also be seen from the microscopic images. The number of cells increased rapidly in the first few days until confluence was achieved around day 5. And then, enlargement in cell size was observed.

However, these observations differ from what we have seen in study 1, in which the number of cardiomyocytes was observed to start decreasing at day 7. This might be caused by different plating densities in the beginning, since Clark *et al*. (1998) demonstrated that cardiomyocyte plating density affects not only the hypertrophic response and features of the differentiated phenotype of isolated adult feline cardiomyocytes, but also plays a significant role in influencing survival *in vitro*. The increase in cell size attributes directly to an increase in cell contact, and high levels of confluence are significant factors influencing the survival of cultured feline cardiomyocytes (Clark *et al*., 1998). In this study, we adopted the isolation and culture conditions with the best outcome from study 1 (isolation method 1 with muscle cell growth media). A much higher cell density was observed at day 1 (Fig. 19a) when compared to study 1, and much bigger confluence was achieved afterwards, that may result in a longer proliferation and survival time of cardiomyocytes in this study.

CAV3 proteins make up sodium channels, which transport Na^+ into cells. And in cardiac muscle, sodium channels are involved in maintaining the heart's normal rhythm (http://ghr.nlm.nih.gov/gene/CAV3). Also, CAV proteins participate in sarcolemma repair mechanism of both skeletal muscle and cardiomyocytes that permits rapid resealing of membranes disrupted by mechanical stress (http://www.genecards.org/cgi-bin/carddisp.pl?gene=CAV3&search=5454f633730438 b3e82fd6986344dffa). In the studies from Xu *et al*. (2009), CAV3 was found to play an up-regulated role in both human ESC-derived cardiomyocytes, fetal heart and adult

heart when compared to embryoid bodies. And Song *et al*. (1996) found that CAV3 protein was strongly induced during mouse myoblast differentiation. A significant increased expression was also found in our study, which probably indicates the building of caveolae and sarcolemma structures (Bastiani & Parton, 2010), and an increasing in plasma membrane area (Song *et al*., 1996).

Furthermore, αMHC protein, a cellular component related to sarcomeric structures, are involved in energy production and primary metabolism, showed a relatively high expression level in human ESC-derived cardiomyocytes (Xu *et al*., 2009). Moreover, similar with GATA4 and MEF2C, Nkx2-5 also plays a key central role in the up-regulated networks in cardiomyocyte-specific differentiation and is important in maintaining the cardiac phenotype (Gan *et al*., 2014). It has been reported that Nkx2-5 had relatively high expression levels in cardiomyocytes and human fetal heart, but much lower levels in adult heart (Xu *et al*., 2009). In our study, it seems that αMHC and Nkx2-5 up-regulated at both early stages of proliferation and the late stages of cell differentiation and maturation. Similar conclusion was found in Gan *et al*. (2014), in their study, slightly down-regulation of gene Nkx2-5 and αMHC was observed at ESCs-derived cardiomyocytes at day 19 when compared to day 12, and a slightly up-regulation at day 26 when compared to day 19, this is consistent with our gene expression results, since ESCs-derived cardiomyocytes completed their maturation process at day 19 and in our study, the process was almost completed around day 5, both Nkx2-5 and αMHC had the lowest expression level around the time of maturation.

In general, this study indicates that newly isolated salmon cardiomyocytes have the ability to proliferate and differentiate *in vitro* by up-regulating gene PCNA, CAV3, GATA4, MEF2C, and the proliferation period can last up to 11 days, depending on the seeding density. In addition, hypertrophic growth was also observed at the late stages of cell culture.
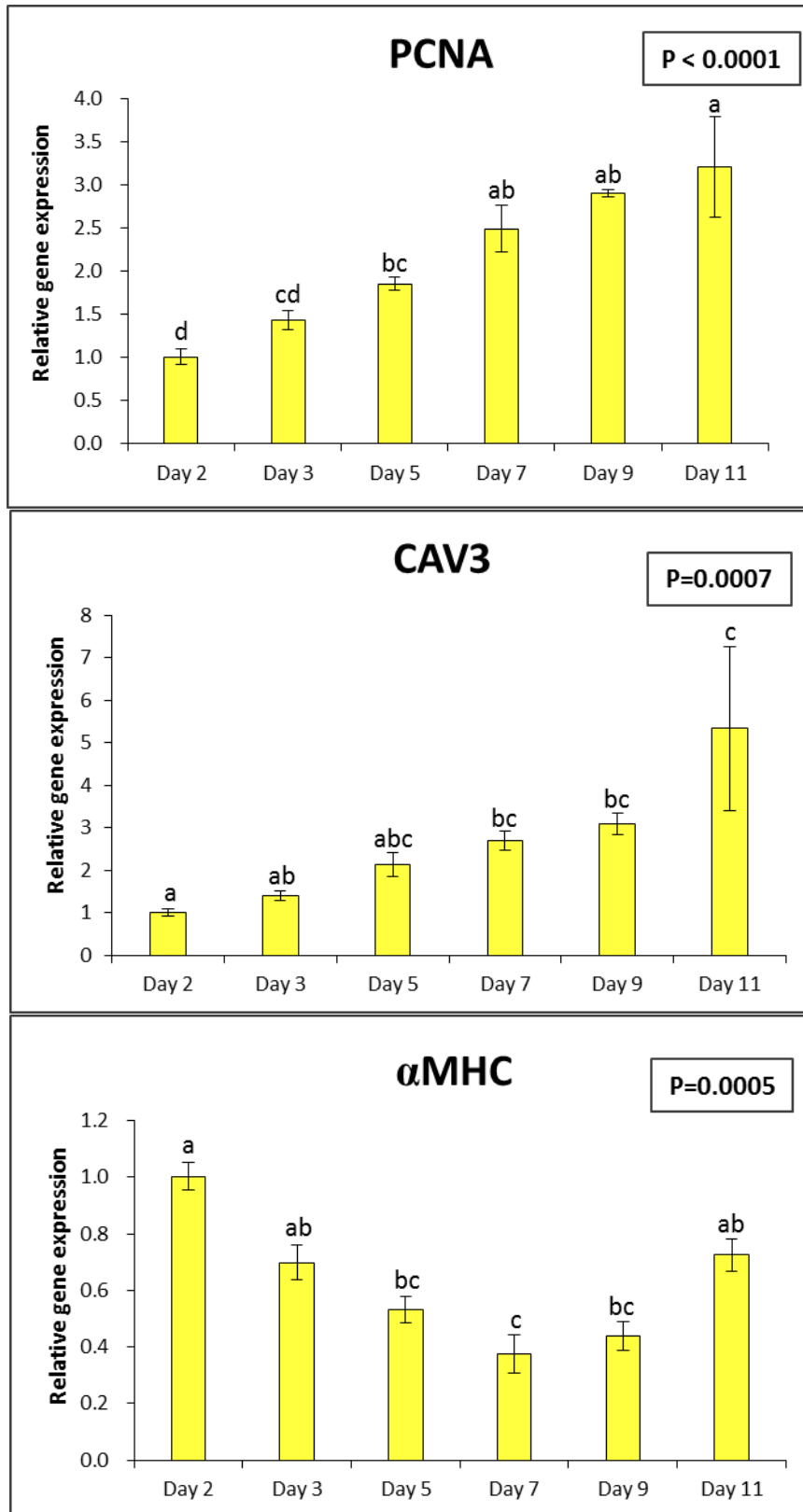
Fig. 20. Relative gene expression of PCNA, CAV3, and αMHC in cardiomyocytes at six different time points. Bars are mean of relative expression from three parallels and are shown with standard error of mean (*n=3*). Different letters denote significance. Mean of day 2 is set to 1 and the data from other groups is relative to 1.

Fig. 21. Relative gene expression of GATA4, Nkx2-5 and MEF2C in cardiomyocytes at six different time points. Bars are mean of relative expression from three parallels and are shown with standard error of mean (*n=3*). Different letters denote significance. Mean of day 2 is set to 1 and the data from other groups is relative to 1.
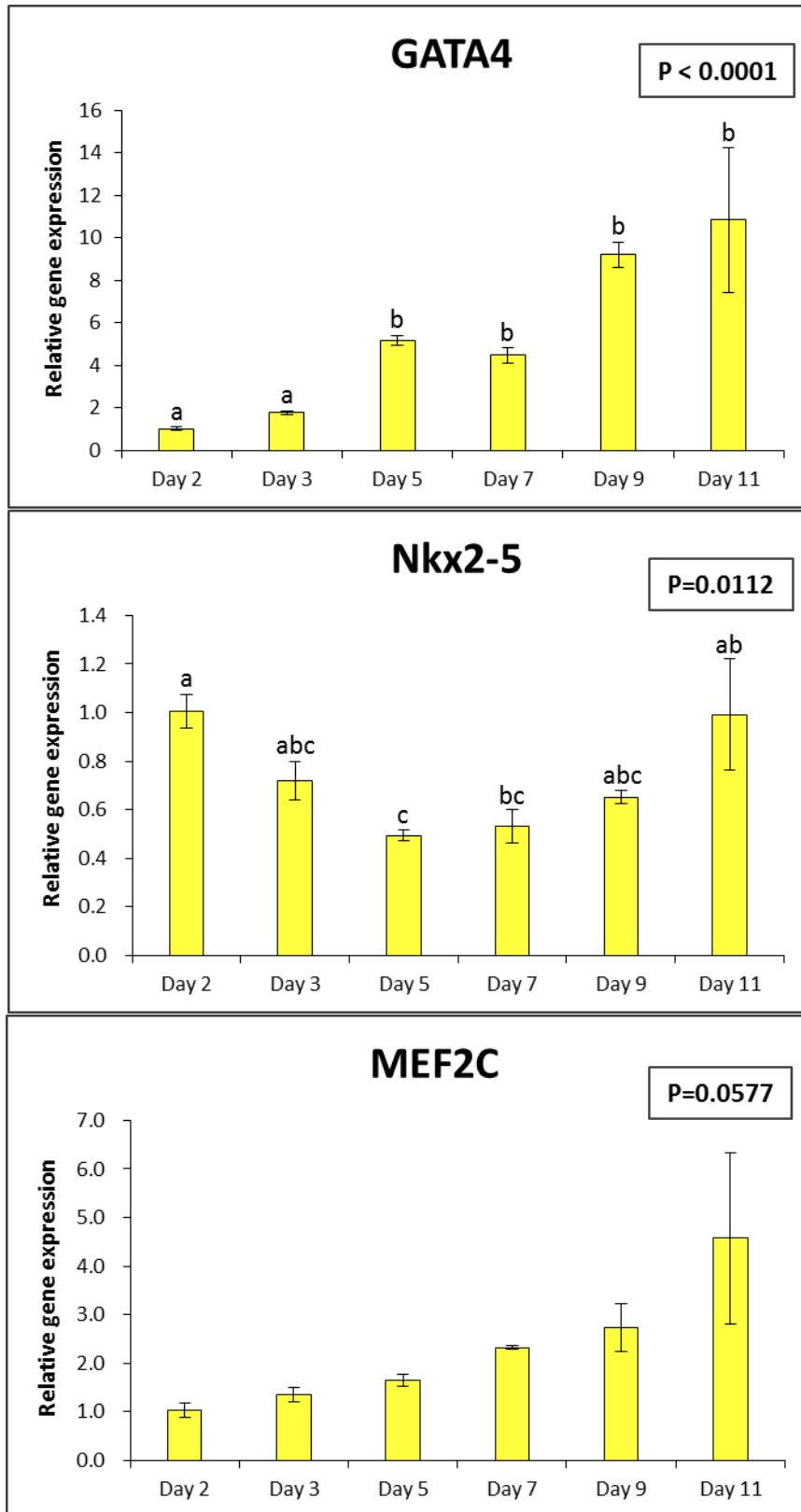
3.3 Study 3: Influence of basic fibroblast growth factor (bFGF) on cell proliferation and development

We observed in study 2 that a good seeding density is probably important for good proliferation capacities of cardiomyocytes in culture. To test if the supplementation of growth factor bFGF (also called FGF-2) to the growth media would increase the proliferation degree of cardiomyocytes in culture, duplicate wells (*Day2+FGF* & *Day7+FGF*) in treatment group were added 15 µL 25 µg/mL bFGF at day 1. And for duplicate wells in the control group (*Day2-Control* & *Day7-Control*), only L-15 muscle cell growth media was added for culturing. Cells from both groups were harvested at day 2 and day 7 respectively for isolation of RNA for gene expression analysis.
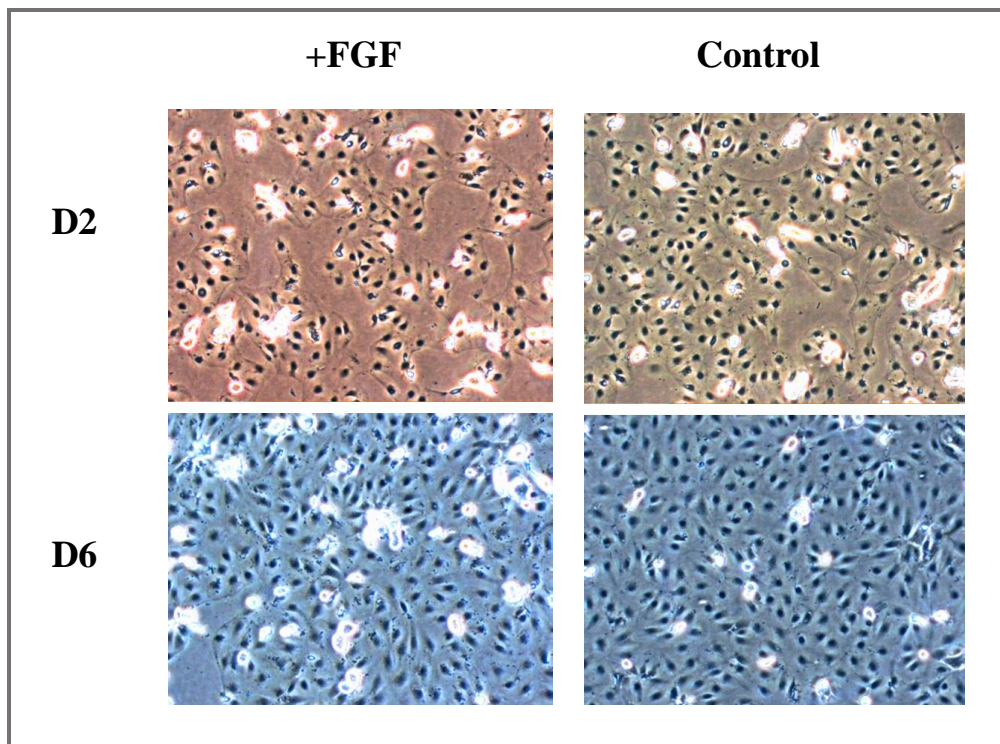

Fig. 22. Phase contrast microscopic images of salmon cardiomyocytes from bFGF treated group and control group (10×magnification) at day 2 and day 6 respectively.

This study was performed without replicate (*n=1*), therefore, no statistics is presented. Still the images and gene expression data give valuable indications on how the cells

respond to the supplementation of bFGF. From the gene expression results (Fig. 23), we can see that in both control and experimental group, four of these genes, PCNA, CAV3, GATA4, and MEF2C showed an increased expression at day 7 comparing to day 2. This is consistent with our gene expression results in study 2, since cardiomyocytes have the ability to proliferate and differentiate *in vitro*.

Basic fibroblast growth factor (bFGF) is a multifunctional protein which has been found in most tissues and organs examined, including the heart (Kardami & Fandrich, 1989; Basilico & Moscatelli, 1992; Liu *et al*., 1995). And it has been demonstrated that adult rat cardiomyocytes express functionally coupled high-affinity bFGF receptors and they are capable of a biological response to bFGF *in vivo* (Liu e*t al*., 1995). Also, bFGF is known to affect cell differentiation and expression of specific genes and to promote cell survival (Basilico & Moscatelli, 1992). In contrast to our study, when comparing the relative expression levels of these four genes at day 7, control group always had a slightly higher expression level than bFGF group. It seems that the supplementation of bFGF to growth media had no positive effect on the proliferation and differentiation status of *in vitro* cultured salmon cardiomyocytes. That can also be seen from the microscopic images (Fig. 22), since cardiomyocytes in both groups had a similar cell density at day 6. Besides, down-regulation of gene Nkx2-5 was observed in both groups at day 7, while a larger reduction was observed in well *Day7-Control*. In study 2, we have found that Nkx2-5 shown up-regulation at both the early stages of proliferation and the late stages of cell differentiation, and its lowest expression was observed around day 5. Combined with the higher expression of PCNA in well *Day7-Control*, it seems that cardiomyocytes in this well were at the early stages of cell differentiation (late stages of proliferation), while cardiomyocytes in well *Day7+bFGF* were still proliferating.

Schuldiner *et al*. (2000) studied the effects of eight growth factors on the differentiation of human ESCs, including bFGF. In their study, embryoid bodies (EBs) that derived from human ESCs were grown in the presence of 10 ng/mL bFGF for 10

days, fibroblast-like cells were observed with an activation of ectodermal and mesodermal markers. In Khezri *et al*. (2007), EBs that produced from mouse ESC line were treat with 10 ng/mL bFGF for the first two days in suspension to investigate the effect of bFGF on the differentiation of ESCs into early cardiomyocytes. And in Rose *et al*. (2013), up to 40 ng/mL of exogenous bFGF was used to explore the role of bFGF supplementation during mouse ESC expansion and differentiation toward the osteogenic lineage. In contrast to the studies above, a much higher concentration of bFGF was applied in our study (around 125 ng/mL). This concentration might be too high for the isolated salmon cardiomyocytes, since no positive effect was observed. Moreover, bFGF may have greater influence on stem cells rather than the newly isolated cardiomyocytes, since the newly isolated cardiomyocytes probably had a higher differentiation degree.

Furthermore, slightly up-regulation of gene αMHC was observed in the control group at day 7, while the expression level of αMHC remained the same in well *Day7+bFGF* at day 7 comparing to day 2. This observation differs from the gene expression results in the time study. Since in study 2, Nkx2-5 and αMHC showed a similar trend of expression during cell culture, and αMHC had its lowest expression level around day 7. In contrast, no down-regulation of αMHC was found in this study at day 7, and even slightly up-regulation was observed in the control group. It seems that the expression level of αMHC is not associated with the maturation status of cardiomyocytes, and there is no correlation between the expression patterns of Nkx2-5 and αMHC. In general, this study demonstrated that the supplementation of bFGF to cell culture media did not promote the proliferation and maturation level of newly isolated salmon cardiomyocytes. Besides, a high concentration of bFGF, may even in turn, slow down the proliferation and maturation process of primary cultured salmon cardiomyocytes. And that is why we decided not to proceed with inclusion of growth factor in the later studies. Moreover, in order to conclude with certainty, the trial should be repeated to check if lower concentrations, than used in this trial, of growth factor would influence cell proliferation and development.
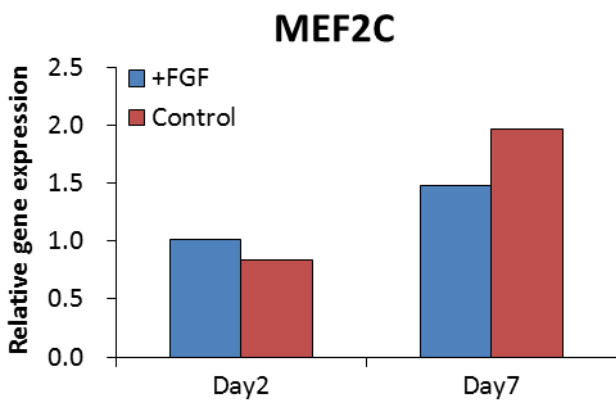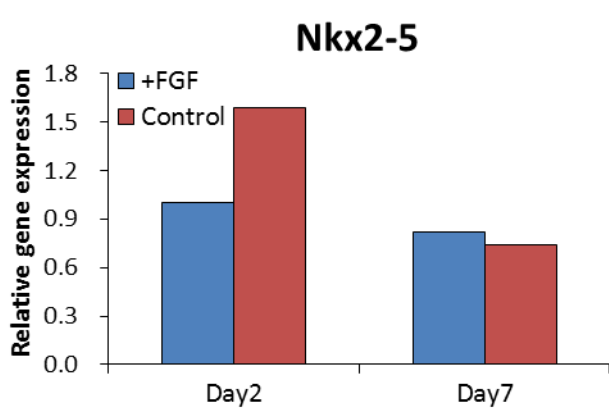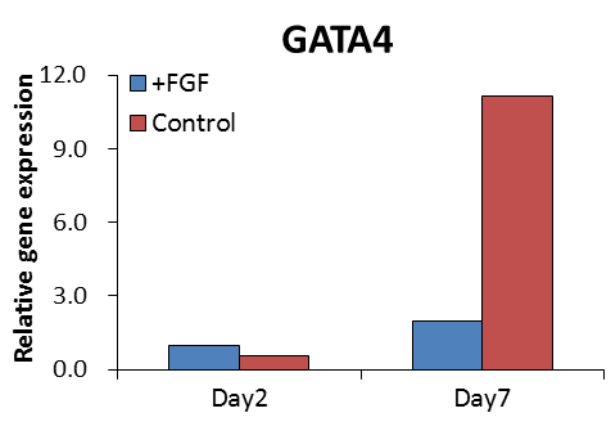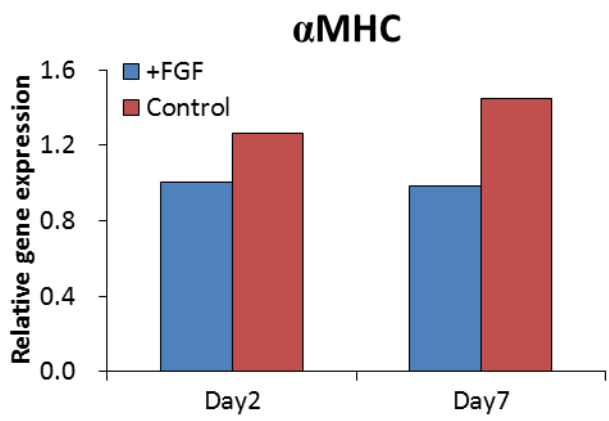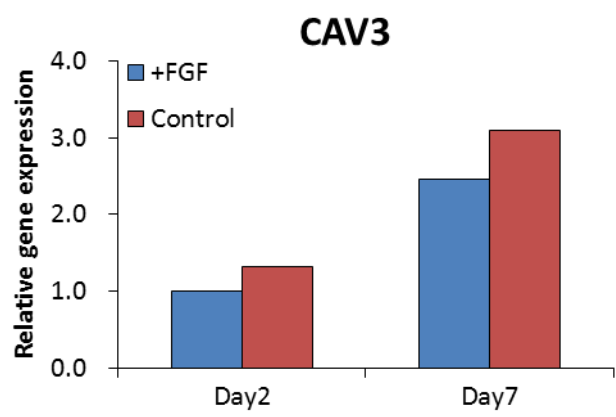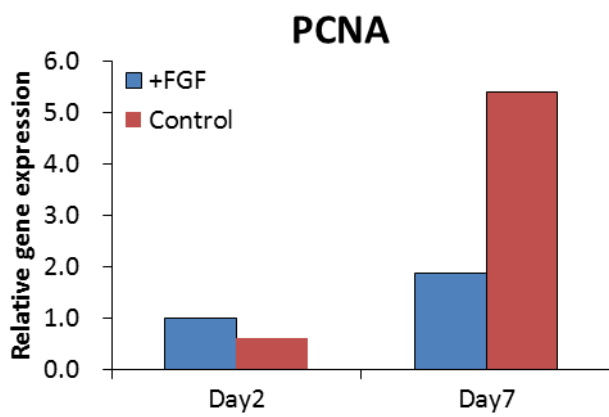
Fig. 23. Relative gene expression of PCNA, CAV3, αMHC, GATA4, Nkx2-5, and MEF2C in cardiomyocytes at day 2 and day 7, respectively (*n=1*). Relative expression of well *Day2+FGF* is set to 1 and the data from other wells is relative to 1.

3.4 Study 4: Long term cultivation of cardiomyocytes

The aim of the microscopy study 4 was to follow the morphology of cardiomyocytes in long term culture. During long term cultivation, different morphologies of the cells in culture were observed. Since cells were isolated from the whole fish heart and filtrated through a 100 μm nylon filter, we are likely to get a culture containing mixed cell types. Cardiomyocytes are thought to be terminally differentiated cells and lose their ability to proliferate shortly after birth (Akazawa & Komuro, 2003), so the cells that multiplied after their adhesions we expect they would be immature cardiomyocytes or stem cells (Fig. 24).

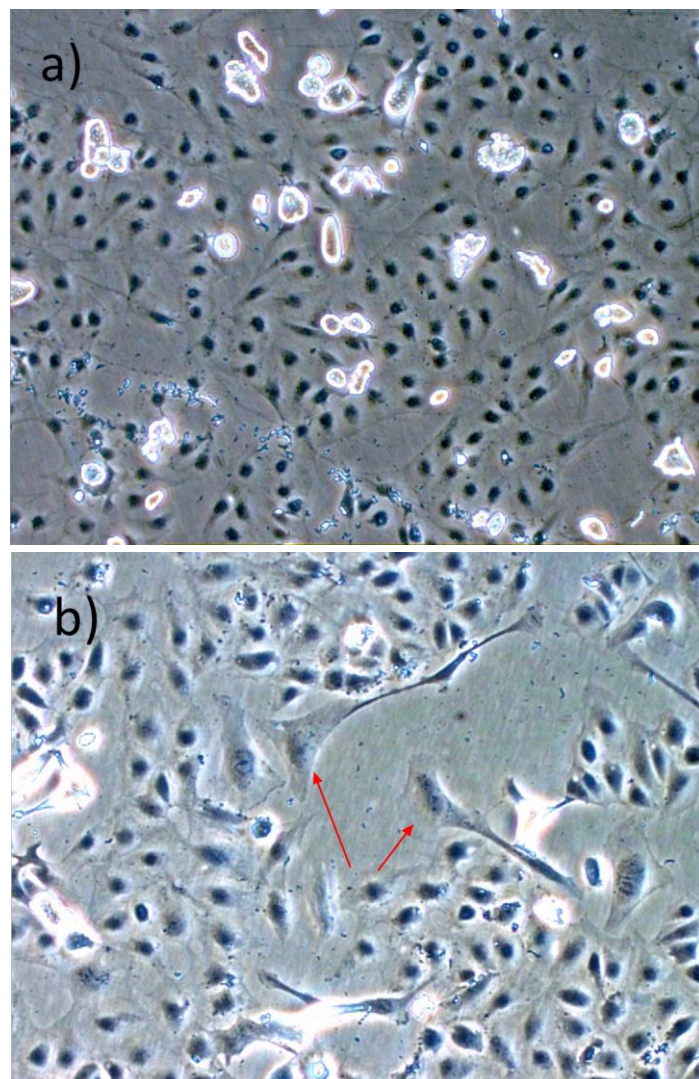**Fig. 24.** Phase contrast microscopic images of salmon cardiomyocytes. **(a)** Confluent monolayer was achieved at day 2; and the spherical cells, which appeared in white translucent color and observed growing on the spread cells, are likely to be unspecialized cardiomyocytes. **(b)** Fibroblast-like cells were observed at day 8 (red arrows), also, some of the spherical cells started stretching toward both ends.

It seems that some fully differentiated cardiomyocytes started dying around day 11 with typical characteristics of cell apoptosis observed (Fig. 25a). While other differentiated cardiomyocytes spontaneously started beating around day 15, and the number of beating cardiomyocytes reached its peak level at day 16. Video of my beating salmon cardiomyocytes was published at Forskning.no, which can be viewed at http://forskning.no/hav-og-fiske/2015/03/laksehjerte.
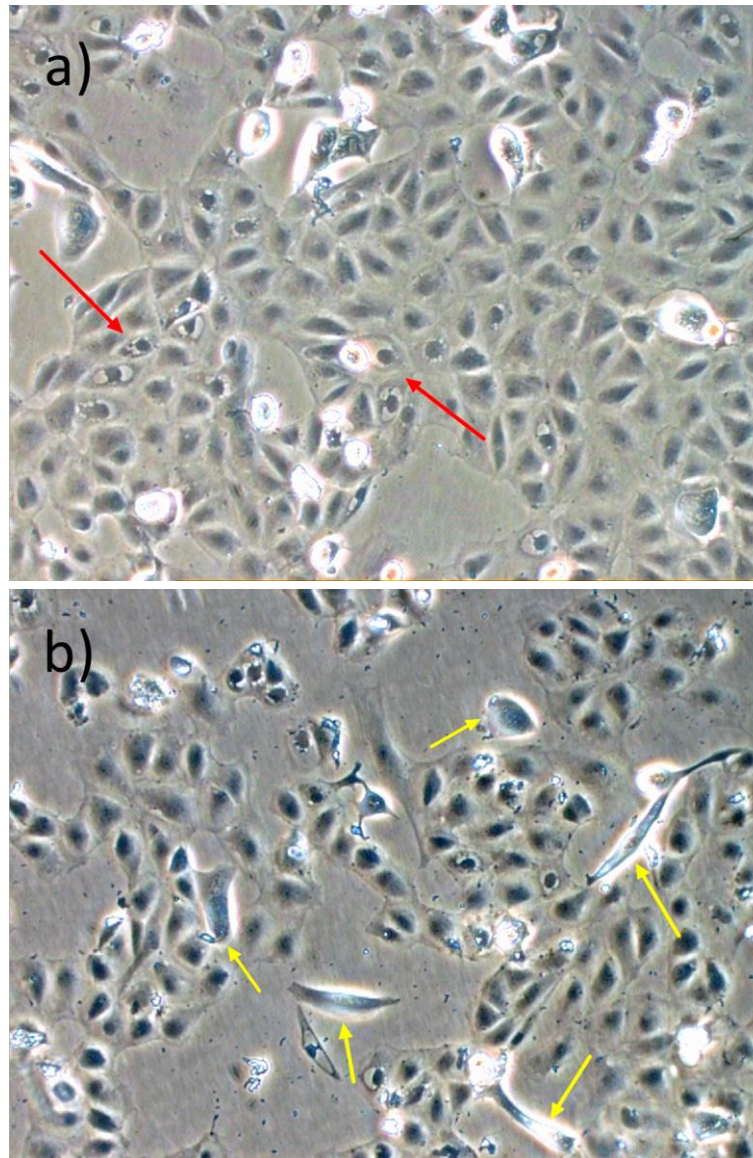
Fig. 25. Phase contrast microscopic images of salmon cardiomyocytes at day 11. **(a)** Nuclear chromatin condensation and cytoplasmic vacuolization were observed (red arrows). **(b)** Some of the cells stretched and elongated into a spindle or oval shape (yellow arrows). We expect they were either developed from translucent spherical cells or mature cardiomyocytes, and these cell structures developed into the beating cell structures at day 15.
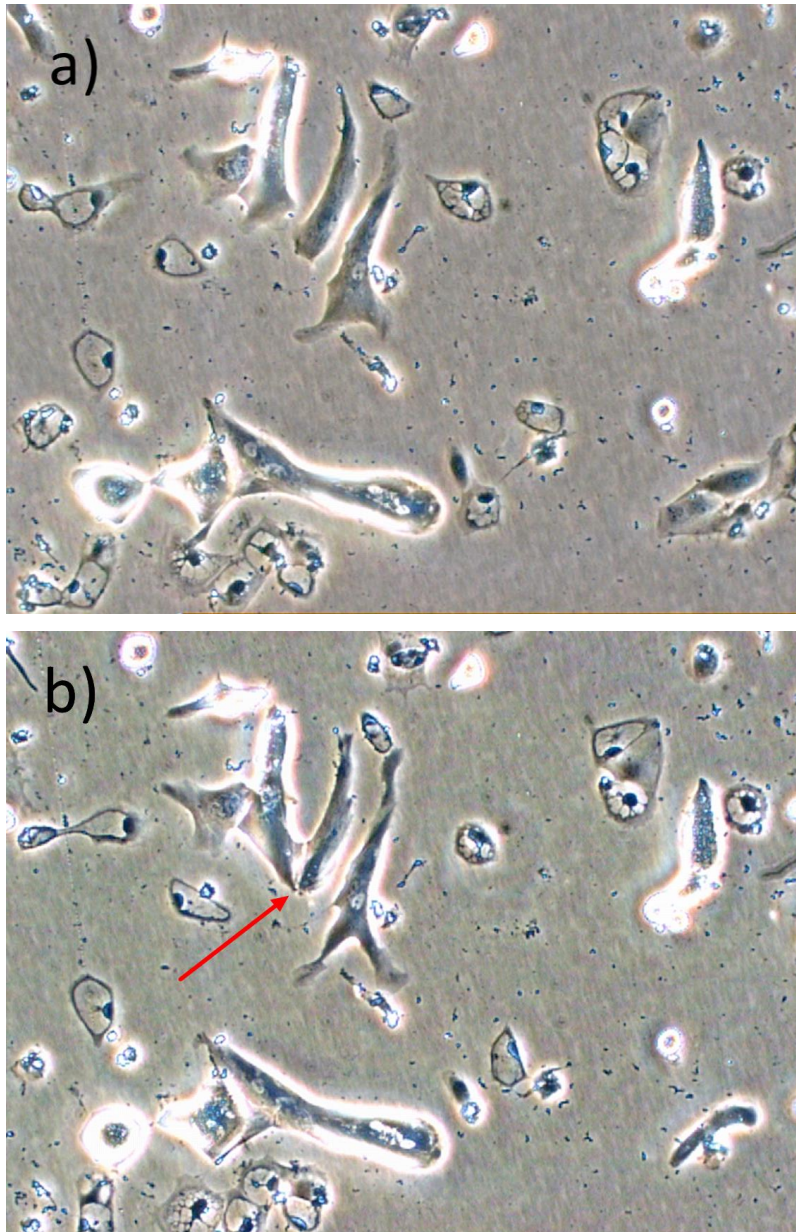
Fig. 26. **(a)** Phase contrast microscopic images of irregular-shaped beating cardiomyocytes at day 18. At this stage, several of the cells that had not developed into beating cells had loosened and died. Cavitation of cytoplasm was observed from dying cells, and after some time, the loosing died cells could be removed by washing. **(b)** The morphological change of two beating cardiomyocytes was observed after one day (red arrow). Beating cells have the tendency to fuse with their neighboring beating cells and once they fused, the beating was synchronized.

The morphological changes of one star-shaped continually beating cell cluster were observed from day 20 to day 57, its beating period lasted for more than 37 days. Since L-15 media contains phenol red, big pH variation was observed in the later period of cell culture. When cells were washed and fed new fresh growth media, media became deep red after one day and pH value of 8 was measured.
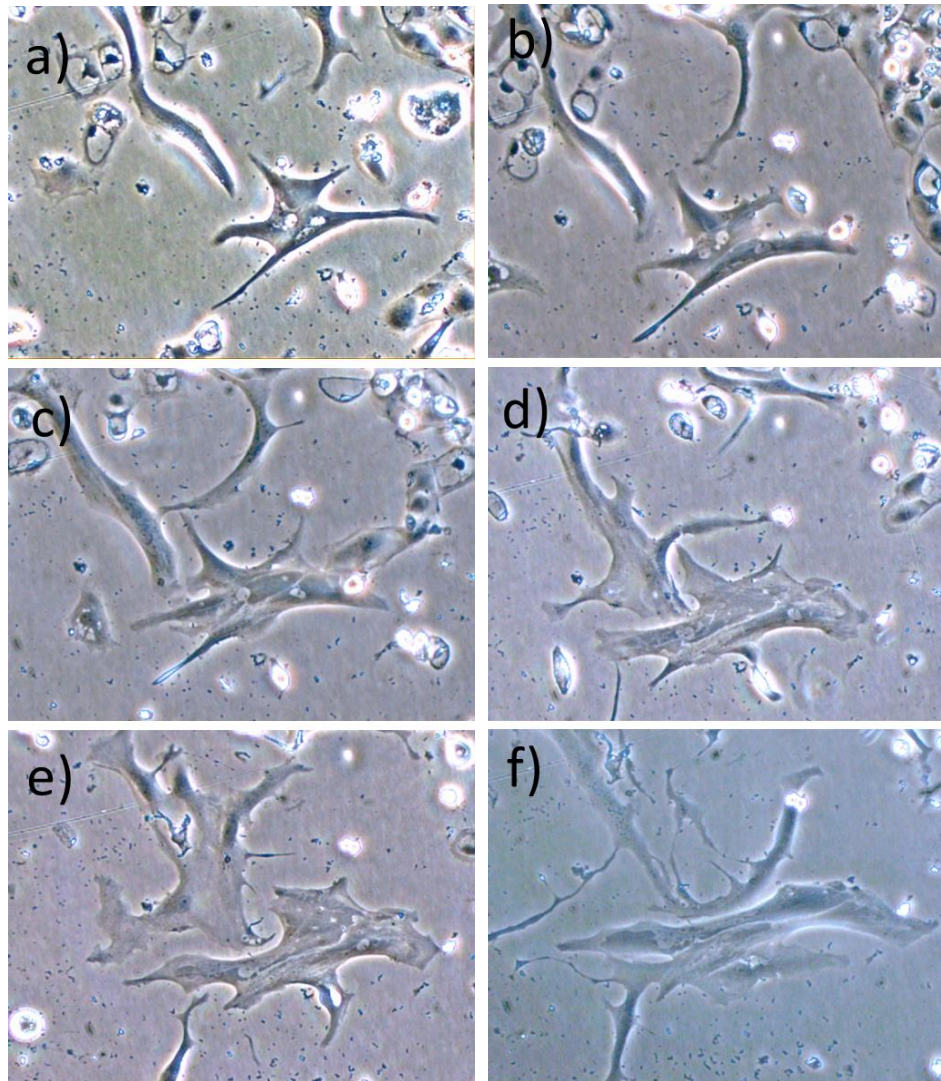
Fig. 27. The morphological changes of one star-shaped continually beating cell cluster were observed (10× magnification). Images were taken at six different time points: **(a)** Day 20; **(b)** Day 23; **(c)** Day 27; **(d)** Day 35; **(e)** Day 42; and **(f)** Day 57. This star-shaped cell cluster was observed beating strongly and regularly at day 20, and its beating rate was around 30 beats per minute. But as the culture time increased, the cell cluster started stretching and its structure became hazy. Also, the beating rate became slower and irregular.
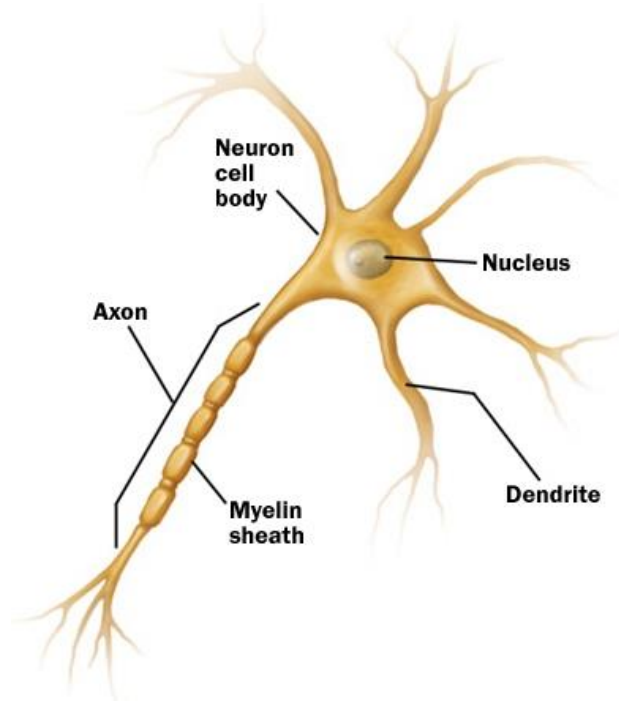
Fig. 28. The basic unit of communication in the nervous system is the nerve cell (neuron). Each nerve cell consists of the cell body, a major branching fiber (axon) and numerous smaller branching fibers (dendrites). Picture from http://www.mayoclinic.org/nerve-cell-neuron/img-20007830

The star-shaped beating cell cluster looks similar with the nerve cell (Fig. 28). Since cardiomyocyte cannot beat without nerve cells, hence we expect the existence of nerve cells in our cell culture. Similar conclusion was found in Tomita *et al*. (2005), their study suggested that cardiac side population cells are cardiomyocyte progenitor cells and neural crest-like multipotent stem/progenitor cells, they can differentiate into cardiomyocytes and typical neural crest-derived cells, including neurons, glia, and smooth muscle under certain conditions.

Furthermore, isolated salmon cardiomyocytes are proven to have the ability to proliferate *in vitro* in our study. It is also reported that zebrafish (*Danio rerio*) has the ability to scarlessly heal the adult heart muscle after injury, which is accomplished by switching the cardiomyocytes from a mature to a partially dedifferentiated state, thereby enabling the reactivation of proliferation (Jopling *et al*., 2010; Kikuchi *et al*.,

2010). And *in vitro* study also shown that adult zebrafish cardiomyocytes, similarly to zebrafish *in vivo* heart regeneration, undergo partial dedifferentiation during primary culture and, in contrast to their mammalian counterparts, are able to proliferate (Sander *et al*., 2013).

Besides, beating cardiomyocytes were observed in our cell culture around day 15, and some beating structures were observed to keep contracting up to 5 weeks. Several similar studies were also found in different fish species. In Grunow *et al*. (2011), long-term (more than one month) spontaneously contracting cardiomyogenic cell aggregates were generated from rainbow trout larva. And in Sander *et al*. (2013), primary cultured adult zebrafish cardiomyocytes started beating as early as 2 days after plating, and the cells kept contracting up to 4 weeks, video can be seen at: http://www.nature.com/nprot/journal/v8/n4/extref/nprot.2013.041-S10.mov. However, this beating structure is a little bit different from our beating cardiomyocytes. Unlike salmon, the primary cultured Zebrafish cardiomyocytes formed network-like connections during cell culture, which resulted in synchronously contracting of the whole culture. But in our culture, salmon cardiomyocytes usually beat in the form of single cells or small cell clusters. That may be due to Sander *et al*. (2013) employed a higher density of cardiomyocytes in their culture, and the stronger ability of Zebrafish cardiomyocytes to dedifferentiate and proliferate, which both lead to the reinforce of cell-cell connections in the culture.

In general, this microscopy study showed us the morphologic changes of salmon cardiomyocytes during long term cultivation. Primary isolated salmon cardiomyocytes can be cultured *in vitro* for a very long time without contamination observed (bacteria and fungi growth), and the observation of beating cardiomyocytes in our culture further reveals the fact that our isolation method and cell culture condition are appropriate for the growing and developing of salmon cardiomyocytes.

3.5 Study 5: Stress response of cardiomyocytes to H_2O_2

To evaluate the H_2O_2 induced stress response in cardiomyocytes, four groups of isolated cardiomyocytes were cultivated in L-15 muscle cell growth media for 7 days. And at day 7, group 2, 3, and 4 were incubated with 100 μM H_2O_2 for 30, 60, and 90 min, respectively before harvesting, while group 1 was set as the control group. The harvested cells were further used for isolation of RNA for gene expression analysis of heart specific and stress response related markers.

For the expression level of gene GATA4, a slightly significant up-regulation was observed in group *H_2O_2-30min* (*P = 0.031*), while slightly significant down regulation was observed in group *H_2O_2-90min*. And for gene αMHC, slightly increased expression was observed in all experimental groups comparing to the control, but the highest increased expression was observed in group *H_2O_2-30min*. It seems that the stress stimulation with H_2O_2 can, to some extent, contribute to the maturation and development of isolated salmon cardiomyocytes, and the growth was optimal with a H_2O_2 treatment for 30 min. A similar conclusion was found in Chen *et al*. (2000). In their study, more than 60% H9C2 or primary cultured neonatal rat cardiomyocytes remained attached and intact after treatment with 200 μM H_2O_2 for 2 h; those cells appeared to be six times bigger in volume and contained three times more protein per cell than untreated cells after 5 or more days in culture, which indicates that while H_2O_2 can cause cell death, the surviving cardiomyocytes undergo hypertrophy. Also, in Ruan *et al*. (2015), it is demonstrated that two weeks of cyclic stress conditioning increased cardiac troponin T (cTnT) intensity per cell by about 1.3 to 1.5-fold over no stress conditioning in both H7 human ESC and IBJ human induced pluripotent stem cell-derived cardiovascular progenitor constructs, and significantly higher normalized cTnT protein levels were found in these constructs over unstressed ones, suggesting that cyclic stress conditioning does have a distinct effect on cardiomyocyte maturation.

Gene SOD1 encodes a soluble cytoplasmic protein which binds copper and zinc ions, and it is one of two antioxidant isozymes that catalyzes the dismutation of free superoxide radicals (O_2^-) into either molecular oxygen (O_2) or H_2O_2. Superoxide radicals are detrimental to the cells if too many are accumulated within the cells. And they are by-products of normal cell processes, particularly energy-producing reactions. Thus, SOD1 plays an important role in protecting the organisms from oxidative stress. The studies from Xie *et al*. (2013) showed that neonatal rat cardiomyocytes treated with H_2O_2 (100, 200, 500 μM) for 24 h significantly suppressed the activity of SOD. And Tiedge *et al*. (1997) revealed that the mRNA levels determine the enzyme activities in the rat tissues. Since they found that the correlation between mRNA and protein levels, as well as between protein levels and enzyme activities of the antioxidant enzymes showed a significant association for SOD1, CAT, and GPx. In our study, significant increased expression of SOD1 was observed in group *H_2O_2-60min* ($P = 0.0404$), when the other two experimental groups, *H_2O_2-30min* and *H_2O_2-90min*, showed a less significant increased expression. In contrast to Xie *et al*. (2013), it seems that the stress stimulation with H_2O_2 up to 90 min did not suppress the enzyme activity of SOD1, and in turn, it significantly enhanced the expression level of SOD1. That might be the result of a short incubation time. But another noteworthy thing is that isolated cardiomyocytes were incubated in CO_2-free conditions, the addition of H_2O_2 to the culture media may, in a short term, result in an acute oxygen super-saturation in the culture medium, and thus promote the antioxidant responses in the cultured cardiomyocytes. A similar conclusion in Ritola *et al*. (2002), since they found that hyperoxia elevated the activity of SOD in juvenile rainbow trout gills following 4 h exposure to oxygen supersaturated water.

Furthermore, enzyme GPx-3 (Se-GPx) and CAT can convert the resulting H_2O_2 into H_2O and O_2, and thereby mitigate the toxic effects of H_2O_2. Slightly significant up-regulation of gene GPx-3 was found in group *H_2O_2-30min* and *H_2O_2-60min*, while slightly significant down-regulation was observed in group *H_2O_2-90min* ($P = 0.0171$).

Concurrently, the expression level of CAT almost unchanged in all experimental groups when compared to the control, even though it has been demonstrated that CAT prevents cardiomyocyte damage during H_2O_2-induced oxidative stress (Janero *et al*., 1991). Also, in Lai *et al*. (1996), glucose oxidase was added to the culture medium to continuously generate H_2O_2, and they found that both the activity and the mRNA for CAT were increased following incubation of neonatal rat cardiomyocytes with low concentrations of glucose oxidase (less than 3 mU/ml). Our results indicate that GPx-3 played a more important role in acute antioxidant response and H_2O_2 removing (Halliwell, 2006) in isolated salmon cardiomyocytes; and CAT were slower at catalyzing H_2O_2 removal, since in Lai *et al*. (1996), the enhanced CAT activity was observed after 12 h of incubation with glucose oxidase. This perhaps due to that most or all CAT in animals is in peroxisomes (Halliwell, 2006), while GPx-3 is a plasmatic enzyme, the latter may have a more direct reaction with H_2O_2 in the culture media.

HSP70 is the major stress-inducible protein in vertebrates, it helps to protect cells from acute stressor insult, especially those affecting the cell's machinery for protein folding. Many studies have shown that HSP70 played an important role in protecting against H_2O_2 induced pro-oxidative stress in cardiomyocytes (Chen *et al*., 2013; Wang *et al*., 2002; Kukreja *et al*., 1994). And in Kukreja *et al*. (1994), over fivefold increase in HSP70 mRNA was observed in rat heart with 100 μM H_2O_2 perfusion for 15 min. But in our study, a significant down-regulation of gene HSP70 was observed in *H_2O_2-90min* (*P = 0.0443*), while less significant down-regulation was found in the other two groups. Since we did not measure the protein level of HSP70, it is hard to explain why down-regulation of HSP70 was observed, further studies are needed in salmon cardiomyocytes, in particular to include enzyme activity assays of SOD, GPx, and CAT, before drawing final conclusion. In general, it seems that short exposure to H_2O_2 can induce the antioxidant responses in cultured salmon cardiomyocytes with increased expression of SOD1 and GPx-3, and the strongest response was induced by a 60 min H_2O_2 incubation. Also, a 30 min incubation resulted in the most obvious hypertrophy in isolated salmon cardiomyocytes.

Fig. 29. Relative gene expression of GATA4, αMHC and SOD1 in cardiomyocytes from stress study. Bars are mean of relative expression from three parallels (*n=3*) and are shown with standard error of mean. Different letters denote significance. Mean of control is set to 1 and the data from other groups is relative to 1.
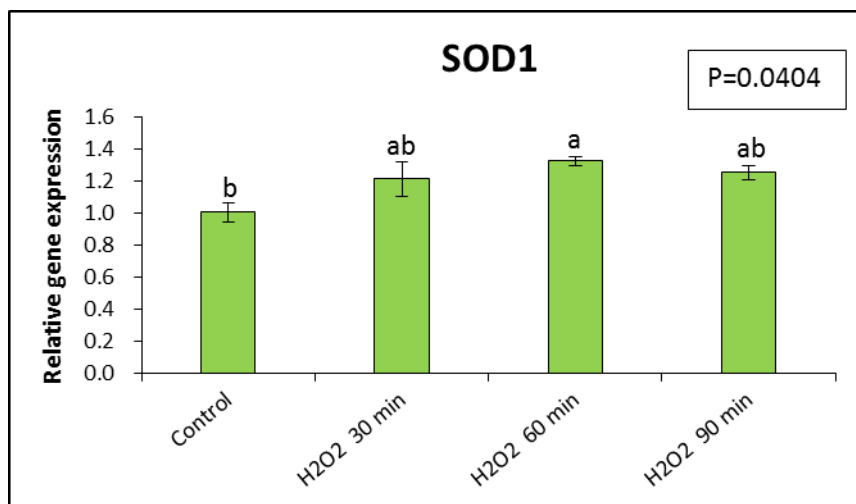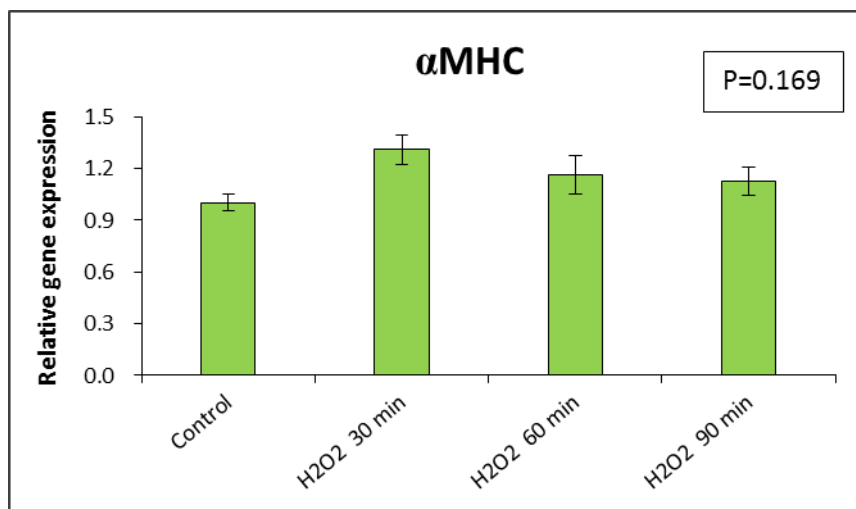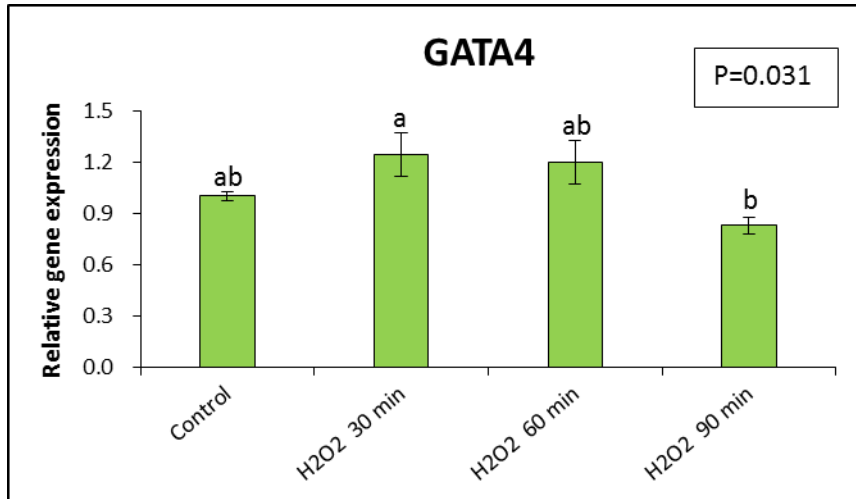
Fig. 30. Relative gene expression of GPx-3, CAT and HSP70 in cardiomyocytes from stress study. Bars are mean of relative expression from three parallels (*n=3*) and are shown with standard error of mean. Different letters denote significance. Mean of control is set to 1 and the data from other groups is relative to 1.
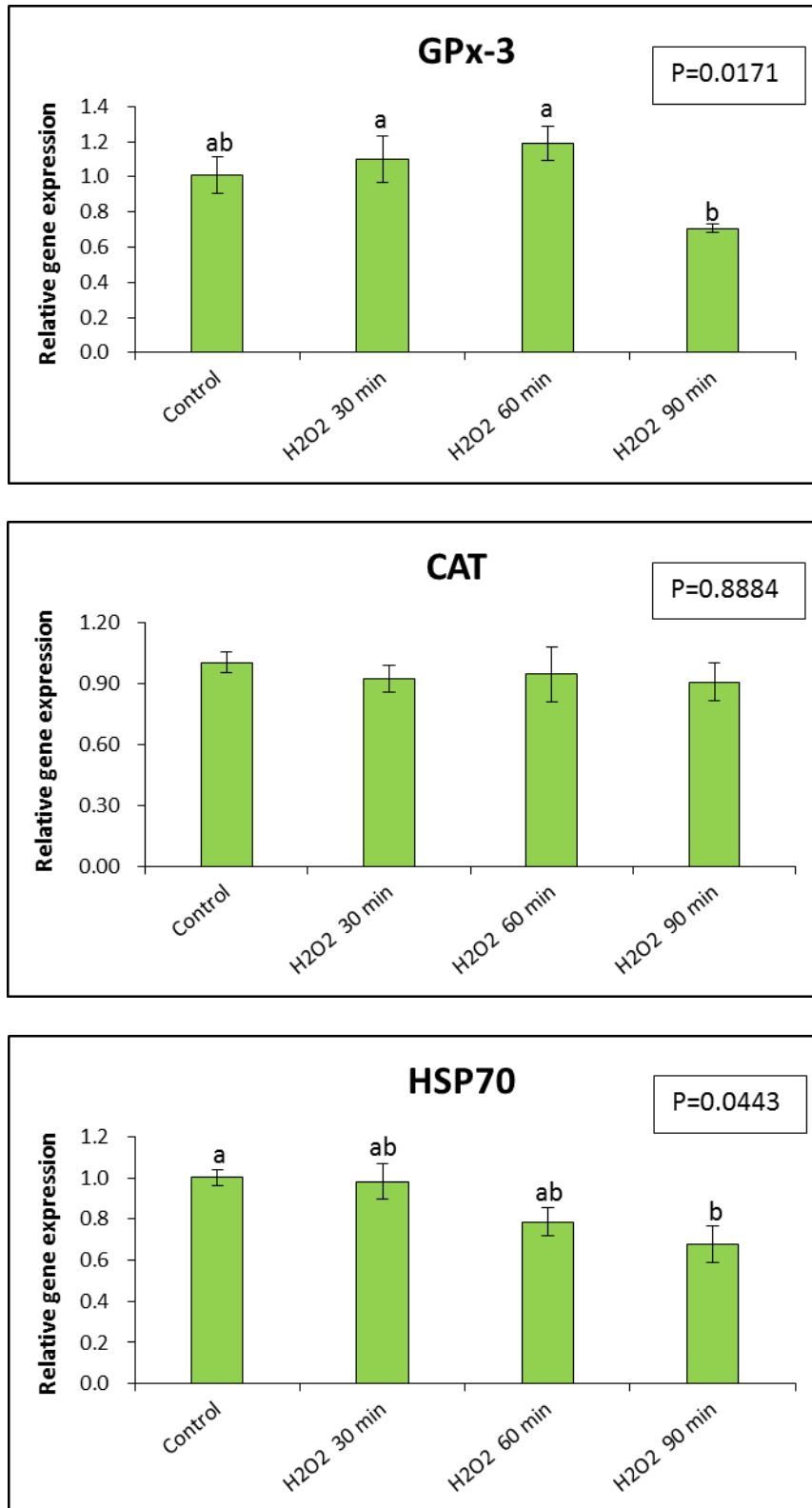
3.6 Study 6: Effects of DHA on immune response

The aim of this study was to test the effects of DHA on the expression of antioxidative and inflammatory gene markers in salmon cardiomyocytes. A further aim was to test if salmon cardiomyocyte is able to respond to LPS. Cardiomyocytes in group 1 (*Control group*) and group 3 (*LPS group*) were cultured in media without DHA, while cardiomyocytes in group 2 (*DHA group*) and group 4 (*DHA&LPS group*) were cultured in media supplemented with 20 µM DHA for 4 days. And at day 8, group 3 and group 4 were incubated with 100 ng/mL LPS for 6 hours before harvesting and isolation of RNA.

From the gene expression results (Fig. 31 to 33), it seems that *in vitro* cultured salmon cardiomyocytes did not really respond to LPS. And cardiomyocytes only responded little to DHA, with significantly ($P = 0.0007$) increased expression of SOD1.

Nrf2 (also known as NFE2L2) transcription factor regulates the expression of antioxidant proteins that protect against oxidative damages triggered by injury and inflammation, such as SOD, GPx, and CAT (Bhakkiyalakshmi *et al*., 2014). Among these genes, only SOD1 showed significantly increased expression in the *DHA* and *DHA&LPS group* when compared to the other two groups, suggesting that the culturing with DHA significantly enhanced the antioxidative response in salmon cardiomyocytes by up-regulating SOD1. Moreover, not much difference in SOD1 expression was found between the *Control* and *LPS group*, but slightly increased expression of SOD1 was observed in group *DHA&LPS* when compared to the *DHA group*, suggesting that DHA may, to some extent, contribute to a stronger antioxidative response in cultured salmon cardiomyocytes towards LPS stimulation, even though LPS itself, did not really enhance the expression of SOD1. Similar conclusion was found in Di Nunzio *et al*. (2011), they demonstrated that human HepG2 cells supplemented with 60 µM DHA appeared richer in antioxidant defenses, with significantly ($P < 0.05$) increased cytosolic SOD activity. And in Kjær *et al*.

(2008), it is demonstrated that in salmon fed with high *n*-3 DHA diet (42% of total FAs), significantly increased SOD activity was observed in their hepatocytes when compared to the fish fed low *n*-3 diet, and that may be due to the oxidative stress from the breakdown process (lipid peroxidation) of DHA. Besides, in Østbye *et al*. (2011), it is demonstrated that moderate doses of DHA (12.18% of total FAs) in salmon diets actually gave the best protection against oxidative damage of mitochondrial membranes and muscle structure. It seems that the concentration of DHA applied in our study (25.4% of total FAs) might be too high for cardiomyocytes in culture. Also, a recent study from Ruyter *et al*. (Don MacKinlay, 2014) showed that DHA addition to adipocytes in culture led to up-regulation of the SOD protein in mitochondria and thereby it prevented LPS induced damage in the mitochondria. It is known that cardiomyocytes are very rich in mitochondria, so that may explain why up-regulation of SOD1 was observed in our study, and the up-regulation of SOD1 may actually protect cardiomyocytes against oxidative damage. Besides, the expression of Nrf2, GPx-3 and CAT was not significantly influenced by the different treatments.

TNFα is characterized as an important early-response pro-inflammatory mediator and it is mainly secreted by macrophages (Roher *et al*., 2011). IL1b is a member of the IL-1 cytokine family that produced by hematopoietic cells such as blood monocytes, tissue macrophages, and skin dendritic cells, it is also an important mediator of the inflammatory response (Garlanda *et al*., 2013; Dinarello, 2011). Stat1a and MX1 are known to be activated in response to interferon signaling. However, in our study, no significant effects on these immune related genes was observed (Fig. 32).

Fish has been known to resistant to endotoxic shock when compared to mammals due to the differences in recognition mechanism (Iliev *et al*., 2005a; Berczi *et al*., 1966). That may explain why no significant difference was observed between the *Control* and *LPS group* in the expression levels of all the genes analyzed. And interestingly, decreased expression was even observed in some of these antioxidative and inflammatory-related genes in the *LPS group* towards 6 h *E. coil* LPS incubation,

including Nrf2, TNFα, Stat1a, and MX1, suggesting that LPS incubation failed to trigger the immune response in salmon cardiomyocytes, as expected. However, many studies have shown that LPS can induce strong immune responses in macrophages from different fish species at μg/mL concentration (Iliev *et al*., 2005b; MacKenzie *et al*., 2003; Pelegr ń *et al*., 2001). In contrast to our study, in MacKenzie *et al*. (2003), macrophages differentiated *in vitro* display a significantly accumulation of rainbow trout TNFα mRNA after 6 h incubation with 100 ng/mL *E. coil* LPS, increasing to a value 60-fold over control non-stimulated cells. It is also reported that purified seabream macrophage monolayers shown a much stronger degree of IL1b expression than total head-kidney cells upon LPS stimulation, suggesting that macrophages are a major source of IL1b in fish (Pelegr ń *et al*., 2001). However, as is well known that heart is not an immune organ in fish, thus, the fish heart may contain less immune cells than other organs or tissues, such as kidney and adipose tissue (Skugor *et al*., 2010), which are reported to shown up-regulation of immune genes after LPS stimulation; also, dispersed cells of a cell culture tend to be more homogeneous after some time of culturing, therefore we believe that our culture would be a relatively homogeneous population of mature cardiomyocytes at day 8, with very few other cell types, including macrophages. Maybe that is why no strong immune reaction was observed in our study. It is also possible that the cultured cardiomyocytes at day 8 were not mature enough to respond to LPS, according to the results from time study, since cardiomyocytes were still under development around this time.

And interestingly, among these genes, GPx-3 showed slightly increased expression towards LPS incubation, which may indicate LPS induced oxidative stress. In contrast to our study, it has been demonstrated that in mice, LPS induces tissue damage and injury partly through the induction of reactive oxygen species (ROS), which partly depends on the suppression of antioxidative enzymes involved in ROS clearance, including GPx-3 (Wiesel *et al*., 2000, Maitra *et al*., 2009). Thus, it seems that incubation with LPS did not really cause cell damage in salmon cardiomyocyte, conversely, it increased the expression of GPx-3 and enhanced the antioxidative

response in salmon cardiomyocytes.

In general, our study shown that *in vitro* cultured salmon cardiomyocytes were tolerant to endotoxin shock. And the supplementation of DHA to culture media, only affected salmon cardiomyocytes with significantly increased expression of SOD1.

Fig. 31. Relative gene expression of Nrf2, SOD1, GPx-3, and CAT in cardiomyocytes from immune study. Bars are mean of relative expression from three parallels and are shown with standard error of mean (*n=3*). Different letters denote significance. Mean of control is set to 1 and the data from other groups is relative to 1.

Fig. 32. Relative gene expression of TNFα, IL1b, Stat1a, and MX1 in cardiomyocytes from immune study. Bars are mean of relative expression from three parallels and are shown with standard error of mean (*n=3*). Mean of control is set to 1 and the data from other groups is relative to 1.
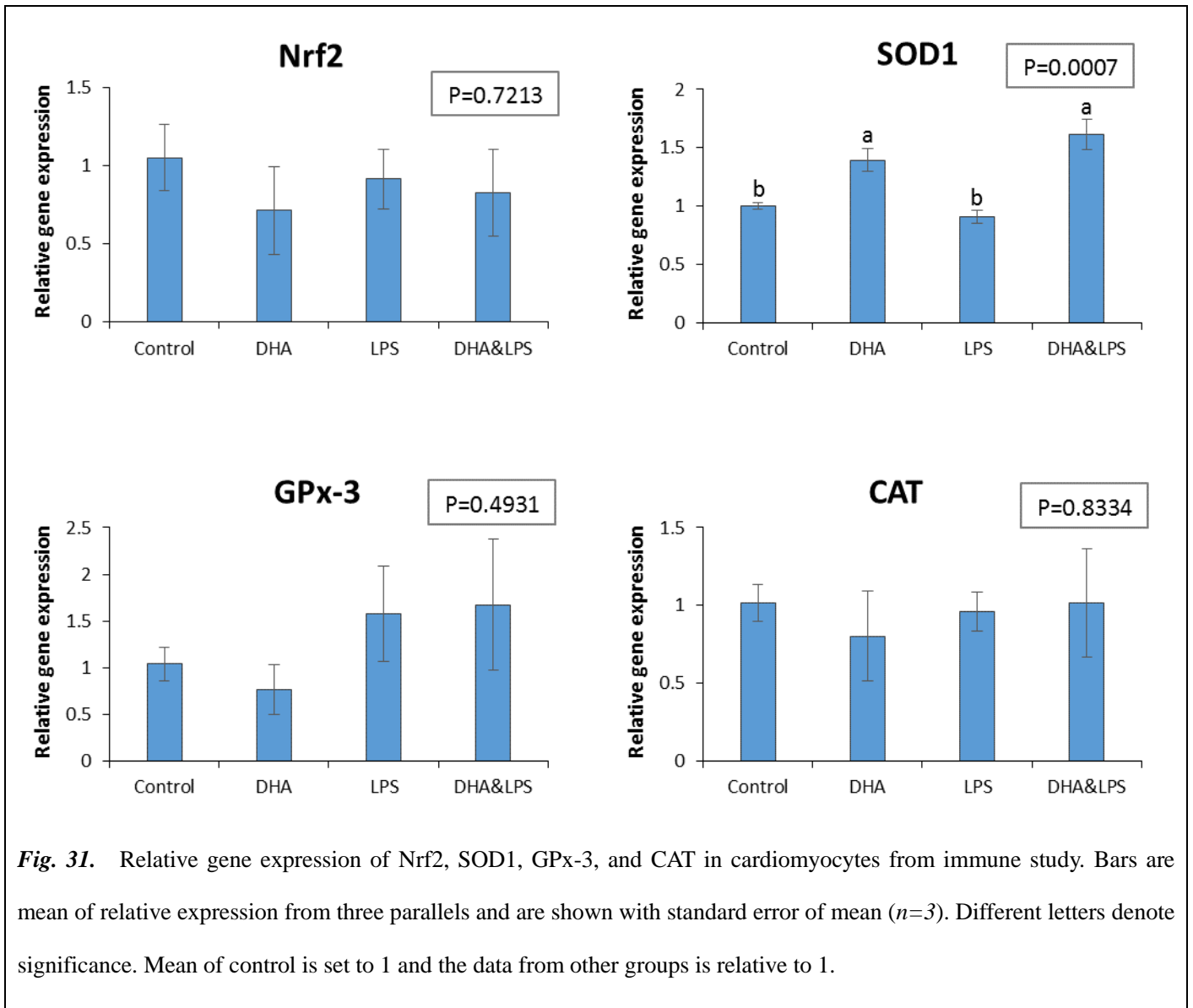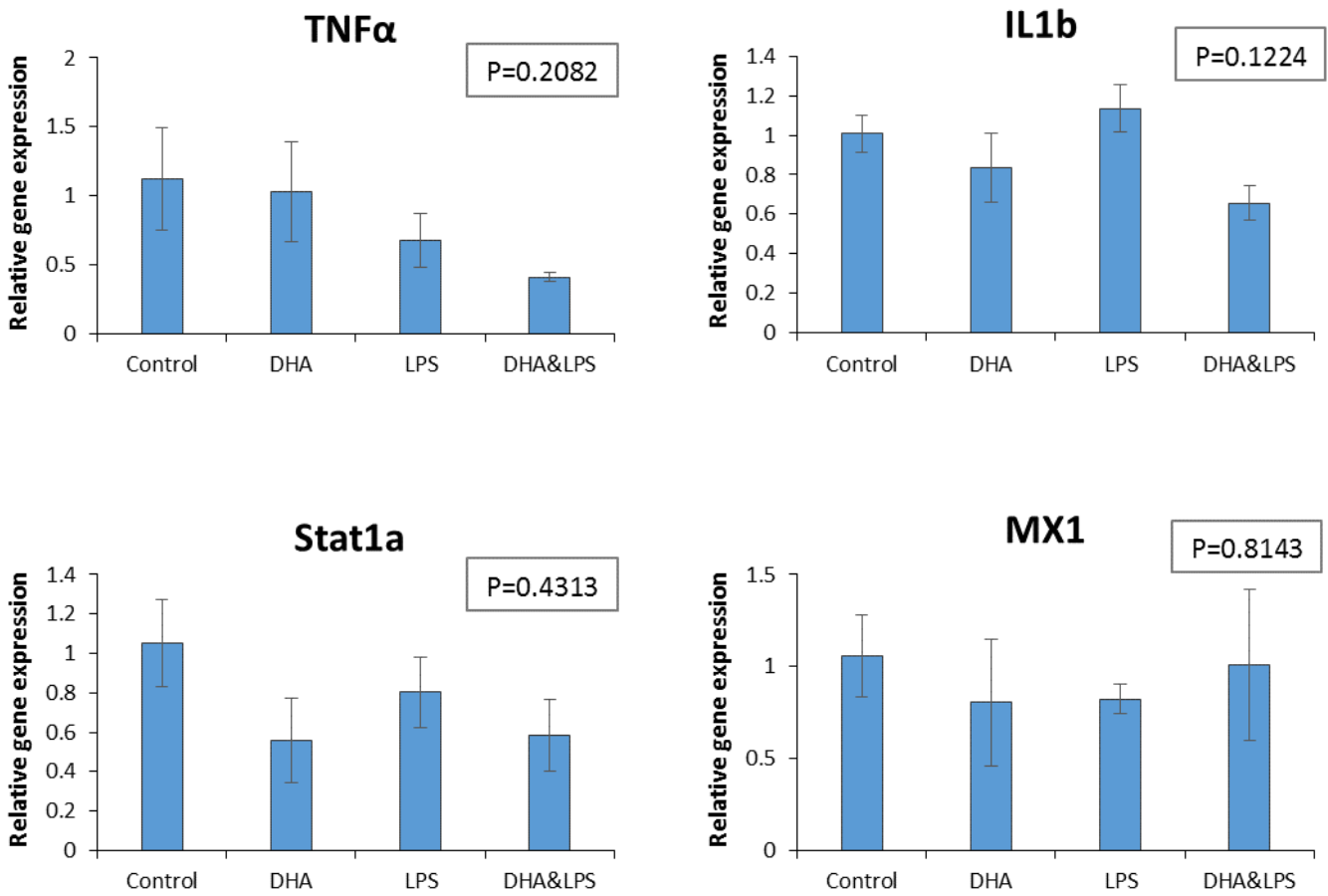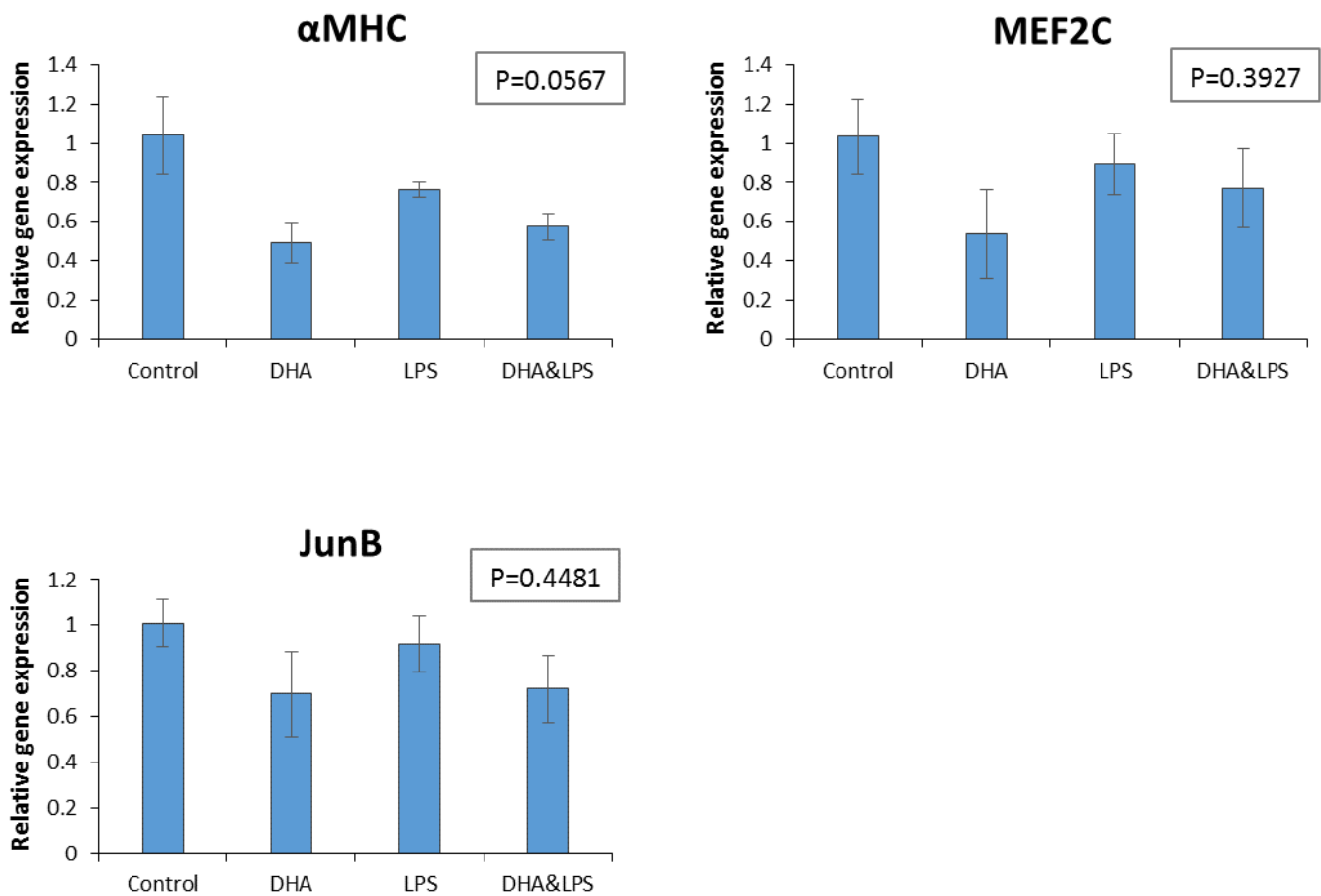
Fig. 33. Relative gene expression of αMHC, MEF2C, and JunB in cardiomyocytes from immune study. Bars are mean of relative expression from three parallels and are shown with standard error of mean (*n=3*). Mean of control is set to 1 and the data from other groups is relative to 1.

3.7   Study 7: Test of four different coating materials for culturing of cardiomyocytes

We have observed in previous studies that the seeding density is very important for a good development and long-term culture of cardiomyocytes. However, it is time consuming to isolate primary cardiomyocytes from several fish in each cell trial with only a few cells achieved from each heart. Therefore, we wish to reduce the number of fish needed by optimizing the proliferation capacities of cardiomyocytes in culture, so that the cells can be seeded a bit thinner and still become confluent because of higher proliferation capacities.

Fig. 34.   Phase contrast microscopic images of salmon cardiomyocytes with four different coatings at day 1, 2, 5, and 7, respectively (10×magnification).

Four different coatings were tested in this trial with one well per experimental group to see how different coating materials influence cardiomyocyte proliferation. Cells were isolated according to isolation method 1 and they were cultured in L-15 muscle cell growth media. Gro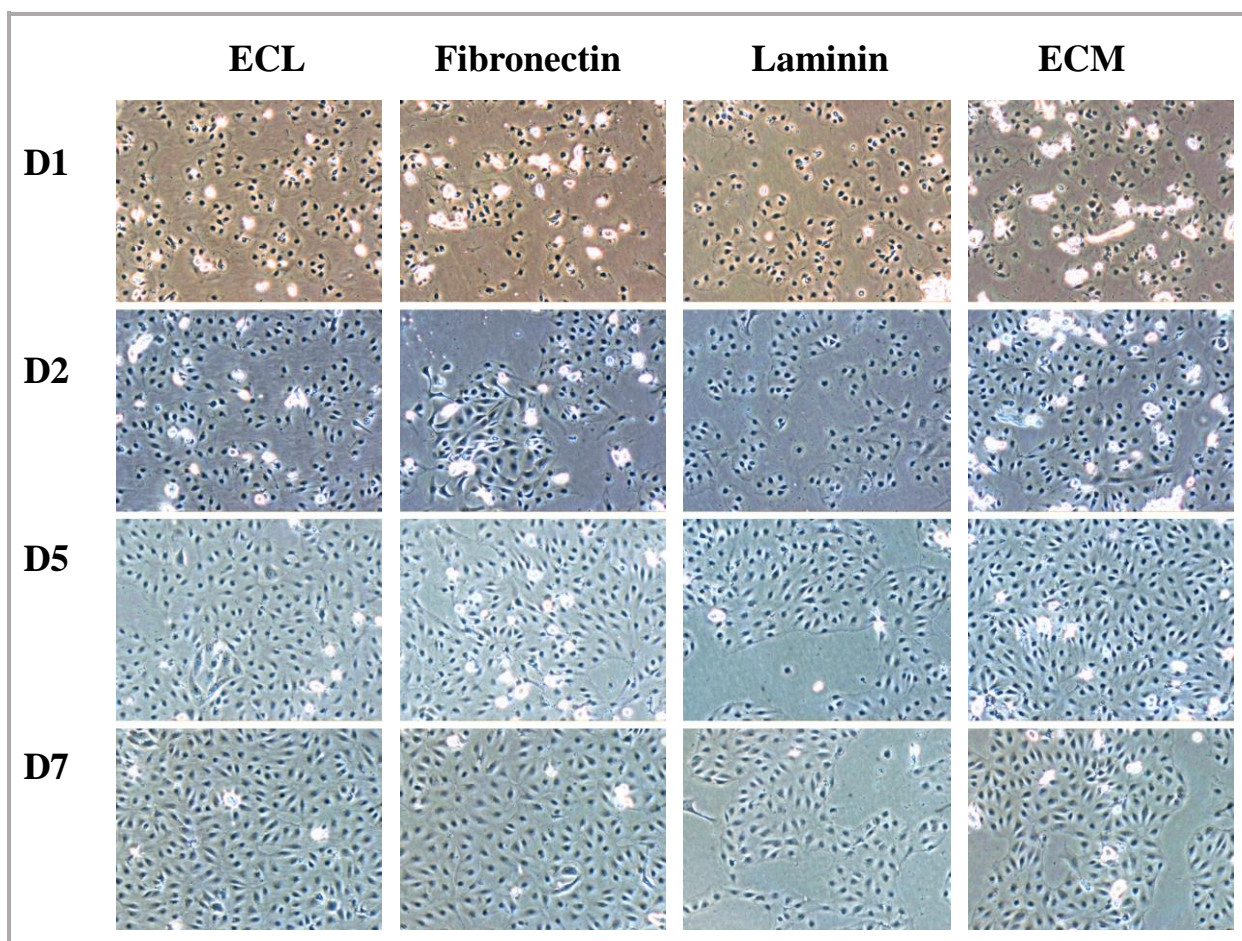up 1, 2, 3, and 4 were pre-coated with ECL cell attachment matrix, bovine plasma fibronectin, mouse laminin, and ECM gel, respectively. Microscopic images were taken at day 1, 2, 5, and 7, respectively to monitor differences in development between the groups. Besides, to evaluate the proliferation of cardiomyocytes, six images were randomly taken from each well at day 1, 2, 5, and 7, respectively to calculate the average number of cells (Fig. 35). And at day 7, cells were harvested for isolation of RNA for gene expression analysis.
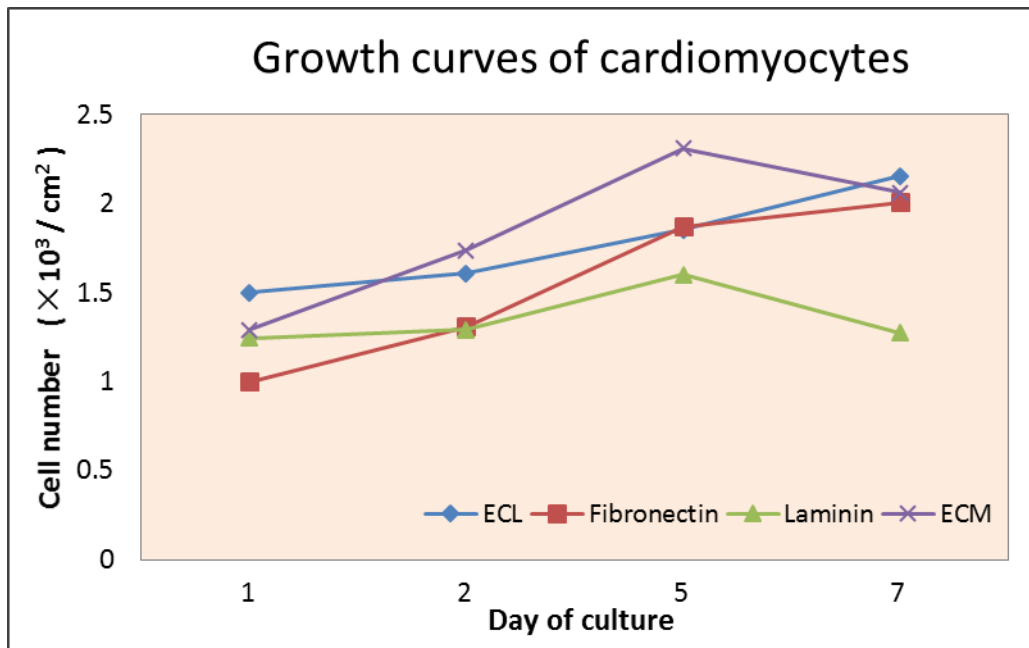
Fig. 35. Growth curves of cardiomyocytes with four different coatings from day 1 to day 7.

Extracellular matrix proteins include: collagen, laminin, fibronectin, vitronectin and tenascin amongst others. These proteins provide an attachment framework for the adhesion and growth of specific cells both *in vivo* and *in vitro* (Harnett *et al.*, 2007). ECL cell attachment matrix contains entactin-collagen IV-laminin. Entactin is a member of the nidogen family of basement membrane glycoproteins. Structurally it connects the networks formed by collagens and laminins to each other (Yurchenco &

Patton, 2009). Plasma fibronectin is a major component of blood plasma and is produced in the liver by hepatocytes (Pankov & Yamada, 2002). ECM gel contains laminin as a major component, collagen type IV, heparan sulfate proteoglycan, entactin and other minor components.

From Fig. 34 and 35, we can see that *in vitro* cultured salmon cardiomyocytes kept proliferating from day 1 to day 5 in all groups. Afterwards, decrease in cell number was observed in laminin and ECM coated wells, this is also consistent with our gene expression results at day 7 (Fig. 36 and 37), since the lower expression of PCNA was found in these two groups, while the highest expression of PCNA was found in group 1 (*ECL*). Combined with the growth curves of cardiomyocytes, it seems that cardiomyocytes plated on ECL coating had the longest proliferation period when compared to the cardiomyocytes in other groups. Besides, only slightly difference was observed in the expression level of gene CAV3, αMHC, GATA4, Nkx2-5 and MEF2C.

Different coatings have been widely used in plating and culturing of cardiomyocytes from different animal species (Sander *et al*., 2013; Gan *et al*., 2014). In Lundgren *et al*. (1988), it is demonstrated that adult rat cardiomyocytes attached equally well to native collagen type IV and laminin substrate that immobilized on plastic surfaces. And metabolic labeling followed by immunoprecipitation demonstrated the synthesis of both laminin and collagen type IV in cardiomyocytes during culture period. In our study, cardiomyocytes from group 3 (*Laminin*) performed the lowest proliferation during whole cell culture, the smallest area of confluence was observed in this group, and its growth curve was much flatter than other groups. That may be due to the employment of collagenase in our cell isolation process, the collagens in the cell surface were digested, and thus, isolated cardiomyocytes may need more time for recovering and producing extracellular collagens. It is also demonstrated in Lundgren *et al*. (1988), since they found that during the isolation procedure, collagen IV was removed from the cell surface. In our study, both ECL and ECM coating contain

collagen component, and cardiomyocytes plated on these two coatings seem to have a better growth than those in group 3 (*Laminin*).

Besides, in Gan *et al.* (2014), fibronectin was used as the coating material for mouse cardiomyocytes. In our study, cardiomyocytes plated on fibronectin coating seem to show a relatively stronger proliferation in the beginning of cell culture. It has been demonstrated that about two thirds of myocardial cells are fibroblast (Deb & Ubil, 2014), and in study 4, we expect our culture contained serveral cell types, including fibroblast-like cells. It is reported that both extracellular signal-regulated kinase (ERK) and c-jun NH_2-terminal kinase (JNK) could by activated by stretch in cardiac fibroblasts that plated on fibronectin, while cells plated on laminin only lead to activation of JNK (MacKenna *et al.*, 1998). ERK are known to be involved in a wide variety of cellular processes such as proliferation and differentiation, that may explain why cardiomyocytes plated on fibronectin coating showed a strong proliferative ability in the early stages of cell culture in our study.

In general, this study let us see the possibility of increasing cell proliferation by improving the coating materials, since cardiomyocytes plated on laminin coating showed a rather poor growth when compared to the cells from other groups, according to the microscopic images, cardiomyocyte growth curves and the gene expression results. But in this study, only one parrallel was set for each experimental group, it is hard to draw a certain conclusion about which coating is the best amongst the four that we tested, thus, further studies are needed before drawing final conclusion.

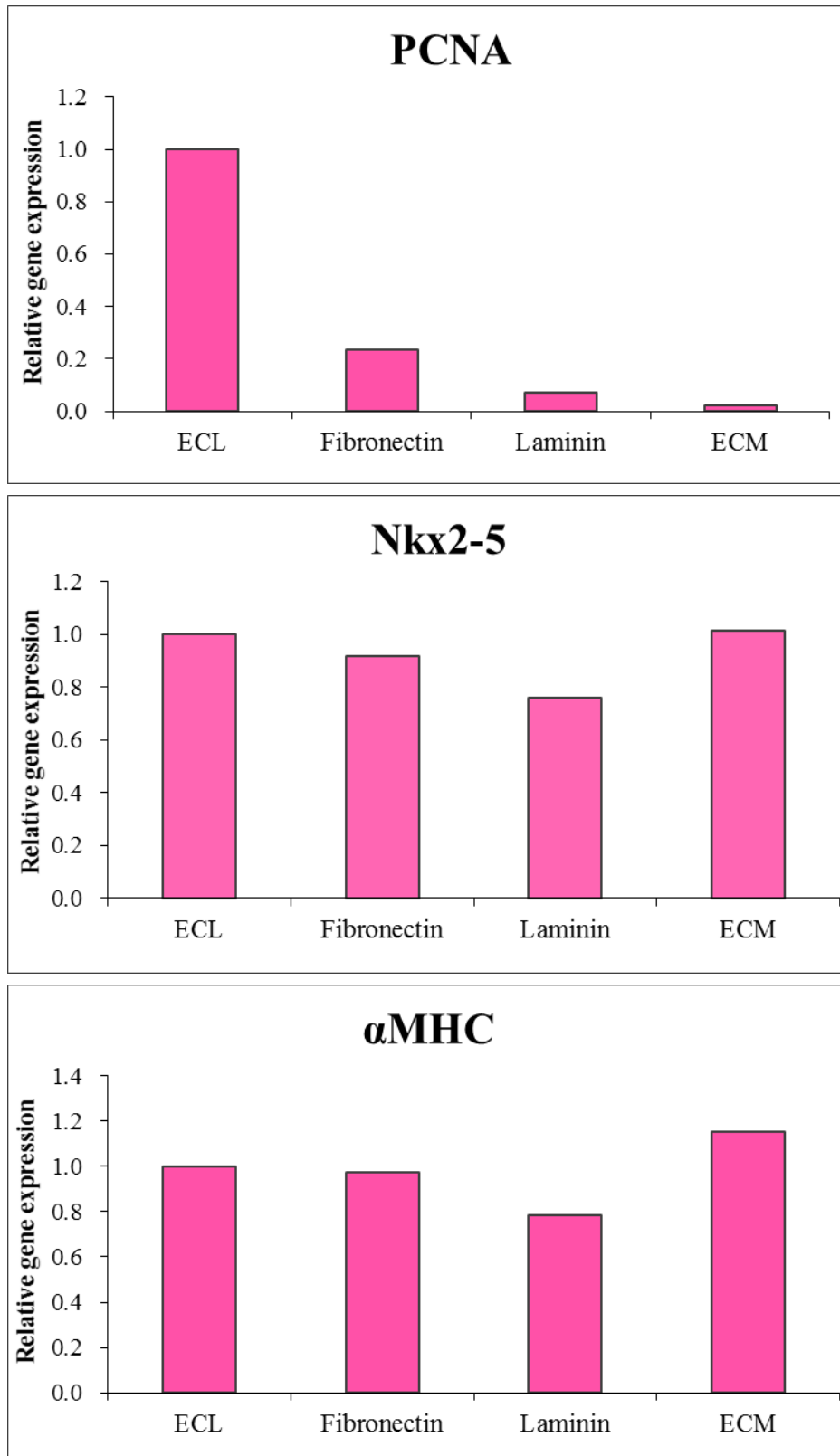***Fig. 36.*** Relative gene expression of PCNA, CAV3, and αMHC in cardiomyocytes from four different coatings at day 7 (*n=1*). Group 1 (*ECL*) was set to 1 and the data from other groups was relative to 1.

Fig. 37. Relative gene expression of GATA4, Nkx2-5 and MEF2C in cardiomyocytes from four different coatings at day 7 (*n=1*). Group 1 (*ECL*) was set to 1 and the data from other groups was relative to 1.
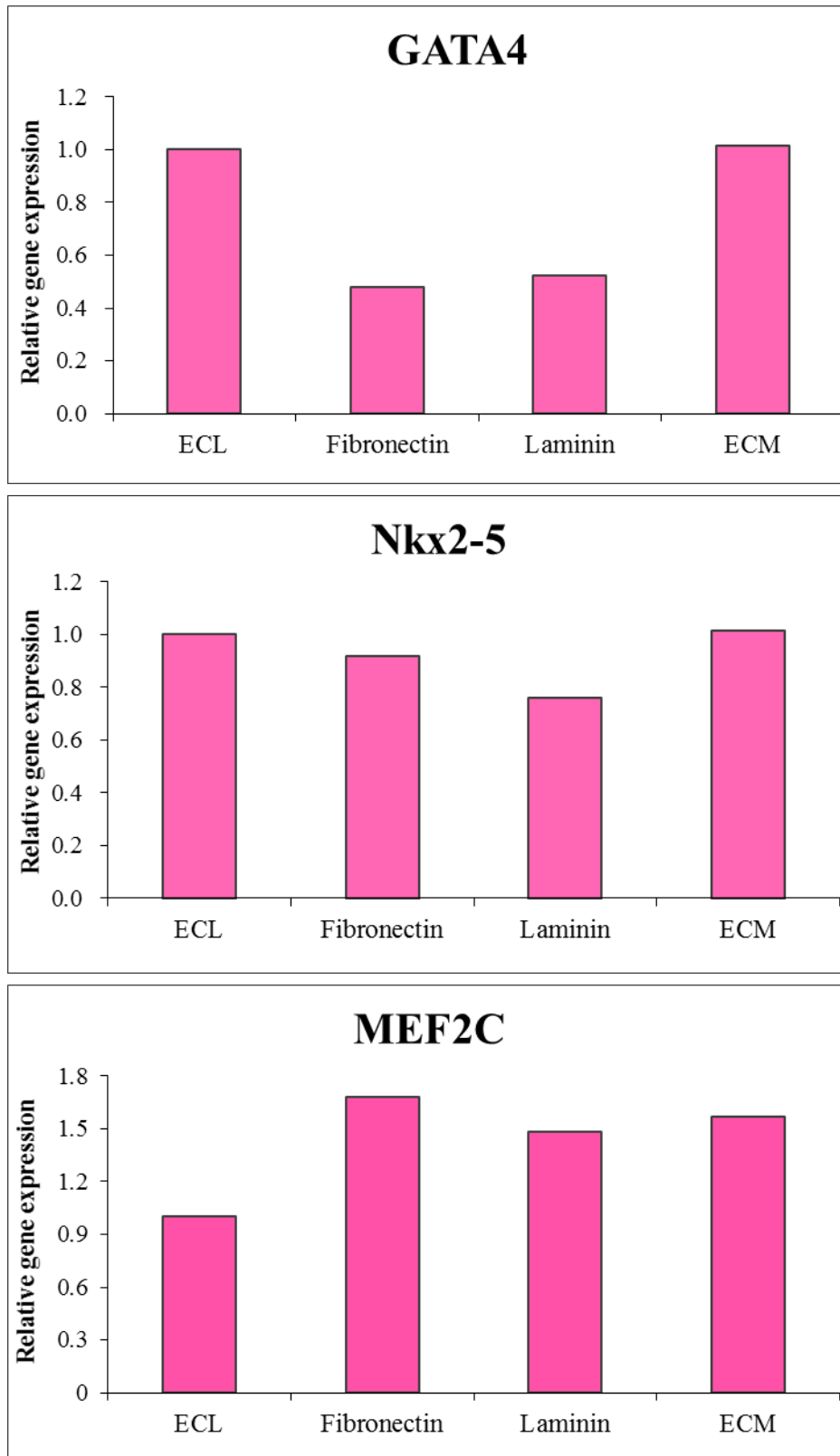
4 Conclusion

In this work, we found that the combination of collagenase and trypsin gave a better outcome in cell isolation than trypsin alone. And primary cultured salmon cardiomyocytes have the ability to proliferate and differentiation by up-regulating several gene markers. Also, the seeding density is important for good proliferation and development of cells in culture.

One surprising finding in our work is that spontaneously beating cell structures were observed during the long-term culture, and some of these contracting structures even kept beating up to 5 weeks without stimuli, which may indicate that the biological characteristics of cardiomyocytes were well maintained in our culture.

Further, we found that the supplementation of growth factor bFGF to culture media did not promote cell proliferation, but it seems possible to increase cell yield by improving the coating materials.

Moreover, salmon cardiomyocytes can respond to H_2O_2 by up-regulating some of the antioxidative-related genes, and an appropriate stress stimulation may even result in hypertrophic growth in salmon cardiomyocytes. However, no strong immune reaction was observed when we stimulated cardiomyocytes with LPS, and salmon cardiomyocytes responded little to DHA as well.

All together, this work provided us great insight into the development of *in vitro* cultured salmon cardiomyocytes, and the successfully developed *in vitro* culture system makes it possiblt to study the reactions of cardiomyocytes under different situations with the reduction of animal experimentation.

Reference

Akazawa, H. & Komuro, I. (2003). Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res*. **92:** 1079-1088.

Banerjee, I., Yekkala, K., Borg, T. K., & Baudino, T. A. (2006). Dynamic interactions between myocytes, fibroblasts, and extracellular matrix. *Ann N Y Acad Sci*. **1080:** 76-84.

Basilico, C. & Moscatelli, D. (1992). The FGF family of growth factors and oncogenes. *Adv Cancer Res*. **59:** 115-165.

Bastiani, M. & Parton, R. G. (2010). Caveolae at a glance. *J Cell Sci*. **123:** 3831-3836.

Bell, J. G., Dick, J. R., McVicar, A. H., Sargent, J. R., & Thompson, K. D. (1993). Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar*). *Prostaglandins Leukot Essent Fatty Acids*. **49:** 665-673.

Berczi, I., Bertók, L., & Bereznai, T. (1966). Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. *Can J Microbiol*. **12:** 1070-1071.

Bhakkiyalakshmi, E., Shalini, D., Sekar, T. V., Rajaguru, P., Paulmurugan, R., & Ramkumar, K. M. (2014). Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2. *Br J Pharmacol*. **171:** 1747-1757.

Borg, T. K. & Baudino, T. A. (2011). Dynamic interactions between the cellular components of the heart and the extracellular matrix. *Pflugers Arch Eur J Physiol*. **462:** 69-74.

Brette, F., Luxan, G, Cros, C., Dixey, H., Wilson, C., & Shiels, H. A. (2008). Characterization of isolated ventricular myocytes from adult zebrafish (*Danio rerio*). *Biochem Biophys Res Commun*, **374:** 143-146.

Bruneau, B. G. (2002). Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ Res*. **90:** 509-519.

Chen, Q. M., Tu, V. C., Wu, Y., & Bahl, J. J. (2000). Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. *Arch Biochem Biophys*. **373:** 242-248.

Chen, Z., Shen, X., Shen, F., Zhong, W., Wu, H., Liu, S., & Lai, J. (2013). TAK1 activates AMPK-dependent cell death pathway in hydrogen peroxide-treated cardiomyocytes, inhibited by heat shock protein-70. *Mol Cell Biochem*. **377:** 35-44.

Clark, W. A., Decker, M. L., Behnke-Barclay, M., Janes, D. M., & Decker, R. S. (1998). Cell contact as an independent factor modulating cardiac myocyte hypertrophy and survival in long-term primary culture. *J Mol Cell Cardiol*. **30:** 139-155.

Dannevig, B. H. & Berg, T. (1985). Endocytosis of galactose-terminated glycoproteins by isolated liver cells of the rainbow trout (*Salmo gairdneri*). *Comp Biochem Physiol*. **82B:** 683-688.

Deb, A. & Ubil, E. (2014). Cardiac fibroblast in development and wound healing. *J*

Mol Cell Cardiol. **70:** 47-55.

Dinarello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*, **117:** 3720-3732.

Di Nunzio, M., Valli, V., & Bordoni, A. (2011). Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells. *Prostaglandins Leukot Essent Fatty Acids*. **85:** 121-127.

Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S. M., & Black, B. L. (2004). *Mef2c* is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development*, **131:** 3931-3942.

Don MacKinlay (Ed.) (2014). Omega-3 fatty acids influence mitochondrial function and inflammatory status in Atlantic salmon *in vitro* differentiated preadipocytes. Presented in Abstract Book: ICBF 2014, Edinburgh. Available at: http://www2.hw.ac.uk/sls/icbf2014/SciProg.htm

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., & Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J*. **16:** 5687-5696.

Edmondson, D. G., Lyons, G. E., Martin, J. F., & Olson, E. N. (1994). *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development*, **120:** 1251-1263.

Fuerstenau-Sharp, M., Zimmermann, M. E., Stark, K., Jentsch, N., Klingenstein, M., Drzymalski, M., *et al*. (2015). Generation of highly purified human cardiomyocytes from peripheral blood mononuclear cell-derived induced pluripotent stem cells. *PLoS One*. **10:** e0126596.

Gan, L., Schwengberg, S., & Denecke, B. (2014). Transcriptome analysis in cardiomyocyte-specific differentiation of murine embryonic stem cells reveals transcriptional regulation network. *Gene Expr Patterns*. **16:** 8-22.

Garlanda, C., Dinarello, C. A., & Mantovani, A. (2013). The interleukin-1 family: back to the future. *Immunity*, **39:** 1003-1018.

Goldstein, J. P., Scott, A., & Currie, S. (2004). Are primary cultures of trout (*Oncorhynchus mykiss*) ventricular cardiomyocytes metabolically viable? *Fish Physiol Biochem*, **30:** 109-117.

Grépin, C., Robitaille, L., Antakly, T., & Nemer, M. (1995). Inhibition of transcription factor GATA-4 expression blocks *in vitro* cardiac muscle differentiation. *Mol Cell Biol*. **15:** 4095-4102.

Grunow, B., Ciba, P., Rakers, S., Klinger, M., Anders, E., & Kruse, C. (2010). *In vitro* expansion of autonomously contracting, cardiomyogenic structures from rainbow trout *Oncorhynchus mykiss*. *J Fish Biol*. **76:** 427-434.

Grunow, B., Wenzel, J., Terlau, H., Langner, S., Gebert, M., & Kruse, C. (2011). *In vitro* developed spontaneously contracting cardiomyocytes from rainbow trout as a model system for human heart research. *Cell Physiol Biochem*. **27:** 1-12.

Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol*. **141:** 312-322.

Harnett, E. M., Alderman, J., & Wood, T. (2007). The surface energy of various biomaterials coated with adhesion molecules used in cell culture. *Colloids Surf B Biointerfaces*. **55:** 90-97.

Haugland, Ø., Mikalsen, A. B., Nilsen, P., Lindmo, K., Thu, B. J., Eliassen, T. M., Roos, N., Rode, M., & Evensen, Ø. (2011). Cardiomyopathy syndrome of Atlantic salmon (*Salmo salar* L.) is caused by a double-stranded RNA virus of the *Totiviridae* family. *J Virol*. **85:** 5275-5286.

Hjeltnes, B. (Ed.) (2014). Fish Health Report 2013. Oslo: Norwegian Veterinary Institute. Available at: http://www.vetinst.no/eng/Publications/Fish-Health-Report/Fish-Health-Report-2013 (accessed: 16.05.2015)

Hong, S., Gronert, K., Devchand, P. R., Moussignac, R. L., & Serhan, C. N. (2003). Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J Biol Chem*. **278:** 14677-14687.

Iliev, D. B., Roach, J. C., Mackenzie, S., Planas, J. V., & Goetz, F. W. (2005a). Endotoxin recognition: in fish or not in fish? *FEBS Lett*. **579:** 6519-6528.

Iliev, D. B., Liarte, C. Q., MacKenzie, S. & Goetz, F. W. (2005b). Activation of rainbow trout (*Oncorhynchus mykiss*) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. *Mol Immunol*. **42:** 1215-1223.

Janero, D. R., Hreniuk, D., & Sharif, H. M. (1991). Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): lethal peroxidative membrane injury. *J Cell Physiol*. **149:** 347-364.

Johansen, R. (Ed.) (2013). Fish Health Report 2012. Oslo: Norwegian Veterinary Institute. Available at: http://www.vetinst.no/eng/Publications/Fish-Health-Report/Fish-Health-Report-2

012 (accessed: 16.05.2015)

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A., & Izpisúa Belmonte, J. C. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature*, **464:** 606-609.

Kardami, E. & Fandrich, R. R. (1989). Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J Cell Biol*. **109:** 1868-1875.

Khezri, S., Valojerdi, M. R., Sepehri, H., & Baharvand, H. (2007). Effect of basic fibroblast growth factor on cardiomyocyte differentiation from mouse embryonic stem cells. *Saudi Med J*. **28:** 181-186.

Kim, Y., Ma, A. G., Kitta, K., Fitch, S. N., Ikeda, T., Ihara, Y., Simon, A. R., Evans, T., & Suzuki, Y. J. (2003). Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. *Mol Pharmacol*. **63:** 368-377.

Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., MacRae, C. A., Stainier, D. Y., & Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by *gata4*$^+$ cardiomyocytes. *Nature*, **464:** 601-605.

Kjær, M. A., Todorcević, M., Torstensen, B. E., Vegusdal, A., & Ruyter, B. (2008). Dietary n-3 HUFA affects mitochondrial fatty acid β-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon. *Lipids*, **43:** 813-827.

Kukreja, R. C., Kontos, M. C., Loesser, K. E., Batra, S. K., Qian, Y. Z., Gbur, C. J., Jr., Naseem, S. A., Jesse, R. L., & Hess, M. L. (1994). Oxidant stress increases heat shock protein 70 mRNA in isolated perfused rat heart. *Am J*

Physiol. **267:** H2213-2219.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., & Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev*. **11:** 1048-1060.

Kurzchalia, T. V. & Parton, R. G. (1999). Membrane microdomains and caveolae. *Curr Opin Cell Biol*. **11:** 424-431.

Lai, C. C., Peng, M., Huang, L., Huang, W. H., & Chiu, T. H. (1996). Chronic exposure of neonatal cardiac myocytes to hydrogen peroxide enhances the expression of catalase. *J Mol Cell Cardiol*. **28:** 1157-1163.

Latchman, D. S. (1997). Transcription factors: an overview. *Int J Biochem Cell Biol*. **29:** 1305-1312.

Liang, Q., De Windt, L. J., Witt, S.A., Kimball, T.R., Markham, B.E., & Molkentin, J. D. (2001). The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy *in vitro* and *in vivo*. *J Biol Chem*. **276:** 30245-30253.

Liberatore, C. M., Searcy-Schrick, R. D., Vincent, E. B., & Yutzey, K. E. (2002). *Nkx-2. 5* gene induction in mice is mediated by a Smad consensus regulatory region. *Dev Biol*. **244:** 243-256.

Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A., & Olson, E. N. (1999). Control of early cardiac-specific transcription of *Nkx2-5* by a GATA-dependent enhancer. *Development*, **126:** 75-84.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., & Harvey, R. P. (1993). *Nkx-2.5*: a novel murine homeobox gene expressed in early heart progenitor cells

and their myogenic descendants. *Development*, **119:** 419-431.

Liu, L., Pasumarthi, K. B., Padua, R. R., Massaeli, H., Fandrich, R. R., Pierce, G. N., Cattini, P. A., & Kardami, E. (1995). Adult cardiomyocytes express functional high-affinity receptors for basic fibroblast growth factor. *Am J Physiol*. **268:** H1927-1938.

Lopez-Jimena, B., Lyons, P., Herath, T., Richards, R. H., Leaver, M., Bell, J. G., Adams, A., & Thompson, K. D. (2015). The effect of dietary n-3/n-6 polyunsaturated fatty acid ratio on salmonid alphavirus subtype 1 (SAV-1) replication in tissues of experimentally infected rainbow trout (*Oncorhynchus mykiss*). *Vet Microbiol*. **178:** 19-30.

Lundgren, E., Gullberg, D., Rubin, K., Borg, T. K., Terracio, M. J., & Terracio, L. (1988). In vitro studies on adult cardiac myocytes: attachment and biosynthesis of collagen type IV and laminin. *J Cell Physiol*. **136:** 43-53.

MacKenna, D. A., Dolfi, F., Vuori, K., & Ruoslahti, E. (1998). Extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J Clin Invest*. **101:** 301-310.

MacKenzie, S., Planas, J. V., & Goetz, F. W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and *in vitro* differentiated macrophages. *Dev Comp Immunol*. **27:** 393-400.

Maitra, U., Singh, N., Gan, L., Ringwood, L., & Li, L. (2009). IRAK-1 contributes to lipopolysaccharide-induced reactive oxygen species generation in macrophages by inducing NOX-1 transcription and Rac1 activation and suppressing the expression of antioxidative enzymes. *J Biol Chem*. **284:**

35403-35411.

Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Ruohonen, K., Vecino, J. L. G., Bell, J. G., & Tocher, D. R. (2012). Functional feeds reduce heart inflammation and pathology in Atlantic salmon (*Salmo salar* L.) following experimental challenge with Atlantic salmon reovirus (ASRV). *PLoS One*. **7:** e40266.

Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Vecino, J. G., Ruohonen, K., Bell, J. G., & Tocher, D. R. (2013). Effect of functional feeds on fatty acid and eicosanoid metabolism in liver and head kidney of Atlantic salmon (*Salmo salar* L.) with experimentally induced heart and skeletal muscle inflammation. *Fish Shellfish Immunol*. **34:** 1533-1545.

Masson-Pévet, M., Jongsma, H. J., & De Bruijne, J. (1976). Collagenase- and trypsin-dissociated heart cells: a comparative ultrastructural study. *J Mol Cell Cardiol*, **8:** 747-757.

McCulley, D. J. & Black, B. L. (2012). Transcription factor pathways and congenital heart disease. *Curr Top Dev Biol*. **100:** 253-277.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M. V., Coletta, M., Vivarelli, E., Frati, L., Cossu, G., & Giacomello A. (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res*, **95:** 911-921.

Miyazaki, M. (1977). Primary culture of adult rat liver cells. I. Preparation of isolated cells from trypsin-perfused liver of adult rat. *Acta Med Okayama*, **31:** 351-360.

Miyazaki, M., Tsunashima, M., Wahid, S., Miyano, K., & Sato, J. (1984).

Comparison of cytologic and biochemical properties between liver cells isolated from adult rats by trypsin perfusion and those isolated by collagenase perfusion. *Res Exp Med*, **184:** 191-204.

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6, ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem*. **275:** 38949-38952.

Molkentin, J. D. & Markham, B. E. (1993). Myocyte-specific enhancer-binding factor (MEF-2) regulates α-cardiac myosin heavy chain gene expression *in vitro* and in vivo. *J Biol Chem*. **268:** 19512-19520.

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M. J. & Izquierdo, M. S. (2003). Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. *Aquaculture*. **225:** 353-370.

Morin, S., Charron, F., Robitaille, L., & Nemer, M. (2000). GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J*. **19:** 2046–2055.

Morrisey, E. E., Ip, H. S., Lu, M. M., & Parmacek, M. S. (1996). GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev Biol*. **177:** 309-322.

Morrisey, E. E., Ip, H. S., Tang, Z., Lu, M. M., & Parmacek, M. S. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev Biol*. **183:** 21-36.

Mukherjee, P. K., Marcheselli, V. L., Serhan, C. N., & Bazan, N. G. (2004). Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc Natl Acad Sci USA*.

101: 8491-8496.

Nurmi, A. & Vornanen, M. (2002). Electrophysiological properties of rainbow trout cardiac myocytes in serum-free primary culture. *Am J Physiol Regul Integr Comp Physiol*, **282:** R1200-R1209.

Olson, E. N. (2006). Gene regulatory networks in the evolution and development of the heart. *Science*, **313:** 1922–1927.

Østbye, T.-K., Kjær, M. A., Rørå, A. M. B., Torstensen, B., & Ruyter, B. (2011). High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquaculture Nutrition*. **17:** 177–190.

Palacios, G., Lovoll, M., Tengs, T., Hornig, M., Hutchison, S., Hui, J., Kongtorp, R. T., *et al*. (2010). Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus. *PLoS One*. **5:** e11487.

Pankov, R. & Yamada, K. M. (2002). Fibronectin at a glance. *J Cell Sci*. **115:** 3861-3863.

Pelegrín, P., García-Castillo, J., Mulero, V., Meseguer, J. (2001). Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines. *Cytokine*. **16:** 67-72.

Peterkin, T., Gibson, A., Loose, M., & Patient, R. (2005). The roles of GATA-4, -5 and -6 in vertebrate heart development. *Semin Cell Dev Biol*. **16:** 83–94.

Ritola, O., Livingstone, D. R., Peters, L. D., & Lindström-Seppä, P. (2002).

Antioxidant processes are affected in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ozone and oxygen-supersaturated water. *Aquaculture*, **210:** 1-19.

Roher, N., Callol, A., Planas, J. V., Goetz, F. W., & MacKenzie, S. A. (2011). Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. *Innate Immun*. **17:** 16-28.

Rojas, A., Kong, S. W., Agarwal, P., Gilliss, B., Pu, W. T., & Black, B. L. (2008). GATA4 is a direct transcriptional activator of *cyclin D2* and *Cdk4* and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. *Mol Cell Biol*. **28:** 5420-5431.

Rose, L. C., Fitzsimmons, R., Lee, P., Krawetz, R., Rancourt, D. E., & Uludağ, H. (2013). Effect of basic fibroblast growth factor in mouse embryonic stem cell culture and osteogenic differentiation. *J Tissue Eng Regen Med*. **7:** 371-382.

Ruan, J. L., Tulloch, N. L., Saiget, M., Paige, S. L., Razumova, M. V., Regnier, M., Tung, K. C., Keller, G., Pabon, L., Reinecke, H., & Murry, C. E. (2015). Mechanical stress promotes maturation of human myocardium from pluripotent stem cell-derived progenitors. *Stem Cells*. doi: 10.1002/stem.2036.

Ruyter, B., Røsjø, C., Einen, O., & Thomassen, M. S. (2000). Essential fatty acids in Atlantic salmon: effects of increasing dietary doses of n-6 and n-3 fatty acids on growth, survival and fatty acid composition of liver, blood and carcass. *Aquacult. Nutr*. **6:** 119-127.

Ruyter, B. & Thomassen, M. S. (1999). Metabolism of n-3 and n-6 fatty acids in Atlantic salmon liver: stimulation by essential fatty acid deficiency. *Lipids*. **34:** 1167-1176.

Sander, V., Sune, G., Jopling, C., Morera, C., & Izpisúa Belmonte, J. C. (2013). Isolation and *in vitro* culture of primary cardiomyocytes from adult zebrafish hearts. Nat Protoc. **8:** 800-809.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., & Estevez, A. (1999). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture*. **177:** 191-199.

Sargent, J. R., Tocher, D. R. & Bell, J. G. (2002). The Lipids. In Halver, J. E. & Hardy, R. W. (Eds.) *Fish Nutrition*, pp. 181-257. California, USA: Academic Press.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A., & Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *PNAS*. **97:** 11307-11312.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol*, **13:** 29-83.

Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N., & Gronert, K. (2000). Novel functional sets of lipid-derived mediators with anti-inflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal anti-inflammatory drugs and transcellular processing. *J Exp Med*. **192:** 1197-1204.

Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., & Moussignac, R. L. (2002). Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med*. **196:** 1025-1037.

Schletter, J., Heine, H., Ulmer, A. J., & Rietschel, E. T. (1995). Molecular mechanisms of endotoxin activity. *Arch Microbiol*. **164:** 383-389.

Simon, M. C. (1995). Gotta have GATA. *Nat Genet*. **11:** 9-11.

Skugor, S., Skugor, A., Todorcević, M., Torgersen, J., Ruyter, B., & Krasnov, A. (2010). Exposure to lipopolysaccharide induces immune genes in cultured preadipocytes of Atlantic salmon. *Fish Shellfish Immunol*. **29:** 817-824.

Song, K. S., Scherer, P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S., & Lisanti, M. P. (1996). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J Biol Chem*. **271:** 15160-15165.

Sultan, K. R. & Haagsman, H. P. (2001). Species-specific primary cell cultures: a research tool in veterinary science. *Vetscite*. **1:** 1-7.

Sun, X., Hoage, T., Bai, P., Ding, Y., Chen, Z., Zhang, R., Huang, W., Jahangir, A., Paw, B., Li, Y. G., & Xu, X. (2009). Cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish. *PLoS One*. **4:** e6596.

Suzuki, Y. J. & Evans, T. (2004). Regulation of cardiac myocyte apoptosis by the GATA-4 transcription factor. *Life Sciences*, **74:** 1829-1838.

Thompson, K. D., Tatner, M. F., & Henderson, R. J. (1996). Effects of dietary (*n*-3) and (*n*-6) polyunsaturated fatty acid ratio on the immune response of Atlantic salmon, *Salmo salar* L. *Aquacult Nutr*. **2:** 21–31.

Thum, T. & Borlak, J. (2001). Butanedione monoxime increases the viability and

yield of adult cardiomyocytes in primary cultures. *Cardiovasc Toxicol*, **1:** 61-72.

Tiedge, M., Lortz, S., Drinkgern, J., & Lenzen, S. (1997). Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes*. **46:** 1733-1742.

Tocher, D. R. (2010). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, **11:** 107-184.

Tomita, Y., Matsumura, K., Wakamatsu, Y., Matsuzaki, Y., Shibuya, I., Kawaguchi, H., Ieda, M., Kanakubo, S., Shimazaki, T., Ogawa, S., Osumi, N., Okano, H., & Fukuda, K. (2005). Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. *J Cell Biol*. **170:** 1135-1146.

Wang, K. K., Deng, G. H., Xiao, W. M., Zhao, Z. Y., Jiang, L., Liu, M. D., & Xiao, X. Z. (2002). The protective effect of heat shock protein 70 on the nucleolar impairment of cultured neonatal cardiomyocyte induced by hydrogen peroxide. *Chin J Arterioscler*. **10:** 384-388.

Wiesel, P., Patel, A. P., DiFonzo, N., Marria, P. B., Sim, C. U., Pellacani, A., Maemura, K., LeBlanc, B. W., Marino, K., Doerschuk, C. M., Yet, S. F., Lee, M. E., & Perrella, M. A. (2000). Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation*. **102:** 3015-3022.

Xie, J., Zhou, X., Hu, X., & Jiang, H. (2013). H_2O_2 evokes injury of cardiomyocytes through upregulating HMGB1. *Hellenic J Cardiol*. **54:** 101-106.

Xu, X. Q., Soo, S. Y., Sun, W., & Zweigerdt, R. (2009). Global expression profile of

highly enriched cardiomyocytes derived from human embryonic stem cells. *Stem Cells*. **27:** 2163-2174.

Yousaf, M. N., Koppang, E. O., Skjødt, K., Hordvik, I., Zou, J., Secombes, C., & Powell, M. D. (2013). Comparative cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD). *Vet Immunol Immunopathol*. **151:** 49-62.

Yurchenco, P. D. & Patton, B. L. (2009). Developmental and pathogenic mechanisms of basement membrane assembly. *Curr Pharm Des*. **15:** 1277-1294.