Norges miljø- og biovitenskapelige universitet

Master's Thesis 2017 30 ECTS
Department of Animal and Aquacultural Science (IHA)

Changes in gene expression induced by hypoxia in cultured gas gland cells from Atlantic cod (*Gadus morhua*)

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Aquaculture

ACKNOWLEDGEMENT

I express my indebtedness, sincere appreciation to my thesis supervisor Øivind Andersen for his constant supervision, valuable suggestion, scholastic guidance for conducting the research and writing this manuscript. For his patience and conscientious, to solve my any problems during my thesis and support in every step makes it possible to conduct my thesis.

I express my gratefulness and gratitude to Eva Veiseth-Kent, Katrine Hånes Kirste, Inger Øien Kristiansen for guiding me in the laboratory work to the topics as well for the support on the way. Their valuable advice, continuous inspiration, and factual comments during the research work was beneficial to me. I am so thankful to them that at any time I get help from them that really means something to me.

My sincere thanks also go to Gerrit Timmerhaus for helping me analysis the final data and give important suggestion in result writing. I am very grateful to Petter Vejle Andersen, Shiori Koga, Tram Thu Vuong for their kind co-operation during the lab work.

Great thanks to all of my respective faculty members for delivering cutting edge lectures during the study period. I thank NMBU for providing me the master position, and Nofima for supporting me throughout the entire experiment. Thanks go to Odd Kriestiansen for providing Atlantic cod several times.

Thanks to all of my friends to help me mentally and for being a part of my daily life, Specially to MD A B M Sharifuzzaman to help me in every step of the thesis and obvious mental support. My special thanks go to Trine Lund, Md. Hafizur Rahman and Md. Akhtarul Alam to help me during writing thesis.

Finally, I acknowledge my gratitude and profound respect to my beloved parents, elder sisters and all other relatives for their blessings and inspirations.

Ifrat Jahan Tamanna

ABSTRACT

Low level of oxygen, called hypoxia, triggers the transcription of regulatory genes that promote O_2 delivery and anaerobic metabolism. The majority of this transcriptional response to hypoxia is mediated by the Hypoxia inducible factor (HIF-1), comprising the two subunits HIF-1α and HIF-1β. The HIF-1α is expressed ubiquitously in all cells and targeted for proteosomal degradation. When fish are exposed to hypoxia condition, HIF-1α degradation is inhibited and subsequently activates or inhibits the expression of specific genes. In hypoxia, prolylhydroxylase domain (PHD) is less active, so the degradation is inhibited and subsequently allows HIF-1α to make Aryl hydrocarbon nuclear translocator (ARNT) - HIF-1α complex and translocates to the nucleus. Finally, this complex binds to the target genes and recruit transcriptional co-activators for full transcriptional activity and either activate or inhibit the expression of specific genes. In this study, I analyzed the expression of hypoxia inducible genes, Hypoxia inducible factor-1α (HIF-1α), Lactate dehydrogenase a (LDHa), Glucose transporter-1 (GLUT-1) and Carbonic anhydrase (CA-6) genes in cultured gas gland cells from Atlantic cod (*Gadus morhua*). Gas gland cells of the swim bladder were cultured in growth medium and treated with the PHD inhibitor FG-9245 diluted in three different concentrations (10, 50 and 90μM) using DMSO. Total duration of cell culture was 16 days. Total mRNA was extracted from the cells and a qPCR analysis was performed by using a Light cycler® 480 platform to quantify the mRNA levels of the four genes. Relative expression of all four genes had increased in the FG-9245 treated group than the control group. However, LDHa and GLUT-1 showed low relative gene expression in 90μM PHD inhibitor treated cells then 50μM inhibitor treatment. In 90μM inhibitor treated cells, there was a sharp increase of relative gene expression of CA-6. The HIF-1α protein was detected in the gas gland tissue of Atlantic cod at normal oxygen level by Western blotting using a specific HIF-1α antibody. Although expected molecular weight was 86kDa, but the molecular weight was observed 28 kDa probably due to degradation in environmental oxygen level. Relative gene expression pattern of HIF-1α, LDHa, GLUT-1 and CA-6 suggests the efficacy of PHD inhibitor on determining the degree of hypoxia in Atlantic cod *in vitro*.

Key words: Atlantic cod, hypoxia, swim bladder, gas gland cell, PHD inhibitor, FG-9245,HIF, qPCR, gene expression

ABBREVIATIONS

ARNT	Aryl hydrocarbon nuclear translocator
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CBP	CREBbinding protein
C_T	number of cycles to cross the threshold
DFO	Desferrioxamine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
FBS	Foetal bovine serum
GLUT	Glucose Transporter
HCL	Hydrochloric acid
HCO_3^-	Bicarbonate
HIF	Hypoxia Inducible Factor
HRG	Hypoxia Responsive Genes
kDA	Kilo Dalton
LDH	Lactate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
ORF	Open Reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PHD	Prolylhydroxylase domain enzyme
qPCR	quantitative polymerase chain reaction
R primer	Reverse primer
RBC	Red blood cell

RPM	Rotation per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
VHL	von Hippel-Lindau

CONTENTS

1. INTRODUCTION

1.1 Atlantic cod

Atlantic cod (*Gadus morhua* L.) is a demersal teleost fish widely distributed in the North Atlantic and is prominent in the Canadian east coast, the southern part of Greenland, the Faeroe Islands, Iceland, the North sea throughout Skagerrak and Kattegat to the Baltic Sea and the Norwegian coast of the Bering Sea (Figure 1.1) (Sundby & Nakken 2008). Atlantic cod usually prefer water temperatures of 0-12^{0}C but also can survive in water temperature below -1^{0}C and above 20^{0}C (Drinkwater 2005). The Atlantic cod lives mainly from the shoreline and down to the continental shelf. The fish return to spawning sites during the winter-spring season (Marteinsdottir *et al.* 2005). The Atlantic cod is normally stayat depths between 10-150 m. They rarely go deeper than 400m. The Atlantic cod is a bottom feeder and prefers to live at the bottom of ocean floor. During the summer, many Atlantic cod move to offshore banks (Fahay *et al.* 1999).

Optimal environmental conditions is important throughout the entire life cycle of fish to achieve fast growth and good health and welfare. Oxygen plays a critical role for the growth of the Atlantic cod. Generally fish can cope with low oxygen at a certain level by increasing gill ventilation. During hypoxic condition, different gases are secrete in swimbladder tissue in deep sea. Besides, two other ways such as oxygen-dependent pathways so called aerobic pathways (oxidative phosphorylation) and oxygen-independant pathways or anearobic pathways (glycolysis) also known to maintain oxigen uptake in oxigen-depleted environment.

In different gas pressure, swimbladder gas gland cells are important gas regulator. There are few studies was done with swimbladder gas gland cells function. And very few genes expression were studied in the Atlantic cod gas gland cells. This is very initial report that attributes the gene expression of cultured gas gland cells of Atlantic cod.

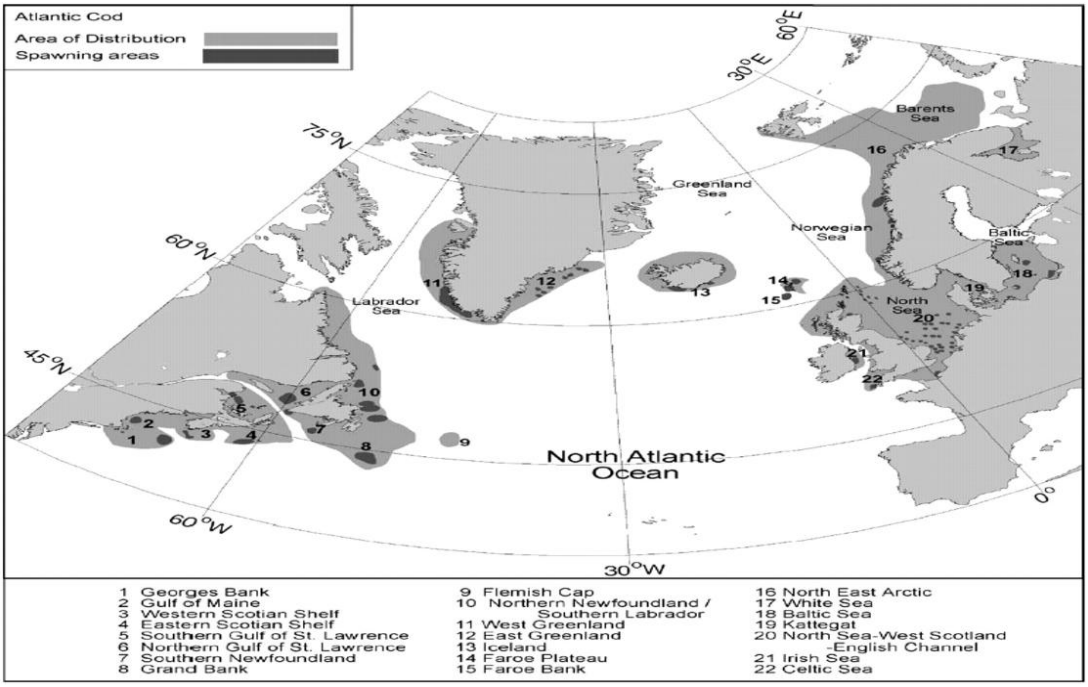


Figure 1.1: Distribution and spawning of Atlantic cod throughout the north Atlantic(Drinkwater 2005).

The Atlantic cod is one of the most important commercial fish species in the North Atlantic and the second highest producing fish in Europe by volume of total catch (Jørstad *et al.* 2007). However, high capture has caused stocks to decline. The total catch per year of Atlantic cod in the North Atlantic decreased from about 3.5 million tons to 1 million tons from 1970 to 2003 (Figure 1.2) (Drinkwater 2005).

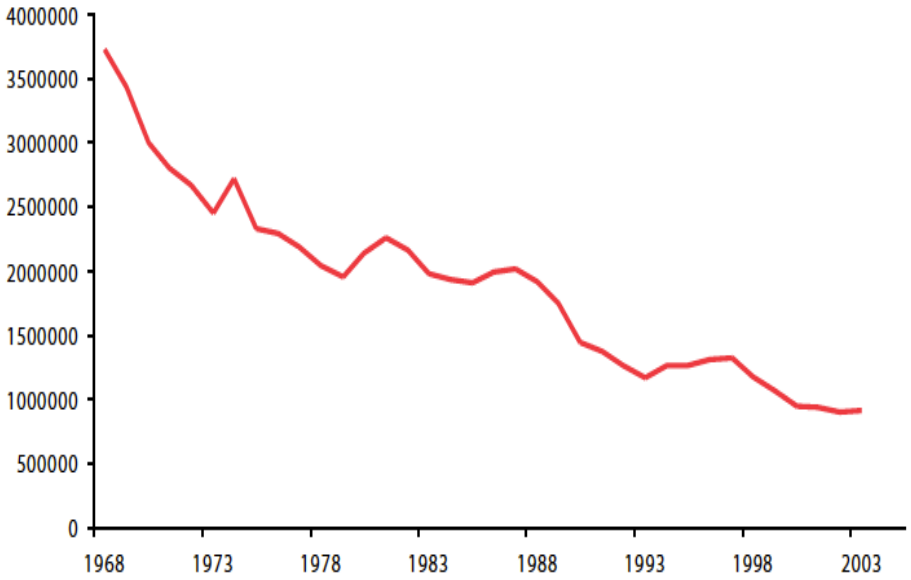


Figure 1.2 Total catch (tons) of Atlantic cod in North Atlantic.

1.2 Function of swim bladder and gas gland of Atlantic cod

The swim bladder is an internal organ of teleost fish (Midling *et al.* 2012; Umezawa *et al.* 2012) (Figure 1.3). This organ is filled with gas, mainly oxygen and carbon dioxide and enables buoyancy (Harden Jones & Scholes 1985; Midling *et al.* 2012) of the fish. The cavity of the swim bladder contains low density of gas which balances high density of fish muscle tissue and skeletal elements. Therefore, fish can stay in right water depth without swimming (Pelster 2004; Umezawa *et al.* 2012). Atlantic cod has a closed swim bladder (physoclist), that occupies 5% of its body volume (Scheid *et al.* 1990). So the gas inside need to be balanced to continue neutral buoyancy in case of depth change (Harden Jones & Scholes 1985; Midling *et al.* 2012).

The Atlantic cod is known to perform vertical migrations (Godø & Michalsen 2000). When the fish ascents, the swim bladder gas expands, thus the swim bladder volume increases and this creates a lifting force. The rate of absorption of oxygen is limited. However, rapid ascents can thus be dangerous to physoclist fish as the swim bladder may expand beyond control or burst (Harden Jones 1951). The swim bladder has three functional components; the oval gland, the gas gland and the *rete mirabile*. The gas gland is located on the ventral side of the swim bladder wall and has thick epithelial cells and capillaries. In Atlantic cod, gas gland cells make cluster (Pelster 2004) and actively pump gas from the blood to the bladder. Gas molecules also move from blood to the swim bladder lumen ((Pelster et al. 1990). Gas gland cells increase the acidity of blood with lactic acid secretion. Increased lactate concentration reduces the oxygen carrying capacity of the blood.

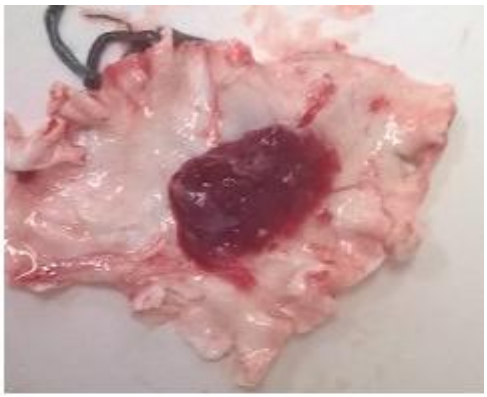


Figure 1.3: Cod gas gland tissue attached at the inner part of the swim bladder.

The swim bladder wall is embedded with many tiny blood vessels where blood is constantly diffused with highly concentrated O_2 (Figure 1.4a). The special arrangement of veins and arteries (*rete mirabile*) in the swim bladder wall prevents O_2 to leave the swim bladder. Each artery and vein are coiled around each other. This ensures the opposite direction of blood flow between two adjacent vessels (Figure 1.4b). The O_2 concentration is higher in the vein than in the artery. Therefore, O_2 is diffused from the vein to the adjacent artery along for their entire length of contact.

This diffused O_2 in the artery is then directly returns to the swim bladder. If the system works efficiently, very little O_2 is lost to the surrounding tissue. This counter current flow limits the diffusible materials (in this case O_2) to a region of high concentration. So an initial increase in gas partial pressure is multiplied by back diffusion of gases and countercurrent multiplication. As a consequence, the fish can survive at the depth of several thousand meters with a gas filled swim bladder (Kobayashi *et al.* 1989; Kuhn *et al.* 1963; Rodin & Jacques 1989).

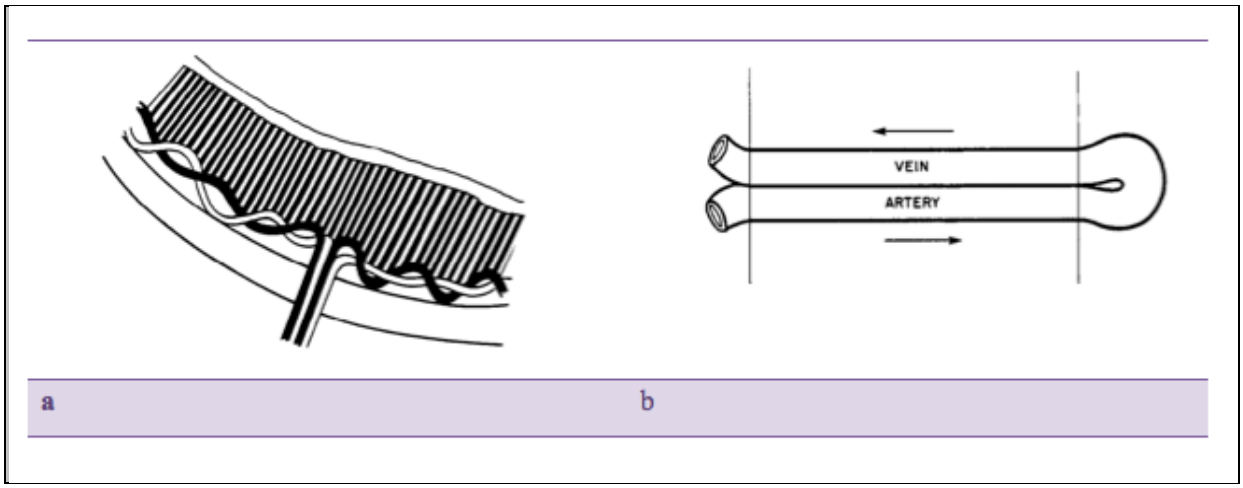


Figure 1.4: a: Blood vessels in the swim bladder wall, black and white color depicts two different types of vessels. b: A single artery-vein pair.

1.3Effects of oxygen concentration on fish

Oxygen is important in the breathing process and energy metabolism of all aerobic organisms (Rimoldi *et al.* 2016). Hypoxia, is a major cause of fish morbidity and mortality in aquatic environments (Li *et al.* 2017). But some teleosts can survive in extremely low oxygen (van der Meer *et al.* 2005). Natural hypoxia occurs frequentlyin many aquatic ecosystems

(Rimoldi *et al.* 2016) and oxygen concentrations in water vary more over time and space than in terrestrial environments (Guillemin & Krasnow 1997).

Over the past decades, in coastal water, the proportion of dissolved oxygen has changed more than any other ecologically important variables over the past decades. The hypoxic areas have increased with an exponential growth rate of 5.54% per year from 1916 to 2006. Excessive production of organic matters have increased the oxygen demand of the coastal ecosystem. As a result, the respiratory oxygen demand of the coastal ecosystem has increased and this has reduced oxygen solubility. Hypoxia in the coastal regions emerges as a major threat to the marine ecosystem (Vaquer-Sunyer & Duarte 2008).

The Atlantic cod is an oxygen intolerable species that requires 28-40% oxygen saturation level for survival (Hall *et al.* 2009). The extremity of hypoxia of a specific species depends upon its hypoxia tolerance (Genz *et al.* 2013; Schurmann & Stehhensen 1997). Hypoxia suppresses major energy consuming processes in vertebrate animals and thereby inhibits their growth and development (Guillemin & Krasnow 1997; Huang *et al.* 2004; Seta & Millhorn 2004). Hypoxia induces many physiological changes in fish like reduced food intake and growth (Bernier *et al.* 2012; Herbert & Steffensen 2005), decreased reproductive capacity and embryonic development (Shang & Wu 2004). In addition, hypoxia causes biochemical changes such as increased blood oxygen-carrying capacity, anaerobic metabolism proportion (Urbina & Glover 2012) and blood parameter changes (Affonso *et al.* 2002). It can disruptthe sex hormone metabolism(Cheung *et al.* 2014), alter sexual differentiation (Cheung *et al.* 2014) and the cardio respiratory function (Belao *et al.* 2011). Atthe cellular level, low oxygenaffects energy generation, damages cells and causes apoptosis (Long *et al.* 2015).

1.4Hypoxia-inducible factor (HIF)

HIF isan important modulator in the transcriptional response to hypoxic stress (Kajimura *et al.* 2006). During hypoxia, the regulatory genesare involved in oxygen delivery and the enhanced anaerobic metabolisms. In hypoxic conditions, the tissue response in eukaryotic organisms is largely coordinated by HIF proteins (Semenza 2007; Semenza 2012).

The heterodimeric HIF-1consists of an O_2-regulated α subunit and a constitutively expressed β subunit complex and mediates most of the transcriptional responses against hypoxia (Wang *et al.* 1995; Wang & Semenza 1995). In the normal oxygen level, HIF-1α is hydroxylated by

proline hydroxylases (PHD1, 2 and 3) in presence of $Fe2^{+}$, 2-oxoglutarate (2-OG) andascorbate. This hydroxylated HIF-1α is recognized by and bound to the von Hippel-Lindau tumor suppressor (pVHL) together with a multisubunit ubiquitin ligase complex.This HIF-1α expressed ubiquitously in all cells and targeted for proteosomal degradation (Figure 1.5) (Maxwell *et al.* 1999; Ohh *et al*. 2000).

However, when fish are exposed to hypoxic condition, HIF-1α degradation is inhibited becausethe enzyme PHD is less active and subsequently allows HIF-1α to accumulate in the cells. At this point,Aryl hydrocarbon nuclear translocator (ARNT)-HIF-1α complex is formed and translocated to the nucleus (Ivan *et al.* 2001; Jaakkola *et al.* 2001; Maltepe *et al.* 1997; Wu *et al.* 2016). Afterwords, ARNT-HIF-1α complex binds to cis-regulatory DNA sequence hypoxia response elements (HREs) of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. Finally activates or inhibits the expression ofspecific genes (Figure 1.5) (Jiang *et al.* 1997; Kajimura *et al.* 2006).

1.5 Genes regulated by HIF-1α during hypoxic condition

During hypoxia, the HIF-1α and HIF-1β heterodimer is translocated to the nucleus and transactivates multiple target genes (Semenza 1999), including glucose transpoters (GLUTs), carbonic anhydrase (CA) (Jamali *et al.* 2015) and lactate dehydrogenas (LDH) (Firth *et al.* 1995). The database Hypoxia Responsive Genes in fishes (HRGFish), which contains gene responses in hypoxic conditions, covers 818 gene sequences and 35 gene types from 38 fishes according to Rashid *et al.* (2017).

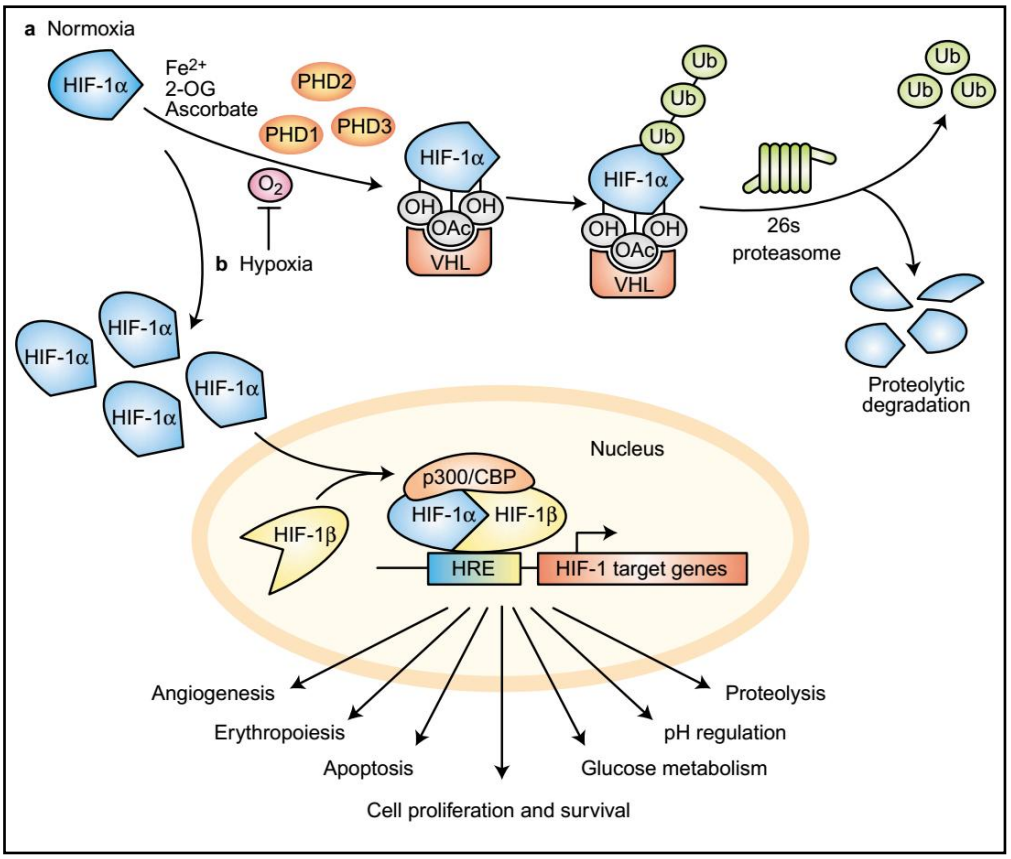


Figure1.5: Regulation of HIF-1α activity during normoxia and hypoxia condition. Modified from (Carroll & Ashcroft 2005). Abbreviations: CBP, CREBbinding protein.

As a result of hypoxia, transactivated genes perform several physiological functions such asangiogenesis, erythropoiesis, apoptosis, cell proliferation and survival, glucose metabolism, pH regulation and proteolysis to adopt in the hypoxic condition (Carroll & Ashcroft 2005).

1.5.1 Glucose transporter-1 (GLUT-1)

The glucose transport through cell membranes of Atlantic cod is facilitated by specific transmembrane proteins (GLUTs), which is important forfish glucose homeostasis (Planas *et al.* 2000). Class 1 sodium-independent GLUTs 1, 2, 3 and 4, which facilitate glucose transporters, are identified in Atlantic cod (Hall *et al.* 2004). Among these four glucose transporters, GLUT-1 is found in most Atlantic cod tissues (Hall *et al.* 2014) and the protein coding sequence has 78.2% sequence similarity with human GLUT-1 (Hall *et al.* 2004). Based on the tissue availability, GLUT-1 is the dominant transcript in gas gland, heart, white muscle and red blood cell (RBC) (Hall *et al.* 2014). In addition, GLUT-1 mRNA is increased,

as a result of enhanced transcription and decreased degradation. That triggers the increase in cell and plasma GLUT-1 content and stimulates the glucose transport (Zhang *et al.* 1999).

1.5.2Lactate dehydrogenase a (LDHa)

Lactate dehydrogenase (LDH) is a cytosolic enzyme which catalyzes the terminal step in the anaerobic glycolysis (Figure 1.6). The capacity of the anaerobic energy production during hypoxia depends upon the LDH activity and functional properties (Fields & Somero 1998; Portner 2002; Zakhartsev *et al.* 2004).

In hypoxic condition, LDH isozymes change in tissue distribution, for example, isozyme A4 changes in heart and brain and isozyme B4 is changes in liver (Almeida-Val *et al.* 1995).LDH is highly expressed in fish in the response to hypoxia (Almeida-Val *et al.* 2011). Like many other fishes, LDH in Atlantic cod is coded by three independent loci. For example, LDH-A and LDH-B have been identified in liver, muscle, heart and eye, while LDH-C is only observed in liver (Zakhartsev *et al.* 2004). The protein (LDH) is a tetramer, composed of four polypeptide subunits. The isoform LDH-A4 preferentially converts pyruvate to lactate under anaerobic condition. This isoform is found predominantly in poorly vascularized tissues with low partial pressure of oxygen (pO2), such as skeletal muscle (Almeida-Val *et al.* 2011). However, an alternative allele of LDH-A is found from Icelandic cod (Mork *et al.* 1985).

1.5.3 Carbonic anhydrase-6 (CA-6)

The acid-base balance in fish relies almost entirely upon the direct exchange of acid-base equivalents with the environment (metabolic compensation). CA is the zinc metallo-enzyme that catalyzes the reversible hydration/dehydration of CO_2 to bicarbonate. Therefore, it is critical to CO_2 excretion, ionic regulation and acid-base balance. The CA is involved in metabolic site of CO2 production (muscle), the circulating red blood cells and the primary respiratory surface (gills/lungs). The CA also reduces some tumor growth in cells *(Mahon et al.* 2014).

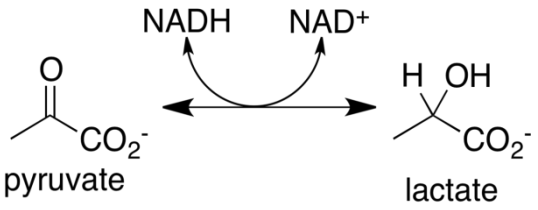


Figure 1.6: Reaction catalyzed by LDH converting pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply. Abbreviation: NAD, Nicotinamide adenine dinucleotide. NAD+ and NADH are oxidized and reduced form of NAD respectively.

This metallo-enzyme plays a crucial role in the systemic acid–base regulation in fish by providing acid-base equivalents for exchange with the environment (Gilmour & Perry 2009). The CA is involved in many physiological processes, but the most important function of the CA is facilitating transportation and excretion of CO_2 (Esbaugh & Tufts 2006). The CA-IX inhibition and expression shows tumor proliferation reduces primary tumor growth (Mahon *et al.* 2014). In hypoxic condition, HIF-1α triggers CA IX, which regulates pH (Iwasaki *et al.* 2015) and the over expression of CA IX in human cancer cells (Ambrosio *et al.* 2016; Jamali *et al.* 2015).

One CA gene family, α-CA consists of 16 CA isoforms (Figure 1.7**).** These isoformes have been identified in mammals and two CA-related protein has been identified in fish (Esbaugh & Tufts 2006; Gilmour & Perry 2009). Three of them (CA VIII, X and XI) shows lack of catalytic activities (Gilmour & Perry 2009).CA VI is the only known secretory mammalian CA isoenzyme with molecular weight of 42 kDa (Murakami & Sly 1987). An immunohistochemical study had done (Parkkila *et al.* 1990) which identifies that in human, the location of CA VI secretion is in the secretory granules of the acinar cells in parotid and submandibular glands.

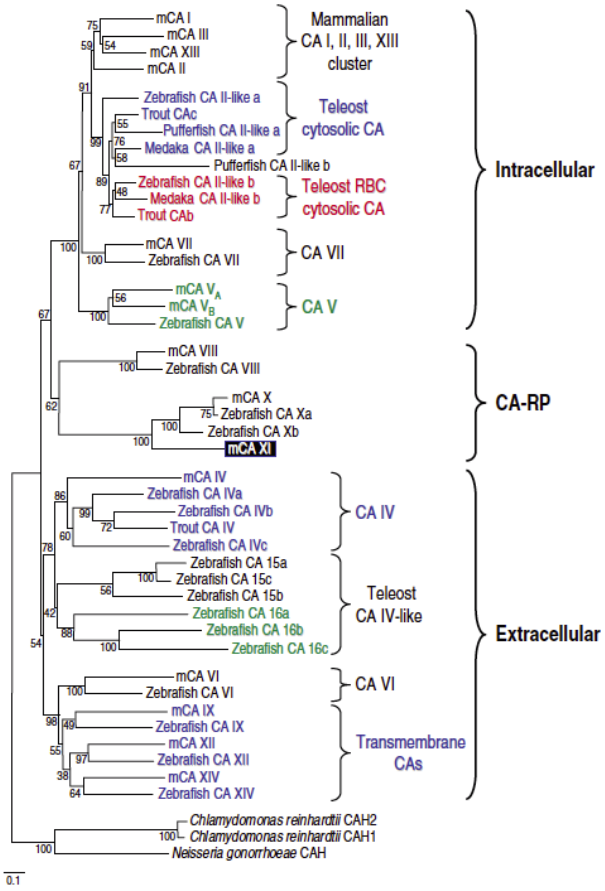


Figure 1.7: The isoforms of α-CA gene family and phylogenetic relationships among mammalian (mouse, *Musmusculus*) and fish (medaka, *Oryziaslatipes*; pufferfish, *Tetraodonnigroviridis*; rainbow trout, *Oncorhynchusmykiss*; Zebra fish, *Daniorerio*). Fish equivalent has not been found for Mouse CA XI. m:mouse. Branches are drawn to scale with 0.1 approximating replacement of 10% of the amino acids in the protein alignment. (Gilmour & Perry 2009; Lin *et al.* 2008)

1.6 Mechanism of the PHD inhibitor FG-4592

Inhibitors of prolyl hydroxylase (PHD) stabilizes HIF-1α and increases tolerance to hypoxia (Wu et al. 2016; Yu, Y et al. 2016). HIF-1α is rapidly degraded in normoxic condition so number of stabilizers are used to reduce degradation, such as Iron chelators Desferrioxamine (DFO) (Demidenko *et al.* 2005), antioxidant NAC (Sanjuan-Pla *et al.* 2005), analog of 2-oxogluterat ((2-OG) FG-4592 (Figure 1.8), IOX2 to deactivate PHD and subsequently prevent HIF-1α degradation (Wu *et al.* 2016).

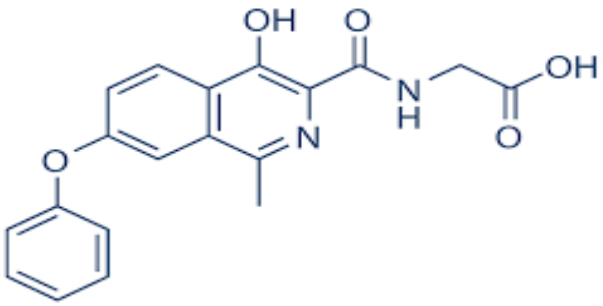


Figure 1.8: Roxadustat (FG4592)(Selleck Chemicals)

The stabilizer Roxadustat FG-4592, a [(4-Hydroxyl-benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid derivative, inhibits PHD and stabilizes HIF-1α. This inhibitor reduces apoptosis and increases the survival of neuronal cells (Wu *et al.* 2016), but one study indicates that inhibition of PHD is the cause of apoptosis (Siddiq *et al.* 2009).

2. AIM OF THE STUDY

Several glycolytic enzymes in the gas gland are responsible for the acidification of the circulating blood to producehigh tension of gas, mainly oxygen, in the swim bladder. The high anaerobic enzyme activity is functional despite highoxygen levels in the gas gland cells. To study the effects of low oxygen levels on the gene expression of glycolytic enzymes in the gas gland of Atlantic codwe mimicked hypoxic conditions by inhibiting PHD, thereby stabilizing HIF-1a. The objectives of this study were:

i. To develop an effective protocol forAtlantic cod gas gland cell culture.
ii. To examine the inhibition of HIF-1α degradation by Western blot analysis.
iii. To study the effect of stabilizing HIF-1α on the expression of the HIF-1α gene.
iv. To study the effect of mimicked hypoxia on the expression of the HIF-1α, GLUT-1, LDHa and CA-6 genes in gas gland cells after treatment with different concentrations of the PHD inhibitor.

3. MATERIALS AND METHODS

3.1 Experimental design

The study was conducted on Atlantic cod captured in Oslofjorden outside Døbak in 18November 2016. Three fish were sectioned and gas gland tissues were collected and proceed to protein analysis by Western blotting. Gas gland cells from the same tissue samples were cultured and treated with PHD inhibitor FG-4592. Then mRNA was extracted to measure the gene expression using real time RT-qPCR (Figure 3.1).

3.2 Atlantic cod gas gland cell culture

3.2.1 Reagents preparation for cell culture

Perfusion buffer I: Chemicals (Appendix-1) were dissolved with redistilled water in a sterile Erlenmeyer bottle and adjusted to the pH 7.4. The solution was transferred to a volumetric flask and diluted to 1L with H_2O. Finally, the buffer was divided into Nunc Easy Flask™ with 150 ml per bottle.

Collagenase: Collagenase was used as a 0.1 % concentration made by mixing 0.1% collagenase, 150mg of lyophilized collagenase with 150 ml of buffer 1.

Buffer II: Trypsin was added at a 0.5mg/ml concentration (2%) to the collagenase mixed buffer I.

1X PBS: 1X PBS was made by diluting 50 ml of 10X PBS into 450 ml Milli-Q water.

Growth medium:10ml of the growth medium was prepared by mixing1ml of PBS(10%), 0.1ml of Hepes(1%),0.1ml (0.01M) of Antibiotic-Antimycotic to 8.8ml of L-15 buffer, supplemented with glutamax™(Thermo Fisher Scientific).

3.2.2 Procedure of cell culture

Gas gland tissue (Figure 3.1) were separated from the inner part of the swim bladder and kept in buffer I. Tissues were chopped into very small pieces and centrifuged (Heraeus SEPATECH centrifuge, Thermo Fisher Scientific, Roskilde, Denmark) at 1250rpm for 5 min. The pellet was then mixed with buffer II followed by careful shaking for half an hour and filtration in the Whatman filter paper. Buffer II was used at a concentration of 5ml/g gas gland tissues.

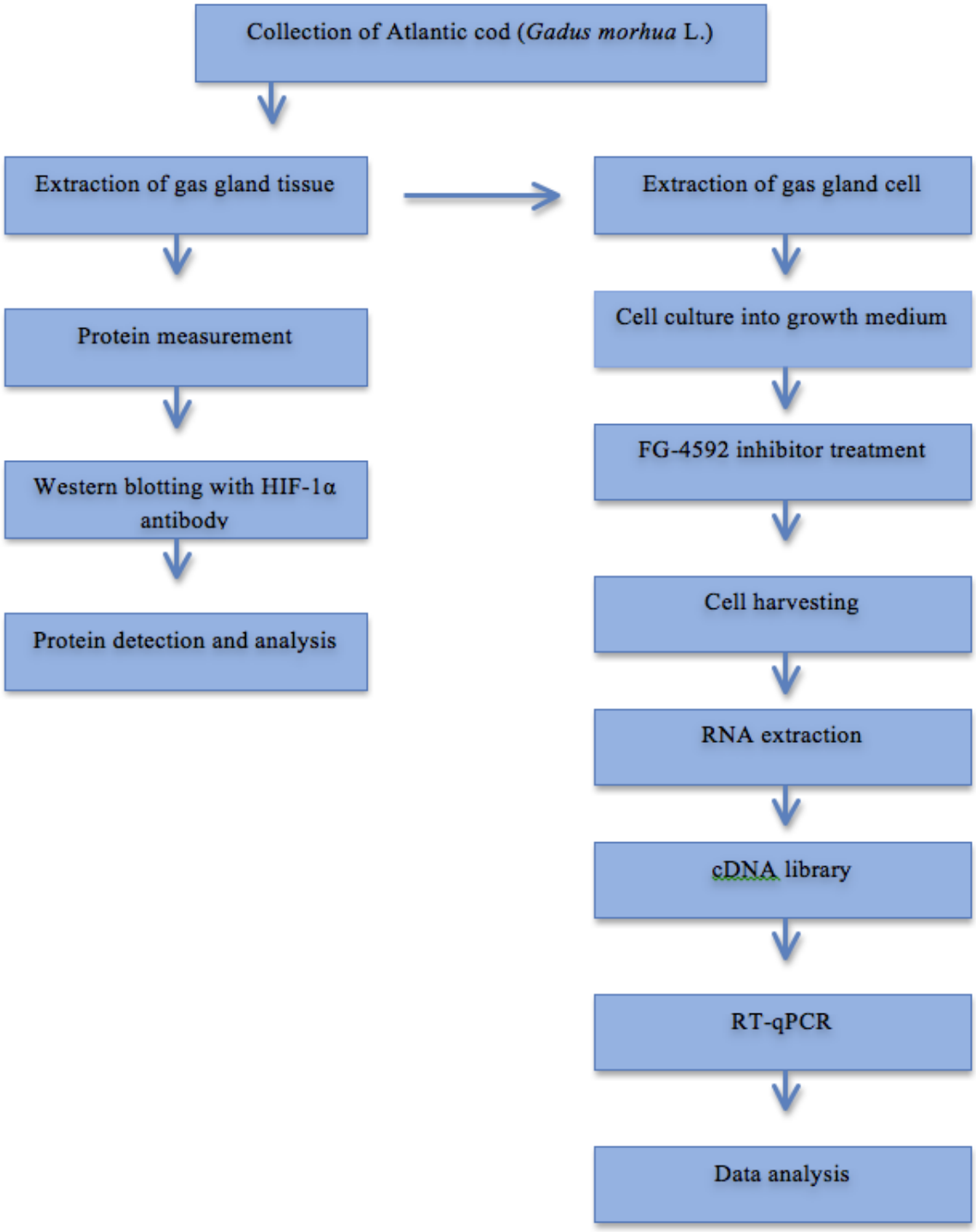


Figure 3.1: Experimental design. All experiments were performed at Nofima, Ås.

Supernatant was centrifuged for 5min at 1250 rpm in room temperature, and the pellet was collected to count the cells in automatic cell counts. Attachment agent, mouse laminin (EMD Millipore, Germany) was poured into the bottom of the well of 4-well plate. Thereafter, the wells were scraped and dried very well. Cells were then seeded into each well with a volume of 3ml of growth medium. The plate was placed in an incubator (37°C) for 6days. In every 2nd day, cells were washed with L-15 buffer and supplemented with fresh growth medium. Trypsin was used in the culture medium to inhibit cell damage.

3.2.3 Cell counts with countess

Cells were counted in an automatic cell countess (Thermo fisher Scientific).The measurement range of the machine was set from $1 \times 10^4 - 1 \times 10^7$ cells/ml. 10µl of the cell suspension was taken in Eppendorf tube and then mixed with 10µl of 0.4% Trypan Blue. Afterwards, 10µl of the Trypan Blue treated cell suspension was taken into both chambers of the automated cell countess. After 30 sec, a cover slide was placed in the slide port of the countess. Both live and dead cells were counted automatically in the countess.

3.3 Inhibitor treatment

The PHD inhibitor, Roxadustat® (FG-4592) was used in concentrations of 10, 50 and 90µM.

3.3.1 Reagents preparation for inhibitor treatment

Growth medium: Same used in culture media.

DMSO: DMSO was used as 0.1% in the growth medium as a control.

PHD inhibitor FG-4592:10mMof stock solution was made using the following equation,

Mass = Concentration * Volume * Molecular weight.

3.3.2 Procedure for inhibitor treatment

Cells were washed with L-15 for two times. In each well, 3ml of growth medium was poured. The control group was treated only with 3µl of DMSO and sample group was treated with 3, 15, and 27µl ofRoxadustat® FG4592 which were adjusted to 10, 50and 90µM respectively. At every second days, cells were replenished with new medium mixed with DMSO and inhibitor for control and sample groups correspondingly.

3.4 Cell harvesting

After 8 days of inhibitor treatment (total 16days of cell culture), cells were washed two times with PBS and lysed by adding 350 µl of DTT/ RLT buffer in each well. 2M DTT was used with RLT buffer. Then skipped and pipetting cell lysate into QIA shredder mini spin column. Finally, centrifugation was performed for 2 min at12000 rpm at room temperature and stored at -70^{0}C.

3.5 cDNA library preparation

3.5.1 Procedure for mRNA extraction

The mRNA mini kit (Thermo Fisher Scientific) was used to extract mRNA from inhibitor treated cells. 350µl cell suspension was mixed with equal volume of 70% ethanol followed by vortexing at high speed until the cell pellet was dispersed completely. After centrifugation (Eppendorf Centrifuge 5415R) at 12000rpm for 30 sec in room temperature, 80µl/sample DNAase solution (Table 3.1) and 600µl of wash buffer I were added to the spin cartridge. Centrifugation was performed again at 12000 rpm for 30 sec.

The flow through and collection tube were discarded. The spin cartridge was placed into a new collection tube. DNAse was used to destroy all cellular DNA from the sample. 500µl of wash buffer II was poured into the spin cartridge and centrifuged at 12000 rpm for 30 sec in room temperature. The bottom solution was discarded and filter was kept carefully. In view of pure mRNA extraction, repeated addition of Buffer II and centrifugation with similar rpm had been performed and spinned again for 10 seconds. At room temperature, 30µlof RNAse free water was added to the spin cartridge and incubated with for 1min and centrifuged for 2 minutes at 12000 rpm. This step was repeated again to confirm complete mRNA extraction from the sample.

Table3.1 :DNAse for purification of mRNA

Reagents	Amount for 1.5ml tube(1X)
10X DNAsw I buffer	8 µl
DNAse	9.9 µl
RNAse free water	62 µl

3.5.2 Measuring mRNA with nano drop

RNAse free water (1.1µl)was dropped onto the sensor and measured at 230 nm. Water was dropped and measured in the same way as RNAse free water, followed by blank measurement to ensure no mRNA. Finally, 1.1µl of sample was loaded on the sensor to measure the sample mRNA.

3.5.3 The procedure of makingcDNA library

150 ng of mRNA sample was taken into five different PCR tubes. Then 1µl of 10x RT buffer, 2.2 µl of $Mgcl_2$, 2.0 µl of dNTP, 0.25 µl of OligodT and Hexamen, and 0.35µl of RNAse inhibitor were mixed as 1X reaction. Reverse transcriptase (RT) was not added together because it is so sensitive to temperature. Then water was added to the mixture to make the final volume of 10µl and finally RT was added. All the reagents were kept on the ice during the mixing time. Thereafter, the program was set in the PCR machine (Veriti 96, Applied Biosystems) (Figure 3.2). The PCR reaction was run for 1hour and 15min.

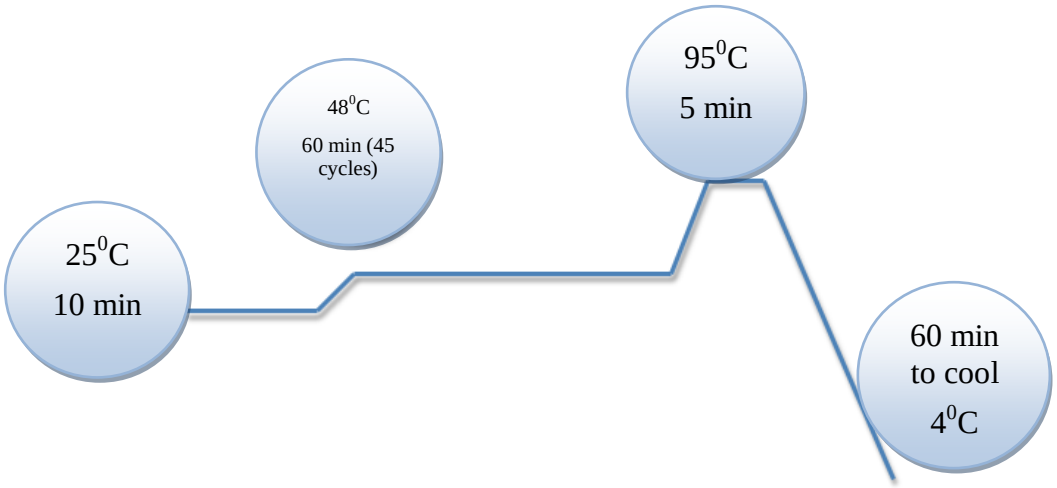


Figure 3.2: Program set up in PCR machine for cDNA library construction.

3.6 Gene expression measurement by real time qPCR

3.6.1 Procedure for real time qPCR run

10µl of cDNA was mixed with 40µl of H_2O into a 1.5ml tube (one per sample). This one is the first dilution (1x). Then 20µl H_2O was mixed with the same volume of cDNA from 1x dilution and this one was 2x dilution. Similarly, the cDNA samples were diluted from 1x to 8x (Figure 3.3).

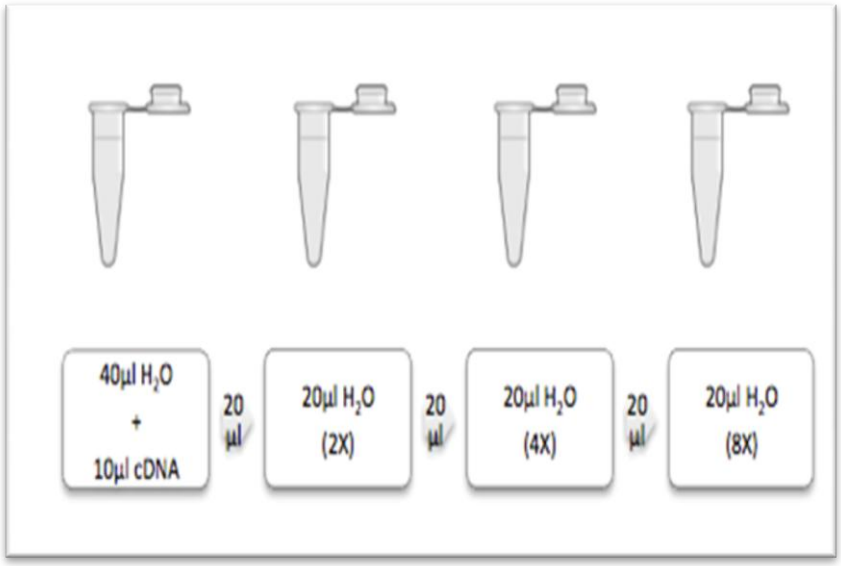


Figure 3.3: Dilution of the cDNA sample by dH_2O(1x-8x).

To make a standard curve (Appendix 2) for a specific gene, 2µl of cDNA from each sample was mixed with 1unit of SYBR Green light cycler buffer with the specific gene. For the final run, In a 96well plate, 10µlof SYBR Green mixed buffer (Table 3.2) along with one selected primer (Table 3.3) were loaded in specific wells. Finally, 2µl of sample cDNA were loaded in each well.

Table 3.2: SYBR Green light cycler buffer recipe (SYBR Green l, Roche)

Ingredients	Volume
SYBR Green I (2X)	6.0 µl
F primer (10mM)	1.2 µl
R primer (10mM)	1.2 µl
cDNA	2.0 µl
dH_2O	1.6 µl

Table 3.3: Primers used in real time qPCR (Invitrogen, Thermo Fisher Scientific).

Primers	Catalog no	Forward primer sequence Reverse primer sequence
HIF-1α	10336-022 L6971D02, L6971D03	F1_AAGTTCACCTACTGCGACGA R1_GAGCGGTTCATCAGGTGTTC
CA-6	132606 L1486D01, L1486D02	F1_TGGACGGCATCCGATACAT R1_CCTCCTTAAAGCTCTTGTACTTGTCA
GLUT-1	132606 L1486D03, L1486D04	F1_GTGTTTGGCATCGAGTCCTT R1_TCGTTCTTGTTGAGCAGCAG
LDHa		F1_GGTATGTCCGTTGCTGACCT R1_GAGGAACACCTCGTCCTTCA
Ubiquitin(The house keeping gene)		F1_GGCCGCAAAGATGCAGAT R1_CTGGGCTCGACCTCAAGAGT

The qPCR reaction (Table 3.4) was performed in a Light Cycler® 480 machine(Roche). A Light Cycler® 480 instrument protocol (Table 3.4) was used to detect the expression value (Ct value, number of cycles to cross the threshold) of the genes.

Table 3.4: The PCR parameters for a LightCycler® 480 system. SYBR Green I Master using a LightCycler® 480 multiwall plate 96.

Program name	Temperature (^{0}C)	Cycles	Hold (mm:ss)	Ramp rate (^{0}C/s)
Pre incubation (Denaturation of cDNA)	95	1	05:00	4.4
Amplification	95	45	00:10	4.4
	72		00:05-00.30	4.4
Melting curve	95	1	00:05	4.4
	65		01:00	2.2
	97		-	-
Cooling	40	1	00:10	1.5

3.6.2 Real time qPCR data analysis method

The log2 Pfaffl value was used to analyze the qPCR data. It is more reliable than the ΔΔCt method to quantify the gene expression. Log2 Pfaffl method is a relative quantification that links PCR signal of the treatment groups transcript with the untreated control (Livak & Schmittgen 2001). Pfaffl value is calculated by following template on excel with one equation. The equation is, Pfaffl values= $(1+ERef)^{\Delta Ct\ Ref\ (Ct\ sample\ -Ct\ Mean\ of\ control\ group)}$/ $(1+E_{Target})^{\Delta Ct\ Target(Ct\ sample\ -Ct\ Mean\ of\ control\ group)}$

Here, Ct value is directly measured by Light cycler® 480. Ct value means the number of cycles of fluorescent signal to cross the threshold (Dorak 2007). Reaction with Ct> 30 indicated lower level of expression and more then 38 was excluded from data as its unreliable. Data were analyzed by R studio,version 3.4.2 (2017-09-28) ($P<0.05$ means significant difference) and presented in mean±SE (Appendix 5).

3.7 Western blotting

3.7.1 Reagents for sample preparation.

Ripa buffer solution: The solution contains 50mM TrisHCl (pH 7.6), 150 mM NaCl, 1% triton-x, 1% sodium deoxycholate, 0.1% SDS. The proteanase inhibitor AEBSE (5µl per 1ml of Ripa buffer, without phosphatase).The SDS detergent denatures the proteins and binds to the positive charges of the protein. Thus giving each protein the same overall negative charge for which proteins are separated based on their size.

PBST buffer: 100ml of Triton-x 100 (Sigma-Aldrich) was mixed with 900ml of deionized water.

Blocking solution: 2% ECL Advance® blocking agent was mixed in 1x PBST. Hence 0.6 gm milk powder was mixed in 30ml of 1X PBST.

3.7.2 Procedure for sample preparation

Ripa buffer was used to denature the tissue. 200µl of the Ripa buffer was used for 10mg of tissue. This solution was always kept in ice to prevent protein degradation. Afterwords, shacked for 30sec for in precellys agitator (Birtin Technology, France), considering the degradation of some fragile proteins and sonicated for 20sec. Then, spinned down by 6000rpm for 5min at 4^0C and took the supernatant.

3.7.3 Dot blot to confirm protein and concentration of antibody

The Dot blot procedure was used to test the antibody and titration of antibody concentrations.

3.7.4 Procedure for dot blotting

The grid was drawn on the PVDF membrane by a pencil to indicate the region that is going to be dotted. Then the PVDF membrane was activated in methanol before Putting 1-2 µl sample of the membrane, spotted small spores at the center of the grid. It was done slowly to minimize the area that the solution can penetrate (usually ¾ mm in diameters). The membrane was activated again into methanol and wet the membrane in 1 x PBST. The membrane was blocked for a few hours in PBST with 2% blocking agent (dry milk). Then washed twice with PBST for 5mineach and incubated with primary antibody (0.2-1 µg/ml) for 2 hours. The primary antibody was diluted with PBST in 3 different concentrations 1:1000, 1:2000 and 1:3000, extensively washed with PBST and incubated with secondary antibody in PBST with 2% dry milk. Finally, washed with PBST and stained by Ponseau S for half an hour.

3.8 Quantification of total protein concentration

3.8.1 Reagents for quantification of total protein concentration

BSA solution: BSA (2mg/ml) stock solution was used in three different dilutions into dH_2Osuch as 2mg/ml, 1mg/ml, 0.5mg/ml and blank try.

Reagent A': 5µl of DC reagent S was added into 250 µl of reagent A. Per sample needed 125 µl of reagent A'.

3.8.2 Quantification of total protein concentration

Tissue samples with Ripa buffer (200µl/10mg tissue) homogenized by precellys agitator and centrifuged at 12000rpm for 4min at 4^0C and the supernatant was taken. Then 125µl of Reagent A' mixture solution was added to each well of a 96 well plate (Falcon). Standards (BSA solution in different dilution) and samples were loaded as duplicates, 5µl of each and incubated for 5 min at normal temperature. 1 ml of DC Reagent B was added to each tube. Then the tubes were vortexed and incubated for 15 minutes. Absorbance was measured at 750 nm. Absorbance measured by spectrostar nano. Bovine serum albumin was used as a protein standard that was serially diluted with the dH_2O at wide range such as 2mg/ml, 1mg/ml, 0.5mg/ml and blank. The standard curve analysis (Y=a+bx, Y=known absorbance,

a=slope, b=y intercept and x=unknown concentration) was used to measure the total protein concentration.

3.9 Steps of Western blotting

Western blotting procedure has three main steps as follows:

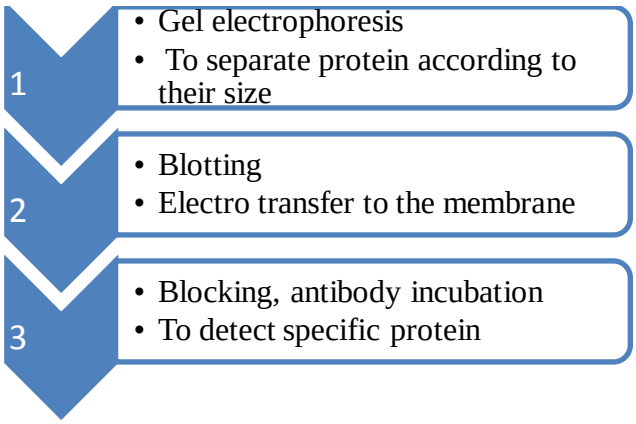


Figure 3.4: Steps of Western Blotting

3.9.1 Gel electrophoresis

3.9.1.1 Reagents for gel electrophoresis

2*Treatment buffer: 1.5M trizma base,pH 6.8, 10%SDS, 87% glycerol and dH_2O. Glycerol increases the density of the samples.DTT and Bromophenol blue solution: 8ml of 2*treatment buffer, 1M DTT was mixed into 500µl of Bromophenol blue. Bromophenol blue was used as an indicator dye.

Running buffer: 500ml of NuPAGE running buffer (Life technology).

3.9.1.2 Sample preparation for gel electrophoresis

Samples were diluted with this 2*treatment buffer, DTT and Bromophenol blue solution as the calculated protein concentration was 2mg/ml in every sample. NuPAGE®(10 % Bis-Tris by Novex®) precast was used to separate the polypeptides by their sizes. After opening the bag, the cassette was washed with dH_2O and the white tape at the bottom of the gel cassette was removed. Then it was placed in the slot in the Criterion™ tank. The chamber of the tank was blocked by a barrage and locked. The chamber was filled with 500 ml of fresh NuPAGE running buffer to fill the the tank. The comb of the wells was removed carefully by pulling upward.

The protein standard rainbow ladder and samples (per sample 10µl) were loaded in the wells of the gel using a micropipette with gel loading tips. The lid was fitted onto the tank and plugged into the power source. The program was set into 200 V, 125 mA, 25 W for approximately 45 min. This duration (some time up to 60 min) depends upon protein separation. Then electrophoresed gel was drawn from the gel cassette and the tank was rinsed with UF (Ultrafiltration) water.

3.9.2 Electro transfer of proteins by using iBlot®

iBlot is a dry blotting system from Invitrogen™. An electric field was employed to transfer protein from electrophoresed gel to nitrocellulose membrane. 'Bottom stack' was opened and entered in iBlot appliance. Electrophoresed gel was separated from gel cassette and cut off the bottom extra space in a gel. Transferred the gel over membrane of the bottom stack and nitrocellulose membrane was placed upon that with moisturizer. A roller was used to remove all the bubbles between gel and membrane and finally 'Top stack' was placed on it, where the sponge was directed in the lid (Figure 3.5). After closing the lid, the program was set and exposed for 6min.

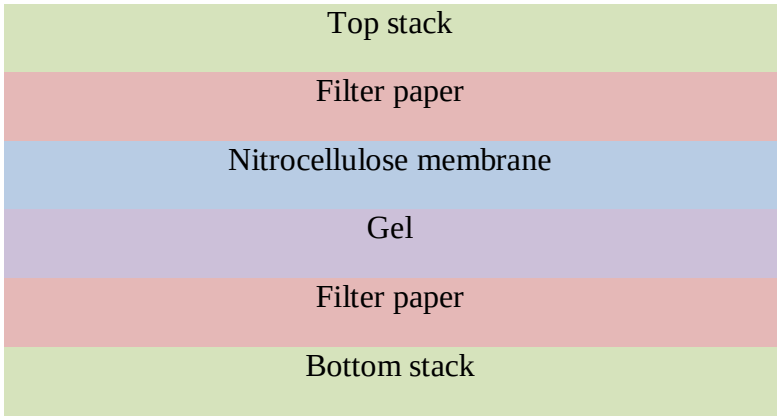


Figure 3.5: The orientation of a gel sandwich (direction top to bottom). Nitrocellulose membrane and gel are placed between soaked filter papers.

3.9.3. Blocking and antibody incubation

3.9.3.1 Reagents preparation for blocking and antibody incubation

Primary antibody: Anti HIF-1α antibody supplied by Aviva Systems Biology, was diluted in the blocking solution at 1:1000 ratio.

ECL*plex rainbow marker: 1µl was used in one well to compare protein band weight.

3.9.3.2 Procedure of blocking and antibody incubation

Stack was removed gently and membrane was incubated in blocking solution on a rocker at room temperature for 1 hour to block the protein free space. Afterwards, the membrane was washed with TPBS 2times for 5 min each on the rocker at room temperature. Then the membrane was incubated with specific primary antibodies for 1hour and 30min on a shaker. Again the blots were rinsed for two times in 1X PBST, 5 minutes each. The secondary antibody (CY5 anti rabbit, GE Healthcare), conjugated with ECL PlexCyDye was diluted in PBST at 1:5000 ratios.

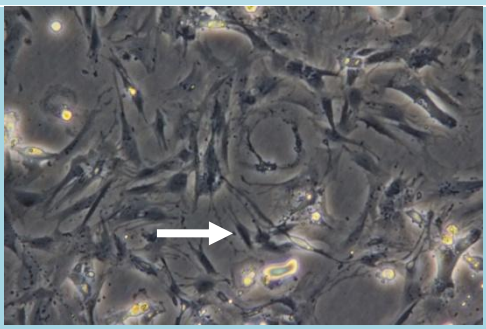
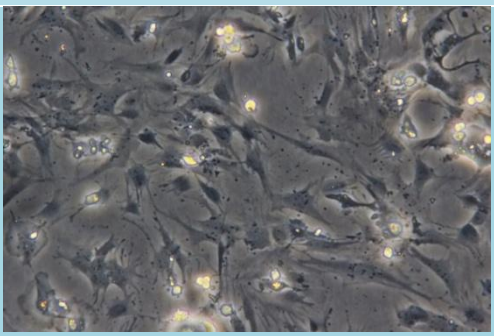
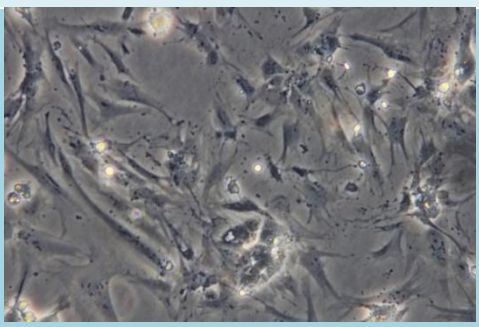
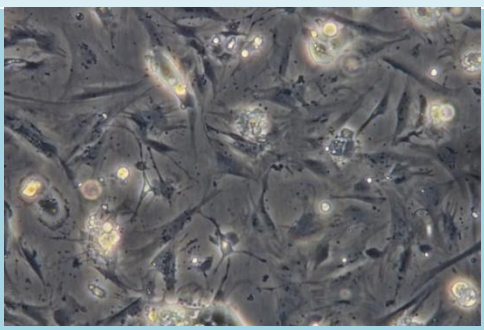
The membrane was then incubated with the diluted secondary antibody for 1 hour at room temperature on the electric shaker. Finally, the membrane was rinsed for three times with TPBS. Then the membrane was wiped with a filter paper for smoother background. Thereafter Epson scanner was used to detect the fluorescence signal by scanning the membrane. Chem-luminescence detection technique, subjected to secondary antibody conjugated ECL PlexCyDye, was used to detect the blotted proteins. A Chem-luminescence product was formed upon addition of ECL substrate that was visualized in a digital imaging system (Image Reader LAS-3000).

4.RESULTS

4.1 Culture of Atlantic cod gas gland cells

The total number of cells was measured by automatic cell counter machine. The percentage of live cells were 74%and the density of live cells was $5.52*10^7$/ml at the start of this experiment.

The PHD inhibitor FG-4592treated cellsshowed faster growth than the control (untreated cells). Cells were in the flat epithelial shape. Lamellar bodies were found in all samples (Figure 4.1a). The growth of the cells was measured at 8, 9, 10, 14 and 16 days of incubation. Cells were proliferated in inhibitor treated cells (Figure 4.1f) but on 14 days of culture cells were started to degrade (Figure 4.1h).

	Cells in the control group treated with DMSO	**Cells in inhibitor group treated with 50µM FG-4592**
Day 8	a 	b 
Day 9	c 	d 

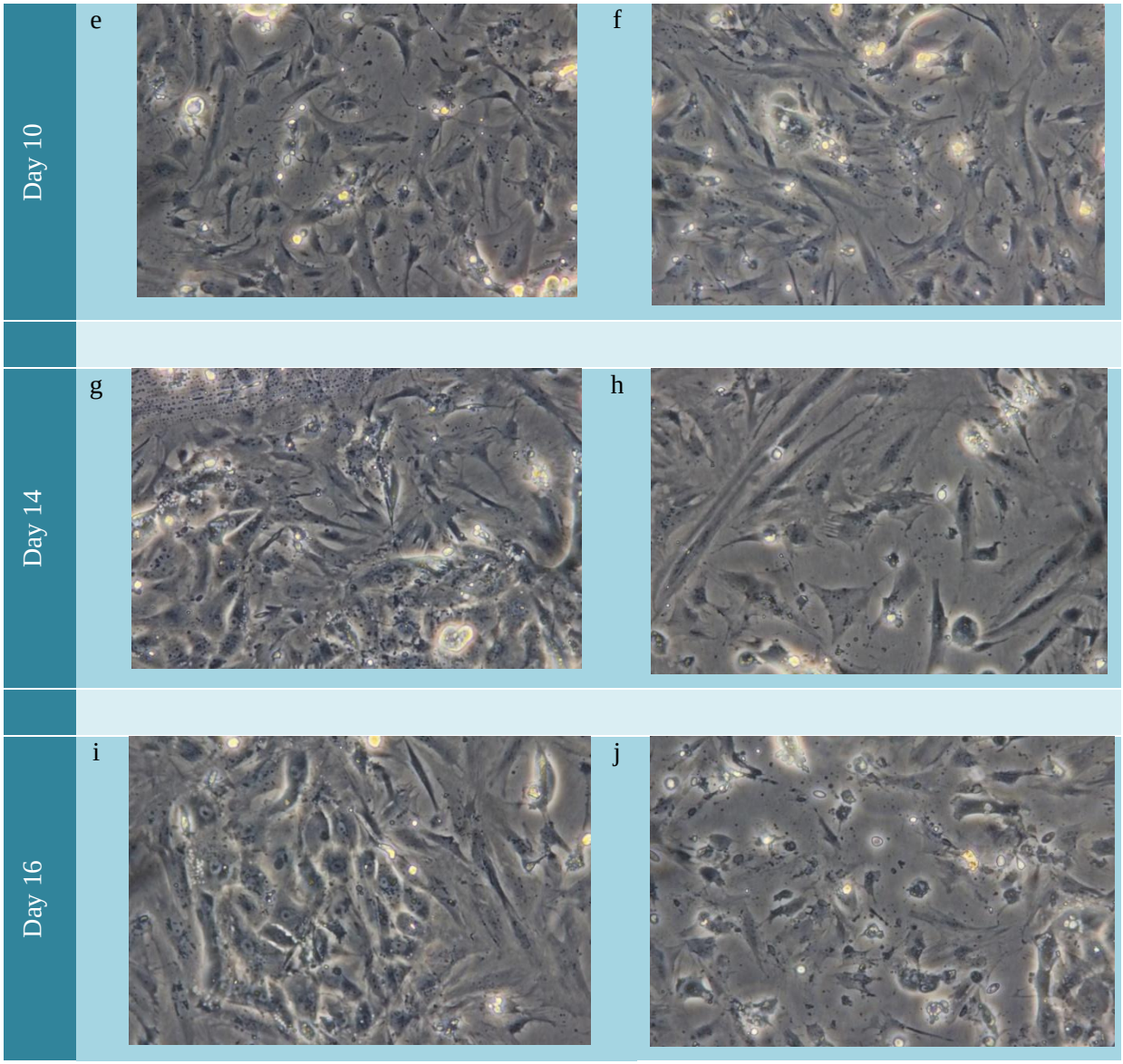


Figure 4.1: Growth of cells in the control group treated with DMSO on day 8 (a), day 9 (c), day 10 (e), day 14 (g) and day 16 (I) after incubation. Cells treated with PHD inhibitor FG-4592 (50µM) on Day 8 (b), day 9 (d), day 10 (f), day 14 (h) and day 16 (j) after incubation. Electronic microscopic view (20x).

4.2. The mRNA level of PHD inhibitor treated gas gland cells

The concentration of mRNA (ng/µl) in the control culture cells and the cells treated with the PHD inhibitor FG-4592 are shown in Figure 4.2. The amount of mRNA was lowest in the culture with the highest concentration of PHD inhibitor tretment (145µg/g tissue). The amount of mRNA in the control culture was 267µg/g tissue, while the amount of mRNA in cultured cells treated with 10 and 50µM PHD inhibitor were 274 and 211µg/g tissue respectively.

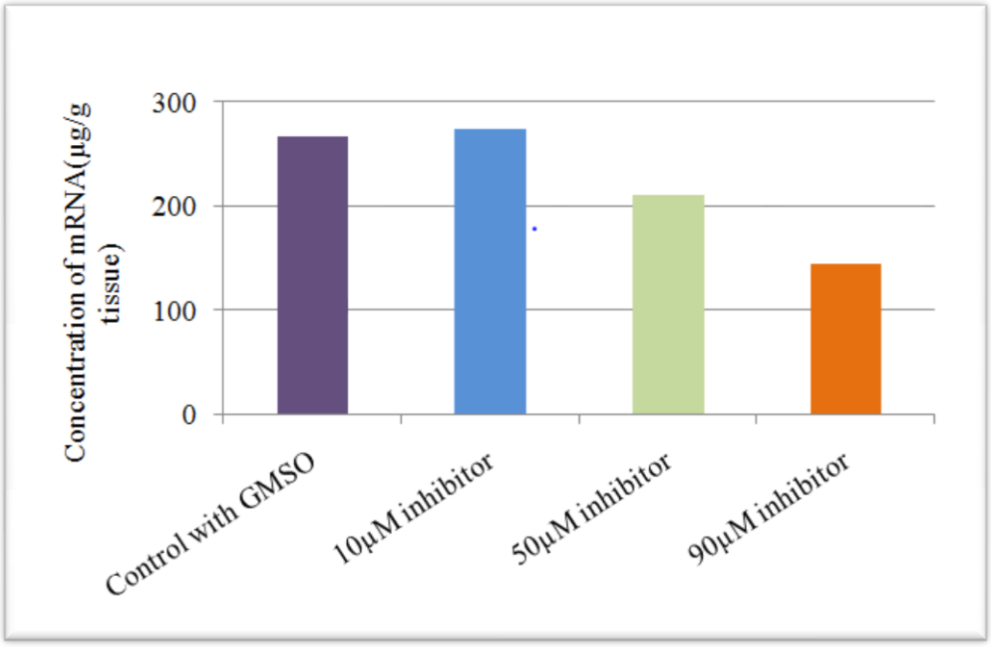


Figure4.2: mRNA (µg/g tissue) levels in cultured gas gland cells with DMSO(control)and three different concentrations of the PHD inhibitor FG-4592.

4.3 Analysis of relative gene expression of HIF-1α, LDHa, GLUT-1 and CA-6

The expression of hypoxia inducible genes were quantified by real-time qPCR from the cultured gas gland cells of Atlantic cod. The expression of the four hypoxia inducible genes HIF-1α, LDHa, GLUT-1 and CA-6 were measured. The genes were expressed differently when treated with different concentration of PHD inhibitor FG-4592, such as 10µM, 50µM and 90µM respectively. Generally, all of them showed higher gene expression then the control. Gene expression was increased with the increase of inhibitor concentrations in 10 and 50µM inhibitor treatment (Figure 4.3).

Relative gene expression of HIF-1α was gradually increased according to the higher inhibitor treatment. It had showed 1.34, 2.42 and 4.80 folds higher relative gene expression than control in 10µM, 50µM and 90µM PHD inhibitor treatment respectively (Figure 4.3). LDHa and GLUT-1 did not follow the same pattern. Both of them showed highest raltive gene expraession at 50µM inhibitor treated culture but lower in 90µM than 50µM treatment. Although gene expression was higher in 90µM than the control. The raltive gene espressions of LDHa were 2.1, 2.95 and 2.34 in 10µM, 50µM and 90µM PHD inhibitor treatment respectively. The relative gene expression of GLUT-1 in 10µM, 50µM and 90µM PHD inhibitor treated culture were 1.27, 3.6 and 0.70 folds higher than control gene. CA-6 followed the similar trend of gene expression of HIF-1α. Interesting, the CA-6 relative gene expression in 90µM inhibitor treatment was increased dramatically than 50µM inhibitor treatment. The relative gene expression of CA-6 in 90µM inhibitor treatment was 6.9,whereas in 50µM inhibitor treatment, relative gene expression was 2.29 (Figure 4.3). Statistical analysis with Rstudio showed singnificant difference in all treatments (P=0.0089) and gene-treatment interaction (P=0.00001).

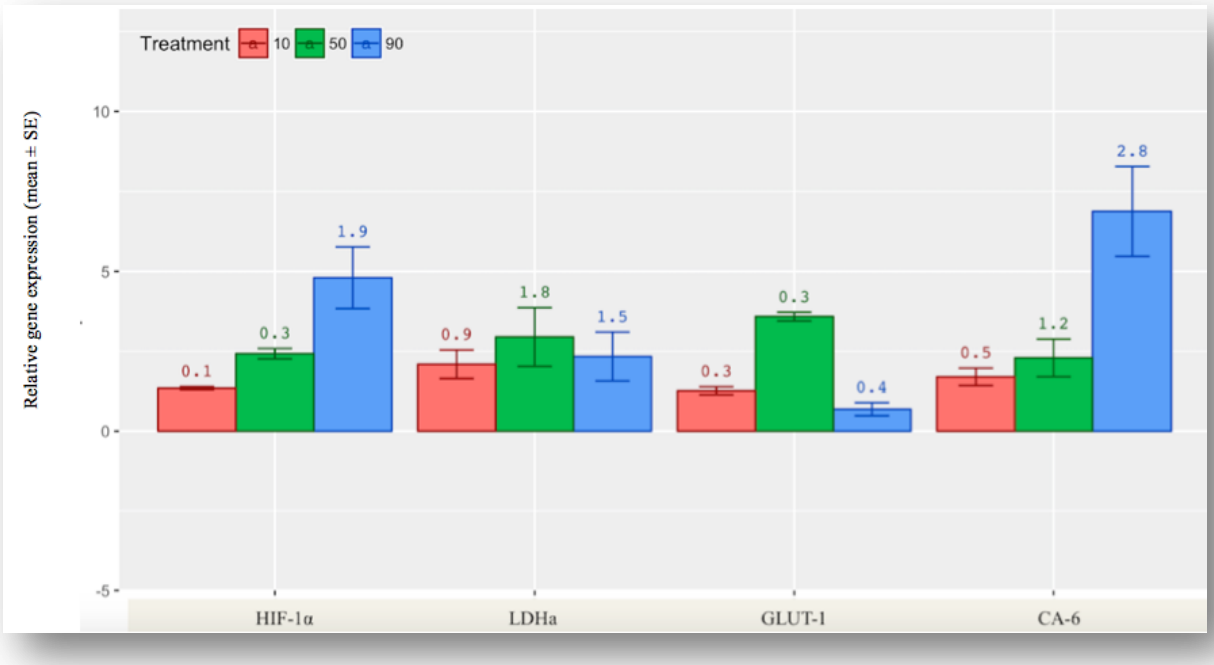


Figure 4.3: Relative gene expression of HIF-1α, LDHa, GLUT-1 and CA-6 in different concentrations of PHD inhibitor treatment. Different color shows differnt treatment. Data were analysed with Rstudio and presented in mean±standard error. P<0.005 meant significatly different.

4.4 Detection of HIF-1α protein by Western blotting

The total amount of protein (appendix 3) was measured from Atlantic cod gas gland tissue by protein assay. The HIF-1α protein was detected (Figure 4.4) by anti-HIF-1α antibody produced in rabbit. This protein was also detected in the pig heart tissue with the same antibody. The HIF-1α in Atlantic cod gas gland tissue and the pig heart tissue were detected at normal oxygen levels and their molecular weight was 28 kDa and 54 kDa respectively (Figure 4.4).

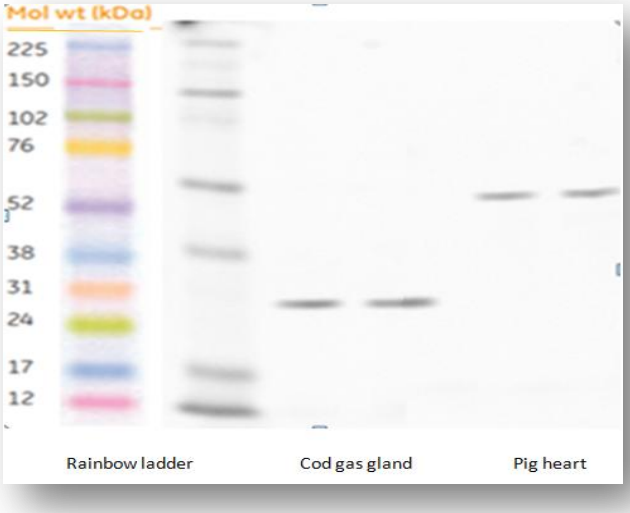


Figure 4.4: Detection of HIF-1α in Atlantic cod gas gland tissue by the Western blot method. Protein was extracted with Ripa buffer and the absorbance of protein solution was measured at a wavelength of 750 nm.

5. DISCUSSIONS

Atlantic cod (*Gadus morhua* L.) is a teleost fish that consists of physoclistswim bladder (Midling *et al.* 2012; Scheid *et al.* 1990). It is in the posterior region of the pharyns from where the swim bladder and lungs of vertibrates were originated (Zheng *et al.* 2011). It was found that, there is an evolutionary relation between swim bladder and tetrapod lungs. In this experiment gas gland cells of swim bladder were cultured in growth medium. It was observed that, Atlantic cods gas gland cells are flattened epithelial cells (Figure 4.1), which are similar to perch swim bladder gas gland cells (Prem *et al.* 2000). The swim bladder maintains the buoyancy with this specialized gas gland cells located in the ventral side of the swim bladder. Atlantic cod is a hypoxia intolerable species, but it can change its respiratory physiology in hypoxia (Hall *et al.* 2009).

In this study, Atlantic cod gas gland cells were treated with the PHD inhibitor Roxadustat (FG-9245) and cultured to determinethe relative expression of the four different hypoxia induced genes HIF-1α, LDHa, GLUT-1 and CA-6. The PHD inhibitor Roxadustat (FG-4592) was used in the cell culture media to stabilize HIF. Several experiments showed the stabilization of HIF with the treatment of PHD inhibitor in human but very few were done with fish. Studies have shown that HIF stimulation and modulation of HIF-regulated angiogenic proteins in human lung cells is feasible through PHD treatment (Asikainen *et al.* 2005; Jaakkola *et al.* 2001). The mRNA extracted from cultured gas gland cells was 267µg/gm of gas gland tissue that is comparatively low amount to experiment with several genes. Hall *et al.* (2014) found more than 2000µg/gm of total RNA in Atlantic cod gas gland tissue. It was also found different level of total RNA in various types of tissue of Atlantic cod whereas gas gland tissue has an intermediate level of RNA compared to other tissues such as lever, heart tissue.

In the current experiment, all four genes showed higher relative gene expression in response to different concentrations of PHD inhibitor in the treated cellsthen the control group (Figure 4.3). Each cell and tissue has different ability to adjust to hypoxia (Wu & Yotnda 2011). Studies clearly showed, different level of gene expression in response to hypoxia (Hall et al. 2009; Yu, Y. *et al*. 2016) in different kind of cells, such as gills and swim bladder of Spotted Gar (Rimoldi *et al.* 2016) and gills and spleen of Atlantic cod (Hall *et al.* 2005). Studies also revealed, changes in gene expression individually *in vivo* or *in vitro* hypoxic condition. For instance, in oxygen level changes of water (Hall *et al.* 2009) and culture medium treated with

inhibitor (Asikainena *et al*. 2005; Wu & Yotnda 2011; Yu, Y. et al. 2016) and in both conditions (Kajimura *et al.* 2006).

In the present study, Roxadustat (FG-9245) treated cells showed higher HIF-1α expression than the untreated cells (Figure 4.3). HIF-1α is a master regulator of hypoxia-induced gene response. Hypoxia alters cellular functions by regulating the master transcription factor HIF-1α (Yu, Y. *et al.* 2016). This induction shows variation among species, tissue types and also exposure time. Earlier studies has revealed the up regulation of HIF-1α in mammals and fish species under hypoxia condition, which also depends upon fish species, tissue (brain, heart, gill, spleen, liver) and the duration of hypoxia (Li *et al.* 2017). According to Yu, Y. *et al.* (2016), hypoxia increases the HIF-1α expression in co-culture of ADMSC (adipose-derived mesenchymal stem cells).

In this experiment, 10µM and 50µM Roxadustat (FG-9245) treated gas gland cells showed higher gene expressionin GLUT-1 and LDHa than the control (Figure 4.3). But in 90µM PHD inhibitor treatment, the relative gene expression of werelower then other two groups. During hypoxia,the oxidative phosphorylationis inhibited, thuscells increase their anaerobic glycolysis for ATP supply. Hypoxia induces stimulation of the Na-independent glucose transporters , and increase 2-deoxy-D-glucose (DG) uptake (Hall *et al.* 2014). Therefore, they are thought to play a major part in glucose trafficking (Ouiddir *et al.* 1999). The gas gland cells of Atlantic cod have the highest rates of glucose metabolism. High levels of GLUT-1 transcript in gas gland cells from Atlantic cod is consistent with high lactate production (Hall *et al.* 2014). It also found in Hall *et al.* (2014) that, there is exceptionally high levels of GLUT-1 protein in the Atlantic cod gas gland cells and possibly involved in high rates of lactate production. The general expression pattern of GLUT-1 in the inhibitor treated group aggress with the earlier research findings, where the expression of GLUT-1 increased in the hypoxia group in Atlantic cod (Hall *et al.* 2009). However, the lower relative gene expression in the highest inhibitor treated group (90µM) might be due to long term incubation or higher dose of the inhibitor in a long term culture medium. Additionally, More sampling within 16 days of culture, might give better expression pattern of LDHa and GLUT-1 in 90µM PHD inhibitor treatment. Levels of GLUT-1 transcripts in a variety of fish follow the similar direction in response to hypoxia (Capilla et al. 2002; Hall et al. 2004; Hall et al. 2005; Hall et al. 2009). Besides, studies have shown transcriptional increase in hepatic GLUT-2 transcript levels during hypoxia in sea bass (*Dicentrarchus labrax*) (Terova *et al.* 2009).

In this experiment, the pattern of the relative gene expression of CA-6 gene was similar to HIF- 1α.This indicatesthe onset of anaerobic respiration and decrease in energy demand in the gas gland tissues.The 90µM PHD inhibitor treated cells showed the highest relative gene expression (Figure 4.3). In Addition, LDHa showed almost similar patter. The activity and functionality of anaerobic energy production depends upon LDH because it catalyzes the terminal step of the anaerobic glycolysis (Fields & Somero 1998; Portner 2002; Zakhartsev *et al.* 2004). Additionally, there is a positive correlation between high level of GLUT-1 transcript and lactate production in gas gland tissue (Bailey *et al.* 1990; Hall *et al.* 2014). In hypoxic condition, LDHa preferentially converts pyruvate to lactate under anaerobic condition (Almeida-Val *et al.* 2011). Almeida-Val *et al.* (2011) demonstrated that the LDHa expression depends upon the time of acclimation in different life stages and tissue type of *Astronotus crassipinis* (the Oscar). During juvenile stage, skeletal and cardiac muscle shows increased LDHa expression upon acute hypoxia, but decreased LDHa expression upon graded hypoxia exposures.

Carbonic anhydrase (CA) is critical to CO_2 excretion, ionic regulation and acid-base balance. Very few experiment was done with CA-6 gene expression in fish. In this study, the relative gene expression of CA-6 gene, treated with Roxadustat (FG-9245) was highest in 90µM inhibitor treated cells than the untreated and other treated cells. Relative expression of CA-6 gene was increased according to the increasing doses of Roxadustat (FG-9245) (Figure 4.3).In case of human,presence of CA-9 considered as a sign of acute tumor behavior, CA-9 functions as a protector of tumor cells from hypoxia (Sedlakova *et al.* 2014). HIF-1α regulates CA-9 over expression in prostate cancer tissue and this over-expression is a marker of tumor hypoxia (Ambrosio *et al.* 2016). There was a sharp increase of relative gene expression of CA-6 in 90µM inhibitor treated cells. However, according to Mayer *et al.* (2014) CA IX may be worth consideration as a marker of biological hypoxia.

In the current experiment, the total protein of the cod gas gland tissue was determined by Protein assay (Appendix 3) and the HIF-1α protein was determined in normoxia condition by the Western blotting method.Theprotein showed band with 28kDa (Figure 4.4) but protein is 86kDa whichwas detected by Robertson et al. (2014). But this protein (HIF-1α) is a highly degradable protein in the oxygenated cells. It degrades within 5 minutes in normal oxygen levels, even after 4 hours of hypoxia treatment (Huang *et al.* 1996).

6. CONCLUSION

Atlantic cod gas gland cells were cultured in a growth medium and treated with PHD inhibitor. Relative gene expression of four different hypoxia inducible genes (HIF-1α, LDHa, GLUT-1 and CA-6) was measured in this study. Relative gene expression was determined by real time qPCR showed efficacy of PHD inhibitor treatment on gene expression in hypoxic condition *in vitro*. Gene expression in three concentrations of inhibitor treatment was measured to differentiate the gene expression in three different hypoxic condition. It was supposed that, all these four genes have been expressed in higher PHD inhibitor treatment than the lower concentration. The reason of the lower amount of mRNA and low gene expression of LDHa and GLUT-1 in maximum (90μM) concentration of inhibitor treatment is not clear. Finally, HIF-1α protein was determined by Western blotting and compared with pig heart tissue. According to this study, there is likely a positive correlation between PHD inhibitor treatment and hypoxia *in vitro*.

7. FUTURE PERSPECTIVE

Due to limitation of time, it was not possible to explore all the conditions of culture from various replicates to reach a conclusive mark about gene expression level especially in higher concentration of inhibitor treatment. Different PHD inhibitor treatment should be adopted in cell culture. Therefore, to understand the relative gene expression pattern of LDHa and GLUT-1 in higher (90μM) PHD inhibitor treated culture, required more sampling. To further research, cell viability assay can be included in different stage of culture. Furthermore, PHD inhibitor along with low oxygen in laboratory condition can be tested to get more sensitive result of gene expression. A clear band of HIF-1α protein in gas gland tissue in normal oxygen indicates a higher level of this protein in hypoxic condition. Assessment of this protein in different concentrations of PHD inhibitor treatment can add another valuable perspective. Previous studies showed that detection of HIF-1α protein in a laboratory environment is a difficult job due to very fast degradable nature of this protein in normoxic condition. So,in this situation a hypoxic chamber can help to assess protein. Finally, further extensive research on the effect of the PHD inhibitor to theorgans including gas gland cells could pave the way to find an effective way to study the effect of hypoxia on Atlantic cod as well as other fishes.

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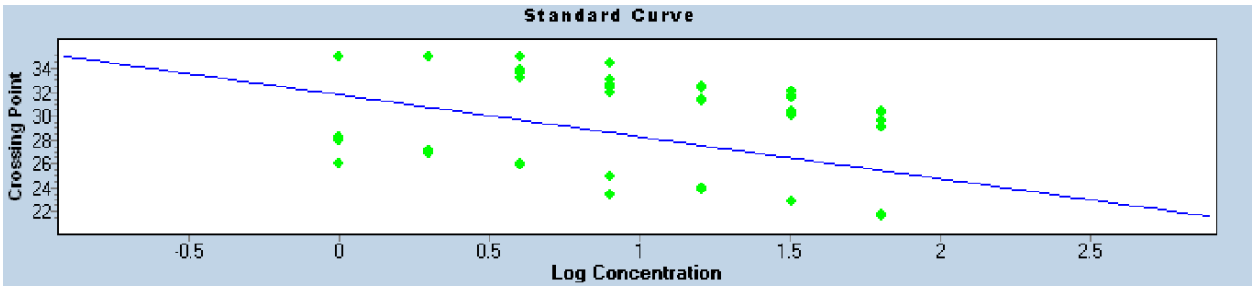
APPENDIX

Appendix 1: Buffer 1 for cell extraction

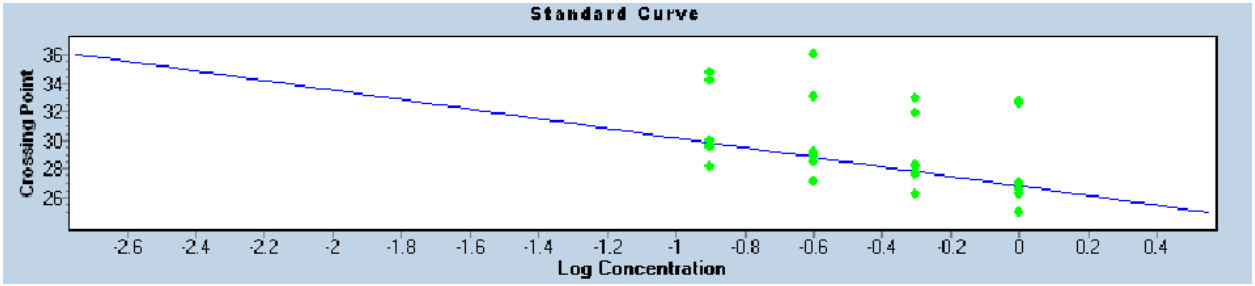
Chemicals	Conc. (mM)	M (g/mol)	g/L
NaCl	100	58.44277	5.844277
KCl	10	74.5513	0.745513
KH2PO4	1.2	136.08554	0.1633026
Glucose	20	180.15588	3.6031176
Hepes ($C_8H_{17}N_2NaO_4S$)	10	260.29	2.6029
BDM ($C_4H_7NO_2$)	10	101.10392	1.011039
MgSO4	4	120.3686	0.481474
Taurine	50	125.14792	6.257396

Appendix 2: Standard curve ofthe genes

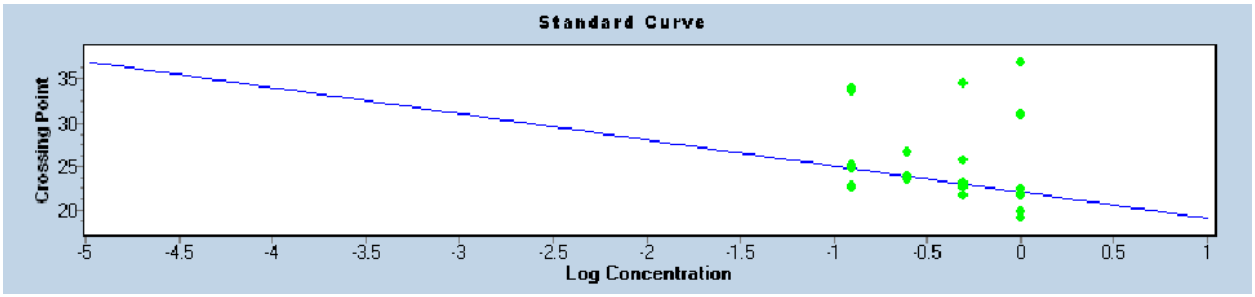
(a) UBI, Efficiency: 1.92



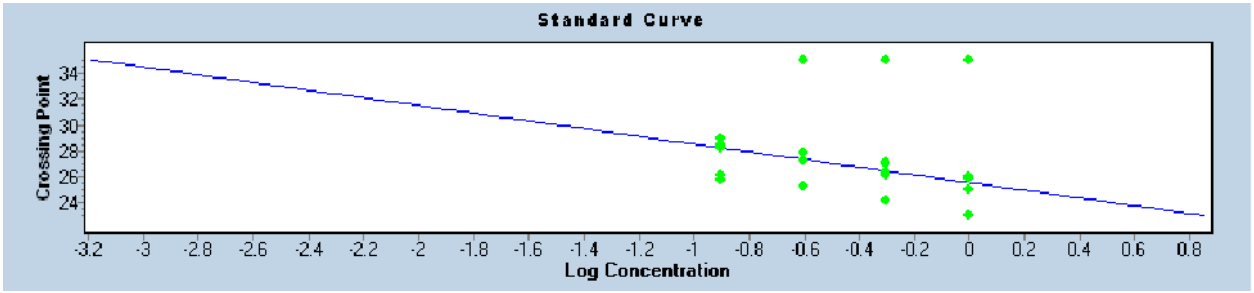
(b) HIF-1α,efficiency= 1.98



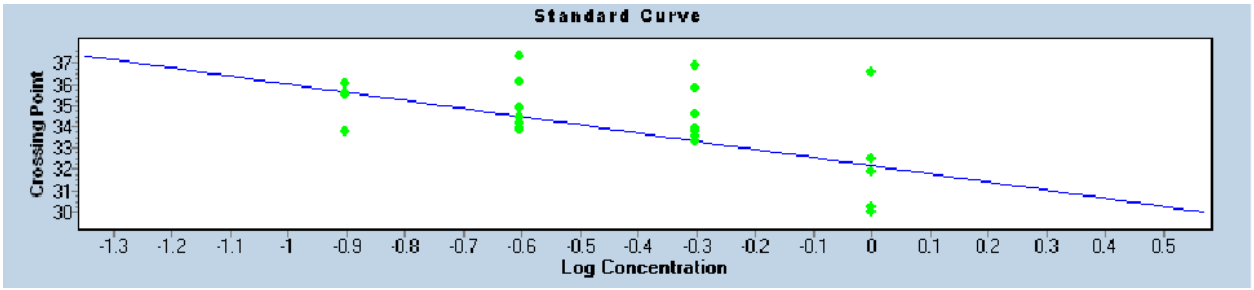
(c) LDHa,efficiency= 2.17



(d) GLUT-1, efficiency= 2.18



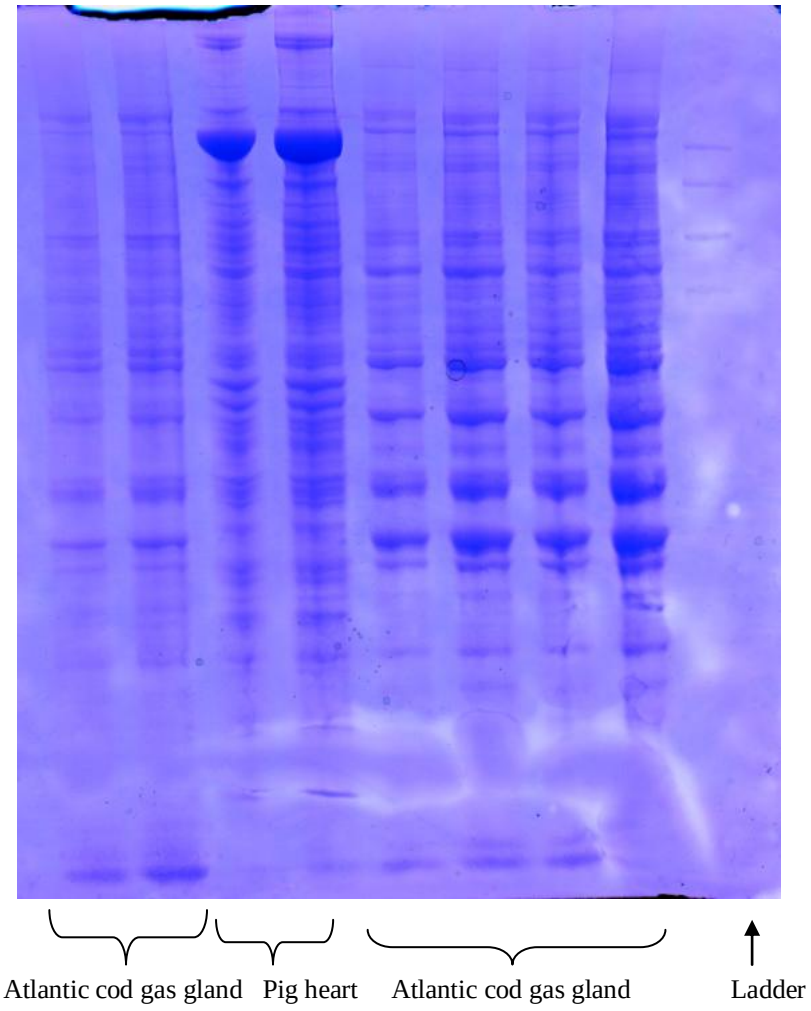
(e) CA-6, efficiency= 1.83



Appendix 3: Measurement of total protein in Atlantic cod gas gland tissue and pig heart tissue

	Sample	Concentration (µg/ml)
22.03.16	PH1	4452.50
	PH2	5126.20
	CG1	4236.80
	CG2	3240.00
27.06.17	PH1	5109.40
	PH2	5080.81
	CG1	5854.93
	CG2	5840.92

Appendix 4:Gel run of Atlantic cod gas gland and pig heart tissue.



Appendix 5: Statistical data analysis by R studio(P<0.05 means significant difference).

```
library(readxl)
StatisticalAnalysis <- read_excel("StatisticalAnalysis.xlsx", sheet = "Sheet4")
StatisticalAnalysis$Gene <- factor(StatisticalAnalysis$Gene)
StatisticalAnalysis$Treatment <- factor(StatisticalAnalysis$Treatment)
StatisticalAnalysis$Dilution <- factor(StatisticalAnalysis$Dilution)


## Some plots
library(ggplot2)

## Plot
subData <- subset(StatisticalAnalysis, Treatment != 0)
ggplot(subData, aes(x = Treatment, y = Expression)) +
  geom_boxplot() +
  facet_grid(. ~ Gene) +
  stat_summary(fun.y = mean, geom = "point", color = "red")

## linear model
mdl <- lm(Expression ~ Gene + Treatment + Gene * Treatment, data = StatisticalAnalysis)
anova(mdl)

## Looking at effect
library(effects)
eff <- allEffects(mdl)
plot(eff)


ggplot(StatisticalAnalysis, aes(Gene, Expression)) +
  stat_summary(fun.y = mean, geom = "bar", aes(fill = Treatment), position = "dodge") +
  stat_summary(fun.data = mean_se, geom = "errorbar", color = "black", aes(group =
Treatment),
               position = "dodge")


library(dplyr)

dataSummary <- StatisticalAnalysis %>%
  group_by(Gene, Treatment) %>%
  do(mean_se(.$Expression)) %>%
  mutate(se = ymax - ymin)

plt <- ggplot(dataSummary, aes(Gene, y, fill = Treatment, ymin = ymin,
                       ymax = ymax, label = round(se, 1), color = Treatment)) +
  geom_col(position = "dodge") +
  geom_text(position = position_dodge(width = 0.9),
            vjust = -1, aes(y = ymax), angle = 0,
            size = 3.5, family = "mono") +
```

```
  geom_errorbar(width = 0.4,
                position = position_dodge(width = 0.9)) +
  scale_y_continuous(expand = c(0, 5)) +
  scale_color_discrete(l = 30) +
  labs(x = "Gene", y = "Expression") +
  theme(legend.position = c(0, 1),
        legend.direction = "horizontal",
        legend.justification = c("left", "top"),
        legend.box.margin = margin(6, 6, 6, 6),
        legend.background = element_blank())

ggsave(filename = "Rplot.png", plot = plt, width = 9, height = 5)
```

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